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THE EFFECT OF SINGLE CLOVE BLACK GARLIC ON THE HEMOSTASIS STATUS AND LIPID PROFILE IN MALE SPRAGUE DAWLEY RATS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) patients have alteration in hemostasis components. Thus, including excess expression of plasminogen activator inhibitor-1 (PAI-1), causing fibrinolysis disorders; the majority of these patients are hypercoagulable state prone to thrombosis. Some evidence suggests that garlic and garlic supplements have antithrombotic and anti-inflammatory properties. Besides, garlic stimulates fibrinolytic activity and normalizes plasma lipid imbalances. Black garlic is processed garlic that is produced through natural aging at a controlled temperature (70 °C) and high humidity (90%) for several days, without other additives. This study aimed to prove the effect of single clove black garlic (SCBG) (*Allium sativum Linn*) on PAI-1 levels and lipid profiles of NAFLD rats induced by a *high-fat fructose diet* (HFFD) containing 1.25% cholesterol and 0.5% cholic acid.The rats were then divided into healthy control group (K_1^+); NAFLD control group without treatment (K_2^+); 0.9 mg simvastatin treatment group (K_1^-); 45 mg metformin treatment group (K_2^-); SCBG 450 mg per 200g BW (X1); 900 mg per 200 g BW (X2); and 1350 mg per 200 g BW (X3). All treatments were administered for 4 weeks via oral gavage. As a result, significant differences in PAI-1 levels and lipid profiles between groups after the administration (p = 0.001) were noted and also by simvastatin and metformin, respectively. There was a correlation between PAI-1 and lipid profile of SCBG treatment. In conclusion, the administration of SCBG (1350 mg per 200 g BB per day) for 4 weeks had a significant effect on PAI-1 levels, and the lipid profiles in *Sprague Dawley* rats modeled NAFLD (p = 0.001). SCBG has provided benefits that can be useful in the management of NAFLD but it's not equivalent to medicine.

Keywords: single clove black garlic; PAI-1; lipid profile; NAFLD; cardiovascular disease

INTRODUCTION

Several mechanisms have been described for the development of atherosclerosis in patients with NAFLD, including genetic predisposition, insulin resistance, and atherogenic dyslipidemia, oxidative stress, chronic inflammation, decreased adiponectin levels, and altered pro- and anticoagulant factor production, which are all present simultaneously **(Francque, van der Graaff and Kwanten, 2016)**. NAFLD, especially in its necro inflammatory form (NASH), can cause atherogenic dyslipidemia **(Kim et al., 2012)**. Also, an increase in procoagulant factors, such as fibrinogen, plasminogen activator inhibitor-1 (PAI-1), and tumor growth factor, all of which increase the risk of atherosclerosis, has been noted **(Alkhouri et al., 2010)**.

To manage NAFLD, lifestyle modifications are strongly suggested, especially dietary arrangements. However, patients usually fail to achieve these changes because they are unable or unwilling to change their dietary habits, they require safe and effective diet management for them. Garlic (*Allium sativum* L.) is a functional food containing an *allyl*-substituted sulfur compound that has been used for a long time both for seasoning and also for prevention and cure of diseases in many cultures **(Rivlin, 2001)**. Preclinical and clinical studies suggest a link between dietary habits and disease occurrence. Previous studies have shown that garlic consumption is inversely correlated to the incidence of hyperlipidemia, atherosclerosis, and thrombosis **(Agarwal, 1996)**. Garlic has many health benefits, but its raw form is not always consumed, because it has a strong odor and taste due to its organosulfur compounds, which makes some people uncomfortable. In recent years, various methods of processing raw garlic have been used, such as fermentation and heating for a long time, which eliminates pungent odors, imparts a sweet taste, and increases the beneficial effects **(Bae et al., 2014)**. Black garlic is processed garlic that is produced through natural aging at a controlled temperature (70 °C) and high humidity (90%) for several days, without other additives **(Toledano-Medina et al., 2019)**. Through

processing, the *S-allyl-L-cysteine* content in black garlic becomes up to five times higher **(Sasaki et al., 2007)**. Also, black garlic had a higher polyphenol content and SOD activity and scavenging activity against hydrogen peroxide were stronger than raw garlic **(Kim et al., 2012; Sato, Kohno and Niwano, 2006)**. The cardiovascular benefits of black garlic administration have been illustrated in various studies. However, so far, few studies have been done on the effect of SCBG administration on PAI-1 levels and lipid profiles in NAFLD. In this study, we evaluated the effective administration of SCBG, simvastatin, and metformin on the hemostasis status and lipid profiles of rats modeled NAFLD induced by a high-fat fructose diet (HFFD) containing 1.25% cholesterol and 0.5% colic acid.

Scientific hypothesis

We investigate several hypotheses in our study:

1. The administration of SCBG at different doses (450 mg per 200 g BW per day; 900 mg per 200 g BW per day and 1350 mg per 200 g BW per day) for 4 weeks have a significant effect on PAI-1 levels, and lipid profiles of *Sprague Dawley* rats modeled NAFLD compared with simvastatin and metformin treatment groups.
2. There are correlations between PAI-1 and Lipid Profiles of *Sprague Dawley* rats modeled NAFLD.

MATERIAL AND METHODOLOGY

This study was part of the research project entitled Study of Pre-clinical Benefit of Black Garlic (A*llium sativum Linn*) in Non-alcoholic Fatty Liver Disease (NAFLD).

Samples

This study used *Rattus norvegicus*, strain Sprague Dawley rats, weighing 180 – 200 g and aged 8 – 10 weeks old.

Chemicals

Plasma PAI-1 levels were determined using Rat PAI-1 ELISA kit, Finetest from Wuhan Fine Biotech Co. Ltd. Lipid profiles were determined using Cholesterol FS (10') 5 x 25 mL/1x 3 mL Standard, Triglycerides FS (10') 5 x 25 mL/1 x 3 mL Standard, HDL Precipitant 250 mL, LDL Precipitant 250 mL from DiaSys, Holzheim, Germany.

Animals and Biological Material:

The male Sprague Dawley rats (n = 42) were purchased from the Center of the Food and Nutrition Studies of Gadjah Mada University, Yogyakarta, Indonesia. An acclimatization period lasting seven days was scheduled before the beginning of the experiment. After the acclimatization period, rats were divided into seven groups (n = 6 per group): healthy rats given standard feed (K_1^+), NAFLD rats without treatment (K_2^+); 0.9 mg simvastatin treatment group (K_1^-); 45 mg metformin treatment group (K_2^-); single clove black garlic (SCBG) 450 mg per 200 g BW (X1); 900 mg per 200 g BW (X2); and 1350 mg per 200 g BW (X3). All animals, except the K_1^+ group, were fed a *high-fat fructose diet* (HFFD) containing cholesterol (1.25%) and cholic acid (0.5%) for 8 weeks.

The standard feed containing 15% crude protein, 3 – 7% crude fat, 12% moisture content, 6% crude fiber, 7% ash, 0.5% phosphorus, and 0.9 – 1.1% calcium, and vitamins. The composition of HFFD as follow was 177.5 $g.kg^{-1}$ pork oil, 172.8 $g.kg^{-1}$ fructose, 19.87 $kJ.g^{-1}$ energy, 20% protein, 35% carbohydrates, and 45% fat.

Black garlic used in this study was produced from the Biotechnology Agency for Technology Assessment and Application (BPPT), Tangerang, Indonesia, with the commercial name of Natural Black Garlic® Lanang, which has passed the test of acute toxicity by the Laboratory Toxicity and Safety Materials, School of Pharmacy Bandung Institute of Technology, Indonesia. The main raw material for Natural Black Garlic® Lanang is single clove garlic. It's processed garlic that is produced through natural aging at a controlled temperature (70 °C) and high humidity (90%) for 26 days without other additives (Figure 1).

Instruments

iMark™ microplate absorbance reader, Bio-Rad. Microprocessor Controlled Spectrophotometer, *OPTIMA SP-300*.

Laboratory Methods

PAI-1 levels were measured using the Enzyme-Linked Immunosorbent Assay (ELISA) method **(Crowther, 2009)**.

Total cholesterol and HDL-C levels were analyzed by Cholesterol Oxidase- Phenol Amino Phenazone (CHOD-PAP) and triglycerides were analyzed by Glycerol Phosphate Oxidase-Phenol Amino Phenazone (GPO-PAP) **(McPherson and Pincus, 2016)**. LDL-C levels were calculated with the Friedewald formula.

Description of the Experiment

Simvastatin, Metformin and Single Clove Black Garlic preparation as follows was 0.9 mg simvastatin and 45 mg metformin per 200g BW as comparative groups and SCBG dose 450 mg, 900 mg, and 1350 mg per 200 g BW, respectively, were mashed and diluted together with 3 mL of aqua bidestilata **(Hatton et al., 2015)**. All samples were administered by oral gavage **(Atcha et al., 2010)**.

Sample preparation: After 4 weeks of administration, all animals overnight fasted. Blood was collected from the *sinus orbital* **(Wolforth, 2000)** and centrifuged at 1,000 rpm for 15 minutes at 2 – 8 °C, then plasma was collected and stored at -80 °C for later analysis.

Number of samples analyzed: forty-two.

Number of repeated analyses: duplo

Number of experiment replication: There is no experiment replication in our study.

Statistical Analysis

Bodyweight gain was recorded weekly, while examination data of PAI-1 levels and plasma lipid profiles were taken *post-test.* All data were analyzed statistically using IBM SPSS Statistics for Windows version 22.0. Shapiro-Wilk test was used to test the normality of data. The analyses used were paired *t*-test, one-way ANOVA, *post- hoc* LSD, Kruskal-Wallis, and Mann-Whitney. For the correlations between PAI-1 levels and lipid profiles used correlation Spearman test.

RESULTS AND DISCUSSION

In this study, the HFFD containing cholesterol (1.25%) and cholic acid (0.5%) – supplied rats showed marked obesity (increases of body weight), hyperlipemia (increases of the plasma total cholesterol, triglyceride, and LDL-C levels with decreases of the HDL-C levels) and hemostasis status (increases of PAI-1 level). Overall, obesity, diabetes, and hyperlipemia were induced by 8 weeks of continuous HFFD supply, which is consistent with the findings of previous studies **(Abe et al., 2019; Jarukamjorn et al., 2016; Oligschlaeger and Shiri-Sverdlov, 2020)**. Administration of HFFD containing cholesterol (1.25%) and cholic acid (0.5%) aim to induced fatty liver in experimental animals. HFFD content is considered the same as a fast-food diet which contains high fat, high fructose, and high cholesterol (i.e. 2% w/w). Free fatty acids are known to trigger mitochondrial dysfunction, oxidative stress, and hepatic stellate cell activation. Fructose is known to be a major activator of lipotoxicity factors in the liver, leading to the development of NAFLD. Fructose consumption was found to be higher in patients with NAFLD compared to controls of the same age, and increased fructose consumption has been associated with the severity of fibrosis in patients with NAFLD **(Abe et al., 2019)**. Free cholesterol directly activates liver stellate cells, besides that high cholesterol in the composition of the fast-food diet also promotes the development of nonalcoholic steatohepatitis (NASH) **(Tomita et al., 2014)**. Thus, we conclude that excess amounts of fat, fructose, and cholesterol in HFFD exacerbate the lipotoxic environment and accelerate the progression of steatohepatitis and fibrosis in obese Sprague Dawley rats.

Administration of HFFD against weight

After administration of HFFD containing cholesterol (1.25%) and cholic acid (0.5%) for 8 weeks, generally, the six groups of rats experienced an increase in body weight as well as the healthy group that was given standard feed. Weighing the rats was performed weekly starting from the acclimatization until the end administration of HFFD. Weighing the mice after acclimatization aimed to ensure that the experimental animal still within the inclusion criteria, namely, having a bodyweight of 150 – 200 g. Table 1 shows the changes in body weight of rats before and after conditioning. These changes probably due to the HFFD administration for 8 weeks during conditioning. HFFD containing high-fat (45%) and high-fructose (172.8 $g.kg^{-1}$ diet), contributing a lot of energy (19.87 $kJ.g^{-1}$) **(Jung et al., 2011)**. Fat also contains high energy compared to carbohydrates and protein. 1 g of fat is equivalent to 9 kcal of energy, while 1 g of protein and carbohydrates each is equivalent to 4 kcal of energy **(Hardinsyah and Supariasa, 2019)**.

In this study, the body weight of the animals significantly increased after HFFD administration in six group treatments ($p = 0.001$). except for the healthy group, which had an increase but not significant. The X2 group does not have significant weight differences before and after conditioning. In this study, the administration of HFFD attempted to apply the diet composition of NAFLD patients. The difference in food intake before and after HFFD administration between groups was related to the differences in the energy composition of the diets of different rats. The dietary intake of 15 g per rat per day was selected according to the nutritional needs of laboratory animals, which is considered the minimum amount needed by rats per day to ensure normal growth of them **(Fakhoury-Sayegh et al., 2015)**.

Effect of single clove black garlic on body weight

In this study, the increases of weights were meaningfully and dose-dependently inhibited by treatment of SCBG and also by simvastatin and metformin, respectively. The X2 and X3 groups experienced a change in obesity status after the SCBG administration. The SCBG group with doses of 900 mg and 1350 mg per 200 g BW per day had a preventive effect on obesity, which had no statistical difference from the healthy group. Meanwhile, the group of SCBG 450 mg per 200 g BW per day only had a slightly reduced Lee index, so they were unable to change the obesity status of the rats to normal (Table 2).

HFFD feeding in this study aimed to induce a fatty liver in rats. The excess consumption of carbohydrates (fructose and sucrose) and fat (fatty acids and cholesterol) plays a key role in the development and advancement of obesity-related NAFLD through the activation of the lipid metabolism pathway modulated on a high-fat diet **(Jarukamjorn et al., 2016)**.

The obesity status of the X2 and X3 groups had changed after the SCBG administration. The treatment group of SCBG 900 mg per 200 g BW per day and 1350 mg per 200 g BW per day provided a preventive effect of obesity and had no statistical difference from the healthy group. Meanwhile, the group of rats given SCBG 450 mg per 200 g BW did not reduce the Lee index. Garlic is a rich source of polyphenol compounds and is highly potent in inhibiting lipid peroxidation. Polyphenols have the potential to prevent obesity by inhibiting enzymes related to lipid metabolism, for example, pancreatic lipase, lipoprotein lipase, and glycerophosphate dehydrogenase, or by regulating lipid homeostasis, for example, by regulating lipogenesis and fatty acid oxidation **(Chen et al., 2014)**. In conclusion that black garlic has an antiobesity effect **(Chang, Wu and Hsu, 2015; Chang et al., 2017; Chen et al., 2014; Lee et al., 2011; Kim et al., 2011)**.

PAI-1 levels

PAI-1 level was measured once at the end of the study. The *post-hoc* LSD test showed significant differences in the PAI-1 levels in all groups of rats ($p = 0.001$). The PAI-1 levels were meaningfully and dose-dependently decrease by treatment of SCBG and also by simvastatin and metformin, respectively. It compared to the NAFLD group, without SCBG treated. However, when compared with the healthy group, the PAI-1 levels in the three treatment groups had not yet reached their PAI-1 levels (Table 3).

Table 1 Effect of single clove black garlic on body weight of rats modeled NAFLD.

Groups	Rats weight (g)					*P*
	Acclimatization	Conditioning	Administration	Δ1	Δ2	
Controls						
Healthy Rats	163.33 ±3.55	208.83 ±3.60^{a}	238.16 ±4.35^{a}	45.50 ±1.22^{a}	29.33 ±2.25	0.005
NAFLD Rats	164.83 ±2.56	242.50 ±2.26^{a}	305.16 ±2.56^{a}	77.67 ±0.81^{a}	62.66 ±1.63^{a}	0.004
Reference						
Simvastatin	164.00 ±3.84	241.50 ±3.73^{a}	271.33 ±3.38^{ab}	77.50 ±1.04^{a}	31.70 ±0.98^{b}	0.002
Metformin	163.33 ±2.80	240.16 ±3.43^{a}	271.30 ±3.40^{bc}	76.83 ±0.98^{a}	31.16 ±1.98^{b}	0.001
SCBG treated						
X1	166.33 ±4.13	244.00 ±4.43^{a}	284.33 ±4.63^{abcd}	77.66 ±1.03^{a}	40.33 ±1.81^{ac}	0.001
X2	165.17 ±2,32	242.33 ±2.58^{a}	272.66 ±3.14^{abe}	77.16 ±1.60^{a}	30.33 ±2.73^{ab}	0.061
X3	163.00 ±2.83	240.33 ±2.42^{a}	270.66 ±3.66^{abe}	77.33 ±0.81^{a}	30.33 ±1.50^{be}	0.002
p*	**0.538**	**0.001**	**0.005***	**0.001***	**0.001***	

Note: Values are expressed as mean ± SD of six rats. Healthy, normal control group; NAFLD, Nonalcoholic fatty liver disease control group without treatment; X1, SCBG 450 mg per 200 g BW; X2, 900 mg per 200 g BW; X3, 1350 mg per 200 g BW. Simvastatin and metformin were administrated at dose levels of 0.9 mg and 45 mg per 200 g BW, respectively. ***p**** = paired t-test; ***p*** = one-way ANOVA test ; ^{abcdef} – different notation shows a significant difference with post-hoc LSD test.

Table 2 Effect of single clove black garlic on obesity status of rats modeled NAFLD.

Groups	Obesity Status (Lee index)			*p*
	Conditioning	Administration	Δ	
Controls				
Healthy Rats	286.32 ±2.82^{a}	287.82 ±3.40	1.50 ±1.55	0.028
NAFLD Rats	319.30 ±5.73^{a}	332.52 ±3.21^{a}	13.22 ±6.75^{a}	0.028
Reference				
Simvastatin	323.80 ±5.62	290.55 ±3.27^{b}	-33.24 ±2.51^{a}	0.028
Metformin	320.19 ±7.86^{a}	287.34 ±7.12^{b}	-32.85 ±2.69^{ab}	0.028
SCBG treated				
X1	321.14 ±6.72^{a}	302.76 ±4.90^{abcd}	-18.38 ±3.68^{abcd}	0.028
X2	318.98 ±4.13^{a}	286.93 ±3.37^{be}	-32.04 ±1.04^{ab}	0.028
X3	318.67 ±8.94^{a}	286.93 ±3.37^{be}	-34.21 ±4.11^{abe}	0.028
p*	**0.009***	**0.001***	**0.001***	

Note: Values are expressed as mean ± SD of six rats. Healthy, normal control group; NAFLD, Nonalcoholic fatty liver disease control group without treatment; X1, SCBG 450 mg per 200 g BW; X2, 900 mg per 200 g BW; X3, 1350 mg per 200 g BW. Simvastatin and metformin were administrated at dose levels of 0.9 mg and 45 mg per 200 g BW, respectively. ***p*** = wilcoxon test ; ***p**** = Kruskal-Wallis test; ^{abcdef} – different notation shows a significant difference with Mann-Whitney test.

Table 3 Effect of single clove black garlic on the plasma biochemical parameters of rats modeled NAFLD.

Groups	Biochemical parameters				
	Hemostatis status (ng.ml^{-1})	Lipid profile (mg.dL^{-1})			
	PAI-1	TC	HDL-C	LDL-C	TG
Controls					
Healthy Rats	1.03 ±1.11	96.17 ±2.94	80.73 ±1.97	25.87 ±2.30	74.56 ±2.94
NAFLD Rats	30.86 ±1.26^{a}	219.93 ±4.80^{a}	24.47 ±2.32^{a}	81.83 ±2.17^{a}	142.44 ±4.75^{a}
Reference					
Simvastatin	4.02 ±3.32^{ab}	117.47 ±3.90^{ab}	64.54 ±2.98^{ab}	41.16 ±1.88^{ab}	95.45 ±2.12^{ab}
Metformin	2.98 ±1.68^{abc}	114.94 ±4.57^{ab}	66.78 ±3.09^{ab}	34.54 ±2.06^{ab}	94.78 ±2.92^{ab}
SCBG treated					
X1	6.84 ±0.22^{abcd}	171.01 ±4.50^{abcd}	36.05 ±3.43^{abcd}	65.78 ±3.57^{abcd}	118.21 ±2.66^{abcd}
X2	5.93 ±0.29^{abcde}	133.33 ±5.63^{abcde}	53.19 ±4.33^{abcde}	40.79 ±1.75^{abde}	106.56 ±3.32^{abcde}
X3	4.86 ±0.24^{abdef}	121.07 ±2.78^{abef}	61.35 ±2.32^{abdef}	33.21 ±2.82^{abcef}	98.39 ±1.95^{abef}
p	**0.001***	**0.001**	**0.001**	**0.001**	**0.001**

Note: Values are expressed as mean ± SD of six rats. Healthy, normal control group; NAFLD, Nonalcoholic fatty liver disease control group without treatment; X1, SCBG 450 mg per 200 g BW; X2, 900 mg per 200 g BW; X3, 1350 mg per 200 g BW. Simvastatin and metformin were administrated at dose levels of 0.9 mg and 45 mg per 200 g BW, respectively. ***p**** = One way ANOVA test; ***p*** = Kruskal-Wallis test; ^{abcdef} – different notation shows a significant difference with Mann-Whitney test.

Significant differences were noted in PAI-1 levels of all groups of rats (p = 0.001). The PAI-1 levels in the healthy group were significantly different from those in the NAFLD, simvastatin, metformin, X1, X2, and X3 groups. The SCBG dose of 1350 mg per 200 g BW more effectively improved the hemostasis status in NAFLD rats than 450 and 900 mg per 200 g BW respectively.

The consumption of HFFD appears to have contributed to NAFLD development and increased the risk of progression to NASH **(Jarukamjorn et al., 2016)**, promote an increase in hemostasis status, namely, PAI-1 level. In this study, X1, X2, and X3 groups had lower levels of PAI-1 than the NAFLD group (HFFD without SCBG administration). In this study, the biological substances of garlic and its processes, which are involved in the antithrombotic function, are unknown. A*llicin*, one of the main components in garlic, is known to have some biological activity, but it does not seem to promote a t-PA activity or reduce PAI-1 levels. Black garlic contains less *allicin* in the production process due to *alliinase* inactivation, so it is odorless. However, whether the *alliin* in the diet was converted to *allicin* after administered in rats and whether SCBG in our study had a specific antithrombotic activity are uncertain. As is known, garlic contains many physiological functions; the composition and amount of each element are responsible for various functions, which may differ between the types and methods of manufacture. It should be noted that the physiological function of garlic is not homogeneous and depends on the number of active components and their combinations in garlic processing **(Fukao et al., 2007)**. Therefore, which components contribute to the antithrombotic activity in NAFLD rats is uncertain. In short, garlic greatly reduces the coagulation activity, which would prevent the formation of blood clots in the thrombus-modeled pathological rats. These results may explain the benefit of garlic and its preparations for blood and blood vessels.

In vitro and in vivo studies have shown that garlic appears to have antiplatelet, fibrinolytic, and antithrombotic effects, in addition to its anti-inflammatory properties **(Chang et al., 2005; Teranishi et al., 2003)**. Garlic also reduced PAI-1 levels in obese subjects compared to the placebo group **(Szulińska et al., 2018)**. Another study showed that administration of Methanolic Extract of Black Garlic (MEBG) reduced levels of PAI-1 (p <0.05) in obese rats fed a high-fat diet **(Chen et al., 2014)**. Regarding the molecular regulation of decreased levels of PAI-1 and inhibition of adipogenesis in obese mice by black garlic supplementation through increased AMPK, forkhead box protein-01(FOXO1), Sirtuin-1(Sirt1), adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), perilipin, acyl-CoA-1 (ACO), carnitine palmitoyltransferase (CPT-1), and uncoupling protein-1(UCP1), thereby reducing lipogenesis, increasing lipolysis and fatty acid oxidation. The expression of cluster of differentiation-36 (CD36) levels decreased, resulting in less free fatty acid uptake in the adipose tissue. Also, black garlic increases adiponectin and downregulates PAI-1, resistin, tumor necrosis factor- α (TNF-α), and glucose transporter type-4 (GLUT4), thereby increasing insulin resistance in rats fed a high-fat diet **(Chen et al., 2014)**. Similarly, the downregulates PAI-1 mechanism of simvastatin and metformin **(Arruda de Faria et al., 2019; Mazza et al., 2012; Nascimbeni et al., 2016; Rodrigues, et al., 2019)**.

Lipid profiles

In this study, the mean of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and triglycerides (TG) levels for all groups were significantly different (p = 0.001). After administration SCBG, the X1, X2, and X3 groups have a significant decrease in triglyceride levels (p = 0.001) compared to the NAFLD group (Table 3).

Consumption of high-fat diet results in the production of acetyl-CoA and malonyl CoA in the liver. Increased levels of malonyl CoA, as fuel for TG synthesis, will inhibit the expression of CPT-1, a key enzyme for lipolysis, thereby reducing fatty acid oxidation. As shown in this study, administration of HFFD and black garlic increases CPT-1 mRNA expression; thus, more TG is broken down (degradation) to supply free fatty acids as an energy source, lowering plasma cholesterol concentrations associated with decreased HMG-CoA reductase expression and ACAT. SREBP-1c levels in the liver also activate the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase and acyl-CoA cholesterol acyltransferase (ACAT) enzymes, which regulate cholesterol synthesis and catalyze cholesterol esterification **(Ha, Ying and Kim, 2015)**.

Calorie intake from fat and fructose promotes excess fat deposits in the adipose tissue. Excessive intake of fat from food will lead to an increase in lipogenesis activity and production of free fatty acids, resulting in the mobilization of free fatty acids from the fatty tissue to the liver and binding glycerol to form triglycerides. Giving a high-fat diet also increased levels of LDL and cholesterol in the circulation, triggering HDL to transport cholesterol from the liver to the circulation (*reverse cholesterol transport*). HDL is esterified into cholesterol esters, which can be taken directly to the liver then directly excreted or exchanged with triglycerides from VLDL and chylomicrons. When cholesterol ester excess, HDL-rich triglyceride (HDL low density) is breakdown by hepatic lipase resulting in lower circulating levels of HDL **(Dissard et al., 2013; Wong et al., 2016)**.

The results showed that the mean of total cholesterol, HDL, LDL, and triglycerides levels for all groups were significantly different (p = 0.001). After SCBG administration, the X1, X2, and X3 groups had lower triglyceride levels (p = 0.001) compared to the NAFLD group. These results supported the study of **Seo et al. (2009)** on db/db model rats given black garlic had lower triglycerides, cholesterol, fasting blood glucose, blood glycated hemoglobin levels, and higher HDL serum compared with rats with no black garlic intervention.

The lipid-lowering mechanism of black garlic in plasma and the liver of rats is described in Ha et al.'s study.
They have measured the expression of liver SREBP-1c mRNA transcription factors' key lipid metabolism and the decreased expression of SREBP-1c in rats fed extract black garlic, resulting in a decrease in liver sterol regulator element-binding protein-1c (SREBP-1c), Acetyl-CoA

Table 4 Correlations between PAI-1 levels and lipid profiles.

Lipid profiles	PAI-1 levels	
	r	*p*
Total cholesterol	0.961	0.005
HDL	-0.968	0.001
LDL	0.871	0.006
Triglycerides	0.961	0.004

Note: r = Spearman correlations.

Figure 1 Natural Black Garlic® Lanang Premium.

carboxylase (ACC), fatty acid synthase (FAS), and G-6PDH mRNA levels, the target gene for SREBP-1c, indicating that the inhibition of the gene expression led to a decrease in the fat synthesis of the liver.

This is associated with a plasma TG concentration decrease. Similar results were reported: the methanolic extract Black Garlic (MEBG) administered in rats fed with a high-cholesterol diet inhibited lipid accumulation through *adenosine monophosphate-activated protein kinase* (AMPK) regulation and regulation of SREBP1c, ACC, and FAS **(Ha, Ying and Kim, 2015; Sobenin et al., 2019)**. Similarly, the lipid-lowering mechanism of simvastatin and metformin **(Arruda de Faria et al., 2019; Mazza et al., 2012; Nascimbeni et al., 2016; Rodrigues et al., 2019; Sobenin et al., 2019)**.

Black garlic and its constituent, *S-allyl-cysteine*, had antioxidant and hypolipidemic effects in rats with a high-fat diet **(Asdaq, 2015)**. Therefore, the high concentration of *S-allyl-cysteine* in black garlic extract induced blood lipid profile changes in *hepatic injury* rats **(Tran, Dam and Tram Le, 2018)**. These data support the results of this study in verifying the effect of single clove black garlic improving the lipid profile in NAFLD rats.

Correlations between PAI-1 levels and lipid profiles

There were strong correlations between PAI-1 levels and lipid profiles, with *r* value = 0.76–0.99. The correlations were positive except for HDL-C levels (p = 0.001; r = -0.968), which means when HDL-C levels decrease, then the PAI-1 levels increase (Table 4).

Decreased plasma HDL cholesterol is the most common dyslipidemia in obese subjects and this condition is caused by hypertriglyceridemia, even if the levels are low or without elevated triglycerides. Hypertriglyceridemia is accompanied by a small increase in solid LDL, is highly atherogenic. The high concentration of non-esterified fatty acid (NEFA) released will be metabolized by the liver to increase the synthesis of liver triglycerides and very-low-density lipoprotein (VLDL), thereby triggering insulin resistance. High circulating levels of VLDL reduce HDL concentrations and increase small solid LDL. Dyslipidemia is also accompanied by hyperactive platelets, hypercoagulability with increased factor VII, and hypofibrinolysis with increased PAI-1. High plasma triglyceride levels increase oxidative stress by increasing superoxide production. Increased NEFA from adipocytes increases tissue factor (TF) and PAI-1 levels and increases platelet aggregation; this condition causes thrombosis. Lipoprotein (a) levels in obesity are increased, especially when associated with hyperglycemia. Lipoprotein (a) has LDL-like properties and is structurally similar to plasminogen. It, therefore, inhibits the binding of plasminogen to endothelial cells and interferes with the generation of plasmin, both of which are atherogenic and thrombogenic. Prothrombin levels and levels of vitamin K-dependent coagulation factor are increased with hyperlipidemia **(Darvall et al., 2007)**.

Significant correlations between PAI-1 levels and lipid profiles were noted in this study. Similarly, where a significant positive correlation was seen between PAI-1 and triglyceride levels; on the other hand, PAI-1 and

HDL-C levels showed a negative correlation in nondiabetic obese patients and the control group **(Festa et al., 1999; Somodi et al., 2018)**. Besides, another study showed a strong and significant positive correlation between the most atherogenic LDL subfraction and the densest concentration of the PAI-1 plasma levels was also noted. This is the initial finding to evaluate the correlation between the *small dense* LDL and PAI-1 plasma levels in obese nondiabetic patients **(Somodi et al., 2018; Väisänen et al., 1997)**. These data support the association between LDL-C and PAI-1 levels in accelerating atherogenesis and increasing the cardiovascular risk of obese patients; however, our hypothesis needs to be verified by further investigation. However, the correlation between HDL and plasma PAI-1 levels are not well understood. Also, there have been few studies on the correlations of PAI-1 with the functional and structural properties of different lipoprotein fractions especially on rats modeled NAFLD.

We were not able to evaluate the long-term effects of SCBG administration on the hemostasis status and lipid profiles in rats modeled NAFLD, as our intervention lasted 4 weeks. Given these limitations and our findings, we believe that additional studies with longer intervention periods are warranted to determine the long-term benefits of SCBG consumption on the hemostasis status and lipid profiles in rats modeled NAFLD. However, further studies are needed to elucidate the underlying mechanisms.

CONCLUSION

The administration of Single Clove Black Garlic 450 mg; 900 mg, and 1350 mg per 200 gram BW for 4 weeks, respectively, affected the changes in body weight, PAI-1 levels, and lipid profile, compared to the healthy and NAFLD rats. The 1350 mg per 200 g BB of Single Clove Black Garlic is an effective dose that reduced PAI-1 levels and lipid profiles, which are equivalent to 15 grams per day when consumed by humans. More studies are needed in humans if Single Clove Black Garlic will be used as a food supplement in NAFLD.

REFERENCES

Abe, N., Kato, S., Tsuchida, T., Sugimoto, K., Saito, R., Verschuren, L., Kleemann, R., Oka, K. 2019. Longitudinal characterization of diet-induced genetic murine models of non-alcoholic steatohepatitis with metabolic, histological, and transcriptomic hallmarks of human patients. *Biology Open*, vol. 8, 11 p. https://doi.org/10.1242/bio.041251

Agarwal, K. C. 1996. Therapeutic actions of garlic constituents. *Medicinal Research Reviews*, vol. 16, no. 1, p. 111-124. https://doi.org/10.1002/(SICI)1098-1128(199601)16:1<111::AID-MED4>3.0.CO;2-5

Alkhouri, N., Tamimi, T. A. R., Yerian, L., Lopez, R., Zein, N. N., Feldstein, A. E. 2010. The Inflamed Liver and Atherosclerosis: A Link Between Histologic Severity of Nonalcoholic Fatty Liver Disease and Increased Cardiovascular Risk. *Digestive Diseases and Sciences*, vol. 55, p. 2644-2650. https://doi.org/10.1007/s10620-009-1075-y

Arruda de Faria, C., Zanette, D. L., Silva Jr., W. A., Ribeiro-Paes, J. T. 2019. PAI-1 inhibition by simvastatin as a positive adjuvant in cell therapy. *Molecular Biology Reports*, vol. 46, p. 1511-1517. https://doi.org/10.1007/s11033-018-4562-4

Asdaq, S. M. B. 2015. Antioxidant and Hypolipidemic Potential of Aged Garlic Extract and Its Constituent, S-Allyl Cysteine, in Rats. *Evidence-Based Complementary and Alternative Medicine*, 7 p. https://doi.org/10.1155/2015/328545

Atcha, Z., Rourke, C., Neo, A. H. P., Goh, C. W. H., Lim, J. S. K., Aw, C. C., Browne, E. R., Pemberton, D. J. 2010. Alternative Method of Oral Dosing for Rats. *Journal of the American Association for Laboratory Animal Science*, vol. 49, no. 3, p. 335-343.

Bae, S. E., Cho, S. Y., Won, Y. D., Lee, S. H., Park, H. J. 2014. Changes in S-Allyl cysteine contents and physicochemical properties of black garlic during heat treatment. *LWT - Food Science and Technology*, vol. 55, no. 1, p. 397-402. https://doi.org/10.1016/j.lwt.2013.05.006

Chang, W. T., Wu, C. H., Hsu C. L. 2015. Diallyl trisulphide inhibits adipogenesis in 3T3-L1 adipocytes through lipogenesis, fatty acid transport, and fatty acid oxidation pathways. *Journal of Functional Foods*, vol. 16, p. 414-422. https://doi.org/10.1016/j.jff.2015.05.002

Chang, W. T., Shiau, D. K., Cheng, M. C., Tseng, C. Y., Chen, C. S., Wu, M. F., Hsu, C. L.2017. Black Garlic Ameliorates Obesity Induced by a High-fat Diet in Rats. *Journal of Food and Nutrition Research*, vol. 5, no. 10, p. 736-741. https://doi.org/10.12691/jfnr-5-10-3

Chang, H. S., Yamato, O., Yamasaki, M., Maede, Y. 2005. Modulatory influence of sodium 2- propenyl thiosulfate from garlic on cyclooxygenase activity in canine platelets: Possible mechanism for the anti-aggregatory effect. *Prostaglandins, Leukotrienes and Essential Fatty Acids*,vol.72,no.5,p.351-355. https://doi.org/10.1016/j.plefa.2005.01.003

Chen, Y. C., Kao, T. H., Tseng, C. Y., Chang, W. T., Hsu, C. L. 2014. Methanolic extract of black garlic ameliorates diet-induced obesity via regulating adipogenesis, adipokine biosynthesis, and lipolysis. *Journal of Functional Foods*, vol. 9, p. 98-108. https://doi.org/10.1016/j.jff.2014.02.019

Crowther, J. R. 2009. *The ELISA Guidebook*. 2nd ed. New York, USA : Humana Press, 566 p. ISBN 978-1-60327-254-4. https://doi.org/10.1007/978-1-60327-254-4

Darvall, K. A. L., Sam, R. C., Silverman, S. H., Bradbury, A. W., Adam, D. J. 2007. Obesity and Thrombosis. *European Journal of Vascular et Endovascular Surgery*, vol.33,no.2,p.223-233. https://doi.org/10.1016/j.ejvs.2006.10.006

Dissard, R., Klein, J., Caubet, C., Breuil, B., Siwy, J., Hoffman, J., Sicard, L., Ducassé, L., Rascalou, S., Payre, B., Buléon, M., Mullen, W., Mischak, H., Tack, I., Bascands, J. L., Buffin-Meyer, B., Schanstra, J. P. 2013. Long Term Metabolic Syndrome Induced by a High Fat High Fructose Diet Leads to Minimal Renal Injury in C57BL/6 Mice. *PLoS One*, vol. 8, no. 1, 14 p. https://doi.org/10.1371/journal.pone.0076703

Fakhoury-Sayegh, N., Trak-Smayra, V., Khazzaka, A., Esseily, F., Obeid, O., Lahoud-Zouein, M., Younes, H. 2015. Characteristics of nonalcoholic fatty liver disease induced in wistar rats following four different diets. *Nutrition Research and Practice*, vol. 9, no. 4, p. 350-357. https://doi.org/10.4162/nrp.2015.9.4.350

Festa, A., D'Agostino, R., Mykkänen, L., Tracy, R., Howard, B. V., Haffner, S. M. 1999. Low-Density Lipoprotein Particle Size Is Inversely Related to Plasminogen Activator Inhibitor-1 Levels. The Insulin Resistance Atherosclerosis Study. *Arteriosclerosis,Thrombosis, and Vascular Biology*, vol. 19, no. 3, p. 605-610. https://doi.org/10.1161/01.ATV.19.3.605

Francque, S. M., van der Graaff, D., Kwanten, W. J. 2016. Non-alcoholic fatty liver disease and cardiovascular risk: Pathophysiological mechanisms and implications.

Journal of Hepatology, vol. 65, no. 2, p. 425-443. https://doi.org/10.1016/j.jhep.2016.04.005

Fukao, H., Yoshida, H., Tazawa, Y. I., Hada, T. 2007. Antithrombotic Effects of Odorless Garlic Powder Both *in Vitro* and *in Vivo*. *Bioscience, Biotechnology and Biochemistry*, vol. 71, no. 1, p. 84-90. https://doi.org/10.1271/bbb.60380

Ha, A. W., Ying, T., Kim, W. K. 2015. The effects of black garlic (*Allium satvium*) extracts on lipid metabolism in rats fed a high fat diet. *Nutrition Research and Practice*, vol. 9, no. 1, p. 30-6. https://doi.org/10.4162/nrp.2015.9.1.30

Hardinsyah, M. S., Supariasa, D. N. 2019. *Ilmu Gizi Teori dan Aplikasi (Nutritional Science Theory and Application)*. 4 ed., Indonesia : Medical Book Publisher EGC. (In Indonesian) ISBN 978-979-044-725-7.

Hatton, G. B., Yadav, V., Basit A. W., Merchant, H. A. 2015. Animal Farm: Considerations in Animal Gastrointestinal Physiology and Relevance to Drug Delivery in Humans. *Journal of Pharmaceutical Sciences*, vol. 104, no. 9, p. 2747-2776. https://doi.org/10.1002/jps.24365

Jarukamjorn, K., Jearapong, N., Pimson, C., Chatuphonprasert, W. 2016. A High-Fat, High-Fructose Diet Induces Antioxidant Imbalance and Increases the Risk and Progression of Nonalcoholic Fatty Liver Disease in Mice. *Scientifica*, 10 p. https://doi.org/10.1155/2016/5029414

Jung, Y. M., Lee, S. H., Lee, D. S., You, M. J., Chung, I. K., Cheon, W. H., Kwon, Y. S., Lee, Y. J., Ku, S. K. 2011. Fermented garlic protects diabetic, obese mice when fed a high-fat diet by antioxidant effects. *Nutrition Research*, vol. 31, no. 5, p. 387-396. https://doi.org/10.1016/j.nutres.2011.04.005

Kim, D., Choi, S. Y., Park, E. H., Lee, W., Kang , J. H., Kim, W., Kim, Y. J., Yoon, J. H., Jeong, S. H., Lee, D. H., Lee, H. S., Larson, J., Therneau, T. M., Kim, W. R. 2012. Nonalcoholic fatty liver disease is associated with coronary artery calcification. *Hepatology*, vol. 56, no. 2, p. 605-613. https://doi.org/10.1002/hep.25593

Kim, I., Kim, J. Y ., Hwang, Y. J., Hwang, K. A., Om, A. S., Kim, J. H., Cho, K. J. 2011. The beneficial effects of aged black garlic extract on obesity and hyperlipidemia in rats fed a high-fat diet. *Journal of Medicinal Plants. Research*, vol. 5, no. 14, p. 3159-3168.

Lee, S. J., Kim, R. J., Ryu, J. H., Shin, J. H., Kang, M. J., Kim, I. S., Sung, N. J. 2011. Effects of the Red Garlic Extract for Anti-Obesity and Hypolipidemic in Obese Rats Induced High Fat Diet. *Journal of Life Science*, vol. 21,no.2,p.211-220. https://doi.org/10.5352/JLS.2011.21.2.211

Mazza, A., Fruci, B., Garinis, G. A., Giuliano, S., Malaguarnera, R., Belfiore, A. 2012. The Role of Metformin in the Management of NAFLD. *Journal of Diabetes Research*, 13 p. https://doi.org/10.1155/2012/716404

McPherson, R. A., Pincus, M. R. 2016. *Henry's Clinical and Management by Laboratory Methods*. 23rd ed. Canada, US : Elsevier, 433 p. ISBN 978-0323413152.

Nascimbeni, F., Aron-Wisnewsky, J., Pais, R., Tordjman, J., Poitou, C., Charlotte, F., Bedossa, P., Ponyard, T., Clément, K., Ratziu, V. 2016. Statins, Antidiabetic Medications And Liver Histology In Patients With Diabetes With Non-alcoholic Fatty Liver Disease. *BMJ Open Gastroenterology*, vol. 3, no. 1, 9 p. https://doi.org/10.1136/bmjgast-2015-000075

Oligschlaeger, Y., Shiri-Sverdlov, R. 2020. NAFLD Preclinical Models: More than a Handful, Less of a Concern? *Biomedicines*, vol. 8, no. 2, 23 p. https://doi.org/10.3390/biomedicines8020028

Rodrigues, G., Moreira, A. J., Bona, S., Schemitt, E., Marroni, C. A., Di Naso F. C., Dias, A. S., Pires, T. R., Picada, J. N., Marroni, N. P. 2019. Simvastatin Reduces Hepatic Oxidative Stress And Endoplasmic Reticulum Stress In Nonalcoholic Steatohepatitis Experimental Model. *Oxidative Medicine And Cellular Longevity*, 10 p. https://doi.org/10.1155/2019/3201873

Rivlin, R. S. 2001. Historical Perspective on the Use of Garlic. *The Journal of Nutrition*, vol. 131, no. 3, p. 951S-954S. https://doi.org/10.1093/jn/131.3.951S

Sasaki, J., Lu, C., Machiya, E., Tanahashi, M., Hamada, K. 2007. Processed Black Garlic (Allium sativum) Extracts Enhance Anti-Tumor Potency against Mouse Tumors. *Medicinal and Aromatic Plant Science and Biotechnology*, vol. 1, no. 2, p. 278-281.

Sato, E., Kohno, M., Niwano, Y. 2006. Increased level of tetrahydro-b-carbolinen derivatives in short-term fermented garlic, *Plant Foods for Human Nutrition*, vol. 61, no. 4, p. 175-178. https://doi.org/10.1007/s11130-006-0028-2

Seo, Y. J., Gweon, O. C., Im, J., Lee, Y. M., Kang, M. J., Kim, J. I. 2009. Effect of Garlic and Aged Black Garlic on Hyperglycemia and Dyslipidemia in Animal Model of Type 2 Diabetes Mellitus. *Journal of Food Science and Nutrition*, vol. 14, no. 1, p. 1-7. https://doi.org/10.3746/jfn.2009.14.1.001

Sobenin, I. A., Myasoedova, V. A., Iltchuk, M. I., Zhang, D. W., Orekhov, A. N. 2019. Therapeutic effects of garlic in cardiovascular atherosclerotic disease. *Chinese Journal of Natural Medicines*, vol. 17, no. 10, p. 721-728.https://doi.org/10.1016/S1875-5364(19)30088-3

Somodi, S., Seres, I., Lőrincz, H., Harangi, M., Fülöp, P., Paragh, G. 2018. Plasminogen Activator Inhibitor-1 Level Correlates with Lipoprotein Subfractions in Obese Nondiabetic Subjects. *International Journal of Endocrinology*,vol.2018,9p. https://doi.org/10.1155/2018/9596054

Szulińska, M., Kręgielska-Narożna, M., Świątek, J., Styś, P., Kuźnar-Kamińska, B., Jakubowski, H., Walkowiak, J., Bogdański, P. 2018. Garlic extract favorably modifies markers of endothelial function in obese patients – randomized double blind placebo-controlled nutritional intervention. *Biomedicine and Pharmacotherapy*, vol. 102, p. 792-797. https://doi.org/10.1016/j.biopha.2018.03.131

Teranishi, K., Apitz-Castro, R., Robson, S. C., Romano, E., Cooper, K. C. 2003. Inhibition of baboon platelet aggregation in vitro and in vivo by the garlic derivative, ajoene. *Xenotransplantation*, vol. 10, no. 4, p. 374-379. https://doi.org/10.1034/j.1399-3089.2003.02068.x

Toledano Medina, M. Á., Merinas-Amo, T., Fernández-Bedmar, Z., Font, R., del Río-Celestino, M., Pérez-Aparicio, J., Moreno-Ortega, A., Alonso-Moraga, Á., Moreno-Rojas, R. 2019. Physicochemical Characterization and Biological Activities of Black and White Garlic: In Vivo and In Vitro Assays. *Foods*, vol. 8, no. 6, 18 p.https://doi.org/10.3390/foods8060220

Tran, G. B., Dam, S. M., Tram Le, N. T. 2018. Amelioration of Single Clove Black Garlic Aqueous Extract on Dyslipidemia and Hepatitis in Chronic Carbon Tetrachloride Intoxicated Swiss Albino Mice. *International Journal of Hepatology*, vol. 2018, 9 p. https://doi.org/10.1155/2018/9383950

Tomita, K., Teratani, T., Suzuki, T., Shimizu, M., Sato, H., Narimatsu, K., Okada, Y., Kurihara, C., Irie, R., Yokoyama, H., Shimamura, K., Usui, S., Ebinuma, H., Saito, H., Watanabe, C., Komoto, S., Kawaguchi, A., Nagao, S., Sugiyama, K., Hokari, R., Kanai, T., Miura, S., Hibi, T. 2014. Free cholesterol accumulation in hepatic stellate cells: Mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice. *Hepatology*, vol. 59, no. 1, p. 154-169. https://doi.org/10.1002/hep.26604

Väisänen, S., Baumstark, M. W., Penttilä, I., Bouchard, C., Halonen, P., Rankinen, T., Berg, A., Rauramaa, R. 1997. Small, Dense LDL Particle Concentration Correlates with Plasminogen Activator Inhibitor Type-1 (PAI-1) Activity. *Thrombosis and Haemostasis*, vol. 78, no. 6, p. 1495-1499. https://doi.org/10.1055/s-0038-1665440

Wolforth, J. B. 2000. Methods of Blood Collection in The Mouse. *Lab Animal*, vol. 29, no. 10, p. 47-53.

Wong, S. K., Chin, K. Y., Suhaimi, F. H., Fairus, A., Ima-Nirwana, S. 2016. Animal models of metabolic syndrome: a review. *Nutrition and Metabolism*, vol. 13, 12 p. https://doi.org/10.1055/s-0038-1665440

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The authors declare no conflict of interest.

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