The Effects of Rice Bran Cereals on Total Cholesterol, Malondialdehyde (MDA) Levels and Histopathology Description of Aortic Organ on Mice Model of Hypercholesterolemia

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ABSTRACT

Rice bran cereals could used as the alternative treatment on hypercholesterolemia condition. Tocopherol as an antioxidant and dietary fiber as cellulose, hemicellulose, and lignin which contained in rice bran cereals have the potential to decrease total cholesterol and malondialdehyde levels. The aims of this study are to determine the role of rice bran cereals to decrease total cholesterol, malondialdehyde levels and improve the histopathology of the aortic organ due to hypercholesterolemia. This study used rats (Rattus Norvegicus) as animals model which were divided into 5 groups: control group (healthy rats), hypercholesterolemia group (sick rat) and 3 therapy groups with variation dose respectively 270, 540 and 810 mg/kg body weight. Data analysis for total cholesterol and malondialdehyde levels used one-way ANOVA with $\alpha = 0.05$ with Tukey test as follow-up test. The results of statistical analysis proved that rice bran cereals with dose of 810 mg/kg body weight affect for the changes of total cholesterol and malondialdehyde levels which are 21.72% and 50.88%, respectively. In addition, results showed that rice bran cereals therapy could significantly influence ($p = 0.05$) decrease of total cholesterol, malondialdehyde levels and improve the histopathology of aortic organ in hypercholesterolemic rats.

Key word: hypercholesterolemia, rice bran cereal, total cholesterol level, MDA level, histopathology aorta

INTRODUCTION

Cholesterol is a group of lipids important for various metabolic functions such as stimulating adrenal hormone formation, the formation of sex hormones, bile acids, and vitamin D [1]. Cholesterol becomes dangerous when the levels are excessive in the body that exceeds the normal limit <200 mg/dL, and causing various metabolic disorders such as atherosclerosis, coronary heart, hypertension and gallstone formation [1, 2]. One of the factors causing high cholesterol levels or hypercholesterolemia is consuming foods with high cholesterol and unsaturated fatty acids content. Foods that contains high cholesterol are foods derived from animals such as meat, liver, brain and egg yolk because cholesterol is typical of animal metabolism products [1]. High cholesterol levels in the body can increase the production of free radicals such as reactive oxygen species (ROS) that cause stress oxidative as the effect of unbalanced amounts of free radical and antioxidants in the body [3]. Moreover, free radical will interact with free fatty acids (polyunsaturated fatty acids or PUFA) causing lipid peroxidation. The side result of lipid peroxidation is several kinds of aldehydes compounds.
such as malondialdehyde (MDA) which toxic to the cell. Increased levels of malondialdehyde is an indicator of lipid peroxidation [4].

Stress oxidative and lipid peroxidation may contribute to endothelium cell injury that are fundamental to the maintenance of tissue integrity and cause the dysfunction of blood vessels [5]. The consequence for the endothelial cell dysfunction may lead to the accumulation of LDL cholesterol in the blood vessels and occurred the oxidation of LDL cholesterol. Moreover, it will enhance the Reaction Oxygen Species (ROS) or free radical production in the aorta that give rise to the acute inflammation and damage the arterial wall [6].

Rice bran is a natural ingredient that contains several active substances such as tocopherol, tocotrienols, oryzanol, β-cholesterol, β-glucan which can lower blood cholesterol levels [7,8]. Tocopherol in rice bran functioned as an antioxidant suppressing lipid peroxidation by reaction with peroxyl lipid radicals. Naturally, Vitamin E is mostly shaped D-α-tocopherol that known as an antioxidant to maintain the integrity of cell membranes and serves as scavenger of free radicals and lipid peroxidation [9]. Vitamin E in bran oil can inhibit lipid peroxidation by free radicals, have a role as antioxidants and scavengers for free radicals that effectively protect lipids and phospholipids in the membrane [10,11].

Dietary fiber contained in rice bran cereals, for instance, cellulose, hemicellulose, and lignin, can improve hypercholesterolemia condition. The dietary fibers bind cholesterol in the body and remove it through the feces, as a result lower cholesterol levels in the body. The content of dietary fiber in rice bran can inhibit the absorption of cholesterol in small intestine and cholesterol synthesis in the liver [7].

According to Hernawati et al. [12], 57 % of dietary fiber supplement in rice bran received to the hypercholesterolemia rats was able to reduce their cholesterol levels in the liver as high as 57.76%. This supplement level was equivalent to 14.30% of the insoluble dietary fiber that has the highest impact to decreased the cholesterol in the liver.

This research was focused on the potency of rice bran cereals to hypercholesterolemia rat by investigating the total cholesterol in the serum, malondialdehyde levels and histopathology description of the aortic organ. Moreover, tocopherol levels contained in the rice bran that plays important role as antioxidant was measured in order to investigate its inhibition toward lipid peroxidation and stress oxidative in the body[13].

EXPERIMENT

Chemicals and instrumentation

The chemicals used in this study were rice bran cereals (Anugrah), ethanol was purchased from Smart Lab Indonesia, pure vitamin E (alpha-tocopherol) from Tokyo Chemical Industry (TCI), phosphate buffer saline (Merck) pH 7.4, aquadest (Hydrobatt), alcohol 70% (OneMed), ethanol 99.8% (SmartLab), NaCl-physiological, HCl, Na-Thio, TCA (Trichloroethanoic acid), 1,1-diphenyl-2-picryl (DPPH) was obtained from Laboratory of Quality and Safety of Food at Agricultural Product Technology Department, Faculty of Agricultural Technology, University of Brawijaya with the concentration of 0.2 mM in 2 mL, cholic acid 98% from Tokyo Chemical Industry (TCI), and MDA standard solution.

The tools used in this study were spectrophotometer UV-Vis (Genesys 10), Easy Touch GCU, blood lancet (OneMed), vortex (Thermoline), vacutainer Non-EDTA and microscope (Olympus BX51).

The animals used in this study were rats (Rattus Norvegicus), male, Wistar strain. Age of the experimental animals is 10–12 weeks with 150–250 grams body weight. The use and all treatment procedures are given to the experimental animals have obtained ethical consent approval from the UB Research Ethics Committee number 946-KEP-UB. One week before the
experiment, rats were acclimated. This study used a complete randomized Design, rats were divided into five treatments with four replications.

Procedure reaction

**Antioxidant Activity Test for Rice Bran Cereals**

The method used for the antioxidant activity of rice bran cereals was conducted by using hydrogen radical 1,1-diphenyl-2-picryl (DPPH) and measurements were carried out using UV-Vis spectrophotometer. Quantitative calculations for antioxidant activity are expressed by IC$_{50}$ (inhibition concentration). IC$_{50}$ values can be obtained from a linear regression of the concentration (mg/mL) versus absorbance value.

**Analysis of Vitamin E content in the rice bran cereals using UV-Vis spectrophotometer**

Standard solution 1000 ppm prepared by dissolving 0.1053 mL of pure vitamin E in 100 mL of ethanol. Furthermore, standard solutions with concentrations of 100, 80, 60, 40, and 20 ppm were prepared. The absorbance of standard solutions was measured using a UV-Vis spectrophotometer (Genesys 10) at a wavelength of 295 nm. Next, the graph of the relationship between absorbance and concentration was plotted, thus obtain linear regression equation $y = ax + b$. Rice bran cereals was measured and the absorbance value obtained was used to calculate the concentrations of tocopherol present in the sample by using the linear regression equation obtained in making the calibration curve.

**Induction of Hypercholesterolemia to the Rats**

Rats were divided into 5 groups: negative control, positive control, and 3 groups of therapy with different doses, which were 270, 540 and 810 mg/kg body weight, respectively. Rats were fed a diet for inducing hypercholesterolemia, except in the negative control group, all treatments for the rats were for 14 days. The composition for hypercholesterolemia diet were 0.02 gr cholic acid, 1 gr of quail eggs, 0.24 mL of waste cooking oil, and 10% of goat fats. The diet was mixed with 3 mL distilled water, and heated at 100 °C. One hour after induction of the diet, the rats were given 16.78 g/tail of standard feed.

**Preparation of Rice Bran Cereals**

The rice bran cereal was weighed and mixed with warm water, then was given to the rats by induction into its stomach. The first therapy group received 270 mg/kg body weight, the second group 540 mg/kg body weight, and the third group 810 mg/kg body weight.

**Total Cholesterol Testing**

Total cholesterol analysis on rats was performed on blood serum. Blood taken from the heart and placed in the vacutainer Non-EDTA with position slanted 45°C. After blood coagulated, it was centrifuged at 5000 rpm for 15 min. The formed serum is separated from the precipitate of blood cells by using a pipette and accommodated in a micro tube. Total cholesterol analysis was tested using the kit from ABX PENTRA CP CHOLESTEROL (HORIBA) with CHOD-PAP (cholesterol oxidase-phenol aminophenazone) method.
Malondialdehyde Analysis for Hypercholesterolemia Rats

The rats aortic was taken and placed into microtube after added 500 mL aquadest, 250 μL TCA 10%, 250 μL HCl 1 N and 250 μL Na-Thio 1.34%. The mixture was homogenized and centrifuged for 15 min at 550 rpm. The supernatant was incubated at 100°C for 30 minutes. Samples were measured with spectrophotometer UV-Vis at 530 nm.

Histopathology Analysis of Aortic Organ

The aortic organ was taken and washed using 0.9% physiological NaCl, then prepared with fixation, dehydration, clearing, embedding and pasting in the object glass. Preparations were stained with hematoxylin-eosin (HE) and observed on the Olympus BX51 microscope with a magnification of 400 times.

Data Analysis

Data obtained in the study were analyzed using SPSS 20.0 software. Analysis of cholesterol and malondialdehyde levels was conducted using ANOVA and continued with TUKEY test, with a significance level of p <0.05.

RESULT AND DISCUSSION

Antioxidant Activity Test

The results of this research showed that antioxidant activity of rice bran cereals had IC₅₀ value of 75.18 μL/mL, this results revealed that the antioxidants contained in the sample (rice bran cereal) appertain to the strong category. IC₅₀ values range of 151-200 mg/mL are included in the low category, 101-150 mg/mL is medium, 50-100 mg/mL is categorized as strong and below from 50 mg/mL specifically indicates that the sample compound contains very powerful antioxidants [14,15]. It suitable with Wulandari et al. [16] that rice bran extracts of red rice reduced free radicals by using DPPH reagent. The antioxidant activity of rice bran and tomato juice was also examined by Damayanti et al. [14], and showed that antioxidant activity in the rice bran was higher than tomato juice, which were 83.89% and 60.74%, respectively.

Analysis of Vitamin E

Vitamin E is natural antioxidant which comprises alpha tocopherol as the most active form. The determination of alpha tocopherol in food was performed using UV spectrophotometric method at 295 nm based on the reported research with minor modification [17,18]. Tocopherol has chromophore groups, therefore is able to absorb radiation at the wavelength of UV.

The analysis result of the sample showed that a 50 mg rice bran cereal contained 24.26% of tocopherol. Buettner revealed that the lower reduction potential of tocopherol (500 mV) than poly unsaturated fatty acid (PUFA) (600 mV) was capable of attacking an electron from PUFA to prevent the formation of lipid peroxidation[19]. Lilik Maslachah [20] reported that vitamin E was effectively reacted with the oxygen radical faster 5 x 10⁴ than poly unsaturated fatty acid (PUFA), therefore it can be a potent antioxidant to inhibit the auto-oxidation of PUFA.

Decrease of Total Cholesterol Level in the Hypercholesterolemia Rats

The rice bran cereals were given to determine the effect of rice bran cereals on total cholesterol and malondialdehyde levels on the model of hypercholesterolemic rats. The variation of the dose which used is 270, 540, and 810 mg/kg body weight. The results can be seen in Table 1.
Table 1. Total Cholesterol Levels in Hypercholesterolemia Rats.

<table>
<thead>
<tr>
<th>Category</th>
<th>Total cholesterol (mg/dL)</th>
<th>Increase of Total Cholesterol (%)</th>
<th>Decrease of Total Cholesterol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Controls</td>
<td>173.50 ± 3.697</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive Controls</td>
<td>247.50 ± 2.887</td>
<td>42.65</td>
<td>-</td>
</tr>
<tr>
<td>Therapy 270 mg/kg body weight</td>
<td>229.75 ± 2.500</td>
<td>-</td>
<td>7.170</td>
</tr>
<tr>
<td>Therapy 540 mg/kg body weight</td>
<td>204.75 ± 2.909</td>
<td>-</td>
<td>17.27</td>
</tr>
<tr>
<td>Therapy 810 mg/kg body weight</td>
<td>193.75 ± 4.574</td>
<td>-</td>
<td>21.72</td>
</tr>
</tbody>
</table>

Total cholesterol in the negative control group was the lowest compared to the other four groups at the range of 173.50 ± 3.697 mg/dL. This value was categorized as the normal range for cholesterol in blood plasma, i.e. <200 mg/dL. A positive control group that received high cholesterol diet have the highest total cholesterol levels among all groups, and exceeded the normal range at 247.50 ± 2.887 mg/dL. This indicates that rats in this group have suffered hypercholesterolemia. The content of unsaturated fatty acids with the high level on the given diet triggered the lipid profiles changes. Gani [21] reported that a diet high cholesterol can trigger an increase of cholesterol levels in blood plasma.

The total cholesterol contained on quail eggs in the diet used was 2139.17 mg/100 g [22]. Used cooking oil is also known to contain unsaturated fatty acids that can increase blood cholesterol, this in accordance with research of Bogoriani and Ketut that stated waste cooking oil can increase cholesterol levels of rats higher than were compared to coconut oil, bulk oil and pork oil (at 86.20 mg/dL) [23]. Used cooking oil is cooking oil that has damaged because it is used repeatedly, the content of saturated fatty acids in cooking oil turned into unsaturated fatty acids when heated over and over. The rats were fed with waste cooking oil of 2.5 mL/200 g body weight for 14 days experienced changes in the lipid profiles, namely increased of total cholesterol, LDL and triglyceride levels and decreased of HDL levels [24]. The addition of cholic acid also contributes to the increase of plasma cholesterol levels. Although cholic acids are generally have more effect on plaque formation but indirectly have an effect on increasing cholesterol levels. The diet atherogenic which inducted to rats in two months cannot increase its cholesterol levels, even cannot form the foam cell if there is no addition of cholic acid [25]. The last component used as diet in this study was goat fat. The cholesterol content found in goat fat is 3.2 mg/g, hence, it causes heart disease and stroke [26,27].

The synthesis of endogenous cholesterol by lysosomes in the liver is partially regulated by cholesterol derived from food, therefore, food is important factor that determining plasma cholesterol levels. The high intake of cholesterol continuously will be the factor causing increased of total cholesterol and LDL cholesterol, an effect of not being compensated by HDL cholesterol to be transported back to the liver [1]. This condition will effect for hypercholesterolemia disorder. Hypercholesterolemia will be resulting the physical altered of the cell membrane, which may facilitate the leakage of the ROS (reactive oxygen species) from the mitochondrial electron system [28]. Mitochondria produce significant amounts of cellular reactive oxidant species (ROS) through the aberrant of O$_2$ reaction during electron transport, electrons escape to react with O$_2$ resulting ROS, the overproduction of ROS in mitochondria is associated with early atherosclerosis and hypercholesterolemia [29]. Besides it, ROS in the body as the effect of hypercholesterolemia condition is from the bile acid synthesis. The exceed of cholesterol in the body will be synthesis be bile acids whit the oxygen (O$_2$), NADPH, and cytochrome P-45 as catalysator [1]. The by-products of bile acid synthesis are free radicals...
(ROS) which are resulting from the reaction of \( \text{O}_2 \) and NADPH produce superoxide anions. The superoxide anions (\( \text{O}_2^- \)) are less reactive and destructive but can react to form the hydroxyl radical (\( \text{OH}^- \)). In human cells superoxide is quickly transformed into hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), then further reduction of \( \text{H}_2\text{O}_2 \) resulting hydroxyl radicals (\( \text{OH}^- \)) which highly reactive [30]. Free radicals attack and destroy major compounds capable of maintaining cell integrity such as fatty acids, proteins and DNA as carriers of cell genetic code, the result is to produce various degenerative diseases such as atherosclerosis and coronary heart disease [1,31].

Group of rice bran cereals therapy with variation dose 270, 540, 810 mg/kg body weigh decreased the total cholesterol levels, at 229.75 ± 2.50, 204.75 ± 2.91, 193.75 ± 4.57, respectively. The results show that rice bran cereals improved hypercholesterolemia disorder. This is in accordance with previous study that showed the addition of rice bran supplements in the diet decreased body weights and improved lipid profiles of male mice that have hypercholesterolemia disorder [30]. Wahyuningrum and Zubaidah also found that the addition of rice bran in red mold rice significantly decreased lipid profiles of hypercholesterolemia rats than red mold rice without the addition of rice bran and red mold rice obtained from the market [32]. Rice bran also has been found to reduce cholesterol levels in blood plasma and in liver significantly [33]. Changes of total cholesterol in groups of rats treated with rice bran cereals may be caused by antioxidants content in the rice bran cereals. These antioxidants are in the form of tocopherol (Vitamin E) which improves lipid profiles in hypercholesterolemic rats. The tocopherol (TocOH) as antioxidant able to maintain the condition of fatty acids, thus not easy to be damaged. The tocopherol will donate hydrogen (H) ions which change the reactivity of peroxyl radicals be less reactive by forming a tocopherol radical (TocO·). In addition, the fiber content in the rice bran can reduce cholesterol levels in the body by binding fatty acids, cholesterol and bile salts and then released through the feces. The fiber will be postponing absorption of foods include carbohydrate in the intestine thus the postprandial glucose levels in the body low and causing the secrete of insulin hormone decreased. The low insulin levels caused the inhibited role of enzyme HMG-CoA reductase which needed in synthesis cholesterol, thus the cholesterol synthesis and cholesterol levels in the body to be decreased [5].

Decrease in MDA Level After Administration with Bran Cereal

The results of MDA concentration of aortic organs on hypercholesterolemia rats model can be seen in Table 2. The negative control group had the lowest MDA levels among other groups at 0.0851± 0.037 \( \mu \text{g/mL} \). This value used as the standard value to determine the increase and decrease of MDA levels of rats after administration of hyper cholesterol diet. In contrast, MDA levels on positive group shows the highest number at 0.2103±0.027 \( \mu \text{g/mL} \) compared to all groups.
Table 2. Malondialdehyde Levels on Aortic of Hypercholesterolemic Rats

<table>
<thead>
<tr>
<th>Category</th>
<th>Average MDA Level (μg/mL)</th>
<th>Increased of MDA Levels (%)</th>
<th>Decrease of MDA Levels (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Controls</td>
<td>0.0851 ± 0.037</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive Controls</td>
<td>0.2103 ± 0.027</td>
<td>147.121</td>
<td>-</td>
</tr>
<tr>
<td>Therapy 270 mg/kg body weight</td>
<td>0.1815 ± 0.024</td>
<td>-</td>
<td>13.70</td>
</tr>
<tr>
<td>Therapy 540 mg/kg body weight</td>
<td>0.1189 ± 0.030</td>
<td>-</td>
<td>43.46</td>
</tr>
<tr>
<td>Therapy 810 mg/kg body weight</td>
<td>0.1033 ± 0.049</td>
<td>-</td>
<td>50.88</td>
</tr>
</tbody>
</table>

The increase of MDA levels caused by the increased cholesterol levels after the induction of diet high cholesterol. High cholesterol levels in the body or hypercholesterolemia condition will increase levels of ROS and oxidative stress. ROS may react with a variety of biomolecules, including lipids, carbohydrates, proteins, nucleic acids, and macromolecules of connective tissue by interfering with cell function while oxidative stress is known to be a component which causing of molecular and cellular tissue damage [32,33]. Free radical (ROS) will react with lipid and result lipid peroxidation. The increased of lipid peroxidation thought to be a consequence of oxidative stress which occurs when the amount of ROS as the prooxidant in the body does not balance (higher) with the antioxidant [35,36]. The side effect of lipid peroxidation is an aldehyde compound like malondialdehyde (MDA) which used as the biomarker from lipid peroxidation and to describe the levels of stress oxidative [37].

The average MDA levels of the hypercholesterolemic rats group that received treatments with rice bran cereals in doses of 270, 540 and 810 mg/kg body weights decreased to 0.1815 ± 0.024, 0.1189 ± 0.030, 0.1033 ± 0.049 μg/mL, respectively. The average value of MDA levels from these three groups was lower than the average MDA levels in the positive control group. Decreased of MDA levels after therapy with rice bran cereals is caused by the content of antioxidants that contained in rice bran cereals. The antioxidant content that contained in rice bran cereals used as therapy can reduce the number of free radicals that increased in hypercholesterolemia conditions. Alpha-tocopherol functioned as antioxidant plays a pivotal role as a chain-breaking antioxidant. It also convert the tocopherol into a new radical, tocopherol-O’. This radical is less reactive and unable to attack adjacent fatty acid side chain, therefore, the chain reaction is stopped and the radical reaction is decreasing. This phenomenon was able to minimize the consequence of lipid peroxidation and oxidative stress conditions, thus MDA levels also getting decreased [38]. The same report was conducted by Agnestiansyah, which investigated D-alfa-tocopherol (vitamin E) with doses of 100, 200 and 300 mg/kg body weight may decrease the levels of MDA of these diabetic rats (Rattus Norvegicus) with MLD-STZ induction [7].

The effect of rice bran cereals on the histopathology of aortic organ

Histopathology in aortic organ to investigate the influence of rice bran cereals with therapy dose of 270, 540, and 810 mg/kg body weight compared to negative and positive control was depicted in Figure 1. Negative control groups (healthy rats) revealed that there is no damage in the tunica intima or outer layer of epithelial cell (Figure 1A). Meanwhile, the positive control groups (sick rats) showed the deformation and damage of endothelial cell (Figure 1 B). It indicated by the irregular form of tunica intima and fatty in the tunica media.
A (Negative Control)  B (Positive Control)

C (270 mg/kg body weight)  D (540 mg/kg body weight)
Figure 1. Histopathology of aortic organ of hypercholesterolemia rats using Hematoxylin-Eosin Staining (HE A) with magnification of 400x. (A) negative control group, (B) positive control group (hypercholesterolemia rats), (C) therapy dose of 270 mg/kg body weight, (D) therapy dose of 540 mg/kg body weight, (E) therapy dose of 810 mg/kg body weight. The structure of aorta consists of Tunica Intima (TI), Tunica Media (TM), Tunica Adventitia (TA), and Lipid (L). Normal epithelial cell (yellow arrow), damaged epithelial cell (red arrow).

Endothelial cell injury in Figure B caused by hypercholesterolemia can enhance the LDL cholesterol level in the blood and accumulation of free radical in the blood plasma. This phenomena can oxidize LDL in the plasma to form LDL oxidation (ox-LDL) that lead to the inflammation reaction that expressed by neutrophil [6]. Neutrophil inhibit the infection by releasing the prostaglandin. Prostaglandin generation may responsible to the vasodilatation and permeability of blood vessel therefore monocyte across the vessel wall and differentiate to macrophage which further contribute to LDL oxidation. Ox-LDL is taken up by macrophages via scavenger receptors and across to the tunica media and tunica adventitia that caused lipid peroxidation in the aorta. Oxidized LDL was shown to promote inflammatory responses including the production and secretion of TNF-α, a proinflammatory cytokine (4,9). Through the inflammatory cascade, TNF-α stimulates the production of adhesion molecules on the endothelium of the aorta [38].

The deformation of endothelial cell caused by hypercholesterolemia was improved after treated with therapy using rice bran cereal with a doses of 270, 540, and 810 mg/kg body weight, respectively. Rice bran cereal has antioxidant for inhibiting the lipid peroxidation and reduce the LDL level therefore the oxidized LDL decreased and capable of tissue refinement [7].
CONCLUSION
The therapy of rice bran cereals can improve the histology of aortic organ, reduce total cholesterol levels on the blood serum and malondialdehyde levels in aortic organs from rats induced high cholesterol diet significantly ($p < 0.05$). The fiber in rice bran cereals can bind the cholesterol, bile acid, and fat on the body and secrete it through feces, while the tocopherol (vitamin E) will be stopped the peroxidation of lipid and lowered the oxidative stress that effect to the decreased of total cholesterol and MDA levels. The decrease in total cholesterol and MDA levels was highest at 810 mg/kg body weight, it is 193.75 ± 4.574 or 21.72% and 0.1033 ± 0.049 μg/mL or 43.46%.

REFERENCES