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Original Research Article

Sorghum Tempeh on Cholesterol Levels and Histopathology of Aorta in High-Fat Diet-Induced Rat Model

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Abstract

Background: Hypercholesterolemia is strongly linked to increased mortality rates and reduced productivity. It is also a major risk factor for the development of cardiovascular disease. Sorghum is recognized for its bioactive components and anti-cholesterol characteristics. Sorghum tempeh involves fermentation, resulting in increased fiber content and improved protein digestibility as compared to white sorghum.

Objective: This study examined the effects of sorghum tempeh on cholesterol levels and the histopathology of the aorta in rats fed a high-fat diet.

Methods: A total of 24 male 8-weeks-old Sprague Dawley rats were randomly divided into four groups: a standard diet group (SD), a high-fat diet control group (FD), a high-fat diet group supplemented with sorghum tempeh dose of 0.75 g/200g of BW (T1), and a high-fat diet group supplemented with sorghum tempeh dose of 1.50 g/200g of BW (T2). Cholesterol levels were measured using the total cholesterol ELISA method. After four weeks of intervention, the histopathology of aorta tissue was analyzed using Hematoxylin-Eosin staining.

Results: Serum total cholesterol levels in the T1 and T2 groups had significant differences ($p<0.05$) against the SD and FD groups. The decreased mean changes of the before-after intervention were observed in the T1 group (-93.195±5.920) and the T2 group (-121.143±4.276), while the mean effect size was observed ($\eta^2 = 0.996$). Histopathological analysis of aortic tissues from rats in the high-fat diet with sorghum tempeh group revealed improved condition of aortic tissues and reduction of foam cells.

Conclusion: Administering sorghum tempeh in the diet can significantly impact changes in body weight and cholesterol levels, with a potential effect of over 90%. The recommended dose of sorghum tempeh is 0.75 g per 200 g of body weight, which may lead to a decrease in body weight and a reduction in cholesterol levels.

Keywords: *sorghum tempeh; cholesterol; high-fat diet; aorta histopathology*
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INTRODUCTION

Based on data from the American Heart Association (AHA), between 2015 and 2018, 38.1% of people had hypercholesterolemia, 27.8% had low-density lipoprotein cholesterol levels ≥130 mg/dL, and 17.2% had low levels of high-density lipoprotein cholesterol, which were below 40 mg/dL. In 2020, cardiovascular disease caused 19 million deaths worldwide.[1] Increased amounts of very-low-density lipoprotein (VLDL) released into the plasma due to heightened liver-mediated cholesterol synthesis are responsible for elevated LDL and total plasma cholesterol levels.

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Elevated plasma cholesterol concentrations are typically attributed to higher levels of dietary cholesterol. Increased concentrations of both total and LDL cholesterol in the bloodstream are linked to hypercholesterolemia, a significant risk factor for the development of cardiovascular diseases.[2,3]

Sorghum is a type of cereal that contains a significant amount of starch and a bioactive compound called 3-deoxyanthocyanin (3-DXA). The concentration of 3-DXA in sorghum is 3-4 times higher compared to other types of grains. The substance also includes phenolic substances, flavonoids, β-glucans, and dietary fiber, which function as antioxidants, anti-inflammatory agents, and cholesterol-lowering agents.[4–6] Shen et al. conducted a study on the impact of sorghum on mice fed a high-fat diet. The group that consumed a diet containing sorghum experienced a 27.27% rise in HDL (high-density lipoprotein) and a 24.47% decrease in serum cholesterol levels.[7]

Sorghum tempeh is a sorghum-based product that undergoes fermentation by Rhizopus sp. This fermentation process produces a protease enzyme with proteolytic activity, which in turn enhances the levels of phenolic acids. As a result, the bioavailability of micronutrients is increased and protein digestibility is improved.[8] The tempeh fermentation process leads to enhanced nutritional quality modifications. Several studies indicate that sorghum may reduce the possibility of developing coronary heart disease by regulating lipid profiles and enhancing insulin sensitivity.[9,10] This study focuses on sorghum tempeh as a functional food product that specifically aims to decrease the risk of hyperlipidemia. The objective of this study was to examine the effects of sorghum tempeh on cholesterol levels and the histology of the aorta in rats that were fed a high-fat diet.

MATERIALS AND METHODS

Sample preparation

The white sorghum seeds were immersed in water at a ratio of 1:3 (w/v) for 24 hours. Following 10 minutes of boiling, the consistency of the sorghum seeds had an adjustment, becoming more tender. The sorghum seeds were inoculated with a 0.1% (by weight) concentration of tempeh yeast. The sorghum was wrapped in permeable plastic and allowed to remain at a room temperature of 29±1oC for 72 hours. Tempeh sorghum was drained at 90°C for 5 minutes to inhibit microbial activity. The intervention was made from 60% sorghum tempeh consisting of 18.7 g of sorghum tempeh, 10 g of skimmed milk, 1.5 g of canola oil, and 1 g of maltodextrin, dissolved in 100 cc of water.

Proximate analysis

An analysis was conducted at the Chem-mix Pratama Laboratory in Yogyakarta to compare the composition of sorghum tempeh and white sorghum. A proximate study was performed on modified sorghum to measure the levels of protein, fat, carbohydrates, antioxidant activity, and dietary fiber. The total carbohydrate content was calculated by subtracting the sum of other components from the total. The Kjeldahl method was applied to do protein analysis proximal. The immediate outcomes were acquired as a percentage (%) value. The examination of fat was conducted using the Soxhlet technique. The AOAC's enzymatic-gravimetric method (1995) was used to measure the total, soluble, and insoluble dietary fiber. Each analysis was conducted twice. Each data point was calculated based on a dry basis. The assessment of antioxidant activity was conducted using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) technique and protein digestibility using the in-vitro method.

Animals and treatment

This study used a true experimental design with a randomized pre-post-test-controlled group design for the total cholesterol levels and post-test-only control group design for the histopathology of aorta. A total of 24 male Sprague Dawley rats, 8 weeks old, were obtained from the Experimental Animal Laboratory of the Center for Food and Nutrition Studies, Universitas Gadjah Mada. Rats weigh 150-200 grams in healthy condition. The conditioning period for rats was eight weeks and the intervention period for rats was four weeks. Additionally, they were divided by simple random sampling into four groups: a standard diet group (SD), a high-fat diet group (FD), a high-fat diet group supplemented with 0.75 g/200g of BW (T1), and a high-fat diet group supplemented with 1.50 g/200g of BW (T2). The cage room individually had an ambient temperature of 28 – 32 ^{o}C with a lighting cycle of 12 hours dark and 12 hours light. Furthermore, the food was measured to 20 g/day using standard feed of Comfeed PARS and high-fat diet consisted of 60% standard feeds, 27.8% wheat flour, 2% pure cholesterol, 0.2% cholic acid, and 10% lard.

During the intervention period, the body weight was measured on a weekly basis. Following the experimental periods, samples of blood and aortic tissue were collected. Cholesterol levels were analyzed using the ELISA method with the total cholesterol kit. Rats had their retro-orbital plexus utilized to withdraw blood ±1% of their total body weight.

Ethical clearance

The study protocol (Ethical Clearance number 52/EC/H/FK-UNDIP/VI/2022) was approved by the Ethics Committee of the Faculty of Medicine, Universitas Diponegoro.

Histopathology of aorta

The aorta was separated and fixed in a 10% formalin buffer solution. The fixed aorta was prepared with paraffin blocks and stained with Hematoxylin-Eosin (HE). The phases involved in the preparation and HE staining process were fixation, dehydration, clearing, and paraffin infiltration. The histopathological slides were examined using a light microscope at a magnification of 100x. The observations identified structural damage to the aorta tissue and foam cells were assigned a score of 0, indicating normal histology. Score 1 indicates the widening of elastic fibers with a small number of foam cells. Score 2 indicates the fragmentation of elastic lamellae with a large number of foam cells. Score 3 indicates the proliferation of smooth muscle, infiltration of lipids in the middle layer, and fibrosis. Score 4 indicates the presence of an ulcerated or lipid-based plaque.[11–13]

Table 1. Comparison of Sorghum Tempeh and White Sorghum

Composition	White sorghum	Sorghum tempeh	*p-value*
Carbohydrate (%)	65.238±0.341	51.054±0.138	0.000*
Fat (%)	4.959±0.035	11.664±0.048	0.000*
Protein (%)	8.661±0.108	16.078±0.044	0.000*
Energy (kcal/100g)	333.107±1.458	370.772±0.087	0.001*
Antioxidant activity (%)	70.079±0.079	79.581±0.079	0.000*
Total dietary fiber (%)	16.328±0.126	19.807±0.177	0.002*
Insoluble fiber (%)	15.206±0.132	18.585±0.137	0.002*
Soluble fiber (%)	1.117±0.006	1.222±0.040	0.067
Protein digestibility (%)	50.915±1.331	63.580±0.200	0.006*

*One-way ANOVA significant difference ($p<0.05$)

Table 2. Food intake throughout the intervention period

Groups	Energy (kcal)	Protein (g)	Fat (g)	Carbohydrate (g)	Fiber (g)
SD	74.922±6.378	3.472±0.297	0.366±0.028[b]	14.255±1.213	1.098±0.919[b]
FD	74.632±7.201	3.460±0.335	0.363±0.034[b]	14.198±1.367	1.091±0.108[b]
T1	71.583±7.720	3.315±0.358	0.500±0.037[a,b]	13.292±1.470	1.323±0.110[a,b]
T2	77.326±4.742	3.578±0.217	0.682±0.025[a,b]	14.053±0.900	1.701±0.070[a,b]
p-value	0.528	0.537	0.000*	0.530	0.000*

*One-way ANOVA significant difference ($p<0.05$), [a]significant difference against control group (*Bonferroni* test), [b]significant difference against intervention group (*Bonferroni* test)

Table 3. Body weight changes of 4 weeks intervention (g)

Groups	W1	W4	*p-value*	Δ	*p-value*
SD	243.67±3.98	269.83±3.54	0.000*	26.17±2.40[a,b]	0.000**
FD	313.83±3.31	365.50±3.51	0.000*	51.67±1.21[a,b]	
T1	304.00±3.46	271.50±7.89	0.000*	-32.50±9.56[a,b]	$\eta^2 = 0.971$
T2	304.50±3.94	307.67±5.57	0.260	3.17±6.11[a,b]	

*Paired t-test, **One-way ANOVA, significant difference ($p<0.05$), [a]significant difference against control group (*Tamhane* test), [b]significant difference against intervention group (*Tamhane* test)

Data analysis

Statistical analyses were performed using IBM SPSS Statistics 25 SPSS software. The mean±SD was used to express all the data. The Shapiro-Wilk test was employed to analyze the normality of research data. The data of proximate analysis, food intake and the mean changes of total cholesterol and body weight were examined using a one-way ANOVA and Tamhane post hoc as a follow-up test. Significance was set at $p < 0.05$. The effect size of the observed differences was evaluated using the eta squared (η^2), along with its 95% confidence interval. The threshold values were defined as 0.01 considered small, 0.06 medium, and 0.14 large effects.

RESULTS

Sorghum tempeh and white sorghum comparison analysis

The proximate analysis results, shown in Table 1, compare the percentage of carbohydrate, fat, protein, energy, and antioxidant activity between sorghum tempeh and white sorghum ($p < 0.005$). Sorghum tempeh also has greater levels of total dietary fiber and insoluble fiber ($p = 0.002$), measuring 19.8% and 18.58%, respectively, compared to white sorghum. However, the soluble fiber of sorghum tempeh and white sorghum were not significantly different ($p = 0.067$). The carbohydrate content of sorghum tempeh was found to be lower (51.05%) compared to white sorghum (65.23%). On the other hand, the fat, protein, and energy content of sorghum tempeh (11.66%; 16.07%; 370.77 cal/100 g) was higher compared to that of white sorghum (4.95%; 8.66%; 333.10 cal/100g). According to the DPPH method antioxidant test results, the antioxidant content of sorghum tempeh was determined to be 79.58%. The protein digestibility of sorghum tempeh ($p = 0.006$) was shown to be enhanced (63.58%) after fermentation, in comparison to white sorghum (50.91%).

Food intake and body weight changes of rats

Table 2 showed the food intake of standard feed and sorghum tempeh administration during the intervention period. The SD and FD groups were only fed a standard diet, whereas the T1 and T2 groups were fed a standard diet together with sorghum tempeh at varying doses. The results of the one-way ANOVA test indicated statistically significant differences in fat and fiber intake ($p < 0.005$). The T1 group consumed 0.5 grams of fat per day, while the T2 group consumed 0.68 grams of fat per day. The T2 group had a higher fiber intake of 1.7 g/day compared to the T1 group, which had an intake of 1.32 g/day. The energy intake of the T1 group was 71.58 kcal/day, while the T2 group had an intake of 77.32 kcal/day. However, there was no significant difference when compared to the control groups ($p = 0.528$). Carbohydrate and protein intake were not significantly different ($p = 0.530$ and $p = 0.537$, respectively).

Table 4. Total Cholesterol Levels Pre and Post Intervention (mg/dL)

Groups	Pre-intervention	Post-intervention	*p-value*	Δ	*p-value*
SD	88.685±1.365	89.986±2.089	0.008*	1.302±0.741[b]	0.000**
FD	217.885±4.225	220.365±3.847	0.001*	2.480±0.929[b]	
T1	214.356±2.757	121.161±4.111	0.000*	-93.195±5.920[a,b]	$\eta^2 = 0.996$
T2	218.980±2.652	97.836±2.504	0.000*	-121.143±4.276[a,b]	

*paired t-test, **One-way ANOVA *p* value significant difference (p<0.05), [a]Significantly different from control group (SD and TD) (*Tamhane* test), [b]Significantly different from intervention group (T1 and T2) (*Tamhane* test)

Table 5. Histopathology scoring

Groups	Histopathology Aorta Score					Median (Min-Max)	*p*
	0	1	2	3	4		
SD	6	0	0	0	0	0 (6-6)[a]	0,003*
FD	0	6	0	0	0	1 (6-6)[b]	
T1	2	4	0	0	0	0.67 (2-4)[c,d]	
T2	1	5	0	0	0	0.83 (1-5)[b]	

*Kruskal Wallis significant difference (p<0.05), [a]Significantly different from FD and T2 (Mann Whitney Post hoc test), [b]Significantly different from normal control (SD), [c]Not significantly different from control group (SD and FD), [d]Not significantly different from intervention (T1 and T2).

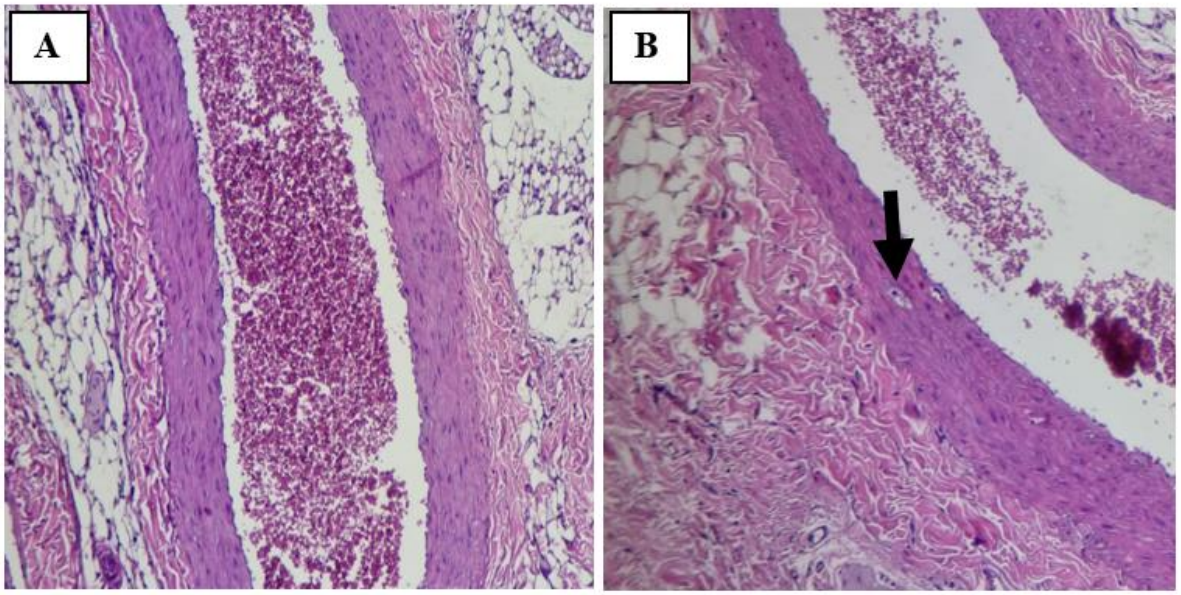

Figure 1. Histopathology of Aorta (Hematoxylin-eosin stain; original magnification: 100x). Microscopic view of a normal aorta cross section (score 0) (A), cross section of aorta containing foam cells (score 1) Histopathology showing the foam cell (arrow) within tunica intima (B).

According to Table 3, after four weeks of intervention, the SD, FD, and T1 groups showed significant differences between week 1 and week 4 of intervention time ($p < 0.005$). Meanwhile, the body weight of the T2 group was not significantly different over the intervention period ($p = 0.260$). The T1 group observed a drop in body weight with a mean change of -32.5 g, while the other group did not see any decrease in body weight.

The One-way ANOVA test showed a statistically significant difference in mean changes of body weight ($p < 0.005$). The effect size was calculated as eta squared ($\eta^2 = 0.971$).

Improvements in total cholesterol levels

The blood samples of rats were analyzed to determine their total cholesterol levels, as presented in Table 4. In the pre-intervention condition, the groups who were induced with a high-fat diet showed greater levels of total cholesterol in FD, T1, and T2 compared to the group that had a standard diet. The paired t-test showed statistically significant differences in the total cholesterol levels of the SD group ($p = 0.008$), FD, T1, and T2 groups before and after the intervention ($p < 0.005$). The One-way ANOVA test showed a statistically significant difference in mean changes of total cholesterol levels among the various groups ($p < 0.005$). The Tamhane test showed a statistically significant difference in the lowering of total cholesterol levels between the T1 and T2 groups compared to the SD and FD groups. The results indicated a reduction in total cholesterol levels in both the T1 and T2 groups following the intervention, with mean reductions of -93.19 mg/dL

and -121.14 mg/dL, respectively. The effect size was calculated as eta squared ($\eta^2 = 0.996$).

Effects of sorghum tempeh on Histopathology of Aorta

Figure 1 demonstrates that the SD group, which was fed a normal diet, displayed a normal microscopic image of the aorta in Sprague Dawley rats. These images showed that the aortic layer had three distinct layers: the tunica intima, tunica media, and tunica adventitia. The preparation displayed a picture of an artery exhibiting the presence of foamy macrophages infiltrating the intima. No pictures of intra/extracellular mucin or thrombus were present. There was no presence of plaque detected over the entire 8-weeks period of the high-fat diet. The histopathology of the aorta in rats given a high-fat diet shows histological abnormalities observed in the tunica intima, including the presence of foam cells. In Table 5, the FD and T2 groups showed statistically significant differences compared to the SD group. Histopathological examination of the aorta showed the normal tissue with no presence of foam cells (score = 0), the T2 group had only one sample of normal tissue (n = 6), while the T1 group contained two samples of normal tissues (n = 6).

DISCUSSION

This study determines that a high-fat diet can lead to hypercholesterolemia, which is defined by elevated levels of total cholesterol in the blood and the production of foam cells at early stages. The total cholesterol levels of hypercholesterolemic rats supplemented with two different doses of sorghum tempeh (0.75g and 1.50g per 200g of BW) were significantly lower compared to the control groups, as in prior research has found that sorghum tempeh effectively reduces LDL and MDA levels in rats after a high-fat diet.[9] Antioxidant activity of sorghum tempeh was slightly higher than white sorghum, which might be related to the fermentation, demonstrating the improvement of sorghum's nutrition characteristics.[14] The fermentation process generates a protease enzyme with proteolytic properties, leading to an elevation in phenolic acids. Consequently, this procedure enhances the bioavailability of micronutrients and improves protein digestibility; according to this study, the protein digestibility of sorghum tempeh was shown to be higher (63.58%) compared to sorghum that was not fermented (50.91%).[8] Study by Murtini et al. reported that sorghum tempeh fermented for 72 hours produced protein (10.27%), starch (45.56%), and fat (0.56%).[6] The protein of sorghum tempeh in this study was also found to be higher (16.07%) compared to the protein of sorghum (8.66%). The fermentation method applied to the production of tempeh additionally improves its bioavailability, but it also breaks down the glycosidic linkages to generate phenolics. The increase in phenolic compounds contributes to a higher level of antioxidant activity.[15] In addition, the antioxidant properties present in sorghum tempeh help reduce the buildup of cholesterol caused by a high-fat diet.

The high fiber content seen in sorghum tempeh is associated with a reduction in blood cholesterol levels. The composition of sorghum tempeh includes two types of fiber: soluble fibers (10.1–25.0%) and insoluble fibers (75.0–90.0%). This research additionally showed that the content of insoluble fiber (18.58%) is higher than soluble fiber (1.22%).[8] Short-chain fatty acids (SCFA) from intestine may lower blood cholesterol levels via decreasing hepatic cholesterol synthesis.[16] These findings propose a potential method to decrease the risk of hyperlipidemia and cardiovascular disease.[17]

The findings of this study demonstrate that the administration of sorghum tempeh for 4 weeks leads to a considerable reduction in total cholesterol levels. When administered at a higher dose of 1.50 g/200g of BW, it shows a significant decrease rate. The average total calorie intake from feed and enteral formula was 71.58 kcal/day in the T1 group and 77.32 kcal/day in the T2 group. Food intake of standard feed and sorghum tempeh administration showed different energy, fat, and fiber intake. The high-dose group received more energy and fat intake than the low-dose group, and the result showed the T1 group experienced a weight loss of 32.5 grams, but the T2 group exhibited a weight gain of 3.167 grams. The high-dose group had a higher fiber intake (1.70 g) compared to the low-dose group (1.32 g). Furthermore, the decreased mean changes before and after intervention were observed in the T1 group (-93.19 mg/dL) and the T2 group (-121.14 mg/dL). The effect size in this study was used to determine the practical significance of the study results and to understand the practical implications of the findings. The administration of sorghum tempeh had a 99.6% impact on changes in cholesterol levels and 97.1% on body weight changes, suggesting a large effect.

The histopathology results of this study demonstrated that the ingestion of a high-fat diet for 8-weeks leads to the development of hypercholesterolemia in rats, as previously observed, and promote the accumulation of foam cells in the tunica intima of the aorta.[13,18–20] Cholesterol buildup leads to a rise in lipoproteins in the inner lining of blood vessels, which are then oxidized by oxygen-free radicals generated by endothelial cells or macrophages. This accumulation occurs specifically in macrophages and other phagocytes, resulting in the formation of foam cells.[21]

After the sorghum tempeh was administered, only one or two out of the six samples exhibited improvement, characterized by the absence of foam cells. A possible explanation is that the samples had different levels of fat accumulation at the start of the intervention. As a result, some samples may have had more advanced conditions that were less susceptible to the intervention, other factors might contribute to this outcome. The rats included in the study may exhibit modest variations in their genetic compositions, which can influence their metabolic processes and responses to dietary intervention of sorghum tempeh.[22] The genetic variations may impact the rats' lipid metabolism, antioxidant defenses, and vulnerability to the formation of foam cells.

The gut microbiota also has a substantial impact on the breakdown of nutrients from food and the subsequent effects on health. Differences in the gut microbes of the rats may cause variations in the availability and efficacy of the active chemicals in sorghum tempeh, leading to varied outcomes.[23] In addition, the immune system, which contributes to the development of foam cells through macrophage activity, can differ across individual rats. Variations in immunological responses and

phenotypic alterations of macrophages during the accumulation of foam cells may result in differences in the cellular mechanisms involved in the formation or reduction of foam cells when exposed to the intervention.[24] In this study, the one rat showing an absence of foam cells could have had a combination of factors that made it more receptive to the positive effects of sorghum tempeh. In contrast, the other rats did not exhibit the same level of responsiveness. Further studies using larger sample sizes and the inclusion of other variables, such as the gut microbiota and macrophage activity, may be required to determine the exact cause.

The outcome of this study had a significant effect on overall cholesterol levels despite the presence of foam cells. Consequently, supplementation with sorghum tempeh may reduce the production of foam cells and enhance the histological appearance of the aorta. However, it was unable to achieve the total same condition as the healthy control group. The results showed that giving sorghum tempeh at a dose of 0.75 g/200g of BW was significant to improve the development of atherosclerosis, including reducing high cholesterol levels and preventing the formation of foam cells. The limitation of this study was that the researcher did not analyze any antioxidant markers to identify the phenolic compound of sorghum tempeh, the organ weight of rats, or histology of other organs, such as the liver. There was a lack of fatty acid analysis to clarify the factors contributing to the greater fat content in sorghum tempeh.

CONCLUSION

Sorghum tempeh is a type of functional food that offers enhanced nutritional benefits, including a higher content of total dietary fiber, predominantly insoluble fiber, increased antioxidant activity, and improved protein digestibility. Administering sorghum tempeh in the diet can significantly impact changes in body weight and cholesterol levels, with a potential effect of over 90%. The recommended dose of sorghum tempeh is 0.75 g per 200 g of body weight, which may lead to a decrease in body weight and a reduction in cholesterol levels. It is possible to restore the normal condition of the aorta histopathology by inhibiting the development of foam cells.

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