Jurnal Gizi Indonesia
(The Indonesian Journal of Nutrition)
Jurnal Gizi Indonesia (The Indonesian Journal of Nutrition)
Vol. 10, No. 2, June 2022 (141-149)
Submitted: 16 November 2021, Accepted: 16 February 2022
Online https://ejournal.undip.ac.id/index.php/jgi

Effect of Moringa (*Moringa oleifera*) Leaf Flour Supplementation on Total Antioxidant Content of *Sprague Dawley* Rat Serum Given High-Fat Diet

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ABSTRACT

Background: Moringa oleifera leaf is high in quercetin which can be a source of exogenous antioxidants. Together with endogenous antioxidants, both the antioxidants will be able to counteract oxidative stress conditions.

Objectives: To analyze the effect of Moringa leaves flour supplementation on Total Antioxidants Content (TAC) of Sprague Dawley (SD) rat serum given a high-fat diet (HFD).

Materials and Methods: A randomized control group post-test design was used on 24 SD rats which were divided into 4 groups, namely healthy control (K_1), HFD (K_2), supplementation with Moringa leaf flour at a dose of 100 mg/100 g BW/day (K_3), and a dose of 200 mg/100 g BW/day (K_4). After 28 days of supplementation, serum TAC was analyzed using the ELISA method. Data analysis used Paired-T Test, One Way ANOVA, and Post-Hoc Bonferroni follow-up test.

Results: The results showed that the TAC of groups K1, K2, K3, and K4 respectively were 4.806 ± 0.239, 1.323 ± 0.292, 4.020 ± 0.239, and 5.123 ± 0.695. There was a significant difference in serum TAC (p=0.000) between supplementation groups. Significant differences in serum TAC were also found in the supplementation group compared to the HFD control group.

Conclusion: Moringa leaves flour supplementation for 28 days at a dose of 200 mg/100 g BW/day increases serum total antioxidant content higher than at a dose of 100 mg/100 g BW/day.

Keywords: High Fat Diet; Moringa Oleifera leaves Flour; TAC

BACKGROUND

Reactive Oxidative Stress (ROS) has beneficial effects at moderate levels, and is involved in various physiological functions such as boosting the immune system. However, at higher levels, it produces oxidative stress, thereby damaging various molecules including lipids, proteins, and DNA. Oxidative stress develops when there is an increase in ROS production on one hand and a lack of antioxidants on the other[1]. A continuous increase in ROS causes the body to remain in a state of oxidative stress[2–4]. Meanwhile, the body has an effective defense mechanism boosted by endogenous antioxidants, thereby preventing excessive ROS formation[5,6]. Endogenous (synthesized by the body) and exogenous (obtained from food) antioxidants work synergistically to protect the body cells and organ systems from further damages due to excessive ROS[7]. Furthermore, less intake of exogenous antioxidants can decrease the endogenous[8,9], which can also be increased by optimizing consuming foods containing antioxidants daily[10]. Good food intake pattern arrangements in supporting the availability of exogenous antioxidants was food that contains polyphenols, such as flavonoids[11]. Moreover, its daily consumption contributes to the production of exogenous antioxidants for the body[12].

Flavonoids are one of the bioactive compounds with antioxidant properties[13] which are found in high content in Moringa (*Moringa oleifera*) leaf. Based on the type of flavonoid, quercetin in Moringa leaf is present in higher amounts than others and contain Provitamin A, Vitamin C, Vitamin E, and minerals, such as selenium and zinc that also act as antioxidants[14–16]. According to Ganatra (2017), Moringa leaf contains 8 times more polyphenols than red wine, 30 times more vitamin A than spinach and four times of carrots, and 7 times more vitamin C than oranges[17]. The antioxidant combination found is more effective compared to the single ones, due to the synergistic mechanism in suppressing ROS[18]. Following research conducted by

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e-ISSN : 2338-3119, p-ISSN: 1858-4942

Nilanjin (2012), the antioxidants contained in Moringa leaf had a beneficial effect on experimental animals fed on a high-fat diet by increasing Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx) levels and reducing the free radicals, thereby inhibiting lipid peroxidation and tissue damage[19].

Moringa leaf flour is one of the processed products that have experienced the initial drying and refining process. It is first ground and stored for months without refrigeration, also this does not reduce the nutritional content significantly[20]. Additionally, In the present study, two doses of Moringa leaf flour were selected as 200 mg/100 g BW/day and 100 mg/100 g BW/day. These doses were selected on the basis of previous reports of the acute toxicity study performed using the dose administered until 2000 mg/kg of dried leaf powder of Moringa which shows no signs of toxicity in rats and based on average daily flavonoid requirements[21,22]. Preliminary research has not been carried out on experimental animals and humans, therefore, this research aimed to prove the effect of Moringa (*Moringa oleifera*) leaf flour on increasing serum total antioxidant contents.

MATERIALS AND METHODS

This experimental research was carried out with a post-test and randomized control design group. The production of Moringa leaf flour, rearing of experimental animals, and biochemical analysis of serum samples were carried out at the Nutrition Laboratory of the Inter-University Center for Food and Nutrition Studies (PSPGPAU), Gadjah Mada University, Yogyakarta, from March to April 2021.

The research subject is a male white rat Sprague Dawley (SD), and the number used was calculated based on the provisions of the World Health Organization (WHO), which postulates the need for a minimum of 5 experimental animals. To anticipate dropout, one experimental animal is added to each group, thereby amounting to a total of 24. Determination of the research subjects considered the inclusion criteria, namely experimental animals aged 8 to 11 weeks, body weight ± 150 g, healthy (active movement), and without defects. The independent variable used is the dosage variation of Moringa leaf flour dose I 100 mg/100 g and 200 mg/100 g BWs of experimental animals/day while the dependent is the TAC of the animal serum.

Research tools include basins, blenders, ovens, 80 mesh sieves, slicers, rat oral sonde, digital animal scales, hand gloves, and masks. The materials used include Moringa leaf, AD II standard feed, High Fat Diet (HFD), and water. High fat diet is a mixture of 10% lard and 2 ml of duck egg yolk in AD II standard feed. The process of making Moringa leaf flour is as follows: 1). The fresh, light, and dark green leaves that are not too dry are separated from the stems, 2). Washed, 3). Drained, 4). Then, dried in an oven at a temperature of 55^{o}C for 60 minutes, 5). After which it is ground using a blender, and 6). Finally, it is sieved with an 80 mesh sieve. Moringa leaf flour used in this research was placed in an airtight container and stored in a refrigerator. Furthermore, primary data were collected from the measured body weight and examination of total serum antioxidant contents which is a comparison of the healthy, and HFD controls, including the treatment groups. Weight measurement data was recorded at the beginning of the research and this continued every week, besides, the total serum antioxidant examination data underwent a post-test.

Body weight was measured every 7 days using a digital animal scale, while examination of the total antioxidant contents was carried out using the ELISA method at the end of the research. In addition, blood samples were taken from the retroorbital plexus of the experimental animals. Subsequently, they were put in different cages and acclimatization lasted for 7 days with the provision of standard AD II feed and *ad libitum* drink daily.

Afterward, the animals were randomly grouped into 4 with 6 in each, namely K_1, K_2, K_3, K_4. Group K_1 was provided standard feed and *ad libitum* drink, while K_2, K_3, and K_4 were fed with HFD. The HFD administration period lasts for 2 weeks and the body weight of the animals was measured every week. This was followed by the intervention stage, which lasted for 28 days with all groups provided standard AD II feed and *ad libitum* drink whereas the intervention group was given additional Moringa leaf flour through an oral probe with a dose of 100 mg/100 g and 200 mg/100 g body weights of rats/day in groups K3 and K_4 respectively. The animals' body weight was measured weekly during the intervention period and samples of their blood were collected from the retroorbital plexus to examine the total serum antioxidants after this stage.

The data were tested for normality using the Shapiro-Wilk test. The first statistical analysis determined the differences between the pre and post-test. The average weight of the experimental animals was normally distributed, with the Paired t-test and One way ANOVA used to examine changes and differences in the

groups. Furthermore, the Kruskal-Wallis test was also used to determine the same attribute among experimental animal groups. The second statistical analysis is the post-test carried out on serum TAC data, which proved that the serum TAC was normally distributed. Furthermore, the One Way ANOVA and Bonferroni Post-Hoc tests were used to examine the difference in the intervention effects in the groups. The significant difference with p-value <0.05 shows the mean ± SD and median (min ± max) for data that were normally and abnormally distributed, respectively.

The analyzed data were computerized using SPSS. Meanwhile, this research was approved by the Health Research Ethics Commission (KEPK) of the Faculty of Medicine, Diponegoro University as stated in the Ethical Clearance NO.25/EC/H/FK-UNDIP/III/2021 dated March 17, 2021.

RESULTS

The body weight characteristics of the experimental animals during the acclimatization period ranged from 182 to 187 g. Besides, none of the animals dropped out during the research and the consumption of HFD for 2 weeks led to a significant increase in weight. The results of statistical tests carried out after its administration (Table 1) showed an increase in body weight before and after being given standard feed AD II K_1 and HFD (K_2, K_3, K_4), although a significant difference was observed in the groups (p = 0.000).

Table 1. Body Weight Value of Experimental Animals (g) Before and After HFD Administration

Group	n	Before	After	*p*	Δ	%Δ
K_1	6	185.00±4.43	196.17±5.12	0.00	11.00(10±12)	6.03±0.29
K_2	6	187.00±2.53	215.33±2.16	0.00	28.00(27±30)	15.16±0.69
K_3	6	185.17±4.36	213.50±4.42	0.00	28.00(28±30)	15.31±0.57
K_4	6	182.83±2.48	211.00±2.61	0.00	28.00(27±29)	15.41±0.46

p=Paired T-Test; [a]=One Way ANOVA Test; [b]=Kruskal-WallisTest

An insignificance difference (p = 0.282) was observed in the average body weight of the experimental animals in all groups at the beginning of the research. After 2 weeks of being fed with HFD, a significant difference was observed among the groups (p = 0.000). The results of the Kruskal-Wallis test showed that there was a significant difference in weight change among the 4 groups (p = 0.002). Descriptively, the highest percentage of weight gain was exhibited by the group that was fed with HFD (±15%) compared to the one that was only given standard feed (±6%).

Table 2. Body Weight Value of Experimental Animals (g) Before and After Moringa Leaf Flour Administration

Group	Before	After	*p*	Δ	%Δ
K_1	196.17±5.12[a]	221.83±5.67	0.000	26.00(24±27	13.09±0.48
K_2	215.33±2.16[a]	266.50±2.74	0.000	51.00(50±53	23.76±0.52
K_3	213.50±4.42[a]	245.50±4.23	0.000	32.00(31±33	14.99±0.60
K_4	211.00±2.61[a]	236.83±3.06	0.000	25.00(25±28	12.24±0.63

p=Paired T-Test; [a]=One Way ANOVA Test; [b]=Kruskal-Wallis Test

The results of statistical tests on experimental animal body weight before and after the administration of Moringa leaf flour (Table 2) showed that all groups including the treatment group which was given standard feed, and HFD, experienced a significant increase in body weight (p = 0.000). The average body weight after 4 weeks of treatment was significantly different (p = 0.000). The average body weight between groups was observed after administering Moringa leaf flour for 28 days. Descriptively, the least percentage of weight gain was shown by the group with the highest dose (±12.24%). Administration of Moringa leaf flour at a dose of 200 mg higher suppressed weight gain in the treatment group compared to 100 mg.

Table 3. Total Antioxidant Contents of Experimental Animal Serum (mmol/L)

Group	n	Serum Total Antioxidant Content	*p*
K_1	6	4.806 ± 0.239[a]	0.000
K_2	6	1.323 ± 0.292[a]	
K_3	6	4.020 ± 0.239[a]	
K_4	6	5.123 ± 0.695[a]	

p=One Way Anova Test, [a]=Post-Hoc Bonferroni Test

The results of the one-way ANOVA statistical test (Table 3) showed that serum total antioxidant contents were significantly different in the 4 groups (p = 0.000). The Bonferroni Post-Hoc Statistical Test showed that the comparison of serum total antioxidant contents in the K3 group to K4 was significantly

different (p = 0.022) after administration of Moringa leaf flour. Treatment with a dose of 200 mg/100 g BW and 100 mg/100 g BW indicated that the total serum antioxidant contents were significantly different to the K_2 group fed with HFD (p = 0.000, p = 0.000). Meanwhile, it was discovered that K_3 treated with a dose of 100 mg/100 g BW was significantly different to the K_1 healthy control group (p=0.001). The treatment of K_4 with a dose of 200 mg/100 g BW showed a higher content compared to K_3 treated with a dose of 100 mg/100 g BW, although it was not significantly different from the K_1 healthy control group (p = 1.000).

DISCUSSION

These results indicate that the least total serum antioxidant contents were discovered in the group given HFD. The storage of excessive fat increases body weight thereby increasing the production of proinflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6)[23]. Furthermore, increased inflammation causes an increase in ROS production and depletion in endogenous antioxidants that enchanged oxidative stress[24–26]. Endogenous antioxidants depletion was caused by its increased consumption in suppression of ROS progression[27], thus endogenous antioxidants are required in sufficient quantities. Intake deficiency of exogenous antioxidants may cause endogenous antioxidants to decrease continuously and the body remains in a state of oxidative stress[28]. Decreased endogenous antioxidants required exogenous antioxidants obtained from food[29]. Endogenous and exogenous antioxidants synergistically maintain or rebalance antioxidants and ROS due to the presence of ROS reducing compounds in exogenous antioxidants such as flavonoids, vitamins, and minerals through mechanism induced enzymes factor transcription, scavenging process by capturing ROS to donate one electron and hydrogen, metal chelating that helps ROS to become relatively stable and unreactive to induce further oxidative stress, and also act as a cofactor of antioxidants enzymes[30,31].

Giving Moringa leaf flour to the treatment group significantly increased the total antioxidant content of serum, therefore, Moringa leaf flour can act as a source of antioxidants that restores or normalizes serum total antioxidant content efficiently. Mabrouki (2020) analyzed the effect of administering Moringa leaf extract on endogenous antioxidants in experimental animals. Endogenous antioxidants were significantly recovered by administration with Moringa leaf extract by mechanism to reduce and maintain ROS in a balanced concentration[32]. In this research, the increased contents are also due to reduced antioxidants used in the suppression of ROS progression. The previous research also showed an increase in the constituents after the extract intervention due to reduced antioxidants use in reducing ROS and the provision of hydrogen to make it more stable[33].

Based on the content of Moringa Leaf flour, flavonoids as a source of exogenous antioxidants are present in high quantities[34]. Furthermore, Rodríguez-Pérez (2015), and Makita (2016), reported that methanol extract of Moringa leaf contained 26, and 14 flavonoids, respectively[35,36]. Compared to vegetables, previous research discovered that the flavonoid content in the dried Moringa leaf was 3 to 12 times more high than other types of vegetables consumed by families, namely 12 times more than cauliflower, 9 times of peas, 5 times of cabbage, 4 times of spinach, and 3 times of broccoli[37]. Another research showed that the experimental animals are given HFD, and dietary intervention containing flavonoids for 4 weeks increased endogenous antioxidants through the mechanism of interacts synergistically with exogenous antioxidant system, then captured free radicals, prevent further oxidative damage, thereby maintaining a balanced ROS system[38,39]. Additionally, quercetin, a flavonol bioactive compound, is a class of flavonoids that was found high in Moringa leaf[40]. In previous studies, it was discovered to have reached approximately 50% of the total flavonoids in Moringa leaf extract[41]. Subsequently, quercetin had an antioxidant function that affected the increase of endogenous antioxidant[42]. Quercetin increased endogenous antioxidant Glutathione Peroxidase (GPx), Catalase (CAT), and Superoxide Dismutase (SOD) by directly or undirectly induced the Nrf2-mediated transcription activity by increase Nrf2 expression of the antioxidants. These antioxidants are regulated by the transcriptional factor Nuclear Factor E2-Related Factor (Nrf2) which responds by binding to the Antioxidant Response Element (ARE) as a promoter of genes that code the antioxidant. Quercetin also regulates levels of endogenous antioxidant Glutathione (GSH). Superoxide Dismutase captures O_2^- of ROS and transforms it into H_2O_2. Catalase and Glutathione Peroxidase further catalyze the decomposition of H_2O_2 to unreactive H_2O. This Reaction requires GSH as a hydrogen donor On the other hand, Quercetin has the ability to act directly as free radical scavengers or hydrogen donors, hydroxyl radical scavenging, and metal-chelating ability[43,44].

Moringa leaf flour also has vitamins such as vitamin E, vitamin C, pro-vitamin A, and complete minerals such as Cu, Mn, Fe, and Zn[45]. These vitamin acts as a free radical scavenger and reduces free radical, donating electrons and hydrogen to free radical to prevents their oxidation and to generate a much less reactive species than most other free radicals[44,45] whereas minerals play an important role as cofactors of endogenous antioxidant that may increase the efficiency of endogenous antioxidant function. Endogenous antioxidants are metal ion cofactor-requiring enzymes that catalyze the dismutation of highly reactive superoxide radicals (O_2^-) into unreactive and relatively stable molecular oxygen (O_2) and hydrogen peroxide (H_2O_2)[48]. However, through this mechanism, all bioactive compounds and micronutrients in Moringa leaf flour become extremely effective so that they may improve the efficiency of endogenous antioxidant function. After 28 days of treatment, the mean increase in serum total antioxidant contents was higher at dose II than dose I, and the increase at dose II was equivalent to the healthy group.

The average weight gain in the group given HFD (±15%) was significantly different compared to the others. This is in line with previous research which proved HFD caused an increase of 9 to 23% [49]. There are two possible causes of weight gain due to the influence of HFD. First, fat as the main composition of HFD that contains high energy compared to others macronutrients[50,51]. Second, types of fat such as saturated fatty acids and cholesterol which are high in pork oil and duck egg yolk increase the HFD energy density and easy of absorption by the body, therefore, it is stored in excess which ultimately increases bodyweight[52–54]. Furthermore, administration of Moringa leaf flour at a dose of 100 mg and 200 mg was able to maintain and suppress the increase in body weight. This study is in accordance with previous studies which showed that administration of 200 mg and 400 mg of Moringa leaf extract were proven to be able to lose and maintain weight because of their high fiber content[55]. Moringa oleifera leaf can provide 41.20 g carbohydrate, 29.40 g protein, 5.20 g fat, and 12.50 g fiber by 100 g dry leaves whereas 100 g dried Moringa leaf powder contains approximately 38.20 g carbohydrate, 27.10 g protein, 2.30 g fat, and 19.20 g total fiber[56]. Due to high fiber content, Moringa can be used to suppress weight gain by mechanism increasing water-binding and swelling capacities. This slows gastric emptying, which in turn increases satiety, longer meal intervals and ultimately decreases food intake[57].

However, there are some limitations associated with this research, such as difficulty in analyzing macronutrient and micronutrient content, especially specific polyphenols in Moringa oleifera leaf flour.

CONCLUSION

In conclusion, the administration of Moringa leaf flour at a dose of 100 mg/100 g and 200 mg/100 g body weight/day increased the total antioxidant content of the serum in Sprague Dawley rats. Furthermore, the dosage of 200 mg/100 g body weight/day had a better effect.

ACKNOWLEDGMENT

The authors are grateful to Mr. Yuli Yanto and Dimas Prihantoro as Laboratory Technicians of the Inter-University Center for Food and Nutrition Studies (PSPGPAU) Universitas Gadjah Mada, Yogyakarta for their assistance during the research.

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