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Dietary Fresh and Boiled Mangkokan Leaves (*Nothopanax scutellarius*) Normalized Body Weight, Serum Lipid Profile and *Malondialdehyde* in Metabolic Syndrome Rats

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Abstract

The antioxidant activity of *Nothopanax scutellarius (Burm. f.) Merr*, an edible plant, can prevent oxidative stress in metabolic syndrome (MetS). Thus, our research aimed to study the effect of dietary inclusion of fresh or boiled *N. scutellarius* on body weight and biochemical markers of Wistar rats with MetS. Twenty-four male Wistar rats were divided randomly into four groups, i.e., normal control group, high-fat-high-fructose diet (HFFD)group, fresh *N. scutellarius* (FNs) group, and boiled *N. scutellarius* (BNs)group. The normal control group was fed only a standard diet during the entire experiment. High-fat and high-fructose (HFHFr) diet accompanied with 20% fructose in drinking water to induce MetS was given to the HFFD, FNs, and BNs groups for 29 days. This was followed by a 29-day intervention diet in which standard normal diet, fresh *N. scutellarius*-containing standard diet, and boiled *N. scutellarius*-containing standard diet were given to the HFFD, FNs,and BNs groups, respectively. HFHFr diet significantly ($p<0.05$) raised fasting blood glucose (FBG), serum triglyceride, total cholesterol, LDL-cholesterol, and *Malondialdehyde* (MDA), and significantly ($p<0.05$) reduced HDL-cholesterol. After 29 days on the intervention diet, serum triglycerides, total cholesterol, and LDL-cholesterol levels were found to

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decrease, and HDL-cholesterol levels were found to increase significantly ($p<0.05$).Thus, it can be concluded that dietary intake of *N. scutellarius* for 29 days can improve MetS components, i.e.,FBG, serum lipid profile, and MDA, similar to those seen in rats on a normal control diet.

Introduction

Metabolic syndrome (MetS) is characterized by conditions of hyperglycemia, decreased serum high-density lipoprotein, increased serum triglycerides, abdominal obesity, and high blood pressure. When three out of the five criteria are met, then the condition is categorized as MetS.[1] The prevalence of MetS increases every year, with approximately 20%–25% of adults worldwide experiencing MetS[2] and cases in Indonesia exceeded 13.3%.[3] People with MetS have a higher risk of suffering degenerative disease such as diabetes mellitus type 2 (DM-2) and cardiovascular diseases because of oxidative stress conditions.[4]

In general, MetS are affected by internal and external factors where by internal factor (genetic) is caused by a genetic mutation that affects leptin (hormone) and its receptor.[1,5] One of the external factors is a lifestyle including the consumption of high-fat and simple-sugar-containing foods and a lack of physical activity.[6] A high fat diets that contains high calorie triggers the accumulation of visceral fat and lipotoxicity that can lead to insulin resistance, increase oxidative stress, and systemic inflammation.[7] Excessive fructose consumption increase glucose metabolism in the liver that can accumulate triglyceride and cholesterol and induce insulin resistance.[1,8]

Consumption of vegetables with a high content of antioxidants are highly recommended in the management of MetS[9,10] as it can improve the oxidative status and MetS components such as lipid profile.[11-14] The consumption of green vegetables with their active components such as carotenoid and flavonoid can play roles in glucose metabolism and prevention of risk of type-2 diabetes mellitus (DM).[15,16]

Mangkokan leaf is an Indonesian term for *Nothopanax scutellarius*,and the Javanese people of Indonesia have a long history of consuming the leaves as part of their daily diets. The leaves are commonly consumed fresh, called “lalapan” or a salad, or cooked. It has potential as an antioxidant source due to its various phytonutrient content such as flavonoid, phenolic, and pigments (chlorophyll, carotene).[17] On the other hand, antioxidant content in green leaves is significantly affected by the cooking method. Brief boiling (30-60 seconds) has been shown to increase the antioxidant content of green leafy vegetables (Murwani *et al*., unpublished). In this study, we aimed to reduce MetS biomarkers by studying the effect of incorporation of *Nothopanax scutellarius* (*Burm. f.*) *Merr*, fresh or boiled,as part of a normal diet of Wistar rats.

Research Design and Method

Research Method

Ethical clearance with No.139/EC/H/KEPK/FK-UNDIP/XI/2019for this study had been granted from our institution the Health Research Ethics Commission of the Faculty of Medicine, Diponegoro University-RSUP.Dr. Kariadi Semarang.

Plant Materials and standard Diet Containing *N. Scutellarius*

Nothopanax scutellarius leaves were purchased from local breeders in Banjarnegara, Central Java, Indonesia. Two forms of Mangkokan leaves (fresh and boiled, Fig. 1a and 1b) were prepared. Fresh *N. scutellarius* were washed with tap water and drained. Boiled *N. scutellarius* were prepared by immersion in 100^0C boiling water for 1 minute and cooled directly by immersion in iced water and then drained.[18] The fresh and boiled leaves were finely cut up and homogenized separately using a blender for 3 minutes.

A standard diet containing fresh *N. scutellarius* leaves were prepared by mixing the homogenized leaves into a standard “semi-purified rodent diet” (69.03% carbohydrate, 13.65% protein, 2.01% fat, and 317.59 kcal energy per 100 g) with a composition of 33.3% (1/3) and 66.7% (2/3), respectively. The mixture was then formed into pellets (Fig.1c). The preparation of a standard diet containing boiled

N. scutellarius follows the same procedure (Fig.1d). The percentage of *N. scutellarius*, i.e., one-third of daily diet intake, was compose based on the My Plate (Piringku) guide from the Indonesian Ministry of Health for a healthy diet.

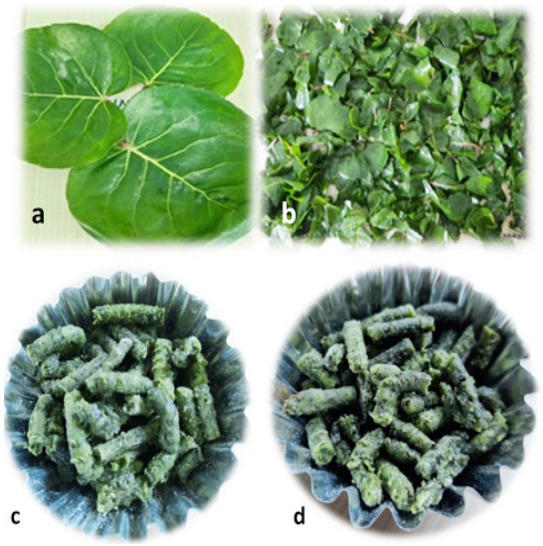

Fig.1. Fresh (a) and boiled (b) *N. scutellarius* leaves, and pellets of a standard diet containing fresh (c) and boiled (d) *N. scutellarius*

Determination of Antioxidant Activity (DPPH) of *N. Scutellarius* Leaves

The fresh and boiled *N. scutellarius* leaves antioxidant activity was determined using the DPPH(2,2-diphenyl-1-picrylhydrazyl)method (Hatano *et al*.,1998).[19] A 0.1 g sample was transferred to a test tube that contained 5 mL of 60 μM DPPH in an ethanol solution, homogenized, and incubated at room temperature under low light conditions for 1 hour. The absorbance of the sample was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm.The percent antioxidant activity was expressed as percent inhibition according to the equation below:

$$\text{Inhibition (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control reaction (DPPH· and ethanol), and A_1 is the absorbance in the presence of substances.

Total Phenolic Determination in *N. Scutellarius* Leaves

Total Phenolic Content (TPC) was determined using the Folin-Ciocalteau assay (Senter et.al.,1989 from Suradi, 1998).[20] Dried leaves powder (20 g) of *N. scutellarius* was packed into a Soxhlet apparatus and extracted with 300 mL methanol at 60–65^{0}C for 3–4 h.The extract was filtered and the filtrate was concentrated under reduced pressure at 40^{0}C and dried.

One g of methanol extract was diluted until 100 mL with distilled water. One mL of diluted sample was transferred to a new tube, added with 5 mL of 2% Na_2CO_3, and incubated at room temperature for 10 minutes. Folin Ciocalteau reagent of 0.5 mL was added and then homogenized and stored at room temperature with low light for 30 minutes. The absorbance of the sample was measured at a wave length of 750 nm using a UV-VIS spectrophotometer (UV-1800 Shimadzu).

Total Flavonoid Determination of *N. Scutellarius* Leaves

Total flavonoid was determined using the spectrophotometer method (IKU/5.4/TF-UV-03). Mangkokan leaves were cut into small pieces, dried in an oven at 45^{0}C for 48 hours and processed into a simplicial powder. One hundred mg of sample was put in 10 mL test tube, added with 0.3 mL of 5% $NaNO_2$. After 5 minutes, 0.6 mL of 10% AlCl3 was added, stood for 5 minutes, then 2 mL of 1 M NaOH and distilled water were added until reaching 10 mL volume. The mixture was diluted 5 times and transferred into a cuvette. Absorbance was determined using UV-Vis spectrophotometer (UV-1800 Shimadzu) at a maximum wavelength of 510 nm.

Phytochemical (Chlorophyll and Carotenoid) Determination

For chlorophyll and carotenoid determination, 1 g of *N. scutellarius* leaves were weighed and mashed in a mortar, then extracted using 100 mL of 80% acetone and stirred until the chlorophyll and carotenoids dissolved (Kurniawan *et al*., 2010).[21] The extract was filtered, and the filtrate was measured using a spectrophotometer at wavelengths of 480, 646, and 663 nm. Total chlorophyll and carotenoid were calculated using the following formula :

Total Chlorophyll (mg/L) = (17,3 x A_{646}) + (7,18 x A_{663})
Total Carotenoid (µmol/L) = A_{480}+ (0.114 x A_{663}) – 0.638 x A_{646} x V.1000 / 112.5 x W

A_{480} : Absorbance in wavelength 480 nm
A_{646} : Absorbance in wavelength 646 nm
A_{663} : Absorbance in wavelength 663 nm
V : volume extract (mL)
W : sample weight (g)

High-Fat-High-Fructose (HFHFr) Diet

Our own HFHFr diet was formulated by mixing a standard "semi-purified diet" (69.03% carbohydrate, 13.65% protein, 2.01% fat, and 317.59 kcal energy per 100 g; code S00202001078) with beef tallow, egg yolk powder, high-fructose corn syrup, and water to obtain a homogeneous mixture. The mixture was formed into pellets and the pellets air-dried at 25^{0}C. The HFHFr diet pellets contain 31.46% fat, 41.95% carbohydrate, 11.93% protein, and 473.96 kcal energy/100 g diet. In addition to the HFHFr diet, during the induction of MetS, rats were also given 20% fructose (corn syrup) in drinking water (100 ml/day). This method was commonly used in previous studies to induce MetS in rats.[8,22]

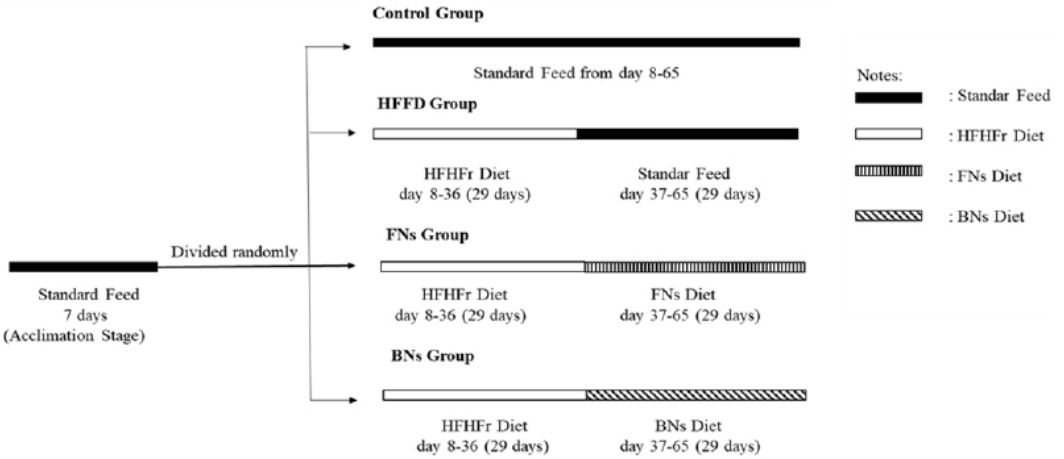

Fig.2. Diagram of the study design. Control group: rats were fed a standard diet (69.03% carbohydrate, 13.65% protein, and 2.01% fat, and 317.59 kcal energy per 100 g, code S00202001078) for 65 days; HFFD Group: rats were fed the standard diet for 7 days as an acclimatization stage, a HFHFr diet (31.46% fat, 41.95% carbohydrate, 11.93% protein, and 473.96 kcal energy/100 g diet) for 29 days with 20% fructose in drinking water, and then the standard diet for 29 additional days; FNs group: rats were fed the standard diet for 7 days for acclimatization, the HFHFr diet with 20% fructose in drinking water 29 days, and then standard diet containing fresh *N. scutellarius* for 29 days; BNs group: rats were fed the standard diet for 7 days as the acclimatization stage, the HFHFr diet with 20% fructose in drinking water for 29 days, and then standard diet containing boiled *N. scutellarius* for 29 days. Blood samples were taken at the end of MetS induction and intervention diets from all groups.

***In vivo* Experimental Design**

Twenty-four 12 week-old male Wistar rats with a mean weight of 150 ± 16 g were obtained from Animal House in Semarang, Indonesia and housed in individual cages with a standard 12 hour light/dark photoperiod at an environmental temperature of 24–25^{0}C. Rats were given 15 g/day standard diet (standard normal diet for a maintenance period of rats that contains 69.03% carbohydrate, 13.65% protein, and 2.01% fat, and 317.59 kcal energy per 100 g, modified from Reeves, 1997) and 100 ml/day water for seven days acclimatization period. After acclimatization, rats were divided randomly into four groups: i) control diet group (C), ii) high-fat high-fructose diet (HFFD), iii) fresh *N. scutellarius* (FNs), and iv) boiled *N. scutellarius* (BNs) with the

same average body weight in each group. The HFHFr diet was given 15 g/day accompanied by 20% fructose in drinking water (100 ml/day) to the HFFD, FNs, and BNs groups for 29 days. After 29 days of administration of HFHFr feeding, 3 mL of blood sampling via the retro-orbital plexus was carried out following an overnight fasting to obtain pre-test data.The HFFD, FNs, and BNs groups were given standard, fresh, and boiled feed containing *N. scutellarius* for 29 days after blood sampling. At the end of *N. scutellarius* diet, blood was resampled to obtain post-test data. Serum was obtained by centrifuging the blood at 4000 rpm for 15 minutes to separate the serum and it was stored at < (-15^{0}C) until analysis. Feed and fluid intake were recorded every day during the study. The animal experimental design is summarized in Figure 2.

Measures of Food and Fluid Intake in Rats

Intake of food and drink of experimental rats was recorded daily by weighing feed (g) and drink (mL) residue daily, and the intake was calculated by subtracting a fixed amount of given feed and drink with the residue. The daily intake during 29 days of MetS induction or after 29 days of diet intervention was calculated by averaging the daily data.

Measures of Metabolic Syndrome Components

Obesity

Obesity in rats was determined based on the Lee Index score; when the result is >300 the rat is categorized as obese.[23]

Lee Index = {Body weight (g)$^{1/3}$ / Naso-anal length (cm)} × 10^3

Fasting Blood Glucose Assay

Fasting glucose from blood serum was measured by the GOD-PAP method (DiaSys). After 12 hours of fasting, whole blood was drawn from the ophthalmic *venous plexus*. First, 10 µL of blood serum was mixed with 1000 µL glucose reagent and incubated at 20–25°C for 10 min. Absorbance against a blank was read within 60 min at a wavelength of 500 nm.

Triglyceride (TG) Assay

After 12 hours of fasting, rat blood samples were drawn from the venous retro orbitalis. Blood was centrifuged to separate the serum. Triglycerides were determined using the GPO-PAP method (DiaSys)[13]

Total Cholesterol (TC) Assay

Whole blood was obtained after 12 hours of fasting from the venous plexus retro orbitalis of rats and then centrifugated to separate the serum. TC was determined by the CHOD-PAP method (DiaSys)[13]

High Density Lipoprotein Cholesterol (HDL-C) Assay

Serum (0.02 mL) was added to 0.5 mL of sediment solution, then the mixture was centrifuged for about 20 minutes at 4500 rpm. The supernatant (0.01 ml) was mixed with 1 mL cholesterol reagent. HDL cholesterol was assayed using the CHOD-PAP (DiaSys) method by the same procedure as for TC.[13]

Low Density Lipoprotein Cholesterol (LDL-C) Assay

LDL was determined directly using the CHOD-PAP method and precipitant LDL. Serum of 100 µL was mixed with 1000 µL precipitating reagent and incubated for 15 minat room temperature, then centrifuged for 20 min at 2500 g. Within one hour after centrifugation, 100 µL of the clear supernatant was transferred to the reaction solution for determination of cholesterol.[13]

Malondealdehyde(MDA) Assay

Serum MDA was determined using the TBARS (2-thiobarbituric acid reactive substance)method.[24] A standard or serum sample of 50 µL was mixed with 750 µL phosphate acid and 250 µL TBA (*thiobarbituric acid*)and homogenized using a vortex. Distilled water(450 µL) was added and the sample heated for 60 minutes, after which it was cooled in an ice bath. The sample absorbance was read by spectrophotometry at 534 nm.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistic version 16 (IBM Corp.,Armonk,NY,USA), and differences were considered significant at $p < 0.05$. To analyze differences between mean values pre- and post-intervention, we used a paired Student's t-test for normally distributed data and a Wilcoxon Test for non-normally distributed data. To assess differences between means among groups, we used a one-way ANOVA for normally distributed data and a Kruskal–Wallis test for data that were not normally distributed.

RESULTS

Proximate Analysis of *N. Scutellarius*

The proximate composition of *N. scutellarius* is presented in Table 1. Moisture is the largest component of fresh *N. scutellariusl* Leaves,comprising 81.895 ± 0.075% per 100 g fresh weight. Crude fiber is the smallest component of fresh *N. scutellarius*, i.e., 1.05%.

Antioxidant Properties and Phytochemical Content of *N. Scutellarius*

Table2 shows the bioactive content of fresh and boiled leaves of *N. scutellarius*. Boiled leaves contain higher moisture, antioxidant activity, and total phenolic than fresh leaves. However, they were lower in total flavonoids, carotene, and chlorophyll than boiled leaves.

Table 1: Proximate Analysis of Fresh *N. scutellarius* Leaves (100 g)

Parameter	Result *
Protein (%)	3.07 ± 0.02
Fat (%)	1.83 ± 0.01
Crude Fiber (%)	1.05 ± 0.02
Moisture (%)	81.90 ± 0.08
Ash (%)	2.77 ± 0.02
Carbohydrate (%)	10.435 ± 0.065
Energy (Kcal/100 g)	70.49 ± 0.27

*average value of the two measurements
Moisture content using the oven method (SNI 01-2891-1992, point 5.1), Ash content according to method (SNI 01-2891-1992, 6.1), protein content using the Kjeldahl method (18-8-31/MU/SMMSIG), Fat content using the Soxhlet method (18-8-5/MU/SMMSIG, Weibull). Carbohydrate content by difference (18-8-9 /MU/SMM SIG). Crude fiber by the gravimetry method (18-11-111/MU/SMM SIG). Total calories were obtained by calculation. SNI, Standard National Indonesia for proximate determination method on the basis of AOAC.

Table 2: Antioxidant Properties of Fresh and Boiled *N.scutellarius* Leaves

	Fresh	Boiled
Moisture (%)	81.90 ± 0.08	86.81 ± 0.63
Antioxidant activity, DPPH (%)	55.52 ± 0.3*	61.64 ± 0.45*
Total Phenolic Content (mg GAE /100gr)	111.32 ± 0.43*	112.815 ± 0.045*
Total Flavonoid (Quercetin Equivalent /%w/w)	0.44	0.41
Total Chlorophyll (mg/100gr)	120.905 ± 0.345*	96.485 ± 0.565*
Total Caroten (ppm)	817.69 ±1.78*	787.67 ± 1.83*

*average value of the two measurements

Food, Fluid, and Energy Intake

During the induction of MetS, food intake in the control group was significantly higher than in the HFFD, FNs, or BNs groups ($p < 0.05$). Meanwhile, fluid intake in the control group was significantly lower than that in the HFFD, FNs, and BNs groups (Table 3). During the 29 days of *N.scutellarius*-containing normal diet feeding (with the HFFD group receiving the normal diet), food and fluid intake did not differ among groups($p > 0.05$).

Table 3: Food, Fluid, and Energy Intake of Rats after 29 Days Induction of Metabolic Syndrome and after 29-Days Intervention with *N. scutellarius* Diet

		Control	HFFD	FNs	BNs	p
Induction Metabolic Syndrome	Food Intake (g)	10.04 ± 0.55[a]	6.2 ± 3.84[b]	4.6 ± 0.90[b]	4.25 ± 0.50[b]	0.009 *
	Fluid Intake (mL)	29.00 ± 8.63[a]	59.00 ± 6.00[b]	56.40 ± 1.95[b]	60.75 ± 4.11[b]	0.026*
	Total Energy (kkal)	33.03 ± 1.74[a]	54.89 ± 1.74[b]	54.12 ± 5.45[b]	55,52 ± 4.03[b]	0.012*
Nothopanax s. diet	Food Intake (g)	11.4 ± 0.90	11.4 ± 1.67	10.4 ± 0.55	10.75.± 1.71	0.629
	Fluid Intake (mL)	26.8 ± 8.82	30.4 ± 7.13	25.0 ± 3.04	23.00 ± 0.82	0.534
	Total Energy (kkal)	36.20 ± 2.84[a]	36.20 ± 5.31[a]	24.79 ± 1.36[b]	26.44 ± 4.22[b]	0.002*

Notes: values are average of five replicates ± SD. * : Significant at $P < 0.05$.P : One-way ANOVA, LSD if data were normally distributed, Kruskal–Wallis and Mann–Whitney if not.
HFHFr diet: High-Fat High-Fructose diet accompanied by 20% fructose drinking water for 29 days induction of metabolic syndrome.
Intervention diet: Standard normal diet in C and HFFD, *N. scutellarius*-containing standard diet in FNs and BNs (29 days).

Energy intake of HFFD, FNs, BNs during MetS is significantly higher ($p<0.05$) than the control group that receives only standard diet during the whole experiment. After 29 days of diet intervention, energy intake FNs, BNs dropped significantly ($p<0.05$) and they were significantly different than control and HFFD groups.

Effect of an *N. Scutellarius*-Containing Diet on Body Weight and Lee Index Scores

After 29 days of induction of MetS with an HFHFr diet in the HFFD, FN, and BN groups, their body weights were not significantly increased nor different ($p = 0.364$) to those of the normal control diet group (Table 4). After 29 days of intervention with a normal diet in the HFFD group and with *N. scutellarius*-containing diet administration in the FNs and BNs groups, body weight of the FNs BNs groups declined significantly ($p < 0.05$) compared with the control or HFFD groups. Meanwhile, body weight of the control and HFFD groups increased significantly ($p>0.05$). The Lee Index score showed a similar pattern to body weight, with the HFFD group showing a significant increase ($p = 0.010$) post-intervention.

Effect of an *N. Scutellarius*-Containing Diet on Fasting Blood Glucose (FBG)

After induction of MetS, the FBG level in HFFD, FNs, and BNs increased significantly compared with the normal control group ($p = 0.009$). After 29 days of intervention, the FBG level in the HFFD, FNs, and BNs groups decreased significantly, while a significant reduction occurred in the FNs and HFFD groups ($p< 0.05$) (Table 4).

Effect of an *N. Scutellarius*-Containing Diet on *Malondialdehyde* (MDA)

Malondialdehyde (MDA) is one of the lipid peroxidation products commonly used as a biomarker of oxidative stress. After induction of MetS with the HFHFr diet, the MDA level in the three groups increased significantly compared with the normal control diet. After 29 days of intervention, the MDA content of the FNs and BNs groups decreased significantly

(p=0.000). Meanwhile, that of the HFFD group increased slightly (p=0.10) to levels similar to those of the normal control group. When the post-intervention values of MDA were compared, those of the HFFD group remained high, while those of the FNs and BNs groups had decreased significantly (p=0.000) (Table 4).

Table 4: Body Weight, Lee Index Score, and Serum Profile of Rats Before and After 29-Day N. scutellarius Diet Intervention

		Groups				P
		Control	**HFFD**	**FNs**	**BNs**	
Body	Pre	188.20±18.83	178.20±9.09	194.80±7.46	197.60±24.95	0.364
Weight	Post	210.40±10.79	199.20±9.89	193.80±7.19	187.60±29.49	0.299
(g)	Δ	22.20±9.63[a]	21.00±6.44[a]	- 1.00±0.70[b]	-10.00±21.44[b]	0.001*
	p	0.007*	0.002*	0.034*	0.356	
Lee	Pre	307.60±15.99	318.40±8.20	320.00±5.70	316.40±3.36	0.413
Index	Post	317.60±11.89	328.60±10.11	319.40±5.69	313.60±8,08	0.110
Scores	Δ	10.00±17.27[a]	10.20±5.02[a]	- 0.60±0.55[b]	- 2.8±5.72[b]	0.026*
	p	0.265	0.010*	0.070	0.335	
Fasting	Pre	113.50±14.78[a]	160.46±15.00[b]	151.02±22.38[b]	132.32±16.39[c]	0.009*
Glucose	Post	127.44±37.50	122.80±14.72	123.30±10.74	118.76±16.45	0.944
(mg/dL)	Δ	13.94±39.56[a]	-37.66±10.99[b]	27.72±11.72[b]	-13.56±21.64[c]	0.010*
	p	0.475	0.002*	0.006*	0.234	
TG	Pre	69.67±2.22[a]	183.05±2.90[b]	180.79±1.69[b]	185.56±2.01[c]	0.000*
(mg/dL)	Post	73.50±13.89[a]	185.42±4.38[b]	120.15±2.91[c]	130.25±3.17[d]	0.000*
	Δ	38.32±0.90[a]	2.37±2.77[a]	60.65±4.44[b]	55.03±4.36[b]	0.002*
	P	0.001*	0.128	0.000*	0.000*	
Total	Pre	78.03±1.38[a]	118.87±2.42[b]	122.40±1.36[b]	120.99±3.18[b]	0.000*
Cholesterol	Post	79.22±1.99[a]	123.45±2.26[b]	96.16±2.86[c]	98.36±2.52[c]	0.000*
(mg/dL)	Δ	1.19±0.79[a]	4.59±2.88[a]	- 26.25±4.11[b]	- 22.64±3.19[b]	0.000*
		0.011*	0.024*	0.000*	0.000*	
HDL-Chol	Pre	82.31±1.08[a]	31.02±1.77[b]	31.84±1.01[c]	32.24±2.29[b]	0.000*
(mg/dL)	Post	80.61±2.07[a]	28.24±1.71[b]	69.47±1.21[c]	55.12±1.98[d]	0.000*
	Δ	-1.69±1.23[a]	-2.78±1.06[a]	37.63±1.09[b]	22.28±2.31[c]	0.000*
	p	0.037*	0.004*	0.000*	0.000*	
LDL-Chol	Pre	22.42±1.26[a]	68.93±1.99[b]	67.96±1.79[b]	67.68±1.79[b]	0.000*
(mg/dL)	Post	24.29±2.75[a]	70.29±2.94[b]	34.33±3.12[c]	48.90±1.68[d]	0.000*
	Δ	1.87±2.15[a]	1.36±1.12[a]	-33.63±3.06[b]	-18.78±3.14[c]	0.000*
	p	0.125*	0.053*	0.000*	0.000*	
MDA	Pre	1.13±0.28[a]	9.12±0.24[b]	9.10±0.32[b]	9.12±0.29[b]	0.000*
(nmol/dL)	Post	1.34±0.22[a]	9.42±0.24[b]	3.04±0.28[c]	3.62±0.38[d]	0.000*
	Δ	0.22±0.72[a]	0.29±0.15[a]	- 6.07±0.49[b]	- 5.50±0.32[c]	0.001*
	p	0.003*	0.010*	0.000*	0.000*	

P: One-way ANOVA if data were normally distributed and Kruskal–Wallis test if not normally distributed
p: Paired t-test if data were normally distributed and Wilcoxon if not normally distributed
*: significant
Pre = before intervention diet, when rats were fed high-fat, high-fructose (HFHFr) diet accompanied by 20% fructose drink for 29 days to induce MetS
Post = after intervention diet,rats were fed standard normal diet in C and HFFD, fresh or boiled *N. scutellarius*-containing standard diet in FNs and BNs respectively for 29 days

Effect of an *N. scutellarius*-Containing Diet on Lipid Profiles

After 29 days of induction of MetS with an HFHFr diet, TG, LDL-C, and TC had increased, but HDL-C had decreased in all groups. After 29 days of intervention, the TG level in the FNs and BNs groups decreased significantly ($p > 0.05$) but there was a slight increase in the control and HFFD group. Total cholesterol (TC) in the FNs and BNs groups decreased significantly after the intervention ($p = 0.000$); meanwhile, the levels of TC in the control and HFFD groups increased slightly but significantly ($p < 0.05$). The LDL-C levels in all groups decreased significantly; this decrease was greater in the FNs and BNs groups ($p < 0.05$).

Serum HDL-C in the control and HFFD groups decreased significantly, but that in the FNs and BNs groups increased significantly ($p = 0.000$). Overall, the FNs and BNs groups showed significant changes in the serum lipid profile by a decrease in TG, TC, and LDL-C, but an increase in HDL-C, whereas the FNs group showed a higher effect than the BNs group.

Discussion

Our results in Table 2 showed that boiling has slightly increased total antioxidant values of *N. scutellarius* with no changes in the content of total phenolic and flavonoid, and a decrease in the total chlorophyll and carotenes. Boiling is a common cooking method used to prepare ready to eat vegetables, and it can affect their chemical composition. It can either decrease or increase the bioactive content and hence the total antioxidant properties, depending on the method and duration of cooking.[25-30]

Induction of metabolic syndrome (MetS) by feeding a high-fat high fructose diet accompanied by fructose drinking water (HFHFr diet) in rats for 29 days can fulfill three out of the five criteria of MetS (hyperglycemia, hypertriglyceridemia, decreased HDL-C, hypertension, and obesity). Obesity in rats measured by Lee Index is the criterion that was not met due to insignificant body weight gain after feeding HFHFr diet for 29 days, while hypertension is not measured. Body weight and Lee Index Scores from groups that were fed with HFHFr diet were not significantly different from control groups. Therefore, although the average values of Lee Index are slightly higher than 300 it cannot be classified as obese. During 29 days of MetS induction, rats from the HFFD,FNs, and BNs groups tended to drink more (20% fructose water), so their food intake decreased. As food intake decreased so does total energy intake and hence no body weight gain. Similar studies found that 20% fructose in drinking water was associated with lower food intake compared with rats that consumed plain water (Khalid *et al*. 2019).[31] Many other studies employing high fructose or sucrose enriched diet in rats requires 8-16 weeks to produce obese rats .[8,32-34] As the duration of our MetS induction is 29 days (approximately 4 weeks), it requires longer duration to induce obesity in rats. However, such shorter period of MetS induction has produced other metabolic syndrome components (fasting glucose-FG, low HDL-C, high TG) being met (Table 4) as an early signs of the syndrome.

After 29 days of intervention with a normal diet in the HFFD group and an *N. scutellarius*-containing normal standard diet in the FNs and BNs, the body weight of all groups was not significantly different ($p<0.05$) (Table 4). Lee Index Scores (as a representation of obesity in rats) are similar to body weight trend, they are not significantly different among groups. This is as consequence of Lee Index determination that depends on body weight which is not different for all groups after diet intervention.

Induction of metabolic syndrome (MetS) by feeding HFHFr diet in rats for 29 days increased significantly ($p>0.05$) fasting blood glucose (FBG) level in the HFFD, FNs, and BNs groups compared to control group (Table 4). After 29 days of intervention diet, the FBG level decreased reaching the same values as the control group ($p>0.05$), and the difference (Δ) between before and after the intervention was significant in the HFFD and FNs groups ($p<0.05$). Our results were similar to Lopez *et al*. (2019), who found that FBG from the group receiving *Moringa oleifera* leaf powder (orally by gavage) for 3 weeks decreased but was not significantly different from that of the MetS control group.[35] The study also showed that oral glucose tolerance test of the intervention diet with *M. olifiera* is significantly higher than MetS control, indicating that inspite of no difference in FBG there is an improvement in glucose tolerance.[35] Our study indicates the role of *N. scutellarius* in the normal diet in normalizing FBG

level, as 29 days of FNs diet intervention is able to improve FBG level reaching the same values as the control group with standard diet.

Phytochemical compounds that have hypoglycemia effects most probably play a role in this result. *N. scutellarius* was given as part of a daily normal standard diet that went through the digestive system with various digestive enzyme activities and microbiota can affect their bioactive compounds.[36] This possibility warrants further studies. An important note that can be underlined in this study is that intervention with a normal diet or lower energy intake in the HFFD group could reduce the FBG level. Other previous studies showed that reduction in energy intake can reduce FBG in healthy and Type-2 DM. The reduction in FBG by energy intake a reduction is mediated by an improvement of pancreatic beta cells that improving insulin sensitivity, reduction of glucose metabolism hormone i.e. adiponectin and leptin level.[37-39]

The decrease in FBG level in the HFFD group also indicated that in addition to reducing calorie intake, the nutritional composition of the food that was ingested could also be influential. During the induction of MetS, the energy source is mostly derived from simple carbohydrates (fructose from drinking water and in the diet) and also comes from fat. After the HFFD group was given a standard normal diet with balanced nutrients composition and no fructose solution, the FBG levels decreased. Therefore, the reduction of calorie intake with a proper macronutrient composition has been proven to be able to control FBG levels.

Induction of MetS by HFHFr diet in this study increased serum TG significantly (Table 4), and diet intervention reduced significantly serum TG in FNs and BNs ($p<0.05$). In HFFD group, normal diet intervention without *N. scutellarius* could not decrease serum TG level ($p>0.05$), suggesting the importance of *N. scutellarius* component in the diet intervention.

MetS induction for 29 days did not reach the *hypercholesterolemic* state (Total cholesterol >129,52 mg/dL)[40] although LDL-C and TC levels in HFFD, FNs, and BNs groups are significantly higher than the control group (Table 3). A possible factor could be due to the absence of pure cholesterol and cholic acid in HFHFr diet. A study by our group (Sukowati *et al*., 2018) and many other groups using high-fat high cholesterol diet with cholic acid in the diet for 7 weeks can increase TC and LDL-C up to 219,8 mg/dL and 165.1 mg/d respectively. Many other studies using high-fat high sugar diet in rats require 8-16 weeks to produce dyslipidemia (Wong *et al*, 2016).[8] These results together indicated that dyslipidemia requires long exposure of high-fat high sugar diet with the presence of exogenous pure cholesterol and the aid of cholic acid in the diet. Results from this study confirmed that 29 days of intake ofan *N. scutellarius*-containing diet can ameliorate the serum lipid profile whereby total energy reduction and antioxidants from *N. scutellarius* in the intervention diet may play a major role in the lipid profile improvement.

The antioxidant activity of chlorophyll and carotenoids as natural pigments and other phytonutrients in the leaves of *N. scutellarius* could assist in reducing serum cholesterol levels through pathways such as preventing the absorption of cholesterol in the presence of saponins, improving liver function to reduce blood cholesterol through bile acid formation, binding of fat and cholesterol by phytonutrients such as phytol and eliminating them from the digestive tract via the excretory system[41] and inhibition of the activity of key enzymes of cholesterol synthesis, i.e., the enzyme HMG-CoA reductase.[42]

Flavonoid in *N. scutellarius* improved the lipid profile, possibly by modulating the activity of different enzymes that are involved in lipid metabolism and the expression of transcription factors involved in TG and cholesterol synthesis, such as the sterol regulatory element-binding proteins i.e. SREBP-1 and SREBP-2.[43] Flavonoids also inhibit the expression of the fatty acid synthase (FAS) enzymes which is associated with hepatic TG secretion, by stimulating AMPK activity in hepatocyte cells.[44] Increased HDL levels could be due flavonoid that induced increased activity of LCAT (lecithin-cholesterol acyl transferase). Increased activity of the LCAT enzyme can lead to an increase in HDL cholesterol.[45] Flavonoids also play a role in increasing the production of Apo A1, which acts as an enzyme cofactor for LCAT as well as a ligand for interaction with lipoprotein receptors in HDL tissue. An increase in Apo A1 can lead to elevation of serum HDL-C levels.[46]

Inclusion of *N. scutellarius* in a normal diet can successfully prevent oxidative stress indicated by a significant decrease of serum MDA level ($p<0.05$) (Table 4). Fresh *N. scutellarius* inclusion had a greater effect on reducing MDA production than boiled *N. scutellarius*,as evidenced by the MDA reduction in FNs group was greater than that in the BNs group ($p<0.05$). This was possibly due to a higher total antioxidant property in fresh than boiled *N. scutellarius* as shown in Table 2. It is interesting to note that after diet intervention the MDA level of rats in HFFD group is significantly higher than before intervention ($p=0.010$), and significantly the highest among groups. This suggests the importance of *N. scutellarius* as a component in the normal diet to reduce serum MDA levels. Dyslipidemia as part of MetS component can trigger a series of a chain reactions from endothelial dysfunction, LDL-C oxidation, vascular inflammation indicated by increase circulating TNFalpha, and foam cell formation that increase reactive oxygen species (ROS) production and lead to cellular oxidative stress.[47-51]

A diet containing fresh *N. scutellarius* is more effective at decreasing body weight, TG, LDL-C, FBG, and MDA levels and increasing HDL-C levels than one containing boiled *N. scutellarius*. Fresh *N. scutellarius* gave a greater effect than that prepared by boiling. The reason why a diet containing fresh leaves was more effective was probably due to higher total antioxidative properties from its flavonoid, chlorophyll, and carotene contents that counter oxidative stress. Furthermore, the moisture content (86,81%) of boiled *N. scutellarius* is higher than that of fresh *N. scutellarius*; therefore, with the same amount of leaves in the diet (1/3 of the daily normal diet), fresh *N. scutellarius* leaves contain more antioxidant compounds than boiled *N. scutellarius*.

Conclusion

N. scutellarius has antioxidant properties due to its phenolic, flavonoid, chlorophyll, and carotenoid contents. Intake of *N. scutellarius* leaves as part of normal diet (at 33.3% of the daily diet) for 29 days in MetS rats can significantly correct the level of fasting FBG, decrease serum TG, TC, LDL-C, and MDA levels, and increase serum HDL-C. Intake of fresh *N. scutellarius* leaves as part of the diet was more effective than that of boiled *N. scutellarius* leaves at improving the serum lipid profile and MDA levels.

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Conflict of Interest

The authors do not have any conflict of interest.

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