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Antioxidant Total and HOMA-IR of Diabetic Rats Given Crocatum piper and Andrographis paniculata Leaf Extracts

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Abstract

Background: type-2 diabetes mellitus (T2DM) is a silent killer which the prevalence continues to increase in every year. Increased oxidative stress occurs in T2DM. Red betel and bitter herb leaf extracts (RBBH) contain a lot of antioxidants. This combination is expected to provide better safety than if used singly because the content of andrographolid in bitter herb has effect such as nausea, vomiting, loss of appetite, and antifertility if consumed in high doses.

Objective: the study aimed to prove the effect of red betel and bitter herb leaf extracts on antioxidant total and *Homeostasis Model Assessment of Insulin Resistance* (HOMA-IR) in T2DM rats given high-fat diet and *Streptozotocin* (STZ) induction.

Methods: experimental randomized study with pre-post-test control group design using 25 *Sprague Dawley* male rats. T2DM model was conducted by providing high-fat feed for 14 days and induction of *Streptozotocin-Nicotinamide*, then given a combination of red betel and bitter herb leaf extracts at doses of 237.5 mg/kg BW, 225 mg/kg BW, and 212.5 mg/kg BW for 21 days. The measurement of antioxidant total used 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) method. HOMA-IR measured by ELISA (Enzyme-linked immunosorbent assay) method using insulin level equation and fasting blood glucose level measured by *glucose oxidase-peroxidase aminoantrypirin* (GOD-PAP). Data analysis used paired t-test, wilcoxon test, and ANOVA test to analyze differences in antioxidant total and HOMA-IR value among groups and followed by Bonferroni post-hoc test.

Results: all treatments could reduce HOMA-IR and significantly increase antioxidant total ($p<0.05$). The most decrease in HOMA-IR and increase in antioxidant total at dose 237.5 mg/kg BW of red betel and bitter herb leaf extracts.

Conclusion: the combination of red betel and bitter herb leaf extracts with dose 237.5 mg/kg BW, 225 mg/kg BW, and 212.5 mg/kg BW can improve blood glucose, insulin, and HOMA-IR levels in type-2 diabetes mellitus rats.

Keywords: *T2DM; red betel leaf extract; bitter herb leaf extract; HOMA-IR; antioxidant total*
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INTRODUCTION

Diabetes mellitus (DM) is one of the health problems with high mortality and morbidity rates in the world including in Indonesia. It is classified in degenerative disease as silent killer and most people do not realize they suffer until complications occur.[1]

The International Diabetes Federation (IDF) reported that in 2017 there were 425 million people in the world have diabetes. Indonesia ranks the sixth highest after China, India, America, Brazil, and Mexico. That number is expected growing up to 629 million people in 2045 and 90% of these cases are type-2 diabetes mellitus (T2DM).[2]

Insulin resistance is the failure of tissues to respond the insulin activity, as well as that is a strong predictor of T2DM and hyperinsulinemia.[3] Insulin resistance can be determined by examining the *Homeostasis Model Assessment of Insulin Resistance* (HOMA-IR) of fasting

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blood glucose and fasting insulin levels.[4] The increasing of *reactive oxygen species* (ROS) formation in hyperglycemia or diabetes mellitus results in oxidative stress.[5] High levels of ROS can reduce antioxidant total. Prediabetic patients have an increase in oxidative stress characterized by a decrease in antioxidant total compared with healthy patients.[6] Antioxidants can protect body from negative effects of oxidants, free radicals, and inflammation by counteracting ROS and preventing the formation of ROS.[7]

Therapy for DM like insulin injection and oral antidiabetic which have side effects such as dizziness, nausea, and anorexia make patients switch it to natural treatment from herbal plants.[8] Medicinal plants claimed as T2DM treatment include red betel (*Piper crocatum*) and bitter herb (*Andrographis paniculata*).[9-11] Red betel leaf extract 200 mg/kg BW can reduce blood glucose levels in diabetic rats. Red betel leaf extract contains high antioxidant like flavonoid which acts as antihyperglycemic agents.[9] Bitter herb leaf extract with a dose of 200 mg/kg BW for 10 days shows a significantly decrease in fasting blood glucose.[10] This combination is expected to provide better safety than if used singly because the content of andrographolid in bitter herb has effect such as nausea, vomiting, loss of appetite, and antifertility if consumed in high doses.[12] A combination of two ingredients like bitter herb and red betel is carried out to provide safety from the effects of consuming bitter leaves without reducing its benefits. This study aimed to determine the combination of red betel and bitter herb leaf extracts against antioxidants total and HOMA-IR in type-2 diabetes mellitus rats..

MATERIALS AND METHODS

Plant material

Red betel (*Piper crocatum*) leaf obtained from Tawangmangu, Karang Anyar Regency, Central Java on October 2018. The extracts of bitter herb (*Andrigraphis paniculata*) obtained from PT. Industri Borobudur Herbal Medicine, Central Java. Red betel leaf was tested for substance identification at the Plant Systematics Laboratory, Faculty of Biology, University of Gadjah Mada, Yogyakarta.

Chemicals and reagents

Streptozotocin (STZ) and *nicotinamide* (NA) obtained from Nacalai Tesque, Japan. The Antioxidant Total Kit purchased from Randox Laboratory, England. The Rat INS kit (Insulin) brand "Fine Test" and blood glucose kit brand "Diasys" obtained from Holzheim, Germany.

Preparation of *Piper crocatum* leaf extract

Extracts made by maceration method using 70% ethanol solvent. The leaves were thinly sliced and dried with good air circulation and were not directly exposed to sunlight. After dried, the leaves were mashed then weighed and put into a container for maceration. Maceration using 70% ethanol solvent with ratio 1:10 for two days protected from sunlight, while stirring, filtered with filter paper to get macerata. The pulp was macerated using 70% ethanol by same procedure. All maceratas were combined and evaporated with *vacuum rotary evaporator* at 40°C until thick ethanol extract obtained and dried with *freeze dryer* to get dry extract (powder).[13,14]

Animal laboratory

Sprague Dawley male rats aged 8-10 weeks, weight 150-200 gram were obtained from the Center for Food and Nutrition Studies Laboratory of Gadjah Mada University, Yogyakarta and placed in individual stainless-steel cages. Room temperature was around 24-27°C and humidity 60-65% with adequate ventilation and lighting using 12 hours cycle of light and dark. Rats fed 20 g of *comfeed II* per day and given water by *ad-libitium*. Caring procedures and research treatments were adjusted to the ethics of experimental animal research at the Center for Food and Nutrition Studies Laboratory of Gadjah Mada University, Yogyakarta.

Design experiments

The study used true experimental study with *randomized pre-post-test with control group design*. Twenty five animals (rats) were divided into 5 groups randomly, such as negative control group (healthy rats) (K1), positive control group (T2DM conditioning without treatment) (K2), combined intervention of red betel and bitter herb leaf extracts 237.5 mg/kg BW (P1), combined intervention of red betel and bitter herb leaf extracts 225 mg/kg BW (P2), combined intervention of red betel and bitter herb leaf extracts 212.5 mg/kg BW (P3).[15] The distribution of RBBH dose combinations based on modification from Thirumalai et al. (2014) showed that effective dose of red betel leaf extract on reducing cholesterol level and Akhtar et al. (2016) showed that effective dose of bitter herb on restoring metabolic profile of obesity with diabetes. The Comparisons of red betel and bitter herb doses were respectively 187.5 mg/kg BW and 50 mg/kg BW (75%:25%) on dose 1, 125 mg/kg BW and 100 mg/kg BW (50%:50%) on dose 2, and 62.5 mg/kg BW and 150 mg/kg BW (25%:75%) on dose 3.

Rats were acclimatized for a week and then given high-fat feed for 14 days, then induced by *Streptozotocin* 55 mg/kg BW and *nicotinamide* 100 mg/kg BW intraperitoneally. Experimental animals were categorized as diabetes mellitus if their blood glucose level more than 200 mg/dl.[16] Technical of extracts administration used feeding tube for 21 days in a row. The study has obtained Ethical Clearance approval from the Health Research Ethics Commission of Faculty of Medicine Universitas Diponegoro (Dr. Kariadi Hospital Semarang) with registered number 114/EC/H/FK-RSDK/X/2018.

Blood sampling

Blood sampling was conducted after 3 days of STZ induction and 21 days after intervention. Rats were fasted overnight before blood was drawn through *orbital plexus*. Blood samples were centrifuged at 4000 rpm for 15 minutes and then serum was taken to analyze antioxidant total, insulin, and glucose levels.

HOMA-IR value

HOMA-IR value was obtained from analysis of insulin and blood glucose levels with the following

formula, HOMA-IR = {fasting glucose (mg/dl) x fasting insulin (μU/ml)}/405.

Statistical analysis

Data were analyzed computerization and displayed in mean±SD. The number of sample was less than 50, so normality test using *Shapiro-Wilk*. The different test of antioxidant total and HOMA-IR value before and after intervention used *paired t-test*, while different test of antioxidant total and HOMA-IR value among groups used *one-way* ANOVA continued with *Bonferroni post-hoc* test. Significant value was stated if $p<0.05$.

RESULTS

Characteristics of experimental animal

The study used 25 *Sprague Dawley* male rats. There were no sick or died rats in this study. The mean of feed intake in all groups did not increase before or after the administration of intervention, while all intervention groups showed that there was an increase in body weight. The analysis results of intervention effect on feed intake showed in Table 1 and intervention effect on body weight could be seen in Table 2.

Blood glucose level

All intervention groups showed significantly decreased in blood glucose level compared to control group (Table 3). The combined intervention of red betel and bitter herb leaf extracts 237.5 mg/kg BW (P1) showed the highest decreased in blood glucose level. *Paired t-test* showed that there were differences in blood glucose levels before and after intervention ($p<0.05$).

1. Insulin level

All intervention groups showed significantly decreased in insulin level. The combined intervention of red betel and bitter herb leaf extracts 237.5 mg/kg BW (P1) showed the highest decreased in insulin level. *Paired t-test* showed that there were differences in insulin levels before and after intervention ($p<0.05$).

2. HOMA-IR value

All intervention groups showed significantly decreased in HOMA-IR value. The combined intervention of red betel and bitter herb leaf extracts 237.5 mg/kg BW (P1) showed the highest decreased in HOMA-IR value. *Paired t-test* showed that there were differences in HOMA-IR value before and after intervention ($p<0.05$).

Table 1. Mean of feed intake changes before and after RBBH intervention

Group	Feed intake (g)		%Δ	*p*	*p'*
	pre-intervention	post-intervention			
Healthy rats	16.4±1.31	18.7±1.14	14.0	0.036*	
T2DM rats + no treatment	17.4±1.73	18.4±2.18	5.7	0.317	
T2DM rats + 237.5 mg/kg BW RBBH	16.5±1.05	16.2±2.80	-1.8	0.838	0.768
T2DM rats + 225 mg/kg BW RBBH	17.6±2.04	18.4±2.18	5.1	0.619	
T2DM rats + 212.5 mg/kg BW RBBH	18.2±0.97	16.9±3.43	-7.1	0.516	

RBBH: combined intervention of red betel and bitter herb leaf extracts. Values are shown as mean±SD (n = 5 rats/group). %Δ: the percentage of changes between pre and post-intervention. *p*: *paired t-test*. *p'*: *one-way* ANOVA test followed by *Bonferroni* post-hoc test. *: significant value at $p<0.05$

Table 2. Mean of body weight changes before and after RBBH intervention

Group	Body weight (g)		%Δ	*p*	*p'*
	pre-intervention	post-intervention			
Healthy rats	202 (187-213)	214 (203-226)	9.4	0.043*	
T2DM rats + no treatment	190 (180-224)	182 (156-194)	-22.6	0.043*	
T2DM rats + 237.5 mg/kg BW RBBH	219 (190-240)	228 (190-247)	6,4	0.500	0.011*
T2DM rats + 225 mg/kg BW RBBH	231 (203-258)	243 (230-264)	5.2	0.042*	
T2DM rats + 212.5 mg/kg BW RBBH	204 (195-225)	221 (204-236)	8.8	0.042*	

RBBH: combined intervention of red betel and bitter herb leaf extracts. Values are shown as median (minimum-maximum) (n = 5 rats/group). %Δ: the percentage of changes between pre and post-intervention. *p*: *paired t-test*. *p'*: *one-way* ANOVA test followed by *Bonferroni* post-hoc test. *: significant value at $p<0.05$

Table 3. Mean of blood glucose level changes before and after RBBH intervention

Group	Blood glucose levels (mg/dl)		%Δ	*p*	*p'*
	pre-intervention	post-intervention			
Healthy rats	55.1±20.93	57.3±2.04	4.0	0.812	
T2DM rats + no treatment	465.5±55.71	374.8±6.23	-19.5	0.024*	
T2DM rats + 237.5 mg/kg BW RBBH	481.6±35.58	127.3±2.36	-73.6	0,000*	0,000*
T2DM rats + 225 mg/kg BW RBBH	466.8±43.37	136.9±1.72	-70.7	0,000*	
T2DM rats + 212.5 mg/kg BW RBBH	457.1±69.73	150.7±3.21	-67.0	0,001*	

RBBH: combined intervention of red betel and bitter herb leaf extracts. Values are shown as mean±SD (n = 5 rats/group). %Δ: the percentage of changes between pre and post-intervention. *p*: *paired t-test*. *p'*: *one-way* ANOVA test followed by *Bonferroni* post-hoc test. *: significant value at $p<0.05$

Table 4. Mean of insulin level changes before and after RBBH intervention

Group	Insulin level (µU/ml)		%Δ	*p*	*p'*
	pre-intervention	post-intervention			
Healthy rats	17.0±0.19	16.8±0.23	1.2	0.012*	
T2DM rats + no treatment	12.2±0.21	12.1±0.15	1.6	0.022*	
T2DM rats + 237.5 mg/kg BW RBBH	12.2±0.29	15.0±0.11	23.0	0,000*	0,000*
T2DM rats + 225 mg/kg BW RBBH	12.4±0.25	14.4±0.33	16.1	0,001*	
T2DM rats + 212.5 mg/kg BW RBBH	12.5±0.14	12.4±0.34	0.0	0.827	

RBBH: combined intervention of red betel and bitter herb leaf extracts. Values are shown as mean±SD (n = 5 rats/group). %Δ: the percentage of changes between pre and post-intervention. *p*: *paired t-test*. *p'*: *one-way* ANOVA test followed by *Bonferroni* post-hoc test. *: significant value at $p<0.05$

Table 5. Mean of HOMA-IR value changes before and after RBBH intervention

Group	HOMA-IR value		%Δ	*p*	*p'*
	pre-intervention	post-intervention			
Healthy rats	2.3±0.87	2.4±0.08	4.3	0.869	
T2DM rats + no treatment	14.1±1.90	11.2±0.23	-21.3	0.022*	
T2DM rats + 237.5 mg/kg BW RBBH	14.5±1.06	4.7±0.09	-67.6	0,000*	0,000*
T2DM rats + 225 mg/kg BW RBBH	14.3±1.31	4.9±0.10	-65.7	0,000*	
T2DM rats + 212.5 mg/kg BW RBBH	14.1±2.19	4.6±0.13	-67.4	0,001*	

RBBH: combined intervention of red betel and bitter herb leaf extracts. Values are shown as mean±SD (n = 5 rats/group). %Δ: the percentage of changes between pre and post-intervention. *p*: *paired t-test*. *p'*: *one-way* ANOVA test followed by *Bonferroni* post-hoc test. *: significant value at $p<0.05$

Table 6. Mean of antioxidant total level changes before and after RBBH intervention

Group	Antioxidant total level		%Δ	*p*	*p'*
	pre-intervention	post-intervention			
Healthy rats	1.8±0.31	1.6±0.26	-11.1	0.280	
T2DM rats + no treatment	0.5±0.13	0.3±0.15	-40.0	0.146	
T2DM rats + 237.5 mg/kg BW RBBH	0.5±0.22	1.2±0.28	140.0	0,001*	0,000*
T2DM rats + 225 mg/kg BW RBBH	0.6±0.18	0.8±0.12	33.3	0.034*	
T2DM rats + 212.5 mg/kg BW RBBH	0.4±0.23	0.7±0.10	75.0	0.012*	

RBBH: combined intervention of red betel and bitter herb leaf extracts. Values are shown as mean±SD (n = 5 rats/group). %Δ: the percentage of changes between pre and post-intervention. *p*: *paired t-test*. *p'*: *one-way* ANOVA test followed by *Bonferroni* post-hoc test. *: significant value at $p<0.05$

Antioxidant total level

All intervention groups showed significantly increased in antioxidant total level mean. The highest increased in antioxidant total level mean occurred in group with combined intervention of red betel and bitter herb leaf extracts 237.5 mg/kg BW (P1). *Paired t-test* showed that there were differences in antioxidant total levels before and after intervention ($p<0.05$).

DISCUSSION

High-fat feed is given to every rat as much as 20 grams for 14 days. High-fat feed for 2 weeks showed an increase in body weight.[17] High-fat feed was also increase percentage of body fat and risk of insulin resistance.[18] The induction of STZ-NA in experimental animals was conducted on day-15. *Streptozotocin* induction caused damage to pancreatic beta cells. *Nicotinamide* was given to slowing down damage, preventing damage, and preventing total damage to pancreatic beta cells.[19] High-fat feed and STZ-NA induction in T2DM conditioning could form obesity and insulin resistance.[16,17]

During the study, weight of rats was weighed every week. The weight of treated rats increased and approached to healthy control group body weight. While, rats in control group had weight loss. This has happened because control group (K2) was in T2DM conditioning group which one of its characteristics was have drastically weight loss due to glycogenolysis and lipolysis which caused decrease in muscle mass and fat tissue. Whereas, the weight of treatment groups has increased because it was able to repair pancreatic beta cells so that it can increase insulin production. Improved insulin availability in tissue triggers an increase in fat tissue and muscle mass, thereby affecting weight gain.[9,20] The administration of red betel leaf extract for 21 days could increase body weight.[20]

High-fat feed and STZ induction have impact on insulin resistance and pancreatic beta cell damage, causing increase in blood glucose level or hyperglycemia.[18,19] In contrast to this study, control group (K2) had decrease in fasting blood glucose levels by -90±57.04 (19.5%), although it was still in diabetic category (≥135 mg/dl). This could be in control group (K2), after the administration of high-fat feed and STZ induction, it continued with standard feeding so that changed in eating patterns in rats resulted in a decrease in blood glucose levels even though the decrease was not as good as compared to the intervention group.

The decrease in blood glucose levels in intervention group was caused by presence of bioactive compounds contained in extract of red betel leaf including flavonoid, alkaloid, tannin, and saponin, while bitter herb extracts contained andrographolid, flavonoid, saponin, phenolic

total, and anthraquinone.[21,22,23] As antidiabetic agent, flavonoid and alkaloid are able to regenerate damaged pancreatic beta cells.[8] Red betel leaf extract could reduce blood glucose level in diabetic rats.[24]Alkaloid works by stimulating hypothalamus to increase the secretion of *Growth Hormone Releasing Hormone* (GHRH), so that the secretion of *Growth Hormone* (GH) in pituitary increased. High GH level will stimulate liver to secrete *insulin-like growth factor-1* (IGF-1). IGF-1 has effect in inducing hypoglycemia and decreasing gluconeogenesis so that blood glucose level and insulin requirements decreased. IGF-1 through negative feedback system will normalize GH level again.[8]

Bitter herb is not only contained flavonoid, but also andrographolid which can inhibit gluconeogenesis and alpha glukosidase in the intestine.[24,25] Andrographolid is the main component of lactone compound in bitter her which belongs to diterpenoid group.[26] Bitter herb extract could reduce blood glucose level in diabetic rats within 10 days.[10] It can be said that bitter herb extract is as good as red betel leaf extract to lower blood glucose level.

The highest increase in insulin level was found in combined intervention of red betel and bitter herb leaf extract with dose 237.5 mg/kg BW (P1), while the lowest increase in insulin level was at dose 212.5 mg/kg BW (P3). Red betel leaf extract could increase blood insulin level up to 41.50% compared with diabetic group.[27] Study on red betel leaf extract has a good effect, but the other hand bitter herb extract is also has effect as good as red betel leaf extract. The administration of bitter herb extract for 10 days could increase insulin level in diabetic rats.[10] The content of alkaloid in red betel leaf extract and bitter herb has hypoglycemic activity by increasing insulin level through regenerate damaged pancreatic beta cells, protect from damage, and stimulate insulin release. Increased insulin secretion is due to stimulation of sympathomimetic nerves by alkaloid compound.[28,29]

Flavonoid compounds also have hypoglycemic activity which have a role in increasing the activity of antioxidant enzymes and improving sensitivity of insulin receptors.[30] Tannin can form a layer that protects intestine and causes intestinal epithelial membrane to constrict, thereby reducing the absorption of nutrients that can inhibit carbohydrate intake and rate of increase in blood sugar is not too high.[8,30] Flavonoid compounds contained in red betel extract and bitter herb are thought to improve sensitivity of insulin receptors. Flavonoids are also protective against damage to pancreatic beta cells and restore insulin sensitivity to cells and can even increase insulin sensitivity. Flavonoids in improving insulin sensitivity act as ROS-trapping antioxidant. The excess ROS can activate several kinases which can disrupt phosphorylation of insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2) which play a role in insulin signaling intracelullar.[31]

Increasing level of antioxidant total occurred in all intervention groups was due to red betel and bitter herb leaf extract which had quite high antioxidant activity content. Antioxidant activity contained in red betel and bitter herb leaf extracts were 84.474% and 87.135%, respectively. Antioxidants have property to protect pancreatic cells from free radicals (*superoxide*, *hydrogen peroxide*, *nitric oxide*, and *hydroxyl radicals*) and prevent damage to pancreatic beta cells due to oxidation.[24] Oxidative stress in diabetes mellitus occurs due to changes in carbohydrate and lipid metabolism so that it can reduce antioxidant defense system. The content of flavonoid and tannin in red betel and bitter herb leaf extract acts as an antioxidant that can protect and capture free radicals, besides that alkaloid compound has ability to stop free radical chain reactions.[30] The main content of bitter herb is andrographolid which can also gradually reduce ROS so that it can improve insulin sensitivity.[32]

CONCLUSION

The combination of red betel and bitter herb leaf extract with dose 237.5 mg/kg BW, 225 mg/kg BW, and 212.5 mg/kg BW can increase antioxidant total and improve blood glucose, insulin, and HOMA-IR levels in type-2 diabetes mellitus rats.

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