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ANTIDIABETIC ACTIVITY OF GMB-4 GREEN TEA CATECHINS IN RATS DEVELOPING TYPE 2 DIABETES MELLITUS MICE WITH INSULIN RESISTANCE

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Abstract

GMB-4 green tea catechins (GMB4-GTC) is extracted from green tea plants of clone GMB-4, which can be used to cope with diabetes mellitus . It is rich in the polyphenols isoflavones and flavonols. The purpose of this research was to determine the potential activity of GMB-4 green tea catechins to decrease blood glucose levels, decrease total cholesterol levels, increase HDL cholesterol, decrease LDL cholesterol, increase insulin levels and reduce apoptosis of pancreatic cells in mice developing type II diabetes mellitus (T2DM) with insulin resistance. This research was conducted using True Experimental design. Test animals used were rats of Wistar strain with T2DM divided into 5 groups: group 1 negative control; group 2 positive control; group 3 administration of GMB4-GTC of 20 mg/Kg B.W.; Group 4 administration of GMB4-GTC of 40 mg/kg B.W.; and Group 5 administration of GMB4-GTC of 60 mg/Kg B.W. Test animals developing T2DM with insulin resistance were created by administering hypercholesterolemic diets for 45 days. Glucose levels were determined using the enzymatic glucose oxidase method. Total, HDL and LDL cholesterol levels were determined using Cobas Mira analyzer. Expression of apoptosis of pancreatic cells was observed the using immunohistochemical technique with apoptosis kit. Results anti-diabetic activity tests showed that administration of GMB4-GTC was effective at a dose of 60 mg/kg B.W., with the value of the post hoc test sig. 0.007 (p <0.05). In conclusion, GMB4-GTC can be used as an anti-diabetic agent through the mechanism of preventing oxidative stress and protecting pancreatic beta cells, resulting in stable production of insulin and normal blood glucose.

Keyword : GMB-4 green tea catechins, Diabetes Mellitus, Insulin Resistance

INTRODUCTION

Diabetes mellitus is a health issues that requires serious attention since it is a degenerative disease affecting productivity and degrading human resources.[1] The estimated prevalence of diabetes mellitus in the world is 0.19% in people aged <20 years and 8.6 % in people aged >20 years, and 20.1% in people aged >65 years in 2000. In 2004 approximately 3.4 million people with diabetes mellitus died due to high blood glucose levels and more than 80% of these deaths occurred in countries with lower middle income.[2]

Polyphenols and flavonols in green tea catechins serve to maintain metal ions and balance carbohydrates in diabetes mellitus, such as by inhibiting activation of α-glucosidase enzymes, inhibiting glucose absorption in the intestinal tract, protecting pancreatic β-cells, increasing insulin secretion, and activating AMPK. Additionally, it is an antioxidant that inhibits oxidative stress and acts as an anti-inflammation on the endothelium and proliferation of endothelial cells.[3] Previous studies indicated that catechins of green tea had ateroprotective effects. Khaira *et al*. (2013) reported that catechins of green tea extracts were capable of inhibiting adipogenesis through decreased expression of C/EBP-α in pre-adipocyte cultures.[4] Susanti *et al*. (2012) demonstrated ateroprotective and vasoprotective effects of green tea catechins on the expression of eNOS in Wistar rats with high-fat diet.[5]

Ratnawati (2010) reported that green tea of GMB-4 clone had an anti-obesity effect by inhibiting differentiation of pre-adipocyte cells on nonergicus wistar rats.[6] The potential of green tea catechins as an antioxidant in Huvec cultured was demonstrated by Peristiowati (2014) through inhibition of NADPH oxidation and increased levels of NO.[7]

The purpose of the present study was to assess the effectiveness of GMB4-GTC administration as an anti-diabetic agent through the mechanism of reduction in blood glucose, total, HDL and LDL cholesterol, increase in insulin levels and decrease in expression of pancreatic cell apoptosis.

METHODOLOGY

Materials

GMB-4 green tea was obtained from the *Research Institute* for *Tea* and Cinchona (RITC) Gambung of West Java, from which catechins were isolated by chromotagraphy in the Chemistry Laboratory of Bandung Institute of Technology (ITB).

Isolation of Catechins from GMB-4Green Tea
A total of 25.1 gr of samples of green tea of clone GMB-4 was soaked in 500 mL of distilled water at 90ºC and then filtered using a plastic tea strainer combined with flannel and a Buchner funnel. The process was repeated twice using the previously soaked sample to obtain 1.5 L of sample extracts.

Sample extracts were eluted into the column, eluates were collected and a small quantity of it was left on the column. Subsequently, the column was eluted with 300 mL of 10% ethanol and eluates were collected. The column was eluted again with 2,100 mL of 10% ethanol; elution was carried out in stages with each elution consisting of 100 mL. Eluates were collected in different containers and then dried in a vacuum oven (10% ethanol fraction).

Subsequently, the column was eluted with 95% ethanol and 300 ml of eluates were collected. The column was eluted again with 1,200 mL of 95% ethanol. Elution was carried out in stages with each elution consisting of 100 mL. Eluates were collected in different containers and subjected to evaporation (95% ethanol fraction). Eluent used for TLC analysis of 10% ethanol fraction was ethyl acetate, while that for 95% ethanol fraction was methanol and chloroform in a ratio of 1: 9.

Ethics
The ethical review has been performed in the Ethics Committee of the Faculty of Medicine, of Brawijaya University.

Creation of T2DM Rats
Rats of Wistar strain were given hypercholesterolemic diets consisting of standard feed (30 g of PARS and 30 g of wheat flour) supplemented with 2 g of duck yolks, 0.06 g of cholic acid, 3.22 g (3.75 cc) of lard, 4 g (4 cc) of goat oil, 0.4 g (0.4 cc) of coconut oil, 25 cc of water for 45 days.[12] The animals were then injected intraperitoneally with a low dose of 30 mg/kg B.W. of Streptozotocin (STZ). At day 3, blood glucose and insulin were examined for the increase in blood glucose levels and presence of insulin resistance.[13]

Administration of GMB4-GTC
Test animals were divided into 5 groups: Group 1, negative control consisting of non-diabetic rats without treatment; Group 2, diabetic rats not treated with GMB4-GTC; Group 3, diabetic mice treated with 20 mg/kg B.W./day of GMB4-GTC; Group 4, diabetic mice treated 40 mg/kg B.W./day of GMB4-GTC; Group 5, diabetic mice treated with 60 mg/kg B.W./day of GMB4-GTC. Test animals were treated for 6 weeks and a blood glucose test was performed every two weeks 8–12 hours post-prandially.

Method for Examination of Blood Glucose Levels
Glucose levels were measured using the enzymatic glucose oxidase method with a Cobas Mira analyzer.

Method for Examination of Insulin Levels
Insulin levels are measured by using the Rat Insulin ELISA Kit.

Method for Examination of Total Cholesterol, HDL and LDL Levels
Total, HDL dan LDL cholesterol levels were measured using the enzymatic glucose oxidase method with a Cobas Mira analyzer.

Method for Observation of Pancreatic β-cells Apoptosis Expression
Expression of pancreatic β-cell apoptosis was observed by Immunohistochemistry usingthe apoptosis detection kit S7101.

RESULTS
Analysis of GMB-4 green tea catechins using High Performance Liquid Chromatography (HPLC) of 95% ethanol fraction showed two main peaks at the retention times of 5.167 and 9.82. Samples contained 80% of two catechin compounds, EGCG and ECG. Measurement of blood glucose levels after administration of hypercholesterolemic diet for 45 days and injection of the low-dose STZ of 30 mg/kg B.W. indicated that the increase in blood glucose levels for the treated mice groups were higher than that for the normal mice. Results of the independent sample *t*-test showed a significant difference (p <0.05). This indicated that the hypercholesterolemic diet and the low-dose STZ injection have significant effects on blood glucose levels of the treated mice relative to the control group. Results of the measurement of blood glucose levels after administration of GMB4-GTC for 6 weeks are found in Table 1. Results of one-way ANOVA showed a significance value of 0.002; thus, H_0 was rejected. Hence, GMB4-GTC had a potential to reduce fasting blood glucose levels in male rats of Wistar strain with diabetes mellitus.

Test animals with insulin resistance showed a decrease in insulin levels pre-prandially and post-prandially as demonstrated the Rat Insulin ELISA Kit. Results of measurements of insulin levels after administration of GMB4-GTC for 6 weeks are found in Table 1.

Results of statistical tests using one-way ANOVA with a sample of 15 Wistar strain rats tail showed a *p*-value = 0.000; thus, H_0 was rejected. This means that there were effects of GMB4-GTC administration.

Measurement of cholesterol levels after administration of hypercholesterolemic diet for 45 days showed that the increase in cholesterol levels for the treated mice were higher than that for the normal mice. Results of the independent sample *t*-tests showed a significant difference (p <0.05). This indicated that administration of hypercholesterolemic diet for 45 days had significant effects on blood glucose levels of the treated mice relative to the control group. Results of measurements of total, HDL and LDL cholesterol levels after administration of GMB4-GTC for 6 weeks are found in Table 1.

Results of one-way ANOVA showed a p-value of 0.000 with α = 0.05; thus, H0 was rejected. In conclusion, GMB4-GTC extracts had effects on HDL and LDL cholesterol levels in male white rats of Wistar strain with diabetes mellitus, as indicated by the presence of at least one different group.

Post-hoc tests that can be simplified in homogeneous subsets showed that the above analyses produced two groups: Group 1, K_Neg, K_P1, and K_P3; and Group 2, K_Post and K_P2.

Results of the observation of pancreatic β-cells apotosis expression using immunohistochemistry with the Apoptosis Detection Kit S7101 after administration of GMB4-GTC for 6 weeks are found in Table 1.

Results of ANOVA showed a probability value of 0.000 at α = 0.05; thus, H_0 was rejected. Hence, administration of GMB4-GTC had effects on the pancreatic β-cell repair in male rats with T2DM.

Post-hoc tests that can be simplified in homogeneous subsets showed that the above analyses produced three groups: Group 1, K_Neg, K_P III and K_P II; and Group 2, K_P I and K_Pos.

Table 1. shows the levels of blood glucose, insulin, total, HDL and LDL cholesterol. Values of blood glucose levels were significant at P <0.05.

			DM + catechin		
Levels	**Normal**	**DM**	**20 mg/kg B.W.**	**40 mg/kg B.W.**	**60 mg/kg B.W.**
Glukosa Darah	116.33 ± 11.44	153.00 ± 30.59	118.66 ± 10.53	184.66 ± 6.86	193.66 ± 13.20
Insulin	7.36	5.33	7.70	12.00	13.40
Kolesterol Total	39.33±2.08	66.67±6.11	34.67±3.21	60.33±5.85	38.67±5.08
HDL	20.00±2.45	21.16±1.77	21.3±6.68	23.43±3.06	22.70±3.45
LDL	5.93±3.06	28.30±1.87	16.03±2.21	28.23±1.58	11.10±1.06
Apotosis Sel Pankreas	1.67±0.57	12.67±1.52	11.67±1.52	3.33±1.52	2.33±1.15

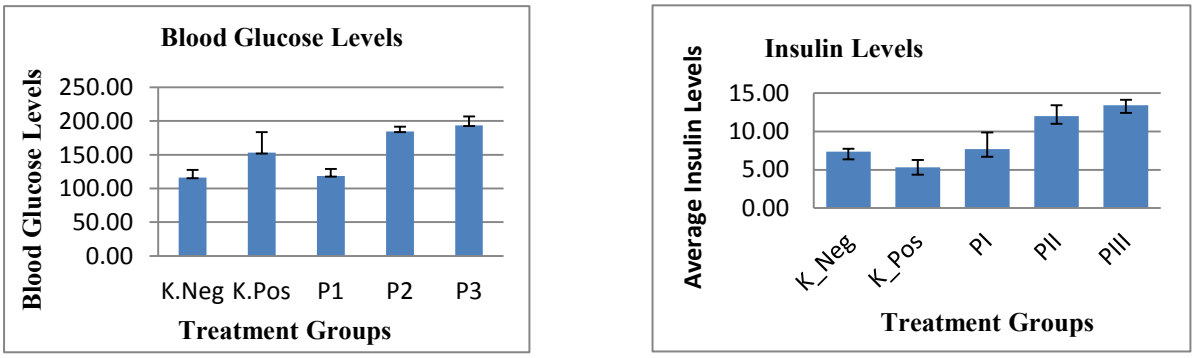

Figure 1. Blood Glucose Levels dan Insulin Levels at *P*< 0.05

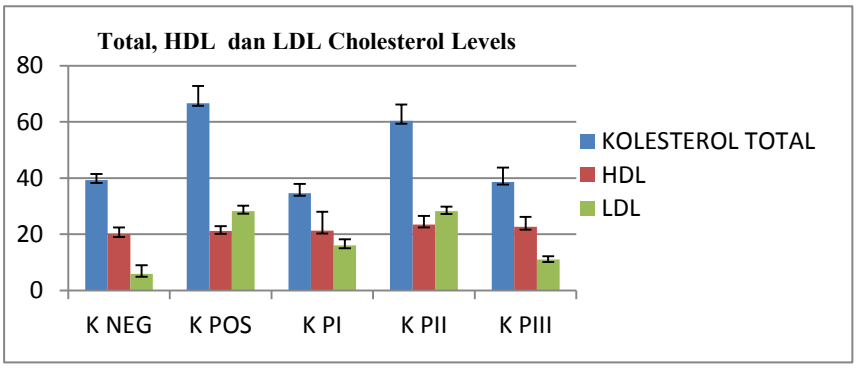

Figure 2. Total, HDL dan LDL Cholesterol Levels at *P*< 0.05

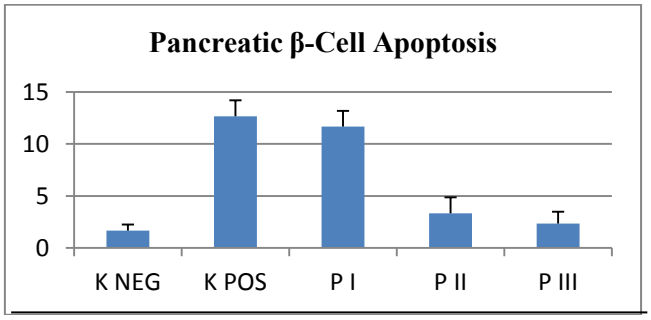

Figure 3. Pancreatic β-Cell Apoptosis at *P*< 0.05

Results of Levene's test indicated a value of 0.067 at α = 0.05; thus, H_0 was accepted. In other words, data was said to be homogeneous and analysis was continued with one-way ANOVA, the result of which was 0,002 at α = 0.05. Thus, H_0 was rejected. In conclusion, GMB4-GTC extracts had the potential to decrease blood glucose levels in male rats of Wistar strain with DM, as indicated by the presence of at least one different group.

Results showed a significant difference in decrease in fasting blood glucose levels after administration of GMB4-GTC in the control group and the treatment groups of male white rats of Wistar strain with DM. One-way ANOVA showed a significance value of 0.002 at α <0.05; then, H_0 was rejected. Hence, administration of GMB4-GTC had a potential to reduce fasting blood sugar levels in male rats of Wistar strain with diabetes mellitus.

With regard to insulin levels, results of one-way ANOVA showed a *p*-value of 0.000 or <0.05; thus, H0 was rejected. It follows that GMB4-GTC had effects on insulin levels.

DISCUSSION

One of the natural ingredients with antioxidant and anti-inflammatory potentials is catechins isolated from the tea plant (*Camellia sinensis*) of clone GMB-4. The *Research Institute* for *Tea* and Cinchona (RITC) Gambung has developed a tea clone, GMB-4, with a catechin level higher than other tea plants. The most dominant source of antioxidants found in green tea is polyphenols (catechins and gallic acid) with greater contents compared to that of black or Oolong teas.[10] Catechins are potent antioxidants that are thought to reduce atherosclerosis. This is supported by a study by Nagao (2005) that showed that the antioxidant properties of Catechins are capable of inhibiting LDL. Additionally, catechins can reduce blood serum levels, proinflammatory factors and LDL cholesterol by inhibiting oxidation.[8]

Mechanism of action of flavonoids, including catechins, is through inhibiting lipid peroxidation in the initiation stage by acting as a scavenger of the free radicals reactive oxygen (O_2^{0-}) and hydroxyl radicals (OH^0). It acts as a donor by donating H atoms to peroxyl radicals to form flavonoid radicals and will react with reactive oxygen (superoxide) to be neutral. With these reactions, the chain reaction of lipid peroxidation can be stopped.[9]

On the basis of the results of the present study and literature, a conclusion can be drawn that the extract of GMB4-GTC can be used as an alternative (non-pharmacological) medicine to reduce blood glucose levels, provided that the doses used are appropriate. The post-hoc tests showed that the doses of extract of GMB4-GTC with the highest potential to reduce blood glucose levels were 60 and 40 mg/kg B.W./rat/day since they showed significant differences with sig. 0007 and 0015, respectively.

Extract of GMB4-GTC at a dose of 20 mg/Kg B.W. was less potential to reduce blood glucose levels since it showed no significant difference with sig. 1000. With the right doses, polyphenols and flavonols maintain metal ions and balance carbohydrates in diabetes mellitus through inhibiting activation of α-glucosidase enzymes, inhibiting glucose absorption in the intestines, protecting pancreatic β-cells, increasing insulin secretion, activating AMPK. Additionally, it is an antioxidant that inhibits oxidative stress and acts as an anti-inflammation on the endothelium and proliferation of endothelial cells.[3]

However, it should be noted that extracts of GMB4-GTC in high doses contain caffeine in an excessive amounts and will be pro-oxidants that damage DNA.[9] Previous studies showed that EGCG (epigallocatechin-gallate) from green tea extracts are cytotoxic. EGCG in green tea actually acts as pro-oxidants, rather than antioxidants in pancreatic β-cells *in vivo.* Therefore, the excessive consumption of green tea can be harmful to health.[11] Doses of 40 and 60 mg/kg B.W. had the same potential to reduce blood glucose levels in mice with type 2 diabetes mellitus. However, the dose of 60 mg/kg B.W. had the highest potential to reduce blood glucose levels since it showed a significant reduction as indicated by the response of the body to the extracts of GMB4-GTC as a potent anti-oxidant.

Green tea contains catechins, the most dominant compound of polyphenols. Catechins are water-soluble, colorless and bitter-tasted compounds. Catechins composed primarily of EGCG, EGC, ECG, and EC. Leaves of green tea contain polyphenols up to 30% of dry weight, in which catechins from green tea account for 80 to 90% of total flavonoids. Epigallocatechin gallate (EGCG) contributes the highest content (48 to 55%), followed by epigallocatechin (EGC) of 9 to 12%, epicatehin-3-gallate (ECG) of 9 to 12%, and epicatehins (EC) of 5 to 7%.[7] Of these four compounds, EGCG constitutes the most abundant antioxidant and has the most potent anti-oxidant effect.

A large number of studies have been carried out to control diabetes mellitus, including by developing a functional beverage with anti-diabetic properties. The efficacy of tea leaves represents a widely-studied subject. Drinking tea is an eastern culture that should be maintained since various studies demonstrated that tea has a relatively potent antioxidant activity. This is due to the fact that polyphenols in green is capable of counteract free radicals in the body. According to Lucas (2007), polyphenols, especially epigallocatechin gallate (EGCG), are capable of protecting pancreatic β-cell from damage due to oxidation effects. In addition, a study indicated that administration of green tea was able to reduce blood glucose levels. EGCG in green tea acts by inhibiting the sodium-glucose transporters on the mucosa.[10]

Administration of EGCG is able to reduce fat and body weight, increase energy use and metabolism, decrease fat absorption and increase fat oxidation, increases insulin action, lower triglyceride accumulation through inhibition of adipogenesis and affect fatty acid synthesizing action of gene SREB-1. Thus, EGCG can be used as preventive therapy for obesity and insulin resistance.[6]

Based on the results of this study a conclusion can be drawn that GMB-4 green tea catechins can be used as an alternative (non-pharmacological) medicine to increase insulin levels. However, the doses used should be appropriate, in which the dose of 40 mg/kg B.W./day was less effective than the dose of 60 mg/kg B.W./day. Catechins have antioxidant, antibacterial and anti-radiation properties. Additionally, it strengthens blood vessels. Thus, catechins are free radicals that can protect pancreatic β-cells and increase insulin secretion.[10]

Free radical scavenging by those compounds in green tea catechins reduce damage to pancreatic tissue; thus, infiltration of mononuclear cells into pancreatic tissue in phagocytosis of damaged beta cells is also reduced. This leads to a reduction in inflammatory process that causes decreased production of TNF-α and repair of insulin-producing pancreatic β-cells.[10]

Polyphenols in green tea play a crucial role to reduce cholesterol levels as exhibited by polyphenol contents in green tea and its concentration. These contents will help absorb LDL levels and return it to the liver, which will be re-neutralized by HDL.[16]

Catechins are potent antioxidants that are thought to reduce atherosclerosis. This is supported by a study by Nagao (2005) that showed that the antioxidant properties of catechins are capable of inhibiting LDL. Additionally, catechins can reduce blood serum levels, proinflammatory factors and LDL cholesterol by inhibiting oxidation. Also, catechins are able to repair pancreatic β-cells.[14]

Tukey's tests for administration of extracts of GMB4-GTC at a dose of 40 mg/Kg B.W./rat/day showed an average cholesterol level of 60.33 mg/dl relative to the negative control group with a significant difference of sig. 0.015, demonstrating a success in lowering cholesterol levels. This is due to the fact that GMB4 green tea catechins are anti-oxidants that can protect the pancreas against free radicals. When free radicals enter the body, antioxidants will bind it to prevent oxidative stress. The pancreas is protected and able to produce insulin for the transport of blood glucose to be absorbed by the cells for energy. It will help lower LDL cholesterol (bad cholesterol) levels and act as an antioxidant that will provide protection against free radicals. With the presence of free radicals, LDL (bad cholesterol) will be conditioned, namely deposited in the walls of blood vessels, initiating atherosclerosis. This has an effect of helping lower cholesterol levels in the blood. The effective dose based on the present study was 40 mg/kg B.W. In the present study, 1 mg of CGT was capable of producing a decrease of 1.51 mg/dl.

On the basis of the results of the present study, a conclusion can be drawn that the extract of GMB4-GTC can be used as an alternative (non-pharmacological) medicine to reduce apoptosis, provided that the doses used are appropriate. The post-hoc tests showed that the doses of extract of GMB4-GTC with the highest potential to reduce apoptosis were 60 and 40 mg/kg B.W./rat/day since they showed significant differences with sig. 0.968 and 0.556, respectively. The dose of 20 mg/kg B.W./rat/day of extract of GMB4-GTC had less potential to reduce pancreatic β-cell apoptosis since it did not a significant difference (sig. 0.000). In an appropriate dose, polyphenols and flavonols in green tea catechins serve to maintain metal ions and balance carbohydrates in diabetes mellitus, such as by inhibiting activation of α-glucosidase enzymes, inhibiting glucose absorption in the intestinal tract, protecting pancreatic β-cells, increasing insulin secretion, and activating AMPK. Additionally, it is an antioxidant that inhibits oxidative stress and acts as an anti-inflammation on the endothelium and proliferation of endothelial cells.[3]

Mechanism of action of flavonoids, including catechins, is through inhibiting lipid peroxidation in the initiation stage by acting as a scavenger of the free radicals reactive oxygen (O_2^{0-}) and hydroxyl radicals (OH^0). It acts as a donor by donating H atoms to peroxyl radicals to form flavonoid radicals and will react with reactive oxygen (superoxide) to be neutral. With these reactions, the chain reaction of lipid peroxidation can be stopped.[9] Free radical scavenging by those compounds in green tea catechins reduce damage to pancreatic tissue; thus, infiltration of mononuclear cells into pancreatic tissue in phagocytosis of damaged beta cells is also reduced.[17]

On the basis of the results of the present study and literature, a conclusion can be drawn that the extract of GMB4-GTC of a dose of 60 mg/Kg B.W./rat/day had less potential to repair pancreatic β-cells than the doses of 40 and 20 mg/Kg B.W./rat/day. The highest dose showed a highly effective effect, in which catechins as anti-oxidants acted faster at a right dose and was able to reduce apoptosis and help repair pancreatic β-cells.

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