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EFFECT OF 7-HYDROXY-2-(4-HYDROXY-3-METHOXY-PHENYL)-CHROMAN-4-ONE (SWIETENIA MACROPHYLLA KING SEED) ON RETINOL BINDING PROTEIN-4 AND PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE EXPRESSION IN TYPE 2 DIABETIC RATS

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Abstract

Background and Aims*: Diabetes mellitus is a metabolic disorder characterized by hyperglycemia due to a defect of insulin secretion, insulin action, or both. There are increasing evidence that active compounds of medicinal plants may be used to treat diabetes. The aim of this study is to investigate the effect of a 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one flavonoid compound of the Swietenia macrophylla King seed on homeostatic model assessment of insulin resistance (HOMA-IR), phosphoenolpyruvate carboxykinase (PEPCK) and retinol binding protein-4 (RBP4) gene expression in diabetic rats.* ***Materials and Method****: Thirty Wistar rats were used with 5 in each group as follows: 1) normal rats; 2) diabetic rats; 3) diabetic rats with metformin; 4), 5) and 6) diabetic rats with a 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one 10, 30 and 90 mg/200 g body weight (BW) respectively. Blood glucose and insulin levels were analyzed before and after treatment. At the end of the study, the liver tissue was removed for quantitative PCR (qPCR) analyses.* ***Results****: Blood glucose levels decreased significantly ($p<0.05$) in diabetic rats with metformin and three different dosages of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one. Insulin levels increased significantly ($p<0.05$) in diabetic rats with metformin and diabetic rats with 10 mg/200 g BW of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one. HOMA-IR value decreased significantly ($p<0.05$) in diabetic rats with metformin and three different dosages of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one. There was a significant decrease of PEPCK gene expression in the group with 90 mg/200 g BW of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one. RBP4 gene expression showed a decline, but the difference between groups was not statistically different.* ***Conclusion****: These results demonstrated that 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one increased insulin and decreased blood glucose level, HOMA-IR value and PEPCK gene expression.*

key words: *Diabetes mellitus***,** *Hyperglycemia, Insulin, PEPCK expression, RBP4-expression, Swietenia macrophyla King seed*

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Background and aims

Diabetes mellitus is characterized by hyperglycemia due to impaired insulin secretion, insulin action or both, which results in abnormalities in the metabolism of carbohydrates, fats and proteins [1]. Diabetes mellitus (DM) is a global health problem. The prevalence of diabetic patients continues to increase yearly and it is estimated more than 80% of people with diabetes are in developing countries [2]. Diabetes mellitus is a metabolic disease that often leads to macrovascular and microvascular complications [1]. Hyperglycemia in diabetes leads to excessive production of free radicals [3]. Excessive production of free radicals is a major factor causing complications in DM [4]. According to Basu *et al*. [5], one of the causes for hyperglycemia is increased gluconeogenesis. High blood glucose levels in diabetic patients might be caused by overexpression of the enzyme phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis, thus reducing PEPCK gene expression may improve the hyperglycemia [6]. Increased expression of PEPCK in DM patients may be due to an elevated expression of the retinol binding protein-4 (RBP-4) in adipocytes [7]. The elevated expression and RBP-4 levels in adipose tissue and liver is known to trigger insulin resistance in humans and animal experimental models [8-10].

Indonesia has about 30,000 species of plants and some of these plants show anti-diabetic activity. Active medicinal plants for treating diabetes include pariah (*Momordica charantia* L.), green tea (*Camellia sinensis*), ginseng (American Ginseng-*Panax quinquefolium* L.) [11], pomegranate (*Punica granatum* L), jamun (*Eugenia jambolana* L) [12] and mahogany (*Swietenia macrophylla* King).

Mahogany (*Swietenia macrophylla* King) is a species of the *Meliaceae* family. Mahogany seeds have been widely used by the community as a traditional medicine for people with DM. Mursiti [13] found that the oil-free extract of mahogany seeds contains alkaloid, flavonoid and saponin compounds. Flavonoids consist of a polyphenolic compound. Some research suggests that flavonoid, or phenol has an anti-hyperglycemic activity. According to a study by Vessal *et al*. [14], quercetin shows antidiabetic activity by regenerating pancreatic beta cells and increasing insulin secretion streptozotocin-induced diabetic rats. Logendra *et al*. [15] showed that PEPCK expression in these rat models was reduced after administration of polyphenols contained in the *Atermisia dracunculus* ethanol extract, namely phenoxy chromon and dihydrochalcone.

This research aimed to assess the potential of antidiabetic flavonoid compounds (7-hydroxy-2- (4-hydroxy-3-methoxy-phenyl) -chroman-4-one) in mahogany seeds.

Materials and methods

Animals

Thirty rats (30) male Wistar rats, (average weight of 200 g, 8 weeks old), were obtained from the Jakarta Food and Drug Control Agency (*Balai POM Jakarta*). They were housed in cages in an animal room (22-25°C room temperature on a 12-hour daylight cycle); food and water were given *ad libitum* during the experimental period using standard diet (AIN 93M). The standard diet in the experiment consisted of casein 24%, DL-Methionin 0.30%, cornstarch 61%, vitamin mix 1%, mineral mix 3.5%, choline chloride 0.2%, alpha cell 5% and corn oil 5%. This study was approved by the Ethics Committee of Faculty of Medicine, University Gadjah Mada.

Induction of type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus was induced by intraperitoneal injection of a 65 mg/kg body weight (BW) of streptozotocin (NacalaiTesque®, Inc, Japan) 15 minute after injection of 230 mg/kg BW of nicotinamide (NA) (Sigma-Aldrich®, USA), following the method outlined by Masiello *et al*. [16]. Fasting blood glucose levels were measured 5 days after induction, and the rats were categorized as diabetic if the fasting blood glucose was ≥170 mg/dL [16].

Preparation of Flavonoid

Seven-hydroxy-2-(4-hydroxy-3-metoxy-phenyl)-chroman-4-one was isolated from *Swietenia macrophylla* King seeds as previously reported by Mursiti S [13].

Experimental studies

After the induction of diabetes, the animals were separated in several groups. Group 1 (normal animals without diabetes induction), group 2 (diabetic animals without treatment), group 3 followed a treatment with metformin while group 4, 5, and 6 treatment with 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one in concentrations of 10 mg, 30 mg, and 90 mg/200 g BW respectively. All treatments were administred by gavage for a period of four weeks. At the beginning (before treatment) and at the end of the experimental period (four weeks), blood samples were collected from *retro orbitalis plexus* after the animals had fasted for 10 hours. Blood samples were collected to analyze plasma glucose, insulin serum and homeostatic model assessment of insulin resistance (HOMA-IR). At the end of the experimental period (post test), animal were anaesthetized and euthanized and the liver was examined for RBP-4 and PEPCK gene expression.

Blood biochemical examination

Plasma glucose level was assayed by enzymatic glucose-peroxidase (GOD-POD) method (Diasys®, Germany). Serum insulin level used the enzyme linked immunosorbant assay (ELISA) method. HOMA-IR was calculated according to the formula: HOMA-IR = Fasting insulin level (mU/L) x Fasting glucose level (mmol/L)/22,5.

Isolation of RNA

Total RNA was obtained using TriZol® reagent. The procedure followed the protocol provided by the TriZol® reagent. [17]

PEPCK and RBP-4 expression analysis by real-time PCR

The cDNA was synthesized using an iScript mix Biorad® kit according to the protocol of the producer. SsoFast™Evagreen® supermix Biorad® was used for q-PCR. The primers used for cDNA amplification were forward 5'-GGTGAGCAGCTTCAGAGTC-3' and reverse 5'– ACCATGTCTGCACACACTTC for RBP4 (204bp); forward 5'–CTCACCTCTGGCCAAGATTGGTA-3' and reverse 5'-GTTGCAGGCCCAGTTGTTGA-3' for PEPCK (189bp); forward 5'-ACGGTCAGGTCATCACTATCG-3' and reverse 5'-GGCATAGAGGTCTTTACGGATG-3'. for β-actin (bp). The q-PCR reaction was conducted individually with each gene using the same internal control beta actin gene. The cycle for the cDNA amplification was 5 minutes at 95^{o}C, followed by 40 cycles at 95^{o}C for 60s, 57^{o}C for 60s, for RBP4; and 60.2^{o}C for 60s for PEPCK. Then, the final extension step at 72^{o}C for 5 minutes was performed, followed by a melt-curve analysis at 65-95^{o}C. The PCR products were subjected to 2% agarose. The cycle of threshold (C_T) value was obtained automatically from the machine's calculation.

Statistical analysis

The results were expressed as the mean ± SE. The effects of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenlyl)-chroman-4-one on blood glucose, serum insulin, HOMA-IR, PEPCK and RBP-4 gene expression were analyzed by one-way ANOVA. Data were considered statistically significant if p values were lower than 0.05.

Results

The effect of flavonoid 7-hydroxy-2-(4-hydroxy-3-mehtoxy-phenyl)-chroman-4-one on blood glucose levels the diabetic rats is shown in Table 1.

Table 1. Blood glucose levels before and after 4 weeks of treatment.

Group	Blood Glucose Level (mg/dL)		Mean Difference (95% CI)	p
	Pretest	Posttest		
Normal	72.354 ± 1.76*	72.93 ± 1.59	-0.58 (-1.09 ; -0.07)	0.035
DM	233.33 ± 4.06	233.96 ± 3.66**	-0.63 (-1.74 ; 0.48)	0.188
DM + metformin	225.07 ± 4.51	104.05 ± 2.74	121.02 (118.68 ; 123.36)	<0.001
DM + 7-hydroxy 10	231.20 ± 3.29	144.40 ± 2.05	86.80 (85.04 ; 88.58)	<0.001
DM + 7-hydroxy 30	224.35 ± 6.07	124.48 ± 5.71	99.87 (97.99 ; 101.76)	<0.001
DM + 7-hydroxy 90	232.18 ± 3.55	109.74 ± 2.82	122.44 (121.18 ; 123.69)	<0.001
p	<0.001	<0.001		

P value in the last row indicate difference between groups. P value in the right indicate difference between pre and post. * difference significant between normal with diabetic rats, diabetic with metformin, diabetic rats with 7-hydroxy with three diference dosage. ** difference significant between diabetic rats with normal rats, diabetic with metformin, diabetic rats with 7-hydroxy with three diference dosage

Table 2. Serum insulin levels before and after 4 weeks treatment.

Group	Insulin Level ng/mL		Mean Difference (CI)	p
	Pretest	Posttest		
Normal	145.29 ± 13.32*	170.98 ±5.53	-25.69 (-48.49 ; -2.88)	0.035
DM	115.10 ± 10.80	119.98 ± 5.12**	-4.87 (-35.08; 25.33)	0.677
DM + metformin	115.67 ± 9.98	157.29 ±22.95	-41.63 (-68.53 ; 14.72)	0.013
DM + 7-hydroxy 10	121.29 ± 10.30	153,73 ± 9.56	-32.44 (-42.61 ; -22.27)	0.001
DM + 7-hydroxy 30	134.23 ± 24.42	158.04 ± 17.05	-23.81 (-67.33 ; 19.70)	0.203
DM + 7-hydroxy 90	141.91 ± 35.33	147.91 ± 6.51	-6.00 (-57.05 ; 45.05)	0.761
p	0.079	<0.001		

P value in the last row indicate difference between groups. P value in the right indicate difference between pre and post. * difference significant between normal with diabetic rats, diabetic with metformin, diabetic rats with 7-hydroxy with three diference dosage. ** difference significant between diabetic rats with normal rats, diabetic with metformin, diabetic rats with 7-hydroxy with three diference dosage

The blood glucose levels in groups 3, 4, 5 and 6 decreased with 53.8%, 37.5%, 44.5% and 52.7% respectively ($p<0.05$) after four weeks treatment with metformin, respectively 7-hydroxy-2-(4-hydroxy-3-methoxy-phenly)-chroman-4-one. The 90 mg/200 g BW dose of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one had the same activity as metformin in reducing blood glucose levels.

The insulin levels of rats treated with flavonoid 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one dose of 10 mg, 30 mg, and 90 mg/200 g BW for 4 weeks s increased by 26.7%, 17.7%, and 4.8%, respectively (Table 2).

In Table 3 we showed that the administration of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one decreased significantly ($p<0.05$) the HOMA-IR value.

There is a significant differrence ($p<0.05$) between the 6 groups in the hepatic PEPCK gene expression after treatment with the 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one

as shown in Figure 1. In this study, the relative expression of PEPCK gene in DM rats that were given a 7- hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one dose of 90 mg / 200g BW was smaller than in DM mice that were given a dose of 10mg, 30mg / 200g BW or DM mice without treatment (Figure 1).

Table 3. HOMA-IR value before and after 4 weeks treatment.

Group	HOMA-IR		Mean Difference (95% CI)	p
	Pretest	Posttest		
Normal	5,2±0,53*	6,1±0,18	-0,96 (-1,74 ; 0,18)	0,027
DM	13,2±1,0	13,9±1,86**	-0,63 (-4,03; 2,77)	0,634
DM + metformin	12,9±1,18	8,1±1,03	4,79 (3,12 ; 6,46)	0,001
DM + 7-hydroxy 10	13,8±1,3	10,9±0,72	2,89 1,71 ; 4,07)	0,001
DM + 7-hydroxy 30	14,9±2,90	9,7±1,26	5,17 (-1,18; 9,16)	0,023
DM + 7-hydroxy 90	16,3±4,14	8,0±0,26	8,28 (2,89 ; 13,67)	0,013
p	<0,001	<0,001		

P value in the last row indicate difference between groups. P value in the right indicate difference between pre and post. * difference significant between normal with diabetic rats, diabetic with metformin, diabetic rats with 7-hydroxy with three diference dosage. ** difference significant between diabetic rats with normal rats, diabetic with metformin, diabetic rats with 7-hydroxy with three diference dosage

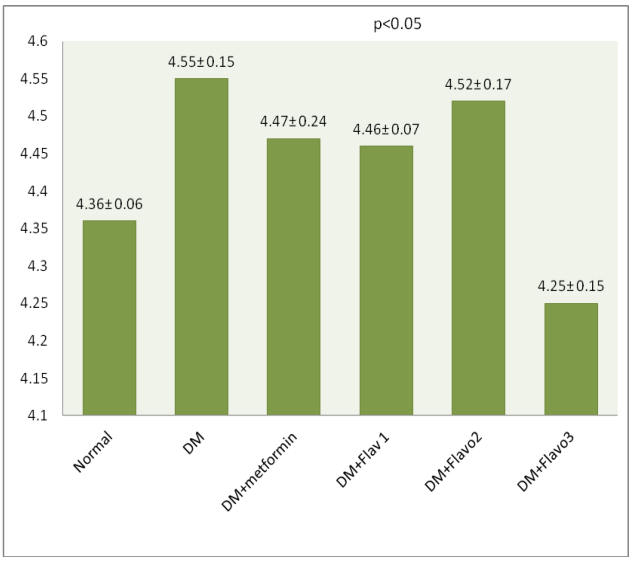
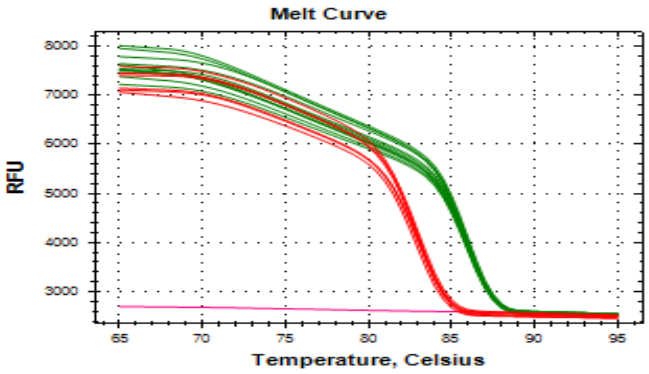


Figure 1. *Liver PEPCK relative expression.*

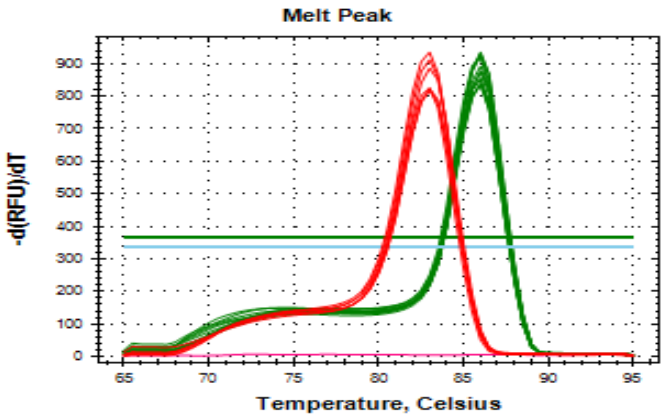


Legend: green - PEPCK gene, red - beta actin control gene, and pink - no template control (NTC)

Figure 2. *PEPCK melting curve.*

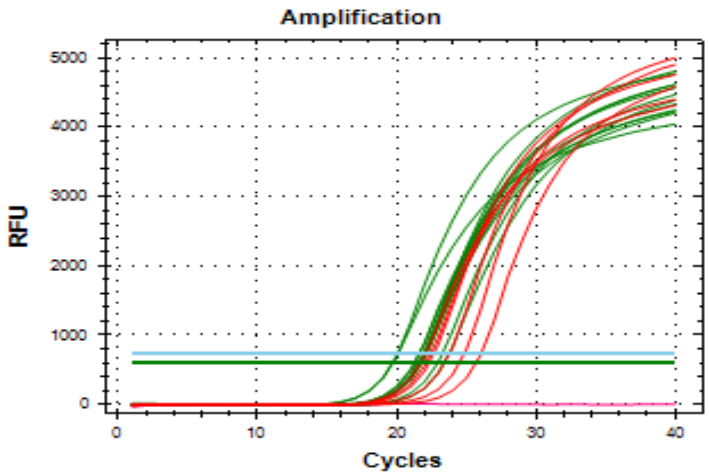
DNA melting curve (Figure 2) after administration of the compound 7- hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one show that at a low temperature, all amplicons (amplified cDNA products) were present as a double-stranded DNA (DsDNA) and began to bind the EvaGreen® fluorescent dye generating a strong signal. Increasing the temperature causes the cDNA products to undergo denaturation and the fluorescence signal decreases until it reaches the melting temperature of the target cDNA product. The melting temperature of the PEPCK gene began to be detected at 84°C and melting temperatures beta-actin began to be detected at 80°C.

Figure 3 shows the melting curve peaks of PEPCK gene after administration of the compound 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one. The melting curve peaks of PEPCK gene is the maximum point where fluorescence signal is detected. Different amplicons generate DNA melting curve peaks at different temperatures. The melting curve peaks of PEPCK gene DNA product was detected at a temperature of 86°C (green) while the peak melting curve of control gene DNA products (beta-actin) is detected at a temperature of 83°C (red).



Legend: green - PEPCK gene, red - beta actin control gene, and pink - no template control (NTC)

Figure 3. *PEPCK melting curve peak.*



Legend: blue - threshold, green - PEPCK gene and pink - no template control (NTC).

Figure 4. *Amplification curve of PEPCK gene.*

The yield curve cDNA amplification products with RT-PCR is represented in Figure 4, having on x-axis the number of PCR cycles and on the y-axis the amount of amplification of the signal that is proportional to the number of fluorescence cDNA products that are amplified. The meeting point of the fluorescence signal over the line threshold (green line) is expressed as the value of C_T. C_T value of PEPCK gene began to be detected at 19.5 while the value of C_T cycle control genes (beta-actin) began to be detected at 22 cycles.

There was no significant difference ($p>0.05$) in hepatic RBP-4 gene expression after four weeks treatment with 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one as shown in Figure 5. In this study, the relative expression of RBP-4 gene that normal mice, untreated DM and metformin treated DM was similar. However, DM rats treated with flavonoid 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one doses of 10 mg / 200 g BW and 90 mg / 200 g BW have a smaller RBP-4 gene expression than the dose 30 mg / 200 g BW or DM mice without treatment.

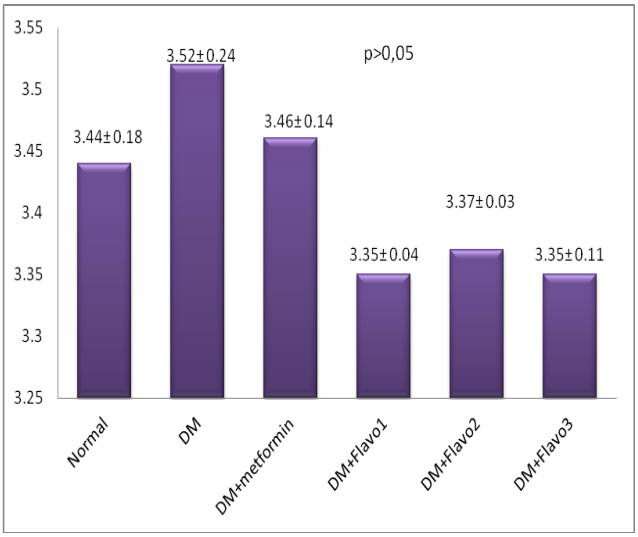
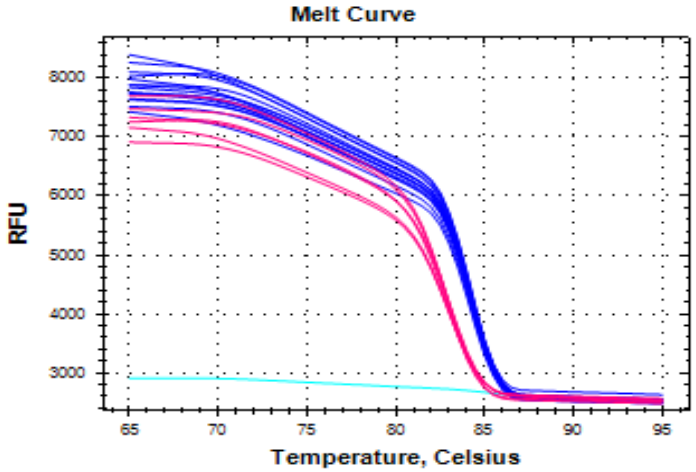


Figure 5. *Liver RBP-4 relative expression*

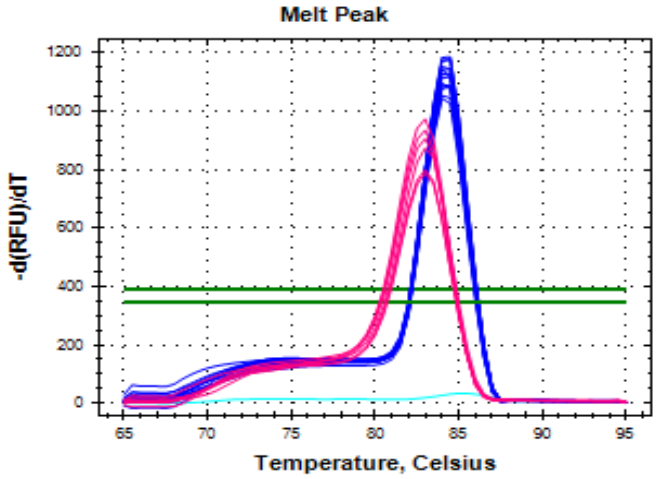


Legend: blue - RBP4 gene, red - Beta actin control gene, light blue - no template control (NTC).

Figure 6. *RBP4 melting curve.*

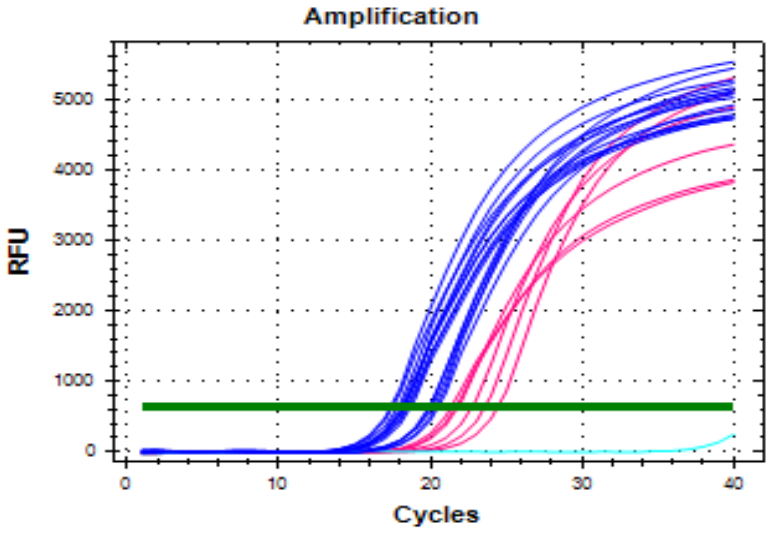
As shown in Figure 6, the DNA melting curve shows that at low temperatures, all amplicons (amplified cDNA product) are a double-stranded DNA (DsDNA) and began to bind the EvaGreen® fluorescent dye generating a strong signal. Increasing the temperature causes the cDNA products to undergo denaturation and fluorescence signal decreases until it reaches the melting temperature of the target cDNA product. The melting temperature of the RBP-4 begins to be detected at 80°C and the melting temperatures of beta-actin begin to be detected at 86°C.

Figure 7 shows the melting curve peaks of RBP-4 gene that is the maximum point of detecting fluorescence signal. Different amplicons generate DNA melting curve peaks at different temperatures. The melting curve peak for RBP-4 gene DNA products is detected at 84,5°C (blue) while the peak melting curve beta-actin is detected at 83°C (red).



Legend: blue – RBP-4 gene, red - beta actin control gene

Figure 7. *Melting peak of RBP-4*



Legend: green - threshold, blue - RBP-4 gene, red - beta actin control gene, and light blue - no template control (NTC).

Figure 8. *Amplification curve RBP-4 gene*

The yield curve cDNA amplification products with RT-PCR is represented in

The yield curve cDNA amplification products with RT-PCR is represented in Figure 8

having on the x-axis the number of PCR cycles and on the y-axis the amount of amplification of the signal that is proportional to the number of fluorescence cDNA products that are amplified. The meeting point of the fluorescence signal over the line threshold (green line) is expressed as the value of C_T. C_T value of RBP-4 gene began to be detected at 18 while the value of C_T cycle for control genes (beta-actin) began to be detected at 21 cycles.

Discussion

In the present study, we examined the effect of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl) chroman-4-one on levels of glucose, insulin, HOMA-IR, and PEPCK and RBP-4 expression in type 2 diabetic rats. Blood glucose levels were significantly reduced. The groups treated with 7-hydroxy-2-(4 hydroxy-3-methoxy-phenyl)-chroman-4-one at doses of 10 mg, 30 mg, and 90 mg/ 200 g BW had the same effect on blood glucose levels as that of metformin. These observations are in agreement with those of Falah *et al.* (2010) [17] who investigated the effect of *Swietenia macrophylla* seed extraction in alloxan induced diabetic rats, and Debasis *et al*. (2011) [18] who reported the effect of aqueous methanolic *Swietenia macrophylla* extract in streptozotocin induced diabetic rats. Based on these results, 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one may stimulate insulin release and therefore produce the observed restoration of metabolic activities.

In our study, in the group treated with the 10 mg/ 200 g BW of the extract there was an increase of insulin levels and a significant reduction in HOMA-IR values (Tables 2 and 3). Increased insulin levels have reached the level found in normal rats, although HOMA-IR value was still not as in the normal group. This indicates that administration of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman 4-one dose of 10 mg/200 g BW was able to increase insulin production in pancreatic beta cells but was not able to completely overcome insulin resistance. The groups treated with the higher doses of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one had actually increased insulin levels but lower than the 10 mg/200 g BW group. Higher doses were more beneficial to overcome insulin resistance even though the decreased HOMA-IR value in the diabetes group that was treated with 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one dose of 90 mg/200 g BW has not reached the HOMA-IR value of the normal group (6.15 versus 13.87). The decrease in HOMA-IR was in line with a decrease in PEPCK expression.

Hyperglycemia in diabetic patients might be caused by over-expression of the enzyme PEPCK through oxidative stress [6]. The group that was treated with 90 mg/200 g BW had the highest ability to suppress PEPCK expression in liver.

Increased expression of PEPCK in DM patients may be due to an elevated expression of the retinol binding protein-4 (RBP-4) in the adipose tissue [7]. The elevated expression of RBP-4 gene in adipose tissue and the liver is known to trigger insulin resistance in humans and animal experimental models [8-10]. Thus, the induction of diabetes enhances RBP-4 expression in liver tissue. RBP-4 increases the expression of PEPCK, which leads to increased hepatic glucose output that serves to raise blood glucose [8]. Treatment with 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one at doses of 10 mg, 30 mg, and 90 mg/ 200 g BW reduced RBP4 expression most effectively compared to metformin. Flavonoid as an antioxidant is capable to inhibit oxidative stress, therefore reducing RBP-4 levels. By reducing RBP-4 level, insulin sensitivity will improve, so PEPCK level will also decrease. Flavonoid is

able to induce reduction of aldose reductase, pancreatic cell regeneration and increase insulin release and Ca^{2+} uptake, making it beneficial for treatment of diabetes mellitus [19].

Conclusion

In conclusion, the results of this study show that the administration of 10 mg, 30 mg, and 90 mg/200 g BW of 7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chroman-4-one significantly ~~($p<0.05$)~~ reduced blood glucose levels. In addition, the dose of 10 mg/200 g BW increased insulin levels and down regulated the gene expression of PEPCK in the liver.

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Conflict of interest. We declare no conflict of interest.

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