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Carica pubescens fruit juice improved superoxide dismutase, triglyceride and high-density lipoprotein levels in type 2 diabetes mellitus Wistar rats

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Abstract

Insulin resistance and insulin deficiency in type 2 diabetes mellitus (T2DM) may increase reactive oxygen species (ROS) production, which can exacerbate pancreatic β-cell. Insulin resistance and insulin deficiency may also reduce lipoprotein lipase (LPL) activity, increase triglycerides (TG) levels and decrease high-density lipoprotein (HDL) levels. This condition could lead to cardiovascular complications. Flavonoids, such as rutin, might be able to improve insulin resistance and increase insulin secretion in T2DM. *Carica pubescens* is one of the typical Dieng fruits that has rutin. This study aimed to investigate the effect of *Carica pubescens* fruit juice (CPJ) on superoxide dismutase (SOD), TG, and HDL levels in high fat diet-streptozotocin-induced diabetic Wistar rats. A total of twenty-five male Wistar rats were divided into five groups; untreated control group (K-); untreated diabetic group (K+); the diabetic group was treated for 30 days with 4 mL/200 g BW of CPJ (X1); 8 mL/200 g BW of CPJ (X2); and 10 mg/200 g BW of rutin (X3). This study revealed a significant increase in SOD and HDL levels and a significant decrease in TG levels in groups X1, X2, and X3, compared to the K+ group ($p < 0.05$). The increased level of SOD and HDL and the decreased level of TG between X2 and X3 were not significantly different. The effect of CPJ at 8 mL/200 g BW was similar to the effect of rutin at 10 mg/200 g BW. These findings suggest the beneficial effects of CPJ on SOD, TG, and HDL levels in type 2 diabetic Wistar rats.

1. Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disorder characterized by an increase in blood glucose level (hyperglycemia) due to inadequate insulin production and the body's inability to respond adequately to insulin (insulin resistance). The International Diabetes Federation (IDF) reported that approximately 425 million adults worldwide suffered from diabetes mellitus in 2017. The IDF estimated that the number of diabetic persons will increase to 629 million by 2045, and more than 90% of diabetes cases will be T2DM (IDF, 2018). In Indonesia, the prevalence of diabetes in 2013 was 6.9% and increased to 8.5% in 2018 (Kementerian Kesehatan Republik Indonesia, 2018). Diabetes mellitus and its complications caused 3.96 million deaths in 2010 and increased to 5 million in 2015 (equivalent to one death every 6 s) (Zheng *et al*., 2018).

Hyperglycaemia may be caused by the body's failure to respond to insulin normally (insulin resistance) and inadequate insulin production (IDF, 2018). Hyperglycaemia may result in excessive production of reactive oxygen species (ROS), which can lead to reduced endogenous antioxidant activity, such as superoxide dismutase (SOD). This condition will cause oxidative stress (Zheng *et al*., 2018). SOD plays a critical role in protecting cells from oxidative stress by converting superoxide radicals into hydrogen peroxide, then further metabolized by CAT (catalase) and GSH-Px (glutathione peroxidase) (Jeong *et al*., 2012; Zheng *et al*., 2018). Subsequently, the high level of ROS can damage pancreatic β cells and worsen T2DM (Gerber and Rutter, 2017). In addition, insulin resistance and insulin deficiency may reduce the activity of LPL. The decreased LPL activity results in increased triglyceride (TG) levels and decreased high-density lipoprotein (HDL) levels (Andrade, 2018). The high level of TG and low level of HDL in people with T2DM can increase the risk of coronary heart disease by as much as 1.54 times and stroke by 2.13 times, compared to people with

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diabetes who have normal TG and HDL levels (Lee *et al*., 2017).

Various studies indicated that polyphenols from food can act as an antioxidant and improve the condition of T2DM (Oboh *et al*., 2014). Flavonoids are one of the largest classes of polyphenol compounds that may improve insulin resistance and increase insulin secretion (Soares *et al*., 2017). *Carica pubescens* L. is one of the typical fruits of Indonesia which contains flavonoids and phenolic acids (Simirgiotis *et al*., 2009; Pinto *et al*., 2009). The *Carica pubescens* or what we call *karika* in Indonesia is a typical fruit plant of Dieng, known as mountain papaya or "*gandul dieng*". The *karika* belongs to the same genus as papaya. The thing that distinguishes papaya and *karika* is where it grows. Papaya requires high temperature and light intensity, while *karika* requires low temperature and lots of rain. This is the reason that *karika* is very suitable to grow in places with a climate in the highlands such as Dieng, Wonosobo Regency, and Central Java (Kusnadi *et al*., 2016). Based on data from the Agriculture and Fisheries Service of Wonosobo Regency (2016) the production of *karika* in Wonosobo reached 18338 kw. The *karika* has the potential to become a major commodity that has high economic value, but this fruit is rarely studied.

Based on the research of Simirgiotis *et al*. (2009), a hundred grams of *Carica pubescens* fresh fruit has 3.1 mg of rutin, which distinguishes *Carica pubescens* from common papayas (Simirgiotis *et al*., 2009). Rutin is a type of flavonoid compound from the flavonol category, which has many health benefits, such as antidiabetic, anti-hypercholesterolemia, antioxidants, anticancer, antibacterial, antiviral, and others (Ganeshpurkar and Saluja, 2017). Previous research by Niture *et al*. (2014) showed that rutin administration of 50 mg/kg BW and 100 mg/kg BW for 3 weeks reduced blood glucose levels, increased HDL and SOD levels, reduced TG levels, low-density lipoprotein (LDL) levels, very low-density lipoprotein (VLDL) levels, and total cholesterol. Research by Al-Enazi (2014) also showed that rutin administration at a dose of 60 mg/kg BW and 100 mg/kg BW for 3 weeks increased SOD levels and reduced blood glucose and TG levels.

Another flavonoid compound in *Carica pubescens* is quercetin (Pinto *et al*., 2009). Quercetin has synergistic benefits with rutin. Rutin and quercetin have antioxidant activity that can neutralize free radicals thereby inhibiting oxidative stress and protecting pancreatic cells from further damage due to oxidative stress. The protective effect of these antioxidants will allow pancreatic cells to remain active in producing insulin (Adewole *et al*., 2007; Sattanathan *et al*., 2011).

In this study, *Carica pubescens* was given in the form of juice. Juicing is an effective method of promoting fruit and vegetable consumption and is very popular in many countries (Zheng *et al.,* 2017). *Carica pubescens* fruit juice administration to T2DM patients has never been investigated. Thus, this study evaluated the effect of *Carica pubescens* fruit juice on SOD, TG, and HDL levels in preventing disease severity in high-fat diet-streptozotocin-induced diabetic Wistar rats.

2. Materials and methods

2.1 Ethical clearance

This experimental research used a randomized post-test only control group design. This study has obtained ethical approval from the Ethical Committee of Medical Research of the Faculty of Medicine, University of Diponegoro, Semarang by number 12/EC/H/FK-UNDIP/II/2019. This research was conducted in April-May 2019 in the Centre for Food and Nutrition of the University of Gadjah Mada, Yogyakarta.

2.2 Preparation of Carica pubescens fruit juice

Carica pubescens fruit was purchased from PT. Carica Gemilang was taken from the Dieng Plateau, Wonosobo Regency, Central Java. A total of 100 grams *of Carica pubescens* flesh were cut into small pieces and then washed and sprinkled with a bit of salt to reduce the protease enzymes in *Carica pubescens* fruit. Then, blanched at 60℃ for 3 mins to stop the activity of protease enzymes contained in *Carica pubescens* fruit and eliminate the bitter taste (Fu and Kim, 2011; Rashima *et al*., 2017). The used of a temperature of 60℃ for 3 mins also to minimize the loss of antioxidant content in it. After that, the flesh was blended without water and put into a homogenizer until it was completely smooth.

2.3 Animals and treatments

This study used male white Wistar rats aged 8-12 weeks with a body weight of 150-200 g. The first randomization was carried out to classify rats that would be used as a model for T2DM. The second randomization was carried out after the rats had confirmed T2DM with blood glucose >200 mg/dL, then divided into 4 groups. The rats were given a standard feed of 20 g per day and drinking water ad libitum during the study. The standard feed is Comfeed II containing 15% crude protein, 3-7% crude fat, 12% water content, 6% crude fiber, 7% ash, 0.9-1.1% calcium, and 0.6-0.9% phosphorus. Rats have free access to food and drink.

A total of 25 male Wistar rats were divided into two

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groups. A total of five rats as a control group (K-) and 20 rats as models of T2DM. The T2DM model group was confirmed to have hyperglycaemia and was divided into 4 groups: untreated T2DM group (K+); T2DM group was treated with 4 mL/200 g BW of CPJ (X1); T2DM group was treated with 8 mL/200 g BW of CPJ (X2), and T2DM group was treated with 10 mg/200 g BW of rutin (X3). The rats were given a standard feed of 20 g per day and water *ad libitum*, while the treatment was administered through oral gavage.

T2DM rats were conditioned by giving high fat diet (HFD) of 20 g/day for 2 weeks. After two weeks, the rats were induced by 110 mg/kg BW of nicotinamide (NA) dissolved with saline solution intraperitoneal, then the rats were induced by streptozotocin (STZ) of 45 mg/kg BW with buffer citrate through intraperitoneal 15 mins later (Veerapur *et al*., 2010). Fasting blood glucose level was measured three days after the induction of NA+STZ, while SOD, TG, and HDL levels were determined 30 days after administration of CPJ and rutin. We used rutin as an antioxidant control, which we assumed contain in *Carica pubescens* fruit. We used rutin from the *Flos sophorae immaturus* plant extract with 95% purity obtained from Xi'an Imaherb Biotech Co. Ltd.

2.4 Data collection

2.4.1 Measurement of blood glucose levels

Fasting blood glucose level was measured by the GOD-PAP (glucose oxidase-peroxidase aminoantipyrine) method with a reagent from DiaSys. A total of 10 µL of blood serum was mixed with 1,000 µL reagent and incubated for 20 mins at 20-25℃. The absorbance value was interpreted with a wavelength of 500 nm. The amount of formed red dye was equivalent to the amount of glucose concentration.

2.4.2 Measurement of superoxide dismutase levels

The level of SOD was determined using the ELISA method from BioVision.

2.4.3 Measurement of triglyceride levels

The level of TG was measured using the GPO-PAP (glycerol-3-phosphate oxidase-phenol aminophenazone*)* method with a reagent from DiaSys. A total of 10 µL blood serum was mixed with a 1,000 µL triglycerides reagent and incubated for 10 mins at 25℃, then measured using a spectrophotometer at a wavelength of 546 nm.

2.4.4 Measurement of high-density lipoprotein levels

The level of TG was measured using the CHOD-PAP (*cholesterol oxidase–p-aminophenazone*) method with a reagent from DyaSis. Before measuring HDL, LDL and VLDL and chylomicrons were precipitated with the addition of phosphotungstic acid and magnesium chloride. A total of 200 µL of blood serum was mixed with 500 µL of HDL precipitation reagent and incubated for 15 mins at room temperature. Then, the solution was centrifuged at 2,000×*g* for 20 mins and the filtrate was left for two hours. A total of 100 µL of supernatant was discarded and mixed with cholesterol reagent. The mixture was left for 10 mins at 25℃ or 5 mins at 37℃, then it was measured using a spectrophotometer at a wavelength of 500 nm.

2.5 Statistical analysis

The data of this study were analyzed normally using *Shapiro-Wilk*. If the data were normally distributed, that was analyzed using One-Way ANOVA and followed by Bonferroni's Post-Hoc test. If the data is not normally distributed, that is analyzed using *Kruskal Wallis* and followed by the Mann-Whitney test.

3. Results

3.1 Fasting blood glucose levels

The fasting blood glucose level of the rats was measured three days after STZ injection. Fasting blood glucose analysis aimed to ensure that the administration of HFD and STZ could lead to T2DM in rats. A rat was considered as T2DM when the fasting blood glucose level was >200 mg/dL (Gheibi *et al*., 2017). Table 1 showed a significant difference in fasting blood glucose between the control and T2DM groups ($p<0.05$). In the T2DM group, fasting blood glucose was significantly higher than in the control group. Administration of HFD and STZ significantly elevated fasting blood glucose compared to the control group by 3.87 times.

Table 1. Fasting blood glucose levels.

Group	Fasting Blood Glucose (mg/dL)	*p*
Control	70.21±1.89	0.000*
T2DM	271.68±7.79	

*p-value < 0.05 = significant

3.2 Superoxide dismutase levels

The effect of CPJ and rutin on SOD level between groups was shown in Figure 1. The K+ group had the lowest level of SOD (29.11±4.58 U/mL), while K- group had the highest level of SOD (84.29±3.43 U/mL). The groups of rats consuming CPJ and rutin had higher SOD levels than the K+ group. A SOD level of the group of rats which consumed 4 mL/200 g BW of CPJ (61.07±6.14 U/mL), 8 mL/200 g BW of CPJ (76.96±2.31 U/mL), and 10 mg/200 g BW of rutin (80.71±3.07 U/mL) were 110%, 164%, and 177% higher than the untreated diabetic rat group, respectively.

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Based on the normality test with the Shapiro-Wilk test, the distribution of the SOD levels data in all groups was normally distributed then we continued to the one-way ANOVA test. The result of one-way ANOVA some groups had a significant difference in SOD levels ($p < 0.05$). Post Hoc Bonferroni showed that SOD levels in K -, X2 and X3 groups were not significantly different. Thus, the administration of CPJ at 8 mL/200 g BW had the equivalent effect to the 10 mg/200 g BW of rutin in increasing the level of SOD.

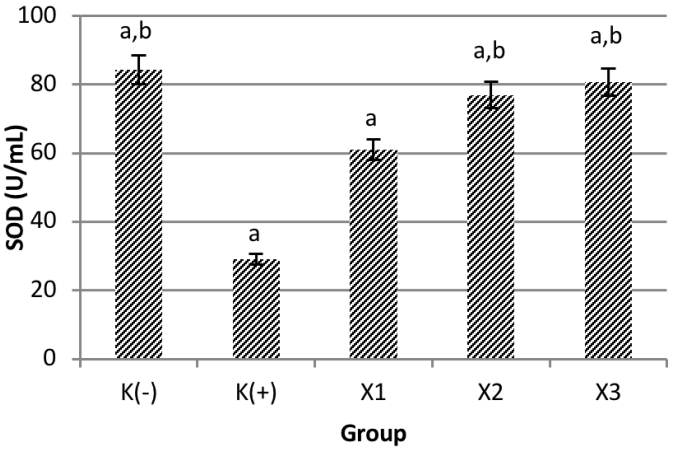

Figure 1. The Level of SOD. K (-) control group, K (+) untreated T2DM, X1 T2DM with 4 mL/200 g BW of CPJ, X2 T2DM with 8 mL/200 g BW of CPJ, X3 T2DM with 10 mg/200 g BW of rutin.
[a] significant difference ($p < 0.05$), [b] not significant difference ($p > 0.05$), [a,b] Post Hoc Bonferroni Test.

3.3 Triglyceride and high-density lipoprotein levels

The levels of TG and HDL between groups after administration of CPJ and rutin are shown in Figure 2. The K- group had the lowest TG level and the highest level of HDL. In contrast, the K+ group had the highest level of TG and the lowest level of HDL. The groups of rats which consumed CPJ and rutin had lower TG levels and higher HDL levels than the K+ group. TG level of the group of rats which consumed 4 mL/200 g BW of CPJ (96.18±2.33 mg/dL), 8 mL/200 g BW of CPJ (85.39±1.67 mg/dL), and 10 mg/200 g BW of rutin (82.55±2.14 mg/dL) were 22%, 31%, and 33% lower than the untreated diabetic rats group (123.45±2.33 mg/dL), respectively. HDL level of the group of rats which consumed 4 mL/200 g BW of CPJ (41.96±3.41 mg/dL), 8 mL/200 g BW of CPJ (61.30±2.09 mg/dL), and 10 mg/200 g BW of rutin (64.83±1.76 mg/dL) were 72%, 151%, and 166% higher than the untreated diabetic rats group (24.37±2.32 mg/dL), respectively.

Based on the normality test with the Shapiro-Wilk test, the distribution of the TG and HDL levels data in all groups was normally distributed then we continued to the one-way ANOVA test. The one-way ANOVA test indicated there were groups with significantly different TG and HDL levels ($p<0.05$). The Post Hoc Bonferroni test showed that the levels of TG and HDL in the X2 and the X3 groups were not significantly different as seen in Figure 2. In other words, the administration of 8 mL/200 g BW of CPJ had the same effect as 10 mg/200 g BW of rutin in decreasing TG and increasing HDL in T2DM.

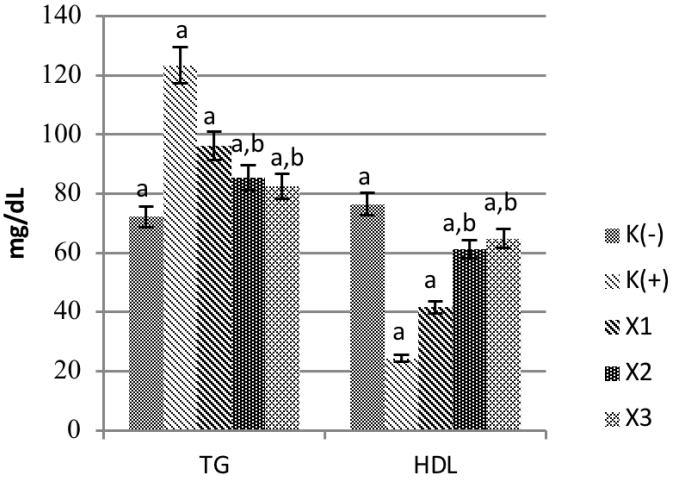

Figure 2. The Level of TG dan HDL. K (-) control group, K (+) untreated T2DM, X1 T2DM with 4 mL/200 g BW of CPJ, X2 T2DM with 8 mL/200 g BW of CPJ, X3 T2DM with 10 mg/200 g BW of rutin.
[a] significant difference ($p < 0.05$), [b] not significant difference ($p > 0.05$), [a,b] Post Hoc Bonferroni Test

4. Discussion

4.1 Fasting blood glucose levels

This study showed that the T2DM group had higher fasting blood glucose than the control group. It was equivalent to the previous research which revealed that the injection of STZ at 45 mg/kg BW after HFD could cause hyperglycemia conditions in rats (Veerapur *et al*., 2010). The administration of HFD aimed to produce insulin resistance then followed by injection of a moderate dose of STZ to reduce β cell capacity resulting in hyperglycemia (Furman, 2015). The excessive amount of energy intake may cause obesity and insulin resistance in bone muscle and liver. Severe adipose tissue extension inflicts adipose inflammation and distortion of adipokines profile indicated by a high level of leptin and a low level of adiponectin. This condition implies the malfunction of adipose tissue. The dysfunction of adipose tissue causes the accumulation of ectopic lipids in non-adipose tissue, such as muscle, liver, and β cells pancreas. This accumulation of lipid intra-myocellular is the direct cause of the dysfunction of glucose intake, which leads to insulin resistance (Skovsø, 2014). Fifteen minutes before STZ injection, rats were injected with NA as much as 110 mg/kg BW dissolved with saline solution intraperitoneally. The combination of NA and STZ can protect cells from apoptosis. The condition of diabetes will be seen 72 hrs after STZ injection

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(Ghasemi *et al*., 2014; Furman, 2015). Three days later the rats were fasted for 6-10 hrs and 2 mL of blood was taken for analysis of blood glucose levels through the retroorbital plexus. Rats were categorized to be T2DM if the fasting blood glucose serum level was > 200 mg/dL (Gheibi *et al*., 2017).

4.2 Superoxide dismutase levels

Chronic hyperglycemia in T2DM can result in increased oxidative stress over time and produce a vicious cycle between reactive oxygen species (ROS) and hyperglycemia (Salas-Salvado *et al*., 2011). The formation of free radicals in T2DM can be caused by the presence of non-enzymatic protein glycation, glucose oxidation, and increased lipid peroxidation. The increased ROS from various pathways in T2DM leads to the degradation of enzymatic antioxidants in the body, including SOD (Ullah *et al*., 2016). In addition, SOD levels decreased in T2DM rats because an increase in H_2O_2 production inactivated SOD activity (Al-Enazi, 2014). This condition caused the K+ group to have the lowest SOD levels among other groups.

If compared to the group K+, the group given CPJ and rutin had higher SOD levels. The higher level of SOD can be caused by the presence of flavonoids (e.g., rutin and quercetin) and phenolic acid (e.g., chlorogenic acid, caffeic acid, and coumaric acid in *Carica pubescens* fruit. Based on previous research, *Carica pubescens* fruit contains rutin at 3.1 mg/100 g fresh weight, quercetin at 519 µg/g dry weight, chlorogenic acid at 233 µg/g dry weight, caffeic acid at 290 µg/g dry weight, and coumaric acid of 169 µg/g dry weight (Simirgiotis *et al*., 2009; Pinto *et al*., 2009). Flavonoids and phenolic acids have synergistic benefits in improving T2DM conditions. Antioxidant activity in these compounds is capable of neutralizing free radicals thereby inhibiting oxidative stress and protecting pancreatic β cells to remain active in producing insulin (Jeong *et al*., 2012).

Rutin affects the direct activation of SOD to catalyze O_2 to H_2O_2, which will be quickly removed by CAT to protect the liver and kidney tissue against OH, which is very reactive and toxic. This mechanism will prevent lipid peroxidation (Al-Enazi, 2014). In this study, the X3 group that was given pure rutin had a higher SOD level than the K+ group. This result was in agreement with previous research, which revealed the potential of rutin administration at 50 mg/kg BW and 100 mg/kg BW for three weeks in increasing SOD levels (Niture *et al*., 2014).

Increased insulin secretion and improved insulin resistance can improve the condition of hyperglycemia and decrease ROS production. Rutin might be a stimulator of insulin action. Rutin protects the integrity of pancreatic β cells by maintaining insulin secretion and stimulating the signalling of insulin substrate 2 receptor (IRS2) in pancreatic β cells. Rutin might be one of the agents that may improve the function of pancreatic β cell secretion during glucose dysregulation in hyperglycemia (Cai and Lin, 2009).

Similar to rutin, quercetin also directly activates antioxidant enzymes, such as SOD, CAT and GPx (Jeong *et al*., 2012). Previous studies have also shown that administration of chlorogenic acid, caffeic acid, and coumaric acid can improve SOD levels in T2DM rats. These compounds are able to prevent the formation of free radicals and increase the enzymes SOD, CAT and GPx (Jung *et al*., 2006; Pari *et al*., 2010; Amalan *et al*., 2015; Ye *et al*., 2016).

4.3 Triglyceride and high-density lipoprotein levels

In this study, the content of rutin at 4 mL CPJ is 0.121 mg and rutin at 8 mL CPJ is 0.243 mg. The content of flavonoids and phenolic acids also plays a significant role in improving the lipid profile in T2DM. Flavonoids such as rutin and quercetin have a positive impact on reducing TG and increasing HDL. Rutin effects on TG and HDL are in line with previous studies. Previous research suggested that rutin administration of 50 mg/kg BW and 100 mg/kg BW for three weeks reduced TG levels and increased HDL levels in diabetic rats (Niture *et al*., 2014). The study by Prince and Kannan (2006) also showed that the administration of rutin at 100 mg/kg BW for 45 days in STZ-induced rats was able to reduce cholesterol, triglyceride, free fatty acid, and phospholipid levels in plasma and tissue significantly. It may happen because the administration of rutin can increase insulin levels. The ability of rutin to scavenge free radicals and inhibit lipid peroxidation, prevented oxidative stress and protected β cells resulting in increased insulin secretion and decreased blood glucose levels. Insulin can inhibit lipolysis in adipocytes, mainly through the inhibition of hormone-sensitive lipase (HSL) which catalyzes the mobilization of free fatty acids from the stored triglycerides. Insulin inhibits lipase activity mainly through the reduced levels of cAMP, due to the activation of cAMP-specific phosphodiesterase in fat cells. Diabetic rats that were given rutin also showed an increase in LPL and LCAT (lecithin cholesterol acyltransferase) activity, which led to decreased levels of LDL and TG, and increased HDL (Prince and Kannan, 2006).

Flavonoids and phenolic acids have synergistic benefits in improving lipid profile in T2DM conditions. Quercetin moderately reduces fat accumulation by

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modulating the expression of genes associated with steatosis by reducing oxidative stress in the liver. In addition, quercetin can also activate AMP-activated protein kinase (AMPK) which functions to prevent the accumulation of lipids in the liver in diabetic rats. The AMPK inhibited acetyl-CoA carboxylase (ACC) activity and SREBP-1c expression. The quercetin also prevented the reduction of PPAR-α expression in the livers of rats fed the Western diet. The PPAR-α mediates the expression of genes that promote -oxidation of fatty acids. The activation of PPAR-α can reduce triglycerides and lipids circulating in plasma (Kobori *et al*., 2011).

Synergistic with flavonoids, phenolic acids also play a role in lowering TG and increasing HDL. The chlorogenic acid regulates lipid metabolism by activating the AMPK pathway (Meng *et al*., 2013). The activation of AMPK is able to inhibit lipid synthesis by inhibiting the activity of glycerol-3-phosphate acyltransferase (GPAT). The GPAT is the first enzyme to catalyze the synthesis of triglycerides. The AMPK also inhibits the enzyme acetyl-CoA carboxylase (ACC) then the synthesis of triglycerides decreases. The ACC is an enzyme that catalyzes the formation of malonyl-CoA. The malonyl-CoA plays a role in increasing fat synthesis and reducing fatty acid oxidation (Henriksen *et al*., 2013). The caffeic acid is effectively able to prevent cholesterol biosynthesis and suppress lipogenesis activity (Liao *et al*., 2013). The research results from Ahmadvand *et al*. (2017) showed that intraperitoneal administration of 50 mg/kg BW of caffeic acid was able to reduce serum cholesterol, LDL-C and atherogenic index levels and was able to increase serum HDL-C in type 1 diabetic rats. Another study showed that caffeic acid was able to reduce plasma and liver triglyceride levels and cholesterol in rats with high-fat diets (Liao *et al*., 2013).

5. Conclusion

Carica pubescens fruit juice has been shown to improve SOD, TG, and HDL levels in T2DM rats. The administration of 8 mL/200 g BW CPJ has a similar effect to 10 mg/200 g BW of rutin in increasing SOD and HDL levels and reducing TG levels in T2DM rats. In this study, it is still necessary to carry out the proximate analysis, the content of flavonoid compounds and phenolic acids in *Carica pubescens* from Dieng Plateau and further research needs to be carried out regarding more effective processing of *Carica pubescens* in order to have maximum results to improve T2DM conditions.

Conflict of interest

The authors declare no conflict of interest.

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