Carica pubescens fruit juice reduces tumor necrosis factor-alpha (TNF-α) and fasting blood glucose (FBG) levels in type 2 diabetes mellitus Wistar rats

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FULL PAPER

Carica pubescens fruit juice reduces tumor necrosis factor-alpha (TNF-α) and fasting blood glucose (FBG) levels in type 2 diabetes mellitus Wistar rats

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Abstract

Chronic inflammation and hyperglycemia in type 2 diabetes mellitus (T2DM) can cause several complications due to organ dysfunctions. *Carica pubescens* (CP) is a typical fruit from Dieng Plateau, Indonesia which contains some rutin that is kind of flavonoid. It is well known that flavonoid acts as anti-inflammation and anti-hyperglycemic which is useful for T2DM conditions. This study was aimed to investigate the effect of CP fruit juice on tumor necrosis factor-alpha (TNF-α) and fasting blood glucose (FBG) levels in rats induced by HFD-STZ, with rutin as a control. The design of this study was a randomized post-test only control group design. A total of twenty-five male Wistar rats were divided into 5 groups: K- = normal group; K+ = diabetic group; X1 and X2 = diabetic groups that received CP fruit juice 4 mL/200 g BW/day and 8 mL/200 g BW/day; X3 = diabetic group that received rutin 10 mL/200 g BW/day. The treatments were administered orally for 30 days. TNF-α and FBG levels were measured using ELISA and GOD-PAP method respectively. The results showed that TNF-α and FBG levels were significantly decreased in the treatment groups (X1, X2, X3) compared with the K+ group ($p<0,05$). Also, there was no significant difference in TNF-α and FBG levels between group X2 and X3, indicating that CP fruit juice 8 mL/200 g BW/day has a similar ability with rutin 10 mg/200 g BW/day. It can be concluded that CP fruit juice can be a recommended fruit juice for a diabetic condition by reducing TNF-α and FBG levels.

1. Introduction

Diabetes Mellitus (DM) is a serious and growing health problem all over the world (Frances *et al.*, 2013). In type 2 diabetes mellitus (DMT2), there is a glucose tolerance disturbance as a result of insulin resistance and pancreatic β-cell damage (Badawi *et al.*, 2010). According to the International Diabetes Federation (IDF), the world's DM prevalence was 463 million cases in 2019 and will continue to increase to 700 million cases in 2045. Indonesia has 10,7 million DM cases in 2019 and occupies 7th ranks as the country with the most DM patients (IDF, 2019). Type 2 diabetes mellitus (T2DM) is closely related to excessive calorie intake history which further leads to excessive body weight. In individuals who experience overweight, hypertrophy and hyperplasia occur in adipose tissue (Monteiro and Azevedo, 2010; Xu, 2013). This condition results in inadequate blood supply impacting the poor oxygenation of the tissues, which is called tissue hypoxia. Hypoxia is one of the factors that play a role in the occurrence of inflammation in adipose tissue. Such inflammation is characterized by an increase in the expression of pro-inflammatory cytokines, one of which is TNF-α. TNF-α relates to the development of insulin resistance in T2DM patients who have excessive body weight history (Xu, 2013).

Subsequent insulin resistance will cause impaired glucose tolerance resulting in increased hepatic glucose production and decreased glucose uptake into cells and tissues that lead to hyperglycemia condition (Ormazabal *et al.*, 2018). Hyperglycemia condition itself can directly worsen inflammatory conditions, wherein the subsequent increase of the pro-inflammatory cytokines can lead to damage to the β-cell pancreas and endocrine malfunctions (Frances *et al.*, 2013). Also, hyperglycemia which occurs chronically can lead to organ dysfunction such as eye, kidney, nervous system, heart, and blood vessels (ADA, 2013). Therefore, by controlling

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inflammatory and hyperglycemia conditions is expected to control the disease progression so that complications can be more prevented. Flavonoid compound which is widely contained in food is well known can improve inflammatory conditions (Panche *et al*., 2016).

Flavonoid is a natural compound with a variety of phenolic structures that are found in plants and beneficial to human health (Panche *et al*., 2016). One of the ingredients that contain flavonoid compounds and not much known and utilized by Indonesian people is *Carica pubescens* (CP) fruit. *C. pubescens* is a typical plant in Indonesia that is found in Dieng Plateau, Indonesia (Laily and Khoiri, 2016). *C. pubescens* contains rutin compound (Simirgiotis *et al*., 2009). Rutin is a type of flavonoid that has properties as anti-inflammatory and anti-hyperglycemia. This makes CP fruit has the potential to control inflammation and blood glucose in T2DM (Niture, Ansari and Naik, 2014; Ghorbani, 2017).

A study conducted by Niture, Ansari and Naik (2014) proved that rats with T2DM showed a decrease in blood glucose and TNF-α levels after given pure rutin at a dose of 50 and 100 mg/kg BW for 3 weeks. Besides rutin, CP rutin also contains other compounds such as quercetin, caffeic acid, chlorogenic acid and coumaric acid which have synergistic effects related to anti-inflammatory and anti-hyperglycemia (Pinto *et al*., 2009). The research on CP fruit juice especially related to T2DM has never been studied before. This study aimed to determine the effect of CP fruit juice on TNF-α and fasting blood glucose levels in T2DM rats, with pure rutin as a comparator.

2. Materials and methods

2.1 Material and reagent

CP fruits were obtained from Dieng Plateau, Wonosobo, Indonesia that naturally grow at 2093 masl altitude. CP fruits used in this study were CP fruits with a 90% ripeness level (90% yellow peel color). Pure rutin powders were obtained from Xi'an Imaherb Biotech Co., Ltd. Streptozotocin (STZ) and nicotinamide (NA) were obtained from Nacalai Tesque, Kyoto-Japan. The TNF-α examination used the TNF-α kit Fine Test brand, China. Fasting blood glucose examination used Diasys kit, Germany.

2.2 Animals and treatments

This research used 25 male Wistar (*Rattus Norvegicus*) rats which were obtained from Central Food and Nutrition Laboratory Gajah Mada University (UGM), Yogyakarta, Indonesia with inclusion criteria: age 2 months, body weight 150-200 grams and healthy conditions with active movements. Rats were acclimatized for 7 days in individual cages at 25°C with a 12-hour lighting cycle and given Comfeed II 20 grams/day as a standard feed that contains crude protein content 15%, crude fat 3-7%, water 12%, crude fiber 6%, ash 7%, calcium 0.9-1.1%, and phosphorus 0.6-0.9% and drinks *ad libitum* were administered during the period of acclimatization to the end of the study. High-fat diet (HFD) was administered for 14 days after the period of acclimatization at 20 g/rats/day, with the composition of com feed II 90%, pork fat 10%, and pure cholesterol was 1.25%. On the 22nd day, rats were intraperitoneally induced with NA 110 mg/kg BW (dissolved with NaCl 1.5 mL/100 g BW) and STZ 45 mg/kg BW (dissolved with sodium citrate buffer 1.5 mL/100g BW) 15 mins after the induction of NA, and three days later the fasting blood glucose levels were examined. Rats have fasted for 6-8 hrs first and then their blood was taken 2 mL through the plexus retroorbital. Rats were declared to have DMT2 when having blood glucose levels > 200 mg/dl (Ghasemi *et al*., 2014). Rat's body weight was measured every 7 days during the acclimatization and HFD administration, and every 3 days during the intervention period.

The design of this study was randomized post-test only control group design. After acclimatization period, rats were divided into two groups: None-HFD (5 rats) as the negative control group (K-), and HFD (20 rats) group that was induced by STZ/NA and divided into 4 groups: diabetic control group (K+); diabetic + Karika fruit juice 4 mL/200 g BW/day (X1); diabetic + Karika fruit juice 8 mL/200 g BW/day (X2); diabetic + rutin 10 mg/200 g BW/days (X3). All treatments were orally administered for 30 days. About 2 mL of blood was collected through plexus retroorbital after rats were fasted for 6-8 hours, and centrifuged at 4000 rpm for 15 minutes to obtain the blood serum to used for examination of TNF-α and final fasting blood glucose levels.

We used rutin as a flavonoid control, that we assumed contain in CP fruit. Based on previous studies, rutin at a dose of 50 mg/kg of BW or 10 mg/200 g of BW may decrease the condition of hyperglycemia in diabetic rats (Niture, Ansari and Naik, 2014). The rutin used in this study was a rutin extract which was obtained from *Flo's plant Sophorae Immaturus* and was purchased from *Xi'an Imaherb Biotech Co., Ltd*. Rutin is powder-shaped and dissolved in aquadest to be administered to rats (X3 Group) orally once per day for 30 days.

2.3 The preparation of CP fruit juice

First of all, CP fruits were peeled and deseeded and then washed thoroughly. Then the fruit is cut into small pieces and rinsed with saltwater. Then the fruit was blanched for 3 mins at 60°C. The purpose of rinsing with

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saltwater and blanching is was to remove the sap found in the fruit. The sap is itchy and bitter, therefore blanching was also done to make the texture of the flesh soft (Adinugraha et al., 2018; Yusmita and Wijayanti, 2018). Furthermore, a total of 100 g of CP fruit was then processed into juice using blender and homogenizer.

2.4 Determination of rutin content in CP fruit juice

Pure rutin of 50 g was dissolved in 50 mL of ethanol to get a stock solution with a concentration of 1000 ppm. Then the stock solution was diluted with ethanol to get various concentrations of standard solutions ranging from 0-50 ppm. 2 mL of each standard solution was taken and transferred into a cuvette, then the absorbance was measured with a spectrophotometer at a wavelength of 359 nm.

CP fruit juice was dissolved in an ethanol solvent with a ratio of 1:1 and mixed using a vortex. Then the solution was centrifuged at 4500 rpm for 15 mins and 2 mL of supernatant was taken and transferred into a cuvette. The absorbance was measured with a spectrophotometer at a wavelength of 359 nm.

2.5 The Examination of TNF-α levels

The examination of TNF-α levels was measured by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) method. Plates were washed two times before adding standard, sample (rats blood serum) and control (blank) wells. Standard or sample (100 µL) were added to each well and were incubated for 90 minutes at 37°C. Plates were aspirated and washed two times. A 100 µL Biotin-labeled antibody working solution was added to each well and incubated for 60 mins at 37°C. Plates were aspirated and washed three times. A 100 µL SABC Working Solution was added into each well and incubated for 30 minutes at 37°C. Plates were aspired and washed five times. A total of 90 µL TMB Substrate was added and incubated for 30 mins at 37°C. Stop solution of 50 µL were added in the final process. The absorbance was measured at a wavelength of 450 nm immediately.

2.6 The Examination of fasting blood glucose levels

The examination of fasting blood glucose levels was done by Glucose Oxidase Phenol 4-AminoPhenazone (GOD-PAP) method. As many as 27 tubes were prepared, with details: 25 tubes containing 10 µL samples (rat blood serum), 1 tube containing a 10 µL standard solution and 1 tube containing 10 µL blank (aquadest). Each 1000 µl of reagent was added into the 27 tubes. The solution was mixed using a vortex and then was incubated for 20 mins at 20-25°C. The absorbance was measured using a spectrophotometer at a wavelength of 500 nm. Blood glucose levels were calculated with the following formula:

$$\text{Blood glucose level (mg/dL)} = \frac{\text{Sample absorbance} \times \text{standard concentration}}{\text{Standard absorbance}}$$

2.7 Statistical analysis

The data of this study were analyzed statistically with a significance value $p<0.05$ and CI 95%. The differences between TNF-α and fasting blood glucose levels were analyzed using the One-Way ANOVA Test and followed by Bonferroni's Post-Hoc test because the data were normally distributed.

2.8 Ethical clearance

This research has obtained the Ethical Clearance approval from the Health Research Ethics Committee (KEPK), Faculty of Medicine Diponegoro University Semarang No.14/EC/H/FK-UNDIP/II/2019.

3. Results

3.1 Rutin content in CP fruit juice

The Rutin content of CP fruit in this study was 13.63 µg/g fresh weight or 13.87 µg/mL (in the form of juice). This result is lower than the previous study conducted by Simirgiotis et al (2009) where the rutin content in CP fruit in that study was 31 µg/g fresh weight.

3.2 The effect of CP fruit juice and rutin on TNF-α levels

TNF-α levels were measured after rats were given CP fruit juice and rutin for 30 days. Based on Figure 1, there were significant differences in TNF-α levels in the treatment groups between before and after the administration of CP fruit juice and rutin ($p<0.05$) in which the group received rutin (X3) had the lowest TNF-α level. TNF-α levels in the group that received CP fruit juice 8 mL/200 g BW/day (X2) was lower than and significantly differed when compared with the group that received CP fruit juice 4 mL/200 g BW/day ($p=0.000$). Figure 1 also shows that there were no significant differences in the TNF-α levels between the healthy rat group (K-) with group X3 ($p=0.113$). Also, the TNF-α levels did not differ significantly between the X2 and X3 groups ($p=1.000$).

3.3 The effect of CP fruit juice and rutin on fasting blood glucose levels

Final fasting blood glucose levels were measured after rats were given CP fruit juice and rutin for 30 days. Figure 2 shows the comparison of fasting blood glucose levels between groups after intervention. There were significant differences in rats fasting blood glucose levels in the treatment groups between before and after

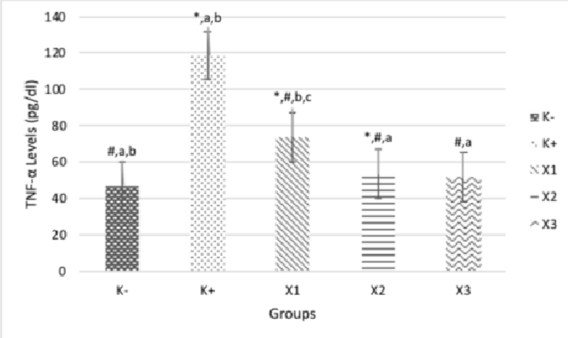

Figure 1. TNF-α Levels after CP Fruit Juice and Rutin administration in T2DM Wistar Rats

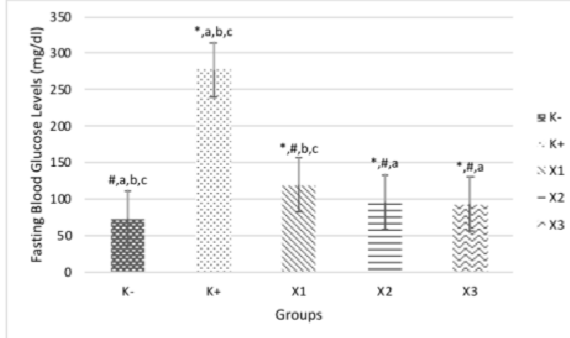

Figure 2. Fasting Blood Glucose Levels after CP Fruit Juice and Rutin administration in T2DM Wistar Rats

K- = normal group; K+ = diabetic group; X1 and X2 = diabetic groups that received CP fruit juice 4mL/200 g BW/day and 8 m/200 g BW/day; X3 = diabetic group that received rutin 10 mL/200 g BW/day. Statistical analysis was measured using One Way ANOVA test and followed by Bonferroni's Post hoc test. *Compared with K-; $p<0.05$; $^{\#}$compared with K+; $p<0.05$; ^{a}compared with X1; $p<0.05$; ^{b}compared with X2; $p<0.05$; ^{c}compared with X3; $p<0.05$.

the administration of CP fruit juice and rutin ($p<0.05$) in which the group that received rutin (X3) had the lowest fasting blood glucose levels. Fasting blood glucose levels in the group that received CP fruit juice 8 mL/200 g BW/day (X2) was lower than and significantly differed when compared with the group that received Carica juice 4 mL/200 g BW/day (X1) (p=0.000). Figure 2 also shows that there was no significant difference in fasting blood glucose levels between the X2 and X3 groups (p=1.000).

4. Discussion

The differences in rutin content in CP fruit can be caused by several factors, one of which is environmental factors due to differences in the growth area. CP fruits used in this study were obtained from the Dieng Plateau region, Indonesia, while the CP fruits in the previous study were from the Chile region (Simirgiotis *et al.*, 2009). According to the research conducted by Liu *et al* (2016), the higher air temperature has an impact on the lower rutin content. Also, the length of sunlight is directly proportional to the rutin content, while rainfall is negatively correlated with rutin content (Liu *et al.*, 2016). Dieng plateau area has an average temperature of around 15°C, while Chile is at 13.6°C. The average sunlight duration in Dieng is <300 hours/year, while in Chile >300 hours/year. The average rainfall in the Dieng area is 2500 mm/ year, while in Chile it is 733 mm/year. Based on the comparison of air temperature, rainfall, and duration of sunlight between Dieng and Chile regions above, it can be concluded that the Dieng region has higher temperatures and rainfall, and lower sunlight duration compared to the Chilean region. This was assumed to affect the rutin content contained in CP fruit in this study, where the value was lower than in previous study (Rusiah *et al.*, 2005; Stolpe and Undurraga, 2016). Also, the lower rutin content of CP fruit in this study compared to previous studies was assumed to be caused by the homogenization of the sample which was still lacking when testing so that its solubility in the solvent was less than optimal. Solubility is defined as the interaction of two or more substances to form a homogeneous molecular disperse. Solubility is related to the particle size of a sample. The greater the surface area of the sample, the greater the interaction with the solvent, so that its solubility will increase. In this study, the homogenization of the sample, CP fruit, was carried out by blending CP fruits. However, when mixed with solvents there was still a small amount of CP fruits deposits at the bottom of the tube (Kumar and Singh, 2016).

The improvement of TNF-α levels in the group that received CP fruit juice is presumed to be caused by the content of various compounds in CP fruit juice, which can act as an anti-inflammatory. CP fruit juice contains a rutin compound (Simirgiotis, 2009). In addition to those compounds, according to Pinto *et al.* (2009), CP fruit also contains quercetin, caffeic acid, chlorogenic acid, and coumaric acid compounds. Rutin, quercetin, caffeic acid, chlorogenic acid and coumaric acid in CP fruit juice work synergistically in controlling inflammatory conditions in T2DM. Rutin and caffeic acid can inhibit the expression of pro-inflammatory cytokine genes, such as TNF-α, which will then reduce its production so that the TNF-α levels decreased (Yang *et al.*, 2013; Niture, Ansari and Naik, 2014). Rutin is also able to reduce the formation of AGEs in diabetes mellitus condition. The high levels of blood glucose that occur chronically can cause glucotoxicity in pancreatic β-cells. Glutotoxicity enhances the occurrence of oxidative stress and accumulation of AGEs. Accumulated AGEs can stimulate the increase of pro-inflammatory cytokines and cell death which then underpins the occurrence of

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diabetes complications (Liang *et al*., 2018; Volpe *et al*., 2018).

Quercetin, chlorogenic acid and coumaric acid compounds in CP fruit juice improve inflammatory conditions through the NF-kB pathway. NF-kB is a complex protein that plays a key role in DNA transcription, cytokine production, and also cell survival (Chen *et al*., 2016; Liang and Kitts., 2016; Zhao *et al*., 2016; Yahfoufi *et al*., 2018). The history of excessive calorie intake and chronic hyperglycemia in T2DM can increase the production of ROS and AGEs. This will then initiate the production of pro-inflammatory cytokines through the activation of the NF-kB pathway. The NF-kB protein was initially inactive because it was tied to IkB in the cytoplasm. The existence of the excessive production of ROS will activate the IKK which further phosphorylating IkB. Phosphorylation of IkB causes protein degradation of IkB itself with the help of proteasome and resulted in the release of NF-kB. NF-kB then translocates into the nucleus and enhances the expression of several genes, including the pro-inflammatory cytokine genes, one of which is TNF-α (Gonzalez *et al*., 2012; Suryavanshi and Kulkarni, 2017; Chen *et al*., 2018).

Besides through the NF-kB pathway, a coumaric acid compound in CP fruit juice is also able to decrease TNF-α levels via the MAPK pathway (Zhao *et al*., 2016; Suryacanshi and Kulkarni, 2017). MAPK is a series of proteins in the cells involved in cell communication. The MAPK pathway plays an important role in signaling the communication of receptors on the cell surface to DNA within the cell nucleus to regulate several cellular functions such as cell proliferation, growth, differentiation, migration, and death. Bonds between several factors with number receptors on the cell surface result in signal transduction and activation of gene expression and then end this signal as negative feedback (Manzoor and Koh, 2012). The stimulus from the outside of cells, such as oxidative stress, stimulates MAPKKK to phosphorylate and activate MAPKK. The activated MAPKK then phosphorylates and activates MAPK. Furthermore, the activated MAPK phosphorylates some transcription factors, thereby causing an increase in the expression of pro-inflammatory cytokine genes, one of which is TNF-α (Chen *et al*., 2018). Also, a decrease in TNF-α levels occurs in a group that received rutin 10 mg/200 g BW/day for 30 days (X3). The results of this study were supported by previous studies stating that rutin administration at a dose of 50 and 100 mg/kg BW for 21 days may significantly reduce TNF-α levels in diabetic rats (Volpe *et al*., 2018).

TNF-α levels in the rutin group were not significantly different compared with the negative control group. It indicates that the administration of rutin 10 mg/200 g BW/day for 30 days in T2DM rats may decrease TNF-α levels equivalent to the normal group. Also, TNF-α levels were not significantly differed between X2 and X3 groups. These results indicate that CP fruit juice 8 mL/200 g BW/day has a similar ability with rutin 10 mg/200 g BB/day in lowering TNF-α levels in T2DM rats. It is presumed because CP fruit juice does not only contain rutin compounds, but also other compounds, such as quercetin, caffeic acid, chlorogenic acid and coumaric acid (Pinto et al., 2009). These compounds work synergistically as anti-inflammatory so they can match the rutin ability to reduce TNF-α levels in T2DM. On the other hand, the high levels of TNF-α in the K+ group at the end of this research is due to the chronic hyperglycemia condition that occurs due to the administration of HFD and STZ without being accompanied by remedial efforts. Hyperglycemia itself then increases the levels of pro-inflammatory cytokines, one of which is TNF-α, through increased macrophage stimulation, oxidative stress and AGEs formation (Giri *et al*., 2018).

The results of this study also show that CP fruit juice can decrease fasting blood glucose levels in T2DM rats. The improvement of blood glucose levels in the group that received CP fruit juice is presumed to be caused by the content of various compounds in CP fruit juice, which can act as an anti-hyperglycemia. CP fruit contains a rutin compound. Besides rutin, according to Pinto *et al*. (2009), CP fruit also contains quercetin, caffeic acids, chlorogenic acid and coumaric acid (Pinto *et al*., 2009). Rutin, caffeic acid and chlorogenic acid in CP fruit juice are presumed to be lowering blood glucose levels through several mechanisms. Firstly, these compounds can reduce glucose absorption in the small intestine by inhibiting α-amylase and α-glucosidase enzymes. The α-glucosidase enzyme is a membrane-bound enzyme located in the small intestinal epithelium that catalyzes glucose breakdown from disaccharides into monosaccharides. Inhibition of glucose absorption from the colon can prevent the occurrence of increased blood glucose levels sharply. Secondly, those compounds can regenerate the pancreatic β-cell damage and protect pancreatic β-cell from glucotoxicity so that insulin secretion and glucose uptake can be increased (Matsuda and Shinomira, 2013; Meng *et al*., 2013; Niture *et al*., 2014; Dhungyal *et al*., 2014; Volpe *et al*., 2018).

Also, rutin, quercetin, and caffeic acid can increase GLUT-4 translocation so that glucose uptake into the cell can be increased (Niture *et al*., 2014; Dhungyal *et al*., 2014; Mukhopadhyay and Prajapati, 2015). Then the

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increase in gluconeogenesis is believed to be one of the causes of hyperglycemia in diabetes. Rutin and coumaric acid in CP fruit juice are known to reduce gluconeogenesis by decreasing the activity glucose-6-phosphatase and fructose-1.6-bisphosphatase enzymes as well as increasing the activity of hexokinase enzyme (Niture *et al*., 2014; Shairibha *et al*., 2014, Amalan et al., 2015). Quercetin and caffeic acid also act as anti-hyperglycemic by increasing the activity of glucokinase enzyme in the liver so that the glucose storage in the liver increases and the production of hepatic glucose decreases (Dhungyal *et al*., 2014; Mukhopadhyay and Prajapati, 2015). Improved fasting blood glucose levels also occur in the diabetic with rutin 10 mg/200 g BW/day for 30 days treatment group. This result is in line with research conducted by Tanko *et al*. (2017) where rutin administration at a dose of 50, 100 and 200 mg/kg BW for 28 days significantly decrease blood glucose levels compared to the control group in diabetic rats (Tanko *et al*., 2017).

The fasting blood glucose levels in the intervention group of CP fruit juice at 8 mL/200 g BW/day were not significantly differed when compared with the rutin group. These results suggest that 8 mL/200 g BW/day of CP fruit juice has a similar ability with rutin at 10 mg/200 g BW/day in lowering fasting blood glucose levels in T2DM rats. It is presumed because CP fruit juice does not only contain rutin compound but also contains other compounds, such as quercetin, caffeic acid, chlorogenic acid and coumaric acid (Pinto *et al*., 2009). These compounds work synergistically to control hyperglycemia so that they can match rutin ability to decrease fasting blood glucose levels in T2DM. On the other hand, the high level of fasting blood glucose in the diabetic control group may have resulted from the continuous production of ROS feedbacks. This group received standard feed during the 30 days intervention period, but the glucose uptake results in the metabolism of feed in the rats' body decreased by due to the incidence of pancreatic β-cell damage and insulin resistance in peripheral tissues due to T2DM condition, that leads to increased ROS and blood glucose levels chronically, and no attempt to improve the condition (Matsuda and Shinomira, 2013; Hurrle and Hsu, 2017).

5. Conclusion

CP fruit juice can be a recommended fruit juice for the diabetic condition by reducing TNF-α and fasting glucose blood levels.

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