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Immunomodulatory Effects of Unripe Sapodilla (*Manilkara zapota*) Fruit Extract Through Inflammatory Cytokine Regulation in Type 1 Diabetic Mice

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Abstract

Background: One of the most prevalent metabolic disorderin the world is diabetes mellitus (DM),marked by chronic inflammation of pancreatic β-cells.To alleviate this disease, the response of the immune system can be manipulated to produce anti-inflammatory molecules.This research aims to evaluate the immunomodulatory activities of unripe sapodilla fruit (USF) extract according to the profile of TNF-αand IFN-γthat expressed from CD4 T cells, $CD4^+CD25^+IL\text{-}10^+$, and malondialdehyde (MDA) in type 1 diabetes mellitus (T1DM).**Methods:**Unripe Sapodilla Fruit (USF) was extracted with water bymaceration and freeze-drying process.Liquid ChromatographyHigh Resolution Mass Spectrophotometry (LC-HRMS)was used to identify the phytochemical content of USF extract. Twenty-five male BALB/c mice were randomized into five groups (n=5). A single high dose (145 mg/kgBW) of streptozotocin (STZ) was administered intraperitoneally to induce T1DM. USF extract was administered orally once a day for 14 days. Blood glucose levels and body weight were assessed once every three days for 15 days. Splenic cells were immunostained with antibodies. The sample underwent a flow cytometric analysis and ANOVA was used to statistically assess all the data with p-value of≤0.05 were significant. **Results:**LC-HRMSrevealed that there were 12 major bioactive compounds in aqueous USF extract, which have activity of anti-diabetic, anti-inflammatory, free radical scavenging, and NF-κβ inhibitor.The results of USF administration showed that glucose levels in diabetic mice were reduced but not significant. $CD4^+TNF\text{-}\alpha^+$, $CD4^+IFN\text{-}\gamma^+$and MDA expression in diabetic mice decreased after USF administration. Meanwhile, $CD4^+CD25^+IL\text{-}10^+$ expression in diabetic mice increased after USF administration.**Conclusion**: USF extract acts as an immunomodulator by regulating inflammatory cytokines in a non-dose-dependent manner. The ability to regulate inflammatory cytokines is shown by enhancing IL-10 as an anti-inflammatory cytokine to suppress TNF-α and IFN-γ. We also show a decrease in MDA expression as a result of USF's antioxidant activity, which aids in the suppression of inflammatory cytokines in diabetic mice.

Keywords: diabetes mellitus, anti-inflammatory,proinflammatory, streptozotocin, unripe sapodilla

1. Introduction

A metabolic disorder known as diabetes mellitus (DM) is marked by hyperglycemia or abnormally high blood glucose levels.Defects in insulin secretion, insulin action, or both contribute to hyperglycemia. One of the most common metabolic diseases in the world, it is constantly expanding. Based on International Diabetes Federation (IDF)data, people with DM reached 463 million in 2019 and increased to 537 million in 2021. If this condition is not properly treated, it is predicted that there would be 643 million sufferers in 2030 and 783 million persons in 2045 (IDF Diabetes Atlas, 2022).

Chronic hyperglycemia in DM patients could cause an energy metabolism disorder, early aging, and cell death. These lead to various microvascular and macrovascular complications (Cicimil *et al.*, 2018).Patients with DM, particularly those with type 1 diabetes mellitus (T1DM), are characterized by the death of the insulin-producing pancreatic islet cells. Hyperglycemia promotes reactive oxygen species (ROS) production such as H_2O_2, $O2^{-\cdot}$ and NO. The increase of ROS in T1DM is a side effect of glucose uptake control failure in muscle and fat tissue. Insufficient insulin causes intracellular glucose concentration, which accelerates glycolysis. Through the tricarboxylic acid (TCA) cycle, the rate of glycolysis contributes to the production of superoxide (O2) in the body (Rahangdale et al., 2009). The accumulation of ROS is shown by malondialdehyde (MDA) expression. ROS will interact with polyunsaturated fatty acids that generate lipid peroxidation to produce MDA and other compounds. MDA is a reactive aldehyde produced by lipid peroxidation that acts as a warning sign that the body is under oxidative stress (Yonny et al., 2016).High expression of MDA can cause cell damage and trigger

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inflammation (Budiwiyono *et al.*, 2021). The increase of ROS will activate nuclear factor kappa B (NF-κβ)to triggerpro-inflammatory cytokinesexpression in T1DM (Fatima *et al.*, 2016). Excessive inflammation would speed up the loss of pancreatic islet cells and worsen immune cell infiltration, which would attack and cause pancreatic cell apoptosis. This mechanism is initiated by enhancingNF-κβ activation from proinflammatory cytokines induction (Szablewski, 2014). NF-κβ is a transcription factor that also targets proinflammatory cytokines such as TNF-α and IFN-γ as its target genes to amplify the inflammatory response by the body. The amplification of inflammatory response could worsen the condition of autoimmune diseases such as T1DM (Bacher and Schmitz, 2004). Proinflammatory cytokines like TNF-α and IFN-γ have been linked to cell destruction in DM patients (Karshenase, 2018). β cell destruction by immune cells is what makes DM considered to be an autoimmune disease. Hence, immunosuppressive activities are needed to maintain homeostasis within the immune system.

Regulatory T cells (T-reg) are lymphocytes known for their suppressive ability. T-reg are generally marked with CD4+CD25+ biomarkers, which modulate immune system with suppress or downregulate effector cell. T-reg are necessary because immune system suppression is critical for preserving immune system homeostasis, particularly in autoimmune disorders like DM. Treg play an immunosuppressive role by secreting IL-10 as one of the cytokine with potent anti-inflammatory properties. IL-10 can reduce the presentation of antigen and the production of proinflammatory cytokines by other immune cells (Bijjiga and Martino, 2013).

Sapodilla (*Manilkarazapota*(L.) P.Royen) comes from Sapotaceae family. It is a popular edible fruit, and it is common in tropical countries such as Indonesia, Brunei, China, India, Malaysia, Singapore, Thailand and Vietnam (Rojas-sandoval & Praci, 2017). The sapodilla fruit contains many phytochemicals with high antioxidant and anti-inflammatory activities, such as epigallocatechin, catechin, 4-o-galloylchlorogenic acid, methyl chlorogenate, chlorogenic acid, and gallic acid (Baky *et al.*, 2016). The aforementioned compounds have strong antioxidant properties, can scavenge free radicals such as H2O2, O2-, and NO, and can also reduce lipid peroxidation. The flavonoids within the extract can also inhibit NF-κβ, which is crucial because it is the transcription factor for proinflammatory cytokines (Leelayungrayub *et al.*, 2019).In this experiment, we used unripe sapodilla fruits with higher polyphenol contents than ripe ones(Shui *et al.*, 2004).This experiment aims to assess the immunomodulatory activities of sapodilla fruit extract in controlling ROS and inflammatory cytokines production.

2. Methods

2.1. Herb extraction

Unripe sapodilla fruits with the specimen number 074/223A/102.7/2020 were collected in Nganjuk City and determined by UPT Materia Medica in Batu City, Indonesia. The extraction of unripe sapodilla fruit refers to (Tamsir *et al.*, 2020) with few modifications. Unripe sapodilla fruits (USF) were peeled and dried before grounded until they became powder. Sapodilla powder was dissolved in distilled water at a 1:10 ratio (sapodilla:solvent, w/v) for the extraction in an Erlenmeyer flask covered with aluminum foil. The solution was mixed at 200 rpm for 24h with a magnetic stirrer. A freezedrying method was used to remove the water after filtering the homogenate extract using Whatman filter paper.

2.2. Phytochemical content screening

The previously prepared sample was put into an autosampler, then injected into the liquid chromatography-high-resolution mass spectrometry(LC-HRMS). To determine the phytochemical composition of the USF aqueous extract, Thermo Scientific Dionex Ultimate 3,000 RSLC nano liquid chromatography (LC) and Thermo Scientific Q Exactive Mass Spectrometry (MS) was used (Thermo Fisher Scientific Inc., USA). Hypersil GOLD aQ serves as the LC instrument's stationary phase, and solvents A and B, which together make up the mobile phase, contain 0.1% formic acid in water and acetonitrile, respectively. The LC was operated at a flow rate of 40 μL/min, a run period of 30 min, and a column temperature of 30 °C (Purwanti et al., 2021). The obtained data were analyzed using MZmine with KEGG Library database. Compound was selected with PASS Online database, if compound has probability to be active (Pa) was more than 0.3, indicating that compound has anti-diabetic, anti-inflammatory, free radical-scavenging, and NF-inhibitory properties.

2.3. Experimental design

All methods in this research were reviewed and verified by the Animal Care and Use Committee of Brawijaya University with ethical approval number 016-KEP-UB-2021.Seven-week-old male BALB/c mice (n=25) were taken from Maulana Malik Ibrahim State Islamic University in Malang, Indonesia. Each mouse was maintained in a pathogen free environment in the Animal Physiology Laboratory at Brawijaya University while being fed a nutritional pellet diet and given proper water hydration. The animals were separated into five groups after acclimatization. Normal mice group (N) is the group of mice that are not injected withSTZ nor given any USF treatment (n=5); diabetic mice group (DM) is the group of mice exposed to streptozotocin (STZ) without any USF treatment (n=5); while in DM treated group, mice were exposed to STZ and were given 3 doses of USF extract 150 mg/kgBW (DM-D1), 250 mg/kgBW (DM-D2), and 400 mg/kgBW (DM-D3) (n=5 for each respective dose). USF extract was given orally once daily for 14 days. Blood sugar levels and body weight were evaluated every three days for 15 days.

2.4. Induction of Diabetes Model

Mice were acclimatized for a week before the experiment. A single high dosage (SHD) of the diabetogenic agent streptozotocin (BioWorld, USA) was injected to induceT1DM. Intraperitoneal (i.p.) injections of STZ at a dose of 145 mg/kgBW were used to generate diabetic mice(Lestari et al., 2022).Prior to receiving STZ injection, mice were fasted for 4 hours. STZ was freshly dissolved in citrate buffer with pH 4.5 for quick infusion within 10 minutes (Lestari et al., 2022). Blood glucose levels were assessed using an Easy Touch glucometer (Bioptik Technology Inc., Taiwan) on the fourth and

seventh day after STZ injection. When the mice's blood glucose level exceeded 200 mg/dL, they were considered in T1DM condition. Previous experiment reported that T1DM in male mice BALB/c could be induced with a single high-dose injection of STZ (Adharini *et al.*, 2020).

2.5. Immunostaining and flow cytometry

The antibodies used in this study include anti-CD4 and anti-MDA conjugated FITC, the others such anti-CD25, anti-TNF-α, anti-IFN-γ, and anti-IL-10 conjugated PE(BioLegend, USA). Spleen and liver were isolated, and then homogenized with phosphate buffer saline (PBS). Spleen cells were isolated to analyze cytokine-secretinglymphocytes, while hepatocytes were isolated to obtain the MDA production. Homogenate was centrifuged at 2500 rpm and 10˚C for 10 minutes. The pellet was resuspended in PBS for a second centrifugation after the supernatant from previous centrifugation was discarded. Extracellular antibodies were used to stain the cells at a concentration of 5 µg/100 µL (CD4 and CD25 antibody). Intracellular flow cytometry staining was carried out by using 50 µL CytofixTM. The suspension was incubated for 20 minutes (4˚C). Wash buffer solution (BioLegend, USA) was added for permeabilization.TNF, IFN, IL-10, and MDA antibodies as intracellular antibodies were used to stain the cells after centrifugation. The samples were prepared for analysis using flow cytometry(BD FACS-Calibur, USA) by adding 400 µL of PBS to the cuvette (Adharini *et al.*, 2020).

2.6. Statistical analysis

Statistical analysis was accomplished by SPSS 21.0 software for the ANOVA and Duncan Multiple Range tests. The probability value of 0.05 was regarded as significant between the two groups. The statistical analysis was done to analyze the blood glucose levels, $CD4^{+}TNF\text{-}\alpha^{+}$, $CD4^{+}IFN\text{-}\gamma^{+}$, $CD4^{+}CD25^{+}IL\text{-}10^{+}$, and MDA expressions. All data were shown in mean ± standard deviation (SD).

3. Results

3.1. Phytochemical content screening of USF extract

This research identifies the bioactive compounds of unripe sapodilla fruit extract. LC-HRMS analysis revealed that there were 12major bioactive compounds in aqueous USF extract, based on the results of the MZmine library analysis, which have biological activities including anti-diabetic, anti-inflammatory, free radical scavenger, and NF-κβ inhibitors. The compounds identified were mostly polyphenolic compounds including chlorogenic acid, caffeic acid, flavonols, luteoliflavan, ampelopsin, luteone, biflorin, catechins, and their derivatives (epicatechin, gallocatechin, leucocyanidin), then proanthocyanidins as derivatives of tannins (Figure 1).

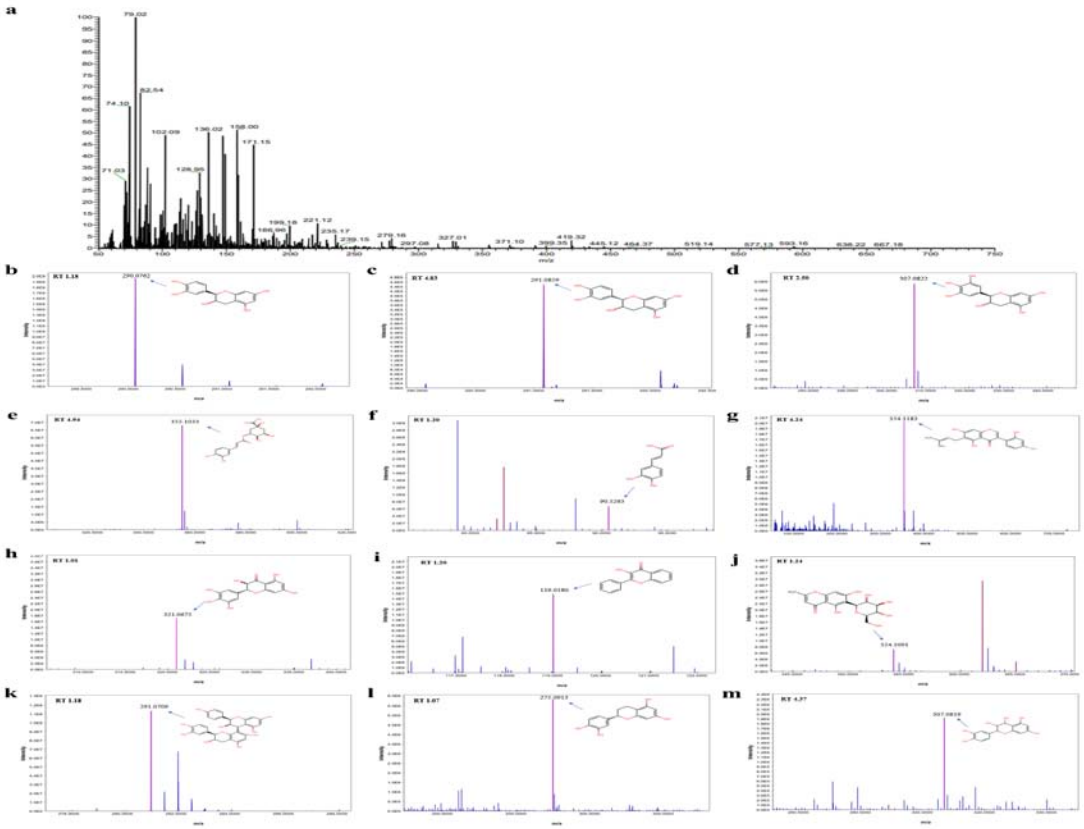


Figure 1.LC-HRMS spectrum of bioactive compounds in the aqueous extract of USF. (a) The LC-HRMS total ion chromatogram was carried out using Thermo Scientific Dionex Ultimate 3,000 RSLC nano liquid chromatography (LC) and Thermo Scientific Q Exactive Mass Spectrometry (MS). Twelve mass spectra were shown b-m. (b) catechin, (c)epicatechin, (d) gallocatechin, (e) chlorogenic acid, (f) caffeic acid, (g) luteone, (h) ampelopsin, (i) flavonol, (j) biflorin, (k) proanthocyanidin, (l) luteoliflavan, and (m) leucocyanidin.

3.2. USF treatment has effect as anti-diabetic in diabetic mice

The glucose level of diabetic mice (DM, DM-D1, DM-D2, DM-D3) was 386 mg/dL in the first measurement, and the glucose level in DM group mice was increased to 432 mg/dL after 15 days (Figure 2). The glucose level in the normal mice group (N) was constantly lower than all groups in 15 days, around 138-154 mg/dL, and greatly lower than DM mice group ($p<0.05$). The administration of USF extract has given an anti-hyperglycemic effect in diabetic mice, as shown by the decrease in glucose level after treatment of 14 days. USF treatment had successfully decreased glucose levels in DM-D1 and DM-D3 group compared to the diabetic control group (DM). However, the glucose levels of DM-D1 and DM-D3 group were still significantly higher than normal group. The DM-D2 mice group's blood glucose level did not differ significantly from that of the normal and DM mice group. This means that USF extract treatment in DM-D2 has a higher anti-hyperglycemic effect than other treated groups. Despite the fact that the value had not yet reached the normal level, the final glucose level in the USF treatment group (DM-D1, DM-D2, DM-D3) was lower.

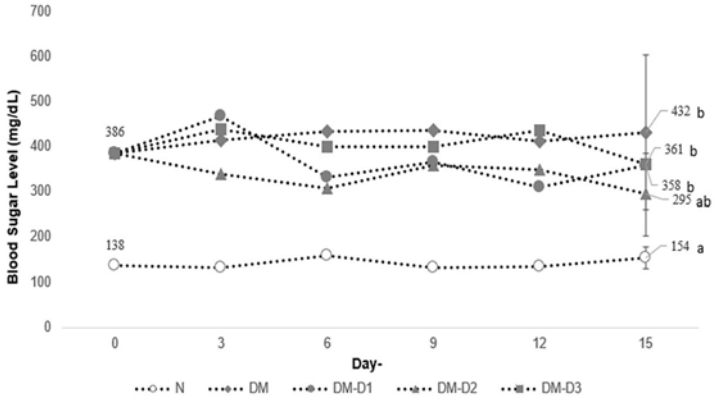


Figure 2. The administration of USF extract has given an anti-diabetic effect, as shown at the decreased blood glucose levels after 14 days of treatment.

3.3. MDA expression levels in diabetic mice are decreased after USF treatment

The expression levels of free radicals in diabetic mice are shown by the MDA expression level (Figure 3). The MDA expression level in the DM mice group increased from 23.65% to 40.87% ($p<0.05$) compared to the N group. The administration of USF extract has given an antioxidant effect in DM-treated mice groups, as shown by the decrease of MDA expression in each treatment group after 14 days. All groups of USF treated mice had significant lower level of MDA ($p<0.05$) with value of 21.75%, 30.81%, 34.14% in DM-D1, DM-D2, and DM-D3, respectively. Among these three groups, DM-D1 was the most effective dosage to reduce MDA level. When compared to the normal mice group, the MDA expression level on DM-D1 treated group was still higher but not significant. However, the two others, DM-D2 and DM-D3 groups were significantly higher. These results implied that USF extract had an antioxidant effect in diabetic mice, and DM-D1 treatment has the highest antioxidant effect.

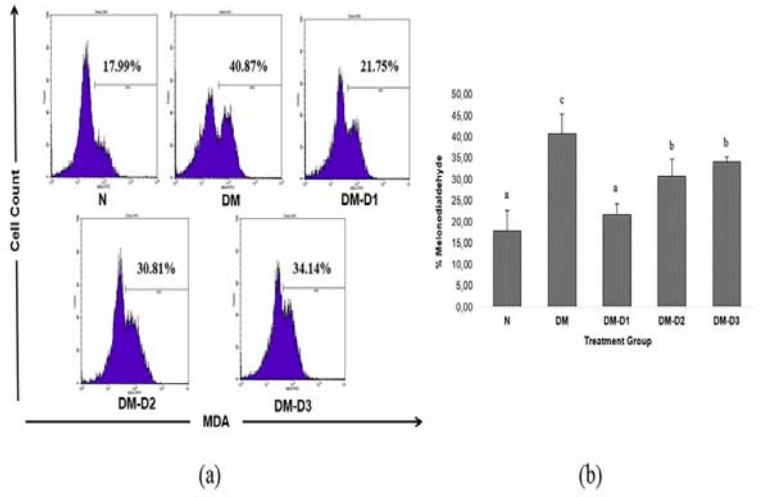


Figure 3. The relative number of MDA in each mice group. (a). Flow cytometry analysis and (b). Results of statistical analysis (p-value≤0.05). N= Normal group; DM= Diabetic mice group (145 mg/kgBW STZ i.p injection/ SHD STZ); DM-D1 group (SHD STZ + 150 mg/kg BW USF treatment); DM-D2 group (SHDSTZ + 250 mg/kg BW USF treatment); DM-D3 group (SHD STZ + 400 mg/kg BW USF treatment).

3.4. $CD4^+TNF\text{-}\alpha^+$ and $CD4^+IFN\text{-}\gamma^+$ expression levels in diabetic mice were decreased after USF treatment

This study has shown that the levels of TNF-α (Figure 4a &4c) and IFN-γ (Figure 4b &4d) secreted by $CD4^+$ T cells between normal (N) and diabetic mice (DM) groups are significantly different ($p<0.05$). DM group had a more incredible amount of TNF-α and IFN-γ expression levels than normal mice (N). Additionally, when compared to the DM mice group without receiving treatment, oral administration of USF extract at all doses for 14 days in diabetic mice significantly lowers the expression levels of $CD4^+TNF\text{-}\alpha^+$ and $CD4^+IFN\text{-}\gamma^+$($p<0.05$).These results imply that USF extract treatments with doses 1(150 mg/kg) and 2 (250 mg/kg) were assessed as effective doses to restore the expressions of TNF-α and IFN-γ close to normal conditions.

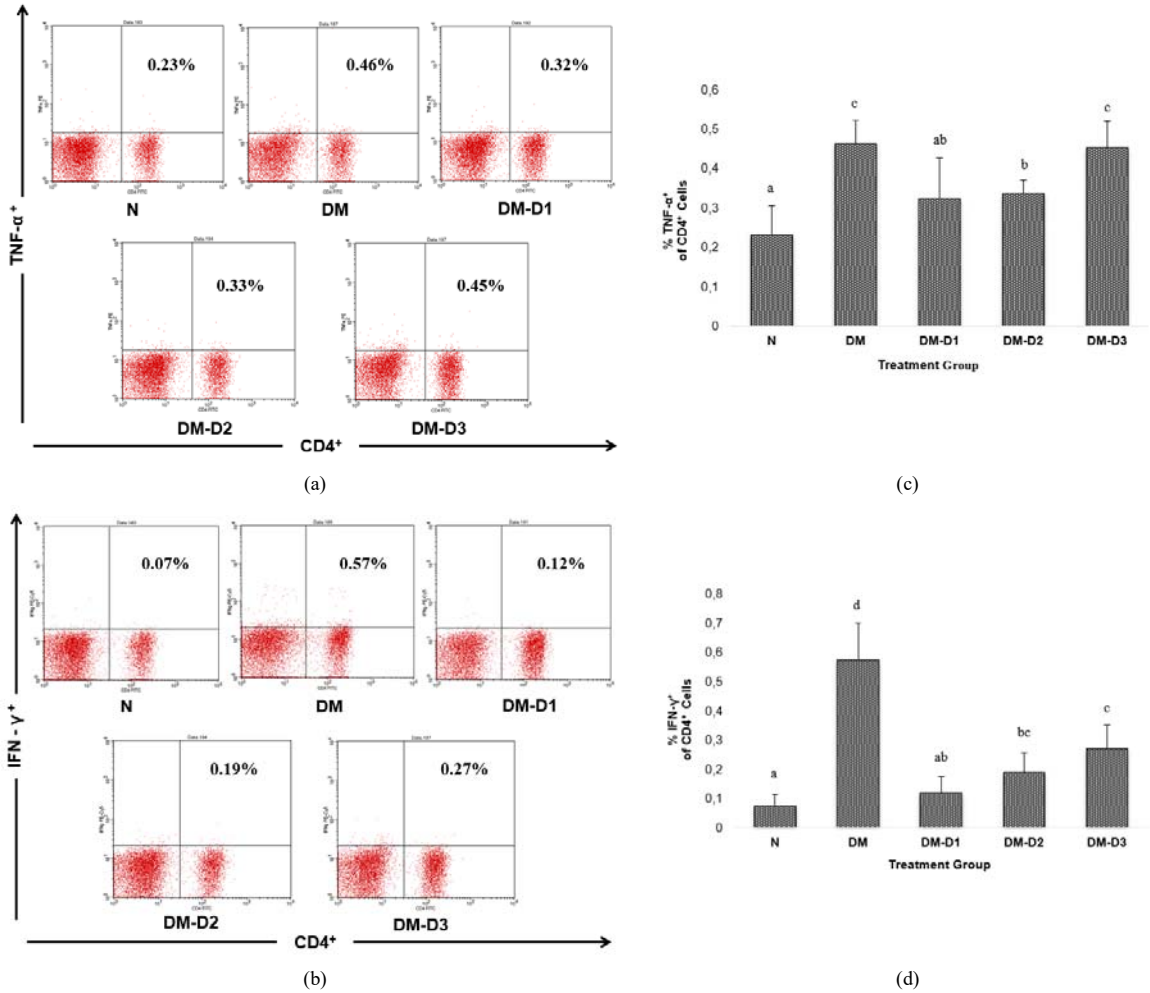
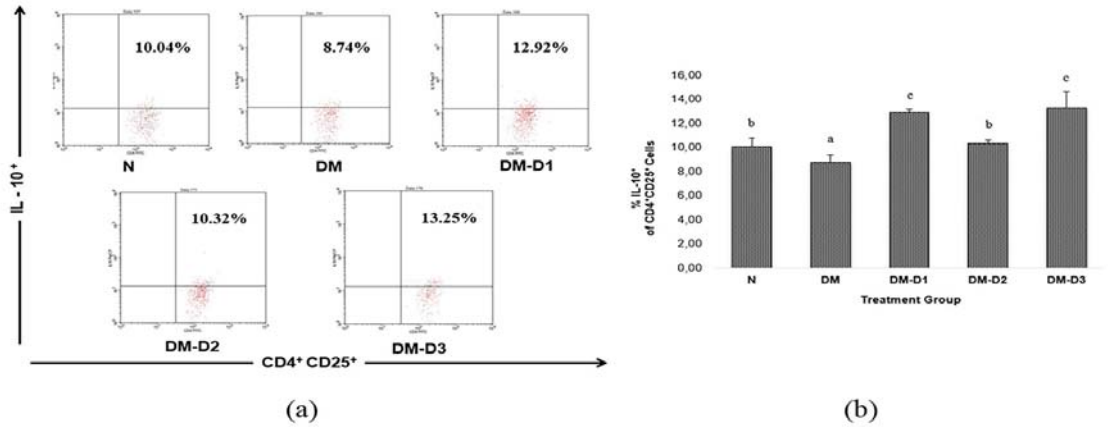


Figure 4. The relative number of $CD4^+TNF\text{-}\alpha^+$ and $CD4^+IFN\text{-}\gamma^+$ cells in each mice group. (a,b) Plot flow cytometry analysis of TNF-α and IFN-γ by CD4 T cells, (c,d) Results of statistical analysis (p-value≤0.05). N= Normal group; DM= Diabetic mice group (145 mg/kg BW STZ i.p injection/ SHD STZ); DM-D1 group (SHD STZ + 150 mg/kg BW USF treatment); DM-D2 group (SHD STZ + 250 mg/kg BW USF treatment); DM-D3 group (SHD STZ + 400 mg/kg BW USF treatment).

3.5. $CD4^+CD25^+IL\text{-}10^+$ expression levels in diabetic mice are increased after USF treatment

The expression levels of regulatory T (T-reg) cells secreting IL-10 in the DM mice group are significantly lower ($p<0.05$) when compared to normal mice group (Figure 5). Meanwhile, all doses of USF treatments result in a significantly higher production of T-reg cells that secrete IL-10 when compared to the group of diabetic mice ($p < 0.05$). Dose 1 and 3 have significantly increased expression levels compared to the animals in the normal group ($p < 0.05$). Dose 2 of USF treatment manages to recover the expression of IL-10 secreting T-reg cells near-normal mice group levels ($p < 0.05$). These results imply that USF extract can increase the IL-10 expression of regulatory T cells, with dose 2 being the best dose to recover IL-10 secreting T-reg cells to near-normal levels.



(a) (b)

Figure 5. The expression of $CD4^+CD25^+IL\text{-}10^+$ T cells in each mice group. (a). Plot flow cytometry analysis IL-10 by T-reg cells and (b). Results of statistical analysis (p-value ≤ 0.05). N= Normal group; DM= Diabetic mice group (145 mg/kg BW STZ i.p injection/ SHD STZ); DM-D1 group (SHD STZ + 150 mg/kg BW USF treatment); DM-D2 group (SHD STZ i.p injection + 250 mg/kg BW USF treatment); DM-D3 group (SHD STZ + 400 mg/kg BW USF treatment).

4. Discussion

Streptozotocin (STZ) is a diabetogenic agent in several studies using mice. STZ induces diabetes by selectively entering the pancreatic β cells through the glucose transporter receptor (GLUT2) and attacking pancreatic β cells. STZ had a structure similar to glucose that could enter the cell through GLUT2 (Graham *et al.*, 2011). We selected strain BALB/c mice because this strain is a widely used animal model in diabetes mellitus research (Adharini *et al.*, 2020). The liver regulates glucose level homeostasis through glycolysis, gluconeogenesis, and glycogen synthesis. In T1DM, the body lacks insulin due to pancreatic -cell destruction, which disrupts glucose level homeostasis. The increase in glucose level also causes the liver to produce excess glucose because the body cannot absorb and process glucose. Homeostasis of glucose levels could be improved by using phenolic compounds (Rajappa *et al.*, 2019).

This study found that USF extract has an anti-diabetic effect by decreasing the glucose level in diabetic mice (Figure 2). The decrease in glucose level in diabetic mice with USF treatment could be associated with phenolic compounds activity within the USF. Sapodilla is rich in polyphenol, and the USF is an excellent source of flavonoid and polyphenol compoundsuch as, catechin and derivatives, chlorogenic acid, caffeic acid, luteone, ampelopsin, biflorin, flavonol, luteoliflavan, leucocyanidin and proanthocyanidin. Based on previous research, unripe sapodilla had a higher total antioxidant capacity (TAC) value and total phenolic content (TPC) than ripe sapodilla. This value decreases during fruit maturation (Shui *et al.*, 2004). Based on the research from Shui et al (2004) and Wang et al (2012), several contents of polyphenols that are reported are the same as the results of this research. Composition of extract act to several mechanism to reduce glucose level in DM condition. Tannin increased glucose uptake through insulin signalings, such as increasing the activation of protein phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK). All the protein activation will increase GLUT-4 translocation to absorb blood glucose (Kumari and Jain, 2012). Moreover, tannins also increase insulin expression by repairing and regenerating pancreatic β cells (Velayutham *et al.*, 2012). Catechin and their derivatives were able to decrease the blood glucose levels by inhibiting the glucose absorption in intestine (Kumari and Jain, 2012).Epicatechin increased insulin production and secretion by modulating cellular signaling on CaMKII activation at GPR40 receptors (Yang and Chan, 2018). Chlorogenic acid is similar to metformin by increasing cells' sensitivity to insulin and glucose uptake in the liver. Chlorogenic acid lowered blood sugar levels by stopping G6-phase activity in glycogenolysis and gluconeogenesis in hepatic cells (Meng *et al.*, 2013).

MDA expression in the DM group is increased because of the hyperglycemia effect in diabetic mice (Facchini *et al.*, 2000). The presence of MDA in the body causes toxicity, changes in DNA, and oxidation of mutagenic lesions leading to cell death and inflammation. ROS accumulation should be suppressed to lower MDA expression (Luzcaj and Skrzydlewska, 2003). MDA expression in USF-treated diabetic mice is shown to be decreased because the compounds in USF could have an antioxidant effect on scavenging ROS (Xie *et al.*, 2017). The decrease in MDA expression is due to USF's phytochemical content that can capture free radicals. Their antioxidant potentials refer to a natural compound's capacity to neutralize free radicals and bind metal ions through functional grouping of their structure **(Dehimat et al., 2021).**It is possible that the chlorogenic acid and caffeic acid contained in this extract can act as antioxidants by releasing hydrogen atoms to scavenge free radicals (Liang & David, 2016). Chlorogenic acid also reported that can generate an endogenous antioxidant enzyme (Stefanello *et al.*, 2015). The decrease of ROS is linked with β cell survival and the reduction of β cell deaths by reducing proinflammatory cytokines.

After 14 days of treatment with USF extract, CD4+ effector T cells expressed lower proinflammatory molecules like TNF-α and IFN-γ, indicating that USF extract was effective in lowering the risk of complications caused on by the high levels of TNF-α and IFN-γ expression associated with diabetes mellitus. Excessive TNF-α production will increase microvascular complications such as increased chronic inflammation of the eye (Gonzalez *et al.*, 2012). High levels of IFN-γ will trigger more severe pancreatic cell destruction (Suarez *et al.*, 1996). The ability to reduce proinflammatory

cytokines expression occurs due to the presence of polyphenolic and flavonoid compounds in the water extract of unripe sapodilla fruit (*Manilkara zapota*). The reduction of oxidative stress contributes to the mechanism of reducing inflammation in the body because the increased expression of proinflammatory cytokines is induced by the increase of ROS (Alipour et al., 2018). Polyphenol compounds, especially tannins derivatives such as proanthocyanidin, have anti-inflammatory effects that play an essential role in cellular protection against inflammation, as well as inhibiting the expression of iNOS genes and NF-κβ transcription factors (Hussain et al., 2016).

Bioactive contents of USF, such as chlorogenic acid,are also reported to have a role in controlling the expression of NF-κβ,which releases proinflammatory cytokines IL-6, IL-1β and TNF-α and reduces AGE-RAGE overexpression by inhibiting ROS formation. Other compounds such as catechins and their derivatives have also been reported to prevent complications in diabetes by reducing the expression of proinflammatory moleculesincluding TNF-α, IFN-γ, IL-18, IL-6, IL-1β and MCP-1. However, high doses of catechins are not recommended because they have side effects that do not have a good impact on the body. This also answers the results of this study which shows that treatment with the water extract of unripe sapodilla fruit (*Manilkara zapota*) is not dose-dependent. Low dose (150 mg/kgBW) is considered more effective because it shows a more significant reduction in TNF-α and IFN-γ inflammation when compared to the use of high doses such as dose 3 (400 mg/kgBW) in this study. Other compounds such as chlorogenic acid are also able to contribute in this mechanism of reducing oxidative stress and inflammation. According to earlier studies, chlorogenic acid is known to contribute to the recovery of DM rats by lowering the lipid content of hydrogen peroxide and enhancing the concentration of non-enzymatic antioxidants such glutathione (GSH), vitamin C, and vitamin E in the blood. This suggests that chlorogenic acid contributes to maintaining homeostasis in STZ-induced DM (Yan *et al.*, 2020) and reduces the production of proinflammatory molecules, such as TNF-α, LPS, IL-1β, and IFN-γ (Liang and Kitts, 2015).

Furthermore, this study shows that USF treatment can increase the anti-inflammatory cytokine expression represented by regulatory T cells secreting IL-10 (Figure 5). The compounds of USF support an anti-inflammatory response (Qiao *et al.*, 2016). The expression of IL-10 secreted by regulatory T cells that increase after USF treatment has strengthened the ability of the compounds within the USF extract as an anti-inflammatory. IL-10secreted by T-reg cells actsas an anti-inflammatory cytokine responsible for inhibiting inflammation. Previous research has reported that the role of T-reg cellsis associated with the effect of suppressive cytokines, including TGF-β and IL-10. Suppressive cytokine therapy is essential to prevent the onset of inflammatory diseases. (Lestari and Rifa'i, 2018).

It is known that DM has lower levels of CD4+CD25+IL-10+ expression (Qiao et al., 2016). Another study has shown that higher IL-10 expression in T1DM patients has better glucose control prediction (Sanda *et al.*, 2008). IL-10 and TGF-β are the two central cytokines secreted by regulatory T cells for immunosuppression. IL-10 can limit the activation of proinflammatory cytokines and decrease immune response mediated by other T cells (Hartati *et al.*, 2017). Unfortunately, higher IL-10 expression does not mean that it is always better. Excessive IL-10 expression could cause diseases related to Th2 cell hypersensitivity, such as systemic lupus erythematosus (SLE) (Biijiga and Martino, 2013). This is why we suggested that dose 2 (250 mg/kg BW) is the best dose to increase IL-10 expressing regulatory T cells. In this experiment dose 2, $CD4^{+}CD25^{+}IL\text{-}10^{+}$ expression significantly increased in the near-normal mice group compared to dose 1 (150 mg/kgBW) and dose 3 (400 mg/kgBW) which $CD4^{+}CD25^{+}IL\text{-}10^{+}$ expression was significantly elevated than normal healthy mice group. Furthermore, this result is also analogous to blood glucose levels data shown previously (Figure 2), demonstrating that dose 2 is the best dose to reduce blood glucose levels in this research.

5. Conclusions

According to this study's findings, USF extract can modulate the immune system by non-dose-dependently regulating inflammatory cytokines. The regulation ability of USF on inflammatory cytokines is shown by the decrease of TNF-α and IFN-γ secreted by helper Tcells and the increase of IL-10 as an anti-inflammatory cytokine secreted by T-reg cells. The decreasing expression levels of MDA showed ROS scavenging ability after USF treatment in diabetic mice and supported the regulation of inflammatory cytokines in diabetic mice. Further research is required to verify and confirm the results.

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