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# Antidiabetic Effects of *Tithonia diversifolia* and *Malus domestica* Leaf Extracts in Alloxan-Induced Sprague Dawley Rats

**Fatmawaty Yazid[1], Shania O. Salim[1], Faizal D. Rahmadika[1], Rosmalena Rosmalena[1*], Nina Artanti[2], Andini Sundowo[2], Vivitri D. Prasasty[3]**

[1]Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia
[2]Research Center for Chemistry, The Indonesian Institute of Sciences, South Tangerang, 15314, Indonesia
[3]Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta,12930, Indonesia

**ABSTRACT**

The limited supply of antidiabetic medication is one of the problems of diabetes medication in Indonesia. The wealth of Indonesian medical plants can be a solution to solve the problems, including the development of *Tithonia diversifolia* and *Malus domestica* as the plants are known to have antidiabetic effects. This research aimed to investigate *T. diversifolia* and *M. domestica* leaf extracts' effect on lowering fasting blood glucose (FBG) levels and histopathological changes in alloxan-induced Sprague Dawley rat livers. Materials and Methods: This study used 24 Sprague Dawley rats that were divided into 6 groups, which was a normal group (non-hyperglycemic group), the positive control group was given metformin, a negative control group was given mineral water, and the other three groups that were given *T. diversifolia* and *M. domestica* leaf extracts at doses of 200, 400, and 600 mg/kg BW. The rats were injected with alloxan at a dose of 120 mg/kg BW via intraperitoneal. Subsequently, after 4 days, the rat FBG levels were elevated. The rats were categorized as hyperglycemic if their blood glucose levels were at > 200 mg/dL and received treatment according to their groups for 16 days. The FBG levels were measured on days 4, 8, 12, and 16. After 16 days, the rats were dissected, and the liver of each rat was taken for histological examination with Haematoxylin and Eosin (H&E) staining. The FBG levels data were tested using one-way ANOVA. Results: The results showed that these plant extracts affected lowering FBG levels and affected hyperglycemic rat histopathology structures. The *T. diversifolia* (600 mg/kg) and *M. domestica* (200 mg/kg) extract showed the most effective lowering of FBG levels. Conclusion: Extract of *T. diversifolia* and *M. domestica* leaf extracts decreased diabetic rat FBG levels and improved the rat pancreas histological structures. It suggests the plant extracts had protective effects on liver complications.

**Keywords:** Antidiabetic agent, *Tithonia diversifolia*, *Malus domestica*, histopathology, hyperglycemia

**Correspondence**:
**Rosmalena Rosmalena**
Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia
**Email**: rosmalena2020@gmail.com

## INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder caused by the pancreas inability to produce insulin hormone. According to the Indonesian Ministry of Health Center for Data and Information (InfoDatin), the prevalence of diabetes mellitus on people aged 15 and older reached the number of 10,9% of the population in 2018, using the diagnosis criteria for DM performed by the Indonesian Society of Endocrinologist (PERKENI). Some efforts have been made to control the uprising cases of DM. Some of them were educating people on the risk factors of diabetes, sports, and consumption of the local traditional medicines [1, 2].

According to International Diabetes Foundation (IDF), the price for curative efforts on diabetes for each person in the Asia-Pacific region, including Indonesia, reached $1265.08 each year [3]. In the consensus by PERKENI, it is written that there are problems in the curative efforts on DM. Notably, there was a limited supply of antihyperglycemic drugs and inadequate health facilities [4]. The results of Basic Epidemiological Health Research (Riskesdas) noted that 9% of diabetic people did not take their antihyperglycemic drugs daily because of taking the traditional herbal medicines [2, 4]. If diabetes were not controlled, it could induce many complications on the human body internal organs, such as the liver [5].

*Malus domestica*, usually known as apple, was one of the popular fruit-bearing plants in Indonesia, with a sum of 1.9 million trees and 319.004 fruits produced in 2017 reached 11th rank on most produced fruit-bearing plants in Indonesia [6]. According to Sowa et al. (2016), apple fruits and leaves contain phenolic compounds such as flavonoids known to have antidiabetic activities [7].

Previous studies reported that flavonoids in paitan leaf (*Tithonia diversifolia*) might contribute as antidiabetic agents. The most effective *T. diversifolia* extract dose as an antidiabetic agent was 500mg/kg BW rats [8]. Other research also proved the most effective dose of paitan leaf extracts as an antidiabetic agent was 100mg/kg BW rat [9]. In addition, a diabetogenic compound used in this study, alloxan, works by a mechanism that damages β-pancreatic cells. Damage to these cells causes a decrease in insulin secretion, which increases blood sugar levels. However, no research discussed the effect of *T. diversifolia* on pancreatic histopathology [10]. Thus, this study aims to determine the effect of *T. diversifolia* and *Malus domestica* as antidiabetic agents on decreasing fasting blood glucose levels and histopathological changes in alloxan-induced Sprague Dawley rat pancreas.

## MATERIALS AND METHODS

### *Experimental design*

This study was conducted at the Laboratory of Medical Chemistry Department FKUI, Laboratory of Experimental Animals and Toxicology Ministry of Health Republic of Indonesia (RI), and Bogor Veteriner Research Institute. In this study, the experimental animals were male Sprague Dawley rats (body weight was 180-200 grams and age ranged from 2-3 months). The rats did not appear anatomically deformed. The subject of this research was selected by consecutive sampling, where all the subjects that met the inclusion criteria and did not have the

exclusion criteria were required. According to the Federer formula calculations, the required sample size of this research is 24 animals. This research was approved by the Ethical Committee Faculty of Medicine, Universitas Indonesia (Letter no. 19-07-09000).

***Preparation of Tithonia diversifolia leaf extract***

Paitan (*Tithonia diversifolia*) leaves was obtained from Pusat Penelitian dan Pengembangan – Lembaga Ilmu Pengetahuan Indonesia, Cibinong. The first step was to wash the paitan leaves thoroughly and proceed with drying the leaves. After drying, the leaves will be cut and ground into powder. This powder will go through the maceration or soaking process. The maceration process was done by immersing 500 g of a plant powder sample in 1 L alcohol solvent (70% ethanol). Not only soaked, paitan leaf powder is also stirred for two days. Stirring was done so that the phytochemical compounds can be dissolved properly. After two days, the supernatant was filtered using filter paper. The pellet was soaked with 70% ethanol solvent at least three times to extract the leftover phytochemical compounds completely. The filtrate from the filter was evaporated using a rotator at 55$^{o}$C. The concentrated sample resulted from 110.7 g has a yield of 22.14%.

***Preparation of Malus domestica Leaf Extract***

The apple (*Malus domestica*) leaves were handpicked from an apple orchard in the Pujon Village, Batu City, East Java, for about 3 kg. The leaves were washed and dried out at room temperature for 24 h. The leaves were ground after it was dried until it became a powder with the weight of 500 g and were soaked in 1 L of 70% ethanol liquid for three days with occasionally stirred. After three days, the soaked powder was filtered using filter paper. The filtrate was vaporized using the rotary evaporator at 55$^{o}$C. The concentrated sample was dried using a 40$^{o}$C oven and yielded 21.28%.

***Preparation of Sprague Dawley Rats and Alloxan***

The Sprague Dawley rats that were needed in this research were 24 animals. The rats were obtained from Laboratorium Departemen Kesehatan Republik Indonesia. Before the alloxan injection process, the rats will have fasted for 12 h. After fasting, alloxan was injected via intraperitoneal at a dose of 120 mg/kg BW.

Distribution of experimental animals based on intervention

rat models were divided into six groups, as follows:

-Normal group : Without intervention
-Positive control group : Metformin
-Negative control group : Distilled water
-Group 1 : 200 mg/kg BW plant extract
-Group 2 : 400 mg/kg BW plant extract
-Group 3 : 600 mg/kg BW plant extract

Rats were placed in one cage according to their group during the experiment. In an amount of 5 g pellet was given to the rats every day. Water also be given to the rats *ad libitum*. All rats were placed inside the cage with bright lighting. After the research was completed, the rats were sacrificed by euthanasia using halogenated anesthetic agents.

***Plant Extract Treatment***

Except for the normal group, all rats were given alloxan by injection to induce fasting blood sugar (FBG) levels were greater than 200 mg/dL for four days. Once FBG levels increased, groups 1, 2, and 3 were treated with plant leaf extracts once a day, orally using a syringe for 16 days [11, 12].

***Blood Collection and Fasting Blood Glucose Levels Examination***

Twelve hours after treatments, the rat blood would be collected. The blood was collected intravenously through the tip of the tail. After the blood was taken, the blood was put into a glucometer strip to observe the glucose level. This process was done every four days.

***Calculation of Decreasing Fasting Blood Glucose Percentage***

The researcher recorded the results of a blood glucose examination, which was done every four days. After that, every data will be calculated with :

$$PPGD = \frac{Go - Gt}{Go}\ x\ 100\%$$

PPGD = Decreasing fasting blood glucose percentage
Go = Blood glucose level before the intervention
Gt = Blood glucose level after 16 days intervention

***Histological Observation of Pancreas***

Histological observation of the pancreas was done after 16 days of intervention. After the rats were decapitated, the pancreas samples were collected and put into the tube. The tube was filled with 10% formalin buffer. The organ samples were dehydrated and cut with a microtome, and placed into the object-glass. The organ was dyed with Hematoxylin Eosin (HE). The dyed cells indicated each cell was damage by observation of Langerhans islet and β-pancreatic cells.

***Profiling of Bioactive Compounds in Tithonia diversifolia and Malus domestica Leaf Extracts***

Mass spectrometry was performed using LC-MS Xevo, G2-XS QTof (Waters MS Technologies) with the ionization type was electrospray ionization (ESI) and all parameters were set by following Fatmawaty et al. method [13].

***Statistical Analysis***

The blood glucose examination was done every day. The data was analyzed using SPSS 22.00 version. The normality test of the data was done with Shapiro – Wilk test. Data with a normal distribution ($p$ > 0.05) was analyzed using the ANOVA test. Meanwhile, data with abnormal distribution ($p$ < 0.05) was analyzed with the Kruskal Wallis test.

Further statistical analysis is needed to find any significantly different ($p$ < 0.05) with a 95% confidence level. Histological observation of rat pancreas was done after measurement of blood glucose on day-16.

## RESULTS AND DISCUSSION

***Rat Fasting Blood Glucose (FBG) Levels***

Table 1 and Table 2 show the results of one-way ANOVA analysis from decreased rat fasting blood glucose (FBG) with $p$ < 0.05 from *Tithonia diversifolia* and *Malus domestica* extract treatments, indicating significant differences between the groups studied.

**Table 1.** The One-way ANOVA analysis results of rat FBG levels treated with *Tithonia diversifolia*.

| No. | Group | % Changes of FBG | *p* |
|---|---|---|---|
| 1 | 200mg/kg BW | 50.48 ± 10.36 | <0.001 |
| 2 | 400mg/kg BW | 66.32 ± 18.15 | |

| | | | |
|---|---|---|---|
| 3 | 600mg/kg BW | 81.87 ± 4.38 | |
| 4 | Normal | 1.62 ± 2.42 | |
| 5 | Positive Control | 75.99 ± 9.67 | |
| 6 | Negative Control | 16.89 ± 22.22 | |

**Table 2.** The One-way ANOVA analysis results of rat FBG levels treated with *Malus domestica.*

| No. | Group | % Changes of FBG | *p* |
|---|---|---|---|
| 1 | 200mg/kg BW | 57.52 ± 9.67 | <0.001 |
| 2 | 400mg/kg BW | 62.76 ± 9.54 | |
| 3 | 600mg/kg BW | 81.23 ± 5.37 | |
| 4 | Normal | 1.62 ± 2.42 | |
| 5 | Positive Control | 75.99 ± 9.67 | |
| 6 | Negative Control | 16.89 ± 22.22 | |

The homogeneity test (Lavene test) from T. diversifolia and *M. domestica* extract treatments were done with $p$ = 0.160 and $p$ = 0.073, respectively, indicating that the data has a minimum variance. Therefore, the Post Hoc test was performed using the Bonferroni test.

The Bonferroni test results in Table 3 dan Table 4 show that each dose (200 mg/kg BW, 400 mg/kg BW, and 600 mg/kg BW) of T. diversifolia and *M. domestica* extract treatments have significant differences with the negative control group ($p < 0.05$).

**Table 3.** Results of Benferonni test from *Tithonia diversifolia* extract treatment.

| Group | Group | *p* |
|---|---|---|
| 200mg/kg BW | 400 mg/kg BW | 1.000 |
| | 600 mg/kg BW | 0.053 |
| | Normal | <0.001 |
| | Positive Control | 0.119 |
| | Negative Control | 0.049 |
| 400mg/kg BW | 600 mg/kg BW | 1.000 |
| | Normal | <0.001 |
| | Positive Control | 1.000 |
| | Negative Control | 0.001 |
| 600mg/kg BW | Normal | <0.001 |
| | Positive Control | 1.000 |
| | Negative Control | <0.001 |
| Normal | Positive Control | <0.001 |
| | Negative Control | 0.049 |
| Positive control | Negative Control | <0.001 |

**Table 4.** Results of Benferonni Test from *Malus domestica* extract treatment.

| Group | Group | *p* |
|---|---|---|
| 200mg/kg BW | 400 mg/kg BW | 1.000 |
| | 600 mg/kg BW | 0.202 |
| | Normal | <0.001 |
| | Positive Control | 0.73 |
| | Negative Control | 0.003 |
| 400mg/kg BW | 600 mg/kg BW | 0.702 |
| | Normal | <0.001 |
| | Positive Control | 1.000 |
| | Negative Control | 0.001 |
| 600mg/kg BW | Normal | <0.001 |
| | Positive Control | 1.000 |
| | Negative Control | <0.001 |
| Normal | Positive Control | 1.000 |
| | Negative Control | 0.049 |
| Positive control | Negative Control | <0.001 |

Those three doses did not significantly differ with the positive control group, where the rats were given metformin. The normal group had significant differences from the positive control and negative control

group. Both 200 mg/kg BW dose and 600 mg/kg BW did not significantly differ with 400 mg/kg BW dose group.

***Histological Observation of Pancreas***

The histological observation was done on the pancreatic organ from Sprague Dawley rats treated for 16 days. The preparations were stained with Hematoxylin Eosin (HE) and observed under a microscope. The observations can be seen in Figure 1 and Figure 2. Based on Figure 1a and Figure 2a, the normal group showed the existence of medium-sized Langerhans islets and did not show any specific changes compared with the normal group. Cells found on the Uniform β-cells dominated Langerhans islets in this group. The positive control group is shown in Figure 1b and Figure 2b showed small-sized Langerhans islets and were dominated by β-cells. Figure 1c and Figure 2c show a negative control group. Langerhans islets were found small-sized with α-cells domination.

The same thing was found in the *T. diversifolia* leaf extract group at doses of 200 mg/kg BW (Figure 1d and Figure 2d) and 400 mg/kg BW (Figure 1e and Figure 2e). Langerhans islets on those groups were found to be small in number and size. The cells that were mostly found in Langerhans islets were α-cells. Unlike the two previous groups, the 600 mg/kg BW group (Figure 1f and Figure 2f) had small Langerhans islets and was dominated by β-cells. It also showed that there was proliferation on the cholangiocytes.

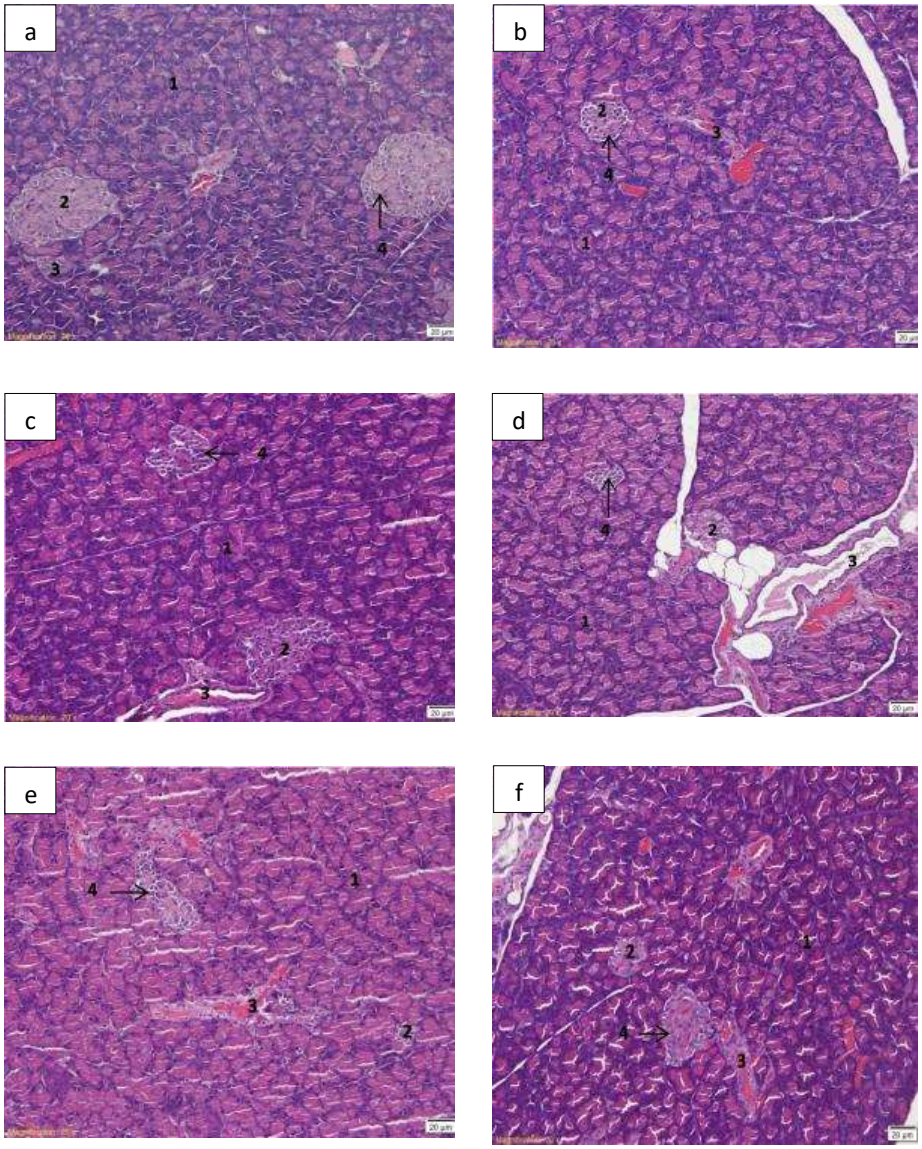



**Figure 1.** The profiles of rat pancreatic histopathology after treated with *Tithonia diversifolia* extracts. The optical magnification was 200x using Hematoxylin Eosin (HE) staining. 1=Pancreatic gland, 2= Langerhans islets, 3=Trabecula. (a) Normal Group, 4=β-cells (light blue); (b) Positive Control Group, 4=β-cells (light blue); (c) Negative Control Group, 4=α-cells (dark blue); (d) 200 mg/kg BW group, 4=α-cells (dark blue); (e) 400 mg/kg BW group, 4=α-cells (dark blue); (f) 600 mg/kg BW group, 4=β-cells (light blue).

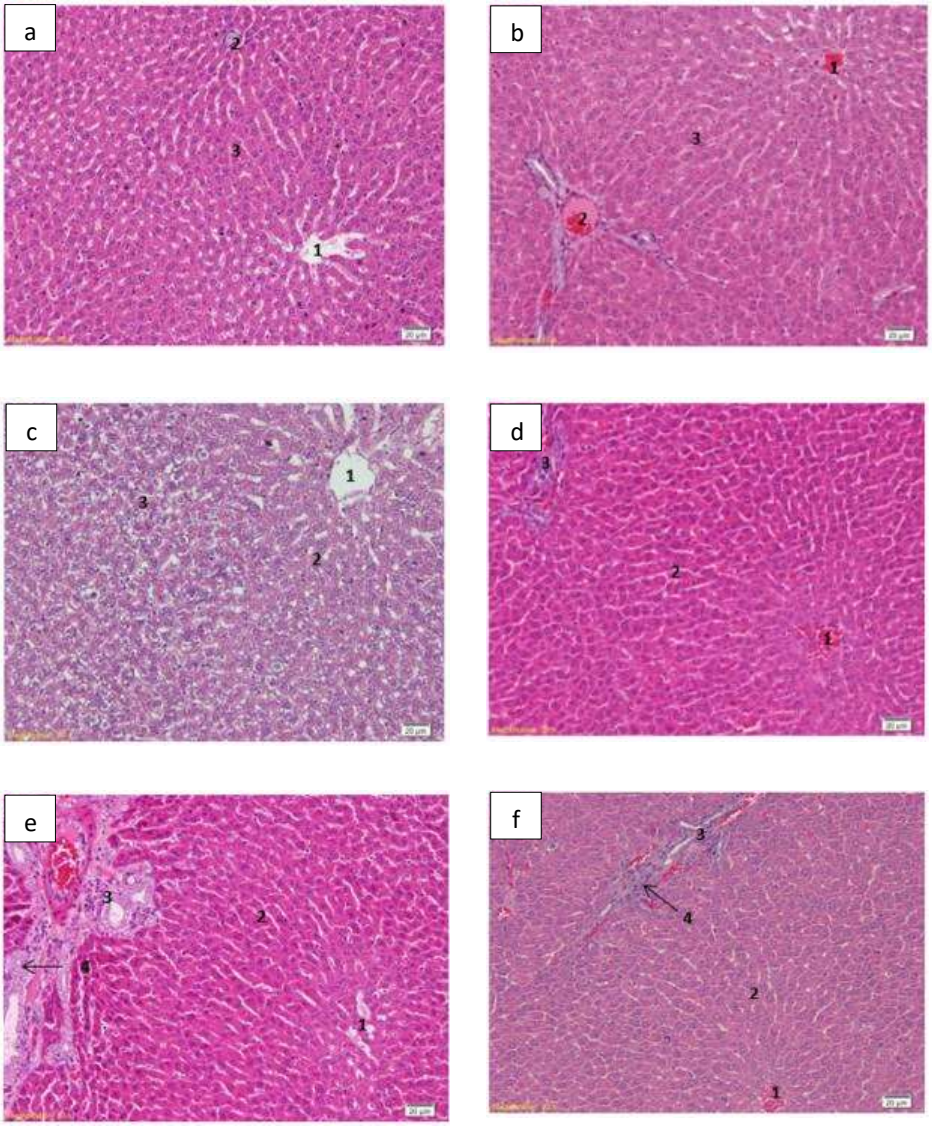
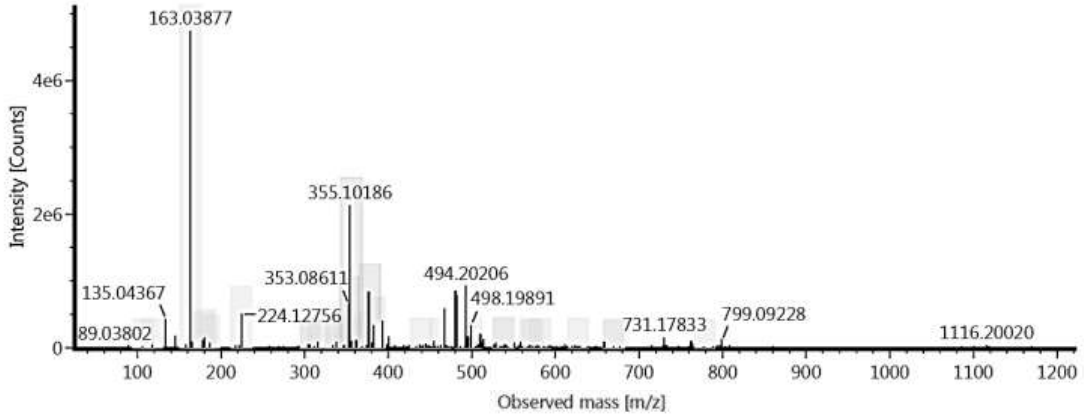


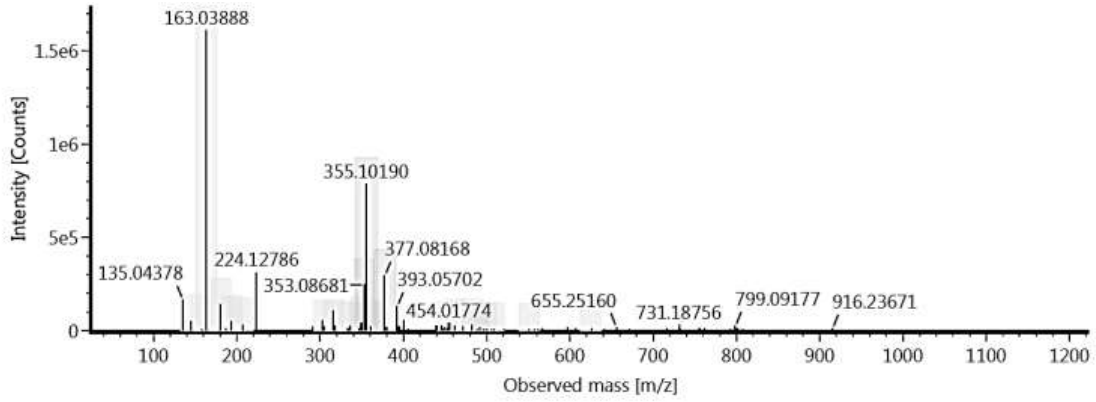

**Figure 2.** The profiles of rat pancreatic histopathology after treated with *Malus domestica* extracts. The optical magnification was 200x using Hematoxylin Eosin (HE) staining. 1=Pancreatic gland, 2= Langerhans islets, 3=Trabecula. (a) Normal Group, 4=β-cells (light blue); (b) Positive Control Group, 4=β-cells (light blue); (c) Negative Control Group, 4=α-cells (dark blue); (d) 200 mg/kg BW group, 4=α-cells (dark blue); (e) 400 mg/kg BW group, 4=α-cells (dark blue); (f) 600 mg/kg BW group, 4=β-cells (light blue).

***Profiling of bioactive compounds in Tithonia diversifolia and Malus domestica leaf extracts***

Figure 3 shows the chromatograms of bioactive compounds from Tithonia diversifolia and Malus domestica leaf extracts performed by LC-MS/MS.




a




b

**Figure 3.** LC-MS/MS Chromatograms of a. *Tithonia diversifolia* and b. *Malus domestica.*

The profiling of bioactive compounds from *Tithonia diversifolia* and *Malus domestica* leaf extracts were listed in Table 5 and Table 6. Most of the bioactive compounds were classified as flavonoids followed by cyclitol carboxylic acid, withanolide glucosides and alkaloids in *T. diversifolia* leaf extract. Meanwhile, *M. domestica* has the highest flavonoids, followed by cyclitol carboxylic acid and glucosides.

**Table 5.** Bioactive compound profiles of *Tithonia diversifolia* leaf extracts.

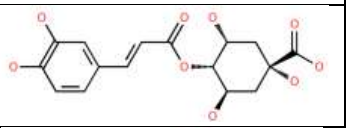
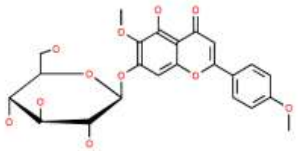
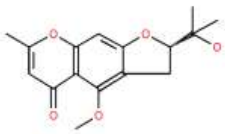
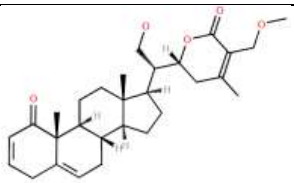
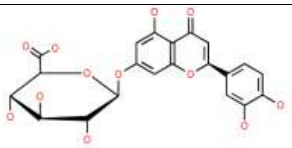
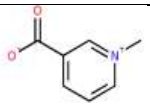
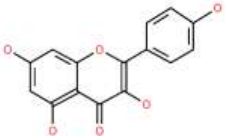
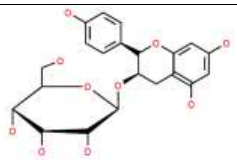
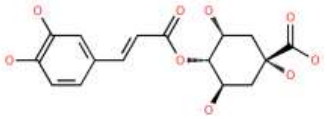
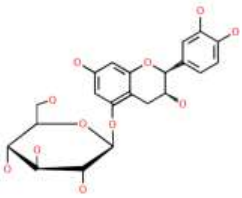
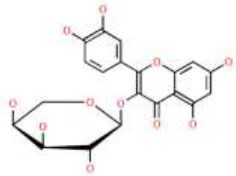
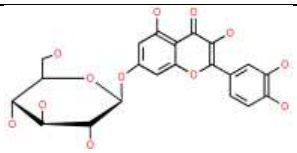
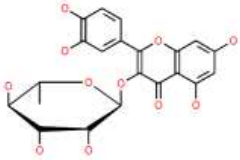
| No. | Compound | Neutral mass (Da) | Classification | Structure |
|---|---|---|---|---|
| 1 | 4-O-Caffeoylquinic acid | 354.09508 | Cyclitol carboxylic acid |  |
| 2 | 5-Hydroxy-6,4'-dimethoxy-flavone-7-O-β-D-glucopyranoside | 476.13186 | Flavonoids |  |
| 3 | 5-O-Methylvisamminol | 290.11542 | Chromones (Flavonoids) |  |
| 4 | Daturametelin C | 468.28757 | withanolide glucosides |  |
| 5 | Luteolin-7-O-glucuronide | 462.07983 | Flavones |  |
| 6 | Trigonelline | 137.04768 | Alkaloids |  |
| 7 | Kaempferol | 286.04774 | Flavonols |  |

**Table 6.** Bioactive compound profiles of *Malus domestica* leaf extracts.

| No. | Compound | Neutral mass (Da) | Classification | Structure |
|---|---|---|---|---|
| 1 | (-)-Epiafzelechin-3-O-β-D-allopyranoside | 436.13695 | Flavonoids |  |
| 2 | 4-O-Caffeoylquinic acid | 354.09508 | Cyclitol carboxylic acid |  |
| 3 | Epicatechin 5-O-β-D-glucopyranoside | 452.13186 | Flavanol glucosides |  |
| 4 | Quercetin-3-O-α-L-arabinopyranoside | 434.08491 | Flavonoids |  |
| 5 | Quercimeritrin | 464.09548 | Glucosides |  |
| 6 | Quercetin-3-O-α-L-rhamnoside | 448.10056 | Glucosides |  |

Percentage change in rat FBG shows the differences of each group. As stated previously, the three-dose groups had significant differences with negative controls. It shows that the intervention in paitan (*Tithonia diversifolia*) and apple (*Malus domestica*) leaf extracts with the three doses can reduce FBG levels. *T. diversifolia* and *M. domestica* leaf extracts with dose groups of 600 mg/kg BW had the highest reduction percentages (81.86% and 81.23%, respectively). However, statistical tests showed that the dosage groups of 200 mg/kg BW, 400 mg/kg BW, and 600 mg/kg BW have no significant differences from each other. In addition, no significant differences were found in the reduction in the three-dose groups with positive controls. These insignificant differences show that the administration of *T. diversifolia* and *M. domestica* leaf extracts have the same effect as metformin administration in the positive control group.

This study results supported the two previous studies conducted that lower doses give better results than high doses [14, 15]. The receptor occupancy theory related with the relationship among dose, effect, and several receptors which forms a hyperbole graph. The graph illustrates that there is a point where a dose reaches its maximum effect ($E_{max}$). Any dose beyond this point will not make a significant difference. Another thing to consider is the ability of a drug to cause undesirable effects. This ability goes hand in hand with increasing administration doses, so high doses are not recommended if the changes are not significant [16, 17].

Another factor that can influence the results of this study is the variation of Sprague Dawley rats. This variation is related to the sensitivity of the four active substances contained in *T. diversifolia* leaves, namely flavonoids, cyclitol carboxylic acid, withanolide glucosides and alkaloids. Meanwhile, *M. domestica* leaf extract has bioactive compounds of flavonoids, cyclitol carboxylic acid and glucosides. Previous studies reported that flavonoids, cyclitol carboxylic acid, glucosides and alkaloids have roles in antidiabetic effects [18-21]. The different levels of sensitivity can cause insignificant differences among the three doses. Thus, it can be concluded that the dose of *T. diversifolia* and *M. domestica* leaf extracts might suggest having the most effective action as antidiabetic in alloxan-induced mice in 200 mg/kg BW due to it has the same effect as well as 400 mg/kg, 600 mg/kg, and positive control groups with lower adverse effects.

Pancreatic histology observation of each group was carried out using Hematoxylin Eosin (HE) staining. Pancreas from mice in the normal group showed medium-sized Langerhans islets and was dominated by pancreatic β-cells. Different results were found in the positive control group which Langerhans islets were found to be small and dominated by β-cells. The diminished Langerhans islets were thought to be caused

by the induction of alloxan, which triggers apoptosis from β-cells. Even so, the Langerhans islets found in the positive control group remained dominated by β -cells as in normal circumstances. This result was thought to be caused by the effects of metformin administration. Xin et al. (2017) reported that metformin could inhibit apoptosis from pancreatic β-cells [22]. This antidiabetic drug is known to prevent mitochondria from opening permeability transition pore (PTP) on intracellular membranes so that cells can stay alive [23, 24]. Langerhans Island was small in number, size, and dominated by α-cells found in the negative control group. Liu et al. revealed that insulin levels influence the proliferation process of cells [25]. Apoptosis from β-cells causes low insulin levels so that the proliferation of α-cells can occur. There are no compounds that protect β-cells from apoptosis in this group either due to ROS or glucotoxicity [26].

Administration of *T. diversifolia* and and *M. domestica* leaf extracts at a 200 mg/kg BW dose and 400 mg/kg BW showed similar histological figures: small-sized Langerhans islets and dominated by α-cells. Different results were found in the 600 mg/kg BW dose group. Rat pancreas in this group contains small-sized Langerhans islets but was dominated by β-cells. Small-sized Langerhans islets in the positive control group were thought to result from alloxan administration. The results of different histological observations in the groups treated by *T. diversifolia* and *M. domestica* leaf extracts were assumed to be affected by the increasing bioactive compounds and the increase in dose responses [27].

*T. diversifolia* leaf extracts contain daturametelin C and kaempferol, while *M. domestica* leaf extracts contain phloretin, quercetin, and epicatechin, which is known to have antioxidant properties to reduce the free radicals inside the human body. According to Jung et al. (2006), phloretin acts as an antioxidant to destroy the ROS formed on the hyperglycemic state and acts as a chelation agent to prevent oxidation reaction from metallic ions could reduce the glycated hemoglobin (HbA1c) levels [28]. Previous studies reported that quercetin structures that have five hydroxyl chains supported destroying the free radicals [29, 30]. Epicatechin is also known to reduce the levels of HbA1c [31, 32]. In addition, daturametelin C and kaempferol can also bind free radicals contained in the cells [33]. These bioactive compounds have roles in preventing apoptosis from cells due to alloxan ROS presence [34, 35].

## CONCLUSION

It was concluded that *Tithonia diversifolia* and *Malus domestica* leaf extracts affected the blood glucose levels of hyperglycemic Sprague Dawley rats. The leaves extract effective dose was 200 mg/kg BW as the dosage increase showed no significant differences. All of the leaves extract dosage showed a protective effect on liver histopathological structure of hyperglycemic Sprague Dawley rats compared with the normal and negative control group. Furthermore, the leaves extract dosage of 400 and 600 mg/kg BW shown protective effects on liver damage complications.

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