DOI: 10.21776/ub.jpacr.2023.012.02.3297 *J. Pure App. Chem. Res.*, **2023**, 12 (02), 80-86
28 August 2023

Effect of Avocado Seed Ethanol Extract (*Persea americana* Mill) on Superoxide Dismutase (SOD1) and Histological Expression of Pancreas in Rats (*Rattus norvegicus*) with Diabetes Mellitus

Fauziah Fauziah,[1]* Nurhaida Nurhaida[1] Binawati Ginting[1] and Aulanni'am[2,3]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala Banda Aceh, Indonesia
[2]Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia
[3]Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia

*Corresponding author: haida_yusuf@usk.ac.id

Received 13 June 2023; Accepted 28 August 2023

ABSTRACT

Diabetes mellitus causes a pancreatic β-cells damage due to the increase of oxidative stress, thereby reducing Superoxide Dismutase 1 (SOD1) activity. This enzyme activity can be increased by utilizing antioxidant compounds from the avocado seed extract. This study aims to determine the increase of SOD1 expression and the repair of pancreatic β-cells tretaed with the ethanol extract of avocado seeds (*Persea americana* Mill). It is also aimed to observe the expression of pancreatic SOD1 and its histopathology change. The expression was measured by using immunohistochemical. This research used 20 Wistar rats aged of 2-3 moths, weighing of 150-200 grams. The diabetic rats were induced by streptozotocin (STZ) with dose of 30 mg/kg BW intraperitoneally. The rats were divided into 5 groups, namely: group 1 is healthy rats, group 2 is diabetic rats, while the group 3, 4 and 5 are therapeutic groups with 300, 350 and 400 mg/kg BW doses of ethanol extract for 14 days treatment. The results showed the ethanolic extract of avocado seeds has a potential as an antidiabetic agents based on the increase of pancreatic SOD1 ($p<0.05$) significantly, increase of SOD1 expression 7.6 ± 1.34, 11.6 ± 1.94 and 13.0 ± 2.82 in three different doses therapy (300, 350 and 400 mg/kg BW), and repairing structure of β-pancreatic cells on the therapeutic groups.

Keywords: avocado seed ethanol extract, Diabetes mellitus, Hematoxylin-Eosin, Superoxide dismutase

INTRODUCTION

There are a lot of chronic diseases that cause a death in the world, for example, diabetes mellitus (DM) [1]. DM would attack anyone regardless of age and socioeconomic limits. Along with the increasing of the economic growth, people suffering the DM would be continuously getting an increase. The International Diabetes Federation (IDF) records 537 million people diagnosed with DM. Diabetes also causes 6.7 million deaths. China is the country with the largest number of diabetics issues in the world at 140.87 million in 2021, then the United States is in second place with 669 thousand deaths. Then, India is in third place with a total of 647 thousand. Meanwhile, Indonesia is the sixth. The number of deaths caused by diabetes in Indonesia is predicted to reach 236 thousand in 2021 [2]. The survey results state that 9.8% of the Aceh people suffer DM. Diabetes mellitus can be triggered by

The journal homepage www.jpacr.ub.ac.id
p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733

diet, such as consuming a sugar excessively. In the cooking process, they often use an excessive sugar. Therefore, it is estimated that by 2025, DM will increase in Aceh area [3]. This can be observed from their food processes, especially the Acehnese's habit to consume sweet foods which is more often and containing high sugar levels.

DM disease has been treated with various chemical drugs. The treatment of DM patients that uses insulin injections have side effects, such as immunopathology, lipodystrophy, hypoglycemia, and seizures as well as coma if left untreated [4]. Herbal medicine has relatively fewer side effects than modern medicine, so people consider herbal medicine to be safer [5],[6]. Therefore, people try to switch the herbal medicine, where the study's results state that one of the herbal medicines that can improve the pancreas is a plant that contains antioxidant compounds. One of the compounds that has antioxidants activity is phenol compounds. There are studies showing that avocado seeds have antimicrobial and antioxidant abilities [4;7],[8], Based on phytochemical tests, the ethanolic extract of avocado seeds contains flavonoid compounds (polyphenols), triterpenoids, quinones, saponins, tannins, monoterpenoids and sesquiterpenoids [9]. These flavonoid compounds can lower blood glucose levels [10]. The ethanol extract of Avocado seed at doses of 300, 600 and 1200 mg/kg body weight (BW) can reduce blood glucose levels in diabetic rats [11].

Superoxide Dismutase (SOD) is found in the extracellular matrix and various tissues including the pancreas which is the main extracellular scavenger of superoxide radicals [12]. SOD activity on islets of Langerhans could result superoxide radicals that secreted into the extracellular space so that they do not contribute for the beta cells destruction [13]. The elevated SOD level is revealed to reduce oxidative stress and decrease the mitochondrial cytochrome C which release at apoptosis in neurons and prevent diabetes-induced glomeruli in DM rats [9; 14]. *In vivo* testing on rats, it shows that there is the glucose blood level decrease and the increase of serum superoxide dismutase (SOD1) antioxidant activity after adding the intake of fermented beans from *Koro Benguk Tempeh* and histologically it shows the tissue repair of pancreatic beta cells after treating with *glycine max* (L.) Merr extract [15]. However, the activity of avocado seed extract in increasing antioxidant compounds in the body, especially the expression of superoxide dismutase (SOD1), as well as repairing pancreatic beta cells has not been reported. Based on this background, the activity of avocado seed ethanol extract as an herbal medicine to increase pancreatic beta cells and SOD1 expression is investigated and discussed in the study.

EXPERIMENT

Chemicals and instrumentation

This research used male Wistar rats of *Rattus norvegicus* (aged 2-3 months) with weight ranges of 150-200 grams, streptozotocin (STZ), distilled water, NaCl, Formaldehyde 4 % (Sigma), xylol, dH_2O, H_2O_2, Phosphate Buffer Saline (Sigma), Tris Buffered Saline (Sigma), Bovine Serum Albumin (Sigma), ethanol absolute (Sigma), ethanol 70%, 80%, 90% and 95% hematoxylin-eosin (Sigma), paraffin block, immunohistochemistry KIT, antibody primer Cusabio USA etellen. The equipment used in this study included feeding tube, a set of glassware, micropipette, microscope (Meiji Techno Trinocular), freezer, microscope (Olympus BX51), SOD1 expression of pancreas in the experimental animal, reaction tube, vortex, scissors, tweezers, Pasteur pipette, incubator, glass objects, spatula, miter chop knife, analytical, hot plate, water bath, gloves, centrifuges, and mortar.

Animal experimental preparation

The animal models used in this study were 20 healthy male Wistar rats, aged of 3 months, which weighed 130–230 g. The rats were divided into 5 groups: (1) the healthy rats, (2) the diabetic rats that induced by injection of STZ (30 mg/kgBW), (3), (4) and (5) were the Diabetic rats treated by avocado seed ethanol extract with dose of 300, 350 and 400 mg/kgBW, respectively. The rat's use is approved by The Indonesian ethics committee (Ref: 189/KEPH/XI/2022).

Plant material preparation

Avocado seeds were weighed as much as 2 kg, then washed with a running water, and sun-dried. The dried avocado seeds were then crushed into powder using blender. A 200 g avocado seed powder was macerated in 2 L ethanol for 48 hours that stirred occasionally. Furthermore, this extract was filtered off and the remaining solid was macerated again with a new ethanol solvent so that the filtrate obtained a clear color. The filtrate then evaporated using rotary evaporator vacuum. A 11 gram of ethanol extract of avocado seed was obtained from the initial 200-gram avocado seed powder.

Administration of avocado seed extract

In the initial stage, the acclimation process was carried out for 7 days while adding 552 pellets. The blood sample, then, was taken out from each rat. The blood glucose was checked as baseline. The group 1 is the untreated group (rats with normal glucose levels), and groups 2, 3, 4 and 5 were induced using STZ at a dose of 30 mg/kgBW intraperitoneally. Seven day after injection of STZ, rats were fasted for 16 hours, then blood glucose levels was measured. After fasting, blood glucose levels of group 2, 3, 4 and 5 reach >200 mg/dL (hyperglycemia). Then, groups 3, 4 and 5 were treated with avocado seed ethanol extract orally (sonde) with doses of 300, 350 and 400 mg/kgBB, respectively. After 14 days of treatment, the blood glucose level was remeasured.

Examination of SOD1 expression by immunohistochemical technique

SOD1 expression in pancreatic tissue used an immunohistochemical method. The preparation step was soaking in xylol 2 times, degraded with alcohol sequentially (96%, 90%, 80%, 70%) for the hydration process. The slide is washed with PBS and dried for a few seconds (without rinsing) and wiped around with tissue paper which was then smeared with primary antibody (SOD1) and diluted in TBSP of 1% BSA. After that, it was incubated for 12 hours (overnight) at 40^0C and then washed with PBS. The HRP secondary conjugate was applied and incubated with PBS for 60 minutes followed by H_2O. Then it was expanded with chromogen for 10 min and counterstained with Mayer's eosin haematoxylins, hydrated, cleaned, and mounted. Then the slide was covered with a cover slip which was observed with a microscope (Olympus BX51).

Histological appearance and Hematoxylin-eosin (HE) staining

A Histological picture is the structure of pancreas that was cut thinly and stained with Hematoxylin-eosin (HE). The histological features are used to differentiate the grade of insulitis from the appearance of the tissue structure. The first step is deparaffinization by soaking in xylol (levels 1, 2, and 3) for 5 minutes and then staining in absolute ethanol (95%, 80%, and 70%) for 5 minutes. Next, it was soaked in distilled water for 5 minutes, put in hematoxylin stain for 10 minutes, and washed with running water for 30 minutes. The sample was then rinsed with distilled water and followed by eosin staining. Furthermore, it put into

eosin alcohol for 5 minutes and soaked in distilled water to remove excess eosin. The next process is dehydration, in which the tool is placed into graded ethanol (80%, 90%, and 95%) solutions. The cleaning process was carried out by putting in it into xylol-1 and xylol-2 and dried it. The sample was mounted with entellan. Ultra-thin sections that have been dried and stained were observed under an optical microscope (Meiji techno trinocular).

Data analysis

The data were analyzed by using the ANOVA Statistical Test (SPSS 22).

RESULT AND DISCUSSION

Pancreatic SOD1 Expression.

The rats injected with streptozotocin (STZ) can trigger cell damage caused by the entry of toxic substances into the body. STZ is a DM agent that causes the formation of free radicals, including NO, O*, and H_2O_2 which can lead to DNA fragmentation. Excess free radicals in the body can cause a damage of various cells such as cell membranes, proteins, and DNA. The β-pancreatic cells damage can reduce the activity of enzyme superoxide dismutase (SOD1). The SOD1 enzyme is one of the endogenous antioxidant compounds that can regulate reactive oxygen species (ROS) levels [12;16]. The study showed that the ethanol extract of avocado seeds (*Persea americana* Mill) increases SOD1 expression and repairs the pancreatic β-cell damage (Figure 1 and Table 1).

The results of SOD1 by immunohistochemistry technique (Figure 1) showed that a positive reaction of SOD1 in the cytoplasm, which was indicated by brown stains on pancreatic cells. The large number of brown stains in pancreatic cells in the islets of Langerhans indicates the high expression of SOD1 in the cytoplasm.

Table 1. The average number of SOD1 expression of β-pancreatic cells in the islet Langerhans

No	Treatment group	Langerhans islet β cell count
1	Healthy rats	6.4 ± 2.07
2	DM rats	2.2 ± 1.30
3	Sick DM therapy with dose of 300 mg/kgBW	7.6 ± 1.34
4	Sick DM therapy with dose of 350 mg/kgBW	11.6 ± 1.94
5	Sick DM therapy with dose of 400 mg/kgBW	13.0 ± 2.82

The healthy rats as a reference for SOD1 expression with the number of β-pancreatic cells in Lagerhans islets are 6.4 ± 2.07 cells, while DM rats has a value of 2.2 ± 1.30 cells. Giving avocado seed ethanol extract with doses of 300, 350 and 400 mg/kgBW can increase SOD1 expression (7.6 ± 1.34, 11.6 ± 1.94 and 13.0 ± 2.82 cells). The results of statistical data show that there is a significant increase of SOD1 expression ($p<0.05$), where the number of pancreatic cells in the islets of Langerhans treated rats is higher than that of diabetic rats. DM rats treated with curcumin with doses of 200 and 400 mg/kgBW shows an increase in SOD1 expression. The increase of SOD1 expression is caused by the antioxidant compounds [17] contained in the ethanolic extract of avocado seeds such as tannins, flavonoids, alkaloids, terpenoids, steroids, tannins and saponins. The alkaloid compounds play a role of cell regeneration, so that they can restore partially the damaged pancreatic-cells and can induce

regeneration of damaged pancreas [18], [19]. Saponins can stimulate insulin secretion from β-pancreatic cells [20].

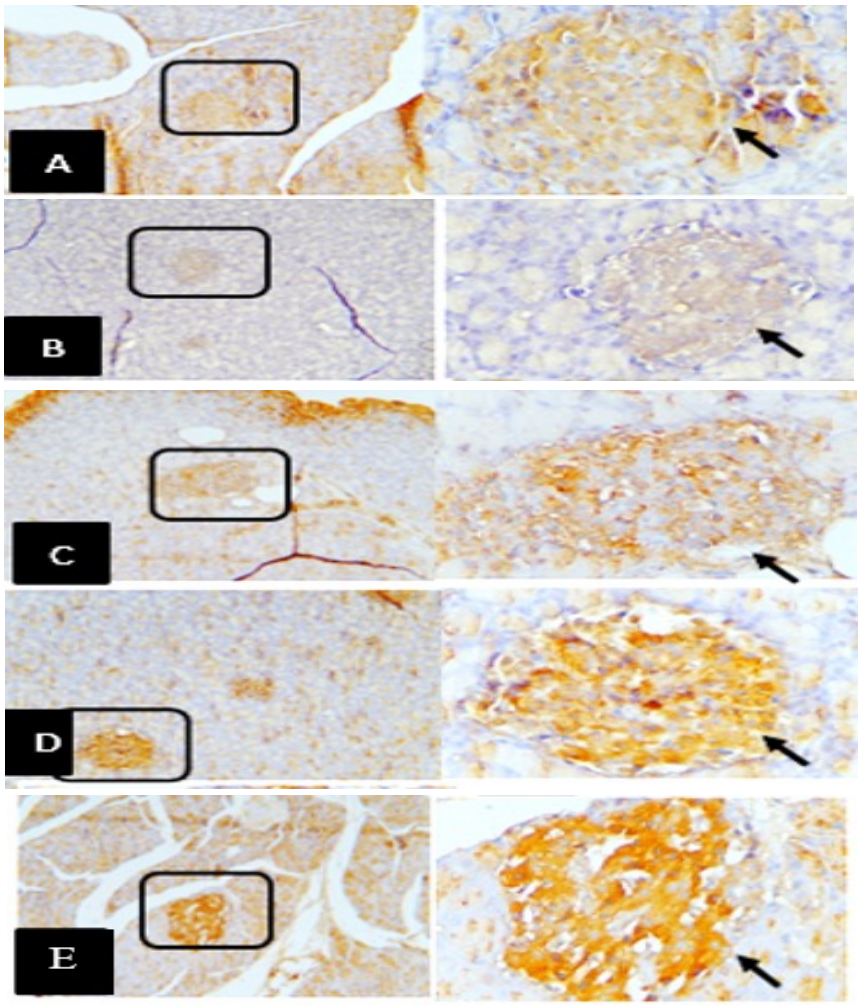

Figure 1. SOD1 A immunohistochemistry results: healthy rats; B: DM rats; C: DM rats adding the ethanol extract at a dose of 300 mg/kgBW; D: DM rats adding the ethanol extract at a dose of 350 mg/kgBW; E: DM rats adding the ethanol extract at a dose of 400 mg/kgBW.

Histopathology of Pancreatic Tissue

The results of the histopathology are shown in Figure 2. Figure 2A shows that the β-pancreatic cells of healthy rats are in good and perfect condition. Figure 2B is a diabetic rat revealing that the area of the islets of Langerhans is imperfect and uneven. Figures 2C, 2D and 2E are pancreatic tissues of diabetic rats treated with ethanol extract of avocado seeds at doses of 300, 350 and 400 mg/kgBW, respectively. Figures 2C, 2D and 2E display improvement of pancreatic tissues after being treated with ethanol extract for 14 days. Figure 2C demonstrates greater necrosis from the amount of free space on Langerhans islands compared to Figure 2D and 2E. Figure 2D shows smaller necrosis compared to Figure 2C, while Figure 2E demonstrates smaller necrosis and no free space on the islets of Langerhans approaching healthy rats that are compared with Figure 2C and 2D, where they show improvement in β-pancreatic cells. This is related to the activity of antioxidant compounds

contained in the avocado seed ethanol extract. These figures exhibit the repair of β-pancreatic cells or regeneration of β-pancreatic cells which was characterized by the cells increase in the islets of Langerhans, in which cell nucleus is in the orderly manner. These results demonstrate similar to healthy rats (Fig. 2A). The β-pancreatic cells damage caused by oxidative stress can be repaired by administering antioxidant compounds such as flavonoids, alkaloids, terpenoids, steroids and phenols.

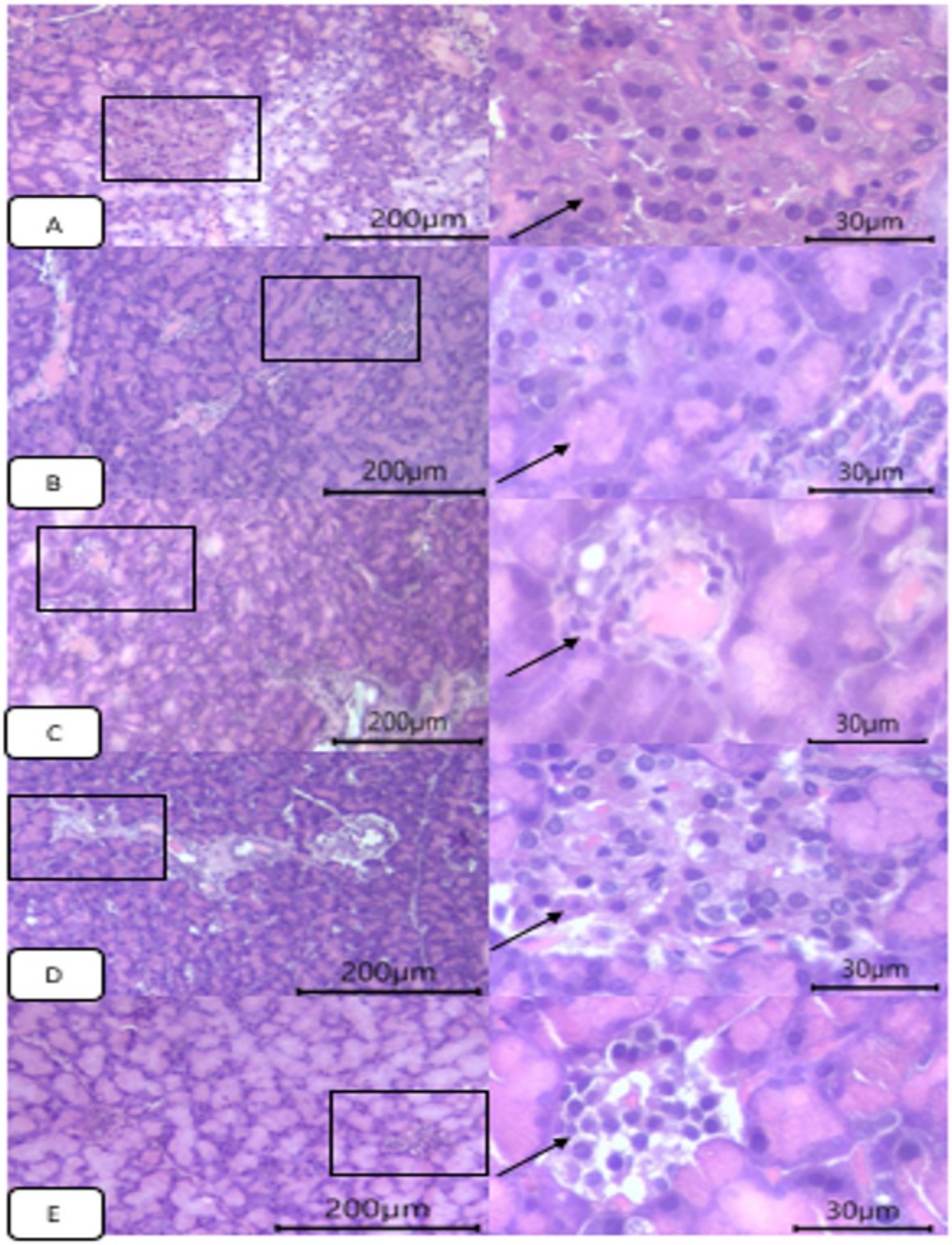

Figure 2. SOD1 A immunohistochemistry results of healthy rats; B: DM rats; C: DM rats adding the ethanol extract at a dose of 300 mg/kgBW; D: DM rats adding the ethanol extract at a dose of 350 mg/kgBW; E: DM rats adding the ethanol extract at a dose of 400 mg/kgBW.

CONCLUSION

The therapy using ethanolic extract of avocado seeds on DM rats shows an increasing of SOD1 expression in the islets of Langerhans and improves the structure of β-pancreatic cells.

ACKNOWLEDGMENT

The author would thank to Dyah Kinasih, and Wibi Riawan for the suggestions and support in finishing this research.

REFERENCES

[1] Temurtas, Hasan, Nejat Yumusak, Feyzullah Temurtas, *Expert Syst. Appl.,* **2009**, 36(4), 8610–8615.
[2] Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B.B., Stein, C., Basit, A., Chan, J.C., Mbanya, J.C. and Pavkov, M.E., *Diabetes Res. Clin. Prac.*, **2022**, 183, p.109119.
[3] Dinas Kesehatan Provinsi Aceh, **2013**, SP2TP Bidang Program, Aceh.
[4] Katzung, B.C, *Basic and Clinical Pharmacology*, **2010**, EGC, Jakarta.
[5] Arsyah, C.D, Thesis, Universitas Islam Negeri Sunan Kalijaga Yogyakarta, Indonesia, **2014**.
[6] Kumar, S., Mittal, A., Babu, D. and Mittal, A., *Curr. Diabetes Rev.*, **2021**, 17(4), pp.437-456.
[7] Nguyen, T.V.L., Nguyen, Q.D., Nguyen, N.N. and Nguyen, T.T.D., *Molecules,* **2021**, 26(24), p.7693.
[8] Kristanty RE, Suriawati J, Sulistiyo J, *J. Pharm*, **2014**, 5(7), 557-559.
[9] Charles, A.C., Dadmohammadi, Y. and Abbaspourrad, A., *Food & Function,* **2022,** 13(13), pp.6894-6901.
[10] Al Duhaidahawi, D., Hasan, S.A. and Al Zubaidy, H.F., *Curr. Diabetes Rev.*, **2021**, 17(6), pp.26-42.
[11] Ojo, O.A., Amanze, J.C., Oni, A.I., Grant, S., Iyobhebhe, M., Elebiyo, T.C., Rotimi, D., Asogwa, N.T., Oyinloye, B.E., Ajiboye, B.O. and Ojo, A.B., *Sci. Rep.*, **2022**, 12(1), p.2919.
[12] Kowluru, R.A., Atasi, L. and Ho, Y.S., *Invest. Ophthalmol. Vis. Sci.,* **2006**, 47(4), pp.1594-1599.
[13] Yin, J., Wang, X., Li, S., Zhu, Y., Chen, S., Li, P., Luo, C., Huang, Y., Li, X., Hu, X. and Yang, W., *Redox Biol.*, **2019**, 24, p.101172.
[14] Luh Putu Gina, Chanif Mahdi, Aulanni'am, *J. Pure App. Chem. Res.*, **2016**, 5 (1), 40-47
[15] Widhiyastuti, E. and Lestari, M.W., *Borneo Journal of Medical Laboratory Technology*, **2020**, 2(2), pp.136-140.
[16] Utomo, H. A. Hanafiah, L.H. Oen, F. D. Suyatna, N. Asikin, *Medika*, **1991**. 5, 373-379.
[17] Maria A, Tesis, Universitas Sumatra Utara, Indonesia, **2017.**
[18] Aguayo-Mazzucato, C. and Bonner-Weir, S., *Cell Metab.*, **2018**, 27(1), pp.57-67.
[19] Murray, R. K., Granner, D. K., Mayes, P. A., Rodwel, V. W., *Harper's Biochemistry*, **2003**, Vol. 25. EGC, Jakarta.
[20] Patel, D., Kumar, R., Laloo, D., Hemalatha, S., *Asian Pac. J. Trop. Dis.*, **2012**, 239-250.