KOMISI ETIK PENELITIAN KESEHATAN

FAKULTAS KEDOKTERAN
UNIVERSITAS WIJAYA KUSUMA SURABAYA

KETERANGAN KELAIKAN ETIK
"ETHICAL CLEARENCE"

No. 65 /SLE/FK/UWKS/2021

KOMISI ETIK PENELITIAN KESEHATAN

PENELITIAN BERJUDUL:
The Administration of Moringa Oleifera Powder to Prevent Trophoblast Cell Damage in Pregnant Rats with Diabetes Mellitus

PENELITI UTAMA:
Dr. dr. Harry Kurniawan Gondo, SpOG (K)

UNIT / LEMBAGA / TEMPAT PENELITIAN:
Lab. Hewan Coba Dan Lab. Histopathologis
Fakultas Kedokteran Universitas Wijaya Kusuma Surabaya

MENYATAKAN:
" LAIK ETIK "

Surabaya, 6 Oktober 2021

Mengetahui,
Dekan

Prof. Dr. Suhartati, dr. MS

Ketua Unit,

Dr. Erny, dr., Sp.A (K)

The Administration of Moringa Oleifera Powder to Prevent Trophoblast Cell Damage in Pregnant Rats with Diabetes Mellitus

Harry Kurniawan Gondo
Faculty of Medicine, Wijaya Kusuma University, Surabaya
Jl. Dukuh Kupang XXV No.54, Dukuh Kupang, Kec. Dukuhpakis, Kota SBY, Jawa Timur 60225
+6285733988210
Email: harrygondo.uwks@gmail.com
Elizabeth Haryanti
Faculty of Medicine, Wijaya Kusuma University, Surabaya
Jl. Dukuh Kupang XXV No.54, Dukuh Kupang, Kec. Dukuhpakis, Kota SBY, Jawa Timur 60225
+62 813-5999-0926
Email: elizabeth.haryanti@gmail.com

ABSTRACT

Background: Preeclampsia is a disease that involves multisystem in pregnancy which is characterized by an increase in blood pressure and proteinuria. This study is to see the effectiveness of moringa oleifera powder to prevent trophoblast cell damage in preeclamptic pregnant rats with diabetes mellitus.

Methods: 30 rats were tested for sugar levels on the 4th day after being induced by alloxan for 18 days to know whether the rats had already been in a hyperglycemic state. They were then grouped into six groups, each group consisted of five pregnant white rats. Group 1 was the negative group (without alloxan being given), group 2 was positive (given alloxan at 150 mg/day/kg BW), groups 3, 4, 5 and 6 were given alloxan 150 mg/day/kg BW and each was given dose of Moringa leaf powder as much as 100; 200; 400 and 800 mg/day/kg body weight. DNA (DeoxyriboNucleic Acid) fragmentation was examined using the TUNEL (Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling) method.

Results: The average calculation of apoptosis results in the Normal and Treatment groups, showed a significant difference in the mean number of trophoblast cells undergoing apoptosis in the control group as opposed to the treatment group.

Conslusion: It can be concluded that administering Moringa leaf powder at a dose of 800 mg/day/kg BW can reduce trophoblast cell apoptosis.

Keywords: diabetes mellitus, stress oxidation, Moringa leaf powder, trophoblast cell apoptosis

INTRODUCTION

The Maternal Mortality Rate (MMR) in Indonesia is fairly high. The maternal mortality rate is beneficial to define the level of mindfulness of healthy living behavior, nutritive status and maternal health, environmental health conditions, and the level of health services, particularly for pregnant women, throughout the periods of childbirth and the perinatal. [1] As stated by the World Health Organization (WHO) in 2016 there was an extraordinary maternal mortality rate in the world, about 830 females died from complications of pregnancy or childbearing around the world every day. It is appraised that in 2015, around 303,000 women died during and after pregnancy and childbirth. [2] One of the factors that affect the complications of pregnancy and childbirth is diabetes mellitus. Diabetes mellitus (DM) triggers complications in 10% of pregnancies and upsurges the danger of hypertension, premature birth, intrauterine growth disorders, perinatal death, acute kidney and liver failure, antepartum and postpartum hemorrhage, and maternal death. [3] In addition, according to Ighodaro et al.,[4] things that often cause a person with diabetes will experience fear if the person is unable to control the life, resulting in stress. Stress conditions in DM patients can affect the pattern of controlling sugar levels. Uncontrolled blood sugar levels in the long term can result in hyperglycemia conditions. DM patients with hyperglycemia are caused by uncontrolled blood sugar levels so that it can cause hypertension.[5, 6] Hyperglycemia can result in disruption of oxidative processes in the body or called oxidative stress. Oxidative stress is a condition of an imbalance between the amount of oxidants and antioxidants in the body. This is due to the excessive production of free radicals such as Reactive Oxygen Species (ROS). According to Francisqueti et al.[7], oxidative stress conditions in DM can lead to serious complications in various organs of the body.

Hypertension in pregnancy can be grouped into chronic hypertension (hypertension that was present before pregnancy), pregnancy-induced hypertension (normal blood pressure before 20 weeks of gestation), preeclampsia (with proteinuria), and eclampsia (with proteinuria and seizures). Preeclampsia is defined as gestational hypertension with proteinuria after 20 weeks of gestation. Proteinuria is defined as the excretion of 300 mg or more of protein in a 24-hour urine collection or a random protein/creatinine ratio of at least 0.3 mg/dL. A urine dipstick can not definitively diagnose preeclampsia unless other methods are not available, in which case a minimum measurement of 1+ must be obtained. [8]

Approximately 2-7% of pregnancies in non-diabetic women are affected by preeclampsia, but women with a history of type 1 diabetes, type 2 diabetes, and gestational diabetes have a greater risk of developing preeclampsia in developed countries. Some of the risk factors for preeclampsia in women with type 1 and type 2 diabetes include nulliparity, advanced maternal age and poor blood sugar control.[2] Based on the results of a preliminary survey conducted by researchers at RSUD DR. H. Abdul Moeloek Lampung Province, it was found that in 2017 there were 129 mothers who experienced preeclampsia with diabetes mellitus, while in 2018 from January to June there were 45 preeclampsia patients with diabetes

mellitus. The pathophysiology of preeclampsia with insulin resistance is almost similar, including endothelial dysfunction, atherosclerosis and inflammation. Therefore, pregestional insulin resistance or mothers who have experienced insulin resistance before pregnancy or have a higher degree of insulin resistance during pregnancy play a coadjuvant role in the development of preeclampsia.[9,2]

Preeclampsia is a disease that involves multisystem in pregnancy which is characterized by an increase in blood pressure and proteinuria. Although most of the cases end well, preeclampsia is one of the leading causes of maternal and perinatal morbidity and mortality. On the observation that the definitive treatment for preeclampsia is delivery of the placenta, the incidence of preeclampsia is high in large placental sizes, for example in multiple pregnancies, and that preeclampsia can occur in molar pregnancies where the placenta develops in the absence of a fetus, suggesting that the placenta is a central focus and an important part of the pathogenesis of preeclampsia.

The normal development of the placenta depends on the differentiation and invasion of the trophoblast. During the process of differentiation and invasion, trophoblast cells rapidly divide to form a link between the mother and the embryo, while another subpopulation of trophoblast invades the decidua to remodel the spiral arteries thereby increasing blood flow to the placenta for fetal development. As a developing organ, the placenta undergoes constant tissue remodeling, which is characterized by a functional apoptotic process. After proliferation and differentiation into specific cell subtypes, aging trophoblast cells are selectively removed and replaced with new trophoblast cells without affecting the surrounding cells. Apoptotic cells are found in normal pregnancy placentas both on the maternal and fetal sides and the apoptotic process plays a role in the occurrence of trophoblast attachment and invasion, the spiral artery transformation process, trophoblast differentiation, and the process of immune tolerance to paternal antigens expressed by trophoblast cells.

Apoptosis is a programmed cell death in which cell death occurs by activating a tightly regulated internal suicide program. Programmed cell death or apoptosis plays an important role in cell homeostasis and tissue remodeling, especially placental growth. Morphological features of apoptosis include cell shrinkage, condensation and fragmentation of chromatin, formation of blisters on cells and their fragmentation into apoptotic objects and phagocytized by macrophages. [3]

In preeclampsia there is inhibited trophoblast invasion, vasculitis, thrombosis and ischemia of the placenta. Abnormalities in the placenta appear to be more influential in the occurrence of preeclampsia than the fetus. Although the etiology remains to be clearly determined, but all center on endothelial dysfunction. According to the theory of placental ischemia, endothelial cell dysfunction occurs as a result of the hypoxic process. Trophoblast exposed to hypoxia in vitro causes excessive apoptotic process so that cytotrophoblast invasion into the myometrium becomes shallow and spiral artery remodeling in the uterus occurs

incompletely causing uteroplacental ischemia. The hypoxic placenta then secretes toxin factors from the placenta into the maternal circulation which cause an inflammatory response and cause endothelial cell damage. Hypoxia can cause apoptosis. [3, 10]

The use of oral glucose-lowering drugs is not recommended for pregnant women according to the American Diabetes Association. Many researchers (e.g., Vargas-Sánchez, et al.,[11], Owens, et al.,[12], Toby et al.,[13]) have investigated the effectiveness of moringa eleifera in lowering blood sugar levels in patients. However, as far as the authors know, no one has investigated the effectiveness of Moringa oleifera in preeclampic patients with GDM (Gestasional Diabetes Melitus). Therefore, it is necessary to study one of the natural ingredients that can be developed as a therapy in overcoming GDM associated with oxidative stress. One of these natural ingredients is Moringa leaf powder. More specifically, this study is to see the effectiveness of moringa oleifera powder to prevent trophoblast cell damage in preeclamptic pregnant rats with diabetes mellitus.

METHODS

This experimental study used experimental white rats with diabetes conditions due to the administration of alloxan for 18 days. RAL method (Completely Randomized Design) was used to select the object of research and to classify and provide treatment. It was due to the homogeneity of experimental animals, ration materials, experimental sites and other research materials. The research design for each treatment carried out in this study followed the procedure used by Gondo.. [9] This study used a rat model with preeclampic pregnancy as a result of alloxan induction. In order to obtain a homogeneous gestational age, uterine cycle synchronization of 30 female white rats using the Leeboth, Pheromone, Whitten effect before mating white rats. In order to obtain a high pregnancy success rate and obtain the same gestational age of white rats, the synchronization method is used first. [14]

In the Lee Both Effect, female rats were isolated (separated) from male rats for 2 weeks in order to condition the unestrus cycle. In the pheromone effect, female rats were stimulated for their lust cycle and conditioned for their estrus cycle by exposing them to cages containing male rat urine husks. And on the Whitening Effect, female white rats were already in oestrus condition after 72 hours of treatment.

Female rats after 72 hours stimulated by pheromones (male rat urine husks) were mated for one night in pairs (1:1). The next day after the mice were mated was considered the first day of pregnancy. . [15] Alloxan was not induced in mice on the first day of gestation. After the rats were pregnant, alloxan was induced for 3 consecutive days as much as 150 mg/day/kg BW so that the rats were in a hyperglycemic condition. The first alloxan induction was carried out on day 4, and on day 18 a blood glucose analysis (post-test) was performed to see if any white rats experienced an increase in blood glucose. Samples were randomized to the treatment group, the positive control group, and the negative control group. At this stage, 30 preeclamptic pregnant female rats were grouped into 6 groups, where each group contained 5

mice., namely: 1) K- is the negative control, in this case this group was not induced by alloxan; 2) K+ is the positive control, in this case alloxan was induced on the second day after pregnancy/marriage with a dose of 150 mg/day/kg BW for the next 3 consecutive days using a probe; 3) Dosage 1 is the alloxan-induced group at a dose of 150 mg/day/kg BW and given Moringa leaf powder at a dose of 100 mg/day/kg BW after alloxan administration, for the next 14 consecutive days; 4) Dosage 2 is the alloxan induced group at a dose of 150 mg/day/kg BW and given Moringa leaf powder at a dose of 200 mg/day/kg BW; 5) Dosage 3 is the alloxan induced group at a dose of 150 mg/day/kg BW and given Moringa leaf powder at a dose of 400 mg/day/kg BW; and 6) Dosage 4 is the alloxan-induced group at a dose of 150 mg/day/kg BW and given Moringa leaf powder at a dose of 800 mg/day/kg BW.

Apoptosis has several characteristics, one of which is DNA fragmentation. To examine DNA fragmentation, the TUNEL (Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling) method was used in this study. The terminal transferase enzyme contained in the TUNEL Reagent was used to recognize the 3'OH end (nick end) generated by DNA fragmentation, while fluorescein-dUTP was used to visualize the 3'OH end for observation using a fluorescence microscope.

The double staining method using TUNEL reagent counterstained with methylene Green was used to visualize the comparison of apoptotic cells with non-apoptotic cells in one field of observation. TUNEL reveals cells undergoing apoptosis and gives a brown color, while methylene Green detects non-apoptotic cells and gives a green color.. [3]

Univariate Analysis

Univariate analysis aims to explain or describe the characteristics of each research variable. The test that was carried out to determine whether the data was homogeneous or not was carried out with the Levene's test of variance and to determine whether the data was normally distributed or not, the Shapiro-wilk test was used.

Bivariate Analysis

Bivariate analysis aims to explain whether there is an effect of giving Moringa leaf powder (Moringa oleifera) on trophoblast cell damage in white rats. The hypothesis test used to assess changes in trophoblast cell is One Way Anova if the data meets the parametric test requirements or the Kruskal Wallis test if the data does not meet the parametric test requirements. To find out the difference between the treatment groups, a follow-up test or post test was carried out.

The rules made by the Institution of Animal Care Use Committee (IACUC) were used as a reference in using animals in this research. The rule specifies some principles to follow, namely ethical principles (respect, beneficiary, and fairness), 3R principles (Replacement, Reduction,

Improvement), and 5F/Freedom (freedom from hunger and thirst, heat and discomfort, pain, trauma and disease, fear and stress and express behavior naturally). The method used in this study has received ethical approval from the Research Ethics Committee of the Faculty of Medicine, Wijaya Kusuma University, Surabaya with Certificate No.: 39/SLE/FK/UWKS/2021.

RESULTS

On average, the results of the calculation of apoptosis in the Normal and Treatment groups showed a significant difference in the mean number of trophoblast cells undergoing apoptosis in the control group compared to those in the treatment group. The results showed a decrease in TUNEL expression after administration of Moringa leaf powder with an average dose of 800 mg/day/Kg BW in the treatment group, as shown in Table 1..

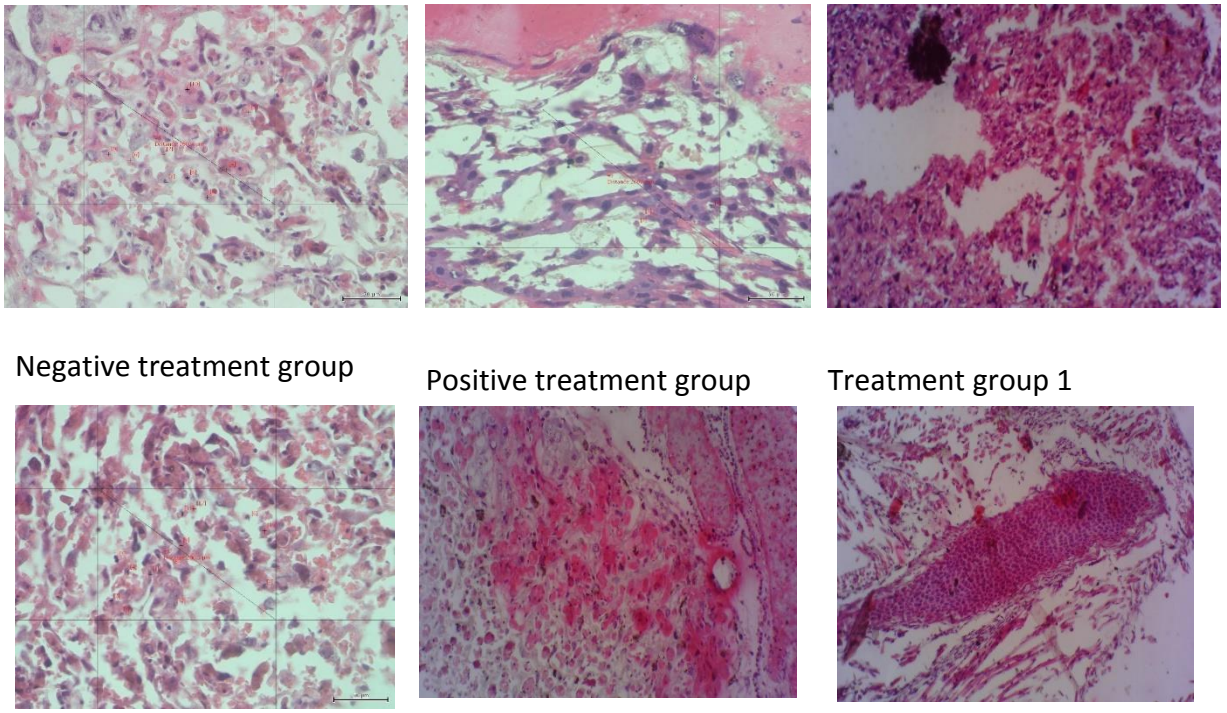
Table 1. Observations of White Rat Trophoblast Cells in Control and Treatment Groups

Group	Apoptotic nucleus	
	R	Average
Negative Control Group	5	5.33
Positive Control Group	5	20
Treatment Group 1	5	15.44
Treatment Group 2	5	8.66
Treatment Group 3	5	7.66
Treatment Group 4	5	6.75

Multiple comparison test with LSD test resulted in significantly different TUNEL levels in the negative control group, positive control group and the treatment group with different doses of Moringa leaf powder. In the treatment group, with Moringa leaf powder at a dose of 100 mg/day/kg BW, the TUNEL expression average was 15.44. Meanwhile, with Moringa leaf powder at a dose of 200 mg/day/kg BW, the average TUNEL expression was 8.66. By giving Moringa leaf powder at a dose of 400 mg/day/kg BW, the average TUNEL expression obtained was 7.66, and by giving Moringa leaf powder at a dose of 800 mg/day/kg BW, the TUNEL expression average was 6.75. The administration of Moringa leaf powder with different doses of 100, 200, 400 and 800 mg/day/kg BW in the treatment group did not show statistically significant TUNEL expression. However, the lowest mean TUNEL expression was found in the group treated with Moringa leaf powder at a dose of 800mg/day/kg BW.

The following is the result of histopathological description of white mouse trophoblast cells.

Figure 1. Histopathology of Trophoblast Cells in White Rats



In this histopathological picture, with 40x magnification around the central vein and Kiernan's triangle in 1 field of view, dilated sinuses and apoptotic trophoblast cells can be found. In treatment 1 there were 4 apoptotic cells, while in treatment 2 there were 3 apoptotic cells, in treatment 3 there were 2 apoptotic cells, and in treatment 4 there was 1 apoptotic cell. In the positive control group there were 4 apoptotic cells, and in the negative control group there were 0 apoptotic cells. From these observations, there was a decrease in the number of cells that experienced the lowest apoptosis from the trophoblast cells of pregnant white mice in treatment group 4.

DISCUSSION

In this study, Transferase (TdT) Kit Mediated dUTPNick End Labeling (TUNEL) with the brand Enogen Lot number 20160302 was used to measure apoptosis by the TUNEL staining method. Trophoblast cells that undergo apoptosis are characterized by the presence of brown color in the nucleus while trophoblast cells that do not undergo apoptosis are characterized by purple or green cell nuclei. Image J software was used to measure the apoptotic expression of trophoblast cells. In this study also found an increase in the expression of apoptosis which was analyzed by TUNEL. DNA fragmentation that occurs in the process of apoptosis lasts for several hours, before finally undergoing phagocytosis. The labeling technique for fragmented DNA (TUNEL) can be seen in Figure 1.

There was no statistically significant TUNEL expression in the Moringa leaf powder treatment group with different doses of 100, 200, 400 and 800 mg/day/kg BW, but in the Moringa leaf powder treatment group at a dose of 800mg/day/kg BW, the mean expression was found the lowest TUNNEL. This is because the increased apoptosis of preeclamptic trophoblast cells involves the role of the inflammatory pathway and the oxidative stress pathway, in regulating the apoptotic process. Free radical reactivity can be inhibited or stopped by an antioxidant substance so that it can inhibit oxidative damage to a molecule. Oxidative stress occurs when there is an increase in free radical formation and a decrease in endogenous antioxidant capacity.

Therefore, additional intake of exogenous antioxidants through various sources is needed to prevent oxidative stress. The production of cytokines as inflammatory mediators is thought to be related to the excessive formation of ROS. Cytokines have an important role in the pathophysiology of being expressed in abnormal conditions in the fetal placenta during pregnancy. Cytokine regulation is affected by oxidative stress. TNF-á (Tumor Necrosis Factor-á) is produced by the placenta and its appearance is associated with several metabolic events. It is known that the apoptotic pathway, in the extrinsic pathway of apoptosis, is mediated by members of the TNF death receptor family which is part of the TNF-receptor (TNF-R) superfamily and has a C-terminal section consisting of 80 known amino acids role in the death process. [18, 10] In the intrinsic pathway, apoptotic signals are mediated directly from the mitochondria in response to stressors such as DNA damage or loss of growth factors.

Moringa leaves have a high flavonoid content, one of the most prominent being quercetine which functions as an anti-inflammatory to inhibit NfkB which is a marker of an inflammatory reaction in endometriosis. [16, 17] Research by Wihastuti et al (2007) proved that the high anti-inflammatory content in Moringa leaves was also able to inhibit TNF-α. Inhibition of NFkB and TNF-. Causes the formation of bonds between TNF and TNF- receptors which are cell death receptors to be inhibited.

The results of observing the histopathological picture of trophoblast cells in the treatment group showed better results than the positive control group. Damage to the cell nucleus in the positive control group can be caused by the process of apoptosis due to induction of alloxan given but without Moringa leaf powder.[19, 20] However, it is known that some of the results that are not suitable can be caused because the initial state of the trophoblast cells from white rats before treatment cannot be known. From the results of data analysis conducted in this study, it can be concluded that administration of Moringa leaf powder can prevent alloxan-induced damage to trophoblast cells in white rats. The administration of Moringa leaf powder in graded doses has also been shown to reduce trophoblast cell damage in white rats.

Limitation of the study

This research was still in the experimental stage on animals (white rats), further research with human subjects would provide more valid results.

CONCLUSION

In conclusion, the administration of Moringa leaf powder in graded doses has also been shown to reduce trophoblast cell damage in white rats. Giving Moringa leaf powder at a dose of 800 mg/day/kg BW is able to reduce trophoblast cell apoptosis.

REFERENCES

1. Dinas Kesehatan. Profil Kesehatan Provinsi Lampung Tahun 2015. Bandar Lampung: Dinas Kesehatan. 2015.
2. WHO. WHO Evidence brief on Maternal Mortality. Geneva. 2016.
3. Gondo, H.K., Haryanti, E. Pengaruh Spirulina terhadap VEGF dari Trofoblast Tikus Putih Hamil Strain Wistar yang Diinduksi Interleukin.Jurnal Ilmiah Kedokteran Wijaya Kusuma. 2020. 9(2):123
4. Ighodaro, OM, Adeosun, AM, Akinloye, OA. Alloxan-induced diabetes, a common model for evaluating the glycemic-control potential of therapeutic compounds and plants extracts in experimental studies. Medicina. 2017. 53(6):365-374.
5. Valdes E, Alvaro SM, Barbara M, Mauro PC. Assessment of Pregestational Insulin Resistance as a Risk Factor of Preeclampsia. Gynecologic and Obsetric Investigation. 2012. 77:111-116
6. Radiansah, R., Rahman, N. and Nuryanti, S. Moringa Leaves Extract (Moringa Oleivera) As An Alternative To Reduce Blood Sugar Levels On Mice. Jurnal Akademika Kimia. 2013.
7. Francisqueti FV, Chiaverini LC, Santos KC, Minatel IO, Ronchi CB, Ferron AJ, Ferreira AL, Corrêa CR. The role of oxidative stress on the pathophysiology of metabolic syndrome. Rev Assoc Med Bras. 2017. 1;63(1):85-91.
8. Borhart, J.. Emergency Department Management of Obstetric Complications. Washington DC: Springer International Publishing. 2017.
9. Elmore, S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007. 35(4):495-516.
10. Docherty, FM and Russ, HA.. Encyclopedia of Tissue Engineering and Regenerative Medicine, Academic Press. 2019
11. Vargas-Sánchez K, Garay-Jaramillo E, González-Reyes RE. Effects of Moringa oleifera on Glycaemia and Insulin Levels: A Review of Animal and Human Studies. Nutrients. 2019; 11(12).
12. Owens, F.S., Dada, O., Cyrus, J.W., Adedoyin, O.O., Adunlin, G., The effects of Moringa oleifera on blood glucose levels: A scoping review of the literature, Complementary Therapies in Medicine, 2020. Vol. 50.

13. Toby, T., Amat, A., & Artawan, I.. Uji Efek Anti Diabetes Ekstrak Etanol Daun Kelor (Moringa Oleifera) Terhadap Tikus Putih Sprague Dawley Yang Diinduksi Aloksan. Cendana Medical Journal (CMJ), 2020. 8(2), 24-35.
14. Keman K, Presetyorini N, Langgar M J. Perbandingan ekspresi p53, Bcl-2, dan Indeks Apoptosis Trofoblas pada Preeklampsia/Eklampsia dan Kehamilan Normal. Majalah Obstetri dan Ginekologi Indonesia, 2009. 33(3),
15. Munim, Abdul, Alwi, MK., and Syam, Aminuddin. Pengaruh pemberian tepung daun kelor (moringa oleifera) terhadap penurunan glukosa darah pada penderita pradiabetes di wilayah kerja puskesmas samata kab. Gowa. Jurnal Ilmiah Kesehatan Diagnosis. 2019. 13(6)
16. Alethea, Talytha and Ramadhian, M. R. Efek Antidiabetik pada Daun Kelor. Majority. 2015. 4(9).
17. Boland, Brandon B.; Rhodes, Christopher J.; Grimsby, Joseph S. The dynamic plasticity of insulin production in β-cells. Molecular Metabolism. 2017. 6(9).
18. Kumar, Abbas, Fausto. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Saunders. 2010.
19. Cook, N. C. and S. Samman. Review Flavonoids-Chemistry, Metabolism, Cardioprotective Effect, And Dietary Sources, J. Nutr. Biochem. 1996. (7): 66-76
20. Cuppett, S., M. Schrepf and C. Hall III. Natural Antioxidant – Are They Reality. In Foreidoon Shahidi: Natural Antioxidants, Chemistry, Health Effect and Applications, AOCS Press. Champaign, Illinois. 1954.