

Endogenous Antioxidant Activity Combination Of Moringa Leaf And Clove Flower Extracts Toward Diabetic Rats (*Rattus Norvegicus*)

Muhammad Fakhrol Hardani¹, Ririen Hardani², Sitti Rahmawati³, Baharuddin Hamzah⁴

^{1,2}*Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Tadulako University, Jl. Soekarno Hatta Km 9 Palu 94118 Indonesia*

^{3,4}*Chemistry Study Program, Mathematics and Sciences Division, faculty of teacher training and education, Tadulako University, Jl. Soekarno Hatta Km 9 Palu 94118 Indonesia*

Abstract— *In pathological conditions such as Diabetes Mellitus (DM), increased oxidative stress can cause a decrease in endogenous activity in the body so the body is unable to detoxify free radicals that cause cell damage. Some of natural antioxidant sources are moringa leaves (*Moringa oleifera* Lamk.) and clove flowers (*Syzygium aromaticum* Merry & Perril). The purpose of this study was to determine the enzyme activity of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) in a mixture composing of ethanol extract of moringa leaf and clove flower towards animals with DM conditions. This research is an experimental laboratory study using a post test only group design. The subjects of this study were 40 white male Wistar rats (*Rattus norvegicus*) strain which were DM conditioned by streptozotocin induction. Rats were randomly grouped into 8 groups, and each group consisted of 5 rats. The combined dosage of the ethanol extract of moringa leaves and clove flowers used is a combination I : 150: 40 mg / kg body weight rat, combination II 100: 80 mg / kg body weight rat and III 50: 120 mg / kg body weight rat. The results showed that the combination of ethanol extract of moringa leaves (*Moringa oleifera* L. and clove flower (*Syzygium aromaticum* Merry & Perril) increased the antioxidant enzyme activity of SOD and GPx in animals with DM conditions.*

Keywords— *Moringa leaves (*Moringa oleifera* Lamk.), clove flowers (*Syzygium aromaticum* Merry & Perril), SOD, GPx, Diabetes mellitus.*

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic disease that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin produced [1]. The International Diabetes Federation (IDF) in 2013 revealed that Diabetes Mellitus sufferers ranked 7th in Indonesia with a total of 8.5 million [2]. Some complications of DM can arise due to lack of control of hyperglycemic conditions, such as cardiovascular disease, diabetic neuropathy, diabetic nephropathy, diabetic retinopathy, erectile dysfunction, oxidative stress state. [3, 4].

Oxidative stress plays an important role in DM complications, where oxidative stress occurs due to an imbalance between free radicals and natural antioxidants [5, 6]. Hyperglycemic conditions result in increased production of reactive compounds through nonenzymatic

glycation pathways in proteins, polyol-sorbitol pathways and glucose auto-oxidation [7]. Antioxidant compounds are needed to prevent oxidative stress. Antioxidants work by stabilizing reactive compounds so as to prevent damage [8]. Antioxidants are generally divided into two, namely endogenous and exogenous antioxidants. Endogenous antioxidants are composed of proteins and enzymes that are synthesized in the body, including the enzyme superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and non-enzymatic such as transferrin, ceruloplasmin. Exogenous antioxidants are antioxidants that come from outside the body's intake, such as vitamin C, vitamin E, phenolics [9].

Studies of natural antioxidant activity have been carried out and show the ability to protect damage from oxidative stress. Antioxidants in DM patients can inhibit the activity of free radicals, which have bioactive components (cynamic acid, coumarin, diterpene, flavonoids, lignin, monoterpenes, phenylpropanoids, tannins and triterpenes) (Chaturvedi, 2007). The combination of ethanol extract of *Morienga oleifera* leaves 500 mg / kg BW and *Ocimum gratissimum* leaves 500 mg / kg BW proves it can normalize blood fasting glucose levels and improve testicular structure and spermatogenesis in sexual dysfunction caused by diabetes [10]. Clove fruit powder at a dose of 1000 mg / kg BW shows an increase in levels of antioxidant enzymes (SOD, GPx, CAT, GST) in dealing with oxidative stress and can act to reduce lipid levels in the High Fat Diet (HFD) [11].

Based on previous research, this can be a reference because it is known that both of these simplicia have chemical contents in them that have potential as antidiabetic and antioxidants. The use of both simplicia as a herbal combination is expected to increase antidiabetic and antioxidant effects. The parameters to be tested are the activity of the antioxidant enzyme super oxide dismutase (SOD), glutathione peroxidase (GPx) on test animals with diabetes mellitus induced by streptozotosin.

2. MATERIALS AND METHODS

2.1 Tools

Maceration container, beaker glass, flannel fabric, 40 mesh sieve, aluminum foil, glass funnel, oven, colorimeter, 50 ml measuring flask, stirring rod, surgical instrument, lancet, weighing device, olympus CX-21 microscope, microtome, evaporator, tweezers , dropper pipettes, weigh paper, filter paper, mouse cages, cutters, syringes and glassware.

2.2 Materials

Ethanol 96%, aquadestilata, Mg powder, glibenclamide, streptozotosin, nicotinamide, Liberman Bouchard reagent, Dragendroff reagent, Glucose Oxidase Phenol Aminoantipyrine reagent, normal saline 0.9%, concentrated sulfuric acid, aluminum chloride, chloride acid, Na-CMC, citrate buffer, formalin, xylen solvents, absolute alcohol, reagent enzyme activity test of SOD, GPx .

2.3 Making Ethanol Extract of Clove Flowers and Moringa Leaves

Dry Cloves and Moringa Leaves that have been pollinated separately each weighed as much as 1 kg then put in a maceration container, added with 96% ethanol as much as 10 L. The container is stirred and closed immediately, then stored in a room protected from sunlight, allowed to stand for 3-5 days and occasionally shaken. After 3-5 days the macerate is filtered with a flannel cloth, the resulting maceration filtrate is then collected in a closed vessel and concentrated using an evaporator.

2.4 Making of Streptozotosin-Nicotinamide Solution

Calculation of STZ dose 60 mg / kg body weight for white rats weighing 200 g. Preparation of Aldrich sigma STZ was dissolved in 0.1 M citrate buffer pH 4.5. And the calculation of the dose of NA 150 mg/kg BW for white rats weighing 200 g. NA is dissolved in 0.9% normal saline [12].

2.5 Making a 1% Na CMC Suspension

A 1% Na-CMC colloid solution is prepared by dissolving 1 g of Na-CMC little by little into 50 ml of hot distilled water while stirring until it is homogeneous. Volume is sufficient up to 100 ml with distilled water.

2.6 Preparation of suspension glibenclamide

Taken glibenclamide tablet powder 0.1 g, then crushed in a mortar and added colloidal solution of Na-CMC 1% w / v gradually eroded until homogeneous. Included in the 100 ml flask and then paid back the volume up to 100 ml with a colloidal solution of Na-CMC 1%.

2.7 Testing the Antidiabetic Effects

For this test the rats were divided into 3 control groups and 5 extract groups. Each treatment consisted of 5 rats. The rats were acclimated for 1 week, then were not given food but were still given a drink for 16-18 hours before treatment. Then the body weight was weighed and fasting blood glucose level was measured on day 1 as the initial blood glucose level (T0) using the Aminoanti-pyrene Glucose Oxidase reagent [13]. Streptozotosin was injected once as much as 60 mg / kg BW, but 15 minutes beforehand it was injected with nicotinamide 150 mg / kg BW intraperitoneally on day 1. After five days (day 5), blood glucose levels and body weight of rats were again measured (T1). The treatments given can be seen in Table 1.

2.8 Tissue preparation and supernatant

On the 28th day after measuring blood glucose levels, mice liver was taken, cleaned, dried, and processed for biochemical testing. Samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing protease inhibitors, 0.2 mM PMSF and 1 mM EDTA, at 4°C for 30 seconds (2-15 seconds) with 15 seconds cooling interval). Homogenates were filtered and the filtrate was centrifuged at 1088 g (at r max 108 mm) for 5 minutes in cold conditions. The resulting supernatant is used to measure antioxidant enzyme activity.

2.9 Testing of SOD and GPx activity

SOD activity in liver tissue was analyzed by the method of Sun et al, (1989). 0.06 ml of liver supernatant was reacted with a mixture consisting of 2.70 ml sodium carbonate buffer 50 mM containing 0.1 mM EDTA (pH 10), 0.06 ml xantin 10 mM, 0.03 ml bovine serum albumin (BSA) 0.5% and 0.03 ml NET 2.5 mM. Next is the addition of xanthine oxidase (0.04 units). The absorbance produced after 30 minutes was measured at a wavelength of 560 nm. As a control used a solution used in the preparation of liver samples, PBS containing 11.5 g / L KCl. The SOD activity (%) is calculated using the following equation: $(A / B) \times 100\%$, where A = absorbance of sample solution and B absorbance of control solution [14].

Measurement of GPx activity was carried out by: 200 µl of clear liver supernatant added 200 µl of 0.1 M phosphate buffer pH 7.0 containing 0.1 mM EDTA, 200 µl reduced glutathione (GSH), 10 mM and 200 µl of the glutathione reductase enzyme (2.4 units). Then incubated for 10 minutes at 37 ° C, added 200 µl NADPH 1.5 mM and incubated again for three minutes at the same temperature, and followed by the addition of 200 µl H₂O₂ 1.5 mM.

Absorbance is measured between one to two minutes with a spectrophotometer at 340 nm wavelength [15]. The calculation is as follows:

$$M \text{ units of GSH-Px} = \frac{\text{Abs} \times V_t \times 2 \times 1000 \times 1/\text{mgprotein}}{6,22 \times V_s}$$

Where : Abs = change in absorbance

Vt = total volume (ml)

6.22 = extrinsic coefficient of NADPH

2 = 2 moles of GSH which is equivalent to 1 mole of NADPH.

1000 = change to milli units

Vs = sample volume

Table 1. The treatment of rats

Groups	Number of rats	Treatment
I (Normal control)	5	Na-CMC 1% suspense solution
II (Positive control)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given Glibenclamide 0,45 mg/kg BW.(P.O)
III(Negative control)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given Na-CMC 1% (P.O)
IV (Test I)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given ethanol extract of Moringa leaves 100 mg/kg BW (P.O)
V (Test II)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given ethanol extract of Clove flower 80 mg/kg BW (P.O)
VI (Test III)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given ethanol extract of Moringa leaves 150 mg/kgBW and Clove flower ethanol extract 40 mg/kg BW (P.O)
VII (Test IV)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given ethanol extract of Moringa leaves 100 mg/kgBW and Clove flower ethanol extract 80 mg/kg BW (P.O)
VIII (Test V)	5	Mice induced by STZ 60 mg/kg BW-NA 150 mg/kg BW (I.P), given ethanol extract of Moringa leaves 50 mg/kgBW and Clove flower ethanol extract 120 mg/kg BW (P.O)

3. RESULTS

The results of an increase in rats blood glucose levels after STZ-NA induction. In this study to make a diabetic animal model, rats were induced using streptozotosin - nicotinamide. Streptozotosin is a chemical used to induce diabetes in animal experiments. Giving streptozotosin is a fast way to produce diabetic conditions because streptozotosin reacts by damaging essential substances in pancreatic beta cells because in cells, streptozotosin is similar to glucose transported by GLUT-2, but is not recognized by other glucose-carrying proteins, this damage causes reduced insulin in the pancreatic beta cells. Therefore we need

another compound that is able to protect pancreatic β cells from the toxicity of streptozotosin, namely nicotinamide. The presence of nicotinamide can reduce the toxicity of streptozotosin because nicotinamide is known to prevent depletion in pancreatic β cells [16]. Streptozotosin and nicotinamide can be given intraperitoneally in experimental animals. An increase in blood glucose levels (Fig.1) causes a decrease will be easily observed after being given treatment.

This study uses streptozotosin as a free radical agent that causes selective damage to the pancreas of rats, this causes hyperglycemic conditions. In this condition, glucose will undergo metabolic processes through the pathway of autooxidation, glycation and formation of dicarbonyl which produces hydroxyl free radicals (OH^\cdot) and superoxide free radicals ($\text{O}_2^{\cdot-}$). The high free radicals produced cause an imbalance between free radicals and endogenous antioxidants so that the free radicals produced cannot be neutralized by endogenous antioxidant enzymes, SOD and GPx.

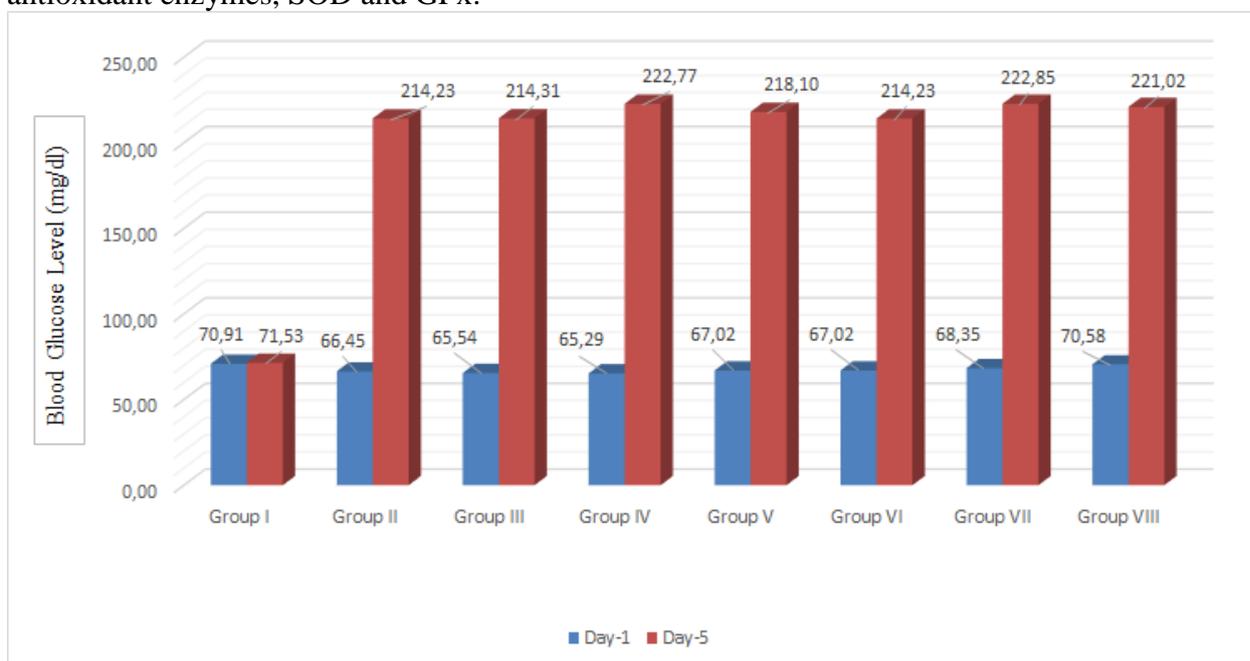


Fig. 1: The profile of Increasing glucose level in blood after STZ-NA induction

Measurement Results of SOD and GPx Enzyme Levels

The ability of Moringa leaf extract and Clove flower extract to increase the activity of antioxidant enzymes was evaluated by measuring the increased levels of the SOD and GPx enzymes as natural antioxidant enzymes in the body in homogenate liver rats that had been treated with extracts for 28 days. The following table results the average measurement of SOD and GPx activity in each treatment group.

Table 2. The average measurement of SOD dan GPx levels

Group	N	The average measurement of SOD and GPx \pm SD	
		SOD (%)	GPx (U/mg)
I	5	85,71 ^g \pm 3,99	71,00 ^b \pm 1,22
II	5	20,36 ^a \pm 3,70	17,75 ^a \pm 1,22
III	5	75,00 ^f \pm 2,82	68,84 ^g \pm 2,00

IV	5	57,14 ^d ±2,82	50,16 ^c ±1,22
V	5	42,86 ^b ±2,82	38,59 ^c ±1,22
VI	5	64,29 ^c ±2,82	64,67 ^f ±1,48
VII	5	51,79 ^c ±2,82	43,06 ^d ±1,48
VIII	5	41,07 ^b ±2,82	33,18 ^b ±1,22

Note :Numbers followed by different letters on the same line show significant differences, the same letters indicate insignificant differences.

The SOD value (%) represents the amount of superoxide anion successfully inhibited or decomposed, the GPx value (U / mg) states the amount of enzyme needed to catalyze the oxidation of 1 nmol NADPH per minute in one mg of protein.

In the normal group gave an average result of normal levels of the antioxidant enzyme activity of SOD and GPx in healthy rats. High SOD and GPx values indicate that rats are in normal conditions. The negative control group was used as a marker of oxidative stress. This study uses streptozotocin as a free radical agent that causes selective damage to the pancreas of rats, this causes hyperglycemic conditions.

The results of the measurement of the average levels of positive control SOD and GPx, a single dose group I, II, a combination of I, II and III (Table 2) increased compared to the average level of the negative control group. Based on the results of statistical analysis of increased levels of SOD and GPx from the positive control group (glibenclamide), the single dose extract therapy group I, II, the combination of I, II and III showed a significant increase in levels ($p > 0.05$) when compared to the negative control group.

Increased levels of SOD and GPx in the positive group, the single dose group I, II, a combination of I, II and III as indicators that show the influence of an increase in defense mechanisms against free radicals and protect the pancreas from oxidative stress conditions [17, 18]. The activity of metabolite compounds contained in the ethanol extract of Moringa leaves and clove flowers is proven to act as an antioxidant, although its activity is not yet close to the normal value in the normal group.

Glibenclamide is an oral antidiabetic of sulfonylureas that works by increasing insulin secretion. Increased insulin secretion from the pancreas goes through the portal vein and then suppresses hepatic glucose production [19]. Glibenclamide also causes the balance in the pancreas to run well which causes endogenous defense enzymes to work to neutralize or capture free radicals that are proven in the positive control group.

The results of phytochemical tests conducted prove that the ethanol extract of Moringa leaves and clove flowers contain alkaloids, flavonoids, tannins and saponins [20]. Alkaloids reduce blood glucose by inhibiting glucose absorption in the intestine, stimulating glycogen synthesis and inhibiting glucose synthesis by inhibiting glucose enzymes. The tannin content can increase glucose uptake by muscle and adipose tissue and prevent the occurrence of glycogenolysis in the liver. Tannins are as astringent, can prescribe protein intestinal mucous membrane and form a layer that protects the intestine, thus inhibiting glucose absorption. Saponin increases permeability of the small intestine, thereby increasing the uptake of substances that are actually less absorbed and causing loss of normal intestinal function [21, 22]

Antioxidants found in *Moringa oliefera* include vitamin C and flavonoids, so that Moringa leaves have a strong activity as an oxidant scavenger that can inhibit the ROS reaction and increase the activity of SOD, GSH and catalase which causes a decrease in oxidative stress in cells [23, 24]. Chemical content in *Syzygium aromaticum* such as eugenol and oleanic acid can act as antioxidants and the main scavenger in counteracting free radicals [25].

Endogenous antioxidants function to ward off free radicals and oxidative stress. Decreased oxidative stress in cells means reducing the process of damage / degeneration of pancreatic β cells so that it will speed up the regeneration process of pancreatic β cells

4. CONCLUSION

Based on the results of research can conclude the following: The combination of *Moringa oleifera* leaf extract and clove flower (*Syzygium aromaticum*) extract can increase the antioxidant enzyme activity of SOD, GPx in rats (*Rattus norvegicus*) with diabetes conditions induced by STZ-NA.

5. REFERENCES

- [1] International Diabetes Federation. 2013. IDF Clinical Guidelines Task Force. Global Guideline for type 2 diabetes. http://www.idf.org/type_2_diabetes.
- [2] World Health Organization. 1999. Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications : *Report of a WHO Consultation*.
- [3] Chaturvedi N. 2007. The burden of diabetes and its complications : *Trends and implications for intervention. Diabetes Res Clin Pract.* 76(3): S3-S12.
- [4] Ceriello A, Testa R. 2009. Antioksidant anti-inflammatory treatment in type 2 diabetes. *Diab Care* 32(2) : S232-S236.
- [5] Giacco F, Brownlee M. 2010. Oxidative stress and diabetic complications. *Circ Res* 107:1058-70
- [6] Nuttal SL, Dunne F, Kendal MJ, Martin U. 1999. Age-independent Oxidative Stress in Elderly Patients with Non-insulin Dependent Diabetes Mellitus. *Q J Med* ; 92:33-8.
- [7] Setiawan B, Suhartono E. 2005. Stres oksidatif dan peran antioksidan pada diabetes mellitus. *Majalah kedokteran Indonesia*, vol. 55 (2).
- [8] Sies H. 1997. Oxidative stress: *oxidants and antioxidants. Exp. Physiol.*, 82(2): 291–295.
- [9] Pisoschi AM, Pop A. 2015. The Role of Antioxidants in The Chemistry of Oxidative Stress: A review : *European Journal of Medicinal Chemistry* 97. 55e74. <http://www.elsevier.com/locate/ejmech>.
- [10] Ebong PW *et al.* 2014. Combined Therapy of *Moringa oleifera* and *Ocimum gratissimum* Reversed Testicular Damage in Diabetic Rats. *British Journal of Medicine & Medical Research* 4(11):2277-2290.
- [11] Shyamala MP, Venukumar MR, Latha MS. 2003. Antioxidant Potential of the *Syzygium aromaticum* L. (Cloves) in Rats Fed with High Fat Diet. *Indian Journal of Pharmacology*. 99-103.
- [12] Szkudelski T. 2012. Streptozotocin-nicotinamide induced diabetes in the rat. Characteristics of the experimental model. Department of animal physiology and biochemistry, poznan university of life sciences, wolynska 35:60-637 Poznan, poland.
- [13] Subiyono, M. Atik Martsiningsih, Denni Gabrela. 2016. Gambaran Kadar Glukosa darah Metode GOD-PAP (Glucose Oxidase – Peroxidase Aminoantypirin) Sampel Serum dan Plasma EDTA (Ethylene Diamin Terta Acetat). *Jurnal Teknologi Laboratorium*, Volume 5, No. 1
- [14] Sun *et al.* 1989. Improved superoxide dismutase assay for clinical use. *Clin Chem*, 35:1265-6
- [15] Pigeolet *et al.* 1990. Glutathione peroxidase, superoxide dismutase and catalase inactivation by peroxides and oxygen derived free radicals. *Mechanism Aging Development*. 51:283–97.

- [16] Tahara A *et al.* 2008. Hypoglycaemic Effects of Antidiabetic Drugs in Streptozotocin-Nicotinamide Induced Mildly Diabetic and Streptozotocin-Induced Severely Diabetic Rats. Nordic Pharmacological Society. *Basic & Clinical Pharmacology & Toxicology Drug Discovery Research*, Astellas Pharma Inc., Ibaraki, Japan.
- [17] Faucher, K., Rabinovitch-Chable, H., Cook-Moreau, J., Barriere, G., Sturtz, F., & Rigaud, M. 2005. Overexpression of human GPX1 modifies Bax to Bcl-2 apoptotic ratio in human endothelial cells. *Mol Cell Biochem* 277(1–2), 81–87.
- [18] Harmon, J. S. *et al.* 2009. beta-cell-specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db Mice. *Endocrinology* 150(11), 4855–4862.
- [19] DiPiro J.T., Wells B.G., Schwinghammer T.L. and DiPiro C. V., 2015, *Pharmacotherapy Handbook*, Ninth Edit., McGraw-Hill Education Companies, Ingggris.
- [20] Hardani M.F., Nurrochmad A., Widodo G.P. 2016. Antidiabetic activity of Combination ethanol extract of moringa leaf (*Moringa oleifera* Lamk) and clove flower (*Syzygium aromaticum* Merry & Perril) towards rats (*Rattus norvegicus*) induce by Streptozotocin-Nicotinamide. Inovasi dan Teknologi Pembelajaran Sains untuk Kemajuan Kualitas Pendidikan. Prosiding Seminar Nasional Pendidikan Sains. Tadulako University.
- [21] Fauziah *et al.* 2014. The Effect of Curry Leaves (*Murayya koenigii* L.) on Blood Glucose Levels of Alloxan Diabetic Mice (*Mus musculus*). *Jurnal Natural* Vol. 14, No. 1
- [22] Tachibana, Y., Kikuzaki, H., Lajis, N. H. and Nakatani, N. 2001. Anti Oxidative Activity of Carbazoles from *Murraya koenigii* Leaves. *J. Food Chem.* 49: 5589-5594.
- [23] Shih MC, Chang CM, Kang SM, and Tsai ML. 2011. Effect of Different Parts (Leaf, Stem and Stalk) and Seasons (Summer and Winter) on the Chemical Compositions and Antioxidant Activity of *Moringa oleifera*. *International Journal Molecular Sciences.* 12(9): 6077-6088.
- [24] Hardani, M. F., Hardani, R., Aanisah, N., Hamzah, B., and Rahmawati, S. 2020. The Reduction Activity of Malondialdehyde (Mda) Level in Combination of Ethanol Extracts of Moringa Leaf (*Moringa Oleifera Lamk*) and Clove Flower (*Syzygium Aromaticum Merry & Perril*). *International Journal of Advanced Science and Technology*, 29(05), 1130–1134.
- [25] Musabayane C., Tufts M., Mapanga R. 2010. Synergistic antihyperglycemic effects between plant-derived oleanolic acid and insulin in streptozotocin-induced diabetic rats. *Renal failure.* 32:832-9