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Research

Effect of Ethanolic Extract of *Moringa oleifera Lam.* Roots on Expression of Cyclin D1 in Testicular Tissue of Metabolic Syndrome Induced Wistar Rats

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ABSTRACT

Background: Metabolic syndrome (MS) impacts on inhibition process of spermatogenesis by damaging various components of cell cycle proteins. Cyclin D1 protein which plays role in regulating the cell cycle of spermatogenic cells is depleted due to the metabolic syndrome. Variation results of *M. oleifera* studies on fertility are interesting for further research. This study aimed to determine the effect of ethanolic extract of *M. oleifera* roots towards expression of testicular cyclin D1 of metabolic syndrome induced Wistar rats.
Subjects and Method: The study was an experimental laboratory conducted at Food and Nutrition Laboratory for Center for Food and Nutrition Studies (PSPG) Gadjah Mada University and Anatomical Pathology Laboratory, Medical Faculty of Universitas Sebelas Maret, Surakarta, Central Java, from August to October 2022. The independent variable was the doses of *Moringa oleifera* roots ethanolic extract. The dependent variable was the expression of cyclin D1 in Wistar rats's testes tissue. Thirty subjects of male Wistar rats were randomly divided into 5 groups. Cyclin D1 data was calculated using Intensity Distribution Score (IDS) and analyzed using one-way ANOVA test followed by Post Hoc Tukey Test. Simple liniar regression test conducted on cyclin D1 data of K2, K3, K4, and K5.
Results: The administration of ethanolic extract *M. oleifera* roots with doses of 150, 250, and 350 mg/kgBW in 28 days increased cyclin D1 significantly ($p<0.001$). There were significant differences between K1 (Mean= 251.76; SD= 17.94) and K2 (Mean=142.75; SD= 32.24) ($p<0.001$), K2 (Mean= 142.75; SD= 32.24) and K3 (Mean= 255.95; SD= 26.29) ($p<0.001$), K2 (Mean= 142.75; SD= 32.24) and K4 (Mean= 280.19; SD= 10.18) ($p<0.001$), K2 (Mean= 142.75; SD= 32.24) and K5 (Mean= 266.33; SD= 54.08) ($p<0.001$).
Conclusion: The administration of ethanolic extract *Moringa oleifera Lam.* roots with doses of 150, 250, and 350 mg/kgBW for 28 days increased cyclin D1 in testicular rats.

Keywords: *Moringa oleifera*, cyclin D1, ethanolic extract, metabolic syndrome, testes

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BACKGROUND

Metabolic syndrome considered a global epidemic because in almost all countries it is a major cause of morbidity and mortality (Leisegang et al., 2019; Saklayen, 2018). The prevalence of metabolic syndrome has been increasing and will continue to rise globally in line with the obesity epidemic

(Carrier, 2017). A meta-analysis study by Zhao and Pang (2020) stated that metabolic syndrome tends to be a risk factor for male infertility. Patients with metabolic syndrome undergo sex hormones dysregulation and oxidative stress due to the excessive accumulation of reactive oxygen species (ROS) in the testes, resulting in damage to cellular components including proteins, lipids, and mtDNA, leading to disruption of spermatogenesis, such as cell cycle arrest, proliferation inhibition and excessive apoptosis of germ cells leading to infertility at the molecular level (Carrera-Chávez et al., 2020; El-Wakf et al., 2020; Widiastini et al., 2022).

Various types of proteins are present in the cell cycle during germ cell division. Among these proteins, cyclins and their kinases play an important role as key regulators of germ cell development (Tolouei Azar et al., 2020). Cyclin D1 regulates spermatogenic cell proliferation during the G1/S phase of the cell cycle (Zamir-Nasta et al., 2018). Thus, dysregulation in cyclin D1 expression can lead to cell cycle arrest in various germ cells, resulting in impaired spermatogenesis (Shamsi-Gamchi et al., 2021).

Moringa oleifera, Lam. is one of the miracle plants, well-known for its various benefits (Muhammad et al., 2020). Moringa extract can be used as a treatment option for people with metabolic syndrome and can be used to prevent adverse effects on spermatogenesis due to metabolic syndrome (Irfan et al., 2020; Mohamed et al., 2019). The consumption of *M. oleifera* leaves extract could reduce excessive accumulation of ROS in microtestes environment and increase spermatogenesis (Alkafafy et al., 2021; Dafaalla et al, 2017; Laoung-On et al., 2021). However, another study reported administration of ethanolic extract of Moringa leaves showed damage to germ cells in the seminiferous tubules (Owolabi and Ogunnaike, 2014).

Variation results of several studies of *M. oleifera* effect on male fertility are interesting for further research. Moreover, previous studies emphasizing on effect of ethanolic extract of M. oleifera roots on cyclin D1 have not been found. The aim of this study was to determine the effect of an ethanolic extract of *Moringa oleifera, Lam.* roots with graded doses at 150, 250, 350 mg/KgBW on the expression of testicular cyclin D1 of metabolic syndrome induced Wistar rats.

SUBJECTS AND METHOD

1. Study Design

This was a randomized controlled trial. The induction of metabolic syndrome conditions and administration of ethanolic extract of M. oleifera roots were conducted at the Food and Nutrition Laboratory of the Center for Food and Nutrition Studies (PSPG) Gadjah Mada University, Yogyakarta. Meanwhile, imunohistochemistry (IHC) staining and histopathological observations of cyclin D1 expression in testes tissue were carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Sebelas Maret, Surakarta on August-October 2022 .

2. Population and Sample

Total samples of this study were Thirty Wistar rats (*Rattus norvegicus) selected by purposive sampling met* the inclusion criteria which were male Wistar rats aged 2-3 months and weighing 150-200 g. Exclusion criteria for this study were rats that showed signs of illness such as piloerection, dull, rough and oily hair, hair loss, loose skin, decreased weight after adaptation state, slightly closed eyelids, sunken eyes, chromodacryorrhea, watery and foulsmelling stools, no appetie and drink, often sleep in caged, often squeaks when touched, sneezing, pale, and wheezing in breathing. The rats were allow-

ed to acclimatize to laboratory environment for a week prior to the experiment.

3. Study Variables

The dependent variable was the expression of cyclin D1 in testes tissue of Wistar rats. The independent variable was the doses of ethanolic extract of *Moringa oleifera, Lam.* roots.

4. Operational definition of variables

The Doses of ethanolic extract of *Moringa oleifera, Lam.* roots is Moringa roots that obtained in dry conditions from Kalasan, Sleman, Yogyakarta extracted by maceration method using ethanol 70% solvent and filtered to obtain filtrate. The filtrate obtained was then evaporated with a rotary evaporator into thick ethanol extract. The thick extract was made into a suspension by CMC-Na 0,5% solvent 2ml/200gBW. Simplicia making and extraction process were carried out at the Food and Nutrition Laboratory of PSPG UGM Yogyakarta. The extract was administered orally using a gastric probe at doses of 150 mg/kgBW/day, 250 mg/kgBW/day, and 350mg/kgBW/day from the 28th to the 56th day.

The expression of cyclin D1 was cyclin D1 protein expressed in the nucleus of cells in microscopic preparations seminiferus tubule of rats testes observed with an Olympus CX 22 light microscope and digital image evaluation software, OptiLab viewer and Image Raster 3. The preparations were stained with immunostaining anti-cyclin D1 antibody and observed in nine fields of view with a magnification of 100x and 400x in each testes.

5. Study Instruments

The assessment was performed semi-quantitatively using the Intensity Distribution Score (IDS) which was the sum of the multiplication of the color intensity scores of the cells with the percentage of the number of cells that were stained positive, ranged from 0-300. The staining intensities of cells were divided into four groups (negative, weak, moderate, strong) according to the color change in the cell nucleus after immunostaining with anti-cyclin D1 antibody as follows: strong (3) for dark brown color, moderate (2) for light brown color, weak (1) for golden-yellow color and negative (0) for purplish blue.

6. Data analysis

Cyclin D1 data was expressed as Mean, SD. Statistical analysis was performed using Statistical Product and Service Solution (SPSS) 22.0 for Windows software. Data analysis was done by one way ANOVA test followed by post hoc multiple comparisons with Tukey HSD test to see the difference of cyclin D1 expression level among all groups. The effect of doses of ethanolic extract of *Moringa oleifera* roots on the expression of cyclin D1 in K2, K3, K4, and K5 group were estimated by a simple linear regression test. The values of $p<0.05$ were taken as significant.

7. Research Ethics

The research ethical clearance approval letter was obtained from the Research Ethics Committee at Dr. Moewardi Hospital, Surakarta, Indonesia, No. 1033/VIII/HREC/2022, on August 4, 2022.

RESULTS

1. Achievement of Metabolic Syndrome

Rats fed with high-fat diet and streptozotocin-nicotinamide (STZ-NA) induced (K2, K3, K4, and K5 group) showed increase in Body Weight >15%, blood glucose level to over 200mg/dL, triglyceride level to over 110mg/dL, low-density lipoprotein (LDL) level to over 75 mg/dL, total cholesterol level to over 200 mg/dL on day 28 as compared with the initial condition, while high-density lipoprotein (HDL) level was decreased to less than 25mg/dL.

2. Differences in Testicular Cyclin D1 Expression

Cyclin D1 expression was observed in the nucleus of all cells seen in the seminiferous tubules of the testes. Interpretation was assessed from the color change reaction seen in the cell nucleus after immunostaining with anti-cyclin D1 antibody, which is strong positive when it stained dark brown, moderate positive when it stained light brown, weak positive when it stained golden yellow, and negative interpretation when it stained purplish blue, as shown in table 1.

Table 1. Overview of cyclin D1 expression in seminiferus tubule with anti-cyclin D1 antibody immunostaining

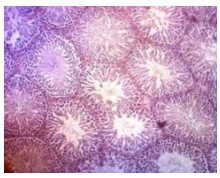
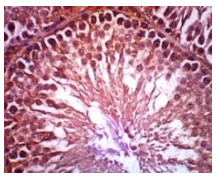
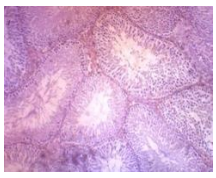
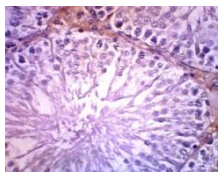
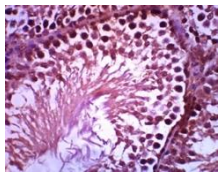
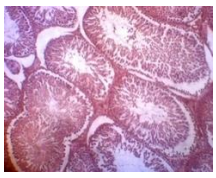
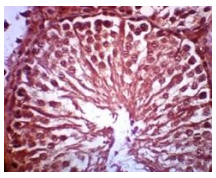
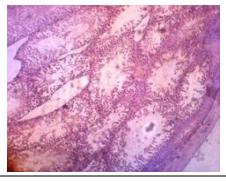
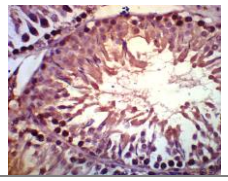
Group	100x	400x
K1		
K2		
K3		
K4		
K5		

Table 2. Mean of cyclin D1 expression in teticular tissue of Wistar rats

Group	Mean	SD
K1	251.76	17.94
K2	142.75	32.24
K3	255.95	26.29
K4	280.19	10.18
K5	266.33	54.08

Testicular tissue cyclin D1 expression in each group measured using IDS presented as mean SD, as shown in table 2. Based on these data, the expression of cyclin D1 in testicular tissue from highest to lowest sorted according to groups, namely K4, K5,

K3, K1, and K2. The mean cyclin D1 expression shown in the K3, K4, and KP5, groups treated with *Moringa oleifera* roots ethanolic extract for 28 days were higher than the MS group, K2. The mean cyclin D1 expression in control (K1) group also showed higher results compared to the MS (K2) group.

One way ANOVA test was carried out to determine whether there was a significant difference in cyclin D1 expression in each group after administration of the ethanolic extract of Moringa roots. The Shapiro-Wilk normality test showed p>0.05 in each group referred normal distribution of cyclin D1 data and qualified for the parametric test. One way ANOVA showed significant difference (p= 0.000) of testicular cyclin D1 expression between groups. Statistical testing was continued with the post hoc multiple comparisons and Tukey HSD test to determine the differences between each treatment group. The results showed a significant difference in the mean expression of cyclin D1 between K2 and K1, K3, K4, and K5 (p< 0.001), while there was no significant difference between K1, K3, K4, and K5. The result of post hoc multiple comparisons with Tukey HSD shown in table 3.

Table 3. Tukey Post Hoc Test Results

Group	Mean	SD	P
K1	251.76	17.94	<0.001
K2	142.75	32.24	
K1	251.76	17.94	0.997
K3	255.95	26.29	
K1	251.76	17.94	0.192
K4	280.19	10.18	
K1	251.76	17.94	0.775
K5	266.33	54.08	
K2	142.75	32.24	<0.001
K3	255.95	26.29	
K2	142.75	32.24	<0.001
K4	280.19	10.18	
K2	142.75	32.24	<0.001
K5	266.33	54.08	
K3	255.95	26.29	0.331
K4	280.19	10.18	
K3	255.95	26.29	0.921
K5	266.33	54.08	
K4	280.19	10.18	0.805
K5	266.33	54.08	

Simple linear regression analysis was carried out to determine the effect of doses of *Moringa oleifera* roots ethanolic extract on cyclin D1 expression level in testicular tissue of metabolic syndrome induced rats. The result of significant value of simple linear regression showed a significant effect of doses of *Moringa oleifera* roots ethanolic extract on cyclin D1 expression level in testicular tissue of metabolic syndrome induced rats, p= 0.000. The correlation between doses of *Moringa oleifera* roots ethanolic extract and cyclin D1 expression level indicated by correlation value (R)

which was 0.80. The value of R square was 0.65. In addition, the equation y= 167.82 + 0.365*x (y=a+bx) where the value of constant (a) was 167.82 and the value of the regression coefficient (b) was 0.365. The results of simple linear regression showed in Figure 1.

3. Effect of Doses of Moringa Roots Ethanolic Extract on Cyclin D1 Expression

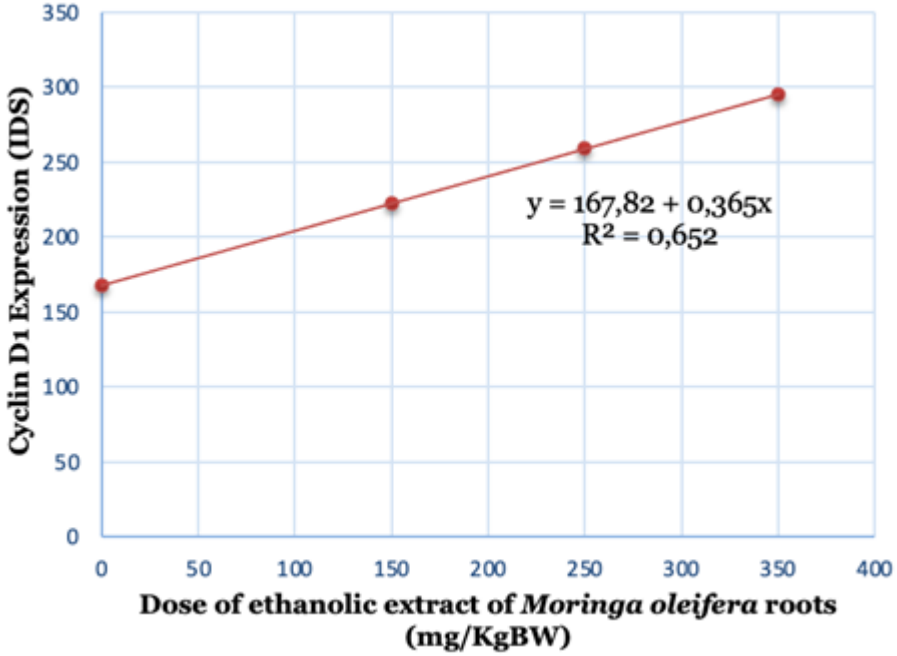


Figure 1. The relationship between the dose of *Moringa oleifera* roots ethanolic extract and cyclin D1 expression.

DISCUSSION

1. Achievement of Metabolic Syndrome

Achievement of metabolic syndrome conditions in accordance with harmonized MS definition from Joint Interim Statement (JIS) which required three out of five risk factors: central obesity, hypertension, low HDL cholesterol, elevated triglyceride, and hyperglycemia (Herningtyas and Ng, 2019). Metabolic Syndrome rats were identified with blood glucose level >200 mg/dL, total cholesterol >110 mg/dL, triglyceride >150 mg/dL, change in body weight 8% of initial weight and HDL levels <35 mg/dL. It could be inferred that rats in K2, K3, K4, and K5 group fed with high-fat diet and induced by streptozotocin-nicotinamide (STZ-NA) have achieved metabolic syndrome condition indicated by an increase in body weight >15%, blood glucose level to over 200mg/dL, triglyceride level to over 110mg/dL, low-density lipoprotein (LDL) level to over 75 mg/-dL, total cholesterol level to over 200 mg/-dL, and decrease HDL level to less than 25-mg/dL.

2. Difference of Cyclin D1 Expression beetwen K1 Group and K2 Group

The results of the post hoc multiple comparisons with Tukey-HSD test between K1 and K2 showed $p<0.001$ ($p<0.005$) which means that there was a significant difference in the cyclin D1 expression in the Wistar rat testes. The mean cyclin D1 expression of K2 was lower than K1, indicated that the administration of high-fat diet and STZ-NA in-

jection performed on K2 mice succeeded in forming a metabolic syndrome condition that significantly affected the decrease in cyclin D1 expression in cells in the seminiferous tubules of testicular tissue.

Metabolic syndrome occurred in K2 group was associated with obesity and insulin resistance. Obesity led an increase in fat tissue resulting in an increase in the secretion of adipokines, such as TNF-α, leptin, resistin, IL-1, and IL-6 which could lead to low-chronic systemic inflammation. Insulin resistance that occurs due to damage to insulin receptors, increased FFA, and inhibition of adiponectin causes an increase in gluconeogenesis resulting in hyperglycemia (Maslov et al., 2019; Samson and Garber, 2014). Increased blood glucose levels were also influenced by pancreatic cells damage which eventually experienced fatigue resulting in decrease of insulin secretion (Nagy et al., 2019).

Hyperglycemia,hyperleptinemia, and hyperlipidemia occured due to obesity led an increase in the production of free radicals and reactive oxygen species (ROS). They were strongly associated with oxidative stress due to the imbalance of antioxidant that resulted in impaired spermatogenesis because of spermatogenic cells are susceptible and sensitive to oxidative stress (Adewoyin et al., 2017).

Oxidative stress which was caused by an increase in H2O2 due to hyperglycemia that occured in the metabolic syndrome led inhibition of phosphorylation of insulin receptors and Akt, resulting in a decrease in PI3K/Akt activity, as shown in Figure 2. The inhibition of PI3K/Akt led an increase in GSK3 activity, resulting a decrease in cyclin D1 levels due to increased phosphorylation of cyclin D1 by GSK3. Upregulation of GSK3 activity led to decrease β-catenin resulting reduction of cyclin D1. Inhibition of PI3K/-Akt caused an increase in FOXO leading to decrease of cyclin D1. Inhibition of PI3K/Akt also resulted in an increase in p21 protein, which was a cdk inhibitor, thereby blocking the formation of the cyclin D1-cdk4/6 complex (Nagy et al., 2019).

3. Difference of Cyclin D1 Expression beetwen K2 Group and K3, K4, K5 Groups.

The results of the post hoc multiple comparisons with Tukey-HSD test between MS group (K2) and treatment gropus (K3, K4, K5) showed $p<0.001$ ($p<0.005$) indicated that there were significant differences in the cyclin D1 expression in the metabolic syndrome Wistar rat testes after administration of ethanolic extract *Moringa oleifera Lam.* roots. The administration of ethanolic extract *Moringa oleifera Lam.* roots caused an increase the expression of cyclin D1 in the testicular tissue of metabolic syndrome Wistar rats. The increase of cyclin D1 expression in testicular tissue of mice with metabolic syndrome showed in K3, K4, and K5 groups may have been caused by the active compounds contained in the ethanol extract of *Moringa oleifera Lam.* roots. Phytochemical compounds found in Moringa root extract include flavonoids, tannins, saponins, terpenoids, and alkaloids (Etejere et al., 2017). Phytochemical compounds contained in the roots of the Moringa plant could be used to treat oxidative stress in testicular tissue due to metabolic syndrome. Research by Abass (2015) showed the results of screening the roots of M. oleifera which contained compounds that were useful as antioxidants, such as flavonoids, tannins, alkaloids, terpenoids, and saponins.

Flavonoids were active compounds that used as antioxidants that could suppress free radicals and reactive oxygyn species (ROS) (Sulistyorini et al., 2015). One type of flavonoid was quercetin which was a strong antioxidant compound with a strength of 4-5 times stronger than vitamin C and vitamin E

as a potential antioxidant (Widiastini et al., 2022). In a study it was found that the flavonoid content in the roots had the highest flavonoid content compared to other plant parts (Abass, 2015). The same thing was also shown in the research of Tshabalala et al. (2020) where the flavonoid content in both parts of the roots of the Moringa plant was higher than in the leaves. The tannin content in the roots was also found to be higher than in the leaves. The content of tannins and flavonoids such as quercetin can cause a scavenging effect on the ROS produced in mitochondria so that it can inhibit oxidative stress.

Inhibition of oxidative stress could trigger the proliferation of type A spermatogonia resulting in an increase in the number of spermatogenic cells (Laoung-On et al., 2021). Increased cell proliferation was indicated by an increase in cyclin D1 expression so that an increase in spermatogenic cell proliferation indicated an increase in cyclin D1 expression. Thus, administration of the ethanolic extract of *Moringa oleifera* roots at a dose of 150 mg/KgBW, 250 mg/KgBW, and 350 mg/KgBW could increase the expression of cyclin D1 in testicular tissue of metabolic syndrome Wistar rats.

4. Difference of Cyclin D1 Expression beetwen K1 Group and K3, K4, K5 Groups

The results of the post hoc multiple comparisons with Tukey-HSD test between control group (K1) and treatment gropus (K3, K4, K5) showed p value, sequentially 0,997; 0,192; and 0,775 ($p > 0.05$), indicated that there were insignificant differences in the cyclin D1 expression in the Wistar rat testes between the control group (K1) and the treatment groups K3, K4, and K5. This could showed that administration of ethanolic extract of Moringa roots at a dose of 150 mg/KgBW, 250 mg/KgBW, and 350 mg/KgBW could increase cyclin D1 expression in testicular tissue of rats with metabolic syndrome to the same level as cyclin D1 expression in normal rats in the control group (K1).

The insignificant difference between the three treatment groups (K3, K4, K5) might occur because the dose range was too narrow. It could also give the possibility that the effective dose of Moringa roots ethanolic extract in increasing cyclin D1 expression in testicular tissue could be given at a dose lower than 150 mg/KgBW. In a study conducted by Laoung-On et al. (2021) showed that with doses of 0.55 mg/KgBW, 1.1 mg/KgBW, and 2.2 mg/KgBW *Moringa oleifera* leaf tea could increase the number of type A spermatogonia and efficiency of spermatogonia but there were no significant difference in the number of spermatogonia between the treatment groups. In another study it was stated that *Moringa oleifera* seed powder with low doses (50 mg/KgBW) succeeded in reducing the level of fat peroxidation and increasing the number of antioxidant enzymes in rats experiencing oxidative stress (Al-Malki and el Rabey, 2015).

5. Effect Doses of Moringa Roots Ethanolic Extract on Cyclin D1 Expression

The result of simple linear regression analysis showed $p<0.001$, referred the regression model has met the linearity criteria. Thus, the dose of ethanolic extract of *Moringa oleifera* roots had a significant effect on the expression of cyclin D1 of testicular metablic syndrome rats. The correlation value was 0.80 referred a very strong relationship between the dose of Moringa roots ethanolic extract and cyclin D1 expression in testicular tissue of metabolic syndrome rats. The value of R square (R^2) established how much the effect of the dose of Moringa roots ethanolic extract on the expression of cyclin D1 of testicular metablic syndrome rats. The value of R square was

0.65 showed that the dose of ethanolic extract of *Moringa oleifera* roots had a moderate effect (65%) on cyclin D1 expression of metabolic syndrome Wistar rats testicular tissue.

In addition, the equation y= 167.82 + 0.365*x (y=a+bx) where the value of constant (a) indicated that the expression level of cyclin D1 in rat testicular tissue is 167.82 when the dose of Moringa roots ethanolic extract was not given or 0. The value of the regression coefficient (b) was 0.365, showed that the dose of Moringa oleifera roots ethanolic extract had a positive effect on the level of cyclin D1 expression so that cyclin D1 expression level of rats testes increased by 0.365 for every 1 mg/kgBW increase in the dose of *Moringa oleifera* roots ethanolic extract.

The increased expression of cyclin D1 in the testicular tissue of Wistar rats with metabolic syndrome was associated with strong antioxidant activity in the ethanolic extract of Moringa roots. Linear regression analysis in the study of Fachriyah et al. (2020) stated that an increase in the concentration of the ethanolic extract of Moringa leaves resulted in an increase in the inhibitory activity of DPPH which was free radical compounds. The increase in DPPH scavenging ability in Moringa root extract could be influenced by the presence of condensed tannins contained in *Moringa oleifera* roots. Tannins contained hydroxyl groups so they could react as antioxidants Tshabalala et al. (2020). In addition, the phenolic and flavonoid content also used as a natural strong antioxidant so that it could inhibit LPO, AGEs, and DNA damage. In a study, it was stated that the infusion of Moringa plants had a better H2O2 scavenging ability than gallic acid (Laoung-On et al., 2021).

Histopathological picture on preparations of K5 group where rats with metabolic syndrome were given Moringa oleifera roots ethanolic extract at dose of 350mg/kgBW/day for 28 days resulted in decrease in quality of the seminiferous tubule structure. It could be seen from decreasing in diameter of seminiferus tubule and maturation of spermatogenic cells despite the high increase in cyclin D1 expression. We could be interpreted that the increase in cyclin D1 expression did not always indicate it was beneficial in fertility because in addition to proliferation it was necessary to look at other factors such as the level of maturation of spermatogenic cells.

Based on this research, the administration of ethanolic extract *Moringa oleifera Lam.* roots with doses of 150, 250, and 350 mg/kgBW for 28 days increased the expression of cyclin D1 in testicular tissue of metabolic syndrome induced Wistar rats. Moringa roots had a potential in preventing infertility due to metabolic syndrome. However, this research need further study on phytochemicals screening of ethanolic extract *Moringa oleifera Lam.* roots so that it could be determined with certainty the active compounds that played a role in influencing the expression of cyclin D1 in the seminiferous tubules of metabolic syndrome Wistar rats testes and more focused research could be carried out in the prevention of metabolic syndrome.

AUTHOR CONTRIBUTION

Sephendra Lutfi, Dyah Ratna Budiani, Novan Adi Setyawan collected and analyzed data and wrote manuscript

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CONFLICT OF INTEREST

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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