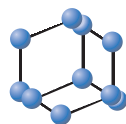
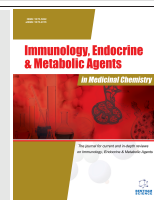


RESEARCH ARTICLE

BENTHAM
SCIENCEEffects of *Moringa oleifera* on Insulin Levels and Folliculogenesis in Polycystic Ovary Syndrome Model with Insulin Resistance

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Abstract: Background: Insulin resistance is a frequent metabolic disorder in Polycystic Ovary Syndrome (PCOS). *Moringa oleifera* has been shown to increase insulin expression and decrease the degree of insulin in diabetes mellitus, therefore it is expected that *Moringa oleifera* could decrease insulin levels and increase folliculogenesis in PCOS.

Objective: To prove the effect of *Moringa oleifera* leaf extract in various doses might decrease the insulin levels and increase folliculogenesis in female PCOS-insulin resistant rats.

Methods: The three month old white rat of Wistar strain (*Rattus norvegicus*) 100-130 grams were divided into five groups (n=8) including normal control, PCOS-insulin resistance, PCOS-insulin resistance given metformin and PCOS-resistance insulin were given *Moringa oleifera* leaf extract in two doses. Then, the PCOS model-insulin resistance by injection of testosterone propionate for 28 days. After 14 days treatment, we analysed insulin levels and folliculogenesis.

Results: The PCOS control group showed a significant increase in insulin levels compared to the normal control group. The insulin levels from group treatment with *Moringa oleifera* leaf extract of 250 mg/kgBW was significantly lower than the PCOS control group. Ovarian histology analysis found that the number and diameter of follicle of PCOS control group showed a significant decrease compared to normal control group. In addition, the treatment with metformin and leaf *Moringa oleifera* dose 250 mg/kgBW and 500 mg/kgBW showed significant increase of folliculogenesis compared to PCOS control group.

Conclusions: *Moringa oleifera* could lowering the blood insulin levels, subsequently decreasing the androgen thus allowed the increasing of folliculogenesis in PCOS.

Keywords: Polycystic ovary syndrome, *Moringa oleifera*, insulin, folliculogenesis, diabetes, testosterone.

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1. INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is an endocrine-metabolic disorder that has severe consequences for women's health that including the of infertility [1]. Polycystic ovary syndrome occurs in

women of reproductive age, which is 6-10% based on US NIH criteria and reaches as high as 15% using Rotterdam Criteria 2003 [2]. According to the NHMRC results in 2015, a PCOS prevalence of 12-21% of women of reproductive age in Australia is based on ESHRE/ Rotterdam criteria 2003.

PCOS is a disorder of heterogeneous, multifactorial, genetic and endocrine complexes [1]. PCOS based on ESHRE/Rotterdam Criteria 2003 is diagnosed if the two of following three criteria are present: polycystic ovary, oligoovulation/anovulation or clinical or biochemical evidence of hyperandrogenism [2]. The etiology and the exact pathogenesis of this disorder could not be ascertained, but in some further studies, it is agreed that this disorder is genetically influenced by the autosomal dominant mode of inheritance (mainly from the paternal origin) [3]. PCOS pathogenesis includes abnormal gonadotropin secretion, abnormal steroidogenesis, insulin resistance, p450c17 and genetic dysregulation [4]. Insulin resistance (71%) is the most common metabolic abnormality in PCOS with the incidence of metabolic syndrome (31.5%) [5]. Insulin resistance leads to hyperinsulinemia compensation, increased androgen production in the ovaries by increasing GnRH frequency and LH pulsation secretion. Insulin and IGF-I are responsible for disrupting ovulation. Insulin and IGF-1 indirectly could also increase androgen levels by decreasing SHBG production in the liver and suppressing IGFBP-1 synthesis directly, rapidly and completely in both the liver and ovaries so that IGF-I, IGF-II and testosterone levels are free increased. This increases the long-term risk of type 2 diabetes mellitus and cardiovascular disease [1]. This excessive androgen production also disrupts folliculogenesis resulting in menstrual disorders and the development of multiple ovarian cysts [6].

Metformin is the first line of PCOS obese treatment by inhibiting hepatic glucose absorption, increasing peripheral glucose uptake, reducing peripheral insulin levels, and improving GLUT-4. However, clinically, finding long-term metformin treatment results with indigestion, diarrhea, and other effects [7]. Metformin treatment might not be suitable as a long-term PCOS treatment.

Herbal therapy became one of the alternative treatments that are being developed in the wider

community, one of it is *Moringa oleifera*, a food plant originating from India which grows in every tropical and subtropical region with temperatures around 25-35°C. Food plants are considered relatively safe as they are likely to contain synergistic and/or side effect neutralizing combinations of activities [8]. *Moringa oleifera*, known to be rich in multiple medicinally active chemicals, may be a good candidate to see if it contains effect enhancing and/or side-effects neutralizing combinations. Medicinal plants are relatively rich in their contents of calcium channel blockers (CCBs) which are known to possess a wide variety of pharmacological activities. But, it remains to be seen whether *Moringa oleifera* have a direct link with the presence of calcium channel blockers [9]. Recent studies of *Moringa oleifera* have potential as antioxidants, anticancer, anti-inflammatory, antidiabetic, and as antimicrobial agents [10]. Flavonol quercetin was found with high concentrations in *Moringa oleifera* leaves [10]. The largest content in *Moringa oleifera* leaf is quercetin known as 3,3',4',5,7-pentahydroxyflavone (chemical name: 4H-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-flavone; chemical formula: C₁₅H₁₀O₇) [11]. Quercetin is one of the flavonoids possessing a strong bioactive element with the effects of free radicals, anti-inflammation, anticancer, antihyperlipidemia, and antiplatelet [12]. Recent studies suggest quercetin is also found to produce PI3K inhibin [13]. Quercetin PI3K inhibin decreases the expression of the CYP17A1 gene in theca cells that might be responsible for decreasing the activation of 17 α -hydroxylase which plays an important role in PCOS [14]. In addition, a study aimed at assessing the effect of quercetin on experimental animal PCOS models showed that quercetin significantly decreased the insulin levels, testosterone and LH levels, and also lipid profile [14]. Furthermore, Quercetin also decreases the ovarian and uterine weight and increases normal follicular and corpus luteum levels in PCOS model animals [14].

In this study, we aimed to perform a research by giving a *Moringa oleifera* which could lower the insulin levels in the blood, then be followed by decrease of androgen levels. It will improve the process of aromatization from androgen hormones into estrogens that finally will improve the folliculogenesis in experimental models of PCOS.

2. MATERIALS AND METHODS

2.1. Plant Material

Moringa oleifera or Moringa is a family of Moringaceas. This plant is widely grown in tropical climates such as Indonesia in the lowlands that height of 700 meters above sea level. The extract of *Moringa oleifera* leaf (Kelorina, Moringa Organic Indonesia, Blora, Indonesia) has been powder-shaped and all the processes done according to the standard for obtaining the *Moringa oleifera* leaf extract [15].

2.2. Animals and Experimental Protocol

The female rat of *Rattus norvegicus* strain Wistar (Biochemistry Laboratory, Faculty of Medicine, Airlangga University, Surabaya, Indonesia) was 3 months old and weighed 100-130 grams [16]. Before the study began, it gives the period of adaptation for a week, in healthy condition, in normal behavior and the results of normal vaginal swab. We excluded rat with anatomical abnormalities and in pregnant during the adaptation. All procedures described were approved by the ethics committee of the Faculty of Veterinary Medicine of Airlangga University.

2.3. Treatment Protocol and Blood Collection Time Points

The female rat *Rattus norvegicus* Wistar strains were randomly divided into five groups ($n = 8$): the normal control group was given aquades for 14 days, PCOS control group-insulin resistance received testosterone propionate injection (Testohormon, Wonderindo Pharmatama, Jakarta, Indonesia) 1 mg/100gBW, intramuscular, in the thigh for 28 days to obtain the PCOS model-insulin resistance and aquades as the treatment. While, PCOS-insulin resistance rat in metformin group received metformin (2mg/100gBW, orally) for 14 days, and the two treatment groups which used rat with PCOS-insulin resistance received treatment with oral *Moringa oleifera* leaf extract (250 mg/kgBW) and (500 mg/kgBW) for 14 days. Before and after the study period, we performed vagina swab to determine the cycle before and after the study.

2.4. Estimation of Serum Insulin Parameters

Before the animal was being sacrificed, 12 hours fasting blood was taken to analyze insulin

levels and then stored at -20°C until the analysis was performed. The serum samples were analyzed for insulin levels by using the Enzyme Linked Immunosorbent Assay/ELISA kit (Elabscience Biotechnology Inc., Wuhan, China) conducted at the laboratory of the Institute of Tropical Disease Airlangga University.

2.5. Estimation of Histological Parameters

Ovarian was removed to measure the folliculogenesis and stored in 10% formalin for fixation. After fixation, ovarian organs were cut a thickness of 0.5 cm, dehydrated and paraffin blocks were performed. Slicing with a thickness of 5-7 μm mounted on the glass object and colored by using hematoxylin-eosin. Histopathologic slides were observed under a microscope to see the number and diameter of primordial follicles, primary, secondary, tertiary, also de Graaf.

2.6. Statistical Analysis

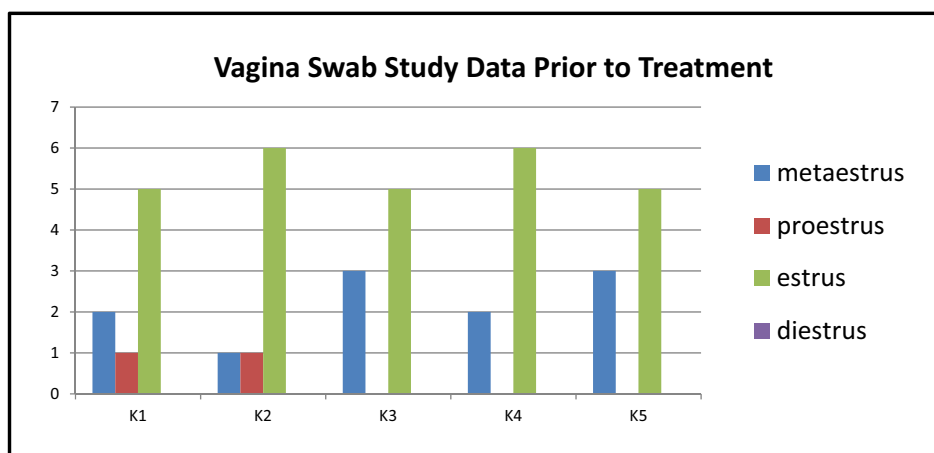
Normality test using Shapiro-Wilk test. All results were statistically analyzed using SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL). One-way factorial analysis of ANOVA variance or Kruskal-Wallis Test were performed based on the distribution data. The data were considered statistically significant at value $p < 0.05$.

3. RESULTS

We conducted vaginal swab examination before and after the treatment. Vaginal swab before treatment showed that the female *Rattus norvegicus* was in the estrous cycle, suggesting in the normal reproduction phase. Subsequently, they testosterone propionate injections for PCOS-insulin resistance models. In contrast, vaginal swab after treatment mostly on the diestrus cycle (Fig. 1).

3.1. Effect of Treatment on Physical Parameters

The PCOS model-insulin resistance in experimental animals showed a significant increase accretion of body weight compared to the normal control group ($P < 0.05$). In addition, only *Moringa oleifera* leaf extract group 500 mg/kgBW significantly decreased accretion of body weight compared to the PCOS-insulin resistance control group ($P < 0.05$) (Table 1).



Information:

K1: normal control group

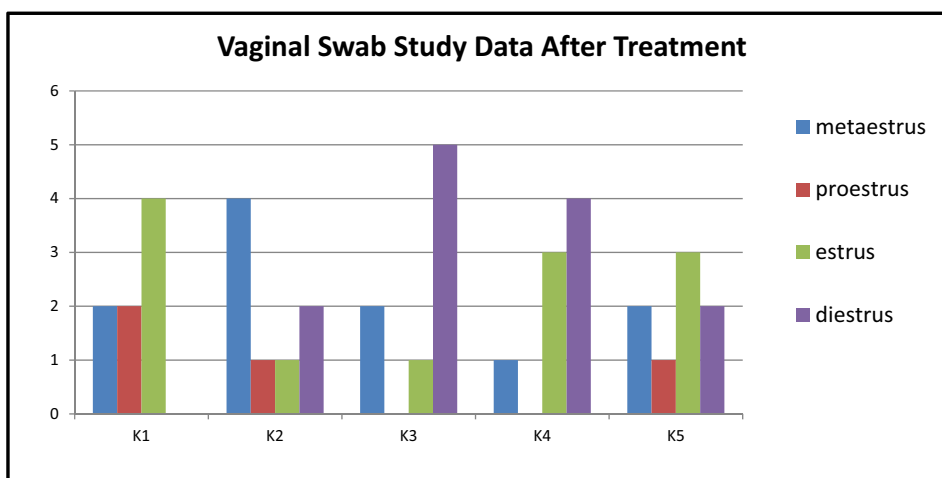
K2: PCOS control group-insulin resistance

K3: PCOS group-insulin resistance given metformin treatment

K4: PCOS group-insulin resistance was given extract of treatment *Moringa oleifera* 250mg / kgBB

K5: PCOS group-insulin resistance given extract of treatment *Moringa oleifera* 500mg / kgBB

(A)



Information:

K1: normal control group

K2: PCOS control group-insulin resistance

K3: PCOS group-insulin resistance given metformin treatment

K4: PCOS group-insulin resistance was given extract of treatment *Moringa oleifera* 250mg / kgBB

K5: PCOS group-insulin resistance given extract of treatment *Moringa oleifera* 500mg / kgBB

(B)

Fig. (1). (A) The Graphs Characteristics of vaginal swab study data prior to treatment; (B) The Graphs Characteristics of vaginal swab study data after treatment.

3.2. Effect of Treatment on Serum Insulin Parameters

The PCOS model-insulin resistance in experimental animals that was injected with testosterone propionate for 28 days showed a significant increase in insulin levels compared to the normal control group ($P < 0.05$). In contrast, there was no statistically significant of the serum insulin levels between group with metformin and *Moringa oleifera* leaf extract treatment and the normal control group. In addition, *Moringa oleifera* leaf ex-

tract group 250 mg/kgBW, 500 mg/kgBW, and metformin significantly decreased insulin-level reduction compared to the PCOS-insulin resistance control group ($P < 0.05$) (Table 2).

3.3. Effect of Treatment on Histological Parameters

Histopathological examination after induction with testosterone propionate to PCOS-insulin resistance showed the decreased of folliculogenesis compared to the normal control group based on the

Table 1. Effect of treatment on physical parameter.

-	K1	K2	K3	K4	K5
Accretion of body weight after the treatment (gram)	58.75±14.17	74±18.28 ^a	77±12.17 ^a	66.25±8.21	55.62±6.32 ^b

^asignificantly different from normal control (p <0.05)^bsignificantly different from PCOS control-insulin resistance (p <0.05)K1: normal control group; K2: PCOS insulin resistance control group; K3: PCOS insulin resistance metformin group; K4: PCOS insulin resistance *Moringa oleifera* leaf extract 250mg/KgBW group; K5: PCOS insulin resistance *Moringa oleifera* leaf extract 500mg/KgBW group.**Table 2. Effect of treatment on serum insulin parameter.**

-	K1	K2	K3	K4	K5
Insulin (ng/mL)	12.92±1.42	16.91±3.75 ^a	13.32±2.04 ^b	11.79±1.26 ^b	13.8±3.02 ^b

^asignificantly different from normal control (p <0.05)^bsignificantly different from PCOS control-insulin resistance (p <0.05)K1: normal control group; K2: PCOS insulin resistance control group; K3: PCOS insulin resistance metformin group; K4: PCOS insulin resistance *Moringa oleifera* leaf extract 250mg/KgBW group; K5: PCOS insulin resistance *Moringa oleifera* leaf extract 500mg/KgBW group.

number ($P < 0.05$) and diameter ($P < 0.05$) of the de Graaf follicles. In addition, an overview of the histology of ovarian follicles of the PCOS control group-insulin resistance (Fig. 2B) showed that number follicular atresia was higher compared to the normal control group still has the de Graaf follicles (Fig. 2A). Thus, the treatment with metformin and extract of *Moringa oleifera* leaf showed a significantly increased folliculogenesis in both number and diameter of the de Graaf follicles in PCOS-insulin-sensitive female rat compared to the PCOS-insulin resistance control group (Table 3). The treatment with metformin and extract of *Moringa oleifera* leaf did not show significant differences in folliculogenesis with normal control groups. This could be seen from the ovarian histologic features of the ovaries in the treatment of metformin that indicating there were primary, secondary, and tertiary follicles (Fig. 2C) but, the treatment with *Moringa oleifera* at a dose of 250mg/kgBW and 500mg/kgBW provides better histologic features indicated by many primary follicles, secondary, tertiary, and de Graaf (Figs. 2D and 2E).

4. DISCUSSION

In our study, the comparisons between normal control group and the PCOS-control group of the body weight was shown a significant difference. The PCOS-control group has a significant increase body weight (p <0.05) compared to normal control

group which may be due to deposition of fat in the abdominal region and insulin resistance. Treatment of *Moringa oleifera* leaf extract dose 250 mg/kgBW and 500 mg/kgBW produced decrease in body weight, but only *Moringa oleifera* leaf extract dose 500 mg/kgBW that showed a significantly decreased body weight compared to PCOS-control group. The body weight decrease because of the quercetin in *Moringa oleifera* has been reported to be useful in redistribution and uptake of fat and reported to be used in obesity [17]. Meanwhile, treatment with metformin has a significant increase body weight (p <0.05) compared to normal control group. Metformin is the first line of PCOS obese treatment by inhibiting hepatic glucose absorption, increasing peripheral glucose uptake, reducing peripheral insulin levels, and improving GLUT-4 [7]. Metformin has an effect on the endothelium and adipose tissue independent of its action on insulin and glucose levels [18]. Based on the evidence, metformin does not help to body weight loss although it may be useful in redistributing adipose tissue [19].

We determined an insulin resistance condition in the PCOS model. Then, the comparisons between normal and the PCOS-control group of insulin resistance was showed a significant difference. The PCOS model-insulin resistance in female rats injected with 1 mg/100gBW intra-muscular testosterone for 28 days increased the insulin levels as evidenced by a significant increase in insulin lev-

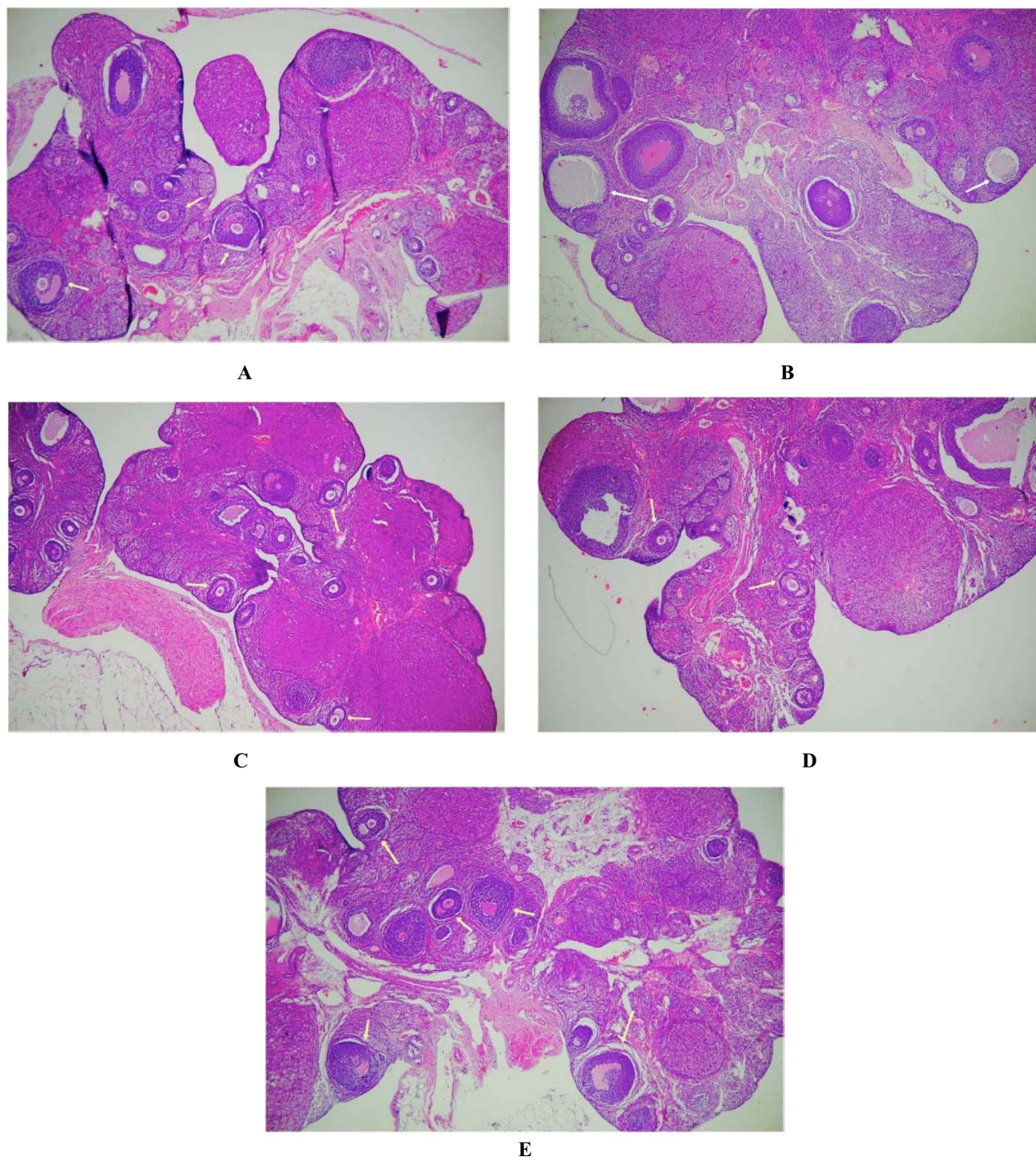


Fig. (2). The Histology of ovarian follicles: (A) normal control group; (B) PCOS control group-insulin resistance; (C) PCOS group-insulin resistance given metformin treatment; (D) PCOS group-insulin resistance was given extract of treatment *Moringa oleifera* 250 mg/kgBW; (E) PCOS group-insulin resistance given extract of treatment *Moringa oleifera* 500 mg/kgBW.

els ($p < 0.05$) in the PCOS-control group of insulin resistance compared with normal control group.

Additionally, the androgens directly inhibit the action of insulin in the periphery, in the liver, and androgen that indirectly also affects the insulin

sensitivity by altering body composition through the fat metabolism [20]. Androgens (testosterone) cause the insulin resistance by decreasing the amount and effectiveness of glucose transport proteins, especially GLUT-4 which was the responsi-

Table 3. Effect of treatment on histological parameters.

-	K1	K2	K3	K4	K5
Number Follicles	3.37±1.68	0.00±0.00 ^a	3.25±1.49 ^b	4.5±1.6 ^b	3.75±1.49 ^b
Diameter follicles	61.44±3.9	0.00±0.00 ^a	52.97±10.86 ^b	67.1±12.0 ^b	56.22±6.26 ^b

^asignificantly different from normal control (p <0.05)^bsignificantly different from PCOS control-insulin resistance (p <0.05)K1: normal control group; K2: PCOS insulin resistance control group; K3: PCOS insulin resistance metformin group; K4: PCOS insulin resistance *Moringa oleifera* leaf extract 250mg/KgBW group; K5: PCOS insulin resistance *Moringa oleifera* leaf extract 500mg/KgBW group.

ble for the transport of glucose in muscle and fat. Testosterone facilitates lipolysis and breakdown of abdominal fat, leading to an increase in free fatty acids. Increased androgens and free fatty acids inhibit the excretion of insulin in the liver and transport of glucose in the muscle, eventually leading to hyperinsulinemia and insulin resistance [21, 22].

Furthermore, the *Moringa oleifera* leaf extract could lower the insulin levels in PCOS models with insulin resistance. The normal control group of *Moringa oleifera* leaf extract's dose was 250mg/kgBW and 500mg/kgBW and metformin group for 14 days in female rat of PCOS-insulin resistance model that showed no significant difference. This suggests that *Moringa oleifera* and metformin were equally good at lowering insulin levels in the normal control group. *Moringa oleifera* leaf extract group of 250 mg/kgBW and 500 mg/kgBW and metformin group showed significantly lower insulin levels than the PCOS control group-insulin resistance. This suggests that administration of *Moringa oleifera* leaf extract and metformin decrease the insulin levels in the female rat of PCOS-insulin resistance.

Flavonol quercetin was found with high concentrations in *Moringa oleifera* leaves [10]. Quercetin was one of the flavonoids possessing a strong bioactive element with the effects of free radicals, anti-inflammation, anticancer, antihyperlipidemic, and antiplatelet [12]. Recent studies suggest quercetin was also found to produce PI3K inhibitor [13]. Quercetin has the potential to directly influence the target pathway of steroidogenesis in ovarian through cells of the PI3K inhibitor, decreasing the expression of CYP17A1 gene in theca cells responsible for decreasing the activation of 17 α -hydroxylase. This decrease of 17 α -hydroxylase activation plays a role in the synthesis of steroids by decreasing the conversion of progesterone to

androgens thereby decreasing androgen levels [1]. Additionally, through the PI3K pathway, it could phosphorylate the Akt protein so that when activated Akt protein plays an important role in the GLUT4 and FOXO1 molecules [23].

Glucose transporter type 4 (GLUT-4) was a transport protein for glucose that aims to bring glucose into cells. The GLUT-4 translocation process to the target cell surface begins with insulin bonding and the α subunit insulin receptor wherein the bond causes the β subunit of the insulin receptor and the other subunit IRS-1 to autophosphorylation. IRS-1 further activates the PI3K mediating GLUT-4 translocation to the surface of the target cell [14]. Increased translocation of GLUT-4 causes uptake of glucose from extra cells into intra-cell also increases. Increased glucose uptake improves insulin resistance conditions.

PCOS model-insulin resistance performed in female rats has occurred insulin resistance by decreasing folliculogenesis. The decreased of folliculogenesis in female rats as evidenced by the number and diameter of de Graaf follicles in the normal control group compared the PCOS-insulin resistance group show that a significant difference (p <0.05).

Insulin resistance leads to hyperinsulinemia compensation then increased androgen production in the ovaries by increasing GnRH frequency and LH pulsation secretion [1]. Decreased steroidogenesis in ovarian tissue through insulin and LH work directly increases intracellular cAMP concentrations by activating StAR which has the potential for direct action of steroidogenesis by decreasing PI3K path activity. Insulin and LH also work directly to increase transcription of LDL-C receptors in ovarian granulosa cells through increased activity on PKA and MAPK pathways and decreased PI3K. In addition, insulin also improves the regulation of aromatization in ovarian granu-

losa cells resulting in increased androgen production in the ovarian cell [1]. This hyperandrogenic state results in the internal environment of the follicle being so dominant androgen that it could not develop and become atresia [24].

Giving *Moringa oleifera* leaf extract could increase folliculogenesis in ovary model of PCOS with insulin resistance. Normal control group compared treatment group extract of *Moringa oleifera* leaf dose 250 mg/kgBW and 500 mg/kgBW and metformin group show that no significant difference. This suggests that *Moringa oleifera* and metformin might increase the near normal folliculogenesis after being given a PCOS model-insulin resistance comparable to the normal control group. Treatment of *Moringa oleifera* leaf extract dose 250 mg/kgBW and 500 mg/kgBW and metformin group significantly increased folliculogenesis compared to PCOS-insulin resistance control group. This suggests that *Moringa oleifera* and metformin were equally good at increasing folliculogenesis in female PCOS-insulin-resistance rats.

Decreased insulin levels in peripheral tissue will lead to decrease androgen production in the ovaries. Decreased insulin levels and IGF-1 indirectly also could decrease androgen levels by increasing SHBG production in the liver and increase the synthesis of IGFBP-1 directly, quickly, and complete both in the liver also ovaries so that the levels of IGF-I, IGF-II, and free testosterone decreases [1]. This androgen decline will affect the environment of the ovaries, androgen aromatization system disorders into estrogen that triggers the absence of early follicular infestation. The decrease in insulin resistance decreases the frequency of GnRH and LH pulsation secretion resulting in an increase in folliculogenesis (early atresia does not occur).

In conclusion, *Moringa oleifera* could reduce the blood insulin levels, subsequently decreasing the androgen thus allowed the increasing of folliculogenesis in the model of female rat PCOS-insulin resistance.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures described were approved by the ethics committee of the Faculty of Veterinary Medicine of Airlangga University.

HUMAN AND ANIMAL RIGHTS

All reported experiments were in accordance with the standards set forth in the 8th Edition of Guide for the Care and used of Laboratory Animals, published by the National Academy of Sciences.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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