Effects of *Moringa oleifera* Leaf Extract to Risk of Endometrial Hyperplasia in Polycystic Ovary Syndrome Model with Insulin Resistance

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Abstract

Polycystic Ovary Syndrome (PCOS) have an increased risk of a number of gynecological neoplasms including endometrial cancer. It is expected that *Moringa oleifera* can decrease the expression of IGF-1, androgen receptor expression and endometrial thickness. We aimed to prove the effect of *Moringa oleifera* leaf extract in various doses to IGF-1 expression, expression of androgen receptor and endometrial thickness in PCOS-insulin resistance model.

We used 40 female *Rattus norvegicus* of Wistar strains weighing 100-130 grams aged 3 months as samples, were divided into 5 groups including normal control group, PCOS control group, PCOS group with Metformin treatment and PCOS were given *Moringa oleifera* of leaf extract in two doses.

Moringa oleifera leaf extract have significantly decreased IGF-1 expression (*P*=.000). *Moringa oleifera* leaf extract have shown significantly decreased the expression of androgen receptors (*P*=.000). *Moringa oleifera* leaf extract has decreased the thickness of endometrium significantly (*P*=.000).

Moringa oleifera of leaf extract could decrease the expression of IGF-1 and expression of androgen receptors so that it could also decrease the thickness of endometrium in PCOS-Insulin Resistence model.

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Introduction

Polycystic Ovarium Syndrome (PCOS) is a frequent problem of reproductive endocrinology that remains controversial. Clinical symptoms vary but usually include oligo-ovulation or anovulation, hyperandrogenism (both clinical and biochemical) and the presence of polycystic ovaries.[1] Based on current PCOS criteria, about 4-6% of women in reproductive years suffer from PCOS and 75% of the population of infertility women with anovulatory case is caused by PCOS. A study conducted in Surabaya found that prevalence of PCOS in women of reproductive age was 4.5%. The prevalence trend of PCOS was increased by 6-18%. This was due to the role of insulin resistance in the pathophysiology of PCOS emergence additionally, an increase in insulin resistance was affected by the lifestyle of people who diet high in calories with the sedentary lifestyle.

Women with PCOS have an increased risk of a number of gynecological neoplasms including endometrial, breast and ovarian cancers. However, it is quite difficult to explore the relationship between the two because cancer remains a major postmenopausal women's

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disease and often occurs long after PCOS disappears, only a small proportion of pre-menopausal women develop cancer along with a PCOS diagnosis.[2]

The hormone estrogen is a mitogen in the endometrium that causes the proliferation of glands and stroma. Estrogen also increases the expression of Insulin-Like Growth Factor-1 (IGF-1), which also affects endometrial proliferation. Progesterone acts as an antimitogenic, anti-proliferate and causes endometrial differentiation. Progesterone also increases the expression of Insulin-Like Growth Factor Binding Protein-1 (IGFBP-1) that inhibits IGF-1 activity. Other researchers extend this theory by suggesting hyper androgen increases the potential neoplastic changes in the endometrium through direct androgenous effects on the endometrium and through effects on levels of Sex Hormone Binding Globulin (SHBG), circulation IGF-1 and estrogen.[2]

Herbal therapy became one of the alternative treatment issues that are growing very rapidly in the community. Researchers in various countries seem to be competing to report the results of his research that corroborates Drumstick Tree as a magical plant. In addition to using traditional medicine, there is an evidentially based PCOS treatment using Metformin. Metformin is the first line treatment of PCOS central obesity by inhibiting hepatic glucose absorption, increasing peripheral glucose uptake, reducing peripheral insulin levels and increasing GLUT-4.[3] Clinical researchers found the results of long-term metformin treatment with indigestion, diarrhea and other effects.[4] There are no studies that prove leaf extract and metformin can decrease the expression of IGF-1, the expression of the androgen receptor and endometrial thickness on the model of PCOS with insulin resistance.

The purpose of this study was to prove the effect of *Moringa oleifera* leaf extract in various doses on IGF-1 expression, expression of androgen receptor and endometrial thickness in PCOS model with insulin resistance.

Materials and methods

Plant material. *Moringa oleifera* leaf extract (Kelorina, PT Moringa Organik Blora Central Java Indonesia), which has been powdered, all the process was done in accordance with the standard to obtain leaf extract of *Moringa oleifera.*[10-13]

Animals and experimental protocol. Female Rattus norvegicus strains of Wistar strains (Laboratory of Biochemistry, Faculty of Medicine, Universitas Airlangga) aged 3 months weighing 100-150 grams was used as samples. Prior to the study, adaptation for 1 week was done, healthy conditions with activity, normal behavior and results of normal vaginal swab. The rat exclusion criteria during adaptation were a visible anatomical abnormality and pregnant. Maintenance of mice was done in plastic cage size 50x30 cm with wood husk pads are changed every 4 days, feeding standard and libitum drinks. The cage was then placed in a natural ventilated room and natural lighting conditions (Day & Night) in Biochemistry, Faculty of Medicine, Universitas Airlangga.[14,15]

Treatment protocol and blood collection time points. White rat Wistar strain (Rattus norvegicus) was divided randomly into five groups (n=8): normal control group was given aquades for 14 days, PCOS control group with insulin resistance received testosterone propionate injection (Testohormon, PT Wonderindo Pharmatama, Jakarta, Indonesia) of 1 mg/100gBB,i.m., on the thigh for 28 days to obtain PCOS model with insulin resistance and aquades as the therapy. The metformin group received a PCOS model with insulin resistance and received metformin (2 mg/100gBB, po) for 14 days, and two treatment groups received PCOS models with insulin resistance and therapy with Moringa oleifera leaf extract (250 mg/kgBB, po) and (500 mg/kgBB, po) for 14 days. Before and after the study period, vaginal swabs were performed on animals trying to find out what cycles were going on. Before the animals were sacrificed, they were administered for 12 hours and then the endometrial organ was taken for examination of endometrial thickness, IGF-1 expression, and expression of androgen receptors.

Preparation of preparats on each parameter. Preparation of histologic preparats was using paraffin method with hematoxylin-eosin staining. The preparation of uterine histological preparats with HE staining included the fixation stage, dehydration stage, cleaning, infiltration, embedding, slicing and staining to determine changes in uterine tissue structure and calculating the thickness of the endometrium.

After the organs in the paraffin blocks were cut and placed on the glass object, dephinorization and rehydration were performed, then they were washed in PBS with pH of 7.4 which followed by incubation. Researchers diluted Primary Antibodies (IGF-1 and Androgen Receptors) in FBS/BSA to desired concentration and volume (IGF-1 BS-0014R, 1: 100 antibodies in FBS, androgen receptor antibody Ser 791, 1: 100 in FBS. Monoclonal Rabbit). Then we diluted the secondary antibody labeled biotin in PBS to the desired concentration and volume. (Anti Rabbit IgG labeled Biotin, SIGMA 1: 500 in PBS). After the steps were done, each slide was labeled and was dropped the medium mounting (Entellan) onto the preparation then it was provided with a mounting medium to calculate the amount of IGF-1 expression and the amount of expression of the androgen receptor in endometrial tissue.

Statistical anayisis. The Shapiro-wilk test was used to test data normality. Statistical test using One Way ANOVA/Kruskal-Wallis depends on the distribution results. The data were considered statistically significant at *P* value <0.05.

Results

Characteristics of experimental animals

The experimental animals in this study were three months old female rat of *Rattus norvegicus* that obtained and treated in the Biochemistry laboratory of the Faculty of Medicine, Airlangga University, Surabaya. The mean initial weight of female rat was 112-122 grams and the final weight of female rat was 174-196 grams (Table 1). Before a 1 mg/100 gramBW testohormon was administered, a vaginal swab was performed on female rat to determine the estrous phase of female rat in each group (Figure 1). After the study, vaginal swab were performed and the results showed that PCOS control group was in the metaestrus phase, metformin group was in the diestrus phase, the leaf extract group of *Moringa oleifera* 250 mg/kgBW was in the diestrus phase, and the leaf extract group Moringa oleifera 500 mg/kgBW is in the estrus phase.

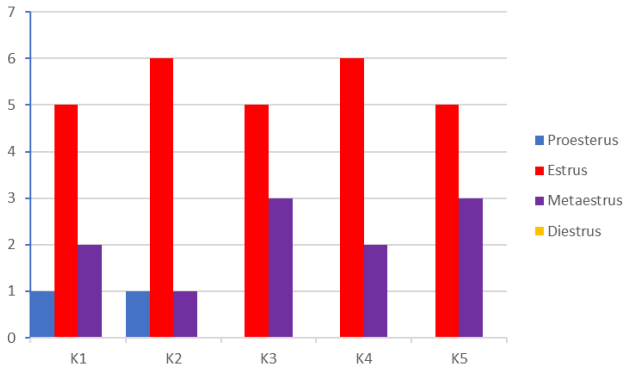

Figure 1. The characteristics of Experimental Animal based on Swab results before giving testohormon. (K1) Normal control group, (K2) Group of PCOS, (K3) Metformin group, (K4) Group of *Moringa oleifera* leaf extract 250 mg/KgBB, (K5) Group of *Moringa oleifera* leaf extract 500 mg/KgBB.

Weight	K1	K2	K3	K4	K5
Initial weight	11.50	121.88	118.63	112.75	119.13
Final weight	174.25	195.88	195.63	179.00	174.75

Table 1. The Characteristics of Experimental Animals Based on Weight. 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 leaf extract treatment of *Moringa oleifera* 250 mg/kgBB; K5: PCOS group-insulin resistance was given leaf extract treatment of *Moringa oleifera* 500 mg/kgBB

IGF-1 Expression in the Female Rat Endometrium of the PCOS Model with Insulin Resistance

Measurements of the amount of IGF-1 Expression using Immunohistochemistry on endometrial tissue (Figure 3) were group of metformin and *Moringa oleifera* leaf extract of 250 mg/kgBW decreased IGF-1 expression significantly (*P*=.000) compared to PCOS control group (Table 2).

Sample	Group				
	K1	K2	K3	K4	K5
Ekspression of IGF-1	13.50± 1.41	14.00± 1.19	8.63± 1.59*	4.13± 1.55*	13.00± 1.60

Table 2. Frequency Distribution Amount of IGF-1 Expression (%) on PCOS Model with Insulin Resistance. Significantly different from PCOS control (P<0.05). 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 leaf extract treatment of *Moringa oleifera* 250 mg/kgBB; K5: PCOS group-insulin resistance was given leaf extract treatment of *Moringa oleifera* 500 mg/kgBB.

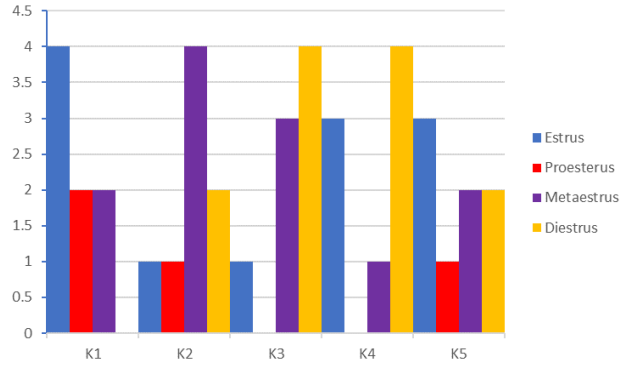

Figure 2. The characteristics of Experimental Animal based on Swab results before euthanasia is performed. (K1) Normal control group, (K2) Group of PCOS, (K3) Metformin group, (K4) Group of *Moringa oleifera* leaf extract 250 mg/KgBB, (K5) Group of *Moringa oleifera* leaf extract 500 mg/KgBB.

Expression of androgen receptors in the female rat endometrium of the PCOS model with insulin resistance

The measurement result of the amount of androgen receptor expression using *Immunohistochemical* (IHC) examination on endometrial tissue (Figure 4) was that PCOS control group significantly increased expression of androgen receptor (P=.000) compared to normal control group. Group of metformin and Moringa oleifera 250 mg/kgBW leaf extract showed significantly decreased expression of androgen receptors (P=.000) compared with PCOS control group (Table 3).

Sample	Group				
	K1	K2	K3	K4	K5
Expression of Androgen Receptor	14.13± 1.13	18.88± 1.81#	13.88± 2.42*	7.13± 1.46*	16.25± 2.87

Table 3. Frequency Distribution Amount of Androgen Receptor Expression (%) on PCOS Model with Insulin Resistance. #Significantly different from normal control (P<0.05). *Significantly different from PCOS control (P<0.05). 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 leaf extract treatment of *Moringa oleifera* 250 mg/kgBB; K5: PCOS group-insulin resistance was given leaf extract treatment of *Moringa oleifera* 500 mg/kgBB.

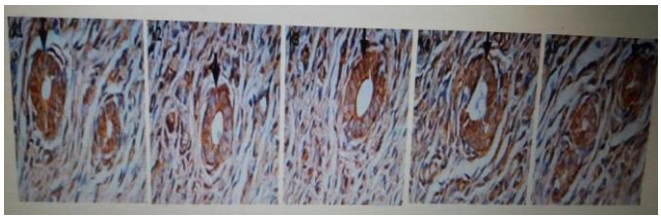

Figure 3. Immunohistochemical results of IGF-1 expression. (K1) Normal control group, (K2) Group of PCOS, (K3) Metformin group, (K4) Group of *Moringa oleifera* leaf extract 250 mg/KgBB, (K5) Group of *Moringa oleifera* leaf extract 500 mg/KgBB.

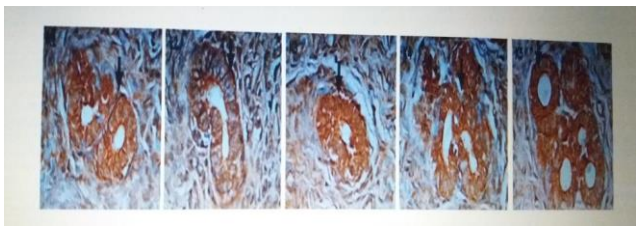

Figure 4. Immunohistochemical results of Androgen Receptor expression. (K1) Normal control group, (K2) Group of PCOS, (K3) Metformin group, (K4) Group of *Moringa oleifera* leaf extract 250 mg/KgBB, (K5) Group of *Moringa oleifera* leaf extract 500 mg/KgBB.

The thickness of the endometrium of the PCOS model with insulin resistance

Measurements of endometrial thickness using HE (*hematoxylin-eosin*) examination on endometrial tissue (Figure 5), Moringa oleifera leaf extract group of 250 mg/kgBW significantly decreased the endometrium thickness (P=0.000) compared to PCOS control group (Table 4).

Sample	Group				
	K1	K2	K3	K4	K5
Endometrium Thickness	36.34± 1.76	32.67± 5.26	26.21± 2.22	21.90± 1.39*	28. 09± 5.07

Table 4. Distribution of Endometrium Thickness Frequency (µm) in PCOS Model with Insulin Resistance. *Significantly different from PCOS control-insulin resistance (*P*<0.05). 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 leaf extract treatment of *Moringa oleifera* 250 mg/kgBB; K5: PCOS group-insulin resistance was given leaf extract treatment of *Moringa oleifera* 500 mg/kgBB.

Figure 5. HE results of endometrial thickness measured at 400x magnification of the microscope and calculated using a micrometer scale. (K1) Normal control group, (K2) Group of PCOS, (K3) Metformin group, (K4) Group of *Moringa oleifera* leaf extract 250 mg/KgBB, (K5) Group of *Moringa oleifera* leaf extract 500 mg/KgBB.

Discussion

Moringa oleifera leaf extract can decrease the expression of IGF-1 in endometrium PCOS model with insulin resistance. The results of the study were the extract of *Moringa oleifera* leaf extract could decrease expression of androgen receptor on PCOS model endometrium with insulin resistance, PCOS control group had significantly increased androgen receptor compared to normal control group. This suggests that the PCOS control group undergoes hyper androgenic. The incidence of hyper androgenic is followed by increasing androgen receptor in the tissues. According to Yongli's study of 17 patients with PCOS and 20 controls, there was an increase in serum testosterone levels, increased expression of androgen receptor and a significant increase in insulin resistance index in the PCOS group compared to the control group and there was a positive relationship between them.[5]

The PCOS control group with the normal control group did not show any significant difference, but the PCOS control group's average outcome improved slightly compared to the normal control group. This is because the presence of hyperandrogen can lead to increased IGF-1 expression in the endometrium. The longer the exposure to androgen resulted in the expression of IGF-1 increased in the endometrium. In this study, androgen exposure was performed for 28 days, so that endometrial target organ which is a long-term complication of PCOS has not been obtained. Moringa leaf extract has many very beneficial ingredients for health, namely vitamins, minerals, amino acids and other antioxidant compounds, especially quercetin, which in some studies has the benefit of reducing IGF-1 directly so that it can inhibit the process of proliferation in the endometrium. This is in accordance with the positive effects of curettin that can interact with IGF-1 signaling so as to inhibit the proliferation of tissue.[6]

The metformin group and the group of *Moringa oleifera* 250 mg/kgBB leaf extract significantly decreased the expression of the androgen receptor compared to the PCOS control group. *Moringa oleifera* leaf extract can decrease expression of androgen receptors in endometrial tissue. In the leaf extract contains polyphenols quercetin which can reduce androgen hormone levels through the path PI3K/akt.

Moringa oleifera leaf extract can decrease the endometrial thickness of PCOS model with insulin resistance. The PCOS control group did not show significant differences compared to the normal control group, but based on the average results there was a slight decrease in endometrial thickness in the PCOS control group compared to the normal control group. It happened because the relationship between endometrial cancer and PCOS is caused by chronic stimulation of endometrial growth due to unopposed estrogen conditions. Although PCOS is the most common cause of anovulation, and anovulation is the most common cause of endometrial hyperplasia, it does not mean that all women with PCOS have a high risk of endometrial malignancies, because the prevalence of endometrial carcinoma in women with PCOS is low.[7] Provision of testohormon for 28 days has not revealed any unopposed estrogen condition caused by the occurrence of

anovulation in the PCOS model in accordance with the results of the study in the PCOS group experienced decreased endometrial thickness. One of the long-term effects of PCOS is endometrial hyperplasia arising from prolonged exposure to estrogen without progesterone support, the duration of this estrogen exposure may be due to hyper androgen.

Long-term unopposed estrogen stimulation of the endometrium will cause patients to have a high risk of endometrial malignancy. The hormone estrogen is a mitogen in the endometrium that causes the proliferation of glands and stroma. Estrogen also increases the expression of Insulin-Like Growth Factor-1 (IGF-1) which affects endometrial proliferation. Progesterone acts as an antimitogenic, antiproliferation, and causes endometrial differentiation. Progesterone also increases the expression of Insulin-Like Growth Factor Binding Protein-1 (IGFBP-1) that inhibits IGF-1 activity. This study did not show the state of unopposed estrogen so it does not appear endometrial hyperplasia.

The group of Moringa oleifera leaf extract 250mg/kgBB showed a significant decrease in endometrium thickness compared with PCOS control group. Moringa oleifera leaf extract containing some minerals, vitamins, proteins and antioxidants such as polyphenols. One of the polyphenols is quercetin which can directly inhibit the work of IGF-1 so that it affects the proliferation of tissue, such as the endometrial tissue. Quersetin can also directly affect the proliferation of tissue. The results showed that leaf extract of Moringa oleifera 500mg/KgBB could decrease expression of IGF-1, expression of Androgen receptor and endometrial thickness compared to PCOS control group, but did not show a significant difference. This may be due to the prooxidant effect of methanol from Moringa oleifera leaf extract 500mg/KgBB which can cause oxidative stress. Other extracts of Moringa oleifera leaf extracts such as quercetin also have a pro-oxidant effect on high-dose administration by producing ROS through auto oxidants and cyclic redox.[8] Pro-oxidant effects are not always dangerous because the formation of ROS may be selective against cancer cells, so it will produce antioxidants to help the body's defence system.[9]

Conclusions

Moringa oleifera leaf extract could decrease the expression of IGF-1 and expression of androgen receptor in the endometrial of PCOS model with insulin resistance, so that endometrial thickness in PCOS model decreased with significant dose in *Moringa oleifera* leaf extract 250 mg/KgBW.

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Declaration of Interest

The authors declared no conflict of interest.

References

1. Fauser BC, Tarlatzis BC, Rebar RW, et al. (PCOS): the Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group. Fertility and sterility. 2012;97:28-38.
2. Balen AH, Conway G, Homburg R, et al. Polycystic ovary syndrome: a guide to clinical management. Taylor & Francis, 2005.
3. Oróstica L, Rosas C, Plaza-Parrochia F, et al. Altered Steroid Metabolism and Insulin Signaling in PCOS Endometria: Impact in Tissue Function. Curr Pharm Des. 2016;22:5614-24.
4. Sun J, Yuan Y, Cai R, et al. An investigation into the therapeutic effects of statins with metformin on polycystic ovary syndrome: a meta-analysis of randomised controlled trials. BMJ open. 2015; 5: e007280.
5. Yongli C, Yongyu S, Hongyu Q. Study of androgen and androgen receptor in relation to insulin resistance in polycystic ovary syndrome. J Huazhong Univ Sci Technol [Medical Sciences]. 2003;23:52-4.
6. Proshkina E, Lashmanova E, Dobrovolskaya E, et al. Geroprotective and radioprotective activity of quercetin,(-)-epicatechin, and ibuprofen in Drosophila melanogaster. Frontiers in pharmacology. 2016;7:505.
7. Barry JA, Azizia MM, Hardiman PJ. Risk of endometrial, ovarian and breast cancer in woman with polycystic ovary syndrome: a systematic review and meta-analysis. Human Reproduction Update. 2014;20:748-58.
8. Chang YF, Chi CW, Wang JJ. Reactive oxygen species production is involved in quercetin-induced apoptosis in human hepatoma cells. Nutrition and cancer. 2006;55:201-9.
9. Touriño S, Lizárraga D, Carreras A, et al. Antioxidant/prooxidant effects of bioactive polyphenolics. Electron J Environ Agric Food Chem. 2008;7:3348-52.
10. Shreelatha S, Jeyachitra A, Padma PR. Anti proliferation and induction of apoptosis by moringa oleifera leaf extract on human cancer cell. food and chemical toxicology. J Br Industrial Biol Res Assoc. 2011;49:1270-5.
11. Ichsan DK, Dewangga GRS and Yaqin RL. Utilization of Moringa Leaf Extract (Moringa oleifera) as Green Material Pipeline Corrosion Inhibitor in API 5L grade B in Sea Water Environment. Surabaya. 2016.
12. Tourkey MJ. Effects of Moringa oleifera aqueous leaf extract in alloxan induced diabetic mice. Interv Med Appl Sci. 2016;8:109-17.
13. Omodanisi EI, Aboua YG, Pguntibeju OO. Assessment of the Anti-hyperglycaemic, anti-inflammatory and antioxidant activities of the methanol extract of moringa oleifera in diabetes-induced nephrotoxic male wistar rats. Molecules. 2017;22:e439.

14. Laswati H, Hendarto H, Irawati D, et al. Tomato juice increase bone density of menopause rats. Jurnal Veteriner Jurnal Kedokteran Hewan Indonesia. 2015;16:457-62.
15. Nur NJ, Bambang W and Merryana A. Combined food (Bekatul dan Lemak) menurunkan kadar kolesterol total, trigliserida, dan LDL pada tikus galur wistar. Jurnal Kedokteran Brawijaya. 2015;28:208-12.