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# *In vivo* Evaluation of Extracted and Fraction of *Moringa oleifera* leaves against Testosterone-Induced PCOS Model in Rattus Norvegicus

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***Abstract***

***Background****: Polycystic ovary syndrome (PCOS) is an endocrine disorder. Parameters characteristic of the disorder include weight gain, insulin resistance and malondialdehyde (MDA). The traditional medicine used is Moringa oleifera.* ***Objectives****: The aim was to determine the effect of weight loss and blood glucose levels, MDA levels and histopathological features of the ovarian follicles in the rat model PCOS-insulin resistance induced by testosterone.* ***Methods****: Extraction process, followed by fractionation using n-hexane, ethyl acetate and water, identification of compound content using TLC, and rats were grouped into 7 groups (n=5), namely normal group, negative group, positive group, extract, fraction water, ethyl acetate fraction and n-hexane fraction by looking at the characteristic parameters and ovarian histopathology. Data analysis using ANOVA and Kruskal-wallis.* ***Results****: The yield of the extract was 30.4%, the water fraction was 85.59%, the ethyl acetate fraction was 6.64% and the n-hexane fraction was 4.05%. Positive for flavonoids, tannins, alkaloids in the ethyl acetate fraction, the water and extract fractions were positive for tannins, the n-hexane fraction was positive for steroids. The modeling sample group obtained extract body weight 195.40 g, water fraction 195.80 g, ethyl acetate fraction 194.00 g, n-hexane fraction 196.00 g, blood glucose level extract 83.00 mg/dL, water fraction 84.27 mg/dL, ethyl acetate fraction 80.00 mg/dL, n-hexane fraction 122.85 mg/dL, MDA extract content 2.704 nmol/mL, water fraction 3.547 nmol/mL, 1.685 nmol/mL, 5.308 nmol /mL and can improve ovarian histopathology.* ***Conclusion****: The most effective value is the ethyl acetate fraction because it has the highest decrease in PCOS characteristics.*

***Keywords****: follicles, malondialdehyde, moringa ethyl acetate fraction, Moringa oleifera, polycystic ovary syndrome*

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## INTRODUCTION

Polycystic ovary syndrome is an endocrine disorder that affects 5%-10% of women of reproductive age and is found in approximately 20%-25% in England and New Zealand (Norman *et al*., 2004). The *European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine* reported a prevalence of 15% -20%. In Indonesia, the highest frequency was found in the age range of 26-30 years (45,7% (Pangastuti & Sumapradja, 2011). Prevalence estimates vary depending on the source population and the *Rotterdam Criteria 2003* diagnostic criteria. There are two of three criteria: polycystic ovaries on ultrasound examination (USG), oligoovulation/anovulation, and clinical or biochemical evidence of hyperandrogenism. Women with PCOS have disturbances in the menstrual cycle (85% -90% with oligomenorrhea and 30% -40% with amenorrhea) and at most 90% -95% infertility (Pate & Sirmans, 2014).

The pathogenesis of PCOS in women is an androgen disorder that can increase the activity of the steroidogenesis pathway in ovarian follicles, which is achieved through theca and granulosa cells. In theca cells, it increases the expression of the enzyme *17β-hydroxysteroid dehydrogenase* (17β HSD), which causes an increase in testosterone levels in granulosa cells (Magoffin, 2006). Increased adrenal activity causes insulin phosphorylation, which causes insulin resistance, affecting approximately 50% -70% of women (Pate & Sirmans, 2014). Insulin resistance increases the production of *Reactive Oxygen Species* (*ROS*) in circulating adipocytes. Increased *ROS* in adipocytes can disrupt the balance between reduction and oxidation reactions, causing *oxidative stress* which can damage cell membranes. The degree of lipid peroxidation was determined using the MDA marker parameters. The treatments available in the market for patients with PCOS include birth control pills, fertility drugs, and diabetes medication (Kashani & Akhondzadeh, 2016). The goal of treatment is to improve ovulation and reduce androgen levels. One of the selective estrogen receptor modulators used in PCOS patients is clomiphene citrate, but it is not suitable for long-term use (Dewi, 2020).

Amelia *et al*. (2017) and Wulandari *et al*. (2017) were shown to increase body weight and MDA levels of the PCOS-insulin resistance model rats induced by testosterone propionate, and moringa leaf extract was able to reduce body weight, MDA, and increase the number of follicles, related to morphometry (length, width, and weight). Ovaries) after administration of moringa leaf extract to mice at doses of 300, 400, and 500 mg/kg BW affected the morphometry of mice (Balumbi *et al*., 2021). Moringa leaves are known in Indonesia as "*the miracle tree*" because they are natural sources of nutrition and medicine, and the secondary metabolites that play an active role are flavonoids (quercetin) (Kementerian Kesehatan RI, 2017). This compound has an -OH group attached to a benzene ring, which allows it to act as an antidote to *ROS*, reduces metal ions, modulates protein phosphorylation (associated with inhibition of enzyme activity), and can inhibit lipid peroxidation, which is mostly found in flavonoid compounds such as quercetin (Halliwell & Gutteridge, 2015). The aim of this study was to evaluate the protective effects of *Moringa oleifera* leaf extract and its fractions against testosterone-induced PCOS in female *Rattus norvegicus*.

## MATERIALS AND METHODS

### Materials

The materials used in this study were 10% formalin and Bouin's buffer formalin solution, acetic acid ($CH_3COOH$) (Marck), and acetone (PT. Brataco), ammonia ($NH_3$) (Marck), ammonium hydroxide ($NH_4OH$) (Marck), and aquades (PT. Brataco), blank paper, and clomiphene citrate ($C_{26}H_{28}ClNO.C_6H_8O_7$) (PT. Kimia Farma) and chloroform ($CHCl_3$) (PT. Brataco), dragendorff reagent, ethanol ($C_2H_6O$) (PT. Brataco), and ethyl acetate ($C_4H_8O_2$) (PT. Brataco), ethyl acetate fraction of moringa leaf extract, ferric chloride ($FeCl_3$), formic acid (HCOOH), gallic acid (Sigma), *glucose test reagent kit*, *Hematoxylin-Eosin* (HE) staining solution, Lieberman Burchard reagent, and methanol ($CH_3OH$) (PT. Brataco), and moringa leaf powder were obtained from PT. Moringa Organik Indonesia (MOI), n-butanol (Marck), and n-hexane ($C_6H_{14}$) (PT. Brataco), n-hexane fraction of moringa leaf extract, silica gel 60 $F_{254}$ TLC plates (Marck KGaA, Darmstadt, Germany), piperine (Sigma), quercetin (Sigma), Sodium Carboxymethyl Cellulose (Na-CMC) (Sigma-Aldrich), stigmasterol (Sigma), *TBARS assay kit*, testosterone (Wonder brand, PT. Wonderindo Phrmatama, Jakarta, Indonesia), female rats (*Rattus norvegicus*) obtained from the Center for Food and Nutrition Studies, Universitas Gadjah Mada, and water fraction of Moringa leaf extract.

### Tools

The tools used in this research were an analytical balance (OHAUS PJ1003), *centrifuge* (D-37520), chamber, Eppendorf tube (OneMed), dissecting set,

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glass slides and coverslips, glass tools (Vyrex), micropipette (Dragonlab), microhematocrit (Nris-Vitrex Medical), Olympus CX23 light microscope, plain tube (OneMed), *rotary evaporator* (Laborota 4000), *Sep-Pak* $C_{18}$ column, syringe (OneMed), and UV-Vis spectrophotometer (Shimatzu 1800).

**Method**

**Animals**

This study used 2-month-old female (the estrus cycle is observed by visual observation methods on external genitalia, such as the presence of vaginal mucus) white rats (*Rattus norvegicus*) weighing 160-200 grams, given pellets and water *ad libitum*. After acclimatization for seven days, the rats were divided into seven groups (n=5). Normal control group (CG), negative control (Na-CMC 1%) (CN), positive control (clomiphene citrate) (CP), moringa leaf extract (500 mg/kg BW) (EM), n-hexane fraction (21.04 mg/kg BW) (FN), ethyl acetate fraction (34.47 mg/kg BW) (FE), and the water fraction (444.49 mg/kg BW) (FW). Modeling uses the hormone testosterone, administered intramuscularly at a dose of 2 mg/200 g BW, volume 2 for 28 days, and giving a 14-day sample. All procedures have been described and approved by the Medical/Health Research Bioethics Commission, Faculty of Medicine, Sultan Agung Islamic University Semarang, Indonesia (350/IX/2022/Komisi Bioetik).

**Extraction**

*Moringa oleifera* leaf powder was obtained from the PT. Moringa Organik Indonesia (Jakarta, Indonesia) (SIG.LHP.II.2022.221405011). Fine powdered moringa leaves (750 g) were added to 70% ethanol at a ratio of 1:10 g/v, and then placed into the maceration vessel. Simplisia was soaked for the first 6 h while stirring occasionally and then allowed to stand for 18 h. The macerate was separated by filtration, and the remaceration process was repeated once using the type and amount of solvent, which was half the volume of solvent in the first solvent. All macerates were collected and evaporated using a rotary evaporator to obtain thick extracts. The percentage yield (w/w) was calculated. The yield must be at least as specified in each monograph (Kementerian Kesehatan RI, 2017).

**Extract characterization**

**Organoleptic**

This was done macroscopically by examining the shape, color, and smell of the Moringa leaf extract.

**Determination of water content**

Water content was determined using the azeotropy method (*Toluene Distillation*) with a sterling-bidwell apparatus, which was strung, and then 10 g of Moringa leaf extract was weighed and placed into a dry pumpkin. Next, 100 mL of water-saturated toluene was added to the flask, boiled over low heat so that no water drips, and the water flowed during the process. The results of the water content are recorded by looking at the volume on the tool scale, the water content is calculated in % b/v.

**Determination of total ash content**

Accurately weigh 2-3 grams of the test material, which has been mashed and placed in a silicate crucible that has been heated and tarred, slowly heated until the charcoal runs out, cooled, and weighed (Kementerian Kesehatan RI, 2017).

**Determination of acid insoluble ash content**

The ash obtained in the determination of the total ash content was boiled with 25 mL of dilute hydrochloric acid for 5 min. The parts that were not soluble in acid were collected, filtered through ash-free filter paper, washed with hot water, and heated in a crucible until the weight remained at ±600° C. The acid-insoluble ash content was calculated against the weight of the test material and expressed as % w/w (Kementerian Kesehatan RI, 2017).

**Fractionation**

The fraction was prepared by weighing 10 g of condensed extract and dissolved in ethanol and water at a ratio (1:9), prepared in 25 mL, which was then separated using a separating funnel with two parts of an immiscible solvent, n-hexane. The n-hexane and water parts were removed. The aqueous fraction was fractionated using an equal amount of ethyl acetate (1:1). The water portion was set aside and the ethyl acetate portion was withdrawn. Fractionate 25 mL of each solvent and repeat the process until the solution becomes clear.

**Identification of the chemical content of the fraction using Thin Layer Chromatography (TLC)**

Phyto components of flavonoids, tannins, alkaloids, saponins, and steroids were identified using TLC and observed at UV wavelengths of 254 and 366 nm. Flavonoid compounds were compared using a mobile phase of chloroform:acetone:formic acid (7:3:0.4), with quercetin as a comparison. The spots were then sprayed with the citroborate reagent in an oven at 100 °C for 5 min. The presence of flavonoids is indicated by green spots, and under visible light, it shows a yellow fluorescent spot. Tannin compounds were prepared using the mobile phase n-butanol:acetic acid:water (4:1:5), with gallic acid used for comparison. The spots were observed under UV light at 366 nm by spraying $FeCl_3$. The presence of tannins is indicated by the presence of brown or black spots. Alkaloid compounds

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were prepared using a mobile phase of methanol:ammonium hydroxide (100:3), with piperine as a comparison. Appearance of spots used by spraying Dragendorff reagent. The presence of alkaloids was indicated by orange-red spots. Saponin compounds were detected using the mobile phase of chloroform:methanol:water (13:7:2), and spots were detected by spraying 10% $H_2SO_4$. The presence of saponin compounds is indicated by purple spots. Steroid compounds using the mobile phase n-hexane:ethyl acetate (18:2), with stigmasterol as a comparison, were sprayed with Liebermann Buchard reagent. The presence of steroid compounds is indicated by blue spots.

**Analysis of rat glucose levels**

Measurement of fasting blood glucose levels using the GOD-PAP method, namely a blood sample taken 10 µL with 1000 µL of reagent, mixed, and incubated at 20-25℃ or 37 °C for 20 min. The intensity was measured using a spectrophotometer at a wavelength of 500 nm.

GOD-PAP $\frac{mg}{dL}$ : $\frac{Sample}{Standard}$ standard × standard concentration (100 mg/dL).

**Examination of *malondialdehyde* levels**

Collection of Blood Specimens Weighing was done before taking Blood samples (1.5 mL) were weighed using a syringe, placed into a plain tube containing EDTA, and then centrifuged at 4000 rpm for 10 min. The liquid blood plasma separated from the solid part of the blood was transferred to an empty tube. The MDA blood test followed the method in *Indonesian food and nutrition progress, 2000 Vol. 7 no. 2*. Prepare a Standard solutions were prepared as follows:50 µL of *1,1,3,3-tetrametoxypropane* (TEP) solution, 450 µL of aquabides, 750 µL of $H_3PO_4$, and 250 µL of *thiobarbituric acid* (TBA). Plasma MDA levels are expressed as nmol/mL. A total of 750 µL $H_3PO_4$ was put into a polypropylene tube containing 250 µL TBA, 50 µL plasma was put into the tube, and 450 µL of aquabides was added. Mix in a vortex mixer for 2 min. The mixture was then placed in a water bath at 100C° for 60 min. Then, it for 1-2 hours until the temperature reached 30 °C. When the solution was cold, it proceeded to the purification stage. The method for making blanks was the same as for making samples, except that samples and standards were not used, and 50 µL of aquabides were used. MDA purification. *The Sep-Pak C18* column was prepared, rinsed with 5 mL methanol, discarded, and rinsed again with 5 mL aquabides (thrown away). The sample was then rinsed with 4 mL of aquabides (discarded), followed by elution with methanol, and the elution results were collected and placed in a cuvette. *TBARS* levels were measured using a spectrophotometer at a wavelength of 532 nm.

This study used descriptive analysis of patient demographic data and cost components. Mann-Whitney and multiple linear regression tests were used to examine the factors affecting the hospital's real costs to see the difference between the real costs and the cost of INA-CBG inpatient schizophrenia.

*TBARS* $\frac{nmol}{mL}$: $\frac{\Delta\, sample - a}{b}$

where Δ sample is the absorbance of the sample, a is the intercept of the regression coefficient and b is the slope of the regression constant.

**Histological analysis**

Ovarian specimen collection, mice were sacrificed using the cervical dislocation technique (neck dislocation), and then placed on a surgical board with pins, beginning with abdominal dissection with curved scissors. The ovarian organs were removed and separated with straight scissors, the ovarian organs were washed with distilled water until no blood was left, and then washed with 0.9% NaCl. The organs were placed on filter paper and then weighed with the organs placed in dry petri dishes, after which the weight was recorded (Amelia *et al*., 2017). For counting the number of follicles**,** tissue ovaries were fixed in 10% formalin in phosphate buffered saline (PBS), pH 7.4. Histological examination of ovaries from all groups was performed using standardized histological methods. Sections of 5 µm thickness were cut into paraffin-embedded blocks and stained with (hetoxylin and eosin HE (*Hematoxylin-Eosin*). Tissue observation was performed using an Olympus microscope to observe the real picture of the tissue and microphotography (4x magnification).

**Statistical analysis**

The data obtained were analyzed statistically using SPSS software version 25.0. The Shapiro-Wilk test was performed to determine whether the data were normally distributed. If the data were normally distributed, a one-way ANOVA statistical test was used. However, if there are data/groups of data that are not normally distributed or homogeneous, the non-parametric Kruskal-Wallis test will be used. This study was considered significant if the analytic variable was $p<0.05$.

## RESULTS AND DISCUSSION

The calculation of the average ethanol extract yield of moringa leaves was 30.4%, which is not less than 9.2% and meets the requirements for extract production. The characterization of the extract included the following.

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**Organoleptic**

The extract obtained was characterized by a thick, green-brown color with a distinctive odor.

**Determination of water content**

The moisture content in the moringa leaf extract was 2.7%, indicating compliance with the requirements of the Indonesian Herbal Pharmacopoeia (FHI), which stipulates that it should not exceed 10.0%. High moisture content (>10.0%) can affect the stability of the extract because water is a medium for microbial growth.

**Determination of acid insoluble ash content**

The percentage obtained was 7.87%, indicating compliance with the FHI, which stipulated that it should not exceed 9.0%.

**Determination of acid insoluble ash content**

The percentage obtained was 0.98%, indicating a lower value than the total ash content because only certain minerals or metals that are insoluble in acid were present.

**Fractionation**

The percentage yield of each fraction of moringa leaf extract had the highest weight for the water fraction at 85.59%, followed by the ethyl acetate fraction at 6.64%, and the n-hexane fraction at 4.05%. Different compounds have different partition coefficients; therefore, if one compound is polar, its relative partition coefficient to the polar phase will be higher than that of non-polar compounds (Najib, 2018).

**Identification of the chemical content of the fraction using Thin Layer Chromatography (TLC)**

Identifying the secondary metabolite content using TLC showed that moringa leaves contain chemical compounds, including flavonoids, tannins, alkaloids, saponins, and steroids (Table 1). This is supported by previous research stating that moringa leaves contain a wide variety of chemical compounds with various methods of identification and analysis.

**Table 1**. Identifying the scondary metabolite contents of moringa leaves

| Chemical Contents (Spray Agent) | Sample | | | | Description |
|---|---|---|---|---|---|
| | Extract | n-hexane Fraction | Ethyl Acetate Fraction | Water Fraction | |
| Flavonoids (Citroborate) | (-) | (-) | (+) | (-) | Yellow |
| Tannins ($FeCl_3$) | (+) | (-) | (+) | (+) | Black |
| Alkaloids (dragendroff) | (-) | (-) | (+) | (-) | Reddish-orange |
| Saponins ($H_2SO_4$ 10%) | (-) | (-) | (+) | (-) | Purple |
| Steroids (Liebermann Buchard) | (-) | (+) | (-) | (-) | Blue |

Information: positive (+), negative (-)

**Table 2**. The levels of *malondialdehyde* in female rats with PCOS-insulin resistance model and administration of extract samples and fractionated extract samples of moringa leaves

| Groups | Levels of *malondialdehyde* (nmol/mL) | |
|---|---|---|
| | D-28 (PCOS-Insulin Resistance) | D-42 (Sample Administration for 14 Days) |
| Normal Control | 1.623±0.328$^{bc}$ | 1.635±1.108$^{b}$ |
| Negative Control | 8.214±0.173$^{a}$ | 8.277±0.178$^{ac}$ |
| Positive Control | 8.629±0.299$^{a}$ | 1.950±0.269$^{b}$ |
| Extract | 8.528±0.340$^{a}$ | 2.704±0.117$^{abc}$ |
| Water Fraction | 8.528±0.300$^{a}$ | 3.547±0.274$^{abc}$ |
| Ethyl Acetate Fraction | 8.227±0.108$^{a}$ | 1.685±0.219$^{b}$ |
| n-hexane Fraction | 8.390±0.134$^{a}$ | 5.308±0.259$^{abc}$ |

$^{a}$vs Normal Control ($p<0.05$), $^{b}$vs. Negative Control ($p<0.05$), $^{c}$vs Positive Control ($p<0.05$)

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**Table 3**. The weight of the right and left ovaries after administration of extract samples and fractionated extract samples of moringa leaves

| Groups | Ovary Weight (gram) | |
|---|---|---|
| | Right | Left |
| Normal Control | 0.17±0.01[b] | 0.17±0.01[b] |
| Negative Control | 0.12±0.01[ac] | 0.12±0.01[ac] |
| Positive Control | 0.17±0.01[b] | 0.17±0.01[b] |
| Extract | 0.15±0.01[abc] | 0.15±0.01[abc] |
| Water Fraction | 0.15±0.01[abc] | 0.15±0.01[abc] |
| Ethyl Acetate Fraction | 0.16±0.01[b] | 0.16±0.01[b] |
| n-hexane Fraction | 0.14±0.01[abc] | 0.14±0.01[ac] |

[a]vs Normal Control ($p<0.05$), [b]vs. Negative Control ($p<0.05$), [c]vs Positive Control ($p<0.05$)

**Table 4**. Number of histopathological ovarian follicles

| Groups | Number of Follicles | | | |
|---|---|---|---|---|
| | Primary | Secondary | Tertiary | Corpus Luteum |
| Normal Control | 3±0.8 | 6±3.1[b] | 4±1.2[b] | 15±6.0[b] |
| Negative Control | 6±1.6 | 16±2.6[a] | 0±0.0[ac] | 1±0.8[ac] |
| Positive Control | 3±0.8 | 10±1.6[b] | 5±1.6[b] | 15±2.1[b] |
| Extract | 3±1.7 | 11±1.6[b] | 2±0.5[b] | 10±5.4[b] |
| Water Fraction | 4±2.2 | 13±1.7[b] | 2±0.8[b] | 10±2.9[b] |
| Ethyl Acetate Fraction | 3±0.8 | 10±3.6[b] | 3±0.9[b] | 13±4.6[b] |
| n-hexane Fraction | 4±1.7 | 12±1.6[b] | 1±0.8[ac] | 8±2.4[b] |

[a]vs Normal Control ($p<0.05$), [b]vs. Negative Control ($p<0.05$), [c]vs Positive Control ($p<0.05$)

The prevalence of schizophrenia in this study, which is domiciled in rural areas, was 95 patients (84.82%) and 17 patients (15.18%) in urban areas. According to data from Kementrian Kesehatan RI (2019), the prevalence of schizophrenia in Indonesia based on residence is more significant in rural areas $7^{o}/_{oo}$ than in urban areas $6.4^{o}/_{oo}$. This contrasts with the population density theory, which states that schizophrenia is higher in areas with high population densities, such as cities. Population density is a factor that influences the occurrence of mental disorders, including psychosis (Sadock *et al.*, 2014). Based on Table 1, the highest class of treatment-experienced schizophrenia, namely class 3, with 91 patients (81.25%), showed that most patients with schizophrenia in Tombulilato Hospital came from middle to lower economic classes.

**Rat body weight**

The average values are shown in Figure 1. of the 28-day model, based on statistical analysis, showed that the data were normally and homogeneously distributed ($p>0.05$). *Tukey's* ANOVA test showed a significant difference between the normal group and the negative and positive groups ($P <0.05$). This is because post-insulin receptors work on tyrosine phosphorylation, but what happens is serine phosphorylation occurs, which subsequently affects glucose transport to muscle or fat cells, leading to changes in body fat distribution and pushing visceral fat accumulation. This leads to insulin resistance. Supported by most cases of overweight and lean PCOS, women showing insulin resistance (Tabrizi *et al.*, 2020). Insulin resistance results in disturbances in insulin signal transduction involving two main pathways: *Phosphatidylinositol 3 Kinase* (PI3K) and *p38 Mitogen-Activated Protein Kinase* (MAPK) (Kusumastuty *et al.*, 2013). Administration of moringa leaf samples for 14 days showed statistically normal and homogenous data analysis, and *Tukey's* ANOVA test showed no significant difference for all sample groups compared to the normal group. This was due to the presence of compounds in the extract, water fraction, ethyl acetate fraction, and n-hexane fraction, in accordance with the results of compound identification. Weight change in insulin-resistant PCOS patients in this study was a contributing factor. Hong *et al.* (2018) reported that the administration of flavonoids (quercetin) prevented weight gain and caused significant weight loss in PCOS rats, whereas other compounds with positive results in compound identification include tannins, alkaloids, saponins, and steroids. Further characteristics of PCOS include blood glucose measurement on the same day as weight measurement.

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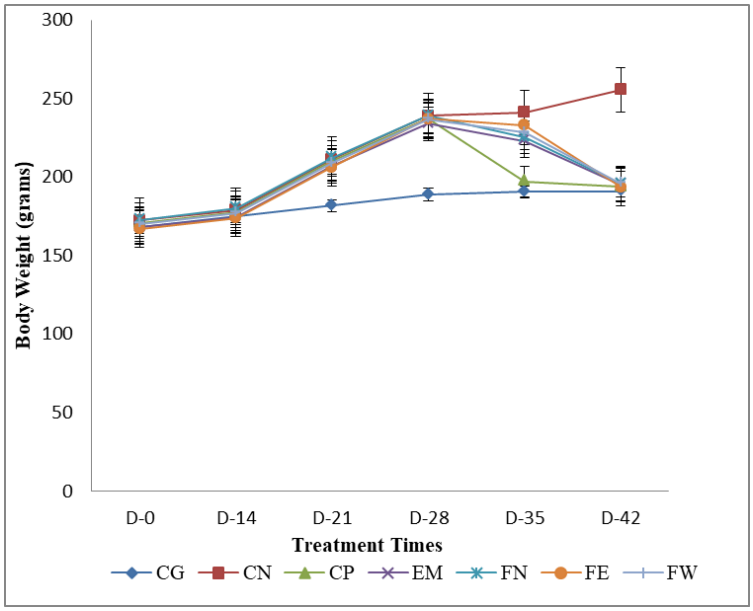


**Figure 1.** The results of the weight test on rats during 28 days of testosterone induction (PCOS-insulin resistance) and sample administration for 14 days (day 42). The values are represented as mean±SD

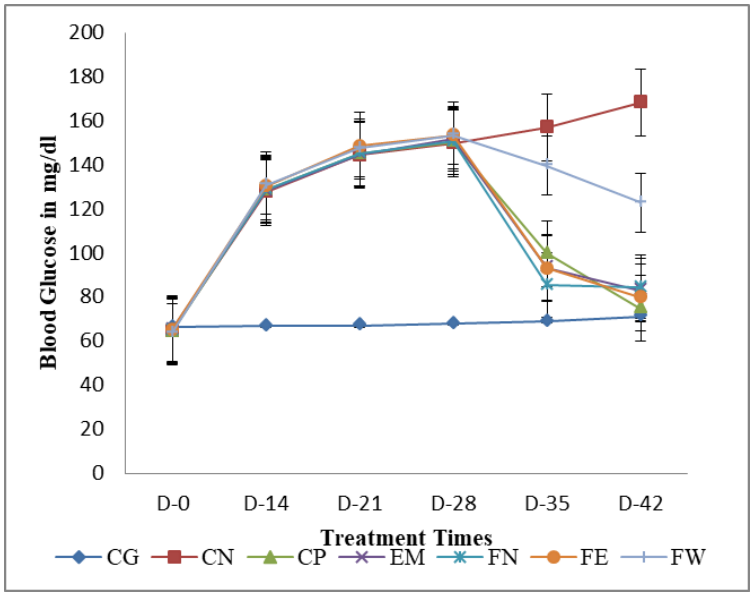


**Figure 2**. The results of he fasting glucose levels of rats during 28 days of testosterone induction (PCOS-insulin resistance) and sample administration for 14 days (day 42). The values are represented as mean±SD

**Blood glucose measurement**

The measurement of rat GDP levels after 28 days of modeling yielded GDP levels between 67.78-153.43 mg/dL. Statistical analysis showed normal and homogeneous data, followed by *Tukey*'s ANOVA test, which revealed a significant difference between the negative, positive, and normal groups ($p<0.05$). Insulin resistance in the PCOS animal model plays a major role in PCOS pathogenesis, as evidenced by the study by Wang *et al.* (2017) who showed a significant decrease in the expression of *Insulin Receptor Substrate-2* (IRS-2) protein in the ovaries of PCOS rats compared to normal control rats, which is consistent with previous research indicating that decreased expression of IRS-2 can cause insulin resistance in PCOS. IRS-2 binds to the SH2 domain of the regulatory subunit p85α of PI3K. A decrease in PI3K activity has been observed in adipose tissue, which can cause insulin resistance. P85α plays an important role and is a crucial component in insulin signaling transduction; therefore, decreased expression of p85α can cause decreased PI3K activity, which in turn inhibits most insulin metabolic functions (Figure 2).

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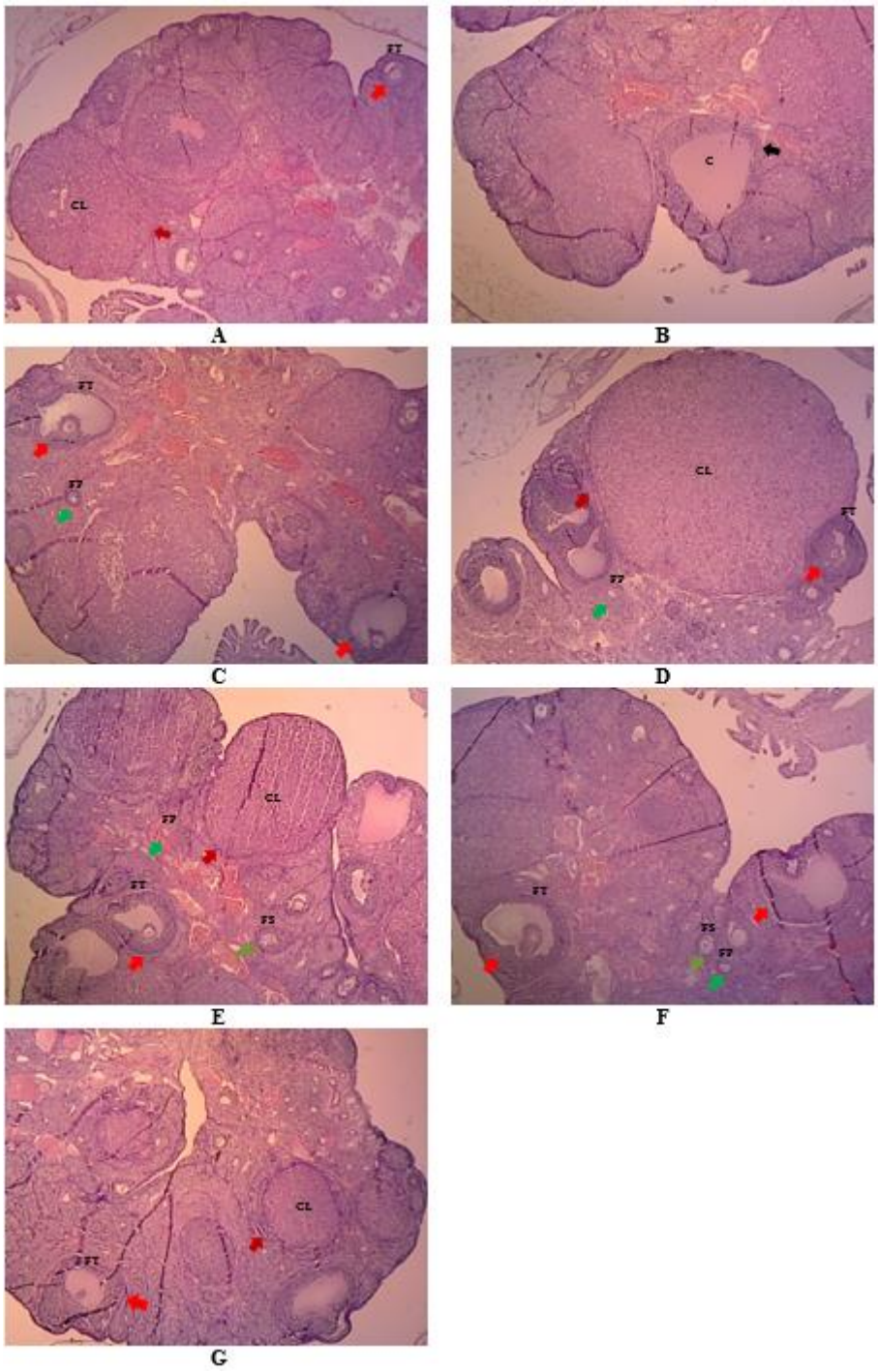


**Figure 3**. Ovarian histopathology of all groups. CG (A), CN (B), CP (C), EM (D), FW (E), FE (F), and FN (G). FP: Follicles Primary, FS: Follicles Secondary, FT: Follicles Tertiary, CL: Corpus Luteum

The administration of samples for 14 days showed a decrease due to the positive presence of flavonoids (quercetin) in the ethyl acetate fraction sample. In the extract and water fraction, there were positive compounds of tannins (phenolics) with antidiabetic effects similar to flavonoid compounds, namely insulin-like effects or insulin secretion stimulation, and the possibility of modulating carbohydrate metabolism enzyme function (Megawati *et al.*, 2021). One flavonoid compound with antidiabetic properties, and high glucose levels in insulin-resistant PCOS rats can maintain glucose homeostasis. In the ethyl acetate fraction, the presence of flavonoid compounds resulted in a higher percentage of decrease than in the other sample groups. Rezvan *et al.* (2017) reported that flavonoids (quercetin) stimulate glucose uptake by activating independent and AMPK-dependent pathways to increase *Glucose Transporter-4* (GLUT-4),

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subsequently decreasing enzyme regulation for gluconeogenesis and protecting pancreatic β-cell function, as well as increasing the expression of the estrogen receptor alpha (ER α) gene.

**Measurement of *malondialdehyde* (MDA) level**

As shown in Table 2, the negative control treatment had an average of 8.695 nmol/mL, which was the highest concentration compared with the normal control group. Based on the statistical analysis of Shapiro-Wilk normality, the treatment of samples on test animals for 28 days was normally distributed ($p>0.05$) but not homogeneous ($p<0.05$). Therefore, *Dunnett's T3* test was conducted, which showed a significant difference between the normal group and the negative, positive, extract, water, ethyl acetate, and n-hexane fractions. This indicates that the modeling of PCOS-insulin resistance was successful owing to an increase in MDA, which was used as an OS indicator. Statistical analysis was conducted 14 days after sample administration, and the data showed normality and homogeneity ($p>0.05$). The *Tukey* test showed a significant difference ($p<0.05$) in the extract sample, water fraction, n-hexane fraction, and ethyl acetate fraction groups, whereas the ethyl acetate fraction sample was not significantly different from the normal group ($p>0.05$), with an average value of 1.685 nmol/mL for the ethyl acetate fraction and 1.635 nmol/mL for the normal group. The ethyl acetate fraction showed positive identification of flavonoids, namely quercetin, which can reduce MDA levels to levels closer to normal. Flavonoids exert antioxidant effects by transferring $H^+$ atoms and forming *ROS*, which can be achieved by inhibiting the activity of xanthine oxidase enzymes, *Nicotinamide Adenine Dinucleotide Phosphate* (NADPH) oxidase, and chelating metals ($Fe^{2+}$ and $Cu^{2+}$), thereby preventing redox reactions that can produce free radicals. This mechanism is similar to that of phenolic compounds, which also have reducing activity in the extract and water fractions. Flavonoids, such as superoxide, peroxyl radicals, and peroxynitrite, are the most effective scavengers of reactive species (Akhlaghi & Bandy, 2009). Based on the statistical analysis in Table 3, the data showed normality and homogeneity ($p>0.05$), and the ANOVA *Tukey* test showed a significant difference between the normal and negative groups ($p<0.05$), whereas the ethyl acetate fraction sample group, extract, water fraction, and n-hexane fraction showed a significant difference ($p<0.05$).

**Histopathology**

The number of follicles in the ovaries of rats affected the weight of the ovaries, as an increase or decrease in weight depended on the number of mature follicles (tertiary follicles), which contained a fluid-filled sac (antrum) that added weight to the ovaries. For the number of rat follicles in the PCOS insulin resistance model shown in Table 4 and Figure 3, data for primary, secondary, and corpus luteum follicles were obtained using statistical analysis, and the data were normally distributed and homogeneous ($p>0.05$). However, data for tertiary follicles were not normally distributed, so the *Kruskal-wallis* statistic was conducted, and the results showed a significant difference between the normal and negative groups ($p<0.05$). There were few tertiary follicles, as ovulation in rats occurred for only a few hours (Mardika *et al.*, 2018). The ovarian histopathology is shown in Figure 3 The diameter of the mature follicles (tertiary follicles) ranged from 0.12-0,70 mm.

The results showed that the ethyl acetate fraction group had follicle numbers that were not significantly different from the positive control group, while the n-hexane fraction group showed differences. The compound content of each fraction may significantly influence the number of follicles. As shown in Table 1, the ethyl acetate fraction contained flavonoid group compounds and the water fraction contained phenolic compounds. The activity that affects the number of follicles is influenced by compounds classified as phytoestrogens, which have two OH groups, similar to those found in estrogen hormones in the body. Flavonoids have a structure similar to 17-α estradiol, which can directly bind to estrogen receptors, thereby restoring development levels and reproductive processes, whereas steroids cannot bind directly and require synthesis (Russell *et al.*, 2000). According to FHI, the marker compound in the moringa leaf is quercetin, and the mechanism of quercetin for treating insulin-resistant PCOS can reduce testosterone levels and reverse low levels of estradiol and progesterone to levels approaching normal. Previous research has also reported that luteum causing estrus cycle recovery. After flavonoid (quercetin) administration, there was a decrease in 17β HSD steroidogenic enzyme activity, a decrease in testosterone concentration, and an increase in estradiol, which occurred because quercetin contains phenolic rings B and flavonoids have been reported to inhibit 17β HSD activity and reduce MDA activity, which acts as an anti-oxidant agent by its ability to

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inhibit xanthine oxidase through free radical reduction, antioxidant modification, and lipid peroxidation inhibition that can restore ovarian structure.

## CONCLUSION

Testosterone induces changes that lead to PCOS pathogenesis. Treatment with moringa leaf extract samples and fraction extracts reduced PCOS-insulin resistance characteristics, with the highest decrease in activity observed in the ethyl acetate fraction group because it had the highest decrease in PCOS characteristics. Testosterone induction does not optimally lead to PCOS-insulin resistance pathogenesis; therefore, further research is needed on the timing and doses used in insulin-resistant PCOS rat models and determining LH, FSH, and testosterone levels to determine the levels that can cause PCOS-insulin resistance.

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## AUTHOR CONTRIBUTIONS

Conceptualization, W. K., N. H., G. P. W.; Methodology, W. K., N. H., G. P. W.; Software, W. K., N. H., G. P. W.; Validation, W. K., N. H., G. P. W.; Formal Analysis, W. K., N. H., G. P. W.; Investigation, W. K., N. H., G. P. W.; Resources, W. K., N. H., G. P. W.; Data Curation, W. K., N. H., G. P. W.; Writing - Original Draft, W. K., N. H., G. P. W.; Writing - Review & Editing, W. K., N. H., G. P. W.; Visualization, W. K., N. H., G. P. W.; Supervision, W. K., N. H., G. P. W.; Project Administration, W. K., N. H., G. P. W.; Funding Acquisition, W. K., N. H., G. P. W.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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