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The Ethanolic Extract of *Moringa oleifera* Lam Leaves for Developing Ovarian Follicles in *Mus musculus*

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ABSTRACT

This study examined the effects of *Moringa oleifera* Lam leaf extract, using ethanol solvent at different doses, supplemented with additional minerals and vitamins, on the development of ovarian follicles in female *Mus musculus*. Thirty-six five-week-old female *Mus musculus* having reproduction disorder were used in the current experiment. The experimental design was a completely randomized design, with four different levels of *M. oleifera* Lam leaf extract (T1=4mg/kg Live weight (LW), T2=8mg/kg LW, T3=12mg/kg LW, and T4 or control=0 mg) fed to the animals. The data were analyzed using one-way ANOVA in SPSS and the histopathological analysis was descriptively conducted. The results indicated that treatments T1, T2, and T3 ensured adequate feed and high daily weight gain in *Mus musculus*. Histopathological examination of the ovaries revealed that a 4mg/kg LW dose of *M. oleifera* Lam leaf extract was sufficient to promote ovarian development and normalize the reproductive cycle.

Key words: *Moringa oleifera* extract, *Mus musculus*, Reproductive disorders

INTRODUCTION

Indonesia has a biodiversity that can be processed into various medicines, one of which is *M. oleifera*. *Moringa oleifera* is believed to have originated in northwestern India and is commonly grown throughout all subtropical and tropical climates in the world (Kuete 2017). Due to its immense potential as a medicinal and non-medical plant, *M. oleifera* is regarded as an essential herbal plant and is also known as the "tree of life" or "magic tree" (Pareek et al. 2023).

M. oleifera has not been thoroughly studied as a feed supplement in animal nutrition that can ameliorate reproductive performance. The leaves contain many active compounds, especially polyphenols and flavonoids, which provide high antioxidant activity (Mickdam et al. 2022; Peñalver et al. 2022). The existing scientific studies confirm that improved animal health (Khan et al. 2021), resistance to diseases (Khan et al. 2022), and higher protein intake promoted the growth and productivity of animals (Shu and Chen, 2020), resulting in higher financial gains for smallholder farmers especially in the tropics (Amad and Zentek 2023). The *M. oleifera* can be supplemented to improve growth and reproductive performance with no adverse effects on ewes' health (Ghattas and Ghada, 2019) and according to Prabsattroo et al. (2015), *M.oleifera* is rich in phenols and flavonoids as natural antioxidants and can enhance spermatozoa density in stressed rats. According to Laoung-On et al. (2021), *M. oleifera* has high total phenol, flavonoid, and antioxidant contents that could enhance sexual function and the male rat reproductive system. Medicinal plants can help with sexual dysfunction and can solve the problem of poor reproductive performance in ruminants due to their antioxidant and antimicrobial activities (Shai et al. 2022; Aslam et al. 2023). However, there is little information about dietary *M. oleifera* as a feed supplement that could improve reproductive performance in female mice that experience ovary hypofunction due to malnutrition.

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White rats are often used as experimental animals (model animals), due to their low cost, rapid reproduction, and anatomical and physiological characteristics that are similar to other mammals, including humans and livestock (Nugraha 2018). Experiments on female white rats have been carried out to increase productivity by using various herbal plants, such as extract ethanol of Pennywort leaves (*Centella asiatica L. Urban*), which have a pharmacological activity to improve the fertility of female mice (Dzulfiqor et al. 2015) and extract of "kumis kucing" (*Orthosiphon aristatus*) leaves which can restore the estrous cycle (Musaddad and Sumarmin 2021). However, there is a need for the study on flavonoid induction formulas based on herbal plants, vitamins, and micro-minerals that are environmentally friendly, available to farmers and easy to apply in the field. Flavonoids are natural polyphenolic compounds in many vegetables, fruits, grains, and tea. As plant secondary metabolites, flavonoids play an essential role in many biological processes and responses to environmental factors in plants (Shen et al. 2021; Mubashir et al. 2022). Likewise, *M. oleifera* also contains chemical compounds such as alkaloids, saponins, polyphenols, fats, tannins, sterols, amino acids, chlorogenic acids, essential oils, potassium, magnesium, aluminum, phosphorus, iron, vitamin C, vitamin A, and flavonoids. Setiasih et al. (2019) reported there are isoflavone compounds in *M. oleifera* that have estrogen-like biological activity, such as daidzein, formononetin, biochanin A and glycetein.

This study aims to determine the effect of supplementing *M. oleifera* using ethanol solvent at various doses, along with additional minerals and vitamins, on the development of ovarian follicles in female *Mus musculus.*

MATERIALS AND METHODS

Location and time of research

Research activities were done at the Herbal Materia Medika Laboratory of the East Java Province Health Service in Batu City, the Testing Services Laboratory of the National Research and Innovation Agency, the Agricultural Products Technology Testing Laboratory, Brawijaya University Malang, and the Pathology Laboratory of Indonesian Center for Veterinary Standard Testing, Bogor. Research activities were conducted from January to December 2023.

Materials and design research

Animals

This study used 36 female *Mus musculus* (26.13±3.4; LW±SD) who experienced reproductive disorders, aged five weeks; were divided into four groups with doses of *M. oleifera* extract (T1=4mg/kg LW; T2=8mg/kg LW; T3=12mg/kg LW; and T4(-)=0 mg; T4 (+)= 0 mg + 0.1 ml PGF2α intra muscularly as controls) with additional minerals and vitamins.

Stages and extraction techniques

Moringa oleifera Lam leaf extraction procedures based on the technique that was previously conducted by Susanty et al. (2019): *M. oleifera* leaf powder was macerated with a polar solvent using 96% ethanol (1:8, w/v), at room temperature for four days and filtered with Whatman paper. Some solvent was added, and the extraction was repeatedly done until the colorless final extract was produced. The concentrated extracts were combined and evaporated at a pressure of 75 mbar at 40℃ in a Buchi brand rotary vacuum evaporator. The thick extract was evaporated in a boiling water bath until a constant weight was obtained.

Determination of total flavonoid content

The technique for determining the total flavonoid content of *M. oleifera* followed (Shraim et al. 2021) procedures. Five milliliters of 2% aluminum chloride ($AlCl_3$) in methanol were mixed with the same volume of sample solution. A 415nm wavelength of absorbency on a UV-vis spectrophotometer were taken after 10min with a blank consisting of 5mL of sample solution and 5ml of methanol without $AlCl_3$. Total flavonoid content was determined using a routine standard curve (10-100mg/mL). The average of three readings was used and expressed as mg of routine equivalents (RE)/100g of extract.

Antioxidant analysis on *Moringa oliefera* Lam extract

The antioxidant activity of the extract against free radical diphenylpicryl-hydrazyl (DPPH) was measured according to the method of Yen and Chen (1995). Extract solution in 2mL of methanol was added to 0.5mL DPPH solution (1 mM in methanol). The mixture was shaken and allowed to stand at room temperature for 30min. The resulting absorption was measured at a wavelength of 515nm. Percent of sample inhibition was calculated based on the absorption difference between the blank and the sample.

Calculation formula: (%) = [(AO-AS)/AO]x100%

Remarks: A0= Absorbance of control

AS= Absorbance of the sample

The percentage of DPPH damping activity was plotted against the sample concentration. The damping value of 50% (IC50) was calculated from the graph of the percentage of damping against the sample concentration. The test was carried out in 2 repetitions, quercetin was used as a comparison.

Formulas of *Moringa oleifera* Lam leaf extract with supplement

The formula consists of *M. oleifera* extract plus vitamin A, vitamin E, vitamin D3, and micromineral (zinc, calcium, and selenium). Formula ingredients were weighed and made in liquid form. The ingredients in the *M. oleifera* extract formulas made for each treatment are in Table 1.

Clinical trials on experimental animals

Clinical trials to determine the dose of *M. oleifera* used by female *Mus musculus* (white mice) which were exposed to the hyperglycemic method (cotton seed meal powder at a dose of 0.07g/head/day given orally for 24 days) until the mice experienced ovarian hypofunction (Novriyanti et al. 2014). Then the white mice were given *M. oliefera* extract according to their treatment groups (Table 1).

Statistical analysis

Differences in parameters measured between the treatments were analyzed using one-way ANOVA in SPSS software program (SPSS Statistic, IBM, New York) version 23. The 5% level significance was used to consider the differences between means. The ovarian histopathology data were descriptively analyzed.

Table 1: The formula treatment doses given to the *Mus musculus* female

	Treatment*				
Ingredients	T1	T2	T3	T4 (-)	T4 (+)
Vitamin A (mg/kg diet)	0.22	0.22	0.22	-	-
Vitamin E (mg/kg diet)	0.22	0.22	0.22	-	-
Vitamin D3 (mg/kg diet)	0.025	0.025	0.025	-	-
Selenium (mcg/kg diet)	150	150	150	-	-
Zinc (mg/kg diet)	10	10	10	-	-
Calcium (g/kg/diet)	0.016	0.016	0.016	-	-
Moringa leaf extract (mg/kg LW)	4	8	12	-	-
PGF2 α (ml/mice)	-	-	-	-	0.1

Source: Nutrient Requirements of Laboratory Animals (1995). * T1=4mg/kg LW; T2=8mg/kg LW; T3=12mg/kg LW; and T4(-)=0 mg; T4 (+)= 0 mg + 0.1 ml PGF2α intra muscularly as controls with additional minerals and vitamins

RESULTS

Moringa oleifera Lam leaf extract

The results of research activities show the use of ethanol as a solvent in *M. oleifera* extract, total flavonoid, and antioxidant levels are in Table 2.

Table 2: Results of *Moringa oleifera* Lam leaf extraction with 96% ethanol solvent

Parameters	Mean±SD
Total extraction results of *M. oleifera* (%)	16.78±2.74
Total flavonoid content (mg QE/g extract)	43.51±0.60
Antioxidant activity (IC50(ug/ml))	172.21±0.22

The content of flavonoid compounds in the sample were calculated in units of mg QE (Quercetin Equivalent)/gram of sample (Shraim *et al.* 2021) and IC50 (inhibition concentration), namely the concentration of the sample solution needed to inhibit 50% of DPPH (diphenylpicryl-hydrazyl) free radicals (Yen and Chen, 1995).

A high concentration of flavonoid was produced from *M. oleifera* with 96% ethanol solvent. While moderate level of the IC50 concentration which was known to include antioxidant, activity was produced in the current study (Table 2).

Nutrient consumption

Table 3 shows that the 4mg/kg LW level shows the highest level of protein consumption, although statistically, it does not show a significant difference.

Nutrients are chemical compounds required by the body for growth, development and maintenance of normal function. Feed nutrient intake between treatments did not show significant differences (Table 3).

Live Weight (LW) of *Mus musculus* females

The results of the study based on body weight in *Mus musculus* females showed that there were no significant differences ($p>0.05$) between treatments. However, the highest mortality of female mice was in the control treatment (T4) (Table 4).

DISCUSSION

The formula in Table 1 was prepared to obtain the best level of *M. oleifera* and PGF2α hormone to develop ovarian follicles in female *Mus musculus***.** Based on Table 2, the results of extracting *M. oleifera* with ethanol solvent produced an IC50 which is known to include antioxidant activity in the moderate level. This was stated by Molyneux (2003), that antioxidant activity is very strong if IC50 is less than 50µg/mL, strong (50-100µg/mL), moderate (100-150µg/mL), weak (150-200µg/mL) and very weak (>200µg/mL). The results in this study were comparable with the results previously reported by Kiswandono and Maslahat (2011) which produced antioxidants in the medium level in *Moringa* extract with ethanol solvent, accounting for 118.19µg/mL. However, another study found that the lC50 from *M. oliefera* extraction with ethanol solvent was 435 ug/mL (very weak activity) which was way too high compared to the results in the current study (Segwatibe et al. 2023). The discrepancy could be caused by many factors, such as plant location or storage, drying procedure, solvent polarity, and the contribution of carbohydrates in the extracts could have influenced the results. Antioxidants can directly neutralize radicals, serve as cofactors for important enzymes involved in cell differentiation and development, and boost the activity of antioxidant enzymes. Supplementing antioxidants can cure infertility (Vašková et al. 2023). Furthermore, Aryal et al. (2019), the antioxidant results from extracting *Moringa* with

Table 3: Feed intake of *Mus musculus* female that were given *Moringa oleifera* Lam leaf extract

Feed Intake (g/head/day)	*Moringa oleifera* leaf extract				
	T1	T2	T3	T4 (-)	T4 (+)
Dry Matter	3.70±0.22	3.63±0.37	3.63±0.22	3.61±0.18	3.52±0.41
Crude protein	0.81±0.05	0.80±0.08	0.80±0.05	0.79±0.04	0.77±0.09
Crude fat	0.22±0.01	0.22±0.02	0.22±0.01	0.22±0.01	0.21±0.02
Crude fiber	0.15±0.01	0.14±0.01	0.14±0.01	0.14±0.01	0.14±0.02

Value =(Mean±SD). There were no differences between treatment ($P>0.05$). fed T1=4mg/kg LW; T2=8mg/kg LW; T3=12mg/kg LW; and T4(-)=0 mg; T4 (+)= 0 mg + 0.1 ml PGF2α intramuscularly as controls with additional minerals, and vitamins

Table 4: Live weight (LW, g) of *Mus musculus* females that were given *Moringa oleifera* Lam leaf extract

Treatment	Mortality (%)	Observation Time (week)			
		1	2	3	4
T1	0.00	28.50±3.39	27.83±2.99	28.17±3.19	29.50±5.01
T2	0.00	29.83±3.54	29.00±3.79	28.50±3.51	28.83±4.79
T3	0.00	29.80±3.57	30.40±3.04	30.20±2.80	30.60±3.13
T4(- control)	33.33	27.90±1.63	26.75±1.26	28.75±1.50	29.50±1.73
(+control)	50.00	26.80±5.02	28.40±3.44	29.00±4.06	30.80±3.56

T1=4mg/kg LW; T2=8mg/kg LW; T3=12mg/kg LW, and T4 (+)= 0 mg + 0.1 ml PGF2α intra muscularly as controls with additional minerals and vitamins.

ethanol solvent are not optimal because herbal plants contain more flavonoids. Isoflavones having high estrogenic-like activity interact with the estrogen receptors that contribute to ovarian disorders (Swathi et al. 2022; Zhong et al. 2023; Mitsunami et al. 2023), to the extent that the mechanism of naturally bioactive ingredients induces a proliferative phase and delays the apoptosis of ovarian granulosa cells. The mechanism promotes follicle and steroidal hormone formation (Zhong et al. 2023). On the other hand, flavonoids enhance the oocyte quality and embryo health in proliferation and apoptosis phases and decline the oxidative stress in ovarian granulosa cells (Rashidi et al. 2021).

Table 3 showed that there was no significant increase in nutritional value from administering *M. oleifera* up to 12mg/kg LW. According to Trisna et al. (2014), providing 5% *M. oleifera* extract through drinking water increased feed intake, water intake, final body weight, and live weight gain of broiler chickens aged 2-6 weeks. Likewise, in female mice the number of follicles in the ovaries affected the weight of the ovaries, as the increase or decrease in ovary weight depends on the number of mature follicles (tertiary follicles), containing fluid-filled sacs (antrum) (Khairi et al. 2023).

However, the results in this study did not support the published data in the literature, there were no significant effects on the increase in live weight of female *Mus musculus* (Table 4). This condition did not affect the competency of mice to conceive as either mice have obesity or normal weight can have normal fertility (Fabian et al. 2022). This argued the concept of being overweight in female's declines oocyte quality and fertility (Wu et al. 2010). The current experiment showed that the death rate of mice in the control group was relatively high. The cause of the death was probably not enough micronutrients provided to the control groups. Lack of nutrition may contribute to the low body's growth, development, and maintenance of normal function. Nutrients can be divided into carbohydrates, proteins, fats, vitamins, minerals, and waters which are required by the body and can be met by eating balanced diets. Nutrient intake refers to the process of taking in and using nutrients to meet functional and metabolic needs. The results of feed nutritional intake of crude protein and crude fat did not differ significantly. Those mice ate the same amount of feed and did not contribute to the differences in the feed intakes (Table 3). The micronutrient intakes were not observed and might be different between treatments and this was the limitation of the current study. However, nutritional compounds such as flavonoids, polyphenols, alkaloids and other bioactive compounds from *M. oleifera* may have potential health and nutritional benefits. Those compounds could be extracted from the plants with the help of ethanol that is in organic solvent. Ethanol can help protect the active compounds extracted from *M. oleifera* because of its properties as a natural preservative. This can prevent the degradation of these compounds during the extraction and storage process of the extract. A study reported that the extraction of *M. oleifera* using 70% ethanol resulted in moderate antioxidant activity with an IC50 value (50.59µg/mL) and an antioxidant activity index value (0.98), which was in moderate range (Riskianto et al. 2021). Ethanol has good solubility for various types of organic compounds, including those found in *M. oleifera* Lam leaves. This allows the solvent to produce large amounts of compounds during the extraction process.

As mentioned previously, all mice experienced ovarian hypofunction induced by feeding cotton seeds before the start of the experiment. An overview of ovarian histopathological tests by administering *M. oleifera* with 96% ethanol solvent plus vitamins and minerals in mice is presented in Fig. 1 to 4.

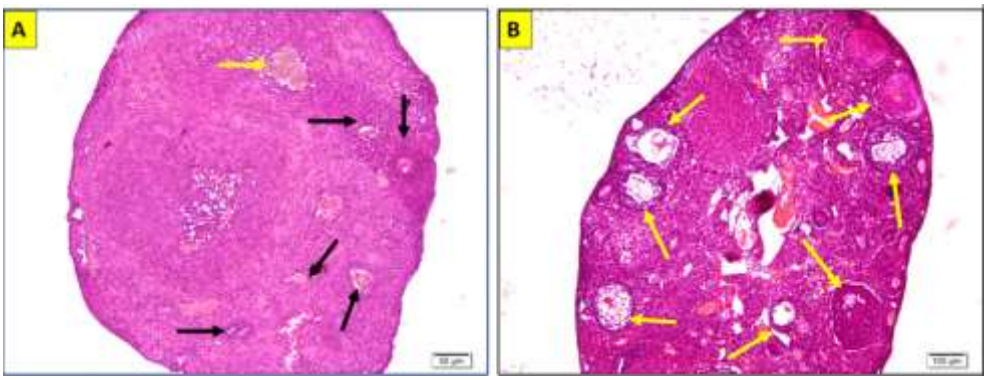

Fig. 1: A. The representative ovary samples of *Mus musculus* treated with *Moringa oleifera* Lam leaf extract at 0 mg/kg LW (negative control) in hematoxylin and eosin staining: ovaries atrophy (black arrow), the number of follicles decreased, some were inactive (yellow arrow). B. Treatment with PGF2α (positive control): ovarian follicles increased and became active.

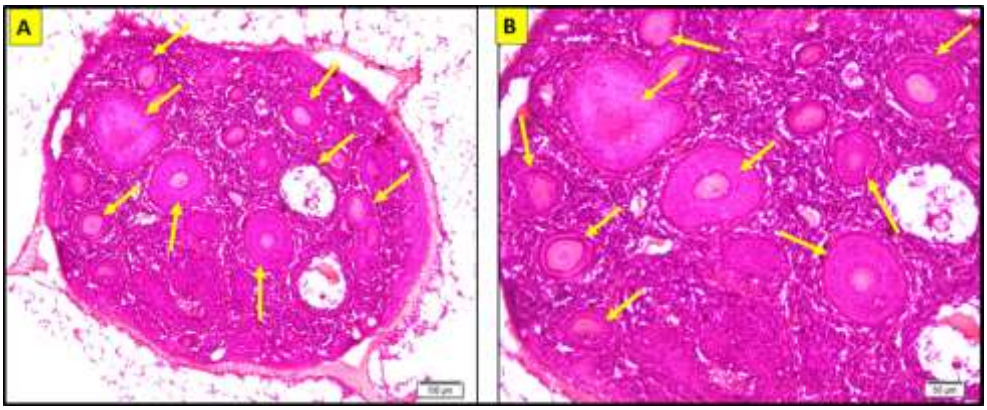

Fig. 2: The representative ovary samples of *Mus musculus* treated with *Moringa oleifera* Lam leaf extract at 4 mg/kg LW in hematoxylin and eosin staining: the number of ovarian follicles was large, active in various phases. (A. Low magnification, B. High magnification).

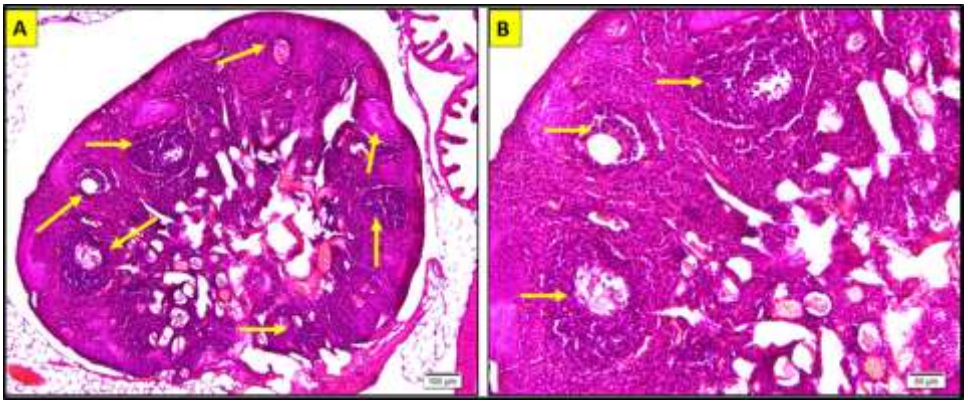

Fig. 3: The representative ovary samples of *Mus musculus* treated with *Moringa oleifera* Lam leaf extract at 8 mg/kg LW in hematoxylin and eosin staining: the number of ovarian follicles was large, active in various phases (A. Low magnification, B. High magnification)

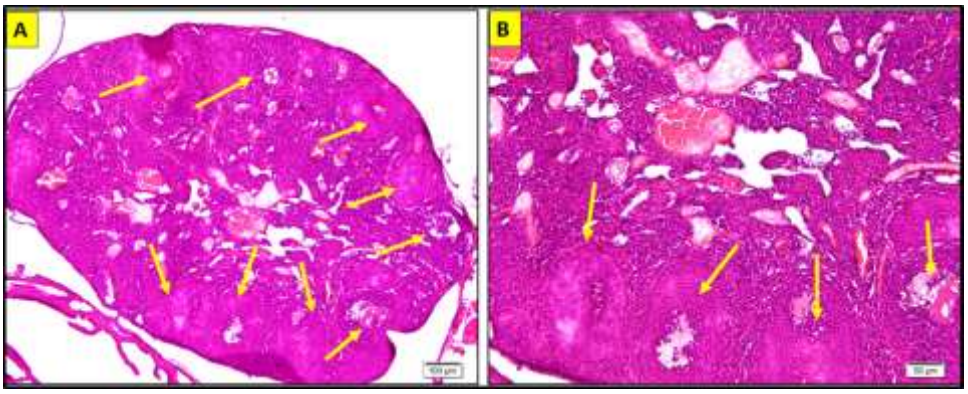

Fig. 4: The representative ovary samples of *Mus musculus* treated with *Moringa oleifera* Lam leaf extract at 12 mg/kg LW in hematoxylin and eosin staining: the number of ovarian follicles is large, active in various phases. (A. Low magnification, B. High magnification)

Those figures depicted the development of the mouse ovaries, a minimum feeding *M. oleifera* Lam leaf extract at 4 mg/LW added with vitamins (A, E, D3) and minerals (zinc, calcium and selenium) was sufficient to prevent potential damage to reproductive organ cells. This result was similar to the previous research results carried out by Armalina et al. (2021) who also found that giving *M. oleifera* Lam ethanolic leaf extract at 30mg/kg BW did not show any effect on the uterus of pregnant mice. Microscopic histology did not show endometrium and myometrium damage in epithelium or uterine glands and no inflammatory cell infiltration. In the current study, a 4mg/kg LW of *Moringa oleifera* Lam leaves was sufficient for ovarian development so that it was expected the reproductive cycle can return to normal. Another experiment also aligned with these results that administration of *Moringa* leaf ethanol extract was proven to be able to reduce the number of mast cells in *Mus musculus* which could cure endometriosis and ovarian hypofunction disorders (Wardani et al. 2017). The flavonoid content from *Moringa oleifera* Lam leaf extract was thought to be beneficial to restore the estrus cycle of female *Mus musculus*. The mechanism of flavonoids ameliorates female reproductive performance was probably through enhancing the endogenous antioxidants and gonadotrophin (luteinizing hormone and follicle stimulating hormone sinthesis) or preventing the reproductive disorders of the animals (Bhardwaj et al. 2021; Shen et al. 2021). Histopathological examination of the ovaries showed that the use of *Moringa oleifera* Lam leaf extract at 500mg/kg LW reduced the thickness of theca cells. The cells contributed to female reproductive disorders that were caused by insulin resistance, hyperandrogenaemia, chronic inflammation, and oxidative stress both in human and animal models. The antioxidant content of *M. oleifera* Lam leaf extract was thought to be the natural cure for those disorders (Wulandari and Hapsari 2020). In mice and humans, normal androgen production by theca cells maintains follicular growth through the promotion of early stage, folliculogenesis and prevention of follicular atresia. Excess androgens will have a detrimental effect on abnormal follicular growth and infertility (Ma et al. 2017). Ovarian hyperthecosis was often found in patients with severe insulin resistance syndrome, which was pathologically characterized by islands of hyperplastic theca cells located throughout the stroma and the presence of relatively few and small atretic follicles (Azziz et al. 2009). Another study reported that *M. oleifera* Lam leave has a significant effect on increasing the diameter of the Graafian follicles of female mice. This is due to the presence of vitamin E in leaves or additional vitamin E, which stimulates granulosa cells to produce estrogen hormone thereby facilitating the process of folliculogenesis (Aminuddin et al. 2024).

Conclusion

The results of extracting *M. oleifera* Lam leaves using 96% ethanol solvent produced antioxidants and total flavonoids at medium recommendation range. Feeding *M. oleifera* Lam leaf extract at 4 mg kg LW with supplement vitamins and minerals (T1) enhanced ovarian development that normalized the reproductive cycle in female mice experiencing ovarian hypofunction.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this article.

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Author's Contribution: LA, AR, DP, ML, RD, EM, RA, and SS designed the experiment, conducted research and collected the data. LA, AR, DP, ML, RD, EM, SS, PWP, DTR, and MC analyzed the data and finalized the write-up of this manuscript. All authors approved and finalized the manuscript.

Informed consent: All procedures were reviewed and approved by the University of Brawijaya Animal Ethics Committee in accordance with the Indonesian Code of Practice for the Care and Use of Animals for Scientific Purposes (074 KEP 2023).

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