ORIGINAL RESEARCH

Ethanol extract of *Cyclea barbata* Miers induces follicle development through 17ß-estradiol level and LHR expression

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ABSTRACT

Objective: Infertility, often linked to anovulation from impaired follicular maturation, affects millions globally. *Cyclea barbata* Miers, a plant with estrogen-like properties, may enhance follicle development through hormonal modulation. This study evaluated the ethanol extract of *C. barbata* leaves for its ability to promote in vitro follicle development by increasing 17ß-estradiol levels and luteinizing hormone receptor (LHR) expression.

Materials and Methods: Molecular docking analysis identified four novel compounds in *C. barbata*—Zearalenone, Bis(2-ethylhexyl) phthalate, Benzanthrone, and Octyl decyl phthalate—with binding affinities of -9.7, -9.6, -9.2, and -7.7 kcal/mol to estrogen receptor alpha (ESRα). Secondary follicles (2-3 mm) from goat ovaries were cultured in vitro using TCM-199 medium supplemented with 10% FBS, PMSG, hCG, and *C. barbata* extract at 25, 50, or 100 ppm for six days. Follicle maturation was assessed via microscopy for size, cumulus-oocyte complex formation, and polar body extrusion. 17ß-estradiol levels and LHR expression were analyzed using one-way ANOVA and LSD tests.

Results: The 100 ppm dose achieved 75% follicle maturation, significantly higher than 25% at lower doses and controls. It increased 17ß-estradiol levels (36.83 ± 2.33 pg/mL, $p < 0.000$) and LHR expression (261.874 ± 54.606, $p < 0.000$), with dose-dependent effects confirmed by statistical analysis.

Conclusion: *C. barbata* extract at 100 ppm enhances follicular maturation by elevating 17ß-estradiol and LHR expression, likely due to its estrogenic compounds. Further in vivo and clinical studies are needed to confirm its therapeutic potential for ovulatory disorders.

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Highlights:

1. Docking analysis revealed that there are four novel compounds in *C. barbata*, including Zearalenone, Bis(2-ethylhexyl) phthalate, Benzanthrone, and Octytldecyl phthalate have stronger affinity -9.7; -9.6; -9,2; and -7.7 kcal/mol, respectively.
2. The extract of *C. barbata* leaves increased follicle development (75%) by elevating estrogen levels and LHR expression at all doses.

INTRODUCTION

The maturation of follicles plays a crucial role in the onset of menstruation. The female reproductive system is integral to successful sexual reproduction, which ensures the continuation of offspring, largely due to its anatomical and physiological characteristics. Infertility, a condition where women are unable to conceive, often arises from disruptions in the reproductive system, such as anovulation.[1] The World Health Organization has identified infertility as a global issue, with its prevalence expected to rise; approximately 48.5 million couples worldwide face infertility, with 57% attributed to male factors and 53.2% to female factors. In Indonesia, the rate is between 15-20% among roughly 50 million couples.[2–4]

Anovulation is linked to the failure of follicle maturation during the antral phase, a process involving several hormones, including Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), estrogen, and their receptors. Insufficient estrogen levels can adversely affect ovulation. Estrogen's significant role in ovulation can be harnessed for in vitro oocyte maturation therapy to produce fertilizable oocytes. A continuous rise in estrogen levels triggers an LH surge.[5]

The ability to ovulate is heavily dependent on the conditions of follicle maturation during the antral phase. During this phase, an antrum forms, containing fluid from biochemical processes essential for the oocyte's maturation. Once the dominant follicle matures sufficiently, the estrogen it secretes creates "positive feedback," leading to the pituitary gland releasing high concentrations of LH (LH Surge). An increase in 17ß-Estradiol can be achieved through steroidogenesis and forkhead (FKHR) in granulosa cell proliferation via estrogen receptors. Estrogen's pivotal role in inducing an LH surge can serve as an alternative for ovarian stimulation. Ovulation disorders may result from disruptions in the positive feedback loop between dominant follicle maturation and estrogen secretion.

The use of plants and herbs for infertility treatment is becoming more common; one such plant is the green grass jelly leaf (*Cyclea barbata* miers), known for its antioxidant, anti-inflammatory, and hormonal properties, including steroids, testosterone, and estrogen.[6–8] Steroids share a similar chemical structure with estrogen, making them potential fertility control drugs. Steroids in *C. barbata* exhibit binding affinity with ESRα.[9–11] This study aims to investigate the potential of *C. barbata* extract for in vitro follicle development, focusing on the estrogen hormone and LHR using laboratory research methods.

MATERIALS AND METHODS

Method

The method used in this study was an experimental laboratory with a pre and post-test-only control group design, carried out in-vitro Growth (IVG).[12] The 3D structure and SMILE information of compounds were obtained from PubChem database (Table 1). The chosen active compounds were based on the finding from GC-MS study. The protein target used in this study was estradiol receptor, retrieved from PDB database (ID 1A52).

Molecular docking analysis

The affinity binding of each compound was obtained from molecular docking analysis to reveal the best interaction score occurs between esterogen receptor (ESR) and compounds, represents the activation of ESR during therapy using *C. barbata* extract. The molecular docking analysis was conduct utilizing PyRx software v0.8, with grid Center X: 89.647, Y: 14.129, Z: 72.484 and Dimension (Angstrom) X: 7.518, Y: 13.988, Z: 10.716. The docking analysis results were visualized using PyMol and Discovery Studio software 2017 R2.

Ovary sample collection

Goat ovaries taken from the Abattoir Sukun Malang. Previously, the researchers selected the condition of the goats, is Capra hircus, healthy, at least 5 months old and weighing between 15 and 20 kg. domiciled on a goat farm in the city of Malang. The slaughtering process meets the existing animal slaughtering standards at the Malang city animal slaughterhouse.

Ovaries were taken to the laboratory by placing them in a water bath filled with 0.9% physiological NaCl solution, which was added with the antibiotic 100 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 g/mL Streptomycin (Sigma-Aldrich) with a temperature of 35 – 37°C. Furthermore, the ovaries are cleaned from adhering to fat tissue.[13]

Table 1. Active compound of *C. barbata* retrieved from PubChem database

No	ID	Compound Name	SMILE
1	190	Adenine	C1=NC2=NC=NC(=C2N1)N
2	247	Betaine	C[N+](C)(C)CC(=O)[O-]
3	288	Carnitine	C[N+](C)(C)CC(CC(=O)[O-])O
4	305	Choline	C[N+](C)(C)CCO
5	500	4-Guanidinobutyric acid	C(CC(=O)O)CN=C(N)N
6	597	Cystosine	C1=C(NC(=O)N=C1)N
7	849	Pipecolic acid	C1CCNC(C1)C(=O)O
8	866	DL-Lysine	C(CCN)CC(C(=O)O)N
9	938	Nicotinic acid	C1=CC(=CN=C1)C(=O)O
10	1135	Thymine	CC1=CNC(=O)NC1=O
11	1174	Uracil	C1=CNC(=O)NC1=O
12	1780	5,8,11,14-Eicosatetraynoic acid	CCCCCC#CCC#CCC#CCC#CCCCC(=O)O
13	3026	Dibuthylphthalate	CCCCOC(=O)C1=CC=CC=C1C(=O)OCCCC
14	3678	Alverine	CCN(CCCC1=CC=CC=C1)CCCC2=CC=CC=C2
15	6140	Phenylalanine	C1=CC=C(C=C1)CC(C(=O)O)N
16	6287	Valine	CC(C)C(C(=O)O)N
17	6306	Isoleucine	CCC(C)C(C(=O)O)N
18	6697	Benzanthrone	C1=CC=C2C(=C1)C3=CC=CC4=C3C(=CC=C4)C2=O
19	7242	O-Toluidine	CC1=CC=CC=C1N
20	7405	L-pyroglutamic acid	C1CC(=O)NC1C(=O)O
21	7894	Isoamylamine	CC(C)CCN
22	7955	Melamine	C1(=NC(=NC(=N1)N)N)N
23	8343	Bis(2-ethylhexyl) phthalate	CCCCC(CC)COC(=O)C1=CC=CC=C1C(=O)OCC(CC)CCCC
24	8380	Octytldecyl phthalate	CCCCCCCCCCOC(=O)C1=CC=CC=C1C(=O)OCCCCCCCC
25	8988	D-proline	C1CC(NC1)C(=O)O
26	10737	4-(Dimethylamino)benzophenone	CN(C)C1=CC=C(C=C1)C(=O)C2=CC=CC=C2
27	19241	N-Butylbenzenesulfonamide	CCCCNS(=O)(=O)C1=CC=CC=C1
28	19241	N-Butylbenzenesulfonamide	CCCCNS(=O)(=O)C1=CC=CC=C1
29	31292	Octadecanamide	CCCCCCCCCCCCCCCCCC(=O)N
30	31292	Octadecanamide	CCCCCCCCCCCCCCCCCC(=O)N
31	69421	hexadecanamide	CCCCCCCCCCCCCCCC(=O)N
32	76468	Docosanamide	CCCCCCCCCCCCCCCCCCCCCC(=O)N
33	81531	Diisopropylethylamine	CCN(C(C)C)C(C)C
34	638011	Citral	CC(=CCCC(=CC=O)C)C
35	5281576	Zearalenone	CC1CCCC(=O)CCCC=CC2=C(C(=CC(=C2)O)O)C(=O)O1
36	5281794	Shogaol	CCCCCC=CC(=O)CCC1=CC(=C(C=C1)O)OC
37	5282309	N,N-Dimethylsphingosine	CCCCCCCCCCCCCC=CC(C(CO)N(C)C)O
38	5283387	Oleamide	CCCCCCCCC=CCCCCCCCC(=O)N
39	5283449	alpha-Linolenoyl ethanolamide	CCC=CCC=CCC=CCCCCCCCC(=O)NCCO
40	5283454	n-Oleoylethanolamine	CCCCCCCCC=CCCCCCCCC(=O)NCCO
41	5365371	Erucamide	CCCCCCCCC=CCCCCCCCCCCCC(=O)N
42	6436624	Ethyl Palmitoleate	CCCCCCC=CCCCCCCCC(=O)OCC

Follicle isolation

The ovaries were taken with sterile tweezers. The ovaries were measured (initial), and the follicles were isolated using a surgical blade on the follicles measuring 2 - 3 mm (Secondary follicles), inserted into a petri dish that contained 0,9% physiological NaCl fluid.[14–17]

In-vitro Growth Culture

There were 16 follicles cultured from 10 ovaries. 16 follicles were cultured in 4 treatment groups so that 1 treatment group consisted of 4 follicles, namely control follicles, 4 *C. barbata* dose 1, dose 2, and dose 3. During culture, 1 follicle was placed in 1 petri dish. 1 group with 4 repetitions. Everything that is done is monitored, namely follicle development, estradiol levels and LHR expression. Invito is carried out in culture using basic culture media, namely TCM-199, FBS 10 etc. In this study, PMSG HCG was used which is analogous to FSH-LH.

Maturation medium used Tissue Culture Medium-199 (TCM-199), serum supplementation (FBS 10%), Paravin oil, and PMSG + HcG as control, while the treatment group added a dose of *C. barbata* leaf extract, namely 25 ppm, 50 ppm, and 100 ppm.[11,14,15] Observation of follicular development was carried out for six days. Follicle monitoring time is divided into 2, namely day 0 and day 6.[15–17] Observations used an inverted microscope and a stereo microscope with 200x magnification.[14,15] On the 6th day, the cumulus expansion and polar bodies were observed using an inverted microscope with 200x magnification.

Table 2. Affinity score between estrogen receptor and ligand using PyRx software in kcal/mol unit

Receptor	Ligand	Binding Affinity (kcal/mol)
Estrogen receptor (ID 1A52)	Estradiol	-10.7
	Zearalenone	-9.7
	Bis(2-ethylhexyl) phthalate	-9.6
	Benzanthrone	-9.2
	Octytldecyl phthalate	-7.7
	Coclaurine	-7.6
	Alverine	-7.6
	4-(Dimethylamino)benzophenone	-7.2
	Shogaol	-7.2
	alpha-Linolenoyl ethanolamide	-7.1
	5,8,11,14-Eicosatetraynoic acid	-7
	Dibuthylphthalate	-6.9
	Erucamide	-6.9
	Oleamide	-6.6
	n-Oleoylethanolamine	-6.5
	Ethyl Palmitoleate	-6.5
	Docosanamide	-6.5
	N,N-Dimethylsphingosine	-6.4
	Octadecanamide	-6.1
	N-Butylbenzenesulfonamide	-5.9
	Phenylalanine	-5.9
	Citral	-5.9
	hexadecanamide	-5.9
	O-Toluidine	-5.2
	Nicotinic acid	-5.2
	Thymine	-5
	L-pyroglutamic acid	-5
	Pipecolic acid	-5
	Adenine	-4.9
	Carnitine	-4.7
	4-Guanidinobutyric acid	-4.7
	Cystosine	-4.7
	Isoleucine	-4.7
	Melamine	-4.6
	DL-Lysine	-4.6
	Uracil	-4.5
	Valine	-4.5
	Diisopropylethylamine	-4.4
	D-proline	-4.4
	Isoamylamine	-3.9
	Betaine	-3.6
	Choline	-3.3

Statistical data analysis and ethical statement

The statistical data analysis used One Way Anova test (F test). the Multiple Comparisons test using LSD (Least Significant Different). To prove the closeness of the relationship between 2 measured variables, the Pearson correlation test was used. This study was approved by the Ethics Committee of Faculty of Medicine, Brawijaya University with clearance no. 167/EC/KEPK-S3/05/2019 on May 23rd 2019.

RESULTS AND DISCUSSION

Molecular docking analysis

Docking analysis (Table 2-3 and Figure 1-2) revealed that there are four novel compounds in *C. barbata*, including Zearalenone, Bis(2-ethylhexyl) phthalate, Benzanthrone, and Octytldecyl phthalate have stronger affinity -9.7; -9.6; -9,2; and -7.7 kcal/mol, respectively, than Co-calurine which is only -7.6 kcal/mol based on our previous finding.[11] Even though the compounds affinity is still weaker than the Estradiol, however, there is assumption of potency in those four compounds. The amino acid involved in the interaction after docking analysis is shown in Table 3.

Table 3. The amino acid involved in the interaction after docking analysis

Receptor	Ligand	Amino acids
Estrogen receptor	Estradiol	GLY521, LEU428, MET343, LEU349 (VDW); HIS524, ARG394, GLU353 (HYDRO); ILE424, LEU384, MET388, LEU387, ALA350, LEU391, PHE404, MET421, LEU525, LEU346 (ALKYL)
	Zearalenone	LEU391, LEU384, MET388, GLY521, LEU525, MET343, THR347, LEU349, GLU353 (VDW); LEU387, HIS524 (HYDRO); ALA350, PHE404, PHE425, LEU428, ILE424, MET421 (ALKYL)
	Bis(2-ethylhexyl) phthalate	MET343, HIS524, GLY521, MET421, PHE425, LEU428, LEU349, GLU353, THR347 (VDW); ARG394 (HYDRO), LEU387, PHE404, LEU391, LEU384, MET388, ILE424, TRP383, ALA350, LEU346, LEU525 (ALKYL)
	Benzanthrone	ARG394, GLU353, THR347, MET343, LEU525, HIS524, MET388, MET421, MET388, LEU428, LEU384, ILE424, GLY521 (VDW), LEU387, LEU391, PHE404, ALA350, LEU346 (ALKYL)
	Octytldecyl phthalate	LEU428, PHE425, MET421, GLY521, TRP383, MET388, LEU384, LEU346, PHE404, ALA350, MET343, THR347, LEU349, GLU353, ARG394 (VDW); LEU391, LEU387, ILE424, HIS524, LEU525 (ALKYL)
	Co-claurine	ILE424, TRP383, GLY521, LEU384, LEU428, LEU387, LEU346, MET421, ALA350 (VDW); THR347, MET343 (HYDRO); LEU525, LEU391, PHE404, MET388 (ALKYL)

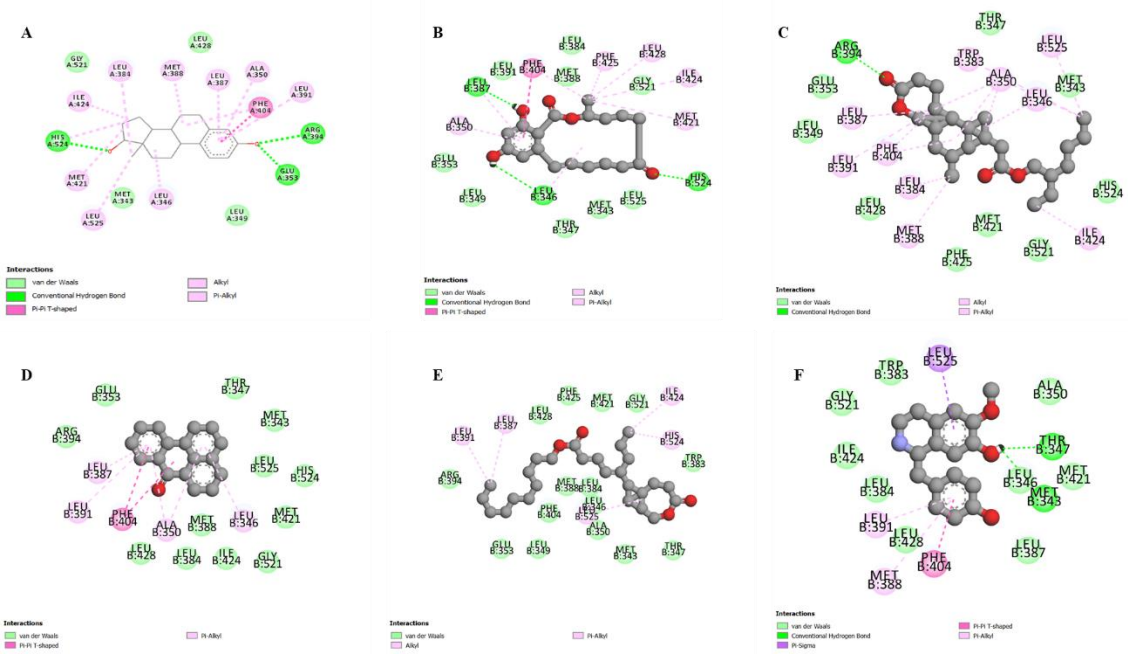

Figure 1. Molecular interaction involved in estrogen receptor and Estradiol (A), Zearalenone (B), Bis(2-ethylhexyl) phthalate (C), Benzanthrone (D), Octytldecyl phthalate (E), and Co-claurine (F)

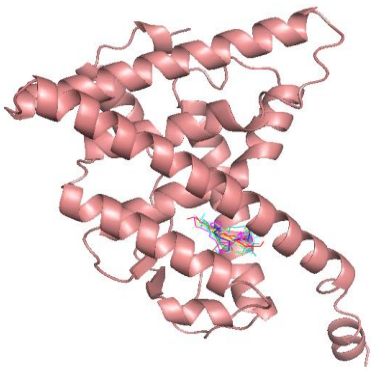

Figure 2. Visualization of molecular docking analysis by PyMol software

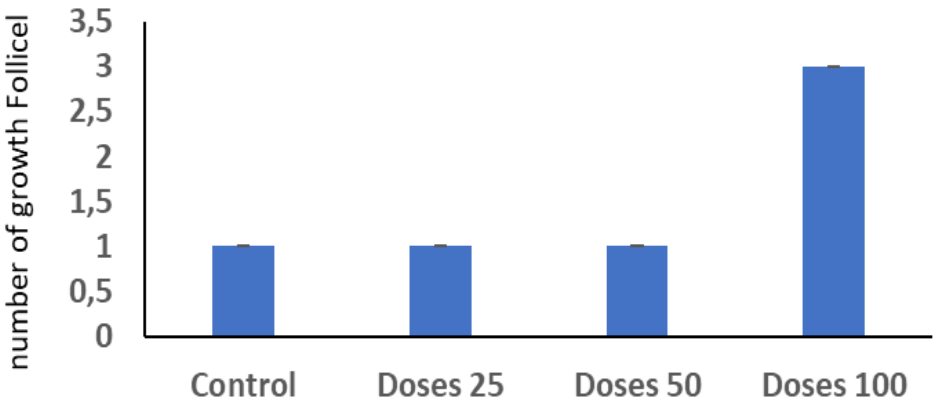

Figure 3. Description of follicle development given by *C. barbata* extract

C. barbata ethanol extract induces follicle development

The results showed that *C. barbata* ethanol extract at a dose of 100 ppm increased oocyte maturation significantly/not significantly compared to control. There were four treatments are as control and three doses of *C. barbata* extract (25 ppm, 50 ppm, and 100 ppm). Observations were made using an inverted microscope and a stereo microscope with 200x magnification.[11] The observations can be seen in Figure 3. The most oocyte maturation was obtained at a dose of 100 ppm, namely three follicles (75%), while the doses 25 and 50 were one follicle (25%).

Estradiol

Based on the Anova one way test results on 17ß-estradiol level data between the control group and three treatment groups of *C. barbata* at a dose of 25 ppm, 50 ppm, and 100 ppm, it was found that there was a significant difference in the mean 17ß-estradiol levels. Estradiol in the four groups of the observation sample is indicated by the p-value = 0.000 <α. The multiple comparison test with the Least Significant Difference (LSD) test is shown in full, presented in Table 4.

Luteinizing hormone receptors

Based on the results of the one-way Anova test on LHR expression data between the control group and three treatment groups of *C. barbata* leaf extract at 25 ppm, 50 ppm, and 100 ppm, there was a significant difference in the mean LHR expression. The four groups of observation samples indicate this: the p-value = 0.000 <α Furthermore, the multiple comparison test with the LSD test is shown in full as presented in Table 5. LHR expression is also explained in Figure 4.

Table 4. Comparison test results of 17ß-estradiol levels in the treatment of *C. barbata* leaf ethanol extract

Observation groups	Mean ± SD	p-value
Control	28.32±1.90[a]	0.000<α
Extract 25 ppm	30.08±1.10[ab]	
Extract 50 ppm	32.28±2.07[b]	
Extract 100 ppm	36.83±2.33[c]	

Note: The column mean ± sd shows the results of the LSD test; if it contains different letters, it means that there is a significant difference (p-value <0.05), and if it contains the same letter, it means that there is no significant difference (p-value> 0.05).

Table 5. The results of the LHR expression comparison test in the treatment of *C. barbata* leaf extract

Observation groups	Mean ± SD	p-value
Control	83820±41669[a]	0.000<α
Extract 25 ppm	152653±8012[b]	
Extract 50 ppm	187302±13617[b]	
Extract 100 ppm	261874±54606[c]	

Note: The column mean ± SD shows the results of the LSD test; if it contains different letters, it means that there is a significant difference (p-value <0.05), and if it contains the same letter, it means that there is no significant difference (p-value> 0.05).

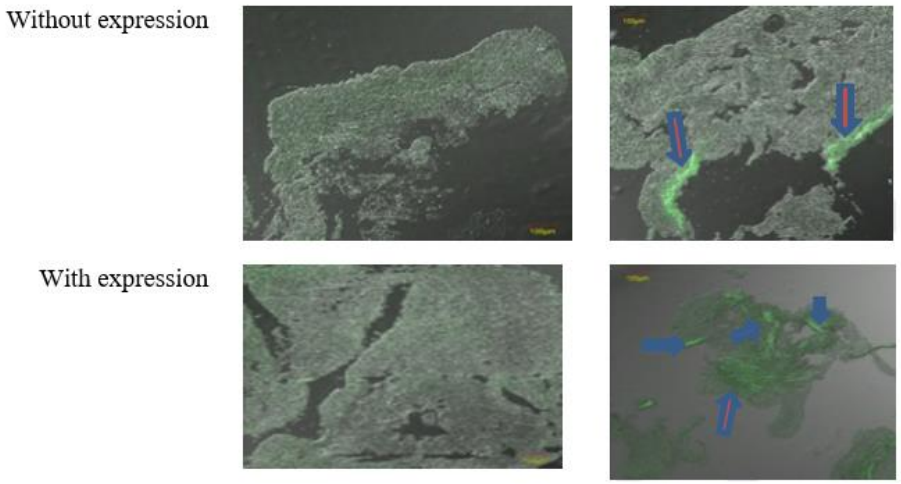

Figure 4. LHR expression comparison test in the treatment of *C. barbata* leaf extract.

This study has several notable strengths, including the successful design of a follicular model that effectively mimics hormonal disturbances, allowing for a controlled assessment of *C. barbata*'s impact on ovarian follicle development. Additionally, the findings clearly demonstrate the efficacy of the ethanol extract in enhancing key pre-ovulatory processes, such as steroidogenesis and follicular maturation. However, certain limitations must be acknowledged. The optimal dosage required to induce a robust positive feedback mechanism in follicular development remains undetermined, highlighting the need for further dose-response studies. Moreover, since the study was conducted exclusively under in vitro conditions, the applicability of these findings to in vivo systems remains uncertain. Future research involving animal models would help validate these results and provide deeper insights into the extract's reproductive effects.

CONCLUSION

C. barbata leaf extract was able to increase follicle development and to induce the development of secondary follicles into mature follicles, as evidenced by the presence of oocytes that have perfect cumulus and the presence of polar bodies. It was obtained that the most optimum dose of *C. barbata* leaf extract to increase the level of estradiol was 100 ppm. *C. barbata* extracts increased LHR expression to induce the development of secondary follicles. Similarly, it was obtained that the most optimum dose of *C. barbata* leaf extract to increase LHR expression was the dose of 100 ppm.

Future studies should explore alternative pathways regulating follicular development and establish an in vivo model using *C. barbata* leaf extract to examine the role of the luteinizing hormone surge in ovulation. Further studies are also needed to determine the optimal dosage of *C. barbata* leaf extract capable of inducing a positive feedback mechanism in follicular maturation. Moreover, clinical trials should assess its effects on antral-phase follicular growth and evaluate its potential

as a standardized herbal therapy for managing follicular development disorders in infertile women.

DISCLOSURES

Acknowledgment

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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Author contribution

NN: Conceptualization, Methodology, Validation, Investigation, Writing- Original draft preparation. YY: Data curation, Writing- Original draft preparation. BR: Visualization, Investigation, Writing- Reviewing and Editing. GWP: Validation, Writing- Reviewing and Editing.

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