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Original Research Article

Antioxidant activity of self-nanoemulsifying drug delivery system (SNEDDS) of Curcuma longa in polycystic ovary syndrome rat model

Alfaina Wahyuni[1,2]*, Ambar Mudigdo[1,3], Sutrisno Soetrisno[1], Brian Wasita[1,3], Uki Retno Budi[4], Vitri Widyaningsih[1,5], Lutfi Chabib[6]

[1]Doctoral Program of Medical Science, Faculty of Medicine, Universitas Sebelas Maret, Surakarta 57126, Central of Java, [2]Department of Obstetrics and Gynecology, Faculty of Medicine and Health Science, Universitas Muhammadiyah Yogyakarta, Bantul 55183, Special Region of Yogyakarta, [3]Department of Anatomical Pathology, [4]Department of Obstetrics and Gynecology, [5]Public Health and Preventive Medicine, Faculty of Medicine, Universitas Sebelas Maret, Surakarta 57126, Central of Java, [6]Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Islam Indonesia, Sleman 55281, Special Region of Yogyakarta, Indonesia

For correspondence:* ***Email:* *alfaina.wahyuni@umy.ac.id;* ***Tel:*** *+6281392624544*

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Abstract

Purpose: *To investigate the effect of SNEDDS containing Curcuma longa extract on hormonal and histological improvement of polycystic ovary syndrome (PCOS) rat model.*
Method: *A total of 36 female Wistar rats, aged between 8 and 10 weeks, were randomly divided into 6 groups, including control (normal rats), PCOS (untreated PCOS rats), PCOS + M (treated with 20 mg/kg bw/day metformin), PCOS + SC25, PCOS + SC50, and PCOS + SC100 (treated with Curcuma longa extract at 25, 50, and 100 mg/kg/day with SNEDDS, respectively). This study used a posttest-only control group design. The rats were euthanized on day 15, and blood samples were taken to examine malondialdehyde (MDA) and anti-mullerian hormone (AMH) levels. Ovarian tissue was prepared on a histological slide, and various follicles were observed. Data was analyzed using analysis of variance (ANOVA) and Kruskal-Wallis tests.*
Results: *Curcuma longa extract, at 50 and 100 mg/kg, incorporated in SNEDDS resulted in a significant reduction in the corpus luteum number and width of the granulosa layer ($p < 0.05$). Also, Curcuma longa significantly reduced malondialdehyde (MDA), anti-mullerian hormone (AMH), preantral follicles, and follicular cysts ($p < 0.05$).*
Conclusion: *Curcuma longa extract in SNEDDS reduces MDA and AMH levels and improves the histology of the ovaries in PCOS model rats. There is a need to conduct further studies of C. longa extract or isolates in SNEDDS using human cell lines of PCOS model.*

Keywords: *Antioxidant, SNEDDS, Curcuma longa extract, Polycystic ovary syndrome*

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a prevalent endocrine condition characterized by a combination of higher levels of androgen and irregular periods that affect 5 to 10 % of women around the world. Approximately 50 to 70 % of women diagnosed with PCOS present with a wide range of symptoms including excess androgen [1]. Also, 65 to 95 % of women with

PCOS showed higher levels of insulin resistance compared to control group [2,3]. Improving lifestyle, diet, exercise, anti-hyperglycemic and hormone drugs are employed to manage PCOS. However, these drugs often present with adverse effects, such as joint or muscle pain, nausea, vomiting, diarrhea, and psychological disorders, reducing quality of life and increasing medication non-adherence.

Developing alternative treatment strategies for women with PCOS is therefore required, especially natural remedies. Turmeric, derived from the stem of *Curcuma longa L*. (Zingiberaceae), is rich in polyphenol curcumin (diferuloymethane). Historically, turmeric has been widely used in traditional medicine to treat various human ailments, such as colic, gynecological disorders, and menstrual issues. Extensive use of curcumin in treating these conditions highlights its significant therapeutic effect as an antioxidant and anti-inflammatory [4]. However, several studies have shown that the limited bioavailability of turmeric results from inadequate absorption, insolubility in water, rapid metabolism, and rapid systemic elimination. Hence, large doses are needed to obtain the expected effect. Many strategies exist to increase its bioavailability, such as providing curcumin in nanoparticles, including SNEEDS [4].

The self-nanoemulsifying drug delivery system enhances the permeation of the formulation through the lipid bilayer membrane, thereby increasing absorption into the systemic circulation. Advantages of SNEEDS are small drug doses, few side effects and gastric irritation, controlled drug release, increased maximum concentration, and increased area under the curve (AUC) [5]. This study investigated the antioxidant activity, safety, and hormonal effect of SNEDDS of *Curcuma longa* extract.

EXPERIMENTAL

Animal

This study used the post-test-only control group design method. A total of 36 female *Rattus norvegicus* strain Wistar rats weighing 100–130 g at 8 – 10 weeks old were used for the study. Furthermore, the rats were kept in an accredited veterinary laboratory at the Faculty of Pharmacy, Gadjah Mada University in Yogyakarta, Indonesia to acclimatize under standard conditions (12 h light-dark cycle, 40 – 60 % humidity and 25 – 28 °C) for 1 week. During this period, all animals were fed with normal feeds and water, and the oestrus cycles were examined. The study was approved by the Health Research Ethics Committee of Muhammadiyah University of Yogyakarta (048/EC-KEPK FKIK UMY/VI/2021). All animals were cared for in compliance with the internationally accepted guide for the care and use of laboratory animals, published by the US National Institutes of Health [6].

Preparation of SNEDDS of *Curcuma longa* extract

The curcumin used in SNEDDS was extracted from *C. longa* (containing 73.95 % curcumin and 90.35 % curcuminoids (Arjuna Natural Extracts Ltd, India)). *C. longa* (250 mg) extract powder was dissolved in capryol 90 (Gattefosse, France) to incorporate curcumin into SNEDDS. Thereafter, 1 mL propylene glycol (Brataco Chemika, Indonesia), was added (co-surfactant) and the solution was stirred using a magnetic stirrer (500 rpm for 15 min). Subsequently, 6 mL of Tween 20 (Merck, Germany) (surfactant) and cremophor RH were added. With this formulation, 1 mL of SNEDDS contained 31.25 mg of *C. longa* extract as nanoparticles.

Induction of PCOS

This study employed a modification from a prior study to establish the PCOS model by mixing letrozole (Tokyo Chemistry Industry Co. Ltd, Japan) with a diet rich in cholesterol and fructose [7]. A total of 36 rats were randomly and equally assigned into 6 groups. The PCOS-model group comprised 30 rats that received daily intragastric administration of letrozole in 0.5 % carboxymethylcellulose (CMC) (Tokyo Chemistry Industry Co. Ltd., Japan) solution (1 mg/kg/day). In addition, the rats were fed a diet rich in fat and fructose, approximately 20 g/day per rat, and had access to free water for 21 days. The estrous cycles were monitored daily by taking vaginal swabs and staining with Giemsa. Polycystic ovarian syndrome (PCOS) developed when the estrous cycle was subjected to a series of modifications, reaching the stage of persistent vaginal cornification.

Study design

The rats were randomly assigned to six groups; control, PCOS (negative control), PCOS + metformin 20 mg/kg/day (Tokyo Chemistry Industry Co. Ltd, Japan) (PCOS + M), PCOS + SNEDDS curcumin 25 mg/kg/day (PCOS + SC25), PCOS + SNEDDS curcumin 50 mg/kg/day (PCOS + SC50), and PCOS + SNEDDS curcumin 100 mg/kg/day (PCOS + SC100). Metformin is a standard therapy for

PCOS used as a positive control [8]. Study by Abuelezz *et al* [9], with modifications based on the findings of earlier studies, was used to obtain the dosage of nanocurcumin. Metformin and curcumin in SNEEDS were administered orally for 14 days. Additional procedures included administering mild anesthetic ether to the rats, allowing the rats to undergo 12 h without food.

Evaluation of parameters/indices

Characterization of SNEDDS

The particle size analyzer and zeta potential analyzer were used to calculate average particle size, polydispersity index, and zeta potential (Horiba SZ-100).

Levels of malondialdehyde (MDA)

Venous blood (3 mL) was drawn from the retro-orbital venous plexus on day 15 using a capillary tube that contained heparin. The samples were kept in tubes, and centrifuged for 10 min to separate the serum and levels of MDA were investigated.

Levels of anti-mullerian hormone

Venous blood (3 mL) was drawn from the retro-orbital venous plexus on day 15 using a capillary tube that contained heparin. The samples were kept in tubes, and centrifuged for 10 min to separate the serum, and then the levels of anti-mullerian were determined.

Ovarian histology

The sample was stored at -20 °C in preparation for ELISA analysis (Bioenzy). The ovaries were surgically extracted, immersed in a 10 % formalin buffer for 48 h, and prepared in paraffin blocks. Thereafter, strips of ovarian tissue were cut and stained with H & E. A 40x and 100x optical microscope was utilized to analyze histology images. Several primary follicles, preantral follicles, antral follicles, corpus luteum, and follicular cysts, including thickness of the granulosa cell and theca layer of the antral follicles, were obtained in five sections.

Data analysis

The data were analyzed using Statistical Packages for Social Sciences (SPSS) version 19.0 (IBM, Armonk, NY, USA). The Shapiro–Wilk test was used to evaluate the normality of the data. Normally distributed data (MDA, AMH number of follicles, and granulosa layer) were analyzed using parametric one-way analysis of variance (ANOVA) and post-hoc LSD tests. Non-parametric tests such as the Kruskal–Wallis and Mann-Whitney tests were used for comparing data that did not follow a normal distribution. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of SNEEDS of *Curcuma longa*

Mean particle size of *C. longa* was 14.8 nm, the average poly-dispersibility index was 0.163, and the average zeta potential was 15.1 mV. This suggests that SNEDDS of *C. longa* formulated were in the nanometer range (Table 1).

Table 1: Characteristics of nanoparticles

Peak no.	Particle size (nm)	Poly-dispersibility index (PI)	Zeta potential (mV)
1	14.9	0.119	15.2
2	14.2	0.261	14.7
3	15.4	0.109	15.5

Malondialdehyde (MDA) levels

As shown in Figure 1, the PCOS group exhibited significantly higher MDA compared to other groups ($p < 0.05$). However, SNEDDS of *Curcuma longa* extract showed dose-dependent reduction in MDA, and higher dose led to a significant reduction in MDA compared to PCOS group ($p < 0.05$).

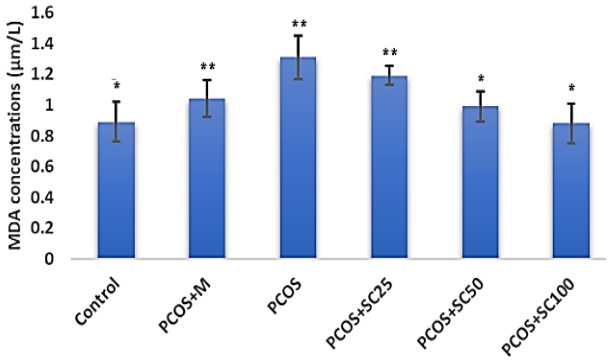

Figure 1: Malondialdehyde (MDA) levels in each group. ***Note:*** **P* < 0.05 vs PCOS group, ***p* < 0.05 vs control group

Anti-mullerian hormone (AMH) levels

The findings indicated that PCOS group exhibited significantly higher AMH levels compared to other groups ($p < 0.05$). However, SNEDDS of *Curcuma longa* extract showed dose-dependent reduction in MDA, and higher doses led to a significant reduction in MDA

compared to PCOS group (p < 0.05), and had same effectiveness as metformin.

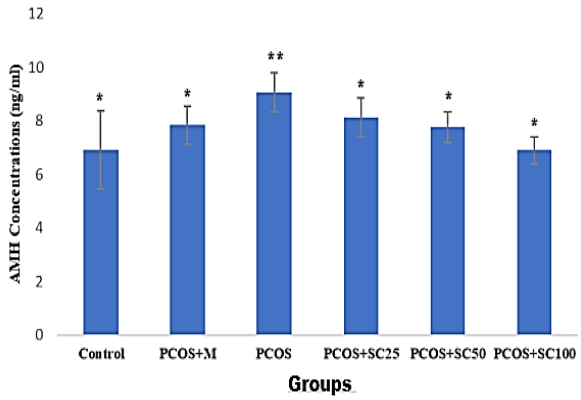

Figure 2: Anti-mullerian hormone levels. ***Note:*** *P < 0.05 vs PCOS group, **p< 0.05 vs control group

Ovarian histopathology

Number of follicles

Several follicles observed were primary, secondary, preantral, antral, corpus luteum, and follicular cysts (Figure 3).

Number of corpuses luteum, follicular cysts, and preantral follicles

There was no significant difference in primary follicle count (Figure 4 A), and antral follicle count (Figure 4 C). Also, there was significant difference in preantral follicles (Figure 4 B), follicular cysts (Figure 4 D), and total number of corpuses luteum (Figure 4 E) in all groups (p < 0.05). Therefore, administration of *C. longa* extract through SNEDDS at doses of 50 and 100 mg/kg BW significantly decreased the number of preantral follicles, antral follicles, and follicular cysts compared to PCOS group (p < 0.05), and corpus luteum was elevated to a level comparable to metformin.

Granulosa and theca layer thickness

Average thickness of the granulosa and theca layer was significantly lower in PCOS group compared to the other group (p < 0.05). Thickness of the granulosa and theca layer in PCOS + SC50 and PCOS + SC100 groups were comparable to control group. These results showed that PCOS reduces the thickness of the granulosa and theca layers, and administration of *C. longa* with SNEDDS at 50 and 100 mg/kg BW thickened both layers (Figure 5).

DISCUSSION

In addition to its potent antioxidant and anti-inflammatory effects, curcumin increases insulin sensitivity [8]. This study demonstrated that SNEDDS of *C. longa* at 50 and 100 mg/kg bw reduces MDA and AMH levels. Furthermore, SNEDDS enhanced the histopathological picture in the ovaries of PCOS-model rats by decreasing the number of preantral follicles, antral follicles, and follicular cysts and increasing the number of corpuses luteum, granulosa cell and theca layer thickness.

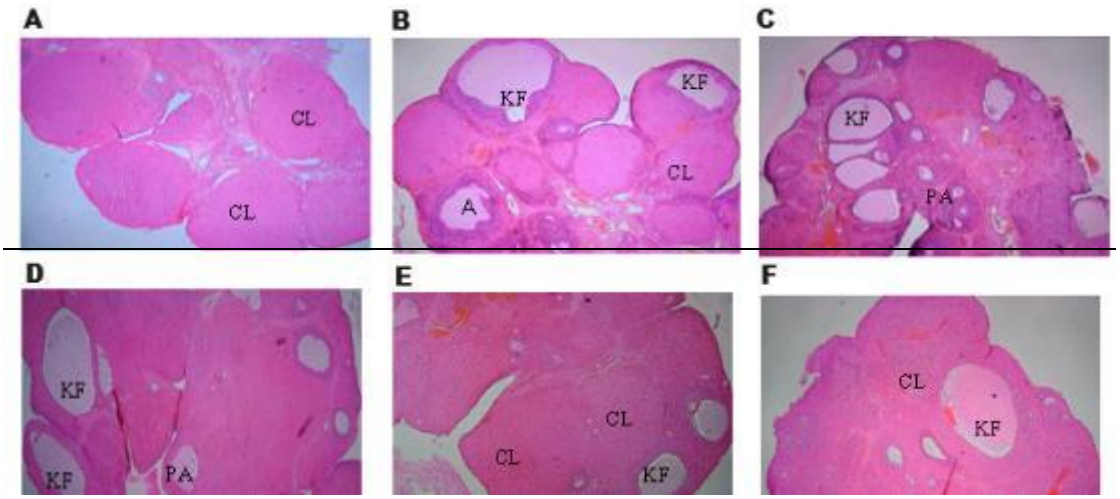

Figure 3: Ovary histology. Corpus luteum predominated in the ovaries of control group (A). The PCOS group had many follicular cysts and atretic follicles. Follicular cysts count decreased, and corpus luteum grew in PCOS rats treated with metformin (C) and curcumin in SNEDDS (D, E, F). (A) control, (B) PCOS + M, (C) PCOS, (D) PCOS + SC25, (E) PCOS + SC50, (F) PCOS + SC100 group rat. ***Note:*** Histological preparations with hematoxylin-eosin staining; 40x magnification. PF: primary follicle; PA: preantral follicle; A: antral follicle; CF: cysts follicular; CL: corpus luteum

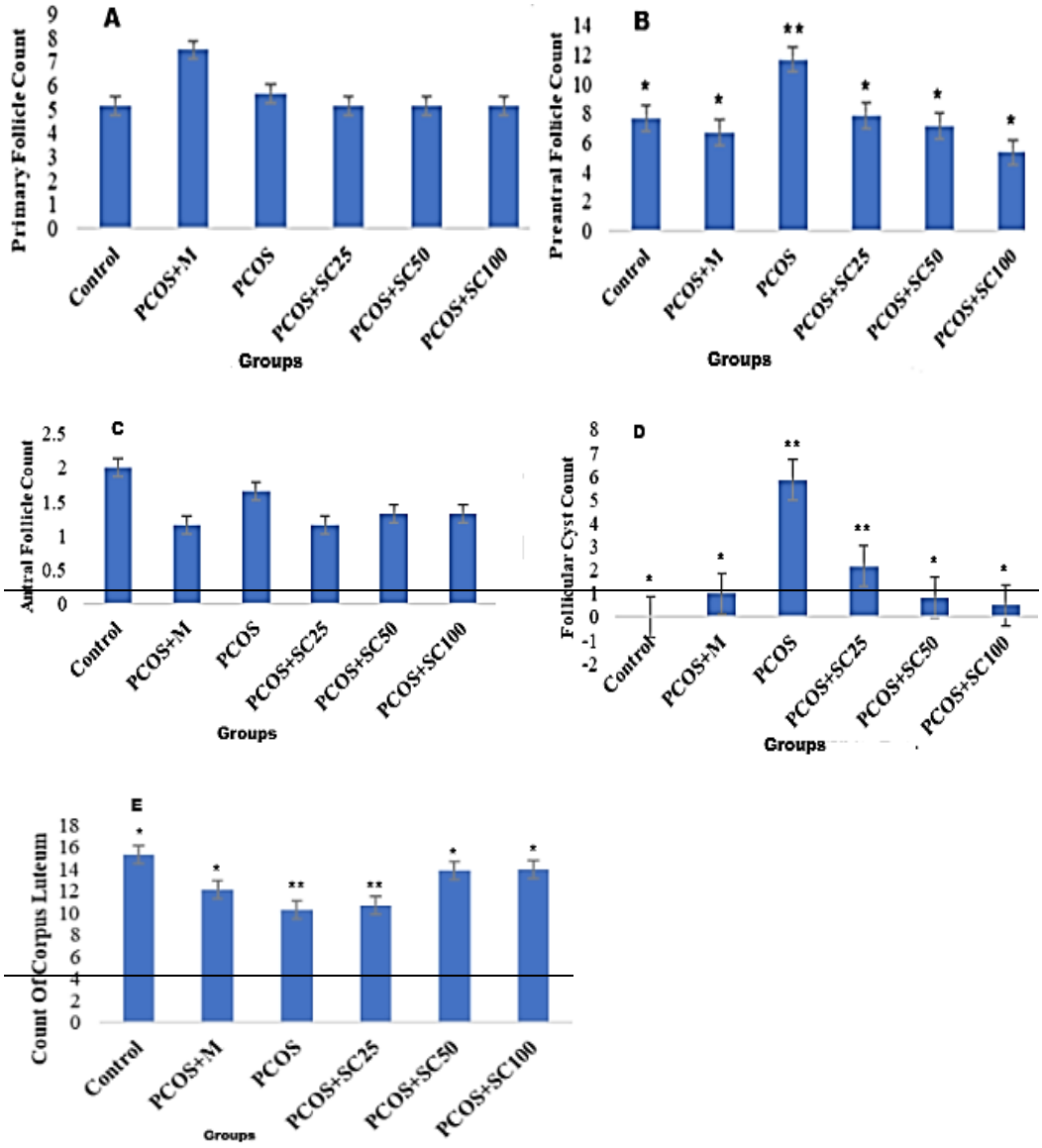

Figure 4: Number of follicles in each group. ***Note:*** *$P < 0.05$ vs PCOS group, **$p < 0.05$ vs control group

The results also showed that *C. longa* extract in SNEDDS was as effective as metformin in improving PCOS abnormalities. The most effective treatment for PCOS was *C. longa* extract in SNEDDS at 50 mg/kg bw daily for 14 days. Increased MDA is one of the parameters of lipid peroxidation of polyunsaturated fatty acids, which increases oxidative stress in the body. Ovarian oxidant-antioxidant status becomes imbalanced due to excess androgen in PCOS, leading to an increase in reactive oxygen species (ROS).

Excess ROS in the ovarian follicular fluid environment inhibits follicle development, ovulation, and oocyte quality [9]. High free fatty acid increases oxidative stress and releases pro-inflammatory cytokines, leading to insulin resistance [10], and exacerbated oxidative stress [11]. Other studies proved that curcumin reduced oxidative stress in PCOS rat models. For example, administration of pure curcumin at 100 and 200 mg/kg for 14 days decreases the activity of SOD enzyme [12], and at the same dose for 30 days, curcumin increases glutathione peroxidase [13].

Granulosa cells of small antral follicles secrete an anti-mullerian hormone (AMH), and production of AMH decreased as the antral follicle grew. Normal androgen levels played a role in decreasing AMH levels. Also, PCOS-related hyperandrogenism increases AMH levels by 2 - 3-fold. Hyperandrogenism and elevated AMH levels alter follicular development, decrease preantral follicle apoptosis, and reduce estradiol levels [14].

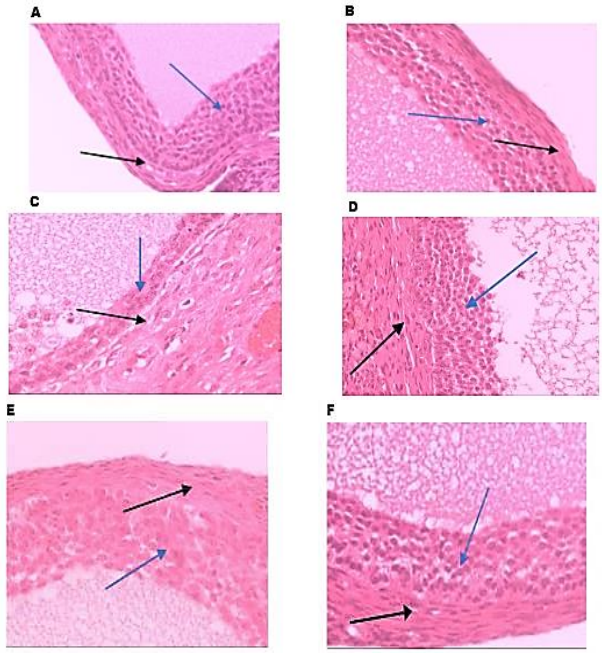

Figure 5: The layer thickness of granulosa cells and theca cells of antral follicle. (A) control group, (B) PCOS+M group, (C) PCOS group, (D) PCOS+SC25 group, (E) PCOS+SC50 group, (F) PCOS+SC100 group. Layer of granulosa cells and theca cells in PCOS group looked the thinnest among other groups. ***Note:*** Histological preparation with hematoxylin-eosin staining (400x magnification). → granulosa cell layer, → theca cell layer)

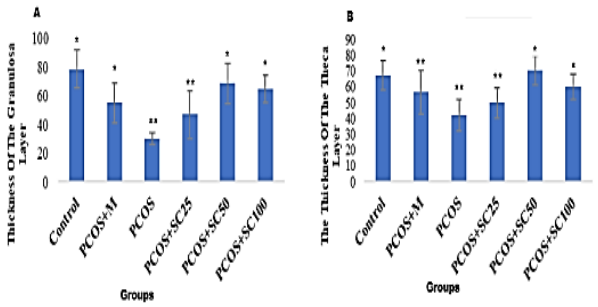

Figure 6: Mean score for granulosa and theca layers thickness. ***Note:*** *$P < 0.05$ vs PCOS group, **$p < 0.05$ vs control group

C. longa extract in SNEDDS at 50 and 100 mg/kg bw improved the histology of the ovaries in PCOS-induced rats by decreasing the number of preantral follicles, and follicular cysts, increasing the number of *Corpus luteum*, and improving the thickness of the granulosa cell and theca layer. In PCOS, hyperandrogenism and insulin resistance played a direct and indirect role in hormonal imbalance due to dysfunction of the hypothalamus-pituitary-ovarian axis [16]. Increased ROS in the ovary inhibited the process of steroidogenesis. This disruption is initiated by an augmentation in the frequency and intensity of GnRH release, resulting in increased LH production compared to FSH, and the follicles failed to grow leading to AMH over-secretion. Follicular dysplasia is related to low-energy supply in granulosa cells due to decreased glycolysis which accelerated granulosa cells apoptosis [17]. Hyperandrogenism affected follicular maturation by inhibiting GC proliferation through increasing PPAR-γ-dependent expression of PTEN/p-Akt [18]. Administration of curcumin and physical exercise reduces this oxidative stress, thereby preventing apoptosis [19]. Studies have shown that nanocurcumin at 55 mg/kg for 20 days reduces ovarian volume, number of follicles, BAX, and caspase-3 and increases BCl2 expression in DHEA-induced PCOS rats [20].

Limitations of the study

No assessment of PCOS severity or IR features was conducted on the PCOS-induced rats, which explains why the results may be inconsistent. To induce PCOS in rats, a combination of letrozole and a diet rich in cholesterol and fructose. This study did not determine if the dietary modification from a diet rich in cholesterol and fructose during PCOS induction to a regular diet during therapy had a beneficial impact on PCOS.

CONCLUSION

Curcuma longa extract in SNEDDS at doses of 50 and 100 mg/kg//day for 14 days reduces malondialdehyde, anti-mullerian hormone levels, number of preantral and cystafollicular follicles, but increases the number of corpus luteum and thickness of the granulosa and theca layers in polycystic ovary syndrome (PCOS) model. However, there is a need to investigate *C. longa* extract SNEDDS using human cell lines of PCOS model.

DECLARATIONS

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Ethical approval

Approval was granted by the Health Research Ethics Committee of Muhammadiyah University of Yogyakarta, Indonesia (048/EC-KEPK FKIK UMY/VI/2021).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Alfaina Wahyuni carried out conceptualization, methodology, and supervision; Ambar Mudigdo performed resources and formal analysis; Soetrisno conducted investigation and resources; Brian Wasita carried out methodology and formal analyses; Uki Retno B participated in project administration and writing the original draft; Vitri Widyaningsih participated in resources and writing the original draft. All authors contributed to the review, editing, and final correction of the manuscript.

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