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Original Article

The Effect of *Syzygium Polyanthum* (Wight) Walp. Extract on Glutathione, Tumour Necrosis Factor-Alpha, Spdl1, And Degraff Follicles Expression in PCOS Rat Models

Renny Aditya[1], Budi Santoso[2,*], Widjiati[3]

ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is an endocrine and reproductive disorder affecting 5% to 13% of women of reproductive age. Approximately 80% of women with PCOS underwent infertility cases. Many studies have shown an association between PCOS and low-grade inflammation. *Syzygium polyanthum* (*S. polyanthum*) contains antioxidants and has antiinflammation activity. **Objectives:** This study aims to investigate the effectiveness of *S. polyanthum* leaf on glutathione, tumour necrosis factor-alpha (TNF-α), sPDL1, and DeGraff follicle expression in PCOS rat models. **Materials and Methods:** The female Wistar rats were divided into five groups (n = 5), K0 (normal control), K1 (PCOS group), and three treatment groups which received three different doses of *S. polyanthum* extract. The treatment group consisted of PCOS rat models with *S. polyanthum* extract supplementation of 150 mg/KgBW (P1), 300 mg/KgBW (P2), and 450 mg/KgBW (P3). **Results:** *S. polyanthum* leaf extract significantly decreased TNF-α expression in the treatment group. The highest TNF-α, GSH, and sPDL1 concentration was found in the P3 group (3.83±0.123, 273.12±12.4 ng/ml, and 3.25 ng/ml, respectively). Moreover, the number of follicles was statistically significant in the P1, P2, and P3 groups (2.0, 3.0, and 3.50, respectively). The number of follicles in the P1, P2, and P3 groups was higher than in the positive control; however, it was lower in the negative control (K0) ($p<0.05$). **Conclusion:** *S. polyanthum* extract was significantly effective in decreasing TNF-α expression, as well as a significant increase GSH, sPDL1 and DeGraff folicle count in PCOS rat model.
Key words: DeGraff follicle count, GSH, sPDL1, *Syzygium polyanthum*, TNF-.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a global health concern for women of reproductive age, affecting 6.5-%8% of women worldwide. Hyperandrogenism, anovulation, menstrual irregularities, and polycystic ovaries are major symptoms of PCOS. Oxidative stress has been identified as a potential PCOS etiological factor. Infertility affects approximately 10% of women with PCOS. In recent years, PCOS has received considerable attention due to its severe effects on female reproduction.[1-3]

A study reported that the high level of reactive oxygen species (ROS) correlates with the pathogenesis of PCOS. A significant increase in oxidative stress was found in PCOS patients compared to women without PCOS.[4] Besides, inflammation is also reported as the hallmark and consequence of PCOS. The endocrine process regulates immunity and inflammatory response, and production of pro-inflammatory cytokines.[5] Inflammation, as well as oxidative stress, is reported to be associated with PCOS pathogenesis. The enhancement of ROS production is caused by peripheral blood leukocytes, pro-inflammatory transcription factor nuclear κappa B (NF-κB), and the increase of pro-inflammatory cytokines and C-reactive protein (CRP).[6,7]

Women with PCOS have been reported an increased prolidase activity in serum and have higher total oxidant status (TAS) and oxidative stress index (OSI) levels. In addition, reduced oxygen (O2) and GSH levels have been found in mitochondrial, along with increased ROS production. These conditions contribute to mitochondrial dysfunction in women with PCOS. Physiological hyperglycemia triggers an elevated level of ROS from mononuclear cells. Therefore, activation of tumor necrosis factor-α (TNF-α) and the inflammatory transcription factor nuclear factor-kappa B (NF-κB) will be increased. As a result, TNF-α, a known mediator of insulin resistance, will be further increased.[8,9]

Oxidative stress (OS) can have detrimental effects on female fertility by affecting ovulation, fertilization, embryo development and the overall outcome of pregnancy.[10-14] A meta-analysis and sensitivity analysis supposed that the higher TNF-α levels of PCOS patients compared to healthy controls was stable and reliable. This meta-analysis suggests that the circulating TNF-α levels in women with PCOS are significantly higher than in healthy controls.[15] Serum TNF-α levels may be elevated in adolescent patients with PCOS. A higher of TNF-α expression is related with infertility, atherosclerosis, and type 2 diabetes.[16]

Significantly elevated levels of serum TNF-α and interleukin 6 (IL-6) were found in patients with PCOS. They exhibited hypertriglyceridemia and chronic inflammation, with elevated peripheral lymphocytes, monocytes, and eosinophilic granulocytes. On the other hand, their ovaries demonstrated persistent chronic inflammation with a higher number of inflammatory cells infiltrating the follicles.[17] Hyperglycemia contributes to inflammation through the production of TNF-α. It is a known mediator of

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IR secreted by mononuclear cells (MNCs). The MNCs generate ROS, which causes cellular damage and activates nuclear factor-B, which stimulates TNF transcription. In this way, OS creates an inflammatory environment that contributes to hyperandrogenism and further increases IR.[2]

Soluble Programmed Death Ligand-1 (sPD-L1) has been identified in more than 20 distinct pathologies and frequently plays a crucial immunoregulatory function. sPD-L1 is a significant multifunctional circulating protein and frequently indicator of an inflammatory state. Expression of sPD-L1 have been observed in various pathologies, including infectious diseases, pulmonary diseases, chronic inflammatory and autoimmune disorders.[18,19] A study reported that the PD-1 gene and PD-L1 gene contribute to the pathogenesis of PCOS.[20] Serum levels of PD-1 and PD-L1 in PCOS patients were also significantly lower than those in the control group, suggesting that abnormalities of PD-1 and PD-L1 in the serum of PCOS patients may be associated with the onset of PCOS.[21]

PCOS is a condition with a significant decrease in serum antioxidant and vitamin levels. Antioxidant supplementations has been shown to improve insulin sensitivity in women with PCOS. The imbalance of oxidative stress (OS) in the follicular fluid environment of the ovaries can cause detrimental issues, such as poor oocyte development, embryo development and the overall outcome of pregnancy. PCOS is the most prevalent cause of anovulatory infertility.[10,22]

Based on the importance of inflammation and the stress-induced oxidative mechanism in PCOS, the use of anti-inflammatory and antioxidant agents as a treatment strategy for PCOS is urgently needed to investigate. The inhibition of herbal medicine shows a promising effect with minimum side effects. Several bioactive compounds in the herb were found to have a curative effect on PCOS, such as flavonoids and polyphenols.[23] Amini *et al.* (2015) reported that antioxidants and vitamins positively affect the management of women with PCOS.[24]

Syzygium polyanthum (Wight) Walp. (family *Myrtaceae*) has been used as folk medicine to treat various diseases related to inflammation and oxidative stress.[25] It is a tropical plant distributed all over Asian countries, including Indonesia, Malaysia, and Thailand. The leaves of *S. polyanthum* have benefit to treat cataracts, diarrhea, diabetes mellitus, hypercholesterolemia, and cardiovascular disease.[26] Besides, the fruits, roots, and barks of *S. polyanthum* are also commonly used for various traditional medicinal and non-medicinal purposes. The pharmacological properties of *S. polyanthum* leaves are anti-inflammatory, antioxidant, antidiabetic, antimicrobial, antihypertensive, antitumor, antidiarrheal, acetylcholinesterase inhibition, and lipase inhibitory properties.[27,28] Raw leaves and fruits of. *S. polyanthum* contain carbohydrates, tannins, alkaloids, steroids, triterpenoids, and flavonoids, while fruits contain saponins, carbohydrates, tannins, alkaloids, steroids, and flavonoids.[29]

Many studies showed the versatile application of herbal such as *S. polyanthum* in promoting health benefits. The herbal shows a promising effect with minimum side effects in treating disease. The pharmaceutical effect of *S. polyanthum* is related to the bioactive compounds, which are influenced by several factors, such as extraction methods, solvent, and part of the plant used for extraction.[30] Extraction is a process to separate active compounds or metabolites such as alkaloids, flavonoids, terpenes, and saponins using an appropriate solvent and extraction method.[31] The extraction method is one of the basic parameters that influence the quality of an extract, which can obtain bioactive compounds optimally other than the plant part and solvent for extraction.[32,33]

There are several methods for medicinal plant extraction, including conventional methods such as maceration, reflux, and Soxhlet, which has a relatively long duration, a large solvent used, and a probability of compound degradation.[34] These conventional extraction methods tend to be replaced by the eco-friendlier method, such as ultrasound-assisted extraction (UAE). The mechanical effect of UAE enhances the internal diffusion, thus affecting the mass transfer penetration of the solvent into the matrix and elevating the content to release.[35] Moreover, the UAE method is used to enhance the extraction efficiency and extractability of bioactive compounds.[36]

Several bioactive compounds in the herb have a curative effect on PCOS, such as flavonoids and polyphenols.[32] In addition, Amini *et al.* (2015) reported that antioxidants and vitamins positively affect the management of women with PCOS. S. *polyanthum* extract contains antioxidant that binds to ROS and inhibits the oxidative stress process in PCOS patients.[37] The ethanolic extract of S. *polyanthum* will reduce inflammatory factors. Therefore, the activation of the JNK and NF-κB pathways decreases.[35]

In the present study, the effect of S. *polyanthum* leaves extract on glutathione (GSH), TNF-α expression, sPDL1 and DeGraff follicle in PCOS rat models were investigated, employing ultra-assisted maceration. Their antioxidant and inflammation activity were also evaluated.

MATERIALS AND METHODS

Animals

This study was conducted from February to July 2022 at the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. Three-month-old female *Rattus norvegicus* of the Wistar strain weighed 150–200 grams were used in this study. *Rattus norvegicus* was selected because of its stable, shorter reproductive life, and short estrogen cycle, as well as its manageability and has been used as a PCOS model in previous studies.[5,37]

A one-week adaption period was given prior the beginning of study. The rats were kept in cages with "ad libitum" food and water at temperatures of 22°C and artificial illumination that established a photoperiod regime of 12 hours of light and 12 hours of darkness, with the light cycle happening between 06:00 and 18:00. All these operations have been authorized by the Faculty of Veterinary Medicine's ethics committee at Airlangga University, Indonesia.

All mice were in healthy condition and freedom from hunger, discomfort, pain, injury, or disease. Rats with three consecutive oestrous cycles were used after an acclimatization period. The ethanol extract of *S. polyanthum* leaves was divided into three doses of 150 mg, 300 mg, and 450 mg. The *S. polyanthum* extract was administered once daily to each group of rats. The rats were divided into five groups, including a negative control group (K0), PCOS rat models as a positive control group (K1), PCOS rat models who received three different doses of *S. polyanthum* extract; 150 mg/g BW (P1), 300 mg/g BW (P2), 450 mg/g BW, respectively. All groups consisted of ten mice.

Plant material and extraction

The leaves of *S. polyanthum* were obtained from UPT. Balai Materia Medika, East Java, Indonesia (7°52'01.2"S and 112°31'13.2"E). UPT Balai Materia Medika deposited the taxonomic identification with a determination number of 074/629/102.7-A/2021. The *S. polyanthum* leaves were air-dried and powdered at room temperature. The extraction of *S. polyanthum* leaves was done by the ultrasound-assisted extraction (UAE) method using SONICA Ultrasonic Cleaner, model SONICA® 2400EP S3 (Soltec Soluzio-ni Technologiche, Italy). First, leaves powder of *Syzigium polyanthum* were soaked in 96% ethanol (1:10, m:v). Extraction was conducted using UAE at 60 Hz for 30 m (230/240V/305 Watt) at room temperature and stirred for every 10 m. The mixture was filtered using filter paper and a rotary evaporator (50°C, 70 rpm). The result of evaporation was heated at 40°C in the oven until dry. The extract was stored in a 4°C refrigerator until further analysis.

PCOS rat model

The PCOS model rats were subjected to vaginal swabs by rotating a cotton swab and inserted into the vagina. The result of vaginal swabs was smeared on a glass object and fixed with 70% alcohol for 5 minutes. Furthermore, the glass object was dripped with Giemsa dye for 2-3 minutes, then washed with water and dried. All samples were observed under a microscope to determine the cycle stages, namely proestrus, oestrus, metestrus, and dioestrus.[5,38]

The PCOS model rats were injected intramuscularly with 0.1 mL of testosterone propionate (Testohormon®) for 14 days. According to previous research, giving Testosterone Propionate (TP) for 14 days results in a condition that resembles PCOS, including the absence of a corpus luteum, the presence of polycystic ovaries, hypertrichosis in the stroma, and granulosa cell thinning or atresia.[39] A vaginal swab was examined to determine the rat's cycle. PCOS rats with the dioestrus stage on vaginal examination were considered to have ovulation disturbance. The intramuscular injection was performed in the back leg within the rats' quadriceps and triceps muscles to avoid pain.

The anatomical pathology of the PCOS rat model is characterized by hyperthecosis and polycystic, while those in control rats are normal.[40] The uterus size in the PCOS model is larger than the control.[38] *S. polyanthum* leaves ethanol extract was administered to the rats on day 14. PCOS rats were sacrificed by ester anaesthesia and dislocation on day 29, and then the ovaries and blood samples were taken for further analysis.

Determination of GSH and sPDL1 concentration by ELISA

The levels of GSH and sPDL1 were determined by ELISA. The kits of rat GSH (Rat GSH ELISA Kit Cat No.MBS265966/Mybiosource, USA) and sPDL1 (sPDL1 elisa kit Cat No.MBS720165/Mybiosource, USA) were used according to the manufacturer's instructions. Each sample was homogenized with 5% metaphosphoric acid and centrifuged at 3,000 x g for 10 min at 4°C. The supernatants were used to determine the GSH and sPDL1 concentration. All samples were measured into microplate reader (Multiskan, Thermo Scientific, USA) at a dual wavelength of 450 nm to obtain the optical density (OD) and concentration values.

The examination of TNF-α expression using immunohistochemical techniques

TNF-α expression is characterized by increased cell signalling protein (cytokine). It plays an important role in the inflammatory process.[40] TNF-α count was measured directly from the ovarium of PCOS model rats.[5,41] The ovarian tissue was deparaffinized for three minutes with xylene. Rehydration preparations were conducted with 100% ethanol for two minutes, 95% ethanol for two minutes, and 70% ethanol for a minute. Then, the further process was conducted using water for a minute, followed by soak in a peroxidase-blocking solution at 27°C for 10 minutes. The samples were incubated in a pre-dilution inhibitor serum at 25°C for 10 minutes, soaked in monoclonal anti-TNFR1 antibodies at 25°C for 10 minutes, and then washed in PBS for 5 minutes. The samples were incubated with secondary antibodies (horseradish peroxidase-conjugated) at 25°C for 10 minutes before being washed with PBS for 5 minutes. After 10 minutes of incubation with peroxidase at 25°C, the cells were washed for 5 minutes with PBS. The preparations were incubated at 25°C for 10 minutes with diaminobenzidine, incubated for 3 minutes with haematoxylin and eosin (H and E), and then washed with water. All samples were placed on mounting media and covered with a coverslip. The expression of TNFR1 (brown in color) was observed on the cells with a 400× of magnification under light microscope, which was previously confirmed at a 1000× magnification. All examinations were conducted with a Nikon H600L light microscope (Tokyo, Japan) outfitted with a 300-megapixel DS Fi2 digital camera and the Nikon Image System image-processing software (Tokyo, Japan). According to the Table 1, a semi-qualitative evaluation of TNFR1 expression was conducted. The histopathological examination was conducted to determine the TNF-α expression in the ovaries. Data for each sample was evaluated semi quantitatively using the modified Remmele method.[37,42,43]

Table 1: Semi-quantitative of Index Remmele Scale (IRS).
The IRS was the result of multiplying the positive cell percentage score (a) with the color reaction intensity score (b). Therefore, the IRS scale =(a×b).

(a)	(b)
Score 0: No positive cells	Score 0: No color reaction
Score 1: Positive cells <10%	Score 1: Low color intensity
Score 2: Positive cells between 11% and 50%	Score 2: Medium color intensity
Score 3: Positive cells between 51% and 80%	Score 3: Strong color intensity
Score 4: Positive cells over than 80%	

IRS=Immunoreactive score

The examination of DeGraff follicle count

The ovarian tissue was deparaffinized with xylene three times for 3 minutes. Rehydrating was conducted with 100% ethanol, 95% ethanol, 70% ethanol, and water for a minute, followed by soak in a peroxidase-blocking solution at 27°C for 10 minutes. The samples were incubated with HE for 3 minutes before being washed with water. Then, all samples were covered with mounting media and sealed with a coverslip. DeGraff follicles were observed on the cells using a light microscope, as previously confirmed in previous studies.[44,45]

Statistical analysis

Data were analyzed using SPSS version 26 and presented as the mean ± standard deviation (SD). Shapiro–Wilk was used to determine the normality of data. If the resulting data were normally distributed ($p>0.05$), the one-way ANOVA test analysis was used to determine the differences between experimental groups. If the data were not normally distributed, the Mann-Whitney test was used to analyse data. A p-value < 0.05 was considered as a significant difference.

RESULTS

GSH, TNF-α, sPDL1, and De Graff follicle expression in PCOS rat model

As shown in Figure 1A, a significant difference was observed in GSH levels in all treatments using *S. polyanthum* extract. Similarly, a significant difference was detected in TNF-α level in all treatments using *S. polyanthum* extract. Moreover, the TNF-α level significantly increased in P1, P2, and P3 groups compared to the K0 group (Figure 1B). There were differences in sPDL1 levels among the K0, K1, P1, P2, and P3 groups. The P3 group had the highest sPDL1 level compared to the other group (Figure 1C). We also observed significant differences in DeGraff Follicle counts in all treatments using *S. polyanthum* extract. The P3 group had the highest DeGraff Follicle counts compared to the P1 and P2 groups (Figure 1D).

Effects of *Syzigium polyanthum* extract on GSH levels

As shown in Table 1, a higher expression of GSH was found in the control group (K0) compared to the K1 group (241.32 0.58 ng/ml, 275.68 ± 0.56 ng/ml, respectively). The GSH expression among the treatment groups was 259.60 ± 0.57 ng/ml (P1), 267.12 ± 0.65 ng/ml (P2), and 273.12 ± 12.4 ng/ml (P3), respectively. K1 had the lowest GSH level compared to the other groups. The highest GSH expression in the treatment group was found in the P3 group, as shown in Figure A. GSH expression was lower in the positive control group (K1) compared to

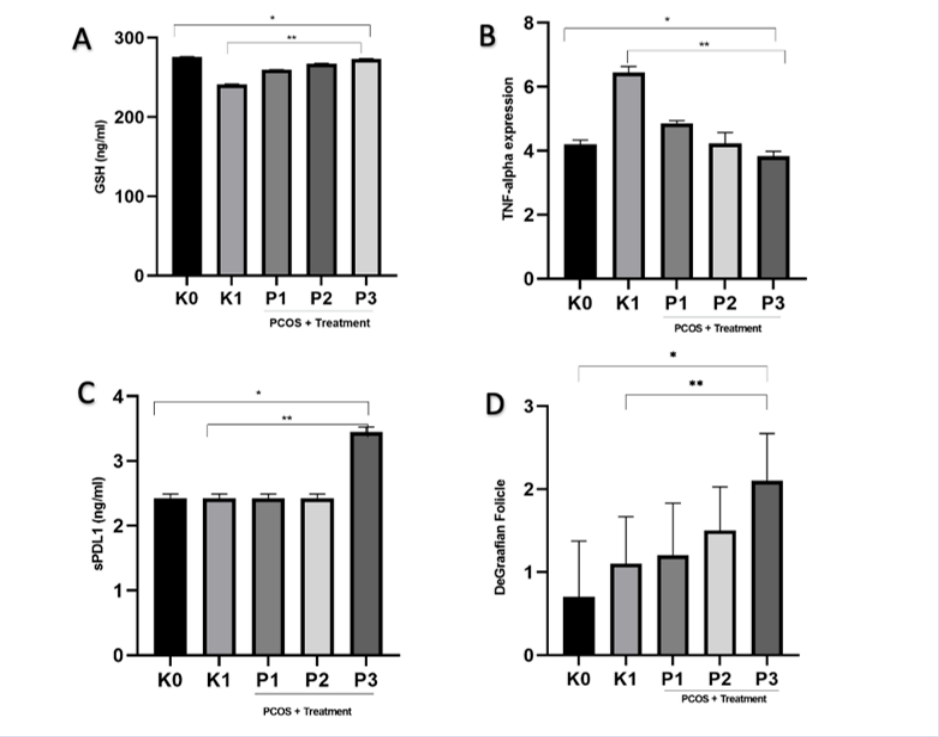

Figure 1: GSH, TNF-α, sPDL1, and De Graff follicle expression in PCOS rat model and normal mice
A. Mean ± SE of glutathione expression, B. Median ± SE of TNF-α expression, C. Median ± SE of sPDL1 expression, D. Median ± SE of DeGraff follicle expression. K0 group: negative control group; K1 group: the group of PCOS rat models; P1, P2, and P3 group: the treatment group (PCOS rat models that received 150 mg/kgBW, 300 mg/kgBW, 300 mg/kgBW of *S. polyanthum* extract). *p< 0.001, **p< 0.001. Different superscripts show significant differences. GSH = Gluthatione, TNF-α = tumor necrosis factor alfa, sPDL1 = soluble programmed death ligand 1.

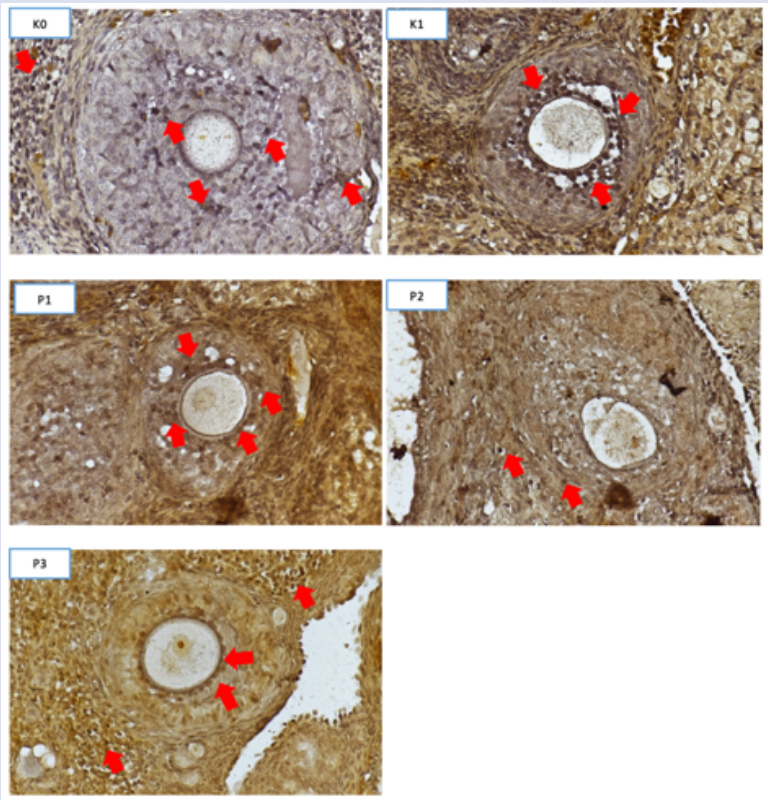

Figure 2: TNF-α expression in group follicles
The red arrow indicates the expression of TNF-α in the follicular granulosa cells which is indicated by the presence of a chromogen brown color. IHC 400x.

the negative control group (K0). However, it was higher in the treatment group. GSH levels increased significantly in the treatment group. The highest GSH levels in the treatment group were found in the P3 group. We observed that P1, P2 and P3 expression had lower GSH levels than the negative control group.

Effects of *Syzigium polyanthum* extract on TNF-α expression by immunohistochemical observation

To further analyze the expression of TNF-α in the ovaries, we conducted IHC examination as shown in Figure 2. Each sample was evaluated semi quantitatively using the modified Remmele method, where the Remmele scale index (Immuno Reactive Score/IRS) is the product of multiplying the percentage score of immunoreactive cells with the colour intensity score on immunoreactive cells (Table 2). We evaluated the mean IRS value at ten distinct fields of 100x and 400x magnification. TNF- α expression was higher in the positive control group (K1) than in the negative control group (K0) as shown on Table 2. In contrast, in the positive control, TNF-α levels were higher in P1 and P2 (3.83 ± 0.10 and 3.27 ± 0.15, respectively compared to the P3 group (2.81 ± 0.12). We found the Immuno Reactive Score results were significantly different among each group's extract administration ($p<0.5$).

Effects of *Syzigium polyanthum* extract on sPDL1 levels

As shown in Table 3, sPDL1 level was lower in the positive control group (K1) levels than the negative control group (K0). sPDL-1 level was also lower in the positive control group (K1) than the sPDL1 treatment group. In the treatment group, sPDL1 levels in P1, P2, and P3 were 2.80±0.53 ng/ml, 3.14±0.02 ng/ml, and 3.44±0.06 ng/ml, respectively. sPDL-1 level in the treatment group was lower than in the positive control group (K0). There was a significant difference in sPDL-1 level between the administration of the extract for each group ($p<0.5$).

Effects of *Syzigium polyanthum* extract on DeGraff follicles

We further evaluated the number of DeGraff follicles to determine the folliculogenesis activity. The number of follicles was determined by observation from all fields of view of ovarian organ preparations under 40x-100x magnification. DeGraff follicles observation was observed under a Nikon E100 light microscope equipped with a 12-megapixel Optilab Advance Plus digital camera and Image Raster image processing software. As shown in Table 4, the number of follicles was higher in the negative control (K0) than in the positive control (K1). The number of follicles in the P1, P2, and P3 groups was 1.2, 1.5, and 2.1, respectively. These results were higher than in the positive control group but it was lower than the negative control (K0). A significant difference in the number of De Graaf follicles was observed ($p<0.5$) in each group.

Table 2: GSH level between groups.

Group	n	Mean ± SD
K0	10	275.68 ± 0.56[a]
K1	10	241.32 ± 0.58[b]
P1	10	259.60 ± 0.57[c]
P2	10	267.12 ± 0.65[d]
P3	10	273.12 ± 12.4[e]

The mean GSH level with different letters indicated a significantly different by ANOVA followed by the post hoc LSD test at $p<0.05$. Data represent mean ± SD (n = 5). K0 group: negative control group; K1 group: the group of PCOS rat models; and P1, P2, and P3 group: the treatment group (PCOS rat models that received 150 mg/kgBW, 300 mg/kgBW, 300 mg/kgBW of *S. polyanthum* extract). GSH = Glutathione.

Table 3: TNF-α expressions between group.

Group	n	Mean ± SD
K0	10	4.18 ± 0.12[a]
K1	10	5.41 ± 0.15[b]
P1	10	3.83 ± 0.10[c]
P2	10	3.27 ± 0.15[d]
P3	10	2.81 ± 0.12[e]

The mean TNF-α expressions with different letters indicated the significantly different by Kruskal Wallis followed the post hoc Mann Whitney test at $p<0.05$. Data represent mean ± SD (n = 50). K0 group: negative control group; K1 group: the group of PCOS rat models; and P1, P2, and P3 group: the treatment group (PCOS rat models that received 150 mg/kgBW, 300 mg/kgBW, 300 mg/kgBW of S. polyanthum extract)

Table 4: sPDL1 level between groups.

Group	n	Mean ± SD
K0	10	2.410.05[a]
K1	10	1.79±0.01[b]
P1	10	2.800.53[c]
P2	10	3.140.02[d]
P3	10	3.440.06[e]

The mean sPDL1 level with different letters indicated the significantly different by Kruskal Wallis followed the post hoc Mann Whitney test at $p<0.05$. Data represent mean ± SD (n = 50). K0 group: negative control group; K1 group: the group of PCOS rat models (positive control group); P1, P2, and P3 group: the treatment group (PCOS rat models that received 150 mg/kgBW, 300 mg/kgBW, 300 mg/kgBW of *S. polyanthum* extract).

Table 5: DeGraff follicle between the control group.

Group	n	Mean ± SD
K0	10	0.700.67[a]
K1	10	1.10±0.56[ab]
P1	10	1.200.63[b]
P2	10	1.500.52[c]
P3	10	2.100.56[c]

The Mean DeGraff Folicle counts with different letters indicated the significantly different by Kruskal Wallis followed the post hoc Mann Whitney test at $p<0.05$. Data represent mean ± SD (n = 50). K0 group: negative control group; K1 group: the group of PCOS rat models (positive control group); P1, P2, and P3 group: the treatment group (PCOS rat models that received 150 mg/kgBW, 300 mg/kgBW, 300 mg/kgBW of S. *polyanthum* extract).

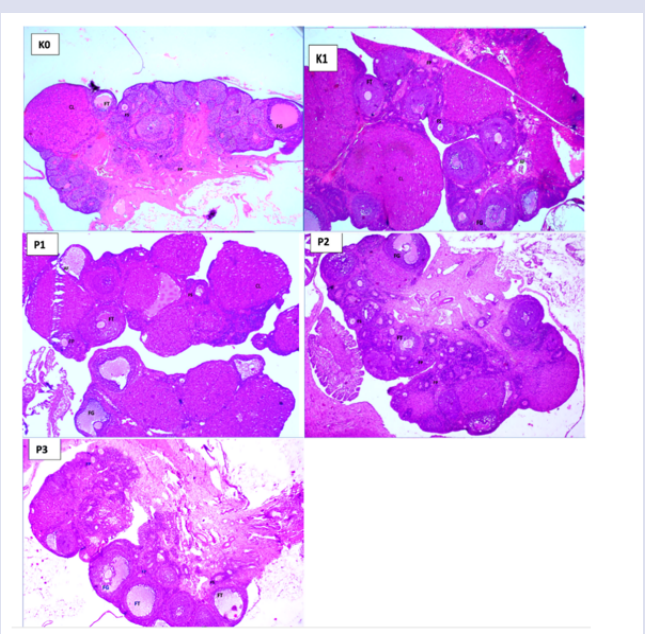

Figure 3: Development of DeGraff follicles in each treatment group using Hematoxylin

Moreover, the various dose of *S. polyanthum* extract significantly affected the number of De Graaf follicles.

DISCUSSION

Polycystic ovarian syndrome (PCOS) is a predominant cause of infertility and gyne-endocrine disorder affecting 5% to 10% of women in their reproductive age. PCOS has been reported with oxidative stress. Alteration of oxidative stress is found in PCOS patients, suggesting that oxidative stress may contribute to the pathophysiology of PCOS. Oxidative stress plays an essential role in PCOS patients by causing altered steroidogenesis in the ovaries. Therefore, this condition triggers an increased level of androgen and infertility. Furthermore, follicular development can be disturbed. Several factors, such as cardiovascular risks, insulin resistance, and obesity, are associated with oxidative stress in PCOS patients.[46,47]

In the present study, an increased level of GSH was found in the treatment group. The highest level of GSH was at P3 with a value of 273.12 ± 12.4 ng/ml, while the control group had the highest GSH levels. This condition occurs due to antioxidant supplementation from the extract of *S. polyanthum* leaves. *S. polyanthum* protects oxidative stress by decreasing ROS formation and modulating cellular signalling pathways. A previous study reported that antioxidant administration in animal models improves metabolic responses and reproductive performance. Antioxidant supplementation has been beneficial for reducing clinical symptoms of women with PCOS by improving their metabolic profiles and attenuating oxidative stress and inflammation. Besides improving mental health parameters, changes in *glutathione* (GSH), high-sensitivity C-reactive protein (hs-CRP), malondialdehyde (MDA), and total testosterone levels in serum have been shown after co-administration of probiotics and selenium for 12 weeks in women with PCOS.[48]

GSH contains abundant antioxidants and plays an important role in protecting cells from oxidative damage. Decreased GSH levels in serum have been found in chronic diseases such as cancer, cardiovascular, and gastrointestinal diseases. Moreover, a reduced level of GSH was also found in women with PCOS compared to non-PCOS women. The higher level of androgen causes more development of ROS formation in women with PCOS, which may lead to the depletion of GSH. The redox GSH cycle, through activating GPx and GR enzymes decrease GSH level in PCOS cases.[46,47]

GSH acts as antioxidant and protects cells from oxidative damage.[49,50] GSH levels in blood have been found to be lower in chronic diseases including cancer, gastrointestinal, and cardiovascular conditions.[51] Previous study showed that PCOS was associated with a significant reduction in GSH levels compared to women without PCOS. A significant decrease found in GSH levels in PCOS women.[52,53] GSH reacts with ROS through its thiol groups to prevent cell damage. In women with PCOS, increased androgen production results in increased ROS formation, which may lead to GSH depletion. An elevated ROS production causes GSH depletion in PCOS.[54] Murri *et al* (2013) discovered that the mean GSH levels of women with PCOS were 50% lower than those of healthy women.[47]

Elevated inflammatory markers and abnormal antioxidant levels have been shown in women with PCOS. Inflammation in PCOS affects atherogenesis, insulin resistance, pancreatic beta cell dysfunction, and ovarian disturbance, which cause antioxidant imbalance. After antioxidant supplementation, a significant decrease in hs-CRP level has been reported in women with PCOS.[48] Chronic low-grade inflammation is one of the primary PCOS features. It contributes to elevated inflammatory markers, such as CRP, IL-6, IL-18, MCP-1, and TNF levels in women with PCOS. Oxidative stress is associated with inflammation in PCOS patients. ROS will release inflammatory markers and response through the activation of hypoxia-inducible factor-1 (HIF-1), nuclear factor kappa B (NF-κB), and activated protein-1 (AP-1). Insulin resistance is also related to inflammation in PCOS patients. Moreover, an increase in adipose-derived TNF-α levels in mice has been reported and related to obesity.[52]

The increased ROS activates transcription factors, such as NF-B, which is involved in the formation of inflammation in cells. Moreover, it is also related with the production of proinflammatory cytokines such as TNF- and IL-6. In PCOS, the initial activation of the systemic inflammatory response in plasma is accompanied by elevated ROS production.[55-58]

Antioxidant has benefits in reducing inflammation and oxidative stress. Several mechanisms can be conducted by producing antioxidant metabolites, reducing ROS enzymes, stimulating the antioxidant activity of the host, and modifying the inflammatory signalling pathways. Moreover, an antioxidant also plays an essential role in elevating glutathione peroxidase (GPx) activity, reducing the activation of NF-κB, inhibiting MAP kinase pathways, and change in the metabolism of arachidonic acid, resulting in the anti-inflammatory effects.[49]

Several studies have revealed that a significant relationship was associated with chronic low-grade inflammation in women with PCOS.[59-62] Leukocytosis in polycystic ovaries may be the first indication that polycystic ovaries are an inflammatory condition.[49,60,63,64] The expression of IFN-c, a cytokine produced by Th1, was significantly elevated and the ratio of Th1/Th2 was significantly higher in PCOS patients than in the control group.[61]

Our results study showed that the highest expression of sPDL1 was found in the P3 group, while the PCOS group (K1) had the lowest level. The P1, P2 and P3 treatment groups had higher sPDL1 compared to the controls. sPD-L1 as soluble immune checkpoint molecules are the developing new immune regulatory mediators. Immunological modulation may be characterized by the serum concentration of sPD-L1 and immune checkpoint molecules. In addition, serum sPD-L1 as immunological regulators has been reported in the pathogenesis of PCOS.[14] The further study on the determination of inflammatory interactions and biological functions by immune checkpoints is necessary to enhance our understanding of the underlying disease mechanism related to the pathogenesis of PCOS. Our results study showed serum levels of PD-L1 were significantly lower in patients with PCOS than in the control group.

Previous study showed serum PD-1 and PD-L1 levels were lower in PCOS patients compared to the control group. The low levels of PD-1 and PD-L1 in the serum of PCOS patients may be related to their inability to effectively inhibit T and B cell function and proliferation, thus leading to the pathogenesis of PCOS. However, whether the decrease in PD-1 and PD-L1 levels can be used as an immunological diagnosis for PCOS disease requires further research.[20] Besides, PD-1 inhibits autoimmunity through two pathways. First, it increases the number of antigen-specific T lymphocytes that undergo apoptosis (programmed cell death). It also reduces regulatory T-cell apoptosis (anti-inflammatory, suppressive T-cells).[65] PD-1 inhibits innate and adaptive immune responses.[66]

Chronic inflammation is a clinical feature of PCOS patients. The PD-1 is an inhibitory signal molecule that prevents T cell activation. PD-1 ligand can induce T-cell apoptosis and stimulate the secretion of IL-10, which has immunosuppressive effects. A previous study showed an elevated PD-1 level in CD4+ and CD8+ T lymphocytes from infertile PCOS patients. The immune system plays an important role in numerous biological components and protects the host from disease. When the body's immune system is compromised, various diseases can manifest. A recent study reports that immune systems regulate PCOS. Women with PCOS have reported an elevated number of leukocytes, endothelial

dysfunction, and an imbalance of proinflammatory cytokines.[67,68] In addition, they also have a chronic condition characterized by low-grade inflammation. Several studies reported that human preovulatory follicles contain substantial numbers of immunocompetent cells, including T-cells, B-cells, macrophages, and dendritic cells are involved in the pathogenesis of PCOS.[69-71]

The number of DeGraff follicle increased according to the dose of *S. polyanthum* extract for each treatment. Our study suggests that the mechanism of *S. polyanthum* extract increases the number of DeGraff follicles by increasing GSH, thereby reducing TNF-α and increasing sPDL1. In addition, our result study reveals that proliferating cells significantly reduced in DeGraff follicles in the PCOS group without treatment. A significant increase of proliferating cells in follicles was also found in the PCOS rat model after receiving *S. polyanthum* extract in the PCOS compared to the PCOS group without treatment. Our results emphasize that antioxidant compound has beneficial in protecting cells from oxidative stress. A study reported that an increase in cell proliferation causes the inhibition of free radicals and oxidative stress damage.[40]

In PCOS patients, there is an impairment in the regulation of granulosa cells (GCs) and immune cells, which may hasten anovulation.[71] Glucose ingestion triggers higher ROS production along with the inflammatory response. Moreover, elevated ROS production, NADPH activity, NFκB, and TNF-α mRNA expression of mononuclear cells (MNCs) were found in PCOS disease.[72]

Granulosa cells supply the oocyte with nutrients and regulate its growth.[73] The maturation of the follicle is affected by an interaction between the oocyte and granulosa cells. Therefore, apoptosis within the granulosa cells is a primary component of folliculogenesis. A higher incidence of apoptosis has been reported in PCOS women.[74] Apoptosis in granulosa cells is a complicated process that could be triggered by numerous factors, including inflammatory cytokines.[75] Programmed cell death-1 (PD-1) is essential for preventing of cancer, autoimmune diseases, and inflammation.[76]

In the present study, *S. polyanthum* extract is strongly associated with a reduction of serum TNF-α level. *S. polyanthum contributes* to reduce inflammation after testosterone injection. A study by Hasan *et al.* (2020), *S. polyanthum* has anti-inflammatory activity. Isolation of active compound, especially flavonoid, *S. polyanthum* is needed to obtain better understanding into its role in inflammation and heart disease. *S. polyanthum* extract might have anti-inflammatory effect in myocardial infarction condition.[77] Research by Hartanti *et al.* (2019) showed that the bay leaves ethanolic extract have a potent activity to reduce the cholesterol serum level by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A.[28]

The abnormal oocyte development observed in PCOS patients may be caused by abnormal activation of T cells and cytokine production. Patients with PCOS and infertility are likely unable to induce T cell activation or recruitment, resulting in an inability to select and develop dominant follicles. The dysfunction of T cells contributes to the immune pathogenesis in the ovary of PCOS patients with infertility. These findings imply that chronic inflammation plays an important role in the pathogenesis of PCOS.[78]

Numerous immune and nonimmune cells, including epithelial cells, endothelial cells, fibroblast cells, monocytes, and macrophages, produce TNF as a pro-inflammatory cytokine. A high level of TNF can stimulate the proliferation of theca intra-cells, leading to hyperplasia and an increase in follicles. The TNF mechanism involves the activation of the JNK pathway. TNF levels in PCOS patients' follicular fluid (FF) are also higher than in non-PCOS women. TNF inhibits the expression of genes involved in progesterone production, thereby reducing progesterone production and ovulation. TNF mediates granulosa cell death through apoptosis and autophagy. By inhibiting adenylyl cyclase, TNF influences thecal and granulosa cell steroidogenesis and luteal regression.[79,80]

Antioxidants contribute to reducing reactive oxygen species (ROS), which stimulate the activation of inflammation signalling and improve the management of PCOS by scavenging ROS. Antioxidants are involved in PCOS women's fertility by reducing the damage caused by free radicals to oocytes, cell maturation, and other physiological mechanisms in female fertility. Morover, ROS also modulates ovulation.[81]

A previous study reported that ovarian steroidogenic enzymes plays an important role in androgen production during in vitro study. It is stimulated by oxidative stress and inhibited by antioxidants. Oxidative stress and antioxidant correlate with theca cells. Antioxidants suppress the proliferation of theca cells, and more pronounced oxidative stress inhibits the proliferation of theca cells. A *in vitro* study showed the modest oxidative stress triggers the proliferation of theca cells. In addition, oxidative stress interferes insulin signaling, causing hyperinsulinemia and triggers thecal steroidogenesis.[32]

A *in vitro* study reported that *S. polyanthum* contains antioxidant and antidiabetic activities. Supplementation of antioxidants helps the body from free radicals and maintains hyperglycaemic in post-prandial hyperglycaemia.[81] Phytochemical studies revealed that the leaves and fruits of *S. polyanthum* contain vitamin C and flavonoids, which may have anti-inflammatory activity. Leaves, fruits, and barks of *S. polyanthum* are traditionally used for various medicinal and nonmedicinal purposes carried out by people in Southeast Asia, including Indonesia. The roots and the fruits of *S. polyanthum* are useful as to reduce the hangover effect of alcohol, while the leaves of *S. polyanthum* have beneficial in treating various diseases.[35] Several reports showed ethanolic extract of leaves exhibited radical scavenging activity.[27,29] Therefore, *S. polyanthum* can be a source of antioxidants for health under PCOS conditions because it protects the body from oxidative disorders caused by ROS.

CONCLUSION

In the PCOS rat model, *S. polyanthum* leaf extract exhibited low TNF-α level and elevated GSH, sPDL1, and DeGraff follicle counts. S. *polyanthum* could be used as an alternative treatment for and management of PCOS.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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