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Original Article

Alteration of IL-6, BMP-15 and GDF-9 Levels on PCOS Rat Models After Treated with *Syzygium Polyanthum (Wight) Walp* Leaves Extract

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is a global health concern for women in reproductive age women. Numerous studies have been reported an association between chronic inflammation and alteration of cytokine in women with PCOS. *Syzygium polyanthum* (*S. polyanthum*) contains antioxidants and has antiinflammation activity. **Objectives:** This study aims to measure the alteration of IL-6, BMP-15, and GDF-9 in rat PCOS model after treated with *S. polyanthum* leaves extract. **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* leaves extract. The treatment group consisted of PCOS rat models with *S. polyanthum* leaves extract supplementation of 150 mg/KgBW (P1), 300 mg/KgBW (P2), and 450 mg/KgBW (P3). **Results:** IL-6 expression was highest in K1 (4,690 ± 0.099) and lowest in the P3 treatment, namely (2,370 ± 0.105). The expression of BMP-15 and GDF-9 was lowest at K1 (2.554 ± 0.04; 4.502 ± 0.050) and highest at P3, namely (2.265 ± 0.072; 4.736±0.074). **Conclusion:** *S. polyanthum* leaves extract was significantly effective in decreasing IL-6 expressions, as well as a significant increase in BMP-15 and GDF-9 expressions in the PCOS rat model.
Keywords: BMP-15, GDF-9, IL-6, *Syzygium polyanthum.*

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a global health concern for women of reproductive age women worldwide.[1] Hyperandrogenism, anovulation, menstrual irregularities, and polycystic ovaries are major symptoms of PCOS. Besides, inflammation is also reported as the hallmark and consequence of PCOS. The endocrine process regulates inflammatory response and production of pro-inflammatory cytokines. Inflammation and oxidative stress are related to the pathogenesis of PCOS. [2] Hyperglycemia contributes to inflammation through the production of TNF-α. Some studies have shown that the higher serum TNF-α and IL-6 levels are related to women with PCOS. These conditions exhibit hypertriglyceridemia and chronic inflammation, followed by elevated peripheral lymphocytes, monocytes, and eosinophilic granulocytes. [3,4,5]

Interleukin 6 is a major proinflammatory cytokine and can be found in a variety of tissues, such as activated adipocytes, endothelial cells, and leukocytes. Women with PCOS have been reported to have persistent chronic inflammation with a higher number of inflammatory cells infiltrating the follicles.[5,6,7] PCOS causes infertility, primary amenorrhea or secondary amenorrhea after pubertal development.[8,9,10] PCOS also causes follicle dysfunction or follicle depletion. [11,12] Among the many extra ovarian and intraovarian factors, the transforming growth factor beta (TGFB) superfamily plays an important role in follicle growth. [13,14,15] Two related members of the TGFB superfamily, such as growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), are involved in the development of the ovary. BMP-15 and GDF-9 play a critical role in follicle development, oocyte maturation, ovulation, and embryo development.[16,17] Moreover, BMP-15 and GDF-9 play a role in promoting early follicle growth.[18,19]

Until now, the therapeutic of PCOS is continuously develop. The potential of anti-inflammatory and antioxidant medicines can be therapy option for women with PCOS. [20,21] The utilization of herbal medicine demonstrates a favorable impact with little adverse reactions. *Syzygium polyanthum (Wight) Walp.* has been widely used as a medicinal plant in several Asian countries, such as Indonesia, Malaysia, and Thailand. [23,24] *S. polyanthum* contains flavonoids and polyphenols that exhibit therapeutic properties for anti-inflammatory, antioxidant, antidiabetic, antibacterial, and antihypertensive. Besides, flavonoids and polyphenols have been observed to possess therapeutic properties for PCOS treatment. [20,21,22] The administration of an ethanolic extract of *S. polyanthum* leaves extract has been found to effectively lower inflammatory factors by inhibiting the JNK and NF-κB pathways. [23,24,25]

Numerous studies showed the versatile application of *S. polyanthum* leaves as health therapy. [26,27,28] The extraction plays important role in the process of obtaining phytochemical components from plant sources. The pharmaceutical effect is related to the bioactive compounds which influence by several factors, such as extraction methods, solvent, and part of the plant used for extraction. [28,29,30] UAE showed effectiveness and extractability of bioactive substances improvement. [31,32,33] The current study examines the effect of *S. polyanthum* leaves extract against IL-6, BMP-15 and GDF-9 levels in PCOS rat models.

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MATERIALS AND METHODS

Study Design

All protocol in this study was approved by the Ethics Committee of the Veterinary Faculty, Airlangga University, Surabaya, Indonesia (No. 2.KE.133.11.2021). All methods in this study were performed in accordance with the relevant guidelines, regulations and animal welfare. This study was used female rat strain Wistar aged three months, with a mean weight of 150–200 grammes. A one-week adaptation time was conducted. The temperature was maintained at 22°C, and an artificial lighting system was used to establish a 12-hour light and 12-hour dark cycle. The light phase of the cycle occurred between 06:00 and 18:00. All rats were treated under the guidance for the Care and Use of Laboratory Animals of the National Institutes of Health. The rats in the PCOS model underwent vaginal swabs. Then, the samples were placed into a glass object and treated with 70% alcohol for a duration of 5 minutes. The glass specimen was subjected to dip in Giemsa dye for a duration of 2-3 minutes, followed by rinsing with water and subsequent drying. The samples were examined using a microscope to identify the cycle stages. The examination of a vaginal swab was performed in order to ascertain the reproductive cycle of the rat. Rats with PCOS exhibiting the dioestrus stage upon vaginal evaluation were identified as having disturbed ovulation.

The rats underwent intramuscular injections in the posterior limb, specifically targeting the quadriceps and triceps muscles. A dosage of 0.1 mL of testosterone propionate (commercially known as Testohormon®) was administered to subjects over a period of 14 days in order to induce a condition that mimics polycystic ovary syndrome. Then, rat received treatment with an ethanol extract of *S. polyanthum* leaves on day 14. The rats were categorised into five groups, which consisted of a negative control group (K0), a positive control group of PCOS rat models (K1), and PCOS rat models that were administered three different doses of *S. polyanthum* leaves extract: 150 mg/g BW (P1), 300 mg/g BW (P2), and 450 mg/g BW (P3) during 14 days, respectively. Rats were euthanized by ester anaesthesia and dislocation on day 29. Subsequently, the ovaries and blood samples were collected for further analysis.

Syzygium Polyanthum (Wight) Walp leaves extractraction

The leaves of *S. polyanthum* leaves 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 conducted by the ultrasound-assisted extraction (UAE) method using SONICA Ultrasonic Cleaner, model SONICA® 2400EP S3 (Soltec Soluzio-ni Technologiche, Italy). First, leaves powder of *S. 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. The ethanol extract derived from the leaves of *S. polyanthum* was partitioned into three different dosages (150 mg, 300 mg, and 450 mg).

Immunohistochemical Analysis

Deparaffinization process using xylene was conducted in ovarian tissue. The rehydration procedures involved soaking the samples in 100% ethanol for two minutes, followed by a further soaking in 95% ethanol for another two minutes, and in 70% ethanol for a minute. Then, samples were soaked with water for a minute, followed by immersion in a peroxidase-blocking solution at 27°C for ten minutes. The specimens were incubated in a pre-dilution inhibitor serum at a temperature of 25°C for 10 minutes. Subsequently, samples were immersed in monoclonal anti-IL6R1 antibodies (Rat anti-IL6R1 IHC Kit No. MBS1751389/Mybiosource, USA), GDF-9 (Rat anti-GDP-9B antibody IHC Kit No. MBS2001862 /Mybiosource, USA) and BMP15 proteins Rat anti-BMP15 antibody (IHC Kit No. MBS2026132/Mybiosource, USA) at 25°C for 10 minutes. Samples were rinsed in phosphate-buffered saline (PBS) for 5 minutes. The samples were incubated with secondary antibodies that were conjugated with horseradish peroxidase at a 25°C for 10 minutes. Subsequently, samples were washed with PBS for 5 minutes. Following the 10 minutes incubation period at a temperature of 25°C, the cells were washed for 5 minutes using PBS. Samples were incubated at 25°C for 10 minutes. Subsequently, samples were incubated for 3 minutes with haematoxylin and eosin (H and E), followed by washing with water. The samples were carefully positioned into the mounting media and covered with a coverslip. The cellular expression of IL-6, GDF-9 and BMP-15 was detected through microscopic examination at a magnification of 400× using a Nikon H600L light microscope (Tokyo, Japan). Histopathological analysis was performed to assess IL-6, GDF-9 and BMP-15 expressions in the ovarian tissue. The Index Remmele Scale (IRS) approach was used to assess the data for each sample in a semi-quantitative basis. 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 colorintensity
Score 3: Positive cells between 51% and 80%	Score 3: Strong color intensity
Score 4: Positive cells over than 80%	

IRS = Immunoreactive score

Statistical analysis

Data were analyzed using SPSS version 26 and Graphad Prism 9. Data were presented as the mean ± standard deviation (SD). Shapiro–Wilk was used to determine the normality of data. If data were normally distributed ($p>0.05$), the one-way ANOVA test analysis was used to determine the differences between experimental groups then followed by Tukey test. 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

Immunohistochemistry of IL-6, BMP-15 and GDF-9

IL-6 Expression

As shown in Figure 1A, a significant difference was observed in IL-6 expressions in all treatments using *S. polyanthum* leaves extract. Similarly, a significant difference was detected in BMP-15 expressions in all treatments using *S. polyanthum* extract. Moreover, the BMP-15 expressions significantly increased in P1, P2, and P3 groups compared to the K0 group (Figure 1B). There were differences in GDF-9 expressions among the K0, K1, P1, P2, and P3 groups. We also observed significant differences in GDF-9 expressions in all treatments using *S. polyanthum* leaves extract. The P3 group had the highest GDF-9 expressions compared to the P1 and P2 groups (Figure 1C).

The mean IL-6 expressions with different letters indicated the significantly different by Anova followed the post hoc Tukey 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, 450 mg/kgBW of *S.polyanthum* leaves extract).

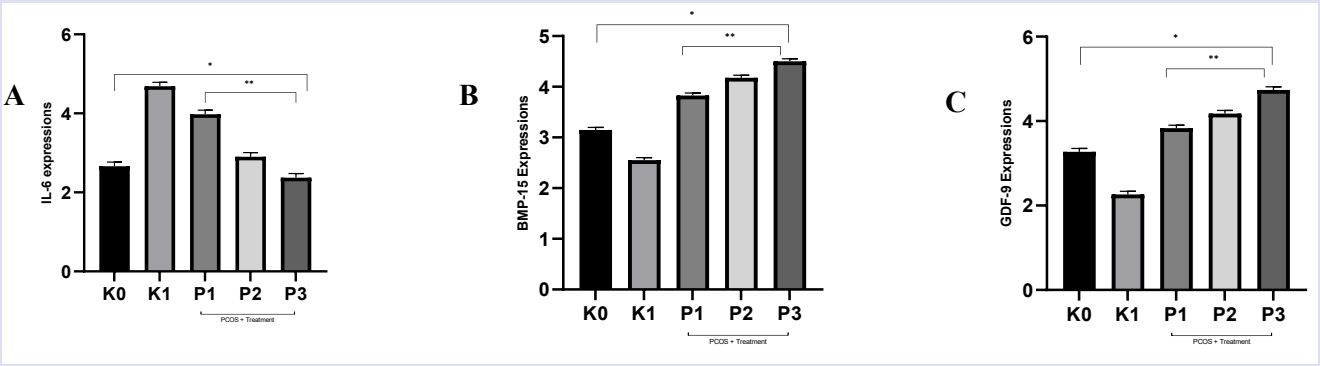

Figure 1. IL-6, BMP-15 and GDF-9 expressions. (A) Mean ± SE of IL-6 expression, (B) Mean ± SE of BMP-15 expression, (C) Mean ± SE of GDF-9 expression. K0 group: negative control group; K1 group: the group of PCOS rat models; P1, P2, and P3 group: the treatment group (PCOS rat models that received 150 mg/kgBW, 300 mg/kgBW, 300 mg/kgBW of *S. polyanthum* leaves extract). *p< 0.001, **p< 0.001. Different superscripts show significant differences.

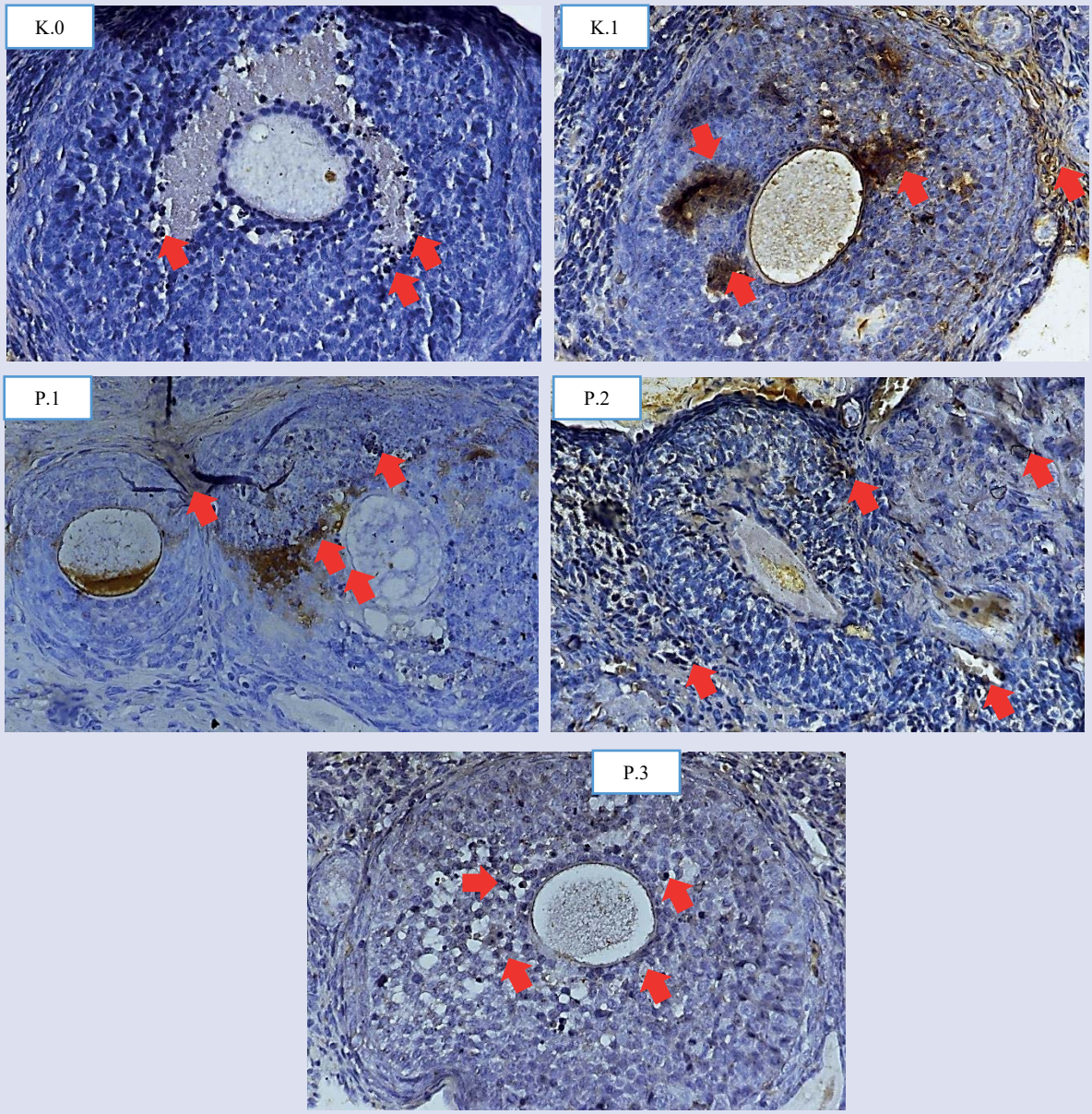

Figure 2. IL-6 expression in each group. The red arrow shows the expression of IL-6 in the granulosa cells of the follicles which is indicated by the presence of brown chromogen (IHC 400x).

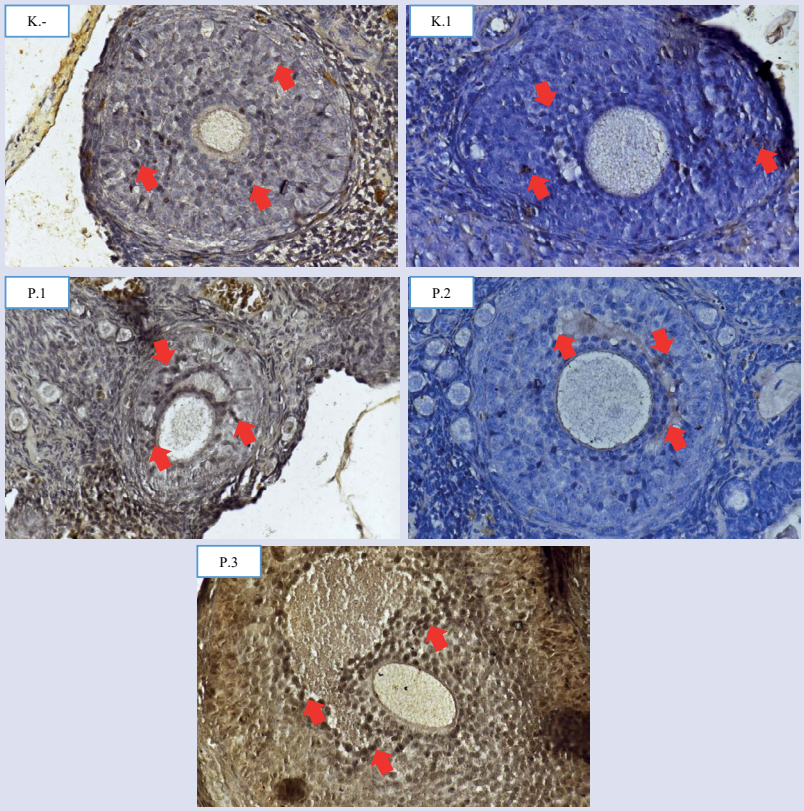

Figure 3. BMP-15 expression in each group. The red arrow shows the expression of BMP-15 in the granulosa cells of the follicles which is indicated by the presence of brown chromogen (IHC 400x).

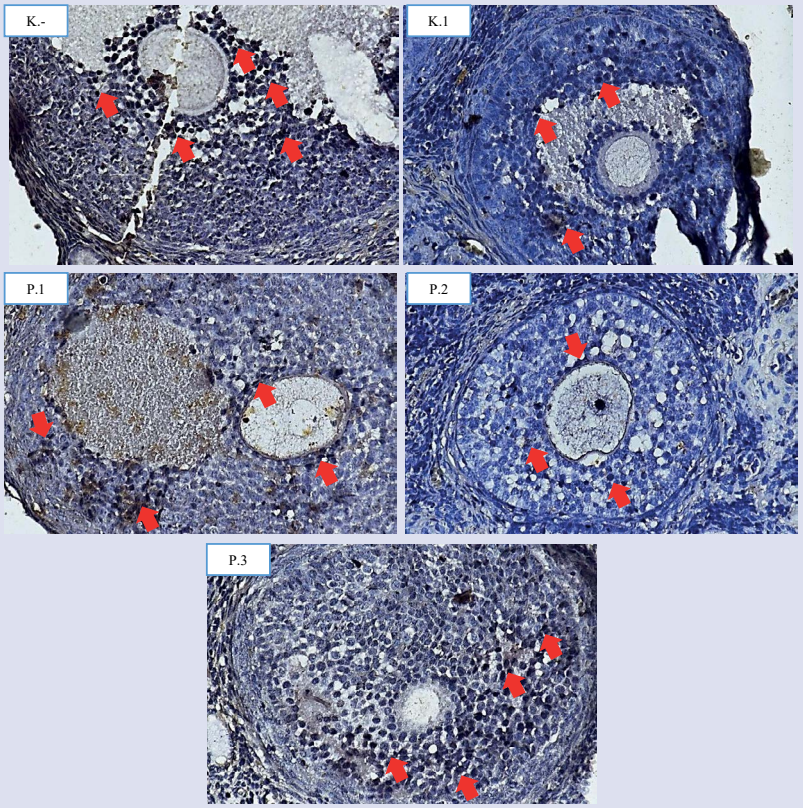

Figure 4. GDF-9 expression in each group. The red arrow shows the expression of GDF-9 in the granulosa cells of the follicles which is indicated by the presence of brown chromogen (IHC 400x).

The mean BMP-15 expressions with different letters indicated the significantly different by Anova followed the post hoc Tukey test at $p<0.05$. Data represent as 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, 450 mg/kgBW of *S. polyanthum* leaves extract).

BMP-15 Expression

To further analyze the expression of BMP-15 expressions in the ovaries, we conducted IHC examination as shown in Figure 3. Each sample was evaluated semi quantitatively using the modified Remmele method. We evaluated the mean IRS value at ten distinct fields of 100x and 400x magnification. BMP-15 expressions was higher in the negative control group (K0) than the positive control group (K1) in as shown on Table 2. In contrast, in the positive control, BMP-15 expressions were higher in the P3 group (4.502±0.050) compared to P1 and P2 (3.828±0.050 and 4.176±0.050).

GDF-9 Expression

The mean GDF-9 expressions with different letters indicated the significantly different by Anova followed the post hoc Tukey test at $p<0.05$. Data represent as 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, 450 mg/kgBW of *S. polyanthum* leaves extract).

The GDF-9 expressions in the ovaries was conducted by IHC examination as shown in Figure 4. Each sample was evaluated semi quantitatively using the modified Remmele method the same procedure as IL-6 and GDF-9 examination. We evaluated the mean IRS value at ten distinct fields of 100x and 400x magnification. GDF-9 expression was higher in the negative control group (K0) than the positive control group (K1) in as shown on Table 3. GDF-15 expression was higher in the P3 group (4.736±0.074) compared to P1 and P2 (3.831± 0.073 and 4.179±0.074). We found the Immuno Reactive Score results were significantly different among each group's extract administration ($p<0.5$).

Table 1. IL-6 expressions between groups.

Group	n	Mean ± SD
K0	10	2.660±0.107[a]
K1	10	4.690±0.099[b]
P1	10	3.980±0.103[c]
P2	10	2.900±0.105[d]
P3	10	2.370±0.105[e]

Table 2. BMP-15 expression between groups.

Group	n	Mean ± SD
K0	10	3.148±0.051[a]
K1	10	2.554±0.049[b]
P1	10	3.828±0.050[c]
P2	10	4.176±0.050[d]
P3	10	4.502±0.050[e]

Table 3. GDF-9 expressions between groups.

Group	n	Mean ± SD
K0	10	3.272±0.078[a]
K1	10	2.265±0.072[b]
P1	10	3.831±0.073[c]
P2	10	4.179±0.074[d]
P3	10	4.736±0.074[e]

DISCUSSION

This study showed an increase in IL-6 expression in the positive control (K1) compared to the negative control (K0). The decrease of IL-6 expression was observed after being given *S.polyanthum* leaves extract in PCOS rat model. Our results demonstrate that *S.polyanthum* leaves alters the expression of IL-6. Inflammatory cytokines like IL-6 is secreted by lymphocytes and macrophages. This cytokine activate macrophages and lymphocytes to promote further cytokines secretion and thus get into vicious circle. Activated lymphocytes and macrophages secrete several cytokines that might induce apoptosis and cause follicle atresis. Elevated macrophages and lymphocytes in the PCOS ovaries could induce cell apoptosis by various cytokines acting on granular and theca cells. [4,5,6] A previous study showed that inflammatory mediators are higher in PCOS patients. The continuous release of inflammatory markers is associated with long-term metabolic complications. Although exact mechanisms are not fully understood yet, there are numerous studies that underline mutual impact of obesity and insulin resistance in increased inflammation, suggesting these states effect on PCOS pathogenesis.[1,2,6]

Our study also demonstrated a decrease in the expression of BMP-15 and GDF-9 in the positive control (K1) compared to the negative control (K0). An increase in the expression of BMP-15 and GDF-9 was also found in the treatment group. The highest level of expression of BMP-15 and GDF-9 was found in the PCOS rat model with the highest doses of *S. polyanthum* leaves extract, while the positive control group had the lowest level of BMP-15 and GDF-9. Our results suggest that *S. polyanthum* leaves extract alters the expression of BMP-15 and GDF-9. This condition trigger local inflammation of the ovary that affects ovulation and induces systemic inflammation.

BMP-15 and GDF-9 is crucial factor in the process of folliculogenesis. and female fertility. The bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) genes are significant constituents of the TGF-β superfamily. These genes encode proteins that are secreted into the ovarian follicles by the oocytes. Within the follicles, these proteins aid in the establishment of a favourable milieu that promotes follicle growth and selection. The primary functions of these entities are to control ovarian maturation, follicular survival/atresia, and cellular proliferation/differentiation. GDF-9 and BMP-15 share substantial homology with regard to their amino acid composition and protein structure. This similarity extends to their overall expression, function, and potential interactions. These factors significantly influenced the function of granulosa cells and cumulus cells in order to enhance oocyte quality. In the preantral and early

follicle stages, GDF-9 inhibits apoptosis and promotes the proliferation of granulosa cells. Additionally, BMP-15 stimulates the expression of a number of other proteins, including EGF, which is essential for the expansion of cumulus cells, and promotes the proliferation of granulosa cells. It is also highly effective against apoptosis in cumulus cells. it assumes a critical function during the phases of cell proliferation, apoptosis, luteinization, and the expansion of the metabolism of cumulus cells. [18,19]

In addition to regulating inflammation, IL-6 controls the synthesis of various cytokines. Serum from women with PCOS contains an excess of IL-6 monocytes, which induces low-grade inflammation. By interacting with these cytokines, flavonoids can exert a potent anti-inflammatory effect. By acting as an inhibitor of the PI3K/AKT/mTOR signalling pathway, it suppresses the expression of IL-6 and various pro-inflammatory cytokines. It is critical for the maturation of the ovary and the implantation process. [3,4,5]

Flavonoids component in *S. polyanthum* extract affects the release of cytokine. Flavonoid has anti-inflammatory effect in *S.polyanthum*

extract. [9,10] Another study showed *S. polyanthum* extract demonstrated free radical scavenging and antidiabetic activity. The fresh juice of *S. polyanthum* has superior in vitro antioxidant and antidiabetic activities. [15] *S. polyanthum* leaves have a potent activity to reduce the cholesterol serum level and the antioxidant activity. [18,19,20] In addition, flavonoid in *S. polyanthum* is abundant and has been long studied for anti-inflammatory.[13,14] The inflammation is regulated by many receptor-mediated pathways, which include Toll-like receptors and the nuclear factor kappa-light chain enhancer of activated B cells (NF-κB).

Inflammatory dysregulations are associated with infertility diseases and affect ovarian function, oocyte quality, and endometrium receptivity. PCOS patients demonstrated significantly higher concentrations of circulating inflammatory cells, such as lymphocytes, neutrophils, eosinophilic granulocytes, monocytes and Th17 cells than women without PCOS, while the percentage of Treg cells was lower.[6,7,8] Besides, a pro-inflammatory condition in women with PCOS impact the ovarian function, sexual hormones, follicular maturation, and ovulation. 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.[1,2,6]

CONCLUSION

The leaves extract of *S. polyanthum* exhibited a significant decreasing of IL-6 expression, while concurrently demonstrating a significant rise in BMP-15 and GDF-9. Due to its versatile application and numerous bioactive compounds, our findings suggested that *S. polyanthum leaves* extract may contribute to and function as a candidate anti-inflammatory and oxidative stress agent, which could have a positive effect on the management of women with PCOS.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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