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ORIGINAL ARTICLE

Quercetin and Flavonoids from *Cuscuta chinensis* Lam. Inhibit Tripterygium Glycoside-Induced Premature Ovarian Failure Progression via PI3K-AKT Signaling Pathway

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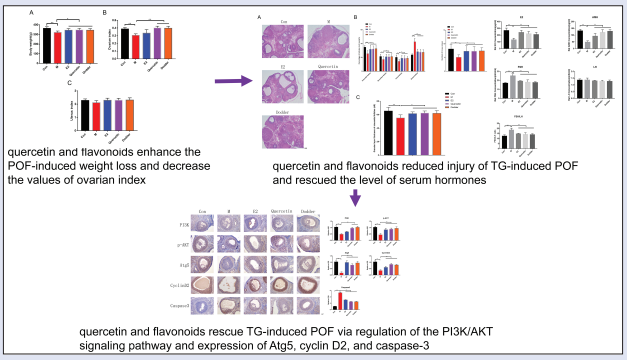
ABSTRACT

Objective: In this study, we aimed to explore the effects of quercetin and flavonoids extracted from *Cuscuta chinensis* Lam. on tripterygium glycoside (TG)-induced premature ovarian failure (POF). **Materials and Methods:** Rats in the POF model were administered with 17β-estradiol (E2), quercetin, or flavonoids that were extracted from *C. chinensis*. Serum levels of anti-Müllerian hormone (AMH), luteinizing hormone (LH), follicular-stimulating hormone (FSH), and E2 were determined via enzyme-linked immunosorbent assay. The mRNA and protein expressions of PI3K-AKT signaling pathway- and apoptosis-related genes in the ovarian tissues were determined by immunohistochemistry, real-time polymerase chain reaction and western blot analysis. **Results:** After the administration of E2, quercetin, and flavonoids, there was a decrease in the estrus cycle, which was accompanied by an increase in weight and ovarian indices. TG reduced the thickness of the layer of granulosa cells in the antral follicles, the ratio of primitive follicles, secondary follicles, and sinusoidal follicles, and the number of cells in the corpus luteum and increased the ratio of atresia follicles; however, E2, quercetin, and flavonoids reversed these effects. In the POF group, serum levels of E2 and AMH were decreased, whereas FSH and FSH/LH levels were increased. TG downregulated the expression of PI3K, p-AKT, Atg5, and cyclin D2 and upregulated the expression of caspase-3. However, the changes in the levels of hormones and proteins were reversed after the administration of E2, quercetin, and flavonoids. **Conclusion:** Quercetin and flavonoids extracted from *C. chinensis* Lam. inhibited TG-induced POF by modulating the PI3K/AKT signaling pathway.

Key words: *Cuscuta chinensis* Lam, flavonoids, premature ovarian failure, quercetin

SUMMARY

- Our study confirmed that quercetin and flavonoids extracted from *C. chinensis* Lam. inhibited TG-induced POF progression, similar to E2. Furthermore, these effects were achieved by regulating the PI3K/AKT signaling pathway.



Abbreviations used: TG: tripterygium glycoside; POF: premature ovarian failure; E2: estradiol; AMH: anti Müllerian hormone; LH: luteinizing hormone; FSH: follicle stimulating hormone; RT-qPCR: real-time polymerase chain reaction; HPLC: high-pressure liquid chromatography; HE: hematoxylin and eosin; IHC: immunohistochemistry.

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INTRODUCTION

Premature ovarian failure (POF) is often diagnosed in women who are <40 years of age. The fundamental manifestations of POF are amenorrhea, high levels of gonadotropin, hypogonadism, and infertility. A common feature of POF is an increase in the serum levels of follicle-stimulating hormone (FSH) along with a decrease in the serum levels of 17β-estradiol (E2). POF can affect the physical and mental health of women and increase their risk of infertility, thrombosis, osteoporosis, autoimmune diseases, cardiovascular diseases, and death.[1] The incidence rate of POF is over 1%, which is increasing year by year.[2]

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Follicular atresia triggered by apoptosis of the ovarian granulosa cells has been reported as one of the primary mechanisms responsible for POF.[3] Apoptosis of the ovarian granulosa cells may be due to the inhibition of the PI3K/AKT signaling pathway. Activation of PI3K/AKT signaling pathway can inhibit the apoptosis of the ovarian granulosa cells and help treat POF.[4] Treatment of POF is primarily accomplished via hormone replacement therapy with estrogen. It promotes the proliferation of granulosa cells as well as inhibits their apoptosis; it also promotes the growth of follicles by activating the PI3K/AKT signaling pathway.[5] However, hormone replacement therapy also increases the risk of complications, such as tumor formation and thrombosis, which limits its clinical application.[6] Phytoestrogens can supplement estrogens as well as prevent breast and ovarian cancers, cardiovascular diseases, and osteoporosis. They have bidirectional regulatory mechanisms and are safer than estrogens.[7-10] Therefore, they are used as alternative and complementary treatment for POF.

Cuscuta chinensis Lam., also known as Chinese dodder, is commonly used in traditional medicine in China and other Asian countries.[8] It is consumed by mixing with alcoholic beverages for the purpose of improving sexual ability and vision. It is also used to treat renal insufficiency, low back pain, impotence, spermatorrhea, frequent urination, infertility owing to cold uterus, threatened abortion, or habitual abortion, as well as to treat hepatic dysfunction with blurred vision.[8] Flavonoids, polysaccharides, alkaloids, volatile oil, and lignans are the chemical components of *C. chinensis*. Flavonoids account for approximately 3.0% of the total chemical components.[8] They have estrogenic effects and can activate the estrogen receptor,[9] upregulate the expression of Bcl-2/Bax, downregulate the expression of caspase-3, and ultimately decrease apoptosis.[10] To the best of our knowledge, so far, there are no studies conducted on the effects of *C. chinensis* extract on POF.

Quercetin, an antioxidative flavonoid, shows estrogenic activity by binding to the estrogen receptor and is a major component of extracts of *C. chinensis*.[11,12] Flavonoids have been reported to prevent spontaneous abortion via regulation of ovulation and hormones.[13] It can directly affect the basic functions of ovarian cells; it promotes proliferation; inhibits apoptosis and hormone release;[14] increases the number of primordial follicles; decreases the number of atresia follicles; and decreases the level of malondialdehyde, caspase, and nuclear factor kappa B in the ovarian tissue.[15] Quercetin has also been reported to improve the antioxidative ability of ovaries and to reduce POF[16] as well as to increase litter size in young mice.[17] Moreover, quercetin activates the PI3K/AKT signaling pathway, which results downregulating the protein expression of caspase-3 and upregulating the protein expression ration of Bcl-2/Bax, thereby inhibiting apoptosis.[18,19] However, it is unknown whether quercetin can alleviate POF via PI3K/AKT signaling pathway.

Therefore, in this study, we aimed to investigate the effects of flavonoids and quercetin from *C. chinensis* on tripterygium glycoside (TG)-induced POF. In this context, we aim to demonstrate that quercetin and flavonoids extracted from *C. chinensis* inhibit TG-induced POF and that these effects may be governed by the regulation of PI3K/AKT signaling pathway. The results of this study may provide deeper insights into the treatment of POF with *C. chinensis* extracts and novel therapeutic directions.

MATERIALS AND METHODS

Flavonoid extracts from *Cuscuta chinensis* Lam

Flavonoids from *C. chinensis* were extracted at the Modern National Centers. In brief, *C. chinensis* was refluxed twice with a tenfold volume of 70% ethanol for 1 h and filtered. Filtrates were combined and added to water and loaded onto an AB-8 macroporous adsorption resin column. The extract was eluted with water followed by 20% ethanol and then 60% ethanol. The eluate was vacuum-dried to remove 60% ethanol. The flavonoids were identified via high-pressure liquid chromatography (HPLC) fingerprint using SinoChrom ODS-BP column (Dalian Elite Analytical Instrument Co., Ltd, Dalian, China; size 4.6 mm × 250 mm and particle size 5 μm, Dalian, China). The separation condition was as follows: flow rate of 1 mL/min, temperature of the column at 30°C, detection wavelength at 220 nm, and binary gradient elution system with 0.1% phosphoric acid aqueous solution and acetonitrile for 130 min. The volume of injection was 10 mL. The HPLC data revealed that flavonoid extracts from *C. chinensis* was extracted successfully [Supplement Figure 1].

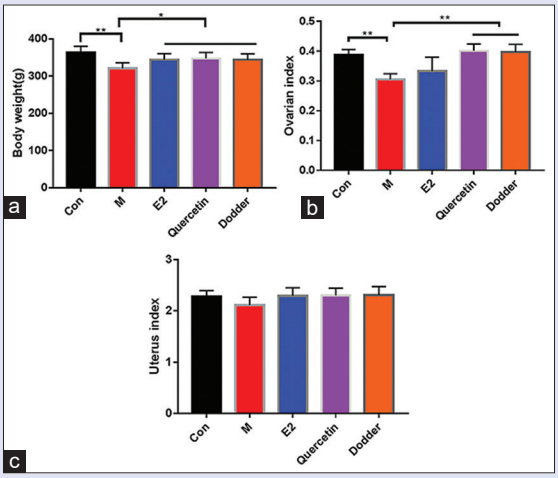

Figure 1: Effects of quercetin and flavonoids extracted from *Cuscuta chinensis* Lam. on the rat weight, ovarian indices, and uterus indices. The weight (a), ovarian index (b), and uterus index (c) for rats in different groups. *$P < 0.05$, **$P < 0.01$

Animals and treatment

Sixty male Sprague–Dawley rats (8 weeks, 220–250 g) were purchased from Guangdong Medical Animal Laboratory Center. All animals were housed in microisolator cages with free access to food and water, according to the Guide for the Care and Use of Laboratory Animals.[20] The rats were kept in a light-controlled room under a 12-h light/12-h dark cycle with controlled temperature (23°C–25°C). All efforts were made to avoid the unnecessary pain of animals. This study was approved by the Institutional Animal Care Committee at Shenzhen Nanshan District People's Hospital.

After feeding the animals for 1 week, their estrous cycle was observed by vaginal smear; only those rats whose vaginal smear was observed were used for further studies. All animals were randomly divided into five groups with 10 rats per group: (1) Control group – Rats were fed with normal saline via oral gavage for 60 days; (2) POF group – Rats were fed with 75 mg/kg/day TG via oral gavage for 30 days followed by oral gavage of normal saline for 30 days; (3) E2 group – Rats were fed with 75 mg/kg/day TG via oral gavage for 30 days followed by oral gavage of 0.1 mg/kg E2 (Sigma-Aldrich, Missouri, USA) for 30 days; (4) quercetin group – Rats were fed with 75 mg/kg/day TG via oral gavage for 30 days followed by oral gavage of 600 mg/kg quercetin (Sigma-Aldrich, Missouri, USA) for 30 days; and (5) flavonoids group – Rats were fed with 75 mg/kg/day TG via oral gavage for 30 days followed by oral gavage of 530.1 mg/kg flavonoids extracted from *C. chinensis*. The dose of flavonoids was determined by the following formula: 30 g/60 kg × 6.25/1000 × extract rate × weight. After treatment, weight, ovarian index (unilateral ovarian weight [mg]/rat's weight [g]), and uterus index (uterus weight [mg]/rat's weight [g]) were measured.

Histological analysis and immunohistochemistry

Ovarian tissue samples were collected immediately after resection and stored at −20°C before use. Then, the specimens were fixed in 10% formalin buffer, embedded in paraffin, and sectioned. Serial sections (5 μm each) were cut along the sagittal plane. Sections were stained with hematoxylin and eosin (HE) for histological analysis. The thickness of granulosa cells in the antral follicles were determined using Image J software (Rasband; NIH, USA). Following tissue deparaffinization and hydration, immunohistochemistry (IHC) staining was performed. Samples were incubated overnight with the following primary antibodies at 4°C: Anti-PI3K (Santa Cruz, USA), anti-p-AKT (Cell Signaling Technology, USA), anti-Atg5, (Proteintech Group, USA), anti-cyclin D2 (Abcam, USA), and anti-caspase-3 (Cell Signaling Technology, USA). Subsequently, all samples were incubated with their corresponding horse radish peroxidase-conjugated secondary antibodies (Abcam, USA) and incubated at 37°C for 30 min and stained with diaminobenzidine. The integral optical density was determined using Image J software (National Institutes of Health, Bethesda, MD, USA).

Measurement of hormones in serum

Briefly, blood (5 mL) was collected from the abdominal aorta and centrifuged at 1200 × *g* for 10 min. Then, the serum levels of anti-Müllerian hormone (AMH), luteinizing hormone (LH), FSH, and E2 were determined via enzyme-linked immunosorbent assay using commercially available kits (CUSABIO Ltd., Wuhan, China).

RNA extraction and quantitative real-time polymerase chain reaction

The mRNA expression of PI3K, Akt, Atg5, cyclin D2, and caspase-3 in the ovarian tissues was measured using the real-time polymerase chain reaction (RT-qPCR) method. Briefly, the total RNA was extracted using TRIzol reagent (Tiangen Biotech, Beijing, China) and the Prime-Script™ one-step RT-qPCR Kit (TAKARA, Dalian, China). Then, the RNA was used to convert to cDNA. PCR was performed in an Exicycler™ 96 (Bioneer, Daejeon, Korea). Following PCR primers were used: Rat-PI3K-219-F: 5'-GTGA CAGGCACAACGACAAC-3', Rat-PI3K-219-R: 5'-GGTAAGCCCTAACGCAGACATC-3'; Rat-AKT-270-F: 5'-AGGCATCCCTTCCTT ACAGC-3', Rat-AKT-270-R: 5'-CAGCCCGAAGTCCGTTATCT-3'; Rat-Atg5-116-F: 5'-AGTTTTGGACCATCAACCGGA-3', Rat-Atg5-116-R: 5'-CAGCTTCTGAATGAAAGGCCG-3'; Rat-Ccnd2-169-F: 5'-GCTGACCAAGATCACCCACA-3', Rat-Ccnd2-169-R: 5'-AACATCCCGCACGTCTGTAG-3'; Rat-Caspase-3-142-F: 5'-AGCTGGACTGCGGTATTGAGA-3', Rat-Caspase-3-142-R: 5'-CATGACCCGTCCCTTGAATT-3'; R-GAPDH-74-F: 5'-GCAAGAGAGAGGCCCTCAG-3', R-GAPDH-74-R: 5'-TGTGAGGGAGATGCTCAG-3'. Relative RNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. GAPDH was used as an internal control.

Western blot analysis

The total protein was extracted from cells and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique. The separated protein bands were transferred onto poly (vinylidene fluoride) membranes. After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were probed with specific primary antibodies (anti-PI3K, anti-p-AKT, anti-Atg5, anti-cyclin D2, anti-caspase-3, and anti-AKT) at 4°C overnight and then incubated with their corresponding secondary antibody at 37°C for 45 min. Target bands were visualized using enhanced chemiluminescence (Bio-Rad). GAPDH served as an internal control.

Statistical analysis

The data were expressed as mean ± standard deviation. Data were compared using one-way analysis of variance, followed by Tukey *post hoc* test. A $P < 0.05$ was considered statistically significant. Calculations were performed using SPSS (18.0, IBM, Armonk, NY, USA).

RESULTS

General observations

Rats in the control group demonstrated the presence of smooth hair and showed normal activity, consumed proper diet, and had regular stools. However, all rats in the treatment groups demonstrated slower responses, mental retardation, reduced activity, lackluster hair, and curled up arched back. Furthermore, several rats had loose stools in the first 30 days after induction with TG. All the aforementioned symptoms improved after administering the animals with E2, quercetin, and flavonoids in the POF group. The vaginal estrus cycles of animals in the POF group were significantly longer than those in the control group [$P < 0.05$, Table 1]. However, the vaginal estrus cycle was remarkably decreased in rats belonging to E2, quercetin, and flavonoid groups when compared with rats in the POF group ($P < 0.05$). These results showed that quercetin and flavonoids extracted from *C. chinensis* might improve the TG-induced POF symptoms and reduce the vaginal estrus cycle.

Effects of quercetin and flavonoids extracted from *Cuscuta chinensis* on body weight and ovarian and uterus indices

The body weight of animals in all the treatment groups (POF, E2, quercetin, and flavonoids) was significantly lower than those in the control group [$P < 0.05$, Figure 1a]. Rats treated with E2, quercetin, and flavonoids had significantly higher weight than that of rats in the POF group [$P < 0.05$, Figure 1a]. The value of ovarian index [Figure 1b] was significantly decreased in the POF group compared with the control group ($P < 0.05$), and the treatment with either quercetin or flavonoids dramatically increased the value of TG-induced ovarian index ($P < 0.05$). Moreover, there was no significant change among the groups in terms of uterus index [Figure 1c]. In summary, our results showed that quercetin and flavonoids extracted from *C. chinensis* Lam. enhance the POF-induced weight loss and decrease the values of ovarian index.

Effects of quercetin and flavonoids extracted from *Cuscuta chinensis* on ovarian histology

Ovarian histology was analyzed by HE staining, and the number of follicles and the thickness of the layer of granulosa cells in the antral follicles were determined. TG significantly reduced the ratio of primitive follicles, secondary follicles, and sinusoidal follicles and the number of cells in the corpus luteum and significantly increased the ratio of atresia follicles [$P < 0.05$, Figure 2a and b]. However, after treatment with E2, quercetin, and flavonoids, the ratio of primitive follicles, secondary follicles, and sinusoidal follicles and the number of cells in the corpus

Table 1: The vaginal estrus cycle of the premature ovarian failure rats

Groups	*n*	Estrus cycle (days)
Control	10	4.5±0.527
Model	10	8.7±1.252**
Estradiol (E2)	10	5.8±1.317**,##
Quercetin	10	5.3±1.159##
Dodder	10	5.9±1.197**,##

**P<0.05 versus control; ##P<0.05 versus model

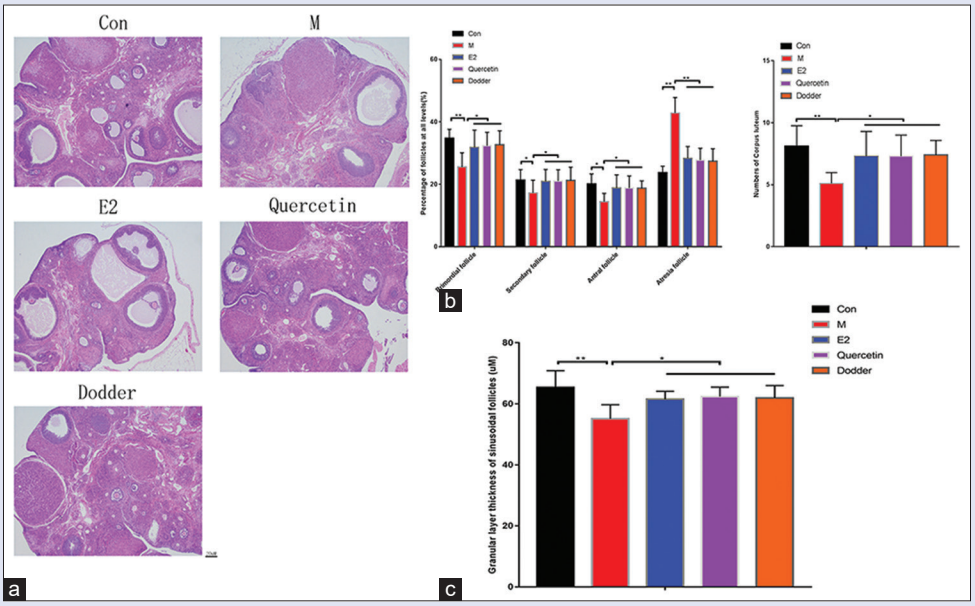

Figure 2: Effects of quercetin and flavonoids extracted from *Cuscuta chinensis* Lam. on ovarian tissue histology. Histology of ovarian tissues was analyzed using HE staining (a), and the ratio of primitive follicles, secondary follicles, sinusoidal follicles, and atresia follicles and the number of corpus luteum were measured (b). The thickness of granulosa cell layer in antral follicles (c) was also measured using Image J software. *$P < 0.05$, **$P < 0.01$

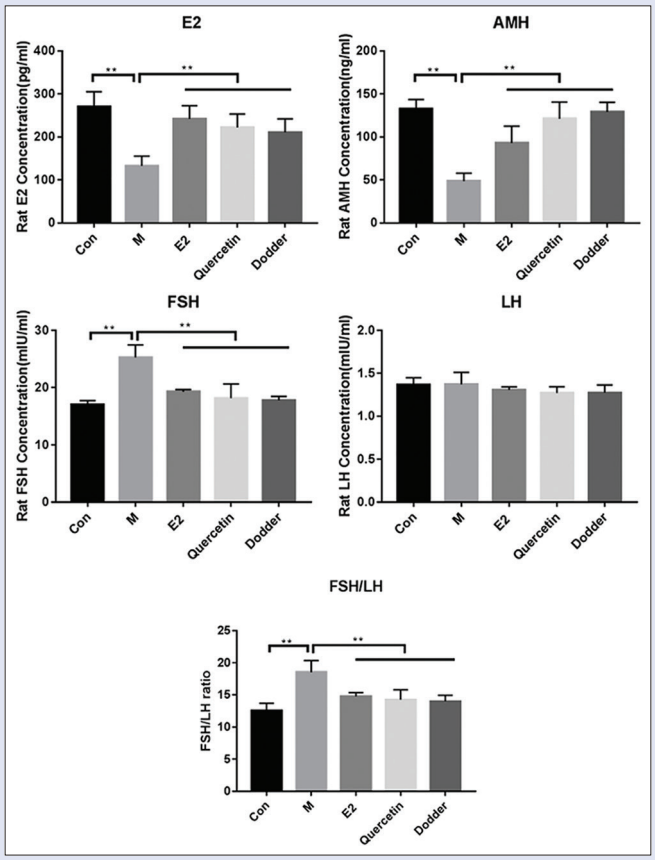

Figure 3: Effects of quercetin and flavonoids extracted from *Cuscuta chinensis* Lam. on serum hormones. Serum levels of E2, anti-Müllerian hormone, FSH, LH, and FSH/LH were determined using enzyme-linked immunosorbent assay. *$P < 0.05$, **$P < 0.01$

luteum were found to be remarkably increased, and the ratio of atresia follicles was significantly decreased ($P < 0.05$). The thickness of the layer of granulosa cells in the antral follicles was remarkably increased in the E2, quercetin, and flavonoid groups but was significantly decreased in the POF group [$P < 0.05$, Figure 2c]. These results show that quercetin and flavonoids reduced injury of TG-induced POF.

Effects of quercetin and flavonoids on serum hormonal levels

In the POF group, serum levels of both E2 and AMH were significantly decreased, whereas FSH and FSH/LH levels were remarkably increased [$P < 0.05$, Figure 3]. However, when treated with E2, quercetin, and flavonoids, the serum levels of E2 and AMH were dramatically increased, whereas the serum levels of FSH and FSH/LH were significantly decreased ($P < 0.05$), suggesting that quercetin and flavonoids extracted from *C. chinensis* might attenuate the abnormal expression of serum hormones in POF.

Effects of quercetin and flavonoids on PI3K/AKT signaling pathway and apoptosis-related genes

In this study, we analyzed the expression of PI3K/AKT signaling pathway and apoptosis-related genes in the ovarian tissues of different study groups using IHC, western blot analysis, and RT-qPCR. Our results demonstrated that the expression of PI3K, p-AKT, Atg5, and cyclin D2 was significantly downregulated and the expression of caspase-3 was significantly upregulated in the POF group compared with the control group [$P < 0.05$, Figures 4 and 5a, b]. However, E2, quercetin, and flavonoids remarkably increased the mRNA levels of PI3K, p-AKT, Atg5, and cyclin D2 protein, and TG significantly reduced the mRNA levels of caspase-3 protein ($P < 0.05$). These results show that quercetin and flavonoids extracted from *C. chinensis* can rescue TG-induced POF via regulation of the PI3K/AKT signaling pathway and expression of Atg5, cyclin D2, and caspase-3.

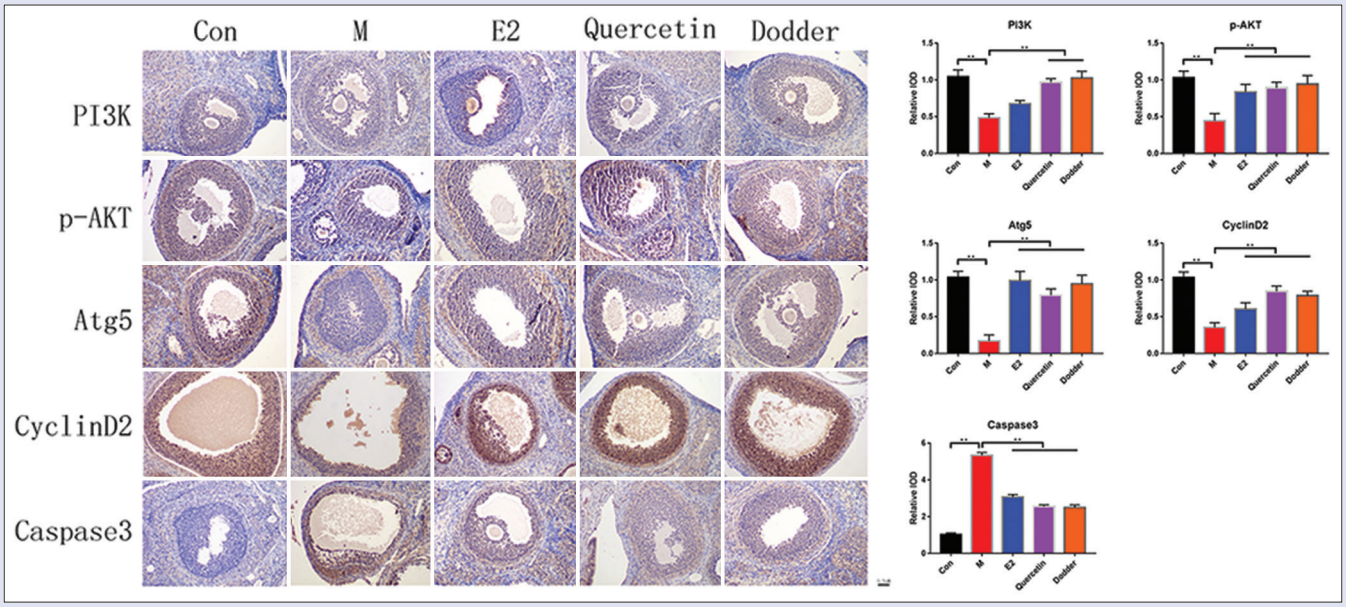

Figure 4: PI3K/AKT signaling pathway and apoptosis-related proteins were detected by immunohistochemistry. Expressions of PI3K, AKT, p-AKT, Atg5, cyclin D2 and caspase-3 were determined using immunohistochemistry in ovarian tissues. *$P < 0.05$, **$P < 0.01$

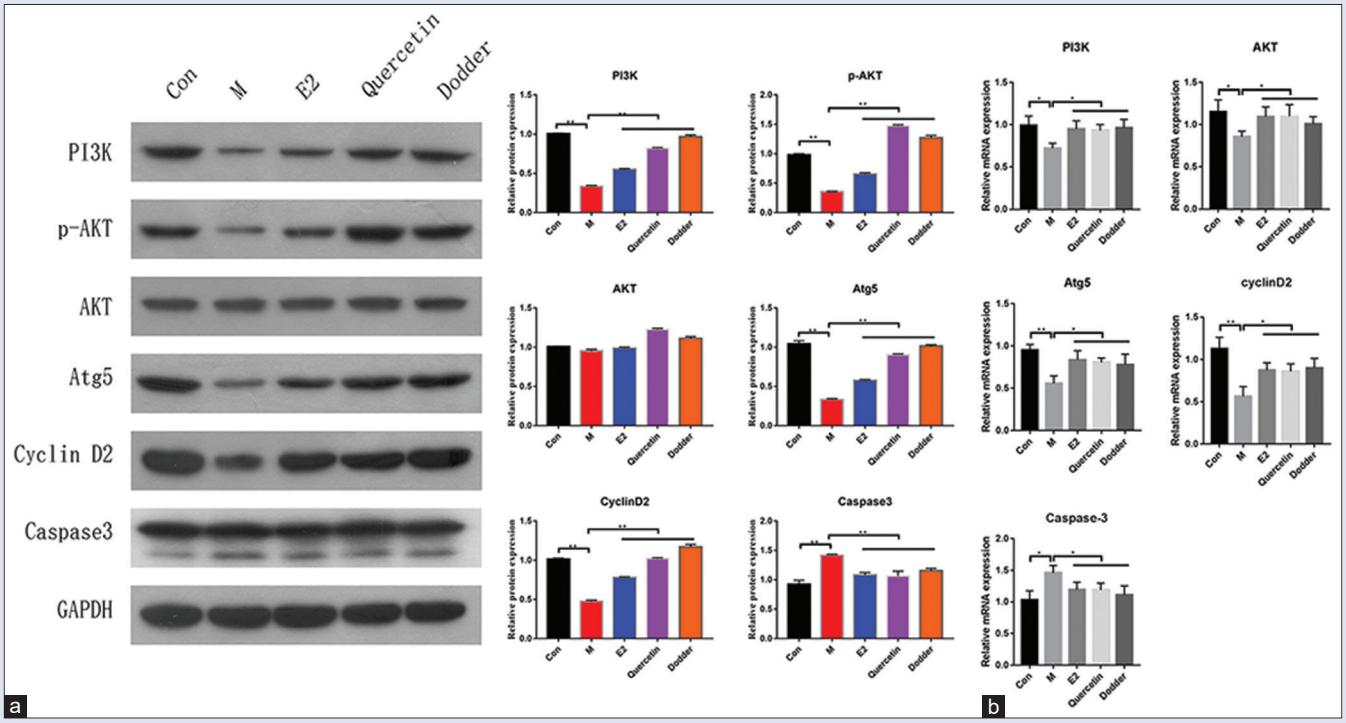

Figure 5: PI3K/AKT signaling pathway and apoptosis-related genes were detected by western blotting (a) and real-time polymerase chain reaction (b) in ovarian tissues. *$P < 0.05$, **$P < 0.01$

DISCUSSION

Numerous studies conducted on POF have demonstrated that apoptosis of the granulosa cells was a major molecular mechanism for POF. The PI3K/AKT signaling pathway is closely related to the proliferation and differentiation of granulosa cells, which is crucial for the growth and development of follicles and ovulation. The inactivation of the PI3K/AKT signaling pathway is important to POF pathogenesis, while the activation of this pathway can reverse POF.[21] Several herbal extracts have been reported to treat POF by activating the PI3K/AKT signaling pathway.[4,22,23] Estrogen is primarily produced by ovarian granulosa cells which regulates follicular development and maturation locally via autocrine and paracrine pathways. Therefore, estrogen is regarded as a direct indicator of follicular reserve function. A decrease in the level of estrogen has been suggested to be closely related to the occurrence of POF.[24] E2 induces the activity of the PI3K/AKT signaling pathway,

and the expression of downstream proteins induces the proliferation of granulosa cells, thereby improving the reproductive capacity of female animals. Loss of the termination signal, phosphatase and tensin homolog, of the PI3K signaling pathway decreases the level of apoptosis of the granulosa cells and their consequent proliferation.[3]

Recently, a study has reported that cell cycle regulatory mechanisms participate in the proliferation of ovarian granulosa cells.[25] In ovarian tissue, cyclin D2 is primarily expressed in the granulosa cells and promote proliferation of the granulosa cells and follicular growth.[26] Selectively knocking out the cyclin D2 gene in mice and blocking the proliferation of the granulosa cells in ovaries result in slow development of follicles and no ovulation.[27] In FSH-mediated proliferation of the granulosa cells, the mRNA of cyclin D2 was upregulated significantly, but the levels of phosphorylated mTOR and cyclin D2 decreased significantly after treatment with rapamycin. This suggests that cyclin D2, as a downstream molecule of the PI3K/AKT/mTOR signaling pathway, plays an important role in the proliferation of the ovarian granulosa cells.[28] Caspase-3 is a member of the highly conserved cysteine protease family and is the primary effector molecule of apoptosis. Activation of PI3K/AKT signaling pathway can inhibit apoptosis. Activated AKT can inactivate caspase-9, leading to the inactivation of caspase-3, thereby inhibiting caspase-3-induced apoptosis.[29] Atg5 is a positive effector of autophagy, and the PI3K signaling pathway is related to the activation of autophagy. As an important process of auto-repair, moderate activation of autophagy may help remove damaged organelles and abnormal proteins, prevent protein aggregation, and thereby protect cells from damage.[30]

Flavonoids are the primary functional components of *C. chinensis* extract. They have an estrogenic effect and activate the estrogen receptor,[9] promote the increase in the level of Bcl-2/Bax, reduce the expression of caspase-3, and reduce apoptosis.[10]

Flavonoids from *C. chinensis* can improve the ovarian endocrine functions in female rats.[31] Quercetin is the active component present in the total flavonoid extract of *C. chinensis*,[11] which can increase the number of primordial follicles, reduce the number of atresia follicles, promote the proliferation of ovarian granulosa cells, inhibit apoptosis, and reduce POF.[16] Moreover, quercetin can also increase the number of offspring of young female mice[17] and activate the PI3K/AKT signaling pathway, downregulate caspase-3 expression, upregulate the Bcl-2/Bax protein ratio, and inhibit apoptosis of PC12 and H9C2 cells.[18,19]

TG is a cytotoxic drug that induces apoptosis of the ovarian granulosa cells, prevents the growth of follicles, and causes follicular atresia, which eventually leads to POF and reproductive toxicity. Induction of POF by TG in rat is a classic study model.[32] In this study, we found that TG-induced animals demonstrated POF syndrome, with a disordered estrous cycle and a depressed ovarian index. The ovarian structure was also changed: the ratio of primitive follicles, secondary follicles, and sinusoidal follicles and the number of cells in the corpus luteum were reduced, whereas the ratio of atresia follicles was enhanced. The thickness of the layer of ovarian granulosa cells decreased. In addition, serum hormonal levels were changed and serum E2 and AMH levels were reduced, whereas FSH and FSH/LH levels were increased. These symptoms were consistent with those of patients with POF, indicating that the POF rat model was successfully developed in this study. Treatment with commercially sourced quercetin and flavonoids extracted from *C. chinensis* reversed the symptoms of POF syndrome. These results show the effectiveness of quercetin and flavonoids extracted from *C. chinensis*. We also found that the expression of PI3K, p-AKT, Atg5, and cyclin D2 was downregulated, and the expression of caspase-3 was upregulated in the POF group. Induction of POF in the rat model by TG may suppress the PI3K/AKT signaling pathway and regulate the downstream factors, i.e., cyclin D2 and caspase-3, leading to the apoptosis of the granulosa cells and eventually resulting in POF. Quercetin and flavonoids extracted from *C. chinensis* may treat POF via activation of the PI3K/AKT signaling pathway and downstream factors to promote the proliferation and inhibit apoptosis of the ovarian granulosa cells. The activation of autophagy may protect granulosa cells from damage, which needs further exploration.

CONCLUSION

We investigated the effects of quercetin and flavonoids extracted from *C. chinensis* in the TG-induced POF rat model. The extracted flavonoids and quercetin suppressed POF via activation of the PI3K/AKT signaling pathway and regulation of caspase-3, Atg5, and cyclin D2. The results of this study may provide deeper insights into the role of *C. chinensis* extracts in POF treatment and offer new therapeutic directions.

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Conflicts of interest

There are no conflicts of interest.

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