



The Effects of R10 Fraction of Garlic (*Allium sativum* L.) on Gonadotropins and Oocyte Maturation in a PCOS Mouse Model

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is a common metabolic, immunological, and reproductive disorder worldwide. Successful therapeutic and management approaches for PCOS remain a challenge. This experimental study was designed to find out the effects of garlic R10 fraction on gonadotropins, oocyte-secreted factors, and oocyte maturation in a PCOS mouse model.

Materials and Methods: R10 fraction was obtained from garlic extract and PCOS-induced mice were prepared using estradiol valerate. Treatment with R10 fraction was performed and compared between two the normal and control PCOS groups. The alterations in gonadotropins (LH and FSH), oocyte-secreted factors gene expression (GDF9 and BMP15), and evaluation of histological changes in the ovary were performed.

Results: After treatment with R10 fraction, the LH level was increased and conversely the FSH level was decreased in the serum of the PCOS model. Furthermore, PCOS-induced ovaries demonstrated high atrophy and a significant decrease in the number of Graaf. There was also a significant reduction in the *GDF9* and *BMP15* gene expression levels in the PCOS model compared to the normal group ($p < 0.001$).

Conclusions: This study indicates that garlic R10 fraction could modulate PCOS in mice at the systemic level in the hypothalamic–pituitary–gonadal axis and the local level in the ovaries. Accordingly, the R10 fraction can be considered a therapeutic approach to improve PCOS conditions.

Keywords: Polycystic Ovary Syndrome, Garlic R10 Fraction, Gonadotropin, Gene Expression, Mouse

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Introduction

Polycystic ovary syndrome (PCOS) is known as one of the most common metabolic and reproductive disorders that involves 6-12% of adult women.¹ The disease is defined by heterogeneous features with widely variable phenotypes and clinical symptoms.² The etiology of this syndrome is still not completely realized. Current evidence suggests that PCOS might be accounted as a complex multifactorial abnormality which is influenced by epigenetic and environmental factors such as diet and lifestyle. The disease is characterized by abnormal functions of the hypothalamic-pituitary-ovarian axis, ovarian dysfunction, hyperandrogenism, chronic anovulation, and aberrant oocyte maturation.³ Women with PCOS have multiple cystic follicles in their ovaries with abnormalities in androgens metabolism and suffer from irregular menstrual cycles, hirsutism, acne, and infertility.⁴ Furthermore, PCOS has been recognized to be frequently associated with multiple pathological conditions such as obesity, hypertension, diabetes, cardiovascular diseases, and endometrial cancers.⁵

Potential therapeutic and management approaches for

PCOS have been challenging. Having considered the side effects, current therapeutic approaches have been focusing on the ameliorating main symptoms⁶ and are usually planned to include different pathological, social, and psychological concerns related to this syndrome. Apart from lifestyle modification requirement, women with PCOS are advised to receive proper pharmacological treatments depending on their symptoms^{6,7} including balancing of sex hormones, menstrual cycle, ovulation, excessive hair growth, and regulation of the insulin levels. Nevertheless, most of the commercially available medications have shown some side effects such as weight gain, hepatic toxicity, and irregular bleeding, which may lead to further problems for patients.⁸ Over the last years, increasing attention has been drawn towards the natural products including plant-based drugs with low adverse effects⁹⁻¹¹ for PCOS treatment.

Many studies have demonstrated immunomodulatory effects of garlic (*Allium sativum*) in different animal models of inflammatory conditions¹²⁻¹⁸ and in vitro experiments.¹⁹⁻²² The extent of research performed on garlic (*Allium sativum*)

is so large that it is beyond the scope of this article to mention the beneficial effects of garlic. However, there are many review articles have been written on it and are still being written to complement each other and cover garlic immunomodulatory effects.²³⁻²⁶ Accordingly, immunomodulatory effects of garlic R10 fraction has been previously shown in PCOS and other inflammatory conditions.²⁷⁻²⁹ Therefore, based on the inflammatory nature of PCOS³⁰ on the one hand and immunomodulatory effects of garlic R10 fraction²² on the other hand, this study was designed to find out the effects of garlic (*Allium sativum*) R10 fraction on gonadotropins, oocyte-secreted factors, and oocyte maturation in mice model of PCOS. It is noteworthy that the mice model of PCOS established in this study has similarities with human cases of PCOS.^{31,32}

Materials and Methods

Animals and Ethical Issues

Sixty adult female NMRI mice (8 weeks old, 25-30 g weight) were purchased from Pasteur Institute, Tehran, Iran and were maintained according to the standard ethical instructions. Mice were housed in a controlled standard environment of temperature and humidity with a 12 h dark:12 light cycle with free access to food and water. Animal experimentation was performed after approval of Imam Khomeini International University authorities (Approval ID: 8567) in compliance with Ethical Committee of Iran Ministry of Health and Education.

Garlic R10 Fraction Preparation

Garlic R10 fraction was prepared as described before.²⁷ Briefly, the bulbs were peeled off, homogenized with two parts of the distilled water in a blender, passed through a Whatman paper under vacuum conditions, centrifuged at 5000 rpm for 30 min, and run through Amicon ultrafiltration system equipped by several different membranes to extract the fraction R10 of garlic including 300, 100, 50, 30, and 10 kDa. The residual sample was obtained from the 10 kDa membrane based on which was nominated as R10 fraction.

Study Design, PCOS Induction, and Treatment with R10 Fraction

The animals were divided into five groups (n = 12) including healthy control, PCOS control, R10 fraction-treated PCOS group 1 (Treat 1), R10 fraction-treated PCOS group 2 (Treat 2), and sham group as a PCOS group treated with saline, as described before.²⁷

PCOS mice model was established in our laboratory using vaginal smear and histological analysis as described before.²⁷ Briefly, the animals were monitored to have normal estrous cycle via vaginal smear assay to determine the stage of estrous cycle.³³ Those animals with several regular estrous cycles were selected³³ and subjected to a single intramuscular

injection of estradiol valerate (40 mg/kg, Sigma Chemical Co., Germany). Two weeks later, estrous cyclicity using vaginal smear analysis was monitored daily. The animals were followed up for eight weeks when abnormal estrous cycles and persistent vaginal cornification (PVC) as the two main signs of PCOS induction were observed.

The PCOS animal models housed in the Treat 1 and Treat 2 groups were treated with R10 fraction via intraperitoneal at a dose of 20 mg/kg weight³⁴ daily for one week (Treat 1) or every-other-day treatment for two weeks (Treat 2). The mice were euthanized with Ketamine/Xylazine (Alfasan, Woerden, Holland) three days after last injection, and serum and ovarian tissues were isolated.

Hormonal, Histological, and Molecular Evaluations

The serum levels of the two gonadotropins including LH and FSH hormones were examined by LH and FSH ELISA kits (Mybiosource, San Diego, CA), respectively.

To evaluate alterations in the structure of ovarian tissues. The ovaries were excised and fixed in 10% (w/v) paraformaldehyde and processed for histological analysis, as described before.²⁷ Briefly, the samples were then dehydrated by different concentrations of alcohol, cleared with xylene solvent and embedded in paraffin. Next step, the tissue sections (5 μ m) were prepared using a microtome (Leica Microsystems SP 1600, Germany). Finally, the ovarian sections were stained with H&E stain (Sigma Chemical Co., Germany), visualized under a light microscope, and photographed (Leica Microsystems AG, Germany).

Expression levels of oocyte-secreted factors genes including *bmp15* and *gdf 9*, as two significant genes involved in oocyte maturation process, were determined using real-time PCR. The total RNA was isolated from the ovarian tissues of all studied groups according to QIAzol lysis reagent protocol (QIAGEN Inc., Valencia, CA) and the cDNA was synthesized using cDNA synthesis kit (Thermoscientific, UK). Primers were designed using Gene Runner software (Version 6.5.52). The primers sequences were as forward 5'-TCAGAGCA AGAGAGGCATCC-3', and reverse 5'-GGTCATCTTCTC ACGGTTGG-3' for *beta-actin*, forward 5'-CTGGCATGTA CAGACCCTGG-3' and reverse 5'-TGGTCCGGCATTAG GAACC-3' for *bmp15*, and forward 5'-ACTGCCATGGAA CACTTGCT-3' and reverse 5'-AGGGTGAATGAGTACGG TGC-3' for *gdf 9*. The gene expression levels were quantitatively measured using ABI system. The *beta-actin* gene was used as the housekeeping gene. The Real-Time PCR program was conducted by initial denaturation at 95 °C for 15 min followed by amplification step including a denaturation step (15 sec for 95 °C), an annealing step (30 sec for 60 °C), and finally, an extension step (30 sec for 72 °C) for 40 cycles. All tests were performed in triplicate. Relative expression of the genes of interest was calculated according to the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

Data analysis was performed with Prism GraphPad Prism (GraphPad Software, San Diego, CA, and Version 6) using one-way ANOVA followed by post hoc Tukey’s multiple comparison. The data were depicted as the mean ± standard error of the mean (SEM), and *p* values ≤ 0.05 were considered statistically significant.

Results

Hormonal Analysis

Gonadotropins play important roles in maturation of oocyte and folliculogenesis. Previous reports indicated perturbation of FSH and LH levels in PCOS.^{34,35} On this basis, we evaluated modulatory effects of garlic R10 fraction on FSH and LH levels. As shown in Figures 1a and b, FSH and LH levels were significantly reduced and increased in PCOS and sham groups compared to normal animals, respectively (*p* <0.001). However, two-week treatment regimens with garlic R10 fraction in the R10 Treat 2 group led to significant

modulation in the serum levels of both FSH and LH in PCOS-treated animals (*p* <0.001). Nevertheless, one-week treatment could only modulate LH level (*p* <0.001) and had no significant effect on FSH level in R10 Treat 1 group.

Gene Expression

Given the modulatory effects of R10 fraction on ovary tissue,²⁷ we next examined expression levels of oocyte-secreted factor genes including *BMP15* and *GDF9* which have a key role in the control of ovarian function.³⁶ As shown in Figures 1c and d, the expression of both genes was significantly reduced in PCOS and sham groups (*p* <0.001). Notably, the levels in the R10 Treat 2 group were markedly modulated comparing with PCOS and sham groups (*p* <0.001) with no significant difference compared to the normal group. However, the one-week treatment in the R10 Treat 1 group could markedly modulate *BMP15* expression compared to PCOS and sham groups (*p* <0.001), though significantly lower than the normal group (*p* <0.001).

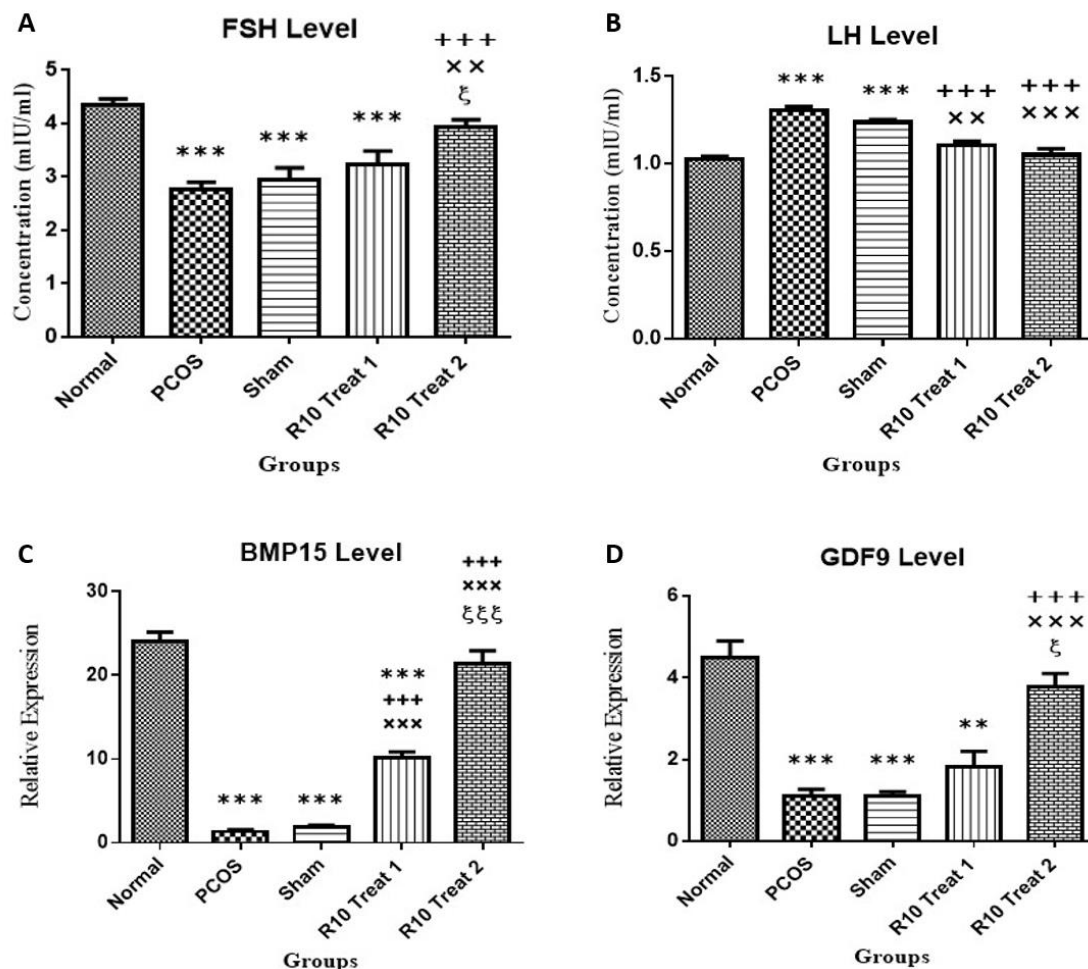


Figure 1. R10 Fraction Effect on Serum Levels of FSH (a), LH (b), BMP15 (c), and GDF9 (d) Were Determined in Normal, PCOS, Sham, R10 Treat 1, and R10 Treat 2 Groups (n = 12). Data are expressed as the mean ± SEM. Significant difference vs. the normal (*), vs. the PCOS group (+), vs. the sham group (‡), and vs. the R10 Treat 1 group (ξ).

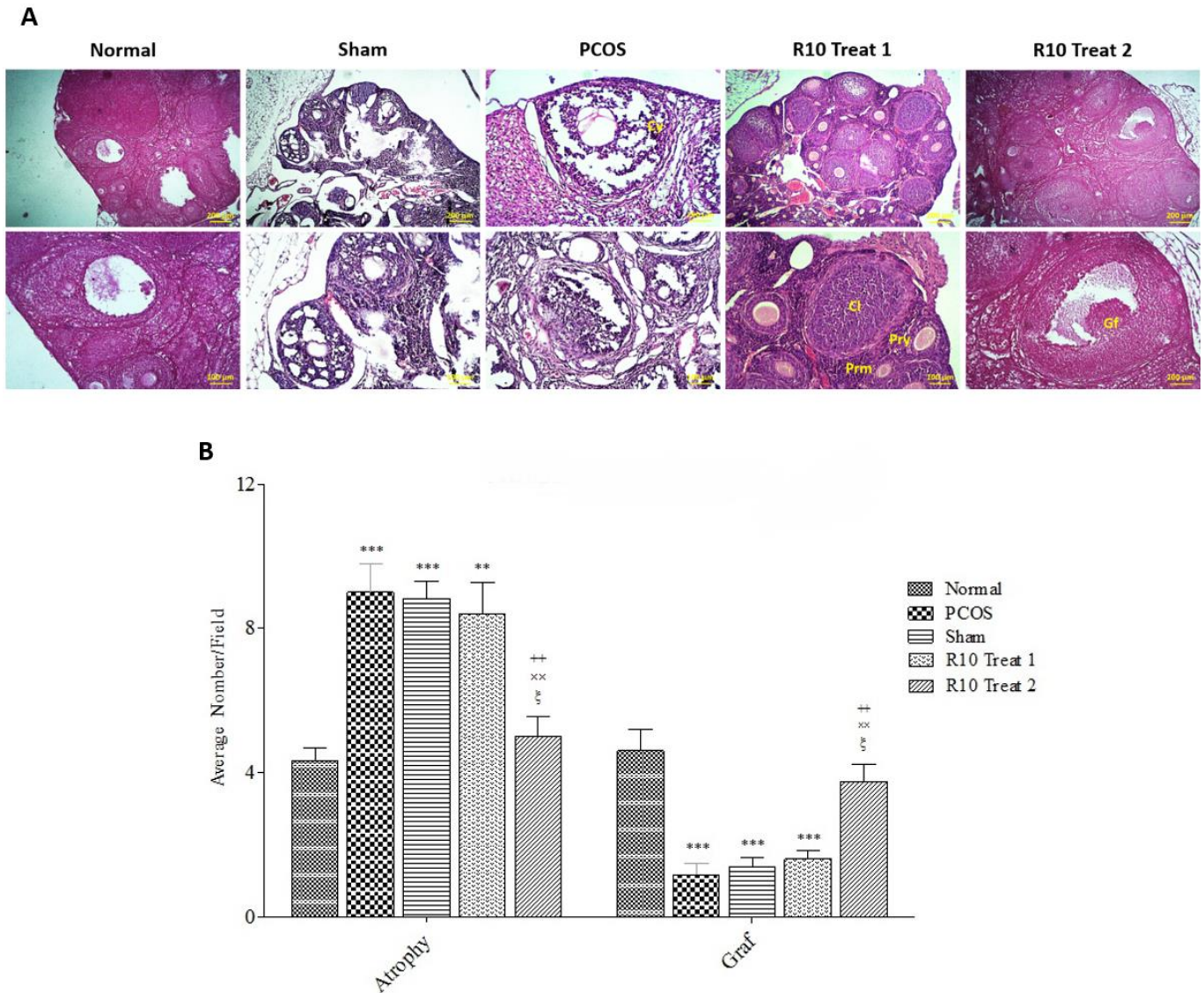


Figure 2. Ovarian Tissues from Normal, PCOS, Sham, R10 Treat 1, and R10 Treat 2 Groups Were Dissected and Stained with H&E Stain (a) and Alterations in the Average Number of Atrophy and Graaf levels (b) Were Examined. Data are expressed as the mean \pm SEM. Significant difference vs. the normal (*), vs. the PCOS group (+), vs. the sham group (Υ), and vs. the R10 Treat 1 group (ξ). Scale bar unit: μm . Cy: Cyst; Gf: Graaf follicle; Cl: Corpus luteum; Pry: Primary; Prm: Primordial.

GDF9 expression levels showed no significant results in R10 Treat 1.

Histologic Test

Considering gonadotropins and oocyte-secreted factors gene expression levels, we then examined levels of Graafian follicles and Atrophy in ovarian tissue. Histological examination of H&E-stained ovary tissues revealed that ovarian atrophy was significantly induced in PCOS and sham groups comparing to the normal group ($p < 0.001$). This was reverted in the R10 Treat 2 group ($p < 0.01$) while R10 Treat 1 group did not significantly change the situation comparing to the normal group ($p < 0.01$) and R10 Treat 2 ($p < 0.05$). As expected, the number of Graafian follicles in PCOS and sham groups was significantly lower than that of the normal group ($p < 0.001$)

indicating abnormal ovulation. Notably, the number of Graafian follicles in the R10 Treat 2 was significantly increased comparing to PCOS and sham groups ($p < 0.01$) implying reactivation of the normal ovulation process (Figure 2a, b). Nevertheless, no significant modulatory effect was observed in R10 Treat 1 compared to normal ($p < 0.001$) or R10 Treat 2 groups ($p < 0.05$), demonstrating the importance of the treatment regimen.

Discussion

Garlic has long been considered as one of the most effective and applicable plants in the traditional medicine. Garlic's anti-inflammatory and anti-cancer properties have been discovered a long time ago.²³⁻²⁶ As the efficient fraction of garlic, previous studies demonstrated that R10 fraction of

garlic possess anti-inflammatory and immunomodulatory properties.²⁷⁻²⁹ Given the immunomodulatory effects of R10 fraction and the inflammatory nature of PCOS,³⁰ in this study, therapeutic effects of garlic R10 fraction, as the garlic effective fraction, was evaluated in mice model of PCOS. The major aim was to optimize the beneficial effects and therapeutic outcome of garlic using its R10 fraction.

PCOS is considered as a multifactorial and complicated disorder on which administration of different medications has caused side effects in turn and predisposed PCOS-induced women to further troubles. Current therapeutic approaches of PCOS mainly are symptoms elimination-based treatments and there is no an ideal pharmaceutical available to treat all PCOS-associated disturbances. Most of the commercially available medications show various side effects, which might in turn cause additional inconvenience for patients. On this basis, the complexity and heterogeneity of the disease in patients PCOS causes different manifestations and makes the situation more complicated to treat.^{37,38}

PCOS can be viewed from systemic and local points of view. From the systemic facet, there is a decrease and an increase in FSH and LH, respectively.³⁴ Assessment of changes in the serum level of the two hormones is a reliable indicator of PCOS as they affect ovaries. These hormones are involved in the regulation of folliculogenesis and sex hormones known as hypothalamic-pituitary-gonadal axis.³⁵ The role of medicinal plants in balancing the levels of sex hormones, including LH and FSH, has been previously reported.³⁹⁻⁴⁰ It is known that the garlic plant is also able to regulate the level of sex-related hormones, e.g. LH, FSH and testosterone. For example, it was shown in one study that the imbalance in the level of sex hormones and testicular damage in rats mediated by cyclophosphamide (as an anti-cancer drug) was modulated after consuming garlic. Presumably, cyclophosphamide causes endocrine defect and induces oxidative stress in the testis. Compensatingly, garlic reduced the damage due to its antioxidant properties.^{41,42} Noteworthy, LH and FSH work together to stimulate normal follicle growth and ovulation.⁴³ In the condition of PCOS, when the level of these hormones changes, the normal growth of follicles as well as ovulation is disturbed. Studies have shown that the number of cystic follicles and ineffective atrophy are increased, and on the contrary, the number of Graafian follicles, i.e. mature follicles, shows a significant decrease in PCOS.⁴⁴ This is consistent with our results in which R10 fraction was shown to modulate FSH and LH level.²⁷ Accordingly, R10 fraction could modulate ovary tissue and cause a decrease in atrophy level and an increase of Graafian follicles in line with systemic changes. In addition, it has been proven that garlic or its compounds are able to change the expression level of some genes.^{45,46} As the first evidence, it has been found that garlic consumption could reduce the expression of two genes including *SIR2* and

ECE1 causing predisposition to vaginal yeast infection.⁴⁷ These beneficial effects have been attributed to the immunomodulatory properties of garlic. As the second evidence, treatment of HepG2 human liver cancer cells with diallyl tetrasulfide, one of the main sulfur-containing compounds in garlic, led to IL-2 and IL6 gene expression and even 4-fold expression of FOS gene.⁴⁸ The two genes including BMP 15 and GDF9 had more expression levels after two weeks of treatment with R10 fraction. These genes have leading roles in the control of ovarian function in female reproduction with synergistic effects.⁴⁹ Modulatory effects of R10 fraction shown in this study under two regimen reveals the importance of dosage and timing of treatment suggested before.²⁷

The advantage of R10 fraction over other cases proposed in traditional medicine, such as *Corylus avellana* seed oil, licorice ethanol extract, and resveratrol, is that R10 fraction affects hormonal and oocyte-related factors along with inflammatory markers. Such an effect has not been reported with other herbal medication candidates.⁵⁰⁻⁵² It is sometimes required to perform combined therapy to achieve better results.⁵³ More kinetic research is required to find out which one of local or systemic changes occur first.

As the last word, it is noteworthy to mention that no animal model is 100% similar to human condition in any cases. Although, mice model of PCOS has many similarities with human cases of PCOS, but they have few differences in terms of weight gain and some morphological and metabolic changes which limits translation of animal studies to human subjects.^{54,55}

Conclusion

The present study showed that R10 fraction as an effective component derived from garlic has a considerable therapeutic function for relieving PCOS symptoms. It could be considered as a potential herbal substance to modulate the hypothalamic-pituitary-gonadal axis, expression of crucial genes in oocyte development, and alleviate histological abnormalities in the ovary associated with PCOS.

Authors' Contributions

SF: Conceptualization, Methodology, acquisition of data, carrying out study, writing-original draft preparation; NP: Methodology, data analyses, writing-original draft preparation; RH: Conceptualization, methodology, interpretation of data. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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