

Silver Nanoparticles Biofabricated from *Cinnamomum zeylanicum* Reduce IL-6, IL-18, and TNF- α in Female Rats with Polycystic Ovarian Syndrome

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Abstract

Polycystic ovarian, or Stein-Leventhal, syndrome (PCOS) is an inflammatory disorder resulting in metabolic dysregulation and ovarian dysfunction as well as women's infertility. Management of PCOS requires multiple approaches. This experimental study was sought to assess the influence of *Cinnamomum zeylanicum* (CZ) derived silver particles (AgNPs) on inflammatory cytokines in rats with PCOS.

In this experimental study, AgNPs were synthesized using CZ bark extract, and characterized by the scanning electron microscope (SEM) and atomic force microscope (AFM). Thirty female rats, *Rattus norvegicus*, were grouped into five groups (6 animals/group). The experimental groups were vehicle control group (received 0.2 ml corn oil only), PCOS (received estradiol valerate of 4 mg/kg only), PCOS group received CZ extract (200 mg/kg), PCOS group received metformin (50 mg/kg) and PCOS group received AgNPs (3.53 mg/kg). After 30 days of treatment, serum concentrations of tumor necrosis factor-alpha (TNF- α), interleukins-18 (IL-18), and 6 (IL-6) were measured using ELISA.

Significant elevation ($P < 0.05$) was noted in TNF- α , IL-6, and IL-18 levels of the PCOS group when compared with findings in the control group (TNF- α : 250.4 ± 32.5 vs. 164.3 ± 34.4 ng/L, IL-6: 169.8 ± 9.4 vs. 77.0 ± 9.3 pg/ml, and IL-18: 45.9 ± 5.5 vs. 35.3 ± 4.1 ng/L). Importantly, AgNPs decreased all three inflammatory biomarkers in the treated group when compared with the PCOS group (TNF- α : 173.9 ± 31.2 vs. 250.4 ± 32.5 ng/L, IL-6: 133.7 ± 9.3 vs. 169.8 ± 9.4 pg/ml, and IL-18: 36.1 ± 6.2 vs. 45.9 ± 5.5 ng/L).

CZ-derived AgNPs may have an anti-inflammatory effect in PCOS rats by decreasing the concentrations of inflammatory cytokines TNF- α , IL-6 and IL-18.

Keywords: Anti-Inflammatory, Cinnamon, Interleukins-6, Silver Nanoparticles, Tumor Necrosis Factor-Alpha

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Polycystic ovarian syndrome (PCOS) is an endocrinologic condition that affects women during reproductive age, with a prevalence of 18% depending on the diagnostic criteria used and the evaluated population (1). Disruption in the pituitary-gonadal axis and ovarian function are the main characteristics of PCOS. The exact etiology of PCOS is unclear yet, however, it probably has an epigenetic basis (2). It manifests differently depending on many interacting factors, including those related to lifestyle (diet, exercise, sleeping, and stress), neuro-endocrine, genetic factors, as well as immunological and metabolic disorders. It causes a variety of reproductive, metabolic, and endocrine disturbances (3). Accordingly, no single treatment is yet available for PCOS (2). Nowadays therapies for the management of PCOS's complications are metformin (4) and oral contraceptive pills (5). Long-term medical therapy's adverse effects and ineffectiveness of current therapeutic approaches in

treating patients with PCOS make complementary and alternative herbs an attractive option in the latest years (6). PCOS was evidenced as an inflammatory condition, with chronic low-grade inflammation and elevated levels of inflammatory cytokines (7). Adipose tissues are mostly responsible for the secretion of free fatty acids, cytokines, and hormones and have effective paracrine and endocrine effects in regulating immunity and inflammatory response, glucose and lipid metabolism, and reproductive capacity. Disruption of these biomolecules may lead to endocrine conditions, such as PCOS (8). In this context, the increased concentrations of inflammatory factors such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and others indicate the presence of systemic or local inflammation in the body. Accumulated evidence showed an association between increased inflammatory cytokines, and PCOS (9). In addition, inflammatory cytokines have a significant



role not only in disturbing fertility but also in numerous reproductive problems that PCOS women may experience. The elevation of certain inflammatory cytokines (IL-6, IL-18, and TNF- α) in PCOS women indicates an alteration in the immune function of those women (10).

Separately, *Cinnamomum zeylanicum* (CZ) anti-inflammatory activity was also studied. The CZ and its compounds may be useful in the treatment of inflammatory diseases (11). Green synthesis of nanoparticles (NPs) is an eco-friendly protocol to synthesis nanomaterials that are safe and cost-effective using biomolecules. Many medicinal plants have been used in the fabrication of NPs (12). Plant extracts, which are rich in bioactive chemicals, have recently been employed in this process. The plant extract biomolecules can serve as reducing agents for metal ions and convert them to NPs in a single-step process. The advantages of biologically produced NPs include lower toxicity and easy scalability. It is expected that phytochemicals responsible for the biological activities of plant extracts can also contribute to the synthesis of the NPs (13). Hence, biomedical applications of NPs in drug delivery to target inflammatory and inflammation-associated disorders have been widely investigated (14).

AgNPs were reported to have antibacterial, antifungal, and immunomodulatory properties among other NPs. Plant-derived AgNPs with immunomodulatory and/or antibacterial activities may improve the treatment of infections that are resistant to antibiotics or chronic inflammation (15). A study by Tyavambiza et al. (16) has shown that AgNPs can attack the lipopolysaccharide (LPS) induced inflammation by inhibiting the secretion of pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β). It is believed that nanoparticles have a higher ability to block pro-inflammatory cytokines and inflammation aiding enzymes because of their larger surface area-to-volume ratio. They reduce the production of pro-inflammatory cytokines like IL-12 and TNF- α , and also reduce COX-2 gene expression (17).

However, the impact of silver nanoparticles (AgNPs) using CZ methanolic bark extract on inflammatory biomarkers is still unknown. Thus, the current study was aimed at evaluating the effect of biologically synthesised AgNPs using methanolic extract of CZ bark on the inflammatory cytokines of estradiol valerate (EV)-induced PCOS.

For the synthesis of AgNPs from CZ bark methanol extract (20g CZ powder:100 mL of 80% methanol), the method described by Gunawardena et al. (11) was used with slight changes. In brief, five milliliters of the CZ bark extract were mixed with 200 ml of 1mM AgNO₃ (silver nitrate) solution. The gained mixture was then shaken with a magnetic stirrer for 20 minutes at room temperature and kept in dark conditions for 72 hours. The darkened brown color, after 72 hours, indicated the formation of AgNPs. Lastly, the sample was centrifuged, washed and the supernatant was discarded several times. The synthesised NPs were periodically monitored and

then characterised using scanning electron microscope (SEM, VEGA III, CZ) and atomic force microscope (AFM, Negara, RU).

In the current experiment, thirty mature female rats (with a weight range of 175-200 g and an age range of 12-14 weeks) were selected and housed at the animal house of the Basrah University College of Veterinary Medicine. They were maintained in standard conditions (temperature: 25 \pm 2°C, humidity: 55 \pm 10%) and fed on a standard pellet diet and water ad libitum. The institutional review board at the Veterinary Medicine College of Basrah University (Basrah, Iraq) approved the Ph.D. project from which this study was extracted (No: 3/18, Date: 01/07/2019).

The estrus cycle of each female rat was determined by vaginal smear for 1 week. Each rat (in groups "PCOS" and treatment groups) received 4 mg/kg of EV, (Sigma-Aldrich, Germany) dissolved in 0.2 ml corn oil through a single intramuscular injection and left for 8 weeks to induce PCOS. While the control group was injected 0.2 ml of corn oil (18).

Before administration of treatments, all the rats used in this experiment developed PCOS at the end of the 8 weeks EV treatment, as evidenced by the estrus cycle's irregularity (persistent estrus phase) and histopathologic features of multiple cysts as well as serum hyperandrogenism (further details can be accessed in our recent paper (18).

The rats were grouped into 5 groups (6 animals in each group). The groups were "control" group (received 0.2 ml corn oil only), "PCOS" group (received EV, intramuscularly, of 4 mg/kg only), "PCOS+CZ bark methanol extract (200 mg/kg)" group, "PCOS+Metformin (50 mg/kg)" group and "PCOS+AgNPs (3.53 mg/kg)" group. Both CZ methanol extract and AgNPs were dry powders at the time of the experiment beginning and they were dissolved in distilled water to be given to rats in the treatment period of 30 days (19). All chemical treatments were administered orally using oral gavage. For comparison, metformin was used as a standard treatment for PCOS and its dose, in addition to the CZ dose, was slightly modified from what was used by Anbu and Venkatachalam (20). The concentration of synthesised AgNPs, and their safety were evidenced in our recent paper (21).

Animals were sacrificed 30 days after the experiment began, and blood samples were collected. To evaluate concentrations of IL-6, IL-18, and TNF- α , commercial kits (ElabScience, USA) of Sandwich-ELISA were used. Test procedures of biomarkers were in accordance with the manufacturer's instructions. Each sample was tested in triplicate.

Graph Pad Prism v8.0.1(GraphPad Software, USA) was used to assess the differences between results, statistically. Results were represented as mean \pm SD. Mean differences of the tested biomarkers were evaluated using ANOVA (one-way) followed by a post-hoc test of Tukey HSD. Differences

were considered statistically significant at $P \leq 0.05$.

Synthesised AgNPs (using *C. zeylanicum* extract) were recognised by clearly changing in color (dark brown) as the first step in the detection of the formation of AgNPs (Fig.1). The SEM results revealed that the AgNPs were smooth and spherical particles with nano-size (Fig.2). Moreover, the findings of AFM analysis showed two-dimensional view of NPs which were spherical in shape, single, or in aggregates ranging in size from size 60 to 80 nm (Fig.3). All these findings confirmed the formation of AgNPs using the green method.

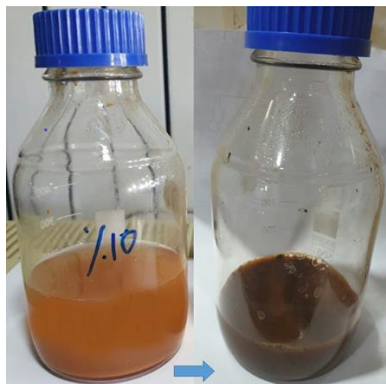


Fig.1: Color change points to the formation of silver nanoparticles (AgNPs).

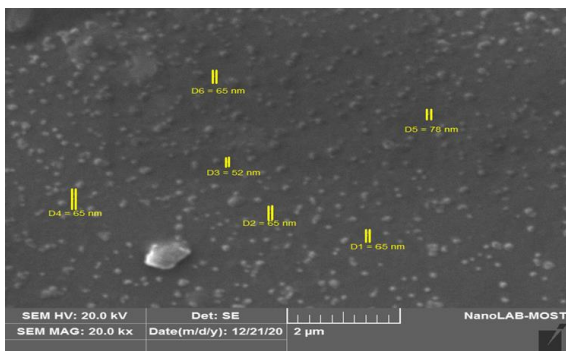


Fig.2: Micrograph of the electron microscope (SEM) demonstrates the size and shape of synthesised silver nanoparticles (AgNPs).

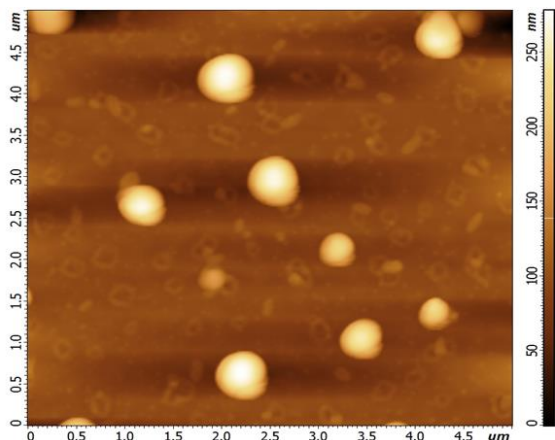


Fig.3: The atomic force microscopy's (AFM) result demonstrates the two dimensions of silver nanoparticles (NPs).

As presented in Figure 4, serum concentrations of TNF- α (250.4 ± 32.5 ng/L), IL-18 (45.9 ± 5.5 ng/L) and IL-6

(169.8 ± 9.4 pg/ml) in PCOS group were all significantly higher ($P < 0.05$) when compared with the control group (164.3 ± 34.4 ng/L, 35.32 ± 4.1 ng/L, and 77.0 ± 9.3 pg/ml respectively). In addition, there were significant decreases in serum levels of TNF- α and IL-6 in all treated groups, one-month post treatment, when compared with the untreated PCOS group. In particular, and when compared with the untreated PCOS group, TNF- α and IL-6 levels in the AgNPs-treated group were 250.4 ± 32.5 vs. 173.9 ± 31.2 ng/L and 169.8 ± 9.4 vs. 133.7 ± 9.3 pg/ml, respectively. The level of IL-18 (36.1 ± 6.2 ng/L) was significantly decreased only after treatment with AgNPs compared with the PCOS group. Furthermore, the AgNPs-treated group had lower concentrations of all three inflammatory biomarkers when compared with other groups that were treated with *C. zeylanicum* or metformin alone.

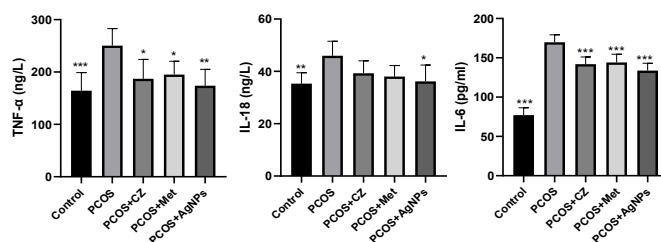


Fig.4: Serum concentrations of TNF- α , IL-18, and IL-6 in all the five groups ["control" group (received 0.2 ml corn oil only), "PCOS" group (received 4 mg/kg estradiol valerate only), "PCOS+CZ" group (PCOS rats received *C. zeylanicum* extract (200 mg/kg))", "PCOS+Met" group (PCOS rats received metformin (50 mg/kg)) and "PCOS+AgNPs" group (PCOS rats received AgNPs (3.53 mg/kg))]. Data presented as mean \pm SD (n=6). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ compared with the PCOS group, TNF- α ; Tumor Necrosis Factor-Alpha, IL-18; Interleukins-18, IL-6; Interleukins-6, PCOS; Polycystic ovarian syndrome, CZ; *Cinnamomum zeylanicum*, and Met; metformin.

In the current study, the anti-inflammatory effects of AgNPs (fabricated by *C. zeylanicum*) on PCOS rats were explored. The untreated PCOS group had significantly higher concentrations of inflammatory cytokines, which is in line with previous literature indicating the PCOS patients have increased TNF- α , IL-18, and IL-6 levels in their plasma and follicular fluid (10, 22). Similar results by Al-Musawy et al. (1) showed that the PCOS rats have significantly increased concentrations of inflammatory cytokines (TNF- α and IL-6). These findings strengthen the hypothesis that the inflammatory process plays a key role in the pathophysiology of PCOS development (7).

According to recent findings, the expression of inflammatory factors such as C-reactive protein, IL-6, and TNF- α is higher in PCOS patients than in normal women. Inflammatory cytokines play a significant role in the development and occurrence of PCOS (9). TNF- α and IL-6 are inflammatory cytokines associated with hypothalamic-pituitary-ovarian dysfunction and anovulation, as well as hyperandrogenism and ovarian hyperstimulation (23). Several physiological reproductive events, such as menstruation, embryo implantation, and the commencement of labor, are influenced by inflammatory processes within normal conditions (24).

Within the ovarian tissue, the immune cells or ovarian cells may produce macrophage and inflammatory cytokines (such as TNF- α , IL-1, IL-6, and IL-8). This will modulate the secretion of steroid hormones, which are necessary for follicle growth and crucial for ovulation, formation, and regression of the corpus luteum in the ovaries. Considering the role of these cytokines in the secretion of steroid hormones, that play an important role in follicle growth and ovulation, disruption of these cytokines can affect steroid hormones and, as a result, the development of follicular cysts (24, 25). TNF- α plays a crucial role in controlling the ovary's normal activity during the follicular growth period. TNF- α induces apoptosis, in granulosa cells of ovarian follicles, and results in forming cystic follicles (26). The results of the present study showed that the levels of TNF- α , IL-18, and IL-6 declined after 30 days treatment especially in the group that was treated with AgNPs. Various studies have demonstrated that the extract of *C. zeylanicum* bark has anti-inflammatory activities (27). Moreover, Tyavambiza et al. (16) explained that in LPS-treated macrophages, AgNPs revealed anti-inflammatory efficacy by decreasing the release of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6). Nanomaterials derived from silver can be employed to directly target the immune system's cells and overwhelm their activity or avoid immune recognition (28). NPs play an important role in blocking molecules that enhance inflammation such as inflammation-assisting enzymes and cytokines. There is evidence that silver also has anti-inflammatory properties (29). Therefore, nanomaterials derived from CZ (AgNPs) may have an anti-inflammatory effect in PCOS rats.

The present study showed that synthesis of AgNPs using *C. zeylanicum* bark extract is a good method since the cinnamon extract acts as a reducing and capping agent. Several methods can be used for the synthesis of AgNPs, such as chemical, physical, and biological methods. Apart from other methods, the mechanism of synthesis of silver NPs using plant extracts relies on the fact that they are normally rich in phytochemical metabolites such as phenolic acids, alkaloids, polyphenols, sugars, proteins, and terpenoids. These phytochemicals have functional groups that are proposed to have a crucial role in the reduction of metallic ions and then stabilizing the NPs. Vijayakumar et al. (13) revealed that the *Nigella sativa*-derived silver NPs can be used as nano-drug with multiple therapeutic effects in treating diabetes and inflammatory disorders as well as bacterial infections. As a result of these findings, biological materials derived from AgNPs can reduce inflammatory biomarkers such as TNF- and IL-6 in female rats with EV-induced PCOS. The color change of biological materials derived AgNPs may be due to the reduction of Ag⁺ into AgNPs after exposure to plant extract's secondary metabolites. The final dark brown color indicated the formation of AgNPs and is in agreement with similar findings by Kumararaja et al. (30). The results concerning the shape and size of AgNPs were in agreement with those of a study by Gunawardena et

al. (11), that demonstrated the presence of Ag particles in nano-sizes using SEM analysis.

However, the present study subjects to some limitations. Firstly, the molecular investigation of the expression of the reported inflammatory cytokines was not carried out to confirm the current results. Secondly, further inflammatory cytokines should be studied to support the efficacy of treatment in the PCOS rats. Thirdly, the exact concentrations of bioactive compounds should be also measured to explain how the concentration can affect the suggested anti-inflammatory effects.

The current study's results confirmed the hypothesis that the synthesised AgNPs (using *C. zeylanicum*) may have anti-inflammatory effects by reducing the inflammatory cytokines (TNF- α , IL-6, and IL-18) in EV-induced PCOS female rats. AgNPs can be considered a therapeutic approach in targeting PCOS.

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Authors' Contributions

S.H.A.; Study design, methodology, and practical parts drafting the manuscript. M.H.A.-S.; Supervised the practical part of this project. All authors read and approved the final manuscript.

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