



RESEARCH ARTICLE

Biosynthesis and Evaluation of *Cinnamomum zeylanicum* Nanomaterials for the Treatment of Polycystic Ovary Syndrome in Mice

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ABSTRACT

Polycystic ovary syndrome (PCOS) or Stein Leventhal Syndrome is an inflammatory metabolic disorder resulting in ovarian dysregulation and dysfunction leading to infertility in 7-20% of women globally. Present experimental study was designed to evaluate the therapeutic effects of medicinal plant (*Cinnamomum zeylanicum*) using chitosan nanoparticles on the hormonal profile and ovaries of PCOS-induced mice. Methanolic extract of *Cinnamomum zeylanicum* was used to synthesize chitosan nanoparticles (CNPs) by ionic gelatin method. The synthesized particles were characterized by FTIR and XRD analysis. Experimental mice were given an intraperitoneal injection of 2 mg/Kg Estradiol valerate in 0.1 ml corn oil to induce PCOS. Results have demonstrated a protective effect of biomolecule-coated chitosan nanoparticles (CNPs) on the improved ovulatory function of PCOS-induced mice. Mice treated with CNPs showed the recovery of the estrous cycle and gonadotropin (FSH, LH) hormones. The FSH titre regain in CZCNPH from 0.58 to 0.75 mIU/ml, similarly, LH levels were reduced from 0.65 to 0.53 mIU/ml. The biomolecules loaded CNPs showed the formation of the oocyte, the formation of zona pellucida, zona granulosa and the dissolution of cysts in PCOS mice. Histomorphometric analysis showed high diameter of oocytes in CZCNPH group. We conclude that the biomolecules loaded CNPs can effectively be used to treat PCOS as compared to synthetic drugs.

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INTRODUCTION

PCOS is a complicated endocrine disorder and a metabolic-gynecological disease which affects an estimated 15-20% of females in their reproductive period leading to infertility, obesity, cardiovascular events and metabolic syndromes (Heydarpour *et al.*, 2020; Chaudhary *et al.*, 2021). The symptoms of PCOS include irregular cycles, anovulation, scalp hair thinning, acne and hyperandrogenism (Nautiyal *et al.*, 2022). Androgenization in animals is used to induce PCOS symptoms similar to humans as androgens are the primary cause of this disease. In animals, prenatal and pre-pubertal treatment can result in anovulation, ovarian cysts, insulin sensitivity, elevation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) ratio (Stener-Victorin *et al.*, 2020).

PCOS may be caused by the significant contribution of both genetic and environmental factors, as it is a

complex heterogeneous disorder. Although the animal PCOS model has various limitations, various PCOS-induced animals are used in research. The etiopathogenesis of PCOS and insight of androgen receptors were studied in rodents and mouse models exposed to dihydrotestosterone (DHT) (Stener-Victorin *et al.*, 2020).

Treatment of PCOS is a global challenge. There are no active protective measures available because of its complex pathophysiology. Even though the newly introduced pharmaceutical drugs have made remarkable strides towards improvement, however their side effects have prompted researchers to resort to natural or herbal alternatives (Thakor and Patel, 2014). Presently, plant extracts are being used for the treatment of common metabolic diseases such as diabetes, asthma, inflammation, PCOS and hypercholesterolemia. Several mammalian models were used to study the pathophysiology of induced PCOS including mice, rats

and rabbits (Jadhav *et al.*, 2013a). These animal models have high evolutionary conservation of the mammalian reproductive system, therefore, are used to study human reproductive disorders like PCOS. These animal models provide clinical and biological insights and many powerful understandings of human reproductive disorders (Stener-Victorin *et al.*, 2020).

Nano-technology is an emerging branch of science that focuses on the preparation, biomanipulation and the use of Nanoscale/Nano complex drugs for clinical studies and treatment of different diseases (Baker and Sathish, 2012; Kavitha *et al.*, 2013). Innovative, natural drug release systems with decreased pharmacological toxicity and a potential to enhance biodistribution are a need of the hour. The low molecular weight, positive charge and biocompatibility of Chitosan as nanoparticles makes it an important candidate for such drug delivery and absorption-enhancement systems (Mikušová and Mikuš, 2021). Chitosan is already being extensively utilized in experimental drug delivery applications (Yi *et al.*, 2020).

Common herbs such as *Cinnamomum zeylanicum*, *Matricaria chamomilla*, *Symplocos racemosa* and *Mimosa pudica* are reported as helpful in the treatment of PCOS (Thakor and Patel, 2014; Yadav *et al.*, 2020). *Cinnamomum zeylanicum* is used to reduce the metabolic parameters and insulin resistance which is one of the most common causes of PCOS (Heydarpour *et al.*, 2020). Cinnamon also reduces the amount of anti-Mullerian hormone in females which is also a cause of PCOS. Cinnamon also regulates the frequency of the menstrual cycle in females having PCOS (Samarasekera *et al.*, 2005).

Cinnamon plants possessing potential pharmaceutical compounds for insulin-sensitizing ability that is associated with the treatment of PCOS are selected to fabricate biomolecules loaded nanocomplex for the present experiment. The major goal of this study was to synthesize and characterize bioactive molecules incorporated TPP (Tripolyphosphate) crosslinked-chitosan nanocomplex from *C. zeylanicum* bark (CZCNPs) methanolic extracts and to evaluate their protective efficacy on *estradiol valerate* (EV) induced PCOS mice models by determining the level of the gonadotropic hormone and examining the histopathological changes in ovaries.

MATERIALS AND METHODS

Ethical approval: All experiments including animal testing were carried out in accordance with International guidelines and with approval obtained from the Research Ethics Committee of Government College University Lahore vide Letter No. GCU/IIB/753.

Preparation of plant extract: Fine sieved 100 g powder of dried bark of *C. zeylanicum* was used to prepare methanol extract using the Soxhlet apparatus. The extract was dried on a rotary evaporator for 15-20 minutes then in an oven at 40°C.

Synthesis and Characterization of silver and chitosan nanoparticles loaded with cinnamon extract: Green synthesis method was used to prepare Ag-NPs. Varying

concentrations of the plant extract were mixed with 1 mM aqueous AgNO₃ solution (Sankar *et al.*, 2013). Low molecular weight chitosan was purchased from Sigma-Aldrich (CAS Number: 9012-76-4). Chitosan nanoparticles were prepared by the ion-gelation method. Sodium tripolyphosphate (TPP) was used as a cross-linking agent (Kunjachan *et al.*, 2015). Absorption bands were demonstrated by excitation of Surface Plasmon Resonance (SPR) and used for the identification of chitosan silver NPs. The absorbance band for chitosan Ag NPs appeared at 400-450nm in UV-Vis Spectroscopy. Structural characterization of chitosan Ag-NPs crystal and composition were analyzed using the X-ray diffraction (XRD) spectrum Bruker system (XRD, D2 Phaser, USA) (Zhou *et al.*, 2009). The interactions of functional groups of biomolecule and metal NPs nanoparticles were investigated using FTIR.

Rearing of mice and induction of PCOS: Swiss albino female mice (6-8 weeks old) with an average weight of 24-28 g were used in current study. Mice were reared and kept in the animal house in the animal house facility at the Department of Zoology, Government College University Lahore. Mice were acclimatized for 48 hours and provided with an appropriate diet in standard conditions of temperature and humidity. Mice (n=60) exhibiting a normal four days estrous cycle were given an intraperitoneal injection of 2mg/Kg *Estradiol valerate* in 0.1 ml of corn oil, to induce PCOS. All induced mice were assessed for 42 days after the injection.

Analysis of vaginal smear: Vaginal smears were carried out to check the estrus stage in induced mice.

Experimental design: PCOS induced mice were methodically divided into 8 groups labeled in alphabetical order. Group A: control group untreated mice; Group B: PCOS untreated mice; Group C: PCOS mice treated with metformin (28 mg/kg); Group D: PCOS mice treated with plant extract, Group E: PCOS mice treated with silver nanoparticles loaded with plant extract low dose (CZNPL) (50 mg/kg); Group F: PCOS mice treated with silver nanoparticles loaded with plant extract high dose (CZCNPH) (150 mg/kg); Group G: PCOS mice treated with chitosan nanoparticles low dose (CZCNPL) (50 mg/kg) Group H: PCOS mice treated with chitosan nanoparticles high dose (CZCNPH) (150 mg/kg). The treatment was given for 28 days via oral administration of respective drugs.

Euthanization, blood and organ collection: After the completion of 28 days of respective treatments, the mice were humanely euthanized by cervical dislocation technique. Blood was drawn via cardiac puncture. Serum was extracted from blood and stored at -20°C till further analysis. Gonadotropin (FSH, LH) levels were measured by commercially available kits. Harvested ovaries were preserved in 10% buffered formalin.

Histopathological studies: Preserved ovary samples were cut into thin 1cm³ pieces prior to histological study. After tissue processing soft tissues were embedded into a hard paraffin block. Tissue paraffin

was sectioned into 3µ ribbons with the help of a rotary motorized microtome (EC- 350). These ribbons were then placed on a glass slide and stained with hematoxylin and eosin for histological examination under a bright microscope (Lone and Liaqat, 2019).

Statistical Analysis: Data obtained from the experiment was analyzed using SPSS software version 17. Mean \pm standard error mean (S.E.M) was used to express results. One-way ANOVA was used for the statistical comparison between control and experimental groups by Turkey's multiple comparison test as post hoc was applied for group comparison. The p-value less than 0.05 suggesting statistical difference.

RESULTS

Adult albino mice were divided into eight groups (A, B, C, D, E, F, G and H) and no mortality was observed during the experimental period in any group.

Effects of estradiol valerate on the estrous cycle: The estrous cycle of all experimental groups was observed through vaginal smears to confirm PCOS. Smear was observed with cornified cells, nucleated epithelial cells and leukocytes. The formation of PCOS was confirmed by irregular estrous cycle.

Characterization of nanoparticles: Interpretation of the X-ray diffraction pattern suggests that Ag-NPs formed by the reduction of Ag⁺ ions by *C. zeylanicum* bark broth are crystalline. The stability of photosynthesized Ag-NPs was studied in terms of interaction among *C. zeylanicum* bark extract biomolecules with AgNO₃ solution using FTIR spectroscopy have appeared (3273, 2924, 1576, 1457, 1039 and 517 cm⁻¹) corresponds mainly flavones and terpenoids that exists in abundance in *C. zeylanicum* bark extract.

Histopathology of ovaries

Normal control, PCOS induced untreated and PCOS treated with metformin group: Ovaries of control group showed primordial follicles, the oocytes were surrounded by a single layer of flat granulosa cells. Secondary follicles contained oocytes in mid-growth stages surrounded by two or more layers of granulosa cells. The Corpus luteum of PCOS mice was of transient endocrine structure that existed in a large number. Furthermore, this group showed ovarian cortical thickening and an increasing number of follicles. Granulosa cell layer in the follicular cysts was thin and thecal layer was thick. The histological analysis of PCOS treated ovaries with metformin showed normal follicular development as well as an increase in the percentage of corpus luteum and a decrease in the percentage of cystic structures.

Treatment groups: PCOS induced mice treated with *C. zeylanicum* bark extract showed the development of follicles and corpus luteum. In group treated with *C. zeylanicum* coated nanoparticles (50mg/kg) the histology of ovaries showed that degeneration of cyst started. Formation of oocytes took place. Thick granulosa cells

were present in it. Thin thecal cells were also present in this ovary. PCOS has also been treated with high doses of *Cinnamon zeylanicum* coated nanoparticles at 100mg/kg. When the histological analysis of such ovaries was done it was observed that proliferation of cells occur. Ruptured cysts were present in this section of the ovary. Regaining of cells started and the formation of granulosa cells occur. The histology of low dose (50mg/kg) treated *C. zeylanicum* chitosan nanoparticles show regeneration of follicular development. While the mice treated with lower doses of *C. zeylanicum* chitosan nanoparticles showed unclear organization of follicles and corpus luteum. Poly cysts started to become disappear. Thecal layers started thinning and granular layers started thickening. When PCOS was treated with *C. zeylanicum* chitosan nanoparticles high dose of 100mg/kg and histological analysis of ovaries was done, it showed that polycystic ovaries were disappeared and the development of normal follicles was increased. The regaining of the corpus luteum was also observed.

Histomorphometric parameters of ovary: The thickness of granulosa cells were significantly ($p < 0.05$) higher in group H (CZCNPH) as compared to control and other treatment groups. The oocyte diameter was almost same in positive control and metformin group but significantly increased in all treatment groups when compared to control group. Histomorphometric results of antral diameter revealed that they were significantly higher in group F (CZCNPL) and group G (CZCNPH) as compared to control and other treatment groups.

DISCUSSION

Polycystic ovary syndrome (PCOS) in humans is a hormone-dependent ovarian disorder. Due to ethical limitations, examination of human ovarian morphology and study of PCOS pre and post clinical trial pathogenesis is carried out on animal models. Although an animal model with complete persuasive replication of all features of human PCOS has not yet been established, rodentia and murinae models are preferred due to their versatility, maintenance ease, short estrous cycles and gestational periods (van Houten and Visser, 2014; Silva *et al.*, 2022). Current study utilized estradiol valerate induced Swiss albino female mice for inhibition of ovulation as simulated models for PCOS. These mice were treated with cinnamon extract, metformin, silver and chitosan nanoparticles. Estrous stages were monitored periodically via examination of vaginal smears. The formation of PCOS was confirmed by irregular estrous cycle.

In present study, the histological investigation of induced PCOS murine ovary exhibited resemblance to human PCOS ovary. Murine PCOS was further characterized by altered gonadotropic hormone ratio along with morphological development of many cysts or poly cysts. A negative correlation between serum estradiol and anti Mullerian hormone (AMH) has been observed by several authors (Andersen and Byskov, 2006) which suggests that although estradiol is essential for human ovarian folliculogenesis, estradiol represses AMH expression (Dewailly *et al.*, 2016), thus the elevated serum estradiol levels lead to an inhibitory effect on AMH

Table 1: Histomorphometry of ovaries. Values represent the Mean \pm SEM of each group

Parameters	NC	PC	Metformin	CZPE	CZNPL	CZNPB	CZCNPL	CZCNPH	p-Value
Granulosa cells	0.021 \pm 0.002 ^{ab}	0.016 \pm 0.004 ^{ab}	0.012 \pm 0.006 ^a	0.030 \pm 0.007 ^b	0.040 \pm 0.008 ^c	0.018 \pm 0.011 ^d	0.018 \pm 0.015 ^{de}	0.047 \pm 0.009 ^c	0.00
Oocyte	0.021 \pm 0.001 ^{ef}	0.016 \pm 0.002 ^f	0.012 \pm 0.003 ^f	0.030 \pm 0.003 ^{de}	0.055 \pm 0.008 ^d	0.076 \pm 0.008 ^c	0.089 \pm 0.006 ^b	0.102 \pm 0.003 ^a	0.00
ANTRAL DIAMETER	0.055 \pm 0.005 ^{bcd}	0.025 \pm 0.005 ^d	0.075 \pm 0.005 ^{bc}	0.045 \pm 0.005 ^{cd}	0.170 \pm 0.030 ^a	0.080 \pm 0.010 ^{bc}	0.175 \pm 0.005 ^a	0.090 \pm 0.010 ^b	0.000

^{a-f} Within the same row, means with different superscripts are significantly different ($p < 0.05$).

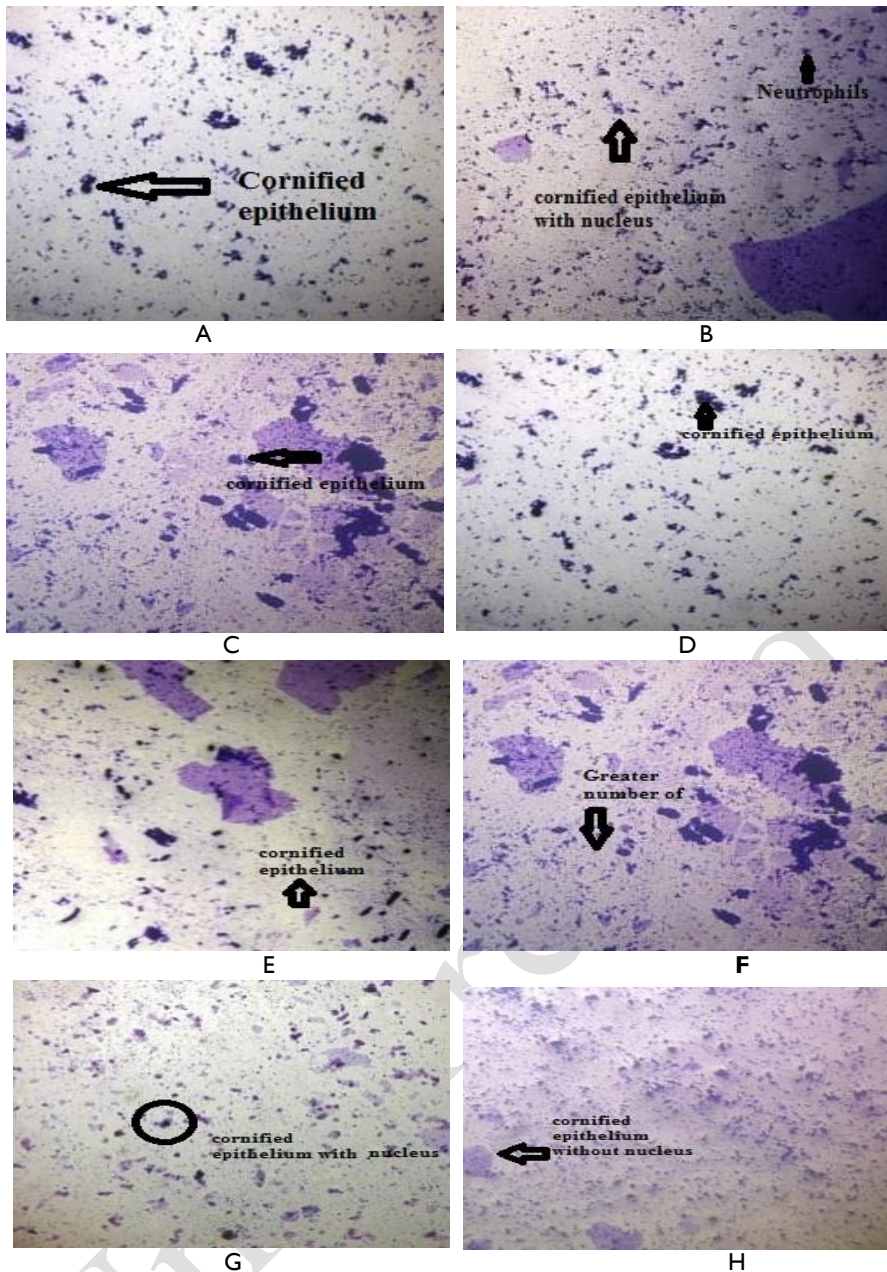


Fig. 1: Estrus cycle stages in all groups. A: Control group in diestrus stage with some cornified epithelium, B: PCOS induces group in metestrus stage with cornified epithelium with nucleus and neutrophils, C: Metformin treated group show diestrus stage, D: CZPE treated group at metestrus stage, E: CZNPL at estrus stage with cornified epithelium, F: CZNPB at diestrus stage, G: CZCNPL at metestrus stage, H: CZCNPH at estrus stage (Giemsa 40X)

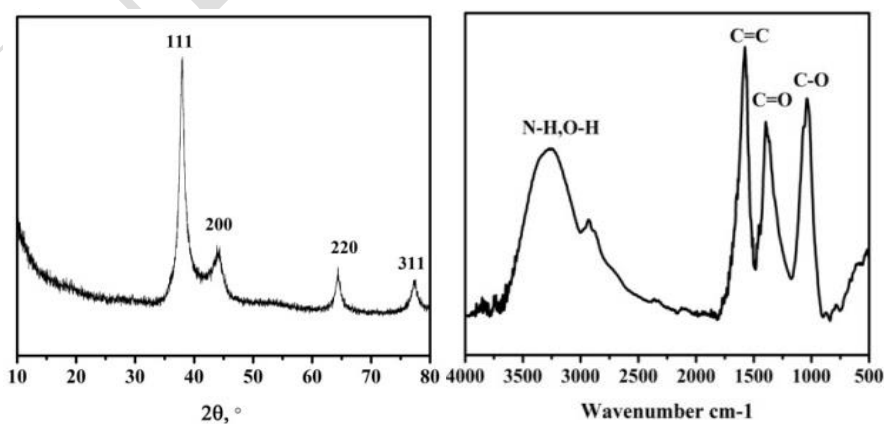


Fig. 2: Representation of XRD of nanoparticles and FTIR of nanoparticles.

Hormonal analysis (FSH and LH): The FSH titer regains in CZCNPH from 0.58 to 0.75 mIU/ml, similarly, LH was reduced from 0.65 to 0.53 mIU/ml.

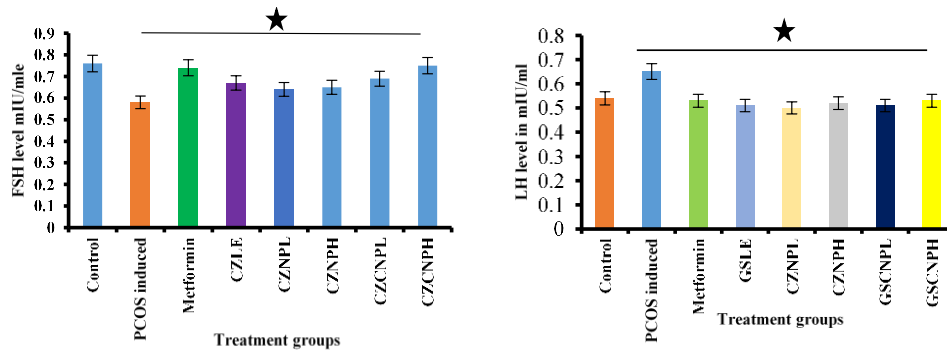


Fig. 3: FSH and LH level in control and treatment groups. The data showed Mean+SEM and analyzed with One-Way-ANOVA and TMRT. Asterisk shows significant difference between PCOS and CZCNPH groups.

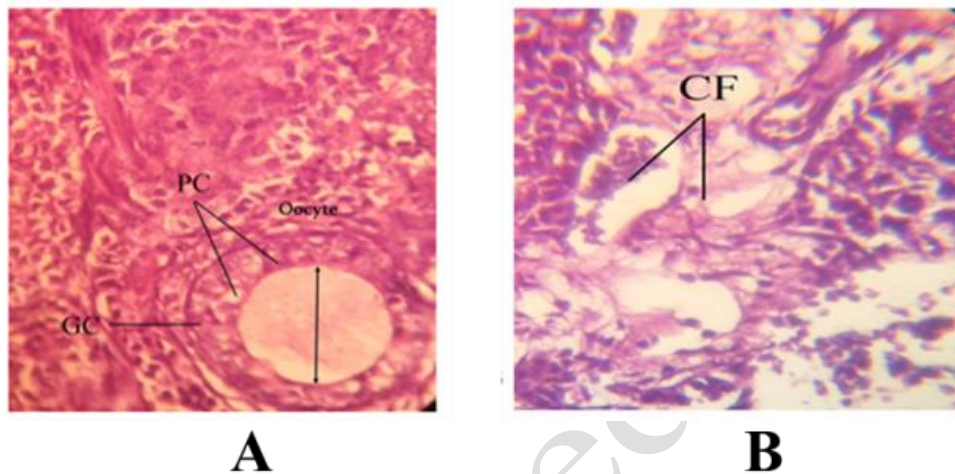


Fig. 4: Micrograph of part of ovary show defined oocyte, primordial cells (PC) and granulosa cells (GC). **B:** PCOS untreated ovaries; Section of ovary shows the cyst formation (CF) (H and E, 40X).

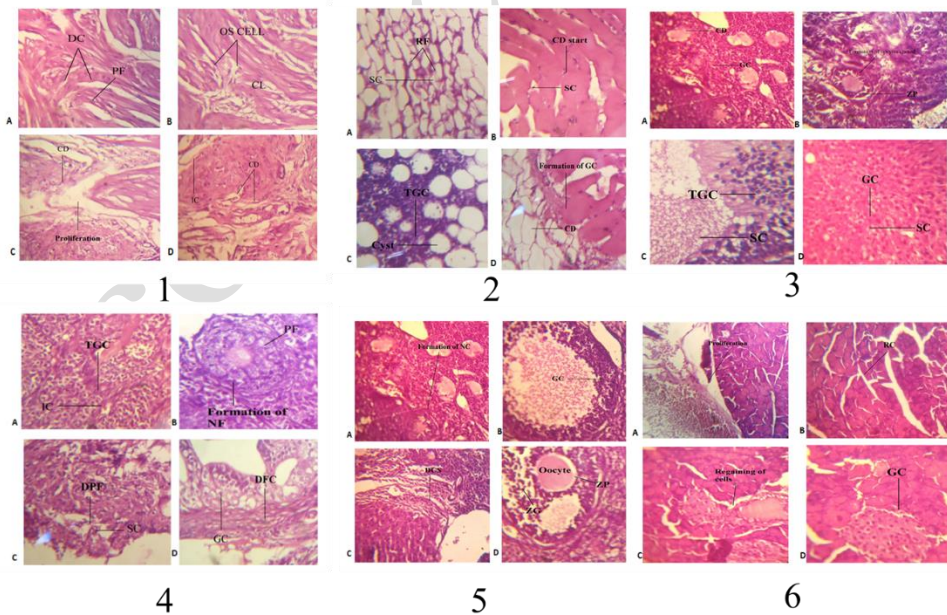


Fig. 5: (1) PCOS treated with metformin, A: disorganized cells (DC) and primordial follicle (PF), B: Ovarian stroma (OS) and corpus luteum (CL) is visible in this section of the ovary, C: cyst degeneration (CD), D: Cyst degeneration (CD), interstitial cells (IC). (2) PCOS treated with *C. zeylanicum* bark extract, A: ruptured follicles (RF) and stroma cells (SC), B: Cyst degeneration (CD) and somal cells (SC), C: Thick granulosa cells (TGC), D: Formation of granulosa cells (GC) occur and cyst degeneration starts. (3) PCOS treated with CZNPL (50mg/kg), A: degeneration of cyst (DC) and granulosa cells (GC), B: zona pellucida (ZP) and stroma cells (SC), D: granulosa cells (GC) and stroma cells (SC). (4) PCOS treated with CZNPH (100mg/kg), A: thick granulosa cells (TGC) and interstitial cells (IC), B: primordial follicle (PF), C: Degeneration of primordial follicles (PDF) and stroma cells (SC), D: Section of ovary showing the distorted follicle cells (DFC) and presence of granulosa cells (GC). (5) PCOS treated CZCNPL (50mg/kg), A: normal cells (NC), B: Normal oocyte and granulosa cells (GC), C: Dissolution of cyst started (DCS), D: oocyte, zona pellucida (ZP) and zona granulosa cells (ZG). (6) PCOS treated with CZCNPH (100mg/kg), A: proliferation of cells, B: Ruptured cysts (RC) are present, C: Regaining of cells started, D: granulosa cells (GC) (H and E, 40X).

and consequently inhibition of ovulation. PCOS has previously been successfully induced in mice by letrozole, estradiol valerate (Manneras *et al.*, 2007; Zurvarra *et al.*, 2009; Amini *et al.*, 2016; Corrie *et al.*, 2021).

Present study identified serum hormonal alteration in PCOS induced female mice. LH and FSH ratios in PCOS mice recapitulated phenotypic PCOS and dysfunctional estrous cyclicity. Higher LH and lower FSH titer levels indicated follicular growth arrest (Jonard and Dewailly, 2004). PCOS mice treated with chitosan nanoparticles high dose (CZCNPH; 150 mg/kg) exhibited a marked regain of FSH hormone levels as well as a lowering of LH levels which was comparable to mice treated with metformin. Metformin is a commonly prescribed insulin-sensitizing drug used for alleviation of hyperinsulinemia, decrease serum androgen levels, oocyte maturation and induction of ovulation in PCOS patients (Wei *et al.*, 2008; Facchinetti *et al.*, 2019). Metformin has also been reported to enhanced oocyte competence (Motta, 2010). Additionally, Mansfield *et al.* (2003) have correlated metformin with inhibitory effects on estradiol and progesterone levels in *in vitro* granulosa cell cultures. Results of current study are consistent with Bayrami *et al.* (2020) who reported chitosan incorporated fennel seed extract phytochemicals as an efficient, safe and stable drug delivery system for encapsulated biomolecules used for enhancing FSH levels and lowering LH levels of PCOS in rodents. Previously it was reported that mehani and omega-3 diet increased FSH levels while simultaneously lowering LH levels (Pushpa and Kalavathy 2013; Ouladsahebmadarek *et al.*, 2014). Similarly, phytochemicals of *Matricaria chamomilla* have been reported as effective alternates in balancing of the FSH and LH ratio in PCOS induced rats (Ghafurniyani *et al.*, 2015).

In present study, PCOS induced untreated mice exhibited cyst formation, reduced follicles and hyperplasia of thecal layer. Previously, workers studying PCOS induced mice have reported development of poly cysts in the ovaries. Furthermore, atretic follicles, cyst with reduced layer of granulosa cells and hyperplasia of the thecal layer were observed in the ovarian cortex (Jadhav *et al.*, 2013a; Jadhav *et al.*, 2013b). Manneras *et al.* (2007) observed similarly large ovaries, thick thecal layer and few corpus lutea displayed in DHT induced PCOS rat model. In current work, the histopathological examination of PCOS induced mice treated with cinnamon-loaded chitosan nanoparticles exhibited a reorganization of ovarian tissue and formation of normal follicles. Observations under a light microscope revealed well-developed normal follicles, normal granulosa cells and thecal layers. Clear antrum with no cellular debris was also observed. Cinnamon-loaded chitosan nanoparticles treatment administered to PCOS induced mice not only induced corpus luteal formation but also normalized the estrus cycle. Cinnamon is a common spice and a flavoring agent with a well-documented historic use as traditional medicine, especially its role in insulin-receptor functions (Gürson *et al.*, 2005; Nikooie and Sedaghat Boroujeni, 2014). The role of cinnamon in anti-obesity and anti-diabetic formulations due to its insulin signaling pathway properties has been a subject of several recent studies (Hayward *et al.*, 2019; Sivaranjani *et al.*, 2021; Keramati

et al., 2022). Oral doses of cinnamon have been shown to significantly improve glucose tolerance, increase stimulation of insulin secretion and reduce lipid accumulation in obese mice (Sheng *et al.*, 2008; Huang *et al.*, 2011). Furthermore, oral administration of cinnamon has the potential to down-regulate testosterone, restore estrous cyclicity and improve insulin sensitivity in murine models (Dou *et al.*, 2018; Maleki *et al.*, 2021).

Current work has revealed that the PCOS-induced mice treated with chitosan nanoparticles (CZCNPs) resulted in regaining of normal follicular structures in ovaries. By administering the biomolecules coated chitosan nanoparticles, poly cysts disappeared in the ovarian tissues and the development of normal follicles was increased. It was investigated previously that the flower extract of *Matricaria chamomilla* not only recovered the PCOS ovaries to their normal morphology but also enhanced the dominant follicle rate (Farideh *et al.*, 2010). Studies have showed that metformin treatment administered to PCOS-induced mice acted on ovarian thecal cells and inhibited the production of androgens by decreasing the secretion of LH from the pituitary leading to ovulation and normal estrus cycle (Anbu and Venkatachalam 2016). Similar results were also observed in letrozole induced polycystic ovary rat model by treating with plant extract of *Symplocos racemosa* (Jadhav *et al.*, 2013b). To date, only one study is available on the effect of CZCNP on a hormonal levels and histopathology of wistar rat ovaries (Anbu and Venkatachalam, 2016). Histopathological and hormonal analysis presented in current study concur with the aforementioned report.

Till date, there are no nanoparticle-based drug treatments are available for PCOS. Current study was designed to investigate the protective role of biomolecules incorporated tripolyphosphate (TPP) chitosan nanoparticles treatment and evaluation of its effectiveness based on histopathological changes against PCOS induced murine model. It is henceforth concluded that CZCNPs not only have a protective role against PCOS but can significantly exhibit a restoration of morphologically damaged ovaries, resulting in regulation of the estrous cycle and hormonal balance.

Conflict of interest: The authors have no conflict of interest.

Authors Contribution: IL conceived, designed and did statistical analysis & editing of manuscript. SN, AA, TZ, MM & SA did data collection and manuscript writing. IL, NA, ASQ & SA did review and final approval of manuscript.

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