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Co-regulative effects of chitosan-fennel seed extract system on the hormonal and biochemical factors involved in the polycystic ovarian syndrome

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ABSTRACT

There is a renewed interest in the application of chitosan-based drug delivery systems over the last few years. In this study, the ionic gelation method was used to prepare chitosan-engaged tripolyphosphate ions, as the cross-linking molecule, (Chit-TPP) and concurrent loading of the biomolecules of the ethanolic extract of fennel, *Foeniculum vulgare*, seed (FEC@NBC). The samples were characterized by SEM, DLS, TGA, FTIR, XRD, GC–MS, and zeta potential, and their effects on the related hormonal and biochemical factors of the rats with polycystic ovarian syndrome (PCOS) were assessed. The estradiol valerate-induced PCOS in female rats was confirmed by vaginal smear test and subsequent histological screening. The PCOS-induced rats were treated by fennel seed extract (FSX), Chit-TPP, and FEC@NBC. The process of treatment was monitored by measuring the serum levels of testosterone, luteinizing hormone, follicle-stimulating hormone, insulin, glucose, high-density lipoprotein cholesterol, total cholesterol, and total triglyceride after 16 days of treatment and compared with healthy control and untreated PCOS-control groups. The FEC@NBC administration contributed to the remarkable hormonal, glucose, and lipid profile regulation in the rats with PCOS. The significance of FEC@NBC performance in dealing with PCOS complications compared to that of the only extract could be resulted from the effective targeted delivery and stability of phytomolecules when encapsulated in Chit-TPP.

1. Introduction

Polycystic ovary syndrome (PCOS) is a complicated endocrine disorder of which debate continues about the underlying causes [1]. Across the globe, about 15% of women experience PCOS in reproductive years and consequently, are at the risk of some other health problems such as metabolic, reproduction, and endocrine disorders [2].The primary concerns associated with PCOS are (i) high risk of infertility, (ii) oligo-amenorrhea, (iii) diabetes mellitus (type II), (iv) acne, (v) hirsuteness, (vi) obesity, (vii) hypertension, (viii) endometrial carcinoma, and (ix) cardiovascular disorders [3,4]. PCOS is generally linked to hormonal malfunctions due to the change in the estrogen, prolactin, luteinizing hormone (LH), testosterone, and androstenedione concentrations. The main indicator of PCOS incident in women is the alteration in gonadotropins, wherein the LH/FSH proportion doubles or triples in PCOS compared to healthy conditions [5]. Under PCOS

Abbreviations: ANOVA, analysis of variance; Chit-TPP, chitosan-tripolyphosphate; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; FBS, fasting blood sugar; FEC@NBC, fennel seed extract-chitosan nanobiocomposite; FSH, follicle-stimulating hormone; FSX, fennel seed extract; FTIR, Fourier transform infrared; GnRH, gonadotropin-releasing hormone; HDL, high-density lipoprotein; LDL, low-density lipoproteins; LH, luteinizing hormone; NIH, National Institutes of Health; PCOS, polycystic ovarian syndrome; PDI, polydispersity index; SEM, scanning electron microscopy; TST, testosterone; TGA, thermo-gravimetric analysis; TC, total cholesterol; TG, total triglyceride; UMA, University of Mohaghegh Ardabili; XRD, X-ray diffraction

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condition, hyperandrogenemia and insulin resistance simultaneously take place, stimulate each other, and jointly provide the basis for developing reproductive and metabolic problems [6]. It should be underlined that the shortage of information on the etiology of PCOS creates an obstacle in PCOS controlling and management. Currently, several clinical drugs such as metformin and gonadotropins are administrated to deal with hormonal changes, irregular menstruation cycle, hyperinsulinemia, and other complications linked to PCOS. However, the side-effects associated with the long-term use of chemical drugs such as stomach pain and excessive growth of hair are of concern.

Over the years, traditional remedies have also significantly contributed to human-health [7,8]. In contrast to ordinary medicines, herbal drugs are multi-component and multi-target in nature [9,10]. For instance, there are about forty medicinal plants, either in a single form or as a component of a composite medicine, reported for treating amenorrhea (absence of menstruation) in Traditional Persian Medicine (TPM) books. The large proportion of these plants displayed anti-dyslipidemic (77.5%) and anti-hyperglycemic (90%) properties, whereas some exhibited anti-obesity effects (37.5%), and a number of them have had positive effects on ovulation induction [11].

In recent years, there has been an increasing concern towards the medicines of herbal origin owing to their valuable assets of being harmless or less-side effects than chemical remedies [12,13]. A vast range of medicinal herbs show therapeutic effects on PCOS correction and rehabilitation, which are due to the various active compounds in them. For instance, *Gymnema sylvestre*, *Cinnamon zeylanicum*, *Aloe barbadensis*, *Matricaria chamomilla*, and *Foeniculum vulgare* contain different groups of biomolecules such as tetracosane, farnasene, aloesin, anthranol, *p*-coumaric acid, cinnamaldehyde, flavonol glycoside, and estragole [14–17].

Foeniculum vulgare (*F. vulgare*) generally known as fennel is one of the ancient medicinal plants in the world from the Apiaceae family [18]. The fruit of fennel comprised of (i) high-value oils including estragole, fenchone and transanethole, (ii) flavonoids such as flavonoid glycosides, (iii) nonflavonoid phenols e.g. hydroxycinnamic acids, (iv) phenolic acids, (v) coumarins and tannins [18–21]. *F. vulgare* has been reported for its wide activities as an antioxidant, anti-inflammatory, antitumor, antibacterial, antifungal, antimutagenic, antithrombotic, cytotoxic, estrogenic, bronchodilatory, emmenagogue, infant colic relieving, galactogogue, oculohypotensive, hypotensive, hepatoprotective, gastroprotective, and memory-enhancing bio-agent [18].

Chitosan-based systems have received considerable critical attention for biomedical applications since it is a safe, biologically compatible, and decomposable polymer with high absorptive capacity, which is quickly solubilized and can be easily engineered [22]. Chitosan has been a subject of research on effective drug delivery systems for developing advanced therapeutic medicine of high bio-distribution and minimal toxicity [23]. In drug delivery systems, the absorption-enhancing effect of chitosan has made it a remarkable option [24]. Moreover, chitosan-based hydrogels can improve the penetration, permeability and sustained release of drug and bioactive molecules [25]. Being non-toxic, bioavailable, and pH-sensitive, chitosan-based systems have shown great potentials as alternative medicines to treat various disorders [26].

Although the positive effects of fennel (*F. vulgare*) seed extract on the health issues associated with polycystic ovary syndrome (PCOS) complications have been testified, the effectiveness of the targeted delivery of its phytomolecules by a safe and green carrier has not been systematically assessed. In the present study, sodium tripolyphosphate (TPP) cross-linked chitosan were synthesized via the ionic gelation method and loaded with the biomolecules present in the ethanolic extract of fennel (*F. vulgare*) seed. The fennel seed extract (FSX) was analyzed by GC–MS to explore some of the phytochemicals present in it. The as-prepared nano-biocomposite (FEC@NBC) and Chit-TPP samples were also characterized by scanning electron microscopy (SEM), thermo-gravimetric analysis (TGA), X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, dynamic light scattering (DLS), and zeta potential. Next, the therapeutic effects of these samples were assessed on the PCOS-induced rats with reference to five groups including Group-I: normal healthy group (Control); Group-II: PCOS rats received saline (PCOS-Control); Group-III: PCOS rats treated by *F. vulgare* seed extract (Extract); Group-IV: PCOS rats treated by chitosan nanocomposite (Chit-TPP); and Group-V: PCOS rats treated by FEC@NBC. The vaginal injection of estradiol valerate induced PCOS in female rats, which was confirmed by vaginal smear test and subsequent histological screening within 60 days. The process of healing was monitored by measuring the serum levels of testosterone (TST), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and insulin together with glucose, and lipid profiles (high-density lipoprotein cholesterol, total cholesterol, and total triglyceride).

2. Materials and methods

2.1. Materials

The reagents involved in the present investigation were all of the analytical grade and utilized with no further purification. Sodium tripolyphosphate ($Na_5P_3O_{10}$), acetic acid ($C_2H_4O_2$), and ethanol (C_2H_5OH) were obtained from Merck (Darmstadt, Germany). Chitosan ($(C_6H_{11}NO_4)_n$ of low molecular weight was purchased from Sigma-Aldrich co. *F. vulgare* seeds were purchased from a local market in Ardabil, Iran. The total triglyceride (TG), total cholesterol (TC), fasting blood sugar (FBS), and high-density lipoprotein (HDL) values were determined using the kits from Pars Azmoun Company (Tehran, Iran). The levels of LH, FSH, insulin, and testosterone hormones were determined in serum with commercial ELISA kits (Mercodia, Sweden). Anaesthesia of rats was carried out by Ketamine and xylazine (Alfasan, India).

2.2. Animals

Overall, 25 female Wistar rats, 8 weeks old, and 180–220 g were delivered from the experimental animal center at the University of Tehran. Rats were housed and fed according to standard conditions. All the experiments involving animals were carried out in compliance with the national institutes of health guidelines for the use and care of experimental animals (NIH Publications No. 8023, revised 1978) and authorized by Ardabil animal care and use committee (UMA-97-19,834). The rats were grouped into five categories with 5 rats per group: (Group-I) the normal healthy rats as Control; (Group-II) the rats with PCOS, which received treated by *F. vulgare* seed extract metabolites anchored on Chit-TPP as FEC@NBC. All the groups were treated via intraperitoneal injection on a daily basis. The amount of nanostructures injected to the rats of groups IV and V was 50 mg/kg body weight of rats (1 mL per day for 15 days). In the case of group III, extract, it was 150 mg/kg (1 mL per day for 15 days). On the day 16, the rats were anaesthetized with intraperitoneal ketamine-xylazine (3:1, 2 mL/1 kg) and the blood samples were collected for thorough analyses. Saline as PCOS-Control group; (Group-III) the PCOS-induced rats treated by *F. vulgare* seed extract as Extract; (Group-IV) the PCOS-induced rats treated by chitosan-TPP as Chit-TPP; and finally (Group-V) the PCOS-induced rats.

2.3. Vaginal smear and PCOS induction

Firstly, the sexual cycle and estrous phase of animals were assessed by vaginal discharge sampling and cellular observation. Estradiol valerate was applied to induce the syndrome when rats were in the estrous phase by single-dose injection of estradiol valerate (2 mg) dissolved muscularly in sesame oil (0.2 mL) [15]. The vaginalization of the rats was done on days 7, 15, 30 and 60.

2.4. Preparation of fennel extract

The fennel seeds were rinsed with tap water and then distilled water, dried and grounded to powder. For the preparation of ethanolic extract, the powder (100 g) was mixed with ethanol (500 mL, 70%) and kept for 48 h in a water-bath at about 40 °C. The extracted solution was filtered and let to get condensed via evaporation at room temperature. The condensed extract (jelly form) was kept below 4 °C for subsequent experiments.

2.5. Synthesis of chitosan nanocomposites

The organic moieties of the condensed extract were imported onto the chitosan using a cross-linking chemical (TPP). At length, 1.5% w/v of the extract was separately mixed with 0.5% w/v TPP under magnetic stirring. This solution was dropwise added into the chitosan solution (2% (v/v) acetic acid and 1% (w/v) chitosan). The chitosan-TPP sample (Chit-TPP) was prepared by the same procedure but in the absence of extract.

2.6. Blood collection and serum analysis

The effectiveness of the as-prepared drug delivery agent was examined on the PCOS-induced rats in reference to the groups I, II, III, and IV on the 16th day of the post-cure via analysis of the serum. Serum FSH, LH, testosterone, and insulin levels in the collected blood samples were determined by ELISA kits (Mercodia, Sweden). In addition to the hormones, FBS, TG, TC, and HDL levels were also measured by commercial kits (Pars Azemon, Iran).

2.7. Characterization of samples

The morphology of the samples was explored through SEM with an accelerator voltage of 15 kV (LEO1430VP). The SEM samples were prepared using the slab staining method [27]. FTIR analysis was performed to survey the chitosan surface functional groups introduced from the extract biomolecules (Perkin Elmer Spectrum RX I). In this regard, a mixture of biocomposite and KBr in a ratio of 1:10 was provided and analyzed in the 400 to 4000 cm^{-1}. TGA was applied on Linseis STAPT1000 analyzer by heating at 10 °C/min from room temperature to 700 °C under airflow. The XRD patterns of the Chit-TPP and FEC@NBC samples were collected to analyze their crystalline and amorphous content with the Phillips X-PERT X-ray machine. DLS was used to determine the particle size distribution (Malvern Instruments, Westborough, USA). The electrical charge of the particles was measured by the Malvern apparatus. Agilent 7890B series GC instrument was employed to explore the secondary metabolites in the studied fennel seed extract (FSX). The differences between the means were measured by performing one-way ANOVA continued by Tukey's multiple comparison post-test using SPSS Software (20). The statistical significance was defined in data analysis ($p < 0.05$). The results were statistically represented through error bars as a Mean ± SEOM (Standard errors of means).

3. Results and discussion

3.1. Characterization of chitosan nanocomposites

The crystallographic characteristics of sodium tripolyphosphate (NaPP), chitosan, Chit-TPP, and FEC@NBC samples are shown in Fig. 1. The XRD trace shows a strong diffraction peak at 2θ = 19.6° in all chitosan-containing samples, indicating that the wurtzite hexagonal crystalline phase (110) was present. The broadening of this characteristic peak in FEC@NBC indicates the presence of biomolecules, which induced an amorphous structure to Chit-TPP particles. Another diffraction peak at 2θ = 11.9°, which is related to the (020) plane corresponds to the anhydrous chitosan crystalline structure. Additionally, two other diffraction peaks emerged in the FEC@NBC at 2θ = 21.7° and 2θ = 23.2°, which indicated that the crystalline structure of chitosan was changed due to the presence of secondary metabolites of fennel extract. The alteration in the crystalline structure of chitosan, when coated with biomolecules, is in line with the findings reported by Perumal et al. [28] and Hosseini et al. [29]. The peaks at 2θ = 19.4°, 20.1°, 32.5°, and 33.3° showed NaPP at $Na_5P_3O_{10}$ crystalline phase. The diffraction peak at 2θ = 26.4° revealed the presence of $Na_4P_2O_7$ (pyrophosphate) phase at minor quantities [30]. The corresponding peaks of NaPP can be also detected in the Chit-TPP sample.

FTIR spectra of fennel extract, bulk chitosan, Chit-TPP, and FEC@NBC were acquired and the corresponding functional sites and intermolecular interactions were examined. As shown in Fig. 2, the bands indicated the presence of phenolic compounds and amino acids in the fennel extract. The bands at 3754 cm^{-1} (asymmetric O—H stretching), 3390 cm^{-1} (stretching vibration of O—H and N—H), 2930 cm^{-1} and 2862 cm^{-1} (alkane C—H stretching), 2374 cm^{-1} (aldehyde C—H stretching), 1634 cm^{-1} and 1550 cm^{-1} (aromatic C=C stretching), 1388 cm^{-1} ($–CH_3$ or phenolic O—H bending) and 1070 cm^{-1} (C—O stretching) were of the main detected peaks. The FTIR spectra of bulk chitosan, Chit-TPP, and FEC@NBC exhibited strong peaks at about 3550–3200 cm^{-1} correspond to intermolecular bonded O—H and N—H stretching modes. In these samples, the peaks at about 2930 cm^{-1} and 2860 cm^{-1}correspond to symmetric and asymmetric stretching of C—H, respectively [31]. The C=O stretching can be detected by the sharp peaks at about 1725 cm^{-1} in the Chit-TPP and FEC@NBC. The peaks at about 1600 cm^{-1}, 1400 cm^{-1}, 1260 cm^{-1}, and 1085 $cm^{-1}$are respectively assigned to C=C stretching vibrations, C—H bending, alkyl/aryl C—O stretching and primary alcohol C—O modes. The appearance of additional peaks at 1338 cm^{-1}, 1160 cm^{-1}, 1104 cm^{-1}, and 692 cm^{-1} could be assigned to aromatic amine C—N stretching, tertiary and secondary alcohol C—O stretchings, and benzene derivative C=C bending, which are the evidence for the linking of extract biomolecules over chitosan sheets. Moreover, the shift and broadening of the N—H band in the FEC@NBC spectrum (3355 cm^{-1} to 3341 cm^{-1}) compared to Chit-TPP could be associated with the formation of hydrogen bonding between the chitosan $–NH_2$/–OH groups and –OH groups of the polyphenols of FSX. Besides, the reduced intensity of the peak at 1060 cm^{-1} and the loss of primary alcohol C—O peak were related to the development of electrostatic interactions between chitosan and extract biomolecules [32].

The morphological appearance of Chit-TPP and FEC@NBC samples are shown in Fig. 3. As reflected in the SEM micrographs, the uneven surface of Chit-TPP has been significantly altered in FEC@NBC after introducing the FSX in the synthesis medium. The resulted smoothen surface could be associated with the capping and stabilizing effects of the extract biomolecules when linked to the surface of chitosan.

The thermal features of Chit-TPP and FEC@NBC were studied by thermo-gravimetric analysis based on the changes in the thermal stabilities during the heating process up to 700 °C. The TGA thermograms and the corresponding weight-loss ratio in each sample are shown in Fig. 4. The first weight-loss of 8% and 11% were observed at about 118 °C in Chit-TPP and FEC@NBC samples, mainly because of the adsorbed aqua molecules. A sudden weight-loss of 40% experienced by FEC@NBC, followed by almost the same percentage of loss by Chit-TPP at 260–340 °C. The rapid thermal damage to FEC@NBC could be associated with the destruction of biomolecules. On the contrary, in the temperature range of 340–700 °C, Chit-TPP lost more weight than FEC@NBC, indicating the higher stability of the latter. The TGA result of bulk chitosan is also given in the figure. The results show that the thermal stability of Chit-TPP was higher than bulk chitosan mainly due to the adequate mechanical quality resulted from sodium tripolyphosphate (NaPP) as the cross-linking molecule. This is evident from the excellent thermal stability of NaPP, as shown in Fig. 4. On the contrary, the extract did not endure higher temperatures and almost decomposed

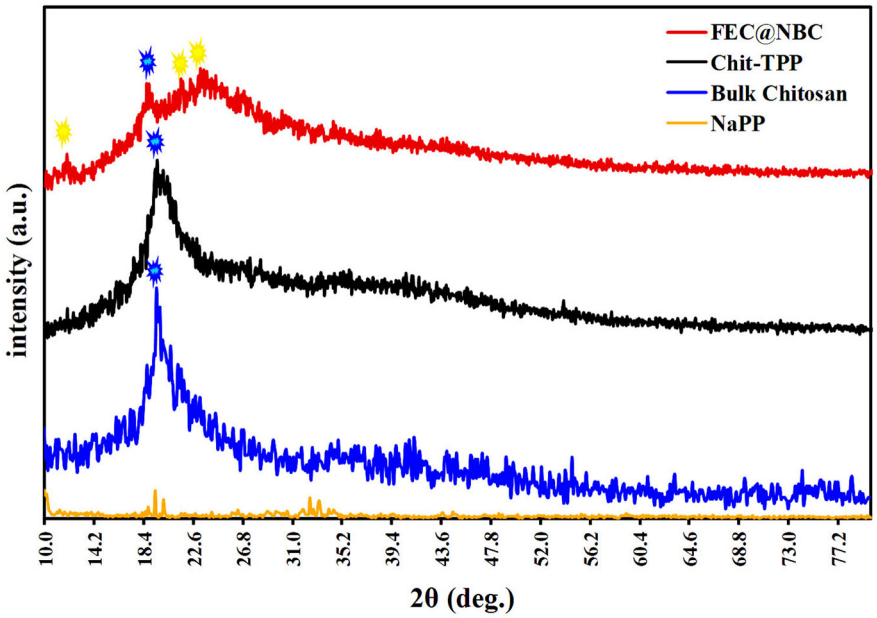

Fig. 1. XRD patterns of the NaPP, Bulk chitosan, Chit-TPP, and FEC@NBC samples.

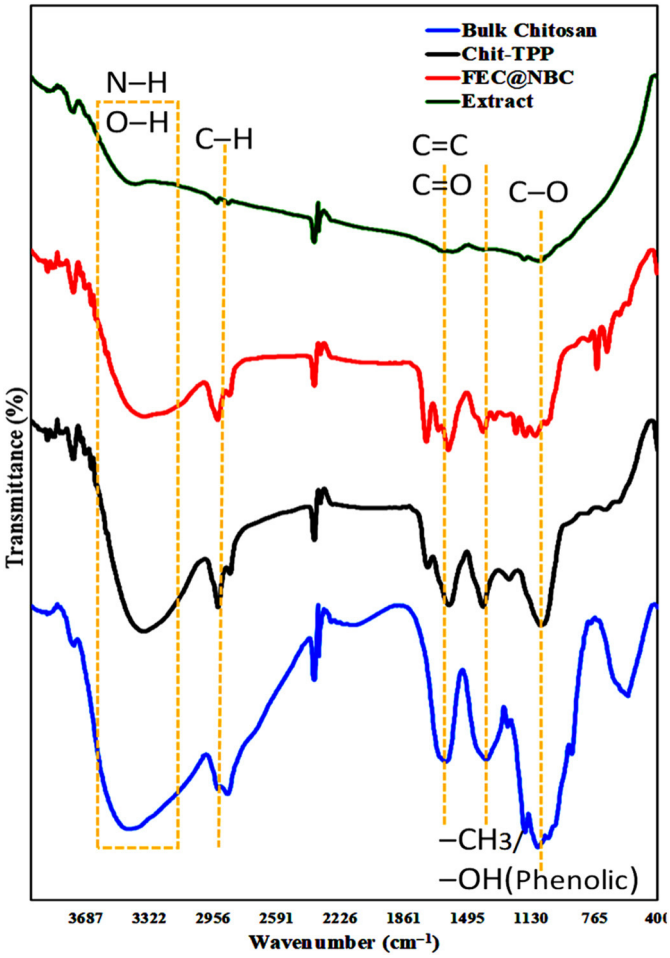

Fig. 2. FTIR spectra of bulk chitosan, Chit-TPP, FEC@NBC, and fennel seed extract.

at about 100 °C since it is mainly composed of organic materials.

DLS data showed the distribution and uniformity of the biostructures (Fig. 5). The Chit-TPP particles had non-homogeneous distribution wherein the majorly of the particles had an average size of 284 nm and a small portion varied between 10 and 15 μm. In comparison, the FEC@NBC particles were uniformly distributed and possessed an average size of 49 nm. An almost the same dispersion value of 0.5 was obtained for both samples using the polydispersity index (PDI). The zeta potential distribution of FEC@NBC was −24.3 mV (Fig. S1(a)). The values of −30 mV or +30 mV are normally considered as high zeta potential and show the high repulsion between the particles [33]. Accordingly, FEC@NBC particles possessed a good stability in the suspension with minor agglomeration. In comparison, Chit-TPP showed lower stability as its zeta potential distribution was −21.2 mV (Fig. S1(b)). On the other hand, the negative zeta potential values indicate that the surface of the particles was negatively charged. The higher negative charge of the FEC@NBC surface could be affected from the anionic groups of the anchored fennel phytochemicals. These phytochemicals not only improved the stability of FEC@NBC in the suspension, but also reduced the agglomeration of the particles owing to the repulsion resulted from the anionic secondary metabolites of FSX.

The results obtained from the characterizing analyses exposed effective interaction between chitosan and biologically active compounds of FSX. This integration not only enhanced the stability of chitosan but also resulted in the effective delivery of biomolecules and regulation of hormonal and biochemical factors in rats with PCOS, which are discussed in detail in the following sections.

3.2. PCOS groups

Based on vaginal examinations, polycystic state stabilization occurred from day 30, and the completion of the polycystic induction was confirmed by histological observation of stability at the estrus stage (Figs. 6 and 7).

3.3. Effectiveness of chitosan nanocomposites on PCOS rats

The principal aim of the current research was to examine the effectiveness of a chitosan-based drug delivery system in dealing with the PCOS complications when carries FSX biomolecules. This study was conducted to assess the regulative effects of the FEC@NBC drug carrier system on the gonadotropins (LH, and FSH), testosterone, insulin, fasting blood sugar, and lipid profile (high-density lipoprotein cholesterol, total cholesterol, and total triglyceride).

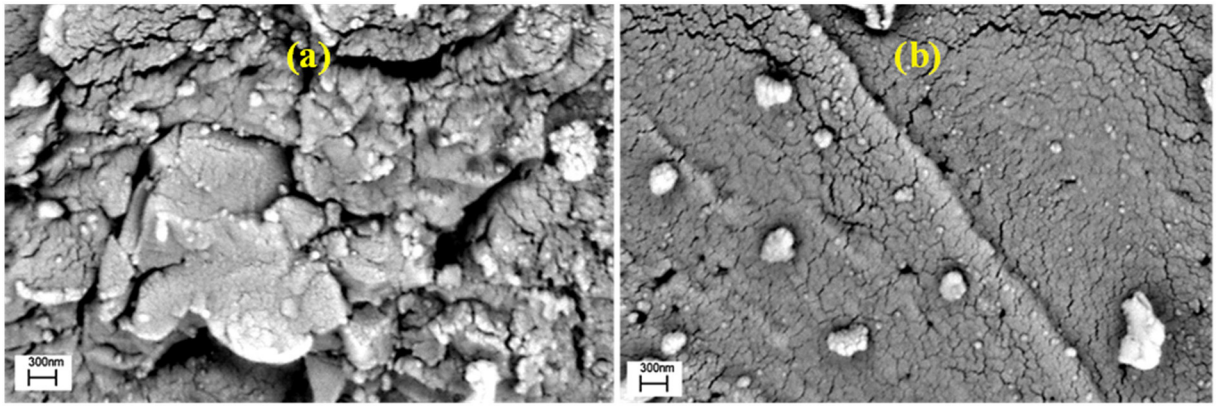

Fig. 3. SEM micrographs of (a) Chit-TPP and (b) FEC@NBC.

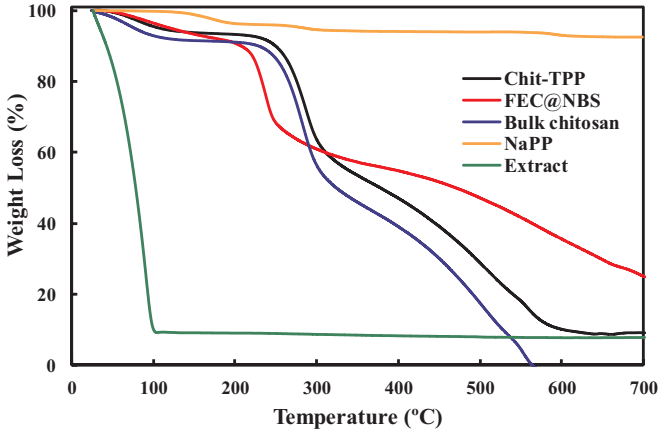

Fig. 4. TGA thermograms of Chit-TPP, FEC@NBC, Bulk Chitosan, NaPP, and extract.

3.3.1. Effects on hormonal regulation

Hormonal alteration is a common abnormality associated with PCOS. The excess secretion of LH is one of the PCOS complications. Together with LH, FSH is also released from the pituitary gland. In PCOS patients, the level of LH is about two- to three-fold higher than that of FSH, which is called elevated LH/FSH ratio. Given that the LH and FSH levels were determined in the studied groups and compared to that of the healthy and untreated groups. Fig. 8a compares the levels of LH in the studied groups. Significant growth in the LH rate was noted in untreated PCOS-Control in comparison with the non-PCOS control group. However, the LH level was reduced in all the treated groups compared to that of PCOS-Control whereby the FEC@NBC and Chit-TPP samples showed the highest significant and lowest insignificant effects on the LH level, respectively. It is noteworthy that a significant decline in the amount of LH was also observed in the extract-treated rats ($p < 0.05$).

The follicle-stimulating hormone affects the function of ovaries through the stimulation of the growth of follicles and the maturity of the eggs. Nonetheless, the reduction of FSH levels inhibits the follicular growth and results in infertility. Subsequently, the immature follicles are converted into small cysts in the ovaries, which is so-called PCOS. Hence, it is important to assess the effects of the fennel extract, Chit-TPP, and FEC@NBC on the FSH levels in the rats with PCOS. Fig. 8b presents the FSH values in the treated rats compared to both control groups. The FSH level in the PCOS-Control was significantly less than the healthy control group. The administration of extract and FEC@NBC enhanced the FSH levels in PCOS-induced rats wherein the differences among the studied groups were significant ($p < 0.05$).

All adult females have testosterone in their bodies at the total values of about 6 to 86 ng/dL [34]. However, the menstruation and ovulation can occur irregularly if testosterone level increases above its normal range. The effects of the Chit-TPP, FEC@NBC, and FSX were examined on the testosterone level in the studied rats (Fig. 8c). The fennel extract and FEC@NBC significantly improved the testosterone levels in PCOS rats when compared to that of the PCOS-Control level ($p < 0.05$).

The results obtained from the hormonal assessment revealed that the rats with PCOS were typically diagnosed with elevated levels of LH and testosterone, and detracted FSH level compared to those of healthy rats. During the course of treatment by extract and FEC@NBC, a significant enhancement in the FSH level along with a reduction in LH and testosterone were detected in the rats with PCOS. The efficacy of the FEC@NBC sample in the normalization of these hormones was much higher than the effects of the only extract. This finding can be associated with the mucoadhesive property of chitosan, which gives rise to higher permeability of FEC@NBC across mucosal membranes and

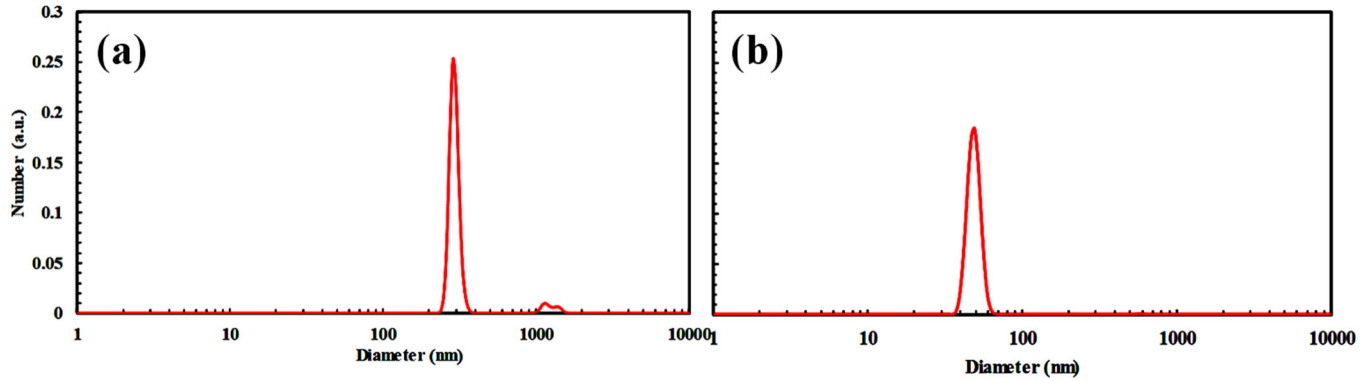

Fig. 5. Dynamic light scattering of (a) Chit-TPP and (b) FEC@NBC.

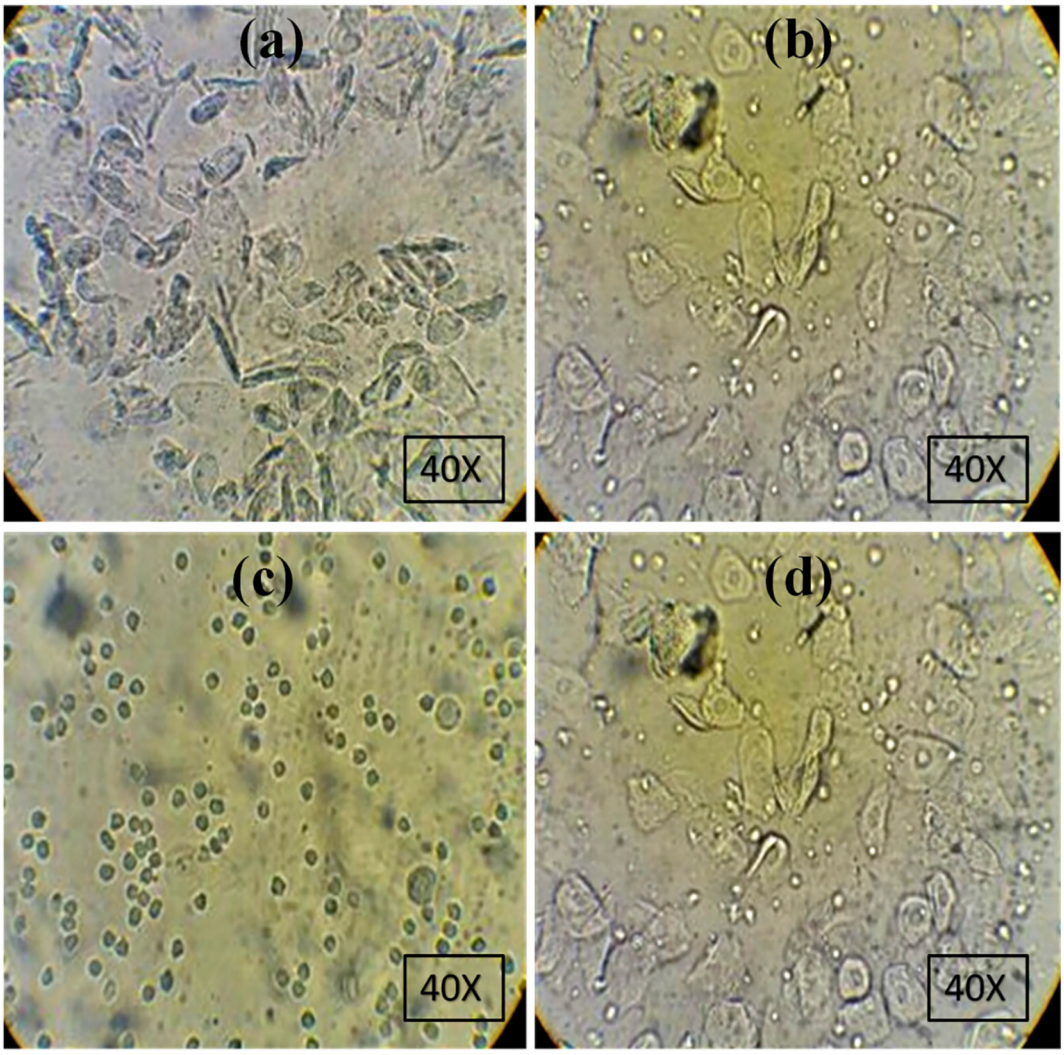

Fig. 6. The estrus cycle phases; (a) estrus, (b) pro-estrus, (c) di-estrus, and (d) met-estrus.

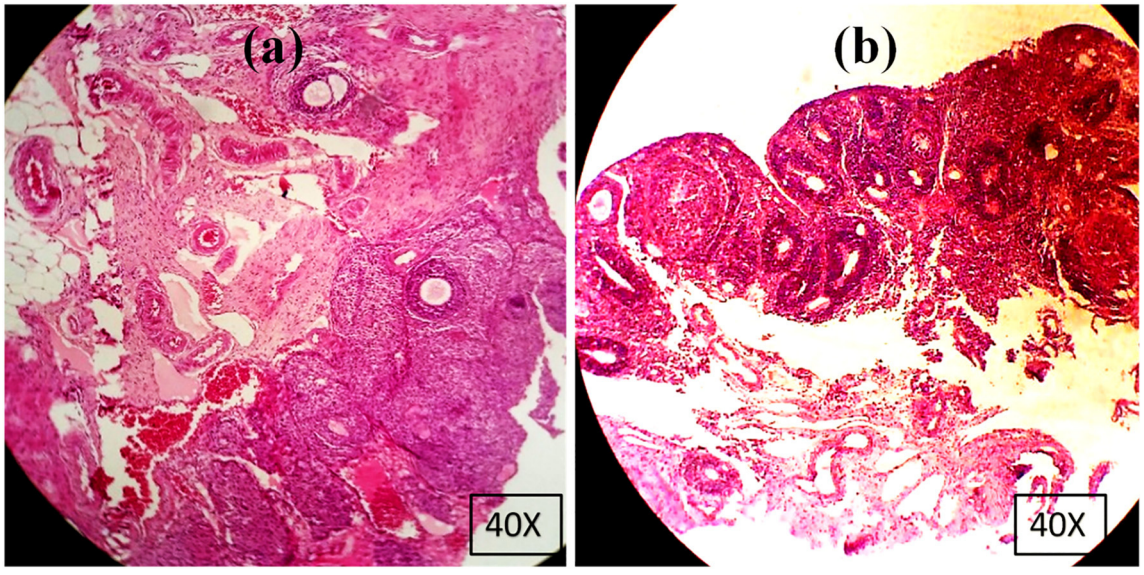

Fig. 7. Histogram of a (a) normal ovary and (b) polycystic ovary.

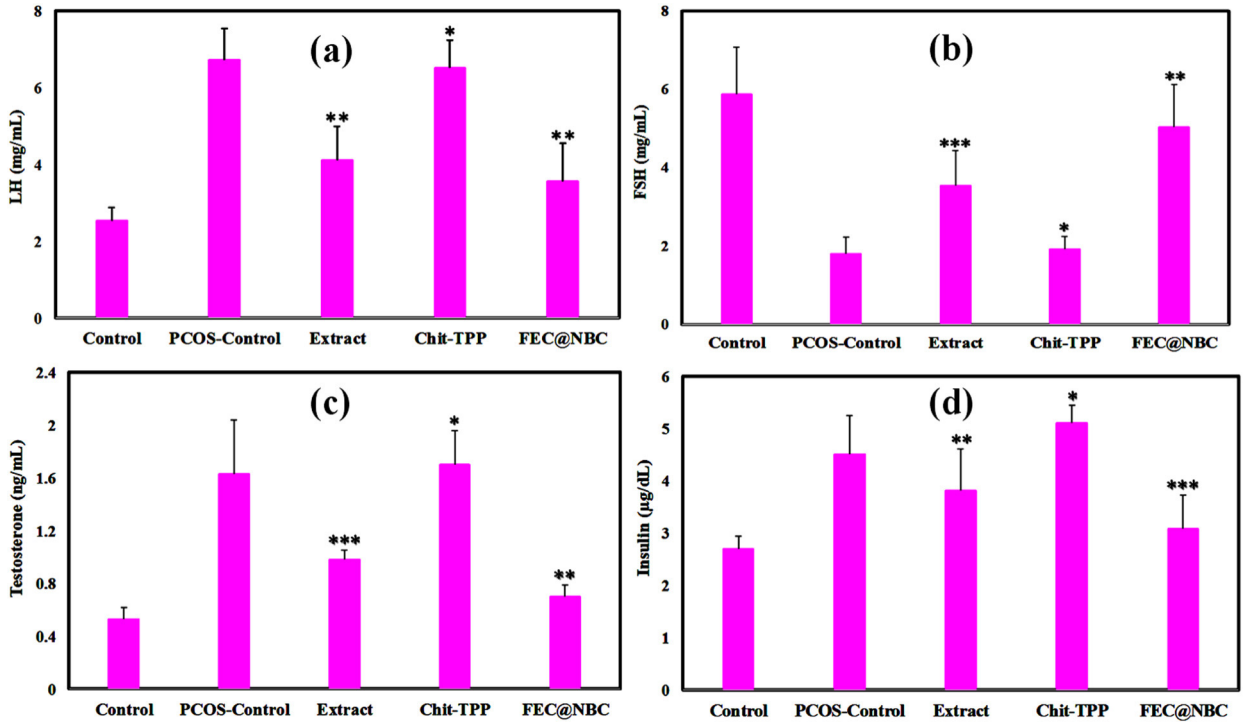

Fig. 8. Serum levels of (a) LH, (b) FSH, (c) testosterone, and (d) insulin in the studied groups.
*Significant difference with Control group.
**Significant difference with PCOS-Control group.
***Significant difference with both Control and PCOS-Control groups.

enhanced pharmacological effects. Moreover, as evidenced by the zeta potential analysis, the encapsulation of bioactive molecules resulted in the higher stability of FEC@NBC and its effective targeted delivery [28]. It is reported that the isoflavone content of the fennel extract (e.g. daidzein and genestein) played a positive role in the reproductive cycle (estrous phase) in female rats [35]. Likewise, the administration of fennel extract has normalized the gonadotropin hormones in rats with PCOS due to the constructional phytoestrogens with anti-androgenic effects [36]. Generally, the phytoestrogens decrease the LH level and consequently result in the testosterone level reduction via the negative feedback effect. It should be pointed out that the molecular and spatial structure of phytoestrogens is similar to that of estradiol 17-β, so it can bind to both α and β estrogen receptors on the cell membranes. Under the elevated levels of estrogen, phytoestrogens diminish the estrogen activity via limiting their access to the receptors, thus inducing folliculogenesis in the ovaries [37]. Phytoestrogens affect the function of the hypothalamic–pituitary–gonadal axis (HPG axis) probably through blocking the estrogen receptors. This function entails the diminution of the LH release from the pituitary. Also, phytoestrogens decrease the gonadotropin-releasing hormone (GnRH) releasing frequency via its gene expression [38].

As reported by Choi & Hwang [39], palmitic acid and β sitosterol present in the fennel seed have anti-androgenic properties wherein they inhibit the formation of the dihydrotestosterone-receptor complex. Moreover, β sitosterol hinders the conversion of testosterone to estradiol via minimizing the aromatase activity, thus reducing the level of estradiol [40]. Another compound in the FSX known as comarine is reported for its inhibitory effects on the aromatase, androgen, and reductase performances, which lead to the reduction in the total testosterone levels [41]. Over and above, the FSX is an abundant source of antioxidants, which prevail the oxidative stress in ovarian tissue and bring about an antioxidant balance in PCOS-induced rats and significantly improve the PCOS-depended complications [42,43].

Increased insulin resistance and subsequent hyperinsulinemia are of the recognized signs of PCOS. Under the excess insulin condition, the repetition of GnRH is intensified, which contribute to bigger LH to FSH ratio. Therefore, it leads to increased androgens and decreased estradiol synthesis, thus preventing the follicles from maturation and developing PCOS. The changes in the insulin levels of the PCOS-induced rats after treating with fennel extract, Chit-TPP, and FEC@NBC in contrast to both control groups are presented in Fig. 8d The results exposed the significant effect of FEC@NBC on the serum insulin down to the levels comparable with the healthy control group ($p < 0.05$). Notably, the reduction in the insulin level in the rats with PCOS after treating with fennel extract was not as significant as FEC@NBC treated PCOS rats.

It has been demonstrated that the elevation in insulin level in patients with PCOS is meaningfully associated with insulin resistance. The first pathophysiological sign of PCOS activates with insulin resistance, which disrupts the insulin function in glucose uptake and metabolism and gives rise to glucose intolerance. The phenolic compounds present in fennel such as quercetin-3-O-glucuronide, trans-anethole, and eriodictyol-7-O-rutinoside have shown great antioxidant and free radical scavenging activities that normalize glucose metabolism via protection of the pancreatic beta cells from any defects [44,45]. Furthermore, several studies have reported bioactive molecules that boost the activity of beta cells to excrete the higher levels of insulin [46,47] and the effects of herbal medicines encompassing phytoestrogens on PCOS complications such as insulin resistance, hyperandrogenism, and weight of ovaries [42]. Considering the highest performance of FEC@NBC in regulating the serum levels of target hormones compared to those of Extract and Chit-TPP, the significant role of chitosan in targeted and efficient delivery of the bioactive molecules can be well-understood.

3.3.2. *Effects on glucose uptake and lipid profile*

As explained above, the bioactive compounds of fennel can suppress insulin resistance condition in rats with PCOS mainly through the enhancement in the glucose uptake and metabolism [48]. To examine this effect, a set of experiments were conducted to measure the fasting blood

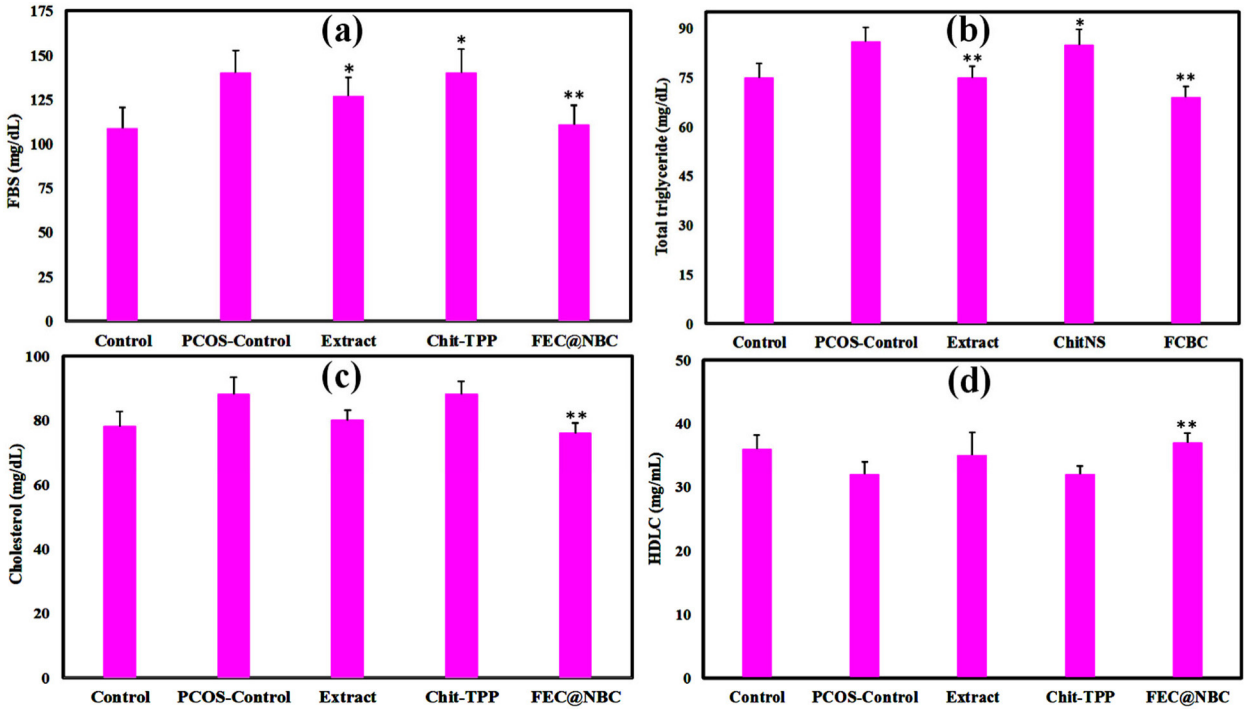

Fig. 9. Serum levels of (a) FBS, (b) TG, (c) cholesterol, and (d) HDL in the studied groups.
*Significant difference with Control group.
**Significant difference with PCOS-Control group.
***Significant difference with both Control and PCOS-Control groups.

sugar (FBS) levels in the treated PCOS-induced rats in comparison with control groups (Fig. 9a). Among the studied therapies, the FEC@NBC sample was the only agent that could significantly diminish the serum FBS compared to that of the healthy control group ($p < 0.05$). Considering the results, it can be argued that chitosan has played a leading role in stabilizing and effective delivery of the bioactive molecules of the extract wherein administration of the only extract was unsuccessful in regulating the FBS level down to the healthy values [28,49].

Hypertriglyceridemia is a crucial concern of insulin resistance, which drives the liver to supply excess low-density lipoproteins (LDL). Since dyslipidemia is significantly associated with the development of cardiovascular diseases, that is of great importance to tackling this condition [50]. After PCOS induction, surplus amounts of total triglyceride and total cholesterol were detected in the serum samples of the rats (Fig. 9b and c). On the contrary, the levels of high-density lipoproteins (HDL) dropped to the levels below the healthy condition (Fig. 9d). The variations in the lipid profile serum levels in the studied rats after 16 days of medical intervention are shown in Fig. 9. From the results, the fennel extract and FEC@NBC sample could actively result in a significant diminution in the serum TG levels of PCOS-induced rats when compared to that of untreated PCOS groups ($p < 0.05$). Moreover, both parties exposed significant anti-cholesterol effects, which was more prominent when FEC@NBC was supplied. The anti-dyslipidemic activity of the samples is mainly attributed to a group of phenolic compounds, anthocyanins, which are proven to have positive effects on serum lipid profile especially on HDL-cholesterol levels [51].

In follow-up, GC–MS analysis was performed to figure out the FSX composition. Table 1 presents some of the identified molecules over the Chit-TPP sample along with the corresponding retention time (min), chemical name, molecular weights, and peak areas (%). As explained earlier, isoflavone and phytoestrogens present in the FSX have significant effects on the reproductive cycle and hormonal regulations of the female rats [35,37]. In addition, a number of the detected organosilicon molecules have found to be medicinally interested [52]. On the other hand, the reports on the various secondary metabolites of FSX demonstrate that the FSX phytochemicals not only regulate the biochemical and hormonal factors altered in PCOS patients but also protects the body from some other health issues such as kidney injuries [53].

Table 1
GC–MS results of the employed fennel seed extract.

Retention time (min)	Compound	Molecular weight (g/mol)	Peak area (%)
2.713	3-Hydroxybutanamide, *N*-phenylmethoxy	209.24	7.67
2.776	3-Hydroxybutanamide, *N*-phenylmethoxy	209.24	28.88
2.844	1-Chloro-2-nitropropane	123.54	5.47
2.879	Dimethylsilanediol	92.17	9.14
3.148	5-Methyl-2-phenyl-1H-indole	207.27	5.38
3.737	Hexamethylcyclotrisiloxane	222.46	0.62
4.681	1-(4-Nitrophenyl)piperazine	207.23	5.22
6.501	Octamethylcyclotetrasiloxane	296.61	6.76
8.332	(1,3)-Bicyclo[2.2.1]heptan-2-one	110.15	5.23
35.557	Hexamethylcyclotrisiloxane	222.46	0.64
38.904	Hexamethylcyclotrisiloxane	222.46	0.38
39.076	Hexamethylcyclotrisiloxane	222.46	1.46
39.242	Decamethyltetrasiloxane	310.68	1.42
39.328	Decamethyltetrasiloxane	310.68	0.99
39.448	Hexamethylcyclotrisiloxane	222.46	2.08
39.511	Decamethyltetrasiloxane	310.68	1.37
39.602	Hexamethylcyclotrisiloxane	222.46	2.12
39.682	Decamethyltetrasiloxane	310.68	3.01
39.791	Hexamethylcyclotrisiloxane	222.46	3.06
39.843	Hexamethylcyclotrisiloxane	222.46	2.11
39.934	Hexamethylcyclotrisiloxane	222.46	2.02
39.974	Hexamethylcyclotrisiloxane	222.46	1.30
40.031	Hexamethylcyclotrisiloxane	222.46	1.21
40.089	Hexamethylcyclotrisiloxane	222.46	1.96
40.186	Hexamethylcyclotrisiloxane	222.46	0.51

PCOS is a multifactorial disorder and its etiology is not clear yet.

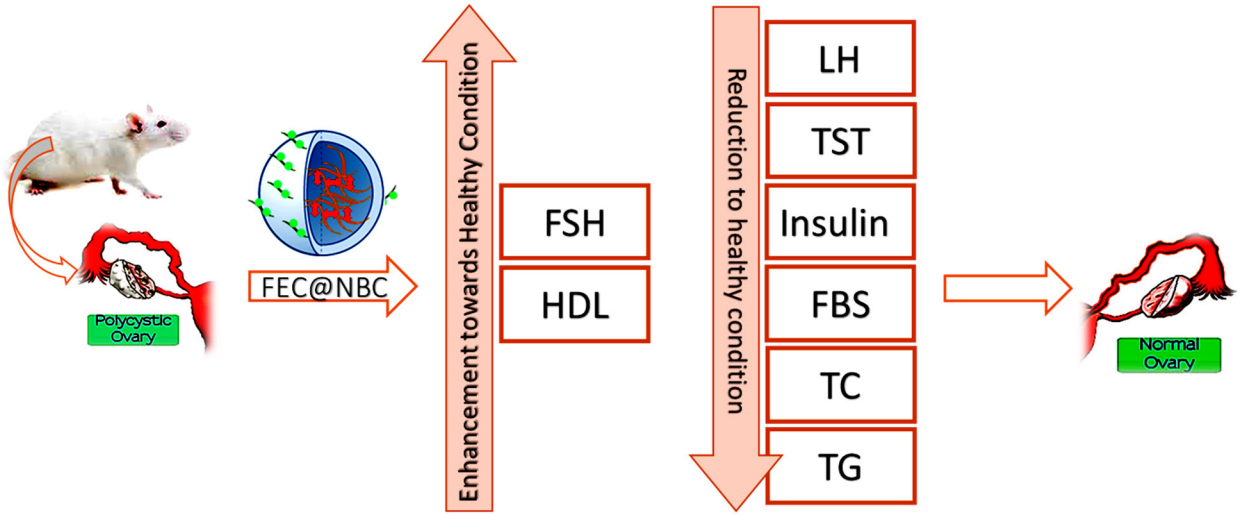

Fig. 10. Overall effects of the FEC@NBC sample on the biochemical and hormonal factors in PCOS rats.

Moreover, the effects of the isolated phytochemicals of the FSX have been rarely studied. Taking into account that these secondary metabolites are generally multi-task, interpretation of the engaged mechanism/s of the action of FSX on the studied PCOS rats is complicated. These phytochemicals can simultaneously take part in various biological processes and help for regulation of the underlying factors under PCOS condition. The joint action of FSX phytochemicals when anchored on Chit-TPP resulted in the elevated levels of FSH and HDL, while the serum levels of insulin, LH, testosterone, TC, TG, and FBS reduced significantly (Fig. 10).

The plausible explanation on the enhanced therapeutic activity of fennel seed extract when carried by chitosan particles (FEC@NBC) could be deduced from the earlier studies [54]. The surface of chitosan is positively charged, which develops the tendency to absorb negatively charged phytomolecules such as flavonoids (e.g. polyphenols) through electrostatic attraction [55]. Being anchored on chitosan surface secures the stability of the secondary metabolites of fennel seed extract and prolongs their retention time in the blood stream, leading to enhanced release of the phytomolecules into the target cells. This is especially resulted from the mucoadhesive property of chitosan wherein it can stick to mucus membranes and discharge the loading in a long-standing mode [56].

4. Conclusion

In this study, the incorporation of fennel seed extract phytomolecules with chitosan resulted in the high stability of both parties and gave rise to an efficient drug delivery system. The characterizing tools exposed the strong interaction of fennel seed biomolecules with chitosan, high stability, and morphological/structural modifications in FEC@NBC. Besides, this approach gave rise to the enhanced stability and effective delivery of the encapsulated biomolecules during the course of the treatment of the rats with PCOS. The various phytochemicals of FSX including isoflavone, phytoestrogens, and antioxidants with multiple activities brought about exceptional outcomes when carried over chitosan nanoparticles. The chitosan particles safeguarded the phytomolecules, enhanced their stability, and effectively delivered them to target cells owing to surface characteristics and mucoadhesive property of chitosan. Altogether, the administration of FEC@NBC was effective in managing the complications associated with PCOS by lowering the serum levels of insulin, LH, FBS, testosterone, total triglycerides, and total cholesterol, while enhancing the FSH and HDL levels. This study introduces FEC@NBC as a safe and effective bioagent to regulate the hormonal and biochemical changes of PCOS and suggests further studies to discover the underlying mechanism(s) of the action of the FSX phytochemicals and driving factors that regulate the levels of the involved hormones and biochemical parameters.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2020.111351.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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