Original Article

Green Biosynthesis of ZnO Nano-Particles, Inhibited Development of Pre-antral Follicles

Maryam Ghorbani [1], Javad Baharara [2*], Akram Eidi [3], Farideh Namvar [4]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran. [2] Department of Biology, Research Center for Applied Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran. [3] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran. [4] Department of Medicine, Mashhad Branch, Islamic Azad University, Mashhad, Iran.

Abstract

Nanoparticles of zinc oxide are widely used in commercial applications and the harmful effects of nanoparticles on the development of oocytes have been studied in previous investigations. In this paper, we aimed to grow an eco-friendly procedure for the green synthesis of zinc oxide nanoparticles (G/ZnO-NPs) utilizing aqueous extract of *Ferula gummosa* gum resin (Galbanum) and evaluated G/ZnO-NPs effects on in vitro pre-antral follicle (PF) maturation compared to Galbanum extract alone and commercial zinc oxide nanoparticles (C/ZnO-NPs). Synthesized, G/ZnO-NPs have an absorbance band at 320 nm and spherical shapes, the mean particle size of 36 nm and are reasonably stable with a zeta potential value of -20 mV. FTIR results indicated that Galbanum extract covered the surface of ZnO-NPs. The results showed that Galbanum extract inhibiting free radicals as well as increasing the production of estradiol and testosterone improves PF growth. Also, the Galbanum extract increased expression of GDF-9 and BMP-15 versus the expression of Foxo1 and VNN1 that associated with atresia of PF were reduced. However, the evidence suggests G/ZnO-NPs and C/ZnO-NPs, have negative effects on PF through increasing the production of free radicals, reducing the expression of estradiol and testosterone, the reduced expression of GDF-9 and BMP-15, as well as the increased expression of Foxo1 and VNN1. Overall, the findings of this study showed that Galbanum extract due to antioxidant effects, supports PF development while C/ZnO-NPs and G/ZnO-NPs inhibit PF growth, and the Galbanum extract covered the surface of ZnO-NPs only slightly offsets the disadvantages of these nanoparticles.

Keywords: Galbanum, Green synthesis, Zinc oxide nanoparticles, Pre-antral follicles, In vitro maturation

Introduction

The applications of nanotechnology are exponentially growing in industry and medicine, due to interesting properties of engineered nanoparticles. The expanded use of nanomaterials led to the increased presence of such materials in our environment which has raised concerns about their harmful effects on human health. The physicochemical properties of nanoparticles affected the biological system and changed or disrupted their normal function. Recently, in green chemistry, natural materials such as plant, fungal, and microbial extracts are used to synthesize nanoparticles. The genus *Ferula* (Apiaceae family) consists of 170 species [1]. *Ferula gummosa* is a native plant, original to the Middle East, especially to Iran, and sometimes rises in the northern and western areas of Himalaya [2]. *F. gummosa's* gum resin extract from the roots and stems is worthy of the name of Galbanum [2]. It is recognized as Barijeh in Iran [2]. The gum resins of the roots from various *Ferula* species are published to be utilized for stomach disorders, rheumatism, headache, arthritis, and dizziness [3]. In Iranian folk medicine, Galbanum is utilized as an anti-spasmodic, digestive stimulant factor for the therapy of colic and flatulence. It is also used as an expectorant for bronchitis and as a uterine tonic [4]. It is recognized that the genus Ferula includes a mixture of coumarin [5] and sesquiterpenes [6]. Several medical properties have been noticed for the important oils of some species of Ferula, such as antioxidant [7], cytotoxicity [8], and antibacterial activities [9]. Among different metal nanoparticles, zinc oxide nanoparticles (ZnO) are widely used for physical and chemical purposes. Zinc oxide nanoparticles are widely applied in pigments and cosmetic, electronic, and chemical fiber industries. Due to their antibacterial and antifungal properties, they are also used in

Address for correspondence: Javad Baharara. Department of Biology, Research Center for Applied Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran.
Email: baharara78@gmail.com

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foods and incorporated into the paints and plastic [10]. In medicine, these NPs show a promising application in cancer diagnosis, prevention, and treatment [11]. However, some studies reported in vivo and in vitro cytotoxicity and genotoxicity of ZnO. After exposure of 0.5h to 72h, nano-ZnOs could cause acute cytotoxicity on various cell types, such as human epidermal cells, liver and retinal cells, and white blood cells via interfering with zinc ion homeostasis or inducing oxidative stress. In vivo, inhalation exposure to nano-ZnOs for 3 days caused severe damage in liver and lung tissues. Also, several studies indicate that ZnO NPs induce DNA damage by oxidative stress in different cell models [12]. It has also been reported that zinc oxide nanoparticles have a deleterious effect on spermatogenesis in NMRI mice. Furthermore, ROS production and oxidative damage are considered the main mechanisms responsible for genotoxicity induced by zinc oxide nanoparticles [13]. However, the effects of this substance on the development of PF have not been studied so far. Many studies have shown that the formation of free radicals can strongly affect the development of PF. On the other hand, Nano-ZnOs can be prepared by various methods, such as the traditional high temperature solid state method, chemical precipitation, sol-gel synthesis, hydrothermal and green method. The aim of the present report was to develop a green method to synthesize G/ZnO-NPs by using *Ferula gummosa* gum resin (Galbanum) aqueous extract and comparing the effects of G/ZnO-NPs and commercial ZnO on PF maturation in NMRI mice.

Material and Method

Reagent and media

Zinc oxide was obtained from Sigma-Aldrich (Poole, United Kingdom). Fetal bovine serum (FBS) and alpha MEM medium were purchased from Invitrogen. The High Pure RNA Isolation Kit and cDNA Synthesis Kit were also purchased from Roche (Mannheim, Germany) and Fermentas Inc. (Vilnius, Lithuania), respectively. Also, the primers were obtained from Bioneer (Daejeon, Korea), and the commercial ZoNPs were purchased from ***Nanozino*** Company. All solutions were prepared using double distilled water and other reagents were of analytical grade.

Collecting of Galbanum gum and extraction

Galbanum gum was collected from Bojnourd (North Khorasan, Iran) in May-June 2017. Recognition of the genus and species of *F. gommusa* has occurred in Agricultural Research Center (Ferdowsi University, Mashhad, Iran) and a voucher specimen (FUMH-E 1014) was located by a botanist in this center. For extraction, Galbanum gum (1 g) was dispersed in 10 ml of distilled water by magnetic stirring and heated at 70 °C for 3 hours. The extract was filtered through a mesh, followed by Millipore filter (0.2 µm), and stored at +4 °C before use.

Extracellular synthesis and characterization of G/ZnO-NPs

The 10ml aqueous solution (0.mM) of zinc acetate dihydrate ($Zn(Ac)^2.2H^2O$) was mixed with 5 ml of the aqueous extract of Galbanum (1 mg/ml) allowed to stand at 70 °C for 24 h. Subsequently, synthesized G/ZnO-NPs were characterized by UV-Vis spectroscopy studies. UV-Vis spectra of G/ZnO-NPs were measured using a Lambda 25 spectrophotometer (Perkin Elmer, Waltham, MA, USA) at wavelengths ranging from 250 to 400 nm.

Dynamic light scattering and zeta-potential measurements: The hydrodynamic size and stability of the G/ZnO-NPs were determined using DLS/zeta potential analysis. The best sample based on UV- visible results was selected for the DLS study. Dynamic light scattering can be used to determine the size distribution profile of zinc nanoparticles at 25 °C using 0.894 cp for the viscosity of the medium, a fixed angle of 90° for the avalanche photodiode (APD) detector, and the wavelength of 657 nm for the 50 mW laser (Cordovan, Vaso particle, France). Zeta-potentials of ZnO-NP in water were evaluated using CAD (zeta compact, France).

FESEM measurements*:* Surface morphology of G/ZnO-NPs was analyzed using a ZEISS DSM-960 microscope working at 25 kV and FE-SEM equipment (FEI-NOVA Nano SEM 230). Sample preparations were performed by adhering particle samples on a carbon tape without requiring a gold conductive coating on the surface.

Fourier transform infrared (FTIR) spectroscopy measurements*:* FT-IR for Galbanum Aqueous Extract and ZnO-NP was obtained in the range of 4000 to 400 cm−1 using a Perkin Elmer spectrophotometer paragon 1000. To remove any free biomass residue or unbound extract from the surfaces of the NPs, the ZnO-NPs were repeatedly washed with distilled water and subsequently centrifuged at 9000 rpm for 15 minutes. The dried powdered zinc oxide nanoparticles were mixed with KBr powder and FT-IR spectra were obtained in the range of 4000 to 400 cm−1.

In vitro maturation of preantral follicles

Animals: In this study, 30 NMRI mice with an age range of 2 to 3 weeks and weight of 10-20 g were used for follicle isolation. Animals were purchased from the Applied Biological Research Center. The mice were kept at a temperature of 23 ± 2 °C, standard light conditions (12 hours of light and 12 hours of darkness), and relative humidity of 68-50%. Food and water were also available freely.

Follicle isolation: Using diethyl ether, NMRI mice were first anesthetized and subsequently the ovaries were cut. The ovaries were placed in 35 mm plastic culture dishes, then they were removed from the waste tissue Intact pre-antral based on morphological properties were mechanically isolated using a tow insulin syringe. The small follicles were collected from the medium. Only intact follicles were selected and categorized for other analyses.

Pre-antral follicles (PF) selection for culture: Healthy PF with the size range of 200-400 µm, which had a central oocyte with no signs of atresia and intact basement membrane, were considered as good quality samples for culture.

The culture of pre-antral follicles: Isolated follicles were placed individually in 100 µl of complete culture medium that consists of α-minimal essential medium (α-MEM, Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, UK), 100 µg/ml penicillin and 50 µg/ml streptomycin in a 96 well culture plate and kept at 37 °C and 5 CO% incubators for up to 12 days. Half of the medium was replaced by an equal volume of fresh medium every 48 hours.

Categories of cultured follicles: The isolated follicles were categorized into equal groups randomly. Control group 1 included the follicles that were evaluated without any treatment. Group 2 were treated with Galbanum extract (10, 50, 100, 200, and 400 µg/ml). Groups 3 and 4 were treated with G/ZnO-NPs and C/ZnO-NPs (5, 7.5, 10, 12.5, 15, and 17.5 µg/ml), respectively.

Morphology and growth evaluation of prenatal follicles: All cultured follicles were morphologically evaluated for 0, 2, and 4 days, using an inverted microscope (Biomed, Korea) to analyze the increase in the diameter, antrum formation, or degeneration.

ROS production assay: ROS levels in different treated groups were measured. Toward this end, 15 follicles were treated in different groups for 4 days with at most five repetitions. After the treatment period, the samples were washed with PBS 1 three times and incubated in 40 mmol of Tris-HCL buffer and 5 µmol/l DCHFDA for 30 minutes at 37 °C. The samples were washed again using PBS, lysed with 100 µl of Tris-HCL buffer, and centrifuged at 10000 rpm for 15 minutes at 4 °C. The fluorescent intensity in the supernatant was recorded using a spectrofluorometer at 488-nm excitation and 525-nm emission. The data were expressed as µM H_2O_2 and the mean dichlorofluorescein (DCF) fluorescence intensity (means ± SEM). The analysis for each sample was duplicated.

Hormone measurement: Estradiol and testosterone concentrations were measured in the collected supernatant of samples using ELISA kits.

Evaluated expression of GDF-9, BMP-15, and Foxo1 and VNN1 genes by Real Time-PCR in pre-antral follicles treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs

The PF was treated with the best concentration of Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs for 4 days. RNA was then extracted using a commercially available RNA extraction Roche kit according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from mRNA by adding one of the samples to 2 random hexamer primers according to the manufacturer's instructions. Reverse-transcriptase PCR Master Mix was created using the SYBR Green, specific primer, GAPDH was used as a housekeeping gene.

Statistical analysis: The data were expressed as mean±SEM and analyzed using SPSS statistical software. Significance was established when the p-value was less than 0.05.

Results and Discussion

Green synthesis of metal oxide nanoparticles utilizing biomass constituents is appealing since these methods are uncomplicated, cheap, and nontoxic compared to chemical and physical procedures. The current essay reports the extracellular synthesis of G/ZnO-NPs via aqueous extract of Galbanum. The biochemical procedure of nanoparticle shaping and stabilization still stays largely undiscovered, except for some investigation groups which have shown that the proteins observed in enzymes releasing by microorganisms are the chief biomolecules included in the establishment of metal/metal oxide nanoparticles [14, 15]. Hydroxyl groups of amino acids are the most operative practical groups which permit the complexation of Zn ions to these molecules adopts with hydrolysis, and eventually of ZnO nanoparticles through thermal disintegration. This construction helps amino acids to steady zinc particles and eventually G/ZnO-NPs that prevents their extreme assemblage or crystal expansion.

Characterization of G/ZnO-NPs

UV-Visible Spectrophotometry: UV–vis spectroscopy is commonly used to examine the size and shape of nanoparticles in aqueous solution. The frequency and width of the surface Plasmon absorption depend on the size and shape of the metal nanoparticles as well as the dielectric constant of the metal itself and the surrounding medium (7). It is also well known that solutions containing ZnO-nanoparticles explain a characteristic absorption peak below 400 nm that arises suitable to surface plasmon resonance in gold nanoparticles [16]. Therefore, G/ZnO-NPs absorbance peak was detected by a UV-Vis spectrophotometer in the range of 250 to 400 nm. The UV-vis absorption spectra of the biosynthesized G/ZnO-NPs specimens are displayed in Figure 1A. It was revealed that the UV–vis absorption spectra of biosynthesized ZnO nanoparticles specimen showed an absorption peak at 320 nm. In this context, Zhang et al. synthesized ZnO nanoparticles by a sonochemical method. They used UV–vis absorption spectra for ZnO nanoparticles characterization and showed that these nanoparticles have a sharp pick at 320 nm, which is in good agreement with the previous works [17].

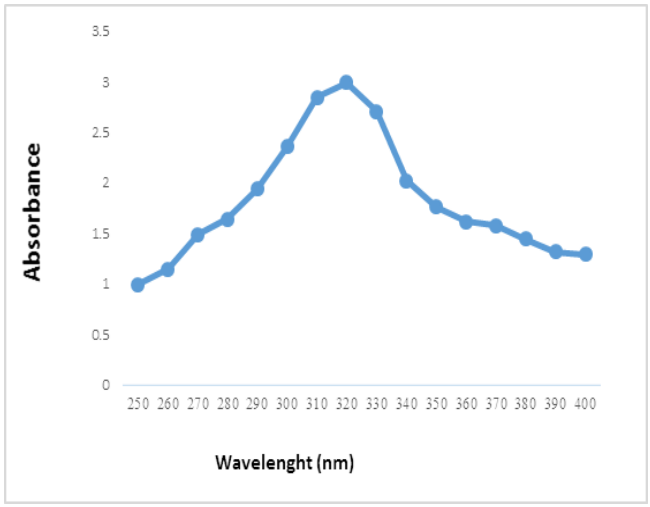
Size dispersion: Dynamic Light Scattering (DLS), a technique often referred to as photon correlation spectroscopy, is a common technique for determining particle size in colloidal suspensions [18]. The application of the DLS method for determining the size distribution of ZnO-NPs in the range of 1–100 nm was discussed before. As can be seen

in Figure 1B, DLS results showed that the distribution of particles ranges approximately from 30 to 110 nm. Then the average size of the ZnO-NPs was found to be 36.60 nm. However, the average size of nanoparticles was about 4-50 nm which is the most appropriate size range for biomedical applications of nanoparticles [19]. According to Azizi et al (2014), their contribution deals with the one-pot method for the synthesis of zinc oxide nanoparticles (ZnO-NPs) through a green process using the brown marine macroalgae *Sargassum muticum* (*S.muticum*) aqueous extract. They showed that the average size of the synthesized ZnO ranged from 30 to 57 nm [20]. Nanoparticles produced by the green method and herbal extracts have a variety of dimensions due to the presence of different reducing agents in the composition of the extracts.

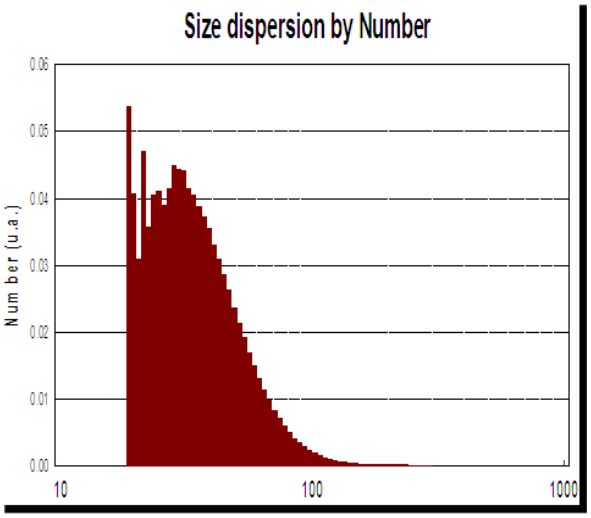
The Zeta potential: The Zeta Sizer was used to measure the electrophoretic mobility of each nanoparticle sample. Complex Zeta potential is a parameter that is used in the study of the surface charges and stability of NPs. These charges can greatly influence the particle distribution, cellular uptake, and adsorption to cellular membranes in vivo. A high absolute zeta potential value indicates a high electric charge on the surface of the NPs. It describes strong repellent forces among particles, prevents aggregation and stabilizes NPs in buffer solution. The zeta potential of the formed nanoparticles was measured only in systems that did not sediment after overnight equilibration [12]. As can be seen in Figure 1C, in natural conditions (pH close to 7.2), the values of zeta potential were equal to −10 to −20 mV. The negative charge revealed that the particles were well disjoined and there was a pellet force between them. Therefore, the particles did not bear conglutination and eluded gathering which guaranteed long term steadiness of the particles. The most considerable extent of zeta potential value reveals a strong repellent force between the particles that increases their steadiness. Therefore, it can be asserted that the synthesized nanoparticles were extremely steady, which is the significant quality of nanoparticles suitable for utilizing medicinal intentions. Baharara et al. (2016), also investigated the stability of the gold nanoparticles produced by the green method, using the Zeta potential measurement. They suggested that the same electric charge on the surface of the nanoparticles would increase their stability [21].

FTIR Analysis: Figure 1E indicates the FTIR spectra of the G/ZnO-NPs. The patterns of absorption peaks in graphs A and B are similar. It is demonstrated that extract is attached on the surface of ZnO nanoparticles. The earned spectrum indicates the ZnO absorption band in the range of 3500 and 500 cm^{-1}. The broad peak around 3453 cm^{-1} showed N-H and O-H stretching vibrations of 1° and 2° amines, amides, alcohol, and H bonded to phenols of the plant extract [22]. The bands at 2967 cm^{-1} correspond to C-H stretching vibrations of alkanes, co-responding to equate with amide linkage between amino acid residues in the proteins and stretching vibrations of amide II [23]. Remaining two bands at 1625 cm^{-1} signified the presence of amide I group (beta-sheets) and carboxylic groups, respectively [24]. The strong intense peaks at 1379 cm^{-1} correspond to C−N stretch vibrations as well as the amide I band of proteins in the extract [24]. FTIR spectroscopic study confirmed that some terpenoids reducing sugar and phenolic group's exhibit in the Galbanum aqueous extract, have reduced qualities that facilitate the quick reduction of the zinc ions in nanostructured ZnO. This phytochemicals exhibit in Galbanum aqueous extract works as a bio-reductant and is important for the direct reduction of zinc ions in their respective nanostructures [23]. Moreover, the existence of amide groups of proteins works as a capping factor to stop agglomeration and aids in ZnO nanoparticle stabilization. Elumalai et al. (2015) described the synthesis of zinc oxide nanoparticles using leaf aqueous extract of *Azadirachta indica* (L.). They discussed that the similarity of the plant extracts spectra and zinc oxide nanoparticles synthesized by the green method indicated the presence of plant extracts on the surface of the nanoparticles. Also, changes in the absorption peaks in two different samples demonstrated that the agent in the extract reduced zinc oxide and produced nanoparticles [25].

FE-SEM analysis: The dimension, form, and dispensation of as-synthesized G/ZnO-NPs were analyzed through FE-SEM monitoring. Figure 1D displays the FE-SEM image of ZnO-NPs, which were prepared after 24 hours of incubation. ZnO-NPs show a spherical shape. This agglomeration is due to polarity and electrostatic attraction of ZnO nanoparticles [20]. Regarding the fact that the specific properties of nanoparticles depend on their shape, synthesis of nanoparticles along with controlling shape is very important. Therefore, the most appropriate shape that can be used for biological purposes is the spherical shape [19]. This was achieved in a study by Sangeetha et al. (2015). They reported that the highly stable and spherical zinc oxide nanoparticles were produced by using zinc nitrate and *Aloe vera* leaf extract. These results are similar to the findings of the current study [26].



(A)



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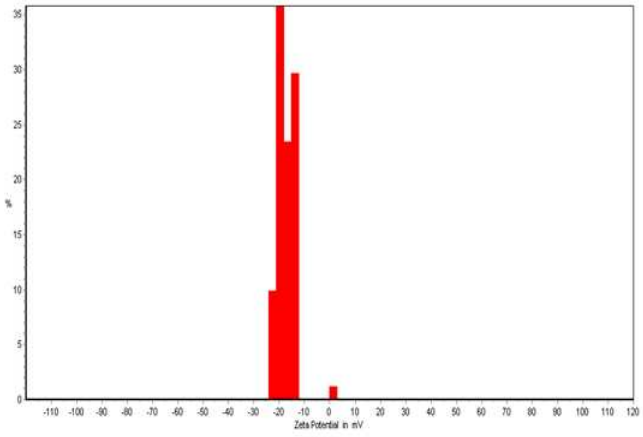

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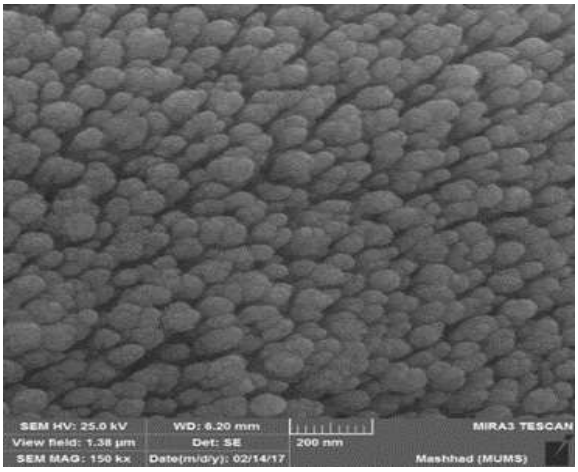

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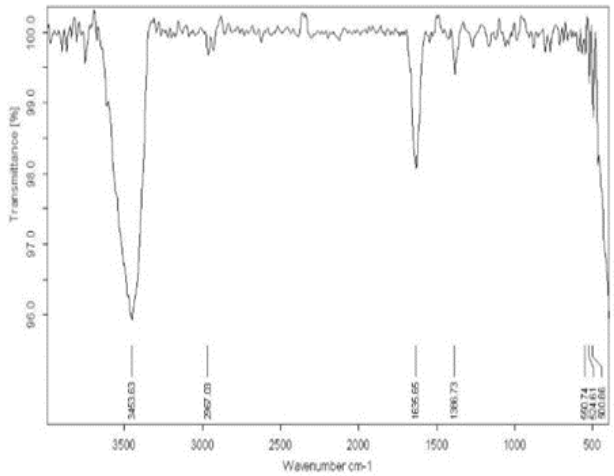

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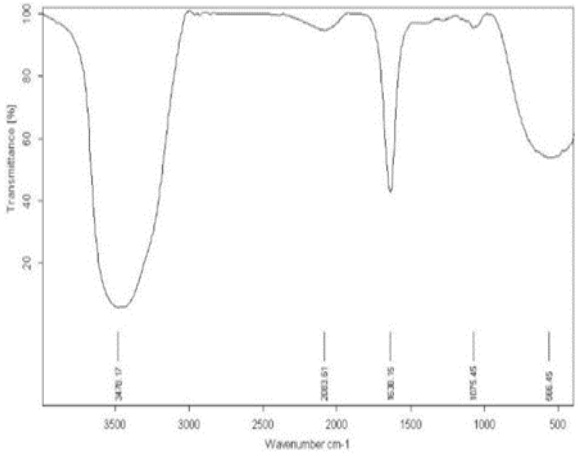

(F)

Figure 1. UV-visible spectra of G/ZnO-NPs (A). DLS graph for determining the size distribution of G/ZnO-NPs (B). Zeta potential of the G/ZnO-NPs (C). FE-SEM micrograph of G/ZnO-NPs at 24 hours incubation time (D). FTIR spectra of Galbanum aqueous extract (a) and G/ZnO-NPs (b) (E).

Developmental parameter of pre-antral follicles treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs

PF consists of oocytes and a layer of squamous follicular cells. These follicles remain in the process of the meiotic division. At the start of the development of follicles, zona pellucida appears, and oocytes and follicular cells are separated. In the late stage of primary follicles development, follicular cells proliferate and form zona granulosa. When the secondary follicles form, follicular antrum appears within granulosa cells, the layer of granulosa cells is increased, and large oocytes are observed. The secondary oocyte, having undergone the first meiotic division, is located centrically [27]. per-antral follicles should reach the above evolution process for fertility preparation. Using IVM, we can study the effects of various materials on the progression of follicle development. Substances that have a positive effect on the progression of follicular development can enhance oocyte maturation and produce a shorter period for maturation, while harmful substances can cause atresia of the follicles or decrease and delay the appearance of the developmental characteristics in the treated follicles. Hence in this study, we first examined the effects of Galbanum extract, G/ZnO-NPs, and C/ZnO on the developmental parameter of PF. According to the results, in Controlled samples, the PF on day zero is surrounded by a relative or complete layer of squamous granulosa cells. The morphology of the follicles during the culture period indicated that the follicles were attached to the bottom of the cultured tissue and were immobilized. Also, on the fourth day, granulosa cells spread around the follicles and gave irregular follicles. During the culture period from the sixth to eights day, cavities around the ovum of follicles appeared that were similar to those of antrum follicles. Due to the growth of granulosa cells, from the seventh day, follicles grew and their details were no longer visible. Pretreatment follicles treated with Galbanum extract, have the characteristics similar to those of the control group on day zero. The follicles were treated with aqueous extract with concentrations of 10, 50, 100, 200, and 400 µg/ml. Results on day two indicated that in the concentrations above 200 µg/ml, follicles were not attached to the bottom of the tissue culture at all and were degenerated. The highest changes were observed in the developmental parameter of the follicles that were treated with 50 µg/ml on day zero to 7, in which granulosa cells on the second day had more expansion compared with the control group. On the fourth day, these follicles had one or two small areas of follicular fluid accumulation and a large antrum. The impact of chromatin was visible above the follicles. On day 6, due to the growth of granulosa cells, follicles development details were not visible. Pretreatment follicles treated with G/ZnO-NPs and C/ZnO-NPs, have an oocyte on day 0 and are surrounded by a partial or complete layer of squamous granulosa cells. PFs were treated with 25, 50, 100, 200, and 400 µg/ml of commercial and green synthesized zinc oxide nanoparticles. The groups were treated with up to 25 µg/ml of commercial and green synthesized zinc oxide nanoparticles. The follicles were not even attached and underwent degeneration. Subsequently, 5, 7.5, 10, 12.5, 15, and 17.5 doses of C/ZnO-NPs were examined to assess the developmental effects of commercial and green synthesized zinc oxide nanoparticles. At all concentrations, on the second day, granulosic cells grew slightly. The granulosa cell layers at concentrations of 5 and 7.5 of C/ZnO-NPs and G/ZnO-NPs were almost similar to those of the control group, and no changes were observed. At higher concentrations in a large number of follicles, oocytes are removed from the inside of the follicle and the follicle is degraded. On the seventh and eighth days, antrum was observed in control group follicles, while in the low concentrations of nano zinc oxide, synthesized zinc (7.5, 5), the antrum was not formed. These results indicated that while the extracts had positive effects on the developmental parameters of PF, C/ZnO-NPs inhibited the development of PF, and plant extract moderated the somewhat hazardous effects of ZnO-NPs. It should be noted that Galbanum extract has estrogenic properties and it has been shown that antioxidant effects have a positive effect on the development of PF. The positive effects of the Galbanum extract on follicle development may be related to its antioxidant effects. Golkar-Narenji et al. (2010) investigated the effect of Papaver rhoeas L. extract on in vitro maturation and in vitro fertilization of pre-antral follicles isolated from mouse oocytes. Obtained results showed that *Papaver rhoeas* L. extract significantly increased in vitro maturation rate and in vitro development. They showed that the positive effect of *Papaver rhoeas* L. extract was related to the doses [28]. Studies by researchers have shown that nanoparticles have harmful effects on follicular development and can aggravate the process. Also, it was shown that ZnO-NPs may damage DNA replication and repairmen machinery in hen oocytes, which subsequently inhibit follicular and embryonic development. Moreover, it was found that the progression of follicular development was significantly decreased in the mice ovary in the zinc oxide nanoparticles groups. According to this study, it seems that the Galbanum extract that covers the surface of the nanoparticles can confirm their negative effects on the developmental parameter.

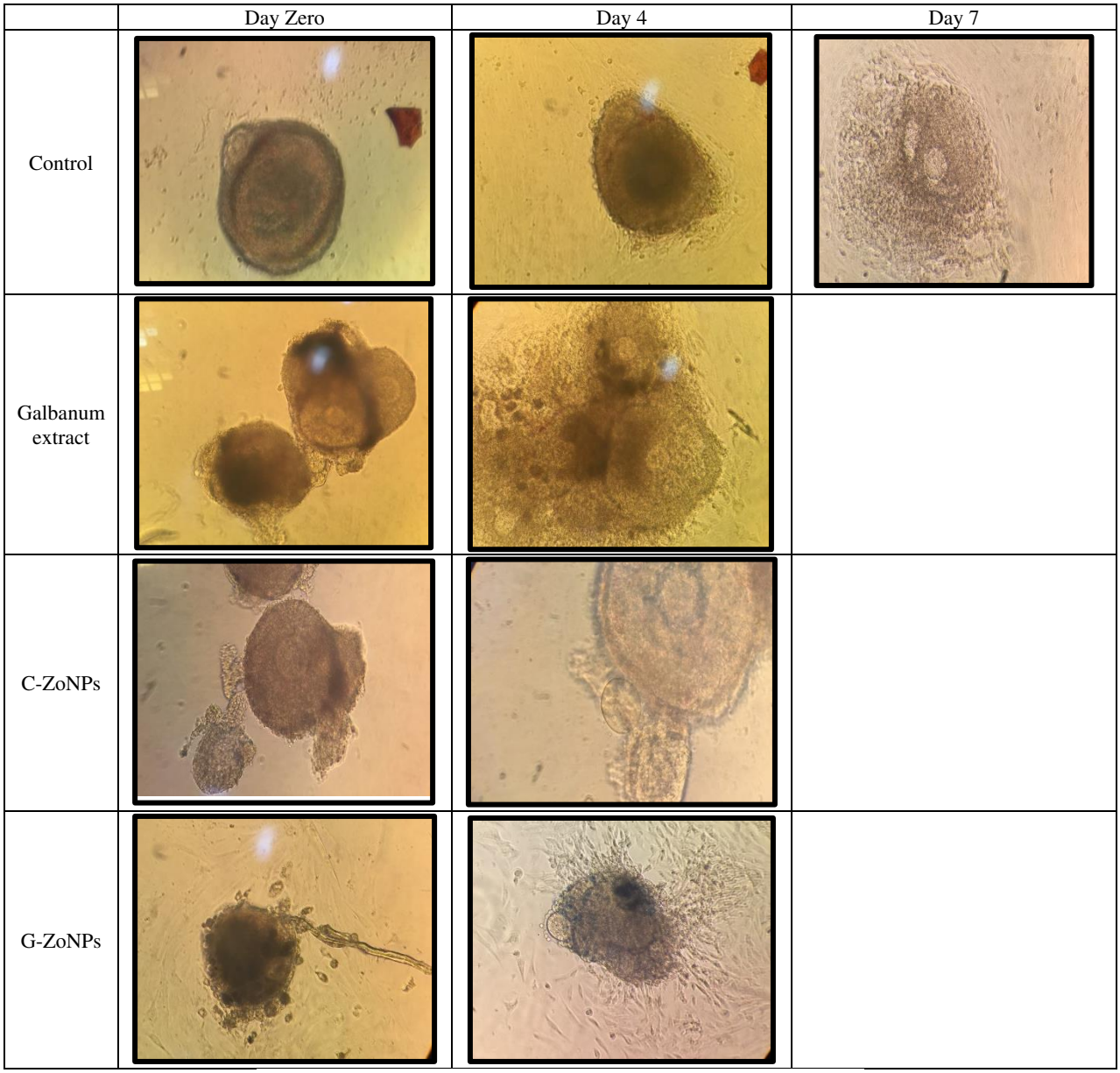

Figure 2. Follicular development in various treated groups.

The diameter of preantral follicles treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs

Primordial follicles are just 25 micrometers. The growth phase of primordial follicles is specifically characterized by increasing the diameter size of the oocyte from 30 µm to 120 µm, along with the proliferation of granulosa cells and the distinction of single cells. For some perspective, an antrum follicle that is now 5 mm is *200 times bigger* than a primordial follicle. Hence, increasing the size of follicles is a major symptom of follicular development. In this study, to investigate the effects of Galbanum extract (figure 3A), G/ZnO-NPs (figure3B), and C/ZnO-NPs (figure3C) on IVM, follicles diameters were measured on the second and fourth days. According to the obtained results, on the second day, the mean diameter of the PF increased in groups treated with the Galbanum extract compared to control groups. Also, the greatest changes were observed in the experimental group 2, while the pattern of follicle diameter increase was nearly lost in concentrations up to 50 µg/ml. Even in the experimental group 4, the decrease of follicle diameter was observed in samples. However, these changes were not still significant in comparison to the control samples. On the fourth day, the mean diameter of the pre-antral follicles treated with Galbanum extract in experimental group 2 increased significantly at the level of $P \leq 0.05$ while the mean increase of the PF diameter in the experimental group 2 was not statistically significant compared to that of the control group. Also, it was observed that the mean diameter of PF in the experimental groups 3 and 4 decreased compared with the control group. It has been shown in numerous studies that plant extracts improve the follicular growth and development due to their antioxidant properties. For example, Barberino et

al. (2016) reported that *Amburana cearensis* leaf extract promotes the progressive and significant increase of follicular diameter throughout the culture period. They consulted that this extract can be an alternative culture medium for "pre-antral follicle development [29]. In this study, we also showed that treating follicles with 10 and 50 µg/ml of Galbanum extract increases their growth and diameter significantly compared to control groups. However, higher concentration leads to a decrease in follicles growth. In other words, at concentrations higher than 50 µg/ml, the growth of follicles is reduced because the compound of Galbanum extract at high concentrations has an inhibitory effect on the growth of the treated follicles. In other studies that used plant extracts to increase the growth of follicles, it was also shown that these extracts at certain concentrations can have positive effects on follicles growth and can be contrasted at higher concentrations. In this context, Abdollahi et al. (2015) studied the effect of Phoenix dactylifera pollen grain on the maturation of PF. Their results indicated that this extract can improve the follicular maturation in certain doses [30]. These results are confirmed by our study too. In the next section, we examine the effect of G/ZnO-NPs and C/ZnO-NPs on follicular maturation. On the second day, the PF diameters treated with G/ZnO-NPs decreased significantly and dose-dependently compared to control groups. After the treatment on the fourth day, the mean diameter of the follicles was decreased by an increased concentration at the level of P≤0.05. In fact, in these treatment groups, some follicles were degenerated, which reduced the average diameter of the treated follicles. The mean diameter of the follicles treated with C/ZnO-NPs on the second day of culture did not change significantly. However, as time passed, significant changes in the mean diameter of the follicles were observed according to the concentration, which resulted in a decrease in the mean diameter of follicles treated with C/ZnO-NPs and indicated the inhibitory effects of commercial nanoparticles on the growth of these follicles. These results showed that the Galbanum extract increased the diameter of the pre-antral follicles, while in the groups treated with G/ZnO-NPs and C/ZnO-NPs the mean diameter of the follicles decreased, which was more pronounced in the follicles treated with C/ZnO-NPs. However, the toxicity of ZnO-NPs has been studied in various organs and tissues including the ovarian in animal models. The results of these studies indicated that ZnO-NPs may adversely impact the female reproductive system and oocytes maturation. Meanwhile, the toxicity of ZnO has been investigated in various organs and tissues, including the testes in animal models. Some of these studies demonstrated that ZnO may adversely impact the female reproductive system and fertility [14]. In this regard, researchers recently found that exposure of chick oocytes to nZnO inhibits their developmental capabilities following fertilization [15]. These findings are consistent with our results in this current study. It seems that the Galbanum extract that coated G/ZnO-NPs can partially inhibit the harmful effects of ZnO-NPs as well as the effects of growth inhibition on the green nanoparticles. In a constant concentration, the average diameter of the follicles treated with G/ZnO-NPs is more than that of the C/ZnO-NPs.

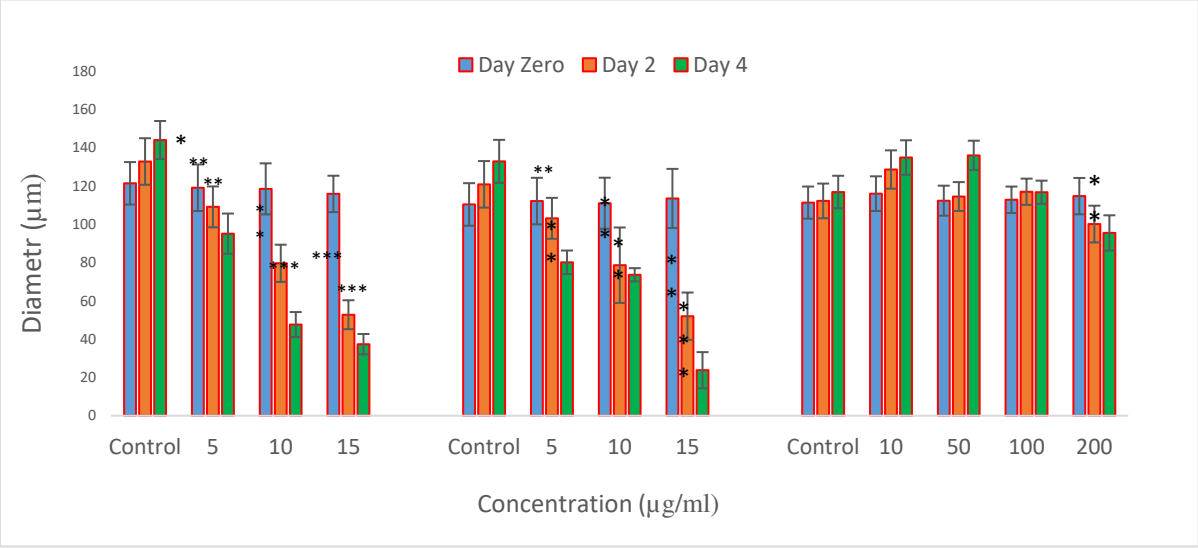

Figure 3. Dimeter of follicle in various treated groups. ***P≤ 0.001 ‘‘**P≤ 0.01 ‘* P≤ 0.05, Mean+ SE

Measurement of ROS level in follicles treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs

Many factors affect the maturation of PF in the in vitro. One of these important factors is oxidative stress. The generation of pro-oxidants such as reactive oxygen species (ROS) is an invariable phenomenon in the cultural development in vitro. Oocyte protection against ROS may play important roles in pre-implantation embryonic development [31]. The effects of antioxidant supplementation on IVM media have been studied in various mammalian species, in the current study. ROS production was assessed in the samples which were treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs (figure 4). Results indicated the ROS level in PF follicles treated with Galbanum extract. Comparison of the presence

percentage of free radicals in treatment groups on days 1 and 5, after treatment with Galbanum extract, showed that free oxygen radicals in experimental groups 1, 2, and 3 were significantly reduced at a significant level of (P≤0.05), which indicates the antioxidant effects of these extracts. In experimental group 4, ROS content has increased in treatment follicles, and the increase in the concentration of extract is likely to cause toxicity and increase free radicals. Comparing the percentage of ROS inhibition in the treatment groups on days 1 and 5 after PF treatment with C/ZnO-NPs and G/ZnO-NPs showed that on these days, free radicals of oxygen in the treatment groups were dependent on the concentration which was increased at a significant level of P≤0.05., Free radicals were increased on day 5 after the treatment compared to day one, which shows the oxidant effects of these nanoparticles. Wang et al. reported that treatment of the PF of bovine with green tea polyphenol as antioxidant increased follicular development and blastocyst formation [31]. It was also shown that the optimum concentration of Papaver rhoseas L. extract in maturation medium caused improvement in the rate of oocyte maturation and subsequent embryo development [31]. Similarly, in the current study, Galbanum extract affected IVM positively and dose-dependently. ROS production and oxidative damage are also considered the main mechanisms responsible for genotoxicity induced by metal oxide nanoparticles. In this study, it was shown that in groups treated with G/ZnO-NPs and C/ZnO-NPs, the concentration of ROS was increasing in the supernatant medium of the treated follicles. However, it seemed that G/ZnO-NPs, created fewer radicals in the medium, due to the presence of the antioxidant coating on the surface of these nanoparticles.

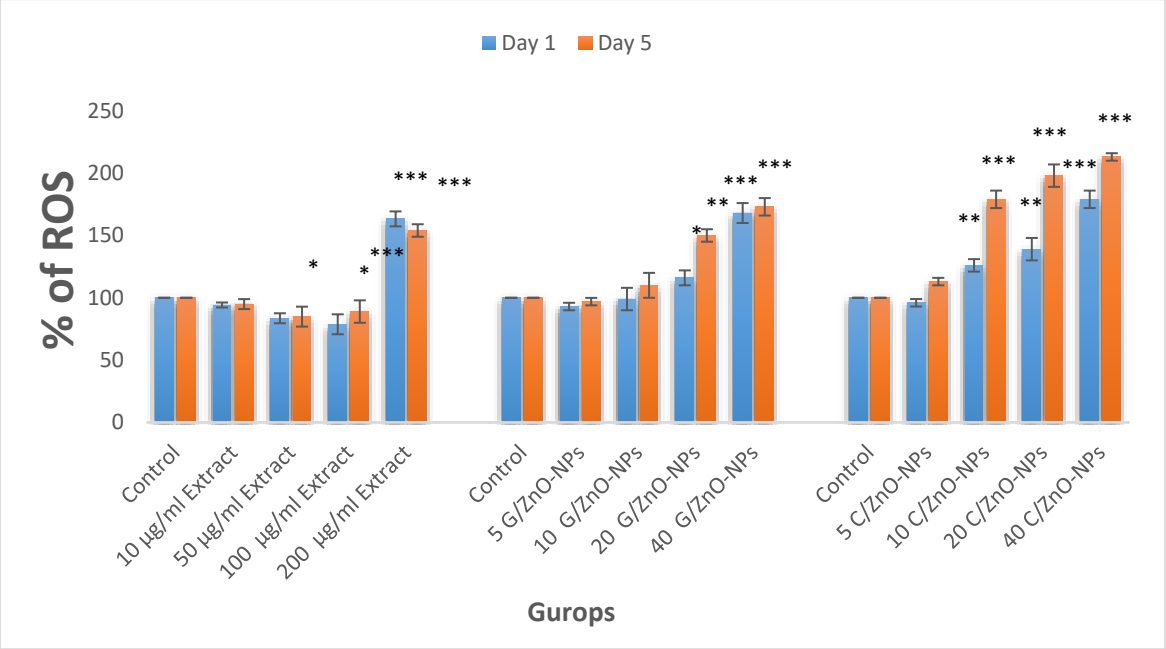

Figure 4. ROS content in various treated groups. ***P≤ 0.001 ‘‘**P≤ 0.01 ‘* P≤ 0.05, Mean+ SE

The effect of Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs on estradiol and testosterone secretion of pre-antral follicles

Estradiol changes: FSH stimulates estradiol production, and researchers reported that it has a marked influence on meiotic spindle organization when the follicles form an antral cavity and produce estradiol, which is normally synthesized at the antral follicle stage in vivo [32]. According to the results of previous studies, estradiol can positively affect the development of PF [32]. In this study, to analyze the evolution effect of Galbanum extract (50µg/ml), G/ZnO-NPs (15µg/ml), and C/ZnO-NPs (15µg/ml) on preantral follicle development, the production of estradiol was assessed in a follicular supernatant medium (figure 5). The percentage of estradiol secretion in PF follicles treated with Galbanum extract on the first day increased compared to control samples, while the percentage of secretion of this hormone decreased in follicles treated with G/ZnO-NPs. The amount of estradiol secretion on the first day in the C/ZnO-NPs was lower than that of the green zinc oxide nano-particles. However, all of these changes were meaningless in comparison to the control samples. After the treatment on the fifth day, the percentage of secretion of estradiol in PF treated with Galbanum extract increased compared to control samples. On the other hand, the secretion percentage of this hormone in groups treated with G/ZnO-NPs or C/ZnO-NPs decreased at P≤0.05. The results of this section showed that Galbanum extract with appropriate concentrations had positive effects on estradiol hormone secretion in pre-antral follicles. Researchers examined the effects of dibutyryl cyclic AMP or forskolin on pre-antral follicle maturation. According to their results, these compounds have positive effects on pre-antral follicle maturation and interestingly stimulate follicle growth and estradiol secretion by follicles from immature mice [32]. These results are similar to the findings of this investigation. Hulshof et al. (1995)

investigated the effects of estradiol on small PFs (mostly primary) mechanically isolated from adult bovine ovaries and cultured for 7 days with estradiol and/or FSH. The increase in follicular diameter during the culture was greater in the presence of either of the hormones. FSH appeared to stimulate granulosa cell proliferation, whereas estradiol increased cell size [33, 34]. In the previous section of this study, it was shown that follicle size in the samples treated with Galbanum extracts was increased, which could be due to an increase in the production of estradiol in these samples. Also, in samples treated with G/ZnO-NPs and C/ZnO-NPs, the reduction in the size of follicles was observed, and the production of estradiol was also reduced, which was consistent with each other as expected.

Changes in testosterone: In vivo and in vitro studies indicated that testosterone promotes primary follicles to secondary transition. This hormone also enhances theca and granulose proliferation and decreases the apoptotic index in antral follicles [33, 35]. In this study, testosterone production in pre-antral follicles treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs were compared. The results of testosterone secretion compared to the control sample showed that in groups treated with Galbanum extract, the testosterone concentration increased compared to the control sample, but this increase was statistically insignificant. In samples treated with G/ZnO-NPs or C/ZnO-NPs, the percentage of testosterone levels decreased significantly compared to the control sample, and this decrease was higher in samples treated with C/ZnO-NPs. On the fifth day, testosterone changes in groups treated with G/ZnO-NPs and Galbanum extract gum showed a significant increase compared to the control group, while the percentage of testosterone secretion in samples treated with C/ZnO-NPs was lower than that of the control sample. However, this reduction was meaningless in terms of the story. The results of this section showed that there is a positive effect on the process of testosterone production, while C/ZnO-NPs harm the process of thawing and secretion of this hormone.

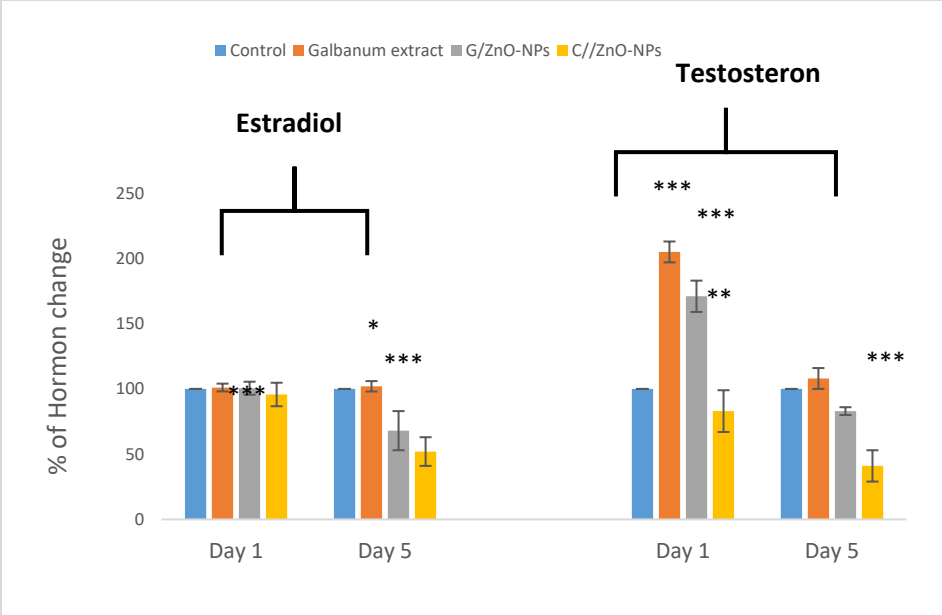

Figure 5. Estradiol and testosterone produced in various treated groups. ***P≤ 0.001 ‘‘**P≤ 0.01 ‘* P≤ 0.05, Mean+ SE

Evaluated expression of GDF-9, BMP-15, and Foxo1 and VNN1 genes by Real Time-PCR in pre-antral follicles treated with Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs

Growth differentiation factor 9 is a member of the TGF-β family, produced by all mammalian oocytes throughout the ovulation. There was evidence that GDF-9 promoted primary follicle progression. Another member of the TGFβ family, Bmp-15, also known as GDF-9B, exhibits an expression pattern similar to that of GDF-9. Bmp-15 production is critically important for early follicular development [36, 37]. In this study, the evaluation effects of Galbanum extract (50µg/ml), G/ZnO-NPs, and C/ZnO-NPs (15 µg/ml) on PF development and expression of BMP15 and GDF-9 were examined (figure 6). The vanin-1 expression had been linked to follicular atresia. VNN1 regulated the response to oxidative stress and decreased resistance to oxidative stress. Its overexpression was consistent with the increase in apoptosis observed during follicular atresia [38]. FOXO3, a forkhead transcription factor, is a downstream effector of PI3K/Akt. In rodents, FOXO3 exerts its transcriptional activity in the nucleus of quiescent primordial follicles and suppresses follicular growth [39]. This factor also enhances the pro-apoptotic factors TP53 and BCL2-associated. Therefore, it can be stated that these two factors decrease expression during normal growth of PF, which is investigated in the present study in Galbanum extract, G/ZnO-NPs, and C/ZnO-NPs treated samples. The results of GDF-9 expression

showed that the expression of this gene was significantly increased in the samples treated with Galbanum extract compared to control samples. On the other hand, the expression of this gene in the PFs treated with G/ZnO-NPs and C/ZnO-NPs was significantly decreased. Similarly, the expression of BMP-15 in the samples treated with Galbanum extract was significantly increased, but this increase was not meaningful in comparison with that of the control samples. In addition, the expression of this gene in the follicles treated with G/ZnO-NPs and C/ZnO-NPs was significantly decreased. Regarding the position that both GDF-9 and BMP-15 genes have positive effects on growth and development, it can be concluded that the Galbanum extracthas a beneficial effect on the growth and development of PF, while G/ZnO-NPs and C/ZnO-NPs have negative effects on the growth and development of PF. In addition, the presence of Galbanum extract on the surface of G/ZnO-NPs inhibited some of the negative effects of zinc oxide. Furthermore, the expression of FOXO1 and VNN 1 in samples treated with Galbanum extract decreased which was meaningless compared to control expression. On the other hand, the expression of this gene in PF follicles treated with G/ZnO-NPs and C/ZnO-NPs increased significantly. In fact, the presence of oxidants such as free radicals during treatment with G/ZnO-NPs and C/ZnO-NPs led to possible injuries in PF follicles and an increase in the expression of antioxidant enzymes, in which the oxidant effects of G/ZnO-NPs caused by their surface coating Galbanum extract were moderated.

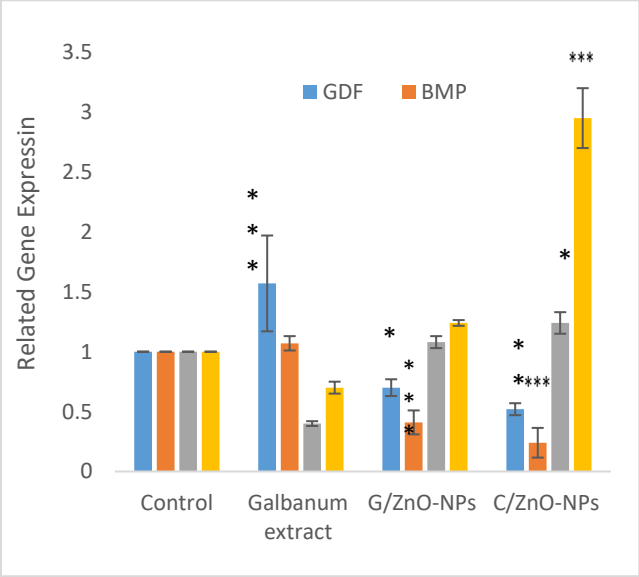

Figure 6. Expression of GDF-9, BMP-15, and Foxo1 and VNN1 genes in various treated groups. ***P≤ 0.001 ‘‘**P≤ 0.01 ‘* P≤ 0.05, Mean+ SE

Conclusions

We described a simple green procedure to synthesize zinc oxide nanoparticles by utilizing Galbanum aqueous extract which also works as a coating agent. These nanoparticles were well-dispersed without adding different physical and chemical capping agents. The diameter of the Zinc nanoparticles is in the average size of 36 nm as shown by DLS. The flavonoid, terpenoid, and proteins constituents which are present in Galbanum aqueous extract may act as stabilizing and reluctant agent. Pre-antral follicles proved that Galbanum extract promotes the development and increases the size of PF at appropriate concentrations, which can be due to antioxidant effects and reduced ROS concentration. Pre-antral follicles that were treated with Galbanum extract produced more estradiol and testosterone. Also, the results of the gene expression analysis showed that during treatment with Galbanum extract, the expression of GDF-9 and BMP-15 increases. These genes have direct effects on the growth of follicles, and the expression of Foxo1 and VNN1 genes has also reduced. These genes are correlated with follicles atresia. All this evidence suggests that Galbanum extract caused the development of PF toward the antral follicle. In the case of C/ZnO-NPs and G/ZnO-NPs, the morphology of PF, as well as the reduction of the average size of follicles, indicated the negative effects of these nanoparticles on the development of follicles. Also, the concentration of ROS in treated samples increased, which caused damage to follicular development. Furthermore, the production of hormones involved in the follicle growth process, estradiol, and testosterone was reduced in nano-particle treatment samples, which could be due to the inhibitory effects of these nanoparticles on the growth of follicles and granulosa cells. Moreover, the expression of progression genes of follicle GDF-9 and BMP-15 was decreased while the expression of Foxo1 and VNN1 was increased. The sum of this evidence showed that C/ZnO-NPs and G/ZnO-NPs inhibited the growth and maturation of PF. However, all these negative effects were moderated in samples treated with G/ZnO-NPs and the severity of the injury was low. It can be argued that the surface coating of green nanoparticles, which is a plant extract with antioxidant properties, can moderately reduce the damage caused by nanoparticles.

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