

Opinion paper

Extract-mediated biosynthesis and characterization of gold nanoparticles: Exploring their protective effect against cyclophosphamide-induced oxidative stress in rat testis

Fahimeh Mobaraki^a, Mohsen Momeni^b, Maryam Barghbani^c, Bahareh Farasati Far^d, Sara Hosseini^{e,f}, Seyed Mousalreza Hosseini^{g,h,*}

^a Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^b Immuno-Biochemistry Lab, Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

^c Department of Medical Biotechnology, School of Allied Medicine, Qazvin University of Medical Sciences, Qazvin, Iran

^d Department of Chemistry, Iran University of Science and Technology, Tehran, Iran

^e Department of Medical Physiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^f Applied Biomedical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

^g Department of Gastroenterology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^h Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

The biosynthesis method is essential for minimizing toxic by-products caused by conventional nanoparticle synthesis. Adding a surface biological coating to gold nanoparticles (AuNPs) makes them durable, adaptable, and cost-effective. Herein, *Nasturtium officinale* leaf extract was used as a reducing and stabilizing agent. UV–vis spectrophotometer, X-ray diffraction, Fourier-transform infrared spectroscopy, transmission electron microscopy, dynamic light scattering, and Zeta potential methods were employed to assess the nanoparticles' formation, crystallinity, size, shape, and surface charge properties. Spherical AuNPs were produced with an average size of 64 nm and demonstrated dose-dependent DPPH scavenging property. To achieve the non-toxic dose of the AuNPs, the MTT assay and AO/PI were performed on the rat bone marrow cells. The co-administration of AuNPs and cyclophosphamide intraperitoneally prevented testicular damages in male rats. In cyclophosphamide-induced gonadotoxicity, the AuNPs' cytoprotective effect was depicted by its reversal of hormonal and morphological alterations toward normality. A reverse transcription-polymerase chain reaction (RT-PCR) analysis was accomplished on apoptotic genes' expression (caspase-3, caspase-9, Bax, Bcl2) to confirm that the biosynthesized nanoparticles could act as a fascinating antioxidant and exert protective effects against apoptosis. These positive results can provide a rational and potential source for a novel conjugation system of chemotherapy drugs with AuNPs in cancer treatment with fewer side effects.

1. Introduction

Sperm production in the testes is a highly complex and well-organized process of stem cell proliferation and differentiation into numerous germ cells through successive mitosis and meiosis divisions [1]. Testicular tissue is, therefore, sensitive to environmental risk factors. Several factors could affect spermatogenesis and lead to infertility or reduced fertility in males [2], including oxidative stress (OS) that can arise due to an imbalance between oxidant and antioxidant systems if there is the accumulation of reactive oxygen species (ROS) [3].

Chemotherapy drugs have recently found more application in cancer treatment. They can also serve in the treatment of various autoimmune disorders, including rheumatoid arthritis and lupus, as well as the prevention of transplant rejection [4]. Cyclophosphamide (CP) is an anti-neoplastic drug that is converted into an alkylated active metabolite in the body and can be easily spread widely across fluids and tissues. It can also be passed through the blood-brain barrier, converted into active metabolites, and finally, excreted by the kidneys. Anemia, neutropenia, thrombocytopenia, formation of secondary tumors, and reproductive system disorders are among the side effects of cyclophosphamide [5,6]. Beside its therapeutic effects on cancer cells, it may have toxic side

* Corresponding author. Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.

E-mail address: HosseiniMR@mums.ac.ir (S.M. Hosseini).

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Abbreviations

AuNPs-NO	Biosynthesis of gold nanoparticles using <i>Nasturtium officinale</i>
CP	Cyclophosphamide
OS	Oxidative stress
ROS	Reactive oxygen species
UV-vis	Ultraviolet-visible spectroscopy
XRD	X-ray powder diffraction
FT-IR	Fourier transform infrared spectroscopy
TEM	Transmission electron microscopy
DPPH	2, 2-diphenyl-1-picrylhydrazyl
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-

	tetrazolium bromide
IC50	Half maximal inhibitory concentration
AO	Acridine orange
PI	Propidium iodide
MDA	Malondialdehyde
GST	Glutathione-s transferase
LPO	Lipid peroxidation
CAT	Catalase
SOD	Superoxide dismutase
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
RT-PCR	Reverse transcription-polymerase chain reaction

effects by inducing oxidative stress in normal body cells, particularly those with a faster division rate, e.g., bone marrow, intestine, and testicle cells [7]. The toxic effects of cyclophosphamide on the testicular tissue have been confirmed in multiple studies on humans and laboratory animals [8–10]; these effects are undoubtedly the most significant aspect affecting this drug's therapeutic applications.

Nanotechnology has major applications in various industries, including pharmaceuticals, healthcare, and medicine [11–13]. Metallic nanoparticles are particularly important owing to their biomedical applications such as bioimaging, biosensor, photothermal therapy, and targeted drug delivery for cancer treatment ranging from chemical compositions to biomolecules. These applications are appropriate in various fields based on the physicochemical properties of nanoparticles, predominantly their structure, size, and shape [14,15]. Among the diverse types of metallic nanoparticles, AuNPs have attracted extreme attention and have also been efficiently exploited due to their unique intrinsic properties. Possibility of combinatory manipulation, ability to precisely control the properties, strong binding affinity to disulfides, amines, and thiols as well as a versatile scaffold for the delivery of different payloads (protein, gene, and drug) trigger drug release to distant places, potent antioxidant activities and significant X-ray absorption coefficient [16–19]. Nanoparticles can be produced through different physical and chemical methods. The chemical method is the common method for this purpose, which usually involves reducing ionic salts in a suitable medium through reducing agents. However, it is a costly process that leaves toxic reagents on the nanoparticles, resulting in widespread environmental damage [11,20,21]. The use of microorganisms and plants in nanoparticle biosynthesis could be potentially useful in producing biocompatible nanoparticles to resolve the critical deficiency [22–24].

Besides being the natural form of the required regenerative materials and stabilizers as well as being non-chemical, green nanoparticle synthesis is affordable and biocompatible, and a suitable alternative for physical and chemical methods [5,6]. Studies have shown that polyphenols, carbohydrates, proteins, particularly plant terpenoids, have all been involved in reducing metallic salt for the synthesis of nanoparticles [25–27]. Plants contain a complex network of antioxidant metabolites and enzymes to inhibit oxidative cellular damage [28]. As flavonoids and other plant biomolecules are non-toxic and biocompatible coating agents, they can be used as natural and eco-friendly agents in nanoparticle synthesis [29,30]. The antioxidant activity of these coating agents helps prevent cancer in normal cells and has specific toxicity for cancer cells [31]. Nanoparticles' effects on cells are influenced by various factors, including dose, treatment time, size, shape, surface chemistry, and cell type [31,32]. So far, various herbs such as black tea (*Camilla Sinensis*) [33], Eucalyptus (*Chapmaniana*) [34], Asteraceae (*Achillea biebersteinii* Afan) [35], and Pomegranates (*Punica granatum*) [36] extracts have successfully been used to synthesize gold nanoparticles (AuNPs).

Herein, the extract of watercress (*Nasturtium officinale*) belonging to the Brassicaceae species was employed to biosynthesize AuNPs [37]. Based on hydrogen capacity and ionic metal precipitation, plants, particularly those high in polyphenols and flavonoids with antioxidant capabilities, have been regarded as a defense agent against chemotherapy drugs [38]. By eliminating free radicals and increasing the host's antioxidant defense system, phenolic compounds with their protective actions defend against the harmful effects of genotoxic carcinogens [39,40]. Although chemotherapy drugs, including cyclophosphamide, are unavoidable in cancer treatment, it is reasonable to use herbal materials and supplementary drugs to mitigate their side effects [41,42]. The current research aimed to determine the efficacy of AuNPs synthesized using *N. officinale* in the treatment of CP-induced testicular damage.

2. Materials and methods

2.1. Materials

Cyclophosphamide (Endoxan®) was purchased from Baxter Company (Germany). Gold salt ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99.9%) was produced by Merck Company (Germany). Fresh Watercress plant (*Nasturtium officinale*) was collected from wetlands in Khorasan Razavi Province, Iran, in spring and was identified in the botanical herbarium of Ferdowsi University of Mashhad with herbarium code 1008 FUMH-E.

2.2. Aqueous extract preparation

After washing the watercress plant leaves with deionized water, they were completely dried under shade and powdered. To prepare the aqueous extract, 10 g of the plant powder was shuffled with 100 ml of deionized water and sealed with a foil lid before boiling on the hot plate for 15 min and cooling for 5 min at room temperature. The colloidal solutions were filtered using Whatman paper and preserved at 4 °C for future experiments.

2.3. AuNP synthesis using the plant extract

To prepare AuNPs, first, all the containers were disinfected, to identify the optimal conditions, gold salt, and plant extract were mixed at different ratios and examined at different times and temperatures. For this purpose, 500, 750, and 1000 µl of the plant extract were poured into 15 ml tubes and the volume was increased to 5 ml with deionized water. Next, 20 µl of the tetrachloroauric acid solution (1 mM) was added to each tube. To study the effects of temperature, the mixtures were set on the magnetic heater-stirrer at 20, 40, and 60 °C and controlled in various time intervals (30 min, 1, 2, and 4 h).

2.4. AuNP characterization

Color changes in the reaction solution were objectively monitored. To control the progress rate of AuNPs synthesis, the UV-Vis spectra of the samples were measured and analyzed using the spectrophotometric method (Shimadzu UV-2500 - Japan) in the range of 400–700 nm at different concentrations, temperatures, and times. The nanoparticles were centrifuged at 14,000 rpm for 10 min and washed with deionized water in triplicate. Finally, they were lyophilized and used as a powder for X-ray diffraction (XRD) (X'pert Pro - the Netherlands). Transmission electron microscopy (TEM) (Leo 912AB Omega - Germany) was used to identify the nanoparticles' shape and size. Size distribution and image analyses were done using ImageJ. The DLS method (Otsuka Electronics Co., Osaka, Japan) was adopted to obtain the nanoparticles' size distribution. The stability of AuNPs was analyzed by Zeta potential measurements at 25 °C. To identify the functional group in AuNP coating agents, Fourier-transform infrared spectrometer (FT-IR) (NICOLET IR100-USA) was used and spectroscopy was performed in the range of 400–4000 cm^{-1} .

2.5. Antioxidant activity

The DPPH radical scavenging assay was applied to assess the inhibitory action of AuNPs-NO free radicals. To this end, 1 mg of DPPH powder was dissolved in 16.9 ml of ethanol and the DPPH reagent solution was made. After dissolving the nanoparticles in distilled water, different concentrations of nanoparticles were prepared. Finally, the reaction solution was made by mixing 500 μl of DPPH with 500 μl of different nanoparticle concentrations. The absorbance was obtained at 517 nm by using UV-spectrophotometer [43]. The negative control was the DPPH solution, ascorbic acid (routine) was used as the positive control (reference antioxidant) and plant extract solution was employed as the blank [35]. The antioxidant activity was determined by the IC50 factor (the material in which 50% of the DPPH free radicals in the reaction environment were inhibited).

2.6. In vitro AuNPs-NO cytotoxicity assessment

In brief, the cells were covered at the aggregation of 1×10^5 cells/ml (1 NEST®, New Jersey, USA) into 96-well plates and were then exposed to diverse concentrations of AuNPs-NO (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 $\mu\text{g/ml}$) for 24 h. Subsequently, each well was filled with a 20 μl MTT solution and incubated at 37 °C for 2 h. After the incubations, 100 μl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was added to formazan crystals in the dark for 4 h. The violet formazan crystals made in the viable cells were dissolved and a homogeneous achromatic liquid was created. The absorbance was measured at 570 nm via a plate reader and microplate spectrophotometer. The IC50% was reported (the concentration that inhibits cell growth up to 50%).

The MTT assay was performed to achieve the toxic and non-toxic dosages of the AuNPs-NO.

The well before the specified IC50 (which had a higher concentration of the AuNPs) was identified as toxic and the well after the specified IC50 (which had a lower concentration of the AuNPs) was identified as the non-toxic dose of the AuNPs-NO. A non-toxic dose was employed in animal studies [44].

2.7. Detecting cell viability by acridine orange and propidium iodide (AO/PI)

The AO/PI assay was used to evaluate the viability of AuNPs-NO-treated RBM-1 cells. Concisely, the test was carried out in a 6-well microplate at the agglomeration of 1×10^6 cells/ml. The RBM-1 cells were treated with different doses of AuNPs-NO (0, 12.5, 26, and 50 g/ml) for 24 h at 37 °C. They were washed twice with PBS after the supernatant was removed. Fluorescent dyes containing AO (10 g/ml) and

PI (10 g/ml) were applied to the cellular pellet. Also, 100 ml supernatant was extracted after 3–5 min and transferred to the Neubauer rhodium hemocytometer slide. The percentage of apoptotic cells was specified using fluorescence microscopy (Olympus CKX41, Tokyo, Japan) [45].

2.8. In vivo treatment schedule

This research was performed on 30 adult male Wistar rats weighing 250–200 g provided from Animal House of Mashhad Medical School (Mashhad, Iran). Animal management and all the correlated procedures were accomplished following the Ethical Committee Acts of Mashhad University of Medical Sciences (the approval ID: IR.MUMS.MEDICAL.REC.1400.603). The laboratory rats were kept at the animal houses under standard conditions at room temperature (21 ± 3 °C) for 12 h light cycle, with 50%–70% humidity. The animals had access to enough food and drinking water. After one week of adaptation, they were accidentally divided into five groups ($n = 6$ rats/each) as follows: 1- Control group: There was no intervention. 2- Sham control group: Only normal saline was received intraperitoneal daily. 3- Experimental group one: 5 mg/kg of CP drug (corresponding to the therapeutic dose) for 16 days were intraperitoneally injected. 4- Experimental group two: 5 mg/kg of CP along with co-administration of non-toxic AuNPs-NO intraperitoneal received for 16 days. 5- Experimental group three: the non-toxic dose of AuNPs-NO was intraperitoneally received for 16 days.

2.8.1. Bodyweight

The animal body weight was accurately measured using a laboratory scale (Marte Scale, EK-3000i -USA) throughout the experiment days.

2.8.2. Weight index and volume of the testis

After resection and washing with physiological saline, the excess parts were removed to calculate the absolute testicular weight.

Testis volume was calculated using the next formula, Eq. (A):

$$V_t = \frac{W_1 - W_2}{D}$$

W_1 = Initial weight (beaker + basket + normal saline)

W_2 = Secondary weight (beaker + basket + normal saline + testis)

D = Density of normal saline (1.0048)

The right testis was used for histological studies. The samples were fixed in formalin for 7 days and then dehydrated in alcohol at different ascending grades (80%, 70%, 50%, 95%, and 100%, respectively). Xylene was used to remove any remaining paraffin from the slides. After molding the samples using molten paraffin, sections with a thickness of 5 μm were provided on the slide using a microtome (Leitz 1512 Rotary Microtome) for histological studies [46].

The left testis was used for molecular studies. The RNA shield solution was employed to preserve the RNA structure of the samples. To perform the real-time PCR, the samples were transferred to -70 °C [47].

2.8.3. Histological methods

After preparing testicular samples, histological slides were stained with hematoxylin-eosin (H&E). Microscopic sections were surveyed with different lenses using optical microscopes (Olympus, Bx50, Tokyo, Japan) to study testicular histomorphometry.

2.8.3.1. Germinal epithelium thickness measurement. An average of 5 visibility is randomly imaged from each sample of testicular tissue using a light microscope (Olympus, model Bx50- Japan). A special surface probe was randomly put on the field of the image. Then, from all the total points collided with the image of the testicular section, the points that collided with the epithelium were calculated, Eq. (B1):

$$V_v = \frac{\sum_{i=1}^n P_i(x)}{\sum_{i=1}^n P_{\text{(total)}}}$$

The density of the germinal epithelium (S_v) was measured via a probe. The total points colliding with images and the number of probes colliding with the luminal surface of the epithelium were counted, and the level of the epithelium was calculated using the following formula, Eq. (B2) [46].

$$S_v = 2 \times \frac{\sum_{i=1}^n I_i}{L/P \times \sum_{i=1}^n P_i}$$

$\sum I$: The number of probes that collided with the surface of the epithelium.

$\sum p$: The total points collided with the testicular image.

L/P : The length of the linear test probe on a real scale.

2.8.3.2. Seminiferous tubule diameter measurement. First, a microscope equipped with a camera and linked to a computer was employed to measure the diameter of the seminiferous tubules. The particular frame was then randomly placed on the images. Motocimage 2000 software measured and recorded the length of the linear in the longitudinal direction of the tube and the small diameter of the seminiferous tubules across the small diameter of the probe interaction.

2.8.4. Sperm count and morphology

To examine the sperm parameters, the epididymis was removed into three parts by sterile scissors and transferred to a 5 ml falcon tube containing normal saline. Next, 10 μ l of the sperm suspension was transferred to the Neubauer slide and sperm counting was performed by using an Olympus BX51 light microscope (Japan) at $\times 40$ magnification and according to World Health Organization's (WHO) guidelines [48].

The first sperm suspension containing 10 μ l was taken and smeared on microscope slides. After fixing the samples in methanol, *aniline blue*, *toluidine blue*, and *Papanicolaou staining* were used to examine the sperms' morphology using standard instructions [49].

2.8.5. Hormonal assessment

The blood samples were stored for half an hour at room temperature and, then, were centrifuged at 3000 rpm for 15 min. After separating serum from blood cells, serum samples were stored and maintained at -20°C and used to measure antioxidant tests and evaluate sex hormones.

2.8.5.1. Determining biochemical parameters. To determine the concentration of malondialdehyde (MDA) in the blood serum sample, 50 μ l of blood was diluted in 1950 μ l of MDA, TCA (trichloroacetic, TBA (thio-barbituric acid) and HCL (hydrochloric acid) at 100°C for 15 min. The solution was centrifuged for 10 min at 2500 rpm. Subsequently, 1 ml of the supernatant was extracted and the absorbance at 535 nm was measured. The MDA concentration in the serum was determined using the extinction coefficient ($\text{mM}^{-1}\text{cm}^{-1}$) according to Birlambert's rule ($A = \text{cdc}$) [50]. To measure glutathione s-transferase (GST) enzyme activity, 50 μ l of GST was added to 50 μ l of a serum sample from 2850 μ l of the phosphate buffer saline (PBS) to measure the GST activity. Then, by adding 50 μ l of CDNB to the serum sample in 5 min, the absorbance was obtained at 340 nm by a spectrophotometer [51]. To measure the activity of the CAT enzyme, the volume of the reaction mixture (490 μ l of hydrogen peroxide (30 mM), 10 μ l of a serum sample, and 1 ml of 50 mM phosphate buffer) was examined in a 1.5 ml cuvette at $\text{pH} = 7$. The CAT activity was assessed according to Aebi's method [52] and the absorbance was read at 240 nm. The rate of SOD enzyme activity was investigated using Ries and Giannopolitis' method. According to this method, a solution containing 50 mM potassium phosphate, sodium-EDTA (0.1 mM), riboflavin (75 μ M), nitrolobotrazolium (75 μ M), and methionine (13 mM) was added to the sample and the absorbance was read at 560 nm [52].

2.8.5.2. Measuring reproductive hormones. Gonadotropin-releasing hormone (GnRH), (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), progesterone and testosterone concentrations were assessed using the ELISA (enzyme-linked immunosorbent) assay.

2.9. Gene expression by real-time PCR

According to the procedure, an RNA isolation kit (Qiagen, Germany) was used to isolate the total RNA from the testicular tissue samples. The samples were homogenized in 750 μ l of RL solution and, then, were placed in 150 μ l chloroform. According to the constructor's guidelines, a first-strand complementary synthesis kit produced cDNA using oligo (dT) (Fermentas, Lithuania). Target primers (Table 1) and a mixer containing the cyber green paint (GENET BIO, Korea) were applied to execute qualitative PCR procedures. A Light Cycler® 96 Real-Time PCR system thermocycler was utilized to complete the 40-step cycle for gene expression levels (Roche, Germany). The $2^{-\Delta\Delta\text{Ct}}$ method was employed to evaluate changes in gene expression. The best thermal profile for *BCL2*, *BAX*, *CASPASE-3*, and *CASPASE-9* included the main denaturation stage at 95°C (10 min), monitored by 45 cycles at 94°C (30 s), specific annealing temperature (30s) and 72°C (30s). The housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was employed as an internal control [53].

2.10. Data analysis

Data were analyzed in SPSS-22 Statistical Software. One-way ANOVA was employed to examine the differences between the experimental groups, followed by Tukey's test, Kruskal–Wallis test, and Duncan's test. Data were presented as mean $\pm$ SD and the differences were regarded as statistically significant at the level of less than 0.05 ($P < 0.05$).

3. Results and discussion

3.1. Characterization of AuNPs-NO

3.1.1. Visual observations and UV-visible spectral analysis

The reduction of Au^{3+} ions to gold nanoparticles using plant extract has been reported to be followed by a color change in the reaction, suggesting the beginning of nanoparticle formation [54]. When the extract of *Nasturtium officinale* was added to the gold salt solution (HAuCl_4), the color of the reaction mixture changed from green to purple (Fig. 1A). The changes could be due to the excitation of surface plasmon oscillation made by electromagnetic field and reduction of gold ions of Au (III) to Au (0) [55].

UV-visible spectroscopy was performed to confirm the formation of biosynthesized AuNPs (AuNPs-NO). The analysis revealed the wide-ranging absorbance peak of AuNPs-NO centered at 540 nm (Fig. 1B).

The nanoparticle formation was observed at different time intervals (Fig. 2). Over time, the intensity of absorbance peaks was enhanced and their broadness was reduced. The best reaction concentration and the fastest duration to create the initial complete peak were related to 1000 μ l of the extract and 20 μ l of gold salt (1 mM) at 60°C for 2 h (Fig. 2C). After attaining the strongest absorption peak in most of the samples, the intensity of SPR bond adsorption did not change over time and peak overlapping was observed. Production of the highest concentration of nanoparticles was considered as the end of the reaction. The result showed that the maximum nanoparticle adsorption occurred at the wavelength of 540 nm; according to Arulkuma et al. [56] and Raveendran et al. [57], the absorption peak (Q-bands) of the nanoparticles is in the range of 540–560 nm.

In the present study, the absorption peaks became more intense and symmetrical as time passed and the temperature and concentration of the extract increased. Song et al. reported that there was ascending linear correlation between the formation of nanoparticles and their

Table 1

Primer sequences of genes' expression.

	Gene	Forward Primer	Reverse Primer	Annealing	Product size (bp)
1	<i>BCL2</i>	5' CAGGATAACGGAGGCTGGGATG 3'	5' GACTTCACTTGTGGCCAGAT 3'	59	157
2	<i>BAX</i>	5' CCCTTTTGCTTCAGGTTTCAT 3'	5' TCAGCTGCCACTCGGAAAAA 3'	59	223
3	<i>CASPASE3</i>	5' ACTGGACTGTGGCATTGAG 3'	5' GAGCCATCCTTTGAAITTCGC 3'	60	138
4	<i>CASPASE9</i>	5' CGGTGACCCAGAAATTGACC 3'	5' CCTGCCCGCTCACGTC 3'	61	164
5	<i>GAPDH</i>	5' GGAAGGTGAAGTTCGGAGTCA 3'	5' GTCATTGATGGCAACAATATCCACT 3'	58	101

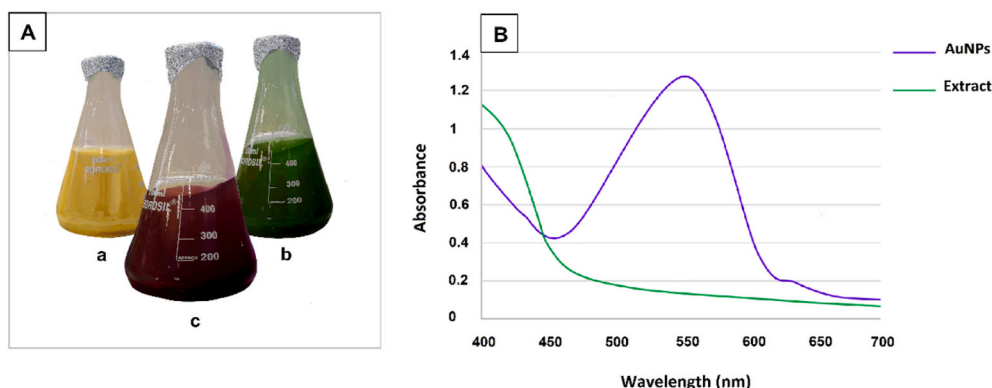


Fig. 1. (A) Visual images of the reduction of gold ions by *N. officinale* extract and biosynthesis of AuNPs-NO. (a) HAuCl₄ solution (1 mM); (b) Aqueous extract of *N. officinale* plant; (c) The color of the reaction solution changed from green to purple. (B) UV-Vis absorbance spectra of plant extract and AuNPs-NO. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

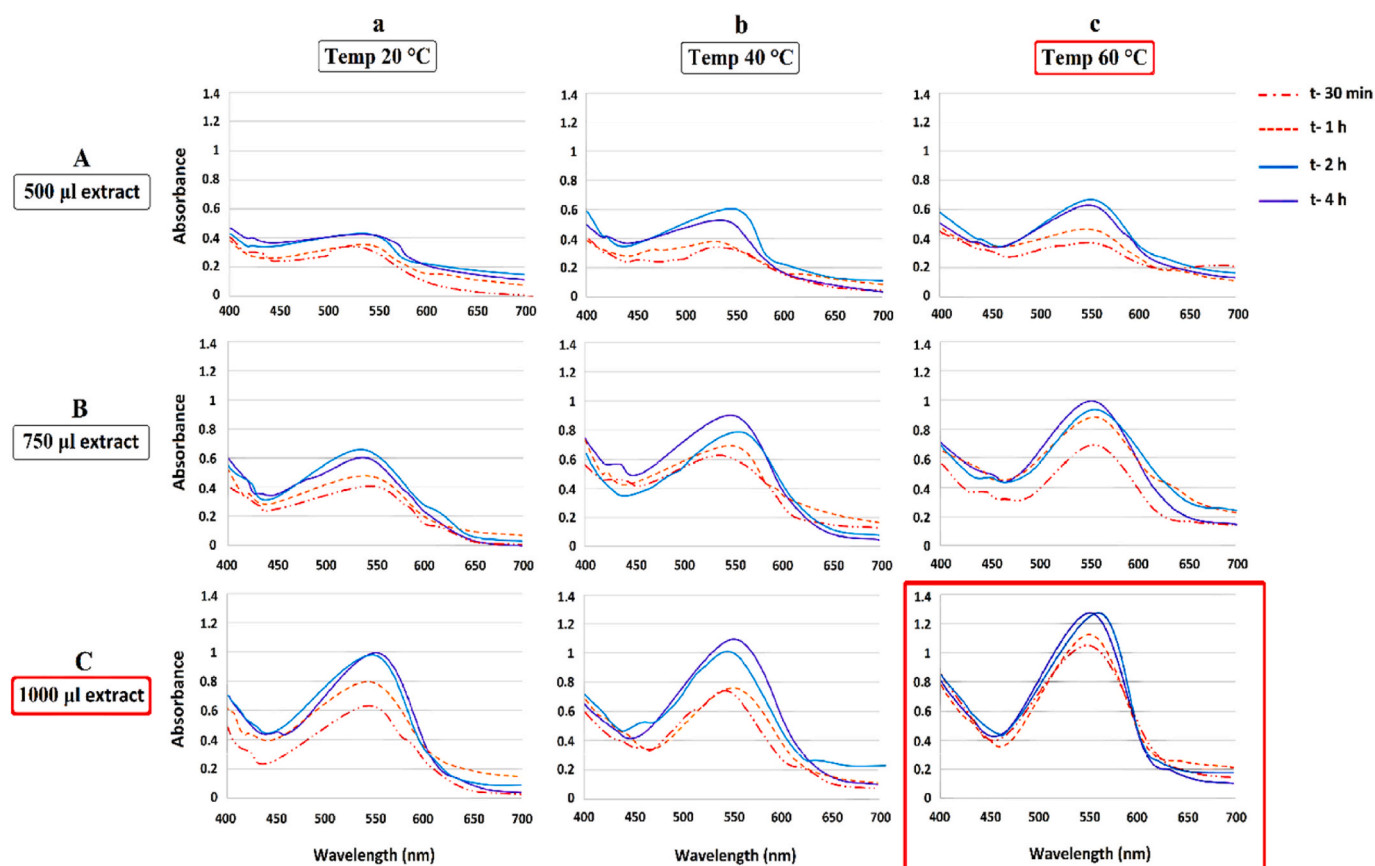


Fig. 2. UV-Vis spectra of AuNPs-NO at diverse concentrations of the plant extract (A) 500 µl; (B) 750 µl; (C) 1000 µl. At each ratio of the plant extract and HAuCl₄ solution (1 mM), various temperatures (a) 20 °C; (b) 40 °C; (c) 60 °C, and different times (30 min, 1 h, 2 h, and 4 h) are shown.

absorption peak at different times. At a specific time, as the concentration of nanoparticles increased, their absorption peak increased and, then, ended (i.e., stopped) [58].

In general, during the formation of gold nucleation (core), there is primary delay time, which is high at low temperatures and low concentrations of the extract. This point is consistent with the present reports [58–60].

3.1.2. XRD analysis

The crystalline nature of AuNPs-NO was authenticated by wide-angle X-ray diffraction (XRD) analysis. The XRD pattern indicated four distinct diffraction peaks with 2θ values at 38.3, 44.4, 64.560, 77.540, could be Miller-indexed to (111), (200), (220), (311) reflections of FCC (face-centered cubic) structure of metallic gold respectively (Fig. 3A). In the literature, similar results were testified for AuNPs synthesized using different biological entities [61–63].

3.1.3. FTIR analysis

FTIR spectrum identified the molecular activities of different functional groups of *Nasturtium officinale* extract and AuNPs-NO (Fig. 3B). The broad peak and strong absorption at 3396.93 were associated with the hydroxyl alcohols, phenols, and carboxylic acid functional groups [64]. The strong peak at 2920.04 cm^{-1} corresponded to the C–H stretching of asymmetry and symmetry vibrations. Another peak at 1739.21 cm^{-1} belonged to C=O carbonyl stretching. The bands at 1635.66 cm^{-1} were attributed to the stretching bonds of C–O in amino

acids. Similar conjugation effects of C=C (aromatic) stretching and N–H stretching vibrations at 1545.55 cm^{-1} and 1432.00 cm^{-1} were observed, which were related to CH–CH bands in lipids or sugars of the extract. The IR bands observed at 1031.63 cm^{-1} might be attributed to C–O stretching vibration in primary alcohol. The bands at 786.02 cm^{-1} were specified to C–H bending or halogen compounds such as chloride and bromide. The absorbance of the IR band revealed at 464.35 cm^{-1} was attributed to the metal peak due to the reduction of Au^{3+} ions to Au^0 of AuNPs-NO [61].

The occurrence of a new bond at 1739.21 cm^{-1} in the spectrum after the reduction of gold salt was related to the stretching vibration of the C=O of a carbonyl or carboxylic acid groups and indicated that polyols were mainly responsible for the reduction of gold ions, whereby they unsaturated carbonyl compounds oxidize to form these bands [65].

There are a variety of chemical factors present in biogenic compounds which can act as reducing agents allowing metal ions to be reduced and leading to metal nanoparticle synthesis. Some theories have been offered to explain the process of gold nanoparticle production. Biomolecules such as protein, phenol, flavonoids, etc. present in the plant extract may contribute to the reduction of metal ions and capping of nanoparticles. In a recent study, aqueous extracts of *Hovenia dulcis* fruit were used for the synthesis of gold nanoparticles. It was shown that the hydroxyl and carbonyl groups of flavonol derivatives bind to gold ions first to form gold complexes, which were then subsequently reduced to nanoparticles (Au^0). In addition, Au clusters formed by nanoparticles could act as nucleation centers to catalyze the decrease of the remaining

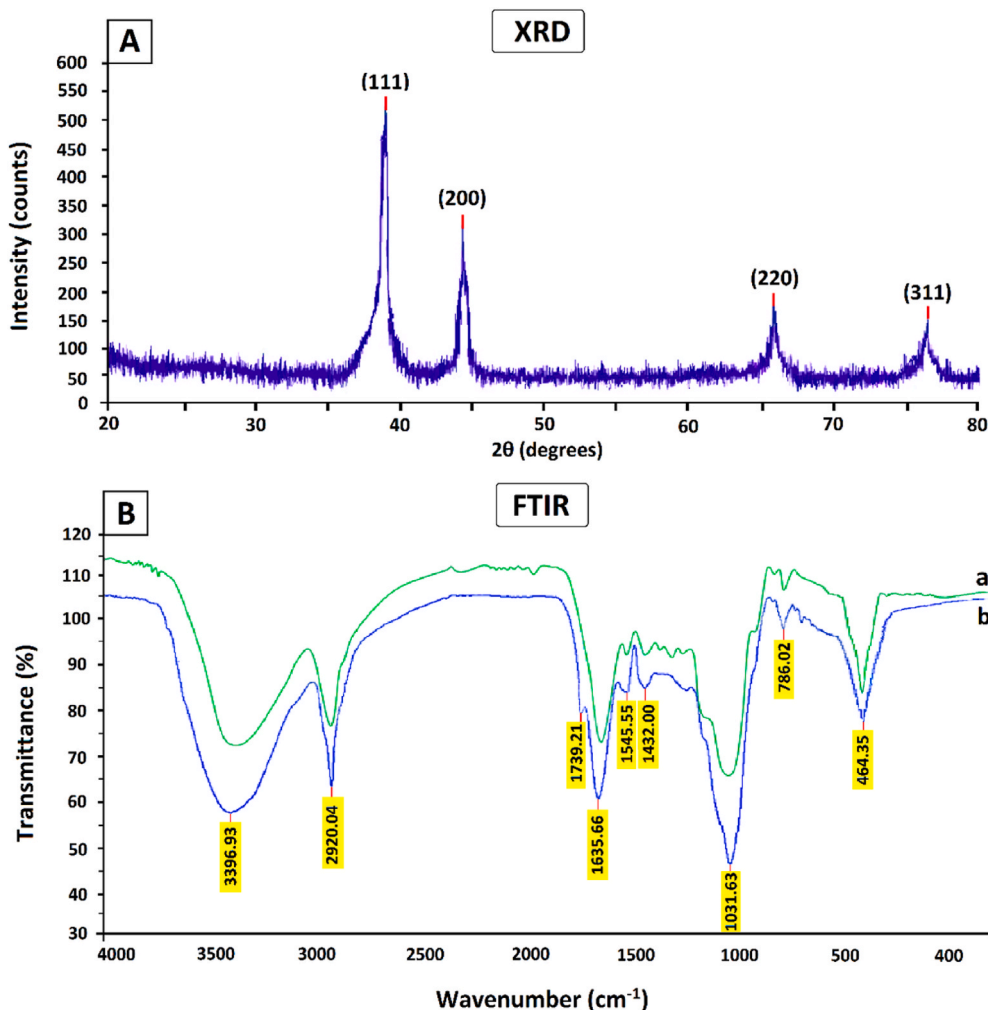


Fig. 3. (A) XRD pattern of AuNPs-NO. (B) FTIR spectra of (a) *N. officinale* plant extract; (b) AuNPs-NO.

ions into nanoparticles [66]. Researchers have found that protein, aromatic and aliphatic amines may be responsible for capping and stabilizing gold nanoparticles synthesized using *Morinda citrifolia* extract [67]. It was also shown that, during the biosynthesis of gold nanoparticles using *Sterculia acuminata* extract, hydroxyl (OH) groups underwent oxidation resulting in the formation of their oxidized/quinone forms that participated in the stabilizing process by entangling with the negatively charged carboxylic (C–O) group to form a complex through electrostatic interactions [68]. Some studies have reported the extract of three parts of the *Nasturtium officinale* plant including flowers, leaves, and stems is a valuable source of bioactive phytochemicals containing carbohydrates, steroids, saponin, reducing sugar, glycosides, flavonoids, and phenolic compounds with different percentages which are mostly responsible for anti-inflammatory, anticancer and antioxidant properties [69–71]. Many studies have also reported that the different compounds of plant extracts can reduce Au^{3+} to Au^0 , forming a biological layer around the nanoparticles and giving them stability and appropriate antioxidant properties [72,73].

Hence, we can expect the bioreduction of gold ions, the growth of Au atoms, the formation of Au clusters, and the biostabilization of gold nanoparticles to occur by a wide variety of plant compounds present in biological agents, such as reducing amines, starch, sugars, gingerol, catechin, phenolic compounds, flavonoids, enzymes, etc (Fig. 4).

3.1.4. TEM, DLS and ZP studies

Transmission electron microscopy (TEM) is the main system to analyze the size and morphology of nanoparticles accurately. The TEM images indicated that the average size of AuNPs-NO was about 64 nm and they had spherical shapes (Fig. 5A and B). Additionally, DLS analysis demonstrated that the nanoparticle size distribution was consistent as the concentration of plant extract increased at each temperature (Fig. 5C). The DLS and TEM results in this study corroborated the UV–Vis spectroscopy results. Substantial differences in the size of nanoparticles measured using TEM and DLS techniques can be attributed to the adsorption of macromolecules (polysaccharides, proteins, etc.) and hydrophilic compounds on the nanoparticles' surface, which resulted in the large volume of water at the surface of nanoparticles.

Consequently, the nanoparticles' hydrodynamic radius increased when measured using DLS [35].

Zeta potential (ZP) is generally employed to identify the information about the surface electric charge and stability of nanoparticles. The direction and velocity of the particles under the influence of an electric field are used to calculate Zeta potential. Nanoparticles with the Zeta potential above ($\pm$) 30 mV have been presented to be stable in suspension [74,75].

In the present study, the negative Zeta potential of colloidal AuNPs-NO biosynthesized by *Nasturtium officinale* extract was above 30 mV (–35.4 mV), representing the high electrostatic stability of the nanoparticles due to the coating of biomolecules present in the plant extract (Fig. 5D). It was affected by the capping of anionic compounds on the surface of nanoparticles which could reflect the electrical potential of particles. Obtained results showed that AuNPs were poly-dispersed and the electrostatic repulsive among nanoparticles prevented the agglomeration of nanoparticles in suspension and caused long-term stability [76].

3.2. Antioxidant activity

The DPPH radical scavenging assay also demonstrated concentration-dependent inhibition by AuNPs-NO (Fig. 6). At the AuNPs-NO concentration of 12.5 $\mu\text{g/mL}$, the inhibition rate of free radicals was about 43% and, with increasing the concentration to 500 $\mu\text{g/mL}$, this rate rose to about 88%. The results showed that these AuNPs-NO at about 40 $\mu\text{g/mL}$ could inhibit 50% of DPPH free radicals, indicating their great free radical-inhibiting potential.

Herein, at lower concentrations (12.5 and 25 $\mu\text{g/mL}$), AuNPs-NO had better antioxidant activity than *N. officinale* extract. At 50 $\mu\text{g/mL}$, both AuNPs-NO and extract had an almost similar DPPH activity. At higher concentrations (100, 200, 300, and 400 $\mu\text{g/mL}$), the antioxidant activity of the extract surpassed AuNPs-NO. Furthermore, at the highest concentration (500 $\mu\text{g/mL}$), both AuNPs-NO and extract displayed DPPH scavenging activity very close to the rutin (Ascorbic acid). Nakkala et al. also evaluated the antioxidant activity of AuNPs synthesized with *Piper longum* fruit and plant extract, and reported that higher concentrations

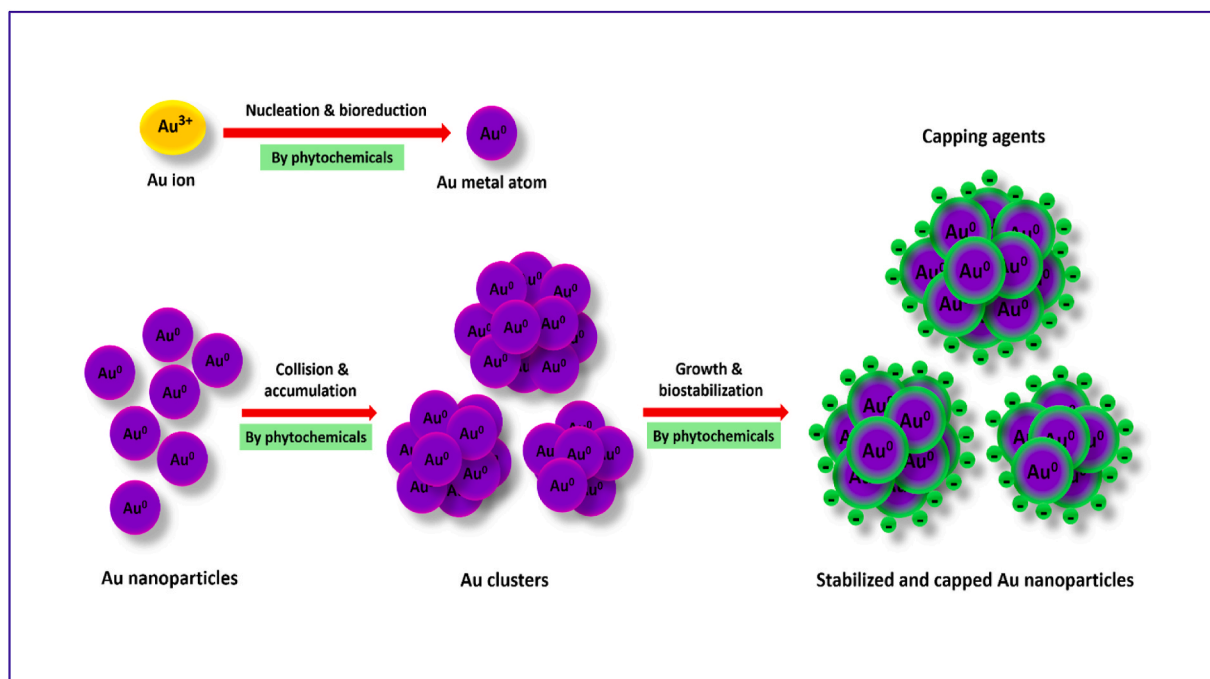


Fig. 4. Mechanism of bioreduction and biostabilization of gold nanoparticles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

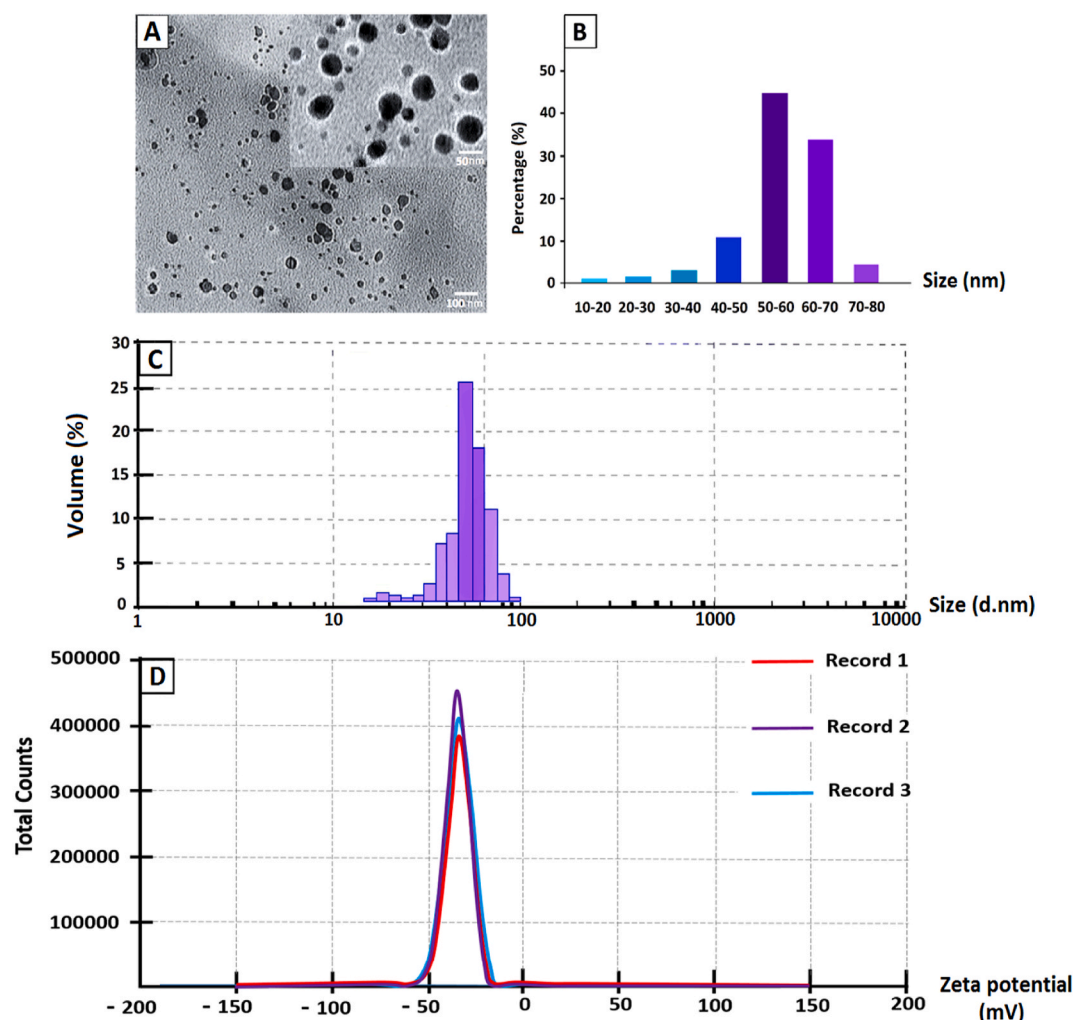


Fig. 5. Size histograms of AuNPs-NO. (A) TEM micrographs; (B) particle size distribution, and (C) DLS. Zeta potential of biosynthesized AuNPs-NO (D).

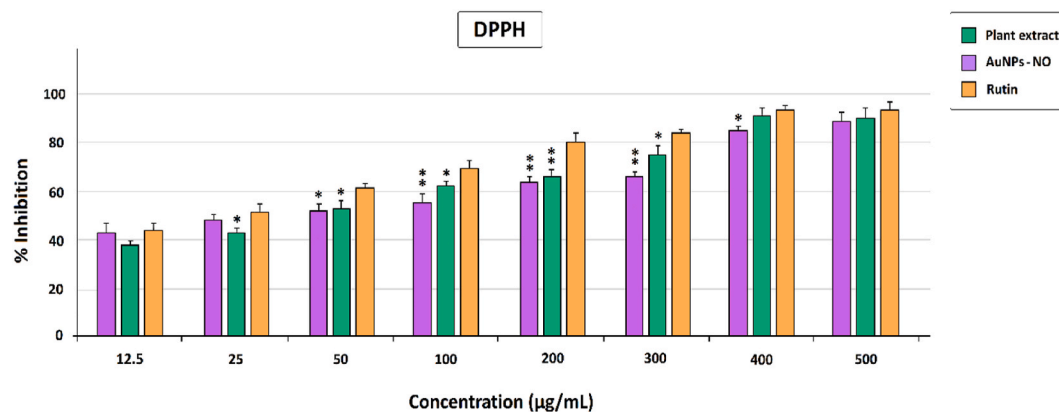


Fig. 6. DPPH radical scavenging assay of *N. officinale* plant extract and AuNPs-NO. Standards are shown as mean $\pm$ SD of triplicate analysis ($n = 3$). The bars represent a significant difference $*p < 0.05$ and $**p < 0.01$ versus control (rutin).

of AuNPs and extract yielded proper DPPH activity [77]. Other studies evaluating the free radical scavenging capacity of AuNPs have represented the concentration-dependent effect of the nanoparticles, which could be compared with the current results [78–80].

Nasturtium officinale extract consists of a high level of polyphenols, carotenoids (B-carotene, lutein), glucosinolates (GSLs), vitamins (B1, B2, B3, B6, E, C), polyphenols (phenolic acids, flavonoids,

proanthocyanidins), and other compounds, which are related to various anticarcinogenic and antioxidant activities to eliminate reactive oxygen and lead to defense against oxidative damage [69].

3.3. In vitro AuNP-NO cytotoxicity assessment

The MTT assay results demonstrated that AuNPs-NO did not affect

the RBM-1 cell line cell viability (rat bone marrow) at the concentrations of 0 and 3.12 $\mu\text{g}/\text{ml}$. A considerable reduction in the number of viable RBM-1 cells was observed in AuNPs-NO-treated cells with higher concentrations than the control cells in a dose-dependent manner. Approximately 50% inhibition of cell viability (IC_{50}) was determined by AuNPs-NO at 26 $\mu\text{g}/\text{ml}$ (Fig. 7), suggesting that the non-toxic dose was the concentration 12.5 $\mu\text{g}/\text{ml}$ and the toxic dose of the AuNPs-NO was the concentration of 50 $\mu\text{g}/\text{ml}$.

In a recent report, the MTT assay results revealed that AuNPs had toxic effects on mouse fibroblast cell lines (NIH3T3), which depended on the targeted doses. IC_{50} was specified in the 5th well. Therefore, the non-toxic dose of AuNPs was in the 6th well and the toxic dose in the 4th well [44].

3.4. Cell population changes using AO/PI staining

The viable and apoptotic cells displayed diffused and condensed chromatin, respectively. There was a noticeable difference in the percentage of apoptotic (orange-green and orange) and necrotic cells (red and reddish-brown cells) in the samples treated with AuNPs-NO compared to the control (Fig. 8). Cell counts exhibited morphologic alterations in RBM-1 cells treated with AuNPs-NO in a dose-dependent manner. The outcomes demonstrated that the percentages of apoptotic and necrotic cell populations in the treatment groups (26 and 50 $\mu\text{g}/\text{mL}$) were 47% and 84%, respectively ($p < 0.01$, $p < 0.001$), whereas these percentages were $<7\%$ in the control cell population and the cells treated with 12.5 $\mu\text{g}/\text{mL}$ concentration of the total cells (Fig. 9). By increasing the concentration of AuNPs-NO from 26 $\mu\text{g}/\text{mL}$ to 50 $\mu\text{g}/\text{mL}$, the population of apoptotic and necrotic cells was enhanced by 27% and 9%, respectively. After a determined period, the necrotic cells only appeared after apoptosis took place. This process was apparent in the cells where apoptosis had begun, e.g., when the cells displayed no evidence of survival factors and when entirely different fatal signals activated several death receptors [81]. Our findings are consistent with the investigations of prior MTT tests in this regard.

3.5. In vivo studies

To obtain the non-toxic dose in the rat model, based on the MTT test results, AuNPs-NO must be diluted 1/64 in the body of the rat. Therefore, for the animal weighing roughly 200 g, there are 20 ml of blood because nearly 10% of the rat's body is blood. By using a specific proportion, the injected non-toxic doses will be 0.312 ml. Based on rat body

weight, the injected dose of nanoparticles was obtained for each animal [44]. The administration was done intraperitoneally for 16 consecutive days.

3.5.1. Bodyweight

As shown in Fig. 10A, a comparison of the average body weights of animals in different groups revealed that all the groups gained weight during the 16-day experimental period, but the CP group gained weight very slightly, with a significant difference from the control group ($p < 0.01$). The administration of AuNPs-NO in the CP + AuNPs-NO group prevented the excessive weight loss caused by CP administration and no significant difference was observed from the control group. There was also a considerable difference between CP and CP + AuNPs-NO groups ($p < 0.05$). Das UB et al. and Haque R et al. have reported that an animal's weight loss is a consequence of low-dose and long-term administration of CP in male mice [82,83]. Furthermore, cyclophosphamide causes loss of appetite, nausea, and vomiting, which could be due to the triggering of chemical receptors at the fourth ventricle in the medulla oblongata [84]. Accordingly, in this study, cyclophosphamide may have reduced body weight by affecting fat due to anorexia and preventing protein synthesis. In this case, co-administration of AuNPs-NO with CP successfully prevented weight loss.

3.5.2. Testis weight index and volume

The mean testicular weight in the CP group was significantly lower than in the control group ($p < 0.01$). There was no significant difference between the control group and the CP + AuNPs-NO group, as the weight loss in the CP + AuNPs-NO group was compensated for by the co-administration of AuNPs-NO. The mean testicular weight of the animals significantly differed across CP and CP + AuNPs-NO groups ($p < 0.05$) (Fig. 10B). The testicular volume also indicated a remarkable decrease in the CP compared to the control group ($p < 0.05$). There was a significant difference between CP and CP + AuNPs-NO groups ($p < 0.05$) (Fig. 10C). The new study confirmed that low-dose, long-term cyclophosphamide treatment in male mice reduced the weight of both body and genital organs, and affected fertility. In addition, as testicular weight was highly dependent on differentiated cell masses, a significant decrease in the testicular weight due to CP administration caused degeneration and reduced the number of germ cells [85]. Reduced testicular weight might also be caused by a decrease in testicular volume, seminiferous tubules' diameter, or epithelial layer, all of which increase testicular damage [86]. Li WQ et al. demonstrated no difference in testicular weight, testicular indices, or epididymis between the

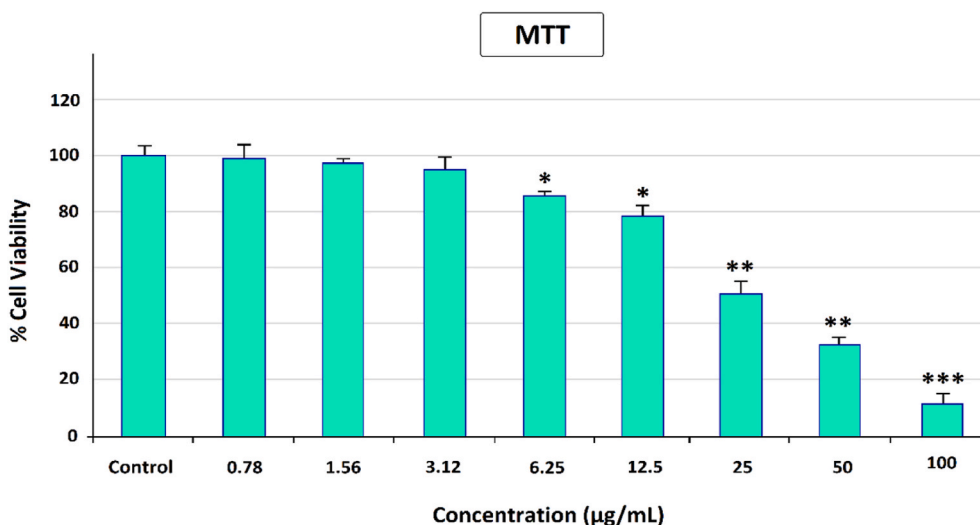


Fig. 7. Assessing the AuNPs-NO cytotoxicity on RBM-1 cell lines for 24 h by MTT assay. Bars indicate the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus untreated group (control).

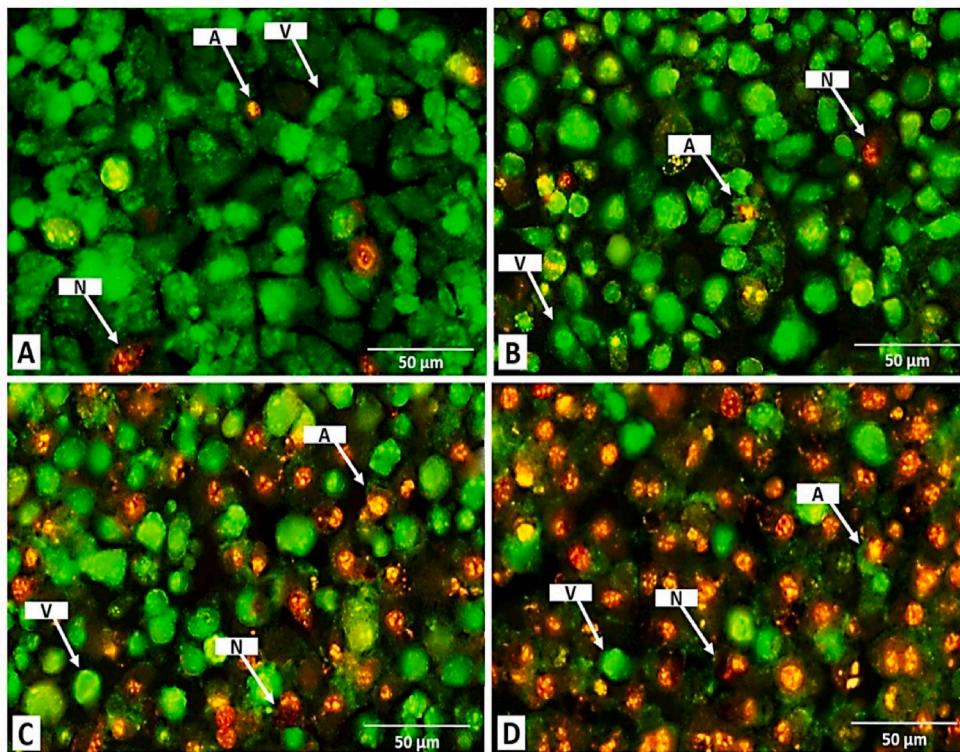


Fig. 8. Fluorescent micrograph of RBM-1 cells stained with AO/PI. Cells were treated with concentrations of A) 0, B) 12.5, C) 26 and D) 50 $\mu\text{g/mL}$ of AuNPs-NO. (A): Most untreated cells (control) and treated cells with a low concentration of AuNPs-NO (12.5 $\mu\text{g/mL}$) were detected with a normal structure with uniformly green, and very few apoptotic and necrotic cells. (C) and (D): Treated cells at the concentration of 26 and 50 $\mu\text{g/mL}$ of AuNPs-NO revealed apoptosis and necrosis. Apoptosis features included orange-green and orange, and necrosis appeared as a red and reddish-brown dye. Cells were presented as the viable cell (V), apoptotic cell (A), and necrotic cell (N) using arrows. The standards are shown as mean $\pm$ SD ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

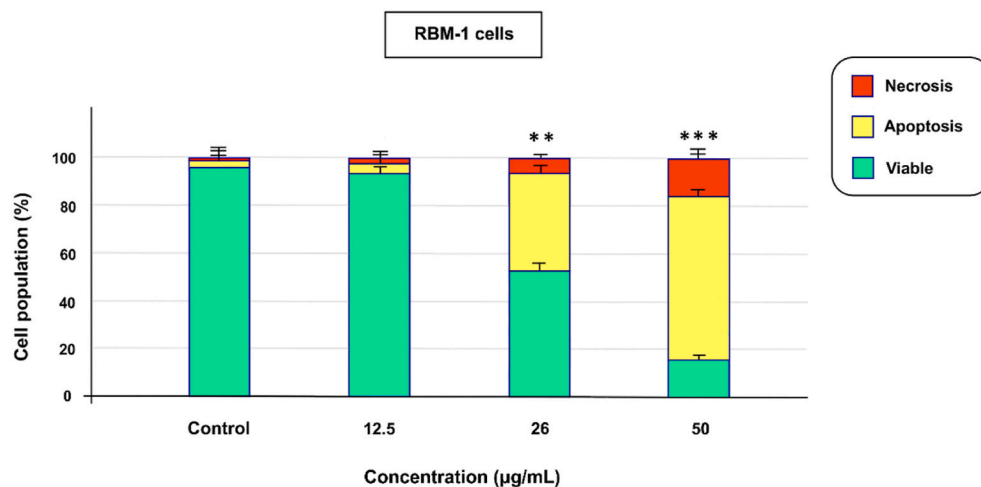


Fig. 9. Evaluating the percentage of viable, apoptotic, and necrotic RBM-1 cells by AO/PI staining. Cells were treated with different concentrations of A) 0, B) 12.5, C) 26 and D) 50 $\mu\text{g/mL}$ of AuNPs-NO for 24 h $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group.

treated and untreated mice after AuNP injection [87]. In the current study, no significant variations in the weight and volume of the testicles were identified between the AuNPs-NO and control groups following the AuNP injection.

3.5.3. Histological examination

Based on Fig. 11, histological observation of testicular sections showed that the seminiferous tubules were regular and compact in the control, sham control, and AuNPs-NO groups. Furthermore, the arrangement of spermatogenic cells and the interstitial tissue was normal. Increased interstitial space, irregularity, and rupture of the seminiferous tubules were observed in the CP group. Moreover, the abnormal arrangement of spermatogenic cells and a reduction in their

number were visible. In most parts of the epithelial and interstitial tissues, connectivity was lost and vacuolation was observed. The cellular arrangement of the seminiferous tubules and interstitial tissue in the CP + AuNPs-NO group improved due to AuNPs co-administration and no vacuolation was observed.

As shown in Fig. 12, a comparison of the mean germinal epithelium thickness in different groups indicated a significant decrease in epithelial height of the CP compared to the control group ($p < 0.001$). There was also a significant difference between CP and CP + AuNPs-NO groups ($p < 0.01$). Comparison of the mean diameter of the seminiferous tubules demonstrated a significant decrease in the CP compared to the control group ($p < 0.01$). A significant difference was also found between CP and CP + AuNPs-NO groups ($p < 0.01$). There was a slight

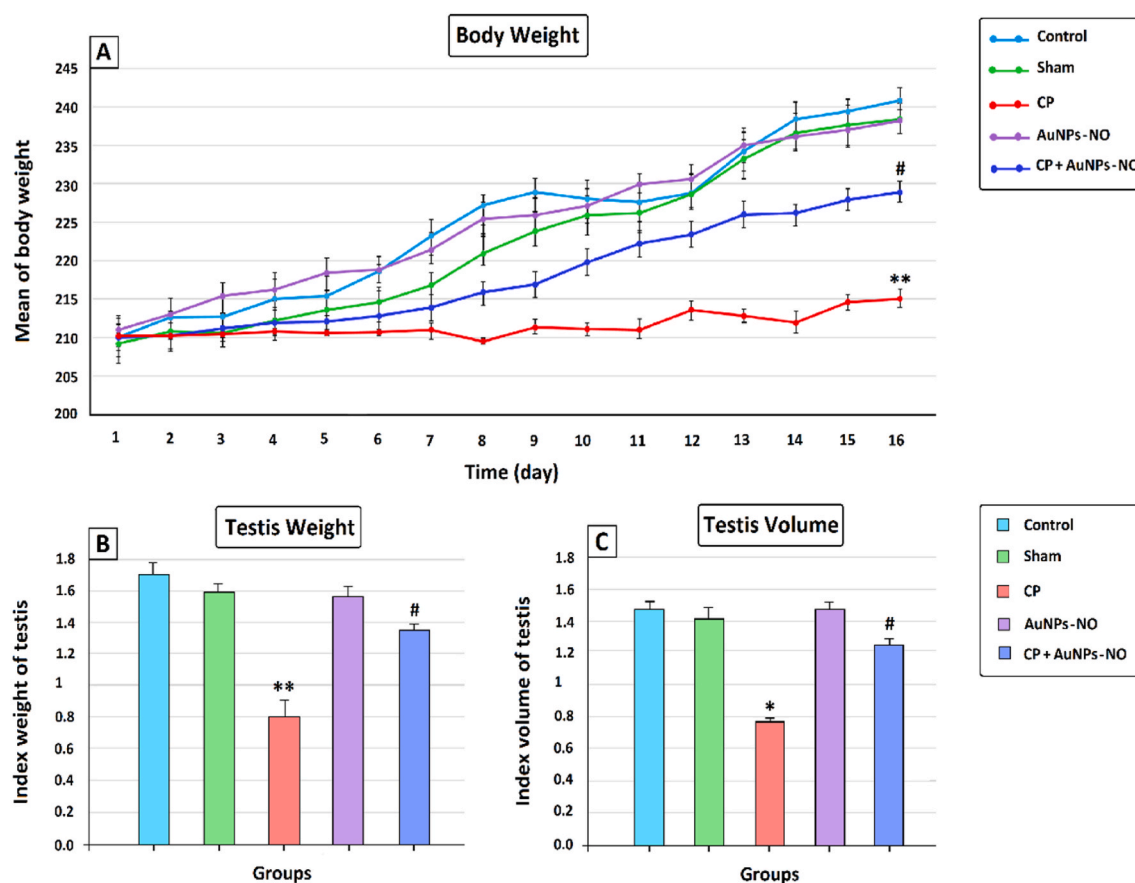


Fig. 10. (A) Comparing the body weight in different groups. (B) Comparing the means of testis weight index in different groups. (C) Comparing the testis volume in different groups. * $p < 0.05$, ** $p < 0.01$ compared to the control group and # $p < 0.05$ compared to the CP group.

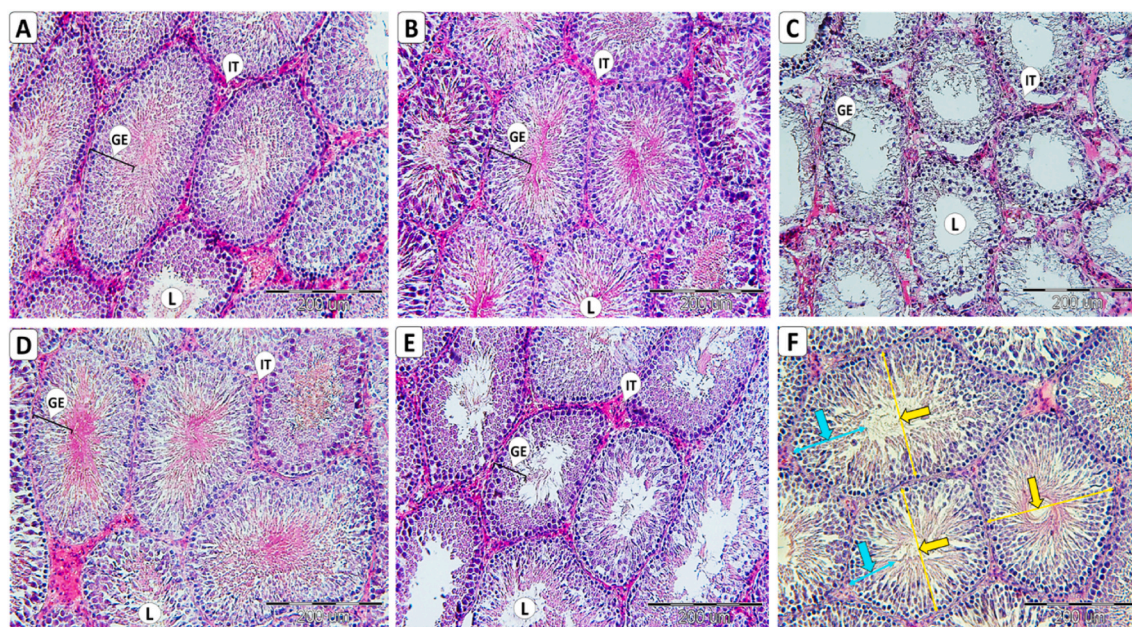


Fig. 11. Photomicrographs of testicular sections using hematoxylin-eosin staining in different groups: (A) control group; (B) sham control; (C) CP group; (D) AuNPs-NO group; (E) CP + AuNPs-NO group and (F) measurement of germinal epithelium thickness (blue arrow) and seminiferous tubule diameter (yellow arrow). Lumen (L), the germinal epithelium (GE), interstitial tissue (IT). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

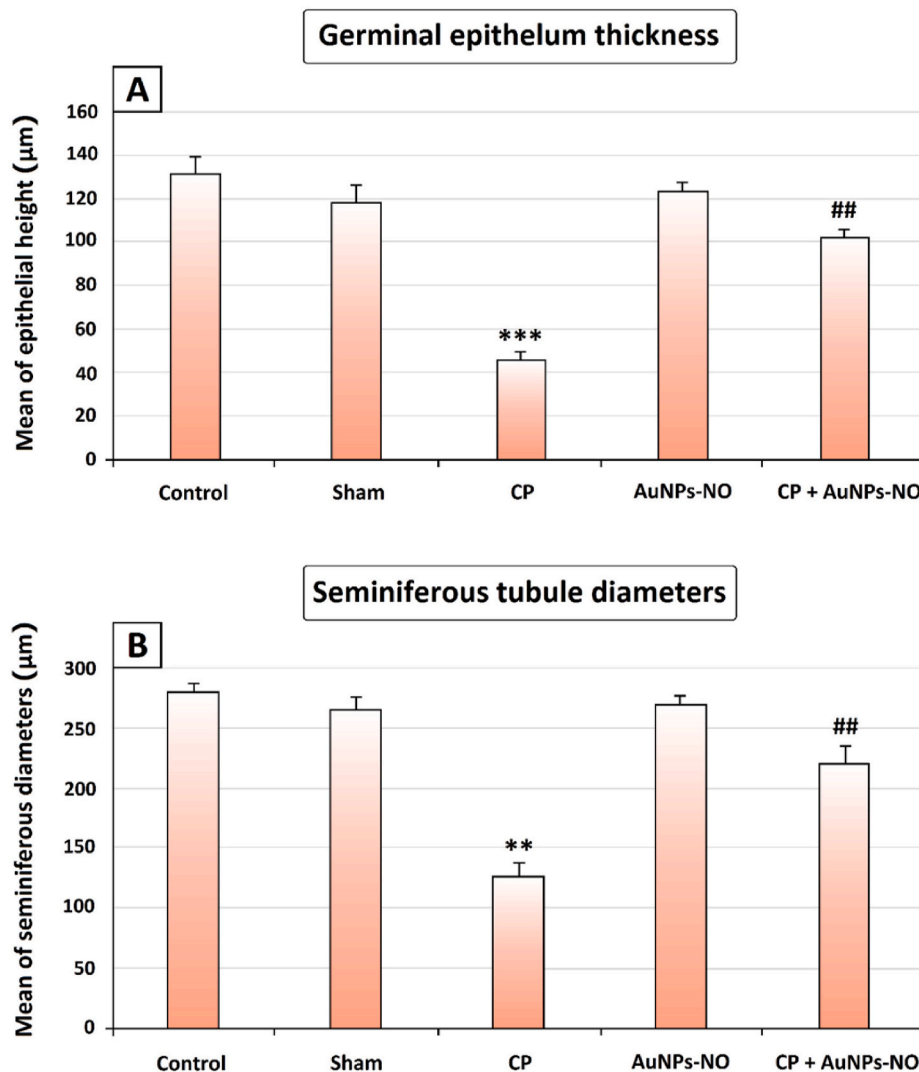


Fig. 12. Comparing the mean (A) germinal epithelium thickness and (B) seminiferous tubule diameter in different groups. *** $p < 0.001$, ** $p < 0.01$ compared to the control group, ## $p < 0.01$ compared to the CP group.

difference in epithelium height and tubules' diameter between the control and CP + AuNPs-NO groups but was not statistically significant.

The histological findings confirmed the results of several *in vivo* studies reporting that CP causes histological-morphological changes in the testes of different male mice and rats [88–90]. Additionally, Niakani et al. found that cyclophosphamide changed the histomorphometric properties of mouse testes. They also observed a decrease in the thickness of the cell layers and a reduction in the diameter of seminiferous tubules [91].

The results indicated that administering a non-toxic dose of AuNPs with the mean size of 64 nm to the rats in the AuNPs-NO group had no detrimental effect on the testicular tissue. By obtaining toxic and non-toxic doses of the nanoparticles, Yahyaei et al. reported that a non-toxic dose of AuNPs (0.32 mg/kg with the size of 50–70 nm) caused no toxicity in rat testicular cells. No change was observed in the seminiferous tubules, spermatogenic, Sertoli, and intestinal cells [44]. It is hypothesized that nanoparticles with small size can penetrate the blood-testicular barrier and, after exposure to the nanoparticles by creating an inflammatory response, the size of the blood-testicular barrier gap increases. Consequently, smaller nanoparticles can pass through the barrier and some larger nanoparticles may remain behind the barrier. However, the molecular mechanisms involved in nanoparticle toxicity on spermatogenesis have not yet been elucidated. In

addition, the nanoparticles are probably endocytosed by spermatogenic cells or Leydig cells; therefore, there are no morphological signs of nanoparticle damage [92].

On the one hand, round AuNPs were significantly able to compensate for the destructive tissue effects caused by cyclophosphamide in the present study. According to the findings, the consumption of antioxidants is one strategy for combating the oxidative effects of chemotherapy medications. Recent studies have reported that antioxidants can be useful in lowering cyclophosphamide cytotoxicity [93,94].

Shuai Pu et al. found that the level of antioxidant activity of AuNPs depended on their size, shape, and quantity, with spherical nanoparticles having higher antioxidant activity than polygonal nanoparticles [92]. Ningaraju et al. described that the resulting green gold nanoparticles had significant anti-inflammatory and antioxidant effects *in vivo* and *in vitro*, through synthesizing green gold nanoparticles using Vitex negundo leaf extract and evaluating their biological activity [95]. Mitra et al. also reported that the co-administration of AuNPs synthesized using *Terminalia arjuna* at 175 g/kg and acetaminophen for 14 days effectively prevented reproductive disorders in rats. These AuNPs protected against the toxicity of acetaminophen, an analgesic drug [61].

3.5.4. Sperm count and morphology

The mean sperm count of the CP group was significantly lower than

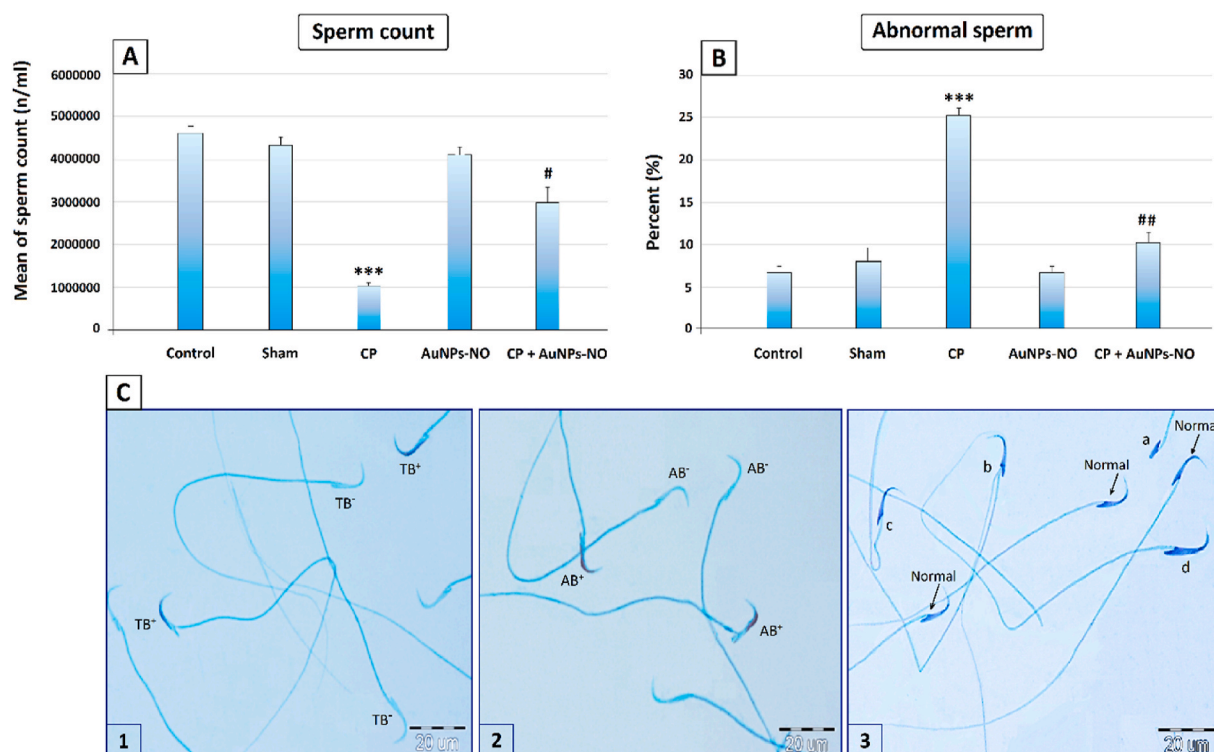


Fig. 13. (A) Comparing the mean sperm counted (B) Comparing the mean abnormal sperm percentage in different groups. *** $p < 0.001$ compared to the control group, # $p < 0.05$ and ## $p < 0.01$ compared to the CP group. (C) Investigating sperm abnormalities using (1) Toluidine Blue, (2) Aniline Blue, and (3) Papanicolaou Staining: (a) Pinhead, (b) Double body, (c) Abnormal middle piece, (d) Giant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

that of the control group ($P < 0.001$). The sperm count of CP + AuNPs-NO did not significantly differ from the control group, but it was significantly enhanced when compared to the CP group ($P < 0.05$) (Fig. 13A). The samples receiving cyclophosphamide had the highest mean proportion of defective sperm. The CP group had a significantly

higher rate of sperm abnormalities than the control group ($P < 0.001$). Co-administration of AuNPs in the CP + AuNPs-NO group significantly reduced cyclophosphamide-induced sperm morphological abnormalities compared to the CP group ($P < 0.01$) (Fig. 13B). Results of sperm staining examination indicated a substantial difference between the

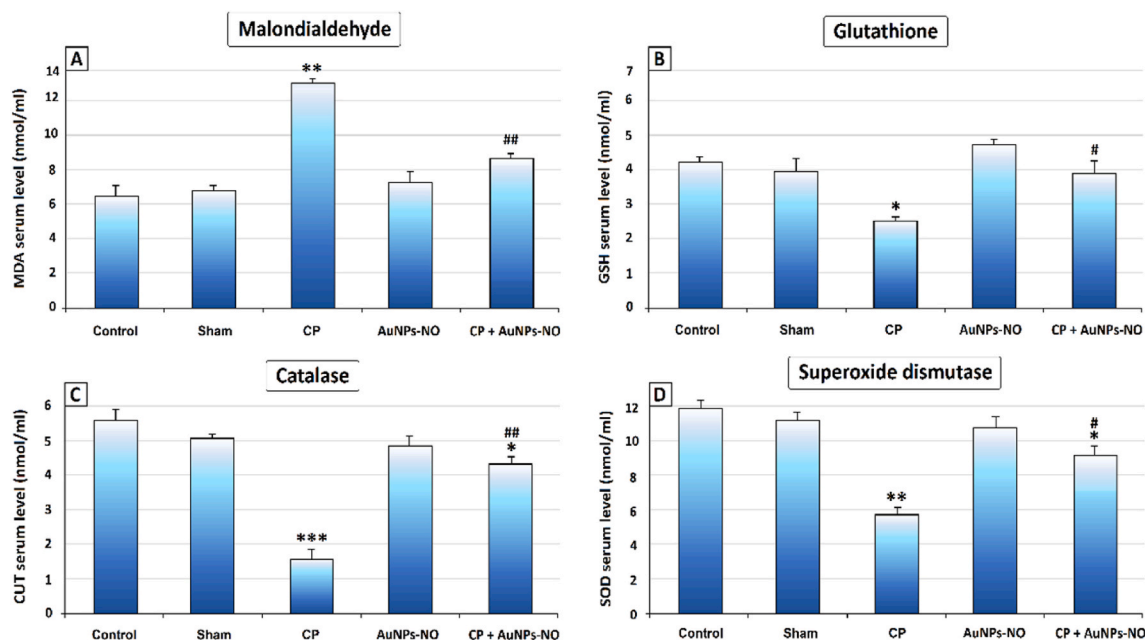


Fig. 14. Measuring biochemical parameters in different groups. (A) Malondialdehyde, (B) Glutathione, (C) Catalase, (D) Superoxide dismutase serum levels were measured. Values are presented as mean $\pm$ SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the control group. # $p < 0.05$, ## $p < 0.01$ compared to the CP group.

experimental groups, with the CP group showing the highest percentage of immature sperm [96]. Several morphological abnormalities of the sperm are illustrated in Fig. 13C, including the abnormal middle piece, double body pinhead, and giant in the CP group. Numerous studies have demonstrated that cyclophosphamide reduces male fertility and increases the rate of oligospermia and azoospermia in males [97–99].

3.5.5. Measuring serum biochemical parameters

Based on Fig. 14, the level of serum malondialdehyde (MDA) in the blood as a marker of lipid peroxidation (LPO) was significantly enhanced in the CP compared to the control group ($p < 0.01$). On the other hand, the amount of serum malondialdehyde in the CP + AuNPs-NO increased compared to the control group, which was not significant, but showed a considerable decrease in the CP group ($p < 0.01$).

In other words, it seems that in the CP + AuNPs-NO group, AuNPs significantly reduced the rate of lipid peroxidation compared to the CP group. Based on Fig. 14, the activity of glutathione-S transferase (GST) in the CP group was significantly reduced compared to the control group ($p < 0.05$). The activity level of this enzyme in the CP + AuNPs-NO group was not significantly different from the control group but demonstrated a significant rise compared to the CP group ($p < 0.05$). Comparison of serum catalase (CAT) activity in the CP and control groups revealed a significant decline in CAT activity in this group ($p < 0.001$). A considerable difference was also observed between CP and CP + AuNPs-NO groups ($p < 0.01$). Furthermore, CP + AuNPs-NO and control groups significantly differed ($p < 0.05$). In the CP group, the activity of this enzyme significantly declined compared to the control group ($p < 0.01$), while the administration of AuNPs in the CP + AuNPs-NO group compared to the CP group significantly elevated SOD activity ($p < 0.05$). There was also a considerable difference between CP + AuNPs-NO and control groups ($p < 0.05$).

In the study by Selvakumar et al. on male Wistar rats, cyclophosphamide injection at the dose of 15 mg/kg once per week for 10 weeks caused abnormal alterations in the surfaces and activity of enzymes such as catalase, glutathione, and superoxide dismutase [100]. In this study, a rise in catalase activity was seen in the CP + AuNPs-NO group. It can be concluded that AuNPs prevent the reduction of this enzyme's activity due to CP administration. Sengani et al. reported that diabetic rats treated with AuNPs synthesized using *C. guianensis* plant extract showed

high lipid peroxidation, superoxide dismutase, and catalase enzyme activity [101].

3.5.6. Measuring reproductive hormones

Fig. 15 demonstrates that the mean concentration of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the CP group significantly decreased compared to the control group ($p < 0.05$ and $p < 0.01$, respectively). The CP + AuNPs-NO group did not significantly change compared to the control group but indicated a significant decrease ($p < 0.05$ and $p < 0.05$, respectively) compared to the CP group. Cyclophosphamide administration in the CP group lowered the concentration of testosterone and progesterone; these changes were significant ($p < 0.01$) compared to the control group. Co-administration of AuNPs in the CP + AuNPs-NO group significantly raised the hormones; this elevation was not significant compared to the control group but was significant compared to the CP group ($p < 0.01$ and $p < 0.05$, respectively). The administration of nanoparticles in the AuNPs-NO group significantly raised testosterone compared to the control group ($p < 0.05$).

Numerous studies have considered cyclophosphamide in the development of reproductive problems. Decrease in blood testosterone, abnormal meiotic divisions during spermatogenesis, and the emergence of an irregular release of gonadotropins are also observed [8,102,103]. Elangovan et al. reported that the levels of LH and FSH decreased via the administration of cyclophosphamide to laboratory rats at the doses of 50, 100, 150, and 200 mg/kg once a week for five consecutive weeks and the decline was also significant in testosterone levels [96]. Wen-Qing Li et al. reported that the administration of AuNPs (mPEG@AuNPs and PEG-NH 2@AuNPs) did not affect FSH and LH levels. Additionally, they observed that treatment with these AuNPs increased blood testosterone levels in male mice, but did not affect their fertility [87].

3.6. RT-PCR analysis of gene expression

Real-Time PCR was accomplished on RNA purified from the testis tissue samples in different groups for the following genes: Bax, Bcl2, caspase-3, and caspase-9 related to apoptotic markers (Fig. 16). A significant part of germ cell apoptosis is changes in the mitochondrial pathway (which involves the interactions between Bcl-2 family

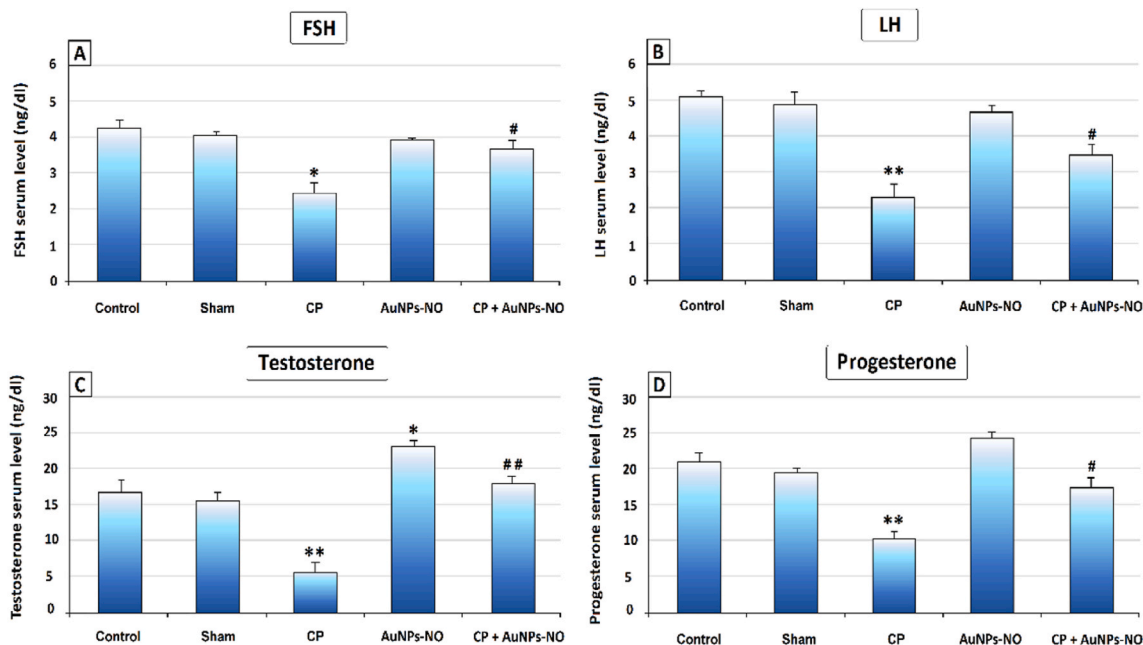


Fig. 15. Hormone analysis in different groups. (A) FSH, (B) LH, (C) Testosterone and (D) Progesterone serum level were evaluated by ELISA. Data are expressed as mean $\pm$ SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$ compared to the control group. # $p < 0.05$, ## $p < 0.01$ compared to the CP group.

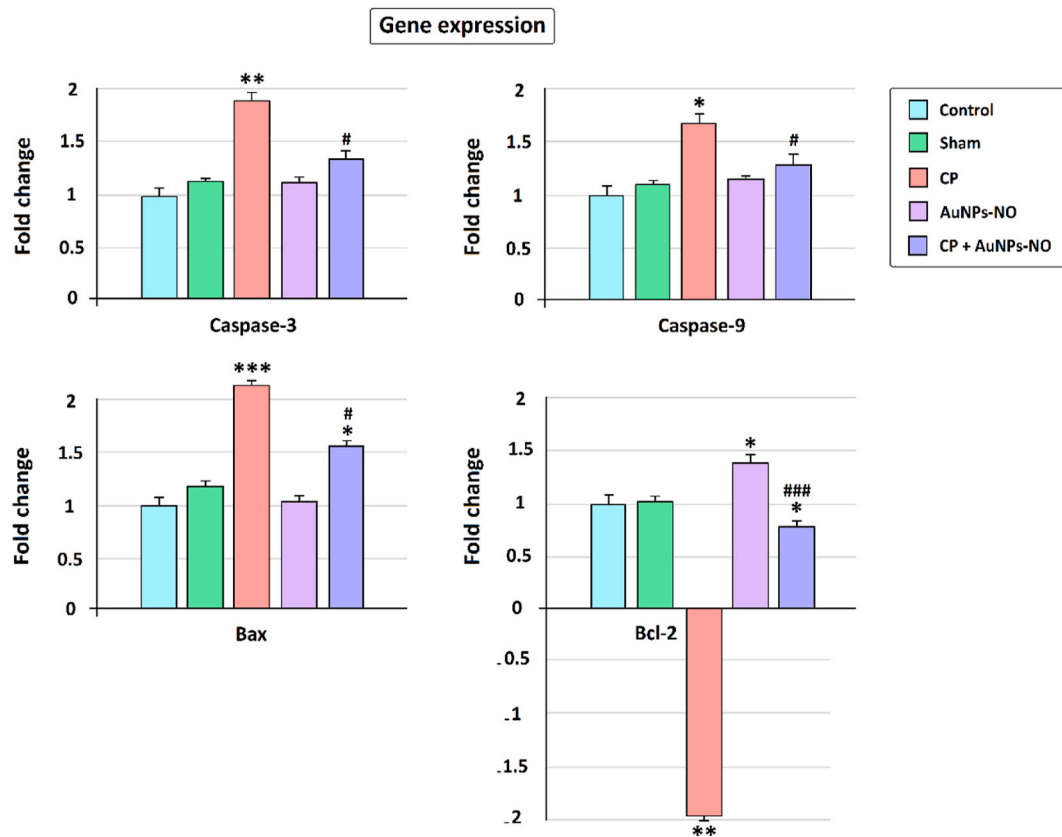


Fig. 16. RT-PCR analysis of apoptotic genes' expression includes caspase-3, caspase-9, Bax, Bcl2 genes in different groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were presented as statistically significant versus control.

members such as Bax and Bcl-2), thereby permitting the release of cytochrome c, which then leads to caspase activation and DNA fragmentation [104].

Herein, significant upregulation of Bax gene expression (2.11-fold change, $p < 0.001$) and downregulation of Bcl2 gene expression (1.98-fold change, $p < 0.01$) were revealed in the CP group compared to the control group. The management of the balance between cell life and death relies on anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) markers [105]. CP administration in male Wistar albino rats markedly increased the Bax mRNA gene expression level and significantly reduced the Bcl-2 gene expression in the testes compared to the control group [106]. The finding demonstrated the higher gene expression of Bax (1.51-fold change, $p < 0.05$) and lower gene expression of Bcl2 (0.78-fold change, $p < 0.05$) in the CP + AuNPs-NO group in comparison with the control group. Mild downregulation of Bax and major upregulation of Bcl2 in the CP + AuNPs-NO group were also observed compared to the CP group ($p < 0.05$ and $p < 0.001$, respectively). Administration of nanoparticles in the AuNPs- NO group upregulated Bcl2 expression compared to the control group (1.38-fold change, $p < 0.05$). There was no confirmation of a significant difference in other genes in the AuNPs-NO compared to the control group. The finding demonstrated that AuNPs can reduce apoptosis by affecting the gene expression of Bax, especially Bcl2. The ratio of anti-apoptotic (Bcl-2) to pro-apoptotic (Bax) proteins is thought to be a major regulator of cell destiny, as increasing Bcl-2 level promotes cell survival, while increasing Bax level promotes cell death [107]. Recent research has demonstrated that co-administration of *S. montana* as an antioxidant agent exerted significant anti-apoptotic effects as measured by the considerable downregulation of elevated Fas and Bax gene expression and significant upregulation of reduced Bcl-2 and PPAR-c gene expression compared to the CP-treated group [106].

In this study, the gene expression of the caspase family (caspase-3 and caspase-9) in the CP group was significantly enhanced compared to

the control group (1.87- and 1.67-fold changes, $p < 0.01$ and $p < 0.05$, respectively). Furthermore, the expression of both caspase-3 and caspase-9 decreased in the CP + AuNPs-NO group compared to the CP group ($p < 0.05$). There was no evidence of a substantial difference in the gene expression of the caspase family in the CP + AuNPs-NO compared to the control group (1.31- and 1.27-fold changes, respectively). Recent studies have found that rats treated with CP show elevated Bax and caspase-3 levels and decreased Bcl-2 levels in their testis [108].

4. Conclusion

Using nanotechnology, the green synthesis of AuNPs is a simple, rapid, eco-friendly, and efficient method. Many researchers have reported different structures, compounds, and inimitable properties of AuNPs for treatment strategies and drug delivery. Herein, the *N. officinale* aqueous extract acted as a reducing/capping agent in AuNP biosynthesis. The AuNPs-NO was assessed in the *in vitro* and *in vivo* models for the first time. The resulting nanoparticles exerted their protection via antioxidant and anti-apoptotic mechanisms. AuNPs-NO had powerful protective and antioxidants effects on rat testicular tissue induced by the cyclophosphamide chemotherapy drug and prevented apoptosis in spermatogenesis. In addition, AuNPs-NO showed excellent blood compatibility. The results indicated the possibility of their use as a novel candidate in therapeutic application. Still, more studies are essential for revealing the effect of nanoparticles on different normal cell lines and diverse organs in animals to discover the clinical utilities and targeting of NP-based therapy. Future studies should concentrate on the novel molecular and cellular mechanism of AuNPs-NO to identify their interaction factors and signaling pathways with target cells. Exploring the fabricated AuNPs-NO as a potential source for the conjugation system of chemotherapy drugs with AuNPs in cancer

treatment is also indispensable. In conclusion, cyclophosphamide-loaded AuNPs-NO may be used shortly as a promising treatment strategy with fewer side effects in normal organs.

CRedit authorship contribution statement

Fahimeh Mobaraki: Conceptualization, Methodology, Investigation, Visualization, Project administration, Writing – original draft. **Mohsen Momeni:** Visualization, Investigation, Methodology, Resources. **Maryam Barghban:** Investigation, Writing – original draft, Formal analysis. **Bahareh Farasati Far:** Writing – original draft, Writing – review & editing. **Sara Hosseini:** Software, Data curation. **Seyed Mousalreza Hosseini:** Supervision, Validation, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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