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Modulation of oxidative damage and genotoxicity of biosynthesized zinc oxide nanoparticles *via* supplementation with modified cinnamaldehyde-loaded chitosan nanoparticles in mice



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ABSTRACT

Synthesis of zinc oxide nanoparticles (ZnO-NPs) *via* green method is an outstanding alternative to conventional/regular methods; however, the safety or toxicity of the biosynthesized ZnO-NPs *in vivo* is not fully explored. This study was conducted to evaluate the protective efficiency of cinnamaldehyde-loaded chitosan nanoparticles (Cin@CSNPs) against oxidative damage and genotoxicity of ZnO-NPs in mice. ZnO-NPs were biosynthesized using the extract of fresh leaves of *Mentha pulegium* L. Cin was extracted from cinnamon essential oil, and was loaded into chitosan nanoparticle (Cin@CSNPs). Both ZnO-NPs, Cin@CSNPs and CSNPs were characterized. The *in vitro* release of Cin@CSNPs was determined. In the biological study, 6 groups of male BALB/c mice were treated by gavage for 3 weeks as follows, control group, the group received ZnO-NPs (25 mg/kg b.w), the groups received Cin@CSNPs at low dose (50 mg/kg b.w) or high dose (100 mg/kg b.w), and the groups received ZnO-NPs plus Cin@CSNPs at the 2 tested doses. Blood and tissue samples were collected for different biochemical, genetical and histological studies. The particle size of ZnO-NPs, CSNPs, and Cin@CSNPs were (20.78 ± 2.60), (170.0 ± 3.7), and (218.23 ± 2.90) nm, and ζ-potential were (32.7 ± 4.6), (8.32 ± 0.27) and (4.80 ± 0.21) mV, respectively. ZnO-NPs disturbed the biochemical and oxidative stress indices, AFP, CEA, TNF-α, chromosomal aberrations in somatic and germ cells, and sperm abnormality along with severe pathological changes in the hepatic, renal, and testicular tissues. Cin@CSNPs improved significantly all the parameters tested and the histological picture in a dose-dependent. Therefore, the biosynthesized ZnO-NPs exhibit oxidative damage and genotoxicity, and Cin@CSNPs have potential protective effects against the risks of ZnO-NPs and may be a promising tool to overcome the challenges of using Cin in food and pharmaceuticals applications.

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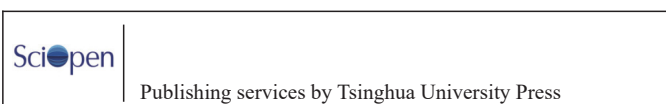
1. Introduction

Zinc (Zn) is a trace element critical for many biological functions, and its imbalance has been linked to a variety of diseases^[1]. Although

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Zn is not stored in tissues, the ingestion of excessive amounts on a chronic basis is connected with detrimental changes in the metabolism of copper, iron, cholesterol, and lipoprotein^[2]. Nowadays, nanotechnology is widely used to improve the properties of functional materials. Nanoparticles (NPs) are synthesized by several methods including chemical, physical, and biological procedures. The synthesis of NPs *via* the green method is a superior alternative to the conventional/regular methods. The physical and chemical methods for the synthesis of NPs have restricted the clinical application due to their toxicity. However, the green synthesis methods are environmentally friendly, feasible, cost-effective, and efficient technologies, with great attributes for NPs^[3]. Additionally, the biologically produced nanomaterials have high sensitivity and biocompatibility and thus have several applications in different fields^[4-6]. The increasing interest in the synthesis of zinc oxide nanoparticles (ZnO-NPs) *via* biological routes is mainly related to the reduction of the toxic chemicals used in biosynthesis^[7-8]. However, the safety or toxicity of the biosynthesized ZnO-NPs *in vivo* is not fully explored.

Cinnamon is the most popular condiment food additive in the world which is isolated from the inner bark of tropical tree species belonging to the genus *Cinnamomum* (Lauraceae family) and about 250 species worldwide are known^[9-10]. Previous reports revealed that cinnamon essential oils have several beneficial properties such as antioxidant^[11-12], antidiabetic^[13], anticancer^[14], antimicrobial^[15], anti-common cold^[16], anticholinergic^[17], antihypertensive^[18], antibacterial^[19], hepatoprotective^[20], lowering blood cholesterol^[16], gastroprotective^[21], antiviral^[22], antifungal^[23], and immunomodulatory^[24].

The main constituents of cinnamon are cinnamaldehyde (Cin), cinnamic acid, and trans-cinnamaldehyde^[25]. Cin constitutes 60%–75% of the total cinnamon essential oil and it was proven to exhibit the most biological activities of this oil^[26]. Cin is used as a food additive, flavoring agent, and food preservative^[27]. However, its application faces serious challenges owing to its high volatility, low water solubility, high-temperature sensitivity, and low redox stability^[28]. Additionally, the direct inclusion of Cin in food has a negative impact on their sensory traits. Therefore, to overcome these challenges, and extend its effectiveness time, this compound should be incorporated into nanostructured hydrophilic systems^[29]. Therefore, the present work primarily focused on the fabrication and characterization of Cin-loaded chitosan nanoparticles (Cin@CSNPs), evaluation of the protective potential of Cin@CSNPs against oxidative damage, chromosomal aberrations in somatic and germ cells, DNA damage, and reproductive impairment resulted from the biosynthesized ZnO-NPs in male mice.

2. Material and methods

2.1 Chemicals and kits

The essential oil of *Cinnamomum zeylanicum* was purchased from the National Research Centre (NRC), Cairo, Egypt (Oil Extraction Unit). High molecular weight chitosan (COS, Mw 165–175 kDa), and zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, CAS 5970-45-6) as the zinc precursor were purchased from Sigma-Aldrich (Paris, France). Kits for aminotransferases (aspartate transaminase (AST), alanine aminotransferase (ALT)), uric acid, urea, and creatinine were purchased from Randox Co. (Antrim, UK). Kits for cholesterol (Cho), triglycerides (TriG), low-density and high-density lipoprotein (LDL, HDL), albumin (Alb), and total protein (TP) were obtained from AR

Diagnostics Co. (Via Fermi, Italy). Kits for nitric oxide (NO), catalase (CAT), total antioxidants capacity (TAC), and superoxide dismutase (SOD) were purchased from Eagle Diagnostics (Dallas, TX, USA). Malondialdehyde (MDA) was supplied by Oxis Research TM Co. (USA). ELISA kit for alpha-fetoprotein (AFP) was supplied by Biochem Immuno Systems Co. (Montreal, Canada). The tumor necrosis factor-alpha (TNF- α) kit was supplied by Orgenium (Helsinki, Finland). A carcinoembryonic antigen (CEA) kit was supplied by Biodiagnostic (Giza, Egypt). All other chemicals used throughout the experiments were of the highest analytical grade available.

2.2 Isolation of Cin

Cin was extracted from cinnamon essential oil according to Suryanti et al.^[30] by the addition of sodium bisulfate through a nucleophilic addition reaction, and diethyl ether was used as solvent extraction for the separation of Cin. After evaporation, Cin was washed with distilled water until neutral then it was dried with anhydrous sodium sulfate.

2.3 Preparation of Cin-loaded chitosan nanoparticles (Cin@CSNPs)

A total of 20 mg of COS was dissolved in 40 mL acetic acid (2.0%, *V/V*) then 20 mL sodium tripolyphosphate (0.75 mg/mL) was dropped slowly with stirring. The suspended CSNPs were collected and stored in deionized water. The supernatant was discarded and CSNPs were air-dried for further use^[31]. Cin (2.4 $\mu\text{g/mL}$) was dissolved in 0.6 mL of methylene chloride (CH_2Cl_2) and the oil phase was gradually dropped into 6 mL of CSNPs aqueous solution under magnetic stirring in an ice-bath condition. Cin-loaded CSNPs (Cin@CSNPs) were spontaneously formed upon drop-wise addition of 4 mL of 0.5% (*m/V*) tripolyphosphate (TPP) according to Piras et al.^[32]. NPs were collected by centrifugation at $9\,000 \times g$ and 4 °C for 30 min and then rinsed with water. The ultrasonication was performed by a sonicator (4 min), to get a homogeneous suspension.

2.4 Biosynthesis of ZnO-NPs

The fresh leaves of *M. pulegium* L. (obtained from the Department of Medicinal and Aromatic Plants, National Research Centre, Cairo, Egypt) were washed with deionized water and allowed to dry at room temperature (32 °C). The leaves were crushed and 5 g of the powder was boiled in 100 mL of deionized water for 10 min and filtered^[33]. ZnO-NPs were biosynthesized according to Rad et al.^[34]. In brief, 50 mL of the extract was heated at 60–80 °C, and when the temperature reached 60 °C, 5 g of zinc acetate dihydrate was added and the mixture was left to boil until it became a paste. This paste was then transferred to a ceramic crucible pot and heated in a furnace at 400 °C for 2 h to obtain ZnO-NPs. The produced powder was gathered (ZnO-NPs) and used for further studies.

2.5 Characterization of ZnO-NPs and Cin@CSNPs

A JEOL JAX-840A and JEOL JEM-1230 electron micro-analyzers were used for scanning electron micrographs (SEM) for ZnO-NPs. Samples of ZnO-NPs were scattered in ethanol and then treated ultrasonically to disperse the individual particles over the gold grids^[35].

However, the morphological determination of Cin@CSNPs was analyzed using SEM (JEOL JSM6300 SEM, Tokyo, Japan) by putting the Cin@CSNPs sample onto a carbon-coated copper grid to form a thin liquid film and was negatively stained by one drop of uranyl acetate. The excess day was removed with filter paper and the film was air-dried before the observation^[36]. An Orius 1 000 CCD camera was used for image acquisition (GATAN, Warrendale, PA, USA). For measuring ζ -potential, the sample of ZnO-NPs or Cin@CSNPs was sonicated for 30–60 min just before assessment. A ZPW 388 software version 2.14 nicomp was used to calculate the average diameter. ζ -Potential and size distribution of ZnO-NPs and Cin@CSNPs was measured using a particle size analyzer (Nano-ZS, Malvern Instruments Ltd., UK). Fourier-transform infrared spectroscopy (FTIR) spectra of COS, CSNPs, Cin, and Cin@CSNPs were determined using an infrared spectrometer (Thermo Fisher Scientific Inc., Nico-let iS10, USA).

2.6 *In vitro* release test

The *in vitro* drug release of Cin was carried out using dynamic dialysis according to Xu et al.^[37]. In brief, Cin@CSNPs were transferred into a dialysis bag and PBS buffer was used as the release medium. The dialysis bag was kept at 37 °C and agitated at 70 r/min and dialysate samples (5 mL) were taken at specified intervals and replaced with the same volume of release medium. The samples were read at 291 nm using a UV spectrophotometer and the data were expressed as mean $\pm$ SD of 3 replicates of each interval.

2.7 *Experimental animals*

Male BALB/c mice (6 weeks old; (25.0 $\pm$ 0.3) g) were purchased from the Experimental Animal House Lab., National Research Centre (NRC), Cairo, Egypt. The rearing of animals was in planned laboratory circumstances of temperature ((25 $\pm$ 2)°C), well ventilation, free supplementation of water and food, and a normal light-dark cycle (12 h light/dark) at the Animal House Lab., NRC, Cairo. All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and the protocol was permitted and approved by the Animal Care and Use Committee of the NRC (ethical approval #13050302/3).

2.8 *Experimental design*

Following the adaptation period for 1 week, animals were divided into 6 groups (10 mice/group) and were treated by gavage for 3 weeks as follows: group 1, untreated normal control animals; group 2, mice that received an aqueous solution of ZnO-NPs (25 mg/kg b.w); groups 3 and 4, mice that received Cin@CSNPs at a low dose (LD) or a high dose (50 or 100 mg/kg b.w); groups 5 and 6, mice that received ZnO-NPs plus the low or the high dose of Cin@CSNPs. Blood samples were gathered from each animal within different experimental groups *via* the retro-orbital venous plexus under isoflurane anesthesia after being fasted for 12 h. Sera were separated using cooling centrifugation and kept at –20 °C until analysis. After collecting the blood samples, 5 mice from each group were i.p.

injected with colchicines (3 mg/kg b.w), 2 h before sacrifice. Thereafter, all mice were sacrificed and liver, kidney, and testis tissue samples were taken. The samples of liver and kidney (approximately 0.05–0.10 g) were weighed and homogenized in phosphate buffer (pH 7.4) and centrifuged for 4 min at 1 700 r/min^[38–39], and the supernatant was used for the estimation of NO, MDA, SOD, TAC, and CAT following the manufacture instructions. Another sample of liver, kidney, and testis from each mouse was used for the histological examination. The samples were fixed in 10% neutral formalin and paraffin-embedded. Sections (5 μ m thickness) were stained with hematoxylin and eosin (H&E) for the histological examination^[40]. Another sample of liver was harvested from each mouse for DNA fragmentation assay; however, somatic chromosomal aberration was conducted in bone marrow collected from the femora of each mouse. Germ cell chromosomal aberration and sperm abnormality were assayed in sperms collected from the cauda epididymis of each testis of mice.

2.9 *Molecular genetic analysis*

2.9.1 *DNA fragmentation assay*

DNA fragmentation in hepatocytes was performed according to Burton^[41], and the modification of Perandones et al.^[42]. The percentage of DNA fragmentation was expressed by the formula:

$$\text{DNA fragmentation (\%)} = \frac{\text{OD of supernatant}}{\text{OD of supernatant} + \text{OD of pellet}} \times 100$$

2.9.2 *Chromosomal aberration in bone marrow cells*

The determination of chromosomal aberrations was performed in the bone marrow of mice previously injected i.p. with colchicine^[43]. A sum of 100 well-spread metaphases per mouse was analyzed and metaphases with gaps, chromosome or chromatid breakage, and fragments were recorded.

2.9.3 *Sperm morphology and chromosome aberrations assay in the germ cells*

Both of the cauda epididymis from each mouse were dissected and smears were made according to WYROBEK et al.^[44]. The smears were fixed in methanol and stained with 10% Giemsa in Sörensen buffer for 10 min and a sum of 1 000 sperms/animal was examined. Abnormalities of sperm head were determined to be of normal or abnormal shape. The dikinase-metaphase I cells collected from the spermatocytes were prepared by the air-drying technique suggested by Evans et al.^[45].

2.10 *Statistical analysis*

All data were statistically analyzed using the computer software SPSS (Statistical Package for Social Science, version 20, Armonk, New York: IBM Corp.). To quantify differences across groups, a one-way analysis of variance (ANOVA) was used, followed by Tukey's post-hoc multiple comparison test and the data were expressed as mean $\pm$ SEM. However, the data for chromosomal aberrations were analyzed by a *t*-test. *P*-values < 0.05 were used to establish significant differences between groups.

3. Results

The DLS of the biosynthesized ZnO-NPs revealed that the average particle size was (20.78 ± 2.60) nm (Fig. 1A) with a ζ -potential of (32.7 ± 4.6) mV (Fig. 1B) and the SEM analysis indicated that the biosynthesized ZnO-NPs have a semi-rounded shape (Fig. 1C). The DLS results of CSNPs showed an average size of (170.0 ± 3.7) nm (Fig. 1D) with ζ -potential of (8.32 ± 0.27) mV (Fig. 1E) and irregular particle shapes were shown by SEM analysis (Fig. 1F). The results also indicated that the loading of Cin to CSNPs (Cin@CSNPs) increased the average size of the particles to (218.23 ± 2.90) nm (Fig. 1G), and also decreased the ζ -potential to (4.80 ± 0.21) mV (Fig. 1H) with an irregular particle shape (Fig. 1I).

The FTIR spectra of the biosynthesized ZnO-NPs (Fig. 2) showed a peak at 3650.7 cm^{-1} which represented the O–H stretching vibration of the intermolecular hydrogen bond. The peak at 1565.87 cm^{-1} represents the amine group (H–H) of the enzymes or proteins in the *M. pulegium* leaf extract. The peak at 1215.97 cm^{-1} represents the C–O bond and the peak that appears at 1043.49 cm^{-1} is attributed to the phenolic groups in the extract; however, the peak that appears at 889.54 cm^{-1} represents the formation of tetrahedral-coordination of zinc and the peaks between 543.83 and 427.77 cm^{-1} are attributed to ZnO stretching bonds.

The FTIR spectra of COS powder (Fig. 3, line 1) showed several peaks at 2343 , 1192 , 1634 , 1382 , 1117 , 990 and 740 cm^{-1} assigned to the –OH and –NH stretching, –CH stretching, the amide I, –CH₂

bending, asymmetrical deformation of CH₃ groups, antisymmetric stretching of C–O–C and C–H stretching, and C–O–C stretching, respectively. The FTIR data presented in Fig. 3, line 2 also showed the interaction of COS with TPP in the synthesized CSNPs which showed several peaks at 3433 , 2955 , 1637 , 1512 , 1339 , 1278 , 1222 , 1164 , 874 , 753 and 653 cm^{-1} . These peaks may be represented by the –OH and –NH stretching, –CH stretching, the amide I, –CH₂ bending, asymmetrical deformation of CH₃ groups, anti-symmetric stretching of C–O–C, C–H stretching, and C–O–C stretching, respectively. The disappearance of the peak at 1637 cm^{-1} and the appearance of the peaks at 1637 and 1512 cm^{-1} represented the amides I and II, respectively. The data also showed that the peak at 1382 cm^{-1} was shifted to 1375 cm^{-1} and 2 new peaks appeared at 1278 and 1222 cm^{-1} representing the C–H asymmetric bending of CH₂ group and C–O–C stretching. These peaks were presented in CSNPs and were not present on COS. Additionally, the bands at 1164 cm^{-1} and 874 cm^{-1} may be attributed to the P=O stretching and the P–O beside the P–O–P of TPP. The spectrum of pure Cin (Fig. 3, line 3) displays characteristic peaks at 2920 cm^{-1} (–CH stretching), 1621 cm^{-1} (–NH bending), 1553 cm^{-1} (–CH bending), 1486 cm^{-1} (–CH₂ bending), 1247 cm^{-1} (C–O–C stretching), 1142 cm^{-1} (C–O–C stretching), and 759 cm^{-1} (C–H out-of-plane bending). The FTIR data (Fig. 3, line 4) also confirmed the presence of Cin in the nanoparticles as shown by the shift of the peaks at 3433 cm^{-1} (–OH stretching), 2955 cm^{-1} (–CH stretching), and 1164 cm^{-1} (–CH stretching) found in the spectra of

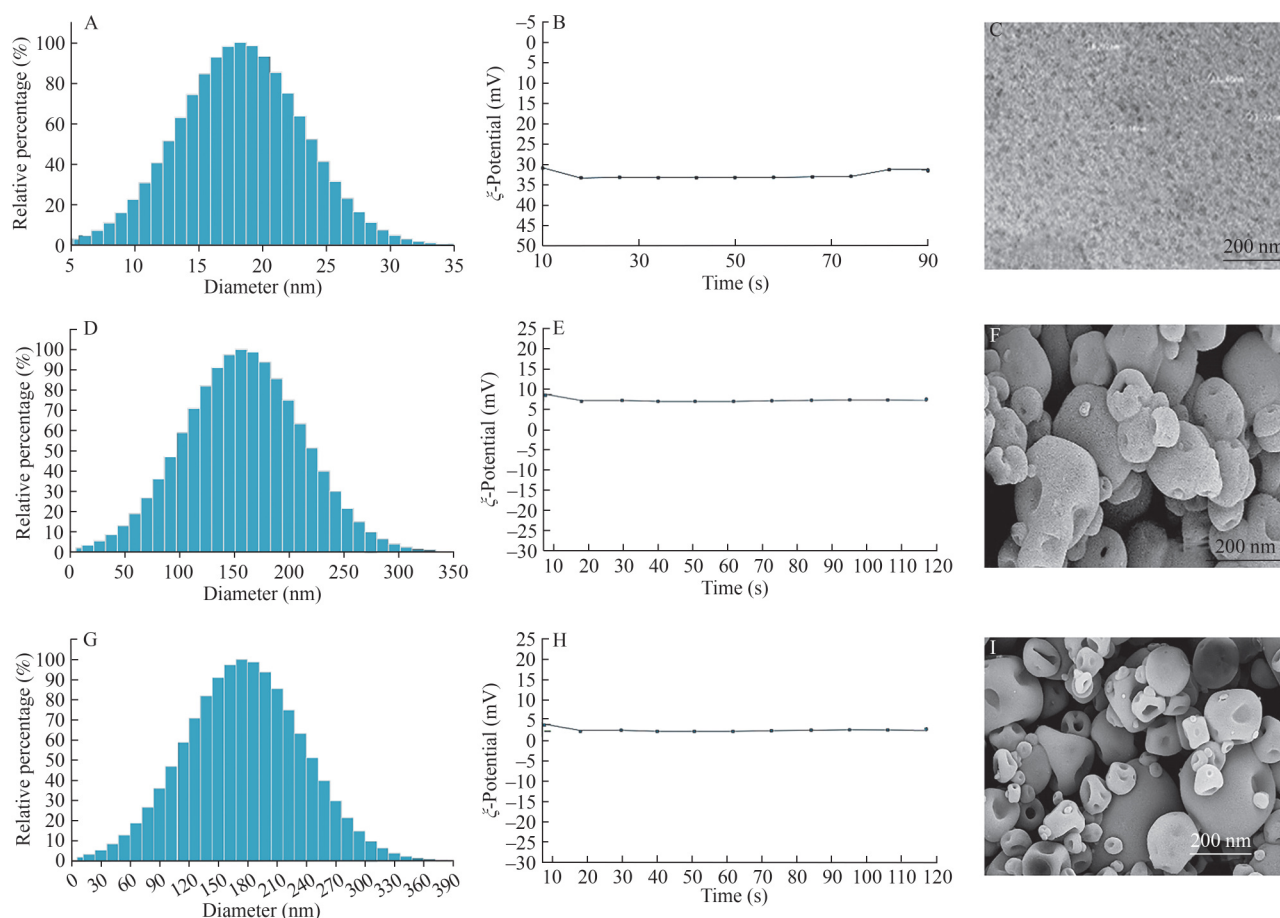


Fig. 1 The (A) size distribution by DLS measurements, (B) ζ -potential, and (C) SEM of ZnO-NPs; the (D) size distribution by DLS measurements, (E) ζ -potential and (F) SEM of CSNPs; and the (G) size distribution by DLS measurements, (H) ζ -potential and (I) SEM of Cin@CSNPs.

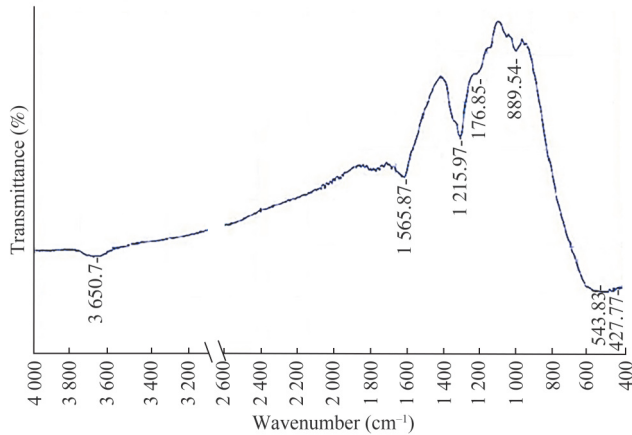


Fig. 2 FTIR spectra of the biosynthesized ZnO-NPs from *M. pulegium* L. leaf extracts and zinc acetate.

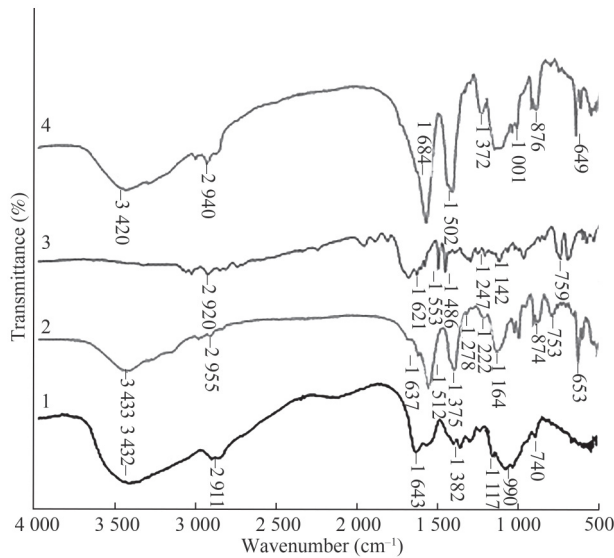


Fig. 3 FTIR spectra of (1) COS powder, (2) CSNPs, (3) pure Cin and (4) Cin@CNPs.

CSNPs to 3 420, 2 940, and 1 001 cm^{-1} in the spectra of Cin@CSNPs, respectively. Moreover, the peak at 1 637 cm^{-1} (amide I) which was present in the spectrum of blank CSNPs was significantly decreased or disappeared in the spectra of Cin@CSNPs. The *in vitro* release of Cin from Cin@CSNPs showed a fast release at first and it reached 50% after 2 h then it increased gradually to reach its maximum after 30 h with a cumulative release amount of 88.6 % at 48 h as shown in Fig. 4.

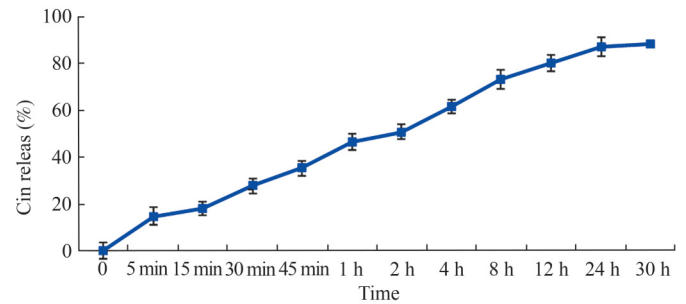


Fig. 4 *In vitro* release of Cin from Cin@CNPs using an initial weight ratio of COS:Cin of 4:1. The data are represented as mean $\pm$ SD of 3 replicates.

The results of biochemical parameters in serum (Table 1) showed that oral administration of ZnO-NPs increased significantly the activity of ALT, AST, and the level of creatinine, uric acid, urea, cholesterol, TriG, and LDL and decreased significantly the levels of Alb, TP, and HDL. All the biochemical indices in the groups that received Cin@CSNPs (LD) or Cin@CSNPs (HD) were comparable to the negative control group except creatinine in both groups which was significantly higher than the negative control group, and LDL in the group that received Cin@CSNPs (HD) which was higher than the untreated control and HDL which was higher than the untreated control group. The combined treatment with ZnO-NPs plus Cin@CSNPs improved significantly all the biochemical parameters tested in a dose-dependent manner and the high dose could normalize most of these parameters.

The antioxidant markers (CAT, SOD, and TAC) in the renal and hepatic tissue revealed a significant decline in the ZnO-NPs alone-treated group compared to the normal control group (Table 2). The

Table 1

Effect of Cin@CSNPs at low or high dose on serum biochemical parameters in mice treated with ZnO-NPs.

Group	Control	ZnO-NPs	Cin@CSNPs (LD)	Cin@CSNPs (HD)	ZnO-NPs + Cin@CSNPs (LD)	ZnO-NPs + Cin@CSNPs (HD)
ALT (U/L)	58.96 $\pm$ 2.32 ^a	74.98 $\pm$ 1.24 ^b	57.72 $\pm$ 1.74 ^a	57.64 $\pm$ 2.53 ^a	63.83 $\pm$ 2.38 ^c	57.97 $\pm$ 2.63 ^a
AST (U/L)	148.28 $\pm$ 2.41 ^a	187.82 $\pm$ 3.81 ^b	147.38 $\pm$ 2.72 ^a	148.82 $\pm$ 2.73 ^a	161.28 $\pm$ 2.71 ^c	149.69 $\pm$ 2.62 ^a
Alb (g/dL)	6.53 $\pm$ 0.26 ^a	4.53 $\pm$ 0.53 ^b	6.58 $\pm$ 0.25 ^a	6.65 $\pm$ 0.85 ^a	5.64 $\pm$ 0.24 ^c	6.52 $\pm$ 0.17 ^a
TP (g/dL)	6.43 $\pm$ 0.28 ^a	5.12 $\pm$ 0.21 ^b	6.43 $\pm$ 0.32 ^a	6.73 $\pm$ 0.28 ^a	6.48 $\pm$ 0.25 ^a	6.42 $\pm$ 0.21 ^a
Creatinine (mg/dL)	0.59 $\pm$ 0.04 ^a	1.32 $\pm$ 0.03 ^b	0.63 $\pm$ 0.06 ^c	0.62 $\pm$ 0.04 ^c	0.77 $\pm$ 0.02 ^d	0.63 $\pm$ 0.03 ^c
Uric acid (mg/dL)	6.12 $\pm$ 0.89 ^a	9.14 $\pm$ 0.28 ^b	6.42 $\pm$ 0.31 ^a	6.32 $\pm$ 0.41 ^a	6.36 $\pm$ 0.21 ^a	6.17 $\pm$ 0.21 ^a
Urea (mg/dL)	38.5 $\pm$ 0.32 ^a	46.72 $\pm$ 2.13 ^b	39.87 $\pm$ 2.68 ^a	38.24 $\pm$ 2.31 ^a	40.16 $\pm$ 1.62 ^c	38.28 $\pm$ 2.24 ^a
Cholesterol (mg/dL)	64.50 $\pm$ 2.42 ^a	84.32 $\pm$ 2.67 ^b	63.93 $\pm$ 2.21 ^a	63.17 $\pm$ 2.71 ^a	76.23 $\pm$ 2.15 ^d	68.36 $\pm$ 2.35 ^c
Tri G (mg/dL)	135.82 $\pm$ 3.42 ^a	149.31 $\pm$ 2.38 ^b	136.37 $\pm$ 2.16 ^a	134.15 $\pm$ 2.61 ^a	141.32 $\pm$ 2.60 ^d	136.32 $\pm$ 3.15 ^a
HDL (mg/dL)	46.43 $\pm$ 1.26 ^a	32.21 $\pm$ 1.49 ^b	48.22 $\pm$ 2.31 ^a	53.21 $\pm$ 2.16 ^c	38.21 $\pm$ 2.32 ^d	45.97 $\pm$ 2.62 ^a
LDL (mg/dL)	54.17 $\pm$ 1.42 ^a	63.17 $\pm$ 2.28 ^b	53.23 $\pm$ 1.26 ^a	48.24 $\pm$ 1.44 ^c	59.28 $\pm$ 2.82 ^d	53.15 $\pm$ 2.31 ^c

Note: Within each row, means superscripts with different letters are significantly different ($P < 0.05$). Data are expressed as mean $\pm$ SEM; LD, low dose; HD, high dose.

Table 2

Effects of Cin@CSNPs on hepatic and renal oxidative stress markers in mice treated with ZnO-NPs.

Group	Liver					Kidney				
	SOD (U/mg)	TAC (mmol/g)	CAT (mU/g)	NO (μ mol/g)	MDA (nmol/g)	SOD (U/mg)	TAC (mmol/g)	CAT (mU/g)	NO (μ mol/g)	MDA (nmol/g)
Control	52.31 $\pm$ 2.41 ^a	26.41 $\pm$ 2.11 ^a	13.14 $\pm$ 1.42 ^a	2.81 $\pm$ 0.31 ^a	39.42 $\pm$ 2.27 ^a	28.31 $\pm$ 1.75 ^a	24.12 $\pm$ 1.62 ^a	11.87 $\pm$ 0.63 ^a	2.32 $\pm$ 0.12 ^a	69.72 $\pm$ 2.38 ^a
ZnO-NPs	31.22 $\pm$ 2.15 ^b	16.12 $\pm$ 1.92 ^b	9.31 $\pm$ 0.76 ^b	5.33 $\pm$ 0.28 ^b	68.84 $\pm$ 3.13 ^b	16.29 $\pm$ 1.72 ^b	17.19 $\pm$ 0.62 ^b	8.18 $\pm$ 0.82 ^b	4.55 $\pm$ 0.31 ^b	82.1 $\pm$ 3.42 ^b
Cin@CSNPs (LD)	53.21 $\pm$ 2.52 ^a	26.77 $\pm$ 1.26 ^a	13.72 $\pm$ 1.62 ^a	2.72 $\pm$ 0.11 ^a	37.25 $\pm$ 3.52 ^a	30.15 $\pm$ 1.82 ^a	26.33 $\pm$ 1.25 ^c	12.14 $\pm$ 1.51 ^a	2.52 $\pm$ 0.12 ^a	68.23 $\pm$ 2.34 ^a
Cin@CSNPs (HD)	55.56 $\pm$ 2.29 ^c	27.13 $\pm$ 1.52 ^a	14.45 $\pm$ 1.32 ^c	2.63 $\pm$ 0.14 ^a	38.21 $\pm$ 2.35 ^a	33.16 $\pm$ 1.72 ^c	27.10 $\pm$ 2.43 ^c	14.17 $\pm$ 1.52 ^c	2.22 $\pm$ 0.13 ^a	57.25 $\pm$ 2.80 ^a
ZnO-NPs + Cin@CSNPs (LD)	41.14 $\pm$ 2.32 ^d	19.31 $\pm$ 1.62 ^c	11.39 $\pm$ 0.82 ^d	4.83 $\pm$ 0.14 ^c	53.21 $\pm$ 1.33 ^d	20.18 $\pm$ 2.17 ^d	19.21 $\pm$ 1.33 ^d	9.88 $\pm$ 0.78 ^d	3.12 $\pm$ 0.25 ^c	76.51 $\pm$ 2.26 ^d
ZnO-NPs + Cin@CSNPs (HD)	48.21 $\pm$ 2.45 ^c	24.17 $\pm$ 1.55 ^d	13.11 $\pm$ 1.22 ^a	3.22 $\pm$ 0.2 ^d	41.83 $\pm$ 2.31 ^c	26.39 $\pm$ 2.43 ^c	23.11 $\pm$ 2.48 ^a	11.02 $\pm$ 0.41 ^c	2.32 $\pm$ 0.51 ^a	71.23 $\pm$ 2.19 ^c

Note: Within each column, means superscripts with different letters are significantly different ($P < 0.05$). Data are expressed as mean $\pm$ SEM; LD, low dose; HD, high dose.

antioxidant parameters in renal and hepatic tissue of mice that administrated Cin@CSNPs (LD) were comparable to the normal control group except for the renal TAC which was significantly higher than that of the normal control group. Additionally, Cin@CSNPs (HD) increased significantly the antioxidant parameters in the liver and kidney except for the hepatic TAC which was in the normal range of the negative control group. Both the low and the high doses of Cin@CSNPs did not affect significantly NO and MDA levels in the liver and kidney (Table 2). The combined treatment with ZnO-NPs plus Cin@CSNPs could improve the antioxidant and oxidative markers and the high dose was more efficacious than the low dose and could normalize hepatic CAT, renal TAC, and NO.

Additionally, administration of ZnO-NPs increased significantly the levels of AFP, CEA, and TNF- α (Table 3); However, administration of Cin@CSNPs (LD) did not affect AFP and TNF- α and decreased CEA. The high dose of Cin@CSNPs did not affect AFP but induced a significant decrease in TNF- α and CEA. The combined treatment with ZnO-NPs plus Cin@CSNPs improved significantly these markers in a dose-dependent fashion.

The results depicted in Table 4 revealed that treatment with ZnO-NPs alone increased significantly the fragmentation of DNA in hepatic tissue. Mice administrated Cin@CSNPs at the 2 tested doses did not show any significant changes in the mean or percentage of DNA fragmentation. The co-administration with ZnO-NPs and Cin@CSNPs at both tested doses exhibited a remarkable improvement in DNA fragmentation. The recorded percentage inhibition of DNA fragmentation in hepatocytes was 42.3% and 53.3% for the low and high doses, respectively.

The present results indicated that treatment with ZnO-NPs increased the chromosomal aberrations in bone marrow cells which reached (21.6 $\pm$ 0.65)% including chromosomal gap, fragmentation, break, gap plus break, and gap plus break or fragmentation (Table 5). No significant alterations in chromosomal aberrations number were noticed in mice that received Cin@CSNPs either at the low or high dose. Moreover, the co-administration of ZnO-NPs plus Cin@CSNPs succeeded in limiting the increase in the total number of chromosomal aberrations in a dose-dependent pattern and the inhibition percentage reached 41.79% and 56.7% in the groups administrated ZnO-NPs plus the low or high dose of Cin@CSNPs, respectively. Moreover, the influences of various treatments on the number and average percentage of different types of chromosomal aberrations in the spermatocytes (Table 6) indicated that administration of ZnO-NPs significantly increased the number of different types of chromosomal aberrations in sperms. Administration of the tested doses of Cin@CSNPs alone did not show any significant alternations

Table 3Effects of Cin@CSNPs on serum AFP, CEA and TNF- α in mice treated with ZnO-NPs.

Group	AFP (ng/mL)	CEA (ng/mL)	TNF- α (ng/mL)
Control	0.06 $\pm$ 0.03 ^a	2.23 $\pm$ 0.17 ^a	0.31 $\pm$ 0.02 ^a
ZnO-NPs	0.09 $\pm$ 0.02 ^b	3.13 $\pm$ 0.31 ^b	0.44 $\pm$ 0.04 ^b
Cin@CSNPs (LD)	0.05 $\pm$ 0.01 ^a	2.14 $\pm$ 0.21 ^c	0.30 $\pm$ 0.02 ^a
Cin@CSNPs (HD)	0.06 $\pm$ 0.04 ^a	2.11 $\pm$ 0.14 ^c	0.28 $\pm$ 0.02 ^c
ZnO-NPs + Cin@CSNPs (LD)	0.07 $\pm$ 0.21 ^c	2.92 $\pm$ 0.11 ^d	0.38 $\pm$ 0.05 ^d
ZnO-NPs + Cin@CSNPs (HD)	0.07 $\pm$ 0.03 ^c	2.41 $\pm$ 0.05 ^c	0.35 $\pm$ 0.04 ^c

Note: Within each column, means superscripts with different letters are significantly different ($P < 0.05$). Data are expressed as mean $\pm$ SEM; LD, low dose; HD, high dose.**Table 4**

DNA fragmentation in the hepatocytes of mice treated with Cin@CSNPs alone or in combination with ZnO-NPs.

Treatment	DNA fragmentation(%)	
	Mean $\pm$ SEM	Inhibition
Control	3.18 $\pm$ 0.20 ^a	0.0
ZnO-NPs	11.8 $\pm$ 0.63 ^b	0.0
Cin@CSNPs (LD)	3.21 $\pm$ 0.20 ^a	0.0
Cin@CSNPs (HD)	3.15 $\pm$ 0.19 ^a	0.0
ZnO-NPs + Cin@CSNPs (LD)	6.80 $\pm$ 0.38 ^c	42.3
ZnO-NPs + Cin@CSNPs (HD)	5.50 $\pm$ 0.32 ^d	53.3

Note: Means superscripts with different letters are significantly different ($P < 0.05$). Data are expressed as mean $\pm$ SEM; LD, low dose; HD, high dose.

in the percentage of chromosomal aberrations in the diakinesis-metaphase I cell when compared with the control (Table 6). Moreover, administration of Cin@CSNPs decreased the average percentage of aberrant cells ($P < 0.01$) induced by ZnO-NPs. The lowering in the percentage of chromosome aberrations in diakinesis-metaphase I cells was noticed in the groups that received ZnO-NPs plus Cin@CSNPs (LD) or Cin@CSNPs (HD) and reached 41.79% and 56.7%, respectively. In addition, the percentage of sperm abnormalities reached 9.8% in animals treated with ZnO-NPs compared with 1.44% for the negative control group (Table 7 and Fig. 5). The results in Table 7 also displayed the reduction of sperm abnormalities percentage which reached 5.22% and 4.82% in the group treated simultaneously with ZnO-NPs plus Cin@CSNPs (LD) or Cin@CNP (HD), respectively.

The histological results of the liver of the untreated control animals showed that liver cells are normally arranged and radiate

Table 5

Number and mean percentage of the different types of chromosomal aberrations in bone marrow cells of mice treated with Cin@CSNPs alone or in combination with ZnO-NPs.

Treatment	No. (%) of metaphases with					Total chromosomal aberrations		Inhibition (%)
	Gap	Fragment	break	Fragment + break	Gap + (Fragment or break)	Including gaps	Excluding gaps	
Control	5 (1.0)	7 (1.4)	2 (0.4)	1 (0.2)	0 (0)	3.20 ± 0.23	2.00 ± 0.22	0.00
ZnO-NPs	19 (3.8)	49 (9.8)	19 (3.8)	12 (2.4)	9 (1.8)	21.60 ± 0.65 ^a	17.8 ± 0.5 ^a	0.00
Cin@CSNPs (LD)	4 (0.8)	8 (1.6)	1 (0.2)	1 (0.2)	1 (0.2)	3.00 ± 0.23	2.2 ± 0.2	0.00
Cin@CSNPs (HD)	7 (1.4)	7 (1.4)	2 (0.4)	0 (0)	0 (0)	3.2 ± 0.2	1.80 ± 0.23	0.00
ZnO-NPs + Cin@CSNPs (LD)	14 (2.8)	30 (6.0)	9 (1.8)	4 (0.8)	4 (0.8)	12.20 ± 0.43 ^b	9.40 ± 0.54 ^b	41.79
ZnO-NPs + Cin@CSNPs (HD)	12 (2.4)	27 (5.4)	7 (1.4)	3 (0.6)	3 (0.6)	10.40 ± 0.33 ^b	8.0 ± 0.4 ^b	56.7

Note: The total number of scored metaphases is 500 (5 animals/group); Frag, fragment. ^aSignificant at 0.01 level comparing to control (non-treated); ^bSignificant at 0.01 level comparing to treatment. Data are expressed as number, percentage and mean ± SEM; LD, low dose; HD, high dose.

Table 6

Number and mean percentage of the different types of chromosomal aberrations in mice treated with Cin@CSNPs alone or in combination with ZnO-NPs.

Treatment	No. (%) of different types of chromosomal aberrations					Total aberrations		Inhibition (%)
	XY univalent	Autosomal univalent	XY+ autosomal univalent	Fragment or break	Fragment or break + XY univalent	No.	Mean ± SEM (%)	
Control	11 (2.2)	3 (0.6)	1 (0.2)	0 (0)	0 (0)	15	3.0 ± 0.2	0.00
ZnO-NPs	41 (8.2)	14 (2.8)	8 (1.6)	2 (0.4)	2 (0.4)	67	13.40 ± 0.65 ^a	0.00
Cin@CSNPs (LD)	10 (2.0)	4 (0.8)	2 (0.4)	0 (0)	0 (0)	16	3.20 ± 0.21	0.00
Cin@CSNPs (HD)	11 (2.2)	3 (0.6)	0 (0)	0 (0)	0 (0)	14	2.8 ± 0.2	0.00
ZnO-NPs + Cin@CSNPs (LD)	23 (4.6)	9 (1.8)	6 (1.2)	1 (0.2)	0 (0)	39	7.80 ± 0.52 ^b	41.79
ZnO-NPs + Cin@CSNPs (HD)	16 (3.2)	9 (1.8)	4 (0.8)	0 (0)	0 (0)	29	5.80 ± 0.32 ^b	56.7

Note: The total number of scored metaphases is 500 (5 animals / group). ^aSignificant at 0.01 level comparing to control (non-treated); ^bSignificant at 0.01 level comparing to treatment. Data are expressed as number, percentage and mean ± SEM; LD, low dose; HD, high dose.

Table 7

Number and percentage of different types of sperm shape abnormalities in male mice treated with Cin@CSNPs alone or in combination with ZnO-NPs.

Treatments	No. of examined sperm	No. (%) of sperms with							No. of abnormal sperm	Abnormal sperms mean ± SEM (%)	Inhibition (%)
		Head abnormalities						Tail abnormalities			
		Amorphous	Triangle	Without hook	Banana	Small	Big				
Control	5 000	24 (0.48)	33 (0.66)	9 (0.18)	0 (0)	0 (0)	0 (0)	6 (0.12)	72	1.44 ± 0.13	0.00
ZnO-NPs	5 000	118 (2.36)	129 (2.58)	98 (1.96)	17 (0.34)	10 (0.2)	4 (0.08)	114 (2.28)	490	9.80 ± 0.31 ^a	0.00
Cin@CSNPs (LD)	5 000	23 (0.46)	31 (0.62)	11 (0.22)	0 (0)	0 (0)	0 (0)	10 (0.2)	75	1.50 ± 0.24	0.00
Cin@CSNPs (HD)	5 000	24 (0.48)	33 (0.66)	12 (0.24)	0 (0)	0 (0)	0 (0)	9 (0.18)	78	1.56 ± 0.20	0.00
ZnO-NPs + Cin@CSNPs (LD)	5 000	60 (1.2)	71 (1.42)	37 (0.74)	7 (0.14)	3 (0.06)	0 (0)	83 (1.66)	261	5.22 ± 0.24 ^b	46.7
ZnO-NPs + Cin@CSNPs (HD)	5 000	55 (1.1)	69 (1.38)	34 (0.68)	3 (0.06)	2 (0.04)	0 (0)	78 (1.56)	241	4.82 ± 0.32 ^b	50.8

Note: ^aSignificant at 0.01 level comparing to control (non-treated); ^bSignificant at 0.01 level comparing to treatment. Data are expressed as number, percentage and mean ± SEM; LD, low dose; HD, high dose.

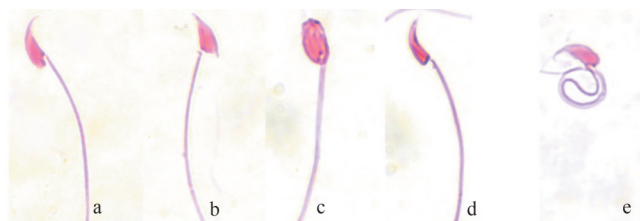


Fig. 5 Sperm shape abnormalities induced in male mice showing (a) normal, (b) triangular (c) amorphous (d) small head and (e) coiled tail.

from the central vein and are separated by blood sinusoids (Fig. 6A). The liver of ZnO-NPs-treated animals showed congested blood vessels, with fibrosis around the blood vessels and blood sinusoids besides focal nodule of infiltrated cells (Fig. 6B). The liver sections in the group treated with Cin@CSNPs (LD) showed nearly normal

hepatocytes except minimal inflammation around them (Fig. 6C). However, the liver of the animals in Cin@CSNPs (HD) group showed nearly normal hepatocytes except few fatty droplets around them (Fig. 6D). The liver of animals in ZnO-NPs plus Cin@CSNPs (LD) group showed congested and coagulative necrosis with few cellular infiltration, the hepatic cells showed normal arrangement with focal vacuolar degeneration, the nuclei have signs of apoptosis in the shape of chromatin fragmentation, shrunken pyknosis and deeply stained (Fig. 6E). However, the liver in ZnO-NPs plus Cin@CSNPs (HD)-treated group showed nearly normal hepatocytes architecture and vesiculated nuclei with few congestion and fibrosis around the main blood vessels (Fig. 6F).

The control kidney showed regular tubular brush borders, intact glomeruli and Bowman's capsule (Fig. 7A). The kidney of ZnO-NPs-treated animals showed focal and mild epithelial necrosis with epithelial cells

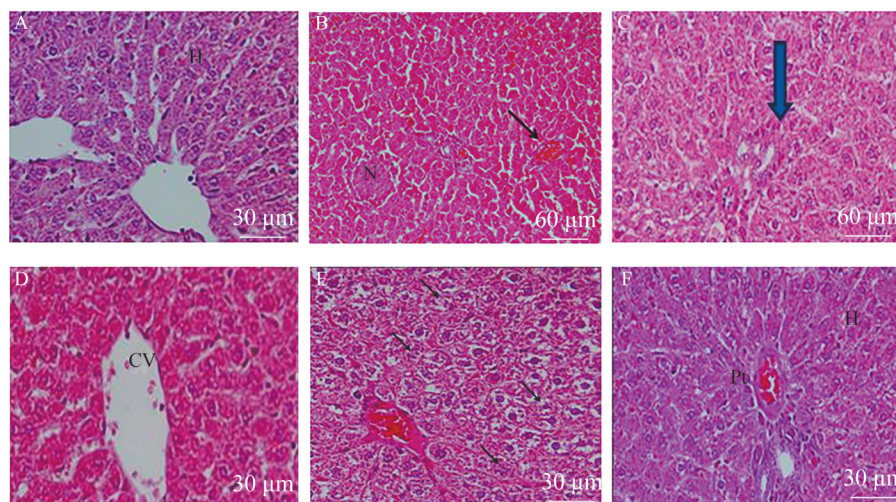


Fig. 6 A photomicrograph of a liver section from: (A) control mice showing the normal arranged hepatocytes (H) radiating from the central vein and separated by the blood sinusoids, (B) mice treated with ZnO-NPs showing congested blood vessels and fibrosis around blood vessels and in blood sinusoids (arrow) and focal nodule (N) of cellular infiltration also scattered, (C) mice treated with Cin@CSNPs (LD) showing the central vein and portal tract (arrow) with nearly normal hepatocytes except minimal inflammation around it, (D) mice treated with Cin@CSNPs (HD) showing the central vein (cv) and portal tract (pt) with nearly normal hepatocytes except few fatty droplets around them, (E) mice treated with ZnO-NPs plus Cin@CSNPs (HD) showing congested and coagulative necrosis with few cellular infiltration, the hepatic cells with normal arrangement with focal vacuolar degeneration and their nuclei have signs of apoptosis (arrow) in the form of chromatin fragmentation, shrunken pyknosis and deeply stained, and (F) mice treated with ZnO-NPs plus Cin@CSNPs (HD) showing nearly normal hepatocytes architecture with vesiculated nuclei with few congestion and fibrosis around the main blood vessels.

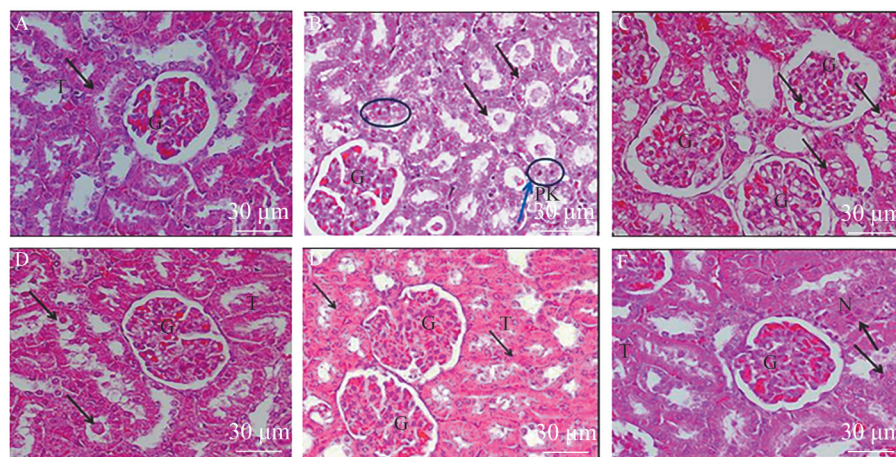


Fig. 7 Photomicrograph of a section of kidney section of (A) control mice showing normal tubular brush borders (arrow) and intact glomeruli (G) and Bowman's capsule, (B) mice treated with ZnO-NPs showing focal and mild epithelial necrosis with epithelial cells pyknosis (PK), hyaline cast formation (arrow) and focal glomerular mesangial damage, (C) mice treated with Cin@CSNPs (LD) showing focal and tubular dilation with vacuolation (arrow), focal glomerular mesangial proliferation with vacuolation (G) and mild hemorrhage with lymphocytic infiltration, (D) mice treated with Cin@CSNPs (HD) showing nearly normal tubules and glomeruli, focal debris of tubular epithelial cells and hyaline cast (arrow), focal glomerular mesangial proliferation (G) and mild hemorrhage with mild degree of venous congestion, (E) mice treated with ZnO-NPs plus Cin@CSNPs (LD) showing marked improvement in kidney morphology, nearly normal tubules and glomeruli, few interstitial hemorrhage are seen, and (F) mice treated with ZnO-NPs plus Cin@CSNPs (HD) showing focal tubular necrosis (arrows), focal glomerular mesangial proliferation (G) and mild hemorrhage with mild degree of venous congestion.

pyknosis, hyaline cast formation and focal glomerular mesangial damage (Fig. 7B). The kidney of Cin@CSNPs (LD)-treated group showed focal and tubular dilation with vacuolation, focal glomerular mesangial proliferation with vacuolation and mild hemorrhage with lymphocytic infiltration (Fig. 7C). However, the kidney of Cin@CSNPs (HD)-treated mice showed nearly normal tubules and glomeruli, focal debris of tubular epithelial cells and hyaline cast, focal glomerular mesangial proliferation and mild hemorrhage with mild degree of congestion in the veins (Fig. 7D). The kidney in ZnO-NPs plus Cin@CSNPs (LD)-treated mice showed marked improvement in kidney morphology with nearly normal tubules and

glomeruli but few interstitial hemorrhage were noticed (Fig. 7E). However, the kidney in ZnO-NPs plus Cin@CSNPs (HD) group showed focal tubular necrosis, focal glomerular mesangial proliferation and mild hemorrhage with mild degree of congestion in the veins (Fig. 7F).

The examination of control testes sections showed the normal appearance of seminiferous tubules, different layers of spermatogenic cells ranging from spermatogonia, spermatocytes, and spermatids to spermatozoa (Fig. 8A). The testis sections of ZnO-NPs-treated group showed disturbance and disappearance of the seminiferous tubules, and spermatogenic series, some tubules have enlarged lumen with exfoliation cells enlarged interstitium with vacuolation and congestion

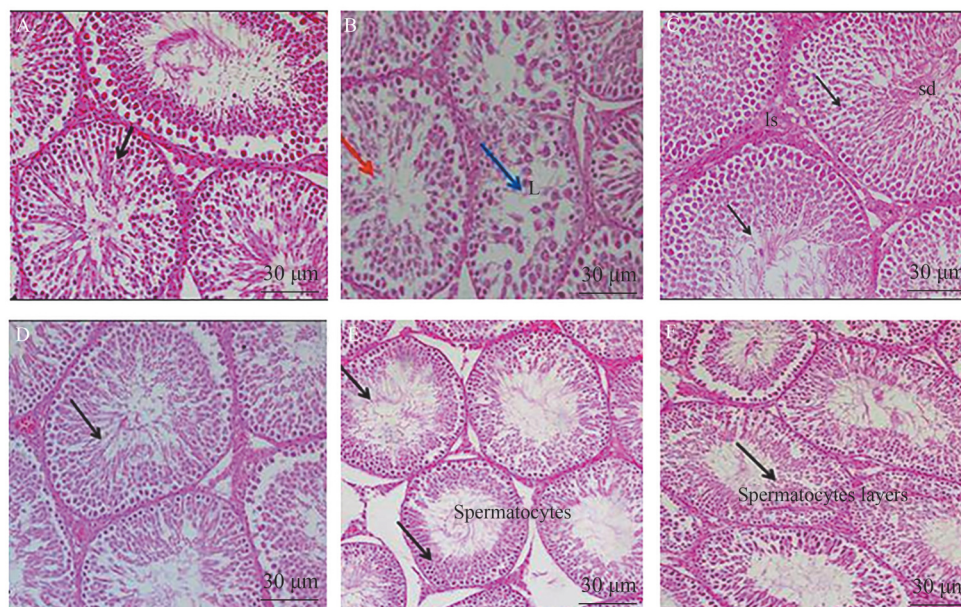


Fig. 8 Photomicrographs of testis sections from (A) control mice showing the normal structure of the seminiferous tubules and different layers of spermatogenic cells ranging from spermatogonia, spermatocytes, spermatids to spermatozoa, (B) mice treated with ZnO-NPs showing disturbance and disappearance of seminiferous tubules spermatogenic series (arrow), some tubules have enlarged lumen (L) with exfoliation cells, (C) mice treated with Cin@CSNPs (LD) showing intact seminiferous tubules with normal spermatozoa in the lumen (Sd) and complete spermatogenic germ cell series (arrow), interstitial edema (IS) and inflammatory cells, (D) mice treated with Cin@CSNPs (HD) showing normal histoarchitecture of seminiferous tubules (arrow), interstitium and the compact arrangement of seminiferous epithelium and lumen filled with sperm, (E) mice treated with ZnO-NPs plus Cin@CSNPs (LD) showing restoration of the normal structure in most of seminiferous tubules and normal interstitium, and (F) mice treated with ZnO-NPs plus Cin@CSNPs (HD) showing compact seminiferous tubules and spermatocytes.

of blood vessels (Fig. 8B). Testis section of Cin@CSNPs (LD) group showed intact seminiferous tubules with normal spermatozoa in the lumen and complete series of spermatogenic germ cells, and few interstitial edema and inflammatory cells (Fig. 8C). The testis of Cin@CSNPs (HD) group showed normal histoarchitecture of seminiferous tubules, interstitium, the compact seminiferous epithelium arrangement and the cavity filled with sperm (Fig. 8D). The testis of ZnO-NPs plus Cin@CSNPs (LD) group showed restoration of normal structure in the majority of seminiferous tubules and normal interstitium (Fig. 8E). Moreover, the testis of ZnO-NPs plus Cin@CSNPs (HD) group showed compact seminiferous tubules and spermatocytes (Fig. 8F).

4. Discussion

Green synthesis of nanoparticles is a safe, cost-effective, and environmentally friendly alternative to existing traditional processes^[46]. In this study, the biosynthesized ZnO-NPs showed a semi-rounded shape, (20.78 ± 2.60) nm average particle size, and (32.7 ± 4.6) mV ζ -potential. Previous reports showed that the major components in *M. pulegium* are pulegone, menthone, and piperitone besides the other phytochemicals such as the phenolic, flavonoid, and terpenoid compounds^[34] which act as reducing and stabilization agents^[47]. The particle size of biosynthesized ZnO-NPs was similar to that reported by Rad et al.^[34] who synthesized ZnO-NPs with an average particle size of 40 nm using *M. pulegium*. Additionally, Shaikhaldeen et al.^[48] synthesized ZnO-NPs with particle sizes ranging from 10 to 50 nm using *Ochradenus arabicus* leaves extract. Similar morphological shape, particle size, and ζ -potential were reported for ZnO-NPs biosynthesized using other plant extracts^[38,49].

It is of interest to mention that the size and morphology of ZnO-NPs may be somewhat different owing to the biosynthesis conditions such as the concentration of zinc, pH variations, the time of reaction, and the temperature^[50]. In our study, irregular particle shapes of CSNPs were manufactured by the ionic gelation method, the average size of particles was (170 ± 3.7) nm and the ζ -potential was (8.32 ± 0.27) mV. In previous work, we synthesized CSNPs with an average size of 100 nm, and the size distribution ranged between 100–600 nm^[51]. In this concern, Mohanraj and Chen^[52] reported that NPs are solid particles ranging from 10–1 000 nm in size; thus, the size of particles reported herein is between the defined ranges. Moreover, the positive ζ -potential of the synthesized CSNPs is owing to the free amino groups which may facilitate the coupling process with other molecules^[53–54]. The loading of Cin onto CSNPs showed irregular particles with an average size of (218.23 ± 2.90) nm and a ζ -potential of (4.80 ± 0.21) mV, this means that the loading process increases the size of particles and also decreased the ζ -potential^[26]. Similar to the current observations, Shao et al.^[55] found that loading of eugenol onto CSNPs increased the particle size than the bulk CSNPs, and this increase was dependent on the increment of CSNPs-eugenol ratio. Moreover, Hadidi et al.^[56] revealed that CSNPs loaded with clove essential oil showed a particle diameter ranging 223–444 nm which was in the average size reported in the current study. It was reported that particle size is a critical factor in determining the release profile, encapsulation efficiency, and storage stability^[57].

The results of FTIR of ZnO-NPs powder showed the appearance of different peaks representing the different groups in the powder. The peak at $3\,650.7\text{ cm}^{-1}$ represented the O–H stretching vibration of the intermolecular hydrogen bond which may be due to the atmospheric moisture^[58]. The peak appears at $1\,043.49\text{ cm}^{-1}$ is attributed to the phenolic groups

in the extract^[59]; however, the peak appears at 889.54 cm⁻¹ represent the formation of tetrahedral-coordination of zinc as suggested by Jayarambabu et al.^[60] and those appeared at 543.83 and 427.77 cm⁻¹ are attributed to ZnO stretching bonds^[61]. The FTIR analysis revealed the successful entrapment of Cin within the polymeric matrix. The bands presented in the FTIR spectra of COS were similar to those reported previously^[26]. Similarly, Kiill et al.^[62] reported that the interaction between COS and TPP was noticed at the range of 860–600 cm⁻¹; which indicated the symmetric stretching modes of these linkages. Additionally, the peak at 1 637 cm⁻¹ (amide I) in the spectrum of blank CSNPs was significantly decreased or disappeared in the spectra of Cin@CSNPs. This can be attributed to electrostatic interactions between the cationic moieties of COS polysaccharide and the hydrophobic anionic moieties of Cin^[63]. The release of Cin from Cin@CSNPs showed a rapid-burst release at the beginning followed by sustained release. This initial burst may be due to the Cin loading on the surface of CSNPs, after that, a continuous pattern occurred due to drug release from the CSNPs^[37].

In the biological study, we evaluate the hazards of ZnO-NPs and the preventive role of Cin@CSNPs in mice. The dose of ZnO-NPs was based on our earlier study^[38], whereas the dose of Cin@COSNPs was based on the literature^[64]. The majority of the effects were the significant alterations in blood chemistry indices, oxidative markers, and antioxidant enzyme activity, the disturbances in AFP, CEA, TNF- α , DNA fragmentation in hepatocytes, the chromosomal aberrations in the somatic cells (bone marrow), sperm abnormalities, and the histological alterations in renal, hepatic, and testicular tissues. Even though micron-sized ZnO is a safe substance based on FDA reports, the safety of ZnO-NPs for animal consumption has not yet been determined. Our data showed that ZnO-NPs disturb serum biochemistry since these NPs increased the liver and kidney indices and decreased Alb and TP. It is well documented that AST and ALT are critical biochemical indices for the detection of hepatocyte damage^[38,65]. Therefore, the increase in these scores in the current study indicates that the biosynthesized ZnO-NPs affected the permeability of the membrane of the hepatocyte^[38,66]. Additionally, the covalent binding of these NPs with the intracellular proteins decreased the level of adenosine triphosphate, which leads to actin disruption and rupture of the cell membrane^[67]. Furthermore, it was found that when ZnO is administered orally, it dissolves in the acidic media in the stomach and forms Zn²⁺ causing significant toxicity^[68].

Creatinine, uric acid, and urea are among the best indicators of renal function which increase significantly during renal impairment. The current results showed that these renal indices were increased in mice administrated ZnO-NPs indicating that these nanoparticles influence the rate of glomerular filtration and the kidney capability to stimulate hemofiltration^[66]. Additionally, the decline in Alb and TP levels in the ZnO-NPs-exposed group indicates renal dysfunction and/or liver necrosis^[69]. Further, the lower Alb level could also be explicated by its exhaustion in the defense against ZnO-NPs-induced oxidative stress^[70]. Mice that administered ZnO-NPs alone exhibited a considerable elevation in triglycerides, cholesterol, and LDL accompanied by a marked decline in HDL indicating a dyslipidemia status which is a high risk factor for coronary heart disease^[71]. Therefore, the present results confirmed that ZnO-NPs possibly disturb the lipid profile, which leads to the risk of atherosclerosis^[72].

The increase in NO and MDA along with the decrease in CAT, SOD, and TAC in the renal and hepatic organs of ZnO-NPs-treated

animals confirmed that these nanoparticles increased lipid peroxidation and induced oxidative stress in these organs. MDA is the main degradation product of lipid peroxidation^[73]; However, NO is a serious free radical that produces severe cell damage when it is excessively produced in serum or tissues^[74]. Exposure to ZnO-NPs has been shown to produce cytotoxicity and DNA damage in human HepG2 cells by generating oxidative damage^[75], implying that the fundamental mechanism of ZnO-NPs toxicity is the development of oxidative stress^[38,76]. These findings are congruent with those presented by Moatamed et al.^[72]. Therefore, the decrease in antioxidants, and the increase in MDA and NO levels affirmed that ZnO-NPs promote ROS generation involving hydrogen peroxide, superanions, and hydroxyl radicals, thereby causing oxidative damage to cells^[77]. Our findings demonstrated that ZnO-NPs exposure increased serum levels of AFP, CEA, and TNF- α , indicating that these particles are bothersome to the immune system. A previous study revealed a decrease in the lymphocyte count, which reflected the inflammatory response and immunological impairment, along with an increase in the count of white blood cells, particularly neutrophils and granulocytes^[78]. When considered collectively, the rise in pro-inflammatory cytokine (TNF- α), tumor markers (CEA and AFP), oxidative indices (NO and MDA), and a decrease in CAT, TAC, and SOD confirmed that the primary mechanism for ZnO-NPs-induced toxicity is the production of ROS and inflammation^[79–80].

DNA fragmentation is an important feature of apoptosis which is the process that regulates the regeneration and elimination of the infected cells^[77], although cell death and tissue degeneration may be triggered by the dysregulation of cell apoptosis resulting in organ dysfunction^[81]. In the current study, exposure to ZnO-NPs dramatically enhanced DNA fragmentation in hepatocytes, suggesting that these particles triggered apoptosis. It is well known that cell apoptosis involves 2 main signal transduction pathways including mitochondrial (or intrinsic), and death receptor (or extrinsic) where both end up activating the executioner apoptotic protein, Caspase-3^[79,82]. The extrinsic pathway begins with death receptor stimulation which is a member of the TNF- α receptors superfamily, resulting in caspase-3 activation^[80]. Oral administration of ZnO-NPs resulted in apoptosis in hepatocytes, as evidenced by increased TNF- α and DNA fragmentation. This suggests that the toxicity of ZnO-NPs is linked to DNA damage, lipid peroxidation, and ROS generation, leading to mitochondrial membrane opening^[83–84]. The current data showed that ZnO-NPs increased the number of chromosomal aberrations in somatic cells (bone marrow) and spermatocytes indicating that these NPs induced genotoxicity mostly *via* oxidative stress mechanism. Previous reports indicated that ZnO-NPs increased the chromosomal aberrations in bone marrow^[80], and primary mouse embryo fibroblast cells^[82] resulting in genotoxicity and cytotoxicity^[83]. Additionally, Ghosh et al.^[84] demonstrated that ZnO-NPs induced genotoxicity and ROS production in Swiss mice. Moreover, the increased number of sperm abnormalities reported herein in mice that received ZnO-NPs confirmed the oxidative damage in the testes induced by these NPs. ROS generation leads to damage in Sertoli and Leydig cells resulting in the imbalance in LH, FSH, and other sex hormones which affect the formation of sperm^[85]. Furthermore, it was reported that there is an association between the low antioxidant levels in semen and the poor quality of sperm in infertile subjects^[87]. The histological evaluation of the liver, testis, and kidney demonstrated that ZnO-NPs caused considerable pathological changes in these tissues. Similar

findings were found in the liver and kidney sections^[67,88], as well as the testis^[86,89], indicating that ZnO-NPs have a toxic impact on the liver, kidneys, and male reproductive organs.

It is accepted now that ZnO-NPs induced their toxicity via the induction of oxidative stress. Supplementation with antioxidants may be the best avenue for protecting against oxidative stress. These antioxidants fight free radicals that harm cells and ultimately the entire human body system^[90]. Cin is the main bioactive compound in cinnamon and was approved by the FDA as a food additive^[91]. Several reports confirmed that Cin possesses a variety of biological activities including antioxidant, immunomodulating, and antitumor ability^[92-94]. In this study, Cin@CSNPs at the 2 tested doses improved most of the biochemical, cytogenetic parameters and the histology of the liver, kidney, and testis in ZnO-NPs-exposed mice. The improvement in biochemical indices, antioxidant activity, and the decrease in AFP, CEA, TNF- α , chromosomal aberrations, and sperm abnormality in this group reflect the antioxidant and radical scavenging activities of Cin@CSNPs. The formula's antioxidant performance can be related to the hydrophobic character of Cin, as well as the benzene ring and aldehyde group, which promotes Cin solubility in lipids and so increases antioxidant efficacy^[95]. These results proposed that the preventative function is related not just to Cin, but also to CSNPs' antioxidant efficacy. It has been observed that COS contains antioxidant and radical scavenging characteristics, as well as the capacity to inhibit lipid accumulation through antilipidemic action. Wen et al.^[96] stated that CSNPs restore the endogenous antioxidants (GPx, SOD, and CAT) activities and upregulate the antioxidant gene expression *in vitro*. CSNPs also were reported to have anticancer, antimutagenic, and immune-enhancing activity^[97-98]. The effectiveness of Cin@CSNPs also can be attributed to the prolonged release of Cin besides the biological activity of the polymers themselves^[99].

5. Conclusion

In this study, Cin@CSNPs ameliorated the detrimental effects of ZnO-NPs on biochemical parameters, oxidative stress markers, cytokines, tumor markers, and chromosomal aberrations in somatic and germ cells in a dose-dependent manner. These results demonstrate the effectiveness of Cin@CSNPs in mitigating oxidative damage caused by ZnO-NPs. As a result, Cin@CSNPs may be a promising delivery technique to limit the hazards of ZnO-NPs while simultaneously addressing the challenges associated with the use of Cin in food and pharmaceutical applications.

Conflict of interest

Wenyi Kang is an associate editor for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. There is no conflict of interest in this study.

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