

Received 15th April 2026,
Accepted 17th June 2026

Cite this: NJBM. 2026, 2(2), 11-20

Keywords: Zinc oxide nanoparticles; *Nigella sativa*; green synthesis; aflatoxin B1; oxidative stress; DNA damage; comet assay; hepatoprotection

Biosynthesized Zinc Oxide Nanoparticles from *Nigella sativa* Mitigate Aflatoxin B1 Induced Oxidative Stress and DNA Damage in Rats

Mohamed Abdul Rida Yaseen¹

¹ Department of laboratory and Clinical Science, College of Pharmacy, University of Al-Qadisiyah, Diwaniyah, Iraq

Correspondence author email: Email: Mohamed.yassen@qu.edu.iq

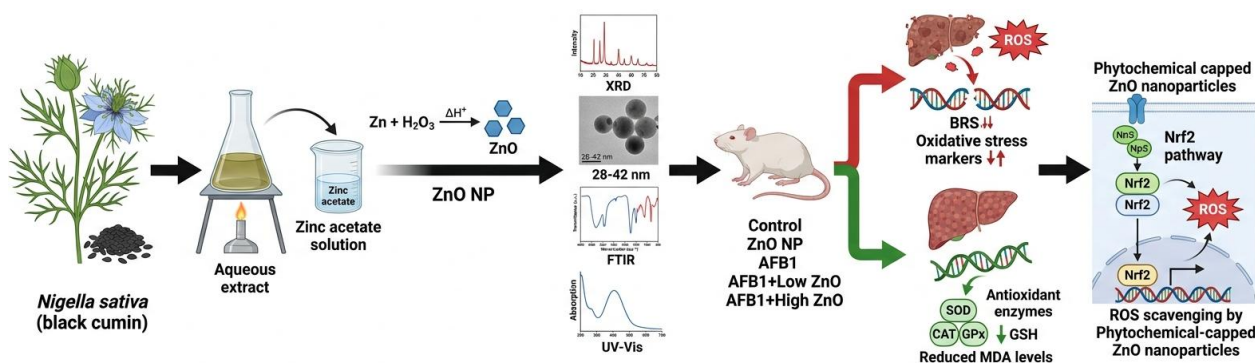
ABSTRACT

Aflatoxin B1 (AFB1) is one of the most dangerous mycotoxins contaminating food and animal feed, causing severe hepatotoxicity, nephrotoxicity, and genotoxicity through oxidative-stress mechanisms. The present work aimed to investigate the protective potential of green synthesised zinc oxide nanoparticles (ZnO NPs) produced from aqueous *Nigella sativa* seed extract against AFB1-induced toxicity in male Wistar rats. ZnO NPs were characterised by UV-Vis, FTIR, XRD, SEM and TEM, which revealed formation of wurtzite-phase crystalline nanoparticles with average diameter of 28–42 nm. Thirty adult male rats were randomly allocated into five groups (n = 6): normal control, ZnO NP control (10 mg/kg b.w.), AFB1 alone (250 µg/kg b.w.), AFB1 + low-dose ZnO NPs (5 mg/kg), and AFB1 + high-dose ZnO NPs (10 mg/kg). All therapies were given orally for 28 consecutive days. AFB1 exposure significantly increased serum ALT, AST, ALP, urea and creatinine and decreased total protein and albumin levels. Hepatic malondialdehyde (MDA) rose sharply alongside marked suppression of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH). The comet assay demonstrated considerable DNA fragmentation in hepatocytes. Co-treatment with ZnO NPs dosage dependently ameliorated these biochemical abnormalities, reinstated antioxidant defences and minimised DNA damage. The histopathological evaluation was in agreement with biochemical results revealing decreased liver necrosis and inflammatory infiltration in ZnO NP-treated groups. Results showed that biogenic ZnO NPs synthesised from *N. sativa* extract are a viable chemoprotective approach against AFB1-induced organ damage probably through free-radical scavenging and activation of endogenous antioxidant mechanisms.

Highlights

- Green synthesis of ZnO NPs using *Nigella sativa* seed extract yielded crystalline wurtzite nanoparticles (28–42 nm).
- AFB1 exposure induced severe oxidative stress, hepato-renal dysfunction, and DNA fragmentation in rats.
- Co-administration of biogenic ZnO NPs dose-dependently restored antioxidant enzyme activities.
- Comet assay confirmed significant reduction in DNA tail length and tail moment following ZnO NP treatment.
- Histopathological findings validated the hepatoprotective efficacy of NS–ZnO NPs against AFB1 toxicity.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Mycotoxins are some of the most persistent risks to food safety around the world, and aflatoxins are the most poisonous and prevalent class of over 400 secondary fungal metabolites known [1]. Aflatoxin B₁ (AFB₁) is a mycotoxin produced mostly by *Aspergillus flavus* and *A. parasiticus* and is designated as a Group 1 carcinogen by the International Agency for Research on Cancer because of its strong mutagenic and hepatocarcinogenic effects [2]. AFB₁ is bioactivated in the liver by cytochrome P450 enzymes on oral consumption to form AFB₁-8,9-epoxide, a highly reactive intermediate that covalently bonds to DNA and proteins that ultimately results in oxidative stress, lipid peroxidation and apoptosis [3,4]. Chronic exposure to AFB₁ is associated with hepatotoxicity, renal damage and immunological suppression [4]. AFB₁ is a multi-faceted health hazard, especially in tropical developing countries where contamination levels often exceed regulatory limits [5]. In recent years, nanotechnology-based therapies have gained increasing attention for their ability to combat mycotoxin toxicity. Zinc oxide nanoparticles (ZnO NPs) are among the most important metal-oxide nanomaterials owing to their good biocompatibility, broad-spectrum

antimicrobial activity, and documented antioxidant capacity [6,7]. Conventional chemical and physical methods for the synthesis of ZnO NPs use toxic solvents, high energy consumption or pricey reagents, which create environmental and biological safety problems [8]. On the other hand, green synthesis methods use phytochemicals present in plant extracts as reducing and capping agents, leading to nanoparticles with better stability and lower cytotoxicity. [9,10].

Nigella sativa L. (Ranunculaceae) popularly called black cumin or black seed has been used as a folk treatment for various maladies for centuries in the Middle East and South Asia. The seeds are rich in thymoquinone, thymohydroquinone, carvacrol, p-cymene and other flavonoids and alkaloids that are linked to considerable antioxidant, anti-inflammatory, and immunomodulatory activity [11,12]. Thymoquinone, a major bioactive component of quinone, has been demonstrated to improve AFB₁ hepatotoxicity through free radical scavenging and Nrf2 pathway activation independently [13,14]. However,

few studies have been carried out to explore if ZnO NPs prepared with *N. sativa* extract can synergistically combine the inherent bioactivity of phytochemical capping agents and the physicochemical properties of nano-zinc oxide to offer improved protection against AFB₁-induced oxidative damage and genotoxicity. Therefore, the present study was designed to green synthesise ZnO NPs using aqueous extract of *N. sativa* seeds, characterise the synthesised nanomaterial and evaluate the chemoprotective efficacy of synthesised ZnO NPs against AFB₁ caused oxidative stress and DNA damage in rat model.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Aflatoxin B₁ (≥98% purity), zinc acetate dihydrate [Zn(CH₃COO)₂·2H₂O], sodium hydroxide, thiobarbituric acid, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione, trichloroacetic acid and all other compounds of analytical grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, urea and creatinine diagnostic kits were obtained from BioMérieux (Marcy-l'Etoile, France). The alkaline comet assay kit was purchased from Trevigen Inc. (Gaithersburg, MD, USA).

2.2. Preparation of *Nigella sativa* aqueous extract

Nigella sativa seeds were obtained from a certified local market in Al-Diwaniyah, Iraq, and authenticated at the Department of Biology, College of Education, University of Al-Qadisiyah. Seeds were thoroughly washed with distilled water, air-dried at room temperature, and finely ground using a mechanical grinder. Ten grams of the seed powder were suspended in 100 mL of double-distilled water and heated at 60 °C under continuous stirring for 30 min. The mixture was allowed to cool, then filtered through Whatman No. 1 filter paper. The filtrate was stored at 4 °C until use [15].

2.3. Green synthesis of ZnO nanoparticles

The synthesis of ZnO NPs was carried out following the approach of Gunalan et al. [16] with certain changes. Briefly, 50 mL of 0.1 M zinc acetate dihydrate solution was added to a magnetic stirrer at 60 °C and 25 mL of the freshly obtained *N. sativa* extract was added dropwise over a period of about 15

min under vigorous stirring. The pH of the reaction mixture was adjusted to ~12 by the dropwise addition of 2 M NaOH. White precipitate developed indicated nucleation of ZnO NPs. The mixture was kept at 60 °C for 2 h. The precipitate was collected by centrifugation at 10,000 rpm for 15 min, washed three times with distilled water and once with ethanol and dried overnight at 80 °C. Finally, the dried powder was calcined at 400 °C for 2 h in a muffle furnace to obtain pure ZnO NPs [17].

2.4. Characterization of ZnO NPs

The optical absorption spectrum was recorded using a UV–Vis double-beam spectrophotometer (Shimadzu UV-1800) over the range 200–800 nm. Fourier-transform infrared spectroscopy (FTIR; Bruker ALPHA II) was used to identify surface functional groups in the range 4000–400 cm⁻¹ using the KBr pellet technique. X-ray diffraction (XRD; Rigaku MiniFlex 600) analysis was performed at $2\theta = 10\text{--}80^\circ$ with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) to determine crystalline phase and average crystallite size via the Scherrer equation. Surface morphology was examined by field-emission scanning electron microscopy (FESEM; TESCAN MIRA3) and transmission electron microscopy (TEM; JEOL JEM-2100) at an accelerating voltage of 200 kV. Particle size distribution was estimated from TEM micrographs using ImageJ software (NIH, USA) by measuring at least 100 individual particles [18].

2.5. Experimental animals and ethical approval

Thirty healthy adult male Wistar albino rats (weight 180–220 g, aged 8–10 weeks) were obtained from the animal house of the College of Veterinary Medicine, University of Al-Qadisiyah. Animals were acclimatized for one week prior to experimentation under standard laboratory conditions ($23 \pm 2^\circ\text{C}$, 12 h light/dark cycle) with free access to standard pellet diet and water ad libitum. All experimental protocols were approved by the Institutional Animal Ethics Committee, University of Al-Qadisiyah (approval no. [PLACEHOLDER]), in compliance with the Guidelines for Care and Use of Laboratory Animals [19].

2.6. Experimental design

After acclimatization, rats were randomly divided into five groups of six animals each and treated daily by oral gavage for 28 consecutive days as follows:

Table 1. Experimental design and treatment groups.

Group	Treatment	Dose & Route
G1 (Normal control)	Distilled water	1 mL/day, oral gavage
G2 (ZnO NP control)	NS–ZnO NPs only	10 mg/kg b.w., oral gavage
G3 (AFB1)	AFB1 alone	250 µg/kg b.w., oral gavage

G4 (AFB1 + Low ZnO)	AFB1 + NS–ZnO NPs	250 µg/kg AFB1 + 5 mg/kg ZnO NPs
G5 (AFB1 + High ZnO)	AFB1 + NS–ZnO NPs	250 µg/kg AFB1 + 10 mg/kg ZnO NPs

AFB1 was dissolved in dimethyl sulfoxide (DMSO) and then diluted in maize oil, the final concentration of DMSO being not more than 0.1% (v/v). Freshly suspended ZnO NPs in distilled water were sonicated for 10 min before each administration. In groups treated with both AFB1 and ZnO NPs, the nanoparticle suspension was provided 1 h before AFB1 dosage.

2.7. Sample collection and preparation

Twenty-four hours following the previous treatment animals were fasted overnight and anaesthetised by inhalation of diethyl ether. Blood samples were collected by cardiac puncture into plain tubes and left to clot for 30 min at room temperature. Serum was isolated by centrifugation at 3000 rpm for 15 min. Serum aliquots were stored at -20°C until biochemical analysis. Blood sample was immediately followed by sacrifice of the rats by cervical dislocation, excising of the liver and kidney tissues, washing with ice-cold physiological saline, blotting dry and weighing. For the assay of oxidative stress markers, 10% (w/v) homogenates of liver tissue were prepared by homogenising portions of liver tissue in cooled phosphate-buffered saline (PBS, pH 7.4). Other liver portions were repaired in 10 %neutral buffered formalin for histopathological evaluation, and fresh hepatocyte suspensions were prepared for the comet assay [20].

2.8. Serum biochemical analysis

Serum levels of ALT, AST and ALP were assayed as markers of hepatocellular integrity. Renal function was measured by serum urea and creatinine levels. Other measures of hepatic synthesis capability were total protein and albumin. All assays were performed using a semi-automated clinical chemistry analyser (Mindray BS-200, Shenzhen, China) with commercial kits according to the manufacturer's guidelines [21].

2.9. Oxidative stress biomarkers

Hepatic malondialdehyde (MDA) concentration, a marker of lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARS) method [22]. Superoxide dismutase (SOD) activity was assayed according to the method of Marklund and Marklund [23] based on auto-oxidation of pyrogallol. Catalase (CAT) activity was measured by monitoring the decomposition rate of H₂O₂ at 240 nm as described by Aebi [24]. Glutathione peroxidase (GPx) activity was determined using the method of Paglia and Valentine [25]. Reduced glutathione (GSH) content was estimated by the Ellman method using DTNB reagent [26]. Protein content in liver homogenates was quantified by the Lowry method [27] for normalization purposes.

2.10. Alkaline comet assay (single-cell gel electrophoresis)

DNA damage in hepatocytes was assessed by the alkaline comet assay following the protocol of Singh et al. [28] with minor

modifications. Freshly isolated hepatocytes were embedded in 0.5% low-melting-point agarose on frosted microscope slides, lysed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) at 4 °C for at least 1 h, and then incubated in alkaline unwinding solution (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min. Electrophoresis was performed for 20 min at 25 V and 300 mA. Slides were washed with 0.4 M Tris solution (pH 7.5), stained with ethidium bromide (20 µg/mL) and seen under a fluorescence microscope (Olympus BX53, Tokyo, Japan). ≥100 cells per animal were evaluated using CometScore™ software. The parameters measured were tail length (µm), tail DNA (%) and tail moment (arbitrary units) [29].

2.11. Histopathological examination

Liver samples were fixed in formalin, dehydrated through graded ethanol, cleaned in xylene, embedded in paraffin wax and cut into 5 µm sections using a rotary microtome. The sections were stained with haematoxylin and eosin (H&E) and inspected under a light microscope at 100× and 400× magnification by a pathologist who was blinded to the treatment groups [30].

2.12. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical analyses were performed using GraphPad Prism version 9.5 (San Diego, CA, USA). Statistical significance was defined as $p < 0.05$ [31].

3. RESULTS AND DISCUSSION

3.1. Characterization of biosynthesized ZnO NPs

The biosynthesised ZnO NPs UV-Vis. absorption spectrum showed a sharp absorption peak at around 365 nm (Fig. 1) which is in the normal range reported for ZnO semiconducting nanostructures, confirming the excitonic absorption due to the wide band-gap energy of ZnO (~3.37 eV) [32]. No other peaks due to contaminants were observed indicating phase purity of the product.

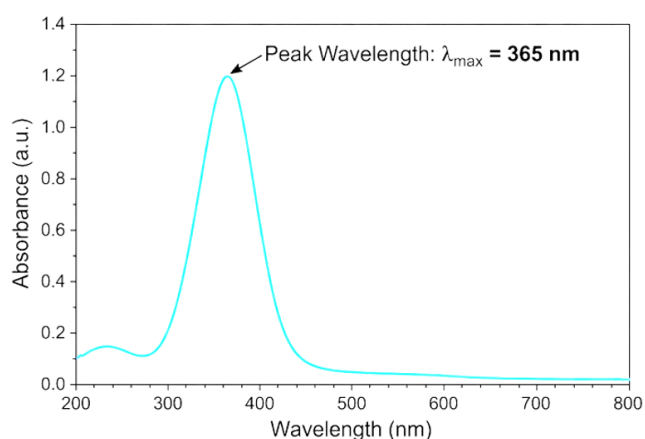


Fig. 1. UV-Vis. absorption spectrum of NS-ZnO NPs synthesized using *Nigella sativa* seed extract.

The FTIR spectrum (Fig. 2) showed a number of bands of interest. A wide absorption at about 3420 cm⁻¹ was attributed to O-H stretching

vibrations of residual and adsorbed water phytochemicals. The bands at 2925 and 2855 cm⁻¹ are attributed to C-H stretching of aliphatic hydrocarbons of the capping biomolecules. A band around 1630 cm⁻¹ was attributed to C=O or C=C stretching possibly due to polyphenolic and flavonoid chemicals from the seed extract. More notably, a prominent absorption band at around 470 cm⁻¹ was assigned to the stretching mode of Zn-O, characteristic of zinc oxide nanocrystals [33,34]. The FTIR findings collectively suggest that the phytochemicals present in *N. sativa* are connected with the surface of nanoparticles, likely acting as stabilizing/capping agents.

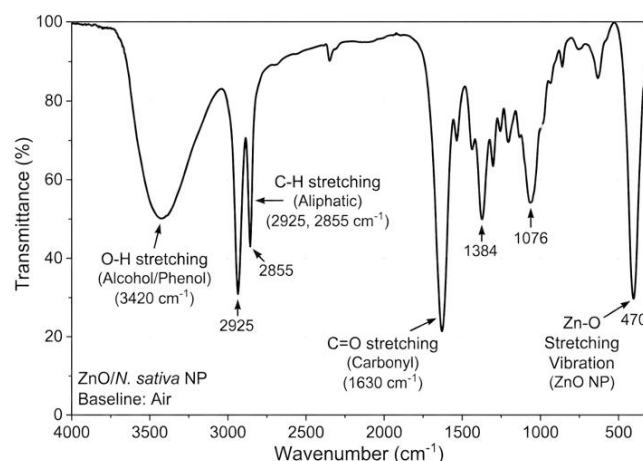


Fig. 2. FTIR spectrum of NS-ZnO NPs showing functional groups from the *Nigella sativa* extract and the characteristic Zn-O bond.

XRD analysis (Fig. 3) displayed diffraction peaks at 2θ values of 31.7°, 34.4°, 36.2°, 47.5°, 56.5°, 62.8°, 66.3°, 67.9°, and 69.0°, which were indexed to the (100), (002), (101), (102), (110), (103), (200), (112), and (201) planes, respectively, of the hexagonal wurtzite structure of ZnO (JCPDS card no. 36-1451) [35]. No diffraction peaks were found from metallic Zn or any other crystalline impurities. The average crystallite size was determined to be 24.6 nm from the whole width at half maximum of the most intense (101) reflection using the Debye-Scherrer equation.

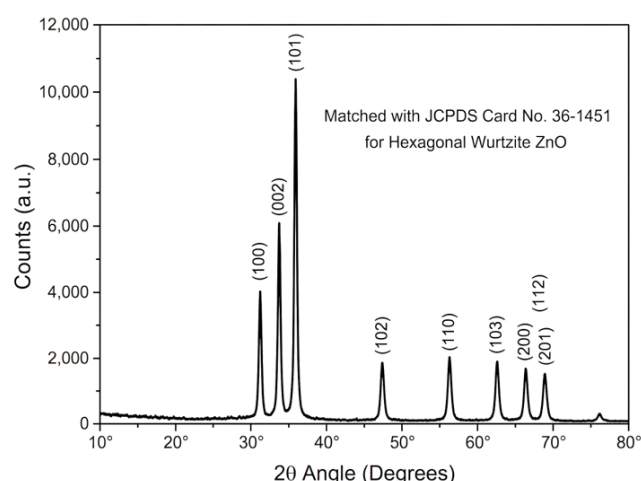


Fig. 3. XRD pattern of biosynthesized NS-ZnO NPs indexed to the hexagonal wurtzite structure (JCPDS 36-1451).

FESEM images showed that the nanoparticles possessed a roughly spherical morphology with some degree of agglomeration, which is a common observation in biogenic nanomaterial preparations (Fig. 4a). TEM analysis provided more precise size information, revealing well-dispersed, nearly spherical particles with diameters ranging from 28 to 42 nm and a mean particle size of 33.5 ± 4.8 nm (Fig. 4b). The slight discrepancy between the XRD-derived crystallite size and TEM-measured particle size can be explained by the fact that individual nanoparticles are often polycrystalline aggregates of smaller crystalline domains [36]. A summary of the physicochemical characteristics is provided in Table 2.

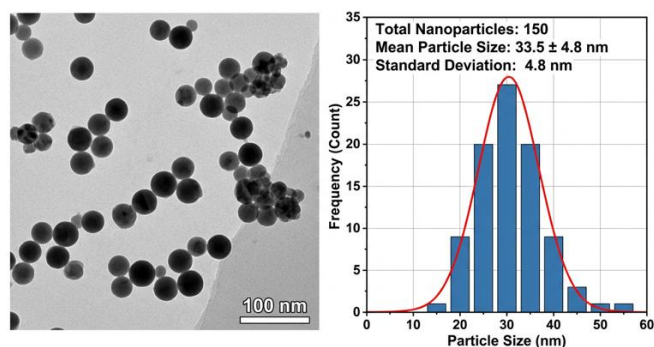


Fig. 4. (a) Schematic representation of NS-ZnO NPs morphology and (b) particle size distribution histogram from TEM analysis (mean = 33.5 ± 4.8 nm, $n = 120$ particles).

Table 2. Physicochemical characterization summary of biosynthesized NS-ZnO NPs.

Parameter	Result
UV-Vis λ_{max} (nm)	365
Crystal structure	Hexagonal wurtzite
Crystallite size (XRD, nm)	24.6
Particle size range (TEM, nm)	28–42
Mean particle size (TEM, nm)	33.5 ± 4.8
Morphology	Spherical to quasi-spherical
FTIR Zn–O band (cm^{-1})	~ 470

3.2. Effect on body weight and relative organ weights

As summarized in Table 3, the initial body weights did not differ significantly among the five groups at the start of the experiment. By day 28, rats in the AFB1-only group (G3) exhibited a markedly lower final body weight compared with the normal control, reflecting the general catabolic impact of chronic mycotoxin exposure that has

been well-documented in earlier literature [37,38]. ZnO NPs administered simultaneously with AFB1 partly prevented this loss of weight. The ultimate body weight of the high-dose group (G5) was comparable to that of the control, demonstrating that the nanoparticles were able to maintain metabolic homeostasis. Relative liver weight was dramatically increased in the AFB1 group (an expected outcome consistent with hepatomegaly related to lipid infiltration and inflammatory oedema) whereas ZnO NP supplemented tended to normalize this parameter. Relative kidney weight did not differ significantly between groups, while a modest rise was detected in G3.

Notably, animals in the normal control group (G1) and the ZnO NP-only control group (G2) exhibited comparable baseline values throughout the study period across all measured parameters (Tables 3–6). Specifically, serum hepatic markers (ALT, AST, ALP), renal markers (urea, creatinine), total protein, and albumin levels in G2 were not statistically different from G1, confirming the absence of any intrinsic toxicity of NS-ZnO NPs at the administered dose (10 mg/kg b.w.) over 28 days. Similarly, oxidative stress biomarkers (MDA, SOD, CAT, GPx, GSH) in liver homogenates and comet assay parameters (tail length, tail DNA %, tail moment) in hepatocytes showed no significant difference between G1 and G2. Histopathologically, liver sections from both control groups displayed well-preserved hepatic lobular architecture, clearly delineated central veins, intact sinusoidal spaces, and uniformly arranged hepatocyte cords radiating from the central vein with no evidence of vacuolar degeneration, necrosis, inflammatory infiltration, or fibrosis. These observations collectively confirm that the biosynthesized NS-ZnO NPs are biocompatible and exert no detectable adverse effects at the tested dose, providing a reliable baseline for evaluating the protective efficacy against AFB1-induced toxicity.

Table 3. Effect of NS-ZnO NPs on body weight and relative organ weights in AFB1-treated rats.

Parameter	G1 (Control)	G2 (ZnO NP)	G3 (AFB1)	G4 (AFB1+Low)	G5 (AFB1+High)
Initial BW (g)	195.4 ± 8.2^a	192.8 ± 7.6^a	194.1 ± 9.0^a	193.5 ± 8.4^a	196.2 ± 7.9^a
Final BW (g)	238.6 ± 10.3^a	235.2 ± 9.8^a	208.4 ± 11.5^c	222.7 ± 10.1^b	231.8 ± 9.4^{ab}
BW gain (g)	43.2 ± 5.8^a	42.4 ± 6.1^a	14.3 ± 4.2^c	29.2 ± 5.5^b	35.6 ± 4.9^{ab}
Rel. liver wt (g/100g)	3.12 ± 0.18^a	3.08 ± 0.21^a	4.25 ± 0.32^c	3.68 ± 0.24^b	3.31 ± 0.19^a

Rel. kidney wt (g/100g)	0.68 ± 0.05 ^a	0.67 ± 0.04 ^a	0.78 ± 0.06 ^b	0.73 ± 0.05 ^{ab}	0.70 ± 0.04 ^a
-------------------------	--------------------------	--------------------------	--------------------------	---------------------------	--------------------------

Values are expressed as mean ± SD (n = 6). Different superscript letters within the same row indicate significant differences (p < 0.05, one-way ANOVA/Tukey's test).

3.3. Serum biochemical parameters

Serum indicators of liver damage are given in Table 4. The rats treated with AFB1 alone (G3) showed significant elevation in the activities of ALT, AST and ALP compared to the control, indicating impaired hepatic membrane integrity and cholestatic injury. These findings concur with certain papers describing the hepatocyte harmful potential of AFB1 metabolites, including AFB1-exo-8,9-epoxide which triggers the membrane disruption by lipid peroxidation [39,40]. Total protein and albumin were concomitantly decreased in G3 suggesting impaired hepatic protein synthesis which is a characteristic of sub-chronic aflatoxicosis [41]. Co-treatment with ZnO NPs at both dosages considerably decreased enzyme leakage in a dose-dependent way. The values of ALT and AST in the high dose group (G5) were not significantly different from the control. This restorative effect could be due to the antioxidant activity of ZnO NPs and the *N. sativa* phytochemicals that decorate their surface, which together hinder the proliferation of membrane-damaging free radicals [42].

Indices of renal function told a similar story. Serum urea and creatinine was increased in G3 as compared to control indicating nephrotoxic injury. Co-administration with ZnO NP brought these levels towards normal, with a more significant recovery in the high dose group. Though AFB1 is largely hepatotropic, its metabolites can reach kidneys through systemic circulation and induce tubular necrosis [43]. The ameliorative effect of ZnO NPs on renal indicators adds up the growing body of evidence that biogenic zinc oxide nanomaterials exert multi-organ protective effects under oxidative stress conditions [44].

Table 4. Effect of NS–ZnO NPs on serum biochemical parameters in AFB1-treated rats.

Parameter	G1 (Control)	G2 (ZnO NP)	G3 (AFB1)	G4 (AFB1+Low)	G5 (AFB1+High)
ALT (U/L)	32.4 ± 3.8 ^a	30.1 ± 3.5 ^a	89.6 ± 8.2 ^c	58.3 ± 6.4 ^b	41.2 ± 4.7 ^a
AST (U/L)	45.8 ± 4.6 ^a	43.2 ± 4.1 ^a	112.5 ± 10.8 ^c	75.4 ± 7.2 ^b	52.6 ± 5.3 ^a
ALP (U/L)	85.3 ± 7.9 ^a	82.6 ± 8.1 ^a	198.4 ± 16.5 ^c	142.7 ± 12.3 ^b	105.8 ± 9.6 ^a

Total protein (g/dL)	7.24 ± 0.42 ^a	7.38 ± 0.39 ^a	5.12 ± 0.48 ^c	6.15 ± 0.51 ^b	6.82 ± 0.38 ^{ab}
Albumin (g/dL)	3.82 ± 0.28 ^a	3.91 ± 0.25 ^a	2.41 ± 0.32 ^c	3.08 ± 0.29 ^b	3.52 ± 0.24 ^{ab}
Urea (mg/dL)	28.3 ± 3.2 ^a	26.8 ± 2.9 ^a	52.4 ± 5.6 ^c	38.6 ± 4.1 ^b	31.5 ± 3.4 ^a
Creatinine (mg/dL)	0.62 ± 0.08 ^a	0.58 ± 0.07 ^a	1.35 ± 0.15 ^c	0.94 ± 0.11 ^b	0.71 ± 0.09 ^a

Values are expressed as mean ± SD (n = 6). Different superscript letters within the same row denote significant differences at p < 0.05 by one-way ANOVA/Tukey's test.

3.4. Hepatic oxidative stress markers

Table 5 and Fig. 5 indicate the status of lipid peroxidation and endogenous antioxidant defences in liver tissue. The MDA concentration, a common marker of oxidative membrane damage, was significantly higher in the AFB1 group than in the control group (≈ 2.8-fold increase), thus confirming the pro-oxidant nature of AFB1 and its metabolites. Meanwhile, the activity of the three primary enzyme components of first line antioxidant defence, SOD, CAT and GPx, were significantly decreased in AFB1 treated rats. At the same time, the decrease in the level of reduced glutathione (GSH) indicated that the cellular thiol pool was largely depleted in detoxifying the electrophilic AFB1 metabolites by glutathione conjugation catalysed by glutathione S-transferase (GST) [45,46].

Co-treatment with NS–ZnO NPs recovered all the observed antioxidant parameters towards the control levels in a dose-dependent manner. The MDA level in the high-dose ZnO NP group (G5) was not substantially different from G1 and SOD, CAT, GPx, and GSH considerably increased in G5 than G3. These findings are in agreement with previous studies by Abdel-Daim et al. [47], who revealed that ZnO NPs alleviated doxorubicin-induced oxidative stress in cardiac tissue and Alkaladi et al. [48], who showed the protective effects of ZnO NPs against lead-induced oxidative damage in the liver and kidneys. The dual antioxidant mechanism apparently at play here is: (i) direct radical scavenging action of the thymoquinone and polyphenolic capping compounds preserved on the nanoparticle surface, and (ii) zinc ion release from ZnO NPs, which upregulates metallothionein and zinc-finger transcription factors involved in Nrf2/ARE signaling pathway activation [49,50].

Table 5. Effect of NS–ZnO NPs on hepatic oxidative stress markers in AFB1-treated rats.

Parameter	G1 (Control)	G2 (ZnO NP)	G3 (AFB1)	G4 (AFB1+Low)	G5 (AFB1+High)
MDA (nmol/g)	18.5 ± 2.3 ^a	17.2 ± 2.0 ^a	52.6 ± 4.8 ^c	34.8 ± 3.5 ^b	22.4 ± 2.8 ^a
SOD (U/mg)	12.8 ± 1.2 ^a	13.1 ± 1.3 ^a	5.2 ± 0.9 ^c	8.6 ± 1.1 ^b	11.3 ± 1.0 ^a
CAT (U/mg)	45.6 ± 4.1 ^a	47.2 ± 3.8 ^a	18.4 ± 2.5 ^c	31.5 ± 3.2 ^b	40.8 ± 3.6 ^{ab}
GPx (U/mg)	8.9 ± 0.9 ^a	9.2 ± 1.0 ^a	3.5 ± 0.6 ^c	5.8 ± 0.8 ^b	7.6 ± 0.7 ^a
GSH (μmol/g)	6.4 ± 0.7 ^a	6.7 ± 0.8 ^a	2.3 ± 0.4 ^c	4.1 ± 0.6 ^b	5.8 ± 0.5 ^a

Values are expressed as mean ± SD (n = 6). Statistical significance assessed by one-way ANOVA followed by Tukey's test. Different superscript letters in the same row indicate $p < 0.05$.

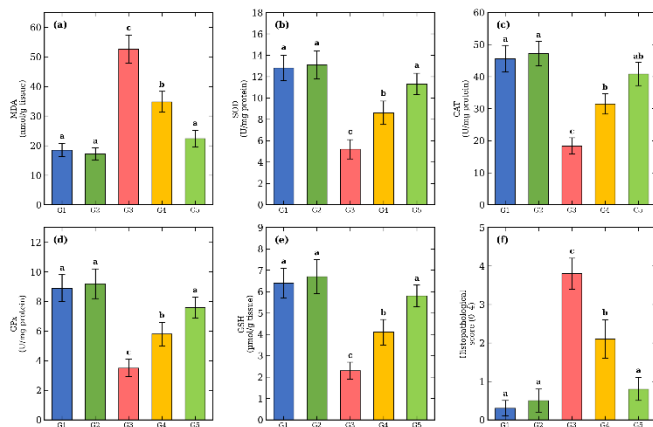


Fig. 5. Oxidative stress markers and histopathological scoring across experimental groups: (a) MDA, (b) SOD, (c) CAT, (d) GPx, (e) GSH, (f) histopathological damage score. Different letters indicate statistically significant differences ($p < 0.05$).

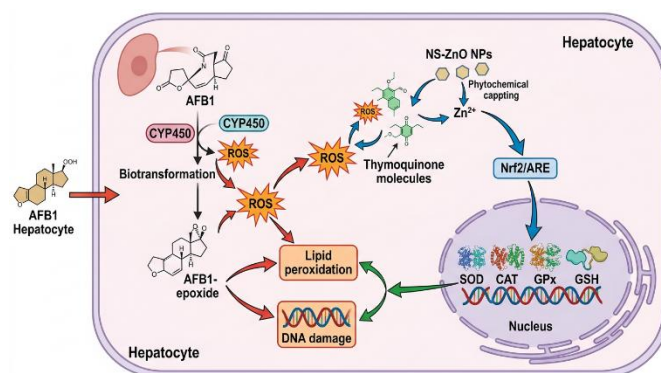


Fig. 6. Schematic illustration of the proposed antioxidant mechanism of NS–ZnO NPs against AFB1-induced oxidative stress, including free-radical scavenging by phytochemical capping agents and Zn^{2+} -mediated upregulation of the Nrf2/ARE pathway.

3.5. DNA damage assessment by comet assay

Single cell level quantification of DNA strand breakage in hepatocytes was performed using the alkaline comet assay. Table 6 presents the quantitative parameters and Fig. 7 shows them graphically. Hepatocytes from the AFB1 group showed a dramatic disruption of nuclear morphology with extended, diffuse DNA tails consistent with widespread single- and double-strand breaks. The mean tail length, percentage of tail DNA, and tail moment in G3 were significantly higher than those in the control (≈ 4.2 -, 5.1 -, and 8.7 -fold increases, respectively). These results are in agreement with previous reports describing that AFB1-epoxide adducts induce depurination at guanine sites leading to abasic sites and subsequent strand breaks when they are attempted to be repaired by base-excision repair enzymes [51,52].

ZnO NPs treatment significantly lowered all the comet properties in a dosage dependent way. However, in the high dose group (G5), the tail length and tail moment reduced by roughly 62% and 71%, respectively, compared to G3 and were near normal levels. The low dose group (G4) demonstrated statistically significant decreases, but less marked. Importantly, animals treated with ZnO NPs alone (G2) had comet characteristics similar to the control, excluding any genotoxic effect of the nanoparticles alone at the dose studied. This is comforting and is in accordance with the genotoxicity study by Sharma et al. [53], who observed no DNA damage with biogenically synthesised ZnO NPs in human cells at concentrations of up to 25 $\mu\text{g/mL}$. The observed DNA protective effect of NS–ZnO NPs may be attributed to their ability to quench ROS upstream of the DNA attack sites, to reduce the intracellular burden of AFB1-epoxide by enhanced phase-II conjugation reactions as well as to possibly facilitate DNA repair processes by zinc-dependent repair endonucleases [54,55].

Table 6. Comet assay parameters in hepatocytes of different experimental groups.

Parameter	G1 (Control)	G2 (ZnO NP)	G3 (AFB1)	G4 (AFB1+Low)	G5 (AFB1+High)
Tail length (μm)	4.2 ± 0.8 ^a	4.5 ± 0.9 ^a	17.6 ± 2.1 ^c	10.3 ± 1.5 ^b	6.7 ± 1.1 ^a
Tail DNA (%)	3.1 ± 0.6 ^a	3.4 ± 0.7 ^a	15.8 ± 1.8 ^c	9.2 ± 1.3 ^b	4.9 ± 0.9 ^a
Tail moment (a.u.)	0.85 ± 0.15 ^a	0.92 ± 0.18 ^a	7.40 ± 1.10 ^c	3.80 ± 0.65 ^b	1.50 ± 0.28 ^a
Damaged cells (%)	8.2 ± 2.1 ^a	9.1 ± 2.4 ^a	62.5 ± 5.8 ^c	31.4 ± 4.2 ^b	14.8 ± 3.0 ^a

Values are expressed as mean ± SD (n = 6, 100 cells scored per animal). Different superscript letters within rows denote statistical significance at *p* < 0.05 by one-way ANOVA/Tukey's test.

alone group (G3) exhibited severe histopathological changes such as diffuse hepatocellular vacuolar degeneration, focal areas of coagulative necrosis, sinusoidal congestion, periportal inflammatory cell infiltration mainly lymphocytes and macrophages and moderate fatty change. Sections also showed biliary hyperplasia and moderate fibrosis surrounding portal triads consistent with the established hepatotoxic profile of chronic AFB1 exposure [56,57].

The liver of the low dosage ZnO NP co-treated group (G4) exhibited remarkable recovery with modest to moderate vacuolar alterations and scattered inflammatory foci. The necrotic regions were also markedly reduced. The high dosage group (G5) presented a near normal hepatic morphology with very few cytoplasmic vacuoles and few inflammatory cells, significantly lower than G3. These histological findings confirm that NS-ZnO NPs exert a dose-dependent hepatoprotective effect, preserving tissue integrity presumably through the attenuation of oxidative damage and subsequent inflammatory cascades. Similar protective histological outcomes have been reported for green-synthesized ZnO NPs against carbon tetrachloride-induced liver injury [58] and against cisplatin nephrotoxicity [59]. The semi-quantitative histopathological damage scores are presented in Fig. 5f, which clearly demonstrate the dose-dependent tissue protection afforded by NS-ZnO NPs.

4. CONCLUSION

The present study demonstrated that zinc oxide nanoparticles biosynthesized using *Nigella sativa* seed extract are effective in counteracting aflatoxin B1 induced hepatotoxicity, nephrotoxicity, oxidative stress, and genotoxicity in male Wistar rats. The green-synthesized ZnO NPs exhibited a hexagonal wurtzite crystal structure with particle sizes in the 28–42 nm range and were capped by bioactive phytochemicals, as confirmed by UV-Vis, FTIR, XRD, SEM, and TEM analyses. Oral co-administration of NS-ZnO NPs at doses of 5 and 10 mg/kg b.w. over 28 days dose-dependently normalized serum liver and kidney function biomarkers, replenished hepatic antioxidant enzyme activities (SOD, CAT, GPx) and GSH levels, reduced lipid peroxidation, and substantially mitigated AFB1-induced DNA strand breaks as measured by the comet assay. Histopathological examination corroborated these biochemical improvements, revealing preserved hepatic lobular architecture in ZnO NP-treated groups. The chemoprotective mechanism is most likely mediated through the synergistic antioxidant effects of the phytochemical corona (primarily thymoquinone and polyphenols) and Zn²⁺-dependent activation of the Nrf2/ARE signaling pathway, which together attenuate ROS generation, lipid peroxidation, and electrophilic DNA adduction. These findings suggest that biogenic NS-ZnO NPs hold promise as a nutraceutical or feed-additive strategy for mitigating mycotoxin-related health risks. However, long-term toxicity profiling, pharmacokinetic evaluation, and investigation of the precise molecular pathways are warranted before clinical or veterinary translation.

Acknowledgments

The author would like to thank the Department of Laboratory and Clinical Science, College of Pharmacy, University of Al-Qadisiyah, for

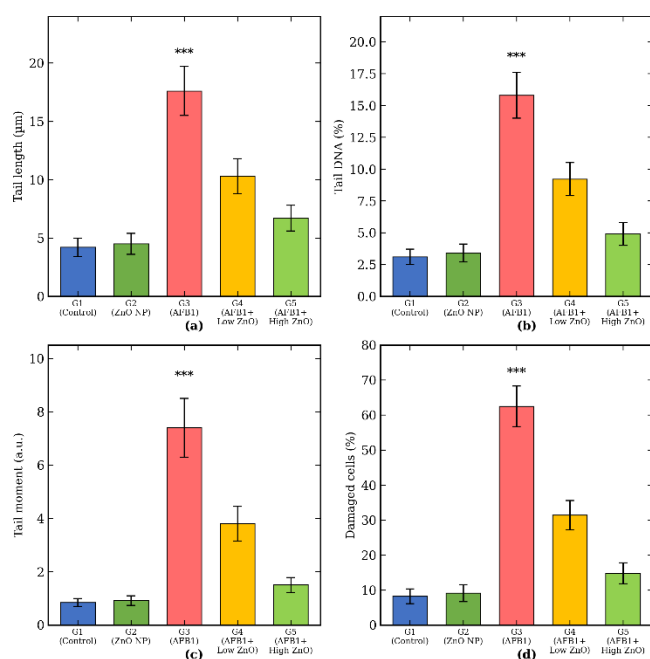


Fig. 7. Comet assay parameters across experimental groups: (a) tail length, (b) tail DNA %, (c) tail moment, and (d) percentage of damaged cells. *** indicates *p* < 0.001 vs. control.

3.6. Histopathological findings

Histological evaluation of liver slices confirmed the chemistry data. The G1 (normal control) and G2 (ZnO NP control) groups showed highly preserved hepatic architecture with intact hepatic lobules, well demarcated central veins and consistently organized hepatocyte cords emanating from the central vein. On the other hand, the AFB1

providing the laboratory facilities and technical support necessary to carry out this work. Appreciation is also extended to the College of Veterinary Medicine, University of Al-Qadisiyah, for supplying the experimental animals and for assistance with histopathological examination. The author gratefully acknowledges the staff of the Central Laboratory at the University of Al-Qadisiyah for their help with nanoparticle characterisation (XRD, SEM, TEM, and FTIR analyses).

CONFLICTS OF INTEREST. The authors declare that they have no conflicts of interest.

ETHICAL APPROVAL. Not applicable.

REFERENCES

- [1] R.A. Ismail, S.A. Aljuboory, Mycotoxin contamination of food and feed: a global concern, *Food Chem. Toxicol.* 153 (2021) 112267.
- [2] IARC, Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, *IARC Monographs Eval. Carcinog. Risks Hum.* 56 (1993) 245–395.
- [3] T.P. Moloi, K. Ziqubu, S.E. Mazibuko-Mbeje, N.H. Mabaso, Z. Ndlovu, Aflatoxin B1-induced hepatotoxicity through mitochondrial dysfunction, oxidative stress, and inflammation as central pathological mechanisms: a review of experimental evidence, *Toxicology* 509 (2024) 153983.
- [4] J.D. Groopman, T.W. Kensler, C.P. Wild, Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries, *Annu. Rev. Public Health* 29 (2008) 187–203.
- [5] V. Balan, L. Mihai, L. Avram, Aflatoxins in food: prevalence, health effects, and emerging trends in its mitigation—an updated review, *Food Saf. Health* 2 (2024) e12030.
- [6] A. Sirelkhatim, S. Mahmud, A. Seeni, N.H.M. Kaus, L.C. Ann, S.K.M. Bakhori, H. Hasan, D. Mohamad, Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism, *Nano-Micro Lett.* 7 (2015) 219–242.
- [7] K. Krishnamoorthy, J.Y. Moon, H.B. Hyun, S.K. Cho, S.J. Kim, Mechanistic investigation on the toxicity of MgO nanoparticles toward cancer cells, *J. Mater. Chem.* 22 (2012) 24610–24617.
- [8] A.K. Mittal, Y. Chisti, U.C. Banerjee, Synthesis of metallic nanoparticles using plant extracts, *Biotechnol. Adv.* 31 (2013) 346–356.
- [9] R. Hamed, R. Obeid, R. Abu-Huwaij, Plant mediated-green synthesis of zinc oxide nanoparticles: an insight into biomedical applications, *Nanotechnol. Rev.* 12 (2023) 20230112.
- [10] P. Rajiv, S. Rajeshwari, R. Venckatesh, Bio-fabrication of zinc oxide nanoparticles using leaf extract of *Parthenium hysterophorus* L. and its size-dependent antifungal activity, *Spectrochim. Acta A* 112 (2013) 384–387.
- [11] A. Alberts, E.-T. Moldoveanu, A.-G. Niculescu, A.M. Grumezescu, *Nigella sativa*: a comprehensive review of its therapeutic potential, pharmacological properties, and clinical applications, *Int. J. Mol. Sci.* 25 (2024) 13410.
- [12] M.N. Nagi, H.A. Almakki, M.M. Sayed-Ahmed, A.M. Al-Bekairi, Thymoquinone supplementation reverses acetaminophen-induced oxidative stress, nitric oxide production and energy decline in mice liver, *Food Chem. Toxicol.* 48 (2010) 2361–2365.
- [13] O.A. Akinrinmade, S.O. Omoruyi, Protective effect of thymoquinone and thymol against aflatoxin B1-induced hepatotoxicity in rats, *Hum. Exp. Toxicol.* 35 (2016) 1251–1258.
- [14] M. Abbas, M.A. Gururani, A. Ali, et al., Antimicrobial properties and therapeutic potential of bioactive compounds in *Nigella sativa*: a review, *Molecules* 29 (2024) 4914.
- [15] S.H. Al-Maqtari, A.A. Alghalibi, Green synthesis of zinc oxide nanoparticles using *Nigella sativa* seed extract and their characterization, *J. Nanomater.* 2021 (2021) 5570389.
- [16] S. Gunalan, R. Sivaraj, V. Rajendran, Green synthesized ZnO nanoparticles against bacterial and fungal pathogens, *Prog. Nat. Sci. Mater. Int.* 22 (2012) 693–700.
- [17] R. Dobrucka, J. Dugaszevska, Biosynthesis and antibacterial activity of ZnO nanoparticles using *Trifolium pratense* flower extract, *Saudi J. Biol. Sci.* 23 (2016) 517–523.
- [18] T. Bhuyan, K. Mishra, M. Khandelwal, R. Prasad, Biosynthesis of zinc oxide nanoparticles from *Azadirachta indica* for antibacterial and photocatalytic applications, *Mater. Sci. Semicond. Process.* 32 (2015) 55–61.
- [19] National Research Council, Guide for the Care and Use of Laboratory Animals, eighth ed., National Academies Press, Washington, DC, 2011.
- [20] B. Halliwell, J.M.C. Gutteridge, *Free Radicals in Biology and Medicine*, fifth ed., Oxford University Press, Oxford, 2015.
- [21] P. Trinder, Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor, *Ann. Clin. Biochem.* 6 (1969) 24–27.
- [22] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal. Biochem.* 95 (1979) 351–358.
- [23] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem.* 47 (1974) 469–474.
- [24] H. Aebi, Catalase in vitro, *Methods Enzymol.* 105 (1984) 121–126.
- [25] D.E. Paglia, W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, *J. Lab. Clin. Med.* 70 (1967) 158–169.
- [26] G.L. Ellman, Tissue sulfhydryl groups, *Arch. Biochem. Biophys.* 82 (1959) 70–77.
- [27] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [28] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell Res.* 175 (1988) 184–191.

- [29] A.R. Collins, The comet assay for DNA damage and repair: principles, applications, and limitations, *Mol. Biotechnol.* 26 (2004) 249–261.
- [30] J.A. Kiernan, *Histological and Histochemical Methods: Theory and Practice*, fifth ed., Scion Publishing, Banbury, 2015.
- [31] R.G.D. Steel, J.H. Torrie, *Principles and Procedures of Statistics: A Biometrical Approach*, third ed., McGraw-Hill, New York, 1997.
- [32] A. Janaki, E. Sailatha, S. Gunasekaran, Synthesis, characteristics and antimicrobial activity of ZnO nanoparticles, *Spectrochim. Acta A* 144 (2015) 17–22.
- [33] N.M. Shamhari, B.S. Wee, S.F. Chin, K.Y. Kok, Synthesis and characterization of zinc oxide nanoparticles with small particle size distribution, *Acta Chim. Slov.* 65 (2018) 578–585.
- [34] H. Agarwal, S. Venkat Kumar, S. Rajeshkumar, A review on green synthesis of zinc oxide nanoparticles – An eco-friendly approach, *Resour.-Effic. Technol.* 3 (2017) 406–413.
- [35] JCPDS, Powder Diffraction File, International Centre for Diffraction Data, Card No. 36-1451, Swarthmore, PA, USA, 2003.
- [36] R. Wahab, S.G. Ansari, Y.S. Kim, H.K. Seo, G.S. Kim, G. Khang, H.S. Shin, Low temperature solution synthesis and characterization of ZnO nano-flowers, *Mater. Res. Bull.* 42 (2007) 1640–1648.
- [37] F.S. Mehrzad, M. Tafvizi, H.R. Kamali, Protective effects of selenium-enriched probiotics against aflatoxin B1-induced toxicity in rats, *Biol. Trace Elem. Res.* 199 (2021) 4625–4635.
- [38] A.E. Altıyar, A.M. Kenawy, M.A. El-Sayed, et al., Acute aflatoxin B1-induced hepatic and cardiac oxidative damage in rats: ameliorative effects of morin, *Heliyon* 9 (2023) e21400.
- [39] L. Wang, Z. Huang, R. Liu, et al., The metabolism and biotransformation of AFB1: key enzymes and pathways, *Front. Pharmacol.* 13 (2022) 1040164.
- [40] T.W. Kensler, B.D. Roebuck, G.N. Wogan, J.D. Groopman, Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology, *Toxicol. Sci.* 120 (2011) S28–S48.
- [41] E.O. Farombi, O.O. Ogundipe, E.S.O. Uhunwangho, M.A. Adeyanju, J.O. Ola-Davies, Prophylaxis of aflatoxin B1-induced liver toxicity by kolaviron, a Garcinia biflavonoid, *Toxicology* 261 (2009) 123–129.
- [42] J. Iqbal, B.A. Abbasi, T. Mahmood, S. Kanwal, B. Ali, M.S. Shah, A.T. Khalil, Plant-derived anticancer agents: a green anticancer approach, *Asian Pac. J. Trop. Biomed.* 7 (2017) 1129–1150.
- [43] R. Verma, Evaluation of the protective effects of the vitamins C and E against aflatoxin B1-induced renal toxicity in mice, *Toxicol. Mech. Methods* 18 (2008) 409–415.
- [44] S.K. Soni, B. Singh, A. Bose, R. Singh, P. Jain, Zinc oxide nanoparticles ameliorate lead-induced hepatorenal damage in rats, *Environ. Sci. Pollut. Res.* 28 (2021) 44750–44762.
- [45] N. Khademi, M.R. Khezri, H. Saadat, M. Ghasemnejad-Berenji, The interplay between glutathione and aflatoxin B1: mechanistic and therapeutic perspectives, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 397 (2024) 4605–4614.
- [46] G.N. Wogan, T.W. Kensler, J.D. Groopman, Present and future directions of translational research on aflatoxin and hepatocellular carcinoma, *Food Addit. Contam. A* 29 (2012) 249–257.
- [47] M.M. Abdel-Daim, S. Siddiqui, A. Akhtar, Cardioprotective effect of zinc oxide nanoparticles on doxorubicin-induced toxicity in rats, *Environ. Sci. Pollut. Res.* 25 (2018) 35076–35084.
- [48] A. Alkaladi, N.A.M. Abdelazim, M. Afifi, Antidiabetic activity of zinc oxide and silver nanoparticles on streptozotocin-induced diabetic rats, *Int. J. Mol. Sci.* 15 (2014) 2015–2023.
- [49] M. Jarosz, M. Olbert, G. Wyszogrodzka, K. Młyniec, T. Librowski, Antioxidant and anti-inflammatory effects of zinc, *Inflamm. Res.* 66 (2017) 311–321.
- [50] A. Cort, T. Ozben, N. Saso, S. De Luca, L. Saso, Synergistic effects of zinc and selenium against oxidative stress, in: *Handbook of Oxidants and Antioxidants in Exercise*, Elsevier, 2020, pp. 187–201.
- [51] L. Ye, H. Chen, J. Wang, K.W.K. Tsim, Y. Wang, X. Shen, H. Lei, Y. Liu, Aflatoxin B1 induces inflammatory liver injury via gut microbiota in mice, *J. Agric. Food Chem.* 71 (2023) 10787–10797.
- [52] Y. Liu, J. Li, W. Kang, S. Liu, J. Liu, M. Shi, Y. Wang, X. Liu, X. Chen, K. Huang, Aflatoxin B1 induces liver injury by disturbing gut microbiota-bile acid-FXR axis in mice, *Food Chem. Toxicol.* 176 (2023) 113751.
- [53] V. Sharma, R.K. Shukla, N. Saxena, D. Parmar, M. Das, A. Dhawan, DNA damaging potential of zinc oxide nanoparticles in human epidermal cells, *Toxicol. Lett.* 185 (2009) 211–218.
- [54] C.-T. Ho, T. Zheng, S. Li, Role of zinc in DNA repair, in: *Molecular Targets and Therapeutic Uses of Spices*, World Scientific, Singapore, 2009, pp. 203–219.
- [55] W. Maret, Zinc and the zinc proteome, *Met. Ions Life Sci.* 12 (2013) 479–501.
- [56] R. Preetha Rani, M.A. Anand, S. Karunakaran, Histopathological changes in aflatoxin B1-treated rat liver, *Asian J. Pharm. Clin. Res.* 8 (2015) 296–299.
- [57] H.S. El-Beltagi, M. Rageb, M.M. El-Saber, R.A. El-Masry, K.M.A. Ramadan, M. Kandeel, A.S. Alhajri, A. Osman, Green synthesis, characterization, and hepatoprotective effect of zinc oxide nanoparticles from *Moringa oleifera* leaves in CCl4-treated albino rats, *Heliyon* 10 (2024) e30627.
- [58] H.M. Hassan, M.A. Gad, E.H. Sayed, Green-synthesized ZnO nanoparticles attenuate carbon tetrachloride-induced hepatotoxicity in rats: role of oxidative stress and apoptosis, *Environ. Sci. Pollut. Res.* 27 (2020) 23987–23998.
- [59] M.S. El-Mleeh, A.A. Negm, Biogenic zinc oxide nanoparticles protect against cisplatin nephrotoxicity in rats through antioxidant and anti-inflammatory mechanisms, *J. Trace Elem. Med. Biol.* 64 (2021) 126704.