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Evaluating the Toxicity of Commercial Chelated Multi-Micronutrient Nanofertilizer

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Abstract

Despite growing interest in nanofertilisers, safety concerns persist. In this study, the toxicity of a chelated multi-micronutrient nanofertiliser was assessed using male albino rats (*Rattus norvegicus*) as non-target organisms. Thirty-five male rats were divided into five groups (n=7): one control and four experimental groups (C1-C4). Rats in experimental groups were orally administered fifteen doses of nanofertiliser (15, 30, 62, and 126 mg/rat, respectively) at one-day intervals. Significant weight loss was observed in C1-C4 (−10.86±10.95 g, −8.43±14.46 g, −38.71±28.70 g, and −34.71±21.55 g, respectively), compared to the control group (31.29±9.62 g). Liver weights were also reduced (C1: 7.35±1.39 g, C2: 6.55±0.88 g, C3: 7.35±1.10 g, and C4: 9.07±1.60 g) versus controls (11.26±2.08 g). Although levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) fluctuated, most alterations were statistically insignificant. Significant gene upregulation was observed: *Casp3* was upregulated by 2.78±0.98 (C2), 2.26±0.96 (C3), and 2.29±0.26 (C4), and *Sod1* by 2.79±1.36 (C2) and 2.43±0.92 (C4). These findings indicate harmful biological effects of the tested nanofertiliser doses, highlighting the need for long-term chronic impact studies.

Keywords: Nanofertilizer, Nanoparticles, Nanotoxicity, RT-qPCR, *Sod1* and *Casp3*

تقييم سمية السماد النانوي التجاري المخلب متعدد العناصر الصغرى

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الخلاصة

رغم الاهتمام المتزايد بالأسمدة النانوية، ولكن المخاوف المتعلقة بسلامتها لا تزال قائمة. تهدف هذه الدراسة إلى تقييم سمّية السماد النانوي التجاري المخلب والمتعدد العناصر الصغرى في ذكور الجرذان البيض ككائن غير مستهدف (*Rattus norvegicus*). قسمت الجرذان البالغ عددها 35 جرذ الى خمسة مجاميع (n=7): مجموعة ضابطة وأربعة مجاميع تجريبية (C1–C4). تلقت المجاميع التجريبية 15 جرعة فموية بفاصل يوم واحد بتراكيز 15، 30، 62، و126 ملغم/جرذ. لوحظ انخفاض كبير في الوزن في جميع المجاميع التجريبية (−10.86±10.95، −8.43±14.46، −38.71±28.70، −34.71±21.55 غم على التوالي) بالمقارنة مع المجموعة الضابطة (31.29±9.62 غم)، كما انخفض وزن الكبد في المجاميع

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التجريبية (7.35±1.39، 6.55±0.88، 7.35±1.10، 9.07±1.60 غم على التوالي) مقارنة مع المجموعة الضابطة (11.26±2.08 غم). وعلى الرغم من تذبذب مستويات الإنزيمات AST، وALT، وALP، فإن معظم هذه التغيرات لم تكن دالة إحصائيا. لوحظ ارتفاع معنوي في مستويات التعبير الجيني: إذ زاد التعبير عن الجين *Casp3* بمقدار 2.78±0.98 (C2)، و2.26±0.96 (C3)، و2.29±0.26 (C4)، والجين *Sod1* بمقدار 2.79±1.36 (C2)، و2.43±0.92 (C4). تشير هذه النتائج إلى وجود تأثيرات بيولوجية ضارة للجرعات المختبرة من السماد النانوي، مما يبرز الحاجة إلى إجراء دراسات طويلة الأمد لتقييم آثاره المزمنة.

1. Introduction

Nanofertilizers represent a novel generation of fertilizing agents, designed by leveraging the distinctive physiological properties of nanoparticles, primarily their nanoscale dimensions and high surface-to-volume ratio, which facilitate improved nutrient transport and assimilation within plant systems [1]. Nanofertilizers are generally classified into three primary groups: those that provide macronutrients, those that deliver micronutrients, and bio-based nanoscale fertilisers. While specific formulations consist of a single nutrient element, others are engineered to carry a complex blend of essential nutrients [1-3]. Their synthesis is achieved through diverse techniques, including physical (top-down) and chemical (bottom-up) methods, as well as biologically mediated methods [4-6].

The appropriate timing, place, quantity, and precise formulation are essential criteria before using nanoparticles in agriculture [7]. Moreover, despite the success that many nano-formulations have achieved in improving and increasing crop yields, the use of nanofertilizers in agriculture remains limited [8] due to challenges related to the dynamic behavior of nanofertilizers in the environment, as well as alterations in their physicochemical properties [9]. Nevertheless, investigating the effects of nanomaterials, including nanofertilizers, presents a significant challenge due to their potential to accumulate in the environment or interact with the biological components of living organisms, which may lead to adverse health effects [10,11].

The internal accumulation of nanoparticles in plants draws attention to the need to reassess fertilizer use through a comparative lens. Conventional fertilizers, though widely used, cause soil degradation, contribute to water pollution through runoff and leaching, emit greenhouse gases, and disrupt microbial communities. In contrast, nanofertilizers offer improved nutrient efficiency but raise concerns due to their persistence in soil, potential toxicity to non-target organisms, disruption of soil microbiota, and ability to enter the food chain [12].

In this context, nanofertilizers tend to accumulate in soil and crops with continuous use [11]. Their accumulation localizes within various plant structures, such as cell membranes and walls, cytoplasm, nucleus, vacuoles, chloroplasts, cortex, pericycle, endodermis, and tonoplast. Additionally, they are transported via vascular tissues to above-ground plant organs, such as stem nodes, leaves, flowers, and fruits [7]. This internal distribution and accumulation support the potential for nanofertilizers to move through trophic levels in the food chain [10]. However, the persistence of nanoparticles in soil and their uptake by plants are governed by intricate interactions involving biological and geochemical transformations, as well as soil-specific parameters like pH, organic matter content, and moisture availability [11]. Therefore, understanding and managing these factors is crucial, as they significantly affect the toxicity of nanoparticles, their movement through the food chain, and their accessibility to other organisms, including humans [7,10].

Conversely, drought conditions influence the dynamics of nanoparticle behavior and their accumulation patterns within environmental matrices. While nanoparticles are used to enhance plant resistance to drought stress, water scarcity exacerbates their accumulation in the soil, negatively affecting soil organisms. Furthermore, prolonged exposure to nanoparticles under drought conditions has led to significant changes in the ecosystem, such as reduced plant growth, declined microbial diversity, deteriorated water quality, and harmful effects on organisms beyond the intended targets [13]. After accumulating and moving through environmental compartments, such as soil, plants, and aquatic systems, nanoparticles can enter living organisms via ingestion [10].

As the liver is one of the principal organs exposed to the bloodstream and circulating nanoparticles, it is therefore one of the organs most susceptible to oxidative stress resulting from excessive production of reactive oxygen species (ROS). In addition to damaging DNA, ROS also damages RNA, as RNA is more susceptible to oxidative-derived modifications than DNA. RNA is less capable of repairing these lesions due to its single-stranded nature and limited repair capacity. Increased levels of ROS lead to deamination of RNA bases, resulting in misfolding, instability, and translation errors during protein synthesis. ROS also affects regulatory (noncoding) microRNAs, which play crucial roles in stress responses and gene regulation. The loss of RNA integrity within hepatic cells disrupts signaling and immune functions. Therefore, ROS-mediated RNA damage constitutes another potential genotoxic mechanism of nanoparticles.
Accordingly, this study investigates the effects of chelated multi-micronutrient nanofertilizer on a non-target organism.

2. Materials and Methods

2.1 Laboratory animals

The experiment was carried out using thirty-five male albino rats (*Rattus norvegicus*), aged between 7 and 10 weeks and weighing 176-350 g. The animals were sourced from two certified facilities: the Iraqi Centre for Cancer and Medical Genetic Research at Al-Mustansiriyah University, and the National Centre for Drug Control and Research/Ministry of Health. Upon arrival, the animals were maintained under standard husbandry conditions in sawdust-lined cages within a controlled animal care facility. Unrestricted access was provided to commercial rodent chow and tap water throughout the study. Before treatment initiation, a fourteen-day acclimatization period was observed to allow physiological stabilization and adaptation to laboratory conditions. After weighing and individual identification, the animals were allocated into a control and four experimental groups (C1-C4), each comprising seven rats. Each group was composed of rats representing all age classes of the studied animals.

2.2 Nanofertilizer and oral gavage

The commercial glycine-chelated multi-micronutrient nanofertilizer, composed of Fe, Zn, Cu, Mn, B, and Mo, was purchased from a specialized agricultural supplier in Baghdad. The recommended concentration of 1 g/L for foliar spraying was adopted as a reference for preparing the nanofertilizer doses of 15, 30, 62, and 126 mg/rat, which were used in this study following the method outlined by Oghenesuvwe *et al.,* [16]. These doses were administered to the experimental rat groups (C1-C4), respectively, as single doses with one-day intervals, using a 38 mm long, 16G ball-tipped needle. After the dosing period, the animals were reweighed to assess body weight changes, anaesthetized with chloroform, and dissected. Blood was drawn from the heart for liver function testing. The liver was weighed, and tissue samples were collected for gene expression analysis.

2.3 Liver function test

Blood samples were collected in 1 mL Eppendorf tubes preloaded with heparin-ether as an anticoagulant. Following the manufacturer's instructions, each tube was gently inverted 8–10 times to ensure thorough mixing of the blood and anticoagulant. Serum levels of ALT, AST, and ALP were then measured using a veterinary dry chemistry analyzer (Seamaty, China). The results were printed after 12 minutes.

2.4 Gene expression by RT-qPCR reaction

Total RNA was isolated from liver tissue samples using the TransZol Up Plus RNA Kit (TransGen, China), in strict accordance with the manufacturer's instructions. The concentration and purity of the isolated RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), with yields ranging from 350 to 3470 ng/μL and A260/A280 ratios within the optimal range of 2.0 to 2.1. Subsequently, four microliters of total RNA from each sample were reverse-transcribed into complementary DNA (cDNA) using the GoScript™ Reverse Transcription System (Promega, USA), according to the supplier's protocol.

Quantitative real-time PCR (qRT-PCR) was performed using the PerfectStar® Green qPCR SuperMix (TransGen, China), along with gene-specific primers for *Casp3*, *Sod1*, and *Hprt1* (Table 1). The primers were synthesized by GenScript (China), with slight modifications based on previously reported sequences in the literature. Amplification was conducted using an Agilent RT-qPCR system (USA), following the thermal cycling conditions detailed in Table 2. Expression levels of the target genes were normalized to the endogenous reference gene Hprt1, which encodes hypoxanthine-guanine phosphoribosyltransferase. Relative gene expression was calculated using the $2^{\wedge-\Delta\Delta Ct}$ method, as described by Livak and Schmittgen [17].

Table 1: Primer pairs employed for quantitative gene expression analysis

Primers	Primer sequence 5′–3′	Amplicon size	Ta ˚C	References
Casp3-F	AATTCAAGGGACGGGTCATGG	182 bp	60	[18]
Casp3-R	ATTGACACAATACACGGGATCTGT			
Sod1-F	ATGTGTCCATTGAAGATCGTGTGA	139 bp	60	[19]
Sod1-R	CTTCCAGCATTTCCAGTCTTTGTAC			
Hprt1-F	CTCATGGACTGATTATGGACAGGAC	123 bp	60	[20]
Hprt1-R	GCAGGTCAGCAAAGAACTTATAGC			

Table 2: qPCR thermal cycling program

Steps	Time (Sec.)	Temperature (˚C)	Cycles
Initial denaturation	30	94	1
denaturation	4	94	
Annealing	15	60	40
Extension	10	72	

3. Statistical Analysis

Data processing and statistical evaluation were performed using IBM SPSS Statistics (Version 23). A one-way ANOVA was used to assess whether significant differences were present among the experimental groups. Post hoc analyses were conducted using the least significant difference (LSD) test to identify specific intergroup differences. Results were reported as means with their standard deviations (mean ± SD). A p-value ≤ 0.05 was considered indicative of statistical significance.

4. Results and Discussion

4.1 Weight change

Figure 1 shows that body weight decreased to a statistically significant extent in all experimental groups compared to the control group ($p < 0.001$). The C3 and C4 groups exhibited the most significant reduction, indicating a dose-response relationship in the physiological reaction to dietary exposure to nanofertilizers. The weight loss observed in this study aligned with reports that demonstrated comparable effects following exposure to different nanoparticles directly in animals. For example, Razooki and Rabee treated mice intraperitoneally with different doses of zinc oxide nanoparticles and reported a significant decrease in body weight two to four weeks later [21]. Likewise, oral administration of copper oxide nanoparticles resulted in significant weight loss in male mice after just two weeks [22]. Other studies have also reported similar effects with molybdenum trioxide (MoO_3) nanoparticles, where direct oral exposure resulted in a measurable reduction in body weight. However, when researchers introduced MoO_3 nanoparticles indirectly via crops such as common beans grown with nanoparticle-based fertilizers, the impact on body weight declined [23]. Conversely, not all studies supported this outcome. In one case, researchers observed weight gain in animals after prolonged oral exposure to iron oxide nanoparticles [24], suggesting that nanoparticle composition, absorption kinetics, and metabolic interactions influence physiological responses.

In the present study, the experimental groups showed a measurable decrease in overall body weight, which researchers linked to various mechanisms of nanotoxicity, including the generation of ROS that damaged cells, enzyme adsorption that disrupted normal digestive processes, reduced digestive efficiency, and changes in the gut microbiota [25].

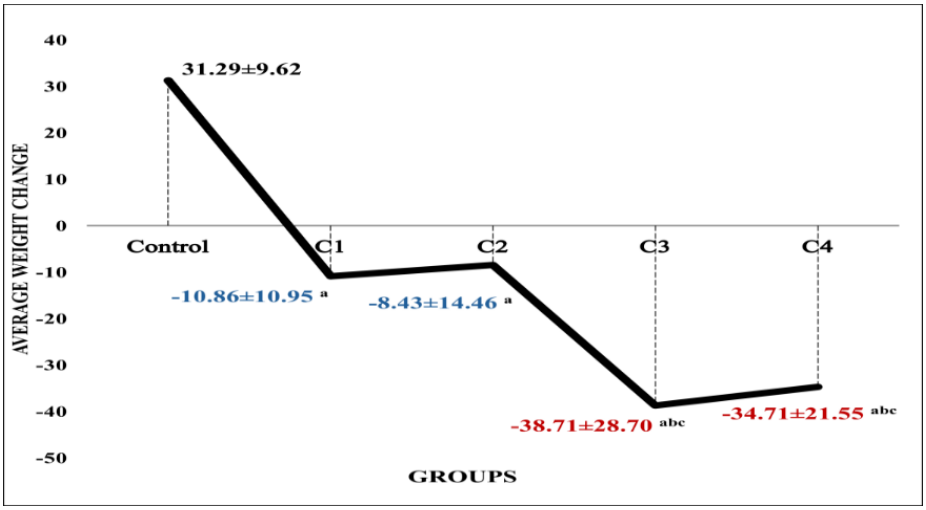

Figure 1: Changes in body weight, presented as mean ± SD. Statistical significance was set at $p \leq 0.05$. Superscript lowercase letters indicate significant differences: 'a' vs. control, 'b' vs. C1, and 'c' vs. C2. The calculated LSD value for body weight was 20.18.

4.2 Liver weight and liver index

In the present investigation, all experimental groups showed a statistically significant reduction in absolute liver weight compared to the control group ($p < 0.001$). While this decline remained consistent across the treatment groups, the liver index did not show a corresponding statistically significant change, as illustrated in Figure 2. Notably, this figure also shows a significant increase in liver weight in the C4 group relative to the C1-C3 groups, indicating a non-linear dose-response relationship. This trend agrees with the observations of

Graham *et al.,* [26], who also reported a non-linear correlation between exposure concentration and biological outcomes. In agreement with the current findings, which showed no significant differences in liver index among the treated groups, previous research reported similar outcomes under varying exposure conditions. For example, in rats administered MoO_3, the liver index remained statistically unchanged, with only slight, non-significant fluctuations observed at concentrations of 10 ppm and 40 ppm [23]. Similarly, a non-significant increase in liver index was reported in animals fed a diet supplemented with Mn_2O_3 [27].

Furthermore, in a study investigating ZnO nanoparticles, a significant reduction in liver index was observed at 25 mg/kg, whereas no significant change occurred at 50 mg/kg. However, a significant reduction reappeared at the higher dose of 100 mg/kg [21]. These results align with the observations of Sołek *et al.,* who noted that the effects extended beyond a specific organ and involved systemic responses throughout the entire body [27]**.**

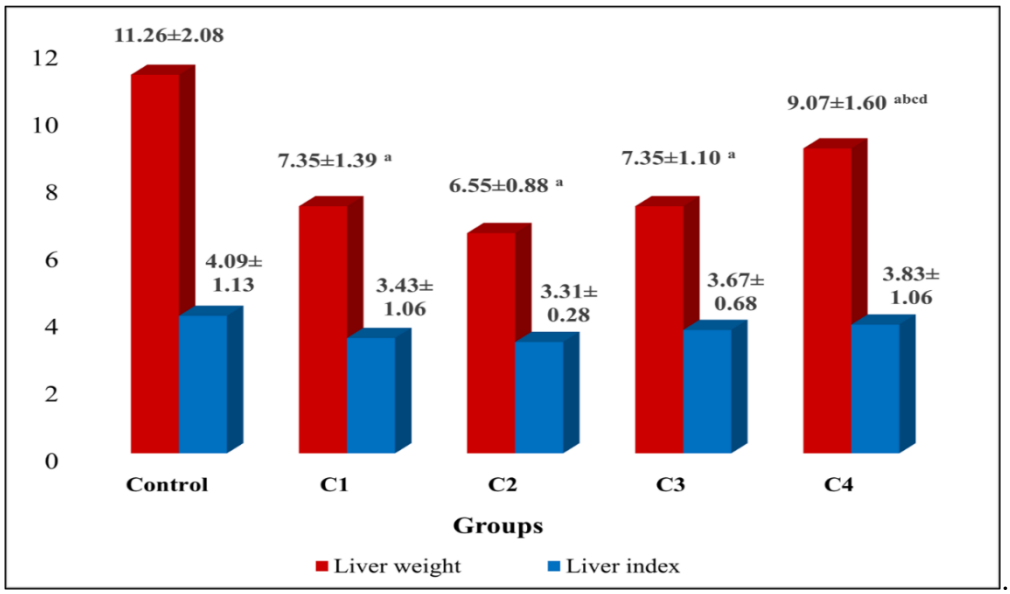

Figure 2: Liver weight and index are reported as means ± SD. Statistical significance was set at $P \leq 0.05$. Different lowercase letters indicate significant differences: 'a' vs. control, 'b vs. C1, 'c' vs. C2, and 'd vs. C3. The LSD values were 1.6 for liver weight and 0.98 for liver index.

4.3 Liver enzymes measurements

As shown in Figure 3, the ALT enzyme level decreased insignificantly in the C1 group and increased insignificantly in the C2 and C4 groups. However, enzyme levels showed a significant elevation in the C3 group compared to the control and C1 groups. Similarly, AST enzyme levels showed a slight, non-significant rise in the C1, C2, and C4 groups, while a statistically significant increase was observed in the C3 group compared to the control. Furthermore, ALP enzyme levels exhibited a non-significant decline in both the C1 and C2 groups, while a non-significant increase was noted in the C3 group. In contrast, the C4 group showed a significant increase compared to the C1 group. These findings aligned with previous studies. For instance, Singh *et al.,* [28] reported a significant increase in ALT levels in rats exposed to MnO_2 at concentrations of 30, 300, and 1000 mg/kg. In the same study, AST levels significantly increased at 300 and 1000 mg/kg, while a non-significant decrease was observed at 30 mg/kg. Additionally, the intraperitoneal injection of mice with ZnO NPs led to a significant elevation in AST levels at concentrations of 25, 50, and 100 mg/kg after two and

four weeks. ALT levels also increased significantly at 50 mg/kg after four weeks, and at 100 mg/kg after both two and four weeks. On the other hand, ALP levels fluctuated between significant and non-significant increases and decreases across all concentrations throughout the study period [21]. In another study, rats treated with 40 ppm of MoO_3 exhibited significantly increased ALT levels and significantly decreased ALP levels, while AST levels remained statistically unchanged [23].

Overall, the observed changes in liver enzyme activity indicate a non-linear relationship with the concentration of nanofertilizer. This trend is consistent with previous findings and those of Graham *et al.,* [26].

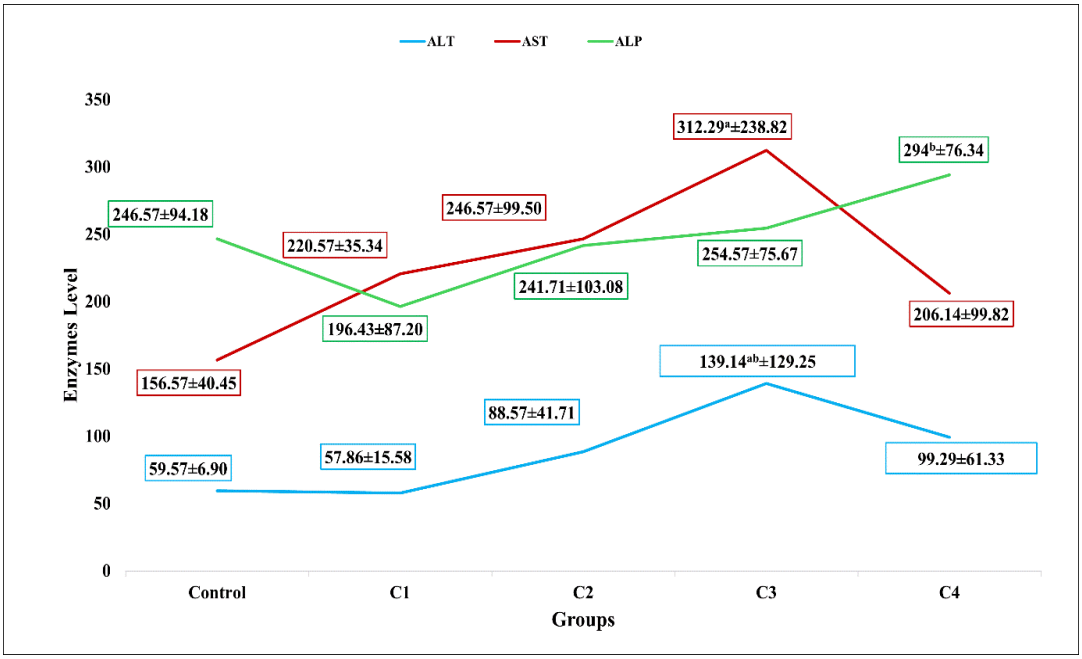

Figure 3: Liver enzyme levels (U/L) are presented as mean ± standard deviation (S.D.), with statistical significance set at $P \leq 0.05$. The lowercase letters denote significant differences as follows: “a ' indicates a difference compared to the control group, and 'b' indicates a difference compared to group C1. The least significant difference (LSD) for ALT was 37.22, for AST was 137.9, and for ALP was 95.98.

4.4 The gene expression

Agricultural chemicals have been shown to induce alterations in the genetic material within cells, even when applied at low concentrations, despite being considered safe [29]. In agreement with these findings, the present study demonstrated a significant upregulation in the expression of the *Casp3* gene in the C2, C3, and C4 experimental groups compared to both the control and C1 groups ($p < 0.001$), as presented in Table 3 and Figure 4. Additionally, *Sod1* gene expression was significantly increased in the C2 group relative to the control. A significant elevation in *Sod1* expression was also observed in the C4 group when compared to both the control and C3 groups ($p = 0.002$). These results were consistent with previous studies that investigated similar genetic targets. Notably, *Casp3* expression was significantly upregulated in rats exposed to ZnO nanoparticles at doses of 100, 200, and 300 mg/kg [30]. A similar increase was reported in mice following oral exposure to CuO nanoparticles at a dose of 100 mg/kg [31]. In contrast, *Sod1* expression exhibited minimal changes in rats receiving intravenous doses of Fe_3O_4@PEG nanoparticles [32]. Nevertheless,

a slight upregulation of *Sod1* was observed in animals fed with nano-zinc-supplemented diets at doses of 25 and 50 mg/kg [33].

The elevation in *Casp3* gene expression indicated liver damage due to oxidative stress. As a result, the liver appeared to activate apoptotic pathways to eliminate irreparably damaged cells and promote tissue regeneration [30]. The upregulation of the *Sod1* gene represented a vital defence mechanism through which hepatocytes responded to oxidative stress by increasing the expression of *Sod1* to convert reactive species into less harmful molecules [34]. The observed downregulation of *Sod1* expression in the C3 group may have resulted from a transient suppression of gene expression triggered by oxidative stress and inflammatory changes affecting transcriptional mechanisms [35].

Table 3: Expression levels of *Casp3* and *Sod1* genes in rat liver tissues. Group differences are denoted by lowercase letters: 'a' indicates significance relative to the control group, 'b' to C1, 'c' to C2, and 'd' to C3.

Groups	*Casp-3* Gene Expression (Mean±S.D.)	*Sod1* Gene Expression (Mean±S.D.)
Control	1.01±0.18	1.05±0.34
C1	1.45±0.50	1.92±0.67
C2	2.78±0.98 ^{ab}	2.79±1.36 ^{a}
C3	2.26±0.96 ^{ab}	1.35±0.27 ^{c}
C4	2.29±0.26 ^{ab}	2.43±0.92 ^{ad}
LSD	0.73	0.89
P value	<0.001**	0.002**

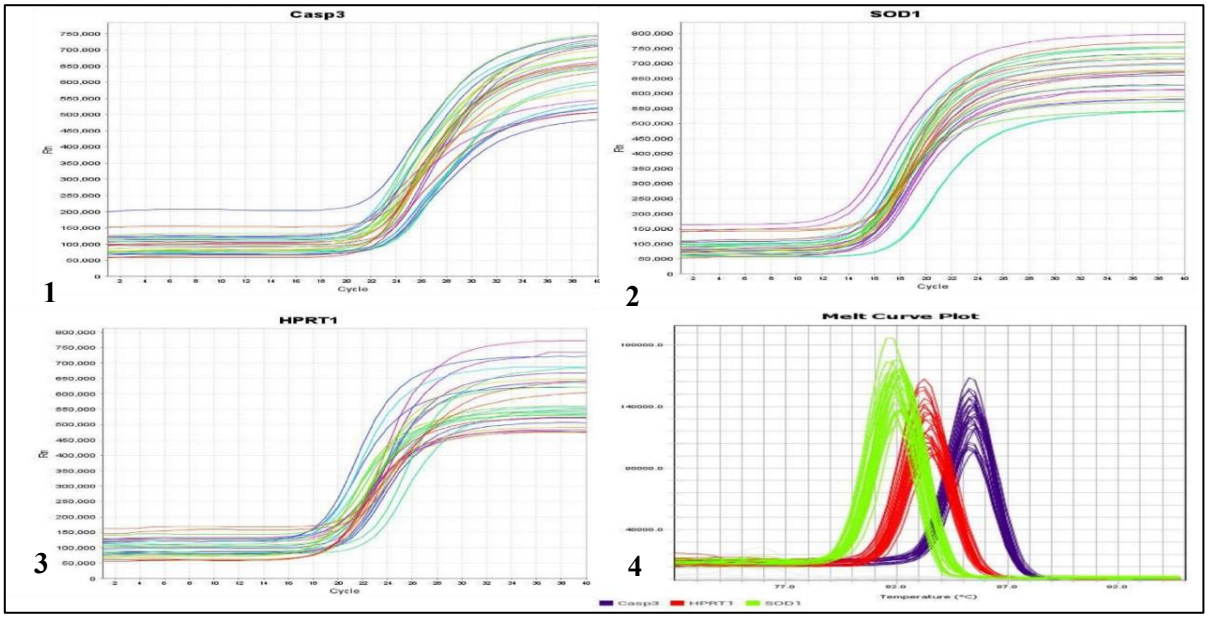

Figure 4: Amplification plots for *Casp3* (1), *Sod1* (2), and the reference gene *Hprt1* (3), and melt curve analysis for all genes (4) obtained by RT-qPCR.

5. Conclusion

In conclusion, although nanofertilizers are generally expected to be safe, the current findings have revealed physiological and genetic side effects resulting from continuous exposure over a relatively short period. These observations shall prompt further rigorous

investigations into the potential risks associated with nanofertilizers. They will require the establishment of strict regulatory frameworks governing their use, particularly in arid environments prone to environmental accumulation and potential transfer through the food chain.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Funding

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Ethics clearance

Ethical approval was obtained from the Ethics Committee of the College of Education for Pure Sciences, Ibn Al-Haitham, University of Baghdad (Reference: EC-77, Date: 1 April 2025). All procedures were conducted in accordance with international ethical standards and adhered to the 'Three Rs' principle (Replacement, Reduction, and Refinement).

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