



RESEARCH ARTICLE

# Metal oxide nanoparticles regulate germination, growth and oxidative stress responses in *Withania somnifera* L. Dunal

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## Abstract

Chemically synthesised nanoparticles are promising growth elicitors for increasing plant production; their dose-dependent effects on medicinal plants have not yet been fully investigated. To bridge this gap, this study evaluated the effects of 3 metal oxide nanoparticles [copper oxide (CuO), zinc oxide (ZnO) and titanium dioxide (TiO<sub>2</sub>)] on the early development of *Withania somnifera* L. Dunal (Arka ashwagandha) seedlings at four different concentrations (CuO: 0.75–100 ppm; ZnO: 25–100 ppm; TiO<sub>2</sub>: 4–10 ppm), with a priority on germination parameter. Germination percentage, biomass and biochemical responses were recorded over the 30-day growth period in which seeds were cultured under controlled conditions. In comparison to the control group, effective concentrations of nanoparticles, mainly CuO at 75 ppm, ZnO at 25 ppm and TiO<sub>2</sub> at 8 ppm, clearly improve germination percentage, fresh and dry biomass, total chlorophyll, protein, carbohydrate, phenolic and flavonoid content. Additionally, as compared to the control, both enzymatic and non-enzymatic tests of plants treated with nanoparticles showed encouraging findings for antioxidant activity. On the other hand, inhibitory effects, decreased growth and reduced biochemical performance were observed at high concentrations of ZnO and TiO<sub>2</sub>, suggesting dose-dependent response. The results of this evaluation indicate a clear two-way response, with higher doses of nanoparticles causing oxidative stress while lower doses enhance metabolic activity. This study highlights the potential of precisely calibrated nanoparticle treatments to improve early growth and biochemical performance of *W. somnifera*.

**Keywords:** copper oxide nanoparticles; oxidative stress; seed germination; titanium dioxide nanoparticles; *Withania somnifera*; zinc oxide nanoparticles

## Introduction

*Withania somnifera* L. Dunal (ashwagandha), renowned for its medicinal properties, widely used for its therapeutic relevance in stress-related disorders and chronic diseases for ages, with distinct mentions in the centuries old Ayurveda and traditional Chinese medicine (1). Its pharmacological properties are mainly related to bioactive metabolites produced by the plant such as withanolides, alkaloids, flavonoids and steroidal lactones (2). These secondary metabolites are considered to have neuroprotective effects that help to adapt to stress and reduce neurodegenerative damage (3–5). In traditional Ayurveda, it is identified as a Rasayana herb with a wide range of different health uses. The effect of nanoparticles on biochemical profiling in *W. somnifera* (including proline and chlorophyll) is a trending research area. Plants treated with nanoparticle suspension have shown differences in stress responses and photosynthetic properties by regulation of secondary metabolite pathways and antioxidant defence systems (6). Nanoparticles have been reported to support plants' health under adverse conditions by improving chlorophyll stability and photosynthetic efficiency by regulation of nutrient uptake and light utilisation (7).

Germination is an important phase in plant life cycle establishment and is remarkably influenced by nanoparticle treatments. Previous studies have shown that nanoparticles can increase germination rates and seedling vigour, however their impact is based upon the type, concentration and plant species involved in the experiment. Nanoparticles, like silica (Si) and zinc oxide (ZnO), have been proven to reduce germination duration and improve germination parameters in different crops. Metal nanoparticles, especially silver (Ag) and zinc (Zn), markedly increase final germination rates and root extension, with lower concentrations it has been recorded that frequent yielding have the most advantageous outcomes (8). The mechanisms involved for this advantage include enhanced physiological processes, increased  $\alpha$ -amylase activity, improved water absorption and modification of the seed coat that promotes germination. Furthermore, some nanoparticles such as ZnO can mitigate abiotic challenges like salt stress, therefore facilitating germination in unfavourable environmental conditions. Regardless of positive findings, the different results in plant reactions between species and growth condition emphasise the requirement for intense field study to confirm lab reports and understand long term effects of nanoparticle exposure during germination (9). Nanoparticles used in low to moderate concentration have been proven to improve seed

germination and early seedling development of different crops in various studies. Systematic analyses and focused seed-priming using nanoparticles in experiments have repeatedly proven that various metal-based nanoparticles enhance germination success, though the effects are dose-dependent and frequently biphasic. For instance, silver nanoparticles (Ag-NPs) increase germination percentage and seedling vigour in a variety of species, through seed-coat modification, antibacterial activity against pathogens and elicitation of metabolic activity (10). Zinc oxide nanoparticles (ZnO-NPs) have been reported to stimulate germination and seedling growth in a size- and concentration-dependent setup by enhancing enzyme activity and nutrient availability to plants. Priming treatments in *Hibiscus* revealed best results at approximately 30 nm and 50 mg L<sup>-1</sup> (11). In contrast, high concentrations of copper oxide nanoparticles (CuO-NPs) cause oxidative and genotoxic stress, which reduce development, whereas low concentrations of CuO-NPs (5–100 ppm) improve germination and vigour through moderate metabolic stimulation. Because nickel is used as a micronutrient while boosting antioxidant and stomatal responses, nickel oxide nanoparticles (NiO-NPs) have also been used as elicitors in a number of experiments to increase wheat crop germination and photosynthetic performance. Overall, these findings imply that nanoparticles used at ideal concentration for nano-priming can be a useful technique for enhancing germination parameters, whereas excessive exposure may cause phytotoxicity because of oxidative stress (12). The aim of this study was to assess the dose-dependent effects of 3 metal oxide nanoparticles (ZnO, titanium dioxide (TiO<sub>2</sub>) and CuO) on the seed germination and early development of *W. somnifera*, to identify both optimum and inhibiting dosage ranges.

## Materials and Methods

### Plant growth materials and nanoparticles

*Withania somnifera* Arka variety seeds were collected from the ICAR-IIHR Hesaraghatta, Bengaluru, Karnataka, India (13° 08' 04.7" N, 77° 29' 27.2" E). The experimental setup was initiated by autoclaving 100 kg of red sandy-loam soil at 121 °C and 15 lb pressure, which was collected from Urban Mali (72, 18<sup>th</sup> Cross, 2<sup>nd</sup> Main, Avalahalli, Anjanapura Post, JP Nagar 9<sup>th</sup> Phase, J. P. Nagar, Bengaluru 560 108, Karnataka). Sterilised soil was then dried under the shade and analysed for micronutrient content, heavy metal concentrations and water holding capacity (Table 1). The quantification of heavy metals in soil and irrigation water was conducted via wet acid digestion, adhering to a modified protocol

(13) and was measured using an Atomic Absorption Spectrophotometer (Shimadzu AA-6880, Japan). Seeds were initially cleansed with a soap solution (1:10) for 2–3 min, subsequently surface sterilised with 0.5 % (v/v) sodium hypochlorite for 4–5 min, rinsed and soaked overnight in distilled water prior to sowing. For germination investigations, sterilised seed trays were filled with soil that had been prepared with nanoparticle suspensions. Three metal oxide nanoparticles (CuO, ZnO and TiO<sub>2</sub>) were chosen as growth elicitors based on prior studies and preliminary screening of varied concentrations (14–16). Germination was measured at weekly intervals and the final germination response was utilised to determine the optimum concentration for the main experiment (Table 2). Nanoparticles stock solutions were prepared in distilled water and ultra sonicated for 25 min at 30 °C. These stocks were used to prepare four concentrations of CuO (0.75, 1, 75 and 100 ppm), ZnO (25, 50, 75 and 100 ppm) and TiO<sub>2</sub> (4, 6, 8 and 10 ppm) for treatment. Each treatment included triplicates, each with 5 plants, for a total of 15 plants (including an untreated control). The seedlings were allowed to germinate in a greenhouse with a temperature of 25 ± 2 °C and a relative humidity of 60–70 %. Under a natural photoperiod (~12–13 hr of light), diffused natural light was provided by the white opaque sheet covering the greenhouse. Nanoparticle treatments were administered weekly for 30 days (Fig. 1).

### Analysis of germination parameters

The germination performance of *W. somnifera* seeds was evaluated over a 30-day period using standard germination criteria. The germination percentage [(No. of germinated seeds / Total no. of seeds sown) × 100] was calculated for each nanoparticle treatment based on the number of seeds that emerged successfully within 30 days. This measurement directly indicated the impact of CuO, ZnO and TiO<sub>2</sub> nanoparticles on seed viability and early establishing potential (17).

### Estimation of biochemical parameters

After 30 days of growth, biochemical parameters were estimated in *W. somnifera* seedlings subjected to 3 nanoparticle treatments and an untreated control. The total protein content was estimated using the Folin-Lowry colorimetric method (18). A modified phenol-sulfuric acid method was used to determine the total carbohydrate content. To hydrolyse carbohydrate in 5 mL of 2.5 N HCl, 1 g of homogenised tissue was first cooked for 3 hr at 95 °C in a water bath. The extract was centrifuged for 10 min at 10000 rpm after cooling. Next, 5 mL of concentrated sulfuric acid (96 % v/v) and 1 mL of 2 % (v/v) phenol were added to 1 mL of the clear supernatant. The absorbance at 490 nm was measured using a UV-VIS

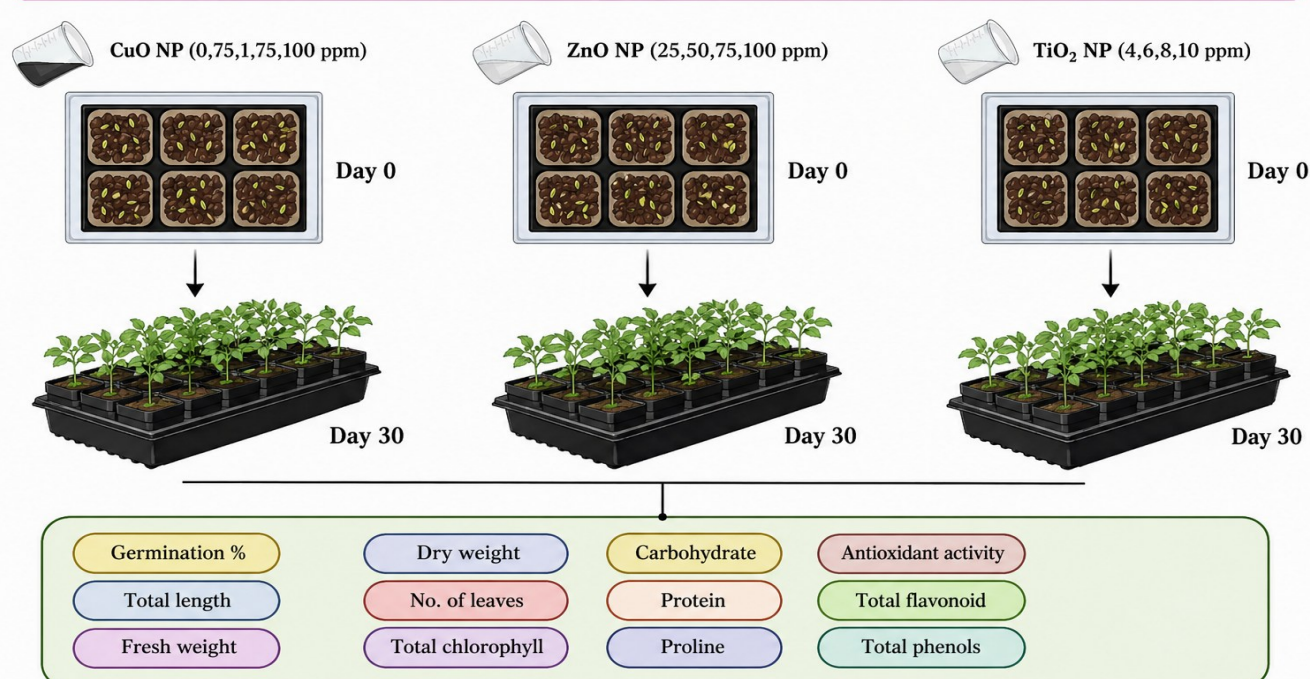
**Table 1.** Initial soil physico-chemical and nutrient profile showing measured values, optimum ranges and diagnostic status relevant to *Withania somnifera* growth conditions

| Parameters                   | Units             | Values | General optimum range | Diagnosis           |
|------------------------------|-------------------|--------|-----------------------|---------------------|
| pH                           |                   | 7.16   | 6.50–7.50             | Normal              |
| Electrical Conductivity (EC) | dSm <sup>-1</sup> | 0.24   | <1.00                 | Non-s-saline (safe) |
| Organic carbon               | %                 | 0.51   | 0.75–1.00             | Medium              |
| Available nitrogen           | ppm               | 102.62 | 125–180               | Low                 |
| Available phosphorus         | ppm               | 13.72  | 5–10                  | High                |
| Available potassium          | ppm               | 127.50 | 62–125                | High                |
| Exchangeable calcium         | ppm               | 722.50 | 800–1500              | Low                 |
| Exchangeable magnesium       | ppm               | 160.00 | 150–250               | Optimum             |
| Available sulphur            | ppm               | 31.60  | 10–15                 | Very high           |
| Available iron               | ppm               | 14.40  | 5–10                  | High                |
| Available manganese          | ppm               | 1.17   | 3–8                   | Low                 |
| Available zinc               | ppm               | 0.95   | 0.75–1.00             | Optimum             |
| Available copper             | ppm               | 0.58   | 0.50–1.00             | Optimum             |

**Table 2.** Preliminary screening of copper oxide, zinc oxide and titanium dioxide nanoparticle concentrations to define treatment ranges based on *Withania somnifera* germination. Germination was recorded at weekly intervals for up to 30 days and the final germination % was used to assess concentration-dependent responses and choose optimal dosages for the main experiment (14–16)

| Nanoparticles    | Concentration (ppm) | Rate of germination (%) | Response                                                                                         | Selected conc.      |
|------------------|---------------------|-------------------------|--------------------------------------------------------------------------------------------------|---------------------|
| CuO              | 0.25, 0.50, 0.75, 1 | 30, 70, 90, 80          | Maximum germination at 0.75 ppm is a concentration-dependent increase.                           | 0.75 and 1 ppm      |
| CuO              | 25, 50, 75, 100     | 40, 70, 99, 90          | Improved germination at increasing dosages, peaking at 75 ppm and suggesting a biphasic effect.  | 75 and 100 ppm      |
| ZnO              | 25, 50, 75, 100     | 80, 70, 75, 60          | Germination is moderate to high at all concentrations and gradually decreases at higher dosages. | 25, 50, 75, 100 ppm |
| ZnO              | 125, 150, 175, 200  | No germination          | The complete suppression of germination indicates toxicity at higher dosages.                    | Not selected        |
| TiO <sub>2</sub> | 4, 6, 8, 10         | 70, 40, 85, 30          | Variable interaction, with optimal germination at 8 ppm.                                         | 4, 6, 8, 10 ppm     |
| TiO <sub>2</sub> | 25, 50, 75, 100     | No germination          | The complete suppression of germination indicates toxicity at higher dosages.                    | Not selected        |

### Germination stage study of *Withania somnifera* L. Dunal for 30 days with nanoparticle treatment



**Fig. 1.** Schematic representation of the 30-day germination experiment of *Withania somnifera* under copper oxide, zinc oxide and titanium dioxide nanoparticle treatments. Seeds were exposed to 4 concentrations of each nanoparticle and seedlings were evaluated on day 30 for germination, growth indices, biochemical parameters and antioxidant responses.

spectrophotometer (Shimadzu UV-1900, Kyoto, Japan) (19). The chlorophyll content was measured using a slightly modified Arnon methodology in which 0.1 g of fresh leaf tissue was grinded using mortar and pestle in 5 mL of 80 % (v/v) acetone, incubated overnight at ambient temperature, centrifuged at 5000 rpm for 5 min and the absorbance of the supernatant was recorded at 645 and 663 nm (20). Proline accumulation, a marker of stress response, was estimated by using Bates' acid ninhydrin technique with minor modification (21).

#### Preparation of crude methanolic plant extract

Plants treated with nanoparticles were harvested then sun dried and powdered (1 g each from the control and nanoparticle-treated samples) were extracted using 10 mL of methanol while being continuously stirred for 48 hr on a rotary incubator. Whatman filter paper No. 1 was then used to filter the methanol solution. The residue was weighed and re-dissolved in methanol to produce a stock solution with a final concentration of 10 mg mL<sup>-1</sup> after the filtrate was evaporated to yield the crude extract. The extract was

bottled, stored at 4 °C until needed and diluted in accordance with the specifications of the experiment (22).

#### Total phenol content determination by Folin-Ciocalteu method

A modified Folin-Ciocalteu assay was used to calculate the total phenolic content (TPC) of treated and untreated plant samples. Sodium bicarbonate solution (0.5 mL of 7.5 % (w/v)) was added after 0.25 mL of the methanolic extract (5 mg mL<sup>-1</sup>) and an equal volume of diluted Folin-Ciocalteu reagent (1:1) were combined. A UV-VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan) was used to measure absorbance at 765 nm after the mixture was incubated for 1 hr at room temperature in the dark (23). Each test sample's phenolic concentration was estimated using a gallic acid standard curve with a range of 0–100 µg mL<sup>-1</sup> and represented as gallic acid equivalents (GAE).

### Total flavonoid content determination by the aluminium chloride method

The total flavonoid content (TFC) in the control and nanoparticle-treated samples was assessed (24), with slight modification. A 0.5 mL aliquot of the methanolic extract ( $10 \text{ mg mL}^{-1}$ ) was combined with 0.1 mL of a 10% (w/v) aluminium chloride solution and 0.1 mL of 1 M sodium acetate. The reaction mixture was incubated for 45 min at ambient temperature to facilitate complex formation. Following incubation, absorbance was measured at 415 nm utilising a microplate reader (BIO-RAD, iMark™, Japan). The content of flavonoids was quantified using a quercetin standard curve ( $0\text{--}100 \mu\text{g mL}^{-1}$ ) and reported as quercetin equivalents (QE).

### Assessment of antioxidant activity

#### Enzymatic antioxidant assays

**Superoxide dismutase activity (SOD):** The SOD activity was estimated using the nitro blue tetrazolium (NBT) photoreduction method. The activity of SOD was measured utilising an NBT reduction assay (25). For each reaction, a 3 mL assay mixture was generated by amalgamating 0.8 mL of phosphate buffer (50 mM, pH 7.0), 0.5 mL of methionine (13 mM), 0.5 mL of NBT ( $75 \mu\text{M}$ ), 0.5 mL of ethylenediaminetetraacetic acid (EDTA) (0.1 mM), 0.5 mL of riboflavin (2 mM) and 0.2 mL of the plant extract. The reaction tubes were subjected to fluorescent light for 10 min at ambient temperature to commence the photochemical reduction of NBT. Following incubation, the absorbance was measured at 560 nm to evaluate the extent of formazan production.

The percentage inhibition of NBT decrease was determined utilising the formula:

$$\% \text{ Inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100 \quad (\text{Eqn. 1})$$

A control denotes the absorbance of the reaction mixture devoid of extract, while A sample signifies the absorbance in the presence of plant extract. One unit of SOD activity is defined as the quantity of enzyme necessary to achieve 50% inhibition of NBT reduction, with enzyme units per millilitre computed depending on the percentage of inhibition observed.

**Ascorbate peroxidase activity (APX):** The activity of APX was assessed (26) with slight alterations. The reaction mixture contains 1.2 mL of potassium phosphate buffer (50 mM, pH 7.0), 0.2 mL of EDTA (0.1 mM), 0.2 mL of ascorbate, 0.2 mL of plant extract and 0.2 mL of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (0.1 mM). At 290 nm, the absorbance decrease caused by ascorbate oxidation was detected. The extinction coefficient of ascorbate ( $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ) was used to calculate the APX activity, which was expressed as micromoles of ascorbate oxidised per minute per milligram of protein. The enzymatic activity was assessed utilising the formula:

APX activity =

$$\frac{(\Delta A/\text{min}) \times \text{Total reaction volume (mL)} \times \text{protein content (mg)}}{\text{Extinction coefficient} \times \text{sample volume (mL)}} \quad (\text{Eqn. 2})$$

Where,  $\Delta A/\text{min}$  signifies the rate of absorbance alteration per minute.

**Catalase activity (CAT):** Catalase activity was evaluated (27) with minor modification. The reaction mixture comprised 1.95 mL of potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL of plant extract and 1.0 mL of  $\text{H}_2\text{O}_2$  solution (0.059 M). The degradation of

$\text{H}_2\text{O}_2$  was seen by measuring the reduction in absorbance at 240 nm. Catalase activity was determined using the extinction coefficient of  $\text{H}_2\text{O}_2$  ( $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ ) and expressed as micromoles of  $\text{H}_2\text{O}_2$  degraded per minute per milligram of protein.

Enzyme activity was assessed utilising the formula:

$$\frac{(\text{Change in absorbance min}^{-1} \times \text{Total reaction volume in mL} \times \text{Amount of protein in g in 1 mL of sample})}{(\text{Extinction coefficient for } \text{H}_2\text{O}_2) \times \text{Volume of sample taken in mL}} \quad (\text{Eqn. 3})$$

### Non-enzymatic antioxidant assays

#### 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH)

The DPPH free radical scavenging activity of the extracts was assessed (28), with slight changes. A 90  $\mu\text{L}$  aliquot of the methanolic extract ( $5 \text{ mg mL}^{-1}$ ) was diluted to 3 mL with methanol, followed by the addition of 1 mL of 0.1 mM DPPH solution. The reaction mixture was incubated in darkness for 30 min at ambient temperature to facilitate colour development. After incubation, absorbance was assessed at 517 nm utilising a UV-VIS spectrophotometer (Shimadzu UV-1900, Kyoto, Japan). Methanol (3 mL) functioned as the blank. The proportion of DPPH radical scavenging activity was determined using the following equation:

Radical scavenging activity (%) =

$$\frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100 \quad (\text{Eqn. 4})$$

#### Ferric reducing antioxidant power (FRAP)

Plant extract (1 mL) was mixed with 2.5 mL of phosphate buffer and potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] each. The mixture was incubated at  $50^\circ\text{C}$  for 20 min. A 2.5 mL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 mL) was then mixed with distilled water (2.5 mL) and  $\text{FeCl}_3$  (0.5 mL, 0.1%). Absorbance was measured at 700 nm using a Shimadzu UV-1900, Kyoto, Japan (29).

#### Phosphomolybdate assay

A 100  $\mu\text{L}$  methanolic plant extract was put in a sterile Eppendorf tube with 1 mL of phosphomolybdate reagent and incubated in a water bath at  $90^\circ\text{C}$  for 90 min. After cooling to room temperature, the absorbance of the resulting greenish shade was measured at 695 nm using a UV-VIS spectrophotometer (Shimadzu UV-1900, Kyoto, Japan). A 1 mL of phosphomolybdate reagent was added to 100  $\mu\text{L}$  of methanol and used as a blank. Ascorbic acid was used as a control (30).

### Estimation of nanoparticles accumulation in 30 days

#### *Withania somnifera*

The nanoparticle concentration in the plant samples was determined utilising a wet acid digestion method with some modification (13). After carefully grinding samples that had been oven-dried ( $35 \pm 5^\circ\text{C}$ ), 0.5 g of the materials were digested using aqua regia ( $\text{HNO}_3$ :  $\text{HCl}$  = 3:1). The mixture was heated to  $45 \pm 5^\circ\text{C}$  on a hot plate under a fume hood until the volume dropped to around 10 mL. After that, the digest was filtered through Whatman No. 42 filter paper after being diluted to 25 mL with deionised water. An atomic absorption spectrophotometer (Shimadzu AA-6880, Japan) was then used to determine the concentrations of the associated nanoparticles ( $\text{CuO}$ ,  $\text{ZnO}$  and  $\text{TiO}_2$ ).

## Statistical analysis

To assess the significance of differences across treatments for each evaluated parameter, a one-way analysis of variance (ANOVA) was conducted on the data after all measurements had been carried out in triplicate. The homogeneity of variance was assessed using SPSS. A post-hoc analysis using Duncan's Multiple Range Test (DMRT) was performed at a significance threshold of  $p < 0.05$  to compare treatment means. All statistical analyses were carried out using IBM SPSS Statistics software version 22.0.

## Results

### Influence of nanoparticles on seed germination of *Withania somnifera*

**Germination percentage:** Germination percentage showed clear concentration-dependent variation across all nanoparticles, with the control maintaining a baseline germination of 65.3 %. CuO nanoparticles showed a significant germination increase, from

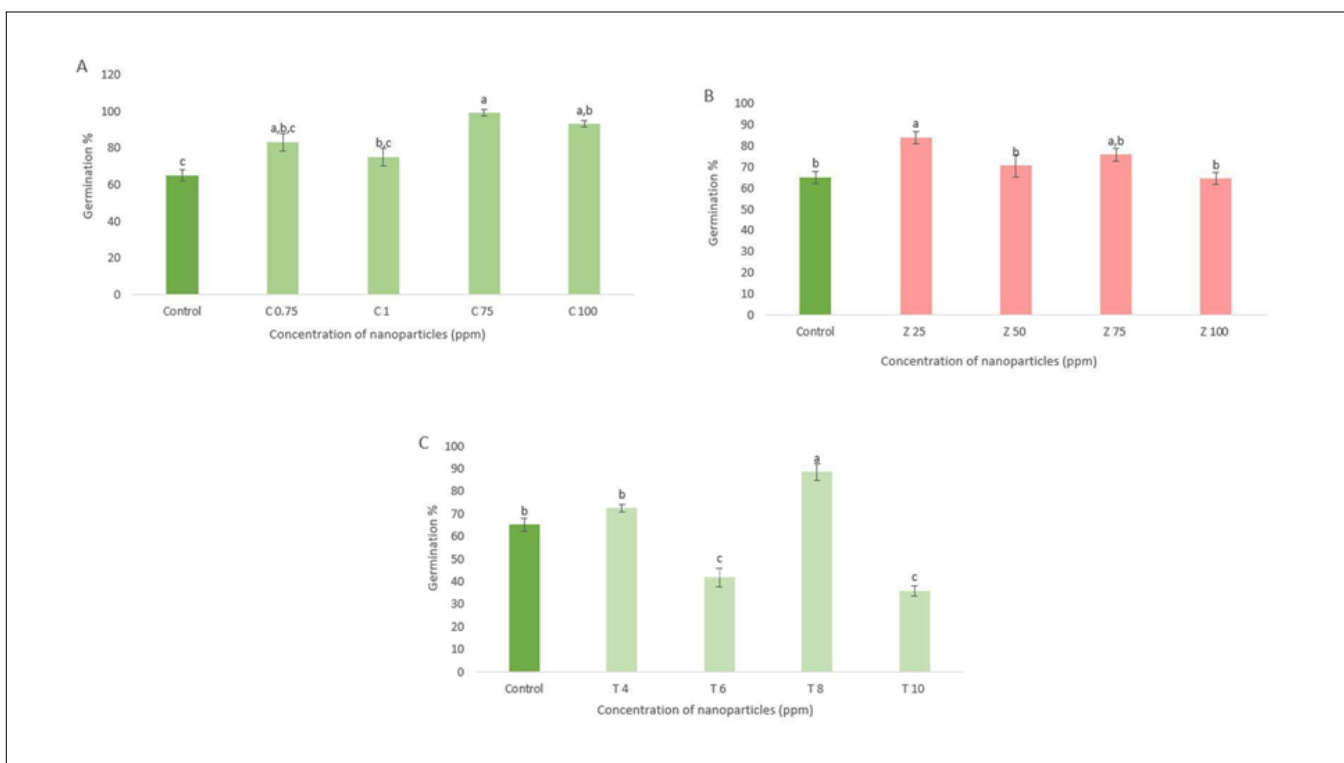
75.3 % at C1 to 99.3 % at C75 and 93.3 % at C100. They also had the most consistent beneficial response across dosages (Fig. 2). The ZnO-NPs showed a restricted hormetic window, with germination peaking at Z25 (84 %) and decreasing at increasing doses (Z50 70.67 %, Z75 76 % and Z100 64.67 %). Increasing ZnO concentration rapidly shifted the response from stimulation to mild phytotoxicity (Fig. 3). High concentration-sensitive TiO<sub>2</sub> nanoparticles had a narrow safe/beneficial range, with germination reaching 88.6 % at T8 ppm and decreasing to 36 % at T10 ppm.

### Length and biomass

The observation of seedling length closely relates with germination trends, confirming that the impacts of nanoparticles extend more than just germination into the initial stages of growth. Control seedlings averaged 8.2 cm, setting the benchmark (Fig. 4). In a concentration-dependent manner, CuO nanoparticles increased seedling length by 19.5 % at C75 (9.7 cm) compared to the control group. In contrast, ZnO-NPs showed a hormetic response, peaking at Z25 (9 cm) and decreasing seedling length to 6.73 cm at Z100. The

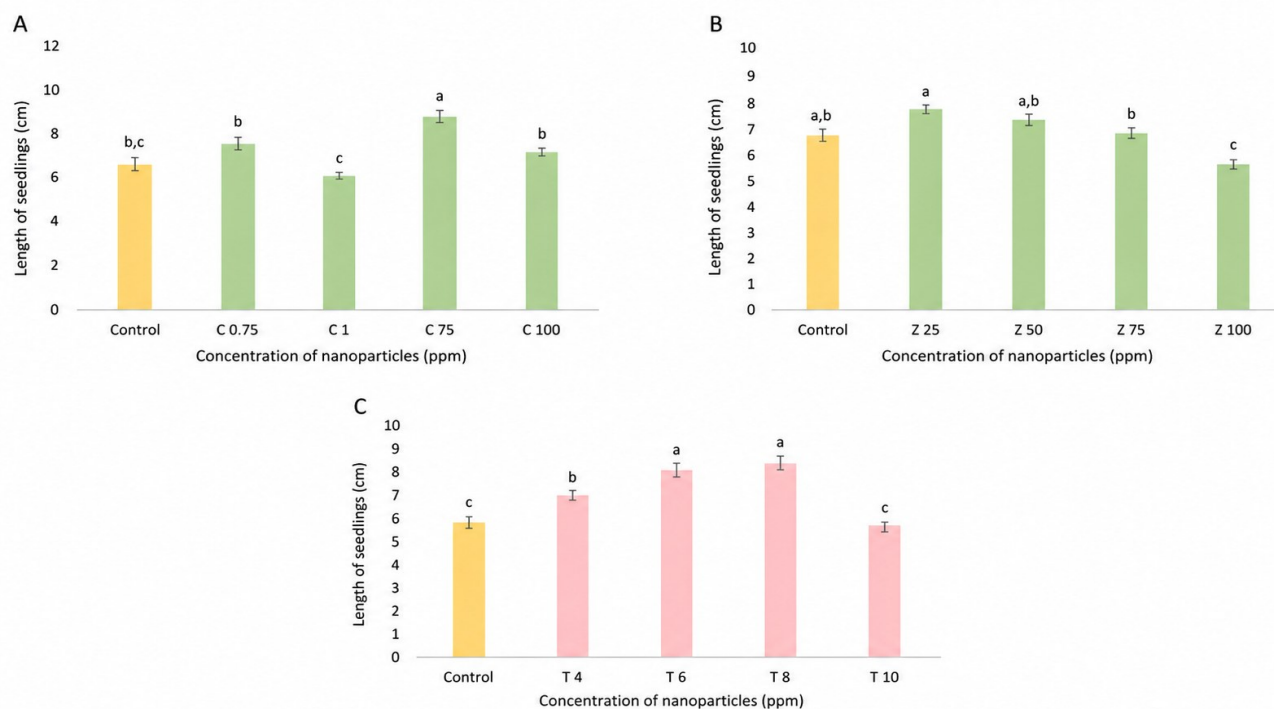


**Fig. 2.** Representative seedlings of *Withania somnifera* grown for 30 days under different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles. Visual differences in seedling vigour, leaf development and root growth illustrate the dose-dependent effects of nanoparticle treatments compared with the untreated control.



**Fig. 3.** Effect of metal oxide nanoparticles on seed germination (%) in *Withania somnifera*. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

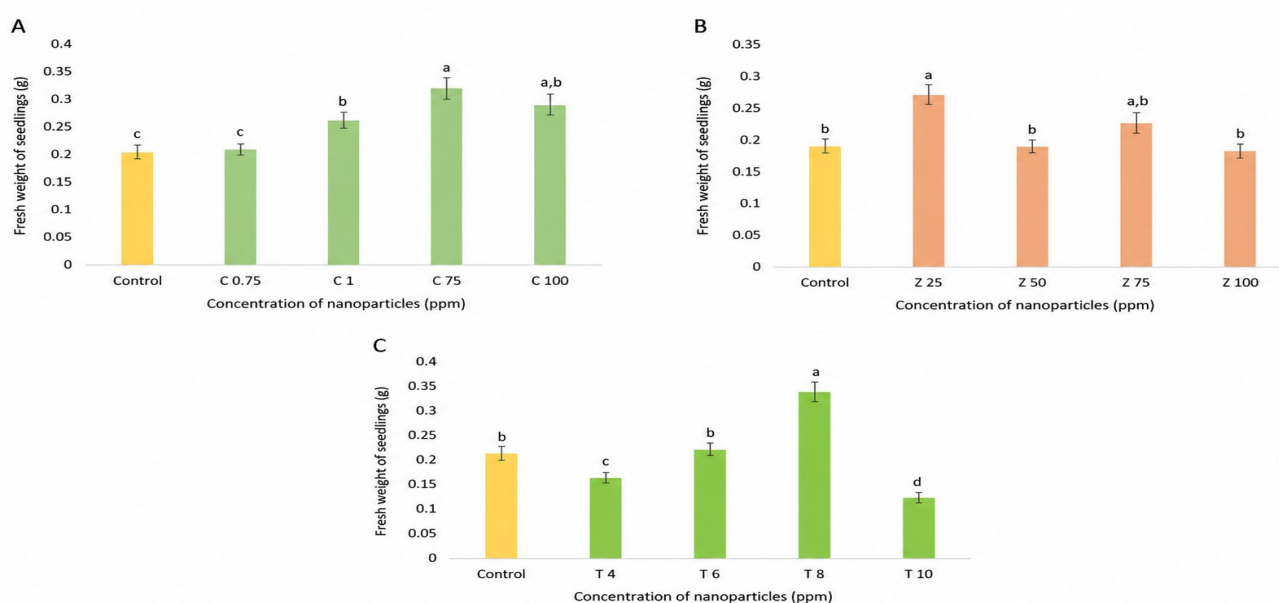
Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



**Fig. 4.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on seedling length (cm) of *Withania somnifera*. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

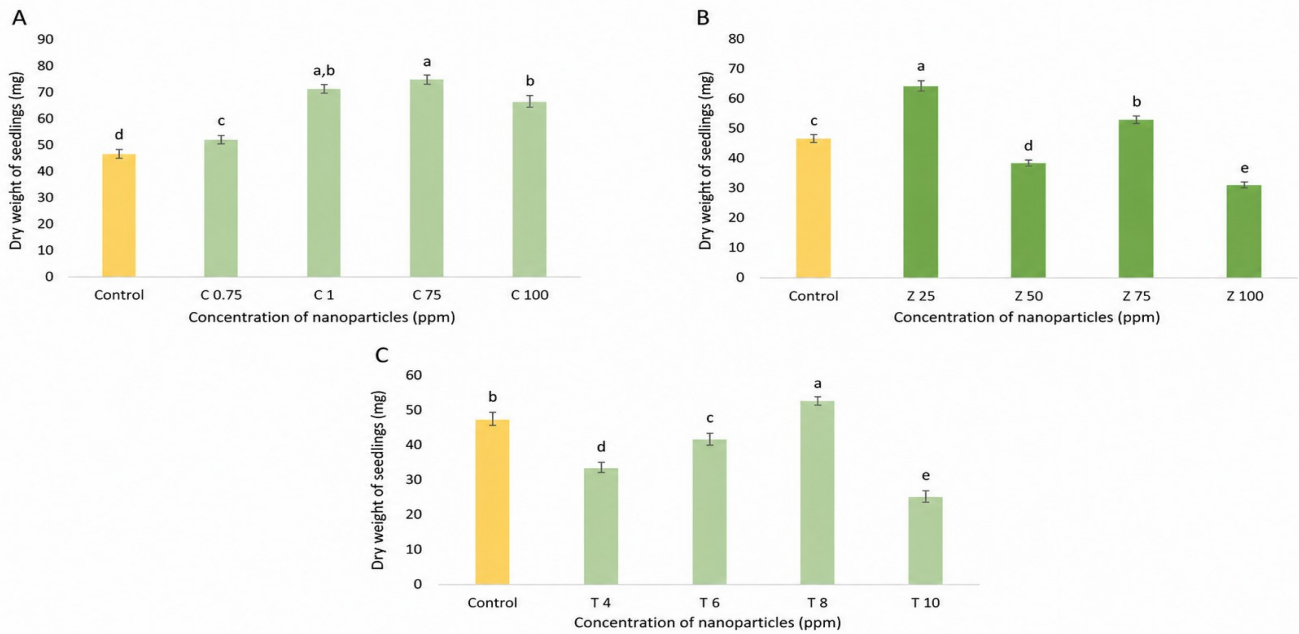
large difference between Z25 and Z100 shows ZnO narrow safety margin and seedlings' dose sensitivity. The  $\text{TiO}_2$ -NPs elicited only moderate stimulation at intermediate concentrations (T6 8.23 cm, comparable to control; T8 8.53 cm) but resulted in significant inhibition at T10 (5.67 cm), correlating with the pronounced reduction in germination at this dosage and suggesting systemic toxicity. The pronounced reduction at T10 presumably indicates increased reactive oxygen species (ROS) production (photocatalytic activity of  $\text{TiO}_2$ ), membrane impairment and disruption of cellular expansion activities, thus constraining seedling elongation. The findings indicate that CuO, particularly C75, is the most efficacious

enhancer of seedling growth, whereas ZnO and  $\text{TiO}_2$  need careful low-dose administration, since elevated concentrations swiftly transition from stimulation to significant developmental suppression. Biomass production, a comprehensive indicator of plant productivity, showed strong concentration-dependent responses to nanoparticles. The fresh weight of control plants was around 0.21 g (Fig. 5) and the dry weight was 47 mg (Fig. 6). The CuO nanoparticles demonstrated optimal performance and confirmed real biomass synthesis rather than just water accumulation, with maximal fresh biomass at C75 (0.34 g) and dry biomass at 75 mg. Zinc oxide exhibited the best performance at Z25, producing 66 mg



**Fig. 5.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on fresh weight of *Withania somnifera* seedlings (g). (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



**Fig. 6.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on dry weight of *Withania somnifera* seedlings (mg). (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

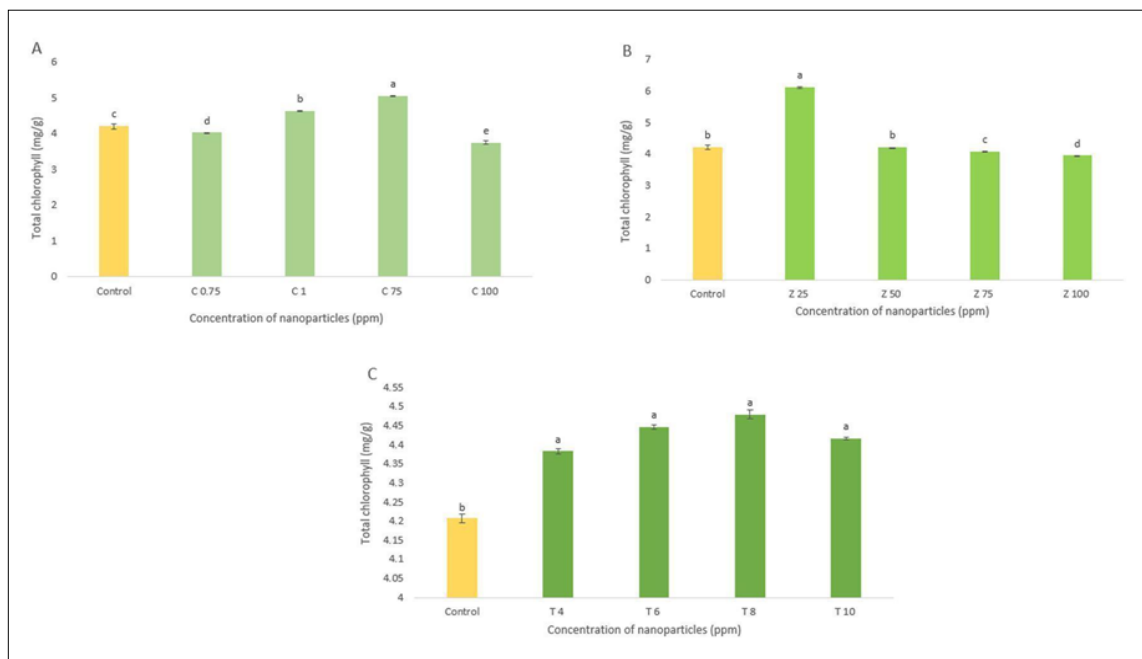
Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

of dry biomass and 0.28 g of fresh biomass. However, at Z100, the dry weight decreased to 32.4 mg, indicating a narrow optimal range and potential adverse effects at higher concentrations. Titanium dioxide also demonstrated pronounced dose sensitivity, with a slight improvement at T8 (52 mg dry biomass) but a significant reduction at T10 (25 mg), suggesting inhibitory effects at elevated levels.

#### Effect of various concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on biochemical response

The biochemical composition of *W. somnifera* exhibited significant change among nanoparticle treatments in comparison to the

control. The state of photosynthetic pigments (total chlorophyll) exhibited considerable variation. The control chlorophyll concentration (Fig. 7) was 4.2 mg g<sup>-1</sup>. The CuO had a biphasic impact, peaking at C75 (5.0 mg g<sup>-1</sup>), presumably attributable to Cu involvement in plastocyanin-mediated electron transport, but decreasing at C100 (3.7 mg g<sup>-1</sup>), suggesting toxicity. Zinc oxide induced the most significant increase of chlorophyll, achieving Z25 (6.1 mg g<sup>-1</sup>). However, Z100 drastically lowered chlorophyll levels from the Z25 peak to around 3.9 mg g<sup>-1</sup>, displaying phytotoxicity. At T8, TiO<sub>2</sub> caused a little rise in chlorophyll content (4.47 mg g<sup>-1</sup>).

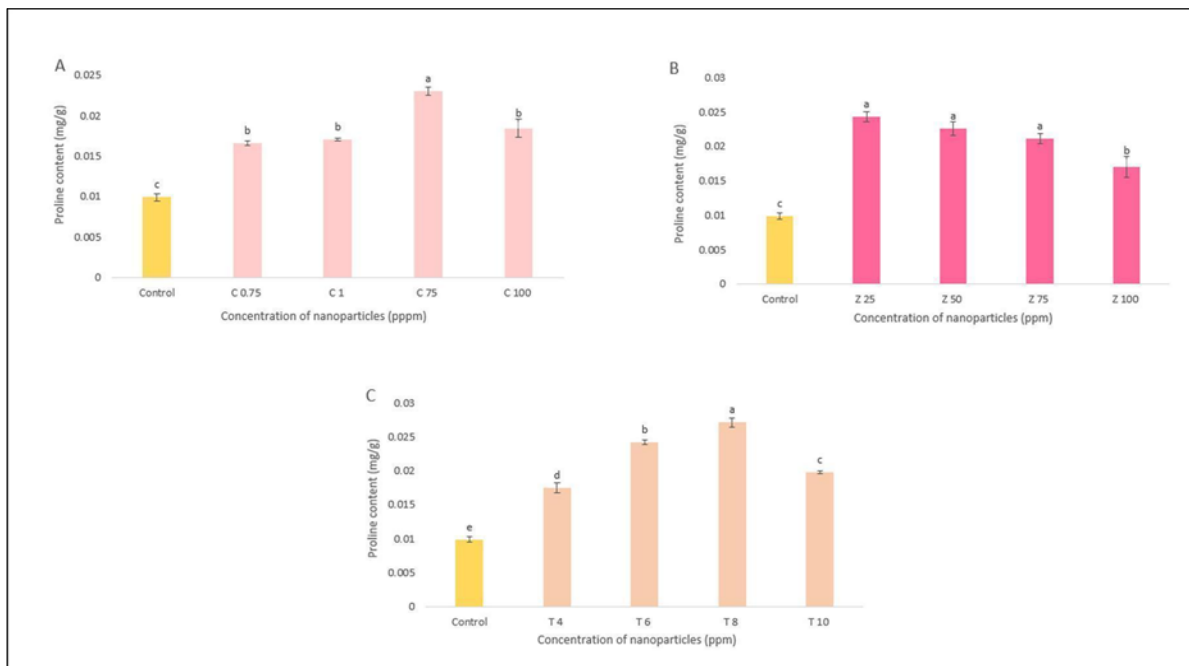


**Fig. 7.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on total chlorophyll content (mg g<sup>-1</sup>) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

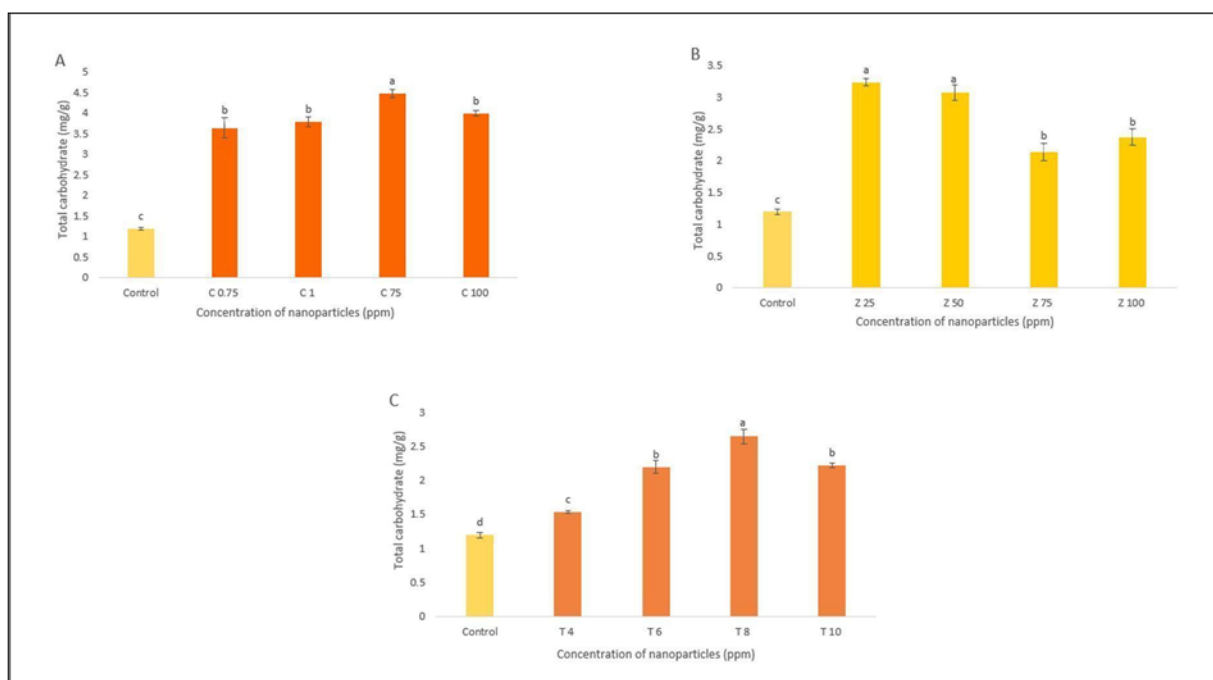
The increase of proline revealed that appropriate nanoparticle dosages elicit moderate stress signals associated with adaptation (Fig. 8). Plants treated with CuO nanoparticles showed increased proline levels to around  $0.023 \text{ mg g}^{-1}$  at C75 ppm, whereas ZnO-NPs treatment display a maximum at Z25 ppm ( $0.024 \text{ mg g}^{-1}$ ) but dropped at Z100 ppm ( $0.017 \text{ mg g}^{-1}$ ), a rise in stress due to high concentration finish the threshold for ability to adapt. The control proline concentration was  $0.009 \text{ mg g}^{-1}$ . The  $\text{TiO}_2$  elicited the greatest proline concentration, measuring around  $0.027 \text{ mg g}^{-1}$ , indicating significant hormetic stimulation.

Primary metabolites exhibited significant increase at optimum nanoparticle concentrations. Copper oxide nanoparticles at C75 produced a maximum total carbohydrate content of  $4.4 \text{ mg g}^{-1}$ , which is suggestive of the dominance of carbon metabolism and significantly matches with its peak biomass and chlorophyll response (Fig. 9). The total carbohydrate of control samples was  $1.19 \text{ mg g}^{-1}$ . In contrast to ZnO-induced metabolic control, ZnO at Z25 ppm increased carbohydrates to  $3.24 \text{ mg g}^{-1}$ , whereas  $\text{TiO}_2$  at T8 increased carbohydrates to  $2.65 \text{ mg g}^{-1}$ . The protein content (control  $0.0032 \text{ mg g}^{-1}$ ) exhibited a comparable trend: the maximum protein level was observed under CuO at C75  $0.0088 \text{ mg g}^{-1}$ , ZnO at



**Fig. 8.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on proline content ( $\text{mg g}^{-1}$ ) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



**Fig. 9.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on total carbohydrate content ( $\text{mg g}^{-1}$ ) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

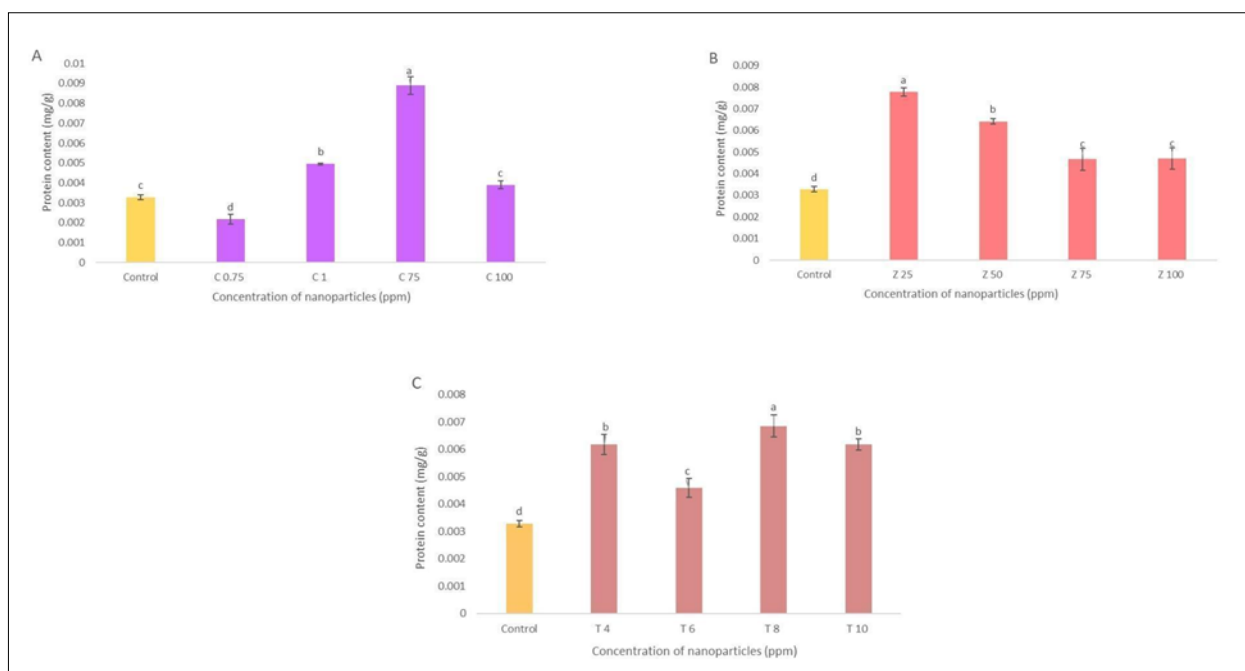
Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

Z25 0.0077 mg g<sup>-1</sup> and TiO<sub>2</sub> at T8 (0.0068 mg g<sup>-1</sup>) (Fig. 10). The evidence indicates that nanoparticles function as dose-dependent metabolic modulators: C75 CuO yields the most significant productivity enhancements, but ZnO and TiO<sub>2</sub> require lower dosage regulation due to limited safety margin.

#### Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on total phenolic content and total flavonoid content

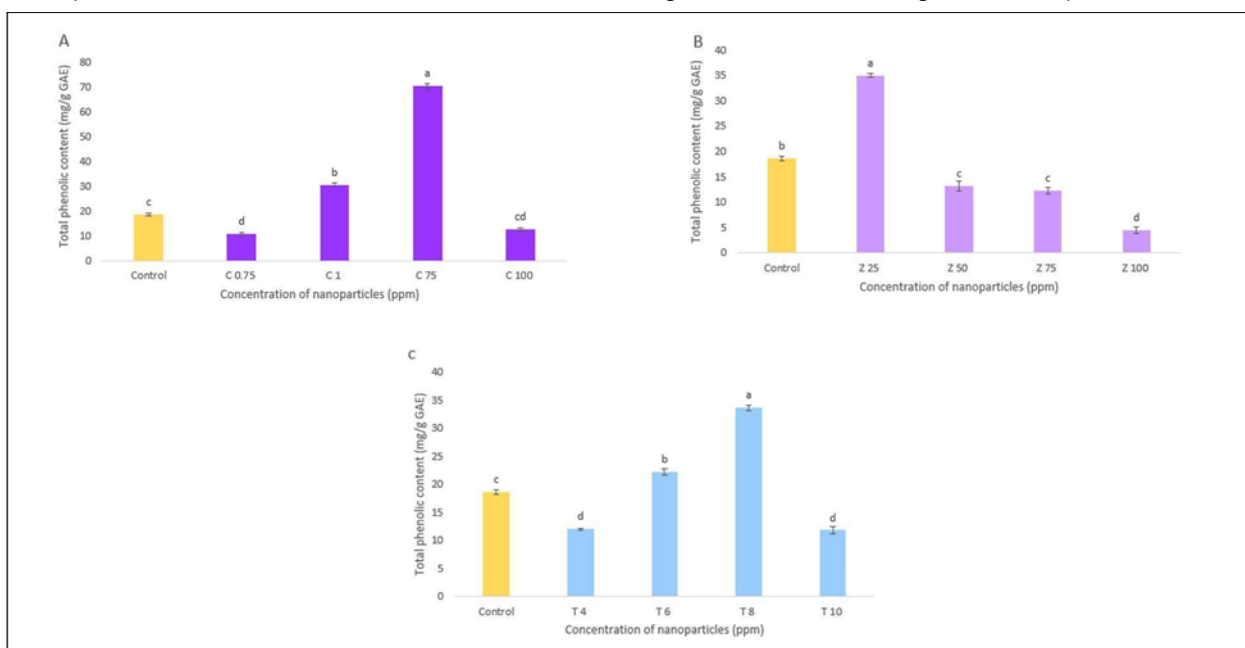
Nanoparticle elicitation significantly increased secondary metabolites, such as phenolics and flavonoids, suggesting that under stress, nanoparticles altered defence metabolism and

antioxidants. Total phenolic content in control plants was 18 mg g<sup>-1</sup> GAE at baseline. The high phenolic boost was caused by CuO, which reached 70 mg g<sup>-1</sup> GAE at C75, a 3.9-fold increase over control (Fig. 11). Copper oxide driven phenolic peak at C75 may be related to stress-induced phenylalanine ammonia-lyase activation. High ZnO concentrations induced toxicity, reducing phenolic content to 4.4 mg g<sup>-1</sup> GAE, nearly seven times lower than Z25, indicating precursor limitation, oxidative/metabolic collapse and impaired gene expression that interferes with secondary metabolism. Titanium dioxide enhanced phenolic content at T8 (33 mg g<sup>-1</sup> GAE), comparable to ZnO optimal impact, demonstrating excellent phenolic elicitation at its stimulatory dose.



**Fig. 10.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on protein content (mg g<sup>-1</sup>) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean ± SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



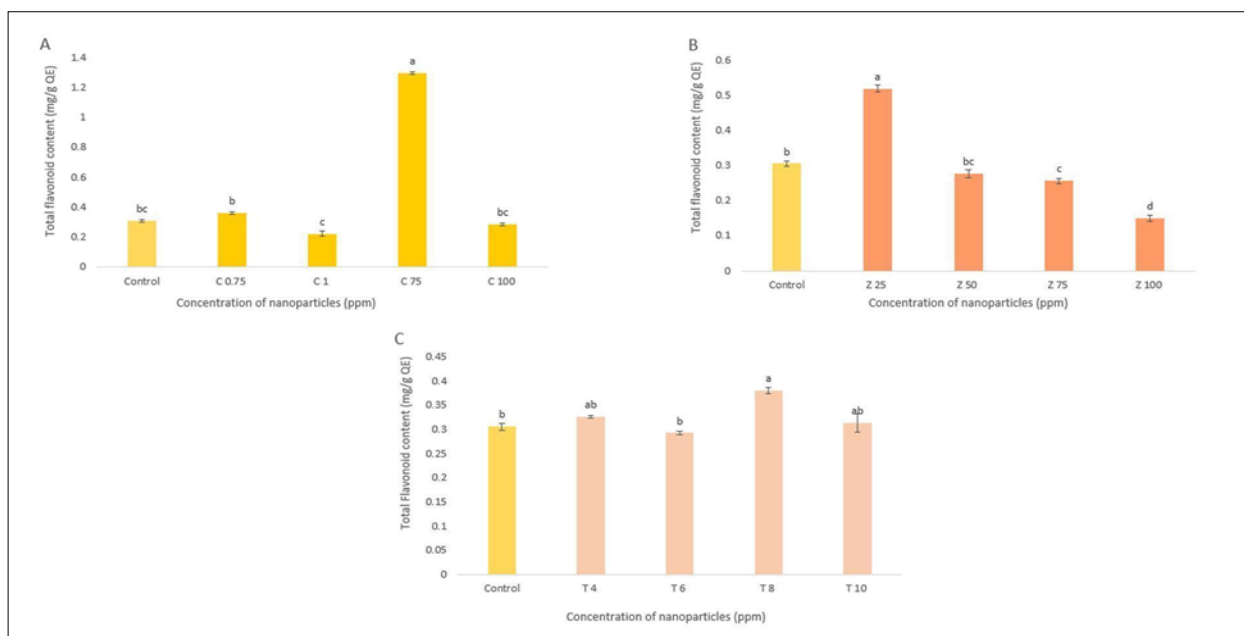
**Fig. 11.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on total phenolic content (mg g<sup>-1</sup> GAE) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean ± SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

The production of flavonoids exhibited a similar but more selective enhancement pattern. Control plants had approximately  $0.30 \text{ mg g}^{-1}$  of total flavonoids expressed as QE (Fig. 12). The strongest reaction was produced by CuO, which peaked at C75 ( $1.29 \text{ mg g}^{-1}$  QE, 4-fold), indicating a notable stimulation of biosynthetic pathways unique to flavonoids. Despite its strong effects on growth and chlorophyll, ZnO showed substantial flavonoid stimulation at Z25 ( $0.51 \text{ mg g}^{-1}$  QE), which is noteworthy but clearly inferior to CuO. This suggests that ZnO is a less powerful flavonoid elicitor. Titanium dioxide showed a small rise in flavonoids at T8 ( $0.38 \text{ mg g}^{-1}$  QE), suggesting that it may prefer to activate non-flavonoid phenolic pools rather than greatly enhancing flavonoid pathways.

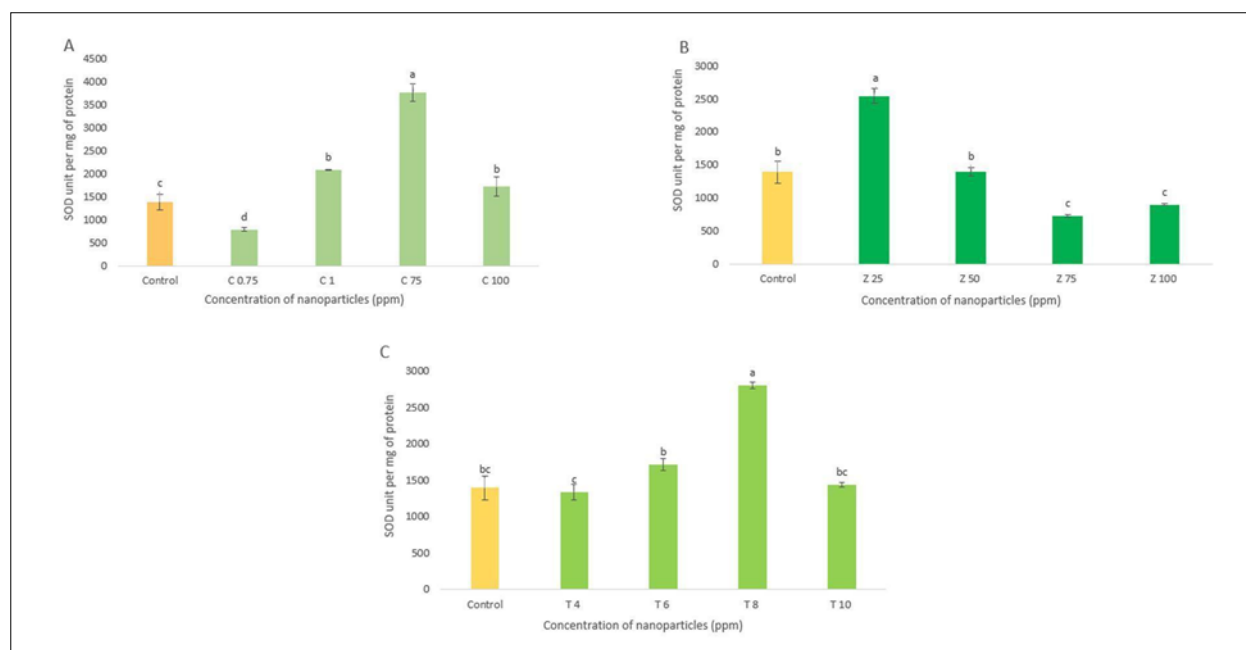
### Enzymatic and non-enzymatic antioxidant activity in nanoparticles treated *Withania somnifera*

The SOD, CAT and APX were activated by nanoparticles, demonstrating that exposure to nanoparticles controls oxidative signalling and improves ROS detoxification. Protein SOD activity in control plants was  $1400 \text{ U mg}^{-1}$ , but CuO increased it the greatest, peaking at C75 with  $3800 \text{ U mg}^{-1}$ , showing strong activation of antioxidant mechanisms and superoxide detoxification (Fig. 13). Copper's direct role as a cofactor in Cu/Zn-superoxide dismutase and reactive oxygen species signalling activation of different isoforms may explain this powerful reaction. Zinc oxide at Z25 ppm concentration increased SOD to  $2550 \text{ U mg}^{-1}$ , whereas  $\text{TiO}_2$  at T8



**Fig. 12.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on total flavonoid content ( $\text{mg g}^{-1}$  QE) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



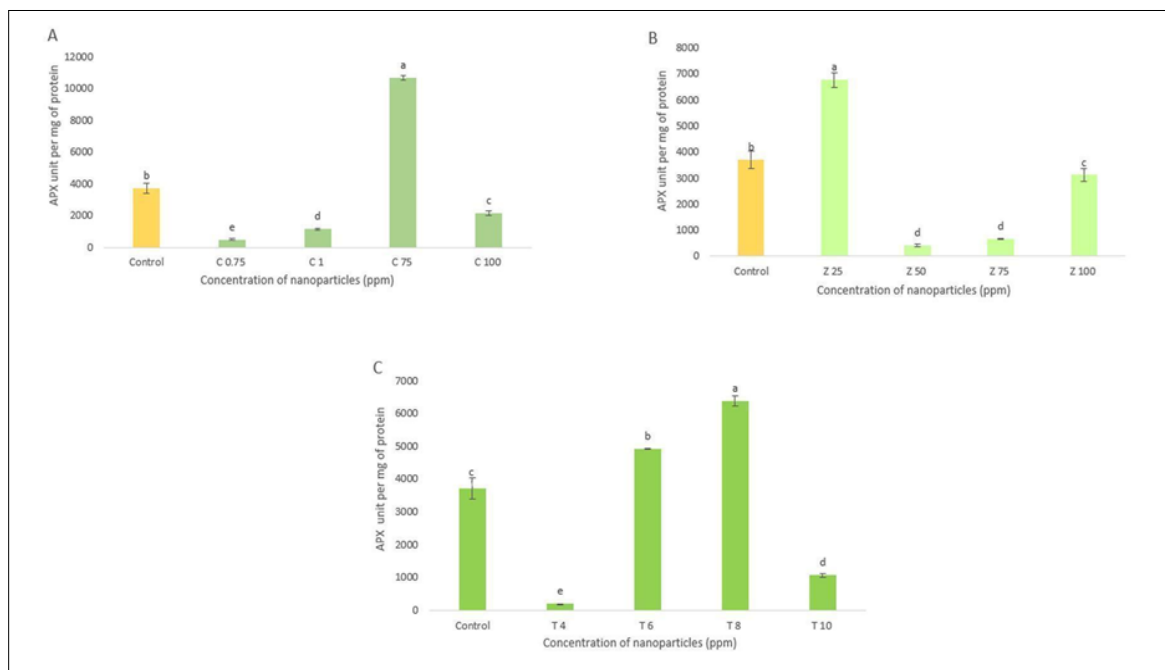
**Fig. 13.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on superoxide dismutase (SOD) activity (SOD unit per mg of protein) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

achieved  $2800 \text{ U mg}^{-1}$ , displaying effective stress signalling due to photocatalytic ROS-induced adaptive responses. At optimal levels, CAT activity in control plants ( $272 \text{ U mg}^{-1}$ ) greatly increased, with CuO C75 ( $1066 \text{ U mg}^{-1}$ ) and ZnO Z25 ( $1018 \text{ U mg}^{-1}$ ) displaying comparable maxima, indicating efficient  $\text{H}_2\text{O}_2$  detox under high stress, ZnO toxicity at Z100 caused a substantial collapse of CAT to  $88 \text{ U mg}^{-1}$ , indicating an antioxidant system failure (Fig. 14). Titanium dioxide T8 showed considerable catalase activation ( $839 \text{ U mg}^{-1}$ ). The CuO NP C75 significantly increased APX levels, with control plants at  $1000\text{--}1500 \text{ U mg}^{-1}$  and CuO C75 at  $10500 \text{ U mg}^{-1}$  (increase by 7-fold), highlighting the importance of ascorbate-dependent  $\text{H}_2\text{O}_2$

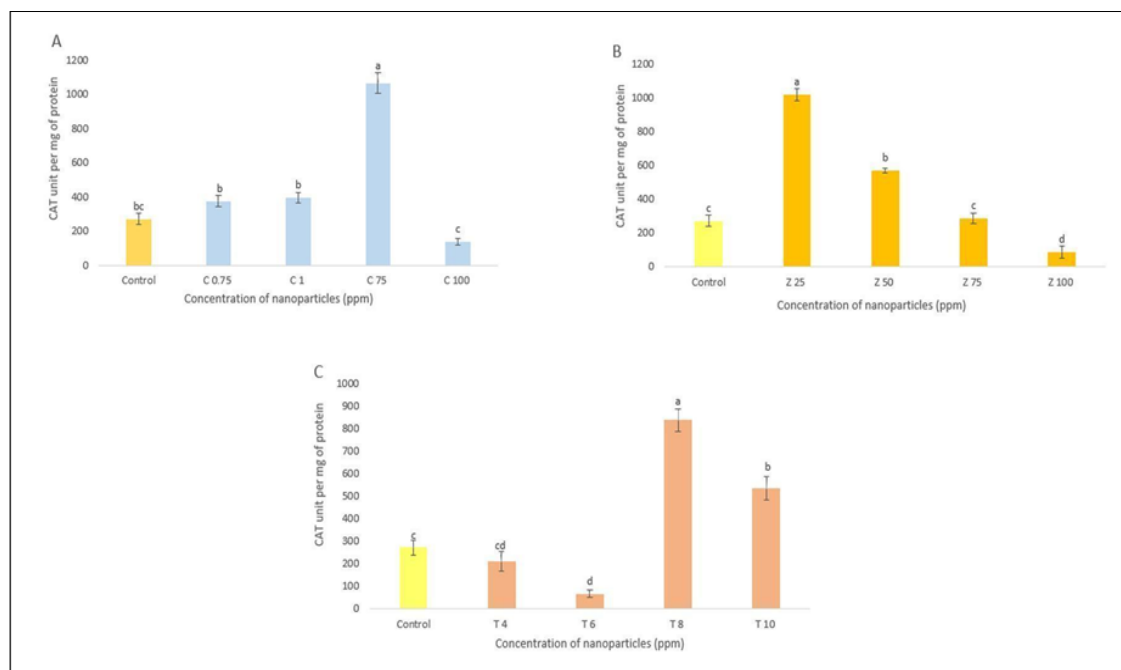
scavenging in maintaining chloroplast integrity and elucidating sustained chlorophyll efficacy under CuO stimulation. At optimal concentrations, ZnO Z25 ( $6800 \text{ U mg}^{-1}$ , increase by 4.5–6.8 fold) and  $\text{TiO}_2$  T8 ( $6400 \text{ U mg}^{-1}$ , increase by 4.3–6.4 fold) showed considerable APX activation, indicating synergistic antioxidant protection (Fig. 15).

The non-enzymatic antioxidant capability was enhanced with optimum nanoparticle treatments. In the DPPH scavenging assay, control plants exhibited approximately 80.5 % (Fig. 16), while optimal treatments yielded consistent moderate increases: CuO C75 approximately 86.3 %, ZnO Z25 approximately 86.7 % and  $\text{TiO}_2$  T6/T8 approximately 86.3 %, indicating the elicitor-induced increase



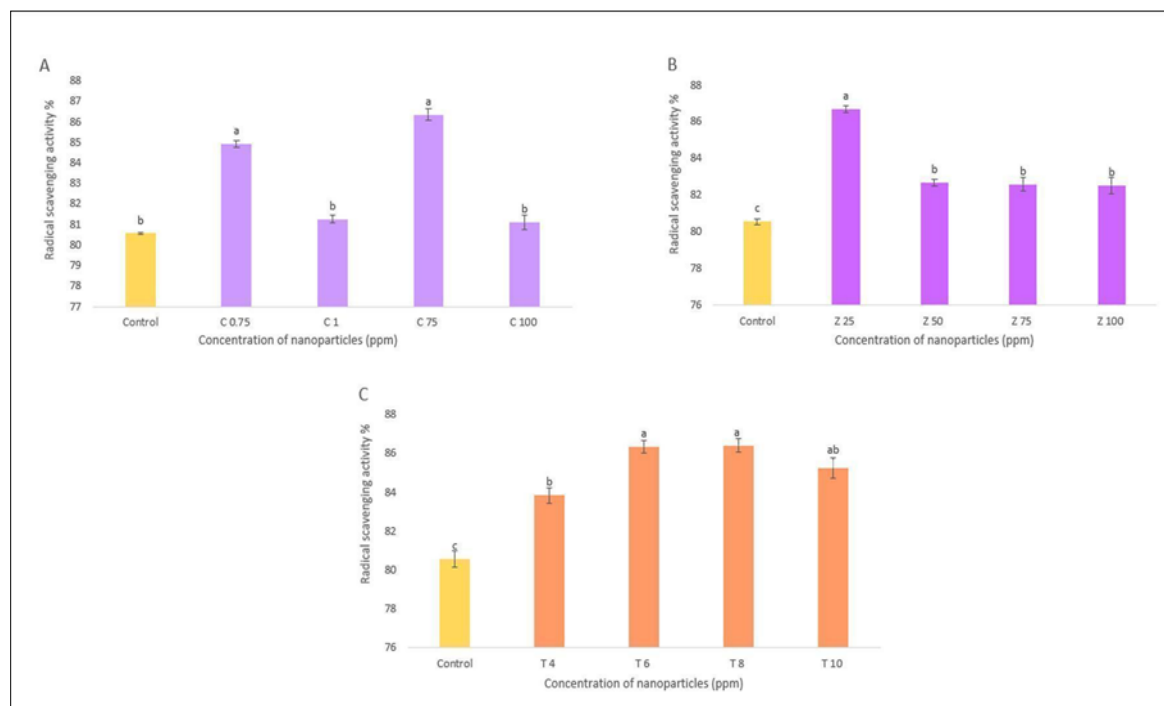
**Fig. 14.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on ascorbate peroxidase (APX) activity (APX unit per mg of protein) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



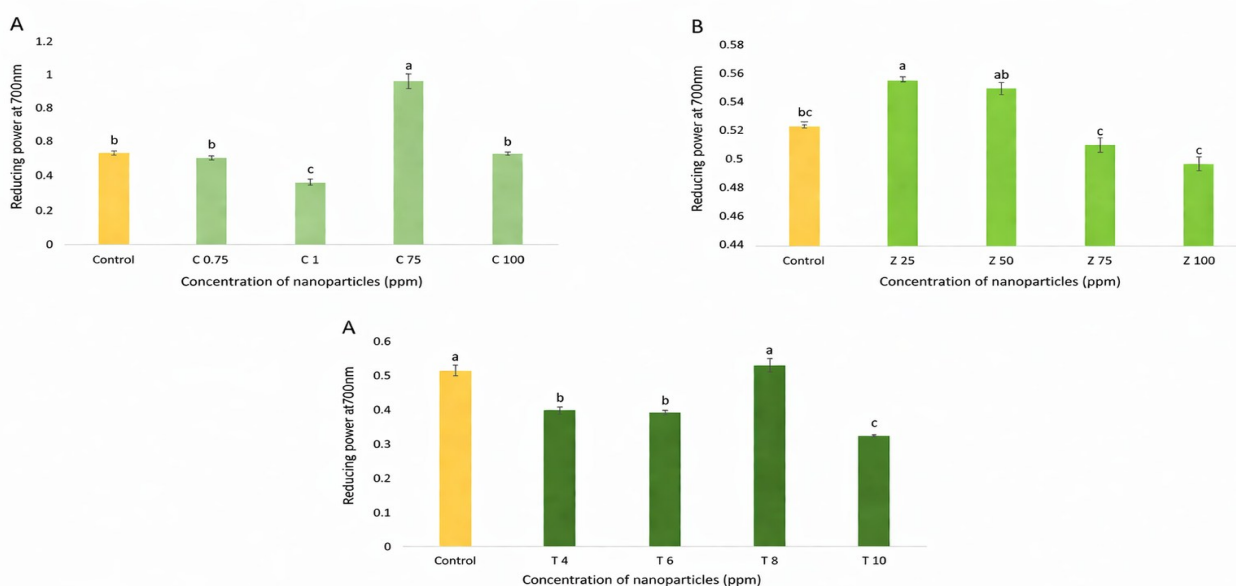
**Fig. 15.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on catalase (CAT) activity (CAT unit per mg of protein) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).



**Fig. 16.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on radical scavenging activity (%) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

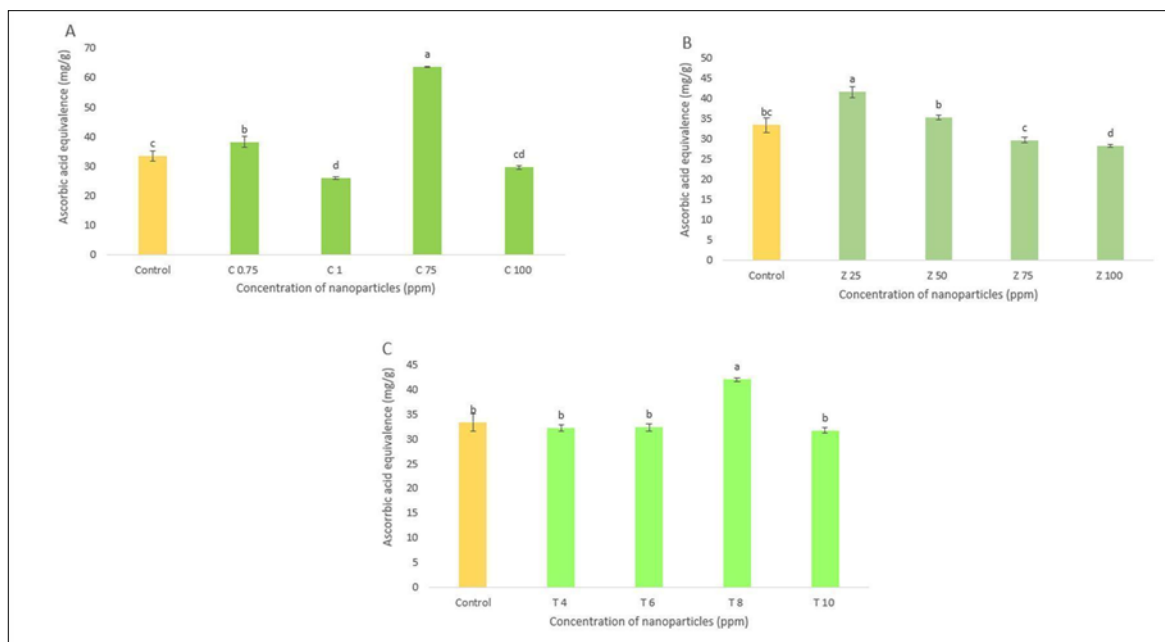


**Fig. 17.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on reducing power (absorbance at 700 nm) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control. Values are presented as mean  $\pm$  SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

of antioxidant reserves. Conversely, reducing power ( $A_{700}$ ) exhibited a distinctly pronounced CuO effect: control 0.52, whereas CuO C75 reached 0.95, while ZnO Z25 (0.55) and TiO<sub>2</sub> T8 (0.54) resulted in only marginal improvement, indicating a potential Cu-phenolic synergy enhancing electron donation (Fig. 17). Similarly, ascorbic acid equivalents increased from 33 mg g<sup>-1</sup> in the control sample to 63 mg g<sup>-1</sup> at CuO C75 ppm, but ZnO Z25 ppm and TiO<sub>2</sub> T8 ppm shown moderate increase that is 42 mg g<sup>-1</sup> (Fig. 18).

#### Accumulation of nanoparticles in *Withania somnifera* after 30 days of growth

In roots and shoots accumulation of nanoparticles were estimated after 30 days of growth, *W. somnifera* displayed dose-dependent nanoparticle absorption and translocation with traits specific to the type of nanoparticles. Root Cu content went up from 3.6  $\mu\text{g g}^{-1}$  (C0.75) to 5.4  $\mu\text{g g}^{-1}$  (C1), 18.2  $\mu\text{g g}^{-1}$  (C75) and 21.9  $\mu\text{g g}^{-1}$  (C100). The CuO treatments displayed no accumulation in any of the control tissues. The rise in shoot Cu from 1.6  $\mu\text{g g}^{-1}$  (C0.75) and 2.3  $\mu\text{g g}^{-1}$  (C1) to 6.5  $\mu\text{g g}^{-1}$  (C75) and 8.4  $\mu\text{g g}^{-1}$  (C100) indicated significant root



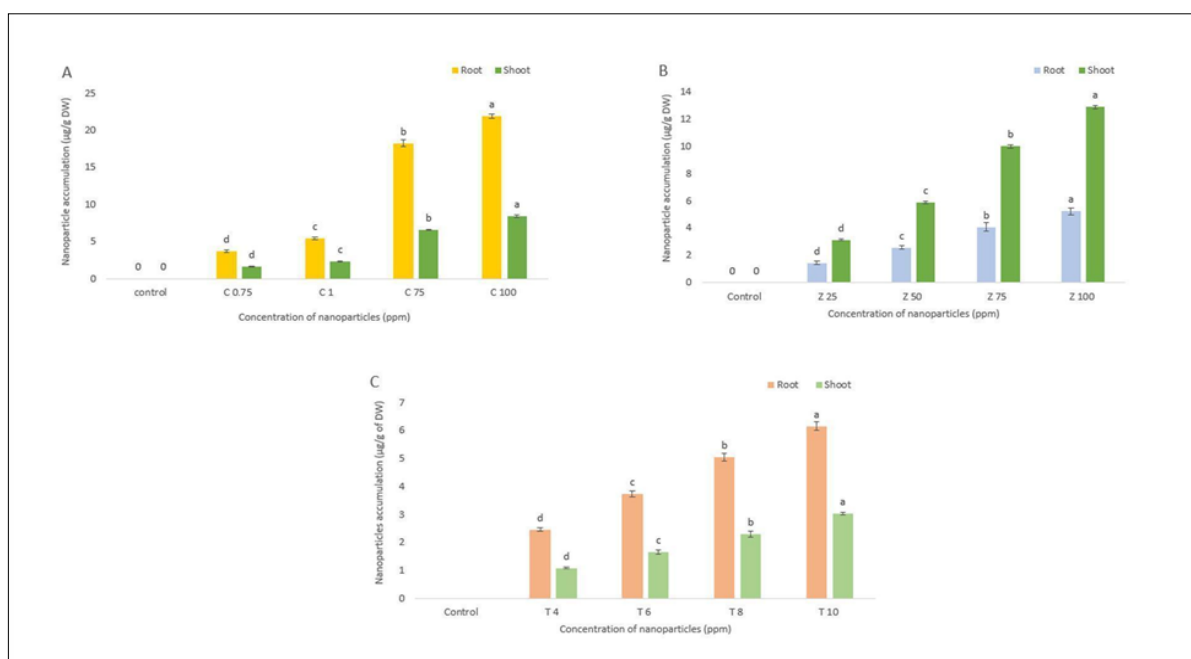
**Fig. 18.** Effect of different concentrations of copper oxide, zinc oxide and titanium dioxide nanoparticles on ascorbic acid equivalent content (mg g<sup>-1</sup>) in *Withania somnifera* seedlings. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control. Values are presented as mean ± SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

sequestration. At all dosages, ZnO treatments exhibit greater shoot accumulation than root accumulation, suggesting increased mobility and translocation; shoot zinc absorption rose from 3.13  $\mu\text{g g}^{-1}$  (Z25) to 5.8  $\mu\text{g g}^{-1}$  (Z50), 10  $\mu\text{g g}^{-1}$  (Z75) and 12.8  $\mu\text{g g}^{-1}$  (Z100), while root zinc increased from 1.4  $\mu\text{g g}^{-1}$  (Z25) to 2.5  $\mu\text{g g}^{-1}$  (Z50), 4.0  $\mu\text{g g}^{-1}$  (Z75) and 5.2  $\mu\text{g g}^{-1}$  (Z100). Titanium dioxide treatments showed high accumulation with dosage, with accumulation in root Ti remaining dominant: 2.46  $\mu\text{g g}^{-1}$  (T4) to 3.74  $\mu\text{g g}^{-1}$  (T6), 5.0  $\mu\text{g g}^{-1}$  (T8) and 6.16  $\mu\text{g g}^{-1}$  (T10), while shoot Ti increased from 1.09  $\mu\text{g g}^{-1}$  (T4) to 1.65  $\mu\text{g g}^{-1}$  (T6), 2.3  $\mu\text{g g}^{-1}$  (T8) and 3.03  $\mu\text{g g}^{-1}$  (T10). The low solubility and high aggregation of TiO<sub>2</sub> contribute to its immobilisation in the root cortex and apoplastic spaces, thereby limiting its translocation across endodermal barriers (Fig. 19).

## Discussion

### Germination and biomass responses

The biphasic reaction observed during germination shows that nanoparticles have a definite concentration-dependent impact. The CuO-NPs boosted germination at optimum concentrations, which is supported by high metabolic activity, increased water absorption through aquaporin control and activation of reserve-mobilising enzymes including proteases and  $\alpha$ -amylase (31, 32). At lower concentrations, controlled production of ROS may also function as signalling molecules that encourage metabolic activity and the breakdown of seed dormancy (31). Copper oxide nanoparticles may also function as a bioavailable supply of copper, assisting vital



**Fig. 19.** Accumulation of nanoparticles ( $\mu\text{g g}^{-1}$  DW) in root and shoot tissues of *Withania somnifera* seedlings treated with different concentrations of metal oxide nanoparticles. (A) Copper oxide: C0.75, C1, C75 and C100 ppm; (B) Zinc oxide: Z25, Z50, Z75 and Z100 ppm; (C) Titanium oxide: T4, T6, T8 and T10 ppm, compared with control.

Values are presented as mean ± SE. Different letters above bars indicate significant differences among treatments at  $p \leq 0.05$  (DMRT).

enzymatic functions related to antioxidant defence and respiration. Zinc oxide nanoparticles, on the other hand, showed a more limited optimum range; higher concentrations caused toxicity, whereas lower amounts enhanced germination by activating zinc-dependent enzymes and improving metabolic activity. Excess zinc ion release may cause this toxicity, which might interfere with development processes by causing oxidative stress, disrupting ion homeostasis and interfering with nutrient uptake (33, 34). Because  $\text{TiO}_2$ -NPs are photocatalytic, they exhibit significant concentration sensitivity. Higher concentrations lead to excessive ROS formation, which causes oxidative damage and decreased germination, whereas lower quantities may promote germination and early metabolic activities (32, 35). Medicinal plants like *Ocimum tenuiflorum* L., *Leonurus cardiaca* L., *Dracocephalum moldavica* L. and *Tanacetum parthenium* (L.) Sch.Bip. have been shown to exhibit similar dose-dependent growth responses (36, 37), which supports the potential use of nanoparticles in plant growth enhancement when applied within safe concentration limits.

### Primary and secondary metabolites

The biochemical reactions seen in *W. somnifera* suggest that plant metabolism is impacted by nanoparticles in a concentration-dependent manner. Zinc serves as a cofactor for carbonic anhydrase and aids in chlorophyll production and photosynthetic efficiency, which may account for the increase in chlorophyll levels after ZnO application (38, 39). Nevertheless, high levels can lead to toxicity by disrupting iron uptake and disturbing metal equilibrium, hindering chlorophyll production. Likewise, CuO-NPs elevate chlorophyll levels as copper is necessary for crucial enzyme functions and electron transfer through plastocyanin. On the other hand, high levels can lead to oxidative stress, causing the degradation of chlorophyll. Increased proline concentrations, particularly following  $\text{TiO}_2$  treatment, suggest the activation of stress-response mechanisms. Proline functions as an osmoprotectant and scavenges ROS, aiding in cell stabilisation during stress (39, 40). Enhanced photosynthetic function and nitrogen processing are indicated by the rise of proteins and carbohydrates in optimal amounts. The CuO enhances respiratory and photosynthetic functions, while ZnO might boost carbon and nitrogen uptake by activating enzymes. Responses triggered by  $\text{TiO}_2$  might be linked to stress-related metabolic alterations that promote initial growth (41). These metabolites, however, significantly declined at elevated nanoparticle concentrations, signalling a shift from adaptive responses to toxicity caused by oxidative injury and metabolic disruption. These results indicate a transition from beneficial metabolic activation at optimal nanoparticle levels to detrimental impacts at increased doses, highlighting the necessity for precise dosage optimisation to improve plant growth and biochemical efficiency.

### Antioxidant responses

The activation of a systematic defence response due to stress from nanoparticles is indicated by increased proline concentrations and lipid peroxidation, as well as enhanced activity of antioxidant enzymes such as SOD, CAT and APX. These responses correspond with a ROS-driven hormetic mechanism, in which controlled oxidative stress at appropriate levels stimulates antioxidant defences, whereas high concentrations may result in oxidative damage (42, 43). The CuO nanoparticles elicited the most pronounced SOD activity, likely due to copper's structural role in Cu/Zn-SOD isoforms. Zinc oxide similarly augmented the antioxidant suite, potentially through zinc-mediated enzyme

stabilisation. In the case of  $\text{TiO}_2$ , the antioxidant surge is largely attributed to photocatalytic ROS generation. While CAT activity effectively neutralised  $\text{H}_2\text{O}_2$  at moderate levels, its subsequent decline at higher concentrations suggests the system exceeded detoxification capacity. The significant rise in APX activity, especially under CuO treatment, highlights the importance of the ascorbate-glutathione cycle in preserving cellular balance (43). The activation of integrated defence systems is further supported by the concomitant rise in non-enzymatic antioxidants, such as phenolic compounds and ascorbate. Improved antioxidant capability is shown by increased DPPH scavenging and reducing power, with CuO exhibiting a greater impact, perhaps because of its participation in redox cycling and interaction with phenolic compounds. Overall, our results highlight the significance of dosage optimisation by showing that nanoparticle treatments cause a coordinated antioxidant response that improves stress tolerance at optimal concentrations, but excessive doses disturb redox homeostasis.

### Nanoparticle accumulation and physiological responses

The physiological trajectory of *W. somnifera* is largely dictated by how these particles move through the plant. Consistent with established models, ZnO-NPs demonstrated high mobility, translocating to aerial tissues, whereas CuO and  $\text{TiO}_2$  remained predominantly sequestered in the roots (44). This localisation is a deciding factor in plant health; the strong root retention of CuO likely provides a sustained, regulated stimulus that boosts antioxidant activity across a wider dosage range without causing systemic oxidative stress in the shoots. In contrast, the rapid migration of ZnO to the foliage puts it in direct contact with the plant's photosynthetic system (45). While beneficial at low doses, this accumulation at higher concentrations triggers immediate oxidative stress and impairs the uptake of essential micronutrients like iron. Titanium dioxide nanoparticles, being largely immobile, localise their stress within the root zone. While this triggered systemic adaptive responses at lower levels, elevated concentrations likely compromised root membrane integrity, inhibiting water transport (46). This confirms a direct correlation: root-dominant sequestration favours controlled adaptation, while shoot-targeted translocation increases the risk of metabolic interference. The accumulation of metal-based nanoparticles in a medicinal plant like *W. somnifera* presents a complex challenge, especially given that these tissues are often consumed directly. From a nutritional standpoint, the accumulation of copper and zinc could be seen as biofortification, as these are essential human micronutrients.

### Ecological and food safety implications of nanoparticle accumulation

The use of nanoparticles in *W. somnifera* raises both possible benefits and risks, especially given that this medicinal plant is utilised directly for extraction and intake. While critical metals such as zinc and copper are recognised to play vital roles in plant development, their buildup in plant tissues must be carefully monitored (47). Although the current study did not look at food safety, the observed nanoparticle absorption suggests that excessive buildup at higher concentrations might constitute a problem. The high efficiency of nanoparticles allows their use at lower concentrations compared to conventional fertilisers, which may reduce overall environmental input. Additionally, the tendency of certain nanoparticles, such as CuO, to remain localised in root tissues may limit their translocation within the plant and reduce potential exposure.

Overall, while nanoparticle application shows promise for enhancing plant growth and metabolic responses, further studies focusing on accumulation, toxicity and safety assessment are necessary to ensure their safe and sustainable use in medicinal plant systems.

## Conclusion

This research work demonstrates through comprehensive analyses that nanoparticles effectively improve the growth performance of *Withania somnifera*, from germination parameters to biochemical modification, in a concentration-dependent manner. Higher concentration caused toxicity and growth suppression, whereas optimal concentration improved growth, antioxidant activity (superoxide dismutase, catalase and ascorbate peroxidase) and metabolic processes. This was clearly a hormetic reaction. Zinc oxide and titanium dioxide showed smaller optimum ranges, whereas copper oxide at 75 ppm demonstrated the most steady and effective reaction with a greater safety margin. The results provide a crucial molecular insight: although excessive reactive oxygen species causes oxidative damage and metabolic disruption, regulated nanoparticle-induced ROS serves as a signalling mechanism to improve physiological and biochemical processes. The total effect of nanoparticles on plant performance is determined by their accumulation and translocation patterns, which are intimately related to these reactions. A framework for using nanoparticles as growth elicitors in the cultivation of medicinal plants is presented in this paper. Future research should focus on understanding the molecular mechanisms causing interactions between nanoparticles and plants, especially using transcriptomic and metabolomic methods. Multi-location field investigations are also required to confirm the reported responses in actual environments. To guarantee safe application, more research into food safety, long-term environmental effects and nanoparticle buildup is crucial for sustainable application. Enhancing the productivity and sustainability of medicinal plant production will also need the optimisation of nanoparticle compositions and stage-specific dosing techniques.

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## Authors' contributions

Both authors contributed equally to this work and have read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interests to declare.

**Ethical issues:** None

## Declaration of generative AI and AI-assisted technologies in the writing process:

During the development of this manuscript, the authors used QuillBot for language refinement and vocabulary improvement. After its use, the authors thoroughly reviewed, revised and validated the text and fully responsible for the integrity and accuracy of the final content.

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