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The high production of tropane alkaloids in hairy roots of *Hyoscyamus reticulatus* L. elicited by ZnO NPs in a bioreactor condition

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Chemical synthesis issue of tropane alkaloids and their lowest production rate along with their extraction costs has made use of new methods necessary to produce valuable pharmaceutical compounds. hairy roots induction end elicitation with biotic or abiotic elicitors such as nanoparticles can be used as a source for high production of worth metabolites compared to intact plants. *Hyoscyamus reticulatus* L. is a rich source of hyoscyamine and scopolamine, which are widely used in pharmaceuticals. Since the in this research, the effect of different concentrations (0, 25, 50, 75 and 100 mg/l) of zinc Nano oxide (ZnO NPs) as a stimulant on the growth rate, antioxidant enzyme activity and antioxidant activity, total phenol content (TPC), total flavonoid content (TFC) and tropane alkaloids in the *H. reticulatus* hairy roots was investigated in a bubble column bioreactor. The highest and the lowest amount of fresh weight (FW) (153.4 and 79.82 g) were recorded in 50 mg/l during 48 h exposure time and 100 mg/l during 72 h respectively. Also, ANOVA results showed that the interaction effect of concentration and exposure time was significant on guaiacol peroxidase (GPX) enzyme activity, TPC and TFC, antioxidant activity and alkaloid content ($p < 0.01$). HPLC analysis revealed that the highest amount of hyoscyamine and scopolamine (400 and 275.1 mg/kg of DW) were observed at ZnO NPs (75 mg/l) during 72 h respectively. According to the results, it can be said that the use of nano zinc oxide as a stimulant is an effective method for increasing tropane alkaloids in a bubble column bioreactor.

Keywords Bioreactor, Phenolic compounds, Catalase, High performance liquid chromatography

Drugs derived from plants one is of the most important sources in the pharmaceutical industry[1]. Due to the fact that the rapid and high production of secondary metabolites through chemical methods is mostly expensive, difficult or impossible. also due to the economic importance of these metabolites and their small production in medicinal plants, the use of alternative biotechnological methods such as growing hairy roots in a bioreactor and using biotic and abiotic stimuli can improve the production of these valuable compounds.

Hyoscyamus reticulatus L. has tropane Alkaloids such as hyoscyamine and scopolamine that are widely used in the pharmaceutical industry. Hyoscyamine and scopolamine are among the most important tropane alkaloids, and synthetic production of these alkaloids is difficult, but still, the global demand for scopolamine is tenfold higher than that of hyoscyamine[2]. Scopolamine is produced in limited species and is preferred due to its more physiological activity and less side effects[3]. One of the important uses of hyoscyamine and scopolamine in medicine is to dilate the pupils of the eyes, increase heart rate, neutralize smooth muscle weakness caused by organic phosphate compounds, reduce sweat and stomach acid secretion[4,5].

In recent years, in order to produce worthwhile metabolites on a commercial scale, hairy roots cultivation method has been used in a number of medicinal plant species. Among the advantages of hairy roots compared to natural roots, we can mention fast growth, high genetic and biosynthetic stability, easy maintenance, and the ability to grow in a culture medium without hormones. Considering these advantages, they can be introduced as a permanent source for the production of phytochemicals[6]. Today, with the expansion of the industrial creation methods of biotechnological products such as the cultivation of plant cells and organs in bioreactors,

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the commercial manufacturing of many compounds with medicinal value has been provided. The hairy roots cultivation in bioreactors can relieve commercial production of valuable metabolites[7]. the use of biological and non-biological stimuli to improve the output of secondary metabolites in hairy roots is an effective solution. Stimulants expand the natural compounds creation by stimulating the plants defense system and the expression of related genes in the biosynthesis pathway[8]. Currently, various types of elicitors are used in cell and different plant organ cultures. Nanoparticles are considered as one of the most important non-biological stimuli due to their favorable characteristics such as high contact surface, easy absorption and better response of plant cells, which increase the growth rate of hairy roots and the production of pharmaceutical metabolites in different plant species have been used[9].

Zinc oxide nanoparticles (ZnO NPs) have emerged as promising abiotic elicitors capable of enhancing secondary metabolite production in medicinal plants[10]. Recent studies have demonstrated that nanoparticles can trigger plant defense responses, activating biochemical pathways that lead to increased biosynthesis of valuable compounds[11,12]. While various nanoparticles, such as silver and iron oxide, have been explored for their elicitor potential, ZnO NPs remain underutilized in plant biotechnology. Iron oxide nanoparticles, for instance, have been shown to enhance biomass accumulation, antioxidant capacity, and bioactive metabolite production in *Artemisia scoparia* callus cultures[13]. However, limited studies have investigated whether ZnO NPs can exert similar or even superior effects on alkaloid biosynthesis in hairy root cultures of *Hyoscyamus reticulatus* L. In medicinal plant biotechnology, the modulation of biosynthetic pathways using nanoparticles has gained significant attention due to its potential to enhance yield and efficiency. For example, Khan et al.[14] reported that ZnO NPs stimulated the biosynthesis of thymoquinone in *Nigella sativa* cell cultures, demonstrating their ability to upregulate key metabolic pathways. Given these findings, ZnO NPs may play a similar role in the biosynthesis of tropane alkaloids, such as hyoscyamine and scopolamine, which are crucial pharmaceutical compounds. While previous research has explored the impact of elicitors like methyl jasmonate and silver nanoparticles, the specific influence of ZnO NPs on tropane alkaloid production in *H. reticulatus* remains unexplored. This study aims to bridge this knowledge gap by evaluating the effects of different concentrations and exposure durations of ZnO NPs on biomass accumulation, antioxidant enzyme activity, phenolic and flavonoid content, and alkaloid production in *H. reticulatus* hairy roots.

Materials and methods

Preparation of hairy roots for culture in bioreactor

Hairy roots were prepared by inoculation of cotyledon explant with A7 strain of *Agrobacterium rhizogenes*, and fast-growing lines were selected based on the growth rate during a period of 3 weeks (Fig. S1 a, b, c, d). Next, the hairy roots were cultured in 250 ml Erlenmeyer flasks containing 30 ml of liquid MS media[15] with a sucrose (50 g/l) and pH = 5.8 on a net. Then, the Eelenmeyer flasks containing hairy roots were kept in the incubator shaker at 120 rpm, temperature 25 °C and in the dark. It should be noted that hairy roots were transplanted every 15 days.

Determine the hairy roots growth curve

In order to determine the hairy roots growth rate of, 2.5 g of the hairy roots were placed inside the 5 l air-lift balloon type bioreactor containing 3 l MS liquid culture media (pH = 5.8) (Fig. S1 a, b, c, d). After incubation, bioreactor was transferred to the growth room at 25 °C and dark conditions and aeration flue was 0.5 l/min per l of media. Then every three days the roots inside the bioreactor were carefully weighed under the hood and the wet weight was recorded. In order to determine the exact growth rate, this work was done for 30 days and drawn as a graph[16].

Stimulation of hairy roots with ZnO NPs

The bioreactor used in this experiment was an air-lift balloon type bioreactor with a capacity of 5 l. 2.5 g of hairy roots were removed from Eelenmeyer flasks and then cultivated in a bioreactor containing 3 l of liquid MS culture medium (pH = 5.8) (Fig. S1 e, f, h). then, the bioreactor was transferred to the growth room at 25 °C and dark conditions and 0.5 l/min per l of media aeration. ZnO NPs used in this research was prepared from Pishgaman Nano Materials Iranian Company, whose size was 30–50 nm. After 18 days culture in bioreactor, hairy roots were treated with different concentrations of ZnO NPs (0, 25, 50, 75 and 100 mg/l) for 48 and 72-hours exposure time with 3 repetitions. Finally, after the end of the treatment time, hairy roots were removed from the bioreactor and after washing with distilled water and removing excess moisture with filter paper, the wet weight of the roots was measured. Then, the roots were air dried for 48 h at 25 °C and the dry weight of the roots was measured.

Preparation of plant extracts

Fresh hairy roots (0.5 g) of were weighed and 50 mM Tris-HCl buffer with pH = 7.5 was added to five times its weight and rubbed on ice for 20 min until a completely homogeneous extract was obtained. The resulting extract was centrifuged at 12,000 rpm for 20 min at 4 °C using a refrigerated centrifuge. The supernatant solution was carefully separated from the sediment and transferred to clean vials and centrifuged again at 9000 rpm for 15 min at 4 °C. At the end, the vials were slowly removed from the device and the supernatant solution was distributed in several small vials. The resulting extract was used to measure the activity of catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) enzymes[17].

Measurement of CAT enzyme activity

First, 50 mM phosphate buffer was prepared at pH = 7, then 2.5 ml of buffer and 20 µL of 3% hydrogen peroxide (H_2O_2) were removed for each sample. These materials were mixed together and then 20 µL of enzyme extract

was added, and the changes in absorption at a wavelength of 240 nm were checked using a spectrophotometer for 1 min. CAT activity was calculated in terms of units changes per min to units per g FW[18].

Measurement of GPX enzyme activity

The activity of GPX enzyme was measured using guaiacol as a substrate and based on the method of Zhang et al.. The reaction mixture contained 25 µL of enzyme extract, 2.77 µL of potassium phosphate buffer, 50 mM with an acidity of 7, 100 µL of 1% hydrogen peroxide, and 100 µL of 4% guaiacol. The increase in absorbance due to the oxidation of guaiacol was measured at a wavelength of 470 nm for 1 min. Then, the GPX activity was calculated in units per g FW[19].

Measurement of APX enzyme activity

After preparing the protein extracts, 2 ml of 50 mM phosphate buffer with pH = 7, 200 µL of 3% hydrogen peroxide (H_2O_2), 200 µL of 50 µM ascorbate were mixed together in an ice bath and immediately 100 µL Enzyme extract was added. The absorbance change curve was read at 290 nm wavelength. Enzyme activity content was calculated in terms of unit changes per g FW[20].

Plant extracts isolation to measure TPC and TFC

This experiment was performed based on the method of Hajimahdipour et al.. with a slight change. hairy root (0.5 g) was placed in a cold mortar, then 10 ml of 80% (V/V) methanol was added and ground completely. Then it was centrifuged (Hettich - MIKRO 22R) for 15 min at 10,000 rpm at 4 °C. To measure the TPC, 50 µL of the extract with 1200 µL of Folin reagent 10% (V/V) were poured into clean glass tubes and after 5 min, 960 µL of 7.5% (V/V) sodium carbonate was added and finally 180 µL Distilled water was added, then the glass tubes were placed in the dark for 30–40 min. Finally, at an absorption spectra were recorded on a Perkin-Elmer Lambda 25, double-beam ultra violet and visible (UV–vis) spectrophotometer with 1.0 cm quartz cuvettes at 760 nm[21].

$$Y = 0.0144X + 0.2303$$

X: total phenol content, Y: amount of absorbance.

To measure the TFC, 200 µL of extract, 150 µL of sodium nitrite, 300 µL of aluminum chloride solution (10% v/v), 1000 µL of sodium acetate solution (1 M) and distilled water was added to the volume of 5 ml. The absorbance was recorded at 380 nm compared to the control. Quercetin (Sigma-Aldrich (MilliporeSigma)) was used to draw the standard curve and the TFC was reported based on mg of quercetin per g FW[22].

$$Y = 0.0094X + 0.8712$$

X: TFC, Y: absorbance.

Preparation of methanolic extract to measure antioxidant capacity

Frozen hairy root samples were completely powdered. Then, in order to prepare the methanolic extract, 5 ml of 80% methanol was added to 0.5 g of hairy root powder and the resulting mixture was stirred for 48 h in the dark. The resulting extract was immediately used to perform the next experiment steps[23].

Measurement of antioxidant activity by DPPH method

To measure the antioxidant activity by DPPH method, a certain amount of methanolic extract of each sample was added in a test tube and 2000 µL of DPPH solution (prepared in advance) was added. The resulting solution was shaken and kept at laboratory temperature and darkness for 30 min and the absorbance was read at 517 nm[24].

$$\mathrm{RSA} = \frac{(Abs\ control)t = 30 - (Abs\ sample)t = 30\ \mathrm{min}}{(\mathrm{Abs\ control})t = 30\,\mathrm{min}} \times 100$$

Abs control: Absorption of blank; Abs sample: sample absorption.

Measurement of antioxidant activity by FRAP method

A certain amount of each extract was mixed with 3 ml of fresh FRAP reagent (300 mM sodium acetate buffer (300 Mm, pH = 6.3) with 6.3 acidity, ferric-tripyridyl-1c-triazine 2 and ferric chloride). The resulting mixture was placed in a hot water bath (temperature 37 °C) for 30 min and its absorbance was recorded at a wavelength of 593 nm using a spectrophotometer. Iron sulfate was used to draw the standard curve[25].

$$Y = 0/0064X + 0/2972$$

X: total antioxidant amount Y: absorbance amount.

Tropane alkaloids extraction

Alkaloid extraction was done by the method of Kamada et al. with a slight modification. According to this method, 0.5 g of dried hairy roots were powdered and transferred into a 100 ml Erlenmeyer flask. Then, chloroform, methanol and 25% (V/V) ammonia were added to the plant samples in a ratio of 5:1:15 and they were extracted under ultrasonic treatment for 10 min. In the next step, the resulting mixture was transferred to a shaker incubator with a temperature of 23 °C for 130 min. After passing through the Whatman and washing

the filter paper twice with 1 ml of chloroform, a rotary evaporator (heidolph −4003) was used to evaporate the chloroform phase. To the remaining and dried extract, 5 ml of chloroform and 2 ml of 1 N sulfuric acid were added and thoroughly stirred (acidification of the environment causes the alkaloids to become salty and charged, and as a result of liquid extraction- The liquid is transferred to the aqueous phase. In the next step, two phases were formed by pouring the obtained mixture into the decanter. The chloroform phase was separated and discarded, and the aqueous phase containing alkaloids was transferred to a beaker, and its pH was adjusted to 10 with 28% (V/V) ammonia on ice. The alkaline solution inside the decanter was added and the alkaloids were extracted once by 2 ml and twice by 1 ml of chloroform. After adding dry sodium sulfate, the obtained chloroform phase was passed through a sieve and the Whatman paper was washed with 1 to 2 ml of chloroform. The filtered solution was evaporated by rotary evaporator at a temperature of 40 C°and the obtained solid substance, which is called total alkaloid, was dissolved in 500 µL of pure methanol. Finally, the obtained solution was poured into 2 ml microtubes for phytochemical analysis[26].

Analysis of tropane alkaloids by high performance liquid chromatography (HPLC-DAD)

In order to measure the amount of tropane alkaloids (hyoscyamine and scopolamine) in extracts extracted from hairy roots, a high-performance liquid chromatography device (Agilent technologies, Palo Alto, California, USA) with an ultraviolet detector with a wavelength of 215 nm was used. The column used was C- C18-ODS (Octadecylsilane) column from Supelco (Bellefonte, Pennsylvania, USA; 250 4.6 mm inner diameter (ID), 5 mm particle size) (250*4 nm) and the flow rate of the solvent was 1 ml/min and the mobile phase consisted of acetonitrile and Acetate buffer in the ratio of 20:80 (V/V). The amount of extract used for each injection was 10 µL and the amount of two alkaloids hyoscyamine and scopolamine was calculated based on the Peak area of the curve obtained using the standard curve. The standard curve was drawn based on the Peak area the curve of hyoscyamine and scopolamine (Sigma, MO, USA) in concentrations of 5, 10, 20, 30, 40 and 50 mg/ml.

Data analysis

ZnO NPs elicitation and exposure time Experiments were conducted as a factorial based on a completely randomized design with three replications, and the data from the experiment were analyzed using SAS 9.2 software. Mean comparisons were done by Duncan's multiple range test and Excel was used to draw graphs.

Results and discussion

Hairy roots growth curve

Before applying the elicitor treatment, the hairy roots growth curve was determined in the bioreactor. The data analysis results revealed that after cultivation, the hairy roots FW increased after a break from the third day and this phase continued until the 15 th day. then, the weight gain decreased and the growth process became imperceptible (Fig. 1). At the end of the logarithmic period and on the threshold of the stationary period (day 19–22), when the hairy root biomass had reached a suitable level, it was chosen as the elicitation time.

The effects of ZnO NPs on biomass growth

In order to investigate the effect of ZnO NPs stimulator on hairy root biomass, the hairy roots FW was measured. The analysis of variance (ANOVA) showed that the effect of treatments was significant ($P \leq 0.05$). The highest and lowest amount of FW (153.4 and 79.82 g) were detected in ZnO NPs (50 mg/L during 48 h exposure time

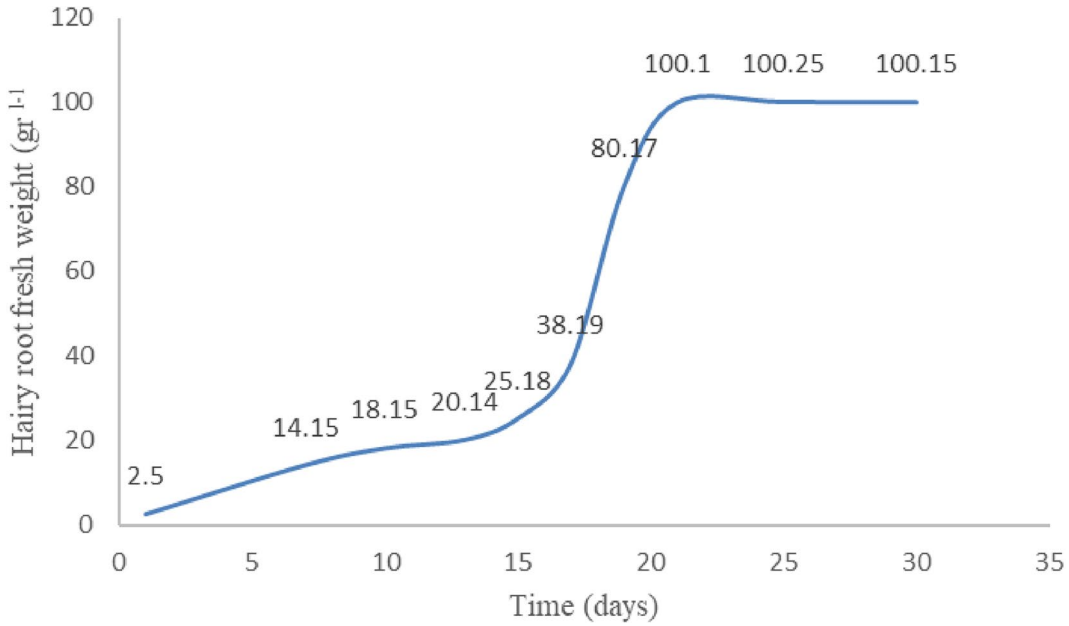

Fig. 1. The hairy root growth rate of *H. reticulatus* in liquid MS culture media.

and 100 mg/L during 72 h) respectively (Fig. 2). Also, different levels of concentration and exposure times had a significant effect on the hairy roots DW ($p < 0.01$). While the interaction effect of concentration and exposure time did not show a significant effect on the DW of treated hairy roots. The highest and lowest amount of DW (14.83 and 7.72 g) was observed in 50 mg/L for 48 h and 100 mg/L for 72 h (Fig. 3A, B).

As the results showed, ZnO NPs up to a concentration of 50 mg/l had a positive effect on *H. reticulatus* hairy roots growth, but with increasing concentration, it is considered a growth-limiting indicator for plants as a heavy metal[27]. excessive concentration of heavy elements including zinc causes toxicity in the culture environment. Heavy elements reveal their toxicity by blocking water channels and quickly reducing the water conductivity of cells[28]. Zinc element is effective in preventing cell divisions by influencing the rate of calcium ion entry, thus reducing plant growth indicators[29]. In zinc stress conditions, among the causes of root growth inhibition, the high sensitivity of the root tip meristem to heavy metals and the effect of zinc on the indole acetic acid oxidase enzyme on the root surface can be mentioned[30]. Yousefzaei et al. reported that the effect of silver nanoparticles on morphological and physiological parameters of *Ocimum basilicum* L. shows the negative effect of increasing concentrations of silver nanoparticles on these parameters[31]. Piatczak et al. reported that with increasing the stimulants concentration, the hairy root biomass of *Rehmannia glutinosa* plant decreased compared to the control[32].

Effect of ZnO NPs on antioxidant enzymes activity

The ANOVA results showed that the interaction effects of zinc nano-oxide concentration and exposure time on GPX activity were significant ($p < 0.01$). nevertheless, the interaction effect of zinc nano-oxide concentration and exposure time on APX and CAT enzyme activity was not significant. However, the simple effects of the elicitor concentration had a significant effect on the activity of APX and CAT ($p < 0.01$). With the enhancing concentration and treatment duration, the enzymes activity was raised. Therefore, the maximum activity of CAT, GPX and APX were recorded at a ZnO NPs (100 mg/l) in 48 and 72 h. the minimum activity level of CAT, APX and GPX enzymes was observed in the control treatment (Fig. 4A, B and C). Among the environmental stresses of plants during their life cycle, we can refer to the stress of heavy metals. Enzyme antioxidant system is one of the main mechanisms to deal with heavy metal stress in plants[33]. The activity level of antioxidant enzymes varies depending on the duration and type of stress, plant species and different plant organs[34]. As the intensity of stress enhance, the antioxidant enzymes activity such as catalase would be increase[35]. In *Fogopyrum esculentom* under different of zinc nano-oxide treatments, reported that the activity of catalase enzyme increases up to concentrations above 100 mg/l[36].

APX is a specific peroxidase that decomposes H_2O_2 into water and monodehydroascorbate through the ascorbate-glutathione cycle using ascorbic acid[37]. The effect of zinc nano-oxide on tomato seeds under salt stress increased the activity of APX and CAT. In another study, the activity of APX raised due to different lead, zinc

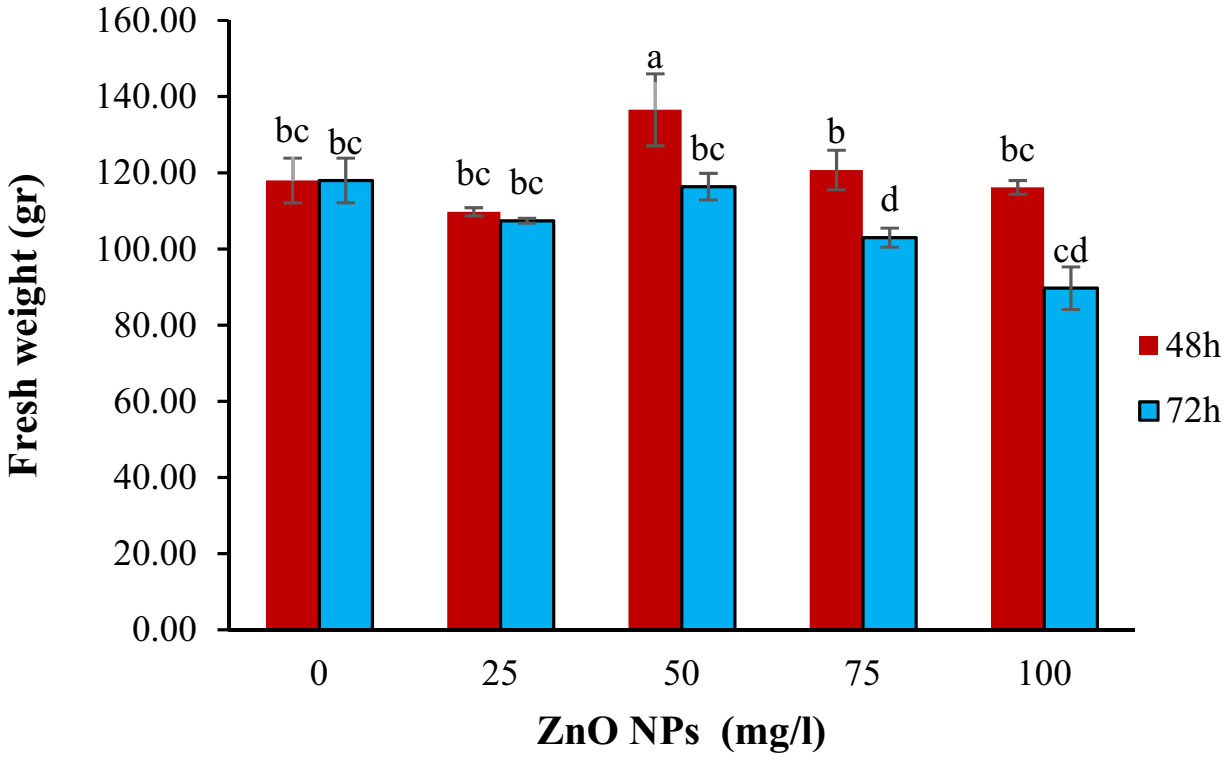

Fig. 2. The effect of zinc ZnO NPs on the fresh weight of the *H. reticulatus* hairy roots. Similar letters indicate the absence of a significant difference at the 5% probability level based on Duncan's test.

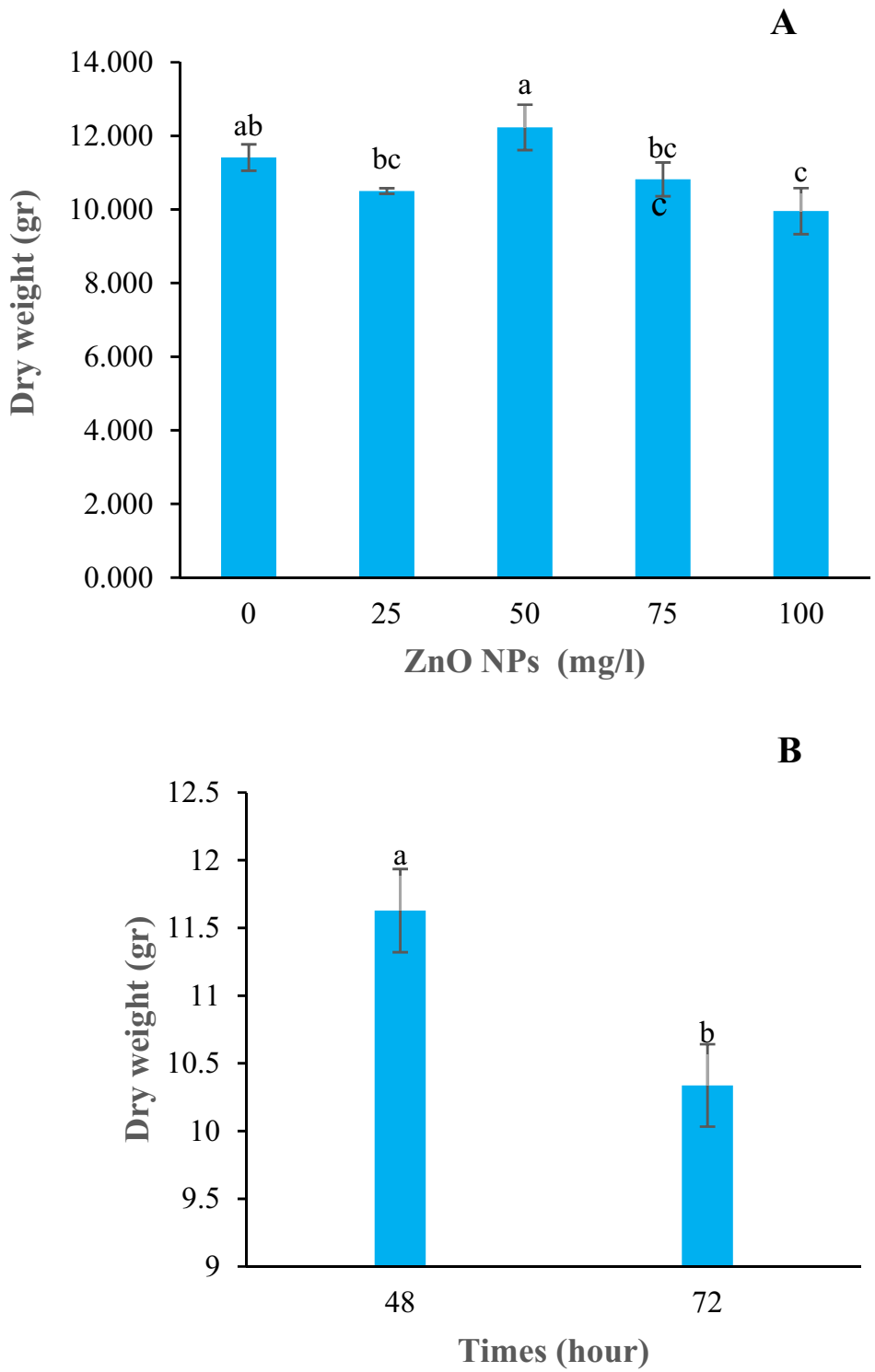

Fig. 3. The effect ZnO NPs concentration (A) and exposure time (B) on the dry weight of *H. reticulatus* hairy roots. Similar letters indicate the absence of a significant difference at the 1% probability level of Duncan's test.

and cadmium treatments on *Silene vulgaris*[38]. Therefore, zinc nano-oxide in high concentrations motivates the production of ROS, which leads to an increase in the level of H_2O_2 in the first hour after the treatment as a stimulating molecule, and as a result, it activates the antioxidant system finally increases the catalase and peroxidase activity and prevent oxidative explosion.

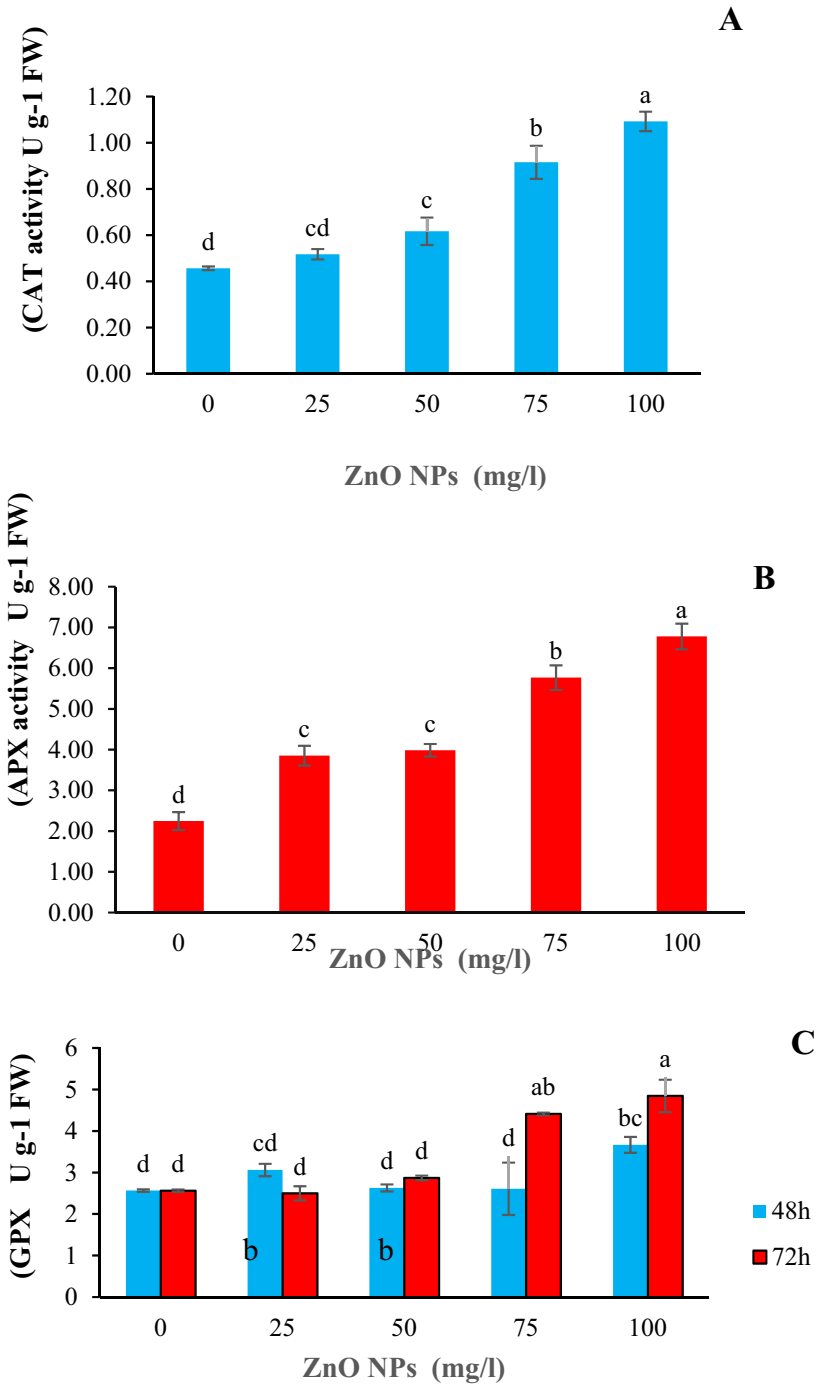

Fig. 4. Effect of ZnO NPs on the activity of catalase (A), Ascorbate Peroxidase (B) and Guaiacol-peroxidase enzyme in *H. reticulatus* hairy roots. Similar letters indicate the absence of a significant difference at the 1% probability level of Duncan's test.

Effect of ZnO NPs on TPC and TFC

The results of this experiment showed that the interaction effect of time and elicitor concentration was significant on TPC and TFC in *H. reticulatus* hairy roots ($p < 0.01$). The TPC enhanced with increasing elicitor concentration compared to the control. But in both exposure times, the content of phenol decreased by 75 mg/l after treatment. The highest amount of TPC (52.612 mg of GAE/g FW) was recorded in the 75 mg/l during 48 h exposure time and the lowest amount of TPC (3 mg of GAE/g FW) was observed in control during 48 and 72 h (Fig. 5A). Phenolic compounds are synthesized in cells under normal conditions, but the metabolism of phenol and phenolic compounds can most likely be caused by environmental factors or stressful conditions[39]. It has been reported that the production of phenolic compounds can boost against the stress of heavy metals and

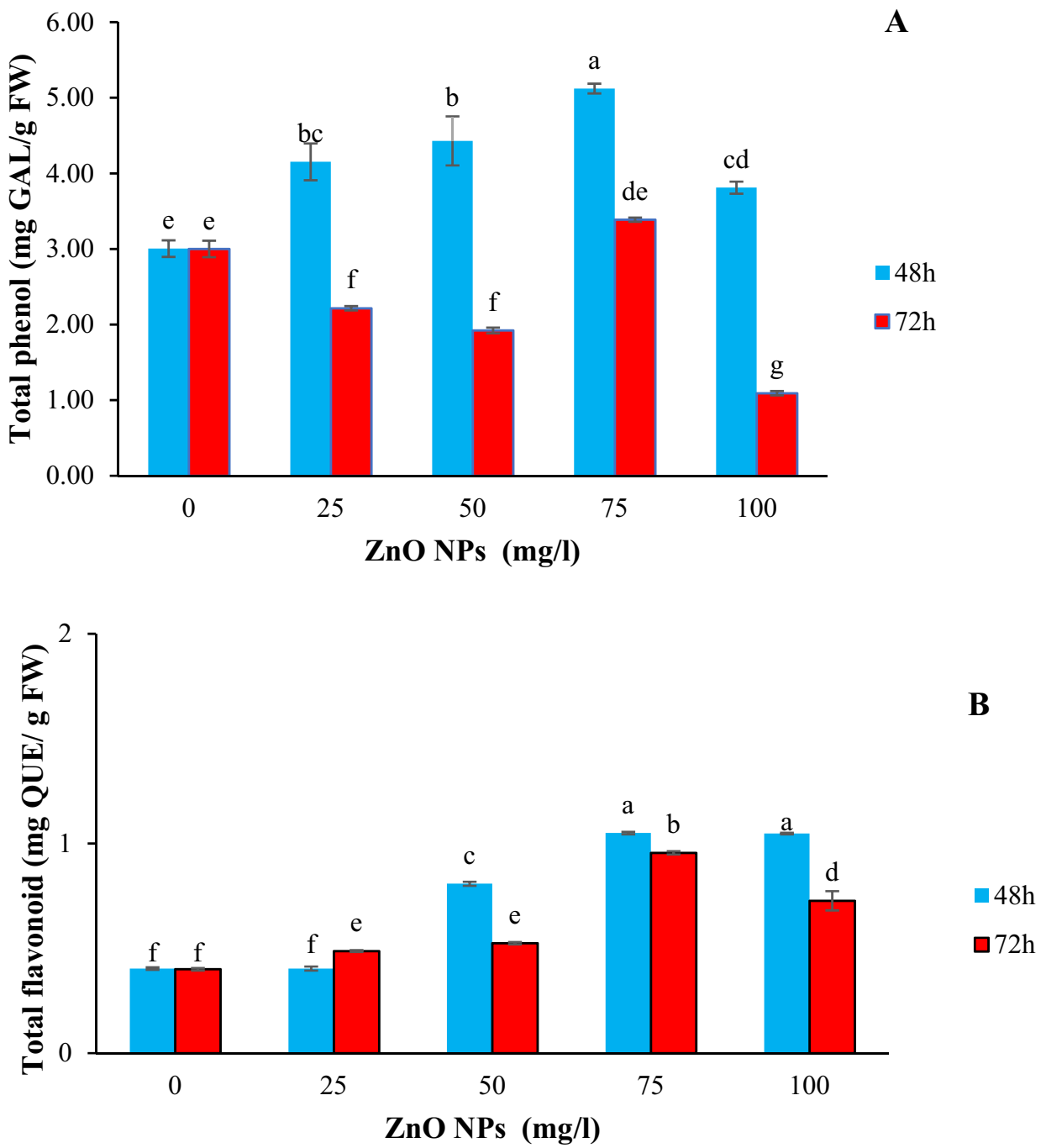

Fig. 5. The effect of ZnO NPs on *H. reticulatus* hairy roots. (A); total phenolic content (TPC) as mg Gallic acid/g FW. (B); total flavonoid content (TFC) quantified based on mg Quercetin/g FW. Similar letters indicate the absence of a significant difference at the 1% probability level of Duncan's test.

show protective reactions. this is done by chelating heavy metals and scavenging reactive oxygen species[40] or by raising enzymes involved in metabolism[41]. The results of the present studies are consistent with the reports of other researchers about nanoparticles in increasing the production of phenolic compounds[42–44].

The results of interaction effects of elicitor concentration and exposure time showed a significant effect on the TFC of *H. reticulatus* hairy roots ($p < 0.01$). The highest TFC (1.05 mg QE/g FW) was recorded in ZnO NPs (100 and 75 mg/l) during 48 h of treatment and the lowest TFC (0.40 mg QE/g FW) in control treatment was observed (Fig. 5B). Also, during 48 h, the TFC enhanced with the rise in elicitor concentration, but it decreased in 100 mg/l ZnO NPs (Fig. 5B). In this study, the accumulation of flavonoid compounds in the first hours after elicitor induction and their decline in the final hours of the treatment indicates the effect of elicitor exposure time on the accumulation of this metabolite, which is similar to Naguib et al.[45]. Also, the main reasons for the reduction of

metabolites in the final hours are the beginning of the hypersensitivity response, the toxicity of the cells due to the greater contact of the stimulus in the culture media, the creation of a feedback effect and the lack of effective genes expression[46]. In this way, reactive oxygen species (ROS) have a destructive effect on DNA and lead to a fall off in the production of effective substances[47]. According to the experiment conducted by Yusefzaei et al. on basil plant, they showed that total phenolic and flavonoid levels increase with increasing concentration of nano copper and copper chloride[31]. Studies have shown that in Low to Moderate Doses (5–100 mg/L) –Elicitation with NPs is Beneficial. Previous studies revealed that in 5–50 mg/L NPs stimulate biomass and cell growth. These results may be the positive effect of Zn + 2 on some enzyme activation such as SOD, catalase. but in high dosage, Zn could be act as inhibitor for more cell growth.

The effect of ZnO NPs on antioxidant activity

The ANOVA results showed that the interaction effect of concentration and treatment time had a significant effect on the antioxidant activity of *H. reticulatus* hairy roots ($p < 0.01$).

In the DPPH method, the maximum and minimum antioxidant activity (29.41% and 6.03%) was observed in 100 mg/L during 72 h of treatment and control treatment. With Increasing the ZnO NPs concentration, antioxidant activity also enhanced (Fig. 6A).

FRAP analysis revealed that the highest and lowest amount of antioxidant activity (6.77 and 1.50 mmol of iron/g FW) obtained in 75 mg/L during 72 h and100 mg/L in a period of 48 h respectively (Fig. 6B).

There are two types of antioxidants; enzymatic (such as glutathione, peroxidase, superoxide dismutase, etc.) and non-enzymatic such as ascorbic acid are electron donors. In fact, in oxidation reactions that have an oxidizing agent and a reducing agent, antioxidants play a reducing role that is their antioxidant capacity depends on their reducing ability. Ayyobi et al. investigated the induction effect of chitosan and colchicine on the production of rosmarinic acid in the hairy root of the *Dracocephalum kotschyi* Boiss. and reported that the antioxidant activity increased with the raising the stimulant concentration[48]. Chung et al. reported that antioxidant activities were significantly higher following AgNPs-elicitation compared with that in Ag^{+}-elicited hairy roots[49]. these results suggesting that AgNPs had a more antioxidant activity than controls.

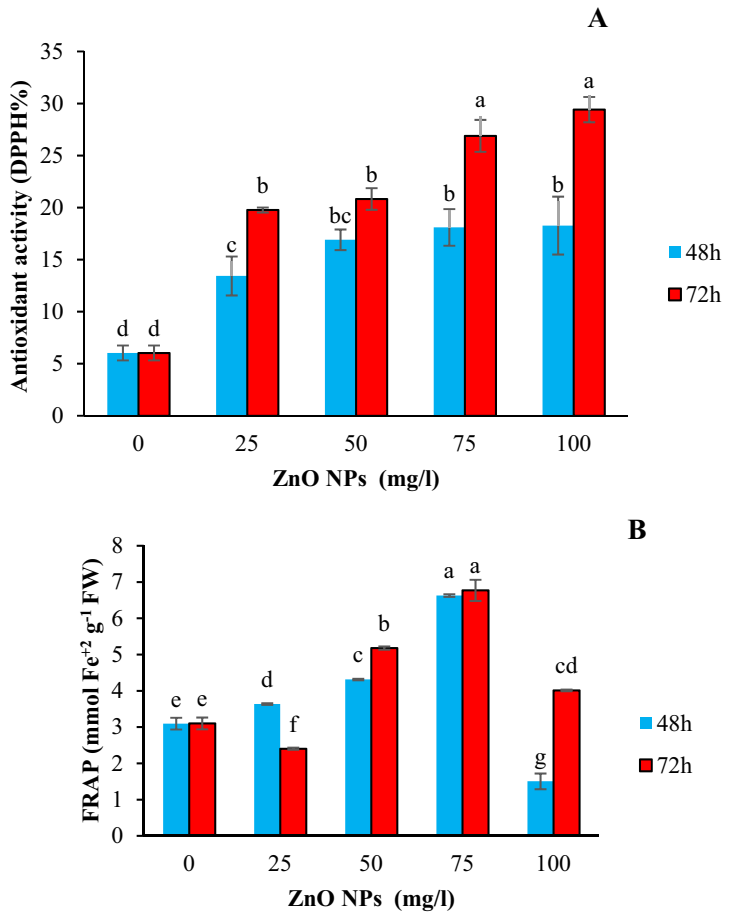

Fig. 6. The effect of ZnO NPs on antioxidant activity of*H. reticulatus* hairy roots (A); DPPH and (B); FRAP. Similar letters indicate the absence of a significant difference at the 1% probability level of Duncan's test.

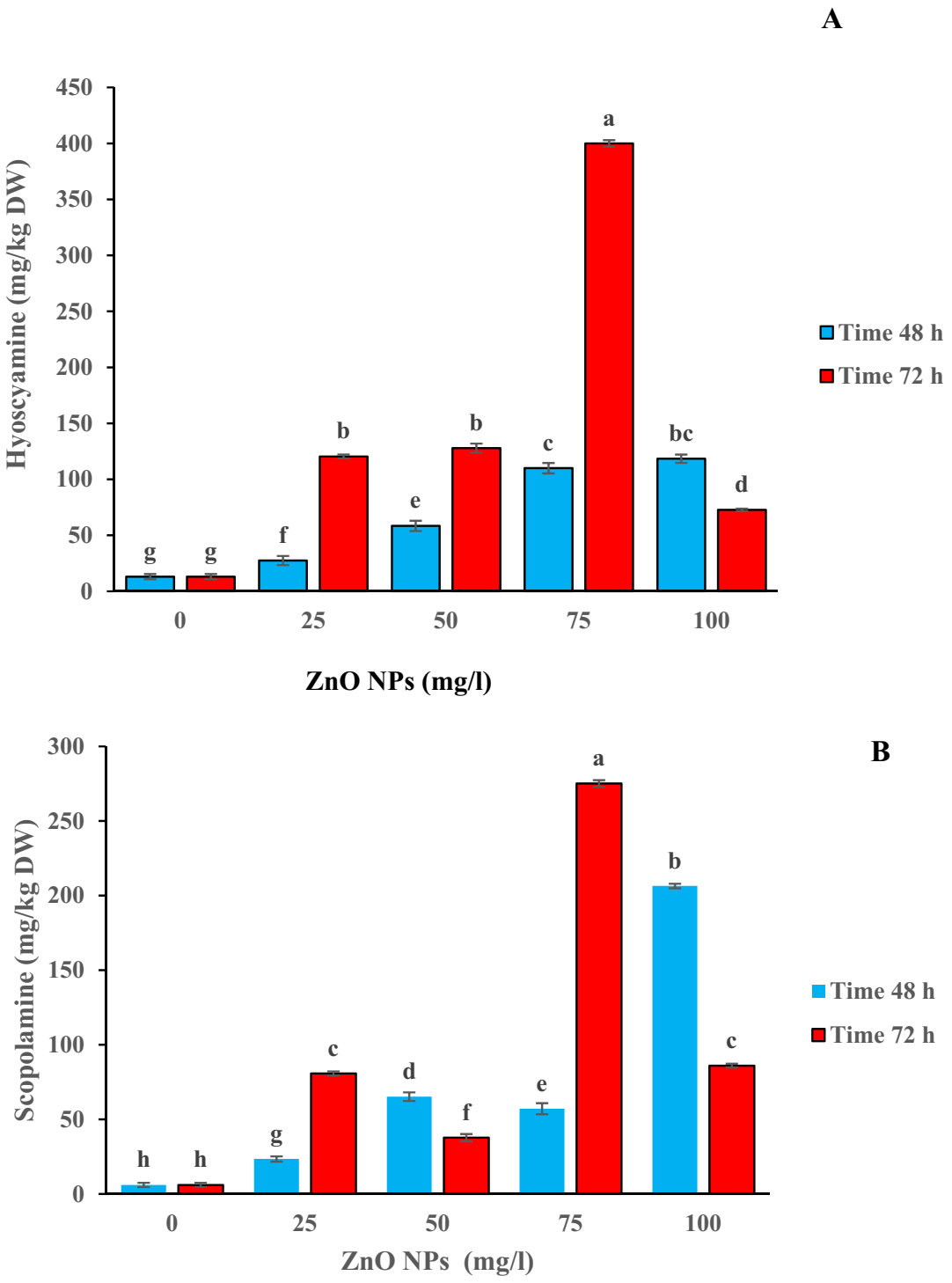

Fig. 7. The effect of ZnO NPs and exposure time on (A); Hyoscyamine, (B); Scopolamine *H. reticulatus* hairy roots. Similar letters indicate the absence of a significant difference at the 1% probability level of Duncan's test.

The effect of ZnO NPs on tropane alkaloids production

The ANOVA results revealed that the interaction effects of various elicitor levels and exposure time was significant on hyoscyamine and scopolamine production in *H. reticulatus* hairy roots ($p < 0.01$). The highest hyoscyamine content (400 mg/kg of DW) was observed in 75 mg/L during 72 h and the lowest content (13.1 mg/kg of DW) was observed in the control treatment (30.53 times compared to the control). in high levels of elicitor, the hyoscyamine production was declined (Fig. 7A).

The highest scopolamine content (275.1 mg/kg of DW) was recorded in 75 mg/L during 72 h and the lowest content (6 mg/kg of DW) was observed in the control treatment. (Fig. 7B, Fig. S2 a, b, c, d, e, f, g, h, k, and n).

It should be noted that the scopolamine content gained 45.85 times compared to the control. tropane alkaloids are mainly synthesized in the root and then transferred to the aerial organs, and the most enzymes synthesis pathway of this group of alkaloids occurs in the young root cells. For this reason, today the effort has been concentrated in hairy root culture systems to produce tropane alkaloids, especially scopolamine[50].

Addition of various elicitors such as salicylic acid, methyl jasmonate or abiotic elicitors such as nano elements as a stimulating factor for the production of secondary metabolites promote the production of valuable compounds in plant cells and organ culture. elicitors accelerate the formation of natural biopharmaceutical components and reduce the process time for gaining high metabolite levels. Various factors such as the elicitor concentration, the age of the culture media, the time of adding the elicitor to the culture medium and the exposure time affect the production and accumulation of natural metabolites. the concentration of used elicitor documented the most effect in the results of this research, which showed a key role in enhancing the tropane alkaloids level. The stimulating effect of iron nano-oxide in different concentrations and treatment times showed that the highest creation of hyoscyamine and scopolamine was observed at 450 and 900 mg/L concentrations of elicitation in 24 and 48 h, respectively 11. It seems that ZnO NPs use 2 different mechanisms to induce plant response; first, stress induction (Elicitation); ZnO NPs act as nano-elicitors, triggering oxidative stress via Reactive Oxygen Species (ROS) generation. Plant cells perceive this stress and activate defense-related pathways, leading to increased secondary metabolite synthesis. In according to the second hypothesis, upregulation of Key Enzymes involved in phenylpropanoid pathway such as PAL (Phenylalanine Ammonia Lyase), CHS (Chalcone Synthase) affect secondary metabolite production and accumulation.

When comparing ZnO NPs with other elicitors, their effectiveness appears to be comparable to previously studied nanoparticles such as silver and iron oxide. For example, iron oxide nanoparticles have been reported to significantly enhance alkaloid accumulation in *Artemisia scoparia*[13], suggesting that metal-based nanoparticles may share a common mode of action in plant secondary metabolism. Additionally, the role of ZnO NPs in triggering plant defense responses and metabolite biosynthesis may be similar to the effect-directed fractionation approach used to identify bioactive compounds in *Typha angustifolia* pollen[51]. These findings reinforce the concept that abiotic elicitors, including ZnO NPs, can serve as powerful tools for enhancing the production of high-value medicinal compounds in plant biotechnology. The results of this study demonstrate that ZnO NPs serve as an effective elicitor for enhancing the production of tropane alkaloids in *H. reticulatus* hairy roots. The observed increase in alkaloid content at 75 mg/L ZnO NPs for 72 h aligns with findings from previous studies on nanoparticle-induced secondary metabolite production. The ability of ZnO NPs to stimulate biosynthesis is likely linked to their role in modulating oxidative stress and activating stress-responsive metabolic pathways. Research on *Silybum marianum* has shown that ZnO NPs influence gene expression involved in secondary metabolism, suggesting a potential mechanism for the observed increase in tropane alkaloids[10]. Furthermore, antioxidant enzyme activity (CAT, GPX, APX) was significantly enhanced under ZnO NP treatment, indicating that reactive oxygen species (ROS) may act as signaling molecules to trigger secondary metabolite biosynthesis, as previously reported in other medicinal plant species[52].

ZnO NPs are competitive elicitors due to their nanoscale efficiency, dual nutrient-elicitor role, and cost-effectiveness in comparison to other biotic and abiotic elicitors such as MeJA or chitosan, but require dose optimization. The effect of zinc nano-oxide and Fusarium mushroom extract on the hyoscyamine and scopolamine production in the hairy roots of *H. reticulatus* displayed that the hyoscyamine and scopolamine in zinc nano-oxide at concentrations of 100 mg/L was 37 and 37.6% respectively. In Fusarium mushroom extract at 5 and 10 mg/L concentrations during 48 h exposure time hyoscyamine and scopolamine boosted by 27.5 and 40.41% respectively[53].

The zinc nano-oxide extract used as an abiotic elicitor in this study, affected the production and accumulation of beneficial compounds in *H. reticulatus*. Our observations have shown that the use of ZnO NPs with a concentration of 100 mg/L can enhance the TPC and TFC accumulation in *H. reticulatus* hairy roots. According to the medicinal properties OF hyoscyamine and scopolamine and the increasing global demand for its production, the use of effective methods of enhancing this valuable metabolite can be considered by industrial markets. In addition, the usage of the current elicitor as an effective factor in bioreactors can be useful on large scale production of beneficial compounds and in the pharmaceutical industries.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Author contributions
B.H. contributed to the study conception and design. Data collection and analysis were performed S.A. The first draft of the manuscript was written by B.H and S.A. R.T. and S.H. prepared HPLC analysis. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Guideline statement
Authors confirm that the use of plants in the present study complies with international, national and/or institutional guidelines.

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