

Impact of cobalt and zinc stress on *Rehmannia glutinosa* growth, bioactive compound production, antioxidant activity, and molecular dynamics simulation

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Research Article

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Abstract

The effects of cobalt (Co) and zinc (Zn) on the growth of *Rehmannia glutinosa*, as well as its antioxidant activity, total phenolic and flavonoid contents (TPC and TFC), targeted polyphenolic compounds, and molecular dynamics simulations were studied. The results revealed that exposure to 0.01 to 0.1 ppm Zn in the growth medium increased plant height, average number of leaves, and root length. Higher concentrations of Zn had deleterious effects, and lower concentrations of Co (0.01 and 0.1 ppm) significantly inhibited growth, as indicated by the retardation and reduction in root length, plant height, and leaf numbers. Higher concentrations of Co (1 ppm) cause early defoliation, necrosis, and plant death. The highest concentration of Zn (1 ppm) caused a significant decrease in Chl a concentration compared to the other concentrations and the control plants. Lower concentrations of Zn and Co (0.01 ppm) showed elevated radical-scavenging activity compared with that of the control plants. Maximum TPC and TFC were achieved at 0.01 ppm of Zn, while a higher concentration of TPC and TFC was obtained with 0.1 ppm of Co. Furthermore, catalase and ascorbate peroxidase activity were significantly affected by Zn and Co. The targeted phenolic compounds were analyzed by LC-MS-MS in the Zn- and Co-treated and control plants. Finally, to evaluate the stability of phytochemicals with high binding affinities and docking scores, a 100-ns molecular dynamic simulation was performed. These findings suggest that exposure to high concentrations of Zn- and Co-contaminated soils may affect the growth and metabolism of *R. glutinosa*.

Key Message

The effects of cobalt and zinc on *Rehmannia glutinosa* development, antioxidant activity, total phenolic and flavonoid contents, targeted polyphenolic compounds, and molecular dynamics simulations were examined.

Introduction

Rehmannia glutinosa Libosch (Family: Scrophulariaceae), a perennial herb, is an important medicinal herb used in Korea, China, and Japan. Fresh, dried, and steamed tuberous roots are traditionally used to treat various health problems including diabetes, insomnia, hypertension, sedation, and hematological conditions. They contain active agents that affect the immune, nervous, circulatory, and cardiovascular systems as well as bone metabolism (Zhang et al. 2008). These activities are attributed to the presence of various phytochemicals, including iridoid glycosides, such as aucubin, catalpol, loganin, phenylethanoid glycosides, polysaccharides, phenolic acids, and rehmannoside A (Wang et al. 2016). Catalpol is an active biochemical compound in *R. glutinosa* that reduces bleeding, prevents liver damage, boosts sex hormone levels, and balances blood sugar levels (Huang et al. 2022).

Heavy metal contamination of agricultural lands is a major environmental issue that threatens ecosystems (Munir et al. 2022). Depending on their presence and concentration, metals can either benefit or harm plants (Rashid et al. 2023). The concentrations of these heavy metal ions in soils are heavily

affected by anthropogenic sources such as sewage sludge, industrial waste, dust smelters, phosphate fertilizers, and natural processes (Briffa et al. 2020). Improperly disposed mine tailings, the dispersion of these metals by heavy rainfall, and anthropogenic activities are the primary sources of water and soil pollution that degrades cultivated land and the environment in Korea (Yun et al. 2020). Thus, high concentrations of these toxic metals continuously enter water sources consumed by animals and humans and have become a major ecological and environmental concern (Mitra et al. 2022). Exposure of plant species to high concentrations of heavy metals causes excessive generation of ROS through Haber–Weiss reactions or by imposing oxidative stress, disruption of the electron transport system, interaction with the antioxidant system, and disruption of the uptake of essential elements (Hasanuzzaman et al. 2020).

Adequate zinc levels are critical for optimal plant development because they regulate secondary metabolism and physiology, and enhance stress tolerance (Fan et al. 2021). Moreover, the absorption of an appropriate amount of zinc enhances the growth parameters of plants by enhancing the antioxidant activity and potassium concentration, and reducing the phosphorus and sodium concentrations (Wang et al. 2015). However, the accumulation of excessive amounts of Zn can induce stress and inhibit yield, growth, and development, while decreasing the levels of photosynthetic pigments, thus leading to plant death (Akladios and Mohamed 2017). The accumulation of heavy metals reduces carbon assimilation, which triggers an increase in the photosynthetic electron flux to molecular oxygen, and increases the production of hydrogen peroxide, superoxide, and hydroxyl radicals (Stohs and Bagchi 1995). Higher ROS levels can damage cellular macromolecules, including pigments, proteins, lipids, and DNA, and are rapidly scavenged by antioxidant enzymes (Sridhar et al. 2020).

Cobalt is generally present at low concentrations but is in high demand in the steel, cement, and paint industries, and it is used extensively in chemical fertilizer production (Sridhar et al. 2020). The presence of this hazardous heavy metal is high in industrial sewage sludge (95.2 mg/kg) and metropolitan sewage sludge (22.9 mg/kg) in Korea (Lim et al. 2006). In addition to nitrogen fixation in leguminous plants, cobalt has no other direct biological or physiological role (Hu et al. 2021). However, it is important to note that elevated levels of Co can lead to DNA damage via oxidative stress (Angelé-Martínez et al. 2023). Moreover, they can damage cells, increase the production of ROS in plants, alter enzymatic activity, impede the absorption of vital nutrients, including phosphorus, sulfur, iron, calcium, manganese, zinc, and copper; hinder photosynthesis; disrupt stomatal conductance, carbon uptake, transpiration rate, chlorosis, and necrosis; and induce chlorosis and necrosis (Sachdev et al., 2021). Elevated concentrations of cobalt induce a decline in various growth parameters of *Raphanus sativus*, including chlorophyll, shoot length, root length, and total leaf area (Jayakumar et al. 2007). Furthermore, an overabundance of cobalt in *Vigna radiata* results in diminished activities of antioxidant enzymes, as well as reductions in the concentrations of plant sucrose, amino acids (Jayakumar et al. 2008), and nutrients in *Lycopersicon esculentum* (Jayakumar et al. 2013). According to Jayakumar et al. (2009), cobalt concentrations that fall within the range of 0.01–1.00 µg/g dry matter are deemed to be safe for the growth and development of plants. Concentrations that exceed this threshold are considered deleterious to plant growth and development.

Hasanuzzaman et al. (2020) report that the antioxidant system significantly mitigates the detrimental effects of reactive oxygen species (ROS) on numerous biochemical pathways and other vital biological processes. Plants have evolved an intricate antioxidant system comprising peroxidase (Pe), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) to prevent oxidative damage. These enzymes protect cellular macromolecules from heavy metals, and exert significant resistance to oxidative stress (Rajput et al. 2021). Moreover, plants exhibit non-enzymatic antioxidant responses, such as phenolic compounds, tocopherols, glutathione, ascorbate, and thiols, which are essential for protecting cellular components from ROS (Hasanuzzaman et al. 2020). Certain phytochemicals, including phenolic compounds, function as excellent ROS scavengers in plants exposed to various abiotic stressors. In particular, phytochemicals such as flavonoids can protect against heavy metal stress by chelating transition metals (Goncharuk and Zagorskina 2023). Moreover, the exposure of plants to abiotic stresses, such as heavy metals, affects the accumulation of phenolic metabolites in plant tissues. These compounds have various plant functions during acclimation, in response to excessive heavy metals. Muszyska et al. (2019) reported that they efficiently scavenge reactive oxygen species (ROS) and bind metal ions via hydroxyl and carboxyl groups. Several studies have shown that *R. glutinosa* root-stock production is affected by disease, low tuber yield, poor rootstock quality, and environmental conditions (Lee et al. 2011). Molecular dynamics (MD) simulation is an effective and cost-effective computational method that enables the prediction of plant biomolecular structures, reaction processes, and binding patterns between substances. Furthermore, it can simulate alterations in the local configurations, functional groups, and bond energies of the biomolecules. These capabilities are highly valuable for investigating the mechanisms underlying plant resistance to abiotic stresses (Wu et al. 2023). We hypothesized that *R. glutinosa* can survive and resist zinc- and cobalt-induced stress, thereby enabling plant species to survive. Currently, there is a lack of information regarding the impact of Zn and Co on the growth, development, and physiology of *R. glutinosa*. These data would be valuable for the development of variable cultivation methods that can improve the yields and functional properties of *R. glutinosa*. The objective of this study was to examine the variations in phenolic compound composition, antioxidant responses, and toxicological behaviors of *R. glutinosa* in response to varying concentrations of Zn and Co. Finally, a 100-ns molecular dynamic simulation was conducted using phytochemicals that exhibited strong binding affinities and docking scores to assess the stability of the phytochemicals identified in the *R. glutinosa* extracts.

Materials and methods

Chemicals

Cobalt chloride (CoCl_2) and zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) were obtained from Daejung Chemicals and Metals Company, Ltd., (Shiheung, Korea). The pH of the Zn and Co solutions prepared with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and CoCl_2 , respectively, was adjusted to 5.75. All analytical standards utilized in this investigation were procured from Sigma Chemical Co. (St. Louis, MO, USA) and analytical grade. The methanol used in the extraction process was acquired from J.T. Baker (Philadelphia, NJ, USA).

Plant growth conditions and heavy metal treatments

R. glutinosa seeds were obtained from the Bioherbal Research Institute of Kangwon National University, Korea. The seeds were washed for 15 minutes under running tap water. They were surface sterilized for 50 seconds in 70% ethanol. After rinsing with sterilized distilled water, the seeds were allowed to soak in 5% sodium hypochlorite and two drops Tween 20 for 10 min. The seeds were then rinsed six times with sterile distilled water. In a full-strength Murashige and Skoog (MS) medium, surface-sterilized seeds were incubated at 25–28°C for 16 hours under $36 \text{ mol m}^{-2} \text{ s}^{-1}$ light intensity from cool-white, fluorescent lights. Half-strength MS medium was used to support germinated plantlets. The pH of all media (MS and 1/2 MS) was adjusted to 5.8 before adding 8 g/L plant agar. All media were autoclaved at 120°C for 20 min. After four weeks in 1/2 MS medium, the plantlets of *R. glutinosa* were removed and planted in 1/2 MS medium supplemented with sucrose (30 g/L), agar (8 g/L), and different concentrations of zinc and cobalt (0.01, 0.1, 0.5, and 1 ppm). The control was a heavy-metal-free basal MS medium. Set at 5.6 for 5.8 with 0.1 N HCl and NaOH, the samples were autoclaved at 121°C for 15 min. Six weeks were spent in a sterile culture environment at 25–28°C under a 16-hour photoperiod ($36 \text{ mol m}^{-2} \text{ s}^{-1}$ light intensity from cool white, fluorescent lights). Fresh weight, plant height, leaf number, leaf area, leaf yield, root number, and root length were recorded.

Effect of heavy metals on photosynthetic pigment concentrations

In order to isolate carotenoids and chlorophyll (Chl a, Chl b), fresh leaf samples (0.5 g) of *R. glutinosa*, both treated and control, were homogenized in 10 mL of acetone at 80% concentration. Ten minutes at 4°C, homogenized samples were centrifuged at $1,968 \times g$. Following this, a cuvette was utilized to capture 2 mL of the supernatant for subsequent use. The absorbance values of chlorophyll a, chlorophyll b, and carotenoids were determined at 663, 645, and 479 nanometers, respectively, utilizing a UV-VS spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan) in accordance with the methodology suggested by Lichtentaler and Wellburn (1985). The concentrations of photosynthetic pigments in the extracts were determined using the following equation:

$$\text{Chlorophyll a (Chl a)} = (12.7 [A_{663}] - 2.69 [A_{645}]) \quad (1)$$

$$\text{Chlorophyll b (Chl b)} = (21.50 [A_{645}] - 5.10 [A_{663}]) \quad (2)$$

$$\text{Carotenoids (car)} = (1000 [A_{470}] - 1.82 \text{ Chl a} - 85.02 \text{ Chl b}) / 198 \quad (3)$$

Electrolyte leakage

The uppermost fully expanded leaf discs (2 g) of the treated and control *R. glutinosa* were collected, immediately cut into segments (2 cm), and rinsed slowly ($1 \times g$) in deionized water using a rotary Orbit Shaker (Lab-Line, Dubuque, USA) at room temperature to remove the surface-adhered electrolytes from the leaf surfaces. After 24 hours of incubation at 15°C, leaf discs were placed in sealed 5 mL Eppendorf

tubes containing 4 mL of deionized water. After 30 minutes of immersion in a boiling water bath at 95°C to extract all electrolytes, the samples were chilled to 25°C. The electrical conductivities of the cooled samples were measured using a digital conductometer (Digimed DM-3, Digicron, São Paulo, Brazil). EL (electrode leakage) was determined applying the subsequent equation:

$$EL (\%) = (EC_1 / EC_2) \times 100$$

The variables EC1 and EC2 represent the initial and final electrical conductivities, respectively.

Antioxidant Enzyme Assay

As described by Aebi (1984), the CAT assay measures the variation in the amount of H₂O₂ caused by the presence of CAT (EC 1.11.1.6) in the test samples, which indicates the potency of the activator/inhibitors. A 0.1 mL aliquot of the extraction solution was introduced into a 1.9 mL reaction buffer, which was composed of 67 mM sodium potassium phosphate and had a pH of 7.4. The reaction mixture was subsequently incubated at 37°C for 15 minutes. Following this, 1.2 mL of 0.6% H₂O₂ was introduced into the reaction mixture. By measuring absorbance at 240 nm for two minutes, the decomposition of H₂O₂ was ascertained. Under the conditions of the assay, one unit (U) of catalase activity was defined as the quantity of enzyme that caused an absorbance change of 0.001 per minute.

The activity of APX (EC 1.11.1.11) was assessed using the absorbance decrease of oxidized ascorbate as a metric, in accordance with the procedure outlined by Nakano and Asada (1981). At the outset, a solution comprising 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ascorbic acid (C₆H₈O₆), 50 mM potassium phosphate (pH 7.0), 2% hydrogen peroxide (H₂O₂), and 0.1 mL of enzyme extract were combined in a volume of 3 mL. At 290 nanometers, the absorbance of the reaction products was quantified utilizing a UV-1800 Shimadzu UV-VS spectrophotometer. A change in absorbance per minute of 0.001 was utilized to denote one unit of enzyme activity in the assay conditions.

Lipid peroxidation

MDA formation was used as an indicator for lipid peroxidation in accordance with the procedure outlined by Heath and Packer in 1968. The final value was obtained by subtracting the nonspecific absorbance at 600 nm from the amount of MDA-TBA complex as measured at 532 nm. Triples of each plant extract were utilized for analysis.

Effect of Zn and Co on total phenolic contents (TPC) and total flavonoid contents (TFC) in *R. glutinosa*

Preparation of plant extracts

Twenty-four hours after collection, the young leaves of the treated and control *R. glutinosa* plants were desiccated at room temperature. In a blender, the desiccated samples were subsequently ground. After combining 2 g of the pulverized sample with 80% MeOH, the resulting homogenate was stirred for 24 hours at room temperature (25°C). Debris was extracted from the homogenate by filtration through a Buchner funnel and filter paper. Following a 40°C rotary vacuum evaporation with a solvent lyophilizer

(SB-1300, Shanghai Eyela Co. Ltd., China), the solvents were lyophilized with a freeze dryer (PVTFD 300R, IIShinBioBase, Yangju, Gyeonggi, Republic of Korea). 100 mL of 80% MeOH was used to dissolve residual extracts, which were then stored at 4°C until further use.

TPC

The TPC of *R. glutinosa* was determined as previously described (Singleton and Rossi, 1965). Briefly, *R. glutinosa* extracts (100 µL) were dissolved in 50 µL of Folin-Ciocalteu (1000 ppm). The mixture was then incubated at room temperature in the dark for five minutes. The mixture was then supplemented with 100 µL of a 20% (w/v) sodium carbonate (Na₂CO₃) solution. Following an additional 30 minutes of incubation at room temperature, the absorbance of the mélange was quantified at 765 nm utilizing a UV-VIS spectrophotometer (UV-1800, Shimadzu). A standard curve was constructed by utilizing solutions of gallic acid ranging from 0 to 250 mg/L. Using the following formula, TPC values are expressed in gallic acid equivalents (GAE) of the dry matter (DM) of the sample (µg/g dry weight):

$$y = 0.3208x - 0.3208,$$

The variables x and y denote the absorbance at 765 nm and the quantity of gallic acid equivalent (µg/mL).

TFC

TFC was determined as previously described (Thiruvengadam and Chung, 2015). Briefly, *R. glutinosa* extracts (500 µL) were dissolved in 100 µL of 10% aluminum nitrate [Al(NO₃)₃] solution and 100 µL of potassium acetate (CH₃CO₂K) (1M). After 5 min, 1 mL NaOH (1 M) was added to the mixture at room temperature. At ambient temperature, 1 mL NaOH (1 M) was added to the mixture after 5 minutes. By comparing the absorbance of each sample to a negative at 510 nm, a UV-VS spectrophotometer (UV-1800, Shimadzu) was utilized. The standard curve was constructed by combining quercetin solutions ranging from 0.5 to 100 µg/L. Using the subsequent formula, TFC was represented as the quercetin equivalent (QE) of the dry matter (DM) of the sample:

$$y = 1.0707x - 1.0183$$

The variables x and y denote the absorbance at 510 nm and the quantity of QE (µg/g).

Estimation of phenolic compounds by LC-MS/MS

Using an Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) system and adhering to a procedure previously documented (Ghimire et al. 2017), the composition of specific phenolic compounds in both the treated and control *R. glutinosa* samples was determined. Connected to an Agilent Technologies autosampler (Agilent 1100 series) and pumps (Agilent 1200, Palo Alto, CA, USA), the LC system incorporated a C18 column (4.6 × 250 mm, 5 µm) for the API 2000 series mass spectrometer (Applied Biosystems, ON, Canada). The negative-ion mode of a mass spectrometer was employed. As operational parameters, the following were established: 70, 40, 2, and 20 psi were specified for the drying

gas pressure, nebulizer gas pressure, collision gas pressure, and curtain gas pressure, respectively. At 350°C and 4.5 kV, respectively, the capillary voltage and drying gas temperature were maintained. Solution A contained acetonitrile (C₂H₃N) in water, while solution B comprised 0.1% (v/v) formic acid (HCOOH) in water. 10–40% B for 0–10 minutes, 40–50% B for 10–20 minutes, 50–100% B for 20–25 minutes, 100–10% B for 25–26 minutes, and 10% B for 26–30 minutes comprised the gradient elution used to efficiently separate the compounds. A volume of 10 L of sample was injected into the column at a temperature of 25°C, while the mobile phase flowed at a rate of 0.7 mL/min. In multiple reaction monitoring (MRM) phases, mass spectrometry data was acquired. Three replicates of each sample were analyzed.

Molecular dynamics simulations

This was applied to explore the flexibility and conformational changes of the hit molecules with the targets. It assists in identifying an appropriate ligand that functions well for the target by demonstrating its compatibility with docked target conformations. During the simulation phase, this raises the possibility of efficient binding by permitting molecular interactions, flexibility, and conformational changes. The complex (ligand and target) was first constructed using the System Builder tool to accomplish this algorithm. There are two phases in System Builder: Solvation and Ions. Orthorhombics were used in solvation to generate a boundary box shape with buffer distances of a:10.0, b: 10.0, and c: 10.0. Salt implantation and ion exclusion were similarly performed within 20 Å. The complex was constructed for further investigation by placing ions and salts. Before the system was neutralized by adding counter ions, the protonation state of the target residues was maintained at pH 7.0. The complete systems were then equilibrated for 100 and 300 ns at a temperature of 300 K and pressure of 1.01325 bar with 1000 frames using NPT (isothermal-isobaric) ensembles.

Effect of zinc and cobalt on antioxidant activity DPPH method

The procedure to carry out the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was as previously described (Thiruvengadam et al. 2014). Briefly, a 1 mL sample of the extracted plant extracts (with a concentration of 0.1 mg/mL) was combined with 4 mL of the DPPH methanol solution (0.004%). After 30 minutes of incubation at room temperature in the dark, the absorbance of the reaction mixture was determined at 517 nm utilizing a UV–VIS spectrophotometer (UV-1800, Shimadzu). The calibration curve was plotted with a Trolox concentration of 20–1000 µM. Trolox equivalents (TE) were employed to quantify the antiradical activity (mg/g dried weight of *R. glutinosa*). BHT was employed in the capacity of positive control. The calculation for DPPH activity was as follows:

$$\text{DPPH \%} = (A_0 - A_1 / A_0) \times 100$$

A₁, denotes the absorbance of the test sample, while A₀ represents the absorbance of the control (which comprises all reagents excluding the test compound). Triplicate of each measurement was performed.

Assessment of statistics

The mean and standard deviation of independent replicates are used to present the data. The findings were subjected to statistical analysis via using the method of one-way analysis of variance (ANOVA) performed with the Statistical Package for the Social Sciences (SPSS, Version 24). The presence of significant differences ($P < 0.05$) was confirmed using Duncan's multiple range test between the control and experimental groups.

Results

Effects of zinc and cobalt on plant development

All zinc-treated plants were grown until the end of the treatment period. At low Zn concentrations (0.01 ppm), the growth of *R. glutinosa* was initially promoted by Zn (Table 1). Application of 0.01–0.5 ppm zinc to the growth medium increased the average plant height. Similarly, 0.01–0.1 ppm of zinc in the growth medium improved the fresh weight, number of leaves and root length. However, higher concentrations of Zn had deleterious effects on the growth of *R. glutinosa*; the average root length, root number, and leaf number decreased significantly with increasing concentrations of Zn (> 0.5 ppm) compared to the control (Fig. 1A-E). A nearly 50% reduction in the FW of *R. glutinosa* was noticed after treatment with 1 ppm Zn (Fig. 2). At 0.01 ppm, cobalt showed no significant difference in fresh weight or root number than control plants. However, lower levels of cobalt (0.01 and 0.1 ppm) significantly inhibited the growth of *R. glutinosa*, as indicated by the retardation and reduction in root length, plant height, leaf number, and node number (Fig. 3). Plants treated with lower concentrations (0.01–0.1 ppm) of Co grew until the end of the treatment. Higher concentrations of cobalt (0.5–1.0 ppm) caused early defoliation, necrosis, and plant death (Fig. 4A-E).

Effects of zinc and cobalt on photosynthetic pigments

Among all treatments, the chlorophyll concentration in the *R. glutinosa* leaves varied significantly ($P < 0.05$) (Fig. 5). Chlorophyll a (Chl a) concentration decreased significantly at the highest zinc concentration (1 ppm) in comparison with the control plants and the other treatments (0.01, 0.1, and 0.5 ppm). A noticeable reduction was observed in chlorophyll b (Chl b) concentrations after treatment with 1 ppm zinc (Fig. 5), followed by the other treatments and control plants. However, lower doses of zinc (0.01–0.1 ppm) had no significant effect on carotenoid concentrations in *R. glutinosa*. The total carotenoid concentrations in the treated *R. glutinosa* decreased with increasing cobalt concentration. In this study, the color of growing *R. glutinosa* leaves transformed quickly from green to yellow, and foliar chlorosis followed by necrosis appeared within a week of transplanting *R. glutinosa* plants to a medium containing a high concentration (1 ppm) of cobalt (Fig. 5).

Antioxidant activity of plant extracts

The variance in zinc concentration in the growth medium had an impact on the DPPH activity of *R. glutinosa*, as indicated in Table 1. The radical scavenging activity of zinc-exposed *R. glutinosa* ranged from 11.84 to 25.72%. Treatment with 0.01 ppm zinc resulted in the highest DPPH activity compared to

the control (16.36%). A negative trend was observed when the concentration of zinc in the growth medium increased. The lowest DPPH scavenging activity was observed for 1 ppm Zn (8%). Cobalt at 0.01 and 0.1 ppm significantly increased the DPPH radical scavenging activity (20.71% and 23%, respectively) compared to the control.

Total phenolic content (TPC) and total flavonoid content (TFC)

TPC of the whole-plant extract of *R. glutinosa* was determined using the linear regression equation of the gallic acid standard calibration curve. The total phenolic content of *R. glutinosa* varied with different zinc concentrations in the growth medium (Table 1). TPC of different plant parts ranged from 1.08 ± 0.09 to 5.14 ± 0.11 mg GAE/g dry sample. In the pre-sent study, 0.01 ppm zinc in the growth medium reduced the TPC content to 5.14 mg GAE/g. The TFC of different plant parts ranged from 2.03 ± 0.03 to 3.89 ± 0.06 mg QE / g dry sample. A higher TFC (3.89 mg GAE/g) was observed in plants exposed to growth medium containing 0.01 ppm zinc. A further increase in cobalt concentration lowered TPC compared to the other cobalt treatments and control plants. TFC increased and peaked (4.04 ± 0.05 mg QE/g) at 0.1 ppm cobalt. Similarly, lower cobalt concentrations (0.01 ppm) produced lower TFC (1.71 ± 0.02 mg QE/g). However, the TPC content of *R. glutinosa* treated with 0.01 ppm cobalt did not differ significantly from that of the control plants.

Lipid peroxidation

The MDA levels in *R. glutinosa* exposed to different concentrations of Zn and Co are shown in Figs. 7A&B. In the present study, there was a significant difference in MDA levels between the treated and control plants. The MDA level remained unaffected at 0.01 and 0.1 ppm zinc administration compared to the control plants. A further increase in zinc concentration in the growth medium increased MDA levels. Higher MDA levels were observed with 1 ppm zinc. Lower concentrations of cobalt (0.01 ppm) resulted in increased MDA accumulation compared with that in the control plants. Treatments with 0.01 ppm cobalt increased the MDA level; however, no statistical difference was observed compared with the control.

Effect of zinc on CAT and APX activity

The activities of antioxidant enzymes, including CAT and APX, in zinc-treated *R. glutinosa* were measured across all treatments (Fig. 8A&B). The relative CAT activity of *R. glutinosa* plants treated with lower concentrations of zinc (0.01 and 0.1 ppm) decreased compared to that of control plants. This trend varied when the zinc concentration in the growth medium was increased. There was no significant difference in CAT activity between the control and *R. glutinosa* grown in medium containing 0.5 ppm of zinc. In contrast, a relatively higher CAT activity was observed at 1.0 ppm of zinc. The APX activity of *R. glutinosa* was significantly affected by zinc treatment. APX activity in zinc-treated plants was higher than that of the control plants. The activity was slightly higher in *R. glutinosa* grown at 0.01 ppm zinc than in that grown at 0.1 ppm zinc; however, this difference was not significant. Higher concentrations of zinc (0.5–1.0 ppm) resulted in higher APX activity in *R. glutinosa*.

Effect of cobalt on CAT and APX activity

CAT and APX activities in cobalt-treated *R. glutinosa* are shown in Figs. 9A&B. In this study, CAT and APX activities of *R. glutinosa* were significantly affected by cobalt treatment. CAT activity of *R. glutinosa* was increased at treatment with 0.1 ppm of cobalt compared to control plants. Similarly, CAT activity was slightly higher in *R. glutinosa* grown under 0.01 ppm cobalt compared to the control. However, this difference was not statistically significant. APX activity was slightly lower in *R. glutinosa* grown under 0.1 ppm cobalt and was not significantly different compared to control plants. APX activity decreased at 0.01 ppm cobalt compared to that in the control plants. Thus, APX activity of *R. glutinosa* was significantly affected by cobalt treatment. A higher positive and significant correlation was observed between antioxidant enzyme activity and TPC and TFC. A positive correlation was also observed between CAT, TPC, and TFC ($r = 0.959$, $P < 0.05$; and $r = 0.783$, $P < 0.01$, respectively). Similarly, a positive correlation was observed between APX and TPC and TFC ($r = 0.951$, $P < 0.05$, and $r = 0.755$, $P < 0.01$, respectively), indicating that antioxidant enzyme activities were primarily mediated by phenolic acids (Table 2).

Effect of zinc and cobalt on electrolyte leakage

Electrolyte leakage (EL) in *R. glutinosa* treated with different concentrations of Zn and Co is shown in Figs. 10A&B. The relative EL declined at all zinc concentrations compared with that of the control plants. This parameter did not change significantly when the zinc concentration was increased from 0.01 to 0.1 ppm. The highest membrane EL was observed in the control plants. Furthermore, the EL of *R. glutinosa* increased under all studied cobalt concentrations compared that with of the control plants. An increased cobalt concentration (0.1 ppm) resulted in an excessive increase in EL. In this study, Pearson's correlation coefficient (r) analysis revealed a strong and positive correlation between the EL, TPC, and TFC of the leaf extracts of *R. glutinosa* ($r = 0.951$, $P < 0.05$; and $r = 0.755$, $P < 0.01$, respectively), indicating that phenolic compounds contributed to resistance against Zn and Co (Table 2).

Effect of zinc and cobalt on the phenolic compounds composition in *R. glutinosa*

We investigated the differences in phenolic compounds in *R. glutinosa* grown under Zn and Co stress. The phenolic compound concentrations and compositions were quantified using LC-MS/MS (Tables 3 and 4). The quantification of specific phenolic compounds presents in 80% methanolic extracts of *R. glutinosa* was achieved by isolating and estimating their retention times in relation to the standard reference compounds. A summary of the phenolic composition of *R. glutinosa* is presented in Table 4. Lower zinc concentrations increased phenolic compound concentrations. Lutein, p-coumaric acid, and apigenin are the phenolic compounds that are most prevalent in *R. glutinosa*. The use of 0.01–0.5 ppm zinc led to a greater increase in apigenin levels compared to the control plants. There were no statistically significant variations in the concentrations of p-coumaric acid, pelargonidin chloride, acetyl glycitin, or caffeic acid. Similarly, as cobalt concentrations in the growth medium increased, so did apigenin concentrations; nevertheless, this disparity did not reach statistical significance. No notable variations

were detected in the levels of pelargonidin chloride and acetyl glycitin with respect to the different concentrations of cobalt. Luteolin was detected in all the treated plants, however completely lacking in the control plant. Similarly, ferulic acid was detected only in the control plants and was absent in the treated plants.

Molecular dynamics simulations of phytochemicals in *R. glutinosa*

To examine the stability of the observed phytochemicals in *R. glutinosa* extracts, phytochemicals that showed potent docking scores with binding affinities in molecular docking were used in a molecular dynamic simulation for 100ns.

Luteolin as a phytochelatin synthase stimulant

MD simulations of luteolin with the phytochelatin synthase complex revealed that luteolin was more stable throughout the simulation trajectory. It had a significant RMSD value of less than 2.4 Å (Fig. 11a). However, it deviated in some places, particularly at 3–5ns, 9–12ns and 25–30ns. However, it was stable in the range of 30ns–100ns. On the other hand, the RMSF plot revealed that the residues SER 58, MET 59, GLN 60, PHE 61, VAL 62, THR 63, ILE 76, ASN 80, TYR 92, TYR 95, VAL 97, GLN 100, ASP 101, LYS 138, LYS 140, ASN 142, GLN 155, GLU158, ASN 159, ASN 165, ARG 204, PHE 236, SER 238, LYS 239, and THR 240 were the mobile regions in the loop regions of phytochelatin synthase (Fig. 11b and Table 5). Of these, MET 59 was the most highly mobilized residue in Phytochelatin synthase, showing fluctuation up to 1.18 Å (Fig. 11b). The interaction fraction plot revealed that the majority of connections were formed by hydrogen bonds. It also had three hydrophobic contacts and two ionic contact fractions, as shown (Fig. 11c). The interaction plot MD simulation revealed that polar residues, such as ASN 142, THR 63, GLN 155, and GLN 100, have the highest binding affinities to luteolin. Specifically, THR63 and GLN 100 had covalent interactions with binding percentages of 46% and 84%, respectively, and 70% and 30%, respectively, while ASN 142 and ASN 155 showed binding percentages of 64% and 55%, respectively. In addition, two interactions with binding percentages of 32% and 30% were observed for the charged residue LYS 140 and the hydrophobic residue TYR 92, respectively (Fig. 11d). It is important to note that luteolin exhibits 88% intramolecular interactions.

Phytochemicals as antioxidant agents under zinc and cobalt stress conditions

Pelargonidin chloride and ascorbate

The protein-ligand complex was subjected to MD simulations after docking, with particular emphasis on the ligand that had the highest docking scores and binding affinities with the target. The results of the MD simulations involving the complex of ascorbate and pelargonidin chloride revealed that the RMSD was remarkably stable. It was stable for the first 0–5 ns. After that, the RMSD deviated by more than 3.0 Å for a duration of 5–65 ns (Fig. 12a). Furthermore, the RMSD was less than 3 Å, indicating that it was

stable in the range of 65 to 100ns. On the other hand, the RMSF plot revealed that the residues GLU 11, LYS 18, PRO 34, ILE 35, LEU 37, ARG 38, TRP 41, HIS 42, ALA 70, PRO 132, ASP 133, ALA 134, HIS 163, THR 164, LEU 165, ARG 167, CYS 168, ARG 172, SER 173, PHE 175, GLU 176, TRP 179, LEU 203, and LEU 205 were the mobile regions in the loop regions of ascorbate (Fig. 12b and Table 6). Of these, PRO 132 was the most highly mobilized residue in ascorbate, showing fluctuation up to 1.3 Å (Fig. 12b). The ligand-protein contact plot revealed two types of contacts, namely hydrophobic and hydrogen bonds, in which hydrogen bond contacts were found to be the predominant contacts in this complex. The interaction plot revealed three types of contacts (backbone, side chain, and π - π stacking) with the protein residues, namely hydrophobic, charged, and polar residues (Fig. 12c). It is known that PRO 34, GLU 11, PHE 175, PRO 132, and HIS 163 have the strongest binding affinities, with percentages of 66%, 88%, 30%, 31%, and 33%, respectively (Fig. 12d).

Ferulic acid and superoxide dismutase

The results of the MD simulations involving the complex of ascorbate and pelargonidin chloride revealed that RMSD was remarkably stable in the range of 0 to 40ns. Later, it became unstable in the range 40–100 ns (Fig. 13a). On the other hand, the RMSF plot revealed that the residues TYR 2, LEU 42, VAL 53, GLU 54, LEU 56, ILE 57, LEU 60, LYS 67, HIS 78, ALA 79, SER 82, LEU 83, LYS 86, SER 148, MET 151, ARG 200, and LYS 205 were mobile regions in the loop regions of superoxide dismutase (Fig. 13b and Table 7). Of these, LYS 67 was the most highly mobilized residue in superoxide dismutase, showing fluctuation up to 1.45 Å (Fig. 13b). The ligand-protein contact plot shows three types of contact: hydrogen bonds, hydrophobic, and ionic contact (Fig. 13c). The interaction plot revealed hydrogen bond contacts between the functional groups of ferulic acid and residues (polar, charged, and hydrophobic). The interaction plot MD simulation revealed that residues, such as SER 148, ARG 200, and TYR 2, have strong binding affinities with ferulic acid. Specifically, ARG 200 had covalent interactions with binding percentages of 86% and 100%, respectively, and SER 148 and TYR 2 had strong binding affinities with percentages of 90 and 91%, respectively (Fig. 13d).

Discussion

Effects of zinc and cobalt on plant growth

Plants require sufficient amounts of trace elements for normal metabolic functions (Andresen et al., 2018). However, excess amounts of these elements in the soil can have deleterious effects and are toxic to plant growth (Seregin and Kozhevnikova, 2006). Zn and Co have long been known as essential elements in plants (Marreiro et al., 2014). These metals accumulate in the soil through several anthropological activities, including sewage disposal and industrial waste. In addition to their beneficial effects on plant life, certain elements have been found to have adverse consequences at elevated concentrations. In the present study, considerable differences in all growth parameters were observed in *R. glutinosa* exposed to different concentrations of Zn and Co. The improvement in average plant height,

leaf count, and root length in response to reduced Zn concentrations indicated that lower Zn concentrations are advantageous for the development and growth of *R. glutinosa*. However, *R. glutinosa* growth parameters, including plant height, fresh weight, leaf count, node count, root length, and root count, were significantly ($P < 0.05$) perturbed by increased Zn concentrations of 1 ppm. In this study, characteristics such as sluggish growth, growth retardation, and defoliation were common features of *R. glutinosa* plants grown under high Zn concentrations (0.5–1.0 ppm). A reduction in the level of Zn within the growth medium has been consistently shown to enhance growth parameters, including grain yield and leaf cell membrane stability in rice (Tufail et al., 2018). Furthermore, higher concentrations of Zn can replace the Mg cofactor in chlorophyll, thereby altering the composition of chlorophyll and limiting photosynthesis and plant development (Hajihashemi et al., 2020). In this study, higher concentrations of cobalt (1 ppm) had a more deleterious effect on the growth of *R. glutinosa* than zinc did. The major symptoms of *R. glutinosa* under higher cobalt concentrations were stunted growth and necrosis, which could be associated with cobalt toxicity due to its higher accumulation in *R. glutinosa* plants. Moreover, a decrease in plant height under cobalt stress has been previously reported in *Vigna mungo* (L.) (Jayakumar and Vijayarengan, 2006). This study corroborates the findings for *Vigna radiata* (Tewari et al., 2020) and *Solanum lycopersicum* (Gopal et al., 2003; Chatterjee and Chatterjee, 2003). Excessive amounts of heavy metals such as cobalt have the potential to hinder cellular division at the tips of roots (Dovgaliuk et al., 2001), obstruct the growth of recently divided cells in the elongation zone of roots (Yuan and Huang, 2016), and obstruct the transportation and absorption of nutrients, water, and minerals in plants (Vijayarengan, 2004). These effects may contribute to the diminished fresh weight and stunted height of the plants.

Effects of zinc and cobalt on photosynthetic pigments

According to Chen et al. (2007), a significant reduction in the quantity of photosynthetic pigments and substantial harm to chloroplasts result from zinc deficiency in the growth medium. Higher concentrations of Zn induce structural changes in the chloroplast membranes of tea through the action of ROS produced by oxidative stress (Li et al., 2011). According to Roa and Shekhawat (2014), higher concentrations of zinc may decrease the concentrations of thylakoids and grana in the chloroplasts. A higher concentration of zinc (1 ppm) drastically decreased the levels of chlorophyll a, chlorophyll b, and carotenoids in these plants compared to those in the control group and other treatments. Furthermore, approximately 40 and 80% inhibition of carotenoid content were observed in *R. glutinosa* treated with 0.5 and 1.0 ppm zinc, respectively. Carotenoids can effectively neutralize peroxy and singlet molecular oxygen ($^1O_2$) radicals. A reduction in the carotenoid content of *R. glutinosa* increases the probability of ROS accumulation (Candan and Tarhan, 2003). Consequently, an overabundance of zinc in an organism tends to diminish its antioxidant capabilities. The major symptoms of *R. glutinosa* under high-zinc treatment were stunted growth, sluggish growth, and disruption in growth parameters. These results suggest that higher concentrations of Zn (> 1 ppm) can cause phytotoxicity in *R. glutinosa* and reduce the levels of photosynthetic pigments. Subba et al. (2014) observed a dramatic reduction in stomatal conduction, transpiration, and photosynthetic rate in zinc-treated plants. According to them, an elevated level of Zn

causes a decrease in the activity of RuBisCO and other enzymes, which probably inhibits the photosynthetic rate of mandarin orange seedlings. In this investigation, as Co concentration increased, chlorophyll a, chlorophyll b, and carotenoids in *R. glutinosa* were reduced. In addition, Abdul Jaleel et al. (2009) found that an increase in cobalt concentration inhibited the assimilation of iron into protoporphyrin molecules, which are precursors of chlorophyll. This results in the depletion of chlorophyll compounds in the plants. Furthermore, excess cobalt inhibits the photosynthesis rate and chlorophyll concentrations (Hermle et al., 2007), lowers RuBisCO activity (Ahmad and Ashraf, 2012), and deteriorates the chloroplast structure (Seregin and Kozhevnikova 2006). Previous studies have reported a significant reduction in the chlorophyll content of cobalt-treated *S. lycopersicum* (Gopal et al., 2003) and *Spinacia oleracea* (Pandey et al., 2009). A significant decrease in photosynthetic pigments in *R. glutinosa* treated with Co (0.1 ppm) indicated that a higher concentration of Co might inhibit plant photosynthesis and decrease the biomass of *R. glutinosa*.

Lipid peroxidation

MDA concentrations in stressed plants often indicate lipid peroxidation (Smirnoff, 1993). In the present study, MDA levels remained unaffected or decreased slightly at lower zinc concentrations. A decrease in *R. glutinosa* MDA concentrations under lower concentrations of zinc stress could be associated with increased antioxidant enzyme activity, thus reducing H₂O₂ levels and cell membrane damage. MDA levels increased at a zinc concentration of 1 ppm in the growth medium. Similar results were reported by mulberry and tea (Mukhopadhyay et al., 2013), indicating that higher concentrations of Zn stimulate ROS production and impose stress on *R. glutinosa* plants. Lower concentrations of Co (0.01 ppm) resulted in increased MDA accumulation, indicating cell membrane disruption (Fig. 3). These findings corroborate those of previous studies (Pandey et al., 2009) on spinach, tomato, wheat (Bakkaus et al., 2005), and *Camellia sinensis* (Mukhopadhyay et al., 2013). A notable elevation in MDA concentration indicated that it was related to a reduction in CAT and APX activity at higher concentrations of Zn and Co. This finding also suggests that membrane peroxidation occurred as a consequence of CAT and APX enzyme dysfunction, leading to cellular component degradation (Monk et al., 1989). There was a negative correlation between chlorophyll a, chlorophyll b, and carotenoid concentrations and MDA accumulation in the zinc- and cobalt-stressed plants. As indicated by the results, the accumulation of excessive ROS under heavy metal stress led to an increase in MDA levels. In turn, increased antioxidant enzyme activity decreased H₂O₂ levels and membrane injury.

Effect of zinc and cobalt on antioxidative enzymes

Plants exhibit a defense mechanism against oxidative damage induced by ROS and heavy metal stress through the upregulation of antioxidative enzyme activity and accumulation of antioxidant compounds (Cho and Park, 2000). Plants generate antioxidant enzymes, including SOD, CAT, and APX, to scavenge radicals produced by biotic and abiotic stresses and to combat oxidative stress (Jiang et al., 2009). Zn exerts protective effects against ROS by modulating membrane permeability, safeguarding critical membranes against oxidative stress (Jan et al., 2017), detoxifying H₂O₂, enhancing nutrient and water

absorption in plants, and augmenting root diameter (Khoshgoftarmanesh and Kalbasi, 2002). Zn plays a crucial role in plants by facilitating the catalysis of $\cdot\text{O}_2^-$ production using NADPH found in SOD, preserving the equilibrium between ROS production and scavenging mechanisms, and safeguarding biomembranes (Marschner, 1995). Higher concentrations of Zn in *R. glutinosa* safeguarded plant cells from oxidative injury via increased APX and CAT activities, suggesting that APX and CAT play a significant role in scavenging ROS in *R. glutinosa*. Our observations agree with those of an earlier study on *Fagopyrum esculentum* (Lee et al., 2013), indicating that CAT participates in ROS decomposition. Moreover, CAT and APX have been shown to be significant enzymes known as antioxidants that are crucial for the scavenging of H_2O_2 (Gill and Tuteja, 2010). These findings align with those of previous reports wherein CAT, APX, and SOD levels were upregulated following treatment with zinc and cobalt (Sinha et al., 2012), suggesting that *R. glutinosa* possesses an efficient ROS-scavenging mechanism to defend against oxidative stress.

Total phenolic, flavonoid contents (TPC, TFC), targeted phenolics, and antioxidant activity of plant extracts

At lower concentrations of Zn and Co, *R. glutinosa* demonstrated increased levels of TPC, TFC, antioxidant activity, and phenolic compound content in this study. It has been reported that *Salvia officinalis* exhibits an increase in TPC, TFC, and antioxidant activity in the presence of cobalt stress (Torun, 2019). Moreover, the strong positive correlation between TPC and antioxidant activity suggests that phenolic compounds possess an effective defence mechanism against cobalt stress in *R. glutinosa*. Guardianship and Ludwigmüller (2009) described flavonoids as heavy metal protectants because of their capacity to withstand oxidative stress induced by heavy metals. The exposure of *R. glutinosa* to high concentrations of Zn and Co resulted in low antioxidant activity, TPC, and TFC. The decrease in the antioxidant potential of *R. glutinosa* leaves under higher concentrations of Zn and Co could be related to a reduction in the concentrations of phytochemicals. In the present study, positive correlations were identified between DPPH activity, TPC, and TFC. These findings suggested that the decrease in phenolic compound levels due to higher concentrations of Zn and Co likely contributed to the reduction in antioxidant enzyme activity. Many studies have demonstrated that phenolic compounds found in plants mitigate the toxicity of heavy metals via a variety of mechanisms, including radical scavenging, chain initiation prevention, decomposition of peroxides, chelation of transition metals, and reduction (Yıldırım et al., 2001). A significant increase in phenolic compound concentrations after zinc treatment indicated their active participation in metal tolerance. Phenolic compounds act as ROS scavengers by donating electrons to peroxidase, which converts hydrogen peroxide into water (Sgherri et al., 2003). In addition, phenolic compounds, such as p-coumaric and ferulic acids, are directly involved in the polymerization of monolignols into lignin using H_2O_2 , which increases ROS elimination and metal sequestration in the cell wall (Barros et al., 2015).

Under zinc treatment, TPC and TFC were moderately and positively correlated with DPPH radical scavenging activity ($r = 0.60$ and $r = 0.75$, respectively). LC-MS/MS was used to identify the phenolic

compound composition of *R. glutinosa* treated with Zn and Co. Apigenin, p-coumaric acid, and luteolin are the most abundant phenolic compounds in *R. glutinosa*. The levels of apigenin, luteolin, and p-coumaric acid were positively correlated with DPPH scavenging activity ($r = 0.89$, $r = 0.90$, and $r = 0.80$, respectively), suggesting that these compounds could play a prominent role in the antioxidant activity of *R. glutinosa* during zinc stress. Moreover, these compounds have been reported to exhibit antioxidant activity and chelate transition metals (Adhikari et al., 2018). Increased antioxidant activity of apigenin has been reported in *Teuricum polium* (Begum et al., 2012), *Chrysanthemum morifolium* (Hu et al., 1994), parsley seeds (Pápay and Antal, 2014), *Alchornea coelophylla* Pax & K.Hoffm (Martínez et al., 2016). Apigenin scavenges ROS and prevents oxidative damage via various pathways, including MAPK-mediated pathways, such as p38, JNK, and ERK1/2, and redox signaling pathways, such as NF- κ B and Nrf-2 (Perez-Leal et al., 2017). The 4-hydroxyl group in apigenin donates its hydrogen atom and electrons to ROS (hydroxyl, peroxy, and peroxyl) to stabilize them (Begum et al., 2012). Additionally, phenolic compounds in *R. glutinosa* may inhibit the effects of zinc and cobalt stress through chelation reactions. The association between metal ion and flavonoid complexes and the oxygen functional groups present in flavonoid compounds has been documented (Bukhari et al., 2009). This finding implies that elevated concentrations of phenolic compounds in *R. glutinosa* might facilitate intense chelation of zinc and cobalt within its cell wall. As a result, higher levels of phenolic compounds in *R. glutinosa* inhibit ROS formation and are essential for the development of heavy metal stress tolerance. ROS produced during heavy metal stress disintegrates cellular structures. Toxic ions degenerate lipid membranes by releasing alkoxy radicals that oxidize lipid membranes and degenerate the ultrastructure of cells. Caffeic acid mitigates metal stress by inhibiting lipid peroxidation and damages cellular structures by scavenging alkoxy radicals (Michalak, 2006). A greater level of caffeic acid increases the synthesis of lignin in cell walls, thus enhancing resistance to heavy metal injury, according to a previous study (Diaz et al., 2001). Elavarthi and Martin (2010) reported that caffeic acid inhibits the function of oxidizing enzymes by forming complexes with deleterious ions that catalyze oxygenation reactions.

Molecular dynamics simulations

Molecular dynamics (MD) simulations are useful computational techniques with which the stability of a protein-ligand complex can be studied within a specified time. When plants face elevated levels of heavy metal stress, various components such as metabolites, receptors, hormones, enzymes, and signaling molecules engage in intricate interactions within the plant system. This response to heavy metal toxicity, as outlined by Tong et al. (2020), operates across multiple scales. Molecular dynamics simulations are instrumental in delving into the inner workings of plants, allowing for an in-depth study of their resistance mechanisms against heavy metal ions. This simulation explored the dynamics at the levels of plant chelates, proteins, and genes (Wu et al. 2023).

In a study by Pochodylo and Aristilde (2017), MD simulation results indicated that the phytochelatin complex with Cu^{2+} exhibited the highest stability. The analysis involved calculating the distances and coordination numbers of the metal cations to specific atom pairs within the phytochelatin molecule,

revealing that Cu^{2+} coordinated with five oxygen atoms, all of which belonged to the carboxylate fraction. In a subsequent study, Zothantluanga et al. (2023) demonstrated that the stability of protein-ligand complexes can be assessed by examining various parameters derived from 100ns MD simulations. These parameters included protein RMSD and ligand RMSD within the protein-ligand complex, protein RMSF and ligand RMSF within the protein-ligand complex, protein-ligand contacts throughout the entire simulation, and ligand properties, such as RMSD, rGyr, intramolecular hydrogen bonds, MolSA, SASA, and PSA.

The roles of Zn and Co in nutrient uptake are poorly understood. Transcriptomic investigations and molecular mechanisms involved in growth, biochemical parameters, and antioxidant parameters under Zn and Co stress are needed in future studies. Moreover, the presence of excess sulfate and chloride added in the form of zinc sulfate or cobalt chloride, respectively, was not assessed with respect to the control treatment. Finally, more investigations are needed to elucidate the physiological changes and alterations in gene expression in *R. glutinosa* exposed to zinc alone or in combination with cobalt at various concentrations.

Conclusions and future perspectives

Contaminations of soil from Zn and Co is increasing due to higher demands in the modern society. Our findings demonstrate that Zn and Co stress had a greater influence on plant growth, resulting in physiological and biochemical changes in *R. glutinosa*. The application of lower concentrations of Zn was more beneficial for the growth of *R. glutinosa*, enhancing its antioxidant properties, antioxidant compound concentrations, and medicinal value. Our results illustrated that low doses of Zn (0.01–0.1 ppm) enhanced the concentration of phenolic compounds, which could be related to the increased antioxidant activities by scavenging ROS and elevating photosynthetic pigments such as Chl a and Chl b. Furthermore, low doses of Zn significantly increased CAT and APX enzyme activities in *R. glutinosa*. Higher concentrations of zinc (greater than 0.5 ppm) are detrimental to the growth and accumulation of antioxidant compounds. Lower concentrations of cobalt (0.01–0.1 ppm) negatively affected seedling growth and inhibited phenolic compound production. Higher than 0.1 ppm Co in the growth medium resulted in the death of *R. glutinosa* seedlings. Increased levels of phenolic compounds, such as apigenin, p-coumaric acid, and luteolin in zinc- and cobalt-treated *R. glutinosa* suggest that these phytochemicals are involved in the elimination of ROS during the growth of *R. glutinosa* under heavy metal stress. These results may be valuable for the growth, selection, and breeding of *R. glutinosa* plants. However, additional research is necessary to assess the specific relationships between metal stress and antioxidant enzymes and compounds, and the mechanisms involved. A comprehensive exploration of the phytochemicals present in *R. glutinosa* extracts was performed, focusing on those that demonstrated robust binding affinities and impressive docking scores. A meticulous 100-ns molecular dynamic simulation was performed to further elucidate their potential therapeutic significance. This simulation aimed to probe the stability of the identified phytochemicals, offering valuable insights into their behavior under dynamic conditions.

Declarations

Conflicts of Interest:

The authors declare no conflict of interest.

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Author Contributions:

Bimal Kumar Ghimire and Seo Jiwon contributed by doing the experiment and writing the manuscript; Chang Yeon Yu supervised the experiment; Prabhu Srinivasan contributed molecular dynamics study; Naiyf S. Alharbi, Shine Kadaikunnan and Seung Hyun Kim contributed by analyzing data and editing the revised manuscript.

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Data will be made available on request.

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Figures

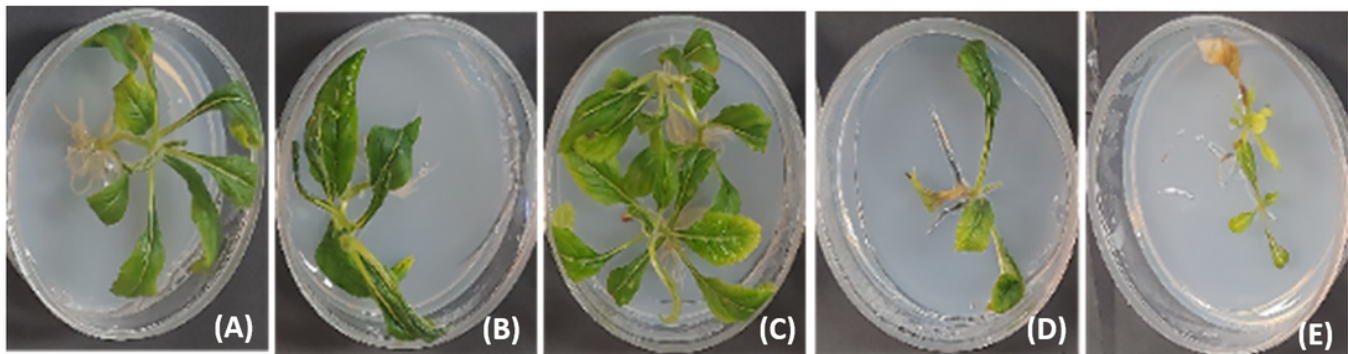


Figure 1

Effect of zinc on growth of *R. glutinosa*. (A) Formation of leaves, shoots, and roots in the control plants. (B) Induction of leaves, shoots, and roots treated with 0.01 ppm of ZnSO_4 , (C) 0.1 ppm of ZnSO_4 , (D) 0.5 ppm of ZnSO_4 , and (E) 1 ppm of ZnSO_4 for six weeks.

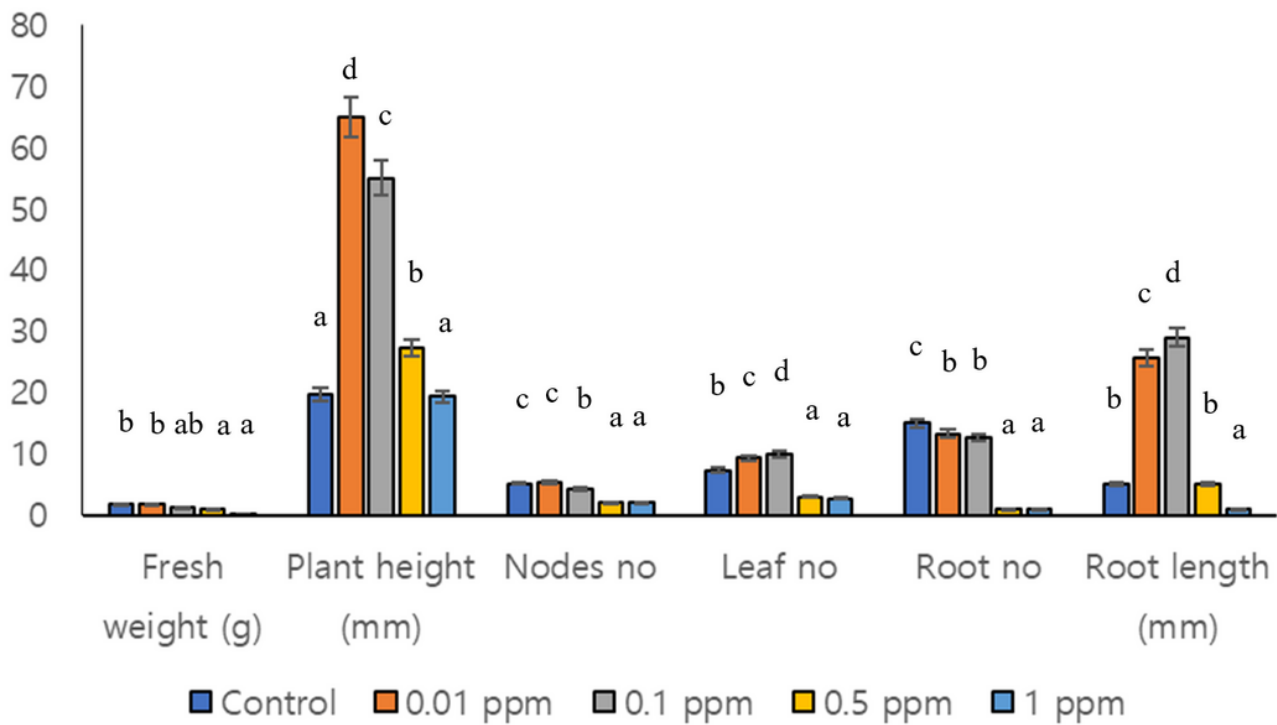


Figure 2

Fig. 2. Effect of zinc on fresh weight, plant height, root length, leaf, node, and root numbers in *R. glutinosa*.

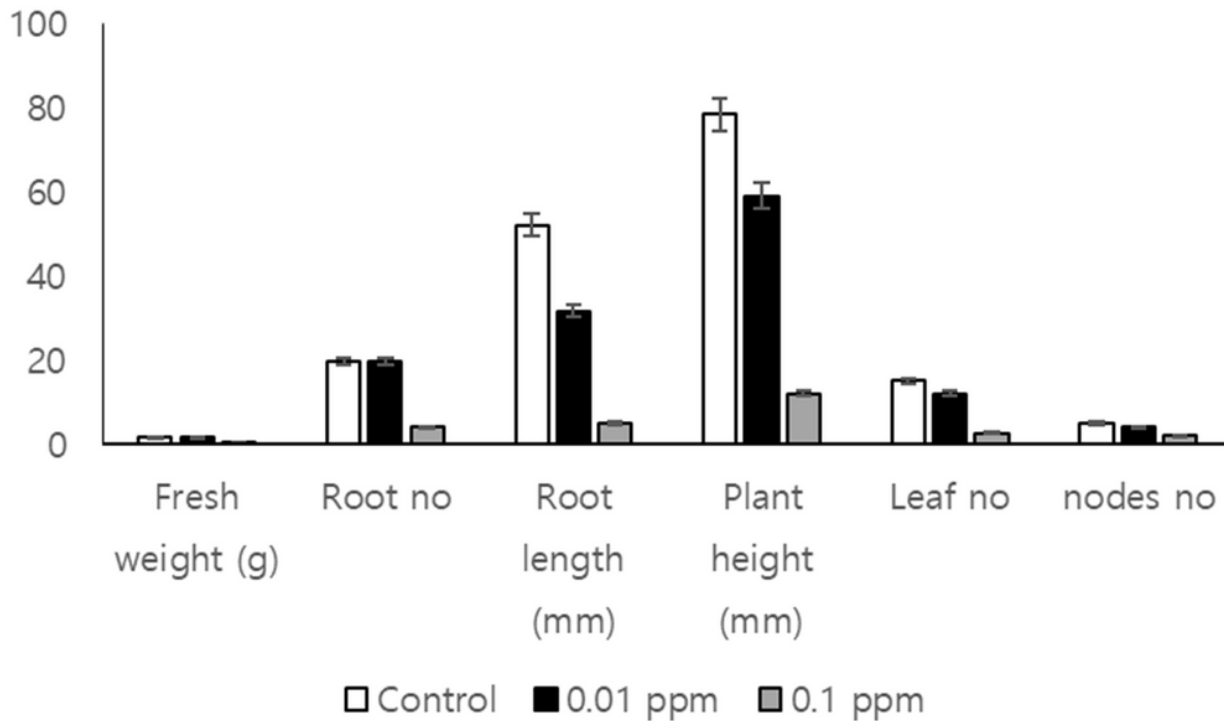


Figure 3

Effect of cobalt on fresh weight, plant height, root length, leaf, node, and root numbers in *R. glutinosa*.

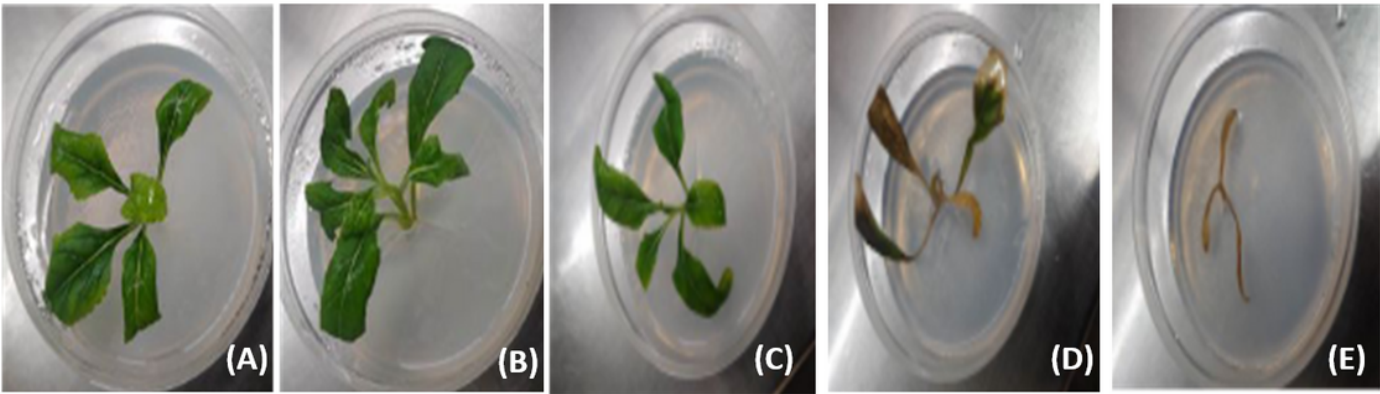


Figure 4

Effect of cobalt on growth of *R. glutinosa*. (A) Formation of leaves, shoots, and roots in the control plants. (B) Induction of leaves, shoots, and roots treated with 0.01 ppm of CoSO_4 , (C) 0.1 ppm of CoSO_4 , (D) 0.5 ppm of CoSO_4 , and (E) 1 ppm of CoSO_4 for six weeks.

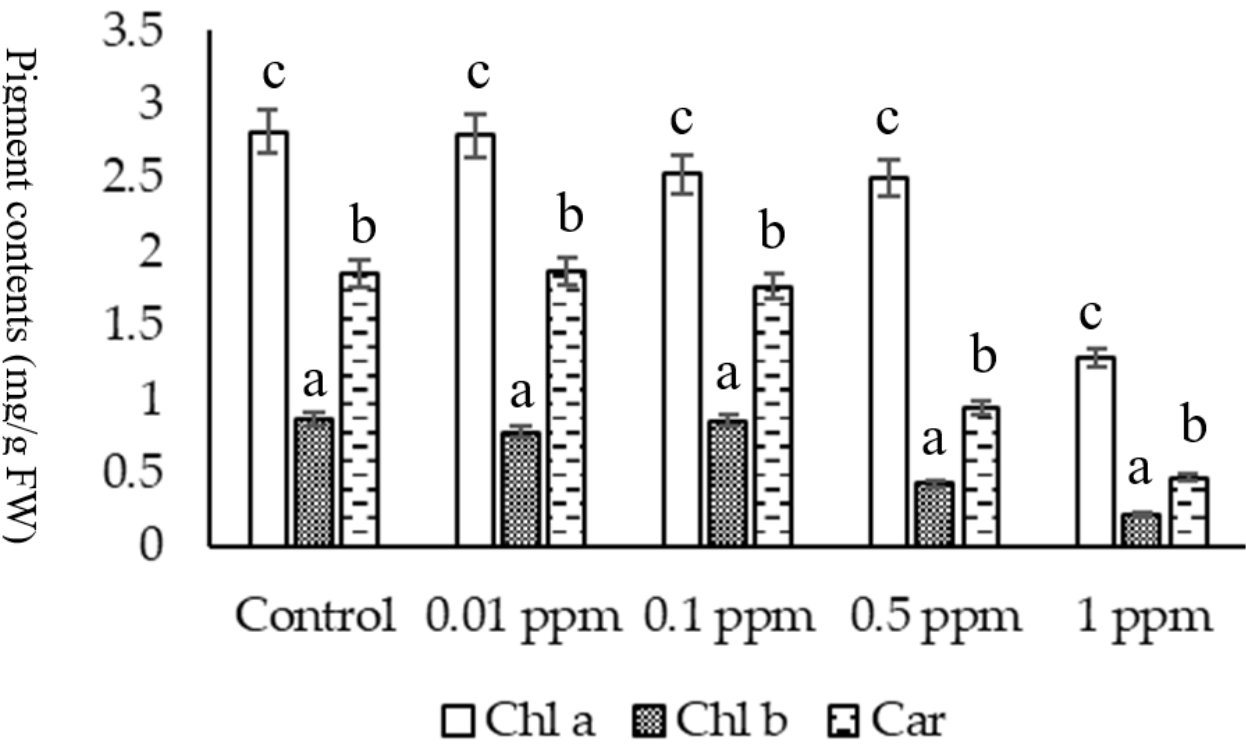


Figure 5

Effect of zinc on the Chl a, Chl b, and carotenoid concentrations in *R. glutinosa*. Means with different letters indicate significant differences at $P<0.05$.

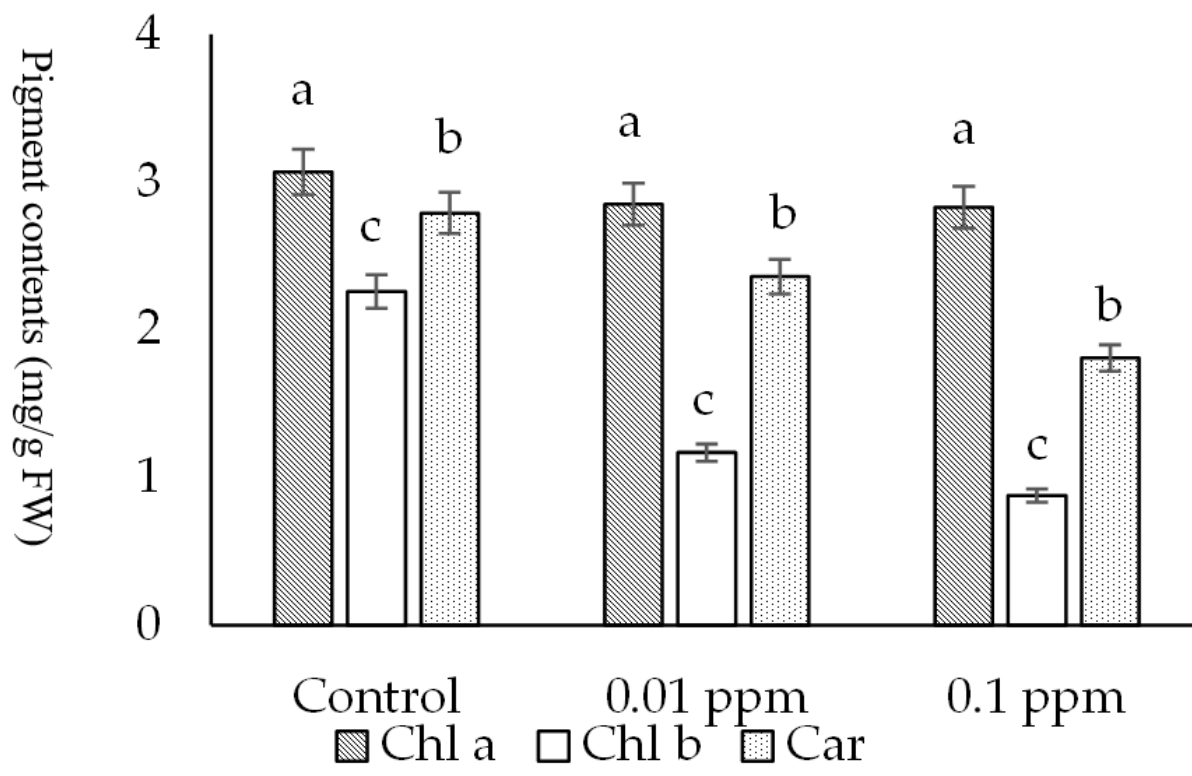
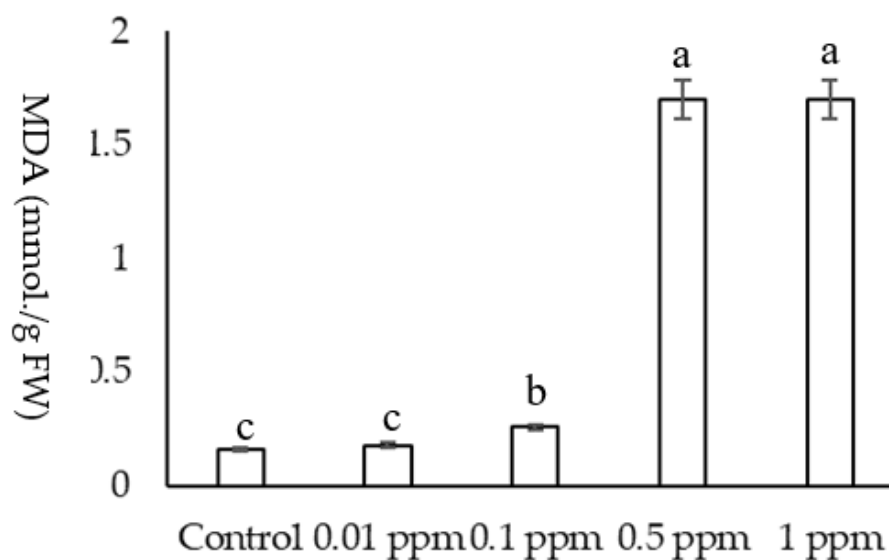


Figure 6

Effect of cobalt on the Chl a, Chl b, and carotenoid concentrations in *R. glutinosa*. Means with different letters show significant differences at $P<0.05$.

(A)



(B)

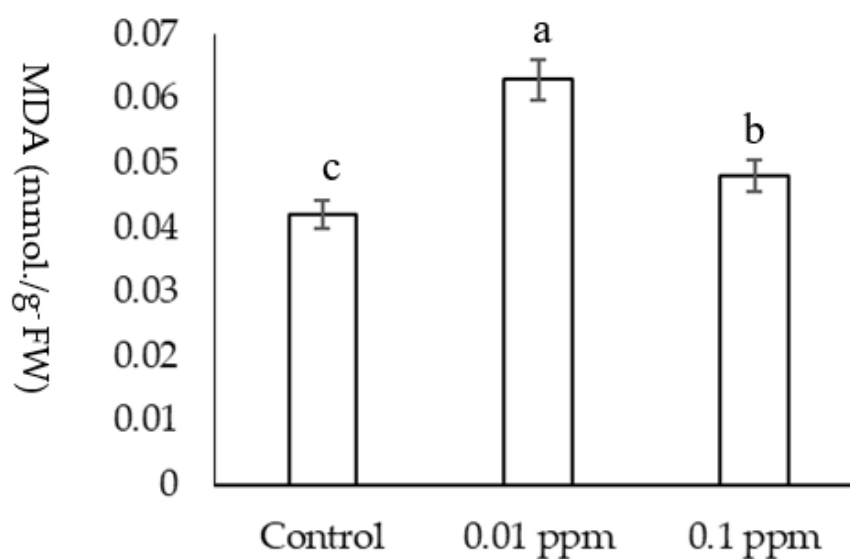
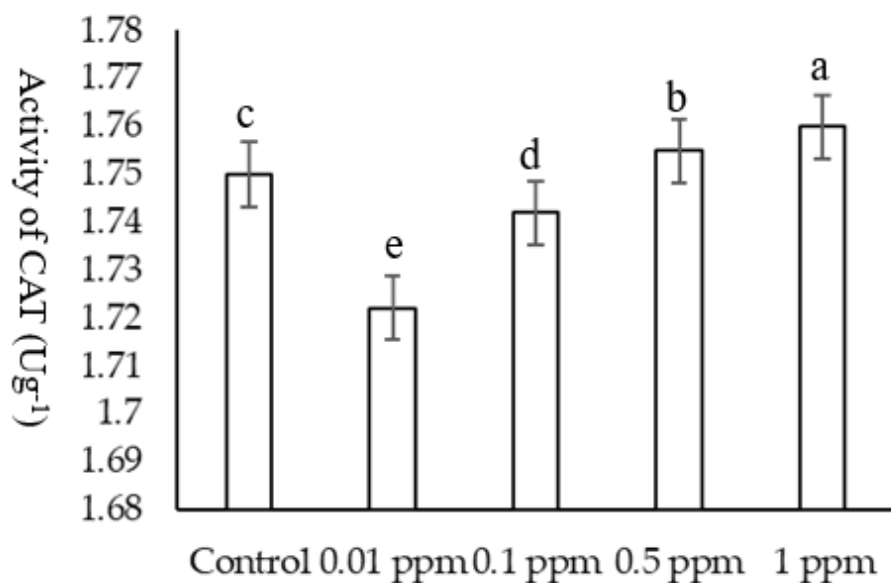


Figure 7

Effect of **(A)** zinc and **(B)** cobalt on the malondialdehyde (MDA) concentration of *R. glutinosa*. Data are presented as the mean $\pm$ standard deviation ($n=3$). Means with different letters show significant differences at $P<0.05$.

(A)



(B)

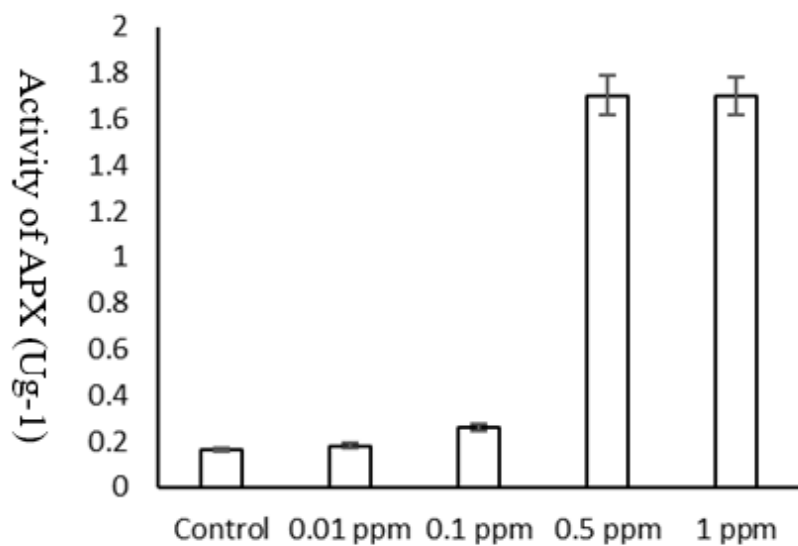


Figure 8

Effect of zinc **(A)** CAT and **(B)** APX activity of *R. glutinosa*. Data are presented as the mean $\pm$ standard deviation (n=3). Means with different letters show significant differences at $P < 0.05$.

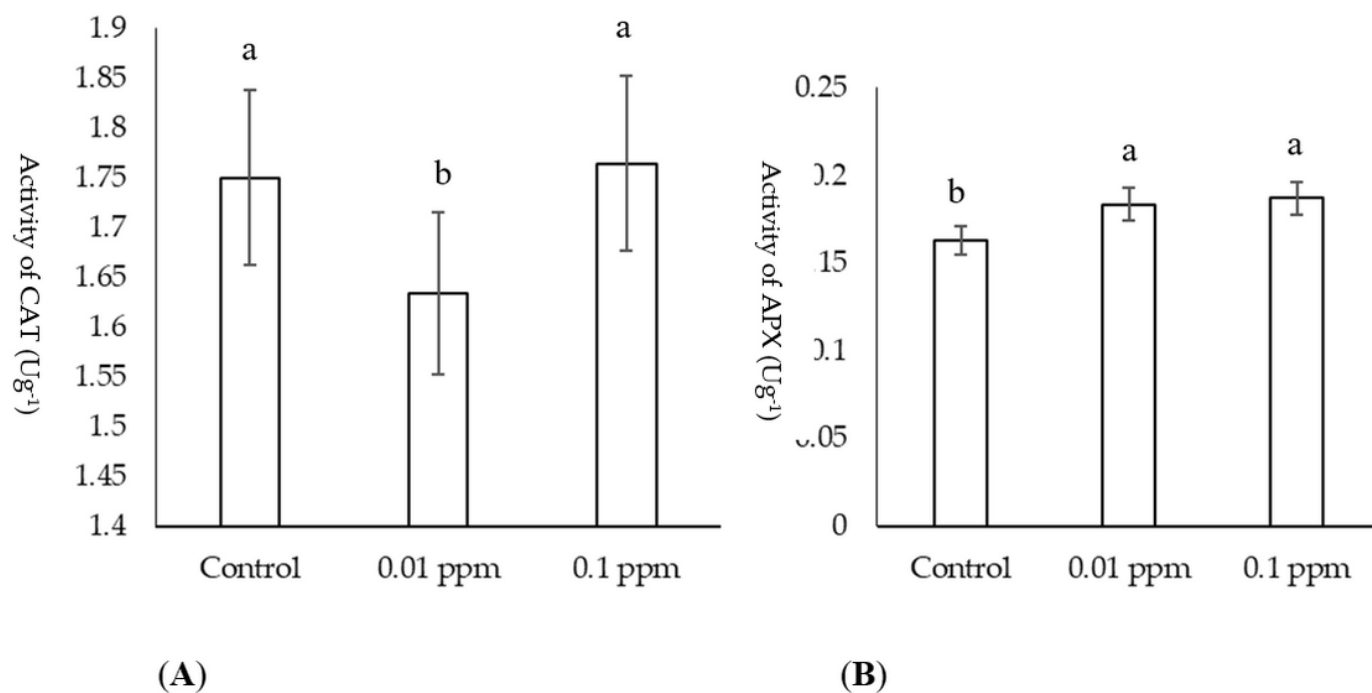


Figure 9

Effect of cobalt **(A)** CAT and **(B)** APX activity of *R. glutinosa*. Data are presented as the mean $\pm$ standard deviation ($n=3$). Means with different letters show significant differences at $P<0.05$.

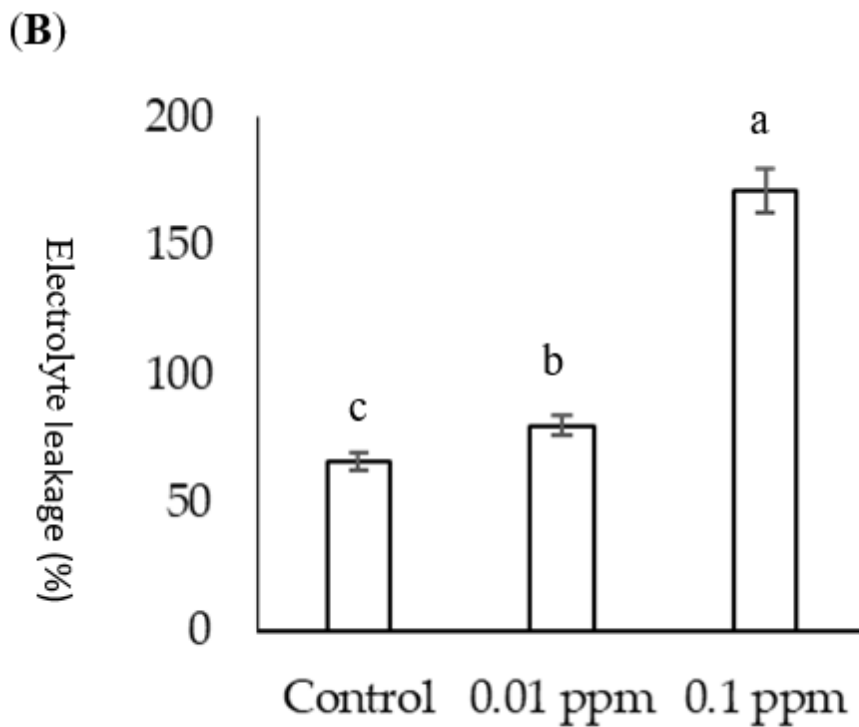
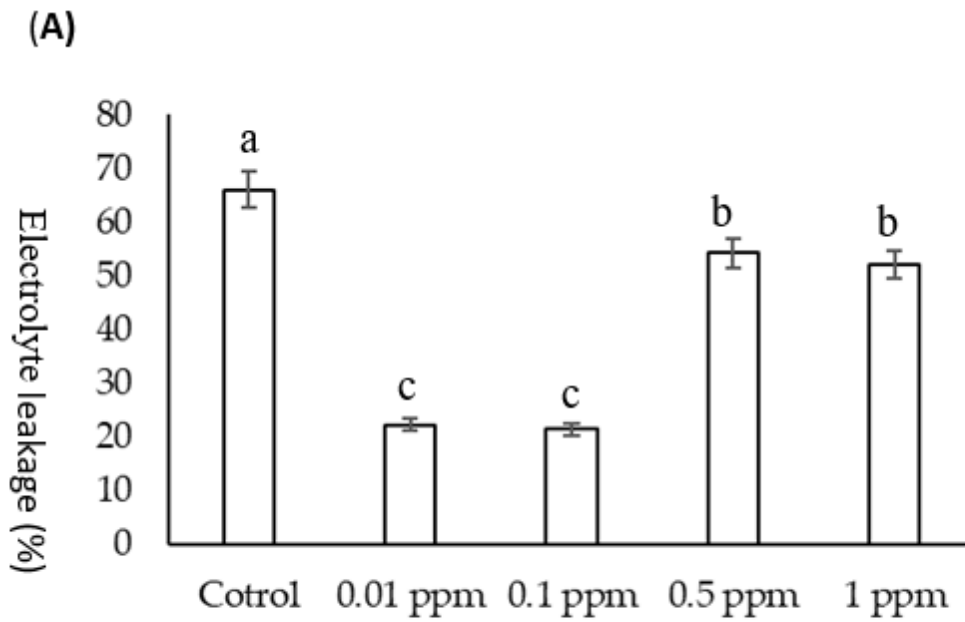


Figure 10

Effect of (A) zinc, (B) cobalt on electrolyte leakage of *R. glutinosa*. Data are represented as means $\pm$ standard deviation ($n=3$). Means with different letters show significant differences at $P<0.05$.

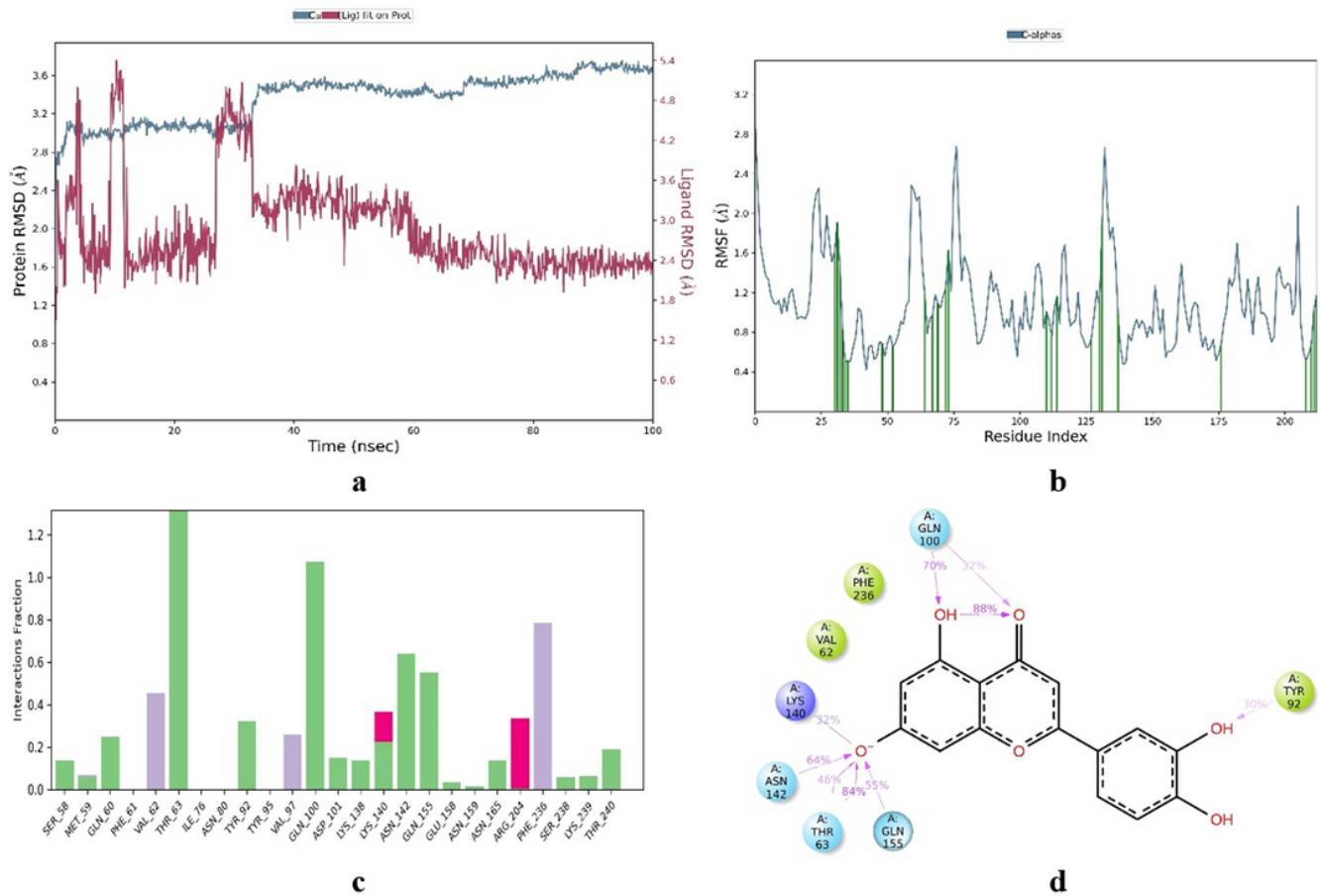


Figure 11

Molecular dynamics simulation of the complex luteolin and phytochelatin synthase: a. Root Mean Square Deviation (RMSD), b. Root Mean Square Fluctuation (RMSF), c). Protein-Ligand Contacts, d). Interaction plot with binding affinity percentage.

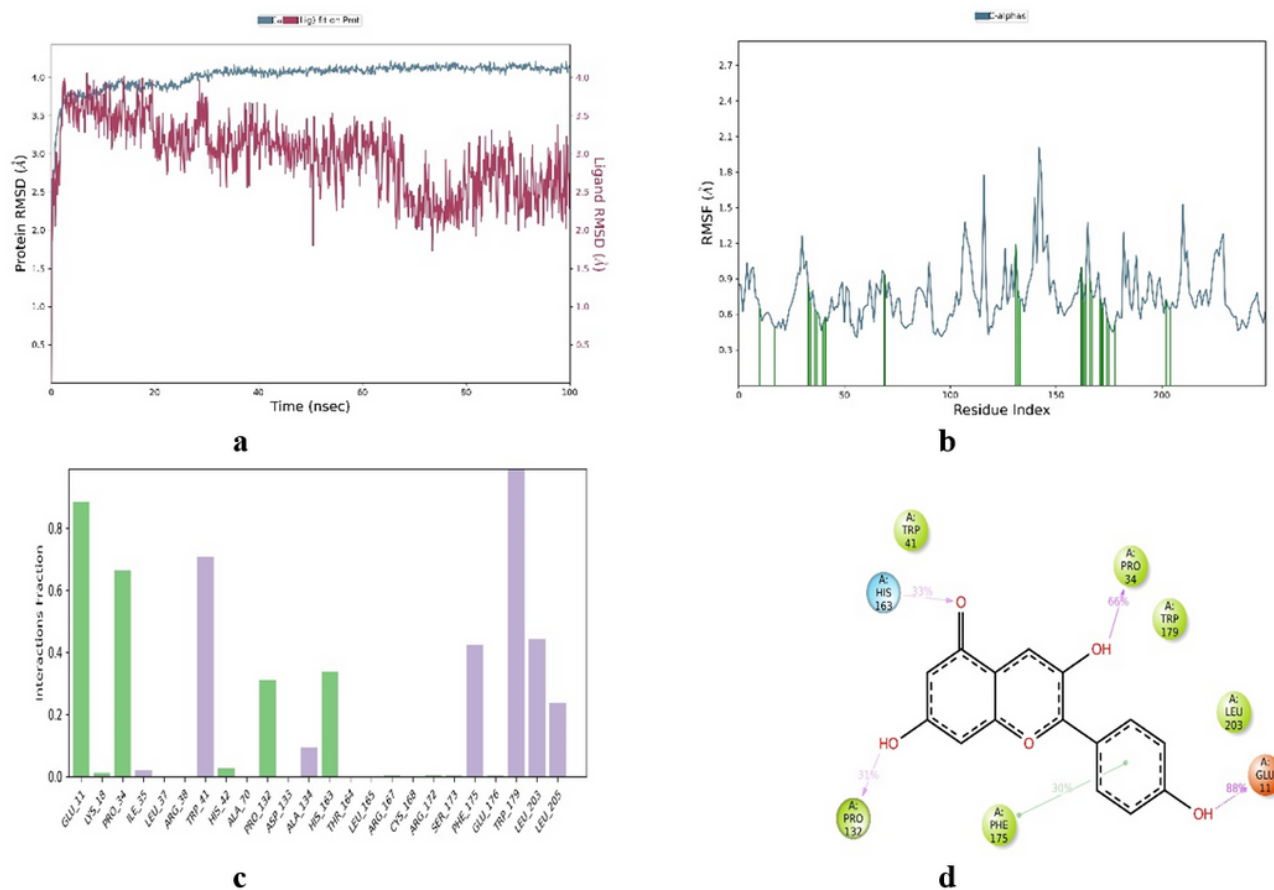


Figure 12

Molecular dynamics simulation of the complex Pelargonidin chloride and Ascorbate: a. Root Mean Square Deviation (RMSD), b. Root Mean Square Fluctuation (RMSF), c). Protein-Ligand Contacts, d). Interaction plot with binding affinity percentage.

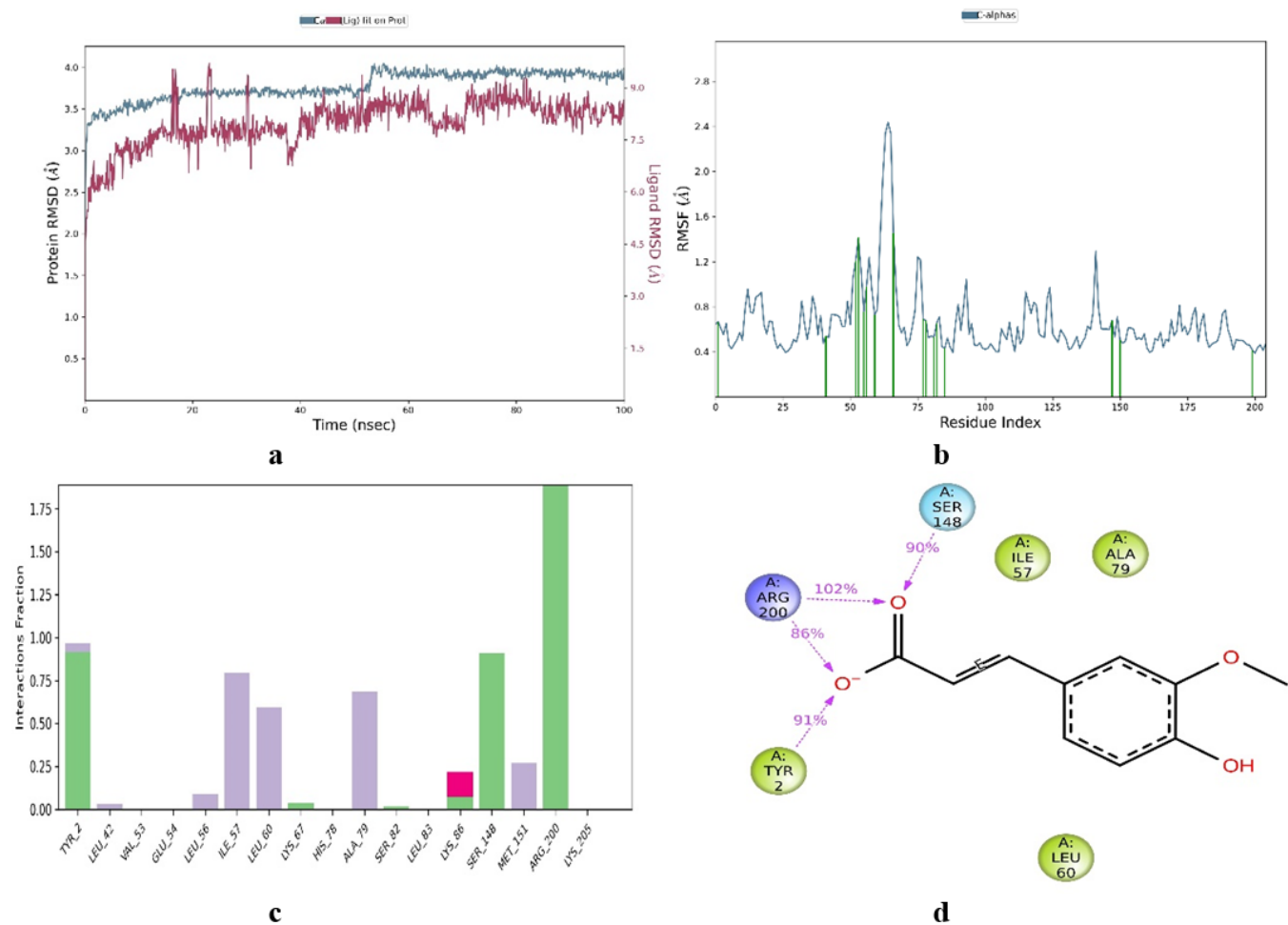


Figure 13

Molecular dynamics simulation of the complex Ferulic acid and superoxide dismutase: a. Root Mean Square Deviation (RMSD), b. Root Mean Square Fluctuation (RMSF), c). Protein-Ligand Contacts, d). Interaction plot with binding affinity percentage.