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Salvia officinalis L. resilience under chromium stress: An integrated study of growth, physiology, biochemical changes and rosmarinic acid production

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Abstract

Medicinal plants are increasingly challenged by rising chromium (Cr) levels in agricultural soil and water bodies due to industrialization and human activities. This research examines the impact of various chromium concentrations on *Salvia officinalis* L., a medicinal herb, over 3 specific time periods: 30, 60 and 90 days. As the duration of Cr exposure increases, various growth parameters showed an upward trend at the lowest concentrations, with the most robust growth observed in the 20 ppm Cr treatment group after 90 days. However, higher chromium concentrations resulted in reduced plant growth compared to untreated plants. Chromium primarily accumulates in the roots, stems and leaves, with the highest accumulation observed at 100 ppm. However, chlorophyll content declined with prolonged Cr exposure, particularly at higher concentrations. Carbohydrate levels initially increased at lower Cr concentrations but decreased with greater exposure, while protein content consistently decreased with elevated Cr levels. Proline levels exhibited mixed responses, rising at lower concentrations and declining at higher ones. Malondialdehyde (MDA) content increased with higher Cr levels and extended exposure. The enzymatic antioxidant system showed an initial increase followed by a decline with prolonged exposure. Rosmarinic acid content increased with chromium (Cr) exposure upto 60 ppm but subsequently decreased beyond that threshold. In the first 30 days, plants treated with Cr demonstrated a 17 % increase in rosmarinic acid production compared to the control (48.9 mg/g DW). However, with continued Cr exposure, there was a decline in rosmarinic acid production ranging from 10 % to 20 % compared to the control level (67.02 mg/g DW) at 90 days post-treatment. These findings underscore the complex and contrasting responses of *Salvia officinalis* to Cr toxicity, highlighting the necessity for extended study into the core mechanisms governing these responses and the development of strategies to alleviate heavy metal stress in plants.

Keywords

Antioxidant enzymes; chromium toxicity; common sage; lipid peroxidation; mitigation strategies; rosmarinic acid

Introduction

As immobile organisms, plants are continuously exposed to various stresses in this challenging environment. Industrialization and climate change, which intrude on agricultural land, increase plant susceptible to abiotic

stresses such as drought, extreme temperatures, heavy metal toxicity, light intensity, salinity and UV radiation. Heavy metal poisoning is one of the primary abiotic stresses on plants, influenced by the physiochemical characteristics of these metals (1). Catastrophic heavy metal pollution is a critical concern due to persistent consumer-driven growth and increasing activity in numerous global industries (2). In India, nearly 718 districts have groundwater contaminated with elements like arsenic, cadmium, chromium and lead (3). According to a research, the average soil values for Zn, Cd, Cu, As, Ni and Cr exceeds what is considered safe for the natural soil background in India (4).

Chromium is a pervasive metal that can contaminate soil, groundwater and surface water, posing risk to the health of people, animals and plants. Chromium primarily exists in 3 oxidation states: Cr^{2+}, Cr^{3+} and Cr^{6+}. Among these, the hexavalent form (Cr^{6+}) is recognized as the most hazardous due to its ability to effortlessly traverse a biomembrane (3). Chemical, metallurgical, mineral, textile dyeing, leather tanning, cement production, electroplating, steel and other industrial operations are the main contributors of chromium to the environment (5). Research indicates that chromium is a hazardous element with detrimental effects on plant growth and metabolism, leading to reduced yield quality (6, 7).

Consuming medicinal herbs can lead to the accumulation of heavy metals in human tissues, resulting in harmful health effects (8). The accumulation of chromium in medicinal plants such as *Ricinus communis, Amaranthus spinosus*, *Coccinia grantis* (9), *Bacopa monierri* and *Withania somnifera* (10) has already been reported. Cultivating medicinal plants in environments contaminated with heavy metals can significantly impact the production of secondary metabolites, causing changes in both the quantity and purity of these molecules (11). According to previous studies, medicinal plants may produce more secondary metabolites when exposed to heavy metals at certain threshold concentrations. However, excessive exposure can be dangerous (12).

Salvia officinalis L., commonly known as common sage, is an aromatic and medicinal herb widely cultivated for its pungent, edible leaves. For thousands of years, traditional and folk medicine have utilized the leaves of *S. officinalis* in various forms, including tea, hydroalcoholic tincture and as a food condiment, to treat a wide range of illnesses (13). According to literature, the plant exhibits antibacterial, anticancer, anti-inflammatory, antioxidant, antinociceptive, hypoglycemic, hypolipidemic and memory-enhancing properties (14). Additionally, due to its well-known antioxidant activity, sage is extensively used in the culinary, cosmetic and perfume industries (14). The plant's secondary metabolites, such as phenolics, terpenoids, polyphenols and flavonoids are the principal bioactive components that significantly contribute to the antioxidant and other medicinal qualities of sage (13).

Rosmarinic acid (RA), an important phenolic acid found in *S. officinalis,* is a bioactive compound synthesized from the amino acids L-phenylalanine and L-tyrosine through a series of enzymatic reactions. Clinical research on RA has shown promise in mitigating allergic diseases, protecting against neurotoxicity and slowing the progression of Alzheimer's disease. RA and its derivatives have garnered attention for their various health benefits, including anti-microbial, antioxidant, anti-angiogenic, anti-tumor and anti-inflammatory properties (15).

To ensure the integrity and safety of herbs like sage, which are widely used in traditional medicines and cooking, it is crucial to investigate how chromium exposure affects the synthesis of secondary metabolites such as RA. Understanding these impacts is vital for evaluating the vegetative growth, biochemical composition and antioxidant properties of sage. Consequently, a hypothesis has been formulated to explore the potential effects of various chromium concentrations on growth patterns, physiological and biochemical parameters and the production of secondary metabolite, with a particular focus on the synthesis of rosmarinic acid in *S. officinalis.*

Materials and Methods

Treatment of soil with heavy metals

Soil for the experiment was collected from CHRIST (Deemed to be University), Bangalore. The soil mixture was prepared by blending soil, sand and cocopeat in a 2:1:1 ratio and subjected to moist heat sterilization using an autoclave. This soil mixture was then treated with 5 different concentrations of chromium sulfate solutions (20, 40, 60, 80 and 100 ppm). Two-months-old stem cuttings of the local variety of *S. officinalis*, sourced from the University of Agricultural Sciences, GKVK, Bangalore, were planted in pots with a capacity of 10 kg of the soil mixture. Each concentration had 3 replications. The plants were regularly watered to maintain soil moisture at 50 % of their holding capacity. Additionally, every 14 days, the plants were irrigated with the respective concentration of chromium sulfate solution. A control group of plants was grown in pots without exposed to heavy metals. The treated plants were harvested at 3 different time points: 30 days, 60 days and 90 days after treatment, for subsequent analysis of their vegetative growth and biochemical parameters.

Assessment of vegetative growth attributes

The number of leaves, shoot length, root length, fresh weight (FW) and dry weight (DW) of the plants were assessed at 30 days, 60 days and 90 days after chromium treatment.

Analysis of biochemical parameters

Determination of total chlorophyll content

The total chlorophyll content was determined using the Arnon technique (16). In this method, 0.1 g of leaf samples were homogenised with 10 mL of 80 % acetone. The absorbance of the resulting solution was measured against the solvent (acetone) blank at 645 and 663 nm using a UV/Vis spectrophotometer (Shimadzu UV-1900, Kyoto, Japan).

Estimation of total protein and carbohydrate content

The protein measurement was conducted using a previously described method (17). In this method, 0.1 g of the sample was mixed with 5 mL of 0.2 M phosphate buffer (pH 7) and centrifuged at 10000 rpm for 10 min. A 10 µL aliquot of the supernatant was combined with 500 µL of alkaline-copper sulfate solution and incubated for 10 min at room temperature. Following this, 50 µL of Folin-Ciocalteu reagent was added, mixed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 660 nm using a microplate reader (BIO-RAD, iMARKTM, Japan). Total carbohydrate content was estimated using the phenol-sulfuric acid method (18). In this procedure, 0.1 g of sage leaves was blended with 5 mL of 2.5 N HCl and heated in a boiling water bath for 3 h. The resulted crude homogenate was cooled and neutralized before being centrifuged at 1000 rpm for 10 min. To 1 mL of the supernatant, 1 mL of 5 % phenol and 5 mL sulfuric acid were added. The absorbance at 490 nm was measured using the microplate reader (BIO-RAD, iMARKTM, Japan).

Analysis of proline content

Proline content was quantified using a previously published method (19). Sage leaves (0.1 g) were homogenized in 5 mL of 3 % aqueous sulfosalicylic acid. The resulting supernatant was obtained after centrifugation at 10000 rpm for 10 min. For proline content estimation, 1 mL of the supernatant was mixed with 2 mL of 1.25 % ninhydrin in glacial acetic acid and boiled at 100 °C for 30 min. After cooling the mixture in an ice bath, the absorbance was measured at 508 nm. A standard curve was prepared using proline concentrations ranging from 0 to 10 µg/mL to determine the proline content in the samples.

Evaluation of lipid peroxidation

Lipid peroxidation in fresh leaves was evaluated by measuring malondialdehyde (MDA) producing using the thiobarbituric acid (TBA) reaction method (20). Fresh leaves (0.5 g) were extracted with 5 mL of 1 % TCA and then centrifuged at 10000 rpm for 5 min. The resulting supernatant (1 mL) was mixed with 0.5 % TBA in 20 % TCA, followed by heating at 95 °C for 30 min. After rapid cooling in an ice bath and subsequent centrifugation at 10000 rpm for 10 min, the absorbance of the supernatant was recorded at 532 nm and 600 nm. The MDA concentration was calculated using the following equation:

$$\text{MDA in mM} = (A_{532}\text{-}A_{600})/155$$

where A_{532} is the absorbance at 532 nm, A_{600} is the absorbance at 600 nm and 155 is the molar extinction coefficient of MDA.

Assay of antioxidant enzymes – extraction of sample

Sage leaf samples (500 mg) were homogenized with 5 mL of pre-cooled phosphate buffer solution (pH 7.8) and centrifuged at 10000–15000 rpm for 20 min at 4 °C. The resulting supernatant was used to analyze the activities of antioxidant enzymes, including Superoxide dismutase (SOD), Ascorbate peroxidase (APX) and Catalase (21).

Ascorbate peroxidase (APX) (EC1.11.1.11)

The activity of ascorbate peroxidase (APX) was measured by assessing the oxidation of ascorbate at 290 nm in a 2 mL reaction mixture. This mixture comprised 1.2 mL of 50 mM phosphate buffer, 200 µL of 0.1 mM EDTA, 200 µL of 0.1 mM H_2O_2, 200 µL of 2 mM ascorbate and 200 µL of the homogenate. The APX activity was quantified using an extinction coefficient of 2.8 mM/cm and expressed as µ moles of APX/min/mg of protein (22).

Catalase (CAT) (EC 1.11.1.6)

The activity of catalase (CAT) was determined by monitoring the decomposition of H_2O_2 at 240 nm in a 3 mL reaction mixture containing 2.85 mL of phosphate buffer (50 mM), 100 µL H_2O_2 (50 mM) and 50 µL homogenate. The activity was quantified as µ moles of catalase/min/mg of protein (23).

Superoxide dismutase assay (SOD) (EC 1.15.1.1)

The superoxide dismutase activity was assessed by the following method with minor modifications (24). A reaction mixture comprising 100 µL of crude enzyme extract, 0.1 mM EDTA, 13 mM methionine, 75 mM NBT, 50 mM potassium phosphate buffer (pH 7.8) and 2 mM riboflavin was prepared and made up to a total volume of 3 mL with distilled water. The mixture was then exposed to light for 20-30 min. The reaction mixture without extract and a blank, without light exposure served as positive control and blank respectively. The photoreduction of NBT was evaluated by measuring the absorbance of the blue-colored formazan at 560 nm. One unit of SOD was defined as the amount of enzyme necessary to inhibit a 50 % reduction in NBT. The results were expressed as units of SOD/mg of protein.

Quantification of Cr accumulation

From each treatment, 1 g of dried and pulverized sage root, stem and leaves were extracted using a wet digestion method in a solution containing 3:1 ratio of HNO_3: $HClO_4$. The resulting solution was then diluted to 25 mL using double distilled water and filtered through Whatman No.42 filter paper. The filtrate obtained was used to determine the Cr concentration using an Atomic Absorption Spectrophotometer (Shimadzu, AA-6880, Japan) (25).

Estimation of rosmarinic acid content using HPLC

For the analysis of rosmarinic acid, samples were prepared by the simple maceration technique, using 0.5 g of dried leaf powder and 10 mL of 80 % methanol with continuous shaking in a shaker for 10-12 h. The methanol-soluble extracts were then subjected to 3 consecutive filtrations, followed by low-temperature concentration and decreased pressure. Each extract was then diluted to a concentration of 10 mg/mL using methanol (26).

The standard used for the analysis was HPLC grade rosmarinic acid (96 % purity) purchased from Ottochemi Pvt Ltd, Mumbai, India. The standard was prepared in different concentrations ranging from 2-10 µg/mL. The analysis was performed using a Shimadzu scientific instrument LCMS-8040 system from Kyoto, Japan, equipped with a Sharpsil-U C-18 (250 × 4.6 mm) column and an SPD40 UV-vis detector, along with analytical software (Lab

Solutions), was utilized for RP-HPLC. The mobile phase consisted of 0.5 % acetic acid in water in pump A and methanol in pump B, with a flow rate of 1 mL/min. The column temperature was maintained at 40 °C. Isocratic elution was performed with monitoring absorbance at 270 nm and the analysis had a run time of 35 min (27).

Statistical analysis

The IBM SPSS Statistics software version 22.0 tool was used to conduct the statistical analysis in triplicate for every experiment replicated thrice. To verify the validity and variability of the findings, one way ANOVA was employed. The means of the control and Cr treated groups were compared using post hoc Duncan's multiple range test (DMRT) at $P \leq 0.05$ to look for any significant differences. The obtained data is presented as means ± SE after the post hoc test (DMRT) letter.

Results

Effect of Cr on vegetative growth

This study explored the adverse effects of chromium (Cr) on common sage by subjecting the plants to 5 different Cr concentrations (Fig. 1). Subsequently, an assessment of growth and biochemical parameters was conducted over 3 time periods: 30, 60 and 90 days (Table 1). Generally, lower concentrations (20 and 40 ppm) of Cr showed minimal effects on growth indices such as shoot and root lengths, the number of leaves, fresh weight (FW) and dry weight (DW), while higher concentrations (60, 80 and 100 ppm) had a negative impact on these parameters. At Cr concentrations of 20 ppm, the shoot length increased by 0.51 %, 10.48 % and 5 % at 30 days, 60 days and 90 days respectively. However, at high concentrations (100 ppm), the shoot length was reduced to 12 %, 20 % and 28 % at 30, 60 and 90 days respectively. Similarly, the root length exhibited a 14-36 % enhancement at the lowest concentration, while it decreased to 6-27 % at the highest concentration over the extension of duration. Likewise, a notable reduction in the number of leaves (5-26 %), fresh weight (5.6 – 27 %) and dry weight (0.5–23 %) was also observed in plants treated with high concentrations of Cr for 30-90 days.

Chromium accumulation

In sage plants, the highest accumulation of chromium (Cr) was observed in the roots, followed by the stems and leaves (Fig. 2). With an extension of the metal exposure period from 30 to 90 days, there was a consistent and substantial increase in Cr accumulation. Over a period of 90 days, Cr accumulation surged up to 188 times in the leaves, 452 times in the stem and 569 times in the roots compared to the control leaf, stem and root respectively. Among the various concentrations tested, the plants treated with 100 ppm of Cr displayed the highest accumulation, reaching levels of 338.85 µg/g in the roots, 211.77 µg/g in the stems and 9.725 µg/g in the leaves.

Effect on chlorophyll content

The total chlorophyll content exhibited a reduction in all chromium-treated sage plants compared to the control plants (Fig. 3). The decline in chlorophyll content was less pronounced in the initial 30 days following treatment. However, with prolonged exposure to Cr, the total chlorophyll content gradually decreased. In plants treated with 20 ppm, the chlorophyll content decreased to 8.5 %, 20 % and 26 % at 30, 60 and 90 days respectively. Similarly, a decline of 14 to 30 %, 17 to 35 % and 18 to 37 % in total chlorophyll content was noted in plants treated with 40, 60 and 80 ppm respectively, over 90 days. In plants treated

Fig. 1. Phenology of sage plants under Cr stress. Plants treated with different concentrations of chromium (**a-f**) and reduced root growth (**g**), leaf size (**h**) and yellowing of leaves (**i** & **j**) under chromium toxicity.

Table 1. Vegetative growth parameters of *Salvia officinalis* under Cr stress.

Days after treatment	Concentration of chromium (ppm)	Shoot length (cm)	Root length (cm)	No. of leaves	Fresh weight (g)	Dry weight (g)
30 days	0	51.93 ± 0.47^{f}	17.87 ± 0.24^{k}	70.33 ± 1.45^{j}	8.37 ± 0.27^{l}	3.13 ± 0.08^{j}
	20	52.20 ± 0.61^{f}	20.47 ± 0.29^{hi}	65.00 ± 1.53^{k}	8.47 ± 0.18^{kl}	3.25 ± 0.03^{j}
	40	51.33 ± 0.57^{f}	18.47 ± 0.26^{jk}	66.67 ± 1.86^{k}	9.16 ± 0.07^{jkl}	3.22 ± 0.02^{j}
	60	46.67 ± 0.81^{g}	18.07 ± 0.12^{k}	66.00 ± 1.53^{k}	9.32 ± 0.36^{jk}	3.17 ± 0.08^{j}
	80	45.33 ± 0.88^{g}	17.30 ± 0.21^{k}	67.33 ± 0.67^{jk}	9.61 ± 0.29^{j}	3.15 ± 0.07^{j}
	100	45.33 ± 0.83^{g}	16.63 ± 0.41^{l}	66.33 ± 1.2^{k}	9.78 ± 0.47^{j}	3.12 ± 0.03^{j}
60 days	0	59.67 ± 1.2^{d}	25.30 ± 0.57^{f}	122.00 ± 1.15^{c}	23.47 ± 0.32^{d}	5.27 ± 0.02^{ef}
	20	65.97 ± 0.5^{c}	34.43 ± 0.83^{a}	116.67 ± 0.67^{d}	26.89 ± 0.21^{c}	5.35 ± 0.06^{de}
	40	60.67 ± 0.88^{d}	27.83 ± 0.33^{d}	112.00 ± 1.15^{e}	23.37 ± 0.41^{d}	5.11 ± 0.02^{fg}
	60	56.57 ± 0.87^{e}	22.70 ± 0.4^{g}	104.00 ± 1.15^{g}	18.26 ± 0.16^{j}	4.98 ± 0.03^{g}
	80	51.37 ± 0.58^{f}	20.80 ± 0.53^{h}	97.33 ± 0.67^{h}	15.95 ± 0.23^{h}	4.79 ± 0.04^{h}
	100	47.73 ± 1.15^{g}	19.40 ± 0.32^{ij}	86.00 ± 1.15^{i}	13.99 ± 0.29^{i}	4.58 ± 0.03^{i}
90 days	0	72.50 ± 0.29^{b}	30.70 ± 0.36^{c}	130.33 ± 0.88^{a}	29.17 ± 0.48^{b}	6.18 ± 0.07^{b}
	20	76.67 ± 0.88^{a}	38.43 ± 0.3^{a}	126.67 ± 0.67^{b}	30.30 ± 0.24^{a}	6.65 ± 0.07^{a}
	40	71.17 ± 0.73^{b}	32.33 ± 0.18^{b}	122.00 ± 1.15^{c}	28.4 ± 0.21^{b}	6.59 ± 0.08^{a}
	60	60.37 ± 1.45^{d}	30.10 ± 0.21^{c}	114.33 ± 0.88^{de}	27.06 ± 0.32^{c}	5.86 ± 0.04^{c}
	80	56.33 ± 0.88^{e}	26.53 ± 0.24^{e}	107.67 ± 0.88^{f}	22.38 ± 0.23^{e}	5.47 ± 0.03^{d}
	100	51.60 ± 0.21^{f}	22.00 ± 0.58^{g}	96.00 ± 1.15^{h}	21.29 ± 0.33^{f}	4.76 ± 0.13^{h}

The provided data displays mean values with standard errors derived from 3 trials followed by the letters obtained from Duncan's multiple range test, which indicates that means with common letters do not exhibit statistically significant differences at significance level P≤0.05.

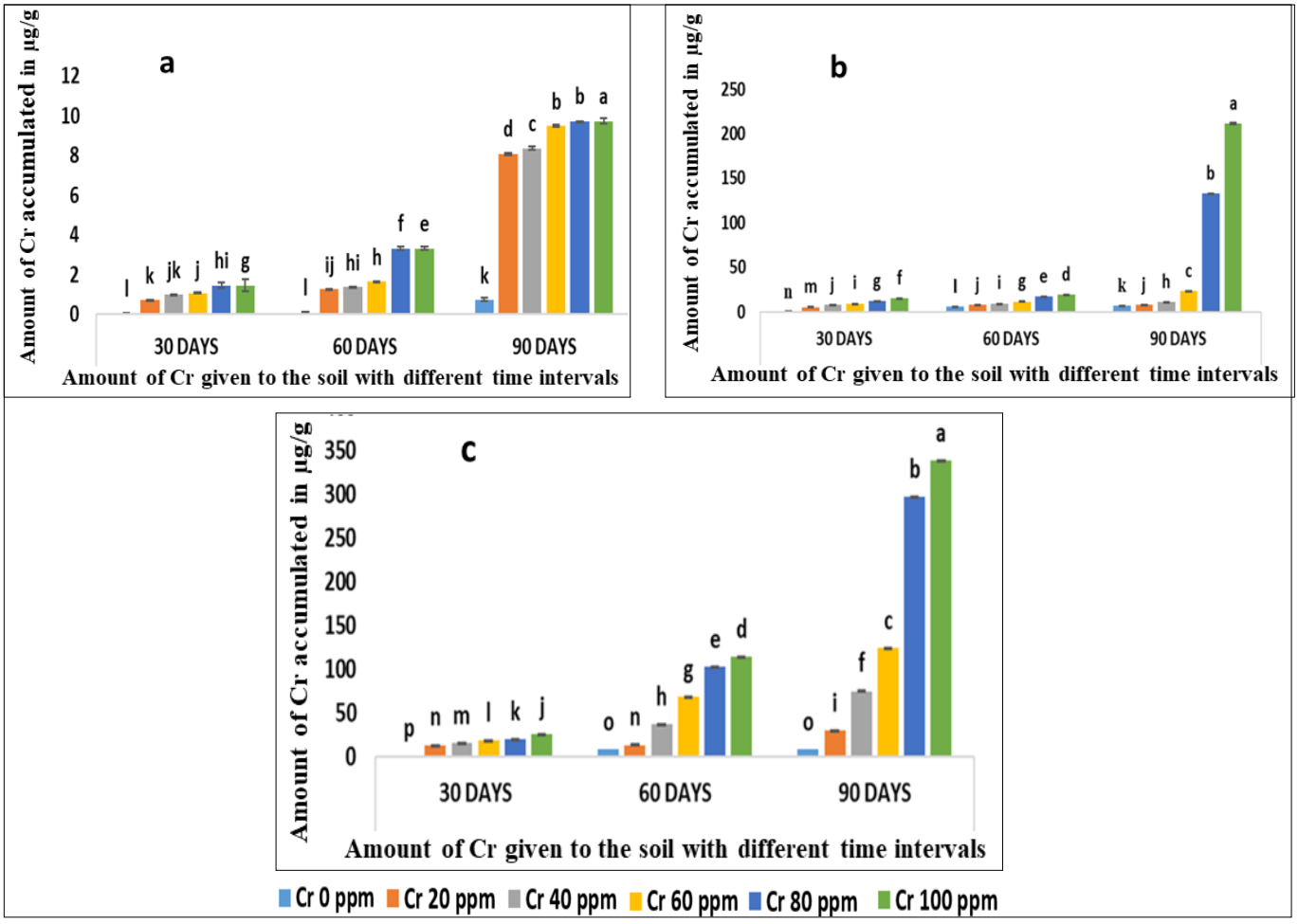

Fig. 2. Accumulation of chromium in different parts of the plant viz., leaf (**a**), stem (**b**) and roots (**c**) with different time intervals of chromium treatment on *S. officinalis*. The provided data displays mean values with standard errors derived from 3 trials followed by the letters obtained from Duncan's multiple range test, which indicates that means with common letters do not exhibit statistically significant differences at significance level P≤0.05.

with 100 ppm of Cr, a reduction of 24 %, 30 % and 41 % in chlorophyll content at 30, 60 and 90 days respectively

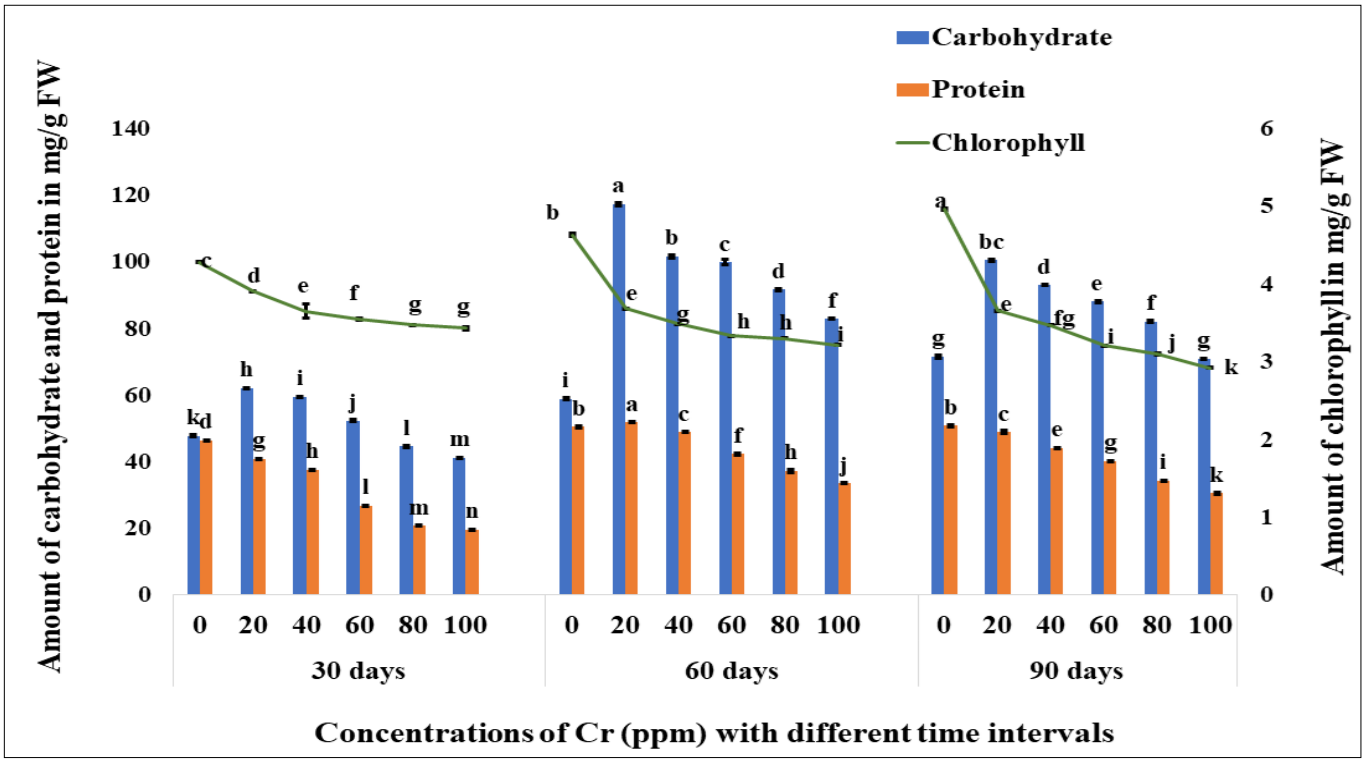

Fig. 3. Effect of chromium on total protein, total carbohydrate and total chlorophyll content in *S. officinalis* at different time intervals. The provided data displays mean values with standard errors derived from 3 trials followed by the letters obtained from Duncan's multiple range test, which indicates that means with common letters do not exhibit statistically significant differences at significance level P≤0.05.

were observed.

Effect of Cr on total carbohydrate and protein content

At 30 days, the total carbohydrate content showed an increment of 29 %, 24 % and 9.6 % in plants treated with 20, 40 and 60 ppm respectively. However, it reduced to 6.7 % and 13.8 % in plants treated with 80 and 100 ppm respectively. By 60 days, carbohydrate concentrations increased in all treatments, with the highest peak of 99.4 % observed in plants treated with 20 ppm of Cr. However, further extension of Cr exposure to 90 days resulted in a decline in carbohydrate content. The percentage increase decreased from 40 % to 14 % in plants treated with 20 to 80 ppm, while a 1 % reduction was noted in plants treated with 100 ppm at 90 days compared to control plants (Fig. 3).

However, the protein levels in sage plants declined as the concentration of chromium increased, with control plants exhibited the maximum protein content throughout all durations (Fig. 3). In untreated plants and those exposed to 100 ppm of Cr, the highest protein content was 46.39 ± 0.12 mg/g FW and the lowest was 19.62 ± 0.08 mg/g FW respectively, after 30 days of exposure to the metal. After 30 days of exposure to 20–100 ppm of Cr, the plants showed a 12–57 % decline in total protein content compared to control plants. At 60 days, Cr treated plants exhibited a reduction of 3- 33 % only. However, the total protein content was again reduced to 3-40 % at 90 days of Cr treatment.

Impact of Cr on proline and MDA production in sage

Proline levels increased at lower Cr concentrations, but slightly decreased at higher concentrations (Cr 80 and 100 ppm). At 30 days of treatment, proline content increased to 65 %, 75 % and 94.5 % in plants treated with 20, 40 and 60 ppm respectively, while in plants treated with 80 and 100 ppm, the percentage increase was 79.6 and 66.5 % respectively. Similarly, at 60 days, a 2.3-fold increase in proline content was observed in plants exposed to 60 ppm, while it reduced to a 1.9-fold increase in plants exposed to 100 ppm. However, at 90 days of treatment, proline content declined in all the concentrations compared to that of 60 days. An increase of 53–98 % was noted in plants treated with 20- 60 ppm, while it declined to 78 % in plants treated with 100 ppm. The highest proline content, measuring 10.85 ± 0.01 mg/g FW, was recorded in the leaves of sage plants subjected to 60 ppm of Cr for 60 days, while untreated plants displayed the lowest proline content (Fig. 4).

Additionally, the MDA (malondialdehyde) content increased with higher metal concentrations and extended metal exposure periods. Sage plant leaves exposed to 100 ppm of Cr for 90 days exhibited notably high MDA content (64.32 ± 0.54 µ moles of MDA/g FW), whereas control plants exhibited the lowest MDA content (Fig. 4). In comparison to the control, the MDA content of plants subjected to 20–100 ppm of Cr increased 2.7–7.4 folds at 30 days, 4.2–8 folds at 60 days and 3.6–7 folds at 90 days.

Effect of Cr on antioxidant enzyme activity

During the initial 30 days of chromium exposure in *S. officinalis* plants, there was a noted elevation in the activity of SOD, APX and Catalase enzymes (Fig. 5). However, as the exposure duration extended to 60 and 90 days, the enzyme activity decreased. For instance, the highest APX activity of 11.83 ± 0.91 µ moles of APX /min/mg of protein was noted in plants treated with 100 ppm Cr for 30 days, but this ac-

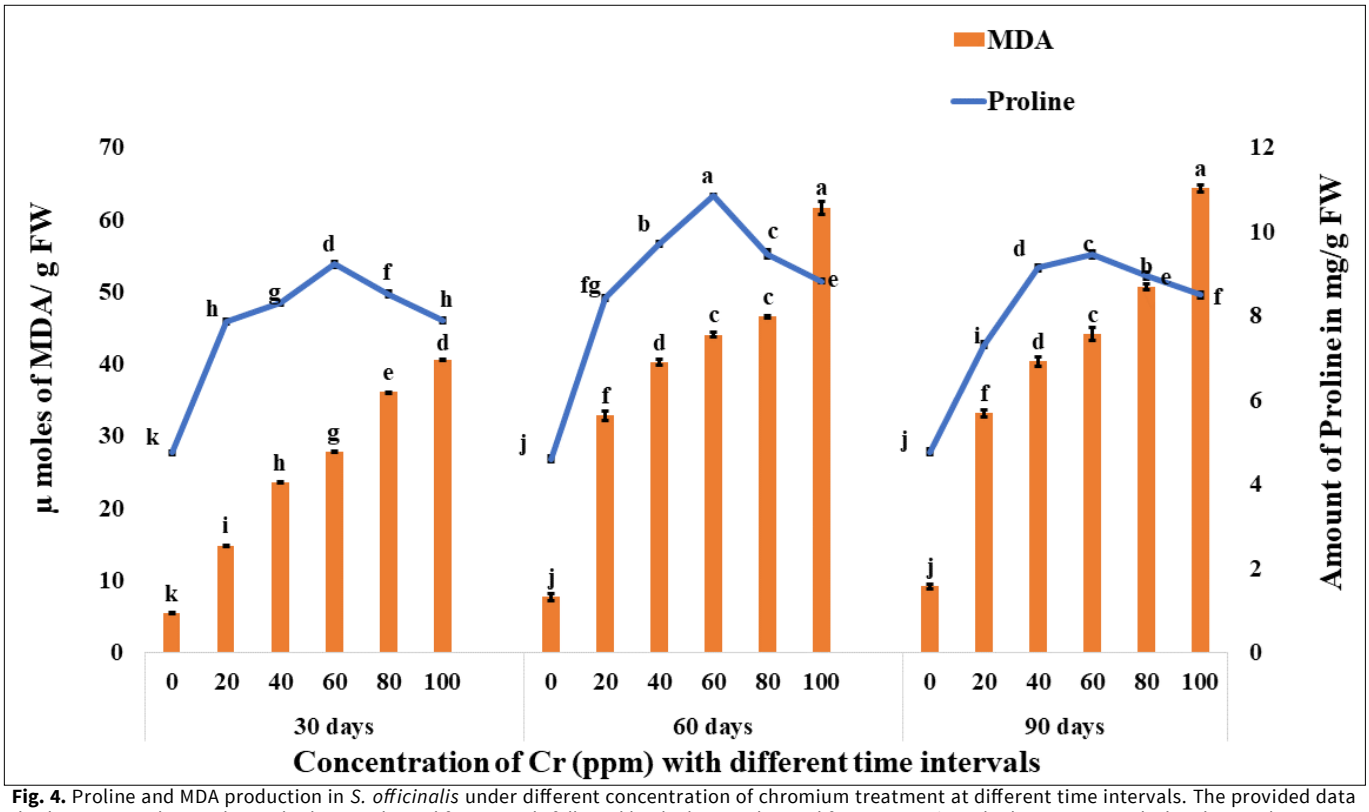

Fig. 4. Proline and MDA production in *S. officinalis* under different concentration of chromium treatment at different time intervals. The provided data displays mean values with standard errors derived from 3 trials followed by the letters obtained from Duncan's multiple range test, which indicates that means with common letters do not exhibit statistically significant differences at significance level P≤0.05.

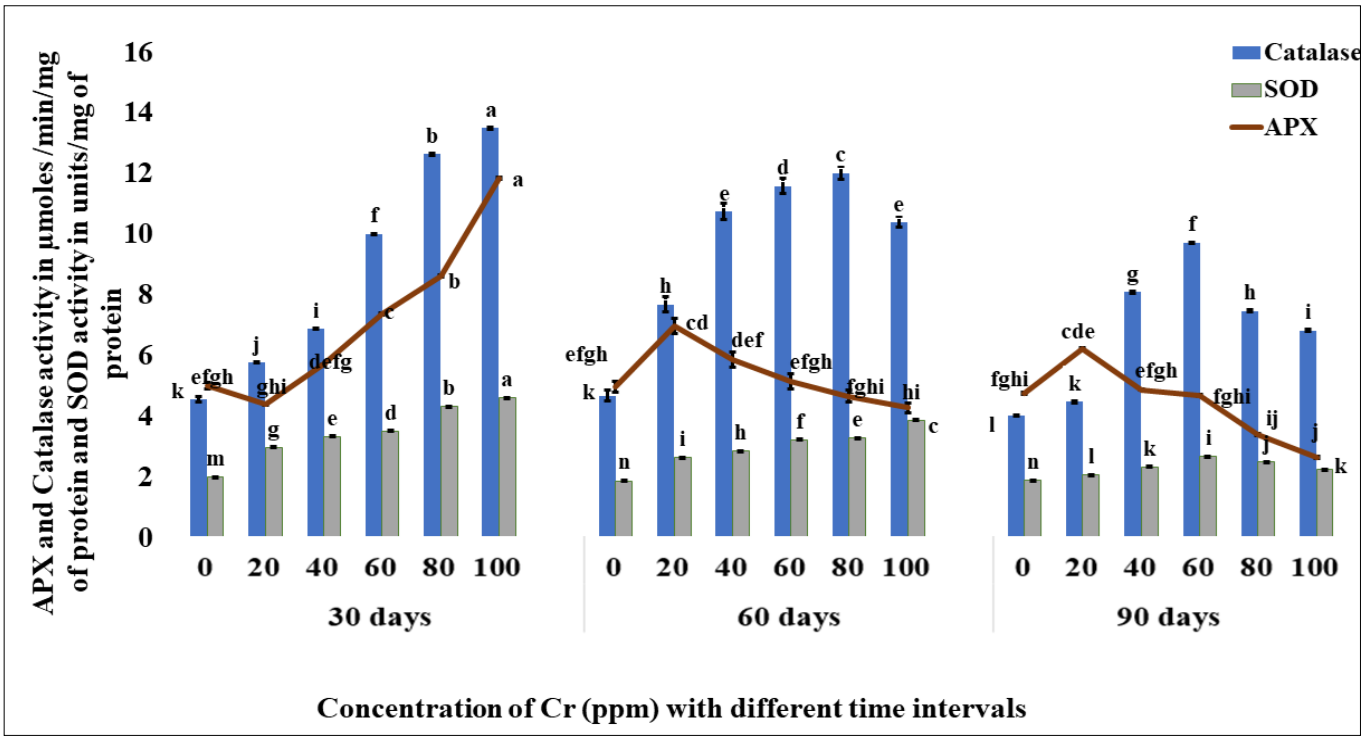

Fig. 5. Antioxidant enzyme activity under different concentrations of chromium at different time intervals in *S. officinalis*. The provided data displays mean values with standard errors derived from 3 trials followed by the letters obtained from Duncan's multiple range test, which indicates that means with common letters do not exhibit statistically significant differences at significance level P≤0.05.

tivity declined by 2.7 and 4.4 times less at 60 and 90 days of Cr exposure at 100 ppm. Similarly, the maximum catalase and SOD activity of 13.48 ± 0.04 µ moles of Catalase/min/mg of protein and 4.59 ± 0.01 units/mg of protein respectively, was seen in plants exposed to 100 ppm Cr for 30 days, while untreated plants exhibited the lowest catalase and SOD activity.

Effect of Cr on rosmarinic acid content

The concentration of rosmarinic acid (RA) exhibited a consistent upward trend with increasing levels of chromium at 30 days, 60 days and 90 days of treatment (Fig. 6). Nevertheless, once the concentration of chromium reached 60 ppm, further increments in metal levels resulted in a decline in the content of rosmarinic acid. The synthesis of rosmarinic acid improved as a response to Cr stress in the initial 30 days. An increment of 1.03–1.23 fold increase in RA was noted in plants treated with 20–100 ppm for 30

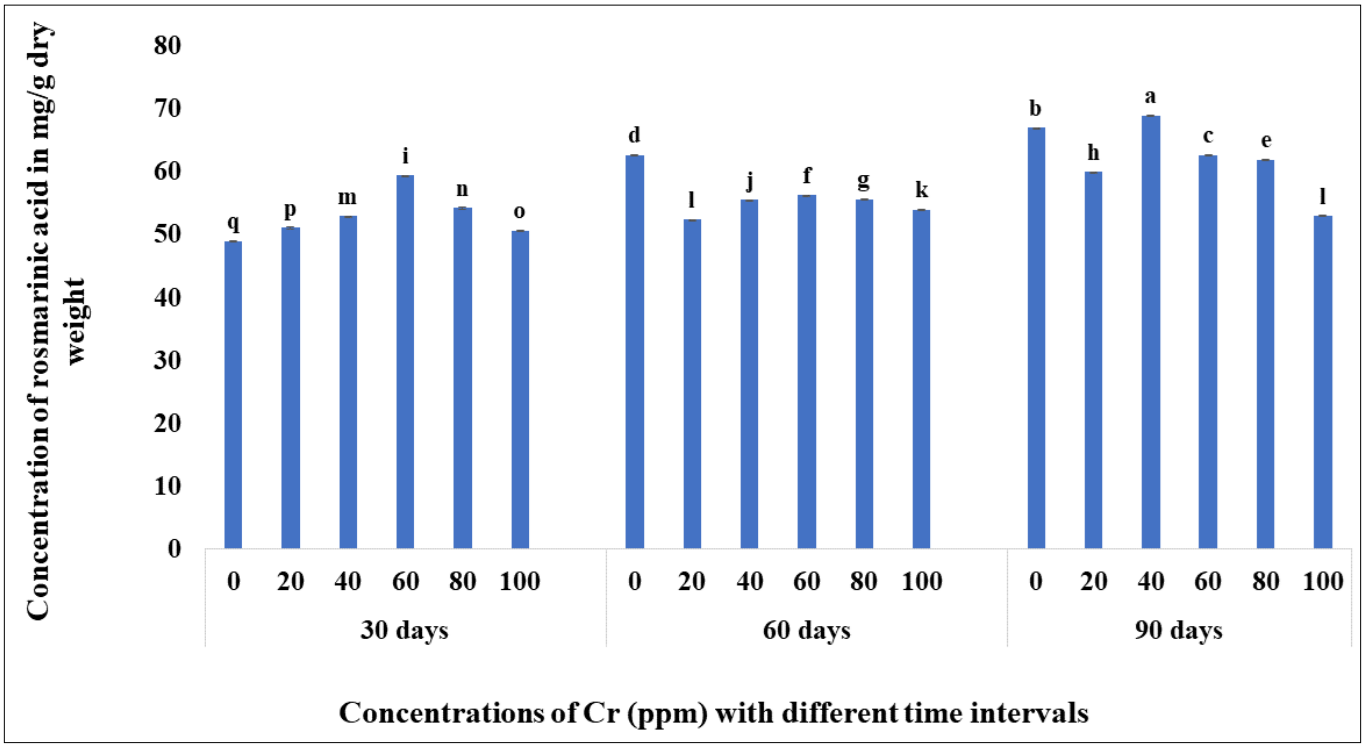

Fig. 6. Rosmarinic acid production under different concentrations of chromium at different time intervals in *S. officinalis.* The provided data displays mean values with standard errors derived from 3 trials followed by the letters obtained from Duncan's multiple range test, which indicates that means with common letters do not exhibit statistically significant differences at significance level P≤0.05.

days. However, it decreased by 10- 16 % at 60 days of treatment across all concentrations. At 90 days of exposure, the RA content again reduced by 20 % in 100 ppm treated plants, while in plants exposed to 40 ppm, there was a 2.9 % increase in RA production.

Discussion

The global issue of heavy metals pollution, particularly chromium, in agricultural land has become increasingly serious, necessitating urgent attention due to its adverse impacts on the environment and ecology. Chromium is among the hazardous heavy metals that harmfully affect plant growth, productivity and metabolic functions, as well as the well-being of animals and humans. This study investigated the detrimental effects of Cr on common sage by subjecting the plants to 5 different Cr concentrations over 3 time periods: 30, 60 and 90 days. Subsequently, an assessment of growth and biochemical parameters suggests that Cr exposure had both stimulatory and inhibitory effects on the growth of common sage plants, with the specific outcome varying with concentration and exposure duration. This study found that a high Cr concentration of 100 ppm resulted in diminished root and shoot length as well as reduced biomass in sage plants. Similar adverse effects of Cr toxicity have been reported in other plant species such as *Cicer arietinum* (28) and *Vigna radiata* (29). The diminished expansion of roots in response to high concentrations of Cr is thought to be a consequence of halted cell division, cell elongation, or both, particularly in the tips of the roots. In transgenic *Arabidopsis* plants, Cr exposure upregulated the expression of low phosphate (Pi) responsive reporter genes *AtPT1* and *AtPT2*, resulting in limited root growth (30). This decline in root growth directly interferes with the uptake of water and nutrients and their conveyance to the upper parts of the plant, consequently restraining the growth of aerial parts (2). The fresh weight of the plants was found to be increasing with the increase of Cr concentrations at 30 days in tandem with the results of a study observed in *Solanum nigrum* and *Parthenium hysterophorus,* might be due to the increased accumulation and vacuolar compartmentalisation of Cr (30, 31).

In this study, *S. officinalis* demonstrated resilience to 100 ppm chromium, showcasing adaptability to chromium stress. Consistently, the roots retained more chromium than the stem and leaves in all treatment scenarios. This pattern of root-based Cr accumulation mirrors results seen in *Brassica campestris* (32), *Oryza sativa* (33) and *Arachis hypogaea* (34). The confinement of Cr accumulation to the roots is likely due to its immobilization within root cells, ultimately impacting plant growth and the plant's ability to withstand Cr toxicity (34).

The estimation of total chlorophyll content in stress studies is a valuable and versatile tool for assessing the impact of stress on plants. We observed a significant decrease in total chlorophyll levels in the leaves as the concentration of chromium (Cr) and the duration of Cr exposure increased. This finding aligns with similar results reported in *Zea mays* (35) and *Vitis vinifera* (36). The decline in chlorophyll levels attributed to chromium exposure is linked to the compromised functions of specific enzymes such as δ-aminolevulinic acid dehydratase (ALAD) and protochlorophyllide reductase, which play crucial roles in chlorophyll biosynthesis (2, 37).

Soluble sugars play essential roles in various stress responses in plants. They act as antioxidants, aid in osmotic regulation, store carbon and stabilize critical proteins. During periods of stress, sugar levels increase, serv-

ing as osmoprotectants, promoting growth and regulating gene expression (38, 39). The observed elevation in carbohydrate content under Cr stress in our study further supports the idea that overall carbohydrate content contributes to Cr tolerance in sage plants. Similarly, our findings indicates a decrease in total protein content in response to Cr stress, consistent with results from studies on *Catharanthus roseus* (40), sunflower (41) and soybeans (42). However, the decline in protein levels in *S. officinalis* could be attributed to heightened protease activity and the activation of other catabolic enzymes in response to chromium stress. Additionally, this stress impedes photosynthesis, causes membrane damage, alters enzyme activity, disrupts nutrient uptake and redirects the plant's energy resources toward stress adaptation, ultimately leading to a reduction in protein content within the plant (40).

Proline is a crucial component in plant cells, acting as both an osmoprotectant and a redox buffer, particularly during osmotic stress (43). It plays essential roles in signaling, metal chelation and antioxidant defense, collectively enhancing a plant's stress tolerance (44, 45). Our study observed an increase in proline production with rising chromium (Cr) concentrations similar to the findings of another study (40). However, the decline at higher concentrations might be attributed to the overwhelming stress and damage caused by excessive metal ions and prolonged exposure, hindering the plant's ability to synthesise and maintain proline levels. Lipid peroxidation serves as an indicator of oxidative stress and previous research has shown that Cr induces MDA production in various plant species like *Ipomoea batatas*, *Lemna minor* and *Mentha arvensis* (46-48). Our study also confirmed a substantial increase in MDA levels in response to high Cr concentrations, signalling the involvement of superoxide radicals in lipid peroxidation (49).

Plants possess a sophisticated and well-coordinated antioxidant defence system, which effectively scavenges and regulates the levels of reactive oxygen species (ROS). This system manages oxidative stress and maintains a stable ROS equilibrium with the aid of several enzymatic antioxidants (50). Among these, SOD is pivotal, leading the defence against reactive oxygen species (ROS) by catalysing the transformation of superoxide ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2). Following this, catalase and APX play crucial roles in eliminating H_2O_2, effectively converting it into molecular oxygen (O_2) and water (H_2O) (51). During the initial 30 days of chromium (Cr) exposure in *S. officinalis* plants, there was a notable increase in the activity of SOD, APX and Catalase enzymes. This surge in enzyme activity likely represents the plant's strategic response to combat the toxic effects of chromium. However, as the duration of Cr exposure extended, the activities of these enzymes gradually decreased. Previous research has indicated that under high Cr toxicity, various plant species exhibit reduced activities of SOD, APX and Catalase (52). This decline in the enzymes' activities could be attributed to the excessive production of reactive oxygen species (ROS) within the plants (53).

Plant secondary metabolites, particularly polyphenolics, plays a crucial role in plant stress responses and the innovative idea of utilizing plant stress to enhance the *in vivo* production of these compounds is gaining attention (54). Among these secondary metabolites, rosmarinic acid (RA) stands out as a potent phenolic antioxidant with a remarkable ability to combat free radicals, surpassing the antioxidant efficacy of tocopherol (55). Various stress-induced responses, such as disturbances in reactive oxygen species (ROS) equilibrium, reduced levels of ascorbate (AsA), the induction of heat shock proteins and the activation of phytohormones, have been identified as effective triggers for enhancing RA production (56). This phenomenon is exemplified by increased RA production observed in other Lamiaceae species such as *Mellissa officinalis* under heat stress (56) and *Ocimum basilicum* (57) under salt stress, highlighting RA's role in bolstering abiotic stress tolerance. Additionally, methyl jasmonate, an important stress-signalling molecule, has emerged as a promising elicitor for promoting RA production in various plants, including tomato (58) and *Solenostemon scutellarioides* (59). This research suggests that plant stress responses to low concentrations of chromium (Cr) can increase the synthesis of valuable secondary metabolites, with rosmarinic acid (RA) as a prime example. Previous studies have revealed the positive effect of Cr on the biosynthesis of specific metabolites such as phyllanthin in *Phyllanthus amarus* (60) and vincristine and vinblastine of *Catharanthus roseus* (40). However, increasing Cr toxicity negatively impacts secondary metabolite production (40).

Additionally, various sources indicate that chromium (Cr) may positively influence the functionality of phenylalanine ammonia-lyase (PAL), the initial enzyme in the RA production process, particularly at lower concentrations in rice plants. However, once a certain Cr concentration threshold is exceeded, PAL activity tends to decrease (61). This observation aligns with our findings, where RA production increased up to 60 ppm of Cr concentration, followed by a decline at higher concentrations. Contrary to this, the findings of an earlier study (62) challenge the conventional idea that PAL solely determines RA biosynthesis, suggesting the involvement of unknown factors in phenolic synthesis in sage plants. An in-depth study of the underlying molecular mechanisms is needed to reveal the actual role of Cr in RA synthesis.

Conclusion

The analysis of the effects of chromium exposure on *S. officinalis* revealed that lower Cr concentrations positively influenced growth parameters, while higher concentrations led to a decline in plant growth. The highest Cr accumulation was observed in plants treated with 100 ppm of Cr, particularly in the roots. Total chlorophyll content decreased in all Cr-treated sage plants, with a more pronounced decline after prolonged exposure. Protein levels declined with increasing Cr concentrations and exposure duration, whereas carbohydrate content increased. Proline levels exhibited a mixed response, increasing at lower Cr concentrations and decreasing at higher concentra-

tions, while MDA content increased in Cr-exposed plants. The enzymatic antioxidant system in sage plants initially showed increased activity in response to Cr toxicity. Meanwhile, rosmarinic acid concentration in sage plants increased with chromium levels up to 60 ppm over 90 days, but higher chromium concentrations resulted in a decline in RA content. These findings highlight the complex and contrasting responses of *S. officinalis* to Cr toxicity, underscoring the need for further exploration of the underlying mechanisms governing these responses and the development of strategies to mitigate heavy metal stress in plants.

Although the study provides valuable insights into the complex responses of *Salvia officinalis* to chromium (Cr) toxicity, several limitations should be noted. Firstly, the specific Cr concentrations used in this study may not accurately reflect real-world conditions of chromium contamination. Moreover, the scope and duration of the study might have missed long-term impacts or interactions with other environmental factors. Additionally, the research focused on biochemical and physiological responses without investigating the underlying molecular mechanisms that could explain the observed effects. Lastly, the results of this study may be specific to the particular characteristics and parameters used and may not be generalizable to other varieties of *S. officinalis* or different environmental conditions.

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Authors' contributions

RR and PN conceptualized and designed the experiment. RR conducted the experiment, performed data analysis, drafted the document, and prepared tables and figures. PN critically reviewed the manuscript. Both authors approved the submission of the final manuscript.

Compliance with ethical standards

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

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