https://doi.org/10.48047/AFJBS.6.13.2024.4934-4958

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

"Understanding Cadmium Toxicity in *Salvia officinalis* L.: Impact on Vegetative Growth, Biochemical composition, and Antioxidant Defense Mechanisms"

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Volume 6, Issue 13, july 2024

Received: 09 May 2024

Accepted: 19 June 2024

Published: 08 July 2024

doi: 10.48047/AFJBS.6.13.2024.4934-4958

Abstract:
The herbal drugs can act as a source for cadmium (Cd) contamination in human beings. *Salvia officinalis*, being a common herb with a wide range of applications in medicine and culinary fields, can be polluted with cadmium during agricultural practices. This study examines the effects of different concentrations of Cd exposure on *S. officinalis*, with a particular emphasis on vegetative and biochemical parameters, over a period of 30 to 90 days. The accumulation studies noted that roots have the maximum Cd content (21.43 mg/kg) compared to shoots. Results show a significant decrease in a number of plant health parameters, such as fresh and dry weight (41% and 44%), leaf count (30%), shoot length (41%), and root length (49%), in the Cd -exposed plants. Furthermore, there is a noticeable toxicity brought on by cadmium, as evidenced by the 8.7-fold increase in malondialdehyde and 18% reduction in chlorophyll levels compared to the control plants. Proline, phenol, flavonoids, total protein, and carbohydrates, as well as the antioxidant enzymes catalase and ascorbate peroxidase, are all comparably higher in Cd treated plants, suggesting that it is adapting and may be able to offset the negative effects of Cd stress. Moreover, rosmarinic acid has a major reduction of 91% in response to 200 ppm Cd for 90 days, raising the possibility that this could have implications for the plant's medicinal properties. This study underscores the intricate response of Sage plants to Cd stress, emphasizing the need for further investigation into the underlying molecular mechanisms. In order to mitigate the detrimental effects of heavy metal contamination on plant ecosystems, future research should focus on clarifying the distinct responses seen at different Cd concentrations and exposure times.

Keywords: accumulation, antioxidant, cadmium, rosmarinic acid, sage, toxic effect

Introduction

Rising global populations lead to enhanced industrial activities, urbanization, and agricultural expansion, all of which contribute to greater heavy metal pollution through emissions, waste disposal, and runoff into waterways. (Long et al., 2021). One of the hazardous metals is cadmium (Cd), which can harm plants and people even in small amounts (Suhani et al., 2021). The main causes of cadmium buildup in agricultural soil are anthropogenic activities like industrial operations and agronomic practices, especially the use of fertilizers and emissions from smelters. (X. Zhang et al., 2020; Zhao et al., 2020). Cadmium's broad distribution in nature and its uptake by plants deliver a substantial health risk to humans due to its toxicity, even at low levels, particularly when it contaminates agricultural produce, food, sewage sludge, and different environmental settings. (Mikhailenko et al., 2020). The detrimental effects of cadmium contamination on plant physiology and agricultural productivity are highlighted by the disruption of nutrient uptake, increased oxidative damage, and inhibition of plant growth and metabolism in plants (Haider et al., 2021).

Human health is seriously threatened by Cd pollution because it can enter the body through a number of different channels, including the food, water, soil, and atmosphere. Cd has a lengthy half-life, damages organs irreversibly, and raises the risk of both carcinogenic and non-carcinogenic health effects. It also tends to accumulate in organs such as the kidneys, liver, and bones (McLaughlin et al., 2021; Wang et al., 2021). According to Kolahi et al. (2020), an adult weighing 70 kg is limited to 70 µg of cadmium per day based on the World Health Organization's provisional appropriate daily intake (Kolahi et al., 2020). Recent research found that herbal medicines also serve as a potential source of cadmium toxicity in human beings (Luo et al., 2020; Yao et al., 2022). Herbal medicines can become contaminated with cadmium from various sources, including cultivation in soil contaminated with trace metal or irrigation with polluted water, as well as unintentional or intentional mixing of trace elements during processing (Nath et al., 2020).

Salvia officinalis, or Sage, is a popular herb used in cooking and medicine characterized by a unique flavor (Pizani et al., 2022). Traditionally, it has been consumed as herbal infusions of fresh or dried leaves as a medication for coughs, colds, asthma, and so on (Farzaneh and Carvalho, 2015). It has been praised for its therapeutic potential, like antidiabetic, antioxidant, anti-inflammatory, and anticancer properties, as well as antimicrobial qualities (Abdollahi et al., 2023;

Zalyhina, 2022). Apart from that, the antioxidant and antibacterial qualities of sage essential oil make it a popular choice for aromatherapy and food preservative (Chrysargyris et al., 2021; Danilović et al., 2021; Mot et al., 2022).

Previous studies already reported the ability of Lamiaceae members as Cd accumulators (Angelova et al., 2006; Azizollahi et al., 2019; Fattahi et al., 2019). Nevertheless, due to a variety of anthropogenic and environmental factors, concerns have been raised about the possible buildup of Cd in sage plants. As Cd contamination poses significant health risks to consumers, there is a critical need to assess and address its presence in sage through enhanced quality control measures. The degree of cadmium toxicity can be understood in part by looking at the biochemical and vegetative alterations as well as the antioxidant response. In order to assure the safety and effectiveness of sage-based products for both medicinal and culinary purposes, this study paper seeks to determine the level of Cd toxicity in sage and advocates for stronger quality inspections.

Materials and methods

Collection of plant material and metal treatment

Two-month-old, healthy stem cuttings of local variety of *S. officinalis* plants were acquired from the University of Agricultural Sciences, GKVK, Bangalore, India. In a 3:1:1 ratio, soil, sand, and cocopeat were combined to create a soil mixture for the experiment. Then, different concentrations of cadmium chloride solution (40, 80, 120, 160, and 200 ppm) were applied to this mixture. After being gingerly moved into pots that were filled with the appropriate soil mixtures treated with Cd, the plants were given a full 90 days to grow. Throughout the experiment, regular irrigation was maintained, and every 14 days, the metal treatments were carried out again. Plants were harvested at 30, 60, and 90 days to evaluate the response of sage plants to Cd stress.

Analysis of vegetative parameters

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

Metal accumulation

Plants were harvested, and the roots, leaves, and stems were separated in order to assess the Cd accumulation. After a thorough cleaning, they were allowed to air dry for three days at a temperature between 45 and 50°C. Aqua regia was used to acid-digest one gram of dried samples of sage plant leaves, stems, and roots that had been treated with Cd. After that, double-distilled

water was used to dilute the resultant samples to 25mL. With the aid of a Flame Atomic Absorption Spectroscopy (Shimadzu, AA-6880, Japan), the Cd concentrations in the corresponding samples were investigated (Turek et al., 2019).

Effect of cadmium on biochemical parameters

Protein content

The protein measurement was done in accordance with Lowry's protocol (Lowry et al., 1951). This method involved centrifuging 0.1 g of the sample for 10 min at 10,000 rpm after mixing it with 5 mL of 0.2 M phosphate buffer (pH 7). Subsequently, 500 µL of alkaline-copper sulfate solution was combined with 10 µL of the sample and left to incubate at room temperature for 10 min. Following this, 50 µL of Folin-Ciocalteu reagent was added, and the mixture was further incubated for 30 minutes at room temperature in darkness. With the aid of a microplate reader (BIO-RAD, iMARKTM, Japan), absorbance was measured at 660 nm. The standard graph prepared using BSA (10 to 250 µg mL^{-1}) was used to calculate the amount of protein.

Carbohydrate content

The phenol sulfuric acid method has been used to estimate the overall carbohydrate content. Initially, 0.1 g of sage leaves was blended with 5 mL of 2.5N HCl and heated to boiling for three hours. After cooling, the neutralized crude homogenate underwent centrifugation for ten min at 1000 rpm. Following this, 5 mL of sulfuric acid and 1 mL of phenol (5% volume) were combined with 1 mL of supernatant. The amount of carbohydrates was ascertained by measuring absorbance of the supernatant at 490 nm with a microplate reader (BIO-RAD, iMARKTM, Japan). Total carbohydrate content was calculated using the standard graph prepared using standard D-glucose concentrations (50 to 500 µg mL^{-1}) (DuBois et al., 1956)**.**

Proline Contents: To estimate the proline content, 0.1 g of sage leaves were homogenized in 5 mL of 3% aqueous sulfosalicylic acid. The mixture was then centrifuged at 10,000 rpm for 10 min. 1 mL of the resultant supernatant and 2 mL of 1.25% ninhydrin in glacial acetic acid were combined and heated to 100°C for 30 min in order to perform the proline assay. At 508 nm, the absorbance was measured following cooling in an ice bath. A standard graph was prepared using standard proline concentrations ranging from 0 to 10 µg mL^{-1}. in order to ascertain the proline content in the samples (Shabnam et al., 2016).

Total phenol content

The Folin-Ciocalteu assay, with certain modifications, was utilized to determine the total phenolic content, following the guidelines provided by (Herald et al., 2012). A volume of 50 µL of the 1 mg mL^{-1} extract was combined with 200 µL of distilled water. The mixture was then mixed with 500 µL of a 7.5% sodium bicarbonate solution and 250 µL of diluted Folin-Ciocalteu reagent. It was then allowed to sit at room temperature for 30 min in the dark. The microplate reader (BIO-RAD, iMARKTM, Japan) was utilized to measure the absorbance at 765 nm. The phenol content of the samples was calibrated using gallic acid standards in the range of 25 to 500 µg mL^{-1}.

Total flavonoid content

The total flavonoid content (TFC) was measured using a modified aluminum chloride technique (Sembiring et al., 2017). Using this approach, 750 µL of 80% methanol, 50 µL of 10% (w/v) aluminum chloride, and 50 µL of 1M sodium acetate were mixed with 250 µL of the 1 mg mL^{-1} extract. After that, the mixture was incubated for 30 minutes at room temperature. The absorbance was then determined using a microplate reader (BIO-RAD, iMARKTM, Japan) at 415 nm. Quercetin standards ranging from 0 to 100 µg mL^{-1} were utilized for calibration in order to ascertain the flavonoid content of the samples.

Total chlorophyll content

The total chlorophyll content was assessed following the Arnon method, in which 0.1g fresh leaves were homogenized with 5 mL of ice-cold acetone (80%). Absorbance measurements were taken at wavelengths of 645 and 663 nm using a UV/Vis spectrophotometer (Shimadzu UV-1900, Kyoto, Japan), with acetone serving as the blank solvent (Arnon, 1949)

Lipid peroxidation

Lipid peroxidation in fresh leaves was evaluated through the thiobarbituric acid (TBA) reaction method, which measures the production of malondialdehyde (MDA) (Dhindsa et al., 1981). 5 mL of 1% TCA was used to extract fresh leaves (0.5 g), and the leaves were centrifuged for 5 min at 10,000 rpm. The resulting supernatant (1 mL) was combined with 0.5% TBA in 20% TCA, followed by heating at 95°C for 30 min. Following a brief cooling period in an ice bath and a 10 min centrifugation at 10,000 rpm, the absorbance of the supernatant was measured at 600 and 532 nm. The calculation of MDA was performed using the below equation.

MDA in mM = $(A_{532}-A_{600})/155$, where A_{532} is the absorbance at 532nm, A_{600} is the absorbance at 600 nm, and 155 is the molar extinction coefficient of MDA.

Effect on antioxidant enzyme activity

Extract preparation: 500 mg of fresh sage leaves were blended with 5 mL of pre-cooled phosphate buffer solution (pH 7.8) and centrifuged at 10000 to 15000 rpm for 20 min at 4^0 C. The supernatant was employed for the assessment of the antioxidant enzymes Superoxide dismutase (SOD), Ascorbate peroxidase (APX), and Catalase (Zahir et al., 2021). The enzymes were quantified per mg of protein.

Ascorbate peroxidase (APX): Ascorbate peroxidase (APX) activity was estimated using the method described by Nakano and Asada (1981). It involved assessing the oxidation of ascorbate at 290 nm within a reaction mixture of 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 homogenate. APX activity was quantified using an extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as μmoles of APX min^{-1} mg of $protein^{-1}$.

Catalase (CAT): The activity of catalase (CAT) was evaluated by observing H_2O_2 decomposition at 240 nm in a 3 mL reaction mixture consisting of 2.85 mL of phosphate buffer (50 mM), 100 µL H_2O_2 (50 mM), and 50 µL homogenate. The activity was quantified as μmoles of catalase min^{-1} mg of $protein^{-1}$ (Aebi, 1984).

Superoxide dismutase assay (SOD): The evaluation of superoxide dismutase (SOD) activity followed the procedure outlined by Dhinsada et al. (1981) with slight modifications. A reaction mixture consisting of 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 adjusted to a total volume of 3 mL with distilled water. This mixture was then incubated under light conditions for 20–30 min. Controls included a reaction mixture without extract (positive control) and a blank without light exposure. By measuring the absorbance of the resultant blue formazan at 560 nm, the reduction of NBT was evaluated. SOD activity was quantified based on the enzyme quantity necessary to prevent a 50% reduction in NBT. The results were given in SOD units mg of $protein^{-1}$ (Dhindsa et al., 1981).

Rosmarinic acid production

The basic maceration method was used to prepare the samples for the rosmarinic acid analysis. 0.5g of dried leaf powder and 10 mL of 80% methanol were combined, and the mixture was

agitated overnight on an orbital shaker. The extracts were filtered and kept for drying using a hot air oven at 35 to 45°C. The resultant extracts were redissolved in methanol to make a 10 mg mL^{-1} solution (Bandoniene et al., 2005) and used for HPLC analysis.

The HPLC grade Rosmarinic acid (96% purity) purchased from Ottochemi Pvt Ltd, Mumbai, India, was used as the standard in different concentrations (2 to 10 μg mL^{-1}). RP-HPLC was conducted using a Shimadzu scientific instrument LCMS-8040 system from Kyoto, Japan. Analytical software from Lab Solutions was utilized in conjunction with an SPD40 UV-Vis detector and a Sharpsil-U C-18 (250 × 4.6 mm). The mobile phase in pump A was 0.5% acetic acid in water, while pump B used methanol at a flow rate of 1 mL min^{-1}. The column temperature was maintained at 40°C. Isocratic elution with a 35-min run time and absorbance monitoring at 270 nm were employed in the analysis (Liu et al., 2013).

Statistical analysis

Every experiment was run in triplicate, and the statistical analysis was carried out using IBM SPSS Statistics software version 22.0. To evaluate the validity and variability of the findings, one-way ANOVA was used. Duncan's multiple range test (DMRT) was used to compare the means of the control and Cd-treated groups at a significance threshold of $P \leq 0.05$ in order to identify any significant differences. The obtained results is presented as means ± SE accompanied by the post hoc test (DMRT) letter.

Results and discussion

Effect of cadmium on vegetative parameters

Cadmium is known to have detrimental effects on the growth and development of plants. This study examined the effects of varying cadmium concentrations on *Salvia officinalis* growth parameters at 30, 60, and 90 days after exposure to Cd (Table 1). The control plants had the maximum levels of every vegetative parameter studied (number of leaves, shoot length, dry weight, fresh weight, and root length). All the examined growth parameters have been shown to decrease with increasing Cd concentrations. The fresh weight was reduced to 20.7 to 41% at 30 to 90 days in high concentration (200 ppm) treated plants compared to the control plants. Similarly, after 30, 60, and 90 days of Cd exposure, plants treated with 40 to 200 ppm showed reductions in dry weight of 3 to 35%, 2 to 30%, and 11 to 44%, respectively. A similar pattern was observed in the length of the shoots, roots, and the quantity of leaves. The number of leaves decreased by 5 to

18% in 30 days, then by 13 to 30% and 12 to 27% in 60 and 90 days after being exposed to 40 to 200 ppm. A maximum reduction in shoot length of 41% was observed in plants treated with 200 ppm Cd for 90 days. In a similar vein, the root length declined to 8.8 to 32%, 22 to 49% and 8 to 34% in 40 to 200 ppm treated plants over 30, 60, and 90 days, respectively.

The analysis of vegetative parameters (shoot length, root length, number of leaves, fresh weight, and dry weight) showed that Cd significantly affects the growth of sage plants, especially at high concentrations. These findings align with those of Marzban et al. (2017) in *Avena fatua*, *Lathyrus sativus*, and *Lolium tementulum*, as well as Varalakshmi and Ganeshamurthy (2013) in *Raphnus sativus*. (Marzban et al., 2017), (Varalakshmi and Ganeshamurthy, 2013). Zhao et al. (2021) hypothesize that the detrimental effects of Cd, such as inhibition of photosynthesis and nutrient uptake, may be the cause of the decline in growth indices (Zhao et al., 2021). The ability of Cd to bind with phosphorus and reduce its availability to the plant is another factor that hinders plant growth (Jawad Hassan et al., 2020).

Table 1. Effect of cadmium on vegetative parameters of *S. officinalis* over different time intervals

Cd concentrations (ppm)	**Days after treatment**	**Shoot length (cm)**	**Root length (cm)**	**No.of Leaves**	**Fresh weight (grams)**	**Dry weight (grams)**
Cd 0	30 days	51.93±0.47cde	17.87±0.24fg	70.33±1.45j	15.69±0.33ij	3.13±0.08ij
	60 days	59.67±1.2b	24.97±0.43c	122±1.15b	23.47±0.32de	5.27±0.02bcd
	90 days	72.5±0.29a	30.7±0.36a	130.33±0.88a	29.17±0.48a	6.18±0.07a
Cd 40	30 days	50.67±1.45def	16.33±0.88hg	62.67±0.67kl	20.95±0.28fg	3.04±0.19ij
	60 days	55.33±2.19c	19.33±0.88ef	106±1.15d	26.41±1.07bc	5.66±0.17b
	90 days	56.1±2.11bc	28±0.58b	114.67±1.76c	29.27±0.58a	5.46±0.09bc

Cd 80	30 days	49±1.15defg	15.33±1.2ghi	66.67±1.33jk	19.57±0.23gh	2.99±0.03ij
	60 days	53±2.52cd	17.67±0.33fg	100±2ef	24.95±1.16bcd	5.13±0.06cde
	90 days	52.5±1.04cd	23±1.15cd	108±1.15d	26.77±0.59b	4.98±0.04def
Cd 120	30 days	47.67±1.33efgh	15±0.58ghi	58.67±1.76lm	17.15±0.43hi	2.8±0.29j
	60 days	49±0.58defg	15.33±0.88ghi	98±2.31fg	22.28±0.83ef	4.95±0.04def
	90 days	49.1±0.32defg	22±1.15de	104±1.15de	24.62±0.75bcde	4.71±0.02efg
Cd 160	30 days	45.33±1.76ghi	13±0.58ij	59.33±0.67lm	15.93±0.15i	2.38±0.27k
	60 days	46.33±1.2fghi	14.33±1.2hij	89.33±1.76h	20.45±1.56fg	4.68±0.07fg
	90 days	47±1.53fgh	21±1.15de	101.33±1.76ef	24.14±1.01cde	4.29±0.05g
Cd 200	30 days	44.33±1.2hi	12±1.15j	57.33±0.67m	12.44±0.38j	2.03±0.28k
	60 days	42.33±0.88i	12.67±1.33ij	84.67±1.76i	19.66±1.76gh	3.66±0.16h
	90 days	42.43±1.27i	20±0.58ef	95±1.73g	17.13±0.52hi	3.44±0.04hi

The values represent means of triplicates ± standard error followed by the alphabets obtained from Duncan multiple range test (DMRT), which indicates that the variance of means with same alphabets are not statistically significant at $p \leq 0.05$

Cadmium accumulation

Different plants exhibit different levels of accumulation patterns. In sage plants, the roots showed a higher cadmium content than the aboveground parts. Compared to control plants, the cadmium

accumulation significantly increased in all the plant parts, such as the leaf, stem, and root (Figure 1). The cadmium accumulation reached a maximum of 21.3 mg kg^{-1} in the roots of sage plants treated with 200 ppm for 90 days. Over the course of 30, 60, and 90 days of treatment, the cadmium content of the Cd-treated plants rose to 13, 7.6, and 9.6 folds in the leaves, while the Cd accumulation in the stems increased to 25.6, 41.5, and 38.6 folds in the control plants. Similarly, after 30, 60, and 90 days of exposure to 200 ppm of Cd, the roots of plants exhibited an increase of 33.3, 10.6, and 11.6 times greater than the roots of control plants. The accumulation pattern of cadmium observed in sage plants was consistent with the results observed in chickpea (Ullah et al., 2020), cucumber (Rombel-Bryzek et al., 2024) and *Canna orchioides* (Zhang et al., 2021), where roots showed the maximum accumulation than shoot. Since roots are the first organs to have direct contact with soil, cadmium accumulation is also found more in the roots of plants (Jawad Hassan et al., 2020; Lin et al., 2024). The capacity of cadmium to hijack the transport systems of micronutrients like Zn, Fe, or Mn facilitates the build-up and translocation of Cd in plants (Sterckeman and Thomine, 2020).

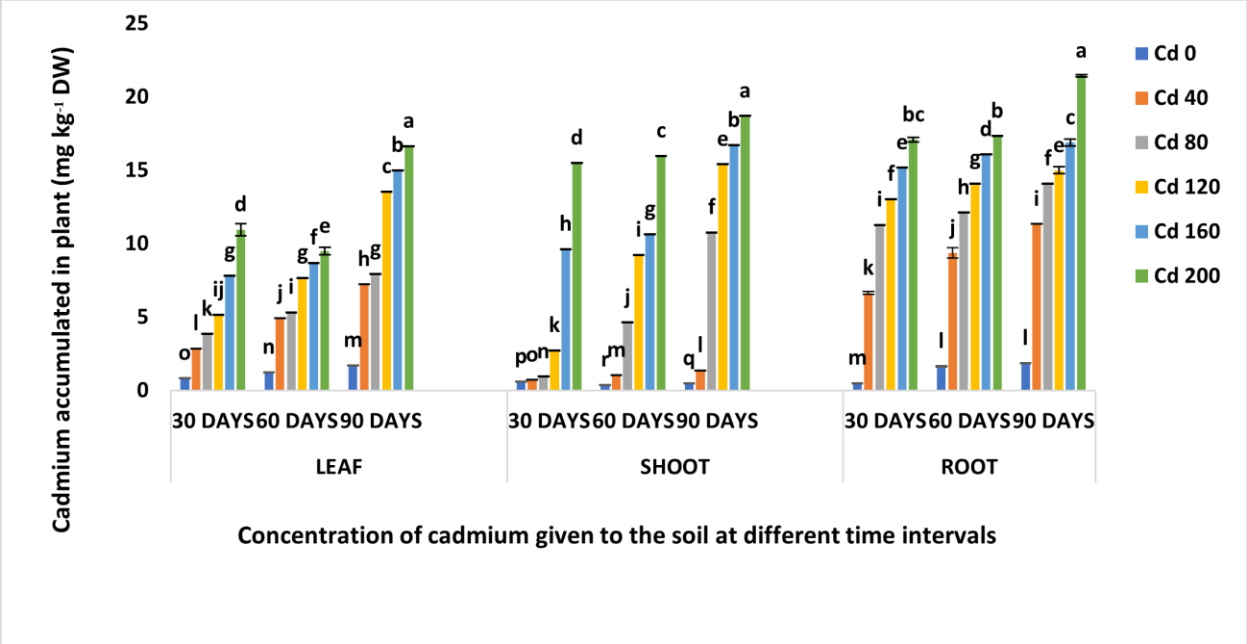

Figure 1. Cadmium accumulation in leaf, stem, and roots with different time intervals of varying concentrations of cadmium treatment on *S. officinalis*.

The provided data displays mean values with standard errors derived from three trails 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 $P \leq 0.05$

Effect on biochemical parameters

Total protein content

The total protein content was observed to be increased in Cd treated plants than that of control plants (Table 2). However, with the increasing concentrations of Cd from 40 to 200 ppm, the percentage of increase of protein content declined to 31 to 11% in 30 days. The protein content decreased from 62.6 mg g^{-1} FW to 49.3 mg FW, and 50.7 mg g^{-1} to 47.1 mg g^{-1} in 60 days and 90 days of Cd exposure. Similar kind of increasing protein content in response to Cd exposure is also reported in sassafras seedlings (Zhao et al., 2021). The increased protein content might be due to the enhanced production of stress and defense related proteins. However, previous studies reported that Cd exposure caused a decline in total protein content of rice and tomato plants due to enhanced protease activity and reduced protein synthesis under Cd toxicity (Faizan et al., 2021a); (Faizan et al., 2021b; Tousi et al., 2020).

Total carbohydrate content

The total carbohydrate content of 40 ppm treated plants was reduced to 25.8, 14.2 and 26.2 % compared to untreated plants in 30, 60 and 90 days respectively. However, further increase in Cd concentrations lead to the enhancement of carbohydrate content, reaching a maximum of 95% in 30 days, 74.9% in 60 days and 29.8% in 90 days compared to control plants (Table 2). Previous reports also observed enhanced carbohydrate content in response to cadmium toxicity in plants like *Malva parviflora,* and *Satureja hortensis.* (Azizi et al., 2020), (Tousi et al., 2020). According to (Azizi et al., 2020), the carbohydrate content can be increased or decreased according to the metal concentrations. At lower concentrations, the decline in carbohydrate content might be due to the cadmium mediated disruption of metabolic pathways engaged in carbohydrate synthesis or utilization, while at high concentrations, the carbohydrate content as a defense response, since it can act as antioxidant as well as an osmolyte, to reduce the toxic effects of cadmium (Li et al., 2020).

Proline content

The proline content in plants is also considered as a stress marker, as it can act as an antioxidant agent (Yaish, 2015). In sage plants exposed to Cd, the proline content increased exponentially (Table 2). An increase of 1.5 to 2.45-fold was observed in 40 to 200 ppm-treated plants in 30 days, while it reached 1.7 to 2.8-fold in 60 days and a 1.2 to 2-fold increase in 90 days of treatment. Similar enhancements in protein content were also observed in *Satureja hortensis*, *Oryza sativa,*

and *Triticum aestivum* (Shen et al., 2020), (Azizi et al., 2020)2020), and (Shavalikohshori et al., 2020). Studies have shown that proline helps to reduce cadmium toxicity by acting as an antioxidant, maintaining membrane integrity, potentially mitigating osmotic stress, and serving as a mechanism to decrease acidity and prevent the accumulation of NADH, thereby supporting plant resistance mechanisms against heavy metal-induced damage and oxidative stress (García de la Torre et al., 2022), (Azizi et al., 2020).

Total phenol and flavonoid content

The total phenol and flavonoid content were found to be increasing with increasing concentrations of cadmium (Table 2). An increment of 3.8, 3.7 and 2.6-fold of phenol content was noted in the current study in 40 to 200 ppm of Cd treated plants compared to untreated plants over 30, 60 and 90 days respectively. Similarly, the flavonoid content also increased in response to Cd stress, by attaining a maximum of 28.7 mg g^{-1} in 200 ppm treated plants for 60 days. Similar kinds of enhanced production of phenol and flavonoids were also reported in *Ocimum sanctum* (Gheshlaghpour et al., 2021), and *Malva parviflora* (Zoufan et al., 2020). Phenols and flavonoids play a significant role in defense mechanisms under stress conditions, because they can act as scavengers of toxic radicals (Azimychetabi et al., 2021; Nizar et al., 2021). Likewise, the enhanced amounts of total phenol and flavonoid contents boosted the antioxidant capacity of sage plants.

Table 2. Impact of cadmium on biochemical parameters of *S. officinalis*

Cd concentrations (ppm)	**Days after treatment**	**Total protein (mg g^{-1})**	**Total carbohydrate (mg g^{-1})**	**Total proline (mg g^{-1})**	**Total phenol (mg g^{-1})**	**Total flavonoid (mg g^{-1})**
0	30 days	46.39±0.12 m	35.41±0.77 d	4.74±0.02 m	39.72±0.28 m	14.69±0.32 m
	60 days	50.45±0.34 j	47.75±0.36c	7.32±0.01 k	117.41±2.7 6k	15.9±0.36l
	90 days	50.78±0.25 ij	50.88±0.39e	8.62±0.03i	130.14±0.3 9ij	18.05±0.02 k
40	30 days	61.09±0.26 b	58.01±0.43f	10.17±0.0 2g	132.86±0.3 9i	21.18±0.1h

	60 days	62.64±0.07 a	61.97±0.8g	11.16±0.04d	137.41±1.44h	25.75±0.06 d
	90 days	60.49±0.17 b	93.22±1.06 g	11.66±0.02c	154±0.26f	27.82±0.16 b
80	30 days	58.7±0.25c d	58.79±0.32 b	4.6±0.04n	56.67±2.68l	13.68±0.2n
	60 days	60.48±0.12 b	50.41±0.68 h	7.87±0.03j	133.55±0.79hi	18.17±0.1k
	90 days	59.12±0.14 c	66.45±0.43f	9.94±0.03 h	150.59±0.6 6f	19.45±0.25 j
120	30 days	57.12±0.65 e	82.23±0.38j	10.66±0.09e	169.91±1.3 1d	22.26±0.23 g
	60 days	58.17±0.1d	97.02±0.45 k	11.98±0.05b	180.82±0.7 9b	24.66±0.25 e
	90 days	54.83±0.12 f	102.85±0.73l	12.88±0.07a	214.68±2.23a	28.71±0.06 a
160	30 days	52.76±0.18 g	71.55±0.7a	5.92±0.04l	53.61±2.47l	18.55±0.16 k
	60 days	53.34±0.15 g	45.64±0.59i	7.25±0.05 k	126.95±0.6 6j	15.64±0.16 l
	90 days	51.41±0.07 hi	59±0.09lm	8.61±0.03i	144.91±0.79g	18.3±0.29k
200	30 days	51.64±0.22 h	75.64±0.23 m	9.85±0.06 h	153.09±0.7 9f	20.36±0.23 i
	60 days	49.34±0.27 k	85.64±0.32 n	10.39±0.01f	163.32±1.1 8e	23.14±0.08 f
	90 days	47.2±0.05l	92.91±0.09 n	11.88±0.11b	174.91±1.0 5c	26.55±0.02 c

The values are represented as mean of triplicates, with ± standard error followed by alphabets obtained from Duncan multiple range test (DMRT), which indicates the variance of values with same letters are not statistically significant at $p \leq 0.05$

Effect on total chlorophyll content

Chlorophyll is the major plant pigment and the site of photosynthesis. Under Cd toxicity, the chlorophyll content in sage plants is affected significantly (Figure 2). At low concentrations, the Cd has a positive effect on chlorophyll content, with an enhancement of 17.7, 14.2 and 5 % at 30, 60, and 90 days respectively. However, a reduction of 2.7 to 17%, 10 to 18%, and 8.8 to 15.3% was noted in 80 to 200 ppm treated plants over 30, 60, and 90 days respectively. The least amount of chlorophyll content (3.2mg g^{-1}) was found in plants treated with 200 ppm of cadmium for 90 days. The reduction of chlorophyll content was also observed in plants like cucumber (Munawar et al., 2022), Brussels (Shah et al., 2020) and wheat (Halim et al., 2021). In this study, chlorophyll content initially increased and then declined with increasing Cd levels, suggesting low Cd enhances chlorophyll formation by facilitating nutrient absorption and porphyrin ring formation, while high Cd inhibits chlorophyll synthesis, likely due to nutrient competition and disrupted photosynthetic processes (Zhao et al., 2021). Apart from that, Cd disrupts the structure of chlorophyll by replacing the Mg, which also contributes to the reduced photosynthetic activity under cadmium stress (Grajek et al., 2020).

Effect on lipid peroxidation

The effect of Cd on lipid peroxidation is expressed in terms of MDA content. In sage plants Cd toxicity significantly induced lipid peroxidation (Figure 2). The MDA production enhanced 1.2 to 4.7fold in 30 days, 3.4 to 8.7fold in 60 days, 3.1 to 8.1 old in 90 days in plants treated with 40 to 200 ppm of Cd than that of control plants. Similar results were also reported in plants like sassafras, barley, peppermint and sunflower (Azimychetabi et al., 2021; Jócsák et al., 2020; Saidi et al., 2021; Zhao et al., 2021), which resulted in disrupted membrane functionality and integrity. The boosted MDA content represents the toxic impact of Cd on sage plants.

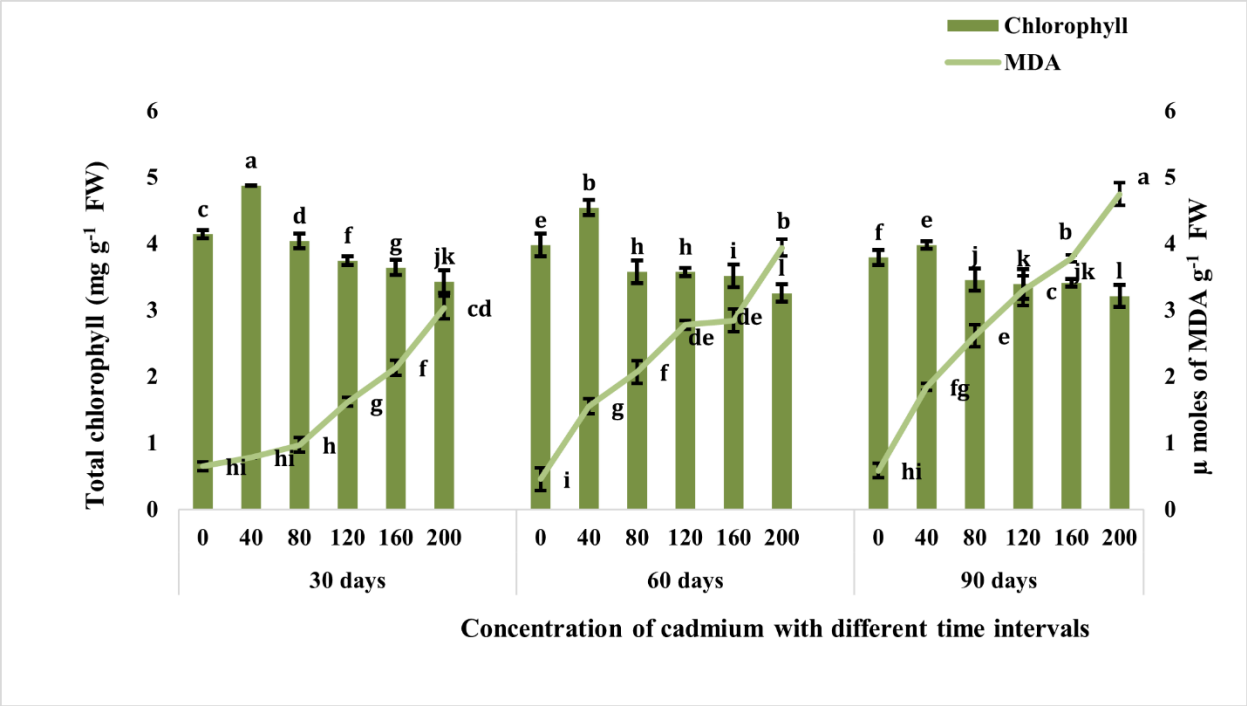

Figure 2. Effect of varying cadmium on total chlorophyll content and MDA production at different time intervals.

The data shows average values along with their standard errors obtained from three replicates, and each experiment was repeated three times. According to Duncan's multiple range test (DMRT), if means share the same letters, they are not significantly different at a significance level of $P \leq 0.05$.

Effects on antioxidant enzymes

Plants use enzymatic antioxidants as their primary defense against reactive oxygen species (ROS). In the study, the effect of Cd on the activity of SOD, APX, and catalase enzymes has been assessed (figure 3). The enzyme activity was found to be increasing from 30 to 60 days in all the treatments, but further extension of Cd exposure, caused a decline in the activity of SOD, APX and catalase in sage plants. A maximum APX activity of 50.79 micromoles min^{-1} mg of $protein^{-1}$ and catalase activity of 9.07 micromoles min^{-1}mg of $protein^{-1}$ were noted in plants treated with 200 ppm for 60 days, while control plants showed a higher SOD activity of 3.62 units mg of $protein^{-1}$ than treated samples.

Similar to our results, plants like *Menta piperita*, *Brassica napus*, and *Lactuca sativa* also exhibited an increase in APX and catalase activity under Cd stress (F. Zhang et al., 2020), (Azimychetabi et al., 2021; Hong et al., 2020). Likewise, in plants like *Pisum sativum* and *Hydrilla verticillata*, catalase is the major enzyme involved in Cd defense. (El-Okkiah et al., 2022; H. Zhang et al.,

2020). It can be inferred that SOD is not involved much in the detoxifying process of sage plants because its activity was lower in the treated plants than in the control group (Rombel-Bryzek et al., 2024). According to (Kolahi et al., 2020) the decrease in SOD activity in lettuce plants under Cd stress could be due to the diminished availability of essential elements necessary for the chemical functioning of SOD, such as zinc, iron, and manganese, which are reduced by high concentrations of Cd in plant tissues. However, plants like sorghum and rapeseeds have high SOD activity in response to Cd stress (F. Zhang et al., 2020), (Jawad Hassan et al., 2020).

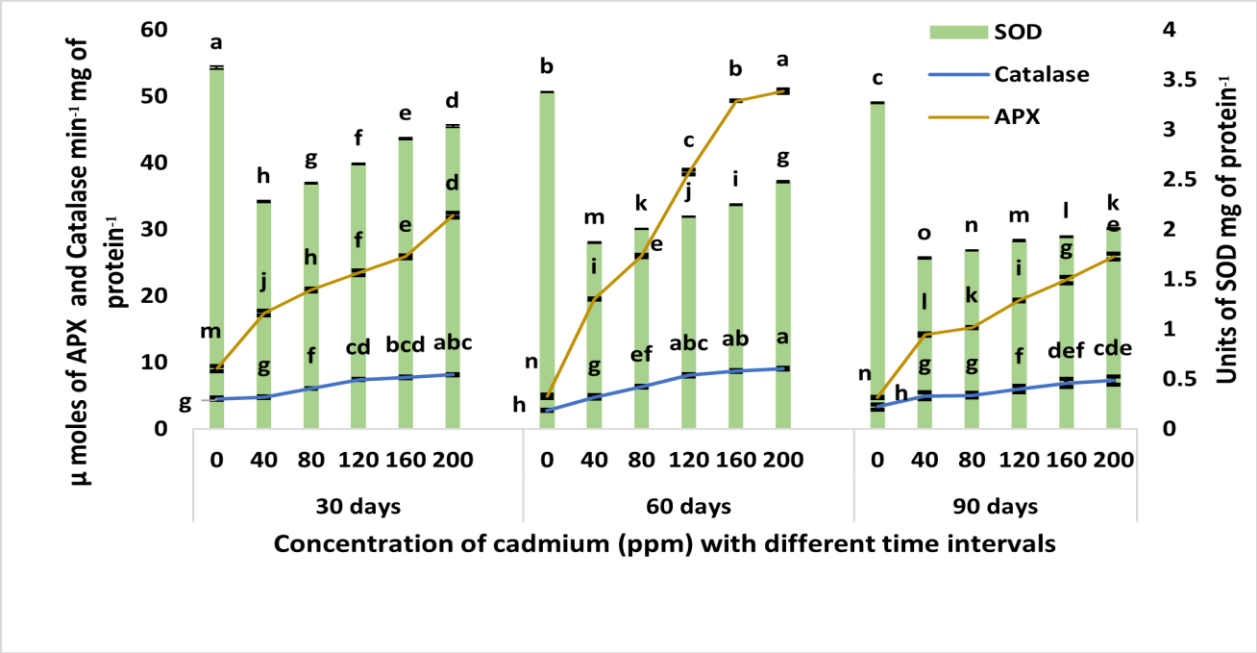

Figure 3. Effect of varying concentrations of cadmium on enzymatic antioxidants at different time intervals.

The data shows average values along with their standard errors obtained from three replicates, and each experiment was repeated three times. According to Duncan's multiple range test (DMRT), if means share the same letters, they are not significantly different at a significance level of $P \leq 0.05$.

Effect on rosmarinic acid content

Plants can respond to stress conditions by manipulating the biosynthesis of secondary metabolites, which serve as chelating agents, metal precipitators, and ROS scavengers (Anjitha et al., 2021). Rosmarinic acid (RA), one of the primary phenolic compounds present in sage plants, is well-known for its potential antioxidant qualities (Su et al., 2020). The effect of different concentrations of Cd on the biosynthesis of RA has been examined in the study. The HPLC analysis of RA has shown that Cd has detrimental effects on rosmarinic acid synthesis (figure 4). The RA content was

reduced 33 to 42% in 30 days, 76 to 88% in 60 days, and 84 to 91% in 90 days in plants treated with 40 to 200 ppm of cadmium. The control plants at 90 days showed the maximum RA production (54.9mg g^{-1}) while the Cd 200 ppm treated plants at 90 days showed the least RA production (4.51 mg g^{-1}).

Cd treatment at a dose of 1.2 mM L^{-1} stimulated the production of RA (1456 mg g^{-1} to 2450mg g^{-1}) in basil plants. (do Prado et al., 2022). Similarly, (Gheshlaghpour et al., 2021) also reported enhanced production of RA (28.87 mg g^{-1}) in *Ocimum basilicum* plants treated with 50 mg kg^{-1} of cadmium. The positive effect of cadmium on RA production in *S. militorhiza* also reported (Fu et al., 2023). However, our current research shows that RA biosynthesis in sage plants may be negatively impacted by high concentrations and exposure times of cadmium ranging from 40 to 200 ppm to 30 to 90 days. Our results are consistent with (Li et al., 2012) observed in *Artemisia annua*, where the secondary metabolite. artemisinin content decreased with the extension of Cd exposure. The ROS generated as a results of unbearable Cd toxicity might be affecting the genes of RA biosynthetic enzymes, which would have caused the reduction in RA content.

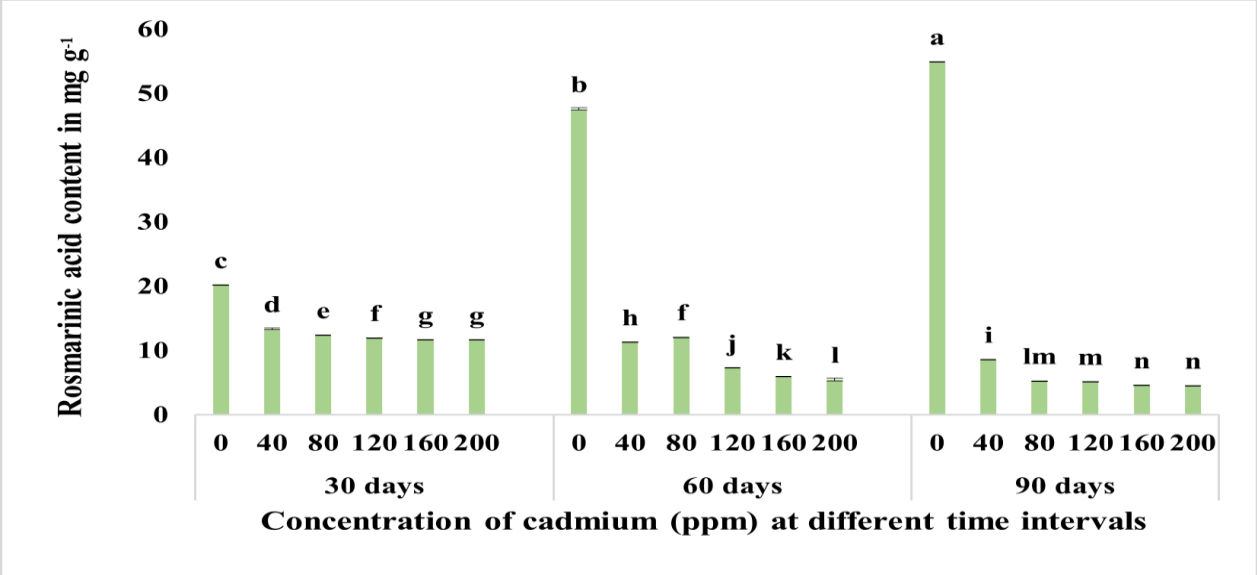

Figure 4. Effect of varying concentrations of cadmium on biosynthesis of rosmarinic acid at different time intervals.

The data shows average values along with their standard errors obtained from three replicates, and each experiment was repeated three times. According to Duncan's multiple range test (DMRT), if means share the same letters, they are not significantly different at a significance level of $P \leq 0.05$.

Conclusion

To summarize, the effects of cadmium on *S. officinalis* highlight its toxic effects, as evidenced by a significant decline in vegetative and biochemical parameters over a 30 to 90 day period. Reductions in fresh and dry weight, leaf number, shoot, and root length, coupled with elevated MDA content and decreased chlorophyll levels, underscore the plant's vulnerability to cadmium toxicity. Nevertheless, the rise in proline, phenol, flavonoid, total protein, and carbohydrate content that has been seen suggests adaptive reactions to lessen the effects of cadmium stress. Notably, the pivotal roles of antioxidant enzymes such as APX and catalase in ameliorating oxidative damage are evident, even though the SOD activity was less pronounced in Cd treated plants. Exposure to cadmium also has a major effect on the biosynthesis of rosmarinic acid. Subsequent studies are necessary to decipher the complex molecular processes that underlie the diverse reactions of cadmium at varying concentrations and times of exposure and its effects on biosynthesis of rosmarinic acid.

Acknowledgement

Rashmi R is grateful for the financial assistance in the form of an SRF (UGC. Ref No. 479/ (CSIR-UGC NET DEC 2018)) from the University Grant Commission (UGC), New Delhi. RR and PN would like to thank Dr. Jyothis Devasia for his invaluable help with the HPLC analysis. RR and PN extend their gratitude to HOD, Department of Life Sciences at CHRIST (Deemed to be University) for providing the required resources and assistance.

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