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Phytoremediation potential of *Calendula officinalis* L. and *Verbena hybrida* for chromium detoxification in tannery wastewater**

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A b s t r a c t. Chromium (Cr) contamination from tannery wastewater (TWW) poses significant environmental and health risks particularly in regions where untreated wastewater is commonly used for irrigation. This study explores the potential of ornamental plants, *Calendula officinalis* L. and *Verbena hybrida*, as promising species for Cr remediation. A completely randomized pot experiment with different TWW concentrations (0, 25, 50, 75, 100%) with and without 5 mM citric acid (CA) was conducted. Seedlings were treated twice weekly with TWW for one month. Increasing the TWW concentration reduced growth parameters, such as shoot and root length, leaf number, and biomass, with the greatest decline at 100% TWW. Photosynthetic pigments decreased, while malondialdehyde, hydrogen peroxide, and proline levels increased. Antioxidant enzyme activities peaked at 50% TWW with CA. The highest Cr accumulation occurred at 100% TWW with CA, with *C. officinalis* L. accumulating 3633 mg kg^{-1} in roots and 2780 mg kg^{-1} in shoots, and *V. hybrida* accumulating 2965 mg kg^{-1} in roots and 3673 mg kg^{-1} in shoots. *C. officinalis* L. was identified as Cr-*tolerant* (translocation factor < 1), whereas *V. hybrida* served as a Cr-*phytoextractor* (bioconcentration and translocation factors > 1). These findings underscore the potential of these plants for sustainable Cr remediation in contaminated environments.

K e y w o r d s: wastewater, chromium, citric acid, phytoextraction, phytostabilization

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1. INTRODUCTION

Kasur is one of the largest centers of tanning in Pakistan, as it is known for its extensive tanning industry (Ali *et al*., 2022; Attique *et al*., 2020). Tannery industries play a leading role in polluting the soil and water bodies with heavy metals, particularly chromium (Cr) posing a global environmental dilemma (Laxmi and Kaushik, 2020; Zaheer *et al*., 2019). Cr is ranked as the 7th most hazardous element according to the Agency for Toxic Substances and Disease Registry (Brasili *et al*., 2020). It is a non-essential heavy metal and quite persistent due to its non-biodegradable nature (Wani *et al*., 2022). Farmers use tannery wastewater (TWW) containing Cr for irrigation because of water scarcity, which depletes soil fertility, making it inappropriate for crop growth and ultimately disrupts the food chain and endangers the health of humans (Sehrish *et al*., 2019; Singh *et al*., 2023). Cr toxicity may reduce membrane stability due to the excessive build-up of reactive oxygen species (ROS), which can also harm morpho-physiological characteristics of plants (Azeez *et al*., 2021). It has been reported to be potentially toxic to plants because it prevents them from absorbing water and nutrients, reduces chlorophyll production, and disrupts enzyme functions leading to plant death (Amin *et al*., 2019).

Phytoremediation is a non-invasive, inexpensive, and aesthetically pleasing method for the treatment of metal-polluted environments (Sharma *et al*., 2023; Wang *et al*., 2020; Khalid *et al*., 2019a). Many factors, such as the characteristics of the soil and rhizosphere, bioavailability of metals, performance of plants, and selection of plant, affect the phytoremediation efficiency (Yan A. *et al*., 2020). The bioavailability of metals can be enhanced by using organic acids of low molecular weight such as citric acid (CA) as chelating agents. Organic acids are thought to be good chelating agents, as they can form complexes with metals and are less toxic and less likely to leach (Ibrahim, 2023). Several studies have reported that citric acid application improved plant growth, enhanced water usage efficiency, chloroplast content, and photosynthesis as well as antioxidant enzyme activity, while reducing ROS and malondialdehyde levels (Chen *et al*., 2020; Parveen *et al*., 2020). Enhanced uptake of metals by the application of citric acid has been demonstrated in many plants like *Brassica rapa*, *Brassica juncea*, and *Kocuria rhizophiliai* growing in metal-contaminated soils (Diarra *et al*., 2021; Hussain *et al*., 2019). The ability of citric acid to chelate and form complexes with metals in the medium allows their rapid and efficient uptake by roots of plants. The small molecular size and superior biodegradability of CA, compared to other chelators, further enhances its effectiveness in this process (Zhang *et al*., 2017).

Different plant species absorb different concentrations of heavy metals owing to differences in occurrence, growth, reproduction, and ability to sustain life in metal-contaminated soils. Due to variations in elemental uptake systems, different plant species show varying tolerance to the same contaminant in the same environmental conditions (Aqeel *et al*., 2021; Khalid *et al.,* 2019b; Zechmeister *et al*., 2003). Various plants have been employed to treat tannery wastewater. Ahmad *et al*. (2020b) highlighted the use of zinc lysine for treating tannery wastewater with maize plants, and García-Valero *et al*. (2020) treated tannery wastewater by constructing a wetland planted with *Phragmites australis*. *Calendula officinalis* L. and *Verbena hybrida* are hardy, fast-growing ornamental plants with potential tolerance to environmental stresses, making them suitable for phytoremediation of tannery wastewater, particularly in regions like Pakistan, where this is a significant concern. Despite their aesthetic value, low maintenance, and adaptability, their remediation potential with citric acid as a chelator remain underexplored. Therefore, this study was conducted to investigate the potential of citric acid to enhance chromium uptake and reduce the toxic effects of tannery wastewater on plant growth and physiological functions. It was hypothesized that the application of citric acid would enhance the chromium uptake, thereby improving the phytoremediation potential of selected ornamental plants. Specifically, our objectives were to: 1) evaluate the impact of tannery wastewater and citric acid on the growth and physiological traits of *Calendula officinalis* L. and *Verbena hybrida*; 2) analyze oxidative stress markers, antioxidant enzyme activities, and proline levels, and 3) assess the efficiency of chromium uptake and accumulation in both plant species. The research aimed to support the development of cost-effective, nature-based strategies for managing wastewater and remediating chromium-contaminated environments.

2. MATERIALS AND METHODS

2.1. Wastewater collection and analysis

Wastewater was collected in plastic containers from a tannery industry outlet located in Niaz Nagar, Kasur, Pakistan (31°06’26.1” N, 74°27’27.2” E), and transported to the laboratory of the University of Education, Lahore. Three wastewater samples were used for physicochemical analysis. The characteristics of the wastewater, analyzed following the APHA (2005) guidelines, are presented in Table 1.

2.2. Soil collection and analysis

For the experiment, the soil was collected from the PHA nursery near the University of Education, Bank Road Campus, Lahore (31°33′46.81″ N, 74°18′18.64″ E). The soil was dried in air and sieved using a 2 mm mesh to eliminate debris and unwanted materials. Prior to the experiment, three samples were randomly collected from the soil to analyze basic soil properties (Table 2). Soil pH was determined by a pH meter, and electrical conductivity was measured using an EC meter. Soil texture was assessed using the hydrometer method (Bouyoucos, 1962). Organic matter content was evaluated following the method proposed by Walkley and Black (1934), while chromium concentration was measured using a digestion protocol outlined by Khalid *et al*. (2021).

Table 1. Physicochemical parameters of wastewater utilized for irrigation

Parameter	Value
pH	3.27
EC	4 000 µS cm^{-1}
COD	2 799 mg L^{-1}
BOD	885 mg L^{-1}
TDS	3 200 ppm
TSS	300 mg L^{-1}
Sulphate	925 mg L^{-1}
Chloride	510 mg L^{-1}
Chromium	350 mg L^{-1}

Table 2. Physicochemical parameters of experimental soil

Parameter	Value
Sand	59%
Silt	19.8%
Clay	21.2%
Organic matter	0.46%
pH	7.68
EC	336 μS cm^{-1}
Chromium	0.27 mg kg^{-1}

Table 3. Treatments applied to *Calendula officinalis* L. and *Verbena hybrida*

Treatment	Concentration
T1	0% TWW (Control)
T2	25% TWW
T3	50% TWW
T4	75% TWW
T5	100% TWW
T6	CA alone
T7	25% TWW + CA
T8	50% TWW + CA
T9	75% TWW + CA
T10	100% TWW + CA

2.3. Experimental layout

A completely randomized pot experiment with three replicates (pots) for each treatment resulting in a total of sixty experimental units was executed outdoors in the experimental area of the University of Education, Bank Road Campus, Lahore, in natural environmental conditions. The average daytime temperatures ranged from 24 to 28°C, while nighttime temperatures ranged from 13 to 17°C during the experimental period. The average relative humidity was around 39%. Each pot (25 cm in length and 20 cm in diameter) was filled with sieved soil, and ten healthy seeds of *Verbena hybrida* and *Calendula officinalis* L. were sown at a depth of 2 cm. The pots were irrigated with equal amounts of tap water to support germination. Two weeks after germination, thinning was performed to maintain three uniform seedlings per pot for the duration of the experiment.

2.4. Treatment application

After thinning, the plants were irrigated twice a week for one month with varying concentrations of tannery wastewater (TWW) alone and in combination with citric acid (CA) at a concentration of 5 mM. Each pot received 500 mL of solution per application, ensuring uniformity across all treatments. Irrigation was carried out in the early morning to minimize evaporation and temperature-related stress. Plants in the control group were irrigated with an equivalent amount of tap water. The treatment combinations applied in the experiment are presented in Table 3.

Tannery wastewater was diluted with distilled water to prepare the respective treatment solutions. The experimental pots were rotated regularly to minimize any spatial effects on plant growth and development. Both plants growing under various treatments are shown in Fig. 1.

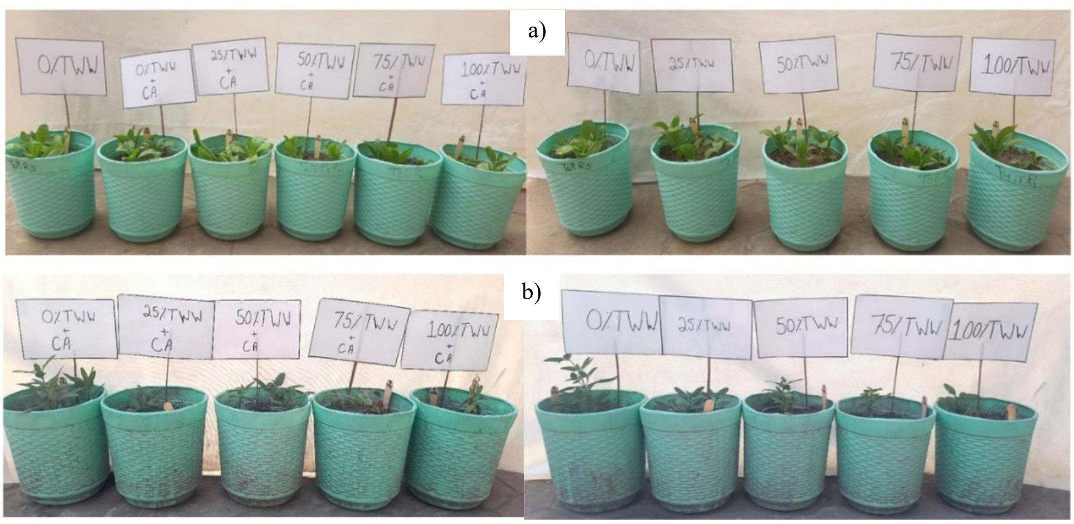

Fig. 1. *Calendula officinalis* L. a), and *Verbena hybrid* b) growing under various treatment combinations of tannery wastewater (TWW) and citric acid (CA).

2.5. Chromium concentration

The chromium content in shoots and roots of both plants was measured following the digestion protocol described by Khalid *et al*. (2021). Plant samples were digested using hydrogen peroxide (H_2O_2) and nitric acid (HNO_3). Dried plant material (0.1 g) was placed in concentrated nitric acid overnight for initial digestion. The mixture was then heated using a hot plate, gradually raising the temperature to 250°C. Hydrogen peroxide was added dropwise until the solution became colorless, indicating complete digestion. After cooling, the digested solution was filtered through Whatman no. 1 filter paper, and the volume was adjusted to 50 mL with distilled water. The chromium concentration in the samples was quantified by an atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan). To ensure accuracy, blank samples were prepared using distilled water and ultra-pure acids, and the relative standard deviation (RSD) remained below 5%. Standard solutions were prepared by diluting 1000 ppm certified metal solutions. Additionally, a standard reference material was analyzed after every ten samples to maintain quality control.

2.6. Translocation factor (TF) and bioconcentration factor (BCF)

The translocation factor was calculated using the equation given by Marchiol *et al*. (2004).

$$\text{TF} = \frac{\text{Metal concentration in shoot}}{\text{Metal concentration in root}}. \quad (1)$$

The bioconcentration factor was determined by following the equation suggested by Gosh and Singh (2005).

$$\text{BCF} = \frac{\text{Concentration of metal in shoot + root}}{\text{Initial concentration of metal in medium}}. \quad (2)$$

2.7. Growth parameters

After one month of treatment application, the plants were carefully harvested from the soil to prevent root damage. The plant samples were carefully separated into shoots and roots. The roots were washed with tap water to eliminate any adhering soil particles, while excess water was removed with paper. The length of shoot and root was recorded using a scale (cm), and the leaves were counted manually. Analytical balance (ArA - 210LC) was used for measuring the fresh weight of shoots. Later, half of the samples were oven-dried at 70°C for three days until constant weight was achieved, after which the same balance was used to determine their dry weights. The dried samples were ground into fine powder using pestle and mortar for further analysis while the remaining samples were stored in a refrigerator for biochemical analysis.

2.8. Photosynthetic pigments

The photosynthetic pigments were evaluated by applying the technique described by Arnon (1949). Fresh leaf samples (0.1 g) were crushed with a pestle and mortar using 10 mL of acetone. Using a spectrophotometer (AE-S60-4V), the absorbance of the supernatant was noted at 663, 645, and 470 nm to measure chlorophyll *a*, chlorophyll *b*, (mg g^{-1}) and carotenoid content respectively. The chlorophyll *a*, *b*, and carotenoid content was measured using the following formulas:

$$Chl_a = \frac{[(12.7 \times A_{663}) - (2.6 \times A_{645})] \times acetone}{leaf\ tissue}, \quad (3)$$

$$Chl_b = \frac{[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times acetone}{leaf\ tissue}, \quad (4)$$

$$C_{x+c} = \frac{1000A_{470} - 1.90\ Chl_a - 63.14\ Chl_b}{214}, \quad (5)$$

where: chlorophyll *a*, chlorophyll *b* (mg g^{-1}), leaf tissue (mg), *x+c* represents xanthophylls and carotenes (Rane *et al*., 2015).

2.9. Proline content

The proline content was determined using the acid ninhydrin method (Bates *et al*., 1973). Leaf samples were minced with 6 ml of 3% (w/v) sulfosalicylic acid and centrifuged at 10,000 g for 5 min. Two mL of acid ninhydrin, glacial acetic acid, and centrifuged extract were combined in a test tube. The reaction mixture was heated in a boiling water bath for 1 h and then cooled in an ice bath to stop the reaction. Subsequently, 4 mL of toluene was added and the organic phase was extracted. This led to the production of a reddish chromophore, which was measured at 520 nm using a UV-vis spectrophotometer (AE-S60-4V), with toluene as the blank.

2.10. Hydrogen peroxide (H_2O_2)

The method proposed by Velikova *et al*. (2000) was employed to quantify H_2O_2. Fresh leaf samples (0.5 g) were homogenized with 5 mL of 0.1% trichloroacetic acid and centrifuged at 12,000 g for 15 min. The supernatant (0.5 mL) was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1M potassium iodide. The absorbance was documented at 390 nm using a spectrophotometer (AE-S60-4V). The H_2O_2 content was determined by referencing a standard calibration curve constructed from various H_2O_2 concentrations.

2.11. Malondialdehyde (MDA)

Malondialdehyde was measured following Heath and Packer (1968). Leaf samples (0.2 g) were homogenized using 5 mL of 1% trichloroacetic acid (TCA) and centrifuged at 10,000 g for five minutes. The supernatant was mixed with 4 mL of 20% TCA containing thiobarbituric acid, incubated at 95°C for 30 min, cooled on ice, and centrifuged again for 10 min. Absorbance was measured at 532 nm using a spectrophotometer (AE-S60-4V), with non-specific absorbance at 600 nm subtracted. The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.12. Antioxidant enzyme activities

The procedure developed by Chance and Maehly (1955) was used to measure peroxidase activity. The reaction mixture (3 mL) contained 0.1 mL of enzyme extract, guaiacol (20 mM), H_2O_2 (40 mM), and sodium acetate buffer (50 mM) at pH 5.0. The absorbance was observed at 470 nm every 20 s using a spectrophotometer (AE-S60-4V).

Catalase activity was assessed following the method developed by Chance and Maehly (1955) by measuring the decomposition of hydrogen peroxide. The reaction mixture contained enzyme extract (25 µL), phosphate buffer (50 mM) at pH 7, and H_2O_2 (15 mM). The decline in hydrogen peroxide was evaluated at 240 nm using a spectrophotometer (AE-S60-4V). The activity was expressed in units, with one unit defined as the amount of catalase that decomposes one µmole of hydrogen peroxide per minute.

The activity of superoxide dismutase was evaluated following the method described by Giannopolitis and Ries (1977) by measuring its ability to inhibit the phytochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture (3 mL) contained potassium phosphate buffer (50 mM) at pH 7.8, riboflavin (2 mM), EDTA (0.1 mM), and NBT (75 mM). The mixture was kept 30 cm below a 30 W fluorescent lamp for 15 min. Using a spectrophotometer (AE-S60-4V), the absorbance was measured at 560 nm.

2.13. Statistical analysis

The data were statistically analyzed using analysis of variance, and various treatment means were compared for significant differences using Tukey's HSD (Honestly significant difference) test at $p \leq 0.05$. Pearson's correlation coefficient analysis ($p \leq 0.05$) was also conducted to evaluate the relationship between the chromium concentration and plant growth as well as biochemical parameters. Statistic 8.1 software was used to perform all the analyses (Analytical Software, Tallahassee, Florida, USA, 2005).

3. RESULTS

3.1. Growth parameters

The shoot and root length, shoot fresh weight, shoot and root dry weight as well as the number of leaves in both *C. officinalis* L. and *V. hybrida* varied highly significantly ($p < 0.001$) under various treatments (Table 4). The shoot and root lengths of both plant species decreased significantly with the increasing tannery wastewater (TWW) concentration, in comparison to the control. The maximum reduction was documented in T5 in both plant species. However, the citric acid (CA) application improved these parameters, compared to the wastewater treatments alone (Fig. 2a-b). Similarly, minimum shoot fresh weight in both plant species was noted in T5, where 100% wastewater was applied, while the highest weight was recorded in T6, where CA was applied (Fig. 2c). A comparable trend was observed for shoot and root dry weights as well as the number of leaves, which also showed a gradual decrease from T2 to T5, compared to the control, and enhancement under the CA application in both plants (Fig. 2d-f).

3.2. Photosynthetic pigments

The chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content in both *C. officinalis* L. and *V. hybrida* exhibited highly significant differences ($p < 0.001$) under various treatments (Table 4). A progressive decline in the chlorophyll *a*, *b*, and total chlorophyll content was noted with the increasing tannery wastewater concentration, compared to the control in both plant species. The minimum chlorophyll content was observed in T5 while the highest value was noted in T6, in comparison to the control (Fig. 3a-c). The application of citric acid alongside wastewater from T7 to T10 further improved the chlorophyll content, compared to the wastewater treatments alone. Similarly, carotenoids exhibited a comparable trend in both plant species (Fig. 3d), with the greatest reduction observed in T5, where 100% wastewater was applied. However, the application of CA in T6 augmented the carotenoid content, in comparison to the control.

3.3. Proline content

The effect of various treatments on the proline content remained highly significant ($p < 0.001$) in both *C. officinalis* L. and *V. hybrida* (Table 4). The proline content in both plant species increased from T2 to T5, showing an increase with the increasing concentration of tannery wastewater, in comparison to the control (Fig. 4a). The application of CA in combination with wastewater further enhanced the proline levels in both plants. The maximum proline content in both plant species was noted in T10, where 100% wastewater in combination with CA was applied, in comparison to the other treatments.

3.4. H_2O_2 and MDA content

The H_2O_2 content in both *C. officinalis* L. and *V. hybrida* exhibited a highly significant ($p > 0.001$) increase with the increasing concentration of tannery wastewater, in comparison to the control (Table 4). The highest H_2O_2 in both plants was noted in T5, where 100% wastewater was applied, in comparison to the other treatments. However, the H_2O_2 levels were found to be reduced when wastewater was applied in combination with CA, compared to the respective wastewater treatments without CA (Fig. 4b). Similarly, the MDA content also exhibited maximum levels

Table 4. Analysis of variance showing F-values for various parameters of *Calendula officinalis* L. and *Verbena hybrida* treated with tannery wastewater alone and in combination with citric acid

Parameter	*Calendula officinalis* L.	*Verbena hybrida*
Shoot length	79.50***	139.6***
Root length	68.42***	78.84***
Shoot fresh weight	153.5***	93.52***
Shoot dry weight	134.1***	80.03***
Number of leaves	75.80***	83.20***
Chlorophyll *a*	56.18***	56.04***
Chlorophyll *b*	2013***	376.9***
Total chlorophyll	428.5***	1766***
Carotenoid	2699***	241.6***
MDA	65.07***	55.28***
H_2O_2	79.77***	57.46***
Proline	154.5***	70.83***
SOD	22.42***	45.75***
POD	69.17***	52.04***
CAT	80.63***	62.73***
Cr in shoot	4820***	14682***
Cr in root	847.7***	4645***

***Significant at $p < 0.001$.

under the 100% wastewater treatment in both plants, while the CA application significantly lowered the MDA levels (Fig. 4c).

3.5. Antioxidant enzymes

The activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) varied significantly ($p < 0.001$) across various treatments in both *C. officinalis* L. and *V. hybrida* (Table 4). The POD activity was found to be increased under all the treatments with wastewater, compared to the control, in both plant species. Among various treatments, the 50% wastewater concentration maximally increased the POD activity in both plant species. The application of CA further enhanced the enzyme activity, and thus the highest POD activity was noted in T8 in both *C. officinalis* L. and *V. hybrida* (Fig. 4d). Similarly, CAT activity also increased from T1 to T3 and then decreased gradually up to T6; yet, it was higher than T1 in both *C. officinalis* L. and *V. hybrida*. The application of CA in combination with wastewater further enhanced the activity of CAT, compared to the wastewater treatments alone as well as the control. The highest CAT activity in both plant species was recorded in T8, where 50% TWW combined with CA was applied (Fig. 4e). The activity of SOD also showed a similar trend in both plant species (Fig. 4f).

3.6. Chromium concentration

The chromium (Cr) concentration in the roots and shoots of *C. officinalis L.* and *V. hybrida* increased significantly ($p < 0.001$) with the rising tannery wastewater concentrations, compared to the control (Tables 4, 5). The shoots and roots of *C. officinalis* L. exhibited the highest Cr concentration in T10, compared to the other treatments. The Cr accumulation in the shoots and roots increased significantly and gradually from T7 to T10 when wastewater was applied in combination with citric acid, compared to the respective wastewater treatments without CA. Similarly, in *V. hybrida*, the application of CA along with wastewater resulted in an increase in the Cr concentration in the shoots and roots, compared to the same wastewater treatments without CA. The highest Cr concentration in the shoots and roots of *V. hybrida* was noted in T10, where 100% wastewater was applied along with CA, compared to the other treatments.

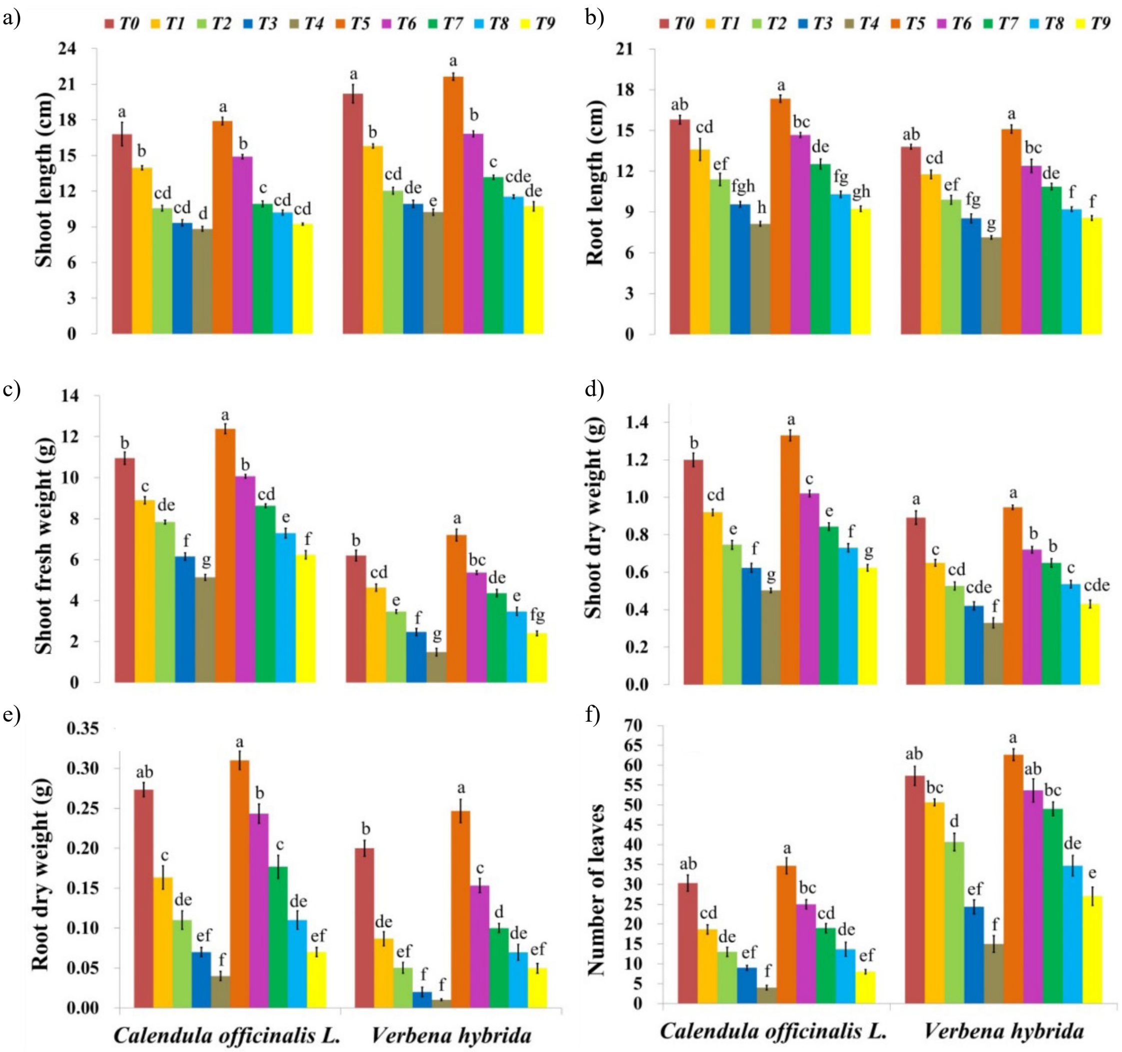

Fig. 2. Effect of tannery wastewater and citric acid (CA) treatments on: a) shoot length, b) root length, c) shoot fresh weight, d) shoot dry weight, f) root dry weight and g) number of leaves of *Calendula officinalis* L. and *Verbena hybrida*. Each bar indicates the average of three replicates (n=3) ± SE. Different alphabets denote significant differences at $p \leq 0.05$.

The translocation factor (TF) and the bioconcentration factor (BCF) for *C. officinalis* L. and *V. hybrida* are presented in Table 6. In *C. officinalis* L., BCF decreased as the tannery wastewater concentration increased. The addition of CA improved BCF across all treatments, with the highest value observed in the 25% wastewater treatment and the lowest in the 100% wastewater variant, compared to the other treatments. In all treatments of tannery wastewater with and without CA, the TF values remained below 1, indicating limited Cr translocation from roots to shoots. In *V. hybrida*, similar trends were observed for BCF and TF. The highest BCF was recorded under the 25% wastewater treatment with CA, with a gradual decrease as the wastewater concentration increased. Unlike *C. officinalis* L., the TF values for *V. hybrida* exceeded 1 in all treatments, indicating greater Cr translocation from roots to shoots.

3.7. Pearson's correlation coefficient

The relationship between the Cr concentration and various growth and biochemical parameters in *C. officinalis* L. and *V. hybrida* treated with tannery wastewater was evaluated by performing Pearson's correlation coefficient analysis (Table 7). In *C. officinalis* L., the Cr concentration exhibited a significant (at $p < 0.01$ and $p < 0.05$) negative correlation with growth parameters, including root and shoot length, shoot fresh weight, root and shoot dry weight, as well as the number of leaves. Photosynthetic pigments, including chlorophyll *a*, total chlorophyll, and carotenoids, also showed a significant ($p < 0.05$) negative correlation with the Cr concentration, while chlorophyll b exhibited a negative but non-significant ($p > 0.05$) correlation. Conversely, oxidative stress markers (proline, hydrogen peroxide, and malondialdehyde) and antioxidant enzymes (superoxide

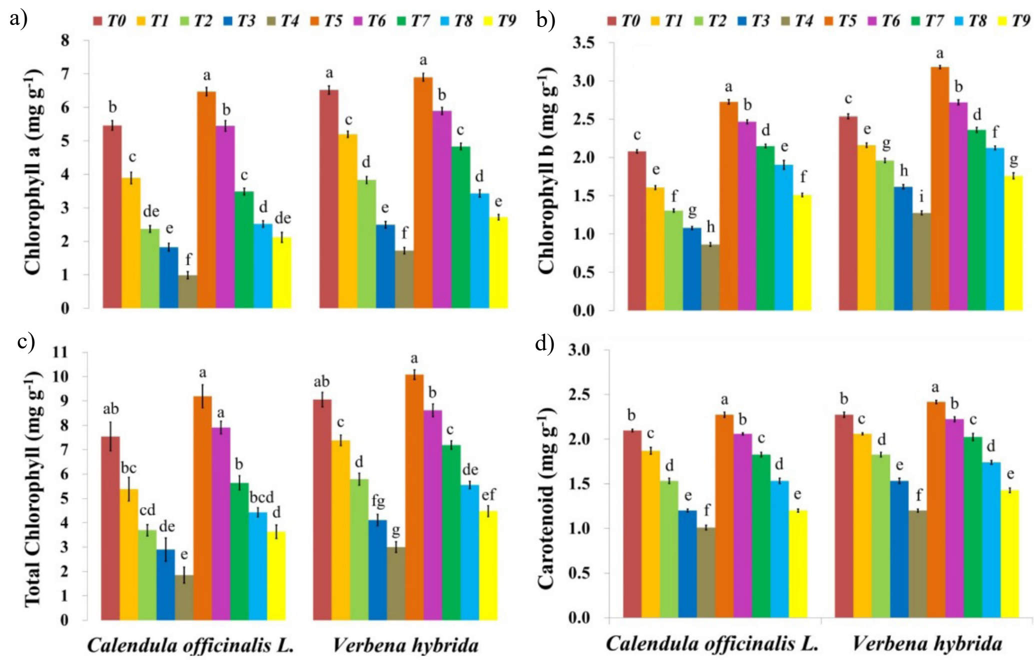

Fig. 3. Effect of tannery wastewater and citric acid (CA) treatments on: a) chlorophyll *a*, b) chlorophyll *b*, c) total chlorophyll and d) carotenoid content of *Calendula officinalis* L. and *Verbena hybrida*. Each bar indicates the average of three replicates (n=3)+SE. Different alphabets denote significant differences at p<0.05.

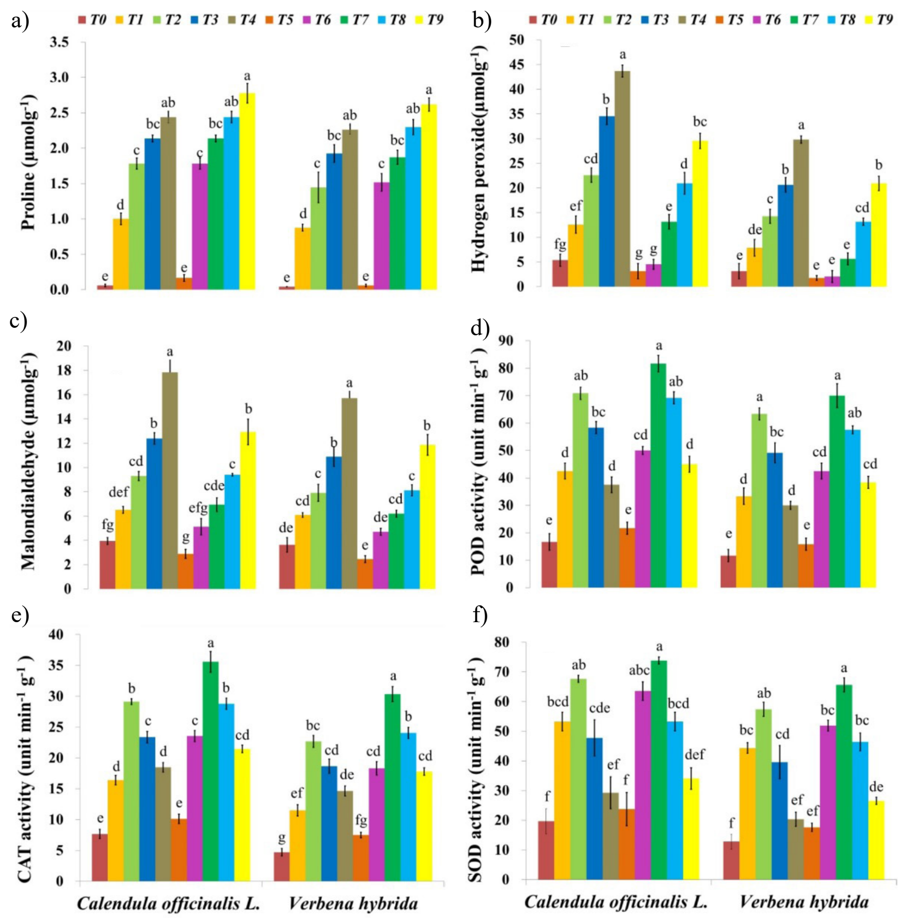

Fig. 4. Effect of tannery wastewater and citric acid (CA) treatments on: a) proline, b) hydrogen peroxide, c) malondialdehyde, d) peroxidase activity, e) catalase activity and f) superoxide dimutase activity of *Calendula officinalis* L. and *Verbena hybrida.* Each bar indicates the average of three replicates (n=3)+SE. Different alphabets denote significant differences at p<0.05.

Table 5. Effect of tannery wastewater (TWW) and citric acid (CA) on chromium concentration in shoots and roots of *Calendula officinalis* L. and *Verbena hybrida*

Treatment	Chromium concentration (mg kg^{-1})			
	Calendula officinalis L.		*Verbena hybrida*	
	Shoot	Root	Shoot	Root
Control	0 ± 0i	0 ± 0h	0 ± 0i	0 ± 0h
25% TWW	1620 ± 36h	1932 ± 34g	2002 ± 25h	1582 ± 25g
50% TWW	1802 ± 23g	2115 ± 20fg	2188 ± 12g	1738 ± 33f
75% TWW	1982 ± 33f	2268 ± 22ef	2350 ± 22f	1900 ± 22e
100% TWW	2195 ± 25e	2445 ± 40de	2585 ± 10e	2118 ± 22d
Control + CA	0 ± 0i	0 ± 0h	0 ± 0i	0 ± 0h
25% TWW + CA	2288 ± 25d	2487 ± 58d	3080 ± 22d	2125 ± 15d
50% TWW + CA	2432 ± 32c	2800 ± 132c	3250 ± 22c	2357 ± 50c
75% TWW + CA	2585 ± 26b	3133 ± 125b	3475 ± 30b	2617 ± 25b
100% TWW + CA	2780 ± 13a	3633 ± 104a	3673 ± 12a	2965 ± 18a

The data represent the average of three replicates ± S.D., and significant differences in the values within the same column at $p<0.05$ are indicated by distinct letters.

Table 6. Translocation factor (TF) and bioconcentration factor (BCF) of *Calendula officinalis* L. and *Verbena hybrida* treated with different concentrations of tannery wastewater (TWW) alone as well as along with citric acid (CA)

Treatment	*Calendula officinalis* L.		*Verbena hybrida*	
	TF	BCF	TF	BCF
Control	0	0	0.01	0
25% TWW	0.84	40	1.35	43
50% TWW	0.95	22	1.29	23
75% TWW	0.96	16	1.24	16
100% TWW	0.98	13	1.22	13
Control + CA	0.01	0.01	0.01	0.01
25% TWW + CA	0.97	56	1.31	56
50% TWW + CA	0.90	30	1.46	32
75% TWW + CA	0.86	22	1.36	23
100% TWW + CA	0.77	18	1.24	19

Each value represents the average of three replicates.

Table 7. Pearson's correlation coefficient between chromium concentration and various growth as well as biochemical parameters of plants treated with tannery wastewater

Parameter	Chromium concentration	
	Calendula officinalis L.	*Verbena hybrida*
Growth parameters		
Shoot length	-0.8278**	-0.8307**
Root length	-0.7745**	-0.7520*
Shoot fresh weight	-0.7653**	-0.7201*
Shoot dry weight	-0.8095**	-0.7515*
Root dry weight	-0.7461*	-0.7336*
Number of leaves	-0.7733**	-0.6100ns
Photosynthetic pigments		
Chlorophyll *a*	-0.7074*	-0.6759*
Chlorophyll *b*	-0.4294ns	-0.5590ns
Total chlorophyll	-0.6489*	-0.65401*
Carotenoids	-0.6599*	-0.6294ns
Antioxidant enzymes		
Superoxide dismutase	0.5618ns	0.5711 ns
Catalase	0.65812ns	0.7906**
Peroxidase	0.7216*	0.7206*
Oxidative stress parameters		
Proline	0.9568***	0.9421***
Malondialdehyde	0.6178ns	0.5909ns
Hydrogen peroxide	0.5544ns	0.4857ns

***, **, * – Significant at 0.001, 0.01 and 0.05 respectively; ns – non-significant.

dismutase, peroxidase, and catalase) were positively correlated with the Cr concentration. Similarly, in *V. hybrida*, a significant negative correlation was observed between the Cr concentration and most of the growth parameters and photosynthetic pigments. In contrast, oxidative stress markers and antioxidant enzyme activities demonstrated a positive correlation with the Cr levels in this plant species.

4. DISCUSSION

In the current study, tannery wastewater significantly reduced the root and shoot length, number of leaves, shoot fresh weight, and dry weights of both root and shoot in *C. officinalis* L. and *V. hybrida*. However, the application of citric acid exerted a positive impact on growth (Fig. 2). The growth suppression is likely due to Cr toxicity in the wastewater (Maqbool *et al*., 2018). Similar results have been obtained for *Spirodela polyrrhiza* L. treated with tannery effluent (Singh and Malaviya, 2019) and in *Spinacia oleracea* under Cr contamination (Dotaniya *et al*., 2018; Sehrish *et al*., 2019). Chromium reduced the growth and production of biomass in castor bean (Qureshi *et al*., 2020), rice (Hussain *et al*., 2018), and cauliflower (Ahmad *et al*., 2020b). The reduction in morphological traits may be attributed to impaired root ultrastructure, which limits nutrient uptake, or Cr competing with essential minerals, reducing their availability to plants (Ali *et al*., 2013). Studies have demonstrated that Cr decreases plant biomass, whereas the citric acid application improves the decreasing effect of Cr on biomass (Farid *et al*., 2019; Mahdavian, 2021; Qureshi *et al*., 2020). The improvement can be attributed to the ability of citric acid to chelate Cr, reducing its toxicity, and to enhance nutrient bioavailability, thereby supporting essential physiological processes (Rodriguez *et al*., 2012).

Chromium contamination significantly reduced photosynthetic pigments in both plants (Fig. 3), consistent with previous studies reporting declines in carotenoids and chlorophyll *a* and *b* in *Parthenium hysterophorus*, *Calotropis procera*, and sunflower exposed to heavy metal stress (Ejaz *et al*., 2022; Khalid *et al*., 2018; Saleem *et al.,* 2015). The observed decrease in chlorophyll may result from the displacement of magnesium, an essential element for chlorophyll biosynthesis, structural alterations in chloroplasts, or impeding enzymes responsible for pigment synthesis (Habiba *et al*., 2015; Rehman *et al*., 2019; Saleem *et al*., 2020a). Additionally, Cr-induced damage to chloroplast membranes further compromised the photosynthetic system (Danish *et al*., 2019; Rana *et al*., 2020). However, the citric acid application enhanced pigment concentrations by chelating Cr, reducing its toxicity, and enhancing antioxidant enzyme activity, thereby protecting chloroplast structures and sustaining photosynthesis (Shahid *et al*., 2017).

As illustrated in Fig. 4, the proline content increased with the rising TWW concentrations, consistent with reports of elevated proline levels under metal stress in various plants, such as trifoliate orange under aluminum or chickpea and olive under cadmium toxicity (Alyemeni *et al*., 2016; Yan L. *et al*., 2020). Proline acts as an osmolyte, stabilizing membranes, detoxifying reactive oxygen species, and facilitating osmotic adjustments, thereby aiding stress tolerance (Sharma *et al*., 2019). Increased proline levels under Cr stress, as observed in *Ocimum tenuiflorum* L., may function as an antioxidant to mitigate metal-induced oxidative damage (Rai *et al*., 2004). Plants treated with citric acid showed higher proline content, in comparison to the tannery wastewater-treated plants, potentially due to the role of citric acid in reducing heavy metal-induced osmotic stress by stabilizing subcellular structures and maintaining water balance (Kavi *et al*., 2015; Kaur *et al*., 2017).

The production of MDA and H_2O_2 increased with the tannery wastewater concentration in both plant species, indicating oxidative stress (Fig. 4). Comparable effects have been reported in *Brassica oleracea, Cymbopogon flexuosus,* and *Oryza sativa* exposed to heavy metals (Ahmad *et al*., 2020a; Patra *et al*., 2019; Yu *et al*., 2018). The oxidative stress caused by tannery wastewater possibly occurs due to an imbalance in the formation and scavenging of reactive oxygen species, which stimulates peroxidation of lipids and ultimately damages the cell membrane (Adhikari *et al*., 2020; Li *et al*., 2018; Saleem *et al*., 2020b). An increase in the level of MDA, which is an oxidized byproduct of membrane lipids, suggests a high risk of membrane damage instigated by Cr toxicity in TWW (Riaz *et al*., 2019). The citric acid application alleviated oxidative damage by increasing antioxidant enzyme activity, reducing ROS accumulation, and promoting plant photosynthetic efficiency and growth (Anjum *et al*., 2012; Farid *et al*., 2017; Islam *et al*., 2016).

The results indicated that mild to moderate Cr concentrations promoted the activities of all antioxidant enzymes, which were reduced at higher concentrations of tannery wastewater (TWW) (Fig. 4). Previous studies have also reported parallel findings for enzyme activity, *e.g*. Gill *et al*. (2015) in *Brassica napus* L. exposed to Cr stress and Mallhi *et al*. (2019) in castor beans under Pb stress. This dual response of antioxidants might propose that firstly they are stimulated to scavenge reactive oxygen species (ROS) but higher levels of stress caused enzyme inhibition owing to extreme oxidative impairment (Mallhi *et al*., 2019; Shahid *et al*., 2012). The scavenging of ROS is promoted by the conjugation of antioxidant enzymes with one another. Superoxide dismutase (SOD) alters superoxide radicals into H_2O_2 and O_2, while catalase (CAT) and peroxidase (POD) restrict H_2O_2 by degrading it into H_2O and O_2 (Shahid *et al*., 2014, 2016). In comparison to the TWW treatments alone, the addition of citric acid (CA) significantly enhanced enzyme activity. This improvement might be due to the role of CA in decreasing oxidative stress and promoting recovery not only through growth improvement but also by increased synthesis of photosynthetic pigments (Al Mahmud *et al*., 2018; Najeeb *et al*., 2009).

The uptake and accumulation of Cr in both *C. officinalis* L. and *V. hybrida* augmented with the concentration of tannery wastewater. The CA application further enhanced this accumulation in both plant species. The results of the present study are parallel with those of earlier studies. For example, Mobin *et al.* (2025) reported increased Pb, Cu, and Ni accumulation in *Helianthus annuus* L. with CA application, while Shakoor *et al.* (2014) observed that applying CA elevated lead levels in *Brassica napus* L. By acting as a desorbent, CA increased the mobility and solubility of metals (Cr, Ni, and Mn) in soil (Qiang *et al*., 2018). *C. officinalis* L. exhibited a higher Cr concentration in roots than shoots. Similar results for metal uptake and accumulation have also been reported for wheat, sunflower, jute, and *Brassica chinensis* L. (Ali *et al.*, 2018; Mallhi *et al.*, 2020; Parveen *et al.*, 2020; Wu *et al.*, 2013). Compartmentalization of Cr in root vacuoles serves as a defense mechanism to lessen toxicity (Kanwal *et al.*, 2014). The accumulation of Cr in roots also helps to shield the photosynthetic machinery of leaves (Conceição Gomes *et al.*, 2017). *C. officinalis* L. exhibited a translocation factor (TF) less than 1, classifying it as a chromium-tolerant non-accumulator species which can be beneficial in phytostabilization, a method that immobilizes heavy metals in the soil, reducing their bioavailability and lowering environmental concerns (Fatnassi *et al.*, 2015; Gil-Loaiza *et al.*, 2016; Guo *et al.*, 2014). *V. hybrida* accumulated higher Cr in its shoots than its roots, in contrast to *C. officinalis* L. Comparable processes have been reported in other plants, including spinach (Eid *et al.*, 2017) and certain metal-tolerant species such as *C. telephiifolia* and *D. thapsi*, which amass higher levels of arsenic (As), copper (Cu), and lead (Pb) in their shoots (García-Salgado *et al.*, 2012). Hyperaccumulators can accumulate heavy metals in their above-ground parts without detrimental effects on their physiological functions or growth (Jacobs *et al.*, 2018). *V. hybrida* exhibited both TF and BCF values higher than 1, categorizing it as a Cr phytoextractor. In phytoextraction, heavy metals are removed from contaminated soils using harvestable plant biomass, which provides a practical solution for the remediation of Cr-contaminated sites (Krzciuk and Gałuszka, 2015).

5. CONCLUSIONS

The results of the present study showed that Cr-induced toxicity from tannery wastewater significantly affected the morphological traits and photosynthetic pigments of both plants. Chromium stress induced oxidative damage by stimulating overproduction of reactive oxygen species (ROS), which elevated malondialdehyde (MDA) and H_2O_2 levels, while antioxidant enzymes (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT)) and proline accumulation helped mitigate the stress. The application of citric acid (CA) enhanced Cr uptake and improved growth and biochemical responses in both species. By sequestering Cr in roots and showing translocation factor (TF) < 1, *Calendula officinalis* L., acted as a Cr-tolerant non-accumulator species. In contrast, by efficiently translocating Cr to shoots and exhibiting bioconcentration factor (BCF) and TF > 1, *Verbena hybrida* functioned as a Cr phytoextractor. In real-world applications, these plants can be used in constructed wetlands, buffer zones near tanneries, or wastewater-irrigated areas to reduce Cr spread. We recommend using *Calendula officinalis* for stabilization of Cr-contaminated soils, while *Verbena hybrida* can be used for phytoextraction of Cr from contaminated sites. Effective post-harvest management of Cr-enriched biomass from *Verbena hybrida* is essential and should involve safe disposal methods, such as controlled incineration or a secure landfill to prevent recontamination. Further research is needed to test the above strategies on a larger scale in field conditions and assess their efficacy for other heavy metals to enhance their practical application in environmental management.

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