Full Length Research Article

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Research Journal of Agricultural Sciences
Volume 15; Issue 01 (Jan–Feb 2024); pp 254–259

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Effect of Chelators on Phytoremediation Capabilities of *Calendula officinalis* L. in Heavy Metal Treated Soils

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Received: 20 Oct 2023; Revised accepted: 30 Jan 2024; Published online: 17 Feb 2024

Abstract

The pot experiment was conducted to evaluate the phytoremediation potential of *Calendula officinalis* L. grown in Cd treated soil, using different chelators. In a pot experiment study, three chelators, including EDTA, citric acid (CA), and tartaric acid (TA), were applied to Cd treated soils (10 and 20 mg kg^{-1}) in *Calendula officinalis* L. growing pots. According to the results of experiment, *Calendula officinalis* L. grew normally without any toxicity symptoms at different Cd concentration in the soil. By increasing the concentration of Cd in the soil, its accumulation in plant also increased but does not increase in the same proportion as the concentration in the soil increases. The application of chelators, EDTA, CA and TA, also enhanced the accumulation of Cd in root and shoot of *Calendula officinalis* L. plant, among the all chelators EDTA application accumulate higher amount of Cd 204.21, 85.27 followed by CA 173.16, 78.25, TA 162.36, 76.14 mg kg^{-1} dry weight of shoot and root respectively. The increasing Cd concentration in soil also decreased dry biomass of plant. The dry biomass of plant reduced by the application of chelators due to enhanced the accumulation of heavy metals in plant parts and toxicity in soil. The maximum dry biomass was recorded in control treatment 3.25, 12.38 mg pot^{-1} root and shoots respectively, followed by CA and TA applied treatment. The results also showed that all the chelators, especially EDTA, significantly increased the Cd mobility factor in Cd treated soils. The bio-concentration factor (0.43–0.96) and translocation factor (1.75-2.39) of Cd, so these results clearly indicate the *Calendula officinalis* L. plant is a hyper-accumulator for Cd. Finally, chelators enhanced the phytoremediation potential of *Calendula officinalis* L. by facilitate the availability of heavy metals.

***Key words*:** Cadmium, *Calendula officinalis* L., Chelators, Heavy metals, Phytoremediation

The accumulation of heavy metals in soils has drawn attention in recent decades because they are non-biodegradable in nature and become hazardous in higher concentrations [39]. Among various components in the ecosystem, soil is the largest receiving body, thus it is mostly exposed to metals pollution which causing an emergent problem of heavy metals contamination in agricultural lands [40]. Heavy metals pollution in soil indicates their entry from both natural and anthropogenic sources [29]. The primary sources of metals in soils are parent materials weathering, and soil erosion moreover existence of parent materials directly associated with total heavy metals concentration in the soil [46]. In addition, various anthropogenic activities related to metals pollution in soils are metal finishing, paint, pigment, battery manufacturing, leather tanning, mining activities, foundries, smelters, human activities, urban composts, municipal waste-water sludge deposition, use of pesticide and phosphate fertilizers, etc. [38]. The pollution of heavy metals in agricultural soils has mostly resulted from the application of waste-water treatment sludge, the use of metals containing fertilizers, deposition of mining waste, and other similar anthropogenic activity [43]. Heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr) and nickel (Ni) not only alter plant biochemical and physiological cycles but also easily entering the food chain and gradually accumulating in living organisms [29].

Many techniques and methods have been tried for the production of food and fodder in polluted soils by stabilizing and/or removing heavy metals from contaminated lands. Conventionally, physical and chemical remediation techniques are used to get rid of metals from contaminated sites. These methods are highly uneconomical, besides disturbing the natural state of the soil [50]. However, phytoremediation techniques are cost-effective and eco-friendly. Whereas, phytoremediation method offers removal of heavy metals by plants thus efficiently decreases the metal content in contaminated sites [2]. Phytoremediation strategies use a particular category of plants known as hyper-accumulators to remove heavy metals from the ecosystem [29]. Hyper-accumulator plant species can accumulate metals 100 folds higher than commonly found plants and their translocation factor (TF) should be >1 [39].

Use of edible crops for phytoremediation is not a viable option as the metals accumulated by them might enter the food chain [30]. Floriculture plants offer alternative proposition

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Citation: Kumar P, Mani D, Bhushan A, Pal S, Verma J. 2024. Effect of chelators on phytoremediation capabilities of *Calendula officinalis* L. in heavy metal treated soils. *Res. Jr. Agril. Sci.* 15(1): 254-259.

being non-edible in nature and, due to their capability to remove heavy metals from contaminated soils. For remediation purposes, use of floriculture plants has additional monitory benefit and aesthetic value [22]. Marigold is an attractive and widely grown ornamental plant with a good ability to absorb heavy metals [42]. Marigold plants can grow rapidly by developing a robust root system which helps them to survive under contaminated soil environment [10]. Thus, marigold could be used to decontaminate polluted sites. The marigold species was selected as a test plant due to its wide adoptability in different soils, hassle-free cultivation practices, rapid growth rate, early maturity, less nutrients requirement, virtuous adaptation under heavy metals stress and its no edible nature. Moreover, semi-arid to sub-humid climatic condition of the experimental site is favorable for marigold cultivation. However, lack of information prompted us to evaluate two marigold species for phytoremediation of heavy metals under soils of varying nature. It was well reported that different species of the same plant (marigold) differ in their ability to accumulate metals under various contaminated soils therefore present study provides an opportunity understand the potential of pot marigold (*Calendula officinalis* L.) for phytoremidiation of heavy metals contaminated soil [41].

Calendula officinalis L. is an ornamental plant, and for this study, it is a candidate plant for the phytoremediation of heavy metal contaminated soil. It belongs to the family Asteraceae and is seldom foraged by herbivores. Its propagation rate is high, life cycle short and growth rapid. Marigolds show rapid phtyoextraction of heavy metals from soil during their initial growth phase [8]. Owing to these properties, marigolds are a good choice for the decontamination of soil; brown field treatment and landfill stabilization [8-10]. Marigolds are also used for phytoremediation of soil contaminated with crude oil [33]. Different species of Marigold plants have been employed for phytoremediation of heavy metal contaminated soils. Low availability and transferability of HMs from the soil (especially for calcareous soils) to plants is a main factor limiting HMs' uptake in accumulator plants, thus reducing the phytoremediation efficiency. In order to solve this problem, various biodegradable and non-biodegradable (synthetic) chelating agents have been widely used in the remediation of HMs-polluted soils [48]. Choosing the suitable chelating agents for phytoremediation depends on their effects on increasing the concentration of metal solutions in the soil and plant tissues, and the environment and plant growth. These chelating agents should be preferably biodegradable in the short term and should not lead to contaminate groundwater. The use of EDTA, citric acid (CA), and tartaric acid (TA) chelators (as chelating agents) in phytoremediation processes has motivated extensive research on enhanced phytoremediation [1-37]. Showed that the application of EDTA (0.5 g kg^{-1} in a single application, 20 days after seed germination and split of 0.5 g kg^{-1} in two applications of 0.25 g kg^{-1}, 20 and 40 days after seed germination) as a chelator increased the translocation index of lead (Pb) by jack beans (especially in the single application) [12]. Assessed the effects of natural low molecular- weight organic acids (NLMWOA: CA, TA, and oxalic acid, in the dose of 62.50 mmol kg^{-1}) and EDTA (0.12 mmol kg^{-1}) application (one day before transplanting to the pot) on Cu and Cd phytoextraction in tobacco (*Nicotiana tabacum*) and reported that a very small amount of EDTA (0.12 mmol kg^{-1}) was more efficient in enhancing the phytoextraction of HMs from the soil compared to a very high amount of NLMWOA (62.50 mmol kg^{-1}) [11].

MATERIALS AND METHODS

Preparation of experimental pots

The pot experiments were conducted at the Sheila Dhar institute of soil science, University of Allahabad, Prayagraj (UP). The Prayagraj is located in junction of Ganga and Yamuna rivers and having alluvial soil deposited by these rivers. The soil was collected from a depth of approximately 0-30 cm from the institute field. Plant residues and other soil impurities were carefully removed from the soil prior to the drying process. The collected soil was properly dried and carefully mixed thoroughly to make soil homogeneous. Then all the soil samples were ground to pass through 2 mm sieve and 5 kg soil filled in each pot. The physic-chemical properties of pot filled soil present in table-01. The experimental pots were treated with Cd heavy metal at the rate of 10 and 20 mg kg^{-1} soil by $Cd(NO_3).4H_2O$ salts solutions with EDTA, CA and TA at the rate 0.50 mmol kg^{-1} in soil. Each treatment was replicated thrice and completely randomized design was used. The treatment details have been present in table-02. The marigold (*Calendula officinalis* L.) was used as test crop. One month old healthy and uniform marigold seedlings were transplanted in each pot in first fortnight of September 2021, and only two healthy seedlings were kept up to maturity. The pots were irrigated regularly to maintain proper moisture for growth of marigold plants.

Determination of soil properties

The determination of total Cd in soil, by take one gram of soil sample was mixed with 5 ml of HNO_3 (16 M, 71%) and 5 ml of $HClO_4$ (11 M, 71%) then soil mixture was heated until dry of sample then added hot distilled water. The dry sample filtrate by filter paper and final sample volume maintains 50 ml the filtrate sample was used to analysis of Cd by Atomic Absorption Spectrophotometer (AAS). For the determination of available Cd from soil, take 5-gram soil was mixed with 20 ml DTPA solution {Di-ethyl-tri-amine-penta acetic acid (DTPA) solution [1.97 g (0.05 M) DTPA powder, 13.3 ml (0.1 M) Tri-ethanol amine and 1.47 g (0.01 M) $CaCl_2$ were dissolved in distilled water and maintain 1 liter sample after adjusting the pH 7.3] was added and the contents were shaken for 2 h and then filtered through Whatman filter paper No. 42. The clean filtrate was used for the estimation of Cd by the spectrophotometer.

Soil pH was determined with 1:2.5 soil–water ratios using digital pH meter at the Laboratory of Sheila Dhar Institute of Soil Science, University of Allahabad, Prayagraj, Uttar Pradesh, India. Double distilled water was used for the preparation of all solutions.

For the determination of organic carbon take fine grind one gram soil sample and digested with 10 ml of 1 N potassium dichromate ($K_2Cr_2O_7$) solution and 20 ml of concentrated sulphuric acid (18 M, 96%). The soil solution was shaken for 2 minutes and kept for half an hour and then diluted with 200 ml of distilled water. Then addition of 10 ml of Ortho-phosphoric acid (15 M, 85%) and 1 ml of diphenylamine indicator were in solution. The solution became deep violet in colour and further it was titrated against N/2 ferrous ammonium sulphate solution, till the violet colour changed to purple and finally to green [7].

The CEC of soil was determined by using neutral 1 N ammonium acetate solution. A known weight of soil (5 g) was shaken with 25 ml of the acetate solution for 5 min and filtered through Whatman filter paper No. 42 [7].

The total nitrogen in soil was determined by take 1 g fine soil sample and digested with 10 ml of digestion mixture containing sulphuric acid and selenium dioxide. Salicylic acid was also added in solution to include the nitrates and nitrites. The digestion carried out till the soil colour changed to white.

The N in the digest was estimated by using micro-Kjeldahl method, Glass Agencies, Ambala, India [7].

For the determination of total phosphorus, 10 g fine grind soil sample was taken with 4 ml $HClO_4$ (11 M, 71%) in a 50-ml beaker covered with watch glass and put on a hot plate and digestion was carried out till the soil colour changes to white. 10 ml HNO_3 (16 M, 71%) was added to the filtrate solution. Ammonia was added to saturate the solution. Then 30 ml standard ammonium molybdate solution was added in the solution to extract the total phosphorus content from soil [18].

Plant sample analysis

Plants were harvested after 90 days of transplanting. Plant samples were firstly washed by tap water than 0.2% detergent solution, 0.1 N HCL, de-ionized water and double distilled water. Plant samples then soaked with tissue paper, air dried for 2-3 days in clean environment, placed in clean paper envelopes, dried in hot air oven at a temperature of 45°C and grind in to a fine powder. Plant dry biomass weight was recorded. 1g of ground plant material was digested with15 ml of tri-acid mixture containing concentrated HNO_3 (16 M, 71%), H_2SO_4 (18 M, 96%) and $HClO_4$ (11 M, 71%) in (5:1:2). The composite was heated on hot plate at low heat (60°C) for 30 min, and the volume was reduced to about 5 ml until a transparent solution was obtained. After Cooling, 20 ml distilled water was added and the content was filtered through Whatman filter paper No. 42 [18]. The Cd was determined by Atomic Absorption Spectroscopy.

Table 1 Physico-chemical properties of studied soil

Property	Value
Sand (%)	55.54
Silt (%)	20.32
Clay (%)	24.25
pH	7.7
EC (dsm^{-1}) at 25°C	0.29
Organic carbon (%)	0.58
CEC [C mol (p^+) / kg]	20.6
Total N (%)	0.08
Total P (%)	0.039
Total Cd (mg kg^{-1})	Trace
DTPA-extractable Cd (mg kg^{-1})	Trace

±Values indicate standard deviation having three replications,
(EC) electrical conductivity
(CEC) cation exchange capacity
(DTPA) diethyl tri-amine penta-acetic acid

Table 2 Treatment details

Symbol	Treatment details
T_1	Cd 10 mg kg^{-1}
T_2	Cd 10 mg kg^{-1}+0.5 mmol kg^{-1} EDTA
T_3	Cd 10 mg kg^{-1}+0.5 mmol kg^{-1} CA
T_4	Cd 10 mg kg^{-1}+0.5 mmol kg^{-1} TA
T_5	Cd 20 mg kg^{-1}
T_6	Cd 20 mg kg^{-1}+0.5 mmol kg^{-1} EDTA
T_7	Cd 20 mg kg^{-1}+0.5 mmol kg^{-1} CA
T_8	Cd 20 mg kg^{-1}+0.5 mmol kg^{-1} TA
T_9	Control

EDTA=Ethylene diamine tetra acetic acid
CA= Citric acid, TA= Tartaric acid

Translocation factor

Translocation factor (TF) was used to determine the efficiency of phytoremediation. TF is an indication of the capacity of particular plant to movement of meals from root to areal portion of plant [24]. It is represented by the formula:

$$\text{Traslocation Factor} = \frac{\text{Metal concentration in shoot}}{\text{Metal concentration in root}}$$

Bio-concentration factor

Bio-concentration factor (BCF) can be used to evaluate the heavy metals accumulation efficiency in plants by comprising the concentration in plant and soil [3-23].

$$\text{Bio} - \text{concentration Factor} = \frac{\text{Metal concentration in shoot}}{\text{Metal concentration in soil}}$$

Statistical analysis

The pot experiment was conducted as a complete randomized design with each treatment replicated thrice. Statistical analysis of data was done following analysis of variance (ANOVA), when the ANOVA was significant that mean were separated using critical difference (CD), at $P \leq 0.05$ level of significance. The diagrams were plotted using the Graph Pad prism9.

RESULTS AND DISCUSSION

Cd concentration in root and shoot

According to the result of ANOVA analysis the effect of Cd levels and chelators on accumulation of Cd in root and shoot of *Calendula officinalis* L. were statistically significant show in figure 01. The accumulation of Cd in root (58.24 to 85.27 mg kg^{-1}) and shoot (101.32 to 204.21 mg kg^{-1}) of *Calendula officinalis* L. plant is significantly increased by increasing the concentration of Cd in soils. The Cd concentration was found higher in chelatores applied treatments 101.32, 85.84, 85.27 and 204.21, 178.46, 173.16 in compared to non-chelators applied treatments 58.24, 66.12 and 101.32, 117.43 mg kg^{-1} root and shoot respectively. The use of chelators at both level of Cd increased the accumulation of Cd in root and shoot of *Calendula officinalis* L. plant compared to without chelator treated treatments. The shoots of marigold accumulate higher amount of Cd (101.32 to 204.21mg kg^{-1}) than root (58.24 to 85.27 mg kg^{-1}). The higher accumulation of Cd in root (101.32, 85.27 mg kg^{-1}) and shoot (178.46, 204.21mg kg^{-1}) were found in EDTA applied treatments followed by CA and TA applied treatments. The results are also in conformity with that of where they evaluated the effect of EDTA and citric acid on heavy metals (Cu, Pb, Zn and Cd) uptake by *Helianthus annuus* and found that EDTA and citric acid, both have the capacity to increase the metal accumulation, but more increase in metal accumulation was noticed with EDTA treatments, than citric acid treatments [20]. The increasing heavy metal concentration in soil the potential of all chelators is reduced due to the translocation of heavy metals in the plants. The higher potential of EDTA to accumulate Cd in *Calendula officinalis* L compared to CA and TA may be due to difference in chemical structure and higher stability of Cd with EDTA than CA and TA in soil. The hydrophobocity of EDTA complex with heavy metals in the soil as a result of which more hydropholic compounds passed through the apoplastic pathway, and thus is less resistance against their entry into the cell [3-13]. The results of present study clearly indicate that *Calendula officinalis* L. potential accumulated the Cd from treated soils and addition of

EDTA, CA and TA had increased the accumulation of Cd than control treatments.

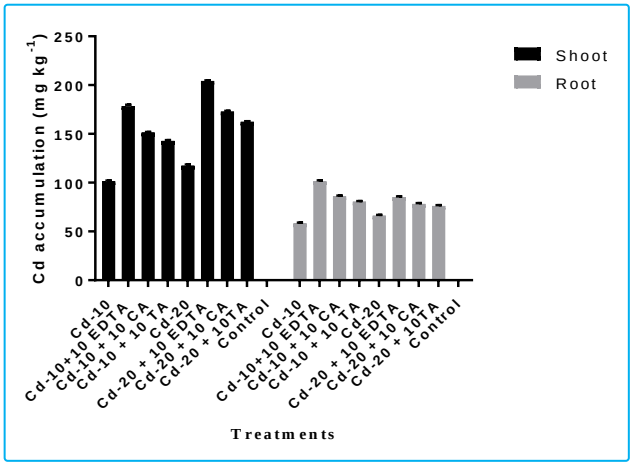

Fig 1 Accumulation of Cd in the root and shoot of *Calendula officinalis* L. under different treatments of Cd and chelators. Error bar represents the standard deviation of three measurements

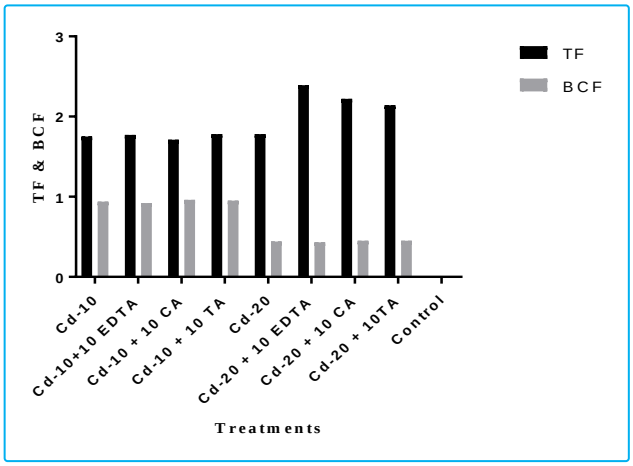

Fig 2 TF and BCF of Cd in *Calendula officinalis* L. under different treatments of Cd and chelators

TF and BCF of Cd in Calendula officinalis L.

Translocation factor and Bio-concentration factor are two vital indexes to determine the phytoremediation capability of plants. Significant effect of Cd level and chelators application on phytoremediation indices, TF and BCF was observed. TF indicates the capability of plant to translocate heavy metals from the root to the shoot and BCF represent the accumulation of heavy metal from soil to aerial portion of plant. According to the classification of hyper accumulators, plants with minimum TF of 1 are defined as hyper accumulator plants [21]. The study results present in figure 02; the TF was found more than one (>1) and BCF is less than one (<1). The highest TF recorded in EDTA (2.39) followed by CA (2.22), TA (2.14) applied treatments and BCF CA (0.96) followed by TA (0.95) applied treatments. The use of chelating agents increased the TF and BCF of Cd in *Calendula officinalis* L. The TF showed increased by the increasing Cd concentration in soil. The result clearly indicates that the *Calendula officinalis* L. plant is hyper accumulator plant for Cd. The selection of plants for phytoremediation of heavy metals depends on TF and BCF [45]. A TF more than one (>1) indicate the transfer of heavy metals from root to the shoot of plant [17]. To tolerate the heavy metals stress marigold species adopted some resistance mechanism such as blocking the absorption of heavy metals, extracellular complexation, cytoplasmic complexation, chelation and the expression of stress-inducible proteins related to heavy metals resistance [50]. The application of chelators increased TF and BCF, which can improve the Cd phytoremediation by *Calendula officinalis* L. plant.

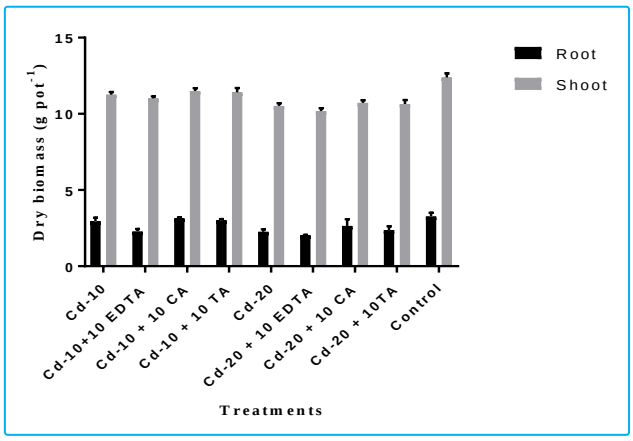

Fig 3 Dry biomass of root and shoot in *Calendula officinalis* L. under different treatments of Cd and chelators. Error bar represent the standard deviation of three measurements

Root and shoot dry weight

The experimental result showing the effect of EDTA, CA and TA chelators on dry biomass of *Calendula officinalis* L. are present in (Fig 3). In figure illustrates the effects of Cd concentration and different chelators on root and shoot dry biomass of *Calendula officinalis* L. The dry biomass of root and shoot was gradually and significantly decreased with the increase in Cd concentration in soil in the presence and absence of chelators, although no toxicity symptoms such as chlorosis or necrosis were observed in any treated plant. According to the literature, the toxic levels of Cd in soils and plants are generally between 3 and 10 mg kg^{-1} and 5 and 30 mg kg^{-1} respectively [9-16]. As a result, *Calendula officinalis* L. can tolerate Cd in the range of 10–20 mg kg^{-1} without any toxic symptoms, but its biomass is markedly reduced. As can be seen in (Fig 3), the biomass of control treatment 3.25 and 12.38 mg pot^{-1} root and shoot dry biomass respectively is higher over the Cd applied treatments (2.95, 11.26 mg kg^{-1}). The reduction of the plant biomass, as a result of Cd application, can be attributed to the accumulation of these elements in the cell wall and its entry into the cytoplasm and, ultimately, the disrupted normal metabolism of the cell [46]. The highest biomass recorded in control (3.25, 12.38 mg kg^{-1}) followed by CA (3.13, 11.48 mg kg^{-1}), TA (3.00, 11.40 mg kg^{-1}) applied treatments root and shoot respectively. Among chelators the CA applied treatments found higher dry bio-mass (3.13, 11.48 mg kg^{-1}) followed by TA (3.00, 11.40 mg kg^{-1}), EDTA (2.27, 11.00 mg kg^{-1}) applied treatments root and shoot respectively. The highest dry bio-mass in control treatment found because of the increased Cd availability to the plant and toxicity in the soil solution. Based on the previous experiment results, EDTA can destroy the root's physiological barriers that control HMs' uptake and cause a high accumulation of metals within the plant, more than its potential [44]. This activates the defense mechanisms against the toxicity of metals and consequently reduces plant growth [32]. According to Hadi et al. investigated the effect of EDTA application on Pb uptake by maize, and showed that EDTA reduces the root and shoot length of the plant[14]. Among the all applied chelators the CA increasing shoot and root dry weight biomass compared to the other treatments in Cd treated soils. It seems that this mechanism and the synthesis of phytochelatin in CA and TA treatments increased the plant dry weight compared to EDTA treatments [25]. Several studies have demonstrated

that the application of CA in heavy metals polluted environments significantly improves plant growth [32]. Reported that EDTA application (0.5 and 2 mmol kg^{-1}) in a Pb-polluted soil significantly decreased the root dry weight of Iris halophile Pall compared to the treatment without the application of a chelating agent, while CA application at the same concentration increased the root and shoot dry weight of the plant [15].

CONCLUSION

The phytoremediation potential of *Calendula officinalis* L. was studied by growing in Cd treated soil with the help of chelators. According to the results of this study, *Calendula officinalis* L. as an ornamental plant, showed a significant potential against stress, toxicity, and accumulation of Cd. The use of chelators EDTA, CA, and TA effectively increase Cd concentration in root and shoot of plant, TF and BCF compared to the non-chelators applied treatments. EDTA application improve the metals accumulation through eliminating the physiological barriers in the root by removing Fe^{2+} and Ca^{2+} cations, which play an important role in the selectivity of the plasma membrane of root cells. *Calendula officinalis* L. is a flowering ornamental plant and does not a component of human and animal food chain, has an appropriate mechanism to overcome the Cd stress in plan and has BCF <1 and TF >1 for Cd; therefore, it can be used as a Cd hyper-accumulator plant in Cd polluted soils. The study result indicates that the application of CA and TA not only increases the Cd accumulation through *Calendula officinalis* L., but also induces lower stress levels compared to EDTA and non-chelators applied treatments. In addition, due to the biodegradable nature of CA and increased efficiency of the TF of Cd than the non-biodegradable EDTA synthetic chelate, finally it is recommended that CA can be selected as the good chelator in order to increase the efficiency of Cd phytoremediation.

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