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Evaluation of multiple applications of EDTA and LMWOAs on phytotoxicity and phytoextraction of Zn, Cd, Pb and Cu in soil with *Tagetes* sp.

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ABSTRACT

Phytorextraction using chelating agents has been developed to enhance the accumulation of trace elements (TEs) by high biomass plants. Synthetic chelates like ethylenediaminetetra acetic acid (EDTA) is most effective to solubilize the TEs in the soil solution and inrease the accumulation in the plants. However, the excessive amount of TEs solubilized by EDTA in the soil can decrease the growth and biomass of plants and increase environmental risk. Low molecular weight organic acids (LMWOAs), on the otherhand are used for enhancement of phytoextraction. However, LMWOAs are less and/or not effective as these are easily biodegradable and hence there is re-precipitation and/or reabsorbtion of TEs on soil particles. In this study, we compared the performance of multiple application of EDTA with LMWOAs in enhancing phytoextraction of TEs and phytoxic effects on *Tagetes* sp., spiked in multi-metal contaminated soils. It was observed that multiple application of LMWOAs were able to enhance the accumulation of Zn, Cd, Pb and Cu without any toxic effect on plants. Net accumulation of TEs by EDTA was higher as compared to LMWOAs, but multiple application of EDTA could not minimised the toxic effect on plants.

1. INTRODUCTION

Soil can be contaminated by TEs from natural sources such as seepage from rocks, volcanic activities, forest fires, atmospheric deposition and anthropogenic activities such as mining, smelting, application of sewage sludge, TEs containing fertilizer, pesticides in agricultural land, disposal of industrial and municipal waste etc. TEs contamination of soil from anthropogenic activities presently are of great environmental concern (Fan *et al.,* 2017).

TEs are potentially toxic elements. They are non biodegradable, very toxic at lower level and they change their mobility with physio-chemical condition depending on the speciation of TEs, oxidation states, living organism, and physio-chemical properties of soil (Hamelink *et al.,* 1994). So, environmental restoration of contaminated soil with TEs is one of the great emerging issues. A number of *ex-situ* and *in-situ* technologies for remediation of TEs contaminated soil have been developed such as landfilling, soil washing, encapsulation, vitrification etc., but they are not economic nor environmental friendly. Phytoextraction is a developing technology that use plants to remove TEs from the soil by accumulating and concentrating TEs in their biomass which is also economical and eco-friendly (Ali and Chaudhury, 2016).There are two basic strategies of phytoextraction, one is the use of natural hyper-accumulating plant species that can tolerate high level of TEs in soil and are able to accumulate the TEs in their tissues. But all hyper-accumulating plants cannot be used because they are endemic, with low biomass and limited growth. The second strategy is chelate enhanced phyto-extraction using high biomass plants. Chelate act as a complexing agent to make metals solubilizes in soil solution (Guo *et al.,* 2019).

Ethylenediamine tetra acetate (EDTA) is probably the most efficient chelating agent that increase the concentration of various metals in above-ground plant tissues (Blaylock *et al.,* 1997; Huang *et al.,* 1997; Vassil *et al.,* 1998). The high efficiency of the chelators relies on the

solubilization of poorly available metals in soils (e.g. lead, chromium, copper), followed by a largely passive accumulation of metal complexes in plant shoots through the transpiration stream (Blaylock 2000; Sarret *et al.,* 2001) depending on the properties of metals and the chelators. According to Turan and Esringü (2007) the limitation in the internal transport system of the plants may be the cause of different concentration of TEs in the shoot and root of the plants. On the other hand Lai and Chan (2004) opined that EDTA form a complex with TE in the soil making it more easily available for uptake and translocation to the aerial parts of plant. However, the slow degradation rate and long persistence of EDTA in soil increases the metal eaching risk and make it unsuitable for use under field conditions (Meers *et al.,* 2005; Wu *et al.,* 2004).To overcome these issues LMWOAs are used as alternative chelating agents that have comparatively higher biodegradability, lower phytotoxicity and chelating strength. However, many researchers have found low effectiveness of LMWOAs on TEs accumulation in plants (Lombi *et al.,* 2001; Kos and Lestan 2004; Wu *et al.,* 2003; Evangelou *et al.,* 2006; do Nascimento *et al.,* 2006) due to rapid degradation of LMWOAs in soil and reprecipitation and/or reabsorbed of solubilized TEs in the soil (Alkorta *et al.,* 2004; do Nascimento *et al.,* 2006; Sabir *et al.,* 2014).

It is observed that multiple application of any chelating agent during growth period minimized the phytotoxicity and environmental problems associated with the use of chelating agent for phytoextraction (Suthar *et al.,* 2014; Wenzel *et al.,* 2003). Our previous study (Ali and Chaudhury, 2016), we observed that the application of 5, 10 and 15 mmol EDTA kg^{-1} of soil significantly increased the TEs accumulation and only 5 mmol EDTA kg^{-1} of soil treated plant was survived. In the present study, 3 mM and 8 mM of EDTA and LMWOAs were used, and phiso-chemical parameters were determined and compared to the earlier phytoextraction studies using the same plant.

The objectives of the present study were to:

- Assess the effect of multiple application of EDTA, citric acid, oxalic acid, malic acid on the phytotoxicity of *Tagetes* sp. exposed to TEs.
- Study the phytoextraction enhancement, phytoextraction efficiency and distribution of TEs in the various part of the *Tagetes* sp.
- Identify the most suitable chelating agent for phytoextraction which would be efficient as well as economic.

2. MATERIALS AND METHODS

Soil Sample Preparation for Pot Experiment

Soil sample, which was used in this study was collected from the nearest agricultural land ($23^0$41'31"N, $87^0$42'07"E) at 5-15 cm depth. The soil sample was properly mixed, dried and sieved through 2 mm to analyse the soil physiochemical properties. The collected was sieved through 4 mm for pot experiments (Dumbrava *et al.,* 2015). Selected soil properties such as soil texture, pH, EC, organic carbon, sodium, potassium, calcium, total nitrogen, available phosphorus and cation exchange capacity were analysed according to the standard procedure by International Soil Reference and Information Centre (ISRIC) and Food and Agriculture Organization (FAO) of the United Nations. Particle size of the soil was analyzed by Pipette method. Soil pH and Electrical conductivity was determined using 1:2.5 soil: water suspension by pH meter (Systonic 361) and conductivity meter (Thermo Scientific conductivity cell, Orion 013605MD). Organic carbon was determined using rapid titration method described by Walkley and Black (1934). Available nitrogen was determinate using Kjedahl instrument (Plican Kelplus-Distyl) as followed by Subbiah and Asija (1956), and available phosphorus was measured by Olsen's method (Olsen *et al.,* 1954). The Sodium, potassium, and calcium concentration in the soil were extracted by 1(N) ammonium acetate (1:5 w/v suspension) and measured by flame photometry (ELICO CL 361). Cation exchangeable capacity of the soil was determined by the method of Harada and Inoko (1980).

For pot experiments, 1 kg of soil was taken in plastic pots and treated with aqueous solution of Lead nitrate [$Pb(NO_3)_2$], Cadmium nitrate [$Cd(NO_3)_2.4H_2O$], Zinc sulphate [$ZnSO_4.7H_2O$], Copper sulphate [$CuSO_4.5H_2O$] to add concentration of 50 mg of a single metal/pot (Ali and Chaudhury, 2016). Soil humidity was maintained using distilled water 3-4 times per week to fulfil 70% water holding capacity. Each pot was fertilized with 200 g of dry and powdered cowdung. The pots were kept in natural environmental condition for 1 month to ensure the equilibrium condition. The range of ambient temperature and relative humidity were 18-30^0C and 65-75%, respectively.

Tagetes sp. was selected in this experiment because of its high biomass yield (dry biomass 2.8 g in control spiked soil) and early production rate, as well as because it can be grown in all climatic conditions with minimum care (Ali and Chaudhury, 2016). Previous studies reported that *Tagetes* sp. is capable to phytoextraction of TEs in the presence of EDTA and LMWOAs (Ali and Chaudhury, 2016; Sinhal *et al.,* 2010).

Plant Sampling and Analysis

The experiments were conducted in November, 2016 in open environment. Plants were collected from commercial market and one plant was planted in each pot. After one month, 250 ml of aqueous solution of EDTA, citric acid, oxalic acid and malic acid were added separately at concentration of 3 mM and 8 m mM, in four equal splits at

30 days, 45 days, 60 days, and 75 days after plantation in the spiked soil. Nine treatments used in the present research work coded were as control, E3, E8, C3, C8, O3, O8, M3 and M8, where E, C, O, M indicated as EDTA, citric acid, oxalic acid and malic acid respectively and 3 and 8 indicates the concentration in mm of the chelating agents.

After 3 months of plantation, the shoots and the roots were collected from each pot and washed with deionized water to remove any impurities attached to the plant surfaces. The plant samples were dried at 70°C for 48 h (Turan and Esringü, 2007). The dried plant samples were ground and digested with 15 ml of tri-acid mixture (HNO_3, H_2SO_4, and $HClO_4$ at 5:1:1 ratio) at 80°C until a transparent solution was obtained (Allen *et al.,* 1986). The concentrations of Zn, Cd, Pb and Cu in the extracts were determined by using Anodic Stripping Voltammetry (Metrohm VA 797 Computraces).

The uppermost fully extended and matured (during booming) fresh leaves were collected for phytotoxicity parameters such as chlorophyll, protein, antioxidant enzymes analysis. Chlorophyll of leaves was extracted by 85% acetone and determined spectrophotometrically at 663 and 645 nm (Arnon, 1949). Total Protein contain of the leaves was determined by Lowry Method (Lowry *et al.,* 1951) using folin as coloring agent and blovin serum albumin (BSA) as a standard. Peroxidase (POD) activity was determined spectrophotometrically at 470 nm with guaiacol (Sadasivam and Manickam, 1991). The enzyme activities were calculated by change of optical density (OD)/min/g fresh tissue. Catalase (CAT) was determined by the method suggested by Aebi (1984) to measure the rate of H_2O_2 disappearance at 240 nm. The reaction mixture contained potassium phosphate buffer (pH-7) and 1/15 M H_2O_2. The reaction was run at 25^0C for 3 min after adding the enzyme extract and the rate of decrease in absorbance per minute at 240 nm was measured to calculate the enzyme activity. The enzyme activities were calculated by the change of OD/min/g fresh tissue.

Statistical Analysis

All experiments were performed with three replicates; and the values obtained were expressed as mean ± standard error (SE). The means and SE were calculated by the Microsoft Office Excel 2013. One-way ANOVA and Duncan test was carried out with SPSS20.

3. RESULTS AND DISCUSSION

Properties of Experimental Soil

Soil physio-chemical properties and initial TEs concentration are given in Table 1. The TEs concentrations in the soil were 165.0 mg Zn kg^{-1}, 17.0 mg Cd kg^{-1}, 33.3 mg Pb kg^{-1} and 50.5 mg Cu kg^{-1}. After artificial addition of TEs solution, the final concentrations of Zn, Cd, Pb and Cu were 221.16 mg kg^{-1}, 61.64 mg kg^{-1}, 84.16 mg kg^{-1} and 109.26 mg kg^{-1}, respectively.

Table: 1
Basic properties of experimental soil

Soil Parameters	Type/ Values
Sand (%)	51.2
Silt (%)	27.7
Clay (%)	21.1
pH	6.6±0.5
Electrical conductivity (mS cm^{-1})	1.02±0.52
Organic Carbon (%)	0.91±0.17
Na (meq 100 gm^{-1})	1.12±0.10
Ca (meq 100 gm^{-1})	4.56±1.2
K (meq 100 gm^{-1})	0.03±0.01
Phosphorus (mg kg^{-1})	11.20±3.80
Available Nitrogen (mg kg^{-1})	290.22±21.50
Cation Exchange Capacity (meq 100 gm^{-1})	11.2±2.1
Zn (mg kg^{-1})	165±10.77
Cd (mg kg^{-1})	17±4.53
Pb (mg kg^{-1})	33.3±4.34
Cu (mg kg^{-1})	50.5±6.87

Effects of EDTA and LMWOAs on Plants

Earlier studies revealed that EDTA and LMWOAs have some impact on plants grown on TE contaminated soil (Wu *et al.,* 2004; Sun *et al.,* 2009, 2011; Najeeb *et al.,* 2009; Anwer *et al.,* 2012; Ali and Choudhury, 2016). Reduction of plant growth and development under various stresses are attributed to inhibition of physiological processes. Growth inhibition, breakdown of enzymatic activity and cell wall elasticity, inhibition of chlorophyll production are some of the most important toxic effects shown by TEs (Kahle, 1988; Vangronsveld and Clijsters, 1994). In the present study, it has been observed that EDTA inhibited the growth of *Tagetes* sp. Black spots were observed on the leaves of the *Tagetes* sp. exposed to 3 mM EDTA and some plant even died at 8 mM of EDTA.

Effect of EDTA and LMWOAs on Plant Growth and Biomass

Plant growth was significantly reduced on application of various chelating agents as reported by Wu *et al.* (2004); Luo *et al.* (2005); Sun *et al.* (2009, 2011). In the present study, it has been observed that EDTA inhibited the growth of the *Tagetes* sp. (Fig. 1a and 1b). After application of EDTA shoot length and biomass of the *Tagetes* sp. decreased significantly ($p<0.05$) about 19 to 35% and 35 to 42%, respectively compared to the control (Fig. 1a and 1b). Sun *et al.* (2011) found similar result with EDTA (1 g kg^{-1}), which showed 17% decrease in *Rorippa globose*as compared to control. Ebrahimi (2013) also reported that root length and shoot length were significantly reduced to about 40.90% and 72.38% and 41.43% and 65.66% under the treatment of 2.5 mmol EDTA kg^{-1} and 10 mmol

EDTA kg^{-1} of soil, respectively. In the present study, the growth reduction of plants after application of EDTA may be due to the mobilization of high amount of TEs into the soil solution and increased uptake of TEs by plants. Excess of free TEs is produced due to application of EDTA in the soil that can severely reduce cell division and plant growth (Johnson and Petras, 1998).

However, application of LMWOAs significantly increased the shoot length of *Tagetes* sp. (Fig. 1a). The plant shoot length increased by 15, 6, 16, 13, 15, and 19% under treatment of C3, C8, O3, O8, M3 and M8, respectively compared to control. On the other hand, biomass of *Tagetes* sp. after LMWOAs application also increased by 4.6, 0.7, 4.5, 2.0, 3.2 and 4.6%, respectively. Similar results were reported by several researches that on application of citric acid in the soil, the plant growth and biomass increased under metal stress in *Juncus effuses* (Najeeb *et al.,* 2009) and in *Zea mays* (Anwer *et al.,* 2012). This increase in plant growth and biomass might be due to enhanced nutrient uptake by plants (Najeeb *et al.,* 2009) or due to synthesis of phytochelation (PCs) in plants (Muhammad *et al.,* 2009).

Effects of EDTA and LMWOAs on Chlorophyll Content of the Plants

Chlorophyll content is one of the key parameters to assess the impact of environmental stress, as changes in pigment content is an indicator of plant illness and photosynthetic activity (Parekh *et al.,* 1990). Previous researchers reported that TEs hinder chlorophyll synthesis by inhibition of an enzymatic step or by reducing the uptake of essential nutrients (van Assche and Clíjsters, 1990).

In the present study, it has been observed that application of EDTA has an inhibitory effect on plant chlorophyll content (Fig. 1c). Under the treatments of E3 and E8 total chlorophyll content significantly ($p<0.05$) decreased in the leaves of *Tagetes* sp. by 83.89% and 88.52% compared to the control. Similarly, in the treatment of LMWOAs chlorophyll of *Tagetes* sp. also significantly deceased under the treatment of C8, O8, and M8 by 12, 17 and 13%, respectively, whereas no significant difference were observed in C3, O3 and M3 treated plants (Fig. 1c). The decrease in chlorophyll content may be due to accumulation of TEs by the plants. Application of EDTA and LMWOAs increased TEs accumulation to the plants (Ali and Chaudhry, 2016; do Nascimento *et al.,* 2006). These TEs stimulate the generation of free radicals and reactive oxygen species (ROS) (Flora *et al.,* 2008) which damage cell membrane, nucleic acid and chloroplast pigments (Fang and Kao 2000; Tewari *et al.,* 2002). The present results are similar with previous research finding which showed that the content of chlorophyll declined due to excess concentration of TEs (Singh *et al.,* 2013; Gill *et al.,* 2012). According to Stiborova *et al.* (1986), TEs reduce the efficiency of photosynthesis by inhibiting the key enzymes such as rubisco and phosphoenolpyruvate carboxylase of the Calvin cycle.

Effects of EDTA and LMWOAs on Protein Content of the Plants

In the present study, it has been found that EDTA significantly ($p < 0.05$) reduced the protein content of leaves in tested plant (Fig. 1d) compared to the control. Under the treatment of E3 and E8 total protein content decreased in *Tagetes* sp. by 38.53% and 58.53% (Fig. 1d) compared to the control, respectively. Plants harvested from polluted soil contaminated with TEs also had reduced protein content as depicted by earlier finding. According to Gupta *et al.* (2009) protein contents decreased probably due to more oxidative injury by TEs. When EDTA was applied

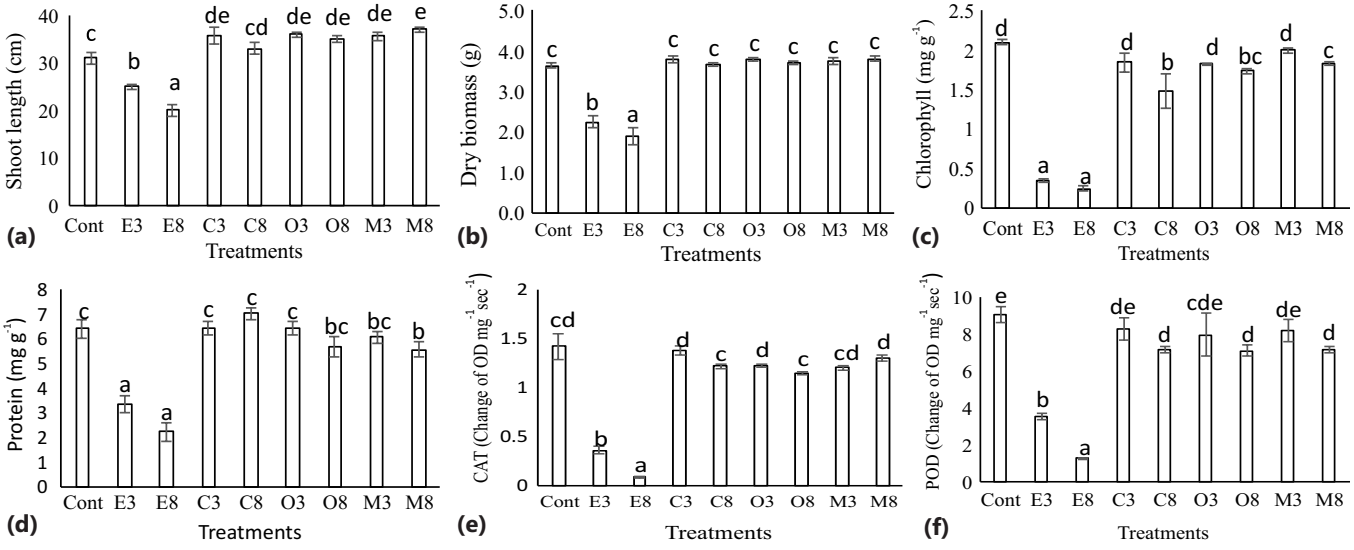

Fig. 1. Phytotoxic effect of EDTA and LMWOA on of *Tagetes* sp. (a) shoot length, (b) dry biomass, (c) chlorophyll content, (d) protein content, (e) CAT and (f) POD

in TEs contaminated soil, plants were exposed to high levels of both free TEs ions and free EDTA. TEs negatively affect the balance of minerals, e.g. Zn, Cu, Fe and Ca, leading to disturbances in cell metabolism and destabilization of biological membranes (Ruley *et al.,* 2004, 2006). Krystofova *et al.* (2009) observed similar result in protein contain in *Helianthus* sp. treated with 0, 10, 50, 100 and/or 500 µM Pb-EDTA for eight day where the total protein contents in all treated plants were much lower compared to control. Alia *et al.* (2015), noted that the protein content in Spinach reduced by 33, 29 and 20% at concentration of 1.5 mg Cd, 500 mg Pb and 700 mg Zn kg^{-1} of soil, respectively. Kanwal *et al.* (2014) also observed that Pb treatment decreased soluble protein in both roots and leaves of *B. napus*. No significant change was observed in protein content under the treatment of LMWOAs.

Effects of EDTA and LMWOAs on Antioxidant Enzyme Activity

TEs can interrupt various physico-chemical process of plants (Siedlecka *et al.,* 2001). Under normal circumstances, concentration of oxygen radicals remains low because of the activity of certain antioxidative enzymes (Asada, 1984). Free radicals or ROS are generated when plants are in stressed condition (Zengin and Munzuroglu, 2005). According to Flora *et al.* (2008) generation of free radicals and ROS are stimulated in the presence of TEs which can damage cell membranes, nucleic acids and chloroplast pigments (Fang and Kao, 2000; Tewari *et al.,* 2002). To mitigate and repair the damage originated by ROS, plants release various antioxidant enzyme such as CAT, POD and superoxide dismutases (SOD), and non-enzymic scavengers, e.g. glutathione, carotenoids and ascorbate (Xiang and Oliver, 1998; Zengin and Munzuroglu, 2005).

In this study the POD activities of *Tagetes* sp. was significantly decreased ($p < 0.05$) after application of EDTA by 63-87% with E3 and E8, respectively compared to control (Fig. 1e). CAT activities also significantly decreased by 76-94% treated with E3 and E8, respectively compared to control (Fig. 1f). The higher concentrations of LMWOAs slightly decreased the POD contain whereas in the lower concentration of LMWOAs no significant difference of POD activity were observed. Similarly, no significantly difference was observed in CAT content after application of LMWOAs. Application of EDTA released high amount of TEs which accumulate in the plants that may have damaged the cells of the leaves. According to Pandey and Sharma (2002), POD and CAT are heam (Fe) containing enzymes and TEs inhibit Fe uptake, so, the CAT and POD activity may have decreased. Another study by Schützendübel and Polle (2002) reported that POD activity can be decreased by the substitution of TEs instead of essential ions in the enzyme structure or because of increase in ROS accumulation which effects the plant signal transduction pathways.

Effects of EDTA and LMWOAs on TEs Concentration of *Tagetes* sp.

The present study revealed that addition of EDTA and LMWOAs can notably increase the concentration of TEs in the various parts of experimental plant compared to their control (*i.e. Tagetes* sp.). EDTA showed highest efficiency in accumulating TEs than any other treatments in the experimental plant.

After application of EDTA, the accumulation of Zn, Cd, Pb and Cu increased from 6.08 to 9.06, 7.29 to 11.51, 1.41 to 2.29, 3.07 to 5.55 times in roots; 8.01 to 12.07, 7.14 to 9.98, 9.23 to 5.13, 7.43 to 5.21 times in shoots and 47.89 to 59.21, 108.38 to 162.81, 16.41 to 22.57, 7.13 to 14.35 times in leaves, respectively compared to spiked control soil (Table 2 to 5). No flower bloomed under the treatment of any concentration of EDTA. Generally, the concentration of TEs in various parts of plant significantly increased with increasing EDTA concentration whereas highest concentration of Pb and Cu were found in 3 mM EDTA.

According to Meers *et al.* (2008) the binding constant of EDTA with Zn, Cd, Pb and Cu are 18, 18.1, 19.7 and 20.49, which are very high and help in dissolution of TEs from soil by increasing the mobility in the soil solution. Nowack *et al.* (2006) reported that the application of

Table: 2
Zn concentration of various plant parts of *Tagetes* sp.

Treatments	Root (mg kg^{-1})	Shoot (mg kg^{-1})	Leaf (mg kg^{-1})	Flower (mg kg^{-1})
Control	65.55±7.15^{a}	23.133±2.24^{a}	27.15±6.05^{a}	24.25±4.35^{a}
E3	398.95±49.15^{f}	185.35±11.75^{g}	1300.4±55.6^{d}	no flower
E8	593.7±16.6^{g}	279.2±6.5^{h}	1607.67±223.61^{e}	no flower
C3	148.65±2.25^{d}	65.75±1.35^{e}	104.35±3.85^{c}	72±1.8^{e}
C8	177.5±1.1^{e}	74.85±0.45^{f}	118.65±5.15^{c}	87.25±3.85^{f}
O3	90.82±3.3^{b}	33.8±0.2^{bc}	74.45±2.75^{b}	45.8±1.8^{b}
O8	121.3±16.8^{c}	45.65±0.95^{d}	103.9±1.5^{c}	63.25±2.35^{c}
M3	89.95±3.15^{b}	29±0.5^{ab}	81.25±2.45^{b}	64.4±1.5^{cd}
M8	113.7±1.6^{c}	38.8±1.4^{cd}	110.35±1.75^{c}	70.35±0.35^{de}

All values indicate Mean ± SE of three replicates. The same letters in the column are not significantly different at p<0.05 between the same tissue of different treatments according to Duncan test.

chelating agent to soil changed the metal uptake mechanism of plants depending on soil type, TEs concentration, plants types, concentration of chelating agent, mode of application, harvesting time etc. Suthar *et al.* (2014) also reported that application of 5.0 mM EDTA kg^{-1} to soil significantly increased the soluble fractions of Cd and Pb by 3.1 and 13.1 fold, respectively compared to control. This could be a reason for higher metal accumulation in the plant parts.

In the present study highest concentration of Zn, Cd and Pb were found in the aerial part of the plant (mainly leaves) after application of EDTA which were about 1300.40 to 1607.67 mg kg^{-1}, 1138 to 1709.55 mg kg^{-1} and 59.9 mg kg^{-1} to 82.4 mg kg^{-1}, respectively. While highest concentration of Cu was found in root zone which was about 212.15 mg kg^{-1} to 383.65 mg kg^{-1}. Ali and Chaudhury (2016) observed that application of EDTA at the concentration of 5, 10 and 15 mmol kg^{-1} of soil significantly increased the Zn, Cd, Pb and Cu concentrations in *Tagetes* sp. but Zn and Cd showed the highest concentration in plants leaves. Another study conducted by Wei *et al.,* (2012) observed that Cd

Table: 3
Cd concentration of various parts of *Tagetes* sp.

Treatments	Root (mg kg^{-1})	Shoot (mg kg^{-1})	Leaf (mg kg^{-1})	Flower (mg kg^{-1})
Control	15.2±0.4^{a}	9±0.96^{a}	10.5±1^{a}	5.1±0.7^{a}
E3	110.85±4.95^{f}	64.25±3.25^{f}	1138±189.95^{e}	no flowers
E8	174.95±13.15^{g}	89.8±1^{g}	1709.55±82.78^{f}	no flowers
C3	24.95±0.75^{b}	21.8±1.2^{b}	36.55±2.55^{bc}	8.3±0.4^{c}
C8	47.95±1.25^{d}	41.4±2.8^{e}	48.65±3.15^{cd}	13.5±0.4^{d}
O3	34.35±0.85^{c}	28.3±0.3^{c}	36.3±1.5^{bc}	6±0.7^{ab}
O8	56.25±4.55^{e}	35.75±2.35^{d}	61.25±3.05^{d}	8.2±0.1^{c}
M3	26.5±1^{b}	12.75±0.25^{a}	25.5±1.2^{ab}	7.05±0.45^{bc}
M8	42.5±0.8^{d}	37.65±0.95^{de}	54.45±3.25^{cd}	8.55±0.45^{c}

All values indicate Mean ± SE of three replicates. The same letters in the column are not significantly different at p<0.05 between the same tissue of different treatments according to Duncan test.

Table: 4
Pb concentration of various parts of *Tagetes* sp.

Treatments	Root (mg kg^{-1})	Shoot (mg kg^{-1})	Leaf (mg kg^{-1})	Flower (mg kg^{-1})
Control	16.2±0.6^{a}	2.934±0.55^{a}	3.65±0.35^{a}	3.95±0.15^{a}
E3	22.9±4.1^{c}	27.05±9.25^{f}	59.9±9.1^{c}	no flower
E8	37.1±1.8^{e}	15.05±2.85^{e}	82.4±8.4^{d}	no flower
C3	20.1±0.5^{bc}	9.05±0.85^{d}	13.6±0.5^{b}	14.85±1.05^{c}
C8	26.05f±0.55^{d}	10.9±0.2^{d}	14±0.4^{b}	14.05±0.35^{c}
O3	17.65±0.45^{ab}	4.9±0.1^{ab}	6.9±0.9^{a}	6.45±0.75^{b}
O8	21.85±1.55^{c}	5.25±0.85^{c}	7.25±0.55^{a}	7.55±0.35^{b}
M3	16.85±0.55^{ab}	4.3±1.5^{ab}	5.95±0.25^{a}	8.2±0.1^{b}
M8	17.75±0.35^{ab}	3.45±1.65^{ab}	7.23±1.23^{a}	6.65±0.45^{b}

All values indicate Mean ± SE of three replicates. The same letters in the column are not significantly different at p<0.05 between the same tissue of different treatments according to Duncan test.

Table: 5
Cu concentration of various parts of *Tagetes* sp.

Treatments	Root (mg kg^{-1})	Shoot (mg kg^{-1})	Leaf (mg kg^{-1})	Flower (mg kg^{-1})
Control	69.05±3.35^{a}	16.133±1.43^{a}	17.35±1.55^{a}	16.1±2.2^{a}
E3	212.15±16.9^{g}	119.9±5.6^{c}	123.65±18.65^{e}	no flower
E8	383.65±31.05^{h}	84±5.1^{d}	249±39^{e}	no flower
C3	132.7±6^{d}	22.15±0.35^{a}	32.95±3.25^{b}	10±0.2^{ab}
C8	165.8±8.8^{e}	36.4±0.5^{b}	40.1±2.2^{c}	19.5±0.3^{b}
O3	199.5±1^{f}	23.5±2.3^{a}	30.7±1.9^{b}	23.25±3.45^{b}
O8	230.75±10.55^{hg}	36±1.8^{b}	52.15±5.15^{d}	64.8±3.9^{d}
M3	86.7±4.6^{b}	20.45±1.65^{a}	29.65±1.65^{b}	33.05±1.65^{c}
M8	119.5±14.1^{c}	20.95±0.65^{a}	41.11±3.01^{c}	37.3±1.4^{c}

All values indicate Mean ± SE of three replicates. The same letters in the column are not significantly different at p<0.05 between the same tissue of different treatments according to Duncan test.

accumulation by french marigold depends on Cd concentration in soil and cultivation period and highest concentration of Cd accumulation was found in plant's aerial parts. In the present study similar trends was observed for TEs accumulation by *Tagetes* sp. where Cd and Zn were mostly accumulated in aerial parts than the roots.

Application of LMWOAs significantly increased the accumulation of TEs concentration in *Tagetes* sp. and accumulation gradually increased with the concentration of organic acids (Table 2 to 5). Among three LMWOAs treatments, the maximum accumulation of Zn and Pb in the *Tagetes* sp. were found under the treatment of citric acid, whereas the maximum accumulation of Cd and Cu in the *Tagetes* sp. were found under the treatment of Oxalic acid. Under treatment of citric acid, Zn concentration increased by 2.71, 3.2, 4.4 and 3.6 times in root, shoot, leaves and flowers compared to spiked control plant, respectively. Similarly, Cd concentration was also increased by 3.2, 4.6, 4.6 and 2.6 times in root, shoot, leaves and flowers compared to spiked control plant, respectively. Compared to the spiked control, the Pb concentration was increased 1.6, 3.7, 3.8 and 3.5 times in root, shoot, leaves and flowers, respectively. After application of citric acid, Cu concentration was increased 2.4, 2.2, 2.3 and 1.2 times in root, shoot, leaves and flowers compared to spiked control plant, respectively.

Under treatment of oxalic acid, Zn concentration increased by 1.5, 2.0, 3.8 and 2.6 times in root, shoot, leaves and flowers compared to spiked control plant, respectively. Similarly, Cd concentration was also increased by 3.5, 4.0, 5.8 and 1.6 times in root, shoot, leaves and flowers compared to spiked control plant, respectively. Compared to the spiked control, the Pb concentration was increased 1.3, 1.8, 2.0 and 1.9 times in root, shoot, leaves and flowers respectively. After application of oxalic acid, Cu concentration was increased 3.3, 2.3, 3.0 and 4.0 times in root, shoot, leaves and flowers compared to spiked control plant, respectively.

Under treatment of malic acid, Zn concentration increased by 1.7, 1.7, 4.0 and 2.9 times in root, shoot, leaves and flowers compared to spiked control plant, respectively. Similarly, Cd concentration was also increased by 2.8, 4.2, 5.2 and 1.7 times in root, shoot, leaves and flowers, compared to spiked control plant, respectively. Compared to the spiked control, the Pb concentration was increased 1.1, 1.2, 2.0 and 1.7 times in root, shoot, leaves and flowers respectively. After application of malic acid, Cu concentration was increased 1.7, 1.3, 2.4 and 2.3 times in root, shoot, leaves and flowers compared to spiked control plant, respectively.

From this result, it was also shown that the Pb accumulation by plants under the treatment of both oxalic acid (only 3 mM) and malic acid did not significantly increase due to less bio availability and less mobile nature of Pb than the other tested TEs as suggested by Prusty *et al.* (1994). It was also reveled that the citric acid was more effective than the other tested LMWOAs. Similar result was showed by do Nascimento *et al.* (2006) where the application of oxalic acid (10 mmol kg^{-1}) increased the Cd, Pb, Zn and Cu concentration in shoot of Indian Mustard by 1.03, 2.15, 1.96 and 3.9 times and application of same concentration of citric acid increased the Cd, Pb, Zn and Cu concentration by 1.61, 5.54, 1.62 and 10.29 times. This may be due to the presence of more carboxylic acid groups in citric acid which is 3 than the oxalic acid and malic acid which is 2 (Agnello *et al.,* 2014).

Enhanced phytoextraction capacity with LMWOAs was observed at all the tested concentrations but the concentration of TEs in parts of plant were significantly less when compared with EDTA-treated plants due to re-precipitation and/or re-absorbtion on soil particles of TEs released from easily biodegradable compounds. This study indicates that EDTA had greater efficiency to enhance the TEs accumulation in *Tagetes* sp. than LMWOAs. The results also confirmed with similar research (Wu *et al.,* 2003; Chaturvedi *et al.,* 2014).

It is clear that application of EDTA as a chelating agent increased the uptake of Zn, Cd, Pb and Cu in the leaves of *Tagetes* sp. (Table 2 to 5). Contrary with EDTA, Zn, Pb and Cu under the treatment of LMWOAs were stored in root zone except Cd. Low translocation of TEs to the aerial parts of the plants with LMWOAs treatment could be due to sequestration of the TEs in the vacuoles of the root cells which reduced the toxicity (Shanker *et al.,* 2005). The present research study suggested that the concentration of Zn and Cu was higher in plants part than the Cd and Pb, which may be due to the presence of higher amount of Zn and Cu in soil naturally.

Phytoremediation Efficiency

Phytoextraction efficiency is determined by the metal accumulation ability of plants and the development of optimal agronomic management practices, including soil management practices to improve metal mobilization and crop management practices to develop a commercial cropping system (Evangelou *et al.,* 2007; Barrutia *et al.,* 2009).

The removal of TEs from soil by phytoextraction is very much dependent on plant biomass, capability to uptake from soil and the fate of the TEs within the plant body. The bioaccumulation factor (BCF), translocation factor (TF) and remediation factor (RF) are used to evaluate the plant efficiency to accumulate TEs and to determine whether the plant can be used for phytoremediation or not. Uptake and translocation of an element from roots to shoots is basically linked to element speciation, soil pH, chelant concentration and degradability etc. (Song *et al.,* 2005; Zhuang *et al.,* 2005). These phytoremediation indicies are calculated as follows (Ali and Chaudhury, 2016):

$$\text{Bioconcentration factor of shoot } (BCF_{shoot}) = \frac{\text{TEs concentration in plant shoot}}{\text{TEs concentration in Soil}}$$

$$\text{Bioconcentration factor of root } (BCF_{root}) = \frac{\text{TEs concentration in plant shoot}}{\text{TEs concentration in Soil}}$$

$$\text{Translocation factor} = \frac{\text{Tes concentration in aerial parts of plants}}{\text{TEs concentration in root of plants}}$$

$$\text{Remediation factor} = \frac{\text{TEs concentration in plant x dry biomass of plant}}{\text{TEs concentration in soil x mass of soil}}$$

In *Tagetes* sp. BCF_{shoot} and BCF_{root} values of Zn, Cu, Pb and Cd highly increased in the treatment of E3 and E8 compared to the control (Fig. 2). Under these treatments (E3 and E8) highest BCF_{shoot} and BCF_{root} value observed for Cu was about 1.45 and 2.83, respectively relative to the control. This result can be in agreement with the hypothesis by Wenzel *et al.* (2003), that few protonated EDTA enters the roots and then make TEs complexes which enhance the TEs transport to the shoot. This transfer might be due to disrupting the plant metabolism via TEs stress which generally regulates the transport of metal to shoot. Application of LMWOAs also have increased the BCF_{root} value. The maximum BCF_{root} was observe for Cu in O8 treated plants about 2.11. The maximum BCF_{shoot} value was observed in the treatment of C3 about 0.35 for Cd. Highest TF value was found to about 10.85 of Cd in the treatment of E3 indicating that plants can accumulate TEs from soil and then translocate them into aerial parts and stored (Norleela *et al.,* 2014). Several fold increase of translocation of TEs from roots to aerial parts by EDTA was noted by earlier workers (Epstein *et al.,* 1999; Barrutia *et al.,* 2010). Among the LMWOAs highest TF was found about 2.67 of Cd in the treatment of C3. RF increased maximum to about 2.46 of Cu under the treatment of E3.

The present study shows that multiple applications of EDTA did not minimize the phytotoxicity nor did it significantly change the accumulation of TEs concentration as compared to the single application of EDTA, as was reported in our earlier findings (Ali and Chaudhury, 2016). On the other hand, multiple applications of LMWOAs significantly increased the accumulation of TEs (except low concentration of Malic acid for Pb) and also increased the plant growth and biomass due to supplying all the mineral nutrients (do Nascimento *et al.,* 2006) compared to the single addition of LMWOAs which could not significantly increase the TEs concentration in soil solution or accumulation by plants (Wu *et al.,* 2003; Evangelou *et al.,* 2006).

4. CONCLUSIONS

Applications of LMWOAs, had a positive effect on TEs bio-availability and enhanced the phytoextraction of TEs by *Tagetes* sp. as well as enhance the growth and biomass. Whereas applications of EDTA caused severe decrease in growth and biomass of tested plants. However, multiple application of EDTA could not overcome this problem. In respect to TEs accumulation, EDTA was much more efficient compared to LMWOAs, due to the biodegradation of the LMWOAs. However, LMWOAs can be used as phytoextraction enhancer which will not likely pose an environmental risk, as the flowers bloomed under these treatments and will also be economical.

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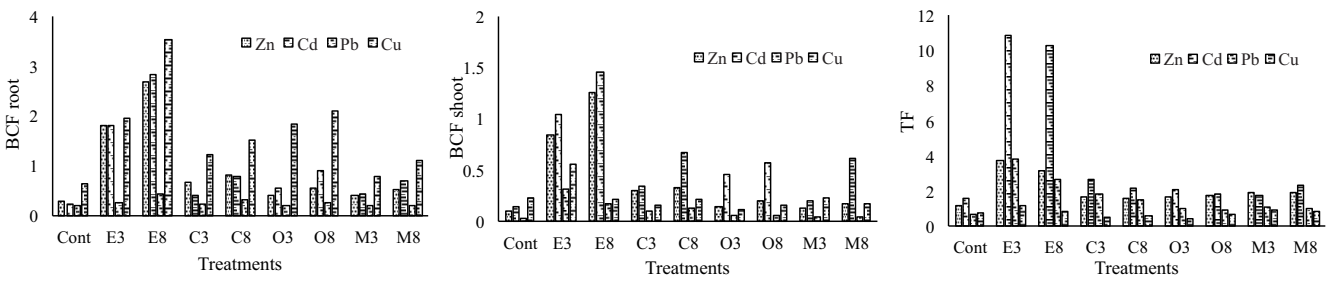

Fig. 2. Phytotoxic efficiency of EDTA and LMWOA on of *Tagetes* sp. (a) BCF root (b) BCF shoot and (c) TF

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