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Assessment of single extraction methods for the prediction of bioavailability of metals to *Brassica juncea* L. Czern. (var. Vaibhav) grown on tannery waste contaminated soil

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Abstract

Various single extractant (DTPA, EDTA, NH_4NO_3, $CaCl_2$, and $NaNO_3$) was used to evaluate the bioavailability of heavy metals from tannery wastewater contaminated soil and translocation of metals to the plant of *Brassica juncea* L. Czern. (var. Vaibhav). The extraction capacity of the metals was found in the order: EDTA > DTPA > NH_4NO_3 > $CaCl_2$ > $NaNO_3$. Cluster analysis between different extractants showed close relationship between DTPA, $CaCl_2$, NH_4NO_3 except EDTA and $NaNO_3$, which showed dispersed relationship. Principal components analysis (PCA) applied to metals extracted with EDTA showed different grouping of metals (i) Na, Co, Pb, Ni and (ii) K, Mn, Zn, Cr, in the loading plot which showed similar availability from contaminated soil. PCA applied on metals accumulation data in the plants also exhibited different grouping of variables (i) Cu, Co, Ni, Cd and (ii) Mn, Zn, Pb, Fe showed almost similar accumulation pattern in the plants. The data displayed positive loading for Mn and negative loading for Cr with PC_2. Cd and Zn have shown high loadings in PC_1 and PC_2, respectively. The translocation of most of the tested metals (Pb, Mn, Cd, Ni, Fe) in the shoot of the plant was found better except Cr, Cu, Co and K. The correlation analysis between different extractable metals and metal accumulation in the shoot of the plant showed significant positive correlation with Pb and Cr. Overall, extraction capacity and cluster analysis augmented that EDTA was found suitable extractant for tannery wastewater contaminated soil to *B. juncea*.

Keywords: Bioavailability; Translocation; Extractant; Metal uptake; Principal component analysis; Contaminated soil

1. Introduction

The prediction of bioavailability of metals is of crucial importance for the assessment of environmental quality of contaminated soil. There are several reports [1–3] to understand the processes involved in metal uptake by the plants and finding the most reliable method for the prediction of availability of an element to the plants from contaminated soil. There are many reports on bioavailability of metals to the crop plants grown on soil amended with tannery sludge [3–7]. The mobility of the metals and their bioavailability are related to eco-toxicity to the plants [8], which depend strongly on their specific chemical forms (exchangeable, carbonate bound) or binding. Thus, it is of utmost important to predict bioavailable metals rather than the total metals in order to assess toxic effects and to study bio-geochemical pathways. In this context, phytoavailability has often been defined through a one-step soil extracting procedure; however, the extent of extracting methods depend on the soil tested [3,6,9–10]. Several methods have been used to evaluate bioavailability of trace elements in soils which are based mainly on extractions by various solutions: (a) acids—mineral acids at various concentrations, (b) chelating agents—e.g., EDTA, DTPA [+TEA], (c) buffered salts—e.g., NH_4OAc, (d) neutral salts $CaCl_2$, NH_4NO_3, and (e) other extractants proposed for routine soil testing.

Recently, it has been reported [5,11] that treated tannery wastewater is being used for the irrigation of agricultural crops. These plants have shown healthy growth, probably due to irrigation with nutrient rich wastewater along with several other organic and inorganic pollutants, Cr in particular. Due to large

Abbreviations: DTPA, diethylene triamine penta acetic acid; EDTA, ethylene diamine tetraacetic acid; NH_4NO_3, ammonium nitrate; $NaNO_3$, sodium nitrate; $CaCl_2$, calcium chloride; PCA, principal component analysis

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amount of organic waste loading *via* wastewater, it may directly or indirectly alter the heavy metal status of the soil which affects the metal mobility or dissociation kinetics [12]. The amount of organic matter in soils affects the binding of heavy metals in soil and speciation in soil solution, its degradation may change the soil pH and thereby indirectly affect the bioavailability of metals [10]. The use of various single extractants (DTPA, EDTA, NH_4NO_3, $CaCl_2$, $NaNO_3$) for the prediction of bioavailability of metals from tannery sludge amended soil has been reported [3]; however, no such report is available on the soil receiving tannery wastewater.

This paper aims to assess the potential of five widely employed single extractants namely, DTPA, EDTA, NH_4NO_3, $CaCl_2$, $NaNO_3$ for the prediction of bioavailability of metals from tannery wastewater contaminated soil to the plants of *Brassica juncea*. Emphasis is put forth to select best method of bioavailability of heavy metals from contaminated soil using various statistical tools.

2. Materials and methods

The agricultural land (2100 acres) in adjoining area of Jajmau, Kanpur (Uttar Pradesh, India) used for the cultivation of various crops and up-flow anaerobic sludge blanket (UASB) treated tannery wastewater is being used for the irrigation since last many decades. The wastewater was collected in acid washed bottles for physico-chemical analysis. Such contaminated soils were collected from the agricultural fields in large plastic bags and brought to the field laboratory which was used for experimental studies. The contaminated soil was air-dried, finely ground and sieved with 2 mm mesh size before use.

2.1. Experimental design

Seeds of *Brassica juncea* L. Czern. (var. Vaibhav) were obtained from Chandra Shekhar Azad Agricultural University, Kanpur. Seeds were sterilized with 0.1% mercuric chloride, and soaked seed were evenly sown in pots (30 cm), which were filled with contaminated soil (12 kg) in three replicates. Ten seeds were sown in each pot to a depth of 0.5 cm and watered daily till seed germination. When the seeds were developing 5 or 6 leaves, they were thinned out to retain 4 uniform ones per pot and allowed to grow. The plants were irrigated with tap water at regular intervals avoiding leakage of water from the pots. The plants were uprooted from the pots after 60 days of sowing with the help of fine jet of water, causing minimum damage to the roots, washed thoroughly with running double distilled water, and blotted dry. Roots and shoots were separated manually, cut in small pieces and oven dried (80 °C).

2.2. Physico-chemical parameters

Various physico-chemical parameters of wastewater (total dissolved solids, biological oxygen demand, chemical oxygen demand) and contaminated soil, i.e., soil texture, water holding capacity (WHC), bulk density (BD), organic carbon (OC), organic matter (OM), cation exchange capacity (CEC) were estimated [13]. Wastewater and soil pH (1:2 soil water suspensions) were measured using Orion pH meter (Model 420). The salinity and electrical conductivity (EC) were measured using Orion Conductivity Meter (Model 150) in the soil (1:2 soil water suspensions).

2.3. Single extraction and total metals

The extractants namely NH_4NO_3, $NaNO_3$ and $CaCl_2$ are mild or cationic exchange extractant, whereas, EDTA and DTPA categorized as complexation. DTPA extractable fraction was obtained by mechanical shaking of sample (10 g) with 20 ml of 0.005 M DTPA, 0.01 M $CaCl_2$, 0.1 M triethanolamine (TEA) buffered at pH 7.3 for 2 h [14]. For $CaCl_2$ extraction, 5 g soil with 50 ml of 0.01 mol l^{-1} $CaCl_2$ solution were mechanically shaken for 2 h [15]. For EDTA extraction, 5 g soil with 25 ml of 0.05 M Na-EDTA solution were mechanically shaken for 1 h [16]. In case of NH_4NO_3 extraction [17], 10 g of soil was added in 50 ml of 1 M NH_4NO_3 and shaken for 2 h at room temperature. For $NaNO_3$ extraction, 10 g soil with 25 ml of 0.1 M $NaNO_3$ solution was mechanically shaken for 2 h [18]. For the estimation of total metals' content in the soil, 0.50 g soil + 10 ml double distilled water + 5 ml HNO_3 + 4 ml HF + 1 ml HCl was used and digested in Microwave Digestion System MDS 2000 in closed Teflon vessels for 120 min at 630 W and 120 PSI. The digested solution (without evaporation) is used for the estimation of metals using GBC Avanta Σ, Atomic Absorption Spectrophotometer (AAS). All the analyses were carried out in triplicates.

2.4. Metal accumulation

After dry weight determination, the oven-dried plant samples were ground and 0.4 g plant samples digested in 10 ml HNO_3 (70%) using a Microwave Digestion System (MDS 2000) for 20 min at 630 W and 40 PSI and metal contents were estimated using Atomic Absorption Spectrophotometer (GBC Avanta Σ).

2.5. Statistical analysis

The analytical results were compiled to form a multi-elemental database using Excel and Statistica. Statistical analysis, including principal component analysis (PCA), was performed using Statistica software. In PCA, the principal component was calculated based on the correlation matrix. Varimax with Kaiser Normalization was used as the rotation method in the analysis.

2.6. Quality control and quality assurance

Method validation (accuracy and repeatability) was performed by analyzing sewage sludge samples of Resource Technology Corporation (EPA certified reference material) (Catalog No. CRM 029-050; Lot No. JC029a) and results were found to be within Prediction Intervals. The blanks were run in triplicate to check the precision of the method with each set of samples.

Table 1
Certified and observed values of elements

S. nos.	Code nos.	Elements	Certified values	Observed values	Accuracy %
1	BND 1101.02	Zn	99.69 ± 0.94	101.34 ± 0.87	98.35
		Fe	100.12 ± 0.78	99.32 ± 0.44	99.29
		Cu	99.95 ± 0.84	101.43 ± 0.51	98.52
2	BND 102.03 (Pb)		2.01 ± 0.02	2.05 ± 0.03	98.51
3	BND 402.02 (Cr)		2.00 ± 0.02	1.98 ± 0.03	99.00
4	BND 1001.02 (Ni)		1.00 ± 0.02	0.99 ± 0.01	99.00

The reference solution of multi-elements and single element was used for calibration of analytical equipment and validation of test methods provided by National Physical Laboratory (NPL), New Delhi and their certified and observed values are given in Table 1.

3. Results and discussion

3.1. Physico-chemical analysis

The physico-chemical properties of treated tannery wastewater (Table 2) analyzed. The texture of soil, i.e., sand (47.3%), silt (37.0%) and clay (15.8%) was analyzed. The level of pH (6.78 ± 0.01), EC (724.12 ± 0.5 μS cm^{-1}) and salinity (1.74‰), CEC (33.24 ± 4.1 cmol kg^{-1}), OM (0.99 ± 0.1%) and OC (0.57 ± 0.0%) of the contaminated soil was found high due to long term irrigation with treated tannery wastewater. The physico-chemical characteristics of soil are known to regulate the fate of the metals, which include: pH, CEC, OM, oxides and clay minerals was largely influence the rhizospheric processes [12]. Soil pH affects not only metal bioavailability, but also vary the process of metal uptake into roots. Gupta and Sinha [3] reported that metal ions can be complexed with organic matter altering their availability to the plants. The COO^- groups in both solid and dissolved organic matter form stable complexes with metals. Thus, the opportunity for forming stable metal-organic matter complexes increases with an increase in the amount of organic matter. In general, the plants are unable to absorb the large metal-complexes and so the bioavailability of metals decreases. Extraction of heavy metals is usually limited by availability of metals from the soil. Liphadzi and Kirkham [10] augmented that high organic matter and cation exchange capacity are some of the most important soil factors which determine the bioavailability of metals to the plants. After harvesting the plants, the root (17.2 ± 2.3 cm) and shoot (64.0 ± 7.2 cm) lengths and dry biomass (8.5 ± 2.1 g) were recorded.

Table 2
Physico- chemical analysis of UASB treated tannery wastewater

Parameters	Data
pH	7.12 ± 0.02
EC (μS cm^{-1})	8.30 ± 0.01
Salinity (‰)	1.74 ± 0.007
Total dissolved solids (mg l^{-1})	7700 ± 112
Biological oxygen demand (mg l^{-1})	270 ± 28
Chemical oxygen demand (mg l^{-1})	1350 ± 106
Metals (μg ml^{-1})	
Fe	3.93 ± 0.58
Cr	3.20 ± 0.52
Zn	0.39 ± 0.03
Mn	0.12 ± 0.007
Cu	Not detected
Ni	Not detected

All the values of mean of three replicates ± S.D.

3.2. Metal accumulation in plants

The plants of *B. juncea* grown on tannery wastewater contaminated soil accumulate appreciable amount of heavy metals (Fig. 1). Among all the tested metals, the accumulation of K was found maximum, whereas, Co found minimum in the plant. Overall, the total metal accumulation was found in the order: K > Na > Fe > Zn > Mn > Cr > Pb > Cu > Ni > Cd > Co. Singh and Sinha [4] also reported that the plants of *Brassica juncea* L. Czern. (var. Vaibhav) grown in different amendment of tannery sludge have shown high accumulation of metals. Recently, Sinha et al. [5] documented that the plants grown on contaminated soil collected from Jajmau (Kanpur) have shown accumulation of metals in all the studied plants, which vary from one plant to another. In the present study, the accumulation of different metals and their distribution in the *B. juncea* were found different. The translocation of K, Cu, Co and toxic metal (Cr) was restricted in lower parts of the plants, whereas, the rest of the tested metals were better translocated to upper parts. The process of metal accumulation by different plants also depends on the concentration of available metals in soils and their mobility and the plant species growing on these soils [6,7,10,19].

Generally, the concentration of metals in shoots is lower than in roots [3–5,7], which may be due to the complexation and sequestration in cellular structures (e.g., vacuole) in the plant and unavailable for translocation to shoot. The transport of metals from roots to shoots includes long distance translocation in the xylem and storage in the vacuole of leaf cells and these processes affected by many factors such as organic acids [20].

The shoot to root ratio of Pb (3.76), was found maximum, and minimum in case of Co (0.60). Overall, the translocation of metals in *B. juncea* have shown following order: Pb > Mn > Cd > Ni > Fe > Zn > Na > K > Cr > Cu > Co. Recently, Sinha et al. [5] reported less translocation of metals in the upper parts of the vegetables/crops grown on contaminated soil than lower parts. In contrast, report on same experimental plant, *Brassica juncea* L. Czern. (var. Vaibhav) grown on different

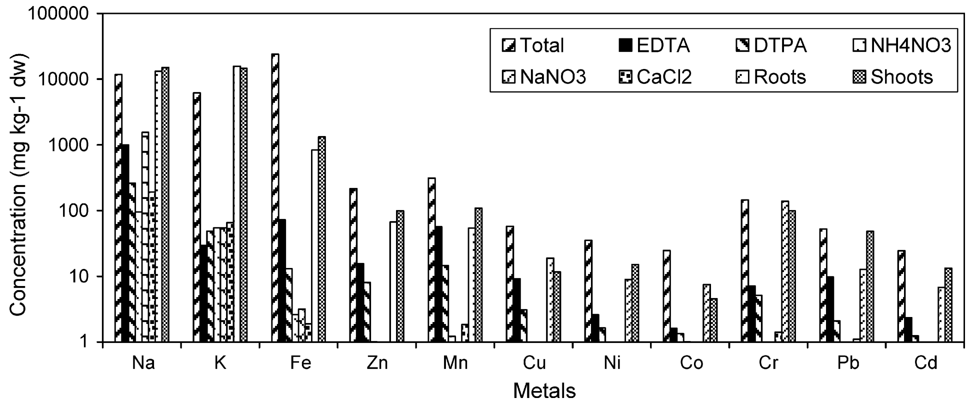


Fig. 1. Comparison between extractable (DTPA, EDTA, NH_4NO_3, $NaNO_3$, $CaCl_2$) and total metals content in contaminated soil and the accumulation of metals in different parts (roots and shoots) of the plants of *B. juncea*.

amendment of fly ash have shown better translocation of metals in upper part than lower part [7].

The present results demonstrated that the translocation of Cr was restricted in lower parts of the plants, which may be due to sequesterization of most of the Cr in the vacuoles of the root cells. The plants may not posses any specific mechanism to transport Cr. The accumulation and translocation of Cr inside the plant also depends on their oxidation state, concentration of Cr in the media and the plant species. Similar to the present findings where Cr restricted in the lower part of the plant, various reports are available for poor translocation of Cr in upper parts of the plants [3,21].

Recently, Gupta and Sinha [3,6] reported poor translocation of metals (Cr, Fe, Mn, Cu, Pb, Cd, Ni) in *Sesamum indicum* and toxic metals (Cr, Pb, Cd) translocation in Chenopodium *album* grown on different amendment of tannery sludge. In contrast, better accumulation of most of the tested metals (Na, K, Zn, Mn, Cu, Cr, Pb, Ni, Cd, Co) and its translocation was recorded in wild plants of *Sida acuta, Ricinus communis, Calotropis procera, Cassia fistula* grown on tannery sludge [19]. These authors reported that the plants found suitable for the decontamination of the tannery waste contaminated sites due to better translocation of metals in the upper part of the plants.

3.3. Extraction capacity

The comparison between extractable (DTPA, EDTA, NH_4NO_3, $NaNO_3$, $CaCl_2$) and total metals content in contaminated soil before the growth of the plants is shown in Fig. 1. Among all the extractants, EDTA extraction showed better extractability of all the tested metals, from tannery waste contaminated soil except, Na and K which was found maximum with $NaNO_3$ and $CaCl_2$, respectively. Overall, extraction capacity was as follows: EDTA > DTPA > NH_4NO_3 > $NaNO_3$ > $CaCl_2$. It can be observed that dilute $CaCl_2$ extracting media (0.01 mol l^{-1}) does not extract very high amount of elements as compared to the concentrated extracting media such as 0.5 M DTPA, 0.05 M EDTA, 1 M $NaNO_3$ and 0.1 M NH_4NO_3. These results are in consonance with earlier report, where EDTA was found best extractants for phytoavailability of metals from tannery sludge amended soil to *Sesamum indicum* [3]. The total metal present in the soil is not available to the plant grown therein and DTPA, EDTA, NH_4NO_3, $NaNO_3$ and $CaCl_2$ extractable metals can be used as an indicator of bioavailability and toxicity of the heavy metals.

In the present study, the level of metals (Zn, Cu, Pb) extracted with most of the extractants showed significant negative correlation with accumulation in roots; however, Cr and Pb have shown significant positive correlation with shoots accumulation in *B. juncea* (data not shown).

In general, higher percentage of metals was extracted with EDTA in comparison with DTPA. EDTA is assumed to extract both carbonate and organically bound fractions of heavy metals which may be due to its low pH. The ratio obtained is generally between 1.5 and 5 for all the metals [2,22,23]. The analysis of the results (Fig. 1) showed that the extraction of most of the tested metals specially, Fe, Mn, Cu and Pb were found many folds higher with EDTA extractant as compared to other tested extractants. Hammer and Keller [22] also reported that the extraction of Fe and Mn was found maximum with EDTA due to solubilization of iron oxides. This resulted in relatively lower correlation coefficient between the EDTA extractable metals and metals in *B. juncea* roots (data not shown). Recently, an author lab [3] reported the use of five widely applied single extractants namely, DTPA, EDTA, NH_4NO_3, $CaCl_2$ and $NaNO_3$. Out of these, EDTA has shown best correlation with metals accumulated in the *Sesamum indicum* grown on different amendment of tannery sludge in comparison to other extractants.

It has been reported that neutral salt solutions may be more effective to estimate plant availability of metals than the more aggressive extractant such as DTPA [22,24]. There tends to be no general agreement as to which neutral salt solution is the most effective. Theoretically, the $CaCl_2$ extractant works by exchanging Ca with metals on the exchange complex thus provides a measure of soil solution plus easily exchangeable metal, i.e., a measure of immediately bioavailable metal plus the buffering capacity of the soil. Although, the use of $CaCl_2$ has been advocated in Europe [25], USA [26], New Zealand [27], and Australia [28]. It has also been reported that other neutral salt extractants such as 1 M NH_4Cl [29] and 0.1 M $NaNO_3$ [24] provide a substantially better indication of plant available concentrations than does $CaCl_2$. In fact, each of the different extractants has been reported to provide various benefits when compared to the others. However, Gupta and Aten [24] recommend the use of 0.1 M $NaNO_3$, examination of their data set suggests that other extractants (such as 0.01 M $CaCl_2$) performed equally well or better. Based on the data sets analyzed in this study and in view of the effectiveness of 0.01 M $CaCl_2$ for a number of other metals, it is possible that this extractant may also be suitable for trace metals such as Cd, Zn, Ni, and Cu. Soils with higher pH and higher cation exchange capacity generally sequestered more Cr [30] due to the ineffective extraction of Cr by $CaCl_2$ from neutral and near alkaline soils.

3.4. Principal component analysis and cluster analysis

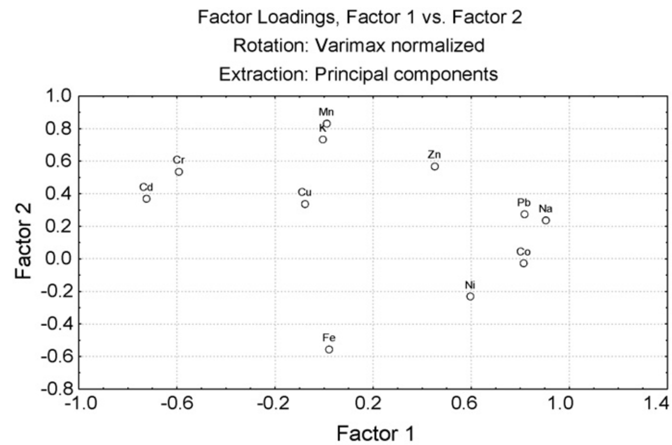
Among all the extractants, EDTA extractant showed maximum extraction capacity for contaminated soil, thus, PCA was performed using this data set. PCA was performed on correlation matrix of EDTA data set in order to identify a reduced set of factor that could capture the variance of data set. Following the criteria of Cattell and Jaspers [31], PCs with Eigenvalue > 1 were retained. Table 3A summarized the results of PCA analysis based on EDTA extractable metals showed two principal components namely PC_1 and PC_2. The first component for the EDTA extractable metals from contaminated soil explain ca. 32.67% of total variance which has strong positive loading (>0.70) on Na, Co, Pb and negative for Cd. The second PC has high positive loading (>0.70) for K and Mn only that explain ca. 23.46% of total variance. However, Zn and Cr showed moderate positive loading (>0.50) in PC_2. The loadings and scores of the first two PCs (PC_1 and PC_2) are plotted in Fig. 2A, showed different grouping of metals (i) Na, Co, Pb, Ni and (ii) K, Mn, Zn, Cr, in the loading plot which showed similar availability from contaminated soil. The PCs score plots described the characteristics of the samples and help to understand their availability pattern in *B. juncea*. Hassett et al. [32] reported that the Pb had synergistic effect with Cd concentration plant as well as in soil. This is also evident from the data of PCA analysis where Cd is negatively (>0.70) and Pb is positively loaded (>0.70) with PC_1.

However, the data of PCA analysis on metal accumulation in the plants of *B. juncea* grown on contaminated soil are presented in Table 3B. The analysis of data set showed two principal components namely PC_1 and PC_2. Principal component 1 explains ca. 61.73% of total variance which has strong negative loading (>0.70) on Na, K, Fe, whereas, Cu, Co and Ni have shown strong

Table 3

Metals	PC 1	PC 2
(A) Rotated factor loading (*varimax normalized*) of EDTA extractable metals		
Na	**0.9038**	0.2381
K	−0.0058	**0.7331**
Fe	0.0201	*−0.5558*
Mn	0.0107	**0.8312**
Cu	−0.0782	0.3390
Zn	0.4501	*0.5694*
Co	**0.8138**	−0.269
Ni	*0.5931*	−0.2301
Cr	*−0.5932*	*0.5365*
Cd	**−0.7249**	0.3707
Pb	**0.8161**	0.2748
Eigenvalue	3.593	2.581
% Total variance	32.666	23.464
Cum Eigen	3.593	6.174
Cumulative %	32.669	56.132
(B) Rotated factor loading (*varimax normalized*) of metals accumulation in *B. juncea*		
Na	**−0.9728**	0.0588
K	**−0.9167**	−0.3294
Fe	**−0.7999**	*0.5467*
Mn	0.0202	**0.8890**
Cu	**0.9667**	0.0511
Zn	0.1453	**0.8932**
Co	**0.9710**	0.1173
Ni	**0.9306**	0.3013
Cr	−0.0425	**−0.9022**
Cd	**0.9271**	0.2903
Pb	*0.5599*	**0.8022**
Eigenvalue	6.7910	3.2231
% Total variance	61.7360	29.3000
Cum Eigen	6.7910	10.0140
Cumulative %	61.7360	91.0370

Bold-faced values are strong loading (>0.7); values in italics are moderate loading (>0.5).



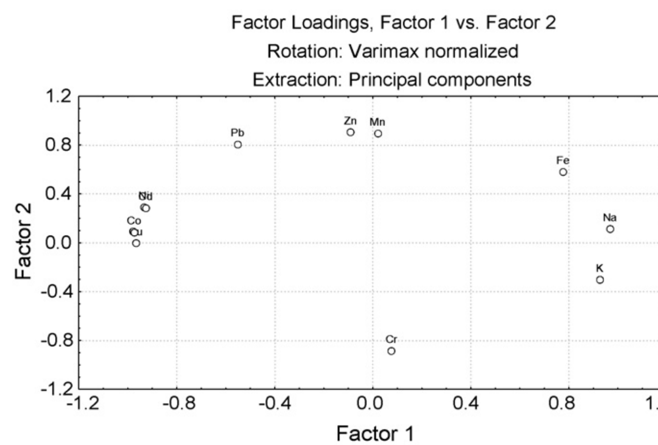


Fig. 2. (A) Principal component analysis applied to EDTA extractable metals. (B) Principal component analysis applied to metal accumulation in the plant.

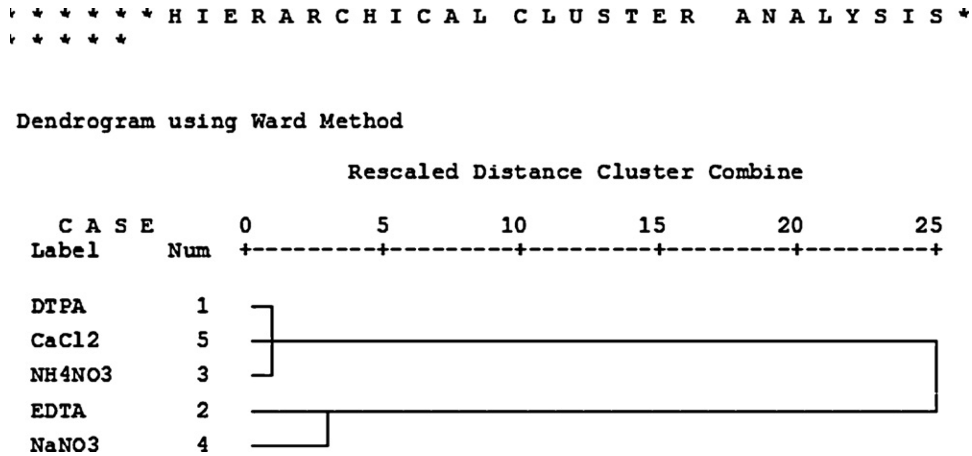


Fig. 3. Cluster analysis between different extractants.

positive loading. The second PC has high loading for Mn, Zn, and Pb (positive) and Cr (negative) that explain ca. 29.30% of total variance. The data (Table 3B) displayed positive loading for Mn and negative loading for Cr with PC_2. This may be due to the competition of Mn with Cr for transport binding in the plants [33]. Further, Cd and Zn have shown high loading in PC_1 and PC_2, respectively, which may be due to competition of metal ions. McKenna et al. [34] reported that increase in Zn concentration in the soil solution reduced the Cd concentration in leaves of lettuce and spinach. The present loading plots (Fig. 2B) clearly showed different set of grouping in the variables.

The information about the relationship among different single extractants used in this study is presented in Fig. 3. The distance cluster represents the degree of association between different extractants. The lower the value on the distance cluster, the more significant was the association. The cluster diagram showed two main clusters: one including NH_4NO_3, $CaCl_2$ and DTPA; second cluster contains EDTA and $NaNO_3$. First group showed close relationship among NH_4NO_3, $CaCl_2$, and DTPA, whereas, EDTA and $NaNO_3$ showed dispersed relationship. These dendrograms explained the grouping of extractants of similar or nearly identical extraction behavior. Thus, EDTA has shown different behavior than other extractants.

4. Conclusions

To evaluate the best extractants for bioavailability of metals to *B. juncea* form contaminated soils, different extractants were used. The results of cluster analysis and level of metals extracted with different extractants emphasized that EDTA was found suitable extractant for tannery wastewater contaminated soil to *B. juncea*. PCA analysis applied to metals extracted with EDTA showed different grouping of metals (i) Na, Co, Pb, Ni and (ii) K, Mn, Zn, Cr, in the loading plot which showed similar availability from contaminated soil. However, PCA applied on metals accumulation data in the plants also exhibited different grouping of variables (i) Cu, Co, Ni, Cd and (ii) Mn, Zn, Pb, Fe showed almost similar accumulation pattern in the plants.

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