

# Copper accumulation, localization and antioxidant response in *Eclipta alba* L. in relation to quantitative variation of the metal in soil

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**Abstract** *Eclipta alba* L. is a well known medicinal herb, found commonly on contaminated roadsides in Kerala, India. To assess its potential for copper tolerance and accumulation, pot culture experiment was carried out. Metal accumulation in the plant in relation to 50–800 mg kg<sup>-1</sup> Cu in soils, administered as CuSO<sub>4</sub>·7H<sub>2</sub>O in solution, was examined. Biomass yield of shoot and root, pigment content, Cu accumulation in the plant, bio-concentration factor, and translocation factor were the parameters studied. At the highest level of treatment, Cu was found accumulated more in the roots than in shoots. A significant increase in lipid peroxidation, proline content, phenolics and flavanoids were observed in Cu treated plants, compared to the control. The activity of antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase was found significantly changed in all the treated plants than in the control. The Bradford assay revealed a significant increase in protein content of the plant at higher levels of Cu treatment. Transmission electron microscopy, images supported the uptake and sequestration of metal particles inside the plant cell. The overall data suggests *Eclipta alba* L. to be a plant with high potential to tolerate Cu toxicity in soil.

**Keywords** *Eclipta alba* L. · Heavy metal stress · Copper accumulation · Antioxidant enzymes · Hyper accumulators · Phytoremediation

## Abbreviations

APX	Ascorbate peroxidase
AAS	Atomic absorption spectrophotometer
BCF	Bio-concentration factor
CAT	Catalase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FRAP	Ferric reducing antioxidant potential
GAE	Gallic acid equivalents
GR	Glutathione reductase
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
QE	Quercetin equivalents
SOD	Superoxide dismutase
TF	Translocation factor
TEM	Transmission electron microscopy

## Introduction

Heavy metal contamination is an environmental problem common in the present day world. The sources of contamination includes industrial emission, improper waste management and chemical fertiliser applications (Cunningham et al. 1995; Raskin et al. 1997). Sustainable development depends on the availability of eco-friendly and economically affordable technologies to deal with heavy metal contamination. Phytoremediation is such an in situ ecotechnology for easy removal of metal contaminants from land, water and air (Chaney et al. 1997). The

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success of phytoremediation depends on the availability of metal tolerant and hyper accumulator species (Ent et al. 2012). Therefore, experimental trials with fast growing and high biomass yielding weeds are essential for the identification of suitable metal tolerant or hyper accumulator plants as promising species for phytoremediation. *Eclipta alba* L. is a fast growing weed of high biomass yield, found commonly on contaminated roadsides in Kerala (Ray and George 2009), with Ni accumulation in higher quantity than that in the contaminated roadside soils (Ray and George 2011). Hussain and Khan (2010) investigated the presence of Cu in different parts of *Eclipta alba* L., which suggested the hyper accumulation tendency of this plant. However, no comprehensive experimental data are available on either its metal accumulation potential or biochemical response to metal toxicity.

Cu—a redox-active metal—is an essential micronutrient required for growth and metabolism in plants. Many enzymes and proteins in plants and animals require Cu as a co-factor (Yruela 2005). An oversupply of Cu is often found toxic to plants (Khatun et al. 2008; Yang and Chu 2011). Cu ion ( $\text{Cu}^{2+}$ ) is the real fungicidal component in the ‘Bordeaux mixture’. However, Cu contamination is quite common in many industrial and agricultural zones in the world (Reichman 2002).

Copper toxicity causes overproduction of reactive oxygen species, leading to oxidative stress in plants (Chai et al. 2014). Plants possess their own defence mechanism such as metal accumulation, metal exclusion, production of secondary metabolites and enzymatic antioxidant responses to overcome the stress (Syta et al. 2013). The assessment of enzymatic antioxidants such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), and glutathione reductase (GR, EC 1.6.4.2) in treated plant biomass is essential to explain the biochemical potential of plants to heavy metal stress. Therefore, experimentation on tolerance to a metal is the first step towards identification of potential species for phytoremediation.

The major objective of the present study was a comprehensive assessment of tolerance in *Eclipta alba* L. under different concentrations of Cu in soil. The metal accumulation potential and capacity of the plant to resist Cu toxicity in relation to different plant parameters are assessed. Various morphological, physiological and biochemical responses of the plant in terms of biomass, change in pigment content, lipid peroxidation, proline, flavanoid, phenolics, protein and enzymatic antioxidant activities were determined in response to the amount of Cu in soil. The study also aimed to Cu localisation and intracellular changes in the leaves of *Eclipta alba* L. by means of transmission electron microscopy (TEM). This is the first experimental work on *Eclipta alba* L. revealing its Cu accumulation characteristics, growth and biochemical response under different levels of Cu in soil.

## Materials and methods

### Preparation of soil and plant material

The experimental soil was collected from Mahatma Gandhi University premises at a depth of 0–20 cm, mixed with dried and powdered cow-dung and sand (3:1:1) for pot culture experiment. The physico-chemical analyses such as pH, electrical conductivity, salinity, available NPK, organic carbon, and Cu content were carried out (Jackson 1958) (Table 1). Each pot was filled uniformly with 1 kg of the ‘pot medium’ for experimental cultivation of the plant. The plant *Eclipta alba* L. was identified as per the type maintained in the Kerala State Regional Herbarium of the Botanical Survey of India at Department of Botany, St. Berchman’s College, Changanacherry and seeds were collected. After normal pre-treatments, the seeds were germinated under natural conditions using coir pith as a substrate to avoid the presence of any external Cu. The seedlings of uniform length (6–8 cm with 3–6 leaves) were collected for planting in the pots of 13 cm length and 15 cm diameter.

### Experimental design and copper treatment

A completely randomized design was followed in the experiment. The five different concentrations of Cu selected for the study were 50, 100, 200, 400, 800 mg  $\text{kg}^{-1}$  dry weight (DW) of the soil. Six replicates of the control as well as each of the treatment groups were maintained. Each metal concentration was prepared by dissolving the respective concentrations of Cu (equivalent to  $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ ) in 1000 mL distilled water and supplied to the plants as a daily dosage (50 mL) for 20 days. This treatment procedure was followed to prevent soil-mineral fixation, which may prevent the bioavailability of the metal as treatment dose and also to avoid metal leaching to an extent. The pots were kept on roof top in plastic trays (to

**Table 1** Physico-chemical characteristics of soil selected for the study

Parameters studied	Soil
pH	6.90 ± 0.01
Electrical conductivity (S $\text{m}^{-1}$ )	28.78 ± 2.11
Salinity (mg $\text{kg}^{-1}$ )	27.98 ± 0.08
Organic carbon (%)	5.20 ± 0.02
Phosphorous (mg $\text{kg}^{-1}$ )	341.97 ± 2.65
Potassium (mg $\text{kg}^{-1}$ )	1314.83 ± 1.57
Available nitrogen (mg $\text{kg}^{-1}$ )	236.50 ± 18.29
Copper (mg $\text{kg}^{-1}$ )	17.00 ± 0.00

Value represents mean ± standard deviation (SD) of three replicates of control soil

collect any leak out fractions), covered by transparent polythene sheets under green net (mesh size to reduce 50% of light intensity), so as to avoid influence of rain and also to provide moderate and uniform culture conditions throughout the experimental period. The pot culture was maintained completely for a period of 30 days, followed by harvesting.

### Chemical analysis of Cu in soil and plant samples

The soil samples (5 g) were taken and dried at 70 °C for 1 h in an oven. It was then powdered using mortar and pestle, sieved and stored in labelled sampling bags for quantitative analysis of Cu by Atomic Absorption Spectrophotometer (AAS, Thermo Scientific iCE 3000). For acid digestion, 500 mg of the soil samples were taken and digested in a microwave digester (Anton paar multiwave), by aqua regia method (Chen and Ma 2001), using 10 mL of concentrated HCl and 65% HNO<sub>3</sub> in the ratio 3:1. The digest was filtered after cooling, and transferred into a 25 mL volumetric flask and made up to the mark with de-ionized water. The Cu accumulated in the respective soil samples (before and after treatment), was determined by AAS and used for further analysis of phytoremediation potential [Bio-concentration factor (BCF)] of the plant species.

The harvested plant material was carefully washed in running tap water and then in de-ionized water to remove soil matter completely. The leaf number per plant in the control as well as treatments was counted. Each plant was separated into leaf, stem and root. Root length, shoot length, fresh weight (FW) and dry weight (DW) were recorded. The separated plant parts were dried in an oven at 40 °C for 12 h, powdered and stored for Cu analysis by AAS. The powdered samples (250 mg) of root and shoot were weighed out from control to treatment groups, and digested in the microwave digestion system with 10 mL of binary acid mixture, 65% HNO<sub>3</sub> and HClO<sub>4</sub> in the ratio 4:1. After digestion, the samples were cooled, filtered, made up to 25 mL using de-ionized water and the accumulation of Cu in the respective samples were done by AAS.

BCF and TF were calculated as follows:

BCF = Concentration of metal in plant root/Initial concentration of metal in substrate.

TF = Metal concentration in shoot/Metal concentration in root (Yoon et al. 2006).

### Preparation of sample for free radical scavenging and other biochemical assays

Fresh plants as a whole were taken from the control as well as treatment groups, shade-dried and powdered. Each sample (10 g) was sequentially extracted in a soxhlet using

300 mL of solvents including hexane, chloroform, acetone, methanol and water. A preliminary qualitative analysis of all the extracts was carried out for alkaloids, flavanoid, tannin, saponin, glycosides and phenolics as per Sasidharan et al. (2011). The methanolic and aqueous extracts were selected for further in vitro antioxidant assays such as DPPH and FRAP.

### DPPH and FRAP assays

The in vitro antioxidant activities of different concentrations of plant extracts from control to various treatment groups were determined (50–400 µg mL<sup>-1</sup>) based on standard procedure (Blois 1958), and expressed in mg ascorbic acid equivalents (AAE) g<sup>-1</sup> of leaf extract, using ascorbic acid standard (5–20 µg mL<sup>-1</sup>). Ferric reducing power of the plant extract was determined using the standard method (Benzie and Strain 1996) with slight modifications. FRAP values were expressed as mM Fe<sup>2+</sup> equivalents g<sup>-1</sup> of sample. All the measurements were done in triplicate.

### Biochemical assays

Total phenolic content in methanolic leaf extract was carried out according to the modified Folin–Ciocalteu method (Herald et al. 2012) and expressed as mg gallic acid equivalents (GAE) g<sup>-1</sup> FW. Total flavanoid content was determined according to the micro titre plate assay (Herald et al. 2012). The methanolic extracts of different concentrations (25, 50, 100, 200, 400 µg mL<sup>-1</sup>) were used to measure the flavanoid content. Quercetin was used as a standard (10–100 µg L<sup>-1</sup>) to generate a calibration curve and the values were expressed as mg quercetin equivalents (QE) g<sup>-1</sup> FW. The chlorophyll content and carotenoid were determined by the standard method (Arnon 1949), and expressed in mg g<sup>-1</sup> FW. Proline concentration in the control as well as Cu-treated plant extract was determined spectrophotometrically (Bates et al. 1973) and expressed as µmol g<sup>-1</sup> FW. Lipid peroxidation was measured spectrophotometrically by determining the malondialdehyde (MDA) content in the samples using extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> and expressed as µmol g<sup>-1</sup> FW (Hodges et al. 1999).

### Assay of antioxidant enzymes

Fresh leaves (500 mg) of each of the control as well as treatment groups were homogenised separately in 5 mL of extraction buffer containing 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) poly vinyl pyrrolidone, (PVP), filtered through a cheese cloth and centrifuged at 15,000×g for 12 min at 4 °C. The supernatant was collected and used for

enzymatic antioxidant assays. The protein content of the supernatants was measured (Bradford 1976). The superoxide dismutase (SOD) activity was assayed as per the standard procedure (McCord and Fridovich 1969). The change in absorbance was calculated for 560 and 532 nm and the enzyme activity was expressed as Units  $\text{mg}^{-1}$  protein. One unit of SOD activity can be defined as the amount of protein needed to inhibit 50% of photo reduction of NBT. The catalase (CAT) activity was determined based on decrease in absorbance at 240 nm, for every 30 s up to 2 min (Aebi 1984) and expressed as units  $\text{mg}^{-1}$  protein, using the molar extinction coefficient of  $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ . The ascorbate peroxidase (APX) activity was measured (Nakano and Asada 1981) with an increase in absorbance at 290 nm and expressed as units  $\text{mg}^{-1}$  protein, with molar extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . Glutathione reductase (GR) activity was recorded based on the increase in absorbance for 5 min at 412 nm (molar extinction coefficient of  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ) and expressed in units  $\text{mg}^{-1}$  protein (Foster and Hess 1980).

### TEM assays

A middle leaf section of 2 mm length, was taken from the control as well as the highest treatment dose ( $800 \text{ mg kg}^{-1}$  soil), fixed in 4% glutaraldehyde (v/v) in  $0.2 \text{ mol L}^{-1}$  phosphate buffer saline (PBS), pH 7.2 for 7 h and post fixed in 1% OSMIUM tetroxide for 1 h. Then the fixed specimen was treated with  $0.2 \text{ mol L}^{-1}$  of PBS (pH 7.2) for 2 h and dehydrated using graded ethanol series (50, 60, 70, 80, 90, 95 and 100%) followed by acetone. The samples were filtered and embedded in Spurr's resin. Ultra thin sections were prepared and the image was viewed under Technai TEM at an accelerating voltage of 60 kV (Hongyun et al. 2005).

### Statistical analysis

All the analyses were performed in triplicate and the results were recorded as mean  $\pm$  standard deviation (SD,  $n = 3$ ). The experimental data were statistically analysed using one-way analysis of variance (ANOVA) by Duncan's new multiple range tests (DMRT) using SPSS version 17.

## Results

### Growth parameters

The overall growth characteristics of the plant under all treatments (Fig. 1) revealed its capacity to tolerate increasing amount of Cu ( $50\text{--}800 \text{ mg kg}^{-1}$ ) in soil. The effect of Cu on growth parameters such as number of leaves, length of shoot

and root, FW and DW of each plant sample were recorded (Table 2). No significant difference in the leaf number was observed among the control and treatment groups.

### Root and shoot length

In *Eclipta alba* L., root length was significantly increased from the control by 14, 27, 32 and 42% and that of shoot length by 8, 12, 15 and 14.7% to treatment levels up to  $400 \text{ mg kg}^{-1}$  of Cu in soil (Table 2). A negative impact of Cu (71% decrease in root length) was noticed in plant roots exposed to  $800 \text{ mg kg}^{-1}$  in soil (Fig. 2). The current observation (Table 2) can be viewed as a critical tolerance limit of the plant root to Cu stress.

The shoot length of *Eclipta alba* L. recorded its maximum at  $400 \text{ mg kg}^{-1}$  and minimum at  $800 \text{ mg kg}^{-1}$  of Cu in soil. However, in shoot length, no significant difference (Table 2) was observed from that of the control to the highest treatment level.

### Plant biomass

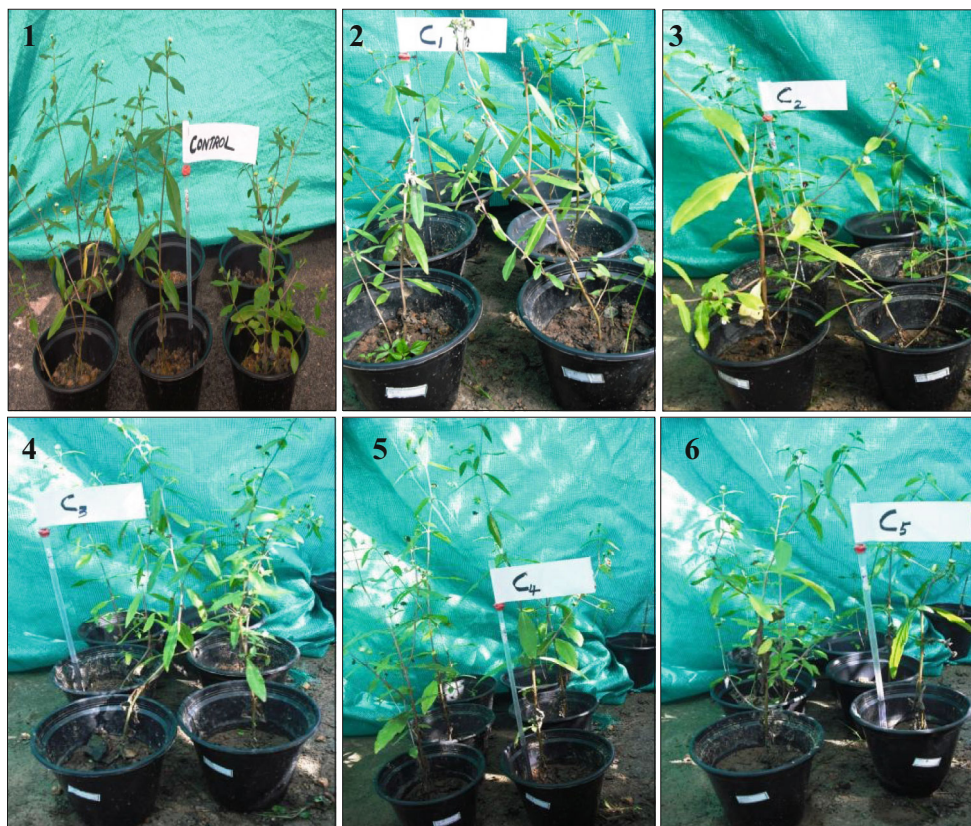
A significant increase ( $p < 0.05$ ) in fresh weight of the shoot (13, 14, 15 and 18%) was observed (Table 2), in treated plants up to  $400 \text{ mg kg}^{-1}$  of Cu in soil. However, fresh weight of the root increased significantly from the control to the treatment levels of  $100\text{--}400 \text{ mg kg}^{-1}$ . But a significant ( $p < 0.05$ ) reduction (18%) in fresh weight was observed in roots exposed to  $800 \text{ mg kg}^{-1}$  of Cu in soil.

The DW of shoot increased significantly ( $p < 0.05$ ) by 19% at  $400 \text{ mg kg}^{-1}$ , but decreased significantly (28%) at  $800 \text{ mg kg}^{-1}$  of Cu in soil (Table 2), when compared to the control. Likewise, DW of the root increased significantly (22%) at  $400 \text{ mg kg}^{-1}$  and reduced significantly (21%) at  $800 \text{ mg kg}^{-1}$  of Cu in soil.

### Chemical analysis of Cu in plant and soil

The accumulation of Cu by the shoot and root of *Eclipta alba* L. increased significantly with increasing concentrations of Cu in soil (Table 3). The highest shoot accumulation of Cu was observed at  $400 \text{ mg kg}^{-1}$  soil, whereas the root accumulation reached its maximum at  $800 \text{ mg kg}^{-1}$  treatment level.

After harvesting, copper content of different soil samples (Table 3) was found significantly different in accordance with the treatment levels. A significant reduction in BCF was observed from the control (Table 4) to the treatment levels of Cu in soil. But the TF was significantly increased ( $p < 0.05$ ) up to the treatment level of  $100 \text{ mg kg}^{-1}$  of Cu in soil than the control. No significant change in TF was observed in the plant exposed to 200 and



**Fig. 1** Experimental plant: growth of *Eclipta alba* L. exposed to different concentrations of Cu in soil. **1** 0 mg kg<sup>-1</sup>, **2** 50 mg kg<sup>-1</sup>, **3** 100 mg kg<sup>-1</sup>, **4** 200 mg kg<sup>-1</sup>, **5** 400 mg kg<sup>-1</sup>, **6** 800 mg kg<sup>-1</sup>

**Table 2** Effect of different concentrations of Cu on the growth parameters of *Eclipta alba* L.

Cu concentrations (mg kg <sup>-1</sup> DW soil)	Number of leaves per plant	Root length (cm)	Shoot length (cm)	Fresh weight (g plant <sup>-1</sup> )		Dry weight (g plant <sup>-1</sup> )	
				Root	Shoot	Root	Shoot
0	28.50 <sup>a</sup> ± 1.80	18.63 <sup>a</sup> ± 0.85	38.00 <sup>a</sup> ± 1.00	5.88 <sup>a</sup> ± 0.05	10.84 <sup>a</sup> ± 0.10	1.03 <sup>a</sup> ± 0.04	2.65 <sup>a</sup> ± 0.03
50	28.20 <sup>a</sup> ± 1.43	21.30 <sup>b</sup> ± 0.57	40.90 <sup>b</sup> ± 0.11	5.95 <sup>a</sup> ± 0.05	12.26 <sup>b</sup> ± 0.12	1.10 <sup>a</sup> ± 0.01	2.78 <sup>ab</sup> ± 0.07
100	27.80 <sup>a</sup> ± 1.32	23.80 <sup>c</sup> ± 0.76	42.60 <sup>b</sup> ± 1.20	6.28 <sup>b</sup> ± 0.13	12.39 <sup>b</sup> ± 0.03	1.16 <sup>a</sup> ± 0.06	2.86 <sup>b</sup> ± 0.40
200	29.30 <sup>a</sup> ± 1.86	24.60 <sup>c</sup> ± 0.57	42.80 <sup>b</sup> ± 1.04	6.21 <sup>b</sup> ± 0.18	12.46 <sup>b</sup> ± 0.26	1.15 <sup>a</sup> ± 0.06	2.83 <sup>b</sup> ± 0.06
400	28.30 <sup>a</sup> ± 0.80	26.50 <sup>d</sup> ± 1.28	43.30 <sup>b</sup> ± 0.60	6.68 <sup>c</sup> ± 0.20	12.78 <sup>b</sup> ± 0.12	1.26 <sup>b</sup> ± 0.06	3.15 <sup>b</sup> ± 0.15
800	27.20 <sup>a</sup> ± 1.16	5.40 <sup>e</sup> ± 1.21	39.30 <sup>ac</sup> ± 2.60	3.50 <sup>d</sup> ± 0.09	9.97 <sup>a</sup> ± 0.16	0.80 <sup>c</sup> ± 0.06	1.90 <sup>ca</sup> ± 0.11

Data represent mean ± standard deviation (SD) of three independent replicates. Lower case letters represent statistical differences between different levels of treatment, in comparison to the control plants. Different superscripts (a–d) indicate significant difference ( $p < 0.05$ )

400 mg kg<sup>-1</sup>, but decreased significantly at 800 mg kg<sup>-1</sup> of Cu in soil. It may also be noted that the TF of *Eclipta alba* L. was found to be greater than 1 in treatment levels up to 400 mg kg<sup>-1</sup> of Cu in soil.

### Free radical scavenging activity

Comparison of IC<sub>50</sub> value (DPPH) and the FRAP equivalents of the methanolic leaf extract of the control and the Cu treated plants (Table 5) revealed that, maximum

antioxidant activity was exhibited by plants treated with 200 mg kg<sup>-1</sup> of Cu in soil. Moreover, at 400 and 800 mg kg<sup>-1</sup> of Cu in soil, no significant change in antioxidant capacity was observed compared to the control.

### Effect of Cu on biochemical parameters

The analysis of pigment content in *Eclipta alba* L. (Fig. 3a, b) revealed a significant increase in total chlorophyll and carotenoid (28, 29%) in 50 mg kg<sup>-1</sup> of Cu treated plants



**Fig. 2** Root morphological changes observed in *Eclipta alba* L. under different treatments (0, 50, 100, 200, 400, 800 mg kg<sup>-1</sup> DW) of Cu in soil

**Table 3** Cu concentrations in stem, leaf and root of *Eclipta alba* L. for 30 days in soil

Cu concentrations (mg kg <sup>-1</sup> )	Stem (μg g <sup>-1</sup> DW)	Leaf (μg g <sup>-1</sup> DW)	Root (μg g <sup>-1</sup> DW)	Total uptake soil Cu (μg g <sup>-1</sup> DW)
0	3.17 <sup>a</sup> ± 0.76	4.33 <sup>a</sup> ± 0.57	6.66 <sup>a</sup> ± 1.00	14.17 <sup>a</sup> ± 0.76 9.17 <sup>a</sup> ± 0.76
50	100.32 <sup>b</sup> ± 12.89	107.33 <sup>b</sup> ± 6.16	81.91 <sup>b</sup> ± 8.50	289.56 <sup>b</sup> ± 9.73 33.60 <sup>b</sup> ± 4.25
100	138.91 <sup>b</sup> ± 1.66	220.08 <sup>c</sup> ± 14.14	162.15 <sup>c</sup> ± 25.91	521.14 <sup>c</sup> ± 13.33 65.54 <sup>c</sup> ± 1.56
200	216.70 <sup>c</sup> ± 15.69	382.08 <sup>d</sup> ± 14.42	471.00 <sup>d</sup> ± 15.19	1069.78 <sup>d</sup> ± 14.75 96.00 <sup>d</sup> ± 7.54
400	260.37 <sup>c</sup> ± 8.94	534.80 <sup>e</sup> ± 14.57	680.06 <sup>e</sup> ± 17.96	1475.23 <sup>e</sup> ± 21.29 233.29 <sup>e</sup> ± 13.87
800	277.78 <sup>c</sup> ± 20.25	613.68 <sup>f</sup> ± 9.37	1295.38 <sup>f</sup> ± 35.93	2186.84 <sup>f</sup> ± 43.18 454.81 <sup>f</sup> ± 28.41

Data represent mean ± standard deviation (SD) of three independent replicates. Lower case letters as super scripts (a–f) represent statistical differences between different levels of treatment, in comparison to the control plants. Different letters indicate significant difference ( $p < 0.05$ )

**Table 4** BCF and TF value in *Eclipta alba* L. grown under different treatment levels of Cu

Cu concentrations (mg kg <sup>-1</sup> )	Bio-concentration factor (BCF)	Translocation factor (TF)
0	0.50 <sup>a</sup> ± 0.01	1.84 <sup>a</sup> ± 0.16
50	0.33 <sup>bc</sup> ± 0.03	2.48 <sup>b</sup> ± 0.50
100	0.31 <sup>c</sup> ± 0.06	2.26 <sup>b</sup> ± 0.45
200	0.35 <sup>bcd</sup> ± 0.01	1.41 <sup>a</sup> ± 0.08
400	0.38 <sup>bd</sup> ± 0.24	1.17 <sup>ac</sup> ± 0.05
800	0.37 <sup>d</sup> ± 0.12	0.69 <sup>c</sup> ± 0.02

Data represents mean ± standard deviation (SD) of three independent replicates. Lower case letters represents statistical differences between different treatments, in comparison to the control plants. Different letters (a–d) as super scripts indicate significant differences ( $p < 0.05$ )

only, compared to the control and reduced significantly thereafter.

A significant increase in MDA (73, 117, 143, 161 and 185%) was observed in *Eclipta alba* L. (Fig. 3c), from control to various levels of treatments. The proline content also increased (105, 260, 391, 471 and 254%) sig-

nificantly ( $p < 0.05$ ) with increasing levels of Cu in soil (Fig. 3d).

In *Eclipta alba* L., the flavanoid and phenolic compounds increased significantly (flavanoids by 28, 48, 87, 128 and 152% and phenolics by 15, 47, 120, 144 and 169%) from control to the treatment groups (Fig. 3e, f).

**Table 5** Antioxidant activity of the control and Cu treated *Eclipta alba* L. were compared

Cu concentrations (mg kg <sup>-1</sup> )	IC <sub>50</sub> value (µg mL <sup>-1</sup> extract) DPPH assay	Ferrous equivalence (µg mL <sup>-1</sup> extract)
0	365 <sup>a</sup> ± 0.00	180 <sup>a</sup> ± 0.05
50	362 <sup>b</sup> ± 0.00	185 <sup>a</sup> ± 0.12
100	349 <sup>c</sup> ± 0.00	170 <sup>b</sup> ± 0.17
200	190 <sup>d</sup> ± 0.00	168 <sup>b</sup> ± 0.09
400	365 <sup>a</sup> ± 0.00	180 <sup>a</sup> ± 0.14
800	366 <sup>a</sup> ± 0.00	182 <sup>a</sup> ± 1.00

Data represent mean ± standard deviation (SD) of three independent replicates. Lower case letters represent statistical differences between different levels of treatment, in comparison to the control plants. Different letters (a–c) as superscripts indicate significant difference ( $p < 0.05$ )

### Effect of copper on antioxidant enzyme activities and protein content

The enzymatic antioxidants estimated in relation to Cu stress were SOD, CAT, APX and GR (Fig. 4). The activity of SOD (Fig. 4a) in *Eclipta alba* L. was concentration dependent. The highest SOD activity was shown by plants that received 800 mg kg<sup>-1</sup> of Cu in soil. The CAT activity (Fig. 4b) decreased significantly with respect to the increasing metal concentrations, and declined markedly at 800 mg kg<sup>-1</sup> of Cu in soils. A significant increase ( $p < 0.05$ ) in peroxidase activity (Fig. 4c) was observed in the treated plants, compared to the control. Maximum APX activity was observed in 800 mg kg<sup>-1</sup> treated groups. GR activity also showed a similar trend (Fig. 4d) with respect to increasing Cu levels in soil. The activity of GR reached its maximum at 200 mg kg<sup>-1</sup> of Cu in soil.

Copper treatment resulted in a significant increase in protein content (Fig. 4e), only in 400 and 800 mg kg<sup>-1</sup> of Cu, when compared to the control.

### TEM assays

Transmission electron microscopy (TEM) was used to determine the localisation of Cu inside the plant as well as the intracellular changes occurred in relation to metal detoxification mechanism. The resin embedded specimens of the control and 800 mg kg<sup>-1</sup> Cu exposed plant leaves were compared. In the control plant leaf, many chloroplasts with a properly defined structure were visible. The chloroplast was observed as the most affected organelle in the plant leaf. The thylakoid arrays in the granum, starch grains in the chloroplast, mitochondria, vacuoles and golgi bodies were evident (Fig. 5a, b). But in 800 mg kg<sup>-1</sup> treated plant leaf, the chloroplast was found distorted with disassembled thylakoid arrays (Fig. 5c–f). The membrane vesiculation and cytoplasmic vacuolation was visible (Fig. 5d, e) in plant leaf exposed to Cu treatment, compared to the control. A tendency for the withdrawal of plasma membrane from its cell wall was observed in leaf

cells of Cu treated plants (Fig. 5f), over the control plant leaf. A large amount of electron dense bodies of reduced metal particles were found deposited in the cell wall (Fig. 6g–j) as well as in the vacuoles (Fig. 6k–l).

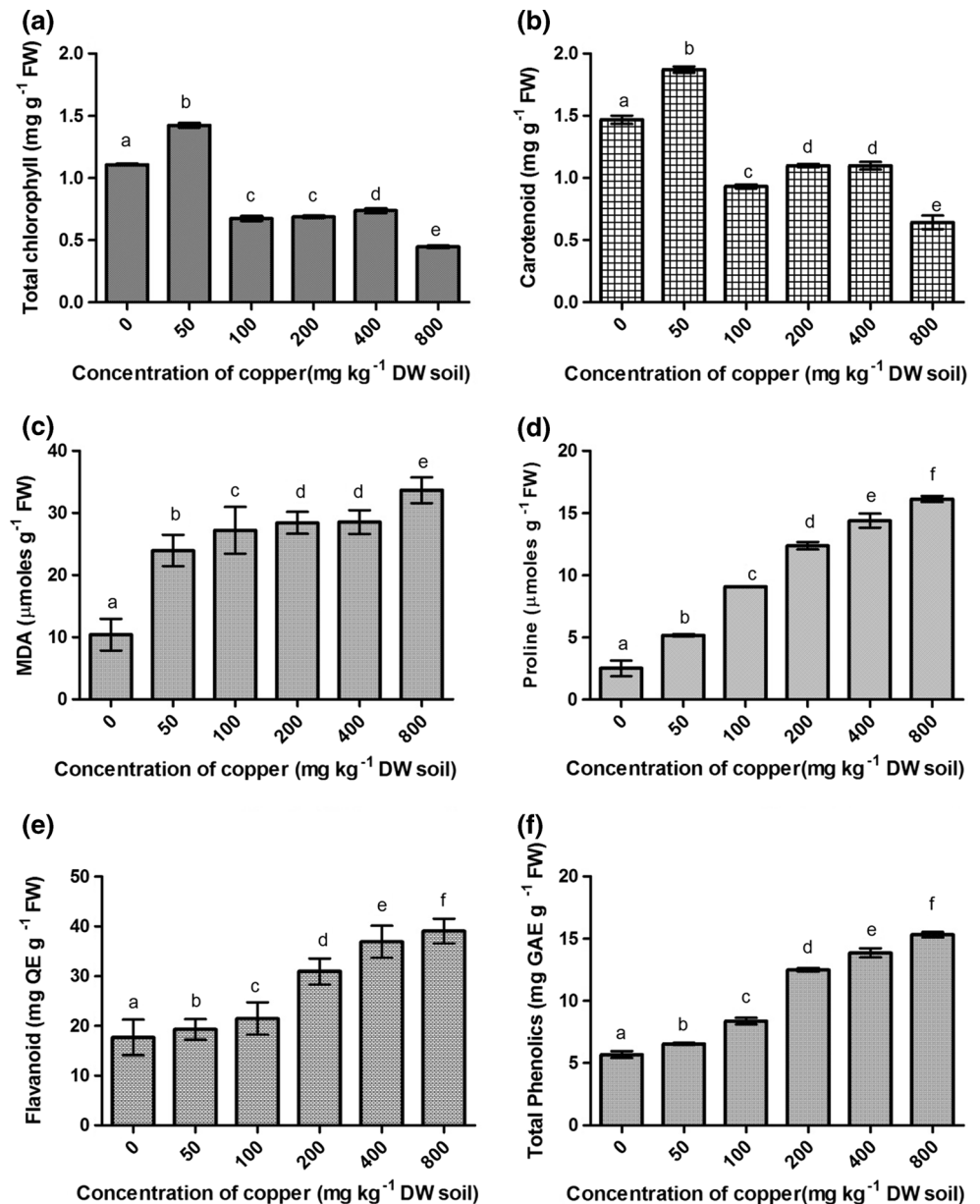
## Discussion

### Growth parameters

The difference in growth pattern and biochemical responses in *Eclipta alba* L. revealed its capacity to overcome Cu stress in soil. *Eclipta alba* L. is a dicot plant with columnar growth pattern. Therefore, measurement of length of the main shoot and that of taproot as well as shoot length, root length and biomass (Table 2) are meaningful information of its growth response to any kind of environmental stress. In general, the different levels of Cu treatments demonstrated a beneficial role in the growth of the plant in soil, (Fig. 1) except visible root toxicity at the highest dose. The result indicated plant's capacity to tolerate Cu stress in soil to an optimum level. A similar response of increase in root length at smaller doses of Cu is already reported (Sako et al. 2016). The selection of a native tolerant species which can survive the toxic conditions is the main criteria for the selection of plants for phytoremediation studies. However, the exact potentials of the plant to tolerate or resist the Cu stress can be revealed from its biochemical responses.

A significant increase ( $p < 0.05$ ) in length and biomass of both shoot and root was found in *Eclipta alba* L. (Table 2) at low levels of Cu in soils. A similar effect has been widely studied (Nair et al. 2014; Chen et al. 2015). A significant decrease in root length and root biomass with darkening of root hairs (Fig. 2) was observed in the highest dose of 800 mg kg<sup>-1</sup>, while shoot biomass remains unchanged. In general, increase in root length at initial doses can be viewed as a supporting factor for better absorption of water and minerals in plants and thereby increasing the plant growth. Therefore, Cu at low level in

**Fig. 3** Effect of different concentrations of Cu on **a** total chlorophyll, **b** carotenoid, **c** MDA, **d** proline, **e** flavanoid and **f** phenolics in *Eclipta alba* L. The values represents mean of triplicates  $\pm$  SD. Different letters indicate significant differences among the treatments ( $p < 0.05$ ) and the control



the soil was found beneficial to the plant. Moreover, the level between 400 and 800 mg kg<sup>-1</sup> of Cu in soils appeared as a critical limit to the plant. A similar darkening of root hairs was also observed in *Lupinus luteus* exposed to Cu in a liquid culture medium (Mourato and Martins 2009).

### Analysis of copper in soil and plant

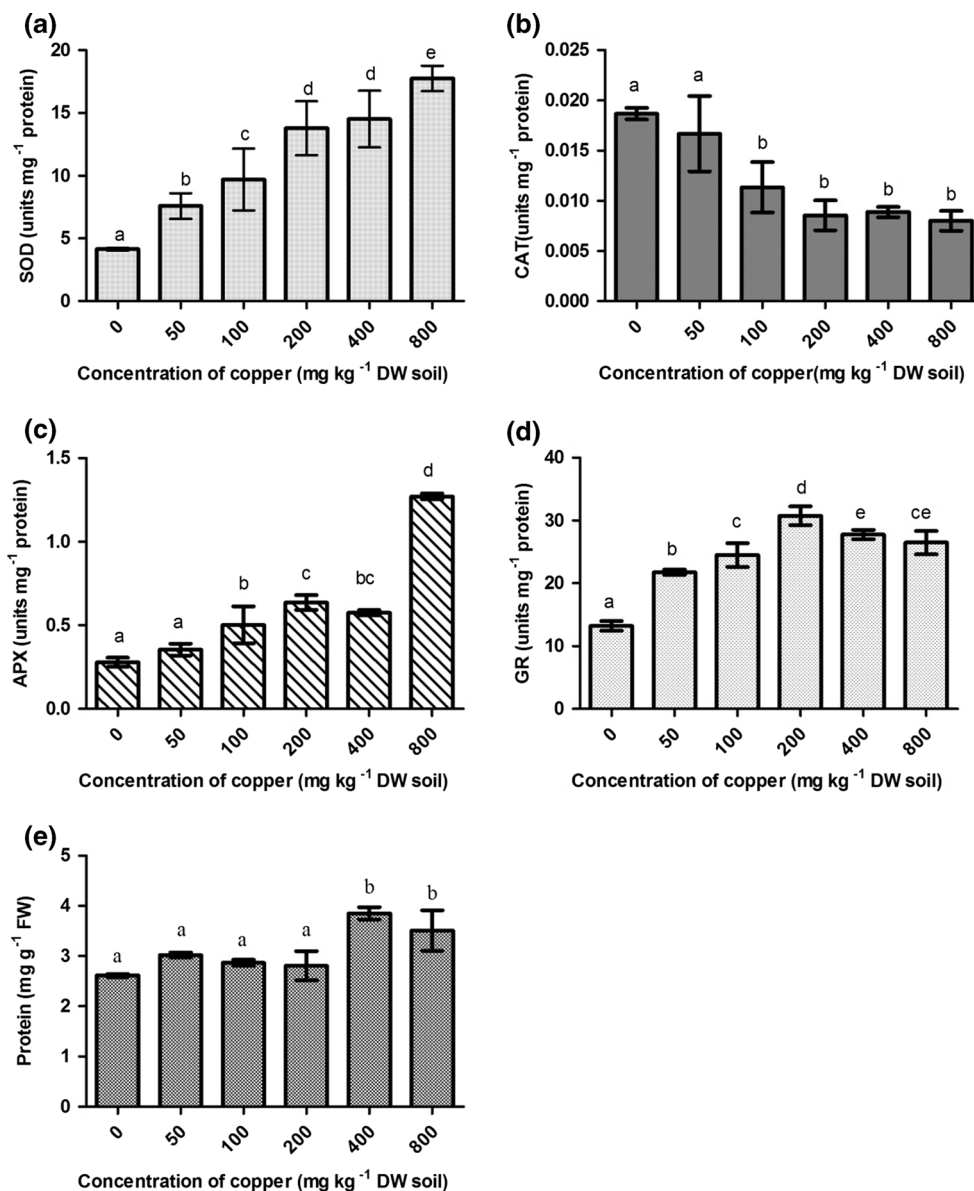
The significantly high amount of Cu observed in different soil samples after harvest were in accordance with the levels of treatment of the metal in these soil samples. The observed trend of accumulation of Cu in different parts of *Eclipta alba* L. (Table 3) proved its ability to survive in Cu

contaminated soils. A similar accumulation pattern is already reported in other plants such as *Lemna trisulca* L. (Prasad et al. 2001) and *Phyllostachys pubescens* (Chen et al. 2015). The current study proved that the plant was able to absorb the added Cu in the soil, but the transport of Cu from root to shoot decreased, when the concentration reached certain critical levels. The concentration dependent accumulation of Cu by the plant appeared as a positive sign of its potential for extracting Cu from the soil.

The BCF value reveals the potential of a plant species to concentrate metals from soil into roots, whereas translocation factor (TF) reveals its capacity to translocate it to the above ground parts. The plants exhibiting BCF or TF values  $\geq 1$  are considered as hyper accumulators suitable for



**Fig. 4** Effect of different concentrations of Cu on activity of SOD (a), CAT (b), APX (c), GR (d) and protein (e) in *Eclipta alba* L. All the values are mean of triplicate  $\pm$  SD. Different letters indicate significantly different values (DMRT  $p < 0.05$ )



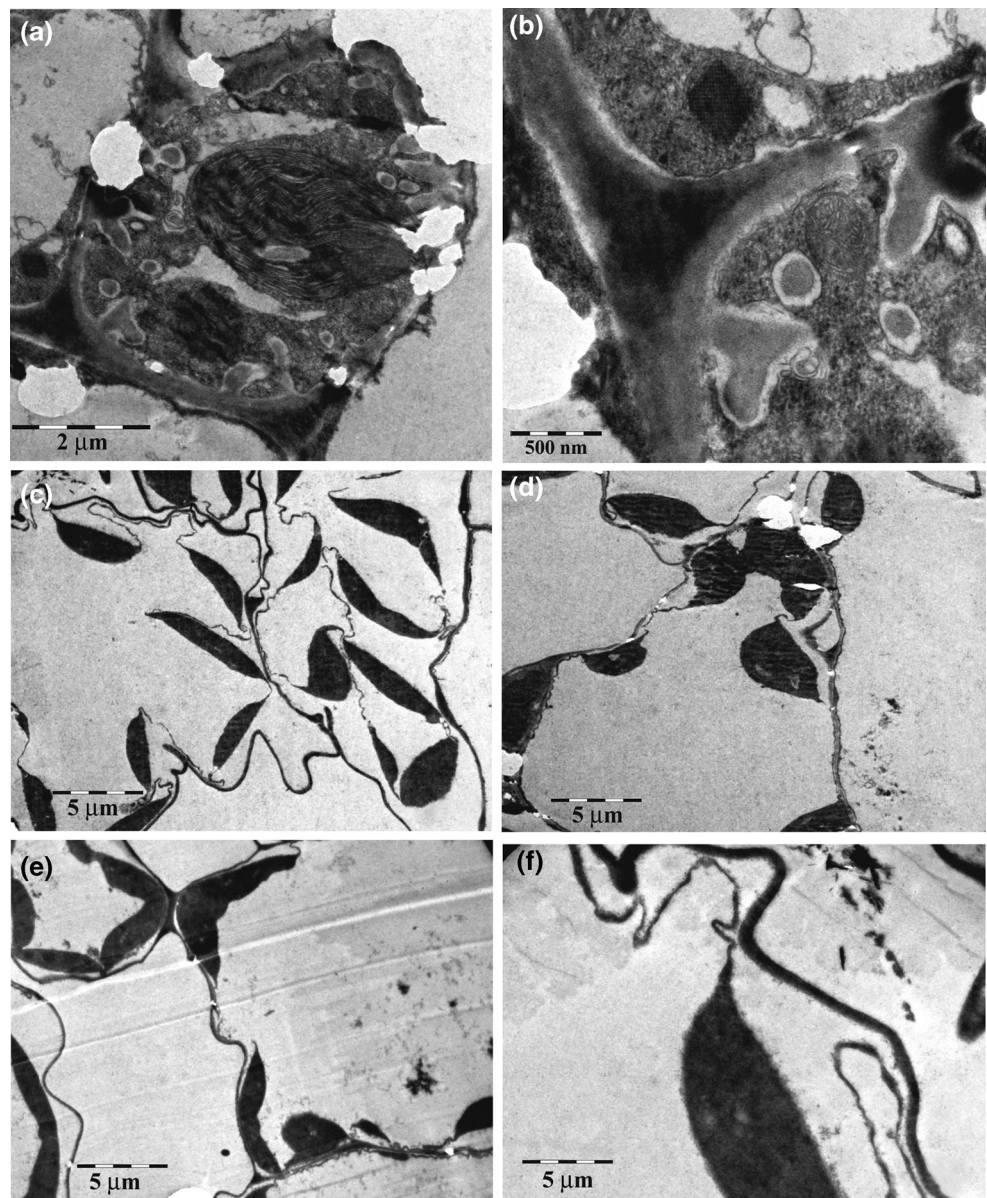
phytoextraction (Yoon et al. 2006). In *Eclipta alba* L. (Table 4), BCF value was  $<1$  in all the treatment groups, whereas the TF was  $>1$  at treatment levels except the highest one, when compared to the control. A similar observation in BCF and TF is already reported (Michaud et al. 2008; Chen et al. 2015). Since the BCF of *Eclipta alba* L. is  $<1$ , it would not come under the category of hyper accumulator. But TF value  $>1$  revealed its capacity to extract Cu from soil and translocate into roots and finally into shoots. Moreover, maximum translocation of Cu from root to shoot was exhibited up to 400 mg kg<sup>-1</sup> soil. The dose dependent accumulation of Cu also attributed a positive sign for the phytoextraction of Cu from soil into roots or shoots. In general, metal tolerance and high biomass yield are the two important factors to be considered for phytoremediation

potential, which was found constructive in *Eclipta alba* L. It may be noted that many soil factors also influence the transport of metals from soil to plant. Therefore, the plant deserves further investigation to confirm its potential in the management of metal contamination.

#### Free radical scavenging assays

The FRAP and DPPH assays (Table 5) showed a significant increase in quenching DPPH in treated ones, compared to the control, which was visible from the progressive increase in free radical scavenging activity. The least concentration of the plant extract to achieve the IC<sub>50</sub> value was obtained under treatment of 200 mg kg<sup>-1</sup> of Cu in soil. No significant change was observed in

**Fig. 5** TEM micrographs of the control plant (a, b) and 800 mg kg<sup>-1</sup> Cu treated *Eclipta alba* L. leaf (c–f) with distortion of thylakoids in the chloroplast, cytoplasmic vesiculation (c, d, e), movement of plasma membrane away from the cell wall (f)



antioxidant activity of further treatments, than the control, which revealed that the plant antioxidant activity was not affected by excess Cu in soil.

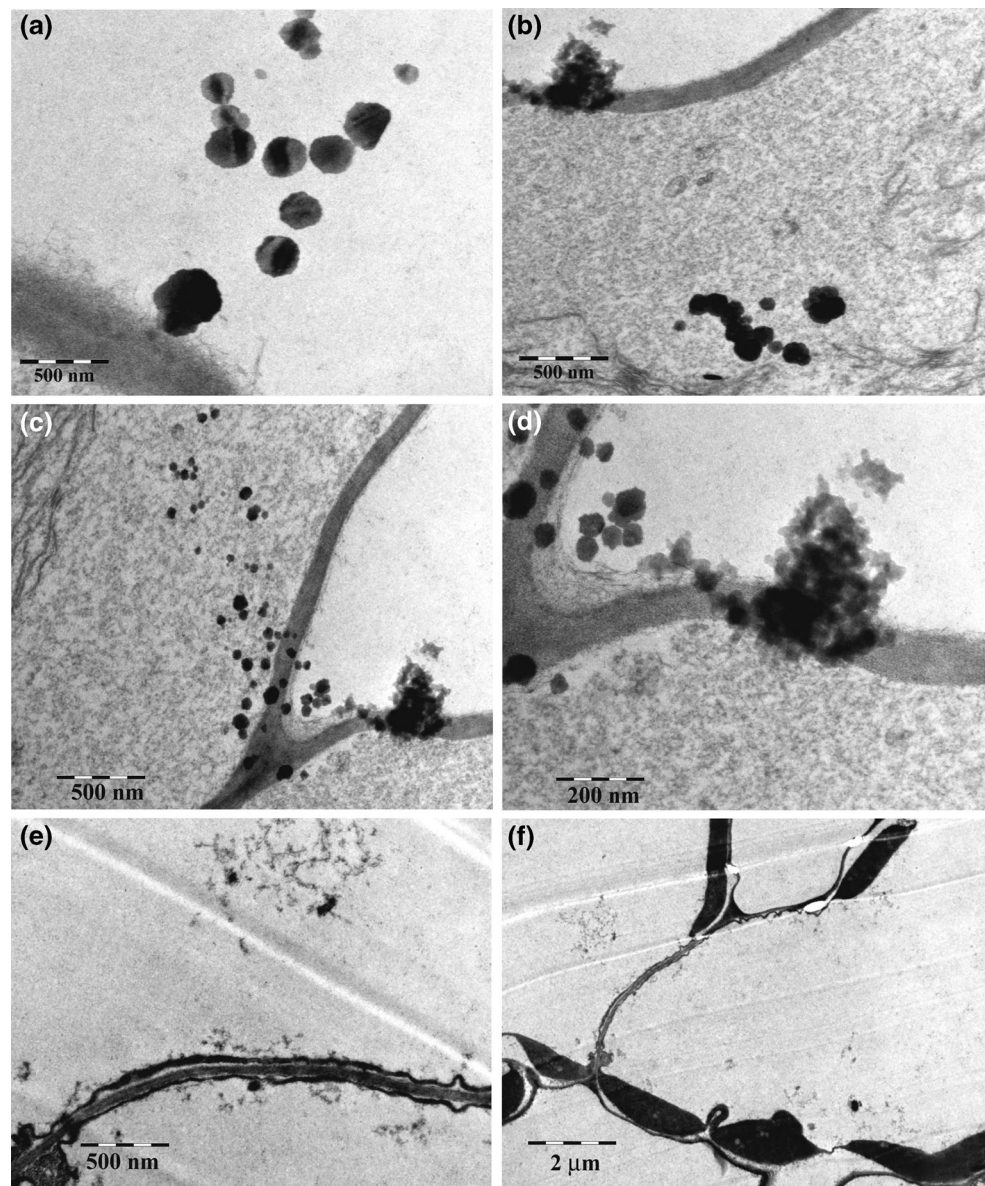
The ferric reducing antioxidant power of the extract (FRAP value) was compared with the absorbance of 1 mM ferrous sulphate and expressed as ferrous equivalents (FE) in  $\mu\text{g mL}^{-1}$  extract. The FRAP value remains unchanged in Cu treated groups, compared to the control, which showed that the reducing property of the plant was not affected by Cu stress in soil. An earlier report on Serbian poplar clones showed a slight increase in FRAP value when subjected to Cu toxicity, but the effect was not significant (Trudic et al. 2013). The FRAP assay is used as a measure to combat Cu induced oxidative stress in *Brassica juncea* L. (Szöllösi et al. 2011). It was observed that

*Eclipta alba* L. was found to be capable of resisting Cu stress, by means of its metal reducing property, even at elevated levels of Cu in soil.

### Biochemical parameters

The change in pigment contents can be used as a parameter to show the impact of Cu on plant physiology, especially photosynthesis (Yruela 2005). In the current study, the inhibitory effect of Cu on pigment contents (Fig. 3a, b), could be due to the significant loss of chlorophyll by Cu induced peroxidation of chloroplast membrane or pigment degradation. A similar decrease in pigment content from Cu stress is already reported in eight populations of *Hau-maniastrum katangense* (Peng et al. 2012).

**Fig. 6** Intracellular localisation of Cu inside the plant leaf. Metal particles clustered around the cell wall (a), as electron dense bodies, membrane transport of reduced metal particles (b–d), vacuolation for the sequestration of the metal (e, f)



The lipid peroxidation is an indication of membrane damage by biotic or abiotic stress (Sytar et al. 2013). The Cu exposed *Eclipta alba* L. exhibited a marked increase in MDA content (Fig. 3c). Hence the enzymatic antioxidant machinery get stimulated to overcome the Cu induced lipid peroxidation. This is an important characteristic feature exhibited by metal accumulating and tolerant plants to overcome the stress. Increased MDA production under Cu stress is also widely reported (Lu et al. 2010; Chai et al. 2014).

The proline accumulation in plants is another indication of plant defence mechanism to various abiotic stress including heavy metals (Schat et al. 1997). However, an increase in proline, which plays an important role in the process of membrane stabilisation, free radical scavenging,

enzyme protection and cytosolic acidity regulation as well as in non-enzymatic free radical detoxification, reveals the capacity of plants to resist metal toxicity (Rejeb et al. 2014). In the study, proline accumulation significantly increased in treated plants (Fig. 3d) with respect to the increasing Cu concentrations in soil. The current study emphasised the stimulatory role of proline as a means of plant defence mechanism, to alleviate Cu toxicity in soil, by stabilising the membrane damage. A similar stimulation of proline synthesis has already been reported (Thounajam et al. 2012).

In *Eclipta alba* L., the significant increase in phenolics and flavanoid, from control to treated plants with respect to increasing Cu levels in soil (Fig. 3e, f) showed its positive biochemical response in relation to Cu accumulation. The

current observation supported a stimulatory role of secondary metabolites against Cu stress, and suggests further analysis on the involvement of different functional groups of bio molecules, which could be a part of plant tolerance mechanism. A similar increase in flavanoid and phenolics against Cu induced toxicity has already been reported in *Nephrolepis biserrata* (Khatun et al. 2008; Manan et al. 2015).

### Antioxidant enzymes

The defensive role of various enzymatic antioxidants was determined in the study. The most effective SOD causes dismutation of superoxide radical into H<sub>2</sub>O<sub>2</sub> and oxygen. The elevated levels of SOD is an indication of plant defence mechanism against the over production of reactive oxygen species induced by heavy metals (Ahmad et al. 2010; Madejón et al. 2009). The present observation proved that the generated enzymatic antioxidant defence mechanism in *Eclipta alba* L. was essential to cope with the free radical production induced by Cu. In *Eclipta alba* L., the significantly ( $p < 0.05$ ) increased activity of SOD (Fig. 4a) than the control shows the plant's tolerant capacity to dismutate superoxide radicals. A similar effect has been reported widely (Srivastava et al. 2006; Thounaojam et al. 2012; Ali et al. 2016).

The principal scavenging enzyme, catalase (CAT), which can dismutate toxic H<sub>2</sub>O<sub>2</sub> produced during endogenous or exogenous stress conditions, is another indication of plant response to metal stress (Ahmad et al. 2010). The present data suggest a significant decrease in CAT activity at the given concentrations of Cu, when compared to the control (Fig. 4b), which might be due to the inhibitory role of Cu. The accumulated Cu in plant tissues showed a negative effect on CAT activity, which could be due to either over production of H<sub>2</sub>O<sub>2</sub> or due to the excessive activation of APX. A similar result is obtained in *Lemna trisulca* L. and also in white lupin and soybean plants (Prasad et al. 2001; Sanchez-pardo et al. 2012).

The ascorbate peroxidase (APX) involved in scavenging of H<sub>2</sub>O<sub>2</sub> in ascorbate–glutathione cycle, plays another important role in plant defence mechanism against different abiotic stress (Ahmad et al. 2010). The significantly increased APX activity reported in the current study (Fig. 4c) showed the defensive role of APX in overcoming Cu stress in soil by the plant. Increase in peroxidase activity as an indication of the adaptive tolerance of plants to Cu stress has been previously reported (Sanchez-pardo et al. 2012; Thounaojam et al. 2012). Thus, APX activity in *Eclipta alba* L. proved to be beneficial for the detoxification of H<sub>2</sub>O<sub>2</sub> induced from Cu toxicity.

The glutathione reductase (GR)—a potential enzyme of ascorbate–glutathione pathway—is another effective

scavenger of free radicals (Ahmad et al. 2010). The significant increase in GR activity (Fig. 4d) observed in treated plants over control, showed the stimulatory role of the enzyme in overcoming Cu induced stress in the plant. A similar response in GR activity is already reported (Thounaojam et al. 2012).

The present investigation confirmed that initial Cu treatments did not cause a change in protein content (Fig. 4e), over the control. However, increase in protein content at treatment levels of 400 and 800 mg kg<sup>-1</sup> might be attributed to the synthesis of some stress related proteins, which can be analysed by further proteomics studies. A similar increase in protein content with response to Cu stress in soil has been previously reported (Doganlar 2013).

### TEM analysis

The large amount of electron dense bodies (metal particles) found deposited near the inner side of the cell wall, cell membrane and also outside and inside of the chloroplast membrane (Fig. 6g–i) of the treated plants stands as evidence for high ability of *Eclipta alba* L. to take up, accumulate and translocate Cu into its leaves, whereas the damage of cell organelles remains evidence to the adverse effect of the metal in its cells. The chloroplast was found as the most affected organelle in response to Cu stress when compared to the control. Disorientation of chloroplast with disrupted thylakoid arrays in the granum was observed in 800 mg kg<sup>-1</sup> Cu treated plant leaf (Fig. 5c–f) compared to the control (Fig. 5a, b). The results were in accordance with Cu tolerance in *Salix purpurea* (Hakmaoui et al. 2007). No change has been observed in other organelles like mitochondria or peroxisomes. The vacuolation observed in TEM analysis revealed this as a means of metal sequestration (Fig. 6j–l), or metal detoxification mechanism by the plant to overcome Cu toxicity. This agrees with previous observations in this regard (Hong-yun et al. 2005; Chen et al. 2015). Thus, TEM analysis confirmed intracellular distribution of metal particles in treated plants as well as organelle changes occurred in them, which are evidence to the adaptive mechanism in plants, in relation to Cu stress in soil.

### Conclusion

*Eclipta alba* L.—a fast growing and high biomass yielding tropical wasteland medicinal herb—growing abundantly on heavy metal contaminated roadsides, was hypothesized as a plant with high phytoremediation potentials. Experimental evidence for its response to increasing levels of Cu in soil is revealed for the first time. Present experimental evidence suggests that the plant has certain promising features for

metal decontamination studies in soils. The tolerance of *Eclipta alba* L. to Cu and its detoxification capabilities are assessed on the basis of its ability to survive, accumulate and sequester excess Cu in soil. Response of the plant in terms of enzymatic antioxidants and secondary metabolites showed its biochemical mechanism to overcome Cu stress in soil. The ultra structural changes and intracellular Cu accumulation supported detoxification process of the plant by vacuolar sequestration of the metal. Disorientation of chloroplast structure revealed Cu toxicity to its photosynthetic apparatus. The present study necessitates further investigations including molecular response of the plant to assess its phytoremediation potential.

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