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Research article

RESPONSE OF LEAVES, STEMS AND ROOTS OF *WITHANIA SOMNIFERA* TO COPPER STRESS

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ABSTRACT: The effects of Cu^{+2} stress (0, 10, 20, 50, 100 and 200 mM as $CuSO_4.5H_2O$) on growth and biochemical parameters of *Withania somnifera* L.Dunal, grown in a field pot experiment were studied. Increasing Cu^{+2} concentrations led to decreased stem length, root elongation and leaf area. Copper (Cu^{+2}) stress decreased chlorophyll and carotenoids content in leaves and stems as compared to controls; this was more pronounced in leaves than in stems. Lipid peroxidation, in terms of malondialdehyde (MDA) content increased. Lipid peroxidation increased significantly with elevation in Cu^{+2} concentrations and was highest in roots followed by leaves and stems at 200 mM. Leakage of electrolytes measured in terms of membrane stability index (MSI), which showed a negative correlation with increasing Cu^{+2} levels. The MSI was lowest in 200 mM Cu^{+2} treated plants. The phenol content of leaves, stems and roots peaked at 50 mM Cu^{+2} treatment and thereafter declined. These results indicate that Cu^{+2} stress negatively impacted nearly all the parameters assayed; particularly in the roots. It is being reported for the first time that stress resistance in *W.somnifera* as observed by increase in total phenol content at low Cu^{+2} levels possibly to combat increased ROS generation.

Keywords: Chlorophyll, Copper, MDA, Total phenol, *Withania somnifera.*

INTRODUCTION

The demand for aromatic medicinal plants is increasing worldwide due to their beneficial effects on long-term health of humans and can be also used to effectively treat human diseases with either low or no side effects [1]. In recent decades, heavy metal stress is one of the major abiotic stresses that cause environmental pollution. They can persist for a long time in the environment and cause soil pollution and produce phytotoxicity in plants and enter the food chain [2]. Agriculture and the environment both get affected from problem of copper toxicity due to sewage sludge application, dust from smelters, industrial waste and bad watering practices in agricultural lands [3]. Among agricultural soils pollutants, Cu^{+2} have become increasingly hazardous as a component of fungicides, fertilizers and pesticides [4] . Heavy metal uptake by the plants is therefore, a main pathway of metal transfer from sediments and water to the food web. Although, Cu^{+2} is essential for growth and development of plants as a component of various proteins [5] and acts as a structural element in certain metalloproteins, many of which are involved in electron transport in chloroplasts and mitochondria as well as in oxidative stress response [6]. Redox cycling between Cu^{+2} and Cu^{+} also contribute to its inherent toxicity and can catalyze the production of highly toxic hydroxyl radicals, with subsequent damage to cellular homeostasis [7]. Exposure of plants to excessive Cu^{+2} has been reported to generate oxidative stress and ROS [8]. Responses in plants to heavy metal exposure vary depending on plant species, tissues, stages of development, type of metal and its concentration. One of the key responses includes triggering of a series of defense mechanisms against ROS which involve enzymatic and non-enzymatic components [9]. Therefore, the tolerance of plants to stress conditions depends on their ability to strike a balance between the production of toxic oxygen derivatives and capacity of antioxidative defense systems. Phenolics may be efficient antioxidants that accumulate in response to heavy metal pollution in plants [10].

Antioxidant action of phenolic compounds is due to their high tendency to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators [11]. In plants, the biosynthesis of phenolic compounds through aromatic amino acids starts with the shikimate/arogenate pathway [12]. The importance of this pathway is that 20% of the carbon fixed by plants flows through it under normal growth conditions [13]. *Withania somnifera*, an aromatic medicinal plant, is widely considered as the Indian ginseng and in Sanskrit called Ashwagandha, belongs to family Solanaceae, and grows in semi-arid regions of India. In Ayurveda, it is classified as a rasayana (rejuvenation) and expected to promote physical and mental health, rejuvenate the body in debilitated conditions. Ashwagandha is used to treat a number of disorders that affect human health which have been well documented [14]. This study was investigates the effects of Cu^{+2} stress on growth parameters, membrane electrical conductivity, chlorophyll and carotenoids content, lipid per oxidation and total phenol content in *W.somnifera*. This may provide a better understanding of tolerance threshold and additional information on the mechanisms of heavy metal tolerance of plants.

MATERIALS AND METHODS

The seeds of *Withania somnifera* were obtained from CIMAP (Central Institute of Medicinal and Aromatic Plants), Lucknow, India. The seeds were surface sterilized using 2% (w/v) $HgCl_2$ and rinsed with deionized water and patted dry with filter paper. The seeds were sown in pots filled with 3.0 kg of soil mixture containing black soil, sand and vermicompost manure (2:1:1). All the pots were watered every two days with deionized water. Fifteen days post-emergence, the triplicate sets of pots were treated with 0, 10, 20, 50, 100, 200 mM of Cu^{+2} as $CuSO_4.5H_2O$ solution respectively at 7 day intervals for 2 months. Fifteen days after the last applied treatment, the plants were harvested. The harvested plants were washed to remove soil and debris, separated the leaves, stems and roots. These were used for determination of growth parameters *viz.* plant height, average leaf area, stem length and root length, and then stored in an -20^{0}C for further biochemical analysis. Double distilled water was used for all solutions and analysis.

Growth parameters:

Plant height, leaves (length and width), stems and roots were measured from scale and expressed in cm. Average leaf area were expressed in cm^2, and calculated by formula:

$$\text{Leaf area} = l \times b$$

Where: l = maximum length of leaf

b = maximum width of leaf (measured in middle).

Chlorophyll estimation:

Extraction and determination of chlorophyll from leaves and stems was performed according to the method of Arnon [15]. Briefly, plant samples (leaves and stems) 0.1 g fresh wt. were finely cut and ground in 2.0 ml of 80% acetone. The homogenate was centrifuged at 3000 rpm for 5.0 min. and supernatant collected. The procedure was repeated until the residue was colourless. The pooled extracts were made up to 10.0 ml with 80% acetone and assayed immediately. Absorbance was read at 645 and 663 nm against 80% acetone as blank. Chlorophyll content was calculated as:

$$\text{Chlorophyll a (mg/g fresh wt.)} = \frac{(12.7 \times A_{663} - 2.69 \times A_{645})}{1000 \times W} \times V$$

$$\text{Chlorophyll b (mg/g fresh wt.)} = \frac{(22.9 \times A_{645} - 4.68 \times A_{663})}{1000 \times W} \times V$$

$$\text{Total Chlorophyll (mg/g fresh wt.)} = \frac{(20.2 \times A_{645} - 8.02 \times A_{663})}{1000 \times W} \times V$$

Where: A = Absorbance at respective wavelengths

V = Volume of extract (ml)

W = Fresh weight of the sample (g).

Carotenoids estimation: Total carotenoids content of plant samples (leaves and stems) were determined by the method of Kirk and Allen [16]. The extract that was used for the chlorophyll estimation was also used for carotenoids estimation.

Absorbance of the extract was read at 480nm in a double beam UV-spectrophotometer (Systronic) against 80% acetone as blank. Carotenoids content was calculated as:

Carotenoids (mg/g fresh wt.) = $A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645})$

Where: A = Absorbance at respective wavelengths.

Lipid peroxidation:

Lipid peroxidation was performed by the method of He *et al.* [17] from leaves, stems and roots of plant samples.0.1 g fresh wt. were finely cut and homogenized in 2.0 ml of 0.2 mol/L citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100. The homogenate was filtered and centrifuged for 15 min at 6000 rpm at room temperature. To the supernatant (1.0 ml) was added to an equal volume of 20% (w/v) trichloroacetic acid (TCA) containing 0.5% (w/v) TBA. The mixture was heated at 95°C for 30 min, cooled quickly in an ice bath and centrifuged at 6000 rpm for 15 min. The absorbance was read at 450, 532 and 600 nm. Lipid peroxidation expressed as nmol MDA/g fresh wt., and was calculated as:

MDA (nmol/g fresh wt.) = $[6.45(A_{532}-A_{600})-0.56\times A_{450}]$/g fresh wt.

Where: A = Absorbance at respective wavelength.

Membrane Stability Index (MSI)

The membrane stability index (MSI) was determined according to Sairam *et al.* [18]. Two sets of plant samples (leaves, stems and root tips) 0.1 g fresh wt. were cut finely, and placed in 10.0 ml of double-distilled water in. One set was kept at 40 °C for 30 min and its conductivity recorded (C1) using a conductivity meter (Systronic). The second set was kept in a boiling water bath (100 °C) for 15 min and its conductivity was also recorded (C2). The membrane stability index (MSI) was calculated as:

$$MSI (\%) = [1-(C1/C2)] \times 100.$$

Determination of Total phenol:

Total phenol was determined according to McDonald *et al.* [19] by Folin Ciocalteu reagent from leaves, stems and roots samples. Methanolic extracts from each plant (0.5 ml of 1:10 g/ml) or gallic acid (for standard) was mixed with Folin Ciocalteu reagent (5.0 ml, 1:10 in water) and 1M Na_2CO_3 (4.0 ml). The mixtures were allowed to stand for 15 min and the absorbance read at 765 nm. Standard gallic acid solution in methanol: water (1:1 v/v) was prepared and calibration curve plotted (conc. range 0- 250 mg/L). Total phenol values are expressed in terms of gallic acid equivalent (mg/g fresh wt.) and content calculated as:

$$T = C \times V/M$$

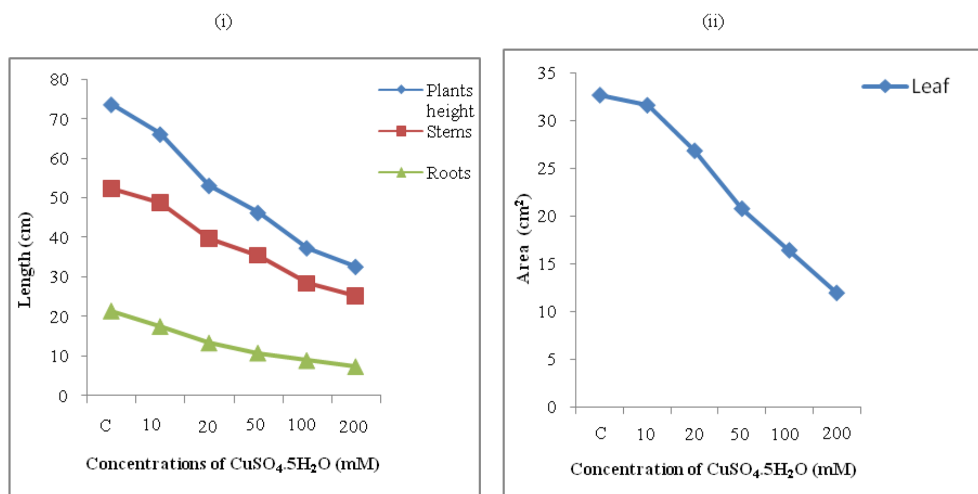
Where: T = Total phenol, mg/g of the extracts as gallic acid equivalent (GAE).
C = Total phenol content of samples expressed in terms of GA calculated from the calibration curve.
V = Volume of extract,
M = Fresh weight of sample extract (g).

Statistical analysis: All the experiments were carried out in quadruplicate and results are given as Mean ± Standard Deviation.

RESULTS

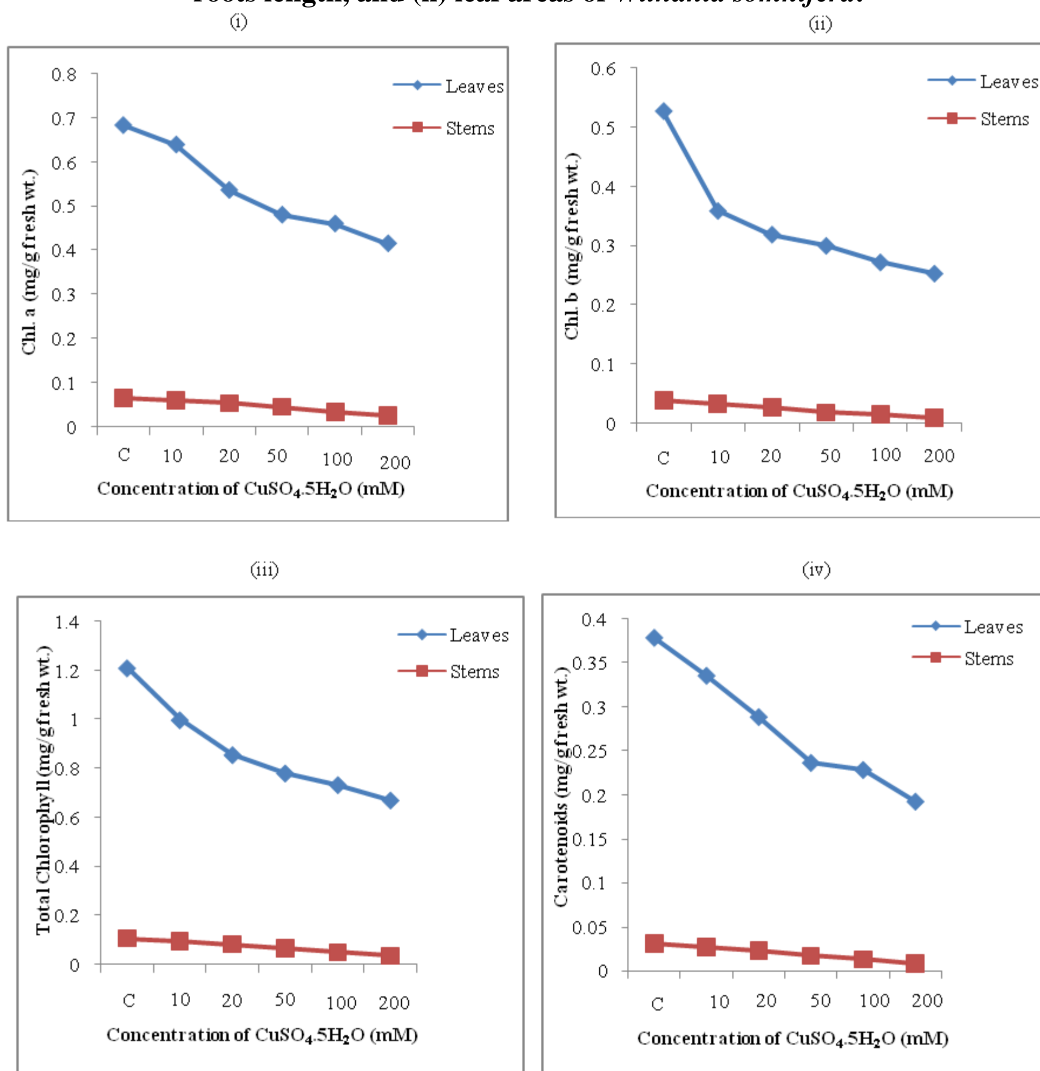
Optimum copper concentration ensures normal growth and development of plants [20] and variations affect growth. From Figure 1, it is clear that height of *W.somnifera* significantly decreased with increasing the concentration of Cu^{+2}. Highest reduction in stem length (25 ±0.74 cm), root length (7 ±0.50 cm) and leaf area (12 ±0.74 cm) was found at 200 mM of Cu^{+2},which was 51.95%, 65.30 % and 63.34% lower than those of the controls (0mM Cu^{+2}) respectively. Plant height decreased from 10.22% (66±0.95 cm) at10 mM to 55.82% (33 ±0.49) at 200mM as compared to control (74±1.17 cm). The chlorophyll and carotenoids content in leaves and stems of plant for different Cu^{+2} treatments are shown in Figure 2.

In control plants, the pigment content was more in leaves as compared to stems. At 200 mM Cu^{+2} concentration, Chl. a (0.42±0.002 mg/g fresh wt.), Chl.b (0.25±0.008 mg/g fresh wt.), total Chlorophyll (0.67±0.010 mg/g fresh wt.) and Carotenoids (0.19±0.004 mg/g fresh wt.) content in leaves were 39.2 %, 52.1 %, 44.8 % and 49.1 % less than those of the controls respectively. However, at 200 mM of Cu^{+2} concentrations in stems, Chl.a (0.026±0.002 mg/g fresh wt.), Chl.b (0.009±0.001mg/g fresh wt.), total Chlorophyll (0.035±.002 mg/g fresh wt.) and Carotenoids (0.008±0.001mg/g fresh wt.) contents were 60.1%, 76.9%, 66.7% and 74.2% lowered as compared to controls respectively. The data pertaining to lipid peroxidation as shown in Table 1 reveals that as compared to control, MDA production in Cu^{+2} treated plants (10 mM-200 mM) increased from 7.7% to 24.9% in leaves, 9.6% to 17.5% in stems and 1.9% to 28.4% in roots respectively.



*All values are MEAN of quadruplicate values

Fig. 1 Effect of different concentrations of $CuSO_4.5H_2O$ on growth parameters (i) Plants height, stems and roots length, and (ii) leaf areas of *Withania somnifera*.



*All values are MEAN of quadruplicate values.

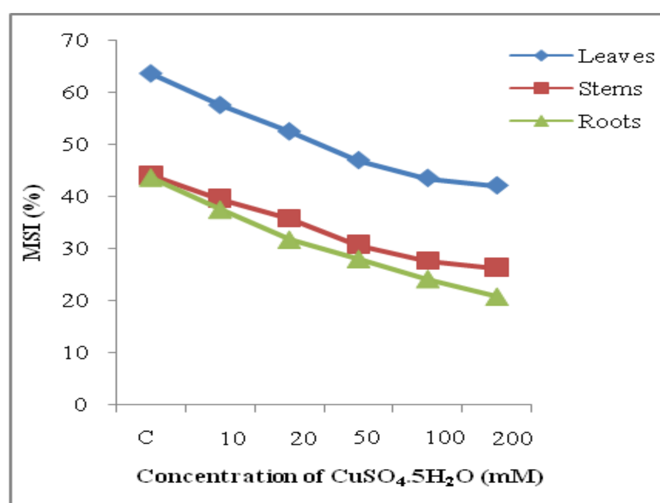
Fig. 2 Effect of different concentrations of $CuSO_4.5H_2O$ on (i) Chl. a (ii) Chl. b (iii) total Chlorophyll (chl. a+b) and (iv) Carotenoids content in leaves and stems of *Withania somnifera*.

Table1. Lipid peroxidation (MDA nmol/g fresh wt.) in leaves, stems and roots of *Withania somnifera* exposed to different concentrations of $CuSO_4.5H_2O$

Treatments	Leaves	Stems	Roots
Cu^{+2} (mM)			
Control (0mM)	1.46±0.143	0.97±0.054	2.12±0.021
10	1.58±0.043	1.06±0.039	2.15±0.102
20	1.61±0.011	1.08±0.027	2.41±0.027
50	1.67±0.054	1.09±0.026	2.50±0.032
100	1.73±0.045	1.11±0.018	2.54±0.043
200	1.83±0.023	1.14±0.033	2.71±0.077

*All values are Mean ± SD of quadruplicate values.

The cell membrane damage is also indirectly assessed by ion leakage from the cells. In control, MSI was highest in leaves (63.6%) followed by stem (43.9%) and root (43.6%) (Figure-3).



*All values are MEAN of quadruplicate values.

Fig. 3 Effect of different concentrations of $CuSO_4.5H_2O$ on membrane stability index (MSI) in leaves, stems and roots of *Withania somnifera*.

At 10 mM Cu^{+2} treatment, MSI was 57.5% in leaves, 39.4% in stems and 37.4% in roots. At 200mM Cu^{+2} level, electrolytic ions leakage significantly get increased by 42.0% in leaves, 26.2% in stems and 20.7% in roots and possibly disrupt membrane stability. It is observed that membrane stability index in leaves, stems and roots found to be significantly decreased with rising concentration of Cu^{+2} stresses as compared to control.
Total phenolic content measured was variably affected by Cu^{+2} concentrations given in Table 2.

Table-2. Total phenolic contents (mg/g fresh wt.) in leaves, stems and roots of *Withania somnifera* exposed to different concentrations of $CuSO_4.5H_2O$

Treatments	Leaves	Stems	Roots
Cu^{+2} (mM)			
Control(0 mM)	0.117±0.006	0.168±0.005	0.132±0.005
10	0.119±0.004	0.187±0.004	0.166±0.010
20	0.133±0.003	0.238±0.004	0.217±0.006
50	0.144±0.003	0.273±0.006	0.239±0.008
100	0.106±0.003	0.156±0.004	0.109±0.006
200	0.087±0.003	0.141±0.003	0.075±0.006

*All values are MEAN ± SD of quadruplicate values.

Compared to their respective control, phenolic content was found to be increased at lower concentration of 10, 20 and 50 mM by 1.7%, 13.7% and 23.1% in leaves, 11.3%, 41.7% and 62.5% in stems and 25.8%, 64.4% and 81.1% in roots respectively. At low Cu^{+2} concentrations, % phenolic accumulation was found highest in roots than leaves and stems compared to their respective control. At higher concentration of 100 and 200 mM, the phenolic content decreases significantly over the controls by 9.4 % and 25.6% in leaves, 7.1 % and 16.1% in stems and 17.4 % and 43.2 % in roots, respectively.

DISCUSSION

Growth analysis is fundamental to the characterization of plant's response to environmental stress**.** It is reported that roots growth is highly affected by Cu^{+2} than stems and leaves. This might be due to a high Cu^{+2} supply usually tends to decrease root growth before stem growth, because of preferential copper accumulation in that organ [21] and is considered as a very sensitive indicator to heavy metal exposure [22]. Our findings suggested that *W.somnifera* is highly sensitive to elevated Cu^{+2} concentrations which strongly inhibit the normal growth and development. Michaud *et al.* [23] also reported that stem and root growth get reduced along with decline in photosynthetic pigment formation in plants under Cu^{+2} stress.

A significant decrease in Chl.a, Chl. b, total Chlorophyll and Carotenoids content of *W.somnifera* was observed with increasing concentration of Cu^{+2} treatments. The decline in the levels of these pigments clearly establishes the effect of Cu^{+2} in pigment metabolism. Similar results of decreased chlorophyll content have been reported in leaves of spinach, Ashwagandha (*W.somnifera*) and wheat (*Triticum durum*) under Cu^{+2} stress [24, 25, 26]. Ouzounidou *et al.* [27] also reported that copper decreased chlorophyll and carotenoids contents in *Thlaspi ochroleum* and oat. The loss in chlorophyll content could be due to peroxidation of chloroplast membranes or replacing of magnesium in chlorophyll molecule by copper [28, 29]. Decreased chlorophyll content is a typical response to injurious levels of copper even in copper tolerant species [30] which is also corroborated in this study.

Copper affects the properties of biological membranes *via* peroxidation of membrane lipids, which is assessed as increased MDA production. The increasing trend of MDA production with increasing Cu^{+2} levels is similar in leaves, stems and roots, but comparatively, roots of *W.somnifera* suffered more oxidative damage. Studies have also reported that cell membrane is the primary target of copper toxicity and cause greatly increases MDA accumulation [25, 31]. Enhanced lipid peroxidation observed in treated plants is probably due to the oxidizing properties of Cu^{+2}, which catalyses the formation of extremely reactive hydroxyl radicals [32] as well as Cu-provoked stimulation of lipoxygenase activity [33]. The peroxidation of unsaturated lipids in biological membranes is the most prominent symptom of oxidative stress in plants by which the functionality and integrity of the membrane is affected and can produce irreversible damage to cell function, hence also considered to be a biomarker of metal-induced oxidative stress [34].

Olga *et al.* [35] found that copper stimulated lipid peroxidation and enhanced membrane permeability. It is reported that electrolyte leakage was increased by the generation of ROS in *Avicennia germinans* and *Hordeum vulgare*, respectively, when exposed to cadmium and copper, which ultimately decreased the membrane stability [11, 36]. The increase of both electrical conductivity and MDA levels of *W.somnifera* under Cu^{+2} may be attributed to injury of plasma membrane caused by reactive oxygen species which induced lipid peroxidation [37].

Effect of stress on plants to abiotic factors can be correlated with the amount of total free phenols. Phenolic compounds are recognized as a class of antioxidant agents which act as free radical terminators [38]. Elleuch *et al.* [39] also reported that total phenol contents were strongly induced by low concentrations of Cu, consistent with the role of these potent antioxidants in scavenging ROS but returned to control levels or below at high concentrations. In MS culture medium grown *W. somnifera* plants, Khatun *et al.* [25] reported that total phenol content get increased even at 200μM concentrations of Cu^{+2} , our findings do not agree with the earlier study. This discrepancy could be due to different growing conditions and concentrations. However, our findings are in agreement with that of Elleuch *et al*. [38] as reported in fenugreek. Diaz *et al*. [40] reported accumulation of phenolics under Cu stress could be possibly due to the induction of shikimate dehydrogenase (SKDH, EC 1.1.1.25) synthesis. An explanation for SKDH induction could be related to the interaction of metals with the SH-groups of the enzyme, which are also necessary for the catalytic activity of SKDHs [41]. It is possible that the plant reacts to copper stress by increasing SKDH synthesis, in order to counteract the metal inhibition of the enzyme. At higher copper concentration, SKDH synthesis would be unable to compensate for the loss of activity by metal inhibition. However, at lower concentration, SKDH synthesis would not be sufficient to cause a great loss in activity by inhibition, but sufficient to induce the enhancement of the synthesis [40]. This study is the first report of on effect of Cu^{+2} on phenolic content in *W.somnifera*.

Although Cu usually binds to proteins it has capacity to initiate oxidative damage and interfere with important cellular processes such as photosynthesis, pigment synthesis, plasma membrane permeability, leading to ion leakage and other metabolic mechanisms, causing a strong inhibition of plant growth [42].

CONCLUSION

Results taken together of decreased growth parameters, chlorophyll and carotenoid content, membrane stability and increased MDA along with phenol to a limited concentration of Cu^{+2}, indicates a shift in the plant response to copper toxicity at low versus high concentrations. It was concluded that *W.somnifera* plants are decidedly sensitive to high levels Cu^{+2} stress as is indicated by an increased MDA production, low membrane stability, and possible loss of ROS scavenging potential due to decreased production of total phenol at higher levels of Cu^{+2} stresses. Further, it is needed to study other enzymatic antioxidant properties also in *W.somnifera* under different concentrations of $CuSO_4.5H_2O$.

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