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EFFECT OF PROLINE AND MANNITOL ON ANTIOXIDANT, SECONDARY METABOLITE, BIOCHEMICAL ANALYSIS OF *LEPIDIUM SATIVUM* L. UNDER ABIOTIC STRESS CONDITION (HEAVY METAL STRESS)

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Abstract

In agricultural ecosystems, heavy metal stress has become immense environmental hazard that cause deterioration of yield and quality of crops. In this study, we examined the effect of proline and mannitol to heavy metal stress tolerance in *Lepidium sativum* L. plants were grown under controlled temperature (25°C) light conditions (16 hours light and 8 hours dark). Antioxidants analysis (catalase, peroxidase, superoxide dismutase, ascorbic acid, and tocopherol) secondary metabolite profile (phenol, glycosides, alkaloid, and saponin) and biochemical analysis (lipid peroxidation) were determined after 45 and 90 days. Exogenous application of proline and mannitol 50μg/l, 100μg/l, and 250μg/l each were standardized and applied to heavy metal stress ($CdSO_4$50 and 100μM). Proline and *mannitol enhanced the* antioxidants analysis*,* secondary metabolite profile*,* biochemical analysis *in stressed plant; these osmolytes play a vital role in cellular osmotic adjustment. Present study indicating that the proline is more effective as compared to mannitol and play a major role to heavy metal stress tolerance in Lepidium sativum* L. Consequently, it found that the plants are able to cope with abiotic stress when exogenous proline and mannitol is applied.

Key words: Abiotic stress, alkaloid, catalase, lipid peroxidation, saponin, superoxide dismutase.

Introduction

Heavy metal stress issues are becoming common in agriculture field. Heavy metal accumulation in soil is concern because it directly affects crop production, which related to food safety (Nagajyoti *et al*., 2010). Elements such as aluminium, cobalt, silicon, sodium and selenium positively affect plant growth and stress resistance (Broadley, 2012). By investigating the plants under stress, we can study about the plasticity of metabolic pathways and confines to their functioning. Cadmium is one of the utmost toxic environmental pollution, which is quite toxic even in minute concentration (Lopez *et al*., 2009). The source of cadmium entrance in environment is industrial processes, mining operations, municipal wastes and phosphate fertilizers (Khan *et al*., 2017). Cadmium has high water solubility, relative mobility and phytotoxicant even in minute amounts (Kashem *et al*., 2007). Cadmium toxicity in plants has also shown to alleviated by interaction with other elements such as zinc (Kukier and Chaney, 2002) and silicon (Neumann and Zur Nieden, 2001; Iwasaki *et al.,* 2002). Cadmium has kept in seventh rank for being most toxic element for both plants and human. Cadmium is a non-essential element but still plants, entering food chain and causing threat to both plant and human life, accumulate it. Cadmium stress in plants shows retardation in various biochemical and physiological processes, which includes chlorophyll synthesis, photosynthesis, nutrient uptake and results in low yield (Farooq *et al*., 2013). Plants show various symptoms of Cd toxicity such as leaf chlorosis, growth inhibition, and disruption of key physiological processes including photosynthesis (Reeves *et al*., 2008). Cadmium interacts with the water balance of plant and disrupts nutrient balance and stomata opening which further make

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disturbance in Calvin cycle enzymes, carbohydrate metabolism and changes the antioxidant metabolism. Cadmium uptake results in inducing oxidative stress via different indirect mechanism. (Sarwar *et al.,* 2010) have studied the interaction of mineral nutrients in reducing Cd accumulation, roles of essential and beneficial plant elements in Cd stress alleviation. Plants possess a number of antioxidant systems that protect them from oxidative damage (Smeets *et al.,* 2005; Pal *et al.,* 2006). Super oxidase is the first enzyme in the detoxifying process that converts O2- radicals to H_2O_2 at a very rapid rate (Polle and Rennenberg, 1994). Cadmium found to result in oxidative stress by either inducing oxygen free radical production (Balaknina *et al.,* 2005; Demirevska-Kepava *et al.,* 2006). By decreasing concentrations of enzymatic and non-enzymatic antioxidants (Sandalio *et al.,* 2001; Balestrasse *et al.,* 2001; Fornazier *et al.,* 2002; Cho and Seo, 2004; Mohan and Hosetti, 2006). These defense systems are composed of metabolites such as ascorbate, glutathione, tocopherol, etc., and enzymatic scavengers of activated oxygen such as peroxidases, catalases and superoxide dismutases (Sandalio *et al.,* 2001; Bor *et al.,* 2003; Panda and Khan, 2003; Demiral and Turkan, 2005; Mandhania *et al.,* 2006). Osmolytes are naturally occurring small molecules accumulated intracellularly to protect plants from various denaturing stresses (Singh and Tiwari, 2003). Organic osmolytes used in plant cell to adapt to hyper and hyperosmolar stress (Moeckel *et al*., 2002). The accumulation of metabolites under heavy metal stress may be "Compatible osmolytes" (Talibart *et al.,* 1994). The osmolytes or so-called compatible solutes are unbiased beneath physiological pH, have a low molecular mass, a high solubility in water, and are non-hazardous to the plants even when accumulated at a high concentration. Proline plays very important role in activating various enzymes activity in responses to environmental stresses and can used as stress indicator. Proline is endogenous carbon-based substance, which regulates normal growth and development of plants, grown under heavy metal stress results in enhancing the activity of enzymes and biosynthetic pathways (Singh *et al.,* 2015). In cadmium

Table 1: Effect of proline and mannitol to water stress on catalase (U (µmol/min)) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test $P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$ significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	0.266±0.103	0.396±0.102
Proline 50µg/l and $CdSO_4$50µM	0.386±0.106	0.526±0.115
Proline 100µg/l and $CdSO_4$50µM	0.497±0.107	0.599±0.103
Proline 50µg/l and $CdSO_4$ 100µM	0.310±0.108	0.498±0.108
Proline 100µg/l and $CdSO_4$ 100µM	0.381±0.106	0.525±0.106
Mannitol 50µg/l and $CdSO_4$ 50µM	0.298±0.110	0.456±0.110
Mannitol 100µg/l and $CdSO_4$ 50µM	0.356±0.109	0.512±0.114
Mannitol 50µg/l and $CdSO_4$ 100µM	0.246±0.102	0.345±0.104
Mannitol 100 µg/l and $CdSO_4$ 100µM	0.312±0.105	0.584±0.113i

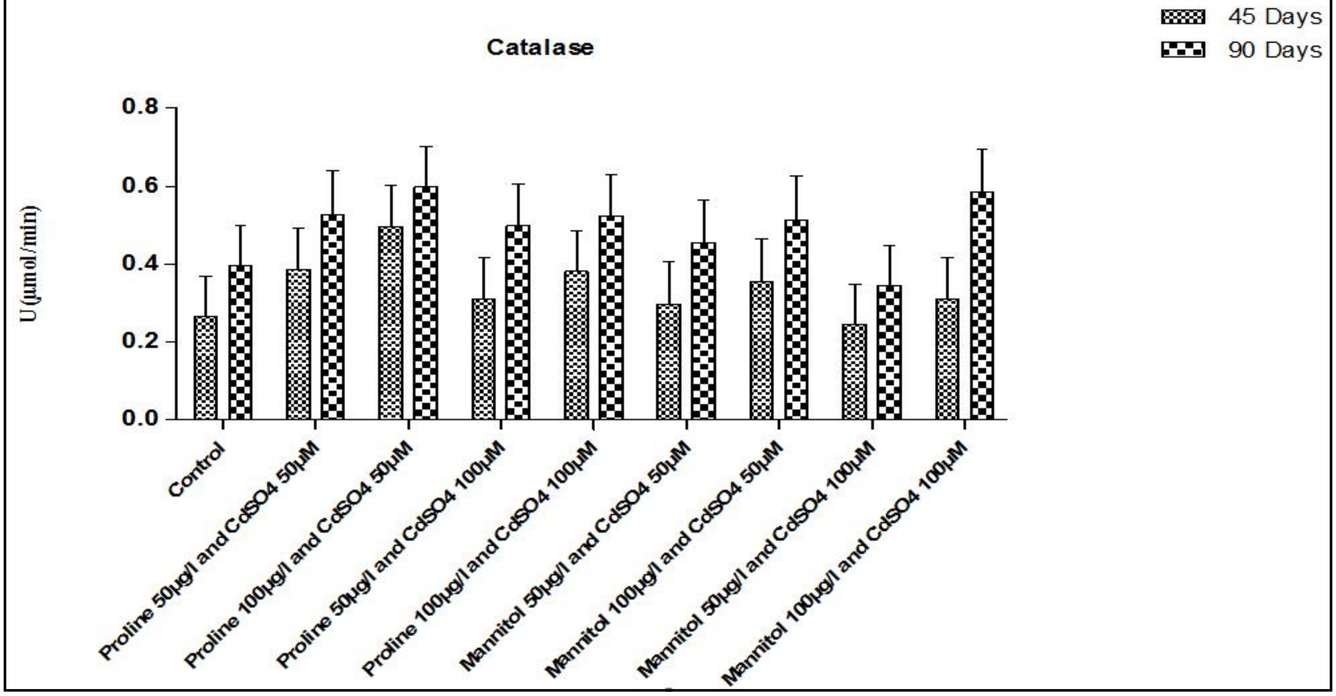

Fig. 1: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on catalase activity.

stressed plants, high proline content plays a protective role. Proline protects plants from heavy metal toxicity (Kavi Kishor *et al.*, 1995). Many researchers consider that proline accumulation is a symptom of injury, which does not deliberate tolerance against metal or other stresses (Lutts *et al.,* 1996). In addition, mannitol is a widely used osmolyte to study plant responses to osmotic stress (Nikonorova *et al.,* 2018; Zhang *et al.,* 2018). Mannitol is a major photosynthetic product in many algae and higher plants (Loescher *et al.,* 1992). Mannitol metabolism plays a role in plants response to equally biotic and abiotic stresses. Mannitol accumulation increases when plants wide-open to low water potential and accumulation controlled by inhibition of competing pathways and decreased mannitol consumption and catabolism. The rate of mannitol use in sink tissues declines during salt stress mainly because of the suppression of the NAD$^+$-dependent mannitol dehydrogenase (Stoop *et al.,* 1995). Mannitol improves growth of transgenic wheat under water stress and salinity both at the callus and whole-plant level (Abebe *et al.,* 2003).

Table 2: Effect of proline and mannitol to water stress on peroxidase (U(µmol/min)) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test $P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$ significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	0.288±0.107	0.379±0.106
Proline 50µg/l and $CdSO_4$ 50µM	0.426±0.101	0.485±0.108
Proline 100µg/l and $CdSO_4$ 50µM	0.541±0.115	0.565±0.102
Proline 50µg/l and $CdSO_4$ 100µM	0.365±0.105	0.394±0.106
Proline 100µg/l and $CdSO_4$ 100µM	0.409±0.120	0.465±0.113
Mannitol 50µg/l and $CdSO_4$ 50µM	0.398±0.104	0.394±0.103
Mannitol 100µg/l and $CdSO_4$ 50µM	0.342±0.107	0.428±0.114
Mannitol 50µg/l and $CdSO_4$ 100µM	0.302±0.103	0.354±0.105
Mannitol 100 µg/l and $CdSO_4$ 100µM	0.333±0.117	0.401±0.112

Materials and methods

Plant growth

The seeds propagated in seed trays comprising sand, soil, farmyard manure (FYM in ratio of 1:1:1) placed in a polyhouse with regulated temperatures ranging among 23 to 25°C, under a long-day photoperiod (16h light/8h dark). 10 days old seedling shifted to different pots, which contain $CdSO_4$ in different concentration 50 and 100µM/kg soil. After shifting of 10 days to pots proline, mannitol 50µg/l and 100µg/l, each were standardized and applied to stress plants exogenously. Plants manured by adding Hoagland nutrient solution to each pot subsequently after every seven days. Plants parts (Leaves) sampled to determined antioxidants analysis (catalase, peroxidase, superoxide dismutase, ascorbic acid, and tocopherol) secondary metabolite profile (phenol, glycosides, alkaloid, and saponin) and biochemical analysis (lipid peroxidation) after 45 and 90 days.

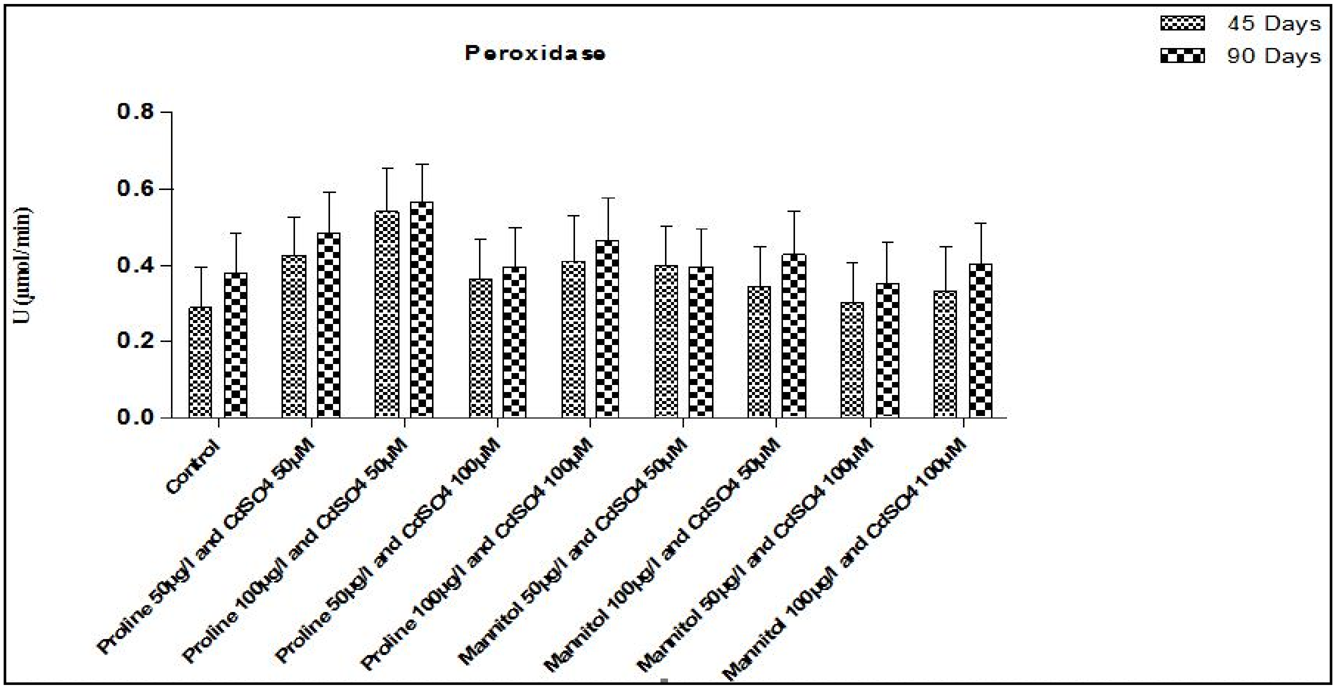

Fig. 2: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on peroxidase activity.

Catalase

Catalase activity assayed following the method of (Luck, 1974). Plant tissue (leaf) homogenized in a blender with (0.067 M, pH 7.0) phosphate buffer (assay buffer diluted 10 times) at 1-4°C and centrifuged. Sediments were stirred with cold phosphate buffer, allowed standing in the cold with occasional shaking and then repeating the extraction once or twice. The final volume for the assay mixture was approximately 3 ml, 240 nm wavelength read against a control cuvette containing enzyme solution as in the experimental cuvette, but containing H_2O_2-free PO_4 buffer. Then sample pipetted out into the experimental cuvette 3ml H_2O_2-PO_4 buffer and mixed in 0.01-0.04 ml sample with a glass or plastic rod flattened at one end. Time was noted require for a decrease in absorbance from 0.45 to 0.4. This value was used for calculations. 1g tissue was homogenized in a total volume of 20 ml, diluted 1 to 10 volumes with water and taken 0.01ml for assay. Concentration of H_2O_2 using the extinction coefficient 0.036/m mole/ml was calculated.

Peroxidase

The method given by (Reddy *et al.* 1995) implemented for assessing the activity of peroxidase. A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) from the plant sample. Centrifuged and the supernatant was cast-off for the assay. To 3.0 ml of pyrogallol solution, 0.1 ml of the enzyme extract added. In a test cuvette, 0.5 ml of H_2O_2 added and mixed. The change in absorbance recorded every 30 seconds up to 3 minutes. One unit of peroxidase defined as the change in absorbance/minute at 430 nm.

Table 3: Effect of proline and mannitol to water stress on super oxide dismutase (U(µmol/min/mg protein)) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	85.563±0.586	90.630±0.834a
Proline 50µg/l and $CdSO_4$50µM	87.402±0.669	93.750±0.798b
Proline 100µg/l and $CdSO_4$50µM	88.153±0.708	95.406±0.869c
Proline 50µg/l and $CdSO_4$ 100µM	86.331±0.711	89.840±0.836d
Proline 100µg/l and $CdSO_4$ 100µM	87.511±0.814	93.580±0.785e
Mannitol 50µg/l and $CdSO_4$ 50µM	85.956±0.826	92.220±0.746f
Mannitol 100µg/l and $CdSO_4$ 50µM	86.475±0.715	93.810±0.653g
Mannitol 50µg/l and $CdSO_4$ 100µM	85.737±0.738	87.490±0.985h
Mannitol 100 µg/l and $CdSO_4$ 100µM	85.164±0.811	92.720±0.850i

Superoxide dismutase

SOD activity was determined according to the method of (Kakkar *et al.,* 1984). The leaves (0.5g), were ground with 3.0 ml of potassium phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants were taken for the assay. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml ofNBT, 0.2 ml of the enzyme preparation and water in a total volume of

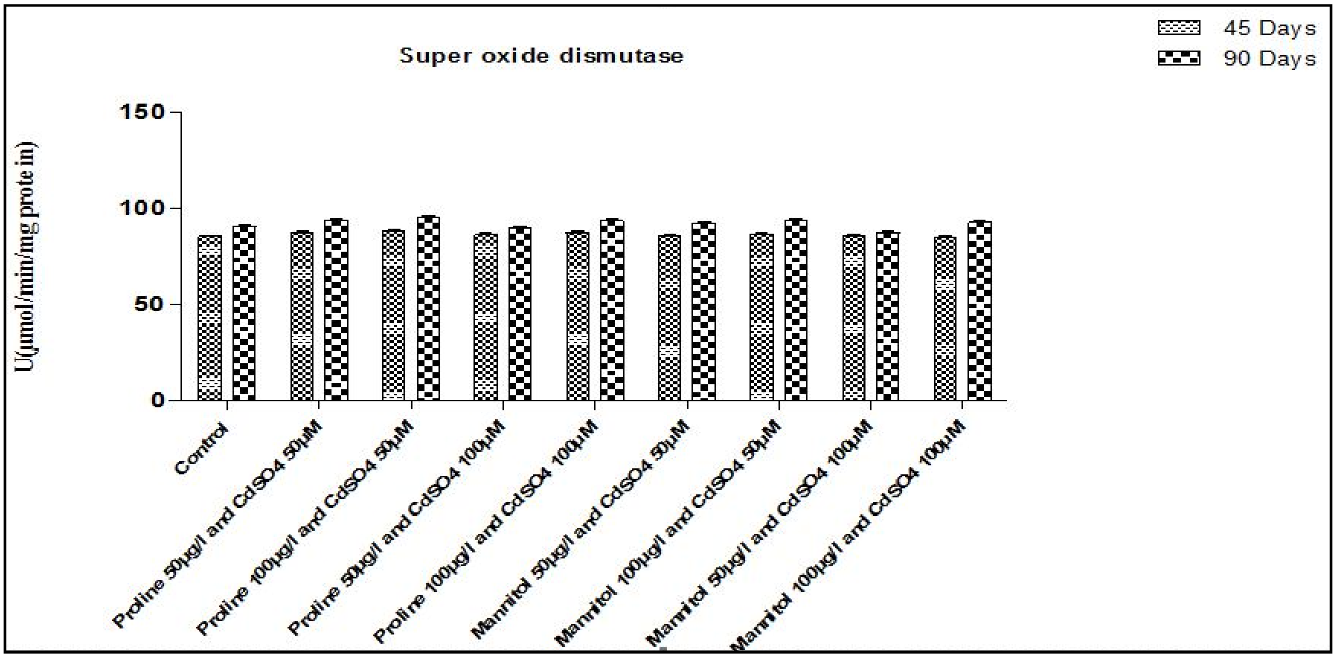

Fig. 3: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on super oxide dismutase activity.

2.8 ml. The reaction started by adding of 0.2 ml of NADH. Mixture incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. Reaction mixture then shaken with 4.0 ml of n-butanol, permissible to stand for 10 minutes and centrifuged. Intensity of the chromogen in the butanol layer recorded at 560nm. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

Ascorbic Acid

Ascorbic acid (AA) analyzed by the spectrophotometric method described by (Roe and Keuther, 1943). Ascorbate extracted from 1g of the plant sample using 4% TCA. The supernatant treated with a pinch of activated charcoal, shaken vigorously using a cyclomixer and kept for 5 minutes. Charcoal particles removed by again centrifugation and aliquots were used for the estimation. Standard ascorbate ranging between 0.2-1.0 ml and 0.5 ml and 1.0ml of the supernatant were taken. The volume was made up to 2.0 ml with 4% TCA. DNPH reagent (0.5 ml) added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5 ml of 85% sulphuric acid, in cold. To the blank alone, DNPH reagent and thiourea added after the addition of sulphuric acid and absorbance read at 540 nm. The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

Table 4: Effect of proline and mannitol to water stress on ascorbic acid (mg/g) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	1.298±0.653	1.852±0.678
Proline 50µg/l and $CdSO_4$ 50µM	2.985±0.893	2.895±0.786
Proline 100µg/l and $CdSO_4$ 50µM	3.754±0.436	3.985±0.698
Proline 50µg/l and $CdSO_4$ 100µM	2.105±0.765	2.105±0.598
Proline 100µg/l and $CdSO_4$ 100µM	2.869±0.953	2.769±0.590
Mannitol 50µg/l and $CdSO_4$ 50µM	2.179±0.658	2.322±0.652
Mannitol 100µg/l and $CdSO_4$ 50µM	2.814±0.726	3.146±0.732
Mannitol 50µg/l and $CdSO_4$ 100µM	1.989±0.654	1.952±0.745
Mannitol 100 µg/l and $CdSO_4$ 100µM	2.431±0.769	2.586±0.687

Tocopherol

Tocopherol assessed in the plant samples by the emmerie-engel reactions reported by (Rosenberg, 1992). Sample (1g) homogenized in 50 ml of 0.1N sulphuric acid and allowed to stand overnight. The contents filtered through Whatman No.1 filter paper aliquots of the filtrate used for the estimation. Into centrifuge tubes, 1.5 ml of plant extract, 1.5 ml of the standard and 1.5 ml of water pipetted out separately. To all the tubes, 1.5 ml of ethanol and 1.5ml of xylene added, mixed well and centrifuged. Xylene (1.0 ml) layer transferred into another stoppered tube. To each tube, 1.0 ml of dipyridyl reagent added and mixed well. The mixture (1.5 ml) was pipetted out into a cuvette and the extinction was read at 460nm. Ferric chloride solution (0.33 ml) was added to all the tubes and mixed well. The red colour developed read after 15 minutes at 520 nm in a spectrophotometer. From the standard

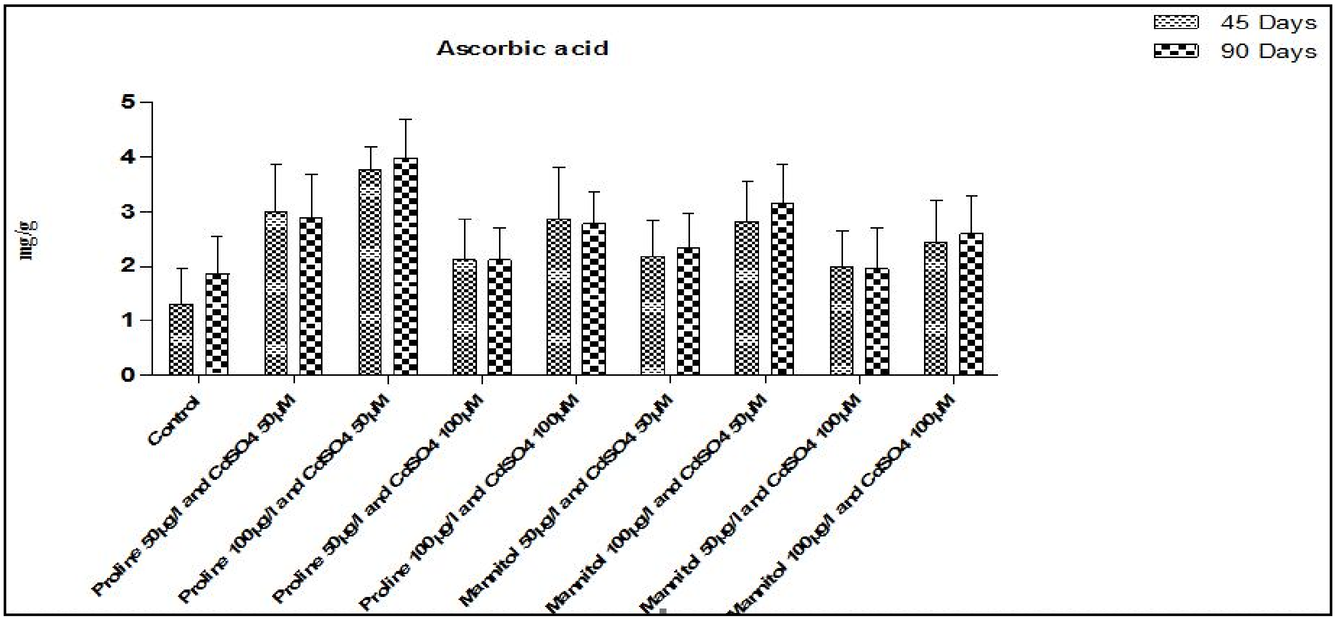

Fig. 4: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on ascorbic acid.

curve, the concentration of tocopherol in the test sample was determined and expressed as µg/g plant material.

Phenol

Phenol content estimated by (Malick and Singh, 1980).1g of the sample (leaf and root) and ground it with a pestle and mortar in 10-time volume of 80% ethanol centrifuged at 10,000 rpm for 20 min. Re-extracted the residue with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. Supernatant then evaporated to dryness residues dissolved in distilled water. Different aliquots (0.2 to 2 ml) into test tubes pipetted out and volume made up in each tube to 3 ml with water. Folin-Ciocalteau reagent added after 3 min, added 2 ml of 20% Na_2CO_3 solution to each tube and mixed thoroughly. Placed the tubes in a boiling water bath for one minute, cooled and measured the absorbance at 650 nm against a reagent blank. Standard curve using different concentrations of gallic acid was prepared. From the standard curve, the concentration of phenols in the test sample was determined and expressed as mg/g material.

Glycosides

Cardiac glycosides estimated by the method given by (El-Olemy *et al.*, 1994). It develop an orange red colour complex with Baljet's reagent. The intensity of colour produced is proportional to the concentration of glycosides. 10ml of the extract and 10ml of Baljet's reagent taken and allowed to stand for one hour dilute the solution with 20ml distilled water and mix. Read the absorbance of the colour obtained against blank at 495nm. The difference between test and control taken for calculation. Standard graph prepared by using standard digitoxin. Concentration (%) = Absorbance×100 g % 17.

Determination of alkaloid

Adopted the method given by (Omoruyi *et al.,* 2012). 5 g of plant sample mixed with 200 mL of 10% acetic acid in ethanol. The mixture covered then permissible toward stand for 4 h. This mixture filtered than the remainder stood concentrated on a hot water bath to a quarter of its original volume. Rigorous ammonium hydroxide added in droplets to the extract until precipitation (cloudy fume) accomplished. The

Table 5: Effect of proline and mannitol to water stress on tocopherol (µg/g) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test $P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$ significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	5.651±0.658	7.233±0.562a
Proline 50µg/l and $CdSO_4$50µM	7.262±0.759	9.414±0.468b
Proline 100µg/l and $CdSO_4$50µM	8.306±0.465	10.522±0.876c
Proline 50µg/l and $CdSO_4$ 100µM	6.417±0.658	8.649±0.687d
Proline 100µg/l and $CdSO_4$ 100µM	7.432±0.796	7.919±0.698e
Mannitol 50µg/l and $CdSO_4$ 50µM	6.242±0.568	8.512±0.698f
Mannitol 100µg/l and $CdSO_4$ 50µM	7.209±0.632	9.363±0.786g
Mannitol 50µg/l and $CdSO_4$ 100µM	5.594±0.659	8.389±0.598h
Mannitol 100 µg/l and $CdSO_4$ 100µM	6.212±0.765	7.441±0.698i

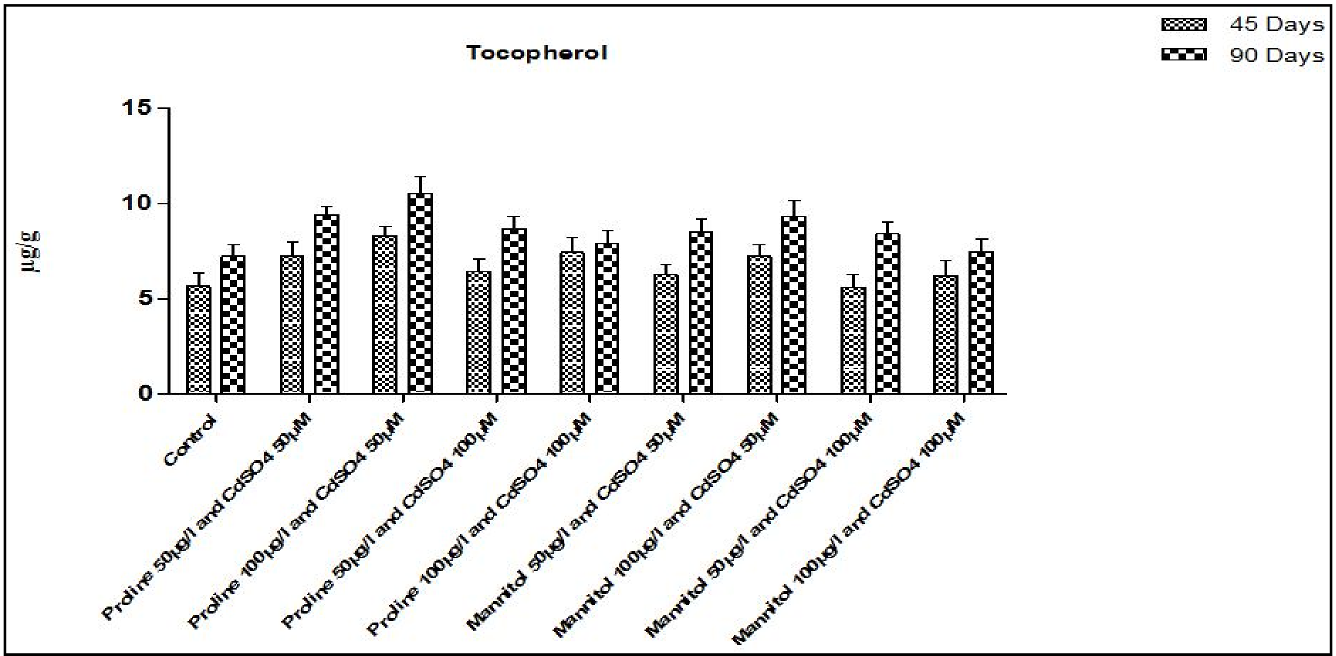

Fig. 5: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on tocopherol.

solution remained permissible to settle, washed through diluted ammonium hydroxide then filtered. The residue collected was dried and weighed then the alkaloid content calculated by means of the equation:

% Alkaloid = Weight of precipitate/Weight of original sample × 100

Determination of saponin

Saponin content estimated as method described by (Obadoni and Ochuko, 2002). 5 g of the crushed plant sample added to 50 mL of 20% ethanol, retained on a shaker aimed at 30 min and then heated in a water bath on 55^{0}C for 4 h. The subsequent mixture filtered and then remainder re-extracted through additional 200 mL of 20% aqueous ethanol. The remainders were collective and condensed to 40 mL in a boiling water bath at 90^{0}C. The concentrate shifted into a splitting funnel, 20 mL of diethyl ether added, and then shaken enthusiastically. The ether film, which was the upper film, discarded and then the aqueous (bottom) layer retained in a beaker. The retained layer re-introduced into a splitting funnel and 60 mL of n-butanol added then shaken enthusiastically. The butanol extract, which is the upper layer, reserved although the bottom layer thrown away. The butanol layer was wash away twice with 10 mL of 5% aqueous sodium chloride. The residual solution collected and heated to evaporation in a boiling water bath, formerly dehydrated to constant weight at 40°C in an oven. The saponin content remained calculated by means of the equation:

% Saponin content = Weight of residue/Weight of original sample × 100

Lipid Peroxidation

Lipid peroxidation estimated from the accumulated malondialdehyde (MDA) following the method given by (Dhindsa *et al.,* 1981). In brief, the plant tissue (200 mg) (leaf) homogenized with 0.1% trichloroacetic

Table 6: Effect of proline and mannitol to water stress on phenol (mg/g) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test $P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$ significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	1.985±0.523	2.721±0.543
Proline 50µg/l and $\text{CdSO}_4$50µM	3.144±0.568	4.622±0.643
Proline 100µg/l and $\text{CdSO}_4$50µM	4.107±0.653	5.942±0.546c
Proline 50µg/l and CdSO_4 100µM	2.592±0.569	3.827±0.645
Proline 100µg/l and CdSO_4 100µM	3.294±0.645	4.307±0.519
Mannitol 50µg/l and CdSO_4 50µM	2.451±0.742	3.129±0.765
Mannitol 100µg/l and CdSO_4 50µM	3.632±0.623	4.109±0.612
Mannitol 50µg/l and CdSO_4 100µM	1.982±0.436	2.752±0.832
Mannitol 100 µg/l and CdSO_4 100µM	2.871±0.562	3.405±0.645

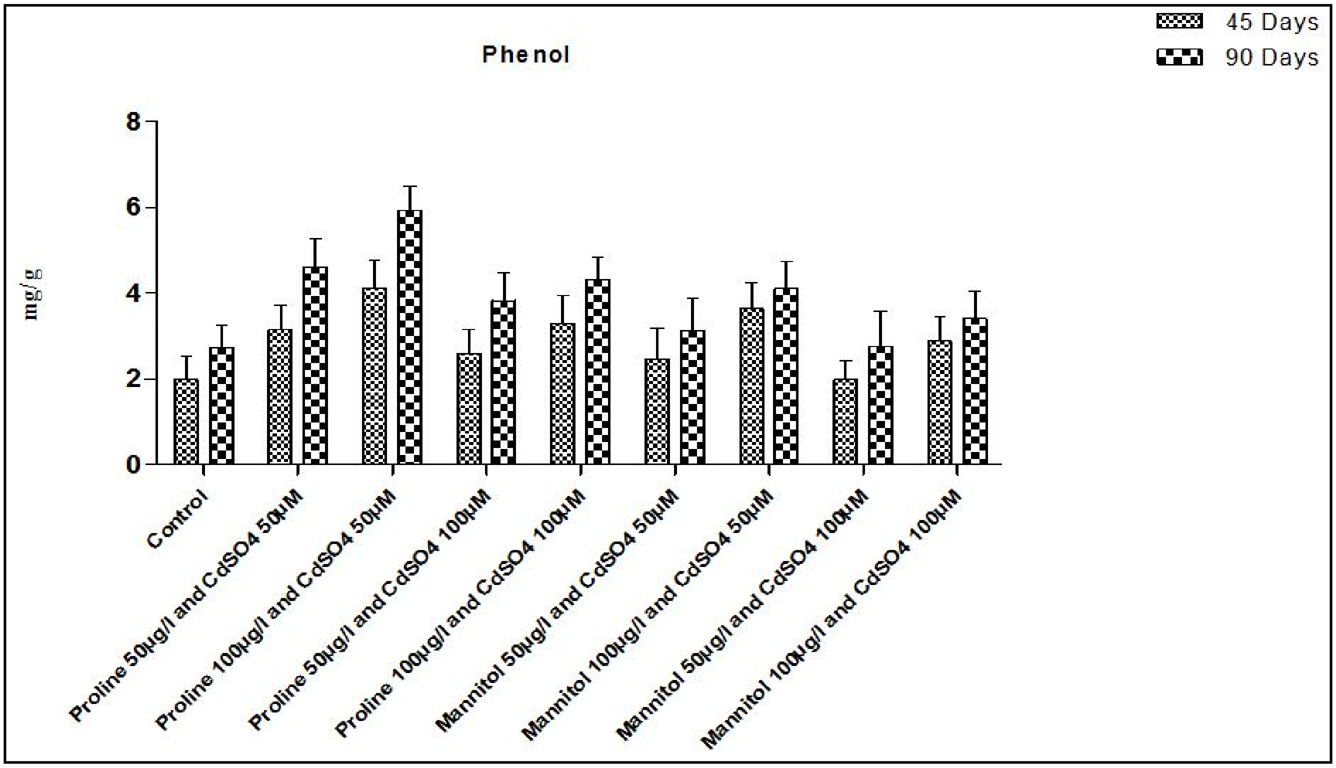

Fig. 6: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress (CdSO_4 50 and 100µM) on phenol.

acid (TCA) (2 ml). The homogenate was centrifuged at 10,000 rpm for10 min. In addition, supernatant collected the supernatant (2 ml) was reacted with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was then heated at 95ºC for 45 min. And rapidly cooled in an ice bath for 5 min. Absorbance was read at 532 nm. Measurements corrected for unspecific turbidity by subtracting the absorbance at 600 nm.

Results

Catalase

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on catalase activity of *Lepidium sativum* L.While different osmolytes 50and 100µg/l applied with cadmium stress ($CdSO_4$), catalase activity enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more catalase activity in stressed plants, which explained in table 1, Fig. 1.

Peroxidase

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on peroxidase activity of *Lepidium sativum* L.While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), peroxidase activity enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more peroxidase activity in stressed plants, which described in table 2, Fig. 2.

Superoxide dismutase

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on superoxide dismutase activity of *Lepidium sativum* L. while different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), superoxide dismutase activity enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more superoxide dismutase activity in stressed plants, which described in table 3, Fig. 3.

Table 7: Effect of proline and mannitol to water stress on glycosides (%/) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	1.68±0.678	1.957±0.568
Proline 50µg/l and $CdSO_4$50µM	2.402±0.548	3.105±0.785
Proline 100µg/l and $CdSO_4$50µM	3.310±0.645	4.252±0.687
Proline 50µg/l and $CdSO_4$ 100µM	1.862±0.571	2.281±0.458
Proline 100µg/l and $CdSO_4$ 100µM	2.713±0.456	3.629±0.659
Mannitol 50µg/l and $CdSO_4$ 50µM	1.951±0.489	2.542±0.745
Mannitol 100µg/l and $CdSO_4$ 50µM	2.623±0.592	3.421±0.659
Mannitol 50µg/l and $CdSO_4$ 100µM	1.631±0.568	1.840±0.587
Mannitol 100 µg/l and $CdSO_4$ 100µM	2.151±0.546	2.881±0.659

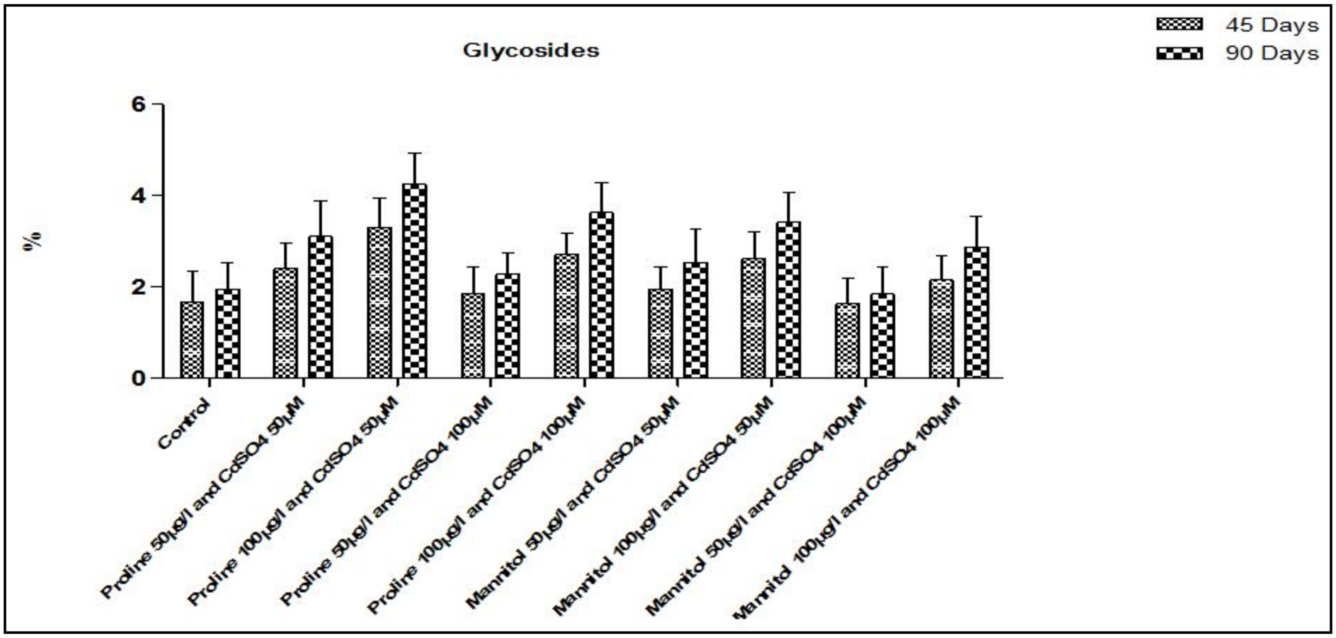

Fig. 7: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on glycosides.

Ascorbic acid

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on ascorbic acid of *Lepidium sativum* L.While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), ascorbic acid enhanced as compared to their respective control at 45 and 90 days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more ascorbic acid in stressed plants, which explained in table 4, Fig. 4.

Tocopherol

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on tocopherol of *Lepidium sativum* L.While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), tocopherol enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more tocopherol in stressed plants, which shown in table 5, Fig. 5.

Table 8: Effect of proline and mannitol to water stress on alkaloid (%/) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	1.674±0.657	2.761±0.658
Proline 50µg/l and $CdSO_4$50µM	2.562±0.675	4.102±0.875
Proline 100µg/l and $CdSO_4$50µM	3.128±0.587	5.285±0.653c
Proline 50µg/l and $CdSO_4$ 100µM	2.105±0.653	3.622±0.698
Proline 100µg/l and $CdSO_4$ 100µM	2.613±0.872	4.682±0.673d
Mannitol 50µg/l and $CdSO_4$ 50µM	1.939±0.658	3.634±0.578
Mannitol 100µg/l and $CdSO_4$ 50µM	2.883±0.745	4.895±0.852f
Mannitol 50µg/l and $CdSO_4$ 100µM	1.255±0.658	2.987±0.659g
Mannitol 100 µg/l and $CdSO_4$ 100µM	2.142±0.764	3.431±0.673

Phenol

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on phenol of *Lepidium sativum* L.While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), phenol enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more phenol in stressed plants, which described in table 6, Fig. 6.

Glycosides

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on glycosides of *Lepidium sativum* L. While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), glycosides enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more glycosides in stressed plants, which explained in table 7, Fig. 7.

Alkaloid

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on alkaloid content of *Lepidium sativum* L.While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$),

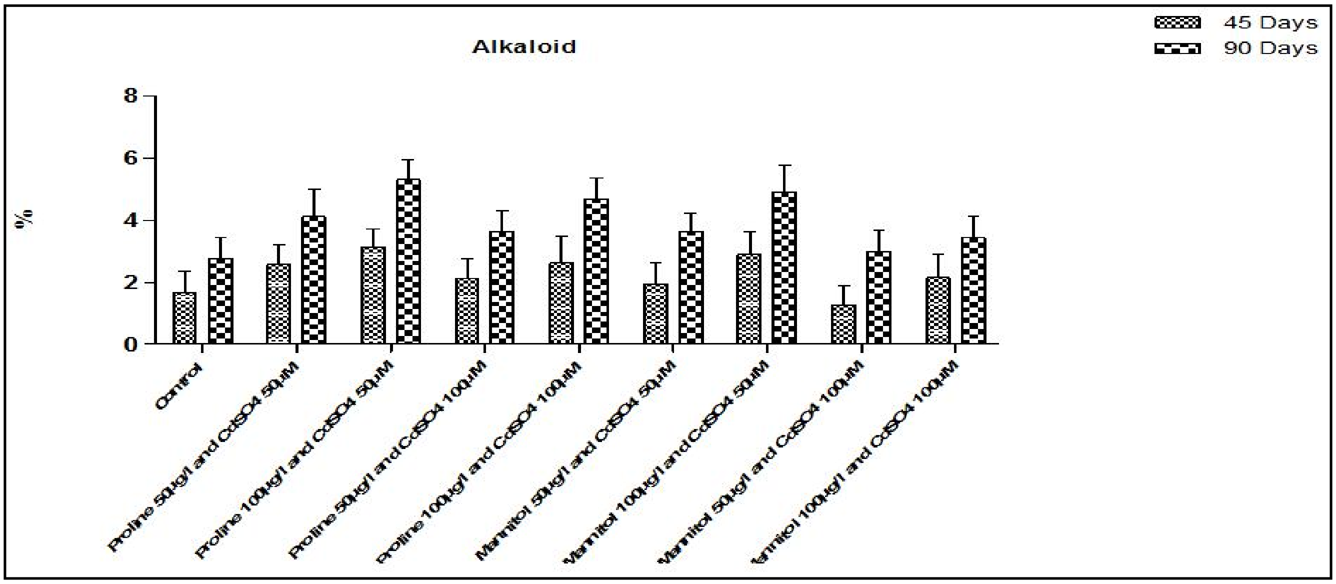

Fig. 8: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on alkaloid.

alkaloid content enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more alkaloid content in stressed plants, which described in table 8, Fig. 8.

Saponin

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on saponin content of *Lepidium sativum* L. While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), saponin content enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more saponin content in stressed plants, which shown in table 9, Fig. 9.

Table 9: Effect of proline and mannitol to water stress on saponin (%/) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test P<0.05*, P<0.01**, P<0.001*** significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	2.102±0.654	2.956±0.879
Proline 50µg/l and $CdSO_4$50µM	3.729±0.458	4.717±0.754
Proline 100µg/l and $CdSO_4$50µM	4.819±0.743	5.837±0.794
Proline 50µg/l and $CdSO_4$ 100µM	2.906±0.645	3.921±0.645
Proline 100µg/l and $CdSO_4$ 100µM	3.822±0.679	4.863±0.657
Mannitol 50µg/l and $CdSO_4$ 50µM	2.721±0.486	3.141±0.749
Mannitol 100µg/l and $CdSO_4$ 50µM	3.278±0.764	4.806±0.698
Mannitol 50µg/l and $CdSO_4$ 100µM	1.423±0.875	2.494±0.749
Mannitol 100 µg/l and $CdSO_4$ 100µM	2.581±0.698	3.837±0.847

Lipid peroxidation

The effects of osmolytes that is proline, mannitol, and heavy metal stress treatment on lipid peroxidation content of *Lepidium sativum* L. While different osmolytes 50 and 100µg/l applied with cadmium stress ($CdSO_4$), MDA content enhanced as compared to their respective control at 45 and 90days in case of both osmolyte proline and mannitol. Present study revealed that proline is more effective as compared to mannitol and enhanced more lipid peroxidation content in stressed plants, which explained in table 10, Fig. 10.

Discussion

In present study increased level of $CdSO_{4,}$ treatments with osmolytes (proline and mannitol) showed enhancement in various aspects antioxidants analysis (catalase, peroxidase, superoxide dismutase, ascorbic acid, and tocopherol) secondary metabolite profile (phenol, glycosides, alkaloid, and saponin) and biochemical analysis (lipid peroxidation) which taken under consideration. Cadmium stress inhibited production of antioxidant, secondary metabolite and biochemical. Results obtained showed that $CdSO_4$ toxicity effects the biochemical and metabolic processes in *Lepidium sativum* L. as in cotton plants

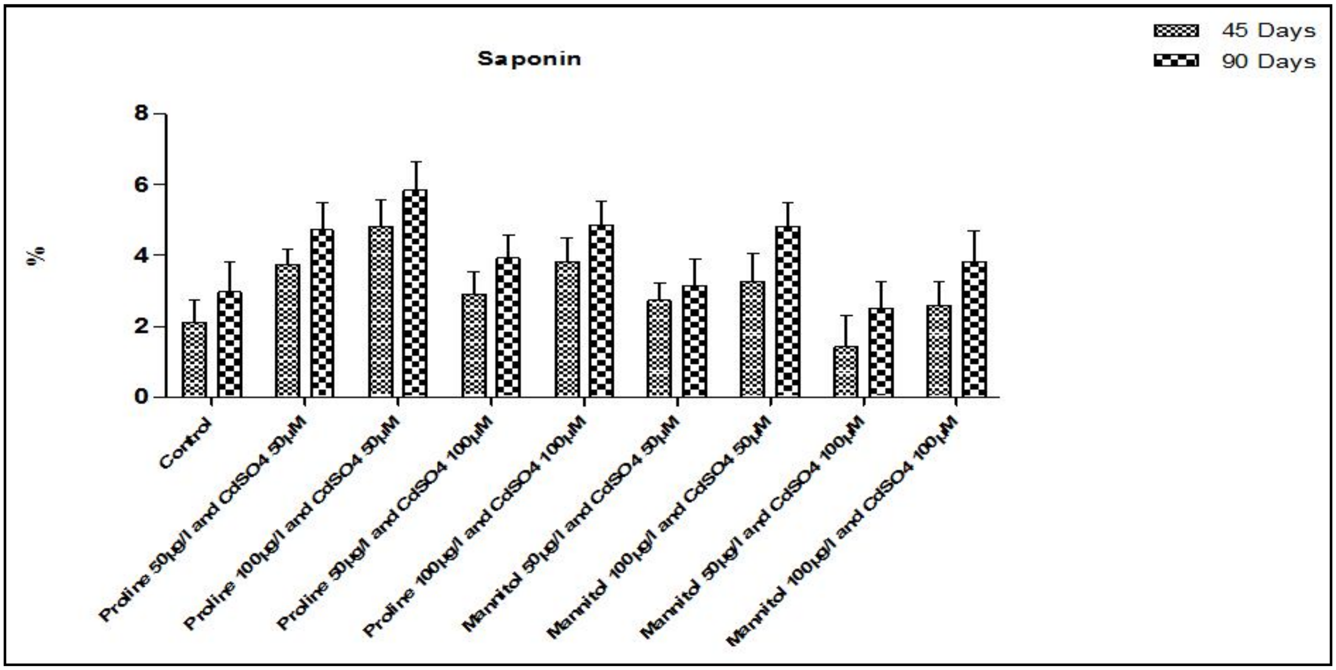

Fig. 9: Effect of proline and mannitol (50 and 100µg/l) to heavy metal stress ($CdSO_4$ 50 and 100µM) on saponin.

(Shi *et al*., 2010; Zhang *et al*., 2008). Heavy metals mainly target the enzymes and prolonged exposure of soils to heavy metals results in decrease in soil enzymatic activity (Tyler *et al*., 1989). The results of present study showed that there was enhancement in catalase, peroxidase, super oxide dismutase, ascorbic acid, tocopherol, phenol, glycosides, and alkaloid, saponin, and lipid peroxidation responses with increase in $CdSO_4$ stress concentration along with exogenous application of proline and mannitol.The enhancement in level of antioxidants, secondary metabolite and biochemical responses was more in proline as compared to mannitol. Proline shows slightly increase in level of antioxidants, secondary metabolite and biochemical than mannitol. Due to the severe stress of oxidative damage to antioxidant enzymes, the reduction in antioxidant enzymes at higher $CdSO_4$ concentrations has found (Mishra *et al.,* 2006). Catalase (CAT) is the principal enzyme that scavenges harmful oxygen species in plants (Pereira *et al.,* 2002). Many reports indicated that CAT activity significantly influenced by cadmium stress and opined that CAT activity plays an important role in the protection against oxidative damage caused by cadmium (Scebba *et al.,* 2006).The degree of enhancement in catalase content was maximum in proline as compared to mannitol. Peroxidase is one of the anti-oxidative enzyme whose activity alters under stress (Devi *et al.,* 2012). Peroxidase involved in several reactions such as ascorbates oxidation, lignification, phenol oxidation, pathogen defense, indole acetic acid oxidation and cell wall elongation (Passardi *et al*., 2007). In present, study the peroxidase activity enhanced by exogenous application of proline and mannitol along with different concentration of $CdSO_4$. The degree of enhancement in peroxidase activity was more in proline as compared to mannitol. It is evident from the results that with an increase in the concentration of heavy metal stress SOD contents enhanced along with the exogenous application of proline and mannitol. The degree of enhancement in SOD content was maximum in proline and minimum in mannitol. A reduction in SOD activity at higher $CdSO_4$ concentrations may result from the inactivation of the enzyme by H_2O_2, which produced

Table 10: Effect of proline and mannitol to water stress on lipid peroxidation((MDA(μmol/l)) of *Lepidium sativum* L. Data are mean ± SD, of three replicates (n=3) were analyzed using graph pad prism 5.2 by Two way Anova followed by Bonferroni multiple comparison post-test $P<0.05*$, $P<0.01**$, $P<0.001***$ significance level. Different lower case letters in a table indicate significant difference between control and treatments.

Treatments	45 Days	90 Days
Control	2.102±0.654	2.956±0.879
Proline 50μg/l and $CdSO_4$50μM	3.729±0.458	4.717±0.754
Proline 100μg/l and $CdSO_4$50μM	4.819±0.743	5.837±0.794
Proline 50μg/l and $CdSO_4$ 100μM	2.906±0.645	3.921±0.645
Proline 100μg/l and $CdSO_4$ 100μM	3.822±0.679	4.863±0.657
Mannitol 50μg/l and $CdSO_4$ 50μM	2.721±0.486	3.141±0.749
Mannitol 100μg/l and $CdSO_4$ 50μM	3.278±0.764	4.806±0.698
Mannitol 50μg/l and $CdSO_4$ 100μM	1.423±0.875	2.494±0.749
Mannitol 100 μg/l and $CdSO_4$ 100μM	2.581±0.698	3.837±0.847

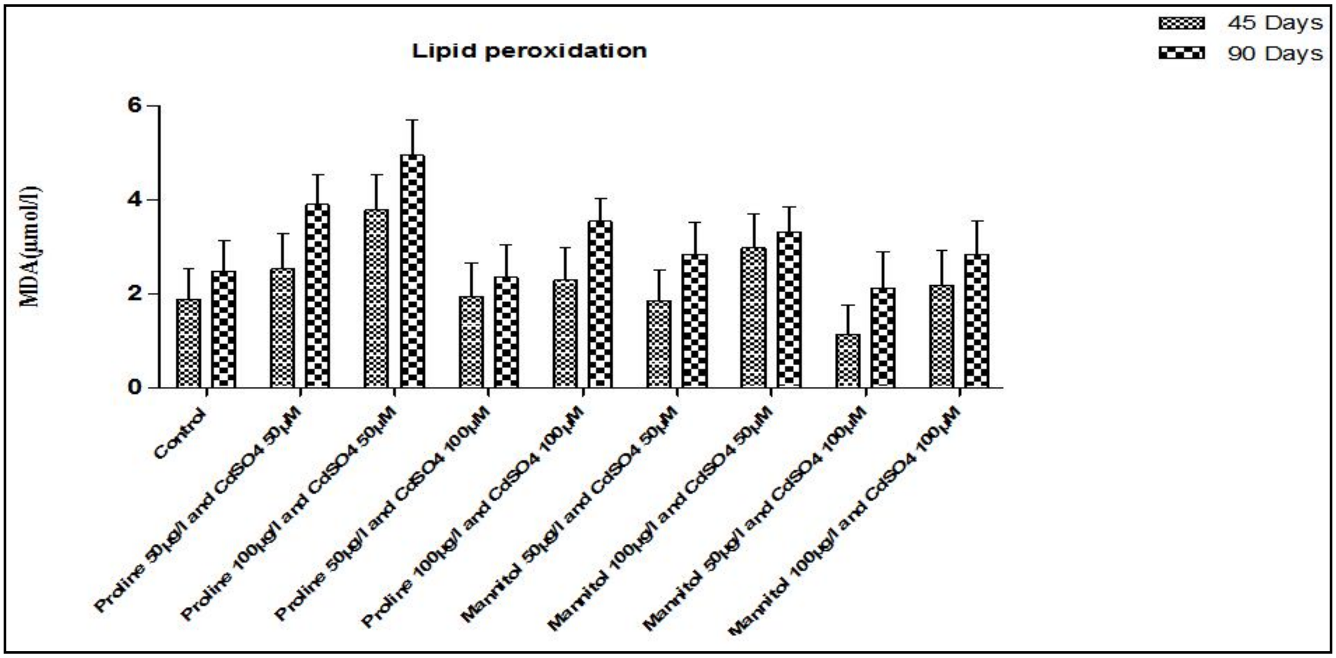

Fig. 10: Effect of proline and mannitol (50 and 100μg/l) to heavy metal stress ($CdSO_4$ 50 and 100μM) on lipid peroxidation content.

in different cellular compartments or from a number of non-enzymatic and enzymatic processes in cells (Dixit *et al.,* 2001; Romero-Puertas *et al.,* 2007). In the detoxification of free radicals phenolics compound play very important role (Ksouri *et al.,* 2007). The accumulation of phenolics and their different compounds vary among plants, tissues, state of development and environmental conditions (Ryan *et al.,* 2002). In the present study, the phenolic content enhanced as compared to their respective control in *Lepidium sativum* L. under heavy metal stress condition when proline and mannitol applied exogenously. The degree of enhancement is maximum in case of proline as compared to mannitol. Against oxidative stress, ascorbic acid plays a very important role, it eliminates ROS through multiple mechanisms, and a donor of electrons for APX- mediated H_2O_2 detoxification (Navabpour *et al.,* 2003). There is decrease in content of ascorbic acid with increase in $CdSO_4$ concentration. In addition, when the exogenously applied proline and mannitol to *Lepidium sativum* L. enhanced ascorbic acid as compared to their respective control. The degree of enhancement is maximum in case of proline as compared to mannitol. Tocopherols, known collectively as vitamin E, are lipid-soluble antioxidants synthesized by plants and other photosynthetic organisms (Mene-Saffrane and Della Penna, 2010; Yusuf *et al.,* 2010). The earlier studies reported that stress-tolerant plants usually display increase tocopherol levels, but the most sensitive ones show net tocopherol loss under stress, which leads to oxidative damage and cell destruction (Munne-Bosch and Alegre, 2002; Munne-Bosch, 2005). In present study, it is evident from the results that with an increase in the concentration of heavy metal stress tocopherol content decreased. In addition, it is enhanced when exogenously proline and mannitol is applied to *Lepidium sativum* L. The degree of enhancement is maximum in case of proline as compared to mannitol. Alkaloids are secondary plant metabolites with a vast array of possible functions, including anti oxidative activity (Havsteen, 2002). Heavy metal stress with an increase in the concentration of cadmium sulphate enhanced the alkaloid content but balance by the exogenous application of proline and mannitol. Plants saponins are a group of naturally occurring triterpene or steroid glycosides, which include a large number of biologically, and pharmlogically active compounds (Lacaille-Dubois *et al*., 2000). It is evident from the results that with an increase in the concentration of heavy metal stress saponin content enhanced when the osmolyte proline and mannitol is applied they decreased the saponin content in concentration dependent manner as compared to their respective control. There was a progressive decrease in the saponin content in case of proline as compared to mannitol. The degree of decrease in saponin content was more in proline and less in mannitol. Lipid peroxidation is a biochemical marker for the free radical mediated injury (Verma *et al*., 2003). By the high level of thiobarbituric reactive species Cd-induced lipid peroxidation at leaf level has been detected (Chien *et al*., 2001, Shah *et al*., 2001) but at thylakoid level, the available information is insufficient and controversial. The level of lipid peroxidation in present study increased with increasing level of $CdSO_4$ in *Lepidium sativum* L. as compared to their respective control. The aim of present study were determined the importance of exogenous application of osmolytes proline and mannitol on the alleviation of cadmium sulphate ($CdSO_4$) induced toxicity effects on antioxidant, secondary metabolites and biochemical responses of *Lepidium sativum* L.

Conclusion

During the current investigation, observed that the exogenous application of proline and mannitol to toxicity of heavy metal stress ($CdSO_4$) were enhanced the antioxidants analysis (catalase, peroxidase, super oxide dismutase, ascorbic acid and tocopherol) secondary metabolite profile (phenol, glycosides, alkaloid, and saponin) and biochemical analysis (lipid peroxidation) responses of *Lepidium sativum* L. The use of exogenously applied osmolytes proline and mannitol showed positive results. Proline and mannitol improved plant physiological activities by lowering the reactive oxygen species damage through increased antioxidant enzyme activities and by lowering uptake and accumulation of cadmium sulphate. In addition, proline is more effective as compared to mannitol. From these observations, it concluded that the exogenous application of proline and mannitol improved the heavy metal stress tolerance in *Lepidium sativum* L. Therefore, it found that the plants are able to cope with abiotic stress when exogenous proline and mannitol is applied.

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