ORIGINAL ARTICLE

Antioxidative defense responses and activation of phenolic compounds in *Brassica juncea* plants exposed to cadmium stress

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Abstract

Background: The present work has explored the effects of cadmium (Cd) on *Brassica juncea* plants. **Materials and Methods:** Malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) content, activities of antioxidative enzymes (superoxide dismutase, guaiacol peroxidase, and catalase), protein content, level of antioxidants (ascorbic acid, tocopherol, and glutathione), antioxidant assays (1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging assay, molybdate ion reduction assay and reducing power assay), total phenolic content, and polyphenols were analyzed in 90-day-old plants. Seeds of *B. juncea* were exposed to 0, 0.2, 0.4, and 0.6 mM Cd concentrations. **Results:** Significant increase in MDA and H_2O_2 content was observed in Cd-stressed plants as compared to untreated control. Cd toxicity positively influenced the activities of antioxidative enzymes and radical scavenging activities of the plant. Results revealed the enhancement in the level of antioxidants. Ultra performance liquid chromatography studies showed the variation in the level of polyphenols with respect to untreated plants. **Conclusions:** This investigation showed that defensive strategies of this plant activated under metal stress and overcame the adverse effects produced due to the generation of reactive oxygen species.

Key words: Antioxidative defense system, *Brassica juncea,* heavy metal stress, lipid peroxidation, polyphenols

INTRODUCTION

In various parts of the world, agricultural soils are slightly to moderately contaminated by heavy metal toxicity such as Zn, Ni, Cd, Cu, As Co, Cr, and Pb. The reason for this might be continuing use of phosphatic fertilizers, dust from smelters, sewage sludge application, bad watering practices in agricultural lands, and industrial waste.[1] Upon exposure to high levels of heavy metals, the primary response of plants is the generation of reactive oxygen species (ROS). Overproduction of ROS and existence of oxidative stress in plants could be the consequences of heavy metal toxicity.[2] The indirect consequences of metal toxicity include their interaction with the antioxidant system disturbance in the metabolism of essential elements and disruption in the electron transport chain. Lipid peroxidation is one of the most deleterious effects produced by heavy metals exposure in plants, which can directly cause biomembrane deterioration. Decomposition product of polyunsaturated fatty acids of the membrane is malondialdehyde (MDA), which is considered as a consistent indicator of oxidative stress.[3]

To protect themselves from ROS, plants have a wide range of defense strategies including efficient antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and antioxidants (ascorbic acid, reduced glutathione (GSH), etc.).[4] SOD helps in removal

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of superoxide radical ($O_2^{\bullet-}$) by catalyzing its dismutation. One $O_2^{\bullet-}$ is reduced to hydrogen peroxide (H_2O_2) and another oxidized to O_2. $O_2^{\bullet-}$ is then removed and consequently the risk of hydroxyl radical (OH•) production decreases via the metal catalyzed Haber Weiss-type reaction. H_2O_2 is further eliminated by the activity of CAT enzyme as it is still toxic, which is dismutated into H_2O and O_2. In ascorbate-GSH cycle, reduced GSH is the probable component and plays a key role in defense system against ROS. An increase in antioxidant defense machinery to protect the plants from Cd stress was noticed by some previous and recent studies.[5] GSH molecules act as substrate for the phytochelatins synthesis which contributes in detoxification of Cd by chelation. Among natural antioxidants, phenolic compounds donate a hydrogen atom or an electron to the free radical, thus help in quenching of oxygen-derived free radicals and also noticed to be neutralizing free radicals in different models system.[6] Therefore, the aim of the present work is to observe the effects of Cd toxicity on lipid peroxidation, H_2O_2 content, and antioxidative defense system of plants including activities of antioxidative enzymes, level of antioxidants, free radical scavenging activities, and quantitative/qualitative estimation of polyphenols in 90-day-old plants of *Brassica juncea*.

MATERIALS AND METHODS

To study the effects of Cd metal on *B. juncea* plants, a field experiment was conducted in Botanical Garden of Guru Nanak Dev University, Amritsar. 20 × 20 feet area was taken for the experimentation and soil: Manure in a ratio of 3:1 was added into it. Certified and disease free seeds of *B. juncea* L. var. RLC-1 were procured from the Punjab Agricultural University, Ludhiana, (Punjab) and surface sterilized with 0.01% mercuric chloride solution, followed by the repeated washings of sterile double distilled water. Seeds were sown in different blocks. Different treatments of Cd metal were given (0, 0.2, 0.4, and 0.6 mM Cd). Plants were then harvested after 90 days of germination to study following parameters.

Membrane damage caused by Cd stress was assessed in terms of MDA content by the method of Heath and Packer.[7] H_2O_2 content was measured by the method given by Velikova *et al*.[8]

Protein estimation was done using method given by Lowry *et al*.[9] Activities of antioxidative enzymes were determined by the standard methods of Kono[10] for SOD, Putter[11] for POD, and Aebi[12] for CAT. Ascorbic acid content was measured by following the method of Roe and Kuether,[13] Tocopherol (Vitamin E) content by the method given by Martinek,[14] and GSH content was determined by the method given by Sedlak and Lindsay.[15] 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was performed according to the method given by Blois,[16] 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay by following the method of Re *et al*.[17] Ferric ion reducing antioxidant power assay (FRAP) by the method given by Oyaizu[18] and Molybdate ion reduction assay was performed by Prieto *et al*.[19] Total phenolic content was determined according to a procedure described by Singleton and Rossi.[20] Qualitative analysis of polyphenols was done by ultra performance liquid chromatography.

Statistical Analysis

Each experiment was conducted in three replicates. Data were expressed in Mean±SE. To check the statistically significant difference between the treatments, one way-analysis of variance was carried out using Assistant version 7.7 beta.

RESULTS

Cd metal induced the formation of MDA and H_2O_2 content [Figures 1 and 2]. The highest level of MDA and H_2O_2 was noticed in the 90-day-old plants of *B. juncea* when exposed to Cd metal. MDA content was recorded maximum at 0.2 mM Cd (12.23 µmol g^{-1} FW) as compared to untreated control plants (5.39 µmol g^{-1} FW). Further with increasing

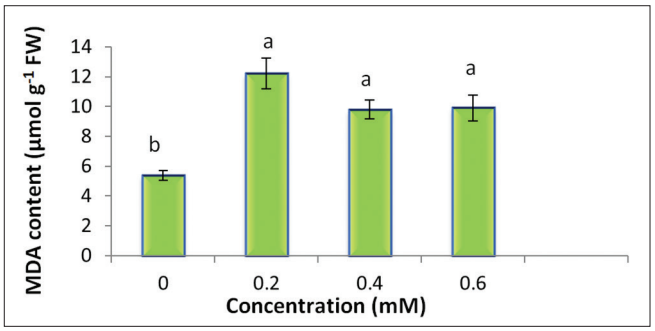

Figure 1: Effect of cadmium on malondialdehyde content (µmol g^{-1} FW) in 90-day-old plants of *Brassica juncea*

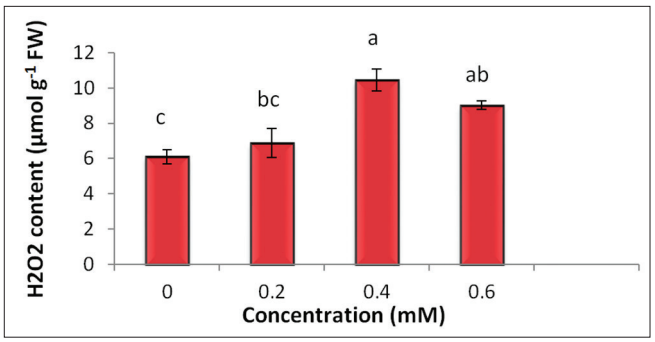

Figure 2: Effect of cadmium on H_2O_2 content (µmol g^{-1} FW) in 90-day-old plants of *Brassica juncea*. Bars presented in mean ± standard error. Different letters (a, b, c, and d) within various concentrations of cadmium (0, 0.2, 0.4, and 0.6 mM) are significantly different (Fisher least significant difference *post hoc test, P*≤0.05) and signify the effect of cadmium metal on malondialdehyde and H_2O_2 content

the concentration of Cd metal, MDA content decreased, but it was higher than the control plants. Whereas H_2O_2 content was recorded maximum in 0.4 mM Cd (10.44 µmol g^{-1} FW) treated plants in comparison to control (6.11 µmol g^{-1} FW).

The protein content was reported to increase with the increasing concentrations of Cd in 90-day-old *B. juncea* plants [Table 1]. Maximum protein content was recorded in 0.6 mM (16.61 mg g^{-1} FW) of Cd treatment. This increase was assessed from 12.62 to 14.84 mg g^{-1} FW in 0.2 mM of Cd treatment and from 12.62 to 14.29 mg g^{-1} FW in 0.4 mM of Cd treatment. In case 0.6 mM, the protein content of stressed plants was noticed to increase from 12.62 to 16.61 mg g^{-1} FW than untreated *Brassica* plants.

Activity of POD increased due to Cd toxicity as compared to control [Table 1]. Control plants of 90 days showed the minimum activity of enzyme (16.89 UA mg^{-1} protein). POD activity was found to enhance with the metal treatment, from 16.89 to 19.91 UA mg^{-1} protein, in 90-day-old plants exposed to 0.4 mM Cd stress. Activity of CAT was altered slightly differently by Cd toxicity [Table 1]. Control plants showed minimum enzyme activity as compared to metal treated plants (9.27 UA mg^{-1} protein). Activity of CAT first enhanced from 9.27 to 13.02 UA mg^{-1} protein at 0.2 mM Cd-treated plants. Rise in enzyme activity was again reported in 0.4 mM Cd-stressed plants. Maximum enzyme activity was noticed at 0.6 mM Cd treatment (14.69 UA mg^{-1} protein). Inhibition in the activity of SOD was measured at 0.2 mM Cd-stressed plants as compared to control plants [Table 1]. Decrease in SOD activity was observed from 5.39 to 5.28 UA mg^{-1} protein, then got increased from 5.39 to 5.73 UA mg^{-1} protein at 0.4 mM Cd and from 5.39 to 6.76 UA mg^{-1} protein at 0.6 mM Cd-stressed plants.

In 90-day-old plants of *B. juncea*, Cd toxicity increased the ascorbic acid content in comparison to control (11.77 mg g^{-1} FW). Very less difference in ascorbic acid (Vitamin-c) content was noticed in between the metal treated plants. 0.4mM Cd toxicity showed the highest level of Vitamin-c (14.84 mg g^{-1} FW). With increasing Cd concentration tocopherol content enhanced in a dose-dependent manner [Table 2]. Maximum value of tocopherol was seen in 0.6 mM Cd-stressed plants (9.39 mg g^{-1} FW) with respect to control (7.09 mg g^{-1} FW). At 0.2 mM and 0.4 mM Cd stress, Vitamin-E content got enhanced (8.18 and 8.81 mg g^{-1} FW, respectively), which was lower than 0.6 mM Cd treatment. 1.43-folds increase in GSH content was observed with 0.6 mM Cd-treated plants in comparison to control (5.93 mg g^{-1} FW). Rise in GSH content was noticed from 5.93 to 6.92 mg g^{-1} FW at 0.2 mM Cd and from 5.93 to 6.52 mg g^{-1} FW at 0.4 mM Cd treatment [Table 2].

Results revealed the significant increase in scavenging of DPPH radical with increasing Cd dose [Table 3]. DPPH radical was scavenged in the dose-dependent manner. At control, 46.97% DPPH was inhibited. % inhibition was increased at 0.2 mM Cd (57.09%), followed by 0.4 mM Cd (71.94%) and maximum radical was scavenged in 0.6 mM Cd-stressed plants. Reduction of molybdate ions was showed by Cd-treated plants [Table 3]. These ions were found to reduce due to Cd stress in comparison to control (71.03%). Inhibition of ions increased to 74.82% compared to control.

Table 1: Effect of Cd on protein content, specific activities of POD, CAT and SOD in 90 days old *B. juncea* plants

Treatments	Protein content (mg g^{-1} FW)	POD (UA mg^{-1} protein)	CAT (UA mg^{-1} protein)	SOD (UA mg^{-1} protein)
0.0 mM	12.62±1.07^{b}	16.89±0.79^{d}	9.27±0.69^{c}	5.39±0.83^{b}
0.2 mM	14.84±0.49^{ab}	18.45±0.75^{b}	13.02±0.91^{ab}	5.28±0.31^{b}
0.4 mM	14.29±1.24^{ab}	19.91±0.92^{a}	10.85±0.42^{bc}	5.73±0.25^{b}
0.6 mM	16.61±0.72^{a}	17.84±0.68^{c}	14.69±0.42^{a}	6.76±0.33^{a}

Data presented in mean±SE. Different letters (a, b, c and d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6 mM) are significantly different (Fisher LSD *post hoc test*, $P \leq 0.05$) and signify the effect of Cd metal on antioxidative enzymes. SE: Standard error, POD: Peroxidase, LSD: Least significant difference, *B. juncea: Brassica juncea*

Table 2: Effect of Cd on ascorbic acid, tocopherol and GSH content in 90 days old *B. juncea* plants

Treatments	Ascorbic acid (mg g^{-1} FW)	Tocopherol (mg g^{-1} FW)	GSH (mg g^{-1} FW)
0.0 mM	11.77±1.04^{b}	7.09±0.48^{b}	5.93±0.84^{c}
0.2 mM	14.24±0.19^{a}	8.18±0.24^{ab}	6.92±0.89^{b}
0.4 mM	14.84±0.79^{a}	8.81±0.58^{ab}	6.52±0.44^{b}
0.6 mM	14.78±0.63^{a}	9.39±0.22^{a}	8.5±0.3^{a}

Data presented in mean±SE. Different letters (a, b, c and d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6 mM) are significantly different (Fisher LSD *post hoc test*, $P \leq 0.05$) and signify the effect of Cd metal on antioxidants. SE: Standard error, GSH: Glutathione, LSD: Least significant difference, *B. juncea: Brassica juncea*

Table 3: Effect of Cd on scavenging activities of DPPH, FRAP, molybdate ion and ABTS in 90 days old *B. juncea* plants

Treatments	DPPH (%)	FRAP (%)	Molybdate ion (%)	ABTS (%)
0.0 mM	46.97±4.65^{b}	57.91±2.18^{b}	71.03±4.21^{b}	58.19±3.86^{ab}
0.2 mM	57.09±4.0^{ab}	71.94±1.68^{a}	74.82±6.49^{b}	52.21±1.85^{b}
0.4 mM	71.94±4.09^{a}	58.9±3.45^{b}	81.77±1.42^{a}	72.18±2.32^{a}
0.6 mM	72.3±4.65^{a}	60.4±1.99^{b}	81.08±4.42^{a}	72.79±4.33^{a}

Data presented in mean±SE. Different letters (a, b, c and d) within various concentrations of Cd (0, 0.2, 0.4 and 0.6 mM) are significantly different (Fisher LSD *post hoc test*, $P \leq 0.05$) and signify the effect of Cd metal on antioxidant assays. SE: Standard error, Cd: Cadmium, CAT: Catalase, DPPH: 1,1-diphenyl-2-picrylhydrazyl, FRAP: Ferric ion reducing antioxidant power assay, ABTS: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), *B. juncea: Brassica juncea*

No significant changes were observed in % reduction at 0.4 mM Cd (81.77%) and 0.6 mM Cd (81.08%) treated plants. Increase in scavenging of FRAP was observed in 90-day-old plants with enhancing Cd concentrations [Table 3]. Minimum reduction of FRAP ion was found in control plants (57.91%). Reduction of ions got maximum from 57.91% to 71.94% in 0.4 mM Cd-stressed plants, which further reduced with 0.6 mM Cd treatment (60.4%). 58.19% inhibition of ABTS was recorded in control plants of 90 days. Then, decrease in % reduction of ABTS was seen in 0.2 mM Cd-stressed plants (from 58.19% to 52.21%). Further significant rise in reduction was observed from 58.19% to 72.18% and from 58.19% to 72.79% at 0.4 mM and 0.6 mM Cd-treated plants, respectively [Table 3].

1.4-folds increase in total phenolic content was observed at 0.6 mM Cd treatment compared to control (from 7.14 to 10.01 mg g^{-1} FW). Increase in phenolic content was seed at 0.2 mM and 0.4 mM Cd stress (8.62 and 9.76 mg g^{-1} FW, respectively) in comparison to control [Figure 3]. It was noticed that gallic acid, catechin, chlorogenic acid, caffeic acid, coumaric acid, ellagic acid, quercetin, kaempferol, epicatechin, and umbelliferone polyphenols were present in untreated control of 90-day-old plants [Figures 4-7]. In 0.2 mM and 0.6 mM Cd-stressed plants, rutin polyphenol became more activated as compared to control plants [Table 4].

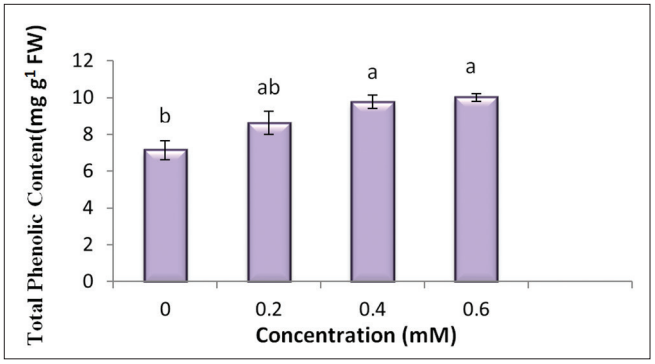

Figure 3: Effect of cadmium on total phenolic content (mg g^{-1} FW) in 90-day-old plants of *Brassica juncea*

DISCUSSION

Cadmium (Cd) is most frequently found in soil which confines the productivity of crops universally as this metal have a propensity to accumulate within plant organs and adversely affects the essential physiological processes.[21] Accumulation of Cd and its toxicity in plants have been reported due to the production of ROS which leads to oxidative damage.[4] In the present study, generation of H_2O_2 had increased in 90-day-old plants exposed to Cd stress. Rise in the level of H_2O_2 and MDA content was recorded as dose-dependent manner, i.e., with increasing stress, production of H_2O_2 radicals were also found to enhance. Plants subjected to 0.6 mM Cd showed the highest H_2O_2 generation. HM stimulates the generation of free radicals such as H_2O_2 and disturbs the redox homeostasis in plants.[22] Mixtures of lipid hydroperoxides are produced when major plant polyunsaturated fatty acids of plant membrane are attacked by free radicals. Peroxidation of membrane lipids decreased the membrane-fluidity and increased leakage during stress conditions.[23] Thus, rise in MDA content shows the generation of oxidative stress, and this might be one of the promising mechanisms by which heavy metal toxicity is manifested in plant tissues. These results are supported by the findings of,[24] where 10 days seedlings of *Phaseolus vulgaris* showed a significant increase in MDA content due to Ni, Co, Zn, and Cr stress.

In the present investigation, protein content, activities of antioxidative enzymes, free radical scavenging activities, and antioxidants were found to enhance. Generation of ROS due to heavy metals stimulates the covalent modifications of protein, therefore, resulting in oxidation of protein which is considered as a diagnostic oxidative stress marker.[23] In plants, location of protein oxidation due to metal stress has been noticed in mitochondria only.[25] The experiment conducted on *Lemna gibba* subjected to Mn and Ni stress, a significant rise in total protein content was recorded by plants under stressed conditions with respect to the untreated control plants[26] SOD is located in all aerobic organisms, where it plays a major role in the defense against toxic reduced oxygen species that are produced as by-products of biological oxidations. During adverse environmental conditions, generation of oxygen radical can be exasperated and hence SOD has been projected to be important for tolerance of stress in plants. SOD acts as the first line of defense in plants that contributes in the

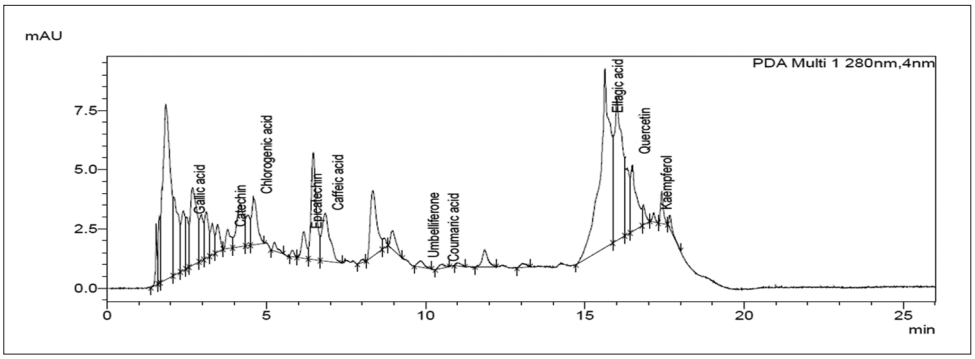

Figure 4: Ultra performance liquid chromatography analysis of polyphenols in 90-day-old plants of *Brassica juncea*

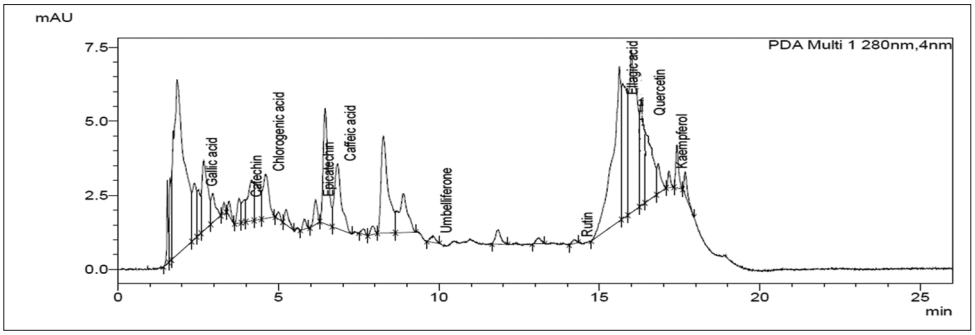

Figure 5: Ultra performance liquid chromatography analysis of polyphenols in 90-day-old plants of *Brassica juncea* treated with 0.2 mM cadmium

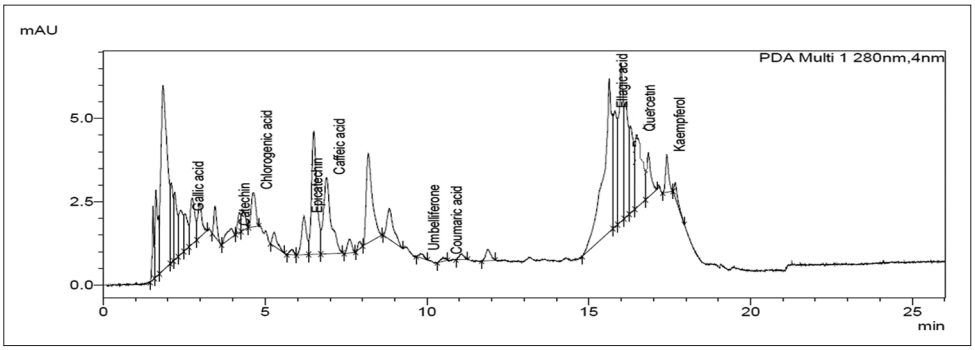

Figure 6: Ultra performance liquid chromatography analysis of polyphenols in 90-day-old plants of *Brassica juncea* treated with 0.4 mM cadmium

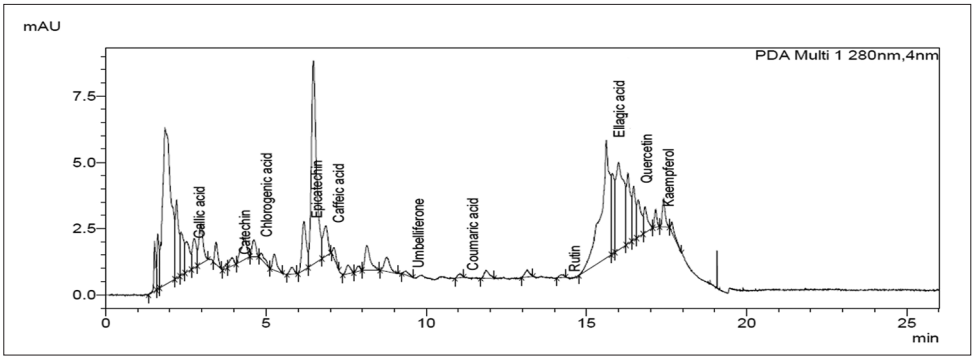

Figure 7: Ultra performance liquid chromatography analysis of polyphenols in 90-day-old plants of *Brassica juncea* treated with 0.6 mM cadmium

conversion of O_2^- into H_2O_2 at a very rapid rate.[27] Alterations in the enzyme activity are crucial for overwhelming the oxidative stress in plants. CAT is tetrameric heme-containing enzymes and helps in degradation of H_2O_2. Increased activity

Table 4: Concentrations of phenolic compounds in 90 days old *Brassica juncea* plants treated with Cd stress

Polyphenolic compound	Percentage			
	Control	0.2 mM Cd	0.4 mM Cd	0.6 mM Cd
Gallic acid	0.882	0.771	0.617	0.758
Catechin	3.491	2.735	1.955	1.271
Chlorogenic acid	3.121	2.556	1.266	0.678
Caffeic acid	2.470	2.321	2.661	0.833
Coumaric acid	0.075	-	0.037	0.044
Ellagic acid	38.063	21.116	18.852	18.715
Quercetin	4.974	5.089	4.142	2.113
Kaempferol	0.878	1.333	3.108	1.440
Epicatechin	2.996	1.685	3.171	4.509
Rutin	-	0.304	-	0.261
Umbelliferone	0.823	0.503	0.285	0.311

Cd: Cadmium, *B. juncea: Brassica juncea*

of POD in metal-stressed plants indicates induction of defense mechanism for the protection of cell against ROS. These enzymes are required for quenching of H_2O_2. The activities of antioxidative enzymes have been recorded to be enhanced in *Raphanus sativus* seedlings exposed to Cr stress.[28] Along with enzymes of the antioxidative defense system in plants, the nonenzymatic radical-scavengers such as ascorbate and GSH have also been interpreted as the key antioxidants for the removal of H_2O_2 in plant cells, accordingly decreasing the accumulation of the free radicals.[29] Phenolic compounds were related to the antioxidant activity, and they play a major role in stabilizing lipid peroxidation. The findings of enhanced scavenging activities in this study suggest that due to rise in Cd stress, plants became potentially active. Modulated activities of antioxidants, enzyme activities, and radicals scavenging activities recorded under stressed conditions demonstrated their active participation in oxidative stress management. Results of present work were also supported by the findings of Mohamed and Akladious,[30] where ascorbic acid, tocopherol, and GSH were recorded to enhance under drought conditions in *Glycine max*. Therefore, it is concluded from the present study that overproduction of ROS is a common outcome of different stress factors. Balance between generation and degradation of ROS is essential to regulate the metabolic functions under stress. Otherwise, oxidative injuries may occur. On the other hand, some of them can also act as signaling molecules if they are produced quickly. Their role as signaling molecules or as toxic ROS at last depends on the balance between their formation and removal by the antioxidative scavenging systems.

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