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HYPER ACCUMULATION OF CADMIUM IN *Phyllanthus amarus* L. A MEDICINAL PLANT

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ABSTRACT

***Phyllanthus amarus* L. treated with different concentration of Cd (w/w) in pot, revealed high accumulation of Cd in plant. The root and shoot growth of plant decrease with the increasing concentration (w/w) of Cd from 50 to 80ppm, while up to 50 ppm growth of plant was unaffected up to 60DAS. Dry biomass of the plant was more influenced as compared to the growth under increased Cd concentrations. Qualitative phytochemical screening of alkaloid, tannins and flavonoids in *Phyllanthus amarus* L. showed that alkaloid production was more as compared to tannins and flavonoids. Maximum proline content was recorded at 50 to 60ppm Cd treated plant after 60DAS. APX and CAT activity (U/mg protein) was increased in fresh leaf of the plant under Cd treated plant *Phyllanthus amarus* L. up to 30- 60ppm over the control. In the plant, maximum Cd accumulated in leaf followed by shoot and root after 30DAS.**

KEYWORDS : *Phyllanthus amarus* L, Cd Uptake, Phyto-Chemicals, APX and CAT

Phyllanthus amarus L. (Family Euphorbiaceae) is a widely distributed small erect, tropical annual herbal shrub. Heavy metal pollution of soils has dramatically increased in recent decades due to the discharge of waste and wastewater from anthropogenic sources (WHO). This situation has become a critical environmental issue owing to the potential adverse ecological effects of the pollutants. Nearly 400 species of terrestrial plants have been identified as hyperaccumulators of various heavy metals in the world (Wang et al., 2008), which are capable of accumulating high levels of heavy metals without suffering metal toxicity or cell damage (Boominathan and Doran, 2003). Cadmium (Cd) is a very toxic heavy metal and an important environmental pollutant, which is present in the soil, water, air, food and in cigarette smoke. Cadmium is not essential to plant growth, and it can interfere with physiological processes including carbon assimilation decrease, chlorophyll synthesis inhibition, oxidative stress generation, etc. (Boominathan and Doran, 2003; Wang et al., 2008). Cadmium toxicity causes oxidative stress, which can take place possibly by generating reactive oxygen species (ROS). The medicinal plants are either naturally grown or cultivated in metal contaminated regions, there is a danger that the heavy metal accumulation by plants of medicinal value may cause serious health hazards to patients using metal adulterated herbal drugs. The objective of this study was to examine the growth response and uptake, distribution, accumulation of Cd and their effect on *Phyllanthus amarus*.

MATERIALS AND METHODS

Set-up of Pot experiment: The soil was collected from an agricultural field of district Jaunpur (25.73°N 82.68°E), UP-India, chemical analysis of the soil showed that organic matter, total N, pH and Cd concentration in this soil were 3.65%, 0.16%, 7.2 and 0.10 mg kg^{-1}, respectively. Surface (0-20 cm) soil samples which were ground to pass through a 4.0mm mesh were used in the pot experiment. The soil samples were air-dried, then mixed with basal fertilizers, at ratios of 100 mgNkg^{-1} dry weight (DW) soil as NH_4NO_3 and 30 mg P kg^{-1} and 80 mg kg^{-1} as K_2HPO_4 (Wang et al., 2008). Eleven Cd treatments were applied, namely C (the control) and treatments (Cd concentrations: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg kg^{-1} DW soil). Each treatment was carried out in six replicates. The tested topsoil samples were mixed thoroughly with $CdCl_2 \cdot 2.5H_2O$ (Wang et al., 2008) at the above-mentioned concentrations, filled into plastic pots (20 cm in diameter, 15 cm in height, 2.5 kg air-dried soil per pot) and equilibrated for 20 days. Seeds of *Phyllanthus amarus* L. Were surface sterilized in 2% (w/v) sodium hypocloride for 3 min, washed several times with sterilized distilled water (SDW), and soaked in SDW overnight. Twenty soaked seeds were sowed directly into the 0.8% agar plate and incubated for three days in dark room. Three similar sizes of sprouted seed were placed in

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the each treatment pots in the greenhouse with natural light (10-12h; photoperiod) and temperature (20-33^{0}C). The tested soils were watered to reach 60% of the water-holding capacity and this level was maintained by watering daily throughout the experiment. The plants were harvested after 15, 30, 45 and 60 DAS (days after showing) for further analysis.

Plant Growth, Phyto-Chemicals and Proline Analysis

Root length, shoot length and dry biomass were examined after 15, 30, 45 and 60 DAS of the plant. Qualitative phytochemicals analysis like alkaloid, tannins, flavonoides in crude powder of the *Phyllanthus amarus* L. was carried out according to the methods earlier described by Trease and Evans (1989). Ten ml of methanol was added in 200 mg of dry plant material and filtered through Whatman filter paper (WFP), 2 ml of filtrate were steamed with 2 ml 1% conc. HCL filtrate and added 5 to 6 drops of Mayer´s reagent/Wagners reagent/ Dragendorffs reagent. Creamish/brown/red/orange precipitate indicates the presence of alkaloids. 10 ml SDW were added in 200 mg of dry plant material and filtered through WFP, 2 ml of filtrate were in 2 ml of $FeCl_3$, blue/black precipitate indicate the presence of tannins. 200 mg of dry plant material were dissolved in 10 ml Ethanol and filtered through WFP, conc. HCL and magnesium ribbon were added in 2 ml filtrates, pink/ red color indicate the presence of flavonoides.

Antioxidant Enzyme Activity of The Plant

Catalase (CAT; EC 1.11.1.6) and APX activity (EC 1.11.1.11) was analyzed by the method earlier described (Upadhyay et al., 2012) in fresh leaf tissue (0.5 g) was homogenized with liquid N_2 in QB buffer (100 mm potassium phosphate, pH 7.8, 1 mm EDTA, 1% Triton X-100, 15% glycerol; to assess ascorbic acid peroxidase (APX) and catalase (CAT) activities. Crude homogenate was centrifuged at 15,000 g for 20 min at 4^{0}C, and the supernatant used for determination of CAT, and APX activity in Umg^{-1} protein. Protein content was determined with the Bradford method using BSA as standard.

Plant and Soil Metal Analysis

The plants were immersed in a 0.01M HCl solution to remove any external cadmium and rinsed with de-ionized (DI) water for 1 min. Subsequently, the plants were separated into three parts: root, stem and leaf. After that, they were dried at 100°C for 10 min, then at 70°C in an oven until completely dry. The plant and soil samples were digested with a solution of 3:1 HNO_3:$HClO_4$ (v/v). The concentrations of Cd were determined using the atomic absorption spectrophotometry by the method (Sun et al., 2007). Bioconcentration factor (BF) and translocation factor (TF) of the cadmium was calculated by the method (Brooks, 1998; Cluis, 2004; Wei and Zhou, 2004 a,b). BF=Metal concentration in plant/Metal concentration in soil and TF=Metal concentration in shoot/Metal concentration in root.

Statistical Analysis

The data obtained were subjected to ANOVA, and means were compared with Duncan's multiple range test. All statistical analyses were conducted using SPSS (Version 14; IBM, Armonk, NY, USA).

RESULTS

Phyllanthus amarus L. plant showed no visual Cadmium toxicity symptoms such as necrosis and whitish-brown chlorosis. The root and shoot growth of plant in pot experiment decrease with the increasing concentration (w/w) of Cd from 50 to 80ppm in pot, while up to 50 ppm growth of plant was unaffected. The growth was observed at 15DAS, 30DAS, 45DAS and 60DAS and result reveled that root and shoot length increased with DAS (Table, 1). Tolerance level of plant was found to be upto 80ppm of Cd treated pot, while at 90 ppm plant could not survived. Twenty eight and thirty percent reduction were found in root length, while 21 and 27% shoot length after 30 and 60DAS respectively, over the control at 50ppm of Cd treated pot. Dry biomass of the plant was more influenced as compared to the growth under increased Cd concentrations, 51% and 56% of reduction was found after 30DAS and 60DAS respectively over the control at 40ppm (Table, 1). Results reveled from qualitative phytochemical screening of alkaloid, tannins and flavonoids in *Phyllanthus amarus* L. showed that alkaloid production was more as compared to tannins and flavonoids (Table, 2). Alkaloid, Tannins and flavonoids was unaffected up to 20ppm Cd treated pot, and was detected up to 80, 70 and 60ppm respectively at both 30 and 60DAS. Proline contents in leaf (µ/g FW) of the plant

Table 1: Growth of *Phyllanthus amarus* L. Under Different Concentration of Cadmium (w/w): Cd (Pot- Experiment)

Treatment	Root length (cm)				Shoot Length (cm)				Total Dry biomass (g)	
	15 DAS	30 DAS	45 DAS	60 DAS	15 DAS	30 DAS	45 DAS	60 DAS	30 DAS	60 DAS
C*	3.5 ±0.8^{a}	5.9±1.5^{a}	6.8±1.5^{a}	7.5±2.9^{a}	8.9±1.3^{b}	15.2±3.8^{b}	36.2 ±2.7^{a}	48.5±4.4^{b}	5.20±1.2^{ab}	8.2±3.8^{a}
10 ppm	3.5±0.2^{ab}	5.9±1.1^{b}	6.4±1.5^{a}	7.2±3.7^{a}	8.5±1.7^{a}	15.2±4.1^{a}	36.0±2.5^{ab}	48.0±3.4^{a}	4.10±1.3^{a}	8.2±2.8^{b}
20 ppm	3.2±0.3^{ab}	5.7±1.6^{a}	6.1±1.9^{b}	7.1±2.3^{ab}	8.5±1.5^{c}	14.1±2.0^{a}	34.6±2.2^{b}	48.0±2.8^{a}	3.86±1.0^{ca}	6.6±2.2^{ab}
30 ppm	2.6±0.2^{c}	5.2±1.5^{ab}	5.4±1.7^{a}	6.5±2.1^{b}	6.6±1.2^{a}	13.0±3.4^{a}	33.5±2.0^{ab}	47.2±2.8^{a}	3.21±1.0^{ca}	5.4±1.1^{a}
40 ppm	2.5±0.2^{b}	4.3±1.5^{b}	5.0±1.5^{a}	6.1±1.8^{a}	6.5±0.9^{a}	12.6±2.1^{a}	25.6±2.0^{c}	35.6±4.5^{a}	3.06±1.1^{ca}	4.2±1.2^{a}
50 ppm	2.5±0.1^{ab}	4.2±1.7^{b}	5.0±1.7^{b}	5.2±1.5^{ab}	6.2±1.6^{b}	12.0±2.3^{a}	25.5±2.2^{b}	35.2±2.8^{ab}	2.52±0.2^{cd}	3.6±0.8^{ca}
60 ppm	2.2±0.1^{b}	3.1±1.2^{bc}	3.2±0.3^{b}	3.7±1.2^{cb}	6.1±1.7^{b}	11.0±2.0^{ab}	22.9±1.3^{ab}	31.0±2.3^{ac}	1.55±0.5^{de}	2.8±0.1^{c}
70 ppm	0.5±0.1^{c}	1.3±1.8^{c}	2.3±0.5^{c}	2.5±1.1^{ce}	4.6±1.4^{ac}	8.2±1.8^{c}	20.4±2.0^{b}	22.0±3.1^{d}	1.43±0.3^{de}	2.5±0.2^{cd}
80 ppm	0.5±0.1^{d}	1.0±0.5^{c}	1.0±0.4^{c}	1.0±0.5^{d}	4.2±1.1^{bd}	5.0±2.1^{c}	6.5±0.7^{a}	11.2±1.1^{cd}	1.25±0.7^{de}	1.5±0.4^{d}
90 ppm	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
100 ppm	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are expressed as mean ± SD. Figures followed by different letters in a same line are significantly different at $P < 0.05$, n= 6. C*= Control (0 ppm cadmium in pot) and ND=Not detected.

Table 2: Qualitative Phytochemical Screening of *Phyllanthus amarus* L. Under Different Concentration of Cadmium (w/w) *:* Cd (Pot- experiment.)

Treatment	Alkaloid				Tannin				Flavonoids			
	15 DAS	30 DAS	45 DAS	60 DAS	15 DAS	30 DAS	45 DAS	60 DAS	15 DAS	30 DAS	45 DAS	60 DAS
C*	++	++++	++++	++++	+	++	+++	+++	++	++	+++	+++
10 ppm	++	++++	++++	++++	+	++	+++	+++	++	++	++	++
20 ppm	++	++++	++++	++++	+	++	+++	++	++	++	++	++
30 ppm	++	+++	+++	+++	+	++	++	++	++	++	++	++
40 ppm	++	++	++	++	+	+	+	+	+	+	+	+
50 ppm	++	++	++	++	+	+	+	+	+	+	+	+
60 ppm	++	+	+	++	+	+	+	+	+	+	+	+
70 ppm	+	+	+	+	+	+	+	+	+	+	+	ND
80 ppm	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND
90 ppm	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
100 ppm	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

++++ = Abundant, +++ = moderately abundant, ++ = moderately presence, + = present and ND=Not detected.

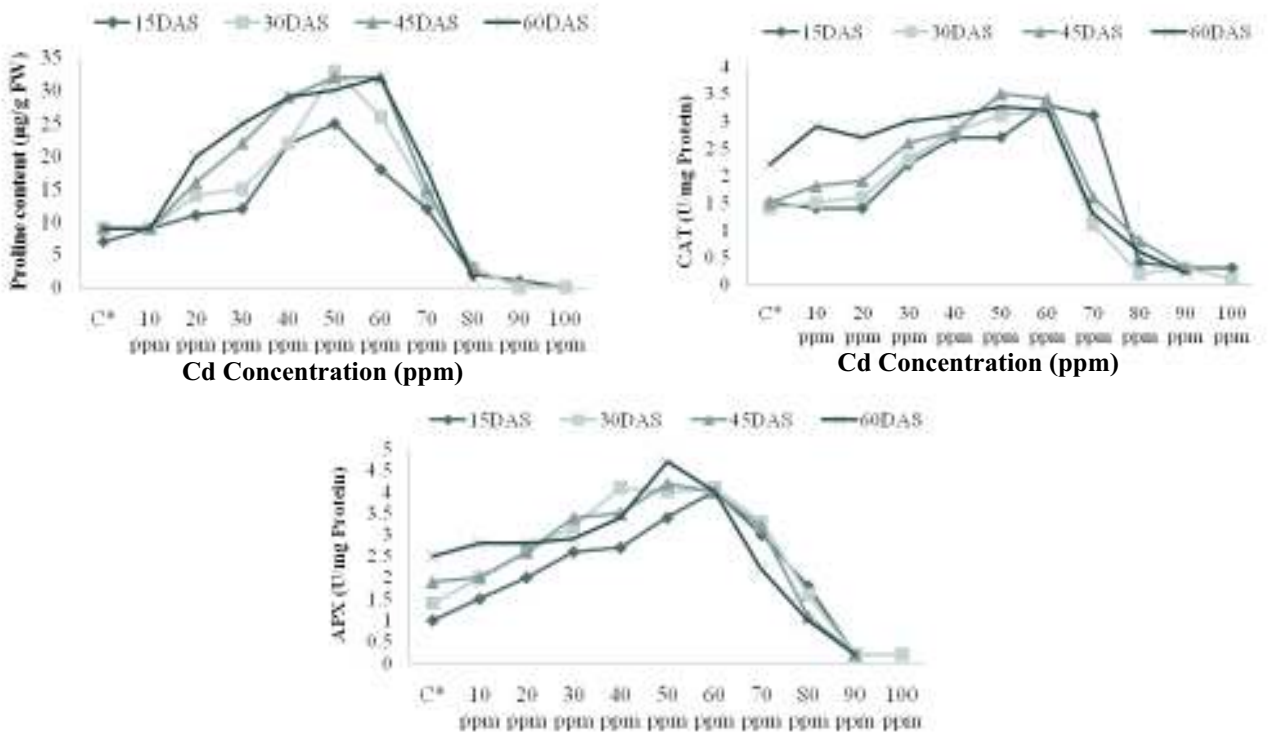

Figure1: Proline, APX and CAT activities in *Phyllanthus amarus* Under Different Concentrations (ppm) of Cadmium After Different DAS

was increased with cadmium concentration and DAS, increasing trends was almost 3 times of the control up to 50ppm after 15,30,45 and 60DAS, four and five times proline content was increased at 40ppm treated plant after 15 and 45DAS respectively (Figure,1). Maximum proline content was recorded at 50 to 60ppm Cd treated plant, which was about three times more than the control 60DAS. APX and CAT activity (U/mg protein) was increased in fresh leaf of the plant under Cd treated plant *Phyllanthus amarus* L. up to 30- 60ppm over the control (Figure,1). Maximum APX activity was found at 30ppm after 60DAS, while maximum CAT activity was found at 50ppm after 60DAS (Figure,1). APX and CAT both the activity was found at 15DAS at all the Cd treatment, and activity was decrease after 45 or 60DAS at >50ppm and >60ppm cadmium treated plant. In the pot experiment, plants were almost grown without any effect of Cd toxicity up to 30ppm treated pot. In leaf, shoot and root accumulates 74, 41 and 32 ppm of Cd after 60DAS at 50ppm treated pot, while maximum uptake of cadmium was recorded at 60ppm treated plant, who survive up to 60DAS, overall in the plant, maximum cadmium accumulated in leaf followed by shoot and root (Figure, 2). More cadmium accumulation tendency of in the plant leaf was found after 30DAS, while in shoot and root showed no such type of pattern. BF and TF for Cd in *Phyllanthus amarus* was almost not significantly to >1.0 at all the treatment after both 30 and 60DAS, while both the factors were more after 60DAS as compared to 30DAS.

DISCUSSION

The root and shoot growth of plant *Phyllanthus amarus* L. In pot experiment decrease with the increasing concentration of cadmium from 50 ppm to 80ppm in pot, while up to 50 ppm growth of plant was unaffected. More recently, Gao et al. (2009) have suggested the possible role of acyl-CoA-binding protein 2, ACBP2, and farnesylated protein AtFP6 in mediating Cu, Cd and Pb transport in A. thaliana roots. A. thaliana plants over-expressing ACBP2 or AtFP6 were more tolerant to Cd than wild-type plants suggesting a similar role in Cu tolerance. Proline contents in

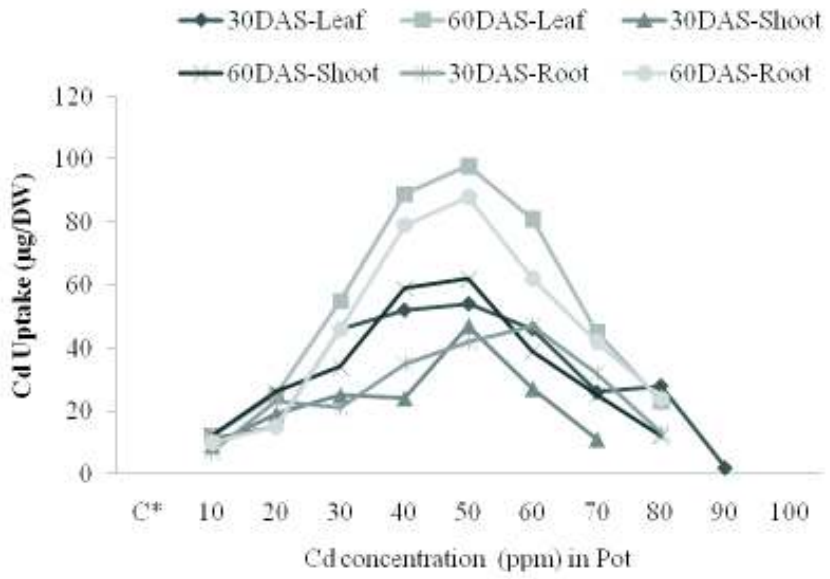

Figure 2: Cadmium Uptake in Leaf, Shoot and Root in The Plant of *Phyllanthus amarus* L. Under Different Concentration (ppm) of Cadmium in Pot Treated Plants. Data ate Average Mean of Six Replicates After 30 And 60das. C*=control

leaf (µ/g FW) of the plant was increased with cadmium concentration and DAS, increasing trends was almost three times of the control up to 50ppm after 15, 30, 45 and 60DAS. Maximum proline content was recorded at 50 to 60ppm Cd treated plant, which was about three times more than the control 60DAS. Proline may act as a mediator of osmotic adjustment, protects macromolecules during dehydration and serve as a hydroxyl radical scavenger (Yoshiba et al., 1997). In the pot experiment, plants were almost grown without any effect of Cd toxicity up to 30ppm treated pot. In leaf, shoot and root accumulates 74, 41 and 32 ppm of Cd after 60DAS at 50ppm treated pot, while maximum uptake of cadmium was recorded at 60ppm treated plant, who survive up to 60DAS, overall in the plant, maximum cadmium accumulated in leaf followed by shoot and root. More cadmium accumulation tendency of in the plant leaf was found after 30DAS, while in shoot and root showed no such type of pattern. Cadmium decreased water potential and transpiration rate as a consequence of decrease of stomata conductance and induced inhibition of both photosystems (PS I and PS II), non-cyclic transport of electrons as well as Calvin cycle including activity of Rubisco and PEP-carboxylase (Siedlecka and Krupa, 1996). The tolerant mechanisms of Cd tolerant plants have been reported previously (Boominathan and Doran, 2003). Bioconcentration factor (BF) and Translocation factor (TF) for Cd in *Phyllanthus amarus* was almost not significantly to >1.0 at all the treatment after both 30 and 60DAS. Results reveled from qualitative phytochemical screening of alkaloid, tannins and flavonoids in *Phyllanthus amarus* L. showed that alkaloid production was more as compared to tannins and flavonoids. Alkaloid, Tannins, flavonoids and total phenolic was unaffected up to 20ppm Cd treated pot. APX and CAT activity (U/mg protein) was increased in fresh leaf of the plant under Cd treated plant Phyllanthus amarus L. up to 30- 60ppm over the control (Fig.1). APX activity was more diverse as compared to CAT activity over the all days. Regulation of antioxidative enzymes can provide plants with an additional protective ability against oxidative stress (Sun et al., 2007; Upadhyay et al., 2012). Accordingly, hyperaccumulators should have an effective Cd tolerance strategy related to the expression of antioxidative enzymes under Cd stress (Boominathan and Doran, 2003, Wang et al., 2008). Overall in the plant, maximum cadmium accumulated in leaf followed by shoot and root (Figure 2). More cadmium accumulation tendency of in the plant leaf was found after 30DAS, while in shoot and root showed no such type of pattern. Therefore, the

result indicates that the levels of metals are more than the permissible limit of WHO and should not advisable to use plant as a medicinal treatment without metal analysis.

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