

Antigenotoxic Potential of Terminalia chebula fruit (myrobalan) Against Cadmium in Allium Test

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Abstract

Allium cepa bulbs were grown in pure tap water (Group I) and in five concentrations (10⁻¹M to 10⁻⁵M) alone (Group II) and all five concentration of cadmium chloride as in group II but each contained myrobalan in it at 0.10 mg/ml (Group III). Parameters of study were mean root length, mitotic index, abnormal mitosis and chromosomal aberrations and morphology of root tips. Cadmium chloride exposure significantly inhibited root growth, declined mitotic index, caused abnormal mitosis and aberrations (Group II). In the presence of myrobalan (Group III) cadmium-induced mitodepression, abnormal mitosis and aberrations could be appreciably prevented. No morphological changes in the root tips of any group could be noticed. Probable protective role of myrobalan is discussed.

INTRODUCTION

Cadmium is environmental pollutant which is both genotoxic (1,2,3) and carcinogenic (4) for human beings and occupationally exposed people are at potentially high health risk (5). Reports on antagonistic herbal compounds towards cadmium toxicity are meager. One report showed cytoprotective role of Glycyrrhizae radix extract and its active component liquiritigenin against Cd-Induced cell death (6) and another revealed attenuation of cadmium chloride - induced oxidative stress and genotoxicity by Pluchea lanceolata (7). Recently myrobalan (fruit of Terminalia chebula) a component of reputed ancient Indian herbal formulation "Trifla" meaning three nuts (Terminalia chebula, Terminalia bellirica (Belliric myrobalan) and Emblica officinalis dried nut) could successfully reduce genotoxicity of lead (8) and aluminium (9) in Allium test. Interestingly, cadmium also exerts genotoxic effects in Allium cepa test model (10,11). The aim of present study was to find out whether myrobalan can also antagonize Cd-genotoxicity.

MATERIAL AND METHODS

ALLIUM CEPA

Equal sized (1.5 to 2.00 cm) healthy dry brown pink onion bulbs of commercial variety onions (2n=16) were obtained from the local market.

TEST HERBAL DRUG

Dried young nuts of medicinal plant Terminalia chebula (myrobalan) locally called "Bal Harad" were procured from local herbal medicine shop. Dried nuts were gently baked for few minutes in steel container. This treatment caused swelling of nuts. After cooling the nuts were grinded in electrical mixer to obtain very fine powder. Powdered material was stored for further use in the present experiment.

SELECTION OF DOSE OF MYROBALAN

Earlier studies from this laboratory (12) had revealed that myrobalan at 0.1 mg/ml concentration did not exert any ill effect. Hence this dose was selected for present study.

TEST CHEMICAL

Cadmium chloride monohydrate as CdCl₂·H₂O, MW 201.32, and purity 99% of Sarabhai India was used. Salt was dissolved in tap water to prepare solutions of different concentrations ranging from M⁻¹ to M⁻⁵. Experimental design was planned as per internationally accepted protocol (13) which consisted of the following steps.

- (i) The pink brown dry outer scales and some of the brownish bottom plate of each bulb were removed carefully leaving root primordial intact.
- (ii) For each concentration of test compound i.e. CdCl₂, a series of 12 test tubes were arranged in a test tube rack. Five series of the test tubes were filled with the different molar

concentrations (M^{-1} to M^{-5}) of solutions of $CdCl_2$ in tap water (Gr II). Twelve tubes were filled with only pure tap water and maintained to provide control (Gr I). 60 tubes were filled with five concentration of cadmium chloride solution as in Gr II but having myrobalan in it at 0.10 mg/ml concentration (Gr III).

(iii) Each descaled onion was placed on the top of each tube with root primordial downward in the liquid.

(iv) After 24 hours test suspension in (Gr III) and test solutions in (Gr II) and tap water in (Gr I) were changed. Change of liquid was repeated after 48 hours.

(v) After 48 hours two onions out of twelve in each series with most poorly growing roots were removed. Same day i.e. after 48 hours distal 2 mm of five roots was cut off from five individual bulbs and fixed in aceto-alcohol (1:3 v/v) for chromosomal study. Every time fixation was done at a fix time 11.00 O'clock.

(vi) After 72 hours length of the 05 root bundles in each series of each onion was measured using a ruler. Mean length of roots for each series was calculated and recorded for further statistical analysis.

(vii) Morphology (shape and colour) of root tips were also recorded after 72 hours.

SQUASHING OF ROOT TIPS

Root tips were squashed in 2% acetocarmine (BDH) + N HCl (9:1 v/v) after gently warming.

OBSERVATIONS

Four fields from each slide were observed to cover about 50 cells i.e. total 200 cells per slide. Total 2000-2500 cells were observed for each group of onions (10-15 slides). Mitotic Index (MI) was calculated percentage of cells in division

$$MI = ((\text{No. of cells showing mitosis}) / (\text{Total No. of cells observed})) * 100$$

Slides were also observed to find out mitotic arrest, chromosome fragmentation, lagging, abnormal orientation, stickiness, polyploidy etc.

STATISTICS

Experiments were done thrice. Student's t-test was applied at 5% level of significance to find out significant difference between Gr I and Gr II or Gr I and Gr III or Gr II and Gr III.

RESULTS

1. MEAN ROOT LENGTH (MRL, TABLE - 1)

Figure 1

Table 1: Mean root length (MRL as mm) of after 72 hr cultivation in different concentrations of $CdCl$ alone or in combination with myrobalan (mean \pm SEM).

S. No.	Concentrations Molarities	Group of Onion Bulbs					Difference Gr-II Vs Gr-III
		Gr-I Control	Gr-II $CdCl_2$ exposed	Gr-III $CdCl_2$ + Myrobalan	% Inhibition Gr-I Vs Gr-II	% Inhibition Gr-I Vs Gr-III	
1	0.00	60.63 \pm 1.65					
2	$10^{-4}M$		45.12 \pm 0.11 ^a	50.30 \pm 1.10 ^a	25.58%	17.03%	8.55% _s
3	$10^{-4}M$		15.63 \pm 0.15 ^a	21.98 \pm 1.12 ^a	74.22%	63.74%	10.48% _s
4	$10^{-4}M$		2.46 \pm 0.13 ^a	3.25 \pm 0.56 ^b	95.94%	94.63%	1.31% _{NS}
5	$10^{-5}M$		(-)	(-)	(-)	(-)	(-)
6	$10^{-5}M$		(-)	(-)	(-)	(-)	(-)

Statistically significant based on t-test at 5% level of significance.
^a = Control Vs Gr. II (p=1.96) S = Significant
^b = Control Vs Gr. III (n=100) NS = Non significant
^c = Gr. II Vs Gr. III (-) = No Growth M = Molarities

All test concentrations of cadmium chloride (except at 10^{-1} M and 10^{-2} M where roots did not grow at all) caused significant inhibition in the growth of roots (Gr. II) in comparison to controls (Gr. I). A comparison between Gr. II and Gr. III ($CdCl_2$ + myrobalan) revealed that myrobalan could partially check cadmium induced root growth inhibition at 10^{-4} M and 10^{-5} M as mean values could not reach up to controls MRL value.

2. MITOTIC INDEX (MI, TABLE - 2)

MI was found significantly lower than controls (Gr. I) at 10^{-3} M, 10^{-4} M and 10^{-5} M cadmium exposure (Gr. II). Drug could not antagonize effect of cadmium at 10^{-3} M but significantly higher MI could be recorded at 10^{-4} M and 10^{-5} M of cadmium chloride having drug Gr. III but still mean values did not reach up to controls mean values. This indicates that drug could partially prevent Cd-induced mitodepression at 10^{-4} M and 10^{-5} M.

Figure 2

Table 2: Mitotic Index (MI) of root tip cells following 48 hrs cultivation in $CdCl$ alone or in combination with myrobalan (mean \pm SEM).

S. No.	Concentrations Molarities	Group of Onion Bulbs					Difference Gr-II Vs Gr-III
		Gr-I Control	Gr-II $CdCl_2$ exposed	Gr-III $CdCl_2$ + Myrobalan	% Inhibition Gr-I Vs Gr-II	% Inhibition Gr-I Vs Gr-III	
1	0.00	34.51 \pm 1.08					
2	$10^{-4}M$		17.87 \pm 0.89 ^a	22.03 \pm 0.40 ^a	48.21%	36.16%	12.05% _s
3	$10^{-4}M$		16.60 \pm 0.38 ^a	21.15 \pm 0.30 ^a	51.89%	38.71%	13.18% _s
4	$10^{-4}M$		14.89 \pm 0.36 ^a	15.43 \pm 1.45 ^b	56.85%	55.28%	1.57% _{NS}
5	$10^{-5}M$		(-)	(-)	(-)	(-)	(-)
6	$10^{-5}M$		(-)	(-)	(-)	(-)	(-)

Statistics and other symbols are same as detailed below Table 1.

3. MORPHOLOGY: COLOUR AND SHAPE OF ROOT TIPS (TABLE - 3)

Morphology i.e. colour and shape of Allium cepa root tips cultivated in all test concentrations of cadmium chloride alone (Gr. II) or cadmium chloride plus myrobalan (Gr. III) did not reveal any change from controls (Gr. I).

Figure 3

Table 3: Morphology of root tip following 72 hrs. cultivation in CdCl alone or in combination with myrobalan.

S. No.	Groups	Morphology i.e. shape of root tips			Colour of root tip			
		Abnormal			Normal	Abnormal		
		Crochet hooks	Bulb	Broken tips	Straight	White	Pale	Dark Brown/Black
	Gr. I Control	No	No	No	Yes	Yes	No	No
	Gr. II CdCl ₂							
1.	10 ⁻⁹ M	No	No	No	Yes	Yes	No	No
2.	10 ⁻⁸ M	No	No	No	Yes	Yes	No	No
3.	10 ⁻⁷ M	No	No	No	Yes	Yes	No	No
4.	10 ⁻⁶ M	(-)	(-)	(-)	(-)	(-)	(-)	(-)
5.	10 ⁻⁵ M	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	Gr. III CdCl ₂ + Myrobalan							
1.	10 ⁻⁹ M	No	No	No	Yes	Yes	No	No
2.	10 ⁻⁸ M	No	No	No	Yes	Yes	No	No
3.	10 ⁻⁷ M	No	No	No	Yes	Yes	No	No
4.	10 ⁻⁶ M	(-)	(-)	(-)	(-)	(-)	(-)	(-)
5.	10 ⁻⁵ M	(-)	(-)	(-)	(-)	(-)	(-)	(-)

(-) = No Growth (n=100)

4. ABNORMAL MITOSIS (TABLE - 4)

In controls no abnormal mitosis or chromosomal aberrations could be observed however, cultivation of Allium bulbs at 10⁻³ M to 10⁻⁵ M cadmium chloride caused chromosome stickiness and scattered chromosomes at metaphase and chromosome fragmentation at anaphase at 10⁻³ M to 10⁻⁵ M (Gr. II). All these effects were found fully prevented at 10⁻⁵ M and significantly less pronounced in the presence of myrobalan at 10⁻⁴ M but drug could not act at 10⁻³ M (Gr. III). Fragmentation mimics apoptotic changes however, it is too early to comment upon without performing tunnel test.

Figure 4

Table 4: Cytological effects of root tip cells following 48 hr cultivation in different concentrations of cadmium chloride alone or in combination with myrobalan (mean shown as percentage, 2000 cells observed in each group).

S. No.	Groups	Treatments	Observed stages of mitosis							Other observations			
			Metaphase			Anaphase				MNC	PKC	Apoptotic	
			N	SC and STC	N	MPA	CB	LC	FRG				
1	Gr. I	Control (Tap water)	100	-	-	100	-	-	-	-	-	-	-
2	Gr. II	10 ⁻⁶ M CdCl ₂	90.12 ± 1.12	9.88 ± 0.09	91 ± 1.68	-	-	-	9 ± 0.08	-	-	-	-
	Gr. III	10 ⁻⁶ M CdCl ₂ + My	98.95 ± 1.31	1.05 ± 0.01	98.77 ± 1.81	-	-	-	1.23 ± 0.03	-	-	-	-
3	Gr. II	10 ⁻⁵ M CdCl ₂	80.38 ± 0.97	19.67 ± 0.87	83.15 ± 1.81	-	-	-	16.85 ± 0.07	-	-	-	?
	Gr. III	10 ⁻⁵ M CdCl ₂ + My	95.24 ± 1.01	4.76 ± 0.51	95.80 ± 1.41	-	-	-	4.20 ± 0.02	-	-	-	-
4	Gr. II	10 ⁻⁴ M CdCl ₂	70.66 ± 0.70	29.34 ± 0.67	85 ± 1.61	-	-	-	15 ± 0.67	-	-	-	?
	Gr. III	10 ⁻⁴ M CdCl ₂ + My	70.15 ± 0.88	29.85 ± 0.54	84.10 ± 1.77	-	-	-	15.90 ± 0.44	-	-	-	-
5	Gr. II	10 ⁻³ M CdCl ₂	NG	-	-	-	-	-	-	-	-	-	-
	Gr. III	10 ⁻³ M CdCl ₂ + My	NG	-	-	-	-	-	-	-	-	-	-
	Gr. II	10 ⁻³ M CdCl ₂	NG	-	-	-	-	-	-	-	-	-	-
6	Gr. III	10 ⁻³ M CdCl ₂ + My	NG	-	-	-	-	-	-	-	-	-	-

NG = No growth (-) = Nil, SC = Sticky chromosomes, STC = Scattered Chromosomes, MPA = Multipolar anaphase, CB = Chromosome bridge, LC = Lagging chromosome, FRG = Fragmented chromosomes, PKC = Polykaryocytes, M = Molurites, My = Myrobalan
 * = Control vs all concentrations
 † = Value in Gr II vs. value of Gr III at each concentration level

DISCUSSION

Earlier studies have shown that cadmium-induced C-mitosis, chromosome stickiness, chromosome lagging, low mitotic index and multipolar anaphases in Allium bulb roots and seed roots (10,11,14). Hence cadmium-induced chromosomal effects in present study are not an unexpected finding. Cadmium induces DNA mismatch repair inhibition, and mediated cell cycle arrest in human cells (3) and apoptotic changes in chromatin structure during cell cycle in CHO cells (15). No root growth was observed at 10⁻¹ M and at 10⁻² M concentrations of cadmium chloride and this might have been due to death of root primordial cells in GO stage of cell cycle. This possible explanation gets support from an ultra structural study which had shown Cd-induced disintegration of cytoplasmic organelles and cell death in Allium sativum (16).

In rat hepatocytes cadmium chloride lowered cell population at GO/G1 and G2/M stages (17). A dose dependent reduction of cell proliferation could be noticed in cultured Chinese hamster ovary (CHO) cells following cadmium exposures (15). The cells were blocked at G2/M and G1/S phases and authors were of opinion that cadmium toxicity was not cell phase specific. During late G1 restriction point gate opens in the presence of a complex molecule at promoters of essential cell cycle genes and unreplicated and/or damaged DNA does not allow cells to go beyond G1 state (18). Cadmium affects both, gene transcription and translation and modulates signal transduction pathway (4). All such known toxic effects of cadmium chloride can be held responsible for causing low mitosis i.e. mitodepression in Allium root tip cells in the

present study.

In *Vicia faba* an increase in antioxidant stress enzymes (Super oxide dismutase, glutathione reductase and catalase), in response to cadmium was evident for enhanced detoxification towards reactive oxygen species. Also, micronuclei induction was interpreted as a consequence of oxidative stress and authors were of opinion that cadmium-induced damage up to certain extent was via generation of ROS i.e. reactive oxygen species (19).

Pluchea lanceolata could reduce Cd-induced oxidative stress and genotoxicity in mice (7). It is likely that cadmium-induced peroxidative damage declined mitosis in *Allium* root tip cells but if myrobalan possesses antioxidant properties it can reduce Cd-toxicity. In fact myrobalan has been shown to exert antioxidant and free radical scavenging activities (17,20,21).

Unreplicated DNA does not allow cells to go beyond G1 stage and Cd damages DNA. It is likely that Cd-induced DNA damage in *Allium* root tip cells could have been remedied by myrobalan. It is known to exert antimutagenic activity in bacteria against direct acting mutagens like sodium azide and 4-nitro-O-phenylene diamine (22). Later on this property was attributed to tannins (23).

The *Allium cepa* root cells also possess certain enzymes, the mixed function oxidases like that of mammalian hepatocytes that can activate promutagens to mutagens (13). In case of metal toxicity detoxification in root cells takes place in the cytoplasm and cell wall within 12-24 hours which was held responsible for the mitotic activity at low concentration exposures (24). Similar action of myrobalan towards cadmium in the present case can not be ruled out.

Individual plant components like sulfhydryl and flavonoid compounds, gallic acid, ellagic acid, mucic acid, citric acid, reducing sugars and tannins can modulate effect of many genotoxicant (25). Myrobalan possesses many of such compounds (26) especially flavonoids which are ideal antioxidants (27) hence can be held responsible for reducing Cd-genotoxicity in *Allium* root cells. Infact polyphenols (tannins, gallic acid and tannic acid) were found to detoxify cadmium in water lily (28). Lastly, the presence of some phytochelatin in myrobalan can also contribute towards antagonizing Cd-toxicity (29).

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