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STUDIES ON AMELIORATIVE EFFECT OF *AEGLE MARMELOS* ON CADMIUM INDUCED SPLENIC TOXICITY IN MURINE MODEL

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ABSTRACT
The present study was targeted to evaluate the antioxidant potential of *Aegle marmelos* against cadmium induced toxicity in mice spleen. Cadmium was administered intraperitonealy (5mg/kg b.wt.) for five days which led to significant elevation in the levels of lipid peroxidation and a significant decrease in the levels of antioxidant enzymes. Histopathological alterations included disorganisation of the red and white pulps with loss of proper margination leading to splenomegaly and an increase in splenic index. Pre and post-treatments of *Aegle* (500mg/kg b.wt.) were able to effectively restore the elevated LPO and decreased levels of antioxidants. Both the treatments effectively attenuated the biochemical and histopathological changes induced by cadmium. Histomicrographs of *Aegle* treated mice spleen revealed well organised red pulp and the white pulp areas traversed by trabeculae. Pre-treatments of *Aegle* were found to be much more effective than the post treatments. These results suggest that oral administration of *Aegle marmelos* extract provides significant protection against cadmium induced splenic toxicity in BALB/c mice and could be used as a potential antioxidant against metal toxicity.

KEYWORDS: Cadmium, *Aegle*, Spleen, Antioxidants, Reactive Oxygen Species.

INTRODUCTION
Cadmium is an extremely toxic heavy metal and a very well-known industrial and environment pollutant. It is found in the earth's crust always in combination with zinc, it is an inevitable end-product of zinc, lead and copper extraction. Generally, occupational and environmental exposure to this ubiquitously present heavy metal occurs from mining, metallurgy, manufacturing of Ni-Cd batteries, cement, pigments and plastic stabalizers. According to ATSDR cadmium is ranked seventh in the top twenty hazardous substances priority list.[1] It is a cumulative toxicant whose bioaccumulation keeps on increasing with time, due to its slow excretion rate from the body. Cadmium affects almost every organ-system of the body namely liver, kidney, testis, spleen, brain and placenta.[2][3] and its occupational exposure has been widely associated with the cancers of many vital organs. It also affects cell cycle progression, proliferation, differentiation, DNA repair as well as apoptotic pathways.[4] But when considering its effect on immune organs the only available evidience is of splenomegaly and thymic atrophy. The tangible reason for these alterations is still vague, as these changes could occur due to oxidative stress or apoptotic/necrotic cell deaths.[5] Few studies have reported cadmium as leading immunotoxic agent which alters spleenic functioning[6] [7] and associated splenomegaly largely with the cadmium induced increase in the red pulp area that causes gain in the spleenic-index.[8] Most plausibly cadmium generates its toxic impact by producing reactive oxygen species (ROS) and disrupting cellular antioxidant systems.[9] These reactive oxygen species are capable of reacting with nucleic acids, membrane lipid enzymes, proteins and other molecules which in turn results in cellular damage, DNA damage and apoptosis.

Despite of huge progress observed in medicinal practices, the side effects of allopathic medications are still a matter of concern. It is generally accepted that the extracts derived from plant products are safer than their synthetic counterparts. These antioxidants terminate these chain reactions by removing free radical intermediates and inhibit the accumulation of toxic radicals. One such antioxidant is *Aegle marmelos* also known as 'bael' belonging to family Rutaceae is indigenous to India and found in abundance in the Himalayan tract. Its leaves have anti-oxidant, anti-inflammatory and anti-cancerous properties[10] and are also useful in various metabolic syndromes.[11] [12] Its major phytoconstituents viz. alkaloids, flavanoids and various other poly phenols formulate it to be a strong free radical quencher.[13] Thus considering these properties, the present study has been designed to investigate the splenoprotective efficacy of *Aegle marmelos* against cadmium induced toxicity in mice.

MATERIALS AND METHODS

Animals

For the present investigations, BALB/c mice (weighing 25-30 gm) were used. Experiments on animals were performed in accordance with the guidelines for the use of laboratory animals approved by the Indian Council of Medical Research, New Delhi, India. Ethical clearance was sought from Panjab University Ethical Committee before the commencement of the study. All animals were acclimatized for a period of 7 days, fed standard pellet diet and water *ad libitum* and divided into 6 groups of 5 mice each.

Chemicals

Cadmium sulphate, and other chemicals used in this work were purchased from Central Drug House (CDH) Pvt. Ltd. New Delhi, India, MERCK & Co. Inc. Kenilworth, U.S.A and HIMEDIA Labs Pvt. Ltd., Mumbai, India. Short-term intraperitoneal cadmium exposures were given to imitate the long term effect of oral exposure in organisms. In this case primary route of absorption of metal is through the mesenteric vessels where it is metabolised via the hepatic portal system and then finally absorbed into the system. The time and route of exposure can greatly affect the absorption and distribution of cadmium to various target sites and therefore its final effect.[14] So, for cadmium exposure an intraperitoneal sub-acute dose of 5mg/kg b.wt. was administered for 5 days.

Preparation of the plant doses

Leaves of *Aegle marmelos* were collected from the Botanical garden Panjab University, Chandigarh, India and identified by a taxonomist (Department of Botany, Panjab University) and the voucher number was obtained 21044. The leaves were shade dried, powdered and extracted in ethanol using Soxhlet apparatus. The extract was filtered and evaporated to dryness under reduced pressure using a rota evaporator (Department of Chemistry, Panjab University) at controlled temperature. 500mg/kg b.wt. of *Aegle* was given orally to the mice for 10 days after dissolving in the groundnut oil.

Phytochemical Screening

To check the presence of different phytoconstituents in the *Aegle* extract, various phytochemical screening tests were conducted. Different chemical tests were performed using protocol of Harbone for establishing the pharmacological profile of ethanolic extract of *Aegle*.[15] Wagner's test for detection of alkaloids, ammonia-HCl test for the detection of flavonoids, ferric chloride test for detection of phenolic compounds, gelatin test for presence of tannins, Molisch test for detection of carbohydrates, foam test for saponins and salkowski test for detection of terpenoids.

Experimental Design

The animals were divided into 6 groups of 5 animals each

1. Control group - Mice were injected with normal saline for 5 days.
2. Cadmium group - Mice were administered 5 mg/kg b.wt. of Cd intaperitoneally for 5 days.
3. Groundnut group- Mice were given 0.5ml oil/kg b.wt. for 10 days orally
4. *Aegle* group - Mice were administered with 500 mg/kg b.wt. of the ethanolic extract of *Aegle* for 10 days orally.
5. Pre-treated *Aegle* group - Mice were administered 500 mg/kg b.wt. of the ethanolic extract of *Aegle* for first 10 days orally and next 5 days with Cd (5 mg/kg b.wt.) i.p. was administered.
6. Post-treated *Aegle* group - 5 mg/kg b.wt. Cd was administered i.p for first 5 days, then 500 mg/kg b.wt. ethanolic extract of *Aegle* was administered orally for last 10 days.

Biochemical Measurements

Several biochemical tests were performed in the 10% homogenate and post mitochondrial supernatants of spleen.

Preparation of Homogenate

10% homogentates were prepared in 50 mM Tris-HCl buffer (pH-7.4) using a homogenizer at 0-4°C. These homogenates were used for the estimation lipid peroxidation (LPO), reduced glutathione (GSH) and for the estimation of the protein content by Lowry's method (1951).

Preparation of post-mitochondrial supernatant (PMS)

The homogenates were centrifuged at 10000 rpm for 20 mins at 4°C, the supernatants were stored at -20°C for the estimation of anti-oxidant enzyme activities.

Oxidative Stress Indicators

Lipid peroxidation (LPO) was estimated by the method of Buege and Aust (1978).[16]

Reduced Glutathione (GSH) was estimated by the method of Beutler *et al.*, (1963).[17]

Anti-oxidant and Detoxifying Enzymes

Catalase activity was measured by the method of Luck (1971).[18]

Superoxide Dismutase activity was measured according to the method of Kono (1978).[19]

Glutathione-S-transferase (GST) activity was assayed by the method of Habig *et al.* (1974).[20]

Glutathione Reductase (GR) activity was measured by the method of Horn (1971).[21]

Estimation of protein content- was done by the method of Lowry *et al.*, (1951).[22]

Histopathological Studies

Histopathological alterations of spleen tissue were studied using light microscopy, which were stained by the process of H and E staining by the method of Pearse (1968).[23]

Statistical Analysis

All values were expressed as mean ± standard deviation (S.D) where n=5. For the comparison of statistical significance between two groups one way ANOVA tukey test was used.

RESULTS

Phytochemical Analysis

Preliminary qualitative estimation of ethanolic extract of *Aegle marmelos* (Table I) revealed the presence of alkaloids, carbohydrayes, phenols, tannins, flavonoids and terpenoids. The pharmacological properties of *Aegle marmelos* are attribute to these phytochemicals.

Table I: Phytochemical evaluation of ethanolic extract of *Aegle marmelos*

Sr. No.	PHYTOCHEMICAL CONSTITUENT	TEST PERFORMED	RESULT
1	Alkaloids	Wagner's test	+
2	Carbohydrates	Molisch test	+
3	Phenol	$FeCl_3$ test	+
4	Tannins	Gelatin's test	+
5	Flavonoids	Ammonia test	+
6	Saponins	Froth test	-
7	Terpenoids	Chloroform test	+

Oxidative Stress Indicators and Antioxidant Enzymes

Five days cadmium exposure (5mg/kg b.wt.) instigated an extremely statistically significant ($p<0.0001$) intensification in the levels of LPO in mice spleen and an increase of 127% was seen as compared to control. Pre and post treatments of *Aegle* (500 mg/kg b.wt.) evidently decreased these elevated levels. The pre-treatment [35% (a*)] was found to be much more effective than the post-treatment [64% (a^*)] with lowering the increased levels of LPO as compared to the control. (Fig. 1). Intoxication of cadmium caused approximately 77% fall in the splenic levels of GSH as compared to control. All the treatments of *Aegle* restored the levels of reduced glutathione to near normal values but the pre-treatment was observed to be more effective in attenuating Cd toxicity than the post treatment. *Aegle* raised the levels of GSH by 49% (b^*) and 42% (b^*) in the pre and post treatments respectively in comparison Cd group. (Fig. 2).

A 60% decline was observed in the levels of CAT and SOD and an 85%, 65% decrease was seen in the levels of GST and GR respectively after 5 days Cd exposure as compared to the control. Both the pre and post-treatments of *Aegle* ameliorated the ill-effects of cadmium and restored the levels of these enzymes. But the pre-treatment of Aegle was better than the post-treatment against Cd induced alterations in the antioxidant enzyme system. Pre-treatment of *Aegle* raised the levels of CAT, SOD and GR by approximately 50% (b^*) and GST by 80% (b*) as compared to the cadmium treated groups. *Aegle* (500 mg/kg b.wt.) alone showed no significant difference in the levels of all the evaluated enzymes when compared to the control group (Table II).

Table II: Effect of sub-acute cadmium exposure on oxidative stress and antioxidant enzyme parameters and its modulation by *Aegle marmelos* (500mg/kg b.wt) in Spleen.

Spleen	Control	Cadmium	Groundnut Oil	Aegle	Pre-treated	Post-treated
CAT	28.964± 0.645	10.984± 0.375a*	28.898±0.092	28.985±0.574 b*	27.667±0.458 a▪b*	21.699± 0.695 a•b•
SOD	14.655± 0.089	5.262± 0.360a*	14.296±0.172	14.905±0.260 b*	12.673±0.229 a▪b*	8.617± 0.096 a*b•
GST	1.594± 0.081	0.232± 0.053a*	1.572±0.734	1.62±0.042 b*	1.508± 0.073a▪b*	1.215± 0.068 a▪b•
GR	43.785±1.432	14.964± 1.534a*	43.701±0.065	43.996±0.739 b*	39.864± 0.832a▪b•	33.931± 0.954 a▪b•

Levels of significance

▪ = p≤ 0.05 (statistically significant); • = ≤ 0.001 (very statistically significant); *= ≤ 0.0001 (extremely statistically significant).

a = comparison with control group; b = comparison between Cd treated group;

ANOVA followed by tukey's honestly significant difference test.

Units

Superoxide dismutase (SOD) – unit/min/mg protein (1 unit enzyme is defined as the amount of enzyme inhibiting 50% nitro blue tetrazolium reduction); Catalase (CAT)- µ moles of H_2O_2 decomposed/min/mg protein; Glutathione-S-transferase (GST)- µ moles GSH adduct formed/min/mg protein; Glutathione Reductase (GR)- n moles NADPH oxidised/min/mg protein.

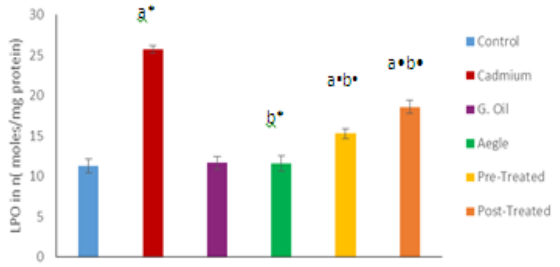

Fig. 1: Lipid peroxidation (LPO) (n moles/mg protein) in Control, Cd, G oil and *Aegle* treated groups.

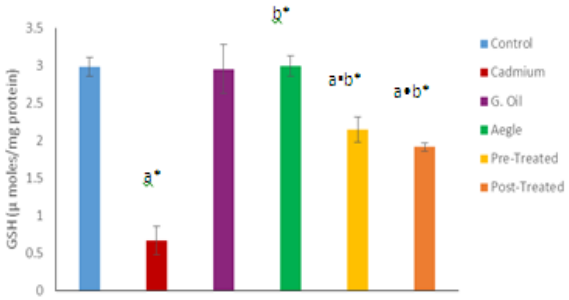

Fig. 2: Reduced glutathione (GSH) - µ moles/mg protein in Control, Cd, G Oil and *Aegle* treated groups.

NOTE: Values are shown as Mean±S.D. (n=5).
Levels of Significance

▪ = p≤ 0.05 (statistically significant); • = ≤ 0.001 (very statistically significant); *= ≤ 0.0001 (extremely statistically significant).
a = comparison with control group; b = comparison between Cd treated group;

Histopathological Analysis

The histopathological evaluation of the control group of spleen revealed a capsule composed of dense fibrous tissue, irregularly spaced trabeculae which emanate from the capsule into the splenic parenchyma. The red pulp was composed of a three dimensional meshwork of splenic cords and sinuses with a proper demarcated marginal zone (Fig. 3, A-B). Five days cadmium intoxication caused disorganisation in the red pulp, and an evident decrease in the size of white pulp with loss of trabeculae. There was a significant increase in the area of red pulp, leading to an overall increase in the splenic index. Margination between the two areas was almost lost (Fig. 2, C-D). Histological micrographs from the *Aegle* pre-treated group revealed prevention recovery in the splenic damage caused by Cd intoxication. Pre-treated mice revealed a well organised red-pulp and white pulp with a proper marginal zone and an evenly distributed range of trabeculae were observed (Fig. 4 A-B). Post-treatment also ameliorated Cd induced changes but still exhibited areas of disorganised red pulp, diminished white pulp and a fewer trabeculae (Fig. 4, C-D).

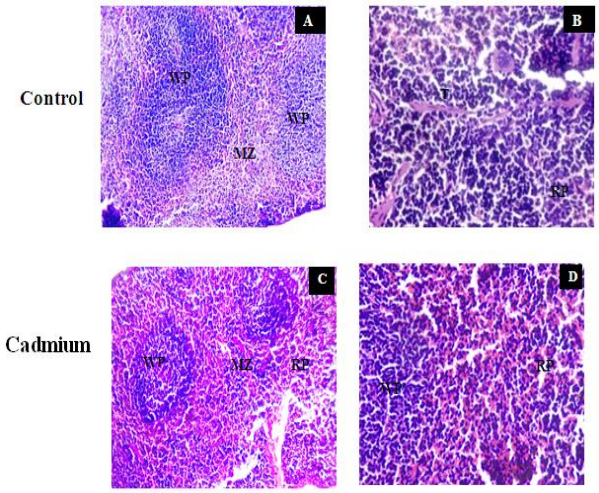

Fig. 3: Light micrographs of control (C) and Cd treated mice spleen.

Control group (A-B): A: a well organised white pulp and red pulp areas with proper marginal zone (100X); **B:** well developed trabeculae in the red pulp (400X).

Cadmium group (C-D): C: Shrinked white pulp and widened red pulp areas with loss of proper marginal zones; **D:** Loss of trabeculae from the red pulp and disintegerated marginal zones.

Abbreviations: WP- white pulp; RP- red pulp; MZ- marginal zone; T-Trabeculae;

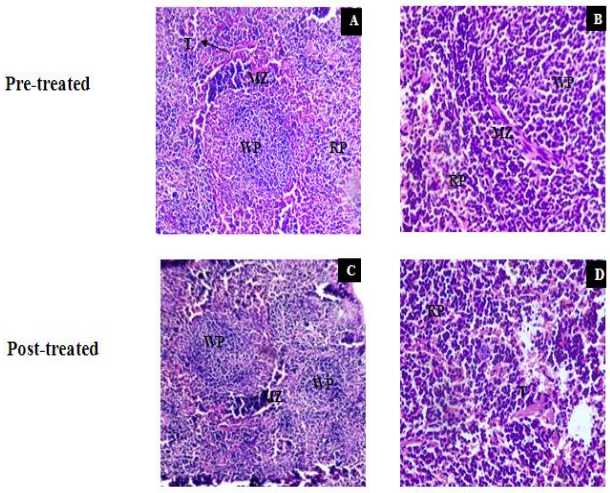

Fig. 4: Light micrographs showing protective effect of pre and post-treatment of *Aegle* 500mg/kg b.wt in Spleen.

Pre-treated group: (A-B): A: well organised red pulp and white pulp areas with a proper marginal zone and evenly distributed trabeculae (100X); **B:** Areas of white pulp, marginal zone and red pulp with proper demarcations (400X).

Post-treated group: (C-D): C: Few foci of diminished white pulp, altered red pulp (100X); **D:** Disinteregated

white pulp and red pulp areas with loss of marginal zones and trabeculae (400X).

Abbreviations: WP- white pulp; RP- red pulp; MZ- marginal zone; T-Trabeculae.

DISCUSSION

Cadmium is an extremely toxic heavy metal and exerts its effects via oxidative damage to the cellular organelles by the induction of ROS production.[24] The reactive oxygen species react with the cellular biomolecules and leads to lipid peroxidation, membrane protein and DNA damage.[25] According to Tahara and Kaneko the splenic cells are quite sensitive to oxidative stress and follow an apoptotic pathway rather than a repair mechanism.[26] A range of endogenous antioxidant enzymes of the body play an important role to protect the tissues against the damage caused by the free radicals.[27] But an overproduction of ROS leads to toxicity, the present study revealed marked elevation in the levels of splenic LPO following cadmium intoxication. The enhanced LPO might have stimulated mitochondrial respiration which is an important source of reactive oxygen species.[28] The levels of the splenic endogenous antioxidant enzymes (SOD, CAT. GSH, GST and GR) are severely affected after cadmium administration in the present study. There was a significant decrease in the levels of almost all the antioxidant enzymes under investigation. The formation of complexes between cadmium and enzyme systems that chiefly contain sulfhydryl groups render them inactive.[29] Another mechanism of cadmium toxicity is the displacement of essential metals such as Zn and Se from the enzymes active site which hence get inactivated.[30] Furthermore, Cd toxicity in the present study caused a disorganisation of the splenic white pulp and the red pulp. There is a reduction in the number of white pulp cells, despite of spleen enlargement. The enlargement of the red pulp might be associated with increased number of damaged RBC's. Oxidative stress plays an integral role in the manifestation of Cd induced splenic damage.[31] [32] These results are in compliance with the finding of Pathak and Khandelwal, who also documented intracellular events leading to damage in spleen cells because of Cd induced thymic regression, oxidative stress and splenomegaly.[33]

In the present study, antioxidant potential of *Aegle* was exploited against Cd toxicity, a 10 days treatment with *Aegle* markedly reduced the levels of lipid peroxidation. Also it significantly restored the lowered levels of endogenous non-enzymatic and enzymatic antioxidants like GSH, SOD, CAT, GST and GR. These results lend support from Lalremurta and Prasanna who demonstrated protective role of *Aegle* by elevating the levels of enzymatic and non-enzymatic cellular antioxidants.[34] A plausible reason for the protective efficacy of *Aegle* in present study might be due to the presence of several phytoconstituents such as quercetin and related flavonoids that are free radical scavengers and antioxidants.[35] Quercetin, inhibits lipid peroxidation by blocking the enzyme xanthine oxidase,[36] chelating iron,[37] and directly scavenging hydroxyl, peroxy and superoxide radicals.[38] Quercetin also protects antioxidative defense mechanism by increasing the absorption of VitaminC.[39] It also inhibits the release and protection of oxidative products generated by the respiratory burst in phagocytes.[40]

As the biochemical insult caused by cadmium has been reduced by *Aegle* its preventive efficacy was also observed in the histoarchitecture of spleen. Pre-treatment with *Aegle* preserved the histoarchitecture of spleen and demonstrated a well organised white pulp, red pulp and marginal zone in splenic tissue. Taken together it can be concluded that *Aegle* represents a promising alternative solution to current state of medications for metal toxicity. Further research is needed to pinpoint which specific phytoconstituents are most effective in rendering *Aegle* its protective efficacy. Thus, *Aegle* can be exploited as a potential drug supplement for ameliorating and preventing metal induced toxicity.

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