Neuroprotective effects of *Aegle marmelos* (L.) Correa against cadmium toxicity by reducing oxidative stress and maintaining the histoarchitecture of neural tissue in BALB/c mice

Era Seth, Surbhi Kaushal, Aitzaz Ul Ahsan, Vijay Lakshmi Sharma* & Mani Chopra

Department of Zoology, Panjab University, Sector-14, Chandigarh-160 014, India

Received 27/06/2016; revised 06/02/2018

Herbal plants have various biological and pharmacological activities and are used as chemotherapeutic agents. *Aegle marmelos* (Lin.) has antioxidant, anti-inflammatory and antigenotoxic properties due to its active phytoconstituents like marmenol, marmin, marmelosin, aegelin, marmelin etc. The present study investigates the protective potential of leaf extract of *A. marmelos* against cadmium-induced oxidative stress and histopathological manifestations involved in the neuronal tissue of BALB/c mice. Mice were given prophylactic and therapeutic treatments (pre and post) of two different doses (250 and 500 mg/kg body weight) of *A. marmelos* to evaluate its protective efficacy against CdSO\(_4\) (5 mg/kg body weight, i.p. for 5 days) induced neurotoxicity by estimating various biochemical and histopathological parameters. Sub-lethal cadmium exposure caused a significant elevation in levels of lipid peroxidation, a noticeable decrease in enzymatic antioxidants (Catalase, Superoxide dismutase, Glutathione reductase, Glutathione –S-transferase) and non-enzymatic (reduced glutathione) antioxidants and marked structural alterations in neural tissue. *A. marmelos* pretreatment significantly (\(P \leq 0.01\)) reversed increased lipid peroxidation and attenuated the decreased enzymatic (\(P \leq 0.05\)) and non-enzymatic antioxidant markers (\(P \leq 0.05\)). Furthermore, pretreatment of *A. marmelos* also protected the histoarchitecture of neuronal tissue. Out of all the treatments, pretreatment was found to be more effective as compared to post-treatment, evidently seen in all biochemical assay (\(P \leq 0.05\), two-way ANOVA). Taken together, these results indicate that *Aegle marmelos* could be used as a potential prophylactic agent for combating adverse effects of cadmium in neural tissue.

**Keywords:** Brain, Metal toxicity, Reactive oxygen species

Cadmium (Cd) is a ubiquitous ecological toxicant and is released into the environment through mining and smelting\(^1\). Since Cd is poorly biodegradable in the environment, human exposure to Cd is constantly increasing through smoking and household dust as well as consumption of cadmium-contaminated water and food\(^2\). Previous studies have shown that Cd is a non-essential element, and could be potentially harmful to animals even at low doses\(^3\). In addition, Cd accumulation in the human body is harmful and may be linked to osteomalacia, infertility\(^4\), hepatotoxicity, nephrotoxicity\(^5\), cardiotoxicity, and neurotoxicity\(^6\).

Cadmium is able to induce neurotoxicity due to its high blood-brain barrier permeability with a wide spectrum of clinical implications including neurological disturbances, behavioral disturbances and changes in the normal neurochemistry of the brain\(^7\). There are several toxicological mechanisms by which cadmium may have an influence on brain development such as inducing oxidative stress in animals, reducing antioxidant levels in humans, and affecting the balance and degree of excitation-inhibition in synaptic neurotransmission\(^8\). Prenatal exposure to cadmium in rodents produces a delay in the sensor motor development and decreases the learning ability of offspring which may result in neurodevelopment defects\(^9\).

Cadmium has the potential to affect cellular antioxidant defenses, damage oxidative DNA repair systems, play a vital role in differentiation and apoptosis with the underlying cause being the heightened production of reactive oxygen species (ROS) which may act as a signaling molecule in apoptosis\(^7\). Oxidative stress is due to the disruption of the balance between the production of oxidant compounds and antioxidant defense system. There are several antioxidant defense enzymes, to antagonize oxidative damage; these include catalase for hydrolyzing hydrogen peroxide, superoxide dismutase for superoxide anions, GST for catalyzing the conjugation of GSH to xenobiotic substrates for their...
detoxification and glutathione reductase (GR), for converting GSSG to again GSH.

The brain tissue is highly susceptible to lipid peroxidation (LPO) because of its high rate of oxygen utilization, an abundant supply of polyunsaturated fatty acids, a deficient antioxidant defense. Cd can induce oxidative stress, cause inactivation of thiol groups in critical molecules, inhibit antioxidant defenses and DNA repair mechanisms by up-regulating the expression of nicotinamide adenine dinucleotide phosphate oxidase 2 (NADPH oxidase 2) and its associated proteins. Oxidative stress that results from the state of imbalance between the concentrations of ROS and the antioxidant defense mechanisms, may be connected to various pathological abnormalities e.g. neurodegenerative diseases, diabetes, cancer. In addition, it causes DNA damage and changes gene expression of pro- and anti-apoptotic genes leading to unregulated apoptosis. Consequently, the studies aimed at the abrogation of Cd toxicity, the generation of oxidative stress has been targeted and the beneficial effects of various ROS scavengers.

Neuroprotective effects of various plants available in literature have to highlight lacuna can be included, why this plant has been chosen for Neuroprotective effects should be included. Plants are natural resources of active components, used in the treatment of various neuronal disorders. Rhizome extract of Zingiber officinalis and flower buds extract of Capparis spinosa have a preventive effect on cognitive impairment and behavioral disabilities. Neuroprotective and anti-apoptotic efficacy of curcumin has also been found effective in an epileptic model of rats. Thus exploring the therapeutic potential of medicinal plants and their extracts may be a promising approach for the treatment of metal toxicity. Aegle marmelos (Lin.) (Rutaceae) commonly known as bael, is an annual herb and is widely distributed throughout India, Bangladesh, Nepal, Sri Lanka and Myanmar. Phytochemical investigations have demonstrated the occurrence of several active components including marmenol, marmin, marmelosin, marmelide, luvangetin, auraptene, psoralen, lloimperatorin, rutaretin, scoopletin, aegelin, marmelin, fagarine, anhydromarmelin, betulinc acid, marmesin, imperatorin, eugenol, marmelosin, luvangentin and auraptene in A. marmelos. The extract of the plant has been reported to possess important pharmacological properties including antioxidant, membrane stabilizing, anti-inflammatory, antipyretic, analgesic, and anti-proliferative effects. Considering these modulatory and protective properties of A. marmelos, it is noteworthy that administration of A. marmelos could be capable of ameliorating Cd-induced oxidative stress and histopathological alterations in mice brain. Thus, the present research work has been targeted to evaluate the neuroprotective efficacy of two doses of Aegle marmelos on Cd-induced neurotoxicity in mice by assessing oxidative stress, antioxidant enzymes, and histopathological alterations.

Materials and Methods

Animals

Experiments were carried on BALB/c female mice (weight 25-30 g), procured from Central Animal House, Panjab University and were acclimatized for 7 days, fed standard pellet diet (Ashirwad Industries, Punjab, Hindustan Unilever Limited, India), and given water ad libitum. After acclimatization, the animals were divided into 8 groups of 5 mice each and housed in polypropylene cages (7 × 10 inches). All animals used in the study were housed, cared and used experimentally in accordance with the National Institute of Health Guide for the Care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Institutional ethical committee Chandigarh, India. (IAEC/411 dated 11-09-2013).

Chemicals

The chemicals of the analytical grade used in this work were Cadmium sulfate; purchased from Loba Chemie Pvt. Ltd., Mumbai, India, 1-chloro-2, 4 dinitrobenzene (cDNB), reduced glutathione (GSH), oxidized glutathione (GSH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) from Sisco Research Laboratory Pvt. Ltd., Mumbai, India, and nitroblue tetrazolium (NBT), bovine serum albumin (BSA) from Central Drug House (P) Ltd., New Delhi, India.

Preparation of extract

Leaves of A. marmelos were collected during August and September from Botanical Garden Panjab University, Chandigarh, India, were identified by Mr. Gurudev (taxonomist) of Department of Botany, Panjab University and got voucher number 21044. Fresh leaves were dried at 37°C for 48 h, powdered
using an electric grinder, and stored. About 200 gm of dried powder was extracted with ethanol (800 mL) by continuous hot percolation, using Soxhlet apparatus. The extract was filtered and evaporated to dryness using a Rota evaporator at controlled temperature (78.5°C). The extract prepared was dissolved in peanut oil as it is a preferable solvent for lipophilic compounds, which reduces the application of synthetic compounds and related side effects.

Selection of Aegle marmelos

*Aegle marmelos* is one of the important plants with several medicinal and nutraceutical properties. In last few decades, *A. marmelos* has been extensively studied for its medicinal properties by advanced scientific techniques and a variety of bioactive compounds have been isolated from the different part of the plant and analysed pharmacologically. The medicinal properties of this plant represent it as a valuable source of medicinal compounds. For studying the modulatory role of *A. marmelos* against Cd toxicity, two doses of *A. marmelos* were selected i.e. 250 and 500 mg/kg body weight. The most effective treatment of *A. marmelos* dosage was also verified by studying the pre and post treatment with cadmium.

Experimental design

The animals were divided into eight groups (n=5). Group I: Control group was administered normal saline (*i.p.*); Group II: Cd-treated mice were administered 5 mg/kg body weight of Cd intraperitoneally for 5 days; Group III: *A. marmelos* treatment (250 mg/kg body weight), mice were administered 250 mg/kg body weight of the ethanol extract of *A. marmelos*; Group IV: Pre-*Aegle* treatment (250 mg/kg body weight), mice were given 250 mg/kg body weight of the ethanol extract of *A. marmelos* for first 10 days orally, and Cd was administered (5 mg/kg body weight) for next 5 days; Group V: Post-*Aegle* treatment (250 mg/kg body weight), mice were administered 5 mg/kg body weight of Cd (*i.p.*) for first 5 days and then were given 250 mg/kg body weight of the ethanol extract of *A. Marmelos* orally for next 10 days; Group VI: *Aegle* treatment (500 mg/kg body weight), mice were given 500 mg/kg body weight of the ethanol extract of *A. marmelos* for 10 days orally; Group VII: Pre-*Aegle* treatment (500 mg/kg body weight), mice were given 500 mg/kg body weight of the ethanol extract of *A. marmelos* for first 10 days orally and 5 mg/kg body weight Cd was administered for next 5 days and; Group VIII: Post-*Aegle* treatment (500 mg/kg body weight) – 5 mg/kg body weight Cd was administered intraperitoneally for first 5 days, then 500 mg/kg body weight of ethanol extract of *A. marmelos* was given orally. This study, for the first time, has evaluated the pre and post-treatment efficacy of two different doses of *A.marmelos*.

Mice were sacrificed and brain tissues was removed and perfused immediately with ice-cold saline (0.9% NaCl solution). Several biochemical and histopathological tests were performed.

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 the Harborne protocol for establishing the pharmacological profile of ethanolic extract of *Aegle*. 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 the presence of tannins, Molisch test for detection of carbohydrates, foam test for saponins and Salkowski test for detection of terpenoids.

Preparation of homogenate and post-mitochondrial supernatant (PMS)

10% homogenates were prepared in 50 mM Tris-HCl buffer (pH-7.4) using a homogenizer at 0-4°C (Remi Tissue homogenizer, 2001). These homogenates were used for biochemical measurement of lipid peroxidation (LPO) and reduced glutathione (GSH) using a spectrophotometer (Jenway UV-visible spectrophotometer, 2003). The homogenates were further centrifuged at 10000 rpm for 20 min and post-mitochondrial supernatants (PMS) were stored for further estimations.

Biochemical assays

Estimation of oxidative stress parameters

The lipid peroxidation and reduced glutathione contents in the homogenate were estimated using standard protocols.

Estimation of antioxidant enzyme activities

The enzymatic activity of catalase was measured in the PMS by the method of Luck (1971). Superoxide dismutase activity was measured in PMS by the method of Kono (1978). The activity of GST was assayed in the PMS by the method of Habig et al.
The activity of GR was measured in the PMS by the method of Horn (1971). Total protein in tissue homogenate and the post-mitochondrial supernatant was estimated using by Lowry’s method.

**Histopathological studies**
Histopathological alterations of brain tissue were studied by double staining method in haematoxylin and eosin stains and examined under light microscopy.

**Statistical analysis**
The data were expressed as mean ± SD for four animals in each group. The statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey’s honesty significant difference test. For comparing the effectiveness of lower and higher doses two-way analysis of variance (ANOVA) was performed followed by Tukey’s post-hoc test. Values were accepted as being statistically significant if the P-value was $P<0.05$, very significant when $P<0.001$ and extremely significant when $P<0.0001$.

**Results**

**Phytochemical analysis**
Preliminary qualitative estimation of ethanolic extract of *Aegle marmelos* (Table 1) revealed the presence of alkaloids, carbohydrates, phenols, tannins, flavonoids, and terpenoids. The pharmacological properties of *Aegle marmelos* are attributed to these phytochemicals.

**Lipid peroxidation and GSH**
Acute exposure of Cd significantly enhanced neuronal lipid peroxidation levels $[F(7, 24) \ df = 75.274, P \leq 0.0001]$ (df, F, P). Pre ($P \geq 0.05$) and post ($P \leq 0.05$) treatments of *Aegle marmelos* markedly decreased the lipid peroxidation as compared to control, $[F(7, 24) \ df = 75.274]$ (df, F, P) but pretreatments were found to be more effective than post treatments and brought down the levels of LPO $[F(3, 12) \ df = 7.732, P \leq 0.05]$ (df, F, P) (Fig. 1).

Cd intoxication significantly ($P \leq 0.001$) decreased GSH (59%) in the brain, while treatment with *A. marmelos* significantly elevated the levels of GSH as compared to control $[F(7, 24) \ df = 67.226, P \leq 0.0001]$ (df, F, P) (Fig. 2). Pretreatments of *A. marmelos* effectively elevated the levels of GSH as compare to post treatments $[F(3, 12) \ df = 8.135, P \leq 0.05$). Thus pre-treatments were found more effective than post treatments in lowering oxidative stress, although both lower and higher doses of pre-treatments were found equivalent in their preventive efficacy.

### Table 1 — Phytochemical evaluation of ethanolic extract of *Aegle marmelos*

<table>
<thead>
<tr>
<th>Phytochemical constituent</th>
<th>Test performed</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Wagner’s test</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Molisch test</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>FeCl$_3$ test</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>Gelatin’s test</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Ammonia test</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Froth test</td>
<td>−</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Chloroform test</td>
<td>+</td>
</tr>
</tbody>
</table>

[+: present & −: absent]

![Fig. 1 — Lipid peroxidation (LPO) (n moles/mg protein) in Control, Cd, and *A. marmelos* treated groups.](image1)

![Fig. 2 — Reduced glutathione (GSH) - µ moles/mg protein in Control, Cd, and *A. marmelos* treated groups. Values are shown as Mean ± S.D. (n=5).](image2)

[Levels of Significance: a = $P \leq 0.05$ (statistically significant); b = $P \leq 0.001$ (very statistically significant) and; c = $P < 0.0001$ (extremely statistically significant). Units: Lipid peroxidation (LPO)-n moles/mg protein and; Reduced glutathione (GSH)-µ moles/mg protein]
Antioxidant enzymes

Five days Cd treatments significantly decreased catalase \([F(7, 24) \text{ df } = 164.793, P \leq 0.0001]\) \((\text{df}, P)\) and SOD \([F(7, 24) \text{ df } = 37.144, P \leq 0.0001]\) \((\text{df}, F, P)\) levels in brain. *Aegle marmelos* pre-treatments were very effective in attenuating Cd mediated reduction in enzymatic antioxidants like catalase \([F(7, 24) \text{ df } = 164.793, P \leq 0.05]\) and SOD \([F(7, 24) \text{ df } = 37.144, P \leq 0.05]\) levels. As *A. marmelos* treatment significantly raised the levels of both catalase and SOD in cadmium-treated groups, approaching control levels in brain.

Cd also caused GSH mediated reduction in the levels of GST (91%) and GR (39%). Pre-treatment of *Aegle marmelos* to Cd-treated mice attempted to normalize the GST \([F(7, 24) \text{ df } = 1454.976, P \leq 0.001]\) \((\text{df}, F, P)\) and GR \([F(7, 24) \text{ df } = 215.391, P \leq 0.05]\) \((\text{df}, F, P)\) levels in brain tissue (Table 2). Post treatments were not able to effectively alleviate the reduced levels of GST \([F(7, 24) \text{ df } = 1454.976, P \leq 0.0001]\) and GR enzymes \([F(7, 24) \text{ df } = 215.391, P \leq 0.001]\) (Table 2). Furthermore, pretreatment of both the doses of *A. marmelos* were more effective as compare to post treatments revealing a notable rise in the levels of GST \([F(3, 12) \text{ df } = 2801.033, P \leq 0.0001]\) \((\text{df}, F, P)\) and GR enzymes \([F(3, 12) \text{ df } = 31.380, P \leq 0.0001]\) \((\text{df}, F, P)\) in preventing Cd-induced toxicity.

Histopathology

Light microscopic examination exhibited normal histoaortic architecture of striatum, cerebellum, and cerebrum in control group. The cerebellum constituted the outermost molecular layer and inner granular layer. Deep in the granular layer was another region that stains lightly called the white matter (Fig. 3A). At the junction of molecular and granular layers were present large flask-shaped Purkinje cells. Each possesses numerous dendrites that arbores in the molecular layer (Fig. 3B). The mice exposed to Cd revealed separation and disorganization of molecular and granular layers (Fig. 3C). Excessive spongioysis in pia mater, disorganization of Purkinje cells, interstitial edema both molecular and granular layers were clearly evident in the cerebellum of Cd-treated mice brain (Fig. 3 C&D). Control mice revealed normal histoaortic architecture in the area of the striatum (Fig. 4A) and cerebrum exhibiting a normal distribution of neuronal cells in the striatum and well-organized pyramidal and fusiform cells in the cerebral cortex of cerebrum (Fig. 4B). The Cd-treated mice revealed congestion in the capillary, nuclear pyknosis, interstitial edema and enhanced fibrosis in the striatum (Fig. 4C). Cerebrum depicted hyperchromitization and swelling of pyramidal and fusiform cells whereas cerebral cortex showed enhanced spongioysis and edema (Fig. 4D).

Pretreatments of *A. marmelos* revealed the protective efficacy of the extract depicting the well-organized cellular structure and well-marked demarcation of the molecular and granular layers in the cerebellum (Fig. 5A&B). Both the pre-treatments exhibited more or less similar histoarchitecture as that of control whereas, slight edema was observed in Purkinje cell at few foci in the pretreatment with

---

Table 2 — Effect of acute Cd toxicity on antioxidant enzyme parameters and its modulation by *Aegle marmelos* (250 and 500 mg/kg body weight) in the brain

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAT</th>
<th>SOD</th>
<th>GST</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.991±0.530</td>
<td>17.942±0.422</td>
<td>1.674±0.07</td>
<td>62.066±2.471</td>
</tr>
<tr>
<td>Cd</td>
<td>13.801±0.368</td>
<td>9.194±0.270</td>
<td>0.153±0.03</td>
<td>38.683±1.963</td>
</tr>
<tr>
<td>Bael 250</td>
<td>37.848±0.837</td>
<td>18.962±0.291</td>
<td>1.770±0.098</td>
<td>63.604±0.974</td>
</tr>
<tr>
<td>Pre 250</td>
<td>29.918±0.808</td>
<td>14.928±0.246</td>
<td>1.156±0.041</td>
<td>50.927±0.564</td>
</tr>
<tr>
<td>Post 250</td>
<td>23.140±0.316</td>
<td>10.730±0.404</td>
<td>0.512±0.043</td>
<td>44.927±0.943</td>
</tr>
<tr>
<td>Bael 500</td>
<td>38.393±0.475</td>
<td>19.397±0.191</td>
<td>1.812±0.074</td>
<td>64.027±0.831</td>
</tr>
<tr>
<td>Pre 500</td>
<td>30.420±0.836</td>
<td>15.144±0.109</td>
<td>1.273±0.026</td>
<td>53.004±0.921</td>
</tr>
<tr>
<td>Post 500</td>
<td>25.965±0.647</td>
<td>10.269±0.015</td>
<td>0.529±0.038</td>
<td>46.723±1.762</td>
</tr>
</tbody>
</table>

[Values are expressed as mean ± SD; \(n = 5\). CAT is in units \(\mu\text{M H}_2\text{O}_2\) decomposed/min/mg protein, SOD is in Units/min/mg protein, GR is in units \(\mu\text{moles GSH adduct formed/min/mg protein}\). *A. marmelos* (250 & 500 mg/kg). Cd (5 mg/kg). Statistical significance: \(a = P \leq 0.05\) (statistically significant); \(b = P \leq 0.001\) (very statistically significant) and; \(c = P < 0.0001\) (extremely statistically significant)]
Fig. 3 — Light micrographs showing comparative account of control and cadmium groups in the brain. Control group A and B: the cerebellum with the outermost pia matter, middle molecular layer and then the granular layer. Deep in the granular layer is the white matter. Purkinje cells are present at the junction of two layers and basal cells in the molecular layer (10X and 40X); Cadmium group C and D: the cerebellum with excessive spongiosis, disorganisation of Purkinje cells, interstitial edema (10X and 40X). [Mol, molecular layer; Gr, granular layer; WM, white matter; Pia, pia matter; Pkj, Purkinje cells and; BC, basal cells]

Fig. 4 — Light micrographs showing comparative account of control and cadmium groups in the brain. Control group A: Area of Striatum with normal distribution and structure of neuronal cells; B: Depicting the cerebrum with pyramidal and fusiform cells embedded in the cerebral cortex (40X); Cadmium group C: Area of striatum showing congestion in the capillary, interstitial edema, nuclear pyknosis; D: Area of cerebrum depicting hyperchromatisation and swelling of the pyramidal cells, spongiosis, and edema (40X). [Mol, molecular layer; Gr, granular layer; WM, white matter; Pia, pia matter; Pkj, Purkinje cells; BC, basal cells; NN, neuronal cells; PC, pyramidal cells; FC, fusiform cells; Pk, nuclear pyknosis and; IE, interstitial edema]

Fig. 5 — Light micrographs showing comparative account of the protective and modulatory effect of pre and post-treatments of A. marmelos (250 and 500 mg/kg body weight) in the area of the cerebellum in the brain. Pre 250 & 500 group A & B: Showing molecular layer and granular layer with well-marked Purkinje cells and basal cells having slight edema (40X); Post 250 & 500 group C: Depicting disorganized Purkinje cells, basal cells, and granular layer and; group D: Disorganised molecular and granular layer of the cerebral cortex with structurally altered Purkinje and basal cells (40X)

Fig. 6 — Light micrographs showing comparative account of the protective and modulatory effect of pre and post-treatments of A. marmelos (250 mg and 500mg/kg body weight) in the area of the striatum of the brain. Pre 250 & 500 group A & B: Normal distribution and structure of neuronal cells with slight capillary congestion (40X); Post 250 & 500 group C: Depicting capillary congestion, slight edema, and destroyed fibres and; D: showing slight interstitial edema, capillary congestion, nuclear pyknosis of the neuronal cells (40X)

250 mg/kg body weight of A. marmelos. Post-treatment of A. marmelos was not effective in
reverting Cd-induced structural alterations as depicted by marked separation of molecular and granular layers and disorganized Purkinje cells (Fig. 5 C&D). Pretreatment of lower and higher doses of A. marmelos revealed well organized neuronal cells but few foci depicted slight capillary congestion (Fig. 6 A&B). Post-treatment of A. marmelos at both the doses were not found preventive against Cd-induced alterations in striatum and cerebellum. The area of striatum demonstrated the presence of capillary congestion and nuclear pyknosis of neuronal cells at multiple foci (Fig. 6C&D). Pretreatment of a lower dose of A. marmelos revealed well-organized pyramidal and fusiform cells in the cerebrum, whereas slightly shrunk pyramidal and hyperchromatised cells were seen at few foci (Fig. 7A). Pretreatment of a higher dose of A. marmelos exhibited more or less normal histoarchitecture of cerebrum with the typical structure of pyramidal and fusiform cells, embedded in the cerebral cortex, while few foci depicted slight hyperchromatisation of pyramidal cells (Fig. 7B). Post-treatment of both the doses of A. marmelos were not found effective in curtailing noxious effect of Cd as these groups revealed interstitial edema, spongiosis, capillary congestion, shrunk pyramidal and fusiform cells in the cerebrum (Fig. 7 C&D). Thus, pre-treatment of both the doses has preventive efficacy against Cd-induced oxidative stress mediated structural changes in the neuronal tissue, while post treatments failed to ameliorate histopathological alterations induced by Cd.

Discussion

Amelioration of oxidative stress parameters by Aegle marmelos
The present study evaluated the ameliorative potential of ethanol leaf extract of A. marmelos against Cd-induced neural toxicity. Cd is a hazardous environmental and occupational pollutant which accumulates in an organism over its lifetime. Cd induces neurotoxic effects by raising lipid peroxidation which alters the integrity and permeability of the vascular endothelium. It thus penetrates the blood-brain barrier and gets accumulated in the brain causing events like oxidative damage an imbalance in pro- and antioxidants and histopathological alterations. In the present study, a significant elevation in MDA levels clearly indicated enhanced lipid peroxidation following short-term Cd administration. Cd might have enhanced lipid peroxidation in the brain by provoking free radical generation leading to membrane instability and altered the efficacy of blood-brain barrier. Plant products are known to exert their protective effects by scavenging free radicals; hence modulating heavy metal-induced oxidative stress and enriching antioxidant defense system. In the present study, A. marmelos markedly reduced Cd mediated lipid peroxidation, thus it could be targeted against Cd-induced neurotoxicity. Moreover, pretreatment with A. marmelos was found to be more effective than post-treatment in curtailing Cd-induced oxidative stress. The prevention of Cd-induced oxidative stress by A. marmelos could be attributed to its antioxidant, ion quenching and membrane stabilizing properties.

Cellular redox potential is largely determined by GSH content and accounts for about 90% of the intracellular non-protein thiol content. The most probable reason for the observed decline (59%) in GSH content could be the binding of divalent metal ions (Cd$^{2+}$) with -SH group of GSH thus resulting in the formation of disulfide compound GSSG (oxidized form). GSH depletion lowers the GSH: GSSG ratio causing the production of free radicals. In the present study, animals subjected to Cd exposure showed a
significant decrease \[ F(7, 24) \text{ df} = 67.226, P \leq 0.0001 \]
in neural glutathione levels with a substantial increase in
levels of lipid oxidation products suggesting that
 glutathione depletion may be a primary reason for
increased levels of oxidative damage. However, oral
administration of \textit{A. marmelos} for 10 days prior to
Cd exposure preserved GSH levels. This protective
efficacy of \textit{A. marmelos} could be due to its free radical
scavenging and anti-peroxidation activities that might
inhibit SH-group oxidation and maintain the cellular
redox balance.

**Enrichment of cellular antioxidant status by \textit{Aegle marmelos}**
An antioxidant status is a useful tool in estimating
the risk of oxidative damage induced by heavy metals.
The brain has relatively low levels of enzymatic and
non-enzymatic antioxidants as compared to lung, liver
and other organs but high amounts of peroxidizable
unsaturated lipids, rendering it more vulnerable to
oxidative stress as compared to other tissues. It was
also demonstrated that Cd-induced changes in
microvascular endothelial cell viability, glutathione
levels, and catalase enzyme activity by producing
ROS\textsuperscript{37}. Enhanced free radical production leads to
proteins and DNA injury, cellular inflammation,
tissue damage and subsequent cellular apoptosis due
to increased expression of stress proteins\textsuperscript{38}. SOD and
CAT are the major enzymes that play important role
in eliminating ROS formed during bioactivation of
xenobiotics and are widely distributed in all the
tissues. SOD scavenge the superoxide radicals
whereas CAT catalyzes the breakdown of hydrogen
peroxide produced in the cells, due to various
enzymatic and non-enzymatic pathways. In the
present study, the activities of SOD (62%) and CAT
(48%) were lowered significantly in neural tissue of
Cd-treated mice. Plausibly the enhanced levels of
lipid peroxidation and free radical generation by Cd
causd the observed decrease in SOD and CAT
activities. Other reports have also suggested that
enhanced LPO is a consequence of decreased SOD
and CAT activities\textsuperscript{39}. Another line of evidence
suggested the direct interaction of Cd with the
enzymes, rendering them inactive and decreasing the
bioavailability of these enzymes\textsuperscript{40}. Thus enhanced
LPO and decreased levels of antioxidants form a
vicious circle of events and each is a consequence of
the other.

Assessment of the toxic response syndrome is
mainly based on detoxification pathways that
primarily depend on GSH and GST levels. Reduced
activity of GST could decrease the ability of the
organism to inactivate the reactive metabolites. In the
present study, 91% decline was observed in neural
GST levels in Cd-treated group. Possibly the over-
production of ROS caused by Cd could be associated
with the depletion of GSH and GST levels. A strong
correlation is suggested between the bioavailability of
GSH and GST\textsuperscript{40}. Thus, decreased cellular GSH levels
and GST activity render cells more susceptible to Cd
metal ions. Glutathione reductase is known to reduce
the GSSG formed by peroxides to regenerate GSH.
GR utilizes the NADPH and maintains the GSH in a
reduced form. A statistically significant decline in the
levels of GR (39%) was seen in the brain. These
observations are in agreement with further studies
which mentioned a decline in the activity of GR along
with decreased levels of GSH in Cd-treated rats\textsuperscript{5}.
Previously we have also reported Cd mediated
elevation in lipid peroxidation and decreased levels of
antioxidants in mice kidney, spleen, and liver due to
overproduction of ROS\textsuperscript{41,42}. In the present study,
\textit{A. marmelos} pretreatment (250 and 500mg/kg body
weight) effectively normalized the levels of SOD and
CAT in Cd-treated groups. Prophylactic \textit{A. marmelos}
treatment protected the levels of GST and GR in
Cd-treated groups. Post-treatment was not very
effective in reducing Cd-induced oxidative stress
probably due to intensive biochemical and structural
damage caused by Cd.

On the basis of present results, the prevention of
Cd-induced reduction in endogenous antioxidant
enzymes could be attributed to the strong antioxidant
properties of \textit{A. marmelos}. Its leaves are a potential
source of antioxidants as it contains several
phyto-components like \( \beta \)-carotene, glutathione,
\( \alpha \)-tocopherol, ascorbic acid, total polyphenols and
flavonoids\textsuperscript{20}. These antioxidants have varied abilities
like free radical scavenging, chain breaking, metal ion
quenching and electron donating properties. Being the
source of so many antioxidants, leaf extract of
\textit{A. marmelos} possibly could have reduced the ROS
load in the system and restored the biochemical
alterations induced by Cd.

**Preventive efficacy of \textit{A. marmelos} against Cd-induced
oxidative stress-mediated histopathological alterations**
The brain has a high rate of oxidative metabolic
activity, intensive production of ROS, relatively low
antioxidant capacity and low repair mechanism,
making it a most vulnerable site for Cd-induced
oxidative damage. Most plausibly, Cd-induced
enhanced the generation of free radicals, lipid peroxidation, membrane instability and low antioxidant capacity of the brain, formed the basis of observed histopathological alterations.

Cd administration in mice caused extensive histopathological alterations in the striatum, cerebral and cerebellar cortices including congestion of capillaries, interstitial edema, neuronal cell death, hyperchromatisation in specialized neuronal cells, pyknosis and fibrosis in some parts. These observations are supported by the findings of Mendez-Armenta et al. who also reported histopathological alterations in different parts of the brain following perinatal Cd exposure in developing rats. In the present study, pretreatments of Aegle marmelos impressively increased brain antioxidant status, and also preserved the histoarchitecture of the brain. It is suggested that A. marmelos, due to its free radical scavenging and metal ion quenching properties, blocked the LPO mediated structural damage. A. marmelos might have scavenged the enhanced oxidative stress thus preventing structural alterations. Notably both the pre-treatments (lower and higher doses) revealed almost equivalent degree of preventive efficacy, however, post-treatments were not found to be much effective in ameliorating Cd-induced histopathological alterations. Both the doses of pre-treatments were able to prevent the oxidative-stress-mediated structural alterations, but the post treatments of same doses were not able to cure already instigated changes. This could be plausibly due to cadmium generated massive oxidative insult and disruption of endogenous antioxidant enzyme system in the brain tissue. Thus, for future studies, higher doses of A. marmelos post-treatment are suggestive for analyzing its protective efficacy.

Taken together it can be concluded that pre-treatment of A. marmelos leaf extract significantly reverted Cd mediated oxidative stress via inhibiting LPO, normalizing proxidant and antioxidant imbalance, scavenging free radical generation (ROS) and restoring the normal histoarchitecture of the brain in BALB/c mice. Thus Aegle marmelos could be exploited as a potential prophylactic agent in curtailling Cd mediated neural injury.

References


