EVALUATION OF ANTIOXIDANT EFFECT OF CITRULLUS VULGARIS AGAINST CADMIUM-INDUCED NEUROTOXICITY IN MICE BRAIN

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ABSTRACT: Cadmium is a potent neurotoxic heavy metal, which induces oxidative stress and membrane disturbances in brain. Oxidative stress has been implicated in the pathology of number of neurodegenerative disorders including Alzheimer’s disease. The present study was aimed to evaluate the antioxidant effect of hexane extract of Citrullus vulgaris seed (CVSO) in the brain of cadmium (Cd) treated mice as an animal model of oxidative stress. The neuroprotective efficacy of CVSO against Cd was assessed by estimating the levels of lipid peroxidation, non-enzymatic antioxidant, enzymatic antioxidant status and acetylcholinesterase (AChE) activity in mice. CVSO was administered along with Cd for four weeks. The results showed that mice intoxicated with cadmium (5 mg/kg/day) for 4 weeks significantly reduced the AChE levels in brain, elevated the levels of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides along with the significant decrease in the levels of non-enzymatic antioxidants (GSH), enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) in the brain tissue. In conclusion, CVSO ameliorated cadmium induced brain damage by reducing oxidative stress and by enhancing AChE activity.

INTRODUCTION: Oxidative stress is a characteristic feature in a number of neurodegenerative disorders such as stroke, Parkinson’s disease and Alzheimer’s disease. Oxidative stress is created when excessive free radicals react with proteins, cell walls and DNA, causing damage to cell structures and thereby leading to degenerative diseases. The brain is particularly vulnerable to oxidative stress injury because of its high rate of oxidative metabolic activity, high content of polyunsaturated fatty acids, relatively low antioxidant capacity, the abundance of redox-active transition metal ions, and nonreplicating nature of its neuronal cells.

Cadmium (Cd) is a heavy metal that has received considerable concern environmentally and occupationally. The main source of exposure to the toxic and carcinogenic metal cadmium is basic foods like cereals and vegetables, which means that the exposure is lifelong. The increase in Cd pollution has become an important public health concern worldwide.

It is well known that long-term exposure to Cd causes various toxic effects in various organ systems such as cardiovascular, kidneys, liver, brain, lung, bones, immune, haemopoietic, endocrine and reproductive systems. Cadmium is a potent neurotoxic heavy metal, which induces oxidative stress and membrane disturbances in brain. Cadmium is able to induce neurotoxicity with a wide spectrum of clinical entities including neurological disturbances, changes in the normal neurochemistry of the brain, oxidative stress and membrane disturbances in brain. Currently, an extensive research has been done to evaluate the...
potential of natural antioxidants to overcome the effects of heavy metal induced toxicities. Antioxidants are becoming popular in combating oxidative stress related diseases. Antioxidants can counteract the decrease in ATPases activity and the increase in oxidative stress that are induced by cadmium.

_Citrullus vulgaris_ (Schrad), water melon, belongs to family cucurbitaceae cultivated throughout the earth. The watermelon fruit has deep green or yellow colored smooth thick exterior rind with gray or light green vertical stripes. Inside the fruit is pink, red or even yellow in color with small black seeds embedded in the middle third of the flesh. Generally, watermelon flesh is the main consumable portion; however, outer rind is also used in some parts of the world.

Watermelon with red flesh is a significant source of lycopene. Its seeds are cooling, diuretic and strengthening, aphrodisiac; seeds are reported also as demulcent, vermifuge and nutritive. Fruit contains carotene, lycopin, mannito, 20-40% of oil from seeds. Seeds are rich source of the enzyme urease. Juice contains citrulline to the extent of 0.17%.

In view of the antioxidant properties of _Citrullus vulgaris_, it was thought to be worthwhile to evaluate the beneficial effects on cadmium induced oxidative stress in brain. Therefore, the present study was designed to evaluate the neuroprotective efficacy of _Citrullus vulgaris_ on cadmium induced oxidative neurotoxicity in the brain of mice.

**MATERIAL AND METHODS:**

**Plant Material and Extraction:**
The seeds of _C. vulgaris_ were obtained from a local market of Kolhapur, Maharashtra (India), which were taxonomically identified and authenticated by Botanical Survey of India, Pune. A voucher specimen was submitted at Institute's herbarium department for future reference. The dried seeds were crushed using mechanical grinder and passed through sieve no. 40. The powder material (200 g) was extracted using n-hexane and the menstrum collected was concentrated till dry to obtain pale yellow coloured oil (Yield 58%).

**Preliminary Phytochemical Screening:**
The n-hexane extract was subjected to phytochemical tests for the presence of different constituents using standard methods.

**Experimental Animals:**
Albino mice weighing around 20-30 g were used in the present study. Animals were housed in polypropylene cages in groups of 5-6 animals per cage under laboratory conditions (alternating light and dark cycle of 12 h each). Animals had free access to food and water. Animals were acclimatized for a week before the commencement of the experiment in order to avoid any stress due to handling. Experiments were carried out between 0900 h and 1800 h. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

**Experimental Protocol:**

**Group 1:** Control mice received normal saline (Cd diluent) orally for four weeks.

**Group 2:** Mice orally received Cd as cadmium chloride in saline (5 mg/kg body weight) for four weeks.

**Group 3:** Mice orally received the cadmium as cadmium chloride (5 mg/kg body weight) in normal saline and administered orally with _Citrullus vulgaris_ seed oil (0.25 g/kg body weight) for four weeks. The _Citrullus vulgaris_ seed oil was administered 60 min before Cd administration.

**Group 4:** Mice orally received the cadmium as cadmium chloride (5 mg/kg body weight) in normal saline and administered orally with _Citrullus vulgaris_ seed oil (0.5 g/kg body weight) for four weeks. The _Citrullus vulgaris_ seed oil was administered 60 min before Cd administration.

**Group 5:** Mice orally received _Citrullus vulgaris_ seed oil (0.25 g/kg body weight) for four weeks for four weeks.

**Group 6:** Mice orally received _Citrullus vulgaris_ seed oil (0.5 g/kg body weight) for four weeks for four weeks. The animals were maintained in their respective groups for four weeks with normal food and fluid intake. Daily weights of the mice were
taken during the experiment. At the end of the experimental period, the animals were anesthetized using ether and sacrificed by cervical decapitation. At the end of the experiment, mice were sacrificed; whole brain was removed, weighted, and used for estimation of lipid peroxidation (LPO), nonenzymatic, enzymatic antioxidants, protein content, and acetylcholinesterase (AChE) activity.

**Preparation of homogenate:**
Brain tissues were homogenized with 10 times (w/v) homogenizing buffer (pH 7.4 + 150 mM KCl). Ten percent homogenate used for LPO and GSH estimations. The remaining 10% homogenate was centrifuged at 9,000 rpm. The supernatant (S) obtained was used for SOD, CAT, GPx and protein estimations.

**Lipid peroxidation (LPO):**
LPO was estimated by the method described by Ohkawa. One milliliter of 10% homogenate was incubated at 37°C for 10 min. One milliliter of 10% chilled (w/v) trichloroacetic acid (TCA) was added to it and centrifuged at 2,500 rpm for 15 min at room temperature. One milliliter of 0.67% thiobarbituric acid (TBA) was added to 1 ml of S and kept in a boiling water bath for 10-15 min. After cooling, 1 ml of distilled water was added to it and absorbance was taken at 530 nm. The results were expressed as nmol MDA/h/g tissue.

**Nonenzymatic antioxidant: Reduced glutathione:**
GSH was estimated by the method described by Ellman. One milliliter of 5% TCA (w/v) was added to 1 ml of 10% homogenate. The suspension was left for 30 min and centrifuged at 2,500 rpm for 15 min. 0.5 ml of S was taken and 2.5 ml of 5, 5'-dithionitrobenzoic acid (DTNB) was added. The suspension was shaken thoroughly and read at 412 nm. The results were expressed as μmol/g tissue.

**Enzymatic antioxidants:**

**Superoxide dismutase:**
SOD was estimated by the method described by Kakkar et al. A total of 650 μl of sodium pyrophosphate buffer was added to 50 μl of brain S fraction; 50 μl phenazine methosulfate (PMS), 150 μl of nitroblue tetrazolium (NBT), and 100 μl nicotinamide adenine dinucleotide phosphate (NADPH) were added and the mixture vortexed thoroughly. The reaction mixture was incubated for 90 s and 500 μl glacial acetic acid was added to stop the reaction. Two milliliter of n-butanol was added, vortexed thoroughly. It was kept at room temperature for 10 min. Absorbance was measured at 560 nm. The results were expressed in terms of μmol/min/mg protein.

**Catalase:**
CAT was estimated by the method described by Sinha. One milliliter of phosphate buffered saline (PBS; 0.01 M, pH 7.0) and 0.4 ml water was added to 100 μl of S fraction. Reaction was started by adding 0.5 ml H2O2. The mixture was incubated at 37°C for 1 min. Reaction was stopped by adding 2 ml of dichromate: Acetic acid reagent and kept at boiling water bath for 15 min. The mixture was cooled, and absorbance was measured at 570 nm. CAT activity was calculated in terms of μmol/min/mg protein.

**Glutathione peroxidise:**
GPx was estimated by the method described by Rotruck. 0.4 ml tris-HCl buffer (pH 7.5, 0.1 M), 0.2 ml GSH, 0.1 ml sodium azide, 0.1 ml distilled water, 0.1 ml H2O2 and 0.1 ml of enzyme (S fraction) was mixed well and incubated at 37°C for 15 min. After incubation, 0.5 ml TCA was added and centrifuged. 0.5 ml of S was taken, and 2 ml Na2HPO4 2H2O and 0.5 ml Ellman's reagent was added. Absorbance was noted at 420 nm. The results were expressed as nmol/min/mg protein.

**AChE activity:**
AChE assay was estimated by Ellman. Brain was weighed and homogenized into 0.1 M phosphate buffer (pH 8.0). 0.4 ml aliquot of the homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 8.0) and 100 μl of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412 nm in a LKB spectrophotometer. When absorbance reaches a stable value, it was recorded as basal reading. Twenty microliter of substrate, that is, acetylthiocholine was added and change in absorbance was recorded for a period of 10 min at an interval of 2min. Change in the absorbance/minute was determined. The results were expressed as nmol/min/g tissue.
**Protein content:**
Protein content was estimated by the method of Lowry et al.\(^23\).

**Histopathological studies:**
The brain tissue was dissected out and fixed in 10% formalin. The paraffin sections were prepared and stained with haematoxylin and eosin and examined using light microscopy.

**Statistical analysis:**
The results of the studies were expressed as mean ± SEM (standard error of mean). The difference between the control and treated means were analyzed using one way analysis of variance (ANOVA) using the software Graph pad prism version no: 5.0. P-values <0.05 were considered as statistically significant. The Dunnett test for multiple comparisons of groups against control was performed to determine the significant differences among the groups.

**RESULTS:**

**Body weight and Brain weight:**
Mice exposed to different treatment groups showed no significant (\(P > 0.05\)) change in body weight and brain weight as compared to control (Table 1). In Cd treated mice, significantly (\(p<0.05\)) decrease in brain weight was observed when compared with control mice. Treatment with CVSO effectively attenuated brain weight, when compared with Cd treated mice. Administration of CVSO alone did not show any alterations in brain weight as compared to the normal control group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight (g)</th>
<th>Brain Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Control</td>
<td>184.3 ± 2.8</td>
<td>217.7 ± 2.5</td>
</tr>
<tr>
<td>Cadmium treated</td>
<td>217.5±3.44*</td>
<td>184.5±2.9*</td>
</tr>
<tr>
<td>Cadmium + CVSO (0.25)</td>
<td>168.7±2.74*</td>
<td>224.2±4.12*</td>
</tr>
<tr>
<td>Cadmium + CVSO (0.5)</td>
<td>179.7±2.7*</td>
<td>238.2±4.1*</td>
</tr>
<tr>
<td>CVSO (0.25)</td>
<td>181.0±2.4*</td>
<td>217.8±3.42*</td>
</tr>
<tr>
<td>CVSO (0.5)</td>
<td>182.0±1.14*</td>
<td>231.8±4.9*</td>
</tr>
</tbody>
</table>

The values represent as mean ± standard error of mean (SEM) for six animals in each group. *\(p<0.05\), **\(p<0.01\) as compared with control value

**Lipid peroxidation (LPO):**
The level of lipid peroxidation in the brain was estimated as the amount of malondialdehyde present in the different tissues. There is significant increase (\(p<0.05\)) in lipid peroxidation in group II as compared to the control group. However, administration of the CVSO restored the levels of lipid peroxidation in group III and IV animals compared to control group (Fig. 1).

**Nonenzymatic antioxidant: Reduced glutathione**
The level of non-enzymatic antioxidants (GSH) in the brain of control and experimental mice were shown in Table 2. The levels of GSH was significantly (\(p<0.05\)) decreased in the brain tissues of cadmium intoxicated mice when compared to control mice. Administration of CVSO in cadmium treated mice significantly (\(p<0.05\)) protected the depleted levels of GSH, in brain when compared with cadmium treated mice (Fig. 1).

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**FIG. 1: EFFECT OF CVSO ON LPO AND GSH IN CADMIUM-INDUCED NEUROTOXICITY IN MICE**
The values represent as mean ± standard error of mean (SEM) for six mice in each group. *\(p<0.05\), **\(p<0.01\), as compared with control value; LPO: Lipid peroxidation, GSH: Glutathione,
Enzymatic antioxidants:
The activities of enzymatic antioxidants (SOD, CAT, GPx) status in the brain of control and experimental mice were presented in Fig 2. A significant ($p<0.05$) decrease in the activities of SOD, CAT, GPx were observed in cadmium intoxicated mice when compared with control mice. Treatment with CVSO in cadmium intoxicated mice showed a significant ($p<0.05$) increase in the activities of antioxidant enzymes in the brain when compared with cadmium intoxicated mice.

Enzymatic antioxidants

![Graph showing enzymatic antioxidants activity](image)

**FIG. 2: EFFECT OF CVSO ON ENZYMATIC ANTIOXIDANT (SOD, CAT, GPx) IN CADMIUM-INDUCED NEUROTOXICITY IN MICE**

The values represent as mean ± standard error of mean (SEM) for six mice in each group. *$p<0.05$, **$p<0.01$, as compared with control value; CAT: Catalase, SOD: Superoxide dismutase, GPx: Glutathione peroxidase.

Acetylcholinesterase activity

The cadmium treatment showed a significant ($P<0.01$) decrease in brain AChE activity compared to control. The CVSO treated group showed significant dose dependent ($P<0.05$) increase in AChE activity as compared to control (Fig. 3).

Protein content: The protein content of brains of different groups is shown in Fig. 3.

![Graph showing protein estimation and acetylcholinesterase activity](image)

**FIG. 3: ACETYLCHOLINESTERASE LEVEL AND PROTEIN CONTENT IN CADMIUM-INDUCED NEUROTOXICITY IN MICE**

The values represent as mean ± standard error of the mean (SEM) for six mice in each group. *$p<0.05$, **$p<0.01$, as compared with control value; AChE: Acetylcholinesterase.
**Histopathological study:**
The results of histopathological study showed that there are no morphological changes of the brain in the normal animals. The cerebral cortex was found to be normal, no oedema and no astrocytic appeared. Hippocampus was found to be normal. In Cd treated group, marked cerebral oedema was observed. Treatment with CVSO effectively reduced the cerebral oedema. There was no astrocytic change appeared, thereby indicating CVSO prevented hippocampal damage (Fig. 4).

![Histopathological changes of hippocampal region of brain against cadmium induced neurotoxicity in brain](image)

**FIG 4: HISTOPATHOLOGICAL CHANGES OF HIPPOCAMPAL REGION OF BRAIN AGAINST CADMIUM INDUCED NEUROTOXICITY IN BRAIN**

**DISCUSSION:** Cadmium represents a dangerous environmental and industrial pollutant. Several reports suggest that oxidative stress is implicated in the toxicity of cadmium. In the present study the protective effect of Citrullus vulgaris seed oil on cadmium-induced oxidative damage in mice brain has been studied. It has been reported that Cd generates reactive oxygen species (ROS) causing oxidative damage in various tissues. Also, exposure to Cd via different routes causes increased lipid peroxidation (LPO) in membranes of erythrocytes and tissues, such as kidney, liver, brain, and testes where thiobarbituric acid reactive substances (TBARS) and hydroperoxides are used as indicators of oxidative damage. Intakes of Cd results in utilization of glutathione (GSH) and protein binding sulfhydryl groups, and consequently enhance the levels of free radicals, such as hydrogen peroxide, hydroxide, and superoxide anions. The brain is vital part of the organism functioning as coordinating and regulating system for body parts. Brain is considered highly vulnerable to oxidative stress than other organs of the body as it consumes high amount of oxygen, contains high amounts of polyunsaturated fatty acid (PUFA) and has low levels of antioxidant enzymes.

Antioxidants are becoming very popular in combating oxidative stress related diseases and as potential therapeutic agents in various ailments. Antioxidants can counteract the decrease in ATPases activity and the increase in oxidative stress that are induced by cadmium. An extensive research has been done to evaluate several natural antioxidants regarding their chemo preventive effects in heavy metal induced toxicities.

In this study, CVSO attenuated the cadmium induced malondialdehyde (MDA) formation and the decreased brain reduced glutathione (GSH) possibly due to its intrinsic antioxidant properties. CVSO may thus prevent peroxidative changes in brain. Also administration of cadmium resulted in pronounced increase of lipid peroxidation in brain of mice accompanied by a depletion of GSH. The result suggests that, cadmium toxicity can cause oxidative stress by an interaction with -SH groups of major intracellular defender glutathione and that...
lipid peroxidation is an early and sensitive consequence of acute cadmium exposure\textsuperscript{35–37}. The increased catalase enzyme activity in group II (treated with cadmium) may be due to increased free radicals, as a result of oxidative stress due to cadmium –toxicity\textsuperscript{38}. In group III & group IV catalase activity decreased significantly compared to group II, confirming the role of CVSO in decreasing the oxidative stress effect and hence liberation of free radicals and so the level of catalase enzyme. GPx is the enzyme which plays a primary role in minimizing oxidative damage. Cadmium exposure depleted GPx level in rat brain in this study. This reduction may be due to depleted level of GSH. In the present study, significant decrease in the GR level of brain was observed in the CVSO exposure group as compared to the control. The study of brain enzyme activities, such as AChE is a key enzyme in detecting the neurotoxic effect of certain heavy metals. Numerous studies have suggested that the free radicals production could at least in partly associated with the decreased activity of brain AChE\textsuperscript{39}. Further, there is significant decrease in AChE activity was observed in this study in cadmium treated group. However treatment with CVSO increased the acetylcholine level as compared to control group. Hence, it was confirmed that treatment with CVSO showed better protective activity against cadmium induced neurotoxicity in brain. Pretreatment with CVSO may have tuned up the antioxidant defense in the brain of rat and conferred protection against oxidative stress.

CONCLUSION: Hence it can be concluded that Citrullus vulgaris protected cadmium induced neurodegeneration attributed to its antioxidant properties and inhibition of AChE activity. This activity of Citrullus vulgaris can be further explored in neurodegenerative conditions for protecting brain damage by reducing oxidative stress and by enhancing AChE activity. However, further studies are recommended for the evaluation of the antioxidant properties of CVSO in different models of oxidative stress.

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