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RESEARCH ARTICLE

Oxidative stress and histomorphological changes in brain and effects of supplementation of *Linumusitatissimum* (flaxseed) and *Emblica officinalis* (amla) against lead toxicity in Wistar rats

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ABSTRACT

Lead (Pb) is a non-biodegradable, ubiquitous, environmental contaminant and is well known for toxicity in both human and animal. This study aimed to investigate the propensity of lead to induce neurotoxicity and changes in histological and oxidative stress in Wistar rats following a 45 days oral exposure and its possible attenuation by amla and flaxseed. Rats were assigned to 6 treatment groups; Group I served as vehicle control and they received distilled water, whereas rats in Group II @ 60 mg/kg bwt. lead acetate, Group III *Emblica officinalis* @ 100 mg/rat/day, Group IV *Linumusitatissimum* @ 300 mg/kg b.wt, Group V lead acetate @ 60 mg/kg b.wt + *Emblica officinalis* @ 100 mg/rat/day, and Group VI lead acetate @ 60 mg/kg b.wt + *Linumusitatissimum* @ 300 mg/kg b. wt. were administered orally. Antioxidant enzyme activities like Catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) were significantly decreased meanwhile, Thiobarbituric acid reactive substances (TBARS) were significantly increased in lead acetate-treated rats (Group-II). Upon light microscopical examination in Group II rats, severe degenerative changes were noticed in the brain. The levels of all the above parameters were significantly improved in the ameliorated group (Group V and VI). The present study revealed that the presence of amla and flaxseed could diminish the adverse effects of lead acetate as shown in the histological analysis of rat brain.

Keywords: Amla, antioxidant enzymes, brain, flaxseed, lead toxicity, wistar rats

INTRODUCTION

Lead is one of the oldest environmental contaminants and is considered to be toxic for virtually all organs of the body. Exposure to lead can occur from a multitude of sources such as soil, air, water and industrial pollutants. Inhalation (lung) and ingestion (GIT) is the major route of entry into the body. Absorbed lead mainly bind with erythrocytes in blood and from there it distributed to target organs, i.e. liver, brain, lungs, spleen, renal cortex, aorta, teeth and bones[1]. In view point of CNS toxicity, lead enters the brain and selectively deposited in the hippocampus and cortex, as well as in non-neuronal elements that are important in the maintenance of the blood brain barrier function. Studies have demonstrated that lead impairs learning, memory processes and cognitive functions both in animal models and human beings[2]. The neuropathlogical effects of lead include nervousness, anxiety and symptomatic encephalopathy. Oxidative stress induces cellular damage through production of Free radicals and inhibitions of enzymatic function are the major contributor in pathogenesis of lead induced toxicity[3].

In recent years, studies have shown the effect of several kinds of plants not only confers protection against heavy metal toxicity but it can also perform a therapeutical role against toxicity due to presence of several bioactive compound and antioxidant properties. Free radicals were generated during the pathogenesis processes induced by lead exposure, it was presumed that supplementation of antioxidants could be an alternative method for chelation therapy[4]. *Emblica officinalis* (Emblica) is also known as amla, is a rich source of tannins[5], polyphenols, flavones[6] and some bioactive substances, among them tannaoids are the active principles present in amla having vitamin C like properties which act as anti-inflammatory and potent antioxidant[7]. *Linumusitatissium* (Flaxseeds), commonly known as linseed is rich in lignan compound. The principle lignan found in flaxseed is Secoisolariciresinol di glucoside (SDG)[8]. Flaxseed also proven to have cardioprotective, chemoprotective, hepatoprotective and anti-inflamatory properties[9,10]. So this present experiment was taken to evaluate

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Table 1. Mean Brain TBARS values (nM of MDA / g of tissue) in rats of different experimental groups.

Weeks	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
2	602.8	1156.1	610.5	606.3	632.7	639.8
4	612.3	1185.6	584.3	594.4	625.3	632.5
6	598.4	1230.4	595.8	601.5	627.9	641.3
Mean ± SE	604.5 ± 4.1^{c}	1190.7 ± 21.59^{a}	596.86 ± 7.58^{c}	600.73 ± 3.45^{c}	628.63 ± 2.16^{bc}	637.86 ± 2.71^{b}

Mean values with different superscripts differ significantly (P < 0.05), ANOVA; S.E. - Standard error

the protective nature of flaxseed and amla in abating the toxic effect of lead in brain of wistar rats.

MATERIALS AND METHODS

Procurement of experimental animals, aqueous extract of *Emblica officinalis* and lead acetate

Female Wistar rats with body weight around 150 to 200 g (procured from Sri Venkateswara Agencies, Bangalore) were used for the present experiment. After one week of acclimatization the rats were grouped randomly and housed in standard polypropylene rat cages (three rats/cage) during the experimental period. They were maintained at 25^{O}C ± 10^{O}C and a 12:12 hour interval light and dark cycle during the 45 days of experimental period by maintaining standard laboratory hygienic conditions with *ad libitum* supply of laboratory animal feed and water. The permission of the institutional animal ethical committee (IAEC) was obtained earlier to commencement of the experiment.

The lead acetate was procured from the Qualigens Fine Chemicals Company, product code No. 27645 with 97% purity. Aqueous extract of Amla (*Emblica officinalis*) with product code C/SVU/EMOF-01 was procured from Chemiloids Company, Vijayawada, India.

Preparation of aqueous methanolic extract of *Linumusitatissimum* (Flaxseed)

Seeds of *Linumusitatissimum* (Flax seeds) were procured from a local herbal shop. After drying in shade, seeds were ground into powder form, which then used for the preparation of aqueous methanolic extract. Flaxseed lignans was prepared as per the method of Zhang *et al*. 2007[11]. Flaxseeds (800g) were ground using a grinder to attain a fine powder form. The powder form of flaxseeds was defatted by blending with hexane (1:6 w/v, 12 h) in room temperature. The defatted flaxseed powder was air dried for around 12 hours. 200 grams of this defatted powder was again blended with 1.2 Litres complex solution of ethanol and water (7:3 v/v) for 24 hours at ambient temperature (25^{O}C). The extract was filtered into flask, and then filtered product was concentrated at 50^{O}C using a Rotary evaporator (Buch, Model 462, Germany) @ 90 rpm. Light yellow colour syrup of flaxseed lignans extract was obtained.

Experimental design

A total 108 number of female adult Wistar albino rats were assigned to 6 groups randomly with 18 rats in each group. Group I served as vehicle control and they received distilled water, whereas animals in Group II, III, IV, V and VI received lead acetate @ 60 mg/kg b.wt., *Emblica officinalis* @ 100 mg/rat/day, *Linumusitatissimum* @ 300 mg/kg b.wt, lead acetate @ 60 mg/kg b.wt + *Emblica officinalis* @ 100 mg/rat/day, lead acetate @ 60 mg/kg b.wt + *Linumusitatissimum* @ 300 mg/kg b.wt. respectively for 45 days. From each group, six rats were sacrificed at fourteen-day intervals.

Histopathology

A detailed post-mortem examination was carried out on the sacrificed rats of all the trial groups. The brain was collected and preserved in 10% NBF for histopathological studies; fixed tissues were processed by standard paraffin embedding technique. 5-6 microns thick tissue sections were cut and sections were stained with Haematoxylin and Eosin stain (H&E)[12].

Lipid peroxidation (TBARS) assay and Antioxidant profile

For oxidative stress brain was collected and stored at -20^{O}C until use. Small tissue pieces of brain tissue were minced in separate containers and homogenized in 0.05 M ice cold phosphate buffer (pH 7.4) and make 10% homogenate by using a virtis homogenizer. 0.2 ml of

Table 2. Mean brain catalase activity (nM of H_2O_2 decomposed/min/mg of protein) in rats of different experimental groups.

Weeks	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
2	0.33	0.17	0.32	0.29	0.26	0.25
4	0.30	0.15	0.29	0.30	0.27	0.24
6	0.31	0.11	0.31	0.32	0.30	0.28
Mean ± SE	0.31 ± 0.008^{a}	0.14 ± 0.01^{c}	0.30 ± 0.008^{a}	0.30 ± 0.008^{a}	0.27 ± 0.01^{ab}	0.25 ± 0.01^{b}

Mean values with different superscripts differ significantly (P < 0.05), ANOVA; S.E. - Standard error

Table 3. Mean brain SOD activity (U/min/mg of protein) in rats of different experimental groups.

Weeks	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
2	15.2	7.2	15.1	15.3	14.7	14.4
4	15.7	6.7	15.4	15.2	14.9	14.5
6	15.5	5.9	15.6	15.5	15	14.8
Mean ± SE	15.46 ± 0.14^{a}	6.6 ± 0.37^{c}	15.36 ± 0.14^{a}	15.33 ± 0.08^{a}	14.86 ± 0.88^{ab}	14.56 ± 0.12^{b}

Mean values with different superscripts differ significantly ($P < 0.05$), ANOVA; S.E. - Standard error

the 10% homogenate was utilized for lipid peroxidation assay[13]. The residual part of homogenate then mixed with 10% trichloroacetic acid in 1:1 ratio, then centrifuged @ 5000 g at 4^{O}C for 10 min and the supernatant was collected and was used for valuation of reduced glutathione[14]. The residual portion of the homogenate was centrifuged for 60 min. at 15,000 g at 4^{O}C and the supernatant attained was used for the valuation of superoxide dismutase[15], catalase[16] and glutathione peroxidase[17] in brain of all rats in all groups.

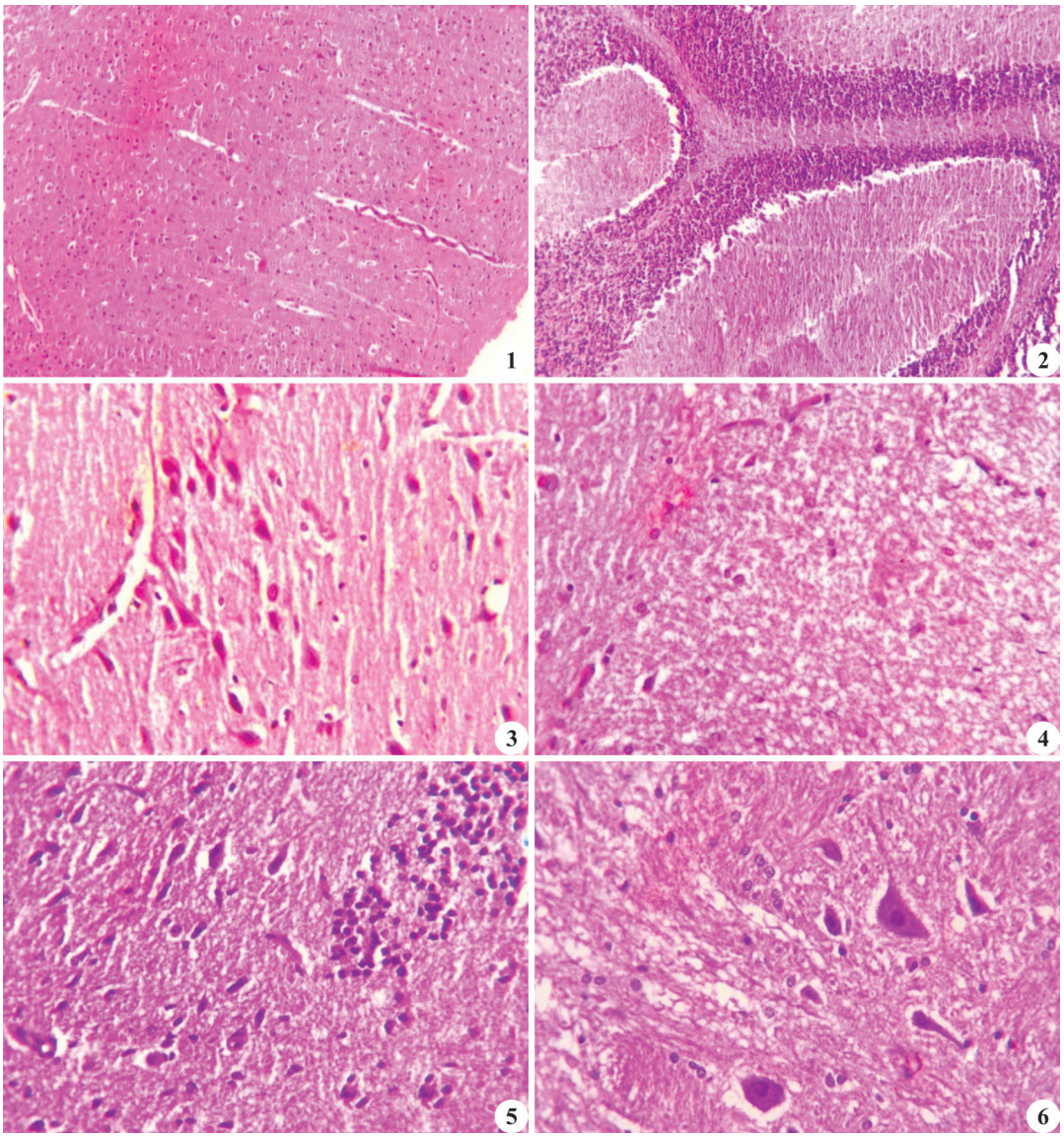

Fig. 1. Cerebrum: Group II: Section showing capillary proliferation (H&E X40); **Fig. 2.** Cerebellum: Group II: Section showing loss of a few Purkinje cells in Purkinje cell layer (H&E X100); **Fig. 3.** Cerebrum: Group II: Section showing shrinkage of neurons and capillary proliferation (H&E X400); **Fig. 4.** Cerebrum: Group II: Section showing demyelinating changes (H&E X400); **Fig. 5.** Brain: Group II: Section showing gliosis and shrinkage of nerve cells in cerebral cortex (H&E X400); **Fig. 6.** Cerebellum: Group II: Section showing nerve cell with central chromatolysis (H&E X400).

Table 4. Mean brain glutathione peroxidase activity (μ of glutathione utilized/min/mg of protein) in rats of different experimental groups.

Weeks	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI
2	25.63	18.28	24.85	24.95	21.26	20.69
4	24.92	13.42	25.37	25.12	21.64	20.91
6	25.47	7.34	26.12	25.53	22.19	21.29
Mean ± SE	25.34 ± 0.21^{a}	13.01 ± 3.1^{c}	25.44 ± 0.36^{a}	25.20 ± 0.17^{a}	21.69 ± 0.26^{ab}	20.96 ± 0.17^{b}

Mean values with different superscripts differ significantly (P < 0.05), ANOVA; S.E. - Standard error

Statistical analysis

The results were analysed statistically by performing one-way ANOVA[18].

Ethical approval

The ethics governing the use and conduct of experiments on laboratory animals were strictly observed and the experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC), Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India (Reference Number 281/go/ReBi/S/2000/CPCSEA/CVSC/TPTY/018/VPP/2016-17). Institutional Animal Ethical Committee (IAEC), Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, is affiliated under The Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA, India) with the Registration Number-281/go/ReBi/S/2000/CPCSEA.

RESULTS

Lipid peroxidation - Thiobarbituric acid reactive substances (TBARS)

Statistically significant (P< 0.05) increase in mean brain TBARS levels was noticed in lead acetate-treated rats (Group II) when compared to the control rats (Group I). The mean brain TBARS values from Group I to VI were 604.5, 1190.7, 596.86, 600.73, 628.63 and 637.86 (nM of MDA/g of tissue) respectively and are shown in Table 1. A significant decrease was observed in mean brain TBARS levels in Emblica ameliorated rats (Group V) and Flaxseed ameliorated rats (Group VI) when compared to the lead acetate-treated rats (Group II). There was no significant alteration in lipid peroxidation levels in

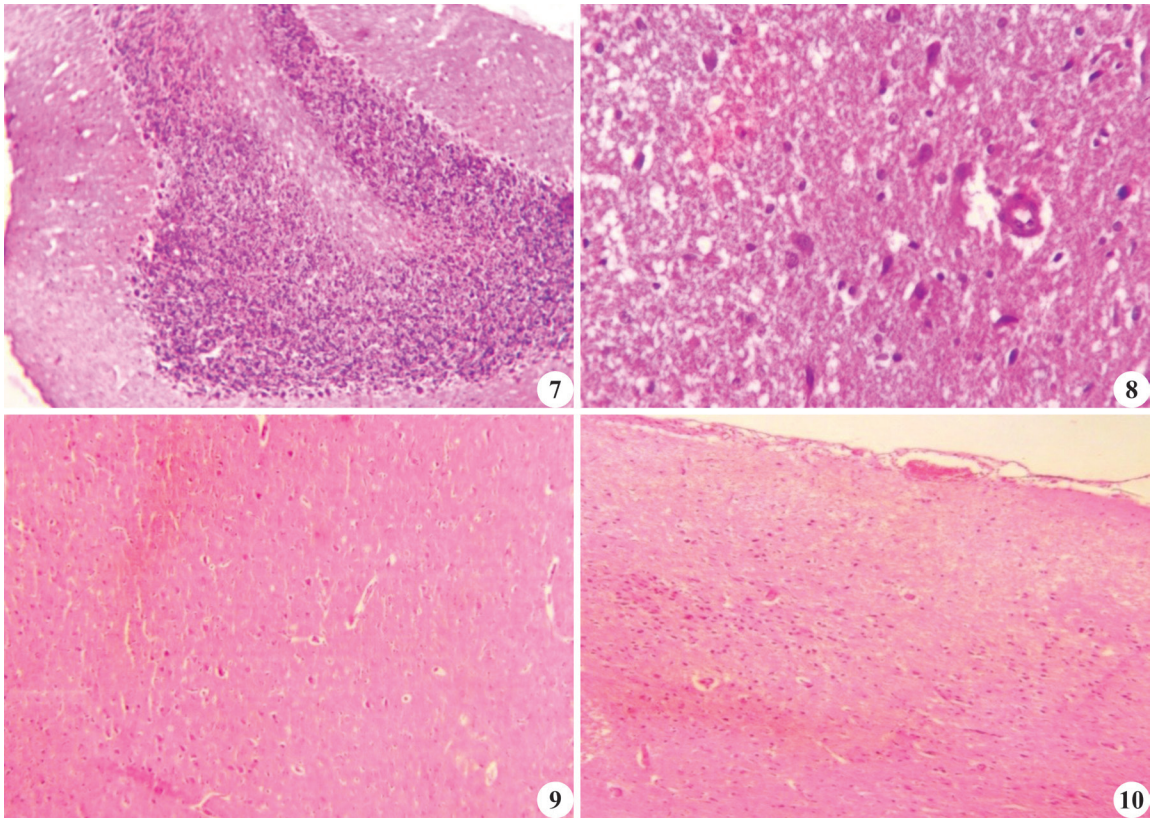

Fig. 7. Cerebellum: Group V: Section showing normal Purkinje cell layer (H&E x100); **Fig. 8.** Cerebrum: Group V: Section showing mild demyelinating changes (H&E x400); **Fig. 9.** Cerebrum: Group VI: Section showing mild capillary proliferation (H&E x40); **Fig. 10.** Cerebrum: Group VI: Section showing mild demyelinating changes and congested sub meningeal blood vessels (H&E x40).

brain of Emblica (Group III) and Flaxseed (Group IV) treated rats.

Antioxidant Profile

Catalase (CAT)

There was a significant ($P < 0.05$) decrease in mean brain catalase activity in lead acetate-treated rats (Group II) when compared to the control rats (Group I). The mean brain catalase activity in Group I to VI were 0.31, 0.14, 0.30, 0.30, 0.27 and 0.25 (nM of H_2O_2 decomposed/ min/mg of protein) respectively and are shown in Table 2. Statistically significant improvement was noticed in mean brain catalase activity in Emblica ameliorated rats (Group V) and Flaxseed ameliorated rats (Group VI) when compared to the lead acetate treated rats (Group II).

Superoxide dismutase (SOD)

In the present study statistically significant ($P<0.05$) decrease in mean brain SOD activity was noticed in lead acetate treated rats (Group II) when compared to the control rats (Group I). The mean brain SOD activity from Group I to VI were 15.46, 6.6, 15.36, 15.33, 14.86 and 14.56 (U/min/mg of protein) respectively and are shown in Table 3. A significant improvement was observed in mean brain SOD activity in Emblica ameliorated rats (Group V) and Flaxseed ameliorated rats (Group VI) when compared to the lead acetate treated rats (Group II).

Glutathione peroxidase (GPx)

There was a significant ($P < 0.05$) decrease in mean brain GP_X activity in lead acetate-treated rats (Group II) when compared to the control rats (Group I). The mean brain GP_X activity in Group I to VI were 25.34, 13.01, 25.44, 25.20, 21.69 and 20.96 (μ of glutathione utilized/ min/mg protein) respectively and presented in Table 4. Statistically significant improvement was noticed in mean brain GP_X activity in emblica ameliorated rats (Group V) and Flaxseed ameliorated rats (Group VI) when compared to the lead acetate treated rats (Group II).

There was no significant alteration in CAT, SOD and GPx level in brains of Emblica (Group III) and Flaxseed (Group IV) treated rats was observed when compared to control rats (Group I).

Histopathology

Light microscopic examination of the brain of lead-treated rats (Group II) revealed mild to moderate capillary proliferation (Fig. 1), and shrinkage of neurons in the cerebral cortex (Fig. 2). In the cerebellum, rounding, degeneration of Purkinje cells and loss of a few Purkinje cells (Fig. 3) were observed by the end of 2nd week. Later stages of experiment (4th week) in addition to the above changes, moderate capillary proliferation with shrinkage of neurons, demyelinating changes and spongiosis (Fig. 4) in cerebral cortex and gliosis were evident. By the end of 6th week, degenerative changes such as thickening of meninges with infiltration of cells, severe congestion of cerebral blood vessels and choroid plexus, severe demyelinating and spongiosis, gliosis and shrinkage of neuron in cerebral cortex (Fig. 5), Severe neuronal degeneration with central chromatolysis (Fig. 6) neurophagia and severe capillary proliferation in cerebral cortex and formation of nodule like structure along with MNCs infiltration in cerebral cortex were conspicuous.In Group V (*Emblica* ameliorated rats) histopathologically, brains revealed, similar lesions like Group II up to 4th week. Later the changes were gradually reduced in intensity like mild capillary proliferation, normal Purkinje cell layer (Fig. 7), mild degenerative changes (Fig. 8) and by the end of 6th week brain regained almost it normal structure. Flaxseed ameliorated rats (Group VI) similar lesions as described in Group II were noticed up to 4th weeks and later stages the changes were gradually reduced in its severity with mild capillary proliferation (Fig. 9), mild demyelinating changes in cerebral cortex and congestion of submeningeal blood vessels (Fig. 10) and almost near to normal appearance of cerebellum with all the three layers having normal arrangement were observed by the end of 6th week of experiment. There was no significant gross and microscopic alteration was observed in brain of *Emblica* (Group III) and Flaxseed (Group IV) treated rats compared to control group (I).

DISCUSSION

In the present study, significant increases in lipid peroxidation values in brain were noticed in lead acetate treated rats (Group II) compared to control rats (Group I). Similar observations were reported by earlier authors[19,20] and the possible explanation in increased lipid peroxidation level may be related to the role of GSH (Glutathione) which helps in the active excretion of lead through bile by binding to the thiol group of GSH, decrease in GSH levels lead to increase tissue burden of lead (Pb), which in turn results in lead induced oxidative stress and a consequent increase in lipid peroxidation[21]. Moreover, lead induces direct depletion of antioxidant enzymes[19]. These antioxidant enzymes like SOD, GPx and CAT take part in maintaining GSH homeostatis in tissues, so depletion of these antioxidant enzymes may contribute to the explanation of the mechanisms responsible for decrease in GSH concentration in tissues due to the exposure to this heavy metal[22]. Emblica ameliorated group (Group V) showed a significant decrease in lipid peroxidation activity and might be due to its antioxidant defence activity and lipid peroxidation inhibition activity of the ascorbic acid content of the Emblica[23]. Similarly, Flaxseed ameliorated rats (Group VI) also showed significant ($P<0.05$) decreased in lipid peroxidation activity when compared to lead treated groups and this might be due to action of lipid lowering effect and reduction of tissue lipid peroxide (MDA) of

flaxseed[24,25,26].

Significant decrease in the SOD, CAT and GPX levels was recorded in brains of lead acetate-treated rats (Group II) compared to control rats (Group I). These findings were in agreement with previous researchers[27,28,29] and the decrease in levels of antioxidant enzymes might be due to lead-induced generation of reactive oxygen species (ROS) (or) by reducing the antioxidant cell defense system by depleting glutathione (or) by inhibiting sulfhydryl dependent enzymes or by interfering with some essential metals like copper needed for antioxidant enzyme activities[30]. Emblica ameliorated group (Group V) showed significant increase in SOD, GPx and CAT levels and this might be due to antioxidant property of the tannoid rich fraction of Emblica includes Emblican in A and B[31]. Significant increase in SOD, GPx and CAT levels were noticed in Flaxseed treated rats (Group VI) and this might be due to presence of Omega-3 fatty acid and SDG in flaxseed which exhibit antioxidant properties and may help in maintaining the normal antioxidant enzymes levels by preventing lead induced cell damage[32].

Microscopically, brain revealed sub meningeal haemorrhages, mild to moderate capillary proliferation in cerebral cortex, rounding and loss of Purkinje cells in the cerebellum, severe congestion of cerebral blood vessels and choroid plexuses, shrinkage of neurons, demyelinating changes and spongiosis and focal loss of cerebral cortex with gliosis, severe neuronal degeneration with central chromatolysis, neurophagia, formation of nodule like structure along with MNCs infiltration in cerebral cortex throughout the experiment. These results were in accordance with[32,33] and the changes in present study might be due to neurotoxic effect of lead by altering certain membrane bound enzymes and might have led to oxidative stress as lead crosses the blood brain barrier quite readily[34,35]. The changes noticed in Emblica ameliorated group (Group V) were mild in intensity when compared to lead treated rats (Group II) and this might be associated with ascorbic acid content of Emblica that promotes elimination of reactive oxygen species (ROS), thereby helping to reduce oxidative stress generated by lead toxicity[30&36]. Microscopically, similar lesions were observed in brain of Flaxseed ameliorated groups (Group VI) but in mild form the reason might be due to the presence of Omega-3 fatty acid and SDG in flaxseed which exhibit antioxidant properties and helps in scavenging the reactive oxygen species (ROS) produced during lead exposure. Thus prevent the oxidative stress induced cell death in brain and explain the less severe degenerative changes in brain tissue of flaxseed ameliorated rats (Group VI)[31,32].

In conclusion, the findings from the present study indicate that lead (Pb) is having deleterious effect on brain and causes various degenerative changes and these changes were aggravated by increase in lipid peroxidation and decrease in antioxidant enzymes levels in brain. Co-administration of flaxseed oramla with lead found to be beneficial against lead induced toxicity as it is evident by improvement in various pathological manifestations in flaxseed and amla ameliorated group. So it can be assumed that supplementation of antioxidant agents like amla and flaxseed in veterinary and human medicine can be useful in heavy metal toxicity as a supportive therapy to minimize the harmful and toxic effects of different heavy metals like lead.

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