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Original Article

Flavonoid naringin inhibits microglial activation and exerts neuroprotection against deltamethrin induced neurotoxicity through Nrf2/ARE signaling in the cortex and hippocampus of rats.

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ABSTRACT

Microglial activation and oxidative stress contribute in the pathogenesis of neurotoxicity elicited by toxicant. This study was conducted to evaluate the potential neuro protective role of naringin on deltamethrin (DLM) induced neurotoxicity in rats. DLM-induced neuronal oxidative stress was evidenced by significantly increased level of lipid peroxidation and significantly reduced levels of antioxidant enzymes were observed in DLM-induced rats. DLM-induced neuronal damage was also evidenced by TEM analysis and histopathological amendments of DLM administered rat brain by H & E and Cresyl violet staining. DLM-induced microglial mediated neurotoxicity was proven by a significant overexpression of GFAP in DLM-induced rats. Thus, it is proposed that the DLM exerts its neurotoxic effects possibly via the mechanism of microgliosis and oxidative stress. In this present study naringin supplementation promoted the Nrf2 dissociation of Keap-1 and its nuclear translocation in the brain is likely to contribute to neuro protective effects of naringin evidenced by up-regulation of antioxidant enzymes, and attenuation of lipidperoxides formation. Naringin supplementation also controls the abnormalities of DLM-induced microglial activation by significantly attenuates the GFAP expression. Altogether, our data clearly indicate that an activation of Nrf2/ARE pathways in brain by naringin protects brain from DLM-induced neurotoxicity.

Keywords: Pyrethroids, deltamethrin, oxidative stress, free radicals, flavonoids, naringin, antioxidants, microgliosis, Nrf2.

INTRODUCTION

Microglia is the tenant innate immune cells of the brain, have been implicated as dynamic contributors to neuronal damage in neurodegenerative diseases like Parkinson's disease [1], Alzheimer's disease [2], amyotrophic lateral sclerosis [3], multiple sclerosis [4], Huntington's disease [5] in which the over activation and dys-regulation of microglia might result in devastating and progressive neurotoxic concerns [6]. Pyrethroid pesticides are the major class of insecticides, are commonly used in agriculture and urban places. Deltamethrin (DLM) is one of the most used type II neurotoxic pyrethroid insecticide. Human exposure to pyrethroids is well recognized to pregnant women, newborns and teenagers [7, 8].

DLM mode of action is thought to be mainly central nervous system in action and originate in higher nerve centers of the brain. Neurotoxicity of pyrethroids is characterized by ataxia, loss of coordination, hyper excitation, convulsion and paralysis [9], sustained opening of Na^+ channels, modulation in the release of neurotransmitters. These facts provide further evidence supporting the hypothesis that environmental and occupational exposure of pyrethroid pesticides produce specific damage to neuronal cells. Although numerous studies have been conducted, the molecular mechanism underlying DLM neurotoxicity is still not well understood. Thus, the present study was performed to find out the molecular mechanism of DLM-induced neurotoxicity in in-vivo. Naringin, a glycoside, is the major flavonoid found in the grapefruit, cocoa, tomatoes, beans, cherries,

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oregano and related citrus species [10]. Naringin have a broad spectrum of pharmacological and beneficial properties including metal chelating, antioxidant [11], anti-lipid peroxidation, anti-inflammatory, anti-apoptotic activities [12, 13] yet no mutagenic activity. Recently, Gopinath and Sudhandiran [14] have reported that naringin possess neuro protective activity against 3-nitropropionic acid-induced neurodegenerative disorders through modulating the oxidative stress and inflammatory responses. In addition, several studies have shown that naringin has neuro protective effects by the induction of brain-derived neurotrophic factor and vascular endothelial growth factor, and by the activation of anti-apoptotic pathways [15, 16]. Although these evidences suggest that naringin may be a potential natural compound involved in the prevention and treatment against neurodegenerative diseases [17, 18], it has not been clarified so far whether naringin has beneficial effects against microglial mediated neurotoxicity by activation of nuclear factor-erythroid 2-related factor-2 in the adult brain. Therefore, we have investigated whether daily supplementation of naringin can have ability of activate Nrf2 to up-regulate the expression of antioxidant genes via antioxidant response elements may provide a neuro protective role in a rat model of DLM-induced neurotoxicity.

MATERIALS AND METHODS

Chemicals: Deltamethrin 97.3 % EC (denotes 97.3 % of technical grade deltamethrin (W/W) was obtained from Bayer crop science limited, Mumbai, India. Naringin were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

Animal model: Adult male albino Wistar rats weighing 200-220g were obtained from Institute of Veterinary Preventive Medicine, Ranipet, Tamilnadu, India. Animals were quarantined and allowed to acclimatize for a week prior to experimentation. Water and feed were supplied ad libitum. Six animals were housed per cage and maintained on 12/12 hr/day and night cycle in a temperature and humidity controlled room. The experiments were conducted according to ethical norms approved (Approval no: IAEC/APCAS/01/2013/01) by the Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines.

Experimental design: Experimental rats were divided into four groups of six rats each as follows.
Group I - served control rats.
Group II - rats induced with deltamethrin dissolved in corn oil 12.8mg/kg BW orally (1/10 LD_{50}) for 21 days.
Group III - rats administered with deltamethrin 12.8mg/kg BW orally and simultaneously administered100mg/kg BW of naringin dissolved in water orally for 21 days.
Group IV - naringin alone.

After the experimental period, the rats were fasted overnight, anaesthetized with sodium pentothal and blood collected from jugular vein for serum isolation and sacrificed by cervical decapitation.

Brain tissue was dissected out by making midline incision. Then, a small incision from the caudal part of parietal bone and a firm cut in the anterior part of the frontal bone were made to remove the brain. Isolated brain sections were dissected on ice using large and small curved serrated forceps to obtain frontal cortex and hippocampus according to the method of [19]. The isolated brain cortex and hippocampi were homogenized using ice cold 50 mM Tris-HCl buffer at pH 7.4. The homogenate (10 % w/v) was then centrifuged at 13,000 rpm for 15 min, and the clear supernatant was recovered and stored at -80℃ and used for further analyses. Biochemical assays were conducted immediately after homogenizing the tissues.

Histological Studies

Hematoxylin and Eosin Staining: Excised brain tissues were isolated and post-fixed with 4 % paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C and embedded in paraffin wax. Coronal sections of 5 µm thicknesses were cut using microtome, and the sections were stained used for hematoxylin and eosin (H&E) staining solution [20].

Cresyl Violet Staining: Cresyl violet staining was performed to assess the extent of neuronal damage in cortical region [21]. Coronal brain sections were stained with 0.1 % (w/v) cresyl violet acetate (Nissl stain) for 5 min, dehydrated through graded concentrations of ethanol, and cleared in xylene. Stained sections were visualized under light microscope. The appearances of Nissl-stained dark neurons were analyzed in the cortex and CA1, CA 2, CA3 of hippocampus of control and experimental group of rats.

Biochemical assays: The activities of serum antioxidants enzymes such as superoxide dismutase (SOD) [22], catalase (CAT) [23], glutathione peroxidase (GPx) [24], glutathione reductase (GR) [25] were assayed. Lipid peroxidation level was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the method of [30]. Further the activities of antioxidant

enzymes were also determined by native-protein polyacrylamide gel electrophoresis (NATIVE-PAGE) of SOD [26], CAT [27], GPx [28] and GR activity [29].

Immunostaining of GFAP: Immunostaining was performed by the method of [31]. Cortex and hippocampus region of brain from experimental group of rats (5 µm) were cut and coated on clean slides. The sections were fixed overnight at 56°C. The sections were de-paraffinized in xylene and rehydrated using graded ethanol solutions. The slides were dipped in freshly prepared solution of 1 % H_2O_2 in methanol for 20 min to quench endogenous peroxidase activity. Slides were rinsed in PBS and incubated for 1 h in blocking solution (3 % BSA, 0.1 % Tween-20 in PBS) at room temperature. Sections were incubated with anti GFAP (1:250) monoclonal antibody, in blocking solution for 12 h at 4°C, re-equilibrated to room temperature and washed with PBS, incubated with horse radish peroxidase (HRP) antibody conjugates (1:2,500) in blocking solution without Tween-20 for 2 h at room temperature. Sections were washed with PBS and incubated with 0.2 % solution of diaminobenzidine (DAB) until desired stain intensity develops at room temperature followed by washing with distilled water. Sections were counterstained with hematoxylin and mounted with di-n-butylphthalate-polystyrene-xylene (DP_X).

Western Blot Analyses of GFAP and Nrf2: The cortex and hippocampus region of brain tissue were homogenized in ice-cold HEPES lysis buffer. The homogenates were centrifuged (10,000 rpm/ 15 min/4°C), and the protein content of the supernatant was assayed and diluted to give equal protein concentrations of 30 µg. Equal concentration of protein from each sample was prepared with 6x Laemmli SDS PAGE Sample loading buffer and boiled for 5 min prior to loading onto 12 % gels, resolved at a constant supply of voltage and transferred onto polyvinylidene fluoride PVDF membranes (Millipore Corp., Bedford, MA, USA).

To assess the expression of GFAP, membrane was incubated overnight at 4°C in 10 ml PBS/Tween (0.1 % v/v) containing 1 % BSA with the rabbit-anti-GFAP primary antibody (Santacruz Biotech, USA). Following incubation, immuno-reactive bands were detected by incubating secondary antibody with HRP conjugates. Protein-antibody complexes were visualized by the addition of DAB as a substrate.

In order to determine the expression of Nrf2, protein extraction from cytosolic and nuclear fractions were performed as follows. The brain tissue was homogenized in 1 ml of ice-cold hypotonic HEPES buffer A. To the homogenates 80 µl of 10% Nonidet P-40 (NP-40) solution was added, and the mixture was then centrifuged for 2 min at 14,000 x g. The supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed once with 500 µl of buffer A plus 40 µl of 10% NP-40, centrifuged, re-suspended in 200 µl of HEPES buffer B and centrifuged for 5 min at 14,800 x g. The supernatant containing nuclear proteins were collected [32]. Prior to western blot analysis, protein concentrations were determined using [33]. Samples (30 µg) were separated by denaturing SDS-PAGE on 12% gel and transferred electrophoretically to a PVDF membrane (Millipore, USA). The membrane was pre-blocked with 5% BSA and 0.1% Tween-20 in Tris-buffered saline at room temperature for 2 h. The membrane was then incubated overnight with the primary antibody (rabbit polyclonal Nrf2, (Santacruz Biotech, USA), at the concentration of 1:1000) at 4°C. Membrane was washed thrice for 15 min and incubated with the secondary HRP-linked antibody (Bangalore GeNei, Bangalore, India) for 2 h. Protein antibody complexes were detected by the addition of DAB as a substrate. Relative intensity of each band was quantified using an image densitometer. The densitometry results were standardized to the intensity of the β-actin band.

Electron microscopic studies: The cortex and hippocampus region of brain tissue from control and experimental group of rats were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 18 hr. Post fixation was done using 2% osmium tetroxide in 10mM sodium phosphate buffer (pH 7.4) and left over night. Then sections were dehydrated using series of ethanol solutions. The tissue was embedded in a mixture of (1:1) 1, 2-epoxy propane and Epon (Epikote resin). The tissue was then hardened using dodecyl succinic anhydride and Methyl Nadic anhydride. A diamine catalyst N-benzyl-N diethylamine was used for hardening. The specimen was kept in a block holder and placed in hot air oven at 60°C for 48 hrs. Ultrathin sections were cut, stained with uranyl acetate and lead nitrate, and collected on mesh grids coated with a thin Formvar film and viewed in a Philips EM201C transmission electron microscope.

Statistical method: All the results were expressed as mean ± SD for six rats in each group. All the grouped data were statistically evaluated with SPSS/12.0 software. Hypothesis testing method included one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test; $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effect of DLM and naringin on Lipid peroxidation: The changes in the levels of TBARS in serum, cortex and hippocampus of brain in control and experimental group of rats were depicted in Fig. 1. It was observed that DLM-induced rats exhibited significantly increased levels of TBARS level, when compared with control (group I) rats. Supplementation with naringin significantly reduced the levels of TBARS ($p<0.05$) when compared with DLM-induced rats. In naringin alone treated rats showed no significant change in TBARS levels when compared to control rats.

Effect of DLM and naringin on antioxidant enzymes: The activities of enzymic antioxidants (SOD, CAT, GPx, GST & GR) in cortex and hippocampus of brain in the control and experimental group of rats were summarized in Table 1. The activities of these enzymic antioxidants were significantly ($p<0.05$) reduced in DLM-induced rats as compared to the control rats. Naringin treated rats showed a significant increase in the activities of antioxidant enzymes ($p<0.05$) when compared to DLM-induced rats. In naringin alone treated rats showed no significant alterations of antioxidant enzyme activities, when compared to control rats. Effects of DLM and naringin on the activities of antioxidant enzymes were further examined by assessing the activity staining of antioxidant enzymes by native gel electrophoresis of SOD, CAT, GPx and GR and the same were depicted in Fig. 2. The expressions of antioxidant enzymes in the brain tissue were significantly decreased in DLM-induced rats (Lane 2) as compared to the control group (Lane 1) of rats, whereas the levels of antioxidant enzymes were significantly increased to normal level in DLM-induced with naringin treated rats (Lane 3) as compared to the DLM-induced rats. In naringin alone treated rats (Lane 4) shown no significant difference compared to control rats.

Effect of DLM and naringin on histological examination of rat brain: Effect of DLM and naringin on histopathological analysis of cortex and hippocampus region of rat brain in control and experimental group of rat by Hematoxylin and Eosin Staining were presented in Fig. 3. The cortex and hippocampus region of brain in control and naringin alone treated rats showed normal cellular architecture with intact cell membrane and regular appearance of cortex and hippocampus (Fig. 3A, D). In DLM-induced rats (Fig. 3B) shown abnormal cellular morphology accompanied by disruption of cell membrane, swelling of neuronal cells, shrinkage of cell nucleus, cellular infiltration, neuronal degeneration and focal gliosis were observed in cortex and severe oedema, vacuolization, pericellular and perivascular edema were observed in the hippocampus region. DLM-induced and naringin treated rats (Fig. 3C) showed marked reduction in number of damaged cells together with protected cellular morphology in cortex and hippocampus, resulted in modulation of the abnormalities in the cortex and hippocampus histopathology near to normal.

Effect of DLM and naringin on histopathological analysis of cortex and hippocampus region of rat brain in control and experimental group of rats by Cresyl violet staining (Nissl bodies) were shown in Fig. 4. Tissue sections of control rats showed normal neuronal architecture of the cortical region and pyramidal neurons of hippocampus (Fig. 4A). DLM-induced rats shown the intensely stained nissl substance evident form Nissl-stained dark neurons (NDNs) together with shrunken cytoplasm, damaged nuclei, amoeboid-like neurons is noted in cortex and hippocampus region, a hallmark of neurodegeneration in DLM-induced neurotoxicity. However, naringin treated group exhibited significantly reduced nissl bodies and marked reduction in neuronal cell loss in cortical region and pyramidal cells of hippocampus (Fig. 4C) was observed. Naringin alone administered rats shown normal architecture of cortex and hippocampus (Fig. 4D).

Effect of DLM and naringin on Glial fibrillary acidic protein (GFAP) expression: Effect of DLM and naringin on immunohistochemical analysis of GFAP expressions in cortex and hippocampus region of rat brain in control and experimental group of rats were shown in Fig. 5. Control and naringin alone treated group shows no immunostaining expressions of GFAP in cortex and hippocampus region (Fig. 5 A & D). In DLM-induced group exhibits increased expressions of GFAP (Fig. 5 B) demonstrated the presence of dark-brown immunoreactive astrocytes and gross hypertrophic processes with ubiquitinated rosenthal fiber-like structures in cortex and hippocampus region. DLM-induced with naringin treated group, naringin significantly reduced the number and the size of GFAP-immunoreactive astrocytes in frontal cortex and hippocampus region (Fig. 5 C).

Effect of DLM and naringin on immunoblot analysis of GFAP expression in cortex and hippocampus region of rat brain in control and experimental group of rats were shown in Fig. 6. DLM-induced rats showed significantly ($p<0.05$) increased expression of GFAP levels (Lane 2) in cortex (Fig. 6A) and hippocampus (Fig. 6B), relative to that of control (Lane 1), while naringin

supplementation significantly ($p<0.05$) reduced the levels of GFAP (Lane 3), during DLM-induced neurotoxicity. Control and drug control groups showed similar levels of GFAP expressions (Lane 1 & 4). Further, the levels of protein expression were quantified using Image-J (NIH software), relative to β-actin (Fig. 6C), and are graphically represented (Fig. 6D).

Effect of DLM and naringin Nrf2 gene expression: Immunoblot analyses of Nrf2 in cytosolic and nucleus fractions of the control and experimental group of rats are presented in Fig. 7. DLM-induced group of rats showed mild increase in nuclear and significant increase in cytosolic accumulation of Nrf2 (lane 2) when compared to the control rats (lane 1). Whereas naringin treatment resulted in significant increase in accumulation of Nrf2 in the nuclear fraction as compared (lane 3) with DLM-induced rats, with relative decrease in cytosol fraction indicating the activation of Nrf2. Naringin alone treatment not shown any significant expression of Nrf2 (lane 4)

Effect of DLM and naringin on Transmission electron microscopic analysis of Cortex, Hippocampus of control and experimental group of rats: Figure 8 shows the effects of DLM and naringin on the electron microscopic analysis of cortex, hippocampus of control and experimental group of rats. In control and naringin alone treated rat ultra-structural analysis of cerebral cortex showed a clear nucleus, heterochromatin along the nuclear membrane, mitochondria and golgi complex (Fig. 8 A & D). The ultra-structural analysis of DLM-induced rat cerebral cortex revealed condensed chromatin within the nucleus, vacuolation, discontinuity in cell membrane, distorted dendritic structure, discontinuity in nuclear membrane, degenerated neuronal cells, microglial cell, foamy inclusions, swollen golgi and swollen mitochondria (Fig. 8 B). DLM-induced and naringin administrated rats (Fig. 8 C) shows mild distortion in the mitochondrial structure, nuclear membrane, and dense cytoplasm. The ultrastructure of the hippocampus of control and naringin alone treated rats exhibited with a distinct nuclear envelope and nucleoli, clear rough endoplasmic reticulum and mitochondria in the cytoplasm with regular cristae (Fig. 8 E & H). In the hippocampus of DLM-induced rats, many neurons appeared shrunken with increased electron density of both cytoplasm and nucleus and swollen mitochondria with fewer cristae having less regular arrangement of nuclear envelope (Fig. 8 F). DLM-induced and naringin administrated rats (Fig. 8 G) shows clear nucleus and normal mitochondrial structure, nuclear membrane and dense cytoplasm.

DISCUSSION

Neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease have been a major focus of neuroscience research to understanding the molecular mechanism and pathophysiological features. DLM appears to have a direct effect on neurons and is often used as a model neurotoxicant. Flavonoid naringin possess potent antioxidant, anti-inflammatory and neuro protective properties. However, the mechanisms of DLM-induced neurotoxicity and neuro protective effect by naringin have remained ambiguous. In this present study, we investigated the potential molecular mechanisms underlying neuro protective effect of naringin on DLM-induced neurotoxicity.

Oxidative stress predominantly occurs through production of ROS and can damage lipids, proteins and DNA. ROS increase the initial attack on lipid membrane of the brain to cause LPO. MDA and TBARS is stable oxidation product of LPO and therefore can be used as an indirect measure of the cumulative LPO [34]. In this present study a significant increase in TBARS was observed in the serum, and brain tissues of DLM-induced rats, which may be due to increased free radical attack and oxidative stress. Increased TBARS level in the rats treated with DLM is in agreement with the findings of [35, 36] who reported that oxidative damage induced by DLM might be due to their lipophilic nature of DLM, whereby they could penetrate the cell membrane easily. Naringin is one of the flavonoid have been proven to have anti-lipid peroxidation activity [13]. In this present study supplementation of naringin significantly inhibited the DLM-induced LPO in serum, and brain tissue homogenate by decreased the levels of TBARS which may be resulting from the scavenging of free radicals and its antioxidant activity. Naringin has been reported to inhibit LPO in iron loaded $HepG_2$ cells, brain, kidney and irradiated liver [37, 38]. Similarly Naringin has also been reported to block the LPO induced by H_2O_2 [39].

The expression of antioxidant enzymes on pesticides toxicity reflects the activation of defense mechanisms in animals to counteract ROS-induced toxicity. Reduced activities of enzymic antioxidants during pesticide exposure are important indicators xenobiotic toxicity [40]. In this present study, significantly decrease in the antioxidants enzymes were observed in DLM-induced rats. The lowered enzyme levels reflects failure of the antioxidant defense mechanisms to overcome the incursion of ROS induced by DLM that leads to the accumulation of free radicals and facilitate augmentation of LPO, which in turn

increases the oxidative damage to the brain tissue [41, 42]. The present outcomes provided strong evidence that oxidative stress is involved in the pathogenesis of DLM-induced neurotoxicity. In normal conditions, the formation of ROS is neutralized by the endogenous antioxidants and always there is balance between the ROS and antioxidants. In this study, the naringin supplementation result in a dramatic increase in the antioxidant enzyme activities compared to the DLM-induced rats. Naringin has been reported to scavenge free radicals [43] superoxide scavenger and hydroperoxide counteragent [44], play an important role in regulating anti-oxidative capacities by increasing the SOD, CAT, and GPx activities by up-regulating the gene expression of antioxidant enzymes. It has also been reported that naringin activates Nrf2 (nuclear factor-erythroid 2-related factor-2) and induces phase II detoxifying and antioxidant genes in an in vivo model [14].

Neuro-histopathology is a major part of the study involved in toxicological and risk assessment of drugs and chemicals. The histological features of DLM-induced rat as observed in this study exhibited irregular damaged cells with condensed pyknotic nuclei, degenerated pyramidal neuron, degenerated neuroglial cells, disruption of cell membrane, shrinkage of cell nucleus together with swelling of neuronal cells, increased spaces with the vacuolation were noticed in the cortex and severe necrotic changes in neuro fibrillar network, degenerative changes in pyramidal neuronal cells, and vacuolated spaces were observed in hippocampus region clearly indicating that DLM is anatomic and biological toxin in the animal brain. This may be due to the toxic effects of DLM and its ability to bind structural proteins and enzymes in neurons. These histological changes could be attributed to increased oxidative stress elicited by DLM. There have been many experimental studies on animals showing that pyrethroids have toxic effects on the nervous system and histological alterations in brain tissue [45, 46]. However, several studies document that pharmacological treatment with naringin could significantly prevent premature ageing and delays the onset of various degenerative disorders. In DLM plus naringin treated rat cortex the cyto-architectural changes were remarkably more distinct, which include slight degeneration of pyramidal neuron, neuroglial cells and normal architecture of nerve fibers, hippocampus has a clear pyramidal neuronal cells, neurofibrillary network with clear cyto-architecture and inter-neuronal cells. Naringin ameliorated the histopathological alterations in DLM-induced rats due to its neuro protective effects. Prakash *et al*., [47] reports naringin as neuro protective against aluminum-induced neurotoxicity in rat's agreement with our results.

The neurodegeneration could be assessed by cresyl violet staining of cortex and hippocampal region which provides assessment of neuronal cell death and extent of pathological change of neurons. In nissl-staining analysis, we have observed that DLM-induced vacuolization, loss of pigmented neurons indicating extensive neuronal damage. The neurons appear to be smaller and shrunken along with formation of Nissl granules, suggestive of neuronal damage. DLM-induced neuronal damage may be attributed to higher susceptibility of free radicals and oxidative stress. Histological analyses reveal that DLM mediates damage to cortex and hippocampus which is in agreement with the previous report of Rui and Yongjian [48]. Naringin treatment ameliorates all the histological changes induced by DLM. These results demonstrate that naringin significantly reduced brain damage and improved functional outcome as observed in behavioral studies.

GFAP a marker for reactive gliosis, an increased expression of GFAP in immunostaining and immunoblot were observed in the DLM-induced rats, confirm the astrogliosis and microgliosis involved in the molecular mechanism of DLM-induced neurotoxicity. In this study, hypertrophied GFAP with rosenthal fiber-like structures indicates DLM-induced astrogliosis. A rise of GFAP expression is related to the development of pathology, such as AD or non-specific gliosis [49]. Little and O'Callagha [50] reported micro-anatomical analysis of pesticide exposure caused a selective loss of nerve cells in frontal cortex and hippocampus and the development of astrogliosis that represents a sign of sufferance of nervous tissue. Thus reactive gliosis cause increase expression of proinflammatory cytokines leads to neuronal injury thereby influencing learning and memory deficits. However administration of naringin attenuated GFAP expression in DLM-induced rats. The attenuated GFAP immunoreactivity during naringin treatment suggests that the flavonoid alleviated reactive gliosis in DLM-induced neurotoxicity. In this study, naringin suppressed the microglial and astroglial mediated neuronal damage in rats induced with DLM, which was consistent with the neuro protective effects of naringin reported by [14, 47].

Nrf2 mediated regulation of cellular antioxidant mechanism play an important role in neuro protection against neurodegenerative diseases. In addition, Nrf2 plays a broader role in the modulation of inflammatory responses in

neurodegenerative diseases [51]. In resting state, Nrf2 is sequestered in the cytoplasm in an inactive form, while in response to an oxidative challenge, Nrf2 translocate to the nucleus. Activation of Nrf2 turns to activate the expression of Nrf2-regulated antioxidant response elements (ARE) genes, which protects neuron against oxidative stress [52]. In this study, DLM-induced animals exhibited slight increase in Nrf2 activation due to the presence of ROS, which by itself has the capability to activate Nrf2. This result is in accordance with the previous study that mitochondria-derived ROS induce the activation of Nrf2 [53]. The maximal Nrf2 activation was observed in naringin-treated animals. Flavonoids are the most potent inducers of Nrf2, resulting in the transcription of cytoprotective genes [54]. Thus, the assenting neuro protective effect of naringin against DLM-induced neurodegeneration is through its potential antioxidant activity and is attributed to Nrf2 signaling cascade. Earlier reports shown naringin up-regulates the expressions of NQO-1, HO-1, GST-P1 and c-GCL at mRNA level. These genes were induced via the mechanism involving the binding of transcription factor Nrf2 to ARE of phase II genes [55]. The ability of Nrf2 to regulate the expressions of these protective genes is associated with its accumulation in nucleus [56]. Our data support this hypothesis, where we have shown the increased accumulation of Nrf2 in nucleus following naringin treatment, suggesting that naringin indeed promotes the transcription of key antioxidant and phase II genes by triggering the Nrf2 nuclear accumulation. Nrf2-mediated ARE genes contribute to cellular protection against oxidative stress induced by DLM and to potentiate antioxidant defense capacity in neuronal cells.

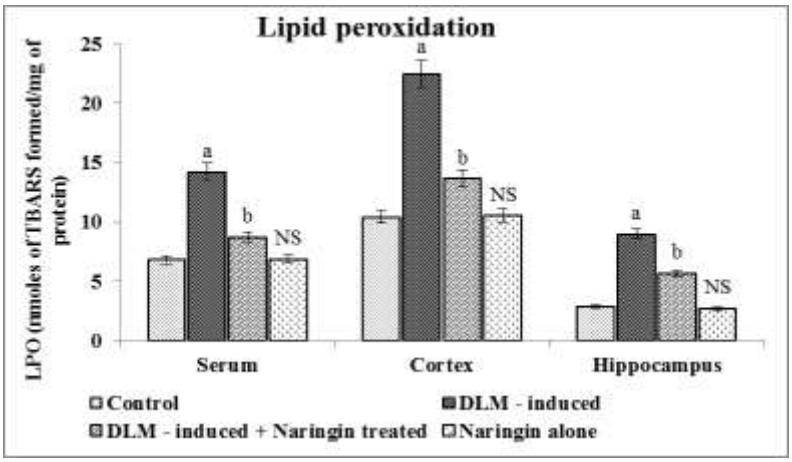
Ultra-structural analysis of DLM-induced cerebral cortex and hippocampus showed general swelling of neurons, and glia. Cellular swelling was represented by local cytoplasm swelling, dilated rough endothelial reticulum fragments and swollen organelles mainly mitochondria. DLM is known to accumulate within various parts of rat brain especially in cortex and hippocampus. Ultra-structural effects of DLM are likely related to excitotoxicity by free radical formation, lipid peroxidation, neuronal inflammation and activating microglia. Naringin supplementation significantly reversed the DLM-induced cerebral injury in cortex and hippocampus to rescues neurons from degeneration and cell death. The antioxidant and membrane protective effects of naringin might be the possible reason behind the decrease in mitochondrial swelling. Also naringin treatment can inhibit apoptosis and neuronal inflammation in the cortex and hippocampus, and reduce neuronal structural damage and maintain BBB integrity, thus protecting neurological functions following DLM-induced neurotoxicity. TEM analysis confirmed the neuro protective action of naringin on DLM-induced neuronal damages.

Conclusions

In conclusion, our findings demonstrate that deltamethrin (DLM) induces oxidative stress, microglial mediated neuronal damage in rat brain, might play an important role in DLM- induced neurotoxicity. Flavonoids naringin can inhibit the adverse effects of DLM-induced oxidative stress, protect the cellular environments from free radical damage by elevating the cellular antioxidant enzyme system. Together, from the results of TEM analysis and histopathological studies it was speculated that naringin exhibits neuro protective role in cortex and hippocampus of rat brain that was experimentally challenged with DLM- induced neurotoxicity. Augmented antioxidant defense system, attenuated reactive gliosis were supposed to be responsible for its neuro protective action of naringin against DLM- induced neurotoxicity. This work for the first time, demonstrate the role of naringin in the activation of Nrf2 signaling cascades that are inhibit in the progression of neuronal damage in DLM- induced neurotoxicity.

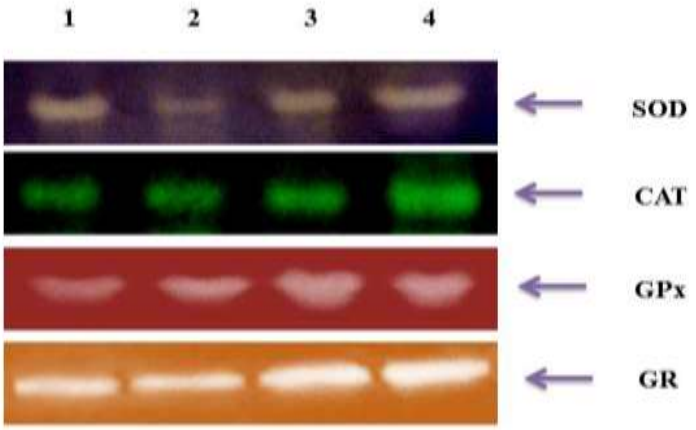
Figure

Fig. 1. Effect of DLM and naringin on lipid peroxidation in control and experimental group of rats.



Results are expressed as mean ± SD for 6 different sets of experiments. Hypothesis testing method included two-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Values are considered significantly different at $P < 0.05$ with post-hoc LSD test. Statistically significant variations are compared as follows: [a] DLM-induced vs control. [b] DLM- induced + naringin treated vs DLM-induced. [a, b] indicates $p < 0.05$ and [NS] indicates Non-significant.

Fig. 2 - Effect of DLM and naringin on the activities of antioxidant enzymes (SOD, CAT, GPx and GR) by Native gel electrophoretic pattern of the brain tissue homogenate of control and experimental group of rats.



Native gel electrophoretic pattern of SOD, CAT, GPx and GR

Lane - 1 Control; Lane - 2 DLM induced; Lane - 3 DLM induced + Naringin treated; Lane - 4 Naringin alone.

Fig. 3- Effect of DLM and naringin on histopathological analysis of cortex and hippocampus region of rat brain in control and experimental group of rats by Hematoxylin and Eosin staining (x 20 magnification).

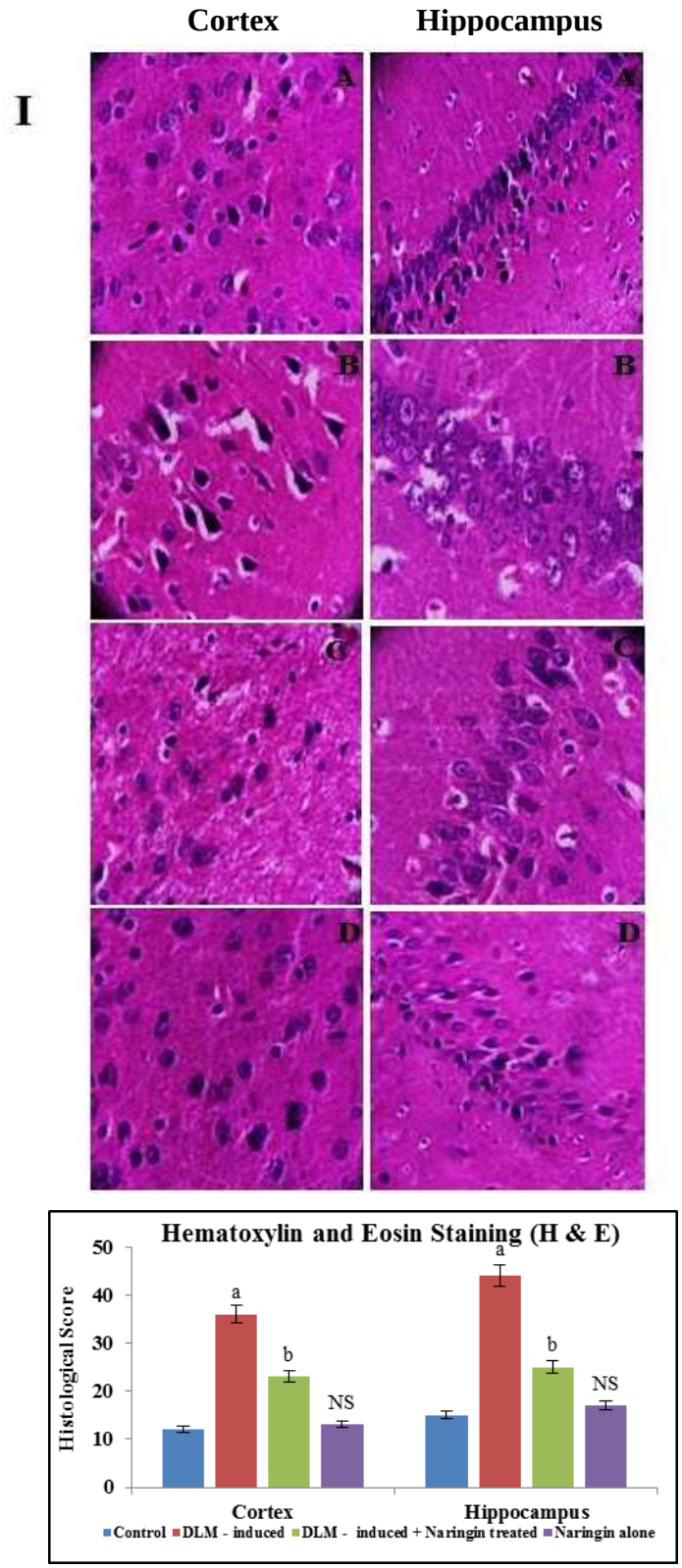


Fig. 3- The representative micrographs of hematoxylin and eosin staining (H & E) (A-D) in cortex and hippocampus region of rat brain in control and experimental group of rats (n=3 rats/group). I. H&E staining of cortex and hippocampus region representative micrographs of histologic sections were shown at x 20 magnifications. To evaluate cellular damage, sections of control and experimental group of rats were evaluated by three independent and blinded observers. Total number of degenerating neurons with pyknotic and condensed nuclear morphology were evaluated. A) Control rats shown normal architecture with intact cell membrane and regular appearance of cortex and hippocampus region. B) DLM-induced rats shown abnormal cellular morphology accompanied by cellular infiltration, neuronal degeneration and focal gliosis were observed in cortex and severe oedema, vacuolization, pericellular and perivascular edema were observed hippocampus region. C) DLM-induced with naringin treated rats showing protected cellular morphology in cortex and hippocampus region. D) Naringin alone administered rats showing normal architecture in cortex and hippocampus region. II. Quantitative analysis of histological score of damaged cells.

Fig. 4- Effect of DLM and naringin on histopathological analysis of cortex and hippocampus region of rat brain in control and experimental group of rats by Cresyl violet (Nissl bodies) staining (x 20 magnification).

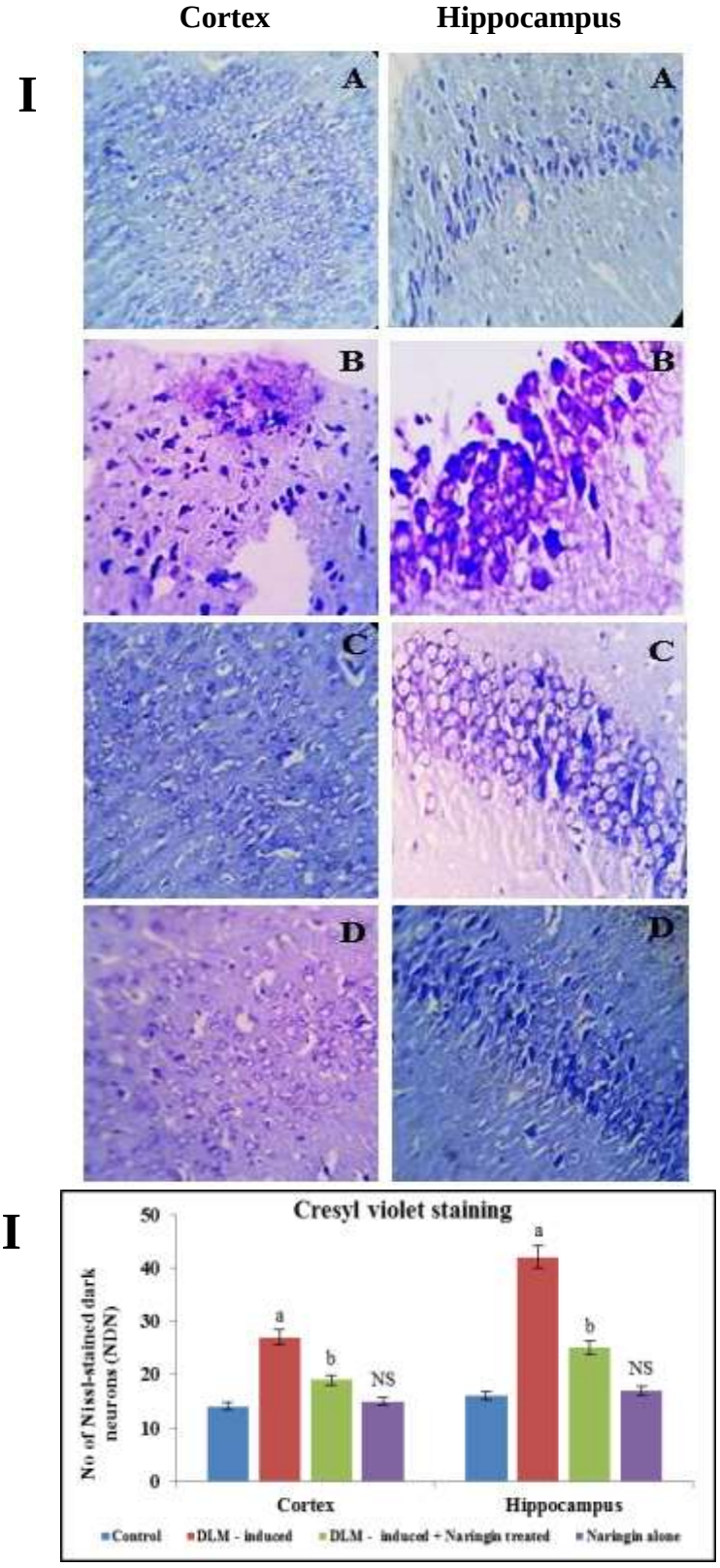


Fig. 4 - The representative micrographs of Cresyl violet staining (Nissl bodies) (A-D) in cortex and hippocampus region of rat brain of control and experimental group of rats (n = 3 rats/group). I. Representative micrographs of histologic sections Cresyl violet staining were shown at x 20 magnifications. To evaluate nissl substance, sections of control and experimental groups were evaluated by three independent and blinded observers. A) Control rats shown normal architecture of neurons in cortex and hippocampus. B) DLM-induced rats shown the intensely stained nissl substance evident form Nissl-stained dark neurons (NDNs) together with amoeboid-like pseudopalisading neurons is noted in cortex and hippocampus region, a hallmark of neurodegeneration in DLM-induced neurotoxicity. C) DLM with naringin treated rats exhibiting significantly reduced NDNs with normal nucleus and cytoplasm in cortex and hippocampus region. D) Naringin alone administered rats shown normal architecture of cortex and hippocampus. To evaluate NDNs, sections of control and experimental groups were evaluated in a similar fashion as described in H & E staining. II. Quantitative analysis of histological score of Nissl-stained dark neuronal cells.

Fig. 5- Effect of DLM and naringin on immunohistochemical analysis of GFAP expressions in cortex and hippocampus region of rat brain in control and experimental group of rats.

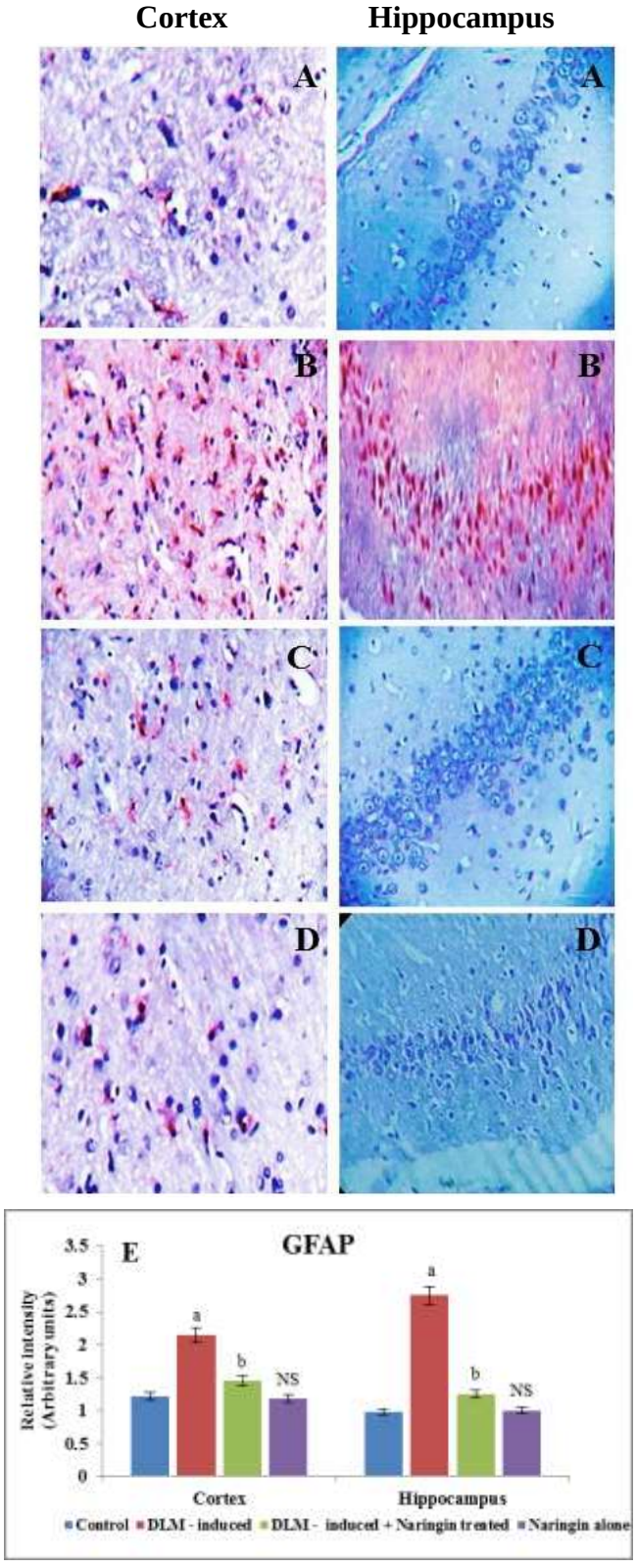


Fig. 5 Representative micrographs of glial fibrillary acidic protein (GFAP) immunostained specimens of cortex and hippocampus region of rat brain in control and experimental group of rats. Brain specimens (cortex and hippocampus) were probed with antibodies specific for GFAP (A-D). A) Control group shows no immunostaining expressions of GFAP in cortex and hippocampus region. B) In DLM-induced group exhibits increased expressions of GFAP as gross hypertrophic processes with ubiquitinated rosenthal fiber-like structures in cortex and hippocampus region. C) DLM-induced with naringin treated group shows reduced immunostaining expressions of GFAP immunoreactive cells in cortex and hippocampus. D) Naringin alone treated group shows no GFAP expressions. E) The results were quantified based on the positive expression of GFAP.

Fig. 6 - Effect of DLM and naringin on immunoblot analysis of GFAP expression in cortex and hippocampus region of rat brain in control and experimental group of rats.

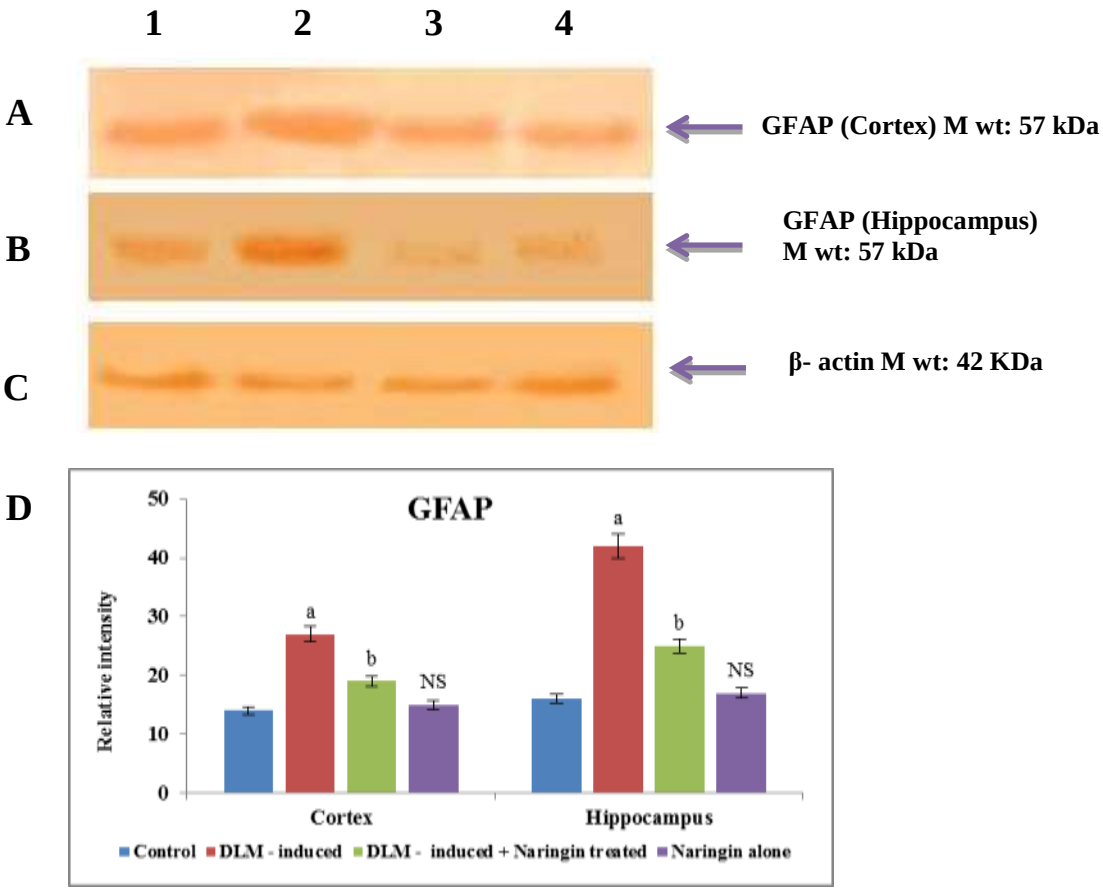


Fig. 6- (A) Immunoblot expression of GFAP in cortex (57 kDa) (B) Immunoblot expression of GFAP in hippocampus (57 kDa) C) β- actin (42 kDa) as positive control in the brain tissue homogenate of control and experimental group of rats. D) Data expressing the respective protein levels was quantitated and expressed in relative intensity (arbitrary units). Values represent relative levels of protein expression. Lane 1: control; lane 2: DLM-induced group; lane 3: DLM-induced + naringin treated; lane 4: naringin alone.

Fig. 7- Effect of DLM and naringin on the immunoblot analysis of Nrf2 in nucleus and cytosolic fractions in control and experimental group of rats.

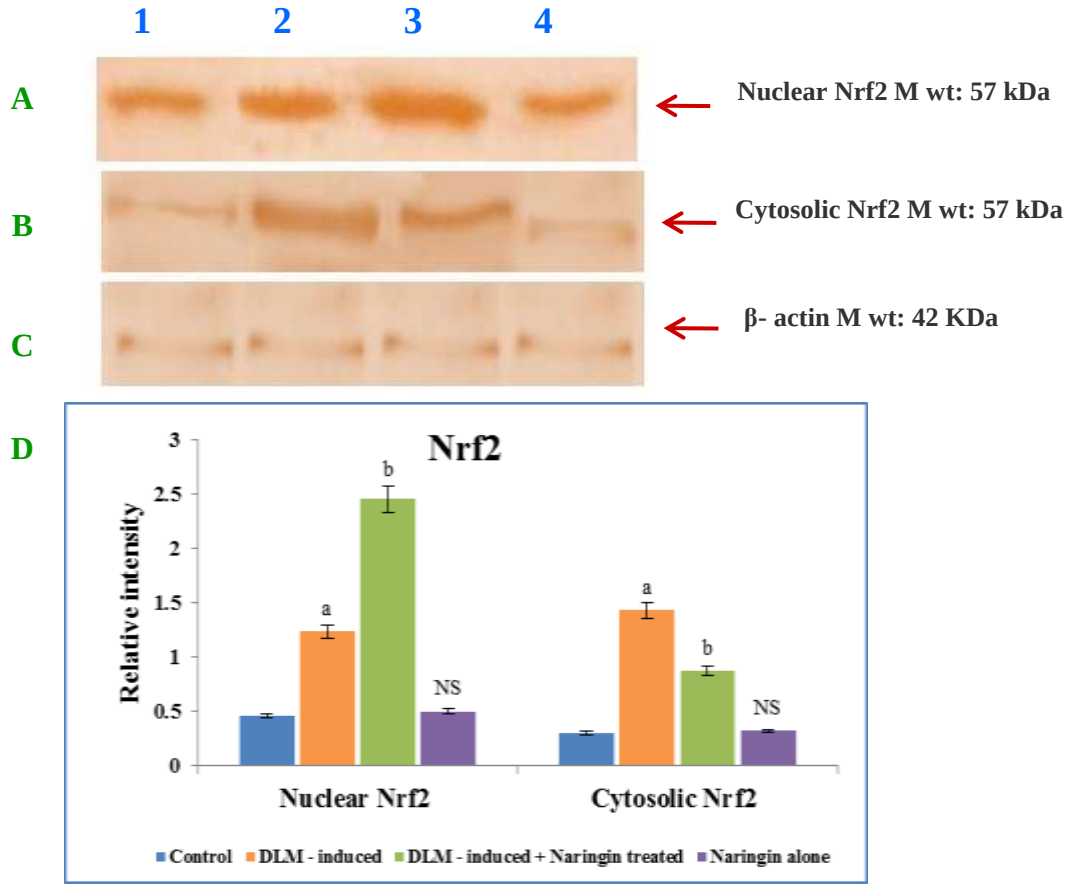


Fig. 7. Effect of DLM and naringin on the immunoblot analysis of Nrf2 in nucleus and cytosolic fractions in control and experimental group of rats (A) Immunoblot expression of Nuclear Nrf2 (57 kDa) (B) Cytosolic Nrf2 (57 kDa) (C) β-actin as positive control in the brain tissue homogenate of control and experimental animals. Lane 1: control; lane 2: DLM-induced group; lane 3: DLM-induced + naringin; lane 4: naringin alone.

Figure 8 - Effect of DLM and naringin on transmission electron microscopic analysis of cortex, hippocampus of control and experimental group of rats.

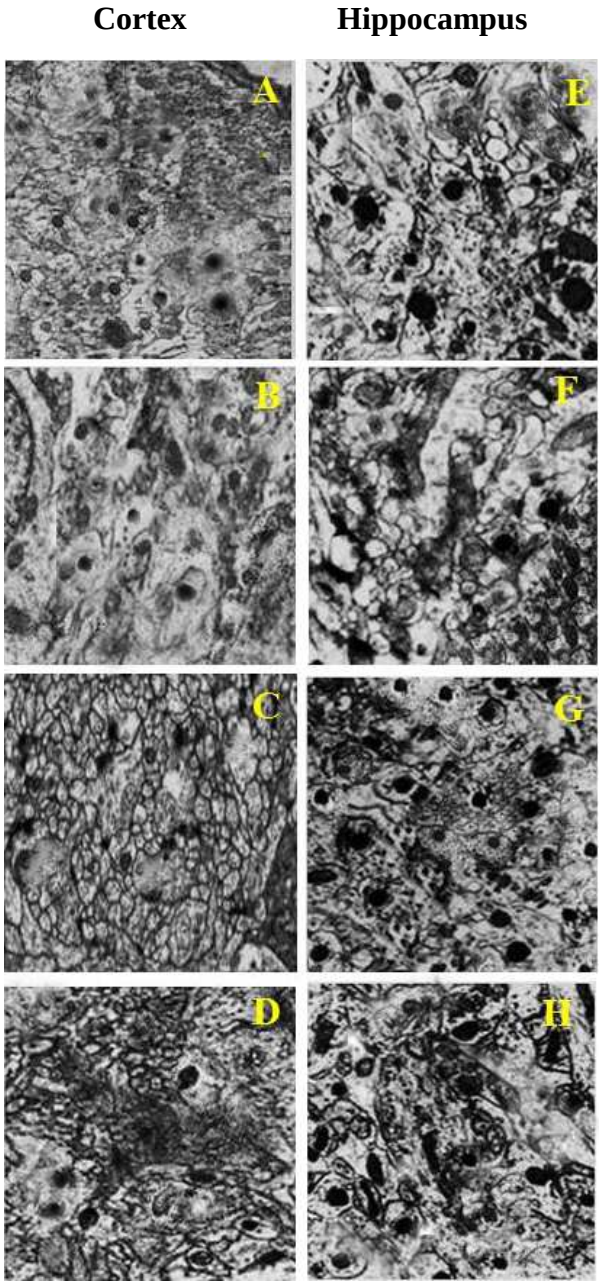


Figure 8. Effect of DLM and naringin on transmission electron microscopic analysis of cortex, hippocampus of control and experimental group of rats. A,E) Normal control rat; B,F) DLM induced rats; C,G) DLM induced + Naringin treated rats; D,H) Naringin alone treated rats.

Table 1 - Effect of DLM and naringin on antioxidants enzyme activities in the serum of control and experimental group of rats.

Particulars	Region	Control	DLM induced	DLM induced+ Naringin	Naringin Alone
SOD	Cortex	46.52 ±3.87	22.58 ±2.38[a]	38.79 ± 3.62[b]	46.47 ± 3.89[NS]
	Hippocampus	58.86±4.86	16.546 ± 3.65[a]	52.634 ± 4.52[b]	59.34 ± 4.63[NS]
CAT	Cortex	1.46±0.05	0.63 ± 0.048[a]	1.25 ± 0.03[b]	1.45 ±0.06[NS]
	Hippocampus	2.94 ± 0.16	1.34 ± 0.18[a]	2.68 ± 0.16[b]	2.96 ± 0.15[NS]
GPx	Cortex	15.74 ±1.95	7.95 ± 1.83[a]	13.84±1.71[b]	15.65 ± 1.91[NS]
	Hippocampus	27.35 ±2.53	11.38 ± 1.79[a]	23.75±1.46[b]	25.674 ± 2.48[NS]
GST	Cortex	92.98 ± 6.62	41.25 ± 4.53[a]	82.43 ± 5.44[b]	94.28±6.97[NS]
	Hippocampus	84.95 ± 5.25	36.58 ± 4.36[a]	72.378 ± 4.21[b]	86.58±4.67[NS]
GR	Cortex	2.82 ± 0.06	0.74 ± 0.08[a]	2.59 ± 0.16[b]	2.86 ± 0.15[NS]
	Hippocampus	1.76 ± 0.08	0.58 ± 0.06[a]	1.63 ± 0.05[b]	1.78 ± 0.05[NS]

SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; Enzyme activities are expressed as SOD: units/min/mg protein (One unit is equal to the amount of enzyme that inhibits pyrogallol auto-oxidation by 50%). CAT: µmoles of H_2O_2 hydrolyzed/min/mg protein. GPx: µmoles of NADPH oxidized/min/mg protein. GR: µmoles of NADPH oxidized/min/mg protein. Results are expressed as mean ± SD for 6 different sets of experiments. Values are considered significantly different at $P < 0.05$ with post-hoc LSD test. Statistically significant variations are compared as follows: [a]DLM-induced vs. control. [b]DLM-induced + naringin treated vs. DLM-induced. [a,b] indicates $p < 0.05$ and [NS] indicates Non-significant.

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