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# WITHDRAWN: Neuroprotective Potential of Flaxseed Oil in Amelioration of Cadmium Induced Neurotoxicity

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**Research Article**

**Keywords:** Cadmium, neurotoxicity, oxidative stress, flaxseed, acetylcholinesterase, α-linolenic acid, NMR profiling

**Posted Date:** June 7th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1694868/v1

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**Additional Declarations:** No competing interests reported.

## EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

## Abstract

# Introduction:

The current investigation aimed to explore the neuroprotective potential of flaxseed oil (FSO) in the amelioration of cadmium-induced neurotoxicity. Cadmium (Cd) is known as a strong neurotoxin heavy metal, which shows an impact on the brain by initiating oxidative stress and causing influences in the membrane. Flaxseeds have healthful attributes and are a rich source of ω-3 fatty acids: short-chain polyunsaturated fatty acids, secoisolariciresinol diglycoside-SDG, solvent and insoluble filaments, α-linolenic acid, proteins and a variety of antioxidants. The neuroprotective effect of FSO was identified in rats along with the expression of caspase − 3 and Bcl-2 to establish the anti-apoptotic potential of FSO.

# Methods

The rats were given the cadmium (5 mg/kg/day) orally for 30 days and simultaneously flaxseed oil (FSO) was also administered at the doses of 2ml/kg/day and 3ml/kg/day for 30 days. Morris water maze test (MWM) and Novel object recognition test (NOR) were conducted to assess the learning and memory parameters. We measured the levels of glutathione (GSH), malondialdehyde (MDA), nitric oxide (NO), acetylcholinesterase inhibitory activity (AChE), apoptosis and histopathology studies in the whole brain homogenate.

# Results

Cadmium intoxication was allied with substantial damage to learning and memory in MWM and NOR tests. The Cd-ingested group presented a rise in the level of MDA, NO and AChE in the homogenate of the brain and a decline in the levels of GSH allied with the control group. The animals, given the dose of FSO, showed restoration of learning and memory capability with oxidative and cholinergic balance in the brain tissue and also decreased levels of caspase − 3 and Bcl-2 alike to the control sample.

# Conclusion

The current study establishes the beneficial effects of flaxseed oil, which increases the level of GSH and antiapoptotic potential, whereas it also decreases the level of MDA, NO, and AChE in the brain which helps in neuroprotection and reduction of neuronal death, which was further confirmed by histopathological observations.

## 1 Introduction

The ingestion of any chemical or physical changes during maturity or at the development of the nervous system initiates an adverse effect on its chemistry, function or structure which leads to neurotoxicity [1].

Among various heavy metals mentioned in the orbital elements of the modern table of periodic, some of them got the topmost value due to pathophysiological consequences like arsenic, cadmium, mercury, and lead [2] because their bioaccumulation in living systems results in the severe impairment of vital organs, specifically, mucous tissues, gastrointestinal tract, reproductive systems and nervous system [3].

The cumulative toxin of the Cadmium (Cd) was due to its enormous extended biological half-life [4]. In many organisms, oxidative stress was induced at the cellular level by the Cd [5, 6], which initiated the damage in the physiology of numerous organs among which bone, placenta, testes, pancreas, lung, brain, liver and kidneys are noteworthy [7–9]. The generation of free radicals is not initiated directly because the Cd is a bivalent cation, however, the production initiated after Cd exposure to reactive oxygen species (ROS) was reported previously. The body has the inadequate capability to show up response against the exposure of cadmium [10], as the metal cannot endure metabolic degradation to less toxic types& is only poorly expelled, resulting in long-term storage as a feasible opportunity for dealing with this toxic component [11]. There is a lot of factor in an occupation which takes part in the Cd exposure like the food which is polluted with it or paints, manures, batteries, vehicle emanations, mechanical squanders, modern effluents, commercial items, mining, groundwater [12] and consumption of tobacco products [13]. The binding of the Cd to the sulfhydryl groups shows the structural alteration of proteins which results in the depletion of glutathione [14]. The Cd also shows to rise in the level of the LPO which shows degradation in brain cells [15].

Thus, to tackle the Cd-induced neurotoxicity flaxseed was used, as it is perhaps the most prominent crop, having been developed since very early [16]. The flax (or linseed, *Linum usitatissimum*), is a significant plant grown worldwide [17]. Flaxseed is a herbaceous plant having a place in the Linaceae family, has a significant industrial yield become global for its fibre and oilseed [18]. Almost 35% of the total mass of flaxseed is consist of oil, thus amongst the total oil α-linolenic acid (ω-3 fatty acid) possesses a higher proportion i.e., 55% of the total value whereas linoleic acid consists 15–18% [19]. It is viewed as a 'superfood' and for the most part, perceived as a sheltered (GRAS) wellspring of peptides, proteins, minerals, and nutrients [20], dietary fibre, lignans, sugars and lipids (supplementing omega-3 and omega-6 polyunsaturated fatty acids) for the synthesis of flaxseed [21]. Flaxseeds have healthful attributes and are a prominent wellspring of ω-3 fatty acids: short-chain polyunsaturated fatty acids (PUFA), α-linolenic acid (ALA) [22], secoisolariciresinol diglycoside-SDG (phytoestrogenic lignans), solvent and insoluble filaments, proteins and a variety of antioxidants [16]. Minor parts included: cyclolinopeptides (CLs), selenium, cadmium, nutrients, minerals, lignans (phytoestrogens), linatine, trypsin inhibitor, phenolics, phytic acid and cyanogenic glycosides [20, 23].

Due to the existence of the various key components in the flaxseed oil, it carries the antioxidant properties, which help against the neurotoxicity, where it decreases the lipid peroxidation value and keeps up the rise in the level of antioxidants [24]. The omega 3 fatty acids constituents extant in flaxseed oil show a defensive outcome in the brain in contradiction to peroxidation by the mechanism of adaptable fluidity of cell membrane as well as enzymes of the cell membrane [25]. Also, it is measured that the flaxseed has the capability of antioxidant properties due to the vitamin E present in it [26], but the effects

were not as comparable to the lignans and omega 3 fatty acids potential against the neurotoxicity [27, 28]. The rich diets of n-3 fatty acids exhibited that the density of neurotransmitter membrane receptors was higher and shows enhanced concentrations of neurotransmitters [29], and increased control of neuronal membrane excitability [30], also in the region hippocampal there is an increase in the nerve growth [31], and the synaptic membranes fluidity also increased, whereas decreased ischemic injury to neurons [32], enhanced blood flow in cerebral, reduced concentrations of lipid peroxides and enhanced concentrations of antioxidant enzymes [33].

This research investigation aimed to measure the neuroprotective action of flaxseed oil against cadmium-induced neurotoxicity in Wistar albino rats.

## 2 Materials And Methods

# 2.1 Chemicals

The powder of Cadmium chloride MW: 183.32g/mol (Sigma-Aldrich) was dissolved in distilled water, whereas the seeds of flax (linseed, *Linum usitatissimum*) Family- Linaceae) were obtained from the Safedabad Lucknow and verified from the CSIR-NBRI herbarium Lucknow (Accession No. LWG 108273). Then with the help of the cold press extraction method Flax seed oil was obtained. Donepezil (Donep 5 - Alkem Laboratories Ltd, Batch No. 20441066) was also utilized as a treatment drug for the comparison of neuroprotective potential and also for significant effects.

# 2.2 Dosage selection

In this study, we have chosen 5mg/kg *p.o.* of $CdCl_2$ [34]. The selected dose of FSO was taken to be 2ml/kg/day and 3ml/kg/day *p.o.* based on the study of Kaithwas *et al.* (2012) [35]. Donepezil 5mg/kg, *p.o.* was administered [36]. Cadmium was administered 1 hour prior to FSO or Donepezil administration [37].

# 2.3 Animals

Wistar rats of weight around 150–220 g were procured from CSIR- Central Drug Research Laboratory (CDRI), Lucknow. Animals were housed in Polycarbonate cages under conventional laboratory conditions (12 hr. light and dark cycle, temperature 23 ± 2ºC and humidity 55 ± 5%). Animals were fed with a commercial pellet diet and water *ad libitum*. The experimental protocols were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and the protocol were reviewed and approved by the Institutional animal ethical committee (IAEC), Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow Campus (AUUP/AIP/M. Pharm./005/2021).

# 2.4 Experiment design

The animals were familiar for seven days before the initiation of the test to keep a strategic distance from any stress because of handling after the rats were randomly separated into 5 cages containing 5 rats in

each cage. Group 1: Control group was administered vehicle (normal saline), Group 2: Negative Control was administered cadmium 5 mg/kg *(p.o.)* for 30 days, Group 3: FSO 2ml was administered 2ml/kg*(p.o.)*. of flaxseed oil and cadmium 5 mg/kg *(p.o.)* for 30 days, Group 4: FSO 3ml was administered 3ml/kg *(p.o.)*. of flaxseed oil and cadmium 5 mg/kg *(p.o.)* for 30 days and Group 5: the standard group was administered as Donepezil dose of 5 mg/kg *(p.o.)*. and cadmium 5 mg/kg *(p.o.)* for 30 days.

# 2.5 Flaxseed oil physicochemical characterization

The organoleptic parameters of flaxseed are studied like the colour and smell. For Quantitative analysis of FSO 1H-NMR spectra were recorded on a 400 MHZ Bruker Switzerland NMR Spectrometer operating at 400MHz, in $CDCl_3$. Coupling constant and chemical shift were recorded.

Also, the specific gravity was calculated using a pycnometer.

# 2.6 Behavioural test

All the rats were utilized in the examination after completing the dosing periods of 30 days. The animals were utilized precisely in both behavioural tests and handled humanely.

# 2.6.1 Morris water maze (MWM)

It is notable that unconstrained variation is a proportion of spatial working memory. The rats were tried in a spatial form of the MWM test which utilized as a proportion of momentary memory, general locomotor action and stereotypic manner. The maze is composed of a round pool constructed of iron. The water (25˚C) was filled in the maze pool and to make them opaque, ink was added to it. An iron escape stage for the rats was kept around 0.5cm from the level of water and the pool was divided into four quadrants. The MWM test was divided into two parts i.e., the training phase and test day. The training phase consists of 4 days where each rat was kept in the water and permitted to discover the stage with a ceiling time of 120 s, also if the rat failed to find the stage, then the animal was placed on the stage for 30 s. The trial interval for each rat was set at around 15 min and 3 times a day, also the position is chosen randomly. So, after the continuous 4 days trial, on the 5th day, the test phase was performed without an escape stage, where the time spent in each quadrant was observed against the target quadrant [38].

# 2.6.2 Novel object recognition test

Ennaceur and Delacour (1988) presented the novel object recognition (NOR) to evaluate the capability of rats in a familiar situation to distinguish a novel object. The test was divided into the period of four days after the accomplishment of exposure of one month in every rat. At the start of the trial, the continuous two days was the phase of habituation in which the test was of 15 min/day. The second phase was of training with a least 10 min in a day and then finally test phase of 5 min in a day. In the habituation phase, the animal was taken for 15 min/day for continuous 2 days in a (50× 50 × 40 cm) rectangular cage without an object which was made of wood. Then, on the second phase of a test during the training, two same items were set close to the edges of the apparatus. Then, the rat was kept in the mid of the apparatus and permitted to discover both objects for 10 minutes. An investigation was characterized as

the rats keeping their nose in the direction of the object and are under 2cm of the object. Lastly, the test phase consists of the 5 minutes of the trial in which one of the old items was replaced by a novel object. To avoid any type of behavioural error due to the scent from the object or surroundings, the apparatus and the objects were cleared up properly with the help of water and then wipe with a cloth to dry the surface. Discrimination amongst two articles was determined utilizing a Discrimination index (DI) which considers singular contrasts in the total exploration time [39].

DI= (investigation time of novel object/total time taken for exploration) −(exploration time of familial object/total time taken for exploration) ×100

# 2.7 Sample Preparation

After the behavioural examinations rats were sacrificed by cervical dislocation technique. Then, the rats were perfused through the heart and brain was dissected by incising the skull from its dorsal side, and then carefully removing the whole brain from each rat. The brain was washed with pre-cooled normal saline and placed on ice to avoid any degradation and afterwards blotted on filter paper. Now, with the help of the homogenizer (IKA, tissue rupture), brain tissue homogenate of 10% v/v by 0.03 M sodium phosphate buffer was prepared. One portion is used for the MDA, GSH, NO, and another portion was used for the estimation of AChE then both the brain homogenate was centrifuged in cooling centrifuged for 10 min at 10000 rpm 4ºC & supernatant was collected and kept at -20˚C for AChE estimation.

# 2.8 Biochemical estimations

# 2.8.1 Glutathione estimation

The hydrolysis of acetylcholine imitates the production of thiocholine, which is the prime objective of the study, where the rate of production is to be estimated. Thus, the production of the yellow anion of 5-thio-2-nitro-benzoic acid (II) was accomplished with the help of prolonging the reaction of the thiol with 5:5-dithiobis-2-nitrobenzoate ion (I)[5] [40]. The supernatant of tissue as 0.20 ml was added to the 1.5 ml precipitating solution comprising 30% NaCl, 0.20% Na-EDTA and 1.67% glacial metaphosphoric acid. The sample was kept at room temperature for 5 min and thereafter the solution was centrifuged for 5 min at 1000rpm. Then, from obtained supernatant, 1ml of the solution was added to the 4 ml of 0.30 M $Na_2HPO_4$ and DTNB reagent (5:5' dithiobis-2-nitrobenzoic acid was taken as 40 mg and it was mixed with 1% sodium citrate) was taken as 0.50 ml. Likewise, to avoid any error, 0.20 ml distilled water was utilized as a blank in the place of the supernatant of the brain. then the colour of the absorbance was examined at 412nm with the help of spectrophotometry [41].

# 2.8.2 Malondialdehyde estimation

Malondialdehyde (MDA) produce various product by developing a 1:2 adduct with thiobarbituric acid (TBA) which can be estimated by spectrophotometry or fluorometry. It is said to be the oxidative stress marker and arises naturally. The presence of MDA was reported in both of the forms i.e., monomer and higher-order oligomers, whereas the MDA estimation was utilized usually as the key source to examine

the level of the lipid peroxidation. The procedure defined by Colado *et al.* (1997) was utilized to analyze the level of MDA in the samples containing the brain homogenate [42]. In this investigation, homogenate of tissue consists of 500 µl in phosphate buffer (pH 7.4), 30% trichloroacetic acid (TCA) as 300 µl, 5N HCl as 150 µl and 2% w/v 2-thiobarbituric acid (TBA) as 300 µl was mixed and thereafter the solution was heated at 90˚C for 15 min. Then, the solution was taken for centrifugation for 10 min at 12,000×g. The obtained supernatant was seen to be pink-coloured and then the supernatant was examined at 532nm with the help of spectrophotometry. Also, with the help of the standard curve which was prepared with malondialdehyde tetrabutylammonium salt, the concentration of MDA was estimated is shown as nmol/mg protein.

## 2.8.3 Nitrite estimation

A bluish violet azo dye forms stably as when N-(1-naphthyl) ethylenediamine dihydrochloride coupled diazonium cation (whereas, the formation of cation initiated in the existence of hydrochloric acid, nitrite reacts with p-amino phenyl-mercapto acetic acid) in an acidic medium[43]. The procedure defined by Berkels *et al.* (2004) was utilized to analyze the level of nitrite/nitrate (NO) in the samples containing the brain homogenate [44]. N-(1–naphthyl) ethylenediamine and nitrous acid diazotized sulfanilamide which is formed by the existence of nitrite in the acid medium joined together and resultant azo dye seen as a bright reddish-purple colour that has been examined at 540 nm.

## 2.8.4 Acetylcholinesterase (AChE) inhibitory assay

The inhibition of AChE activity to increase central cholinergic function is an important therapeutic strategy for the treatment of cognitive decline. The AChE hydrolyses the acetylthiocholine iodide in tissue samples to produce the thiocholine from which the DTNB react. So, this helps to calculate the activity of AChE because the formation of thiocholine amount indicates the adduct of DTNB by the intensity of absorption[40]. The Ellman method was used for the quantitative measurement as per the standard procedure to calculate the level of AChE in the brain [40]. The mixture of assays consist supernatant as 0.05 ml, 0.01 M sodium phosphate buffer (pH 8) as 3 ml, acetylthiocholine iodide as 0.10 ml and 5,50 dithiobis-(2-nitro benzoic acid) (Ellman reagent) as 0.10 ml. Then the samples were analyzed for 5 min at 412 nm to estimate the change in absorbance. The calculation of results was performed by utilizing the molar extinction coefficient of the chromophore ($1.36*10^4$ $M^{-1}$ $cm^{-1}$) and shown as the control percentage.

## 2.8.5 Protein Estimation

The samples were analyzed in the range of 0.01–0.1 mg/ml by taking the Bovine Serum Albumin (BSA, 1 mg/ml) as standard, whereas the Phosphomolybdic agents help to develop the blue colour in it. Phosphotungstic components of Folin & Ciocalteus reagent (FCR) by the amino acid Tryptophan and Tyrosine existing in the protein along with the colour established by the reaction of a biuret of protein with Alkaline Cupric Tartrate, which estimated at 760nm. After the preparation of brain tissue homogenate, 100µl of homogenate was taken in test tubes and 1900µl of distilled water was added. Then to this mixture, added 400µl of the lowry reagent and for 30 min at room temperature, this solution was

incubated. After that, Folin & Ciocalteus reagent solution was added and again for 30 minutes at room temperature it was incubated. Then readings were taken on UV-spectrophotometer at 760nm [45].

## 2.8.6 Apoptosis studies

Apoptosis arises generally in ageing and also at the phase of development and as a mechanism of homeostatic is to sustain populations of cells in tissues. Apoptosis similarly arises as a mechanism of defence for example as any noxious agents or disease damage the cells or in the reactions of the immune. Whole mouse brain proteins were extracted using RIPA buffer (25mM tris HCl, 150mM NaCl, 5mM EDTA, 0.1% TX-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (#78429; Invitrogen). The protein concentration of the lysate was measured by the BCA method, equal amounts (40 µg) of protein were added to Laemmli buffer and boiled for 10 minutes at 950C. Proteins were divided into 10% SDS-PAGE gels and added to 0.45µm nitrocellulose membrane at 250mA for 1.5h. The blockage of membranes was executed with non-fat dry milk (3%) in TBST and then after the samples were incubated with the primary antibody on a shaker at 40˚C overnight. Caspase-3 (#9665; CST) Bcl-2(#2876; CST) antibodies were diluted in 1:1000 ratios for immunoblotting. HRP-conjugated secondary antibodies were used in the ratio of 1:10000 for chemiluminescent detection of primary antibody binding. Immunoblots were developed by Super signal Femto substrates and imaged using a MyECL gel imager. The intensity of protein bands was quantified using ImageJ. The immunoblots with unsaturated exposure were used for quantification with appropriate loading controls (tubulin) as standards [46, 47].

## 2.8.7 Histopathological studies

The brain of the rats was collected after the sacrifice and the complete undamaged brain was kept in the solution of formalin (10% v/v) to avoid any degradation. The tissue of the brain samples was cut precisely into the thickness of 3 mm and thereafter the dissected slabs of the tissue were fixed in paraffin. The segments of the brain were prepared at the size of 5–10 µm thick, and then the samples were stained with the help of the haematoxylin and eosin (H&E). The staining of H&E was executed as per the standard protocol [48].

## 2.8.8 Statistical analysis

The statistical analysis was done by calculation of the mean ± SD among the result using One-Way analysis of variance (ANOVA) and Two-Way ANOVA trailed by Tukey's test and Sidak's test. All the Statistical data were calculated using the software Graph Pad Prism *Ver.* 8.02.

# 3 Result

## 3.1 Flaxseed extraction and physicochemical characterization

The seeds of the flax were washed and air-dried before the extraction of the oil from it. Then the cold press methods were used for the extraction to avoid any kind of degradation in the quality of the oil. The oil was stored in a refrigerator in an airtight bottle throughout the research protocol. Then, the physiochemical characteristics of the flaxseed oil were performed on Day 1, which shows the baseline of the oil and then on Day 15 and Day 30 to estimate the findings. The organoleptic characteristics showed a light yellow to yellow in the colour of the flaxseed oil. The oil had a distinctive smell of flaxseeds, deprived of an odd odour. Also, the flaxseed oil was found to have non-significant changes in specific gravity over a period of 30 days. The specific gravity was obtained at the baseline (immediately after the extraction) and was compared with the standard values. The base value is compared with the value obtained at subsequent periods (15 days and 30 days). There was no significant difference between physicochemical values on Day 1, Day 15 and Day 30. These findings further support the physicochemical stability of the oil samples for 1 month. Data were analysed using one-way ANOVA.

The NMR spectrum of flaxseed oil was obtained in $CDCl_3$ in which the following characteristic peaks were observed. The $^1H$ NMR spectra of the oil exhibited a signal of 0.8 -1ppm which can be attributed to the $CH_3$ proton present in the oil. The presence of a signal in the region of 1.2–1.5 ppm is mainly because of the presence of aliphatic protons which are higher in numbers. The allylic proton tends to appear between 1.8-3ppm. SDG hydroxy proton seems to be present between 4-4.5 ppm. The signal corresponding to olefinic protons appears between 5.1–5.5 ppm. The overall $^1$ H spectra indicate the presence of omega 3 fatty acids Alpha-linolenic acid, linolenic acid, secoisolariciresinol diglycoside-SDG in the oil.

# 3.2 Behavioural test

# 3.2.1 Effect of test drug in Morris’s water maze test (MWM)

The MWM test was executed to determine whether the flaxseed oil is capable of attenuating learning and memory impairment induced by cadmium. The Cd-induced rats showed escape latencies were longer throughout the sessions of training than the control and FSO-treated groups. According to the 5-day procedure plan, on day 1 there were no noteworthy variations observed in any group. On Day 2, the Standard group ($p < 0.01$) had shown improved performance when compared to the negative control. On Day 3, ($p < 0.001$), FSO 3ml ($p < 0.0001$) and Standard ($p < 0.0001$) groups shows enhanced performance when compared to the negative control. On Day 4 of the consecutive trials of the MWM test shows ($p < 0.0001$) shows against the control group, whereas the FSO 2ml ($p < 0.0001$), FSO 3ml ($p < 0.0001$) and Standard ($p < 0.0001$) groups were reported to have improved performance against negative control.

On Day 5 of the MWM test, the mean time spent in each quadrant (sec) was observed, where the Q2 was the target quadrant of the test. The target quadrant of the MWM test ($p < 0.0001$) showed against the control, whereas the FSO 2ml ($p < 0.0001$), FSO 3ml ($p < 0.0001$) and Standard ($p < 0.0001$) groups. The Cd-induced animal's retention time in each quadrant was similar throughout the session, whereas the remaining groups show higher retention time in the target quadrant i.e., Q2, which’s shown in Fig. 3.

## 3.2.2 Effect of test drug in Novel Object Recognition test (NOR)

## 3.2.2.1 Training phase

The training phase of the test includes that each rat was permitted to discover the two similar objects for 10 min. Thus, the data revealed that the time taken for the exploration of both objects was almost equal in every group.

## 3.2.2.2 Test phase

On the test day, amongst the two similar objects, one of the objects was taken out and in place of that object, we had put the novel object and kept the rat for 5 min to discover the objects. Thus, the data revealed that the higher time taken for exploration of the novel object was in the rats of control, FSO 2ml, FSO 3ml and standard group.

## 3.2.2.3 Discrimination index (DI)

Discrimination amongst familiar and novel objects was estimated with the help of DI. The data shows that the DI in the negative control group rat was reduced compared with the other groups. This finding indicates that the negative control group rats are not able to distinguish a novel object from the previously placed object which was familiar to them.

## 3.3 Biochemical Estimation

## 3.3.1 Glutathione estimation

The activity of GSH levels was examined in all rat brain homogenates. A noteworthy reduction in the levels of GSH was detected in the Cadmium group (146.148 ± 12.781µg/mg protein) as compared to the control (425.265 ± 12.348µg/mg protein) and standard (409.821 ± 44.709µg/mg protein). There was a substantial rise in the level of GSH in the groups given the FSO 2ml/kg (308.104 ± 52.935µg/mg protein) and FSO 3ml/kg (342.306 ± 19.936µg/mg protein) flaxseed oil. The rise in the level of GSH in comparison to the group given the cadmium represents enrichment of cadmium-ingested neurotoxicity.

## 3.3.2 Malondialdehyde estimation

The activity of MDA levels was examined in all rat brain homogenates. A noteworthy rise in the levels of MDA was detected in the Cadmium group (116.783 ± 13.290 nmol/mg protein) as compared to the control (57.688 ± 5.096 nmol/mg protein) and standard (74.281 ± 8.387 nmol/mg protein). There was a substantial fall in the level of MDA in the groups treated with FSO 2ml/kg (84.347 ± 15.118 nmol/mg protein) and FSO 3ml/kg (85.679 ± 3.989 nmol/mg protein) flaxseed oil. Decrease in MDA level in comparison to the group given the cadmium, representing enrichment of cadmium-ingested neurotoxicity.

## 3.3.3 Nitrite estimation

The activity of NO levels was examined in all rat brain homogenates. A noteworthy rise in the levels of NO was detected in the Cadmium group (201.007 ± 21.641 µg/mg protein) as compared to the control (65.703 ± 9.939 µg/mg protein) and standard (106.916 ± 12.344 µg/mg protein). There was a substantial fall in the level of NO in the groups treated with FSO 2ml/kg (172.083 ± 34.087 µg/mg protein) and FSO 3ml/kg (149.079 ± 13.353 µg/mg protein) flaxseed oil. Decrease in NO level in comparison to the group given the cadmium, representing enrichment of cadmium-ingested neurotoxicity.

### 3.3.4 AChE inhibitory assay

The activity of AChE inhibitory was examined in all rat brain homogenates. A noteworthy rise in the levels of AChE inhibitory activity was detected in the Cadmium group (0.091 ± 0.032 µmol/min/mg protein) as associated with the control (0.044 ± 0.004 µmol/min/mg protein) and standard (0.051 ± 0.001 µmol/min/mg protein). There was a substantial fall in the level of AChE inhibitory activity in the groups treated with FSO 2ml/kg (0.067 ± 0.005 µmol/min/mg protein) and FSO 3ml/kg (0.056 ± 0.018 µmol/min/mg protein) flaxseed oil. Decrease in AChE inhibitory activity level in comparison to the group given the cadmium, representing enrichment of cadmium-ingested neurotoxicity.

## 3.4 Apoptosis Studies

The anti-apoptotic activities of FSO were determined by examining the appearance of Bcl-2 and Caspase-3 in the brain tissue by western blot analysis. 40µg protein was electrophoresed on a 10% SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). Protein levels were quantified using ImageJ and were normalised with loading control (tubulin). Fold change (below the blots) was measured by comparing each value with the value of the standard. (*) indicates the Bcl-2.

## 3.5 Histopathological Studies

In this investigation, the neuroprotective effect was estimated of the drug by taking samples of the brain and exposed to tissue H&E staining. In the Control group, microscopic examination revealed the presence of a normal cerebral cortex, normal hippocampus and normal cerebellum structure (Fig. 14A&B). In Cd treated group, microscopic examination revealed degeneration of pyramidal cells of the hippocampus (Fig. 14C). In Cd + FSO 2ml treated group, microscopic examination revealed the presence of normal grey matter in the cerebral cortex but there was degeneration of pyramidal cells in the hippocampus as well as mild degeneration of granular cells in the cerebellum (Fig. 14D&E). In Cd + FSO 3ml treated group, microscopic examination revealed the presence of normal grey matter in the cerebral cortex (Fig F&G). In the Standard group, microscopic examination revealed the presence of mild degeneration of granular cell bodies in the cerebellum, whereas other structures appeared normal (Fig. 14H).

# 4 Discussion

In this study, we examined the behavioural parameters, biochemical analysis, apoptosis studies and histopathological alteration instigated by exposure to cadmium and the protective action of flaxseed oil treatment. The extracted oil of flaxseed was also evaluated for the specific gravity, where they show non-

significant changes on Day 15 and Day 30 from the baseline, which results in the physicochemical stability of the oil.

The stability of the oil was obtained with the help of a pycnometer, the relative density confirms the study and the characteristic peaks of active constituents present in FSO were observed by $^{1}H$ NMR spectroscopy.

Chronic exposure to Cd initiates the deposition of its toxic element in all the regions of the brain whereas the prominent region for the deposition is the hippocampus which is responsible for memory and learning [49, 50] because as the $Cd^{2+}$ starts migrating towards the brain it enhanced the production of free radical and alter antioxidant defence systems [51, 52]. As a consequence, dysfunction at the cellular level, and oedema of cerebral and lipid peroxidation can occur. Whereas the exposure of $Cd^{2+}$ subsequently causes impairment of proteins, lipids and DNA *via* oxidation reactions which lead to cell death in certain brain regions [12, 52, 53]. The chronic ingestion of Cd results in decreased memory and learning capability which was observed in the MWM and NOR test for spatial memory.

The animals administered with Cd developed impairment in the capability to retain memory and lose learning power. The foremost significant involved in deficits of spatial memory and learning is the loss of the cholinergic neural system in the hippocampus. In the current investigation, the flaxseed oil showed its potent activity against the Cd-induced neurodegeneration in rats, and the results were supported by the noteworthy reduction in latency time, and enhanced target quadrant exploration as revealed by the spent time data in the target quadrant zone in Morris's water maze trial. On other hand, flaxseed oil enhanced recognition capabilities in Cd-ingested rats as demonstrated by a noteworthy increase in the exploration rate of novel objects. The finding strengthens with a higher discrimination rate amongst the familiar and novel objects in the treated. The treatment with the oil of flaxseed revealed a significant enhancement in cognitive performance against Cd-induced neurotoxicity. The activity was better proven in the group treated with the 3ml/kg of flaxseed oil, as the central cholinomimetic agent. Thus, it has been confirmed that the Cd-ingested groups showed disruption in the cholinergic function, which was affirmed by the rise in the escape latencies and similar object recognition of both familiar and novel objects by negative control rats, whereas flaxseed oil reverts the effect. So, these actions of the flaxseed are said to be accountable for the development of spatial memory by strengthening the cholinergic system in the current investigation.

To confirm the mechanism underlying Cd neurotoxicity, we measured the levels of GSH, MDA, NO and AChE in the homogenate of a rat's brain. Our findings showed that exposure to Cd (5mg/kg BW) for 30 consecutive days induced neuronal alterations through the depletion of antioxidant defence mechanisms, which disturbs the cellular redox and leads to oxidative stress. This was confirmed by a rise in the levels of MDA, NO and AChE activity and a reduction in the GSH levels in cadmium treated group. Flaxseed oil-treated groups showed a significant reduction in MDA, NO level and AChE activity as increased GSH levels in a dose-dependent fashion.

The intake of flaxseed oil is allied with the high content of catecholamine in the rat's brain because of its n-3 fatty acids content. Whereas, the content of n-3 fatty acids is responsible for the changes in the function of the brain by altering neurotransmitters function and production like dopamine and serotonin [54], protein kinase C (PKC) inhibition as well phospholipase A2 inhibition [55]. Additionally, if the level of n-3 fatty acids is reported as low, then it expects low levels of cerebrospinal fluid 5-hydroxy indole acetic acid (known to be the foremost metabolite of serotonin), which is recognized to be defensive in contradiction to depression, and the n-3 fatty acid deficit is allied with declines in dopaminergic and serotonergic neurotransmitters and release of acetylcholine, serotonin and dopamine [56].

Also, some of the researchers reported that it has the capabilities to ineffective the oxidative stress alleviating by free radical level or free radicals scavenging (particular OH radical) [57]. Whereas it has been affirmed that the lignans of the plant are said to be creating such a prominent effect [24]. Similarly, a protective effect against peroxidation was also reported due to the flaxseed oil constituents i.e., omega 3 fatty acids by adaptable enzymes of the cell membrane and fluidity of cell membrane [25]. Certainly, it is recognized that Cd-ingested variations in structures of the cell membrane [58]. Specific fatty acids probably stop such consequences [25]. Also, it is measured that the flaxseed has the capability of antioxidant properties due to the vitamin E present in it [26], but the effects were not as comparable to the lignans and omega 3 fatty acids [27, 28].

The enzyme of the antioxidant level where declines may be attributed to the accumulation of Cd in the brain tissue which leads to the fall in the level of GSH [59]. Depletion in the GSH content inactivates the antioxidant enzymes; in addition, Cd binds to the sulfhydryl (–SH) group of the oxidative enzymes, which leads to their inhibition [60]. On the other hand, measuring the level of MDA which is termed the key to estimating lipid peroxidation [61], showed that there is an increment in the value in the negative control group, [62, 63]. [64, 65]. Additionally, it has been suggested that Cd also takes an important part in the generation of NO[63, 64]. The NO has been said to be protective or regulatory functions, when the concentration is low in the cells, whereas if the concentration increases, it will lead to a toxic effect [66]. In this investigation, the data interpretation shows that the level of NO was marked high in the negative control group compared to the control one, which suggests that the Cd-induced neurotoxicity may involve variations in the production of NO. The investigation also marked the rise in the activity of the AChE inhibitory assay, which revealed that the AChE enzyme (AChE) is accountable for the degradation of the acetylcholine (ACh). Thus, for the symptomatic treatment of cognition decline, it is necessary to inhibit the AChE inhibitors formed due to the Cd-ingested neurotoxicity [67, 68].

The behavioural decline in cognition is influenced by cholinergic transmission get deficits in majorly the region of hippocampal and cortical of the brain [69]. Several AChE inhibitors are now being used to inhibit the AChE like tacrine, physostigmine, donepezil, rivastigmine, neostigmine and carbamates [50, 70, 71]. We observed that cadmium rise the activity of AChE in the rat brain compared to the healthy group, whereas treatment with flaxseed oil decreases the activity of the AChE in the brain in cadmium-induced neurotoxicity.

Processes of cell death were known to be apoptosis which is activated by various stimuli. Activation of caspases is the crucial step that occurs once a cell is destined for apoptosis [47]. Upon death signal, initiator caspases (caspase-2, -8, -9 and − 10) stimulate effector caspases (caspase-3, -6, -7) by cleavage on specific sites. Caspase-3 remains in the cells as a pro-caspase of 32kDa which is cleaved into 20kDa and 10kDa active forms as apoptosis proceeds. On contrary, the inhibition of the caspase's activation with the help of Bcl-2 protects cells from apoptosis. When a cell is committed to apoptosis, the anti-apoptotic function of Bcl-2 is downregulated either by its cleavage or by its decreased expression [72]. We have demonstrated that treatment with FSO protects cells from death. Negative control induced apoptosis which is evident by reduced Caspase-3 and Bcl-2. While treatment with FSO rescued the decreased levels of caspase − 3 and Bcl-2 alike to control sample was taken as standard which also upregulated the caspase-3 and Bcl-2. The present investigation establishes that the oil of flaxseed has the capability of improving the effect on Cd-ingested oxidative stress.

Further, this study opens multiple aspects for studying the involvement of FSO in modulating the molecular pathways in cadmium-induced neurodegeneration in various types of memories like aversive memory and emotional memory in different animal models.

# 5 Conclusion

Overall, our results showed that when we administered the cadmium at a stated dose for the stated period, Cd initiates the disturbance in the balance of the antioxidant/ prooxidant in rats, which has been confirmed by the fall in GSH level and rise in MDA, NO and AChE level. Also, the behavioural activities showed cognition decline in the cadmium-induced rats compared to the healthy control rats. Thus, the flaxseed oil exerts protective effects against Cd-induced neurotoxicity through its potent antioxidant, anti-apoptotic activities and action on the AChE enzyme. This was also supported by the histopathological evaluation of brain tissue. Flaxseed oil also helps to establish the cognition ability which was confirmed from the behavioural activity. Thus, it has been concluded that the neurodegeneration caused due to the free radicals scavenge activity by ingestion of Cd can be reverted with the help of flaxseed oil. Therefore, flaxseed oil might be relatively beneficial against Cd-ingested neurotoxicity.

# Declarations

**Authors' Contributions**: D.K.M, DS, ZF, LM and H.A: Conceptualization, Methodology, Data collection, Writing- Original draft preparation. All the author(s) read and approved the final manuscript.

**Funding:** No funding source

**Data Availability:** All data generated or analyzed during this study are included in this article.

**Compliance with Ethical Standards**: We obtained approval from approved by the Institutional animal ethical committee (IAEC), Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow Campus.

**Competing Interests**: The authors declare that they have no competing interests.

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# Figures

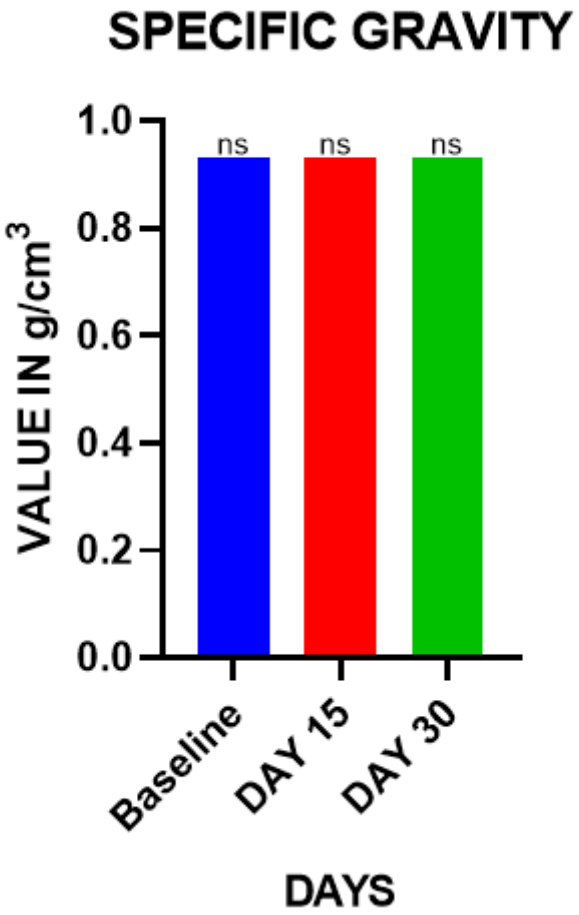
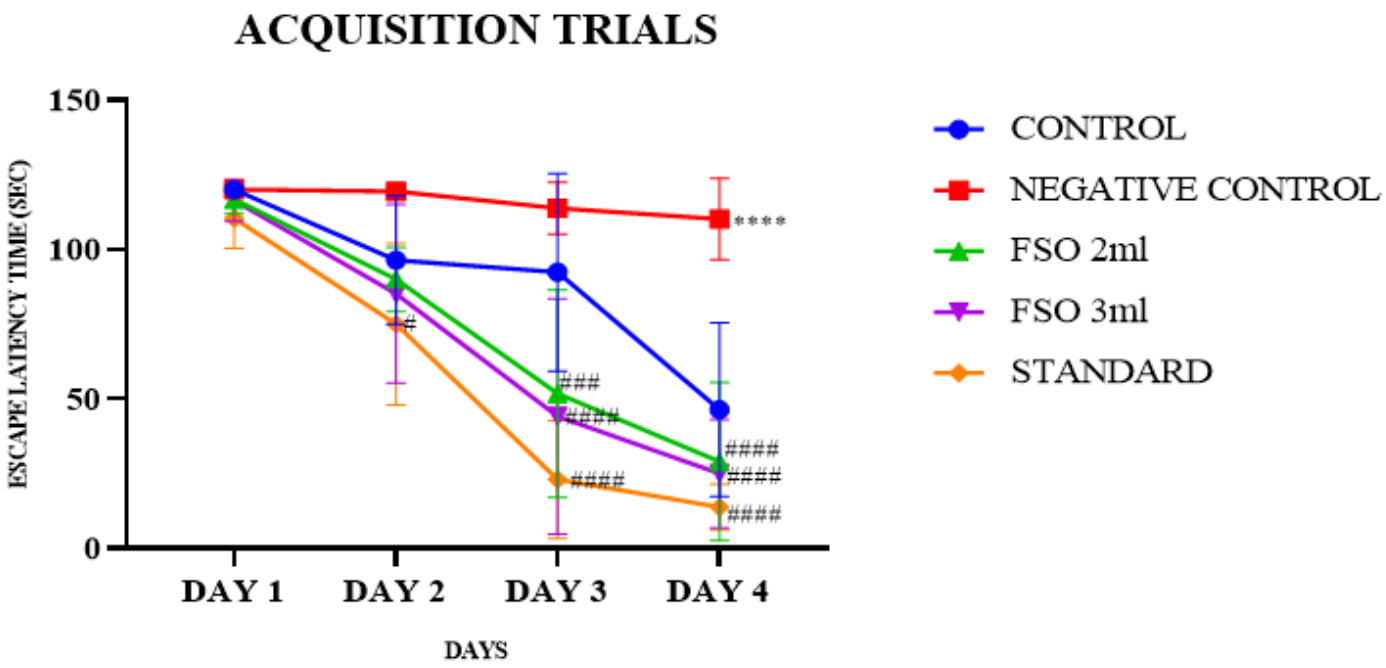



**Figure 1**

The specific gravity of the Flaxseed oil

ns P values are considered non-significant *versus* standard reported values; data were estimated with the help of one-way ANOVA (multiple comparisons). The Standard reported value at baseline is 0.92 g/cm³ of oil; whereas the observed value at baseline is 0.932 g/cm$^3$ of oil.




## Figure 2

Effect of flaxseed oil on escape latencies (seconds) on Cd-induced rats in the acquisition trials.

All data were analysed *via* two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests using GraphPad Prism software. Data presented are Mean ± SD (n =5). $^{****}$P<0.0001 when compared to control group and $^{\#}$P<0.01, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 when compared to negative control.

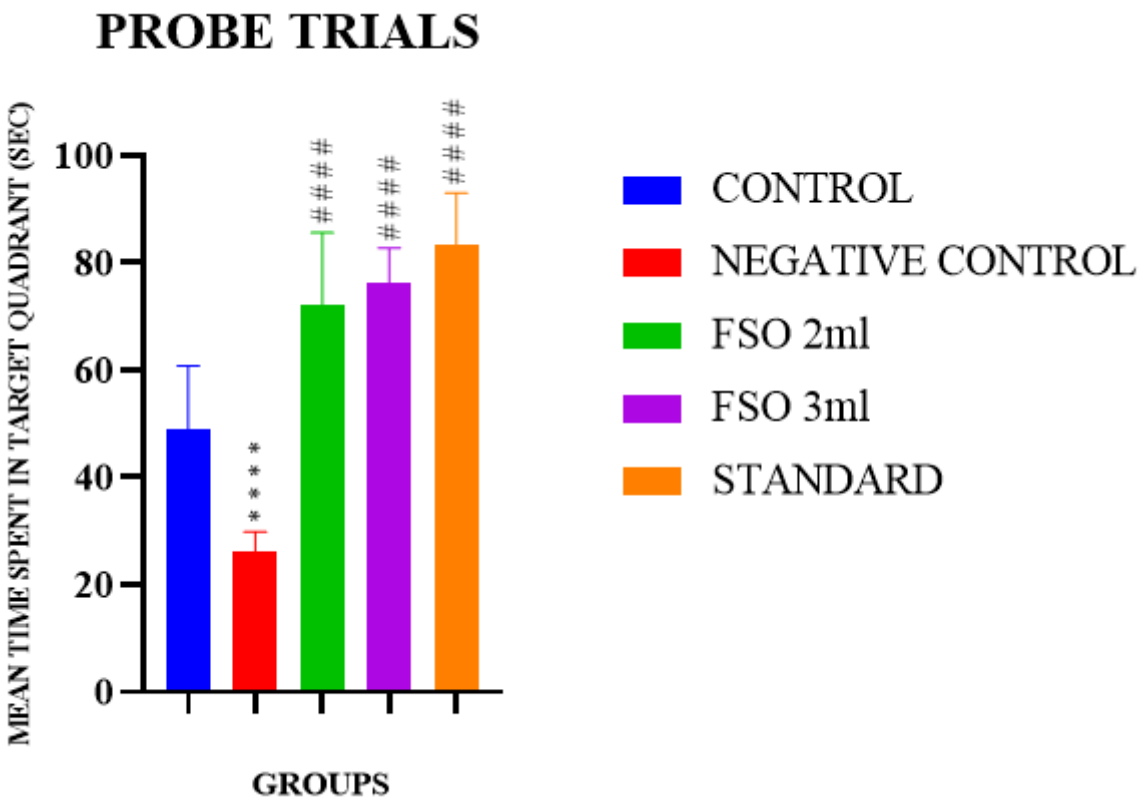



**Figure 3**

Effect of flaxseed oil on mean time spent in the target quadrant (seconds) on Cd-induced rats in the probe trials.

All data were analysed *via* two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests using GraphPad Prism software. Data presented are Mean ± SD (n =5). $^{****}$P<0.0001 when compared to control group and $^{\#\#\#\#}$P<0.0001 compared to negative control.

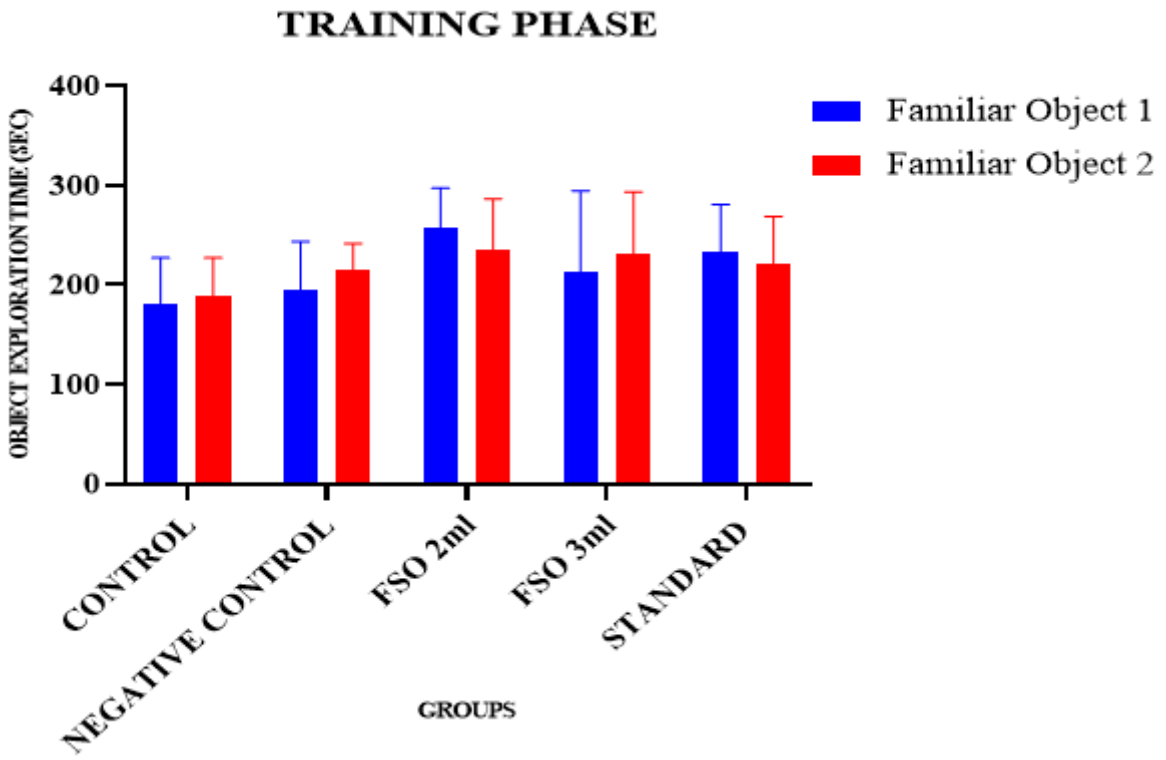



**Figure 4**

Effect of flaxseed oil on object exploration time in the training phase of novel object recognition test on Cd-induced rats.

Each bar represents the mean ± SD (n= 5).

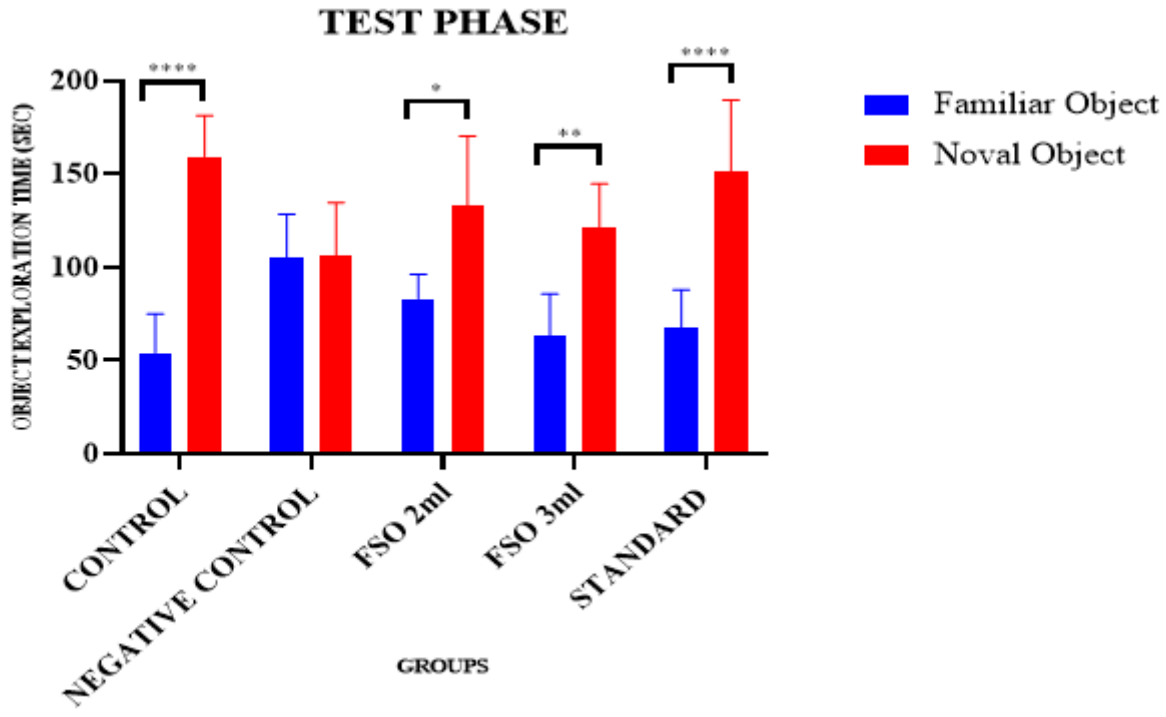



**Figure 5**

Effect of flaxseed oil on object exploration time in the test phase of novel object recognition test on Cd-induced rats in the test trials. All data were analysed *via* two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons tests using GraphPad Prism software. Data presented are Mean ± SD (n =5). ($^{*}$P<0.01, $^{**}$P<0.001, $^{****}$P<0.0001).

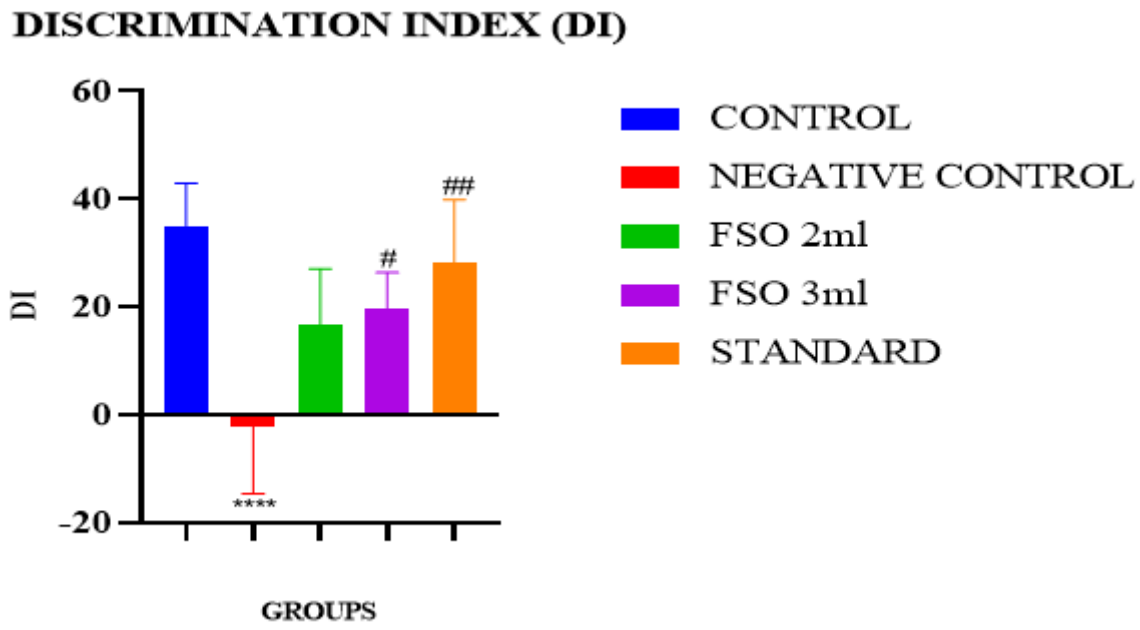



**Figure 6**

Effect of flaxseed oil on discrimination index (DI) in novel object recognition test on Cd-induced rats. All data were analysed *via* one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests using GraphPad Prism software. Data presented are Mean ± SD (n =5). $^{****}$P<0.0001 when compared to the control group and $^{\#}$P<0.01, $^{\#\#}$P<0.001 when compared to the negative control.

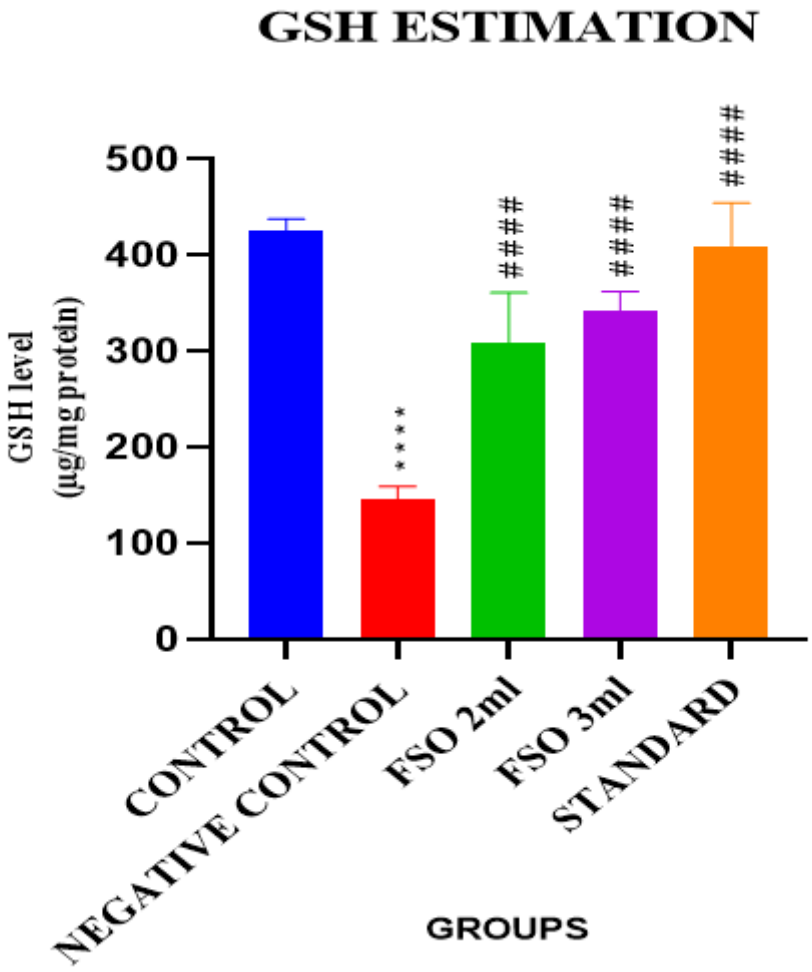
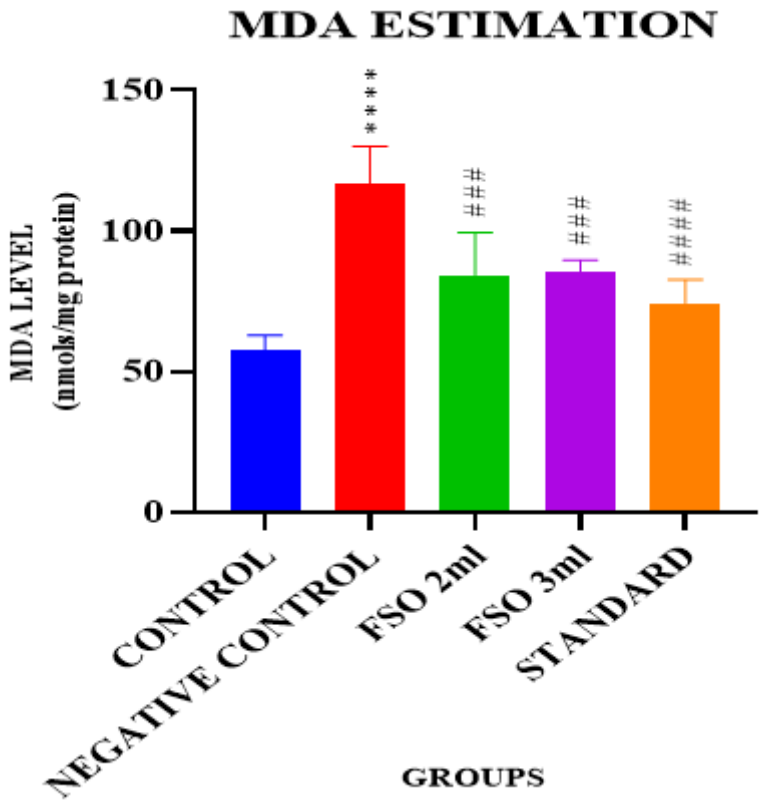



**Figure 7**

Ameliorative effects of the flaxseed oil on the levels of GSH in the brain homogenate of rats exposed to cadmium.

All data were estimated with the help of one-way ANOVA trailed by Tukey's multiple comparisons tests. Data presented are Mean ± SD (n =5). $^{****}$P<0.0001 when compared to the control group and $^{####}$P<0.0001 when compared to the negative control.




## Figure 8

Ameliorative effects of the flaxseed oil on the levels of MDA in the brain homogenate of rats exposed to cadmium.

All data were estimated with the help of one-way ANOVA trailed by Tukey's multiple comparisons tests using. Data presented are Mean ± SD (n =5). $^{****}$P<0.0001 when compared to control group and $^{###}$P<0.001 and $^{####}$P<0.0001 when compared to negative control.

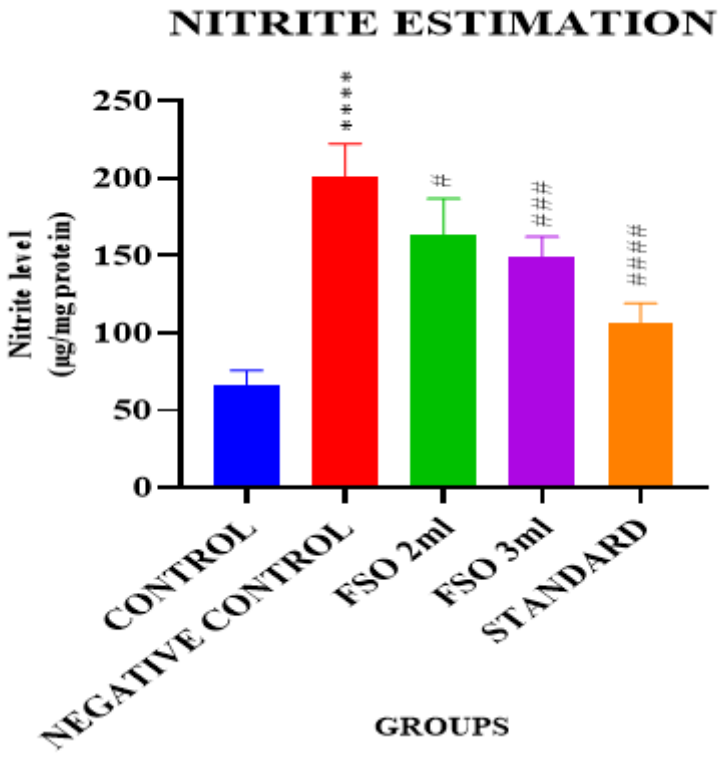
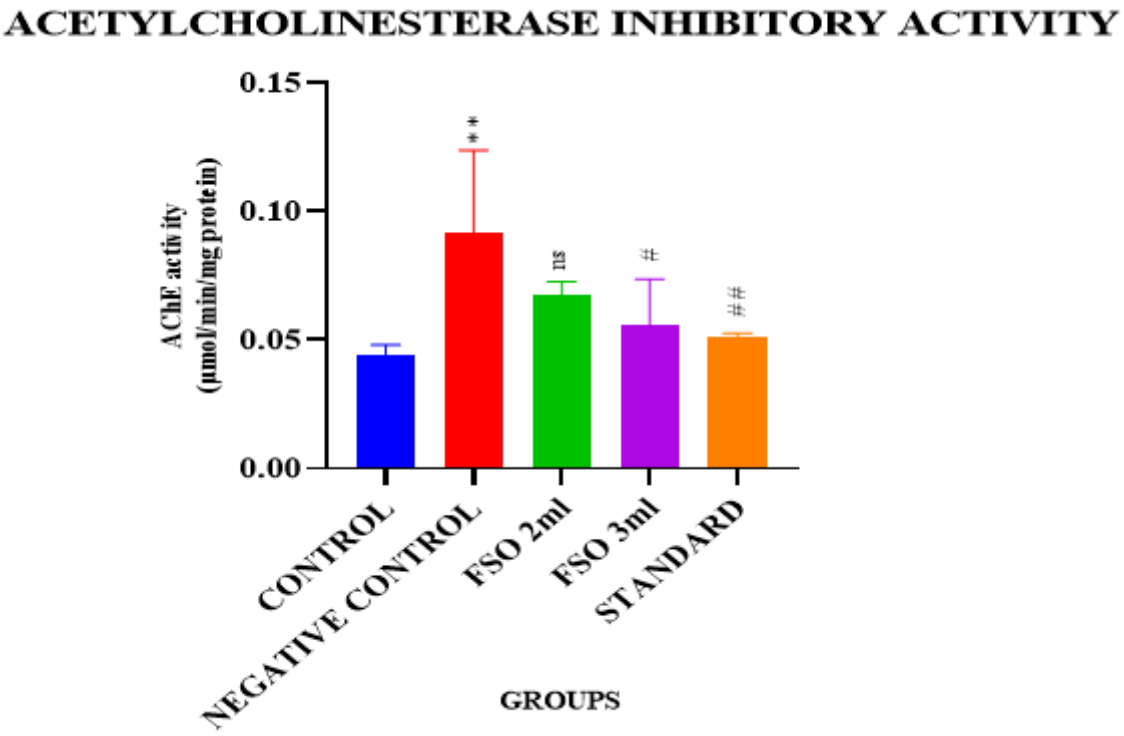



**Figure 9**

Effect of flaxseed oil on levels of NO level (µg/mg protein) in Cd-induced neurotoxicity in rat whole brain.

All data were estimated with the help of one-way ANOVA trailed by Tukey's multiple comparisons tests. Data presented are Mean ± SD (n =5). $^{****}$P<0.0001 when compared to control group and $^{\#}$P<0.01, $^{\#\#\#}$P,0.001 and $^{\#\#\#\#}$P<0.0001 when compared to negative control.




## Figure 10

Ameliorative effects of the flaxseed oil on the levels of AChE inhibitory activity in the brain homogenate of rats exposed to cadmium.

All data were estimated with the help of one-way ANOVA trailed by Tukey's multiple comparisons tests. Data presented are Mean ± SD (n =5). $^{**}$P=0.001 when compared to the control group and $^{\#}$P<0.01 and $^{\#\#}$P<0.001 when compared to the negative control.

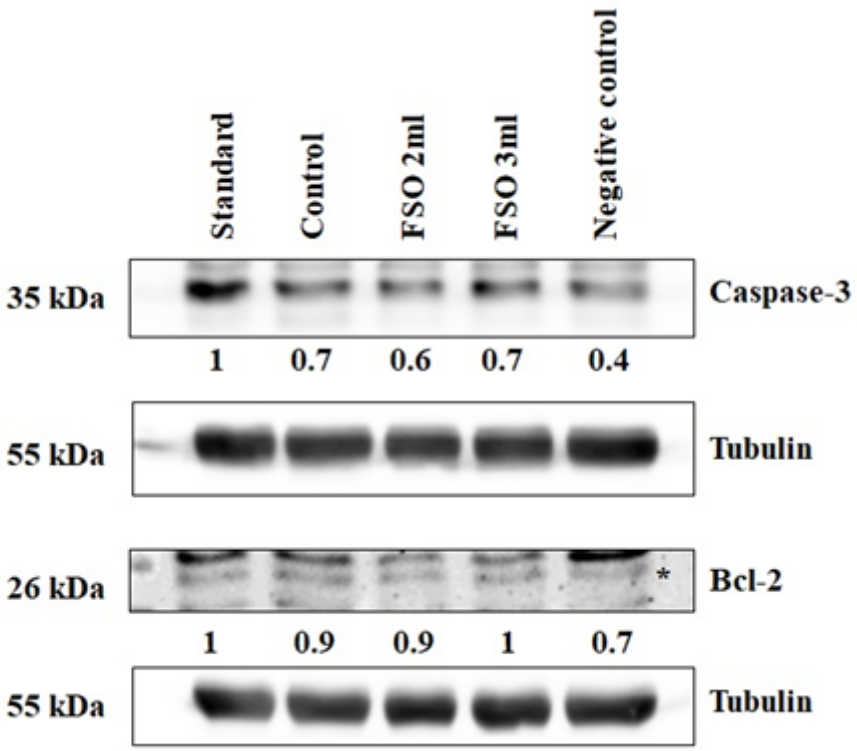



Figure 11

**Figure 13.** Anti-apoptotic effect of FSO

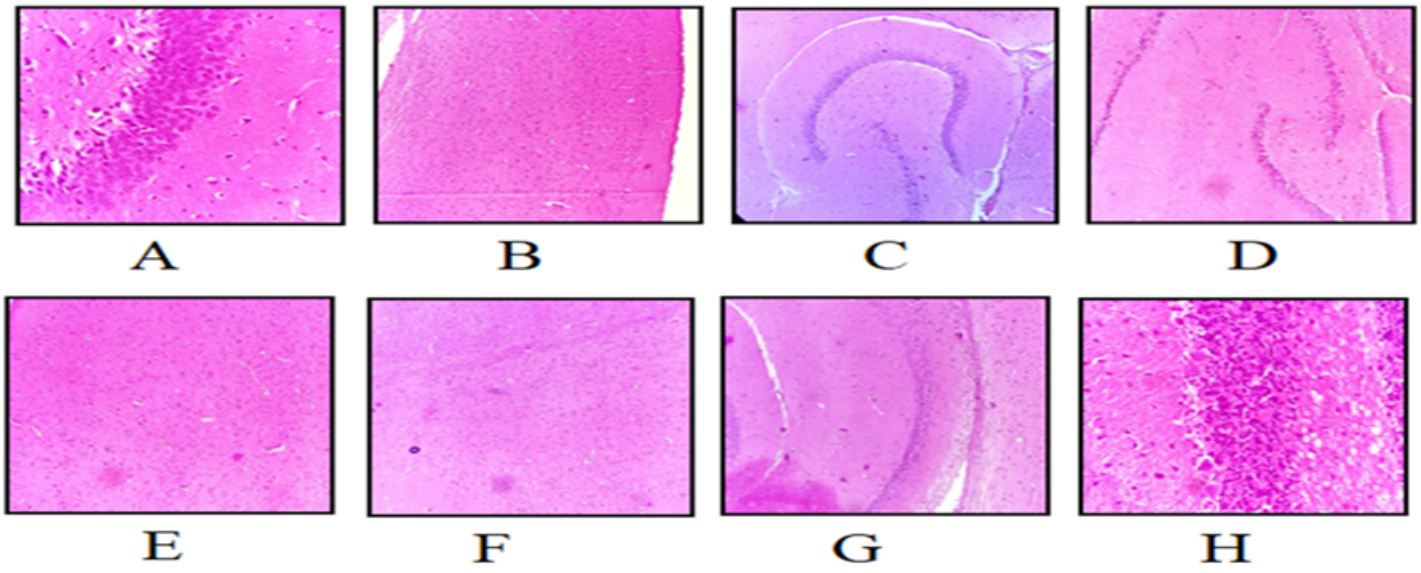



Figure 12

**Figure 14.** Histopathology of the brain of Wistar albino rat. Control group (A & B): It revealed the normal hippocampus and cerebral tissue of rats in the brain, Negative Control (C): It shows the existence of degenerative changes in neurons and the hippocampus showed degeneration of pyramidal cells, FSO 2ml (D & E): It shows the degeneration of pyramidal cells in the hippocampus, and degeneration of neuronal cell bodies, FSO 3ml (F & G): It shows the normal grey matter in the brain and the pyramidal cells were also normal in the hippocampus, Standard (H): It shows the mild degeneration of granular cell in the cerebellum while further structures looked normal.