

Original Research Article

Therapeutic potential of *Vitex negundo* against fluoride-induced cognitive and neurobehavioral impairments in male wistar albino rats

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Article history:

Received: Sep 26, 2025

Received in revised form:

Sep 03, 2026

Accepted: Sep 03, 2026

Epub ahead of print

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Keywords:

Vitex negundo leaf

Sodium fluoride

Cognition

Acetylcholinesterase

Oxidative stress

Abstract

Objective: Fluoride is an excitotoxin that overstimulates excitatory receptors and increases reactive oxygen species, leading to cognitive dysfunction. This study investigated the protective effects of *Vitex negundo* leaf hydroalcoholic extract (VNL) against fluoride-induced cognitive damage through behavioral and biochemical testing in male Wistar albino rats.

Materials and Methods: The GC-MS was used for identification of compounds present in the VNL. In treatment protocol, Groups I and II were designated as the normal and sodium fluoride (NaF) control groups, respectively, Group III as the VNL control (400 mg/kg body weight) group, and Groups IV and V as treatment groups which received VNL orally at a dose of 200 and 400 mg/kg body weight, respectively. All groups except I and III received 100 ppm of sodium fluoride in their drinking water.

Results: GC-MS analysis of the VNL extract revealed a total of nine major compounds, such as glyceraldehyde, d-glucosamine, DL-arabinose, catechol, acetic acid, methyl 6-oxoheptanoate, 2,2'-[oxybis(2,1-ethanedioxy)] bis-, melezitose, diethyl phthalate, and d-mannose. Fluoride administration altered neurobehavioral parameters as evidenced by changes in locomotor activity, forced swim test, and Y- and T-shaped runway performance. Additionally, it significantly increased lipid peroxidation and decreased brain acetylcholinesterase, superoxide dismutase, reduced glutathione, and catalase levels. Treatment with VNL lowered brain lipid peroxidation while elevating levels of acetylcholinesterase, superoxide dismutase, reduced glutathione, and catalase. Behavioral changes also improved significantly in the treatment groups.

Conclusion: Treatment with VNL significantly improved cognition in fluoride-exposed Wistar albino rats, potentially through its antioxidant properties.

Please cite this paper as:

Raghavendra M, Rajesham V, Raghuvver Yadav P, Ravichandra Sh. Therapeutic potential of *Vitex negundo* against fluoride-induced cognitive and neurobehavioral impairments in male Wistar albino rats. Avicenna J Phytomed, 2026

Introduction

Cognitive processes include thinking, awareness, problem solving, and memory formation. When someone has trouble with their dynamic abilities and remembering items from their daily lives, it is called cognitive impairment (Trivedi 2006). The primary facts concerning cognitive impairment are memory loss, observation, expansion of mentality, and not acquainting with custom-made individuals or areas. Age, traumatic brain damage, and exposure to heavy metals, herbicides, or poisons are the main risk factors for cognitive decline (Sharbafshaaer 2018; Mavroudis et al. 2024).

The Intelligence Quotient (IQ) is a score assesses cognitive capacity relative to the general population. It is typically used to gauge a person's aptitude for idea comprehension and problem-solving (Mohn et al. 2014). Numerous neurophysiological characteristics have been linked to IQ, including the possibility that IQ may be affected by reductions in frontal projection size, grey matter volume, and cortical width. Although a variety of factors can affect an individual's IQ, including age and environmental contaminants such as fluoride, excitotoxicity is the common mechanism underlying most neurodegenerative illnesses (Burgaleta et al. 2024).

Excitotoxicity occurs when glutamate and aspartate accumulate at synapses. These neurotransmitters overstimulate glutamate receptors on the postsynaptic neuron. If not cleared quickly, this overstimulation damages the neuron, leading to dendritic shrinkage, synaptic loss, and ultimately cell death (Gillissen et al. 2003).

Excitotoxicity is a major cause of neuronal death, and fluoride plays a significant role in this process (Blaylock 2004). Fluoride increases free radical production, damages dendrites, synapses, and mitochondria, reduces glutamate reuptake, and elevates lipid peroxidation while lowering antioxidant enzymes such

as superoxide dismutase, glutathione reductase, and catalase. It also disrupts mitochondrial energy production by impairing glycolysis and the Krebs cycle, which can lead to Alzheimer's and dementia. Furthermore, fluoride activates microglial cells which release harmful substances such as glutamate and quinolinic acid. These combined effects, particularly its accumulation in the hippocampus, make fluoride a potent driver of neurotoxic and excitotoxic events (Adkins and Brunst 2021).

Vitex negundo, sometimes referred to as a humble tree with five leaves, is a large, fragrant shrub found in India, East Africa, Malaysia, the Philippines, and the warmer regions of Taiwan (Venkateswarlu 2017). From the literature on *V. negundo* have shown that the leaves can strengthen cells due to the high levels of total phenolic content which includes quercetin, myricetin, epicatechin, and catechin (Wu et al. 2024). *Vitex negundo* also contains lignans having anti-cholinesterase activity (Hung et al., 2007; Vannur et al. 2022). Therefore, this study evaluated the cognitive effects of *V. negundo* on oxidative stress and psychological impairment caused by fluoride in male adult Wistar albino rats.

Materials and Methods

Ethical issues

This study used animals from Sai Thirumala Enterprises in Hyderabad, India. The Institutional Animal Ethics Committee (IAEC) of CMR College of Pharmacy, Hyderabad, India, approved the experimental protocols (Approval No. CPCSEA/1657/IAEC/CMRCP/COL-19/72).

Drugs and chemicals

Drugs and chemicals used in the study were procured from Himedia Laboratories Pvt. Ltd, while diagnostic kits were

obtained from Erba Diagnostics Mannheim, India.

Collection and botanical identification of plant material

Vitex negundo leaves were collected from the Kushaiguda, Medchal District, Telangana, India. Dr. P.V. Prasanna of the Botanical Survey of India in Hyderabad identified the plant material (Voucher specimen number: BSI/DRC/2019-20/Tech./294).

Preparation of the plant extract

Vitex negundo leaves were washed, shade-dried, and ground into a coarse powder. The powdered material was subsequently extracted with a 50:50 water-alcohol mixture. For one week, the blend was incubated at room temperature with gentle mixing from time to time. Following filtration, a rotary flash evaporator concentrated the extract before storage at 4°C.

Gas chromatography-mass spectrometry (GC-MS) analysis

Thermo DSQ GC-MS was used to identify the phytochemicals in the *V. negundo* leaf hydroalcoholic extract (VNL). The equipment had a DB-35ms GC column, and helium was used as the carrier gas at a flow rate of 1.2 ml/min. The injection port was set to 250°C, and the oven temperature gradient was as follows: the initial temperature was 60°C for 53.5 min, then increased to 280°C over 3 min, and finally maintained at 280°C for 12 min. Component identification relied on the Willey and NIST libraries and comparison of their retention indices.

Experimental animals

The study used male adult Wistar albino rats (200–230 g, 8–10 weeks old) supplied by Sai Tirumala Enterprises, Hyderabad, India. Rats were placed in the animal facility and acclimatized to the laboratory environment (25 ± 3°C, 60 ± 5% relative

humidity, and 12 hr light/dark cycle) as per CCSEA guidelines, New Delhi.

Experiment protocol

A blocking technique was used to randomly divide 30 male adult Wistar albino rats into 5 groups of 6 animals each. The test doses were selected based on our laboratory's earlier studies (Raghavendra et al. 2022)

Group I: Received distilled water for four weeks (Normal control).

Group II: Received sodium fluoride (NaF) in distilled water (100 ppm) for four weeks (NaF control).

Group III: Received VNL at a dose of 400 mg/kg, b. wt daily, per oral for four weeks (VNL control).

Group IV: Received a dose of VNL 200 mg/kg b. wt daily, per oral with NaF water (100 ppm) for four weeks.

Group V: Received a dose of VNL 400 mg/kg b.wt daily, per oral with NaF water (100 ppm) for 4 weeks.

After completing the treatment schedule, under mild ether anesthesia, cervical dislocation was performed to euthanize the rats for brain tissue collection. One brain tissue sample from each group was fixed in 10% formalin for histopathological investigation, and the remaining was used to assess oxidative stress markers.

Design of the behavioral experiments

Locomotor activity, T- maze, Y- maze, and Forced swim test were performed on the 1st, 7th, 14th, 21st and 28th day.

Locomotor activity

An Actophotometer (Slisco, PL-153) was used to track the animal's locomotor activity. Thirty minutes after therapy, each animal was placed in an Actophotometer for five minutes to record its basal activity (Gosavi et al. 2020).

T-maze test

The T-maze test consisted of acquisition and retention phases. Animals were trained for seven consecutive days

during the acquisition phase. On the first day of the subsequent retention/treatment phase, food was placed in the previously selected left arm before the trial began. Following overnight food deprivation, each animal was kept in the start box for 60 sec, after which the sliding door was opened to allow the animal to enter the goal arms. Entry into the previously baited left arm was recorded as a correct choice. Working-memory performance was assessed based on the number of correct choices and working-memory errors, in accordance with the method described by Lazarova *et al.* 2024. The working-memory index (WMI) was calculated as the number of correct choices divided by the total number of trials.

Y-maze test

To test the rats' spatial memory, a three-arm-Y- maze (55 × 10 × 15 cm) was set up at 120°. Normal rats tend to favor different arms of the maze because they can recall which arm they visited on their previous visit. Each rat was allowed to roam freely in arms A, B, and C for five minutes, during which their natural inclinations towards each arm were recorded. The order in which the rats entered each arm was monitored to quantify behavioral alterations and evaluate the effect of VNL on the memory of fluoride-amnesic rats (Kim *et al.* 2023). The indicator of improved memory, the spontaneous alternation (SAP), was computed as:

$$\% \text{ SAP} = \frac{\text{[Number of alternations)]}}{\text{Total arm entries} - 2} \times 100$$

Forced swim test

Rats were trained on the apparatus for five days to become familiar with the task prior to the fluoride administration. The maze has a circular tank-shaped pool measuring 120 cm in diameter and 50 cm in height. The water in the tank was kept at 27 ± 2°C and filled to a depth of 30 cm. Four equal quadrants were used to divide the maze. Throughout the experiment, a tiny, rectangular platform was hidden in the

southeast quadrant. The platform was buried 1 cm below the water surface during the evaluation period, but it remained 1 cm above the water surface during the training season (Rezvani-Kamran *et al.* 2017).

Preparation of tissue homogenate

Brain tissue was homogenized in ice-cold 1.15% KCl using a homogenizer, and the resulting homogenate was centrifuged at 800 × g for 5 min at 4°C (REMI C-24) to pellet cellular debris. The supernatant was further centrifuged at 10,000 rpm for 20 min at 4°C to yield the post-mitochondrial supernatant (PMS). The PMS was used to quantify acetylcholinesterase (AChE) levels and the levels of superoxide dismutase, reduced glutathione, lipid peroxidation, and catalase (Dorado-Martínez *et al.* 2024).

Estimation of AChE

AChE level was measured in brain tissue using Ellman's method (Abomosallam *et al.* 2024).

Estimation of oxidative stress markers

The Lipid peroxidation (Ohkawa 1979), superoxide dismutase (Muthulakshmi *et al.* 2018), reduced glutathione (Mohammadzadeh *et al.* 2018) and catalase (Afolabi *et al.* 2019) levels were measured by analyzing the brain tissue homogenate with standard procedures.

Histopathological study

The histopathology of the brain hippocampus was performed by the Hematoxylin and Eosin (H&E) staining process.

Statistical analysis

Kolmogorov-Smirnov test was used to check data normality. Results show Mean ± SEM. One-and two-way ANOVA, with post hoc Dunnett's test, were used to evaluate statistical significance using Graph Prism software.

Results

The result of GC-MS investigation of VNL is presented in Table 1 and Figures 1A and 1B. The analysis identified a total of 9 compounds such as glyceraldehyde, d-

glucosamine, DL-arabinose, catechol, acetic acid, methyl 6-oxoheptanoate, 2,2'-[oxybis(2,1-ethanediylxy)] bis-, melezitose, diethyl phthalate, and d-mannose.

S. No.	Retention Time	Area %	Compound	Formula	M.W g/mol
1	2.404	1.82	Glyceraldehyde	C ₃ H ₆ O ₃	90.08
2	2.961	1.17	d-Glucosamine	C ₆ H ₁₃ NO ₅	179.17
3	6.945	1.50	DL-Arabinose	C ₅ H ₁₀ O ₅	150.13
4	8.525	2.25	Catechol	C ₆ H ₆ O ₂	110.11
5	9.560	0.80	Acetic acid, 2,2'-[oxybis(2,1-ethanediylxy)] bis-	C ₈ H ₁₄ O ₇	222.19
6	10.335	2.42	Methyl 6-oxoheptanoate	C ₈ H ₁₄ O ₃	158.19
7	14.273	5.90	Melezitose	C ₁₈ H ₃₂ O ₁₆	504.4
8	18.192	47.10	Diethyl Phthalate	C ₁₂ H ₁₄ O ₄	222.24
9	18.654	2.19	d-Mannose	C ₆ H ₁₂ O ₆	180.16

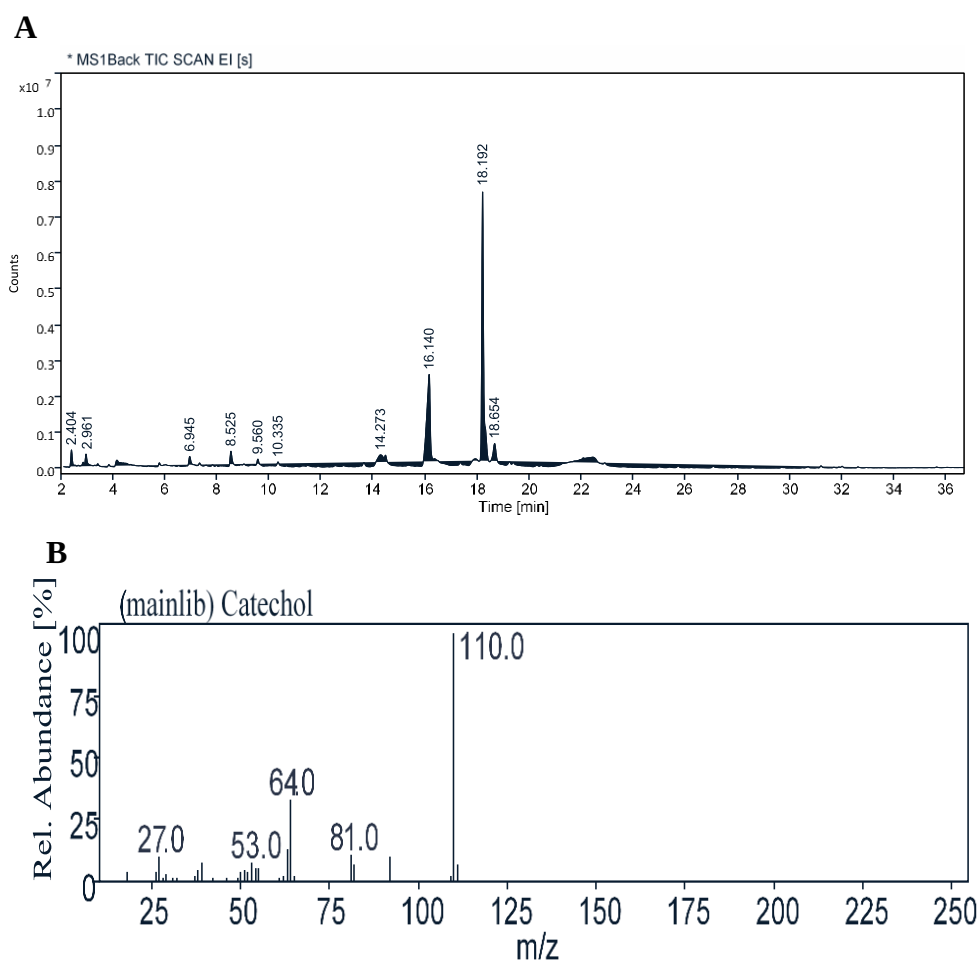


Figure 1. A. GC-MS total ion chromatogram of VNL. B. Mass spectrum of GC peak with retention time 8.525 identified from mainlib as Catechol (110.0)

Effect of VNL on locomotor activity

Figure 2 shows the effect of VNL on weekly changes in locomotor activity. No significant variations were observed in NaF-exposed rats up to day 14 compared with the normal control. However, locomotor activity significantly decreased on days 21 and 30 in the NaF control group compared with the normal control. Treatment with VNL at 200 and 400 mg/kg b. wt. doses showed a significant ($p<0.01$) improvement in the locomotor activity on the days 21 and 30 of the study as compared to the NaF control. The VNL control showed much more locomotor activity than the normal control group from day 14 through the end of the study.

Effect of VNL on working memory index (T- and Y- Maze)

The working memory index decreased significantly in the NaF control compared with the normal control. Compared to the NaF control group, treatment with VNL (200 and 400 mg/kg b. wt.) significantly increased ($p<0.01$) the working memory index on days 14, 21, and 30 of the study. No significant variation was observed in the VNL control compared to the normal control. Weekly changes in the working memory index are shown in Figures 3 and 4.

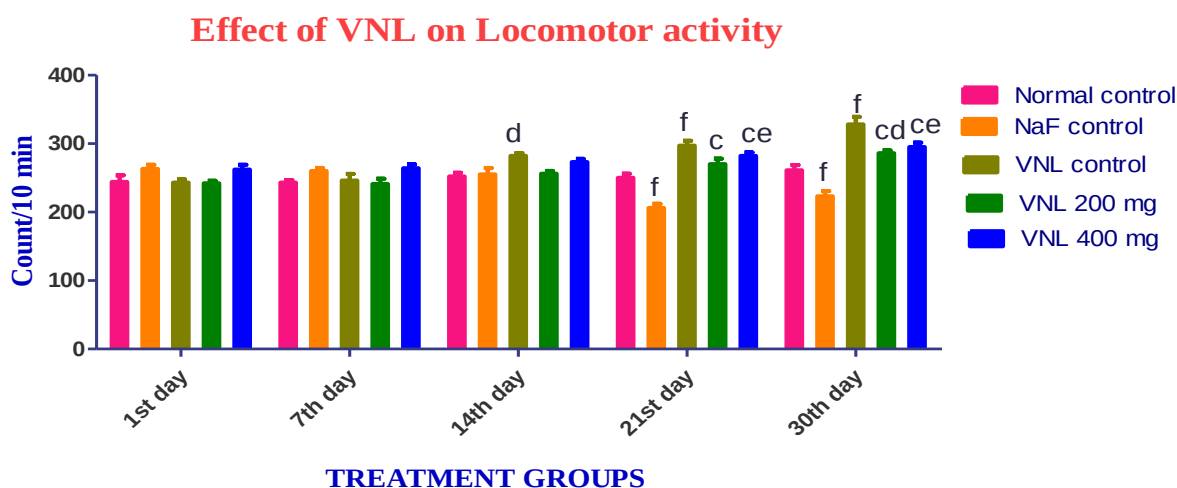


Figure 2. Effect of VNL on locomotor activity. ^a $p<0.001$, ^b $p<0.01$ and ^c $p<0.05$ Vs NaF control; ^d $p<0.001$, ^e $p<0.01$ and ^f $p<0.05$ Vs Normal control.

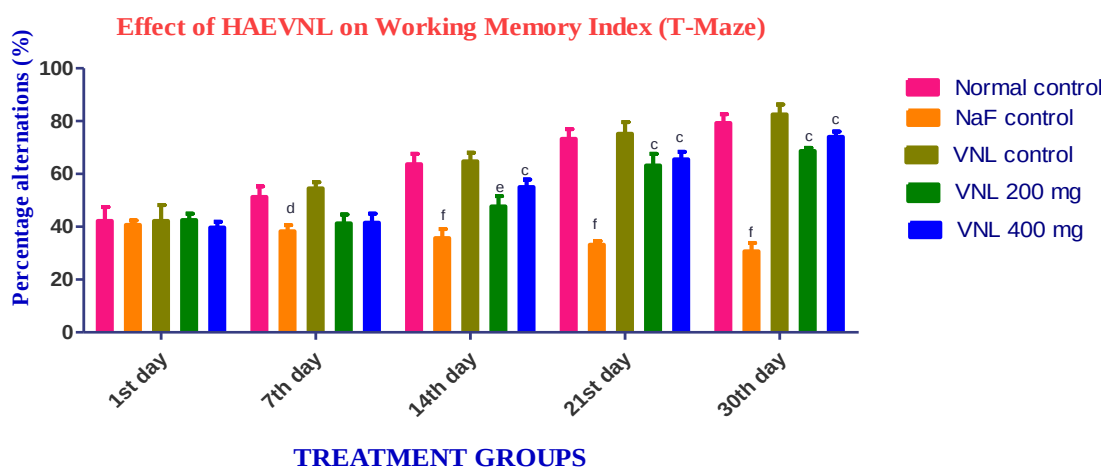


Figure 3. Effect of VNL on working memory index (T-Maze). ^a $p<0.001$, ^b $p<0.01$ and ^c $p<0.05$ Vs NaF control; ^d $p<0.001$, ^e $p<0.01$ and ^f $p<0.05$ Vs Normal control.

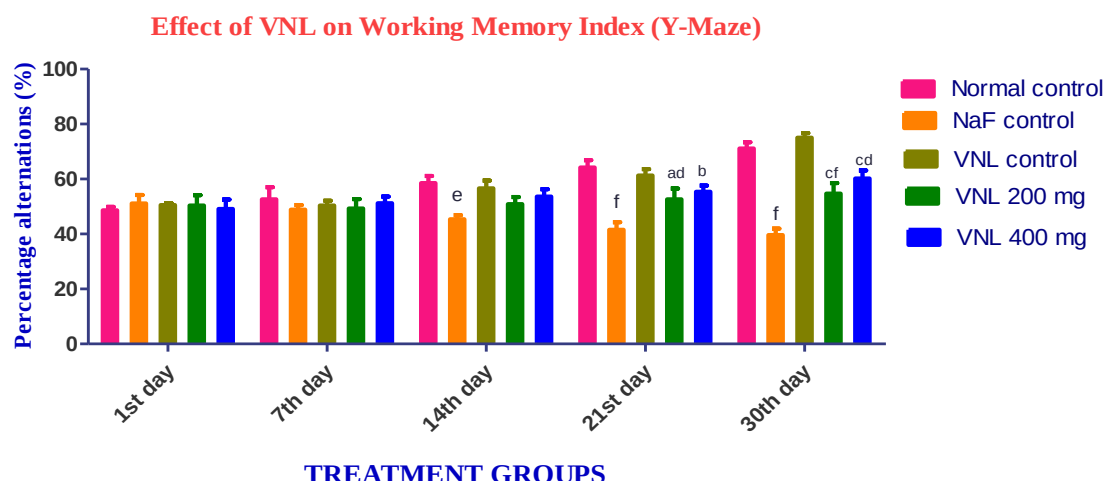


Figure 4. Effect of VNL on working memory index (Y-Maze). ^a $p < 0.001$, ^b $p < 0.01$ and ^c $p < 0.05$ Vs NaF control; ^d $p < 0.001$, ^e $p < 0.01$ and ^f $p < 0.05$ Vs Normal control.

Effect of VNL on forced swim test

The effect of VNL on weekly changes in the immobility time is specified in Figure 5.

Through day 7, NaF exposure did not significantly alter animal immobility time relative to the normal control. Immobility time increased significantly in the NaF control compared with the normal control on days 21 and 30. Compared to the NaF control group, VNL treatment (200 and 400 mg/kg b. wt.) significantly decreased immobility time on days 14, 21 and 30 ($p < 0.01$). From day 7 onwards, the VNL

control showed a significant decrease in immobility time compared to the normal control group.

Effect of VNL on brain AchE level

Figure 6 shows the effect of VNL on AchE levels in the brain tissue homogenate. AchE levels decreased significantly in the NaF control compared to the normal control. Compared to the NaF control group, VNL treatment at 400 mg/kg b. wt dose significantly increased AchE levels ($p < 0.01$). No significant change in AchE level was observed in the VNL control when compared to the normal control.

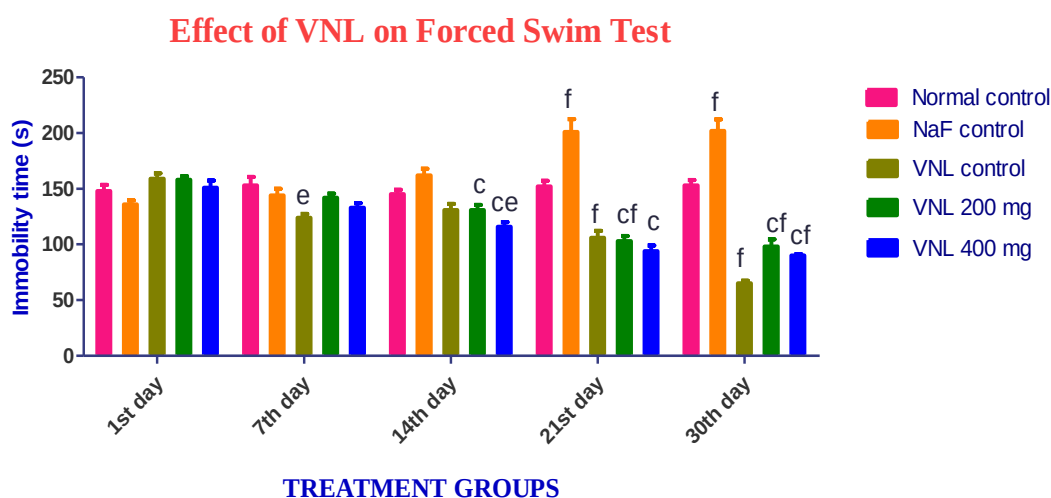


Figure 5. Effect of VNL on forced swim test. ^a $p < 0.001$, ^b $p < 0.01$ and ^c $p < 0.05$ Vs NaF control; ^d $p < 0.001$, ^e $p < 0.01$ and ^f $p < 0.05$ Vs Normal control.

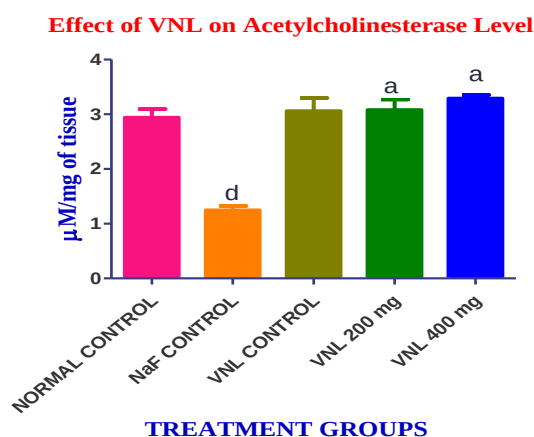


Figure 6. Effect of VNL on brain acetylcholinesterase level. ^a $p < 0.001$, ^b $p < 0.01$ and ^c $p < 0.05$ Vs NaF control; ^d $p < 0.001$, ^e $p < 0.01$ and ^f $p < 0.05$ Vs Normal control.

Effect of VNL on brain oxidative stress markers

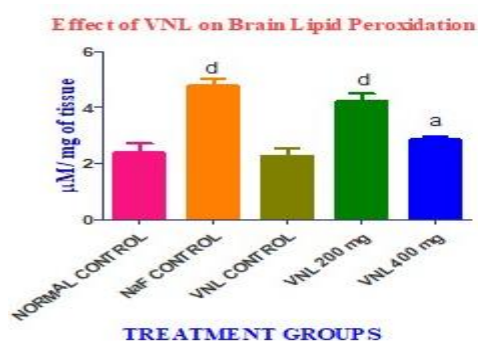
Figure 7 summarizes how VNL affected brain tissue homogenate oxidative stress markers including lipid peroxidation, superoxide dismutase, reduced glutathione, and catalase. The NaF control showed higher lipid peroxidation and lower levels

of superoxide dismutase, reduced glutathione, and catalase than the normal control. Compared to the NaF control, VNL treatment at 400 mg/kg b. wt. dose significantly reduced lipid peroxidation ($p < 0.05$) and concurrently boosted superoxide dismutase, reduced glutathione, and catalase levels.

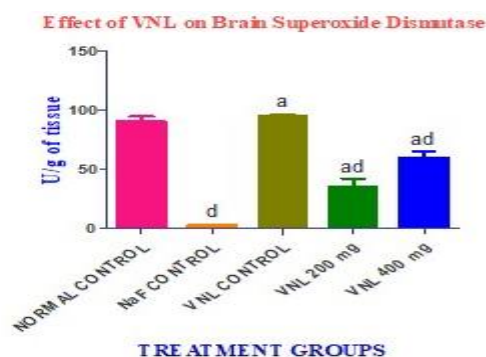
Histopathology study

Brain tissues from the normal and VNL control groups showed no hippocampal pathology.

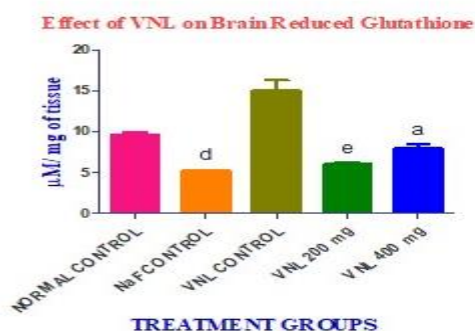
The NaF control group exhibited 90% degeneration in the hippocampal CA1, CA3, and CA4 regions. The hippocampus tissue from the VNL 200 mg/kg treatment showed moderate (60%) damage in the CA1 and CA3 and mild damage in the CA4 region, while the VNL 400 mg/kg treatment showed a smaller extent of neuronal damage (10%) in the CA4 region, while the CA1 and CA3 regions remained normal. The histopathological changes are shown in Figure 8.



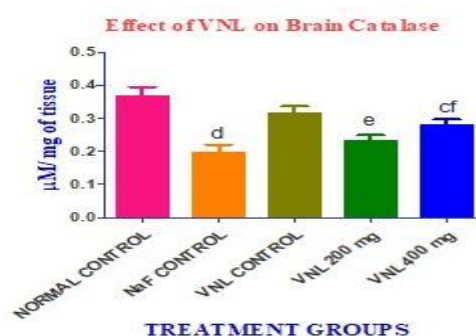
(a)



(b)



(c)



(d)

Figure 7. Effect of VNL on brain oxidative stress markers (a-d) (a: Brain Lipid Peroxidation level, b: Brain Superoxide Dismutase level, c: Brain Reduced Glutathione level, d: Brain Catalase level). ^a $p < 0.001$, ^b $p < 0.01$ and ^c $p < 0.05$ Vs NaF control; ^d $p < 0.001$, ^e $p < 0.01$ and ^f $p < 0.05$ Vs Normal control.

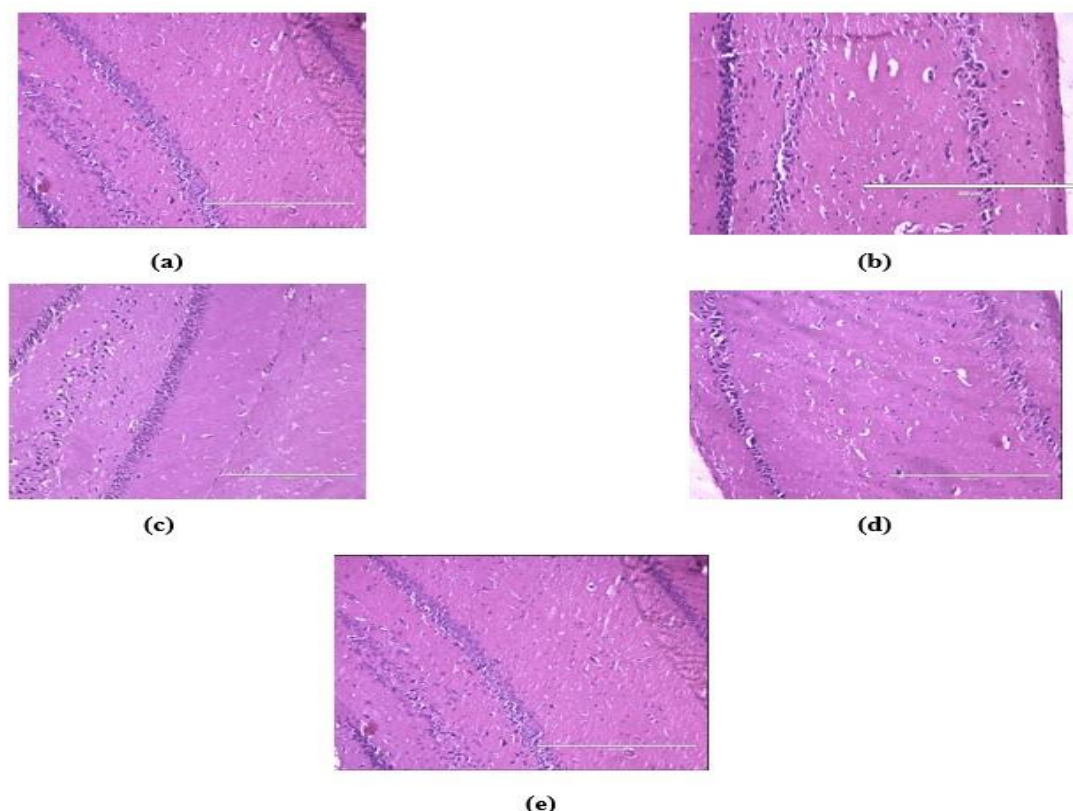


Figure 8. Effect of VNL on hippocampus structure (a-e). (a: Normal control group, b: NaF control group, c: VNL control group, d: VNL 200 mg/kg group, and e: VNL 400 mg/kg group) (Magnification 40x and Scale bar 400 μm)

Discussion

The primary findings of this investigation reveal that fluoride exposure profoundly affects the brain, inducing behavioral alterations, changes in AchE, oxidative stress, and histopathological changes. Fluoride easily moves through the blood-brain barrier, allowing it to accumulate in the brain. Long-term exposure to high levels of fluoride has been shown to alter brain architecture, leading to potential neurological consequences (Zwierello et al. 2023). The prefrontal cortex, a brain area, helps control attention, choices, memory, emotional processing, and behavioral adaptability (Dec et al. 2017). Epidemiological studies in regions with elevated fluoride levels in drinking water have revealed a statistically significant correlation between fluoride exposure and reduced cognitive function in children, compared with their counterparts

in non-contaminated areas (Taylor et al. 2025). According to Niu et al. 2015, continuous exposure to elevated fluorine concentrations (10, 25, and 50 mg/L) led to a substantial accumulation of fluoride in mice brain, with levels reaching 250 times those observed in the control group. A study revealed that adult mice with 100 mg/L fluoride in their drinking water had reduced expression of tubulin heterodimers in the hippocampal region (Dec et al. 2017). Fluoride exposure (5 mM) induces neuronal dysfunction in the adult rat and mouse hippocampus by perturbing chloride ion homeostasis and modulating the activity of MAP kinase family proteins. Consequently, this results in reduced neuronal volume and impaired neuronal function (Lee et al. 2016). Variations in animal body weight were reported in our previously published research article (Raghavendra et al. 2022).

The brain weights of animals exposed to fluoride in this study did not change much.

Fluoride exposure has been unequivocally linked to cognitive impairment, particularly in learning and memory abilities. Epidemiological investigations and animal behavioral studies have consistently demonstrated these adverse effects. The hippocampus is particularly vulnerable to fluoride-induced toxicity. T- and Y-maze tests, locomotor activity, and the forced swim test were used to assess cognitive and memory abilities (Yuan et al. 2019; Harquin Simplicite et al. 2014; Bittencourt et al. 2023). The T- and Y-maze tests are widely employed to evaluate short-term memory in animals. These assessments enable measurement of spatial working memory which is mediated by the prefrontal cortex, and spatial reference memory which is subserved by the hippocampus (d'Isa et al. 2021; Kraeuter et al. 2019). Administration of 100 ppm/L NaF resulted in substantial cognitive deficits, as evidenced by increased error rates, prolonged reaction times, and decreased correct response rates. These results imply that fluoride exposure may contribute to impairments in intelligence, learning, and memory, as well as altered neuronal activity and locomotor function (Mullenix et al. 1995; Niu et al. 2008). Administration of VNL at 400 mg/kg b. wt dose was found to be devoid of adverse effects and notably facilitated significant improvements in learning, memory, and locomotor function. These results corroborate the earlier findings reported by Swamy et al. 2024 and Raghu et al. 2013.

Fluoride toxicity could result in brain tissue damage, characterized by reduced choline acetylase activity, leading to impaired memory retention. Acetylcholine, a key neurotransmitter, plays a vital role in recognition and memory processes. High fluoride levels damage brain tissue and may lead to a deficiency in cholinergic neurotransmission, contributing to the observed poor learning ability in fluoride-exposed animals (Zhang et al. 2007). AChE

stops cholinergic transmission by hydrolyzing acetylcholine at the synaptic cleft. AChE is essential for maintaining normal brain physiology, learning, and memory. Our study revealed decreased AChE expression in fluoride-treated groups, implying a potential link between fluoride exposure and AChE inhibition (Pal and Sarkar 2014). Furthermore, VNL extract treatment restored AChE levels in rats exposed to sodium fluoride.

The ensuing buildup of reactive oxygen species precipitates a cascade of deleterious effects, including metabolic dysregulation, inflammatory responses, and impaired cellular differentiation, maturation, and proliferation, culminating in tissue damage. The accumulation of fluorine in the central nervous system triggers oxidative stress in both juvenile and adult specimens. *In vitro* studies utilizing hippocampal neuron cultures exposed to fluorine (40-80 mg/L) for 48 hr revealed a significant increase in reactive oxygen species production and lipid peroxidation, accompanied by a decrease in antioxidant enzyme activity (Superoxide dismutase and glutathione peroxidase) and glutathione levels, ultimately contributing to cognitive impairments in learning and memory (Shalini et al. 2015). Chronic administration of 20 mg/kg fluorine to adult rats every 24 hr resulted in a significant depletion of reduced glutathione levels in the brain, accompanied by an increase in OH⁻ radical production and enhanced lipid and protein oxidation in the cerebral cortex, cerebellum, and medulla oblongata. Comparable effects were reported in rats exposed to fluorine-contaminated drinking water at concentrations of 50 and 150 mg/L (Akinrinade et al. 2015). Administration of VNL at 400 mg/kg b.wt dose resulted in significant normalization of lipid peroxidation and a marked increase in the levels of superoxide dismutase, glutathione, and catalase. These biochemical changes were paralleled by improvements in cognitive function, as evidenced by enhanced performance in maze studies,

compared with animals exposed to NaF alone. Histopathological changes also further agreed with the behavioral and oxidative stress parameters of the study.

The present study reveals that exposure to sodium fluoride may alter behavioral patterns, learning, and memory abilities. Treatment with *V. negundo* significantly mitigates fluoride-induced neurotoxicity, restoring normal behavioral patterns and learning and memory. This beneficial effect might be due to catechol which acts as a potent antioxidant and neuroprotective agent. Molecular studies are required to know the exact biological reasons.

Acknowledgment

The authors are thankful to Secretary and Correspondent, Dr. Ch. Gopal Reddy, CMR College of Pharmacy, Kandlakoya (V), Medchal, Hyderabad, India.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical Considerations

This study used animals from Sai Thirumala Enterprises in Hyderabad, India. The Institutional Animal Ethics Committee (IAEC) of CMR College of Pharmacy, Hyderabad, India, approved the experimental protocols.

Code of Ethics

CPCSEA/1657/IAEC/CMRCP/COL-19/72

Authors' Contribution

Conceptualization and Methodology, RM; Investigation, RM, VVR; Software and Formal analysis, RM, SR; Writing original draft, RM; Writing-review and editing, PRY. All authors have read and approved the manuscript.

Abbreviations

VNL: *Vitex negundo* leaf hydroalcoholic extract

NaF: Sodium fluoride

GC-MS: Gas chromatography–Mass spectrometry

AchE: Acetylcholinesterase

CA: Cornu Ammonis

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