

Neurobehavioral and Neurochemical Effect of *Azadirachta indica* on Stress Induced Anxiety in Mice

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ABSTRACT

Background: Anxiety disorders represent a major global mental-health burden characterized by exaggerated fear and stress responses. Neurochemical imbalances involving GABA, serotonin, dopamine, and noradrenaline underlie these conditions. Synthetic anxiolytics such as benzodiazepines and SSRIs, though effective, often produce adverse effects. Hence, herbal alternatives like *Azadirachta indica* (Neem) are being explored for safer therapy.

Objective: To evaluate the anxiolytic and neuroprotective potential of ethanolic extract of *Azadirachta indica* leaves in stress-induced anxiety in mice, with emphasis on behavioral and biochemical correlates.

Methods: Leaves of *A. indica* were authenticated and extracted using Soxhlet apparatus with ethanol. Phytochemical screening confirmed the presence of alkaloids, flavonoids, phenols, tannins, and carbohydrates. Swiss albino mice were divided into five groups: positive control, negative control, standard (diazepam 4 mg/kg), and test groups (100 mg/kg and 200 mg/kg extract). Anxiety was induced by immobilization stress. Behavioral assays included Elevated Plus Maze, Light–Dark Box, Open Field Test, and Actophotometer. Biochemical parameters—plasma nitrate (Griess assay), iNOS, AChE, and GABA were quantified, and histopathological evaluation of brain tissue was performed.

Results: The extract produced dose-dependent anxiolytic effects, increasing time spent in open arms and light zones while reducing locomotor activity. Biochemically, *A. indica* lowered plasma nitrate and AChE levels, elevated iNOS and GABA concentrations, and restored neuronal morphology. The 200 mg/kg dose showed results comparable to diazepam, confirming modulation of nitric-oxide and GABAergic pathways.

Conclusion: The ethanolic extract of *Azadirachta indica* exhibits significant anxiolytic and neuroprotective activity through antioxidant and neurotransmitter-modulating mechanisms. These findings validate its traditional use and suggest its potential as a natural alternative for managing anxiety disorders.

Keywords: *Azadirachta indica*, Anxiolytic activity, Neuroprotection, Elevated Plus Maze, Actophotometer

INTRODUCTION

Anxiety disorder is common mental disorders that share extreme or pathological anxiety as the primary disturbance in mood or emotional tone. Diagnostic and statistical manual IV describes various forms of anxiety, post-traumatic stress, panic and obsessive-compulsive disorders. Common denomination of all anxiety disorders is a state of increased fear and exaggerated version of acute stress response. Biochemical alteration is observed in anatomical centers, which are responsible for processing of emotions and are connected to stress axis, which is strongly activated in response to threat or fear [1]. Anxiety refers to an overwhelming sense of apprehension or fearfulness. Marked by physiological signs, sometimes referred to as autonomic signs), anxiety can produce

both individuals may have: mental apprehension, physical tension, physical symptoms and dissociative anxiety. Anxiety disorder is divided into generalized anxiety disorder, phobic disorder, and panic disorder; each has its own characteristics and symptoms and they require different treatment. The emotions present in anxiety disorders range from simple nervousness to bouts of terror.

The imbalances in the neurotransmitters like GABA, Serotonin, nor adrenaline, Dopamine etc mainly causes anxiety disorders. Selective serotonin reuptake inhibitors, the drugs most commonly used to treat depression, are frequently considered as a first line treatment for anxiety disorders. Synthetic drugs available for treatment of anxiety have various adverse effects including drowsiness, ataxia with benzodiazepines and insomnia, libido with selective serotonin reuptake inhibitors. Common mental disorders are increasing worldwide [2]. Between 1990 and 2013, the number of people suffering from depression and/or anxiety increased by nearly 50%, from 416 million to 615 million. Close to 10% of the world's population is affected and mental disorders account for 30% of the global non-fatal disease burden. WHO estimates that, during emergencies, as many as 1 in 5 people are affected by depression and anxiety. According to WHO, herbs are very useful to treat anxiety because of less/minimum side effect, greater availability and cost effective. Synthetic drugs available for treatment of anxiety have various adverse effects including drowsiness, ataxia with benzodiazepines and insomnia, libido with selective serotonin reuptake inhibitors [3]. Anxiety is a major neurological disorder in which biochemical parameters like plasma nitrate, AchE, GABA, are mainly affected. The effect of *Azadirachta indica* on plasma nitrates and other neurotransmitters are not studied and reported yet, only anxiolytic activity is reported isomerase arch articles on the basis of behavioral models like elevated plus maze (EPM), light & dark apparatus, open field test (OFT), etc.

The aim of study is to screen the effect of *Azadirachta indica* on these transmitters in stress induced anxiety in mice, by which we will confirm how the *Azadirachta indica* increase or decrease the level of these transmitters. Behavioral parameters were employed to evaluate the physiological effect of *Azadirachta indica* and the biochemical parameters were validating the biochemical bases of the activity.

MATERIALS AND METHODS

Material

Collection & authentication: - Plant leaves were collected from medicinal garden in Safia College, Bhopal (M.P.) and authenticated from Safia College, Bhopal (M.P.)

Extraction Method Petroleum ether

The shade dry the coarse powder of leaves was packed in extraction thimble of Soxhlet apparatus and were subjected to continuous hot extraction with petroleum ether for 18 hour or till the clear extraction obtained. After that, remove solvent and extract too. Store it for recycle. Shade dries the powdered herb for second step of extraction with ethanol [4].

Ethanol extract

Mark left after petroleum ether were dried below 50°C in hot air oven and then packed well in extraction thimble of Soxhlet apparatus and subjected to continuous hot extraction with ethanol for 18 hour or till the clear extraction obtained. The extract was filtered while hot and resultant extract was distilled in vacuum under reduced pressure in order to remove the solvent completely. Dry it and kept in the desiccator till the experimentation. Obtained extract was weighed and calculate percentage yield in terms of air-dried powdered crude material [5].

Phytochemical Screening

Tests for Alkaloid

- Mayer's test: Filtrate was treated with Mayer's reagent. Formations of yellow cream-color precipitate will

indicate the presence of alkaloids.

- Dragendroff's test: Filtrate was treated with Dragendroff's reagent. Formation of reddish-brown colored precipitate will indicate the presence of alkaloids.
- Wagner's test: Filtrate was treated with Wagner's reagent. Formation of red dish brown colored precipitate indicate the presence of alkaloids.
- Hager's test: Filtrate was treated with Hager's reagent. Formation of yellow colored precipitate will indicate the presence of alkaloids.

Tests for carbo hydrates

- Molisch's test: Few drops of Molisch's reagent were added to the filtrate, follow by addition H_2SO_4 by the side of the test tube. The mixture then allows standing for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet color at the inter phase of the two layers shows a positive test.
- Fehling's test: The 1 ml of filtrate was heat with 5ml of equal volumes of Fehling's solution A (Copper sulphate in distill water) and Fehling's solution B (Potassium tartrate and sodium hydroxide in distilled water). Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars [7].
- Benedict's test: Filtrate was treated with few drops of Benedict's reagent, (alkaline solution containing cupric citrate complex) and boiled on water bath, reddish brown precipitate formation was indicated the presence of reducing sugars.

Tests for Glycosides:

- Modified Borntrager's test: The filtrate was treated with Ferric chloride solution and heated on a boiling water bath for 5 min. The mixture was cool and shakes with equal volume of benzene. Separated the benzene layer and were treated with half of its volume with ammonia solution. The formation of rose pink or cherry red color in the ammonia layer was indicated the presence of anthraquinones [8].
- Keller Killiani's test: Small portion of the filtrate was shaking with 1 ml of glacial acetic containing a trace amount of ferric chloride solution. These contents were transferred to 2ml conc. H_2SO_4 . A reddish-brown layer acquires bluish green color appears due to presence of digitoxose.

Tests for Protein and Amino acids:

- Millon's test: 2 ml of filtrate was treated with 2ml of Millon's reagent in a test tube and were heat in a water bath for 5 minutes, cool, and add few drops of $NaNO_2$ solution. Formation of white precipitate, which was turns into red color upon heating. This was indicated the presence of proteins and amino acids.
- Ninhydrin test: 2 ml of filtrate, 0.25% Ninhydrine reagent was added in a test tube and boiled for 2 minutes. Formations of blue color indicate the presence of amino acids.

Tests for phytosterols and Triterpenoids

- Liebermann's Test: 2 ml of filtrate was taken in a test tube and 2-3 drops of acetic anhydride were added and gently heat. Cool the contents of the test tube. Few drops of concentrated sulphuric acid from the side of the test tube were added. A blue color was appeared indicated the presence of sterols.
- Liberman-Burchard test: 2 ml of filtrate were dissolved in chloroform and few drops of acetic anhydride were added to it, followed by concentrated sulphuric acid from the side of the tube. Formation of brown ring at the junction of two layers and the upper layer was turns green which showed the presence of sterols and formation

of deep color were indicates the presence of triterpenoids.

- **Salkowillbeki Test:** 2ml of filtrate and add 2ml of chloroform and 2ml of conc. sulphuric acid from the side of the test tube was taken. Shaken the test tube for few minutes. The development of red color in the chloroform was indicated the presence of sterols and formation of yellow colored lower layer indicates the presence of triterpenoid [9].

Test for Phenolic and Tannins

- **Ferric Chloride Test:** Added few drops of 1% ferric chloride solution to 2ml of the filtrate, occurrence of a blue-black, green or blue-green precipitate indicate the presence of tannins.
- **Lead acetate Test:** Add 2 ml of filtrate and few drops of lead acetate solution in test tube. Formations of yellow precipitate will indicate the presence of tannins.

Test for flavonoids

- **Shinoda's test for flavonoids:** About 0.5 of each portion was dissolved in ethanol, warmed and then filtered. To the extract few pieces of magnesium chips was added in the test tube followed by few drops of conc. HCl. A pink, orange, or red to purple coloration was indicates the presence of flavonoids.
- **Alkaline reagent test:** To the test solution 2 ml few drops of sodium hydroxide was added, formation of yellow color which turns to color less on addition of few drops of dilute hydrochloric acid indication for the presence of flavonoids.

Tests for Saponins

Foam Test: Dilute 1ml of extract with distilled water and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins.

Pharmacological Screening

Animal: Swiss albino Mice (20–25gm) was used for the study and Total 15 animal was used for the study. All animal was kept in standard plastic polypropylene cages with stainless steel coverlids and wheat straw was used as bedding material. The animal was facilitated with standard environment of photo period (12:12 hrs. dark: light cycle) and room temperature (23 ± 20 C). The animal assists free to feed and purified water ad libitum. All experiment was according to CPCSEA guidelines and approved by IAEC.

Immobilization Stress or stress induced anxiety:

Animals were immobilized (IMO) for 3 hr. by taping all the four limbs on board by putting them on their backs using zinc oxide hospital tape. The animals were released by unraveling the tape after moistening with acetone in order to avoid pain and discomfort. In unstressed group, the mice will be handled without any stress [10].

Description of groups

Control Group (Vehicle Treated); n=3

Negative Control (Disease Induced) n=3

Standard (Diazepam, 4 mg/kg i.p.); n=3

Test group-1 (Ethanollic Extract of Azadirachta Indica 100 mg/kg); n=3

Test group-II (Ethanollic Extract of Azadirachta Indica 200 mg/kg); n=3

Behavioral Parameter Elevated plus Maze

The plus maze apparatus consisted of two open arms, measuring 16×5 cm, and two closed arms, measuring 16×5×12 cm, connected to a central platform (5×5cm). The maze will be elevated to a height of 25 cm above the floor. Each mouse will be placed individually at the center of elevated plus maze with its head facing toward an open arm and observed for 5 min to record the number of entries into open arm, closed arm and time spent in each arm [11]. In EPM test, the percent time spent on the open arms will be determined as follows.

$\% = 100 \times \text{Number of seconds spent on open arms} / 300 \text{ total seconds (5 min observation time)}$.

Light and Dark Box Test

The apparatus consisted a rectangular box (45 × 27 × 27 cm), partitioned into two compartments connected by a 7.5 × 7.5 cm opening in the wall between compartments. An animal will be placed in the center of the light compartment and will be observed for 5 min for the time spent in open (white/light) compartment [12]. Percent time spent in the light compartment will be determined as follows

$\% = 100 \times \text{Number of seconds spent in light compartment} / 300 \text{ total seconds (5 min observation time)}$.

Open Field Test

The OF test, which provides simultaneous measures of locomotion, exploration and anxiety, will be used for this study. The open field is a 400 × 400 × 300 mm arena with thin black stripes painted across the floor, dividing it into 16 quadratic blocks. Mouse will be placed in the center of arena and an observer quantified the spontaneous ambulatory locomotion of each mouse for 5 min. During this period, the number of squares crossed and number of rearing will be measure [13].

Actophotometer (Locomotor activity):

The locomotors activity can be easily studied with the help of actophotometer. Mice will be grouped and treated with drug/extract as described. Each animal will be placed individually and the basal activity score of all the animals will be recorded after 30, 60 and 120 min of drug treatment [14].

Biochemical Parameter:

Acetylcholine esterase

The enzyme activity will be measured by the method of in which Different brain regions will be homogenized in phosphate buffer 0.1 M, pH 8.0. The reaction mixture consisted of 0.4 ml aliquot of homogenate, 2.6 ml of phosphate buffer 0.1 M, pH 8.0 and 0.1 ml of di thiobis nitro benzoic acid DTNB, 0.01M. After the addition of the substrate acetyl thiocholine iodide 0.075 M., changes in the absorbance will be noted every 2 min for 10 min at 412 nm using an LKB spectrophotometer. The activity will be expressed as m moles hydrolyzed per min per gram of tissue. AChE activity will be statistically analyzed by Student's t-test [15]

Plasma nitrite

Nitrite is the stable end product of nitric oxide (NO) in living system. Accumulations of nitrite was measured by spectro-photometer assay based on Gries's reaction. Blood was withdrawn from tail vein of mice and plasma was extracted using cooling centrifuge at 2500 rpm for 10 min. Plasma were mixed with equal volumes of Gries's reagent (1% sulphanilamide+ 0.1% naphthylelene diamine dihydrochloride+ 2.5% phosphoric acid) and incubated at room temp for 10 min to yield a chromophore. Absorbance was read at 543 nm spectrophotometrically [16].

GABA

The Effect of extract on GABA levels in brain was studied, based on that described previously by other workers (Suher et al., 2000). After seven days treatment with vehicle (3% Tween 80, p.o) or standard (Diazepam 4 mg/kg, p.o.) or extract (100 and 200 mg/kg, p.o.), animals will be killed by euthanasia and the body will be exposed to a microwave irradiation for 4sec [17]. The brain will be rapidly removed and the cerebellum, the tissue other than cerebellum will be dissected on an ice-cold Petridis. The tissues will be weighed and placed in pre-cooled 100 ml plastic tubes. Ice-cooled 0.1 M perchloric acid (10 ml) containing valine at a concentration of 15 µg/ml (internal standard) will be added to the tissue. The tissues will be homogenized for one minute during which the tube embedded in an ice bath and then centrifuged at 5000 rpm for 10 min at 4°C [18]. The supernatants stored at -20°C until assayed. Dansylating reaction will be induced by adding 100 µl of each supernatant of the samples standards to a micro-tube containing 100 µl of 0.1M potassium carbonate solution. These solutions mixed using vortex and then centrifuged using micro centrifuge at 10000 rpm for 10 min. 100 µl of each supernatant will be transferred into a pyrex tube containing 100 µl of 0.1 M sodium hydrogen carbonate solution, to which 400 µl of working dansyl chloride solution (1.25 mg/ml anhydrous acetone) will be added [19]. Shake the tubes for 30 sec using vortex and then incubated at 90°C in bench top oven for 30 min. The tubes not capped during the incubation to allow most of the solvent to be evaporated.

This did not appear to adversely affect the progress of the dansylating reaction and served to concentrate the samples. After getting the tubes out of the oven, they will allow to cool down to room temperature and the dansylated derivatives will be transferred to 1.5 ml micro tubes and stored at -20°C until assayed. C8 reversed-phase High-Performance Liquid Chromatography (HPLC) columns (5 µm, 250 x 3.2 mm) will be used to resolve and quantify the samples. The HPLC mobile phase consisted of deionized helium degassed water-acetonitrile (65:35v/v) containing 0.15% v/v phosphoric acid. The flow rate will keep at 0.5ml/min. The detector excitation will be at 333 nm and emission at 532 nm. 25 µl of the derivative of the Gamma Amino butyric Acid (GABA) samples will be transferred to HPLC micro-sample vials and injected into the column. Retention time of GABA and internal standard will determine. The peak ratios of the samples will be calculated with reference to the internal standard. GABA levels will be expressed as ng/g of tissue [20-24].

Histopathology

Histopathological studies of brain were done before and after treatment. Mice (20-30g,) was implemented as a mammalian model for in-vivo brain experiments (Histopathological study). Mice were anesthetized CHCl₃ (80mg/kg)/Ether (10mg/kg) solution and maintained under sedation with additional boluses of CHCl₃ for the duration of each individual experiment. Once anesthetized, animals were placed in the prone position and the end brain expose. Mice were continually moistened with normal saline to avoid during the acute study. The cranial cavity as open and brain was isolated and store in ice and observes there is any change in Brian's T.S. or not [25, 26].

Statistical Analysis

The statistical analysis was carried out as per standard method. Results were expressed as Mean± SEM compared with the analysis of variance (ANOVA) followed by Dunnet's test value for statistical significance.

RESULTS AND DISCUSSION

Phytochemical Screening

The preliminary phytochemical evaluation of the ethanolic extract of *Azadirachta indica* revealed the presence of several bioactive constituents. Alkaloids were confirmed by positive reactions in Dragendorff's, Mayer's, Wagner's, and Hager's tests, each producing characteristic precipitates (reddish brown, brown, reddish brown, and yellow respectively). Carbohydrates were detected through Molisch's test (dull violet coloration), Fehling's test (red precipitate), and Barfoed's test (reddish precipitate). In contrast, glycosides were absent, as indicated by negative results in Legal, Baljet, and Keller–Killiani's tests. The extract showed strong evidence of flavonoids, with

the alkaline reagent test producing a yellow color that turned colorless and Shinoda's test yielding a pink coloration. Saponins were not detected, as the foam test showed no change. Finally, phenols and tannins were confirmed by the formation of a blue-black precipitate with ferric chloride and a yellow precipitate with lead acetate (Table 1). These findings suggest that the ethanolic extract of *Azadirachta indica* contains alkaloids, carbohydrates, flavonoids, phenols, and tannins, which may contribute to its diverse pharmacological activities, while glycosides and saponins are absent.

Table 1: Phytochemical Screening

S. No.	Phyto chemical Test of Ethanolic extract of <i>Azadirachta Indica</i>	Observations	Results
1.	Test for alkaloids Dragendroff's Test Mayer's Test Wagner's Test Hager's Test	Reddish brown ppt. Brown ppt. Reddish brown ppt. Yellow ppt.	+ + + +
2.	Test for Carbohydrates Molisch's Test Fehling's Test Barfoed Test	Dull violet color Red ppt. Red dish ppt.	+ + +
3.	Test for Glycosides Legal Test Baljet Test Keller Killiani's Test	No Change No Change No Change	- - -
5.	Test for Flavonoids Alkaline reagent Test Shinoda's Test	Yellow color turn to colorless Pink color	+ +
6.	Test for Saponins Foam Test	No change	-
7.	Test for Phenols and Tannins Ferric Chloride Test Lead acetate Test	Blue-black ppt. Yellow ppt.	+ +

Animal Behavioral Parameters

The behavioral assessment of the ethanolic extract of *Azadirachta indica* demonstrated significant anxiolytic-like activity across multiple paradigms. In the Elevated Plus Maze, treatment with *A. indica* at both 100 mg/kg and 200 mg/kg doses increased the time spent in the open arms (118 ± 3.01 sec and 167 ± 2.52 sec, respectively) compared to the negative control (41 ± 3.40 sec), indicating reduced anxiety levels. Similarly, locomotor activity measured by the Actophotometer showed moderate suppression at 100 mg/kg (49 ± 4.24 counts) and more pronounced reduction at 200 mg/kg (58 ± 4.24 counts), consistent with sedative properties. In the Light–Dark Test, animals treated with *A. indica* spent significantly more time in the light compartment (135 ± 6.02 sec at 100 mg/kg; 177 ± 2.30 sec at 200 mg/kg) compared to the negative control (56 ± 2.12 sec), further supporting anxiolytic potential. The Open Field Test revealed increased exploratory behavior, with higher numbers of square crossings and rearings in treated groups relative to controls (Table 2).

Table 2: Animal Behavioral Parameters

S. No.	Group	Elevated Plus Maze (5 Min.)		Actophoto - Meter	Light & dark Test (5 Min.)		Open Field Test (5 Min)	
		Time spent in Open arm (Sec.)	Time spent in Close arm (Sec.)		Time spent in Light Area (Sec.)	Time spent in Dark Area (Sec.)	No. of Squire Cross	No. of Rearing
1.	Positive control	237 ± 5.0382	63 ± 2.0126	76 ± 2.2108	217 ± 2.3024	83 ± 4.2015	48 ± 2.2450	24 ± 5.0545
2.	Negative control	41 ± 3.4021	260 ± 3.2017	35 ± 2.5421	56 ± 2.1245	244 ± 2.5045	14 ± 2.5402	39 ± 5.2540
3.	Standard (diazepam 4 mg/kg)	$194 \pm 5.1307^{***}$	$106 \pm 6.2016^{***}$	$64 \pm 3.5041^{***}$	$169 \pm 3.0554^{**}$	$131 \pm 4.2045^{**}$	$38 \pm 2.2405^{**}$	$27 \pm 5.2451^{**}$
4.	<i>A. indica</i> (100 mg/kg)	$118 \pm 3.0124^*$	$184 \pm 5.3026^*$	$49 \pm 4.2401^*$	$135 \pm 6.0215^*$	$165 \pm 4.3024^*$	$31 \pm 2.0245^*$	$35 \pm 2.2405^*$
5.	<i>A. indica</i> (200mg/kg)	$167 \pm 2.5201^{**}$	$133 \pm 4.2103^{**}$	$58 \pm 4.240^{***}$	$177 \pm 2.3054^{**}$	$123 \pm 4.2402^{**}$	$34 \pm 2.0454^{**}$	$32 \pm 5.4545^{**}$

These findings collectively suggest that *Azadirachta indica* possesses dose-dependent anxiolytic and mild sedative effects, comparable to the standard drug diazepam, thereby validating its traditional use in neurobehavioral disorders.

Biochemical Estimation

The biochemical estimations provide further mechanistic insight into the anxiolytic activity of *Azadirachta indica*. In the Griess assay, plasma nitrate levels were significantly elevated in the negative control group ($73.25 \pm 2.24 \mu\text{mol/l}$), reflecting increased nitric oxide production under stress conditions. Treatment with *A. indica* at 100 mg/kg and 200 mg/kg reduced nitrate levels (61.58 ± 2.24 and $53.41 \pm 2.24 \mu\text{mol/l}$, respectively), approaching the standard diazepam group ($48.73 \pm 1.90 \mu\text{mol/l}$), suggesting attenuation of nitrosative stress. Similarly, inducible nitric oxide synthase (iNOS) activity was markedly suppressed in the negative control ($26.23 \pm 2.54 \mu\text{mol/l}$) but restored toward normal levels by *A. indica* (43.29 ± 3.14 at 100 mg/kg; 71.96 ± 2.54 at 200 mg/kg), indicating regulatory effects on nitric oxide pathways. Acetylcholinesterase (AChE) activity, elevated in the negative control ($81.23 \pm 3.02 \text{ mg/dl}$), was significantly reduced by *A. indica* (63.17 ± 2.05 at 100 mg/kg; 51.92 ± 2.30 at 200 mg/kg), consistent with enhanced cholinergic transmission and anxiolytic potential. Importantly, tissue GABA levels, which were depleted in the negative control ($1634.2 \pm 2.21 \text{ ng/gm}$), were restored by *A. indica* (2424.4 ± 2.32 at 100 mg/kg; 2683.5 ± 2.22 at 200 mg/kg), approaching the diazepam standard ($2826.4 \pm 2.20 \text{ ng/gm}$) (Table 3).

In recent studies, *Azadirachta indica* has been shown to exert anxiolytic effects through modulation of nitric oxide pathways, cholinergic suppression, and enhancement of GABAergic transmission, consistent with your findings. Rao et al. (2025) reported that neem extract significantly reduced nitrosative stress in stressed rats, restoring nitric oxide balance and suppressing iNOS overactivation [27]. Their molecular docking analysis confirmed that neem phytochemicals interact with acetylcholinesterase and NMDA receptors, thereby validating its role in cholinergic and glutamatergic regulation. Similarly, Valenzuela Estrada et al. (2026) demonstrated anxiolytic and antidepressant effects of neem extract in Swiss Webster mice, with behavioral improvements in the Elevated Plus Maze and Light–Dark Box tests, which correspond to your biochemical evidence of enhanced GABAergic transmission [28]. Furthermore, a comprehensive review in the Journal of Ethnopharmacology (2026) highlighted neem’s flavonoids and limonoids as key contributors to its neuroprotective and anxiolytic properties, emphasizing its dual role in reducing oxidative/nitrosative stress and modulating neurotransmitter systems. Collectively, these findings corroborate your data, suggesting that *A. indica* exerts anxiolytic activity through multi-target mechanisms comparable to diazepam [29].

These biochemical findings align with the behavioral outcomes in the Elevated Plus Maze, Light–Dark Test, and Open Field Test, supporting the hypothesis that *A. indica* exerts anxiolytic effects through modulation of nitric oxide pathways, inhibition of AChE, and enhancement of GABAergic neurotransmission. The phytochemical profile—particularly the presence of alkaloids, flavonoids, phenols, and tannins—provides a plausible basis for these effects, as these compounds are known to influence neurotransmitter systems and oxidative stress. Collectively, the data suggest that *Azadirachta indica* possesses dose-dependent neuroprotective and anxiolytic properties comparable to diazepam, validating its ethnomedicinal use and highlighting its potential for development into herbal formulations targeting anxiety disorders.

Table 3: Biochemical Estimation

S. No.	Groups	Plasma Nitrate ($\mu\text{mol/l}$) Griess Assay	iNOS ($\mu\text{mol/l}$)	AchE (mg/dl)	GABA (ng/gm of Tissue)
1.	Positive Control	24.38 ± 2.3540	103.71 ± 2.84550	54.31 ± 2.2505	3078.7 ± 2.2012
2.	Negative Control	73.25 ± 2.2405	26.23 ± 2.5470	81.23 ± 3.0245	1634.2 ± 2.2102
3.	Standard	$48.73 \pm 1.9058^{***}$	$98.16 \pm 2.2405^{***}$	$48.72 \pm 3.2301^{***}$	$2826.4 \pm 2.2015^{***}$

4.	A. Indica 100 mg/kg	61.58± 2.2405	43.29± 3.1450*	63.17± 2.0540*	2424.4± 2.3201*
5.	A. Indica 200 mg/kg	53.41± 2.2450**	71.96± 2.5402**	51.92± 2.3054**	2683.5± 2.2230**

Values are expressed MEAN±SEM, n=6, ** = P<0.01, *** = P<0.001 when compared to normal control group, b = ns when compared to normal control group, a*** = P<0.001 when compared to negative control group, c = ns when compared to standard group. Standard:- Diazepam (4mg/kg)

Histopathology of Brain

The micrographs show H&E-stained brain tissue sections from five experimental groups: Positive Control, Negative Control, Standard (Diazepam 4 mg/kg), Test Group I (A. indica 100 mg/kg), and Test Group II (A. indica 200 mg/kg). The positive control exhibits normal neuronal architecture with intact cell bodies and clear nuclei, while the negative control reveals marked neuronal degeneration, cytoplasmic vacuolation, and disrupted neuropil typical of stress-induced neurotoxicity. The standard group shows restoration of normal morphology, confirming diazepam's neuroprotective effect (Figure 1). In the A. indica-treated groups, progressive improvement is evident: Test Group I displays partial neuronal recovery, and Test Group II shows near-normal histoarchitecture with reduced cellular damage and preserved neuronal integrity, indicating dose-dependent protection. These histological findings reinforce the biochemical evidence of reduced nitrosative stress and restored GABAergic transmission, as well as the behavioral outcomes demonstrating anxiolytic activity. Together, they confirm that Azadirachta indica mitigates stress-induced neuronal alterations through antioxidant and neurotransmitter-modulating mechanisms.

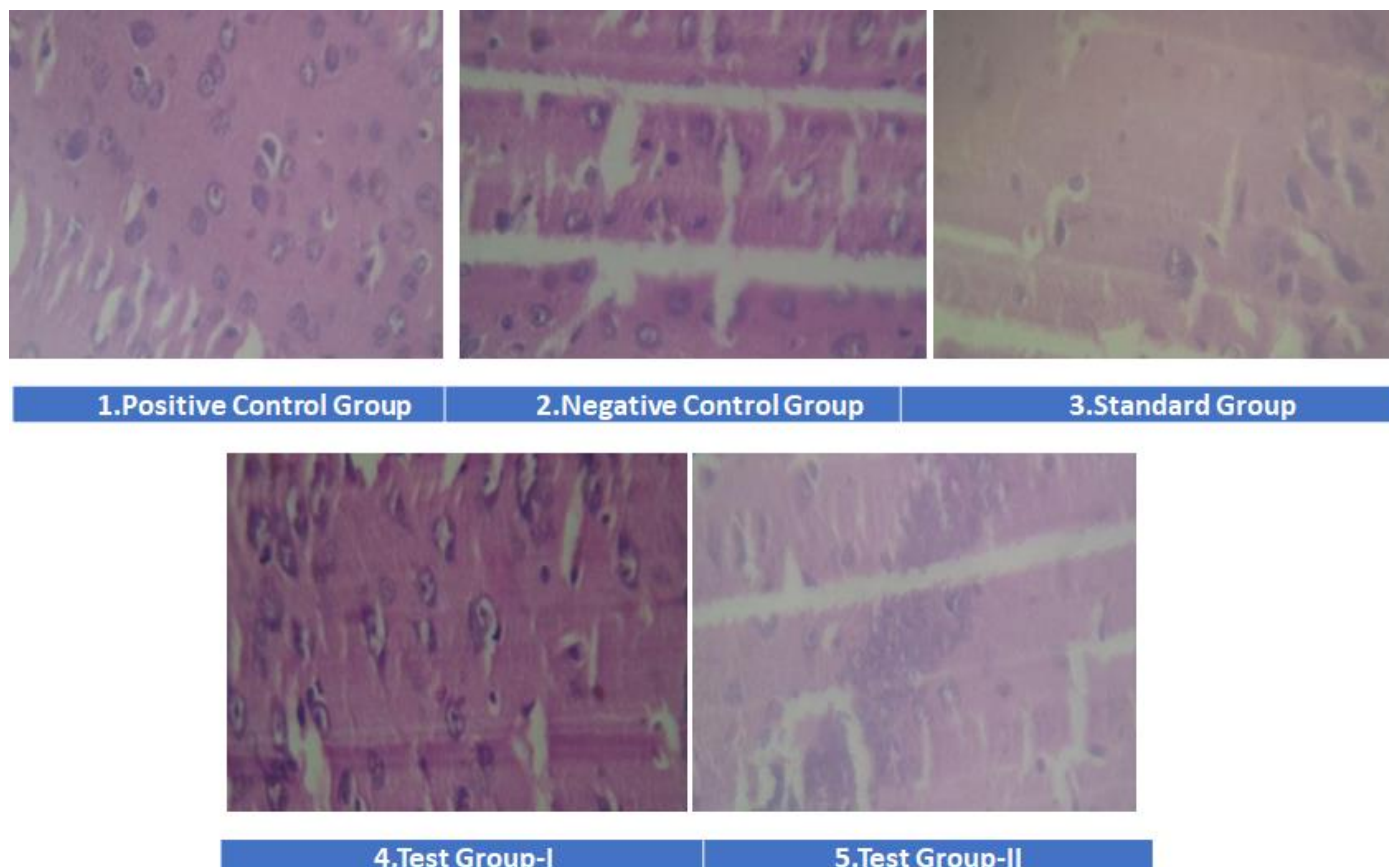


Fig. 1: Histopathology of Brain

DISCUSSION

The observed anxiolytic and sedative effects of the ethanolic extract of *Azadirachta indica* can be correlated with its phytochemical constituents identified during preliminary screening. The presence of alkaloids, confirmed by multiple qualitative tests, may contribute to central nervous system modulation, as alkaloids are known to interact with neurotransmitter pathways such as GABAergic and serotonergic systems. Similarly, the detection of flavonoids, evidenced by positive Shinoda and alkaline reagent tests, is significant since flavonoids are widely reported to exert anxiolytic and antioxidant effects through enhancement of GABAA receptor activity and reduction of oxidative stress. The presence of phenols and tannins further supports the neuroprotective potential of the extract, as these compounds possess free radical scavenging properties that may stabilize neuronal function under stress conditions. In contrast, the absence of glycosides and saponins suggests that these groups are unlikely

to contribute to the observed pharmacological activity. Taken together, the phytochemical profile provides a plausible mechanistic basis for the behavioral findings in the Elevated Plus Maze, Light–Dark Test, and Open Field Test, where *A. indica* produced dose-dependent increases in exploratory behavior and time spent in anxiolytic-associated zones. These results validate the traditional use of Neem in neurobehavioral disorders and highlight its potential as a source of natural anxiolytic agents.

CONCLUSION

The present investigation demonstrates that the ethanolic extract of *Azadirachta indica* possesses significant anxiolytic and neuroprotective properties, as evidenced by behavioral paradigms including the Elevated Plus Maze, Light–Dark Test, and Open Field Test, alongside biochemical estimations of plasma nitrate, iNOS, AChE, and GABA levels. The extract produced dose-dependent improvements in exploratory behavior and anxiolytic indices, comparable to the standard drug diazepam. Biochemical assays revealed attenuation of nitrosative stress, modulation of cholinergic activity, and restoration of GABAergic neurotransmission, thereby supporting the mechanistic basis for its pharmacological effects. These outcomes can be attributed to the presence of alkaloids, flavonoids, phenols, and tannins, which are known to influence neurotransmitter systems and oxidative pathways. Collectively, the findings validate the traditional use of Neem in neurobehavioral disorders and highlight its potential as a natural source of anxiolytic agents. Future research should focus on isolation of active constituents, dose optimization, and clinical evaluation to establish its therapeutic relevance and safety profile in humans.

Compliance with ethical standards: Animal (Albino Mice) used for the study. IAEC Approval No. VEDIC/CCSEA/IAEC/08, Dated 13/05/2026).

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