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Neuroprotective and antioxidant effects of *Azadirachta indica*: A comprehensive GC-MS and preclinical study

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Abstract: **Background**: Herbal and Ayurvedic remedies are increasingly explored for mental health disorders such as anxiety and depression. *Azadirachta indica* (Neem) has long been recognized for its medicinal value. **Objectives**: The current study evaluated the neuroprotective, anxiolytic, antidepressant, antioxidant, and toxicological effects of the ethanolic fruit extract of *Azadirachta indica* in preclinical models. **Methods**: Anxiolytic activity was assessed through the open field test, elevated plus maze, and hole board test, whereas forced swimming and tail suspension tests were used to evaluate antidepressant effects. Corticosterone and acetylcholinesterase levels, brain histopathology, and GC-MS analysis of bioactive constituents were also performed. **Results**: *Azadirachta indica* fruit extract exhibited strong antioxidant activity (68.6%) and a high safety margin (LD_{50}> 5000 mg/kg). *Azadirachta indica* produced notable behavioral changes across multiple tests. *Azadirachta indica* at 400 mg/kg reduced locomotor activity, increased exploratory behavior, and improved open arm preference, while also reducing head-dipping and immobility in depression models. The histopathological evaluations showed no morphological changes. *Azadirachta indica* at 200-400 mg/Kg also significantly decreased corticosterone levels and improved acetylcholinesterase levels. GC-MS analysis identified 51 metabolites that may contribute to these pharmacological effects. **Conclusion**: Overall, these findings suggest that *Azadirachta indica* fruit extract possesses anxiolytic and antidepressant potential, supporting its role as a natural therapeutic candidate for neuropsychiatric disorders.

Keywords: Acetylcholinesterase; Antioxidant; Anxiolytic; Anti-depression; Corticosterone GC-MS; Neuroprotective

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INTRODUCTION

Neuropsychiatric disorders such as anxiety and depression are major contributors to the global burden of disease (Kieling *et al.,* 2024). Synthetic medications, including tricyclic antidepressants and benzodiazepines, are effective but are frequently associated with adverse effects and dependence, reinforcing the need for safer alternatives (Pannu & Goyal, 2025). In this context, the use of herbal medicines as complementary options is gaining attention. *Azadirachta indica* (neem) is of particular interest because of its diverse pharmacological properties and long-standing use in traditional medicine (Sharma and Sharma, 2022). Previous studies have reported the antihyperglycemic, anti-inflammatory, hypolipidemic, antioxidant, antibacterial, anxiolytic, and antidepressant activities of *Azadirachta indica* (Asghar *et al.,* 2022). Most studies have explored these effects individually, without integrating behavioral, biochemical, and histological findings alongside comprehensive metabolite profiling. This limitation restricts a deeper mechanistic understanding of the role of *Azadirachta indica* bioactive constituents in its neuroprotective and psychotropic potential.

The present study was designed with the hypothesis that bioactive metabolites of *Azadirachta indica*, identified through GC-MS, contribute to its observed neurobehavioral and neuroprotective effects. A multi-level approach was employed, incorporating behavioral models for anxiety and depression, in vitro antioxidant assays, Lorke's toxicity evaluation, biochemical markers (corticosterone and acetylcholinesterase), and histopathological analysis.

MATERIALS AND METHODS

Preparation of Azadirachta indica fruit extract

Fresh fruit of *Azadirachta indica*, which is also known as nimboli, was collected from the University of Karachi. June and August were the fruiting seasons of neem. The fruit was authenticated in the Department of Pharmacognosy, University of Karachi. *Azadirachta indica* specimen # AIF-01-21 was granted for future reference. As the extract was prepared directly from freshly collected fruit, no commercial batch number was

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applicable; however, this authenticated batch was consistently used throughout the study. The fruit was washed, deseeded, and dried, which was then immersed in 95% ethanol, a polar solvent, at room temperature (25 ± 2) º C for 21 days. The mixture was filtered and evaporated in a rotary evaporator (under reduced pressure) and stored in a refrigerator (in an airtight container). The extract was soluble in water and then administered orally in the dose range of 100, 200, and 400mg/Kg based on body weight (Hawiset *et al.,* 2022).

Animal selection and grouping

Healthy adult albino male mice (n=60) weighing 20-30g, of 6-8 weeks old. Experimental mice were obtained from animal house facility of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan. Animals were acclimatized for a week before starting the experimentation and were also examined before starting; only healthy animals without any visible signs of illness were included. The animals were selected and kept in cages at a controlled temperature (24 ± 1 ºC) with unrestricted access to food/water according to the guidelines provided by the National Advisory Committee for Laboratory Animal Research for the care and use of experimental animals (Jones- Bolin, 2012). Humane endpoints were considered in case of severe distress, persistent abnormal behavior, or marked deterioration in health condition. Potential confounding factors were minimized by using animals of the same sex, similar age and weight range, maintaining similar housing conditions, and performing assessments under consistent experimental conditions. The study protocol was approved by the Institutional Animal Ethics Committee of the University of Karachi (IBC KU-430-A2024) and conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

A formal a priori sample size calculation was not performed; animals were randomly assigned into six groups of 10 each, with the sample size guided by precedents in rodent behavioral pharmacology literature and established experimental practice to obtain sufficient biological variability while minimizing animal use. Investigators were blinded to treatment group during behavioral and pharmacological assessment. One control group received Water for Injection (WFI); two standard groups received diazepam 3mg/Kg (Cheng *et al.,* 2013) Imipramine 30mg/Kg (Kawai *et al.,* 2018); and three groups received doses of *Azadirachta indica* 100, 200, and 400mg/Kg (Hawiset *et al.,* 2022). The treatment regimen involved daily oral administration of either Water for Injection or *Azadirachta indica* fruit extract at designated doses for 15 consecutive days. Behavioral assessments for anxiolytic activity were conducted on days 7 and 14, whereas antidepressant activity was evaluated on days 15 and 16. Following behavioral assessments, animals were euthanized by decapitation, and blood samples were collected into trisodium citrate tubes. Plasma was separated by centrifugation using a Humax 14 K centrifuge (Human, Germany) at 2000 × g for 10 minutes. Corticosterone levels were quantified using an electrochemiluminescence immunoassay on the Elecsys 2010 system. Brain samples were used to evaluate acetylcholine (ACh) and acetyl cholinesterase (AChE) using a fluorescence microplate reader or fluorometer. The hippocampus was isolated and preserved in 10% formalin within glass containers for subsequent histopathological examination.

DPPH radical scavenging assay

The DPPH assay was conducted to determine the % RSA of compounds by comparing them to a DMSO-containing control and calculating their IC50 values. Each DPPH test was conducted in triplicate to ensure reproducibility *(Harini et al.,* 2012).

Phytochemical evaluation

Using established protocols, preliminary phytochemical analyses of *Azadirachta indica* fruit extract were carried out to identify the principal phytochemical ingredients (Balamurugan *et al.,* 2019; Mosić *et al.,* 2020).

Gas chromatography and mass spectrometry

The extract was dissolved in ethanol and filtered using established protocols. The analysis was done (Agilent 7890) with an autosampler and a Triple quadrupole mass spectrometer (Yeshanew & Ingkaninan, 2020).

As a chemical molecule elutes from the GC column into the detector, each peak in the chromatogram reflects the signal produced. The retention period is displayed on the x-axis, and the signal's intensity (abundance) is displayed on the y-axis with its retention times.

Gross toxicity and mortality rate assessment (GT and MRA)

GT and MRA were conducted on 12 male albino mice after acclimatization, divided into two phases: Phase I and Phase II as described by (Lorke, 1983). Phase I comprised three groups of mice (three animals each) at doses of 10, 100, and 1000mg/Kg of *Azadirachta indica* extract (Chinedu *et al.,* 2013), and signs of acute toxicity symptoms and mortality were monitored. While in Phase II, three animals (one animal in each group) were given extreme doses as per protocol i.e., 1600, 2900 and 5000 mg/Kg of extract and signs of behavioral toxicity (tremors, lacrimation, diarrhea, salivation, writhing, convulsions, tonic extension, drowsiness, ataxia, muscular spasm) were monitored (Lorke, 1983; Wahid *et al.,* 2020).

Histopathological examination

The research involved histopathological analysis of the kidney, liver, and brain after a period of fifteen days, as described previously in the literature (Maxammadzokirovna, 2023; Mondal, 2017).

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Table 1: DPPH radical quenching analysis

Compound	Concentration	% Inhibition	IC_{50}±SEM
Azadirachta indica	0.5 µg/ml	24.8	-
	1mg/ml	68.6	344.9±1.48 µg /ml
Gallic Acid	0.5 µg/ml	95.3	21.7±0.287 µg /ml

Values represented as mean ± SEM (n = 3)

Table 2: Phytochemical analysis of *Azadirachta indica* fruit extract

Phytochemicals	*Azadirachta indica*
Phenols	+
Alkaloids	-
Glycosides	+
Flavonoids	+
Tannins	-
Anthraquinone glycosides	-
Carbohydrates	+
Terpenoids	+
Fixed oil and fats	-
Quinone	-
Steroids	+
Saponins	+
Protein	-

Present = +; Absent = -

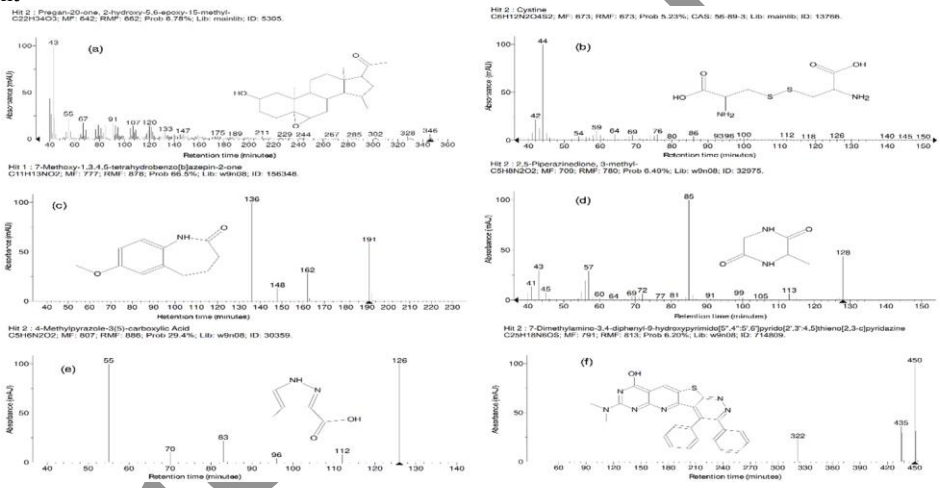


Fig. 1: Peaks of distinct analytes of *Azadirachta indica* isolated through GC-MS: (a) Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl-,(b) 7-Methoxy-1,3,4,5- tetrahydrobenzo[b]azepin-2-one ,(c) 2,5-Piperazinedione, 3-methyl , (d) 7-Dimethylamino-3,4-diphenyl-9-hydroxypyrimido[5'',4'':5',6']pyrido[2',3':4,5]thieno[2,3-c]pyridazine, (e) cysteine,(f) 4 methyl pyrazole -3.

Table 3: Chemical constituents of *Azadirachta indica* extract by GC-MS along with their chemical structures, molecular formulae, and biological activities.

S #	Retention time(min)	Name of the compound	Area	Height	Molecular formula	Molecular mass (g/mol)
1	5.797	Cystine	372143.89	74913.81	C6H12N2O4S2	240.3
2	8.65	4-Methylpyrazole-3(5)-carboxylic acid	3052254.33	557449.85	C5H6N2O2	126.11
3	8.16	2,5-Piperazinedione, 3-methyl3(5)-carboxylic Acid	531950	76911.85	C5H8N2O2	128.12
4	27.674	7-Methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one	499971.26	28512.16	C11H13NO2	191.23
5	52.331	7-Dimethylamino-3,4-diphenyl-9-hydroxypyrimido[5'',4'':5',6']pyrido [2',3':4,5]thieno[2,3-c]pyridazine	1425689.75	247009.2	C25H18N6OS	450.52
6	54.142	Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl	373454.99	27032.06	C22H34O3	346.50

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Anxiety-modulating potential

Open field test (OFT)

Rodents' innate curiosity was used in OFT to investigate novel surroundings. In neuroscience research, this test is used to evaluate mice's locomotor, exploratory, and anxiety-like behaviors as described by (Bishnoi *et al.*, 2021; Seibenhener & Wooten, 2015; Perals *et al.*, 2017). *Azadirachta indica* extract and WFI were administered orally daily for 15 days, with OFT performed twice for 5 minutes on days 7th and 14th (Knight *et al.*, 2021). An assessment was conducted of the variables. Such as total distance travelled (TDT), number of entries in the center square (NECS), total number of rearings (TNR), and duration of rearings (DR).

Elevated plus maze (EPM)

Another model to determine anxiolytic activity used in the current study was the EPM. The mice were placed in the Elevated Plus Maze for a duration of five minutes. The apparatus consisted of two arms. One open (OA) and one closed (CA) that are, joined by a central square. Behavioral parameters were recorded, including the number of entries into closed arms (nECA), number of entries into open arms (nEOA), time spent in open arms (TOA), and time spent in closed arms (TCA). (Goni *et al.*, 2021). The percentage of open arm entries (%age EOA) and the percentage of interval spent in open arm (%age TOA) were calculated (Riaz & Khan, 2017).

Hole board test (HBT)

This model assessed mice's inquisitiveness and anxiety; i.e., a decline in the number of head dips (nHD) exhibits an anxiolytic behavior (Casarrubea *et al.*, 2023; *Acikgoz et al.*, 2022). The number of head dips was noted by placing animals for five minutes.

Anti-depressant activity

Forced swim test (FST)

The depressed behavior of mice is measured by FST, as explained by (Poleszak *et al.*, 2019). The animals were given five minutes to swim individually in a cylindrical glass vessel. A decrease in duration of immobility (dImm), i.e., when the mouse gave up trying to move in water, was considered an antidepressant effect (Goni *et al.*, 2021). The antidepressant effect of *Azadirachta indica* and imipramine on percentage immobility inhibition (%ageImmInh) was calculated by the formula described by (Riaz & Khan, 2017), i.e., control dImm -test dImm = a, a × 100 = b, b/control.

Tail suspension test (TST)

This model assesses the central nervous system's activity, specifically in relation to depression-like behaviors and antidepressant activity (Yousuf *et al.*, 2020; Wu *et al.*, 2021). Mice were suspended by the tail using adhesive tape affixed to a horizontal bar positioned 50 cm above the ground. Animals were kept visually and auditorily isolated during the 5-minute test (Goni *et al.*, 2021; Wang *et al.*, 2019).

Estimation of acetylcholine and acetylcholinesterase levels

The concentration of acetylcholine and acetylcholinesterase was measured by the Amplex® Red Acetylcholine/ Acetylcholinesterase Assay kit (Invitrogen, Paisley, UK). Brain samples were collected from a separate cohort of animals specifically allocated for biochemical analysis, with 10 biological replicates per group. Brain tissue was processed to obtain the supernatant and 0.1ml of diluted supernatant was transferred in duplicate onto 96-well microplates. Add 0.1 mL of a 400 M Amplex Red Reagent (ARR) working solution containing 200 µL ARR stock solution, 100 µL HRP, 100 µL choline oxidase, and 100 µL acetylcholinesterase/10 µL acetylcholine in 1X reaction buffer to each well. The absorbance was measured in a fluorescence microplate reader (VarioskanTM, Thermo Fisher Scientific Inc., USA) at 560 nm and 590 nm (Sifringer *et al.*, 2013), and concentrations were calculated via a factor derived from an acetylcholine standard curve in each experiment. Biochemical assays were performed on 10 biological replicates per group, with each sample analyzed in duplicate technical replicates for accuracy.

Estimation of corticosterone level

Blood was collected by decapitation on the 15th day; serum was separated by centrifugation (Humax 14k, Human Diagnostic, Germany), and the radioimmunoassay method was used to analyze corticosterone levels (Gong *et al.*, 2015). Biochemical assays were performed on 10 biological replicates per group, with each sample analyzed in duplicate technical replicates for accuracy.

Histopathology of brain sample

The rodents were decapitated on day 16th to remove the mice's brains, which were subsequently submitted to a lab for a gross assessment of the hippocampus. Slides were then prepared to assess neuronal alterations in the brains of control and treated animals.

Statistical analysis

The data were analyzed using SPSS-25 and subjected to ANOVA followed by post hoc Tukey test, with statistical significance determined at *P < 0.05 and **P < 0.005.

RESULTS

DPPH radical quenching assay

The ethanolic extract of *Azadirachta indica* exhibited a dose-dependent increase in DPPH activity. *Azadirachta indica* exhibited 68.6% inhibition by Radical Scavenging analysis through DPPH, as shown in Table 1, at 1 mg/ml, indicating substantial antioxidant potential at higher doses. The calculated IC_{50} for *Azadirachta indica* was 344.9 ± 1.48 µg/ml, suggesting moderate radical scavenging efficiency compared to the standard.

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Table 4: Safety evaluation of *Azadirachta indica.*

Dose phase	Treatment	Dose (mg/Kg)	Number of mice tested	Mortality observed
Phase I	*Azadirachta indica*	10	3	0/3
	Azadirachta indica	100	3	0/3
	Azadirachta indica	1000	3	0/3
Phase II	*Azadirachta indica*	1600	1	0/1
	Azadirachta indica	2900	1	0/1
	Azadirachta indica	5000	1	0/1

*No. of death of animals/no. of animal survived

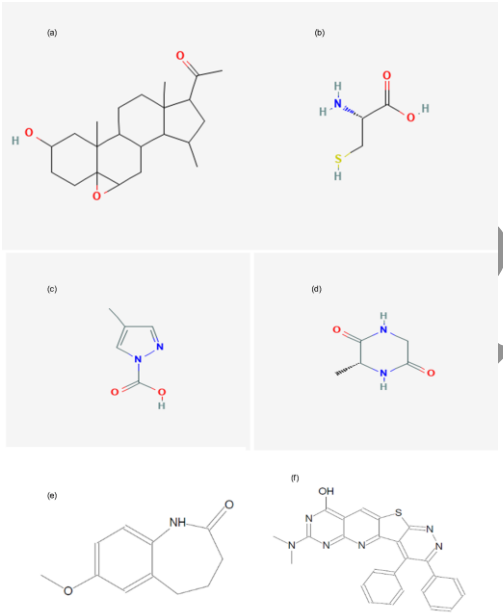


Fig. 2: Chemical structure of the compounds of *Azadirachta indica* identified by GC-MS:(a)Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl-,(b) cysteine,(c)4 methyl pyrazole -3, (d)2,5-Piperazinedione, 3-methyl-,(e) 7-Methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one, (f) 7-Dimethylamino-3,4-diphenyl-9-hydroxypyrimido[5'',4'':5',6']pyrido[2',3':4,5]thieno[2,3-c]pyridazine.

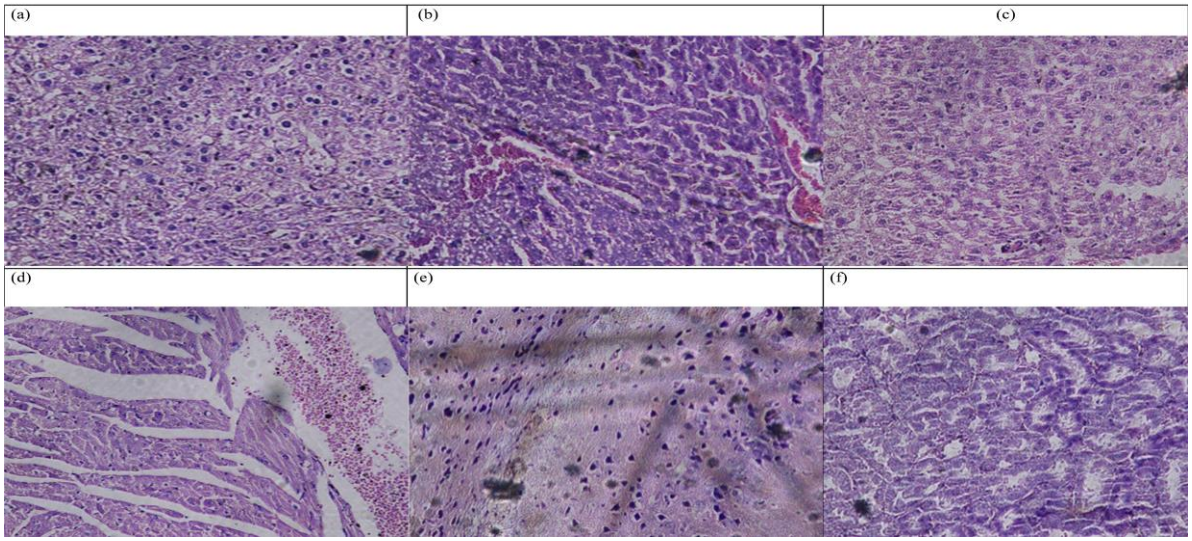


Fig. 3: Photomicrographs of liver and kidney histopathology: (a) *Azadirachta indica* 100mg/kg mice liver slice, (b) *Azadirachta indica* 100mg/kg mice kidney slice, (c) *Azadirachta indica* 1000mg/kg mice liver slice, (d) *Azadirachta indica* 1000mg/kg mice kidney slice, (e) *Azadirachta indica* 5000mg/kg mice liver slice, (f) *Azadirachta indica* 5000mg/kg mice kidney slice.

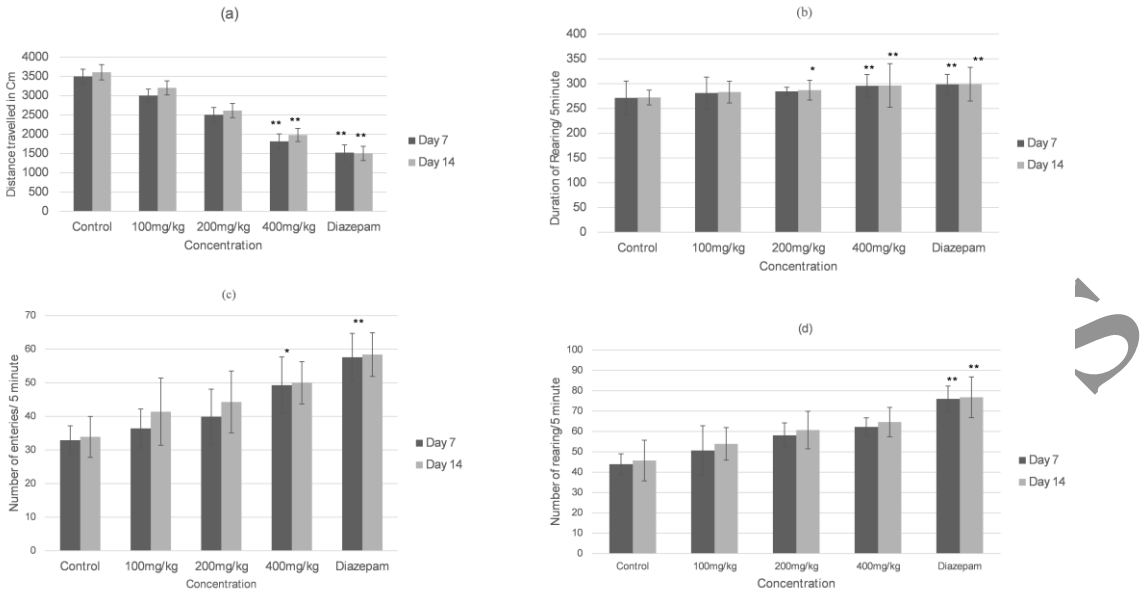


Fig. 4: Impact of *Azadirachta indica* on behavior of mice via OFT.(a) TDT=distance travelled (cm),(b) DR=duration of rearings (sec),(c) NECS=number of entries in center square,(d) TNR=total number of rearing.
*P ≤ 0.05, **P ≤ 0.005, in corelate to control group; n=10, mean ± S.D.

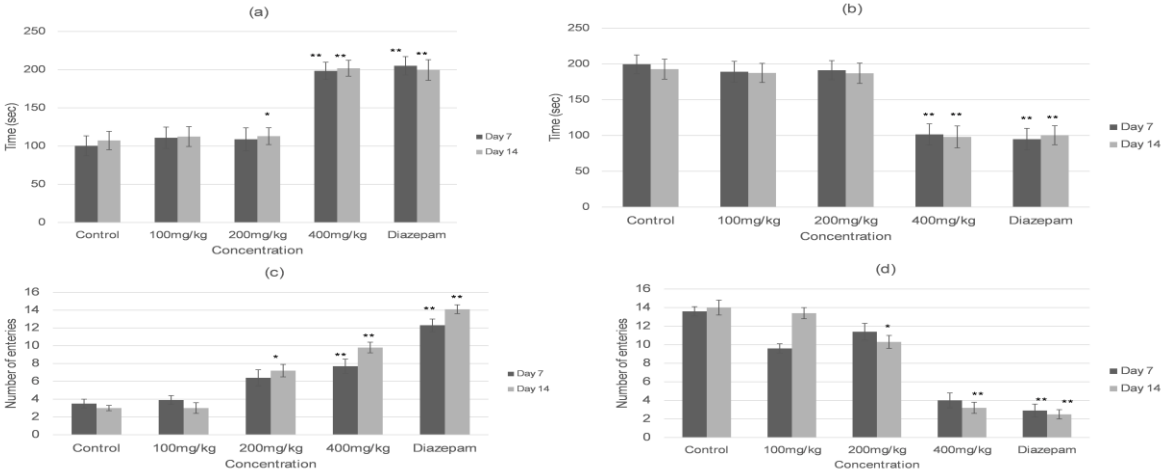


Fig. 5: Impact of *Azadirachta indica* on behavior of mice via EPM.(a) TOA=time spent in open arm,(b) TCA=time spent in close arm,(c) nEOA=number of entries in open arm,(d)nECA=number of entries in close arm.
*P ≤ 0.05, **P ≤ 0.005, in corelate to control group. n=10, mean ± S.D.

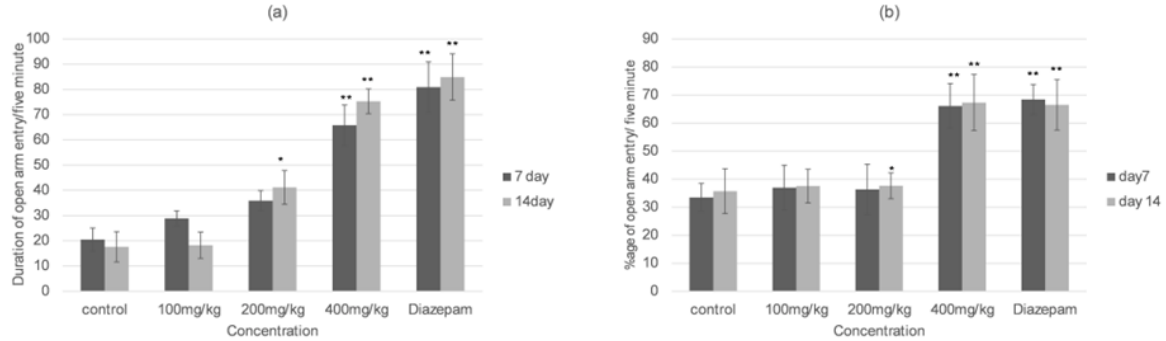


Fig. 6: Impact of *Azadirachta indica*, diazepam and control as %age TOA & %age EOA via EPM.(a) %age TOA=percentage of spent time in, b) &%age EOA=percentage of entries in open arm.*P ≤ 0.05, **P ≤ 0.005, in corelate to control group; n=10, mean ± S.D

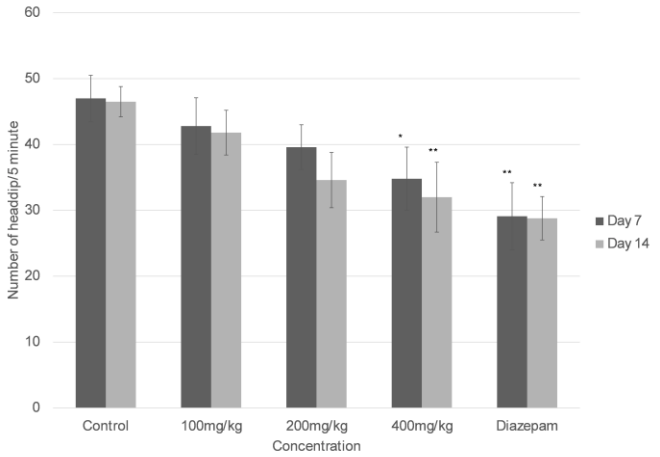


Fig. 7: Impact of *Azadirachta indica*, diazepam and control as nHD via HBT. *P ≤ 0.05, **P ≤ 0.005, in corelate to control group; n=10, mean ± S.D. nHD=number of head dips.

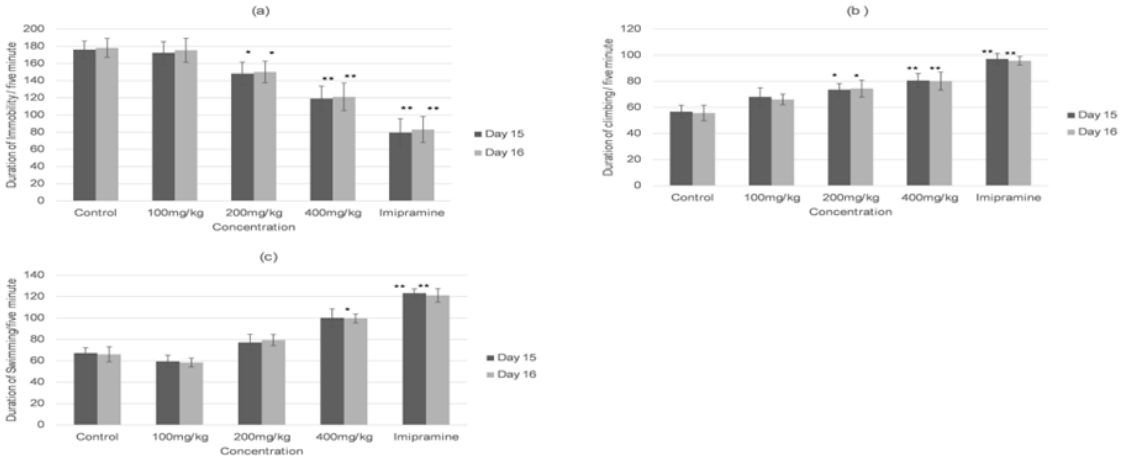


Fig. 8: Impact of *Azadirachta indica* on the behavior of mice via FST.(a)Duration of immobility(dImm),(b)Duration of Climbing(dCIm),(c)Duration of swimming(dSwm).n=10, mean ± S.E.M, *P ≤ 0.05, **P ≤ 0.005, in corelate to control group.

Phytochemical analysis

Phenols, saponins, flavonoids, Carbohydrates, glycosides, and terpenoids were qualitatively found in phytochemical analysis of the ethanolic extract of *Azadirachta indica*, as shown in Table 2.

Gas chromatography and mass spectroscopy

Fig. 1 showed the constituents identified through gas chromatography and mass spectroscopy in the extract of *Azadirachta indica* are cystine, 7-methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one, 4-methylpyrazole-3(5)-carboxylic Acid, 3-methyl3(5)-carboxylic Acid, 7-dimethylamino-3,4-diphenyl-9-hydroxypyrimido[5'',4'':5',6']pyrido[2',3':4,5]thieno[2,3-c]pyridazine2,5-piperazinedione and pregan-20-one,2-hydroxy-5,6-epoxy-15-methyl. The names of compounds and structures were assigned based on the original GC-MS study and compared with NIST library spectra. Table 3 shows the chemical constituents of *Azadirachta indica* extract, including their retention time, area, height, and molecular formula. Fig. 2 shows the chemical structure of the compound of *Azadirachta indica* extract identified by GC-MS.

Acute toxicity testing by the Lorke method

Table 4 shows *the in vivo* profile of acute toxicity of *Azadirachta indica* extract administered orally. The findings clearly showed the extract's safety profile. During the research period, no acute toxicity symptoms were observed.

Histopathological analysis of acute toxicity

Histopathological analysis of kidney and liver tissues of *Azadirachta indica* treated animals were performed after

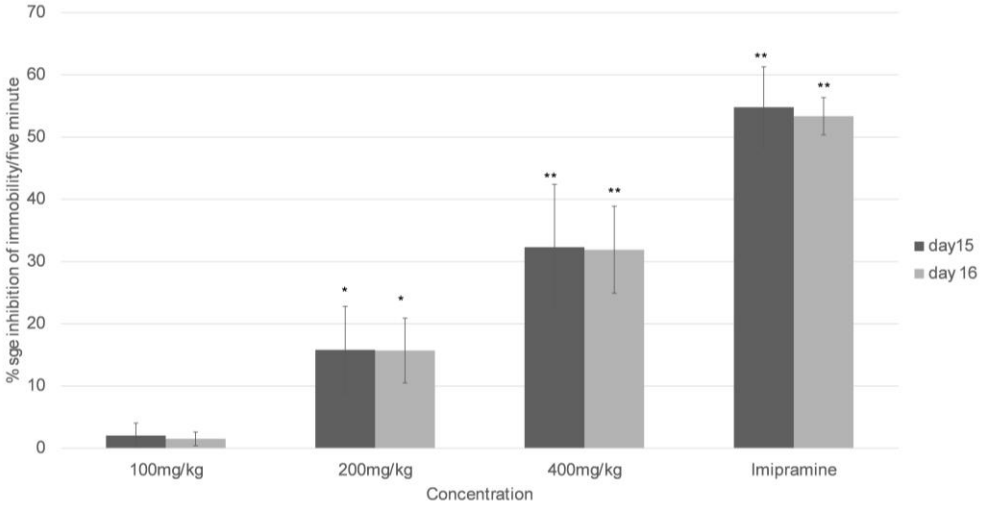


Fig. 9: Impact of *Azadirachta indica*, imipramine and control as %ageImmInh via FST
*P ≤ 0.05, **P ≤ 0.005, in corelate to control group; n=10, mean ± S.D. ImmInh=immobility inhibition

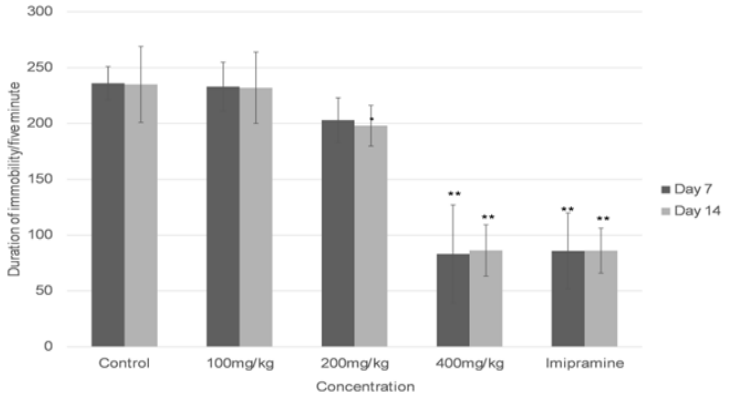


Fig. 10: Impact of *Azadirachta indica*, Imipramine and control as dImm via TST
*P ≤ 0.05, **P ≤ 0.005, in corelate to control group; n=10, mean ± S.D. dImm=duration immobility.

acute toxicity. At higher doses of 5000 mg/Kg, the liver showed mild portal inflammation and decongestion, although Fig. 3 showed normal lobular architecture of hepatocytes and no signs of lobular hepatitis in the lowest dosages of treated animals. Similarly renal biopsy sections showed no apparent glomerular alterations or evidence of acute tubular necrosis.

Anxiety-modulating potential

Open field test (OFT)

Fig. 4 demonstrated the impact of *Azadirachta indica* on mouse behavior by OFT. At 400 mg/Kg, there was a highly significant rise in DR and a reduction in TDT on the seventh and fourteenth days of testing as compared to control group. On the fourteenth day of testing, there was a notable improvement in DR at 200 mg/kg as compared to control. On the seventh day of testing, NECS at 400 mg/kg showed a significant improvement as compared to control.

Elevated plus maze (EPM)

Fig. 5 showed *the effect of Azadirachta indica* on the behavior of mice via EPM. As compared to the control group, 400mg/Kg dose exhibited a highly statistically significant rise in TOA and nEOA and a highly statistically significant reduction in TOA and nEOA on days 7th and fourteen days of the experiment as compared to control group. In contrast, the 200mg/Kg dose of *Azadirachta indica* exhibited a significant rise in TOA and nEOA and a reduction in nEOA on day 14, while TCA was reduced on the seventh day when compared with the control animal.

Fig. 6 revealed comparative anxiolytic effects of *Azadirachta indica*, diazepam, and control by EPM as %ageEOA and %ageTOA, respectively. Fig. 6 shows the impact of *Azadirachta indica* 400mg/Kg caused a highly significant increase in %ageEOA and %ageTOA in comparison to 100 and 200mg/Kg.

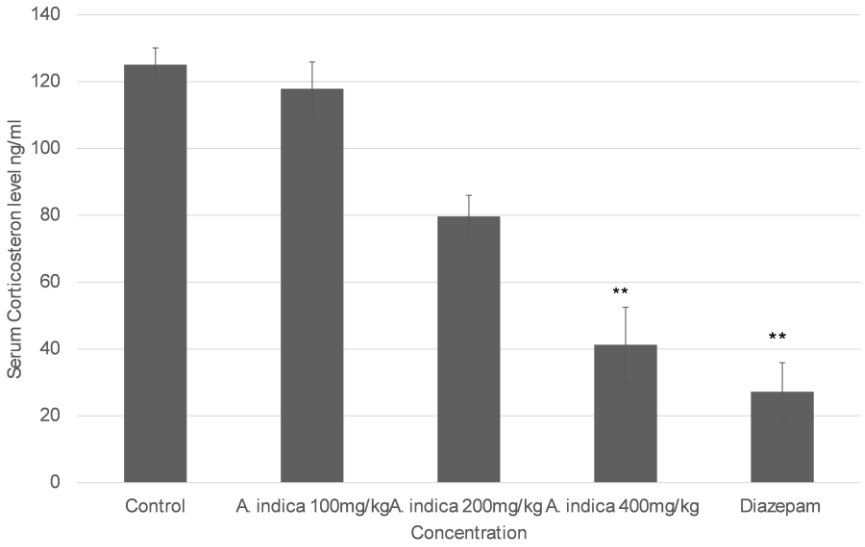


Fig. 11: Estimation of corticosterone blood level of *Azadirachta indica*, diazepam and control n=10, mean ± S.D, *P ≤ 0.05, **P ≤ 0.005, in corelate to control group.

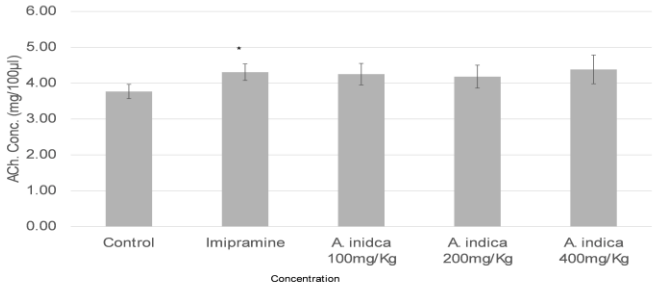


Fig. 12: Evaluation of acetylcholine concentration of *Azadirachta indica* rat's brain n=10, mean ± S.D, *P ≤ 0.05, **P ≤ 0.005, in corelate to control group.

Hole board test (HBT)

Fig. 7 reveals the comparative anxiolytic impact of *Azadirachta indica*, diazepam, and control via HBT expressed as nHD. *Azadirachta indica* at 400mg/Kg showed a substantial reduction in nHD as compared with the control group.

Anti-depressant activity

Forced swimming test (FST)

Fig. 8 demonstrates *the impact of Azadirachta indica* on the behavior of mice via FST. *Azadirachta indica* fruit has shown a highly significant reduction in dImm and a rise in dClm (duration climbing) by 400mg/Kg on the 15th and 16th day as compared to control; however, an upsurge in dSwm (duration swimming) was observed on the 16th. The study found a significant decrease in dImm and an increase in dClm by 200mg/Kg on both 15- and 16-day testing compared to the control.

Fig. 9 revealed comparative antidepressant effects of *Azadirachta indica* and imipramine by FST as %age ImmInh. *Azadirachta indica* 400mg/Kg showed a significant increase in %ageImmInh.

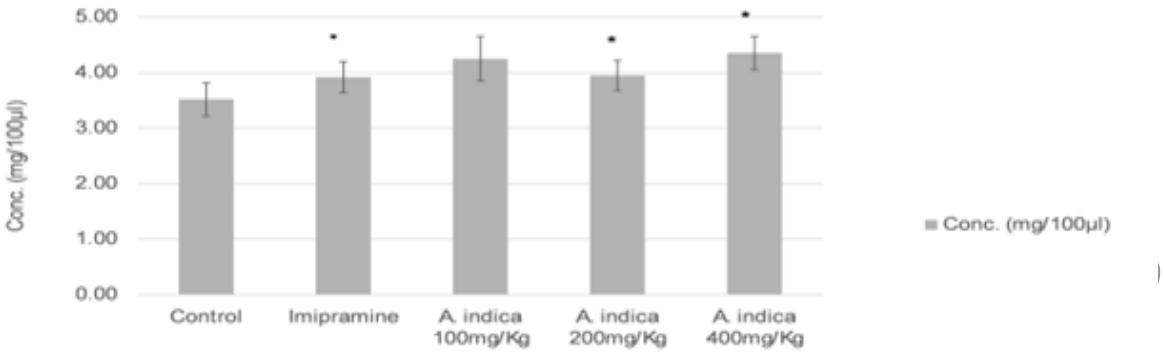


Fig. 13: Estimation of acetylcholinesterase level of *Azadirachta indica* rat's blood n=10, mean ± S.D, *P ≤ 0.05, **P ≤ 0.005, in corelate to control group.

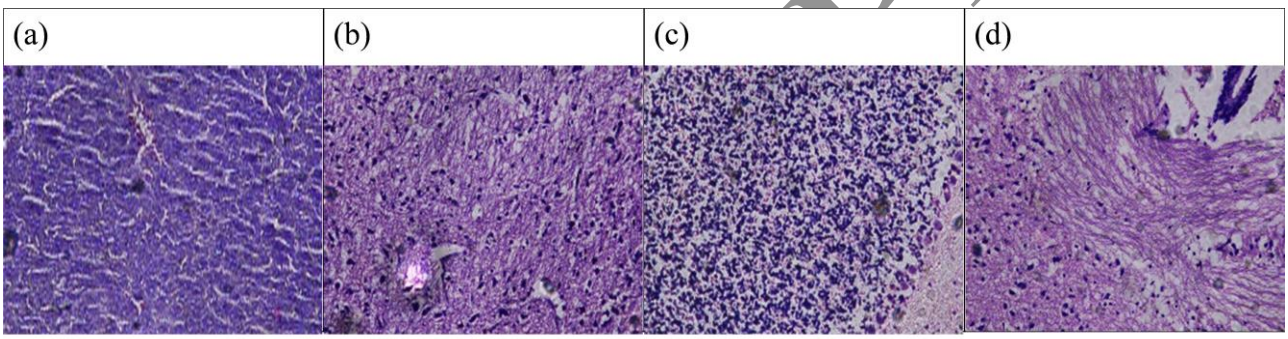


Fig. 14: Photomicrographs of brain histopathology: (a) Section of control showed normal cells, (b) Section of *Azadirachta indica* 100mg/Kg, (c) Section of the *Azadirachta indica* 200 mg/Kg, (d) Section of *Azadirachta indica* 4000mg/Kg.

Tail suspension test (TST)

Fig. 10 revealed comparative antidepressant effects of *Azadirachta indica*, imipramine and control as dImm (duration of immobility) by TST. *Azadirachta indica* 400mg/Kg and imipramine showed a significant decrease in dImm when contrasted with control on both the 15th and 16th days of testing.

Estimation of corticosterone level

Fig. 11 exhibited corticosterone blood concentration of *Azadirachta indica*, diazepam, and control. *Azadirachta indica* at 400mg/Kg and diazepam significantly reduced corticosterone concentration compared to the control group over fifteen days.

Estimation of acetylcholine (ACh) and acetylcholinesterase level (AChE)

Figure 12 shows that imipramine produced a significant increase in hippocampal acetylcholine (ACh) levels compared to the control group ($p < 0.05$). In contrast, the *Azadirachta indica* treatment groups did not show a significant change in ACh levels compared to either control or imipramine.

Fig. 13 showed the impact of *Azadirachta indica* on AChE levels in the brains of all test groups. Post hoc Tukey test showed *Azadirachta indica* significantly increased AChE levels in the hippocampus of the brain at doses of 200 and 400mg/Kg compared to control group.

Histopathology of brain

Fig. 14 showed histopathology of brain tissues. No significant morphological changes were revealed in all treated groups of *Azadirachta indica* and control animals.

DISCUSSION

Herbal phytochemicals are widely recognized for their diverse biological activities, making them valuable in therapeutic research. In this study, the phytochemical profile of *Azadirachta indica* was examined, revealing constituents such as phenols, saponins, flavonoids, glycosides, carbohydrates, and terpenoids. These

compounds are notable for their neuroactive and antioxidant properties, which likely contribute to the anxiolytic and antidepressant effects observed in the behavioral models.

51 phytochemicals have been identified in *Azadirachta indica* through GC-MS analysis with diverse biological functions. The current investigation has found an abundance of phytochemicals in *Azadirachta indica*, some of which have been previously reported by other researchers. These include cholest-5-en-3-ol (3β)-, 9-octadecenoate, 7-dimethylamino-3,4-diphenyl-9-hydroxypyrimido[5'',4'':5',6']pyrido[2',3':4,5]thieno[2,3-c]pyridazine, 2,3-bis(3',4'-dimethoxyphenyl)-5,6-dimethoxybenzo[b]furan, 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde, 9-[2-deoxy-β-d-ribohexopyranosyl]purin-6(1H)-one, 7-methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one, d-arabino-hex-1-enitol, 1,5-anhydro-2-deoxy-, lactose, desulphosinigrin, 1-nitro-β-d-arabinofuranose, tetraacetate, 2-myristynoyl pantetheine, permethylated and reduced product of intact H3-glycolipid, α-d-Glucopyranoside, o-α-d-glucopyranosyl-(1.fwdarw.3)-β-d-fructofuranosyl, 1,2,3-propanetriol, monoacetate. These chemicals have been shown to possess several pharmacological characteristics, including anti-inflammatory, anti-microbial, cytotoxic, anti-cancer, anti-oxidant, neuroprotective, memory-enhancing, anti-depressant, anticonvulsant, sedative, hypnotic, anti-epileptic, and neurodegenerative diseases (Cetin, 2021; Kumar *et al.,* 2019; Levonen *et al.,* 2004). Table 3 offers a complete list of all metabolites along with their biological activities, whereas only a few bioactive components out of 51 discovered metabolites are listed above with their recognized pharmacological activity.

For instance,7-Methoxy-1,3,4,5tetrahydrobenzo[b]azepin-2-one, a benzoazepin derivative, has been reported to possess anxiolytic and antidepressant properties via modulation of serotonergic and dopaminergic pathways (Ullah *et al.,* 2018). Piperazine derivatives such as 2,5-dioxo-3-methylpiperazine are known antagonists at dopamine D2 and 5-HT2 receptors, contributing to antipsychotic and anxiolytic effects (Qadir *et al.,* 2022). Cysteine derivatives play a critical role in glutathione synthesis and redox regulation, consistent with the antioxidant activity detected in the DPPH assay (Liu *et al.,* 2020). Similarly, β-sitosterol and stigmasterol, both sterols detected in the extract, are well-documented for their neuroprotective and antidepressant-like actions through modulation of oxidative stress and serotonergic signaling (Ahmed *et al.,* 2023). Finally, phytol, a diterpene alcohol, is known to act on GABAergic pathways and may contribute to both anxiolytic and sedative effects, which could partially explain the reduced locomotor activity at higher extract doses (Islam *et al.,* 2025). Overall, these metabolites likely underlie the observed neurobehavioral and antioxidant effects, though some metabolites like phytol may be reported for their sedative influences at higher doses. Future studies should validate these mechanisms through receptor binding and long-term pharmacological evaluations.

The present study results revealed significant antioxidant effects of *Azadirachta indica* by DPPH at an increase in concentration of 68.6%. Antioxidant effects of cystine and Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl were previously identified in numerous studies (Piste, 2013). Such antioxidant activity is highly attributable to cystine and flavonoid compounds, which both contribute a crucial role as substrates in the synthesis of glutathione. These findings align with ethno-pharmacological evidence supporting the use of plants as an alternative to synthetic antioxidants, which may be beneficial in managing various neuropsychiatric and neurodegenerative conditions (Doreddula *et al.,* 2014; Harini *et al.,* 2012).

The acute lethal study by Lorke's toxicity test of *Azadirachta indica* extract on mice revealed no major signs of toxicity or other symptoms noted in any dose of *Azadirachta indica* after oral administration. The predicted LD50 values, as per Lorke's guidelines, are over 5000 mg/Kg, which is considered safe (Elufioye & Onoja, 2015). However, at the maximum dosage, histopathological results showed moderate hepatic inflammation, which may indicate subclinical organ stress with continued usage. The requirement of evaluating long-term toxicity prior to clinical translation is highlighted by this observation. The extract's neuroprotective nature was supported by the absence of structural abnormalities seen in brain histopathology.

The present study investigated the anxiolytic effects of *Azadirachta indica* via OFT, EPM, and HBT. Administration of *Azadirachta indica* at doses of 200 and 400mg/Kg resulted in highly significant increases in DR and decreases in TDT and significant improvement in NECS at 400mg/Kg via OFT. Hence, 400mg/Kg effects were quite like the effects of diazepam. *Azadirachta indica* may exhibit improved animal behavior based on these behavioral changes, which indicate a high degree of low anxiety and increased exploratory behaviour. Hence, animals' decreased time in the middle zone during OFT indicates their worry about exploring and going into a new place, which may be linked to higher levels of anxiety (Simpson & Kelly, 2011). The results of this investigation confirm the therapeutic benefit of *Azadirachta indica* in lowering anxiety symptoms.

The current study indicates a much larger frequency of TOA, nEOA, %ageEOA, and %ageTOA than TCA and nECA via EPM, which might be regarded as an anxiety-inducing effect of *Azadirachta indica* at 200 and 400mg/Kg. This observation is similar to how diazepam

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works (Ankur *et al.*, 2013). The EPM was also utilized to identify behavioral alterations in mice (Shaida *et al.*, 2019).

The results of the present study, after a 14-day testing period, revealed that the administration of 400mg/kg of *Azadirachta indica* and diazepam(3mg/kg) led to a notable reduction in nHD. Additionally, a significant decrease in nHD was observed with the administration of 200mg/kg on the 14th day of testing. The evaluation employed the HBT to gauge rodent anxiety based on exploratory activity in various environmental settings (Abreu *et al.*, 2018; Martínez-Vázquez *et al.*, 2012). Although the decrease in locomotor activity at 400 mg/kg raises the possibility of sedative effects, this might complicate the interpretation of anxiolysis in OFT and EPM. This restriction should be considered while assessing behavioral results. The above-stated anxiolytic outcomes of *Azadirachta indica* extract may be due to the occurrence of 7-Methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one as identified in the extract by GC-MS (Geneste *et al.*, 2006; Giri, 2021).

The results based on experiments conducted on two animal models of depression i.e., TST and FST, revealed *Azadirachta indica* antidepressant-like effects. Compared with the control, TST *Azadirachta indica* 400mg/Kg showed an effect similar to imipramine, i.e., significant decreases in dImm on both the 7th and 14th days of testing. While in FST, *Azadirachta indica* showed potential antidepressant qualities by a highly significant decline in dImm and an increase in dClm at 400mg/Kg on the 7th and 14th days. Comparative antidepressant effects of *Azadirachta indica* and imipramine: *Azadirachta indica* 400mg/Kg showed a significant increase in % age ImmInh, quite near to imipramine. This outcome is consistent with other studies showing less immobility in behavioral depression brought on by stress *(Hao et al.*, 2019*;* Zhang *et al.*, 2019). This may be associated with the presence of flavonoids, which are polyphenols and the most effective functional components with biological activity. The presence of flavonoids may be responsible for showing antidepressant properties, as the antidepressant effect of flavonoids is already proven (Ganaie *et al.*, 2020; Hritcu *et al.*, 2017). A significant amount of polyphenols is found in *Azadirachta indica* fruit (Dambhare *et al.*, 2020). The above-mentioned antidepressant effect is confirmed by the presence of 7-methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one,7-dimethylamino-3,4 diphenyl-9-hydroxypyrimido[5'',4'':5',6'] pyrido[2',3':4,5]thieno[2,3-c]pyridazine, as confirmed by GC-MS (Alghamdi & Asif, 2023).

The results of the study on corticosterone levels, compared with the depressed rat exhibited that rats treated with *Azadirachta indica* displayed low levels of corticosterone, which is due to the occurrence of 7-methoxy-1,3,4,5-tetrahydrobenzo[b]azepin-2-one as detected by GC-MS. According to Reamtong, the compound is categorized structurally as benzoazepin derivative. It exists in various mood-alleviating drugs and is important in reducing anxiety and depression (Reamtong *et al.*, 2021).

AChE levels in Figure 12 showed that the rats treated with *Azadirachta indica* at the doses of 100 and 400mg/Kg as compared with the control and disease control animals, showed significantly increased AChE levels when compared to the control, as increased AChE levels indicate a decrease in anxiety and depressive symptoms in an individual (Mitić & Lazarević-Pašti, 2021).

Brain histopathology revealed no morphological changes in the hippocampus at any dose of *Azadirachta indica*, consistent with previous experimental studies demonstrating the neuroprotective effect of flavonoids (Bellavite, 2023). The experimental data also confirmed that *Azadirachta indica* is rich in flavonoids and exhibits neuroprotective effects.

This study integrates GC-MS-based metabolite profiling with behavioral, biochemical, and histopathological assessments to establish a clear link between specific metabolites (e.g., benzoazepin and piperazine derivatives) and neurobehavioral outcomes in neem. It provides a stronger translational basis for the neuropharmacological potential of *Azadirachta indica*, unlike previous reports that assessed isolated effects of neem.

Despite these promising findings, some limitations should be acknowledged. The absence of molecular assays (e.g., receptor binding or neurotransmitter quantification), lack of bioavailability studies, and no evaluation of long-term or chronic toxicity restrict the strength of mechanistic claims. Moreover, the possibility of sedative confounding at higher doses complicates the interpretation of anxiolytic results in OFT and EPM. Future work should address these gaps through chronic administration studies, pharmacokinetic profiling, and molecular validations to confirm the precise pathways involved.

CONCLUSION

This study exhibited that *Azadirachta indica* extract validates antidepressant, anxiolytic, and antioxidant properties in preclinical experimental models. GC-MS profiling revealed 51 bioactive components that have already been reported to have anti-inflammatory and neuroprotective qualities, which might be responsible for the pharmacological action observed. Additionally, *Azadirachta indica* neuro psycho pharmacological significance comes from the alteration of corticosteroid and acetylcholinesterase levels.

Further research is necessary for transforming these findings into clinical application, including dosage optimization, mechanistic investigation, and long-term

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safety assessment in several animal models before moving on to clinical trials. Future studies should also differentiate between anxiolytic and potential sedative effects at higher dosages. While highlighting the need for thorough validation and translational research, the study suggests a scientific foundation for investigating *Azadirachta indica* as a potential option for natural treatments for neuropsychiatric disorders.

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Authors' contributions
Mariam Razzak and Azra Riaz: Conceptualized and designed the study; Mariam Razzak: Performed the experimental work and data collection. Muhammad Ali: Interpreted the GC-MS analysis and interpretation of phytochemical data; Shahana Wahid: Co-supervised the research project, validated the methodology and critically reviewed the manuscript; Bushra Sikandar and Saira Saeed Khan: Contributed to statistical analysis and data interpretation; Tabbassum Zehra and Maham Nawaz: Assisted in laboratory experimentation and literature review; Anusha Ahmed: Contributed to manuscript drafting and formatting. All authors reviewed, revised, and approved the final version of the manuscript.

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Data availability statement
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. All relevant data supporting the findings of this study are included within the article.

Ethical approval
The study protocol was reviewed and approved by the Ethical Review Committee of the University of Karachi (IBC KU-430-A/2024). All experimentation was conducted in accordance with the institutional ethical guidelines. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The authors declare that they have no conflicts of interest related to this work.

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