RESEARCH PAPER

Anxiolytic and Neuroprotective Effects of a Clozapine Liqui-Solid Formulation in a Caffeine-induced Anxiety Model in Wistar Rats

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Abstract

Anxiety disorders are complex neuropsychiatric conditions associated with oxidative stress, neuroinflammation, neurotransmitter imbalance, and impaired neuroplasticity, and the therapeutic utility of clozapine is often limited by poor aqueous solubility and variable oral bioavailability. The present study evaluated the anxiolytic and neuroprotective potential of an optimized clozapine liqui-solid formulation in a caffeine-induced anxiety model in Wistar rats. The study was conducted for 14 consecutive days using four groups: control, negative control treated with caffeine (30 mg/kg, i.p.), standard treated with diazepam, and a test group treated with clozapine liqui-solid formulation (10 mg/kg, p.o.). Anxiety-like behavior was assessed using the elevated plus maze, open field test, and light–dark box test, while biochemical and neurochemical investigations included estimation of malondialdehyde, glutathione, superoxide dismutase, catalase, corticosterone, inflammatory cytokines, monoamine neurotransmitters, GABA, brain-derived neurotrophic factor, tropomyosin receptor kinase B, and glial fibrillary acidic protein. The clozapine liqui-solid formulation significantly improved behavioral performance by increasing open-arm exploration, central-zone activity, and time spent in the light compartment compared with the caffeine-treated negative control group. Treatment also reduced lipid peroxidation, corticosterone, and pro-inflammatory cytokines, restored endogenous antioxidant defenses, normalized serotonin, dopamine, noradrenaline, and GABA levels, enhanced BDNF and TrkB expression, and reduced GFAP levels. These findings demonstrate that the clozapine liqui-solid formulation exerts marked anxiolytic and neuroprotective effects through multi-mechanistic pathways involving behavioral modulation, antioxidant activity, anti-inflammatory action, neurotransmitter restoration, and neuroplasticity enhancement, suggesting that liqui-solid delivery may improve the therapeutic performance of clozapine in anxiety-related disorders.

Keywords: Anxiety; Clozapine; Liqui-solid formulation; Caffeine-induced anxiety; Wistar rats; Oxidative stress; Neuroinflammation; Neurotransmitters; BDNF; Neuroprotection.

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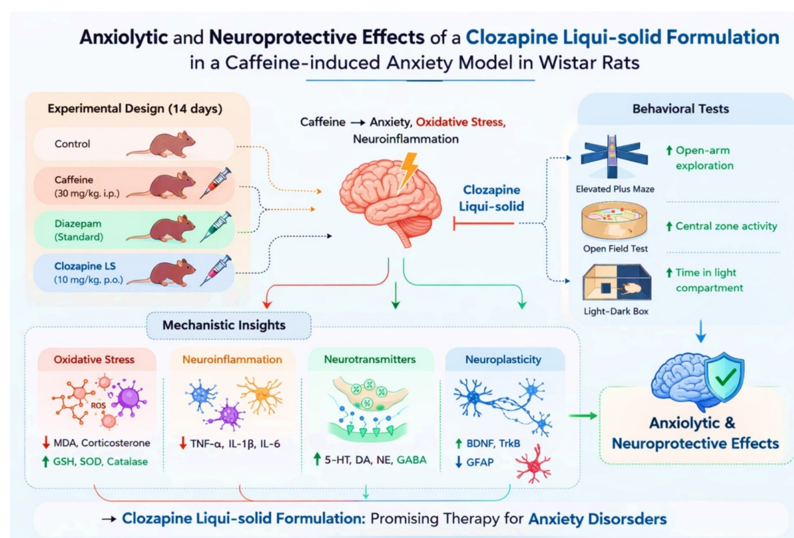
GRAPHICAL ABSTRACT



1. INTRODUCTION

Anxiety disorders are among the most prevalent psychiatric illnesses worldwide and remain a major contributor to

disability, impaired quality of life, and reduced social and occupational functioning. The World Health Organization reported that approximately 359 million people were living with an anxiety disorder in 2021, corresponding to about 4.4% of the global population, with women affected more often than men. Clinically, anxiety disorders are characterized by persistent and excessive fear or worry, frequently accompanied by irritability, restlessness, autonomic arousal, sleep disturbance, impaired concentration, palpitations, sweating, trembling, and avoidance behavior. Despite the availability of effective treatments, only a minority of affected individuals receive adequate care, highlighting the need for improved therapeutic strategies and more effective dosing systems [1].

The neurobiology of anxiety is multifactorial and involves dysregulation across interconnected neurotransmitter, endocrine, inflammatory, and neuroplastic pathways. Abnormal signaling in serotonergic, dopaminergic, noradrenergic, and GABAergic systems contributes to heightened arousal and maladaptive fear processing, while hypothalamic–pituitary–adrenal (HPA) axis overactivation increases glucocorticoid release and reinforces stress responsivity. In parallel, oxidative stress and neuroinflammatory mediators such as TNF-α, IL-1β, and IL-6 can impair synaptic function and reduce neurotrophic support, including brain-derived neurotrophic factor (BDNF)-dependent signaling, thereby promoting anxiety-like phenotypes and limiting adaptive neuronal plasticity. Recent reviews continue to support this integrated mechanistic framework and emphasize the importance of linking behavioral findings with molecular and biochemical endpoints in preclinical anxiety research [2,3].

Because anxiety is expressed through both emotional and exploratory behavior, well-validated animal paradigms remain central to translational screening. Among these, the elevated plus maze (EPM), open field test (OFT), and light–dark box (LDB) are widely used for evaluating anxiety-like behavior in rodents. These assays are particularly useful because they capture avoidance of open or illuminated spaces, risk assessment, and exploratory drive, all of which are highly sensitive to anxiogenic and anxiolytic manipulations. Caffeine is a suitable pharmacological inducer in such models because, at sufficiently high doses, it can provoke anxiety-related symptoms and alter behavioral performance in rodents; previous studies have shown caffeine-induced increases in anxiety-like behavior in paradigms such as the EPM and related tests, supporting its use in preclinical anxiety studies [4-7].

Clozapine is a unique atypical antipsychotic with a broad receptor profile involving serotonergic, dopaminergic, muscarinic, histaminergic, and adrenergic systems. Although its established clinical role is in treatment-resistant schizophrenia rather than primary anxiety disorders, its multimodal central pharmacology makes it relevant to pathways that influence stress responsiveness, affective behavior, and behavioral inhibition. A major limitation of clozapine, however, is its poor aqueous solubility, slow dissolution, and extensive first-pass metabolism, all of which contribute to variable and often low oral bioavailability. Recent formulation studies continue to identify these biopharmaceutical constraints as a major obstacle to optimizing clozapine delivery and therapeutic performance [8-10].

For poorly water-soluble drugs, the liquisolid technique has emerged as a practical and scalable approach for improving dissolution behavior and, potentially, oral absorption. In this strategy, the drug is dissolved or dispersed in a non-volatile solvent and then converted into a dry, free-flowing, and compressible system using appropriate carrier and coating materials. Recent reviews describe liquisolid technology as a promising platform for hydrophobic drugs because it can enhance wetting, increase the surface area available for dissolution, and improve release performance while remaining compatible with conventional solid dosage manufacturing [11,12].

In the present work, a clozapine-loaded liquisolid formulation developed to overcome the drug's dissolution limitations was evaluated in a caffeine-induced anxiety model in Wistar rats (180–250 g) over a 14-day study period. Caffeine was administered intraperitoneally at 30 mg/kg, while the test formulation was assessed at 10 mg/kg clozapine. Anxiety-like behavior was evaluated using the EPM, OFT, and LDB, and treatment effects were further explored through biochemical and molecular markers related to oxidative stress, neuroinflammation, stress-axis activation, and neuroplasticity. The study was designed to determine whether improving clozapine delivery through a liquisolid system could produce measurable anxiolytic-like and neuroprotective effects in this preclinical model [4,6,11].

2. Materials and Methods

2.1 Drugs, formulation, and reagents

Clozapine was used as the active pharmaceutical ingredient and was administered as the optimized liquisolid formulation at a dose equivalent to 10 mg/kg, p.o. Caffeine was used as the anxiogenic agent at 30 mg/kg, i.p., and diazepam was used as the standard anxiolytic drug at the selected experimental dose. Normal saline or a suitable vehicle was used for preparation of injectable and oral suspensions. All chemicals and reagents used were of analytical grade. Common reagents required for oxidative stress assays included thiobarbituric acid, sodium dodecyl sulfate, acetic acid, trichloroacetic acid, reduced glutathione, 5,5′-dithiobis-(2-nitrobenzoic acid), epinephrine, hydrogen peroxide, potassium phosphate buffer, and EDTA. ELISA kits were used for corticosterone, TNF-α, IL-1β, IL-6, and BDNF estimation. For protein expression studies, RIPA

buffer, protease inhibitor cocktail, bicinchoninic acid assay reagents, SDS-PAGE reagents, PVDF membrane, TrkB and GFAP primary antibodies, HRP-conjugated secondary antibodies, and enhanced chemiluminescence substrate were used [18-23].

2.2 Experimental animals

Healthy adult Wistar rats weighing 180–250 g were used for the study. Animals were housed in polypropylene cages under standard laboratory conditions at controlled temperature and humidity, maintained on a 12 h light/12 h dark cycle, and provided standard pellet diet and water ad libitum. Prior to the experiment, animals were acclimatized to the laboratory environment. Such environmental control is essential in rodent anxiety studies because behavioral paradigms like the elevated plus maze, open field, and light–dark box are sensitive to external stressors and handling conditions [13,14].

2.3 Experimental design

The study was conducted for 14 consecutive days, during which the animals were randomly allocated into four experimental groups, with six rats in each group. Group I served as the control group and received only the vehicle. Group II served as the negative control group and was administered caffeine at a dose of 30 mg/kg intraperitoneally to induce anxiety-like behavior. Group III served as the standard treatment group and received caffeine along with diazepam, which was used as the reference anxiolytic drug. Group IV served as the test group and received caffeine along with the clozapine liquisolid formulation at a dose of 10 mg/kg by the oral route. Caffeine is a well-recognized pharmacological inducer of anxiety-like behavior in rodents, whereas diazepam is widely employed as a positive control in preclinical anxiolytic screening models 15,16].

2.4 Induction of anxiety and treatment schedule

Anxiety-like behavior was induced by intraperitoneal administration of caffeine (30 mg/kg) during the study period. The standard group received diazepam along with caffeine treatment, whereas the test group received the optimized clozapine liquisolid formulation orally along with caffeine induction. Since liquisolid formulations are intended to improve dissolution and oral performance of poorly water-soluble drugs, the optimized formulation was administered as a freshly prepared oral dispersion or suspension [16,17].

2.5 Behavioral assessment

Behavioral assessment was carried out in a quiet room under controlled illumination. Apparatuses were cleaned between animals to avoid odor-based interference. Anxiety-like behavior was evaluated using the elevated plus maze (EPM), open field test (OFT), and light–dark box (LDB). These tests are standard behavioral paradigms for assessing open-space avoidance, exploratory activity, and light aversion in rodents [13,14].

Elevated plus maze

The elevated plus maze consisted of two open arms and two closed arms elevated above the floor. Each rat was placed at the center of the maze facing an open arm and allowed to explore for the specified test period. The principal parameters recorded were time spent in the open arms and number of open-arm entries. Increased open-arm activity was considered indicative of anxiolytic-like behavior [13].

Instrument used: Elevated plus maze apparatus with stopwatch or video-tracking system.

Open field test

For the open field test, each rat was placed in the center of the arena and allowed to explore freely. The main parameters recorded were time spent in the central zone and total locomotor activity. Reduced center exploration reflects anxiety-like behavior, while locomotion helps distinguish anxiolysis from sedation or nonspecific motor effects [14].

Instrument used: Open field apparatus with manual grid scoring or automated video-tracking system.

Light–dark box test

The light–dark box test was carried out in an apparatus with one illuminated and one dark compartment. Each animal was placed in the apparatus and allowed to move freely between the two compartments. The parameters recorded were time spent in the light compartment and number of transitions between compartments. Increased time in the light chamber and more transitions were considered indicative of reduced anxiety-like behavior [14].

Instrument used: Light–dark box chamber with stopwatch, photocell counter, or video-tracking system.

2.6 Sample collection and tissue preparation

After completion of the behavioral studies, animals were anesthetized and blood samples were collected for serum separation. The brains were rapidly excised, washed with ice-cold saline, blotted dry, and homogenized in chilled buffer using a glass-Teflon homogenizer or motorized tissue homogenizer. The homogenates were centrifuged using a refrigerated centrifuge, and the supernatants were used for biochemical, neurochemical, and molecular estimations [24].

Instruments used: Tissue homogenizer, refrigerated centrifuge, micropipettes, vortex mixer, deep freezer, and pH meter.

2.7 Estimation of oxidative stress markers

Oxidative stress in brain homogenates was assessed by estimating malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) using a UV–Visible spectrophotometer.

- MDA was estimated by the thiobarbituric acid reactive substances (TBARS) method using thiobarbituric acid, sodium dodecyl sulfate, acetic acid, and trichloroacetic acid. Absorbance was measured at 532 nm [18].
- GSH was estimated by the DTNB method using 5,5′-dithiobis-(2-nitrobenzoic acid), phosphate buffer, and

protein-precipitating reagent. Absorbance was measured at 412 nm [19].

- SOD activity was estimated by the epinephrine auto-oxidation method using epinephrine, carbonate buffer, and EDTA [20].
- Catalase activity was determined by measuring the decomposition of hydrogen peroxide in phosphate buffer [21].

Instruments used: UV–Visible spectrophotometer, centrifuge, water bath, micropipettes, vortex mixer.Chemicals used: TBA, SDS, acetic acid, TCA, DTNB, epinephrine, hydrogen peroxide, phosphate buffer, EDTA.

2.8 Estimation of corticosterone

Serum corticosterone was estimated as a marker of hypothalamic–pituitary–adrenal axis activation using a rat corticosterone ELISA kit according to the manufacturer's protocol. Serum samples were added to antibody-coated wells, followed by incubation with enzyme conjugate and substrate solution, and absorbance was measured using a microplate reader at the recommended wavelength [22].

Instruments used: Microplate reader, incubator/plate shaker, micropipettes.Chemicals/reagents used: Rat corticosterone ELISA kit, wash buffer, substrate solution, stop solution.

2.9 Estimation of inflammatory markers

Brain homogenate supernatants were analyzed for TNF-α, IL-1β, and IL-6 using rat-specific sandwich ELISA kits. Tissue samples were homogenized in ice-cold buffer, centrifuged, and the supernatants were processed according to the kit instructions. Cytokine quantification by ELISA is a standard approach for assessment of neuroinflammation in rodent studies [23].

Instruments used: Tissue homogenizer, refrigerated centrifuge, microplate reader, plate shaker, micropipettes.Chemicals/reagents used: Rat TNF-α, IL-1β, and IL-6 ELISA kits, phosphate-buffered saline or lysis buffer, protease inhibitor cocktail.

2.10 Neurotransmitter estimation

Brain levels of serotonin, dopamine, noradrenaline, and GABA were estimated using high-performance liquid chromatography (HPLC/UHPLC). Brain tissue was homogenized in cold perchloric acid, centrifuged, and the supernatant was filtered before analysis. Monoamines were quantified using an electrochemical detector, while GABA could be estimated after derivatization with appropriate reagents, such as o-phthalaldehyde [24].

Instruments used: HPLC/UHPLC system, autosampler, analytical column, electrochemical or fluorescence detector, centrifuge, membrane filters.Chemicals/reagents used: Perchloric acid, mobile-phase buffers, methanol/acetonitrile, EDTA, ion-pairing reagents, o-phthalaldehyde.

2.11 Estimation of BDNF, TrkB, and GFAP

BDNF was estimated in brain homogenate using a commercial rat BDNF ELISA kit and a microplate reader, as ELISA is a commonly used and validated technique for BDNF quantification in brain tissue [25]. TrkB and GFAP expression were estimated by Western blotting. Briefly, tissue lysates were prepared in RIPA buffer containing protease inhibitors, protein concentration was determined by the BCA assay, equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with primary antibodies against TrkB and GFAP followed by HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence substrate [25,26].

Instruments used: Tissue homogenizer, refrigerated centrifuge, microplate reader, gel electrophoresis unit, blot transfer unit, chemiluminescence imaging system.Chemicals/reagents used: Rat BDNF ELISA kit, RIPA buffer, protease inhibitor cocktail, BCA reagents, acrylamide, SDS, PVDF membrane, primary antibodies for TrkB and GFAP, HRP-conjugated secondary antibodies, ECL substrate.

2.12 Statistical analysis

The data were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test for comparison among groups. A value of $p < 0.05$ was considered statistically significant [14].

Effect on neurotransmitters (5-HT, dopamine, NA, GABA)

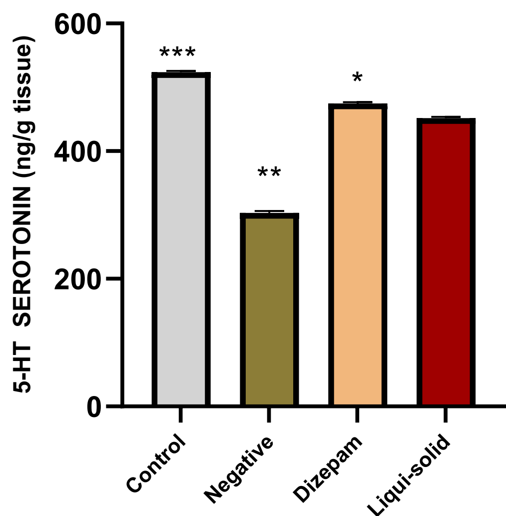
The Negative group showed a pronounced reduction in monoaminergic neurotransmitters compared with the Control group. Specifically, 5-HT, dopamine, and noradrenaline (NA) were markedly decreased in the Negative group, indicating significant neurochemical impairment. Treatment with diazepam and the Liqui-solid formulation significantly restored neurotransmitter levels compared to the Negative group. Although both interventions improved neurotransmitter deficits, diazepam generally produced a slightly higher recovery than the Liqui-solid formulation.

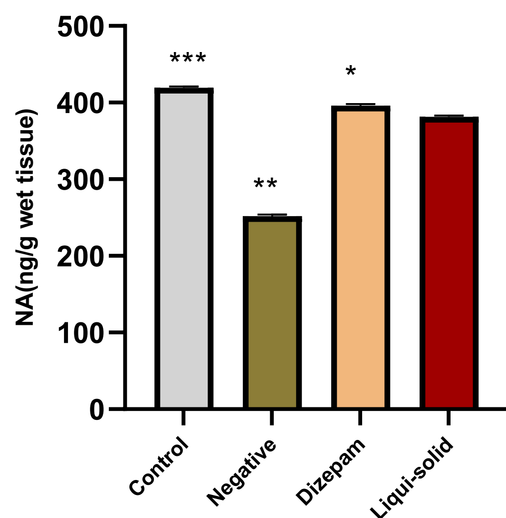
For inhibitory neurotransmission, GABA levels were also reduced in the Negative group compared with Control. Both diazepam and Liqui-solid significantly elevated GABA versus Negative, consistent with anxiolytic/anti-stress neurochemical normalization.

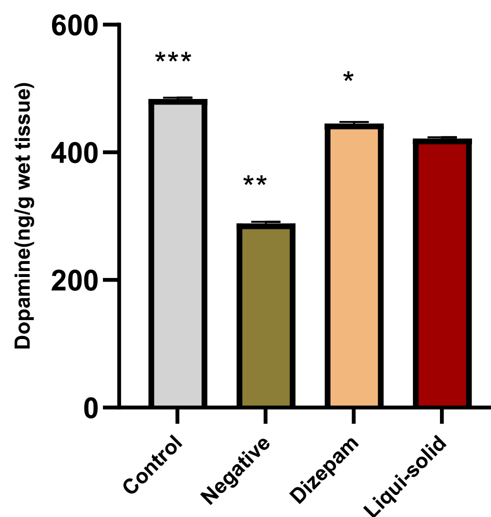
Table 1. Neurotransmitters (Mean ± SEM, n = 6)

Parameter	Control	Negative	Diazepam	Liqui-solid	ANOVA p-value
5-HT	523.67 ± 1.84	303.33 ± 2.78	474.50 ± 2.28	451.67 ± 2.17	$2.80×10^{-24}$
Dopamine	483.83 ± 1.90	288.83 ± 2.51	445.33 ± 2.25	421.67 ± 2.17	$1.41×10^{-23}$

NA	419.50 ± 1.48	251.67 ± 2.17	396.17 ± 1.90	381.33 ± 1.93	5.78×10^{-24}
GABA	6.90 ± 0.06	3.75 ± 0.08	6.48 ± 0.06	6.15 ± 0.04	2.33×10^{-19}







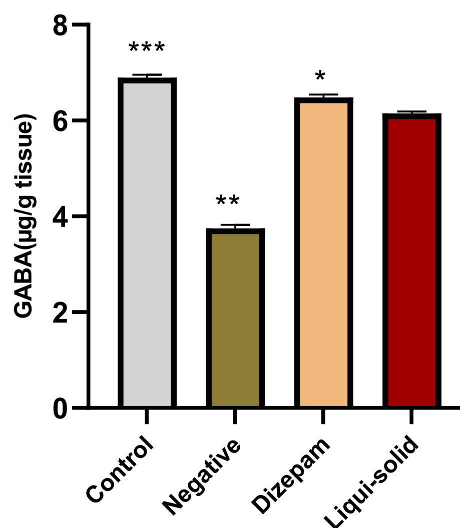


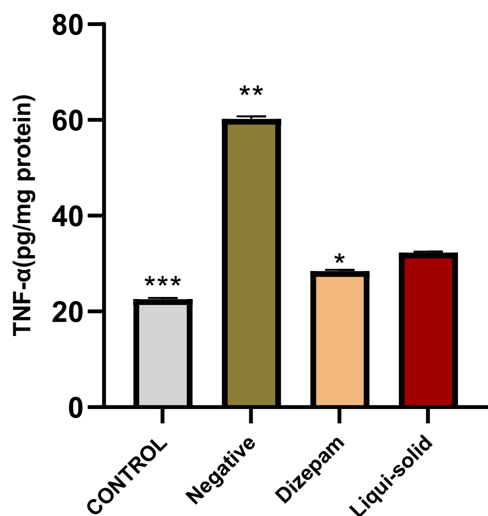
Figure 1 : *Effect of treatments on neurotransmitters (5-HT, dopamine, NA, and GABA). Data are mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. Significant differences were observed between Negative vs Control and treatment groups vs Negative ($p < 0.05$).*

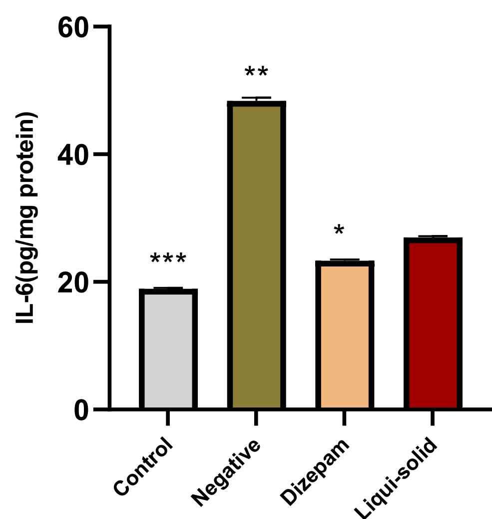
Effect on inflammatory cytokines (TNF-α, IL-6, IL-1β)

Pro-inflammatory cytokines were markedly elevated in the Negative group, indicating a strong inflammatory response. TNF-α, IL-6, and IL-1β increased substantially versus Control. Both diazepam and Liqui-solid significantly reduced cytokine levels compared to the Negative group, demonstrating anti-inflammatory protection. However, cytokines in treated groups remained higher than Control, suggesting partial normalization under the current conditions.

Table 2. Inflammatory cytokines (Mean ± SEM, n = 6)

Parameter	Control	Negative	Diazepam	Liqui-solid	ANOVA p-value
TNF-α	22.55 ± 0.28	60.20 ± 0.58	28.40 ± 0.29	32.27 ± 0.23	4.95×10 ⁻²⁵
IL-6	31.65 ± 0.32	72.57 ± 0.48	38.05 ± 0.31	42.18 ± 0.23	5.02×10 ⁻²⁶
IL-1β	20.15 ± 0.31	55.27 ± 0.41	26.15 ± 0.34	29.57 ± 0.33	1.88×10 ⁻²⁵





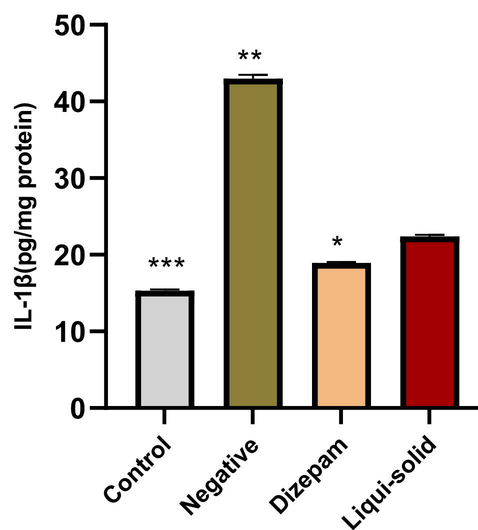


Figure 2 :*Effect of treatments on inflammatory cytokines (TNF-α, IL-6, and IL-1β). Data are mean ± SEM (n = 6). One-way ANOVA with Tukey's multiple comparisons test. Negative group showed significant elevation vs Control; diazepam and Liqui-solid significantly reduced cytokines vs Negative ($p < 0.05$).*

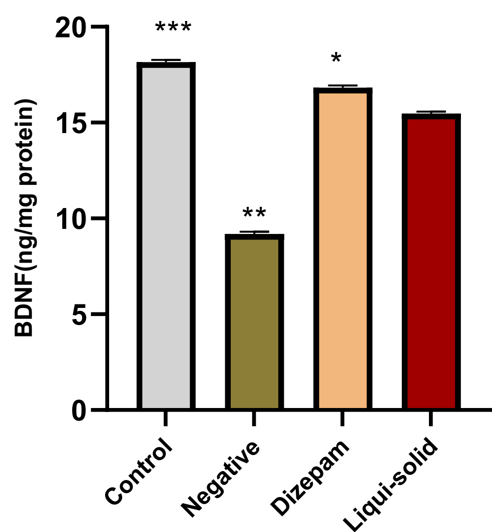
Effect on neurotrophic and glial markers (BDNF, TrkB, GFAP)

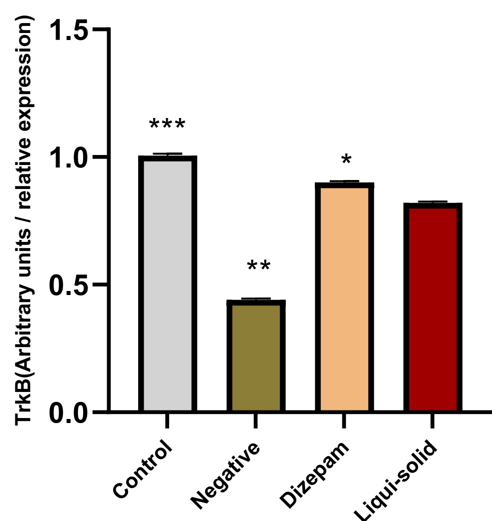
The Negative group showed a significant decline in neurotrophic signaling markers BDNF and TrkB, consistent with impaired neuroplasticity. Treatment with diazepam and Liqui-solid significantly increased BDNF and TrkB compared with Negative, indicating restoration of neurotrophic support.

In contrast, GFAP, a marker of astrocyte activation, was increased in the Negative group, suggesting glial reactivity. Both diazepam and Liqui-solid significantly reduced GFAP versus Negative, supporting attenuation of stress/inflammation-linked gliosis.

Table 3. Neurotrophic and glial markers (Mean ± SEM, n = 6)

Parameter	Control	Negative	Diazepam	Liqui-solid	ANOVA p-value
BDNF	79.70 ± 0.25	45.28 ± 0.33	73.23 ± 0.33	68.12 ± 0.36	1.16×10 ⁻²⁵
TrkB	68.57 ± 0.34	39.12 ± 0.26	64.05 ± 0.34	59.35 ± 0.33	2.47×10 ⁻²⁵
GFAP	18.57 ± 0.24	44.70 ± 0.37	23.90 ± 0.25	26.82 ± 0.28	1.20×10 ⁻²⁵





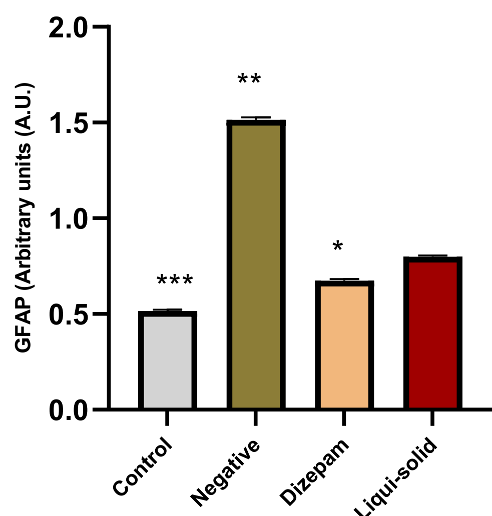


Figure 3 : *Effect of treatments on BDNF, TrkB, and GFAP. Data are mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. Negative group showed reduced BDNF/TrkB and elevated GFAP vs Control; treatments significantly normalized these markers vs Negative ($p < 0.05$).*

Effect on oxidative stress markers (MDA, GSH, SOD, catalase)

The Negative group exhibited a classical oxidative stress profile: MDA (lipid peroxidation marker) increased, while antioxidant defenses (GSH, SOD, and catalase) decreased compared with Control. Both diazepam and Liqui-solid significantly reduced MDA and restored antioxidant markers compared to the Negative group. The extent of improvement suggests that the Liqui-solid formulation provided meaningful antioxidant protection, though values did not fully return to Control levels in all parameters.

Table 4. Oxidative stress parameters (Mean ± SEM, n = 6)

Parameter	Control	Negative	Diazepam	Liqui-solid	ANOVA p-value
MDA	2.12 ± 0.03	5.57 ± 0.05	2.74 ± 0.04	3.05 ± 0.04	$3.22×10^{-24}$
GSH	8.67 ± 0.09	4.15 ± 0.08	7.95 ± 0.07	7.48 ± 0.08	$1.18×10^{-22}$
SOD	24.10 ± 0.21	13.57 ± 0.17	22.82 ± 0.19	21.28 ± 0.18	$2.03×10^{-22}$
Catalase	38.78 ± 0.25	22.28 ± 0.24	36.83 ± 0.26	34.45 ± 0.23	$5.70×10^{-24}$

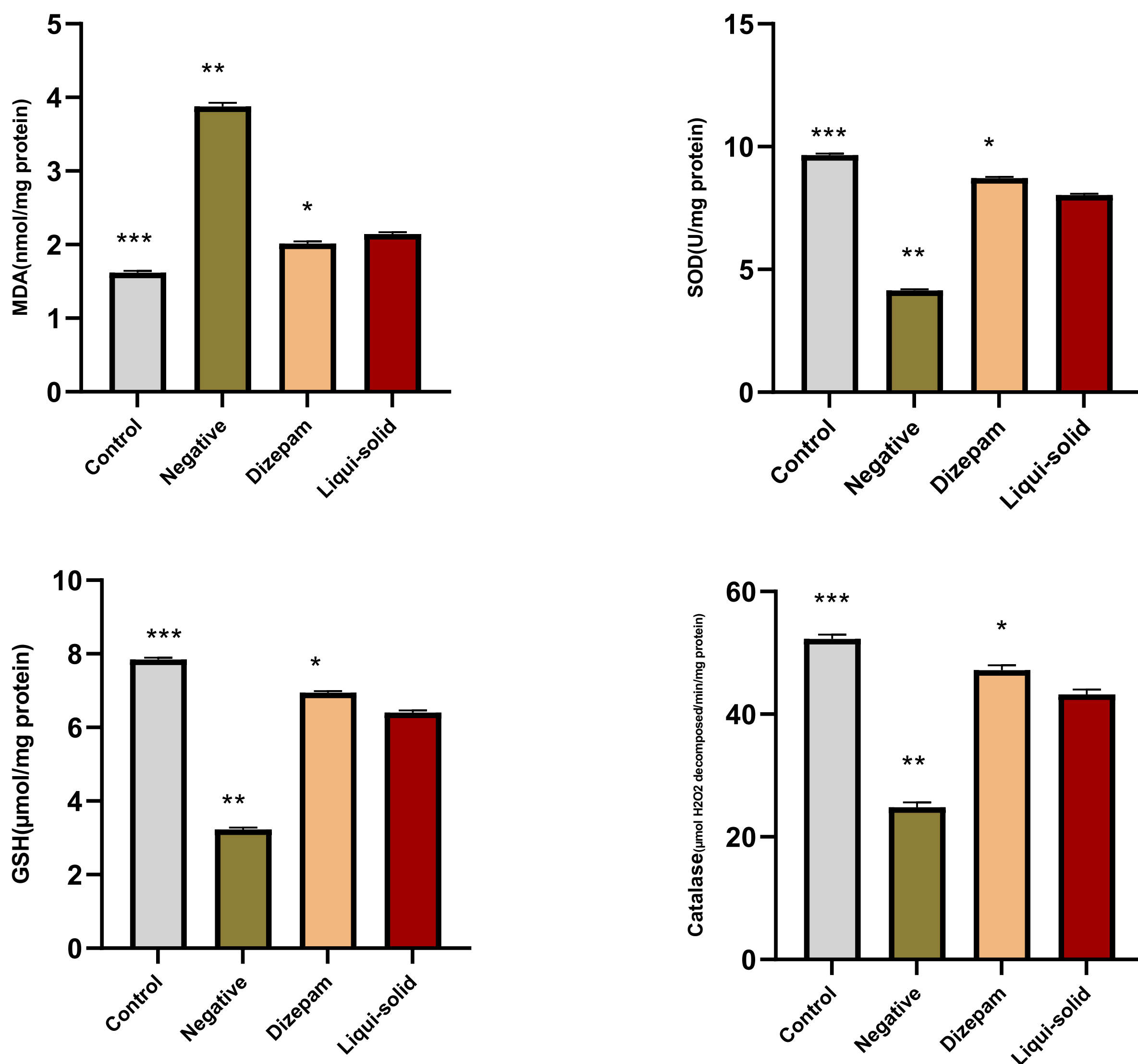


Figure 4 : *Oxidative stress and antioxidant status across groups (MDA, GSH, SOD, catalase). Data are mean ± SEM (n = 6). One-way ANOVA with Tukey's post-hoc test. Negative group showed increased MDA and reduced antioxidants vs Control; diazepam and Liqui-solid significantly improved these markers vs Negative ($p < 0.05$).*

Effect on corticosterone

Corticosterone levels were significantly elevated in the Negative group compared with Control, confirming activation of the stress axis. Both diazepam and Liqui-solid significantly reduced corticosterone levels compared with Negative, indicating a mitigation of the stress response.

Table 5. Corticosterone (Mean ± SEM, n = 6)

Parameter	Control	Negative	Diazepam	Liqui-solid	ANOVA p-value

Corticosterone	55.38 ± 0.44	109.82 ± 0.52	63.55 ± 0.43	70.32 ± 0.42	4.20×10⁻²⁶

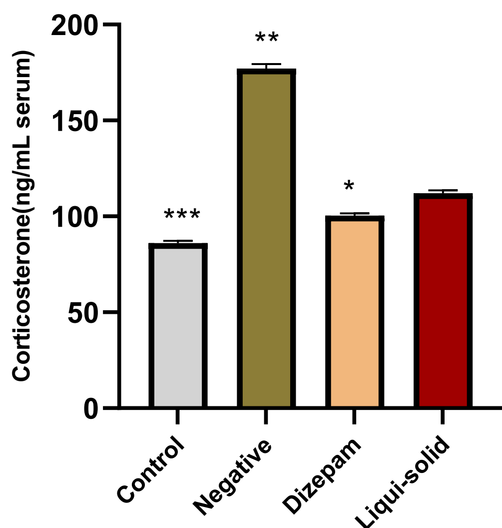


Figure 5 : *Effect of treatments on corticosterone. Values are mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. Negative group showed significant elevation vs Control; treatments significantly reduced corticosterone vs Negative ($p < 0.05$).*

Behavioral parameters (EPM, OFT, Light–Dark box)

Elevated Plus Maze (EPM)

The Negative group demonstrated anxiety-like behavior, evidenced by a marked reduction in time spent in open arms and number of open arm entries compared with Control. Diazepam and Liqui-solid significantly increased both parameters versus Negative, indicating an anxiolytic-like behavioral improvement.

Open Field Test (OFT)

In the Negative group, time spent in the central zone decreased significantly, reflecting increased anxiety/avoidance behavior. Total locomotor activity also decreased, suggesting reduced exploratory behavior. Both diazepam and Liqui-solid significantly improved central zone time and locomotor activity compared to Negative.

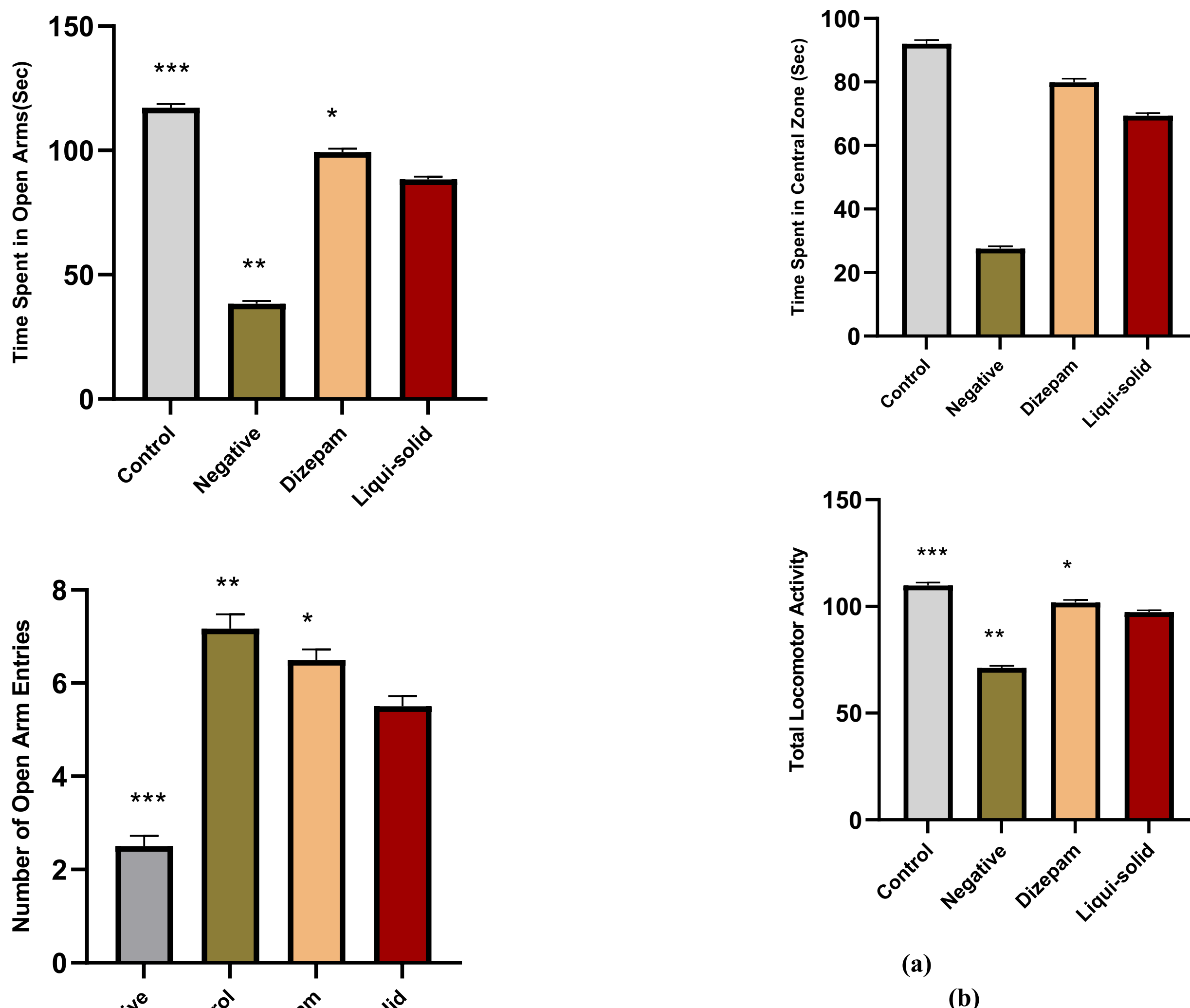
Light–Dark Box

The Negative group spent significantly less time in the light compartment and showed fewer transitions, confirming anxiety-like phenotype. Both diazepam and Liqui-solid significantly increased time spent in the light compartment and transitions versus Negative.

Table 6. Behavioral parameters (Mean ± SEM, n = 6)

Parameter	Control	Negative	Diazepam	Liqui-solid	ANOVA p-value
EPM: Time in open arms (s)	117.17 ± 1.47	38.33 ± 1.05	99.33 ± 1.36	88.33 ± 1.05	$1.29×10^{-20}$
EPM: Open arm entries	9.50 ± 0.34	2.33 ± 0.21	8.00 ± 0.26	6.83 ± 0.31	$2.67×10^{-19}$
OFT: Time in central zone (s)	76.50 ± 0.56	28.83 ± 0.65	66.00 ± 0.58	60.67 ± 0.61	$2.03×10^{-23}$
OFT: Total locomotor activity	142.50 ± 1.15	79.33 ± 0.95	130.50 ± 1.02	121.17 ± 0.98	$2.25×10^{-23}$
LDB: Time in light (s)	122.50 ± 1.18	52.83 ± 1.05	109.17 ± 1.22	98.33 ± 1.11	$1.19×10^{-22}$
LDB: Light–dark transitions	15.33 ± 0.33	6.17 ± 0.31	13.50 ± 0.34	12.00 ± 0.37	$3.16×10^{-21}$

Elevated Plus Maze (EPM)



(a)

(b)

Figure 6. Behavioral assessment using EPM, (a) Time Spent in Open Arms and (b) Number of Open Arms . Data are mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. Negative group showed significant anxiety-like behavior vs Control; diazepam and Liqui-solid significantly improved behavioral indices vs Negative ($p < 0.05$).

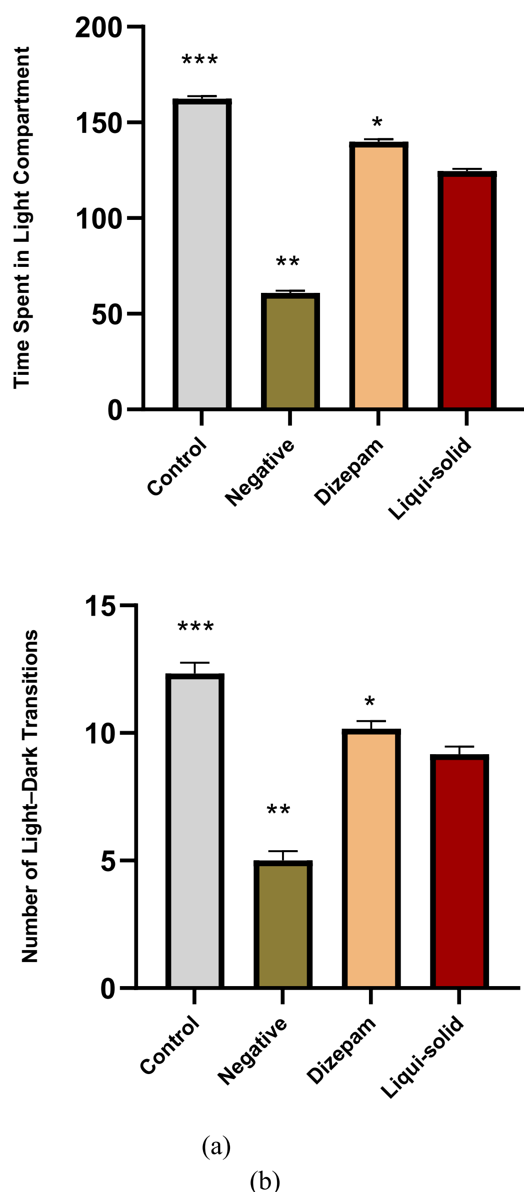
Open Field Test (OFT)

(a)

(b)

Figure 7 *: Behavioral assessment using Open Field Test (OFT) (a) Time spent in central zone(sec) and (b) Total Locomotor activity . Data are mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. Negative group showed significant anxiety-like behavior vs Control; diazepam and Liqui-solid significantly improved behavioral indices vs Negative ($p < 0.05$).*

Light–Dark Box (LDB)



(a)

(b)

*Figure 8: Behavioral assessment using **Light–Dark Box (LDB)**(a) Time spent in Light Compartment(sec) and (b) Number of Light-Dark Transitions . Data are mean ± SEM (n = 6). One-way ANOVA followed by Tukey's test. Negative group showed significant anxiety-like behavior vs Control; diazepam and Liqui-solid significantly improved behavioral indices vs Negative ($p < 0.05$).*

4. DISCUSSION

The present study evaluated the neuroprotective and anxiolytic-like potential of the Liqui-solid formulation in comparison with diazepam, using a model that produced marked neurobehavioral and biochemical disturbances in the Negative group. Across behavioral paradigms (EPM, OFT, and Light–Dark box) and multiple molecular readouts, the Negative group consistently demonstrated features of anxiety-like behavior, oxidative stress, neuroinflammation, impaired neurotrophic signaling, and neurochemical imbalance. Importantly, treatment with diazepam and Liqui-solid improved nearly all measured parameters, indicating strong pharmacological and biological reversal of the induced pathology.

Behavioral improvement confirms anxiolytic-like activity

In the Elevated Plus Maze, the Negative group showed a pronounced reduction in open arm time and open arm entries, which is a classical anxiety-like pattern reflecting avoidance behavior. Similarly, the Open Field Test showed reduced time in the central zone along with decreased locomotor activity, while the Light–Dark box showed reduced time in light and fewer transitions. Together, these behavioral outcomes confirm that the Negative condition induced robust anxiety-like behavior.

Both treatment groups significantly improved behavior compared with the Negative group. Diazepam showed slightly greater normalization in most parameters, which aligns with its established anxiolytic mechanism. However, the Liqui-solid group also produced substantial improvements, demonstrating that the test formulation can reduce anxiety-like behavior and improve exploration and risk assessment behaviors.

Restoration of neurotransmitters suggests normalization of neuronal signaling

Neurochemical assessment showed that the Negative group exhibited substantial reductions in 5-HT, dopamine, and noradrenaline, along with reduced GABA. This pattern is consistent with disrupted monoaminergic and inhibitory neurotransmission, which can directly contribute to anxiety phenotypes, impaired coping behavior, and altered locomotor exploration.

Both diazepam and Liqui-solid significantly restored these neurotransmitters toward control levels. The increase in GABA is particularly relevant because inhibitory GABAergic signaling serves as a key regulator of stress reactivity, neuronal excitability, and anxiety behavior. Diazepam's effect is expected due to positive modulation of $GABA_A$ receptor activity. The improvement observed with Liqui-solid indicates that the formulation may enhance neurochemical stability either by improving drug availability/brain delivery, reducing oxidative-inflammation cascades that suppress neurotransmitter synthesis, or supporting neuronal survival and function.

Reduced corticosterone indicates attenuation of stress-axis hyperactivation

The Negative group displayed markedly elevated corticosterone, confirming activation of the hypothalamic–pituitary–adrenal (HPA) axis, which is a hallmark of chronic stress and anxiety-like states. Both diazepam and Liqui-solid significantly reduced corticosterone compared with the Negative group, indicating a reduction in stress-axis overdrive. This finding is strongly aligned with the behavioral improvements, suggesting that the treatment groups helped restore physiological stress regulation.

Anti-inflammatory effects contribute to neuroprotection

Neuroinflammatory markers (TNF-α, IL-6, IL-1β) were markedly elevated in the Negative group, confirming a robust inflammatory response. These cytokines can impair synaptic signaling, reduce neurotrophic support, and increase oxidative damage, collectively worsening behavioral outcomes. Diazepam and Liqui-solid significantly reduced all cytokines compared with Negative. Although treated groups did not always fully return to the Control baseline, the magnitude of reduction indicates meaningful neuroimmune modulation.

This anti-inflammatory response is mechanistically important because inflammation and anxiety-like behavior can reinforce each other: stress elevates cytokines, cytokines worsen neurotransmitter function and neuroplasticity, and impaired neuroplasticity sustains anxiety phenotypes. Therefore, the cytokine-lowering effect of Liqui-solid supports its broader neuroprotective action.

Oxidative stress correction supports improved neuronal integrity

Oxidative stress analysis revealed a classic pattern: the Negative group showed increased MDA (lipid peroxidation) and decreased antioxidant defenses (GSH, SOD, catalase). This confirms that oxidative damage was strongly induced in the Negative state. Oxidative stress can damage membrane lipids, reduce neurotransmitter availability, impair mitochondrial function, and activate inflammatory cascades, ultimately contributing to both molecular and behavioral dysfunction.

Both diazepam and Liqui-solid significantly reduced MDA and restored antioxidant enzymes and GSH levels. The antioxidant restoration observed with Liqui-solid is particularly valuable because it indicates broader cytoprotective potential beyond symptom control. These effects also explain, at least in part, the improvements in neurotransmitters and neurotrophic signaling—since oxidative damage directly suppresses neuronal biosynthetic and signaling pathways.

BDNF–TrkB pathway recovery and reduced GFAP suggest improved neuroplasticity and reduced gliosis

The Negative group showed reduced BDNF and TrkB levels, reflecting impaired neurotrophic signaling and reduced neuroplasticity, which are strongly linked with stress-related behavioral disorders. In parallel, GFAP increased in the Negative group, indicating glial activation/astrogliosis, often seen in neuroinflammation and injury-like states.

Both diazepam and Liqui-solid significantly increased BDNF and TrkB compared to Negative and significantly reduced GFAP, suggesting that treatment improved neuroplasticity while reducing reactive glial activity. This triad (↑BDNF, ↑TrkB, ↓GFAP) provides strong mechanistic support for the behavioral improvements because enhanced neurotrophic signaling promotes synaptic stabilization, neuronal repair, and adaptive stress resilience.

interpretation and role of the Liqui-solid system

Across all endpoints, the Liqui-solid formulation demonstrated meaningful protective and restorative effects. While diazepam generally produced the strongest normalization (expected for a standard anxiolytic), Liqui-solid produced a broad-spectrum improvement across neurochemistry, stress hormones, antioxidant status, inflammation, and neuroplasticity markers. This pattern suggests that Liqui-solid's benefit may not be limited to one receptor pathway; instead, it likely acts by reducing the underlying biological burden (oxidative stress + neuroinflammation), thereby indirectly restoring neurotransmitter function and neurotrophic signaling.

From a formulation perspective, Liqui-solid systems are known to potentially enhance dissolution and absorption of poorly soluble drugs, which may increase therapeutic efficiency at the same dose. Therefore, the observed biochemical and behavioral outcomes may reflect improved bioavailability and stronger central pharmacodynamic effects. However, confirming this requires pharmacokinetic or brain distribution data.

5. CONCLUSION

The study concludes that the Negative group developed marked anxiety-like behavior with clear biochemical disturbances, including reduced 5-HT, dopamine, noradrenaline and GABA, elevated corticosterone, increased pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), increased lipid peroxidation (MDA) with depleted antioxidant defenses (GSH, SOD and catalase), and impaired neuroplasticity markers (decreased BDNF/TrkB with increased GFAP), whereas treatment with diazepam and the Liqui-solid formulation significantly reversed these changes by improving EPM, OFT and Light–Dark box performance, lowering corticosterone and cytokine levels, reducing oxidative stress while restoring endogenous antioxidant enzymes, and normalizing BDNF–TrkBsignaling with reduced GFAP; overall, the Liqui-solid formulation demonstrated a broad neuroprotective and anxiolytic-like profile comparable to the standard treatment in many endpoints, supporting its potential as an effective strategy to mitigate stress-induced neurobehavioral and neurochemical dysfunction.

6. REFERENCES

1. World Health Organization. Anxiety disorders [Internet]. Geneva: WHO; 2025 [cited 2026 Mar 9]. Available from: https://www.who.int/news-room/fact-sheets/detail/anxiety-disorders
2. Merkouris E, Brasinika A, Tsiptsios D, Tzanetakou IP, Karapanayiotides T. Molecular basis of anxiety: a comprehensive review of 2014–2024 clinical and preclinical studies. Int J Mol Sci. 2025;26(11):5417.

3. Bertollo AG, et al. Hypothalamus-pituitary-adrenal and gut-brain axes in affective disorders: mechanistic insights and therapeutic implications. Int J Mol Sci. 2025;26.
4. Kraeuter AK, Guest PC, Sarnyai Z. The elevated plus maze test for measuring anxiety-like behavior in rodents. Methods Mol Biol. 2019;1916:69-74. doi:10.1007/978-1-4939-8994-2_4.
5. Danduga RCSR, et al. Elevated plus maze for assessment of anxiety and memory in rodents. Bio Protoc. 2024;14(5):e4926.
6. Garcia AMB, Cardenas FP, Morato S. The effects of pentylenetetrazol, chlordiazepoxide and caffeine in rats tested in the elevated plus-maze depend on the experimental illumination. Behav Brain Res. 2011;217(1):171-177. doi:10.1016/j.bbr.2010.09.032.
7. Prajapati SK, et al. Repeated caffeine administration aggravates post-stress anxiety-like behaviour and cognitive impairment in rats. PhysiolBehav. 2019;journal details pending verification.
8. Haidary HA, Padhy RK. Clozapine. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023.
9. Abdallah MH, et al. Evaluation of mucoadhesive nano-bilosomal in situ gels for improved clozapine delivery. AAPS PharmSciTech. 2024;25.
10. Qadir A, et al. Formulation development and evaluation, in silico PBPK analysis, and oral bioavailability considerations of clozapine systems. 2025.
11. Prusty A, et al. Liquisolid technique for solubility enhancement of poorly water-soluble drugs: current advances and applications. Curr Drug Deliv. 2025.
12. Xie B, et al. Solubilization techniques used for poorly water-soluble drugs. Acta Pharm Sin B. 2024;14.
13. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc. 2007;2(2):322-328. doi:10.1038/nprot.2007.44.
14. Arrant AE, Schramm-Sapyta NL, Kuhn CM. Use of the light/dark test for anxiety in adult and adolescent male rats. Behav Brain Res. 2013;256:119-127. doi:10.1016/j.bbr.2013.05.035.
15. File SE, Andrews N. Low doses of caffeine induce anxiety in rats. J Psychopharmacol. 1997;11(3):287-294.
16. Garcia AMB, Cardenas FP, Morato S. The effects of pentylenetetrazol, chlordiazepoxide and caffeine in rats tested in the elevated plus-maze depend on the experimental illumination. Behav Brain Res. 2011;217(1):171-177. doi:10.1016/j.bbr.2010.09.032.
17. Spireas S. Liquisolid systems and methods of preparing same. Pharm Res. 1992;9(10):1351-1358.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351-358. doi:10.1016/0003-2697(79)90738-3.
19. Ellman GL. Tissue sulfhydryl groups. Arch BiochemBiophys. 1959;82(1):70-77. doi:10.1016/0003-9861(59)90090-6.
20. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972;247(10):3170-3175.
21. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121-126. doi:10.1016/S0076-6879(84)05016-3.
22. Bekhbat M, Glasper ER, Rowson SA, Kelly SD, Neigh GN. Measuring corticosterone concentrations over a physiological dynamic range in female rats. PhysiolBehav. 2018;194:73-76. doi:10.1016/j.physbeh.2018.04.033.
23. Barichello T, Generoso JS, Simões LR, Faller CJ, Ceretta RA, Petronilho F, et al. Role of TNF-α, IL-1β, and IL-6 in brain inflammation in experimental models. Neurosci Lett. 2010;474(1):40-45.
24. Reinhoud NJ, Brouwer HJ, van Heerikhuize J, Korte-Bouws GAH, den Heijer J, Olivier B. Analysis of glutamate, GABA, noradrenaline, dopamine, serotonin, and metabolites using microbore UHPLC with electrochemical detection. ACS Chem Neurosci. 2013;4(5):888-894. doi:10.1021/cn400044s.
25. Elfving B, Plougmann PH, Wegener G. Detection of brain-derived neurotrophic factor (BDNF) in rat blood and brain preparations using ELISA: pitfalls and solutions. J Neurosci Methods. 2010;187(1):73-77. doi:10.1016/j.jneumeth.2009.12.017.
26. Zhang S, Li Y, Chen S, Li X, Wang Z. GFAP expression in injured astrocytes in rats. Exp Ther Med. 2017;14(3):1905-1908. doi:10.3892/etm.2017.4760.