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RUTIN ATTENUATES ALCOHOL WITHDRAWAL-INDUCED ANXIETY IN RATS VIA NMDA RECEPTOR MODULATION, ANTIOXIDANT DEFENSE, AND MULTI-TARGET NEUROINFLAMMATORY PATHWAY REGULATION: AN INTEGRATED NETWORK PHARMACOLOGY AND PRECLINICAL STUDY

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ABSTRACT

Background: Alcohol withdrawal syndrome (AWS) is a clinically relevant syndrome associated with anxiety, neuroinflammation and glutamatergic excitotoxicity, in which there is a limited number of safe, effective and non-addictive treatments available. There is no systematic study on the effect of the bioflavonoid glycoside rutin on the anxiety induced by AWS using an integrated in silico and in vivo approach.

Objective: To explore the anti-anxiety, neuroprotective and anti-inflammatory activity of rutin and its combination with the NMDA receptor antagonist memantine in ethanol withdrawal-induced anxiety in rats by network pharmacology and molecular docking studies.

Materials and Methods: A network pharmacology approach was carried out with the GeneCards, OMIM, Swiss Target Prediction, STRING, Cytoscape and GeneCodis databases. Molecular docking was done using PyRx and the visualization presented in Biovia Discovery Studio. Male Wistar rats were made alcohol dependent via oral ethanol administration for a period of 21 days (2.4% v/v to 4.8% v/v to 7.2% v/v). Seven experimental groups were treated for 7 days post withdrawal – Normal control, Ethanol control, Rutin 50 mg/kg p.o., Rutin 100 mg/kg p.o., Rutin 200 mg/kg p.o., Memantine (20 mg/kg p.o.), and Rutin + Memantine (50 + 20 mg/kg p.o.). The Elevated Plus Maze (EPM), Light/Dark Box Test (LDT), and Open Field Test (OFT) were used to assess anxiety. Oxidative stress markers (GSH, SOD, CAT, MDA, NO), neurotransmitters (GABA, serotonin, dopamine, glutamate, norepinephrine), TNF-α and NMDA receptor expression were measured in brain tissue. Histopathological analyses of the hippocampal tissue were conducted by H&E staining.

Results: Network pharmacology revealed that there were 69 common targets between rutin and AW-anxiety, including the hub protein of TNF, TP53, EGFR, PTGS2 and GSK3B. The molecular docking studies showed that rutin has good binding affinity with PTGS2, TNF, HSP90AB1, and GSK3B with −10.1 kcal/mol, −9.1 kcal/mol, −9.1 kcal/mol, and −8.4 kcal/mol binding energy respectively. In vivo, rutin dose-dependently reduced anxiety-like behavior in all three behavioral paradigms, significantly increased depleted GABA, serotonin and dopamine, decreased elevated glutamate and NE, decreased MDA and NO, increased GSH, SOD and CAT activity and decreased elevated TNF-α, and decreased NMDA receptor expression. The rutin + memantine association (50 + 20 mg/kg) was the most effective treatment, almost normalizing all parameters. The simultaneous neuroprotective effects were confirmed by hippocampal histopathology.

Conclusion: Rutin has dose-dependent anxiolytic and neuroprotective activities in ethanol withdrawal, primarily via modulation of NMDA receptors, restoration of GABAergic/glutamatergic balance, antioxidant reinforcement, and suppression of neuroinflammation. The synergistic effects of the rutin–memantine combination indicate potential for translation in the management of AWS.

KEYWORDS: Rutin; Alcohol withdrawal syndrome; Anxiety; NMDA receptor antagonism; Network pharmacology; Neuroprotection; Oxidative stress; Neuroinflammation

1. INTRODUCTION

Alcohol use disorder (AUD) is one of the largest public health problems globally and is the most costly of all causes of death. Worldwide, alcohol abuse accounts for the deaths of about 3 million people every year and has been linked to a range of harmful health effects, such as liver disease, cardiovascular diseases, and neuropsychiatric disorders.[1] In India AUD has become an increasing health problem and around 5% of the population have been found to have the problem of alcohol consumption.[2] The northeastern states, southern India, and the Eastern Peninsular belt (Chhattisgarh, Odisha, Jharkhand, Telangana, Tamil Nadu and Kerala) are identified as high consumption areas, consumption patterns are influenced by multidimensional and complex

sociodemographic, cultural and behavioral factors.[3] Alcohol withdrawal syndrome (AWS) occurs in ~50% of people with AUD when they suddenly stop or drastically reduce their chronic excessive alcohol use, and it ranges in severity from mild autonomic symptoms (tremors, diaphoresis, increased heart rate) to severe symptoms like hallucinations, tonic-clonic seizures, and the most severe cases — delirium tremens (DTs) — which can be fatal if left untreated (5–15%).[4-5] Anxiety is one of the first and most distressing symptoms of AWS that can begin to happen 6–24 hours after alcohol use stops and may last several days to weeks, making it a significant factor in relapse.[6] AWS is a disorder thought to be primarily characterized by a severe imbalance in inhibitory (GABAergic) and excitatory (glutamatergic) neurotransmission in the brain. Chronic ethanol exposure causes compensatory increases in NMDA receptors and decreases in GABA-A receptors in the CNS.[7] When this neuroadaptation is removed abruptly, it is unmasked and leads to hyperactivity of the glutamatergic excitatory system with clinical symptoms of autonomic hyperactivity, anxiety, seizures and delirium.[8] At the same time, oxidative stress and neuroinflammation (characterized by increased malondialdehyde (MDA), nitric oxide (NO), and tumour necrosis factor-alpha (TNF-α)), play a role in the neurodegeneration of the hippocampus and exacerbate the anxiety during withdrawal.[9] Pharmacological treatment of AWS is currently largely based on benzodiazepines, which are effective but highly addictive, can lead to tolerance, respiratory depression and misuse.[10] Various drugs have been studied, including alternative agents (acamprosate, naltrexone, gabapentin, and carbamazepine), but none of them are effective at completely treating the multi-dimensional pathophysiology of AWS-induced anxiety.[11] Memantine, an uncompetitive NMDA receptor antagonist, has demonstrated early efficacy in the attenuation of glutamatergic excitotoxicity during ethanol withdrawal, but has not been proven to be effective in ethanol withdrawal alone.[12] These constraints highlight the critical need for new multi-target drugs. Rutin (quercetin-3-O-rutinoside), a glycoside form of the flavonoid quercetin, that is found in a variety of foods of plant origin such as buckwheat, citrus fruits and asparagus, has been a major focus of pharmacological interest because of its wide potential bioactivities with strong antioxidant, anti-inflammatory, neuroprotective and anxiolytic activity.[13] Preliminary investigations have shown that rutin can regulate the activity of GABA-A receptors and serotonergic receptors, inhibit the lipid peroxidation, block neuroinflammation, and prevent damage to neurons during ischaemia.[14] Although rutin has many of these desirable properties, no studies have been specifically designed to evaluate the effects of rutin on anxiety due to AWS, and the interaction of rutin with memantine has not been examined. The present study uses an integrated approach by network pharmacology, molecular docking and in vivo pharmacological evaluation to explore the treatment effects of rutin, either alone or in combination with memantine, against ethanol withdrawal-induced anxiety in a rat model. To gain mechanistic information for the in vivo experiments, the molecular targets and signaling pathways involved in rutin's anxiolytic activity were identified using network pharmacology. The dual in silico–in vivo approach was designed to obtain mechanistic information and in vivo data to support rutin as a natural, multi-target molecule for AWS management.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and reagents used in this study are listed in Table 1. Rutin (quercetin-3-O-rutinoside) was procured from Loba Chemie Pvt. Ltd. (Mumbai, India). Memantine hydrochloride (10 mg tablet) was sourced from certified local pharmaceutical distributors (Pune, India). Ethanol and all other reagents—including methanol, perchloric acid, β-nicotinamide adenine dinucleotide (β-NAD), chloroform, 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium nitroprusside, Griess reagent (A and B), phosphate buffer, hematoxylin, and eosin—were of analytical reagent (AR) grade and were obtained from Research Lab Fine Chem Industries (Mumbai, India). The ELISA kit for TNF-α estimation was procured from Krishgen Biosystems (Mumbai, India). Paraformaldehyde and DPX mountant were sourced from Sigma-Aldrich (St. Louis, MO, USA). Alcohol dehydrogenase and all other biochemical reagents were of the highest purity grade available.

Table 1. List of chemicals and reagents used in this study

Sr. No.	Name of Chemical/Reagent	Manufacturer/Supplier
1	Rutin	Loba Chemie Pvt. Ltd., Mumbai
2	Memantine	Local distributor, Pune
3	Methanol	Research Lab Fine Chem Industries, Mumbai
4	Perchloric acid	Research Lab Fine Chem Industries, Mumbai
5	Alcohol dehydrogenase	Research Lab Fine Chem Industries, Mumbai
6	β-NAD	Research Lab Fine Chem Industries, Mumbai
7	Ethanol	Research Lab Fine Chem Industries, Mumbai
8	Chloroform	Research Lab Fine Chem Industries, Mumbai
9	DTNB reagent	Research Lab Fine Chem Industries, Mumbai

10	Phosphate buffer	Research Lab Fine Chem Industries, Mumbai
11	TBA	Research Lab Fine Chem Industries, Mumbai
12	TCA	Research Lab Fine Chem Industries, Mumbai
13	Sodium nitroprusside	Research Lab Fine Chem Industries, Mumbai
14	Griess reagent A and B	Research Lab Fine Chem Industries, Mumbai
15	Haematoxylin and eosin	Sigma-Aldrich
16	ELISA kit (TNF-α)	Krishgen Biosystems, Mumbai

Abbreviations: β-NAD, β-nicotinamide adenine dinucleotide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TBA, thiobarbituric acid; TCA, trichloroacetic acid; TNF-α, tumor necrosis factor-alpha.

2.2 Instruments and Software

The instruments and software platforms employed are listed in Table 2.

Table 2. Instruments and software used in this study

Sr. No.	Instrument/Software	Purpose	Manufacturer/Source
1	UV-Visible Spectrophotometer	Absorbance measurement	Shimadzu, Japan
2	FTIR Spectrophotometer	Functional group identification	PerkinElmer/Shimadzu
3	Microplate Reader (ELISA)	ELISA absorbance at 450 nm	BioTek, USA
4	Mechanical Homogenizer	Tissue homogenization	Remi Equipments Ltd., Mumbai
5	Centrifuge	Supernatant preparation	Remi Equipments Ltd., Mumbai
6	Incubator	Biochemical assay incubation	CM 101, Remi Equipments Ltd., Mumbai
7	Light Microscope	Histopathological analysis	Olympus, Japan
8	Elevated Plus Maze	Behavioral anxiety assessment	CM 101, Remi Equipments Ltd., Mumbai
9	Light/Dark Box	Behavioral anxiety assessment	Locally fabricated
10	Open Field Arena	Locomotor/anxiety assessment	Locally fabricated
11	GeneCards/OMIM	Disease target retrieval	www.genecards.org
12	Swiss Target Prediction	Compound target prediction	www.swisstargetprediction.ch
13	STRING	PPI network construction	www.string-db.org
14	Cytoscape 3.8.1	Network visualization/analysis	www.cytoscape.org
15	GeneCodis	GO and KEGG enrichment analysis	geneCodis.dacya.ucm.es
16	PyRx	Molecular docking	Sanner, Scripps Research
17	Biovia Discovery Studio	Molecular interaction visualization	Dassault Systèmes
18	GraphPad Prism	Statistical analysis	GraphPad Software Inc.

Abbreviations: FTIR, Fourier-transform infrared spectroscopy; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

2.3 Network Pharmacology Analysis

2.3.1 Target Identification

Targets associated with alcohol withdrawal (AW)-induced anxiety were retrieved from GeneCards (www.genecards.org), Entrez Gene, and OMIM databases using the search terms "alcohol withdrawal," "alcohol withdrawal anxiety," and "alcohol withdrawal syndrome." A total of 3,168 non-redundant disease targets were

collected. Concurrently, putative molecular targets of rutin were predicted using the SwissTargetPrediction platform (www.swisstargetprediction.ch), yielding 100 non-duplicate targets.

2.3.2 Identification of Common Targets

A Venn diagram was constructed to identify the intersection between AW-anxiety targets and rutin targets. The overlapping targets, representing potential pharmacological nodes of rutin in AWS-induced anxiety, were selected for downstream network analyses (Fig. 1).

2.3.3 Protein-Protein Interaction (PPI) Network Construction

The 69 common targets were imported into the STRING database (Version 11.0; www.string-db.org) to construct a protein-protein interaction (PPI) network. The network was exported to Cytoscape 3.8.1 for visualization and topological analysis. The degree distribution of each node was calculated using the Network Analyzer plugin. Hub proteins were defined as those with a degree value ≥20, corresponding to the top 10 most interconnected targets.

2.3.4 Gene Ontology and KEGG Pathway Enrichment Analysis

Gene Ontology (GO) functional enrichment analysis was conducted using GeneCodis to categorize the 69 targets into biological processes (BP), cellular components (CC), and molecular functions (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed to identify the signaling pathways significantly associated with rutin's mechanism of action in AW-anxiety. Pathways with a corrected p-value < 0.05 were considered statistically significant.

2.3.5 Compound-Target-Pathway Network

A compound-target-pathway (C-T-P) network was constructed in Cytoscape 3.8.1 incorporating rutin, the top 10 hub protein targets, and the top 20 enriched KEGG pathways. The network was analyzed to identify multi-target regulatory relationships.

2.4 Molecular Docking

2.4.1 Protein and Ligand Preparation

The three-dimensional crystal structures of the four most prominent hub proteins—PTGS2 (COX-2; PDB ID: 5KIR), TNF (PDB ID: 2AZ5), HSP90AB1 (PDB ID: 1UYM), and GSK3B (PDB ID: 1Q5K)—were downloaded from the Protein Data Bank (PDB; www.rcsb.org). Water molecules and co-crystallized ligands were removed, and hydrogen atoms were added using AutoDock Tools prior to conversion to PDBQT format. The three-dimensional structure of rutin (PubChem CID: 5280805) was retrieved from PubChem and energy-minimized using the MMFF94 force field.

2.4.2 Docking Simulation

Molecular docking was performed using PyRx (AutoDock Vina). Grid boxes were centered on the active sites of each receptor protein with dimensions set to encompass the binding pocket. An exhaustiveness setting of 8 was applied for all docking runs. Binding affinity values were expressed in kilocalories per mole (kcal/mol), where lower (more negative) values indicate stronger binding interactions. Molecular interaction visualizations, including two-dimensional and three-dimensional interaction maps, were generated using Biovia Discovery Studio 2021.

2.5 Drug Characterization

2.5.1 UV-Visible Spectrophotometric Analysis

Rutin solutions were prepared in methanol at concentrations of 2, 4, 6, 8, 10, and 12 µg/mL. The UV-Visible absorption spectrum was recorded between 200 and 400 nm using a UV-Visible spectrophotometer. A standard calibration curve was constructed by plotting absorbance against concentration to determine linearity, and the wavelength of maximum absorbance (λmax) was identified.

2.5.2 Fourier-Transform Infrared (FTIR) Spectroscopy

The FTIR spectrum of rutin was recorded over the range of 4000–400 cm^{-1} using a Fourier-transform infrared spectrophotometer with KBr pellet preparation. Characteristic absorption bands were assigned to specific functional groups to confirm the chemical identity of the compound.

2.6 Animals

Male Wistar rats (200–250 g) were obtained from certified animal suppliers and housed under standard laboratory conditions (12-h light/dark cycle; temperature 22 ± 2°C; humidity 55 ± 10%) with ad libitum access to standard rodent chow and water. Following 10 days of acclimatization, rats were introduced to a Modified Liquid Diet (MLD) over 7 days as a transitional period before ethanol administration. All experimental procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, and were approved by the Institutional Animal Ethics Committee (IAEC) of the institution.

2.7 Induction of Alcohol Dependence

Ethanol dependence was induced by oral administration of a liquid ethanol diet over 21 consecutive days, following the method described by Kumar et al. (2016), with modifications.[15] The ethanol concentration was escalated incrementally: 2.4% (v/v) for the first 3 days, 4.8% (v/v) for days 4–7, and 7.2% (v/v) for days 8–21. On day 22, ethanol was withdrawn to precipitate AWS. Blood ethanol concentration (BEC) was monitored on

days 3, 7, 14, and 23 (post-withdrawal) using the alcohol dehydrogenase enzymatic method to confirm successful induction and clearance of ethanol.

2.8 Experimental Design

Animals (n = 48) were randomly allocated to seven groups (n = 6–8 per group):

- Group 1 (Normal Control): Standard diet + oral vehicle (distilled water) for 30 days
- Group 2 (Ethanol Control): Ethanol liquid diet (21 days) + oral vehicle (distilled water) for 7 days post-withdrawal
- Group 3 (Rutin 50 mg/kg): Ethanol liquid diet (21 days) + Rutin 50 mg/kg p.o. for 7 days post-withdrawal
- Group 4 (Rutin 100 mg/kg): Ethanol liquid diet (21 days) + Rutin 100 mg/kg p.o. for 7 days post-withdrawal
- Group 5 (Rutin 200 mg/kg): Ethanol liquid diet (21 days) + Rutin 200 mg/kg p.o. for 7 days post-withdrawal
- Group 6 (Memantine 20 mg/kg): Ethanol liquid diet (21 days) + Memantine 20 mg/kg p.o. for 7 days post-withdrawal
- Group 7 (Rutin + Memantine 50 + 20 mg/kg): Ethanol liquid diet (21 days) + Rutin 50 mg/kg + Memantine 20 mg/kg p.o. for 7 days post-withdrawal

All treatments were administered by oral gavage once daily. Behavioral assessments were performed on day 30 (7 days post-withdrawal, at the end of treatment). Body weight was recorded on days 1, 4, 8, 15, 21, and 23 (pre-treatment period) and on days 1, 3, 5, and 7 during the treatment period.

2.9 Assessment of Alcohol Withdrawal Symptoms

Alcohol withdrawal symptoms were evaluated on day 23 (24 hours post-ethanol withdrawal) and recorded at 2, 4, 6, and 8 hours after ethanol discontinuation using a modified Clinical Institute Withdrawal Assessment for Alcohol (CIWA-Ar) scale adapted for rodents. Behavioral signs scored included tremors, piloerection, abnormal posture, tail stiffness, agitation, vocalization, and wet-dog shakes. Scores were averaged per group.

2.10 Behavioral Assessment

All behavioral tests were conducted on day 30 between 9:00 AM and 12:00 PM to minimize circadian variability. Equipment was thoroughly cleaned with 70% ethanol between trials.

2.10.1 Elevated Plus Maze (EPM) Test

The EPM apparatus consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) arranged in a cross-shape, elevated 50 cm above the floor. Rats were placed individually in the center of the maze, facing an open arm. Locomotor behavior was recorded for 5 minutes. The number of open arm entries and time spent in open arms were recorded. An increased frequency of open arm entries and duration therein are indicative of reduced anxiety.[16]

2.10.2 Light/Dark Box Test (LDT)

The Light/Dark Box apparatus comprised a light compartment (30 × 30 × 30 cm, illuminated) and a dark compartment (30 × 30 × 30 cm, covered), connected by a central opening. Each rat was placed in the light compartment and observed for 5 minutes. The number of entries into and the time spent in the light and dark compartments were recorded. Anxiolytic activity was indicated by an increased number of light-compartment entries and greater time spent therein.[17]

2.10.3 Open Field Test (OFT)

The open field arena (60 × 60 cm) was divided into central and peripheral zones. Each rat was placed in the center of the arena and allowed to freely explore for 5 minutes. The time spent in the central zone and the total number of line crossings (locomotion) were quantified. Reduced center time and crossing frequency indicate heightened anxiety-like behavior.[18]

2.11 Biochemical Estimations

Following behavioral assessment on day 30, animals were sacrificed under ether anesthesia. Brains were rapidly dissected on ice, weighed, and homogenized (10% w/v) in ice-cold phosphate-buffered saline (PBS; pH 7.4) using a mechanical homogenizer. The homogenate was centrifuged at 5,000 × g for 10 minutes at 4°C, and the resulting supernatant was collected for all biochemical and neurotransmitter analyses.

2.11.1 Oxidative Stress Parameters

Glutathione (GSH) levels were estimated using the DTNB (Ellman's reagent) method. The reaction of GSH with DTNB produces a yellow color (5-thio-2-nitrobenzoic acid), quantified spectrophotometrically at 412 nm. Results were expressed as µg/mg protein.[19] Superoxide dismutase (SOD) activity was determined based on the inhibition of pyrogallol auto-oxidation, measured at 420 nm, and expressed as µmol/mL.[20] Catalase (CAT) activity was measured by quantifying the decomposition of hydrogen peroxide (H_2O_2) at 240 nm, expressed as µmol/mL.[21] Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) method; the pink chromogen formed between MDA and TBA was measured at 532 nm and expressed as nmol/mg protein.[22] Nitric oxide (NO) levels were estimated using the Griess reagent method, measuring nitrite/nitrate at 540 nm and expressed as µmol/mL.[23]

2.11.2 Neurotransmitter Estimation

Brain GABA, serotonin (5-HT), dopamine, glutamate, and norepinephrine (NE) levels were quantified using validated ELISA-based assay kits following the manufacturers' protocols. Results were expressed as pmol/mL (GABA, glutamate, NE) or ng/mL (serotonin, dopamine).

2.11.3 Estimation of TNF-α

TNF-α levels in brain homogenates were quantified using a sandwich ELISA kit (Krishgen Biosystems, Mumbai, India) following the manufacturer's instructions. Brain tissue was homogenized in PBS, centrifuged at 5,000 × g for 10 minutes at 4°C, and the supernatant collected. Standards (100 µL) and tissue samples (100 µL) were added to the 96-well ELISA plate, incubated at 37°C for 90 minutes, followed by washing, addition of biotin-conjugated detection antibody (60 min, 37°C), HRP-streptavidin (30 min, 37°C), and TMB substrate (10–20 min, 37°C). The reaction was terminated with stop solution, and absorbance was read at 450 nm. Results were expressed as pg/mL.[24]

2.11.4 NMDA Receptor Expression

NMDA receptor (NMDAR1 subunit) protein levels in brain tissue were quantified using a sandwich ELISA kit, following identical sample preparation as described for TNF-α estimation. Results were expressed as ng/mL.[25]

2.12 Protein Estimation

Total protein content in brain homogenates was determined by the Lowry method using bovine serum albumin (BSA) as the standard. Absorbance was measured at 660 nm.

2.13 Histopathological Analysis

2.13.1 Hematoxylin and Eosin (H&E) Staining

Following behavioral testing, selected animals from each group were deeply anesthetized and perfused transcardially with 5% formalin through the left ventricle. Brains were carefully removed and fixed in 10% neutral buffered paraformaldehyde for 72 hours. Brain tissue samples from the hippocampal region were trimmed to approximately 3 mm thickness, dehydrated, cleared in xylene, and embedded in paraffin. Sections of 3–5 µm thickness were cut using a microtome and stained with hematoxylin and eosin (H&E). Six slides per animal were examined under a light microscope. Neuronal integrity was assessed by identifying normal (intact) and dark (pyknotic/degenerating) neurons in the hippocampal subregions, including Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3), and the dentate gyrus (DG).[26]

2.14 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Behavioral, biochemical, and neurotransmitter data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Body weight and BEC data were analyzed using two-way ANOVA followed by Bonferroni's post hoc test. Withdrawal symptom scores were analyzed using two-way ANOVA with Bonferroni's correction. Statistical analyses were performed using GraphPad Prism (Version 8.0; GraphPad Software Inc., San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Network Pharmacology

3.1.1 Target Identification and Overlap

A total of 3,168 non-redundant targets associated with alcohol withdrawal-induced anxiety were retrieved from GeneCards, Entrez Gene, and OMIM databases. SwissTargetPrediction yielded 100 predicted pharmacological targets of rutin. Comparative analysis of the two target sets identified 69 common targets, which were designated as candidate therapeutic nodes for rutin's action in AWS-induced anxiety (Fig. 1).

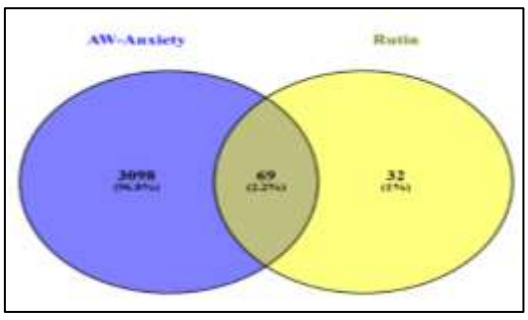


Figure 1 — Venn diagram of overlapping targets

3.1.2 PPI Network Analysis and Hub Target Identification

The 69 common targets were used to construct a PPI network in STRING, subsequently imported into Cytoscape 3.8.1 for topological analysis. The network contained multiple interconnected nodes representing signaling crosstalk. Degree analysis using the Network Analyzer plugin identified the top 10 hub proteins with the highest connectivity (Table 3).

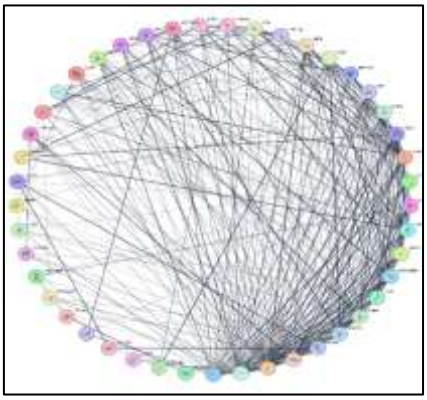


Figure 2 PPI network visualization

Table 3. Top 10 hub proteins identified from PPI network degree analysis

Protein	Degree
TNF	34
TP53	33
EGFR	32
ESR1	29
PTGS2	26
GSK3B	26
MMP9	24
KDR	23
HSP90AB1	22
IGF1R	20

Abbreviations: TNF, tumor necrosis factor; TP53, tumor protein p53; EGFR, epidermal growth factor receptor; ESR1, estrogen receptor 1; PTGS2, prostaglandin-endoperoxide synthase 2 (COX-2); GSK3B, glycogen synthase kinase 3 beta; MMP9, matrix metallopeptidase 9; KDR, kinase insert domain receptor (VEGFR2); HSP90AB1, heat shock protein 90 alpha family class B member 1; IGF1R, insulin-like growth factor 1 receptor.

3.1.3 GO and KEGG Enrichment Analysis

Gene Ontology analysis of the 69 targets revealed significant enrichment in biological processes including astrocyte activation, presynaptic modulation of chemical synaptic transmission, positive regulation of MAP kinase activity, peptidyl-serine phosphorylation, and protein autophosphorylation (Fig. 3). KEGG pathway analysis identified EGFR tyrosine kinase inhibitor resistance, HIF-1 signaling, AGE-RAGE signaling in diabetic complications, glioma, and MAPK/NF-κB signaling pathways as the most significantly enriched (Fig. 4).

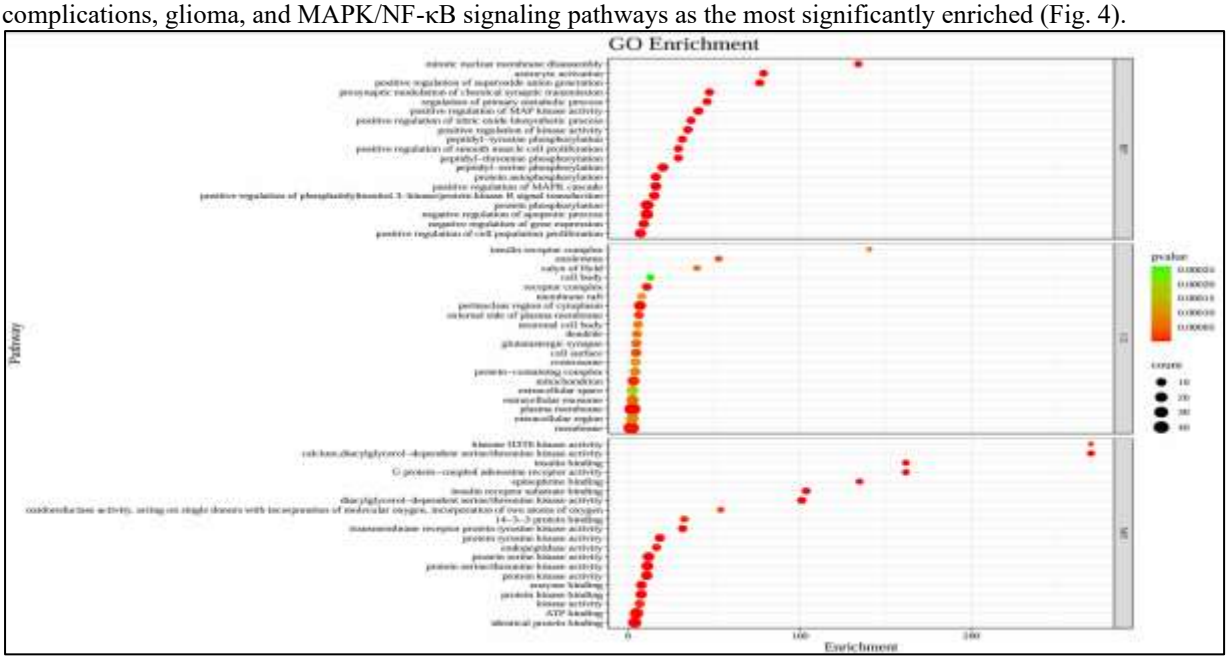


Figure 3 GO enrichment analysis

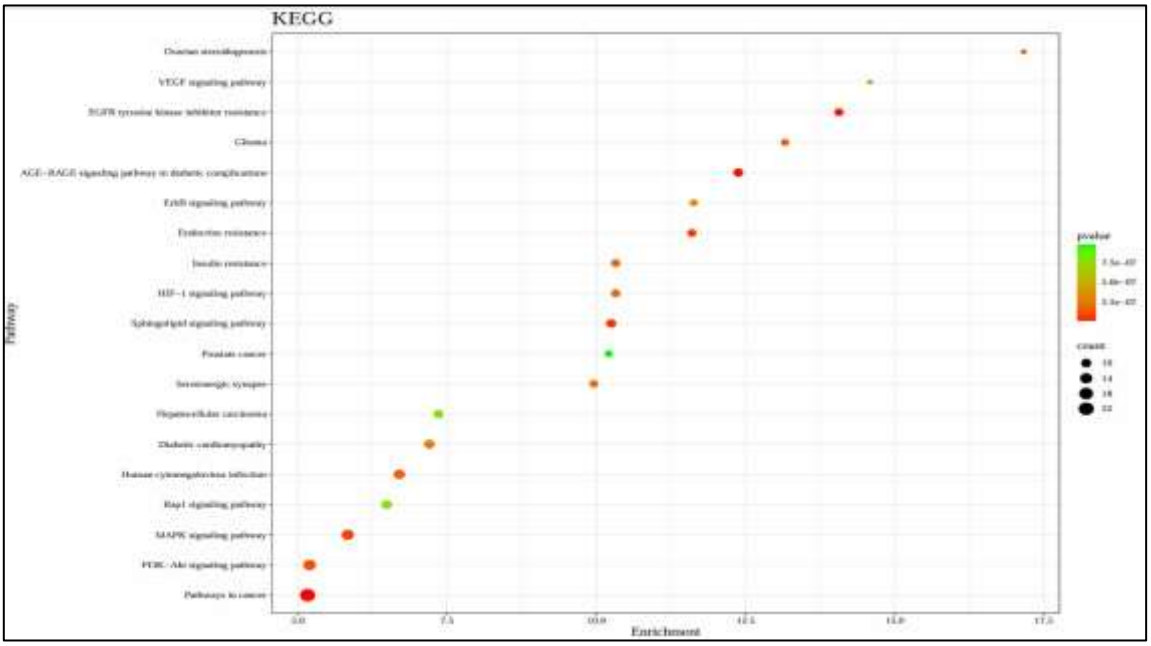


Figure 4 KEGG pathway enrichment analysis

3.1.4 Compound-Target-Pathway Network

The C-T-P network constructed in Cytoscape 3.8.1 comprised rutin, 10 hub protein targets, and the top 20 enriched pathways (Fig. 5). The multi-target, multi-pathway interactions of rutin within this network indicated its capacity to modulate AWS-induced anxiety through synergistic engagement with diverse signaling axes.

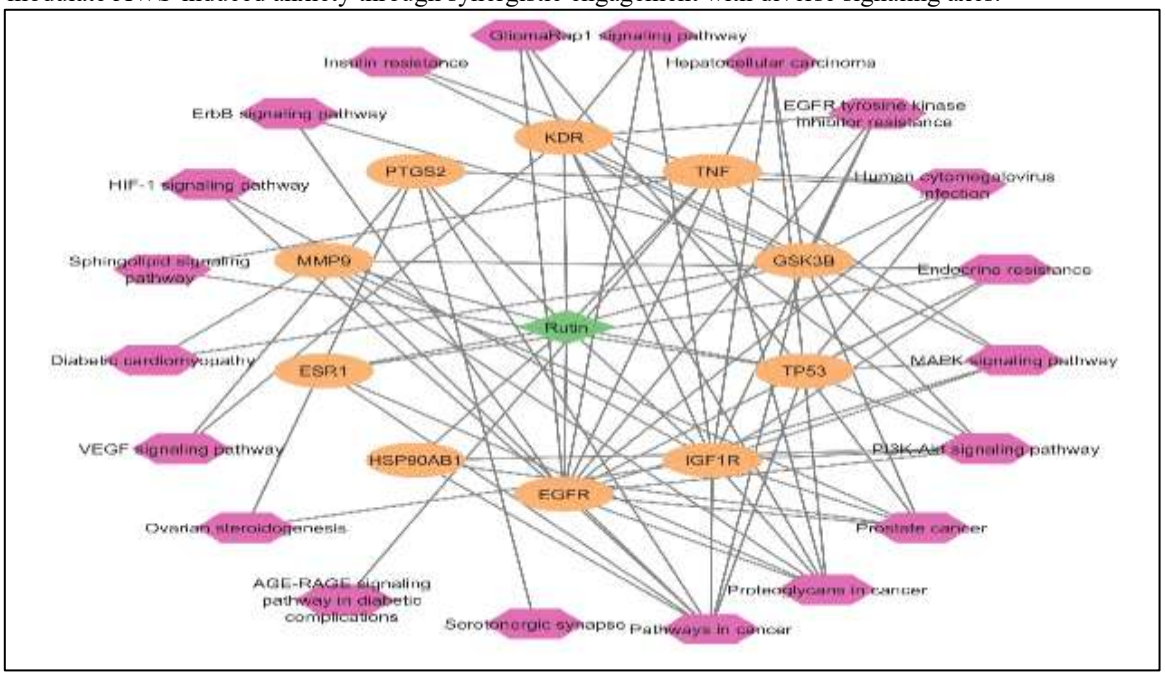


Figure 5 Compound-Target-Pathway network

3.2 Molecular Docking

Rutin was docked against the four most significant hub proteins from the PPI analysis: PTGS2, TNF, HSP90AB1, and GSK3B. The binding affinities, representative interaction diagrams, and key interacting residues are presented in Table 4 and Figure 6. The highest binding affinity was recorded for PTGS2 (−9.1 kcal/mol), consistent with rutin's known anti-inflammatory activity. Stable binding interactions were also confirmed with TNF and HSP90AB1. Docking against GSK3B supports additional modulation of MAPK/NF-κB and Wnt signaling. Collectively, these docking results supported the multi-target anti-anxiety mechanism identified by network pharmacology.

Table 4. Predicted binding affinities of rutin with key target proteins from molecular docking

Protein	Binding Affinity (kcal/mol)
PTGS2 (COX-2)	−10.1
TNF	−9.1

HSP90AB1	−9.1
GSK3B	−8.4

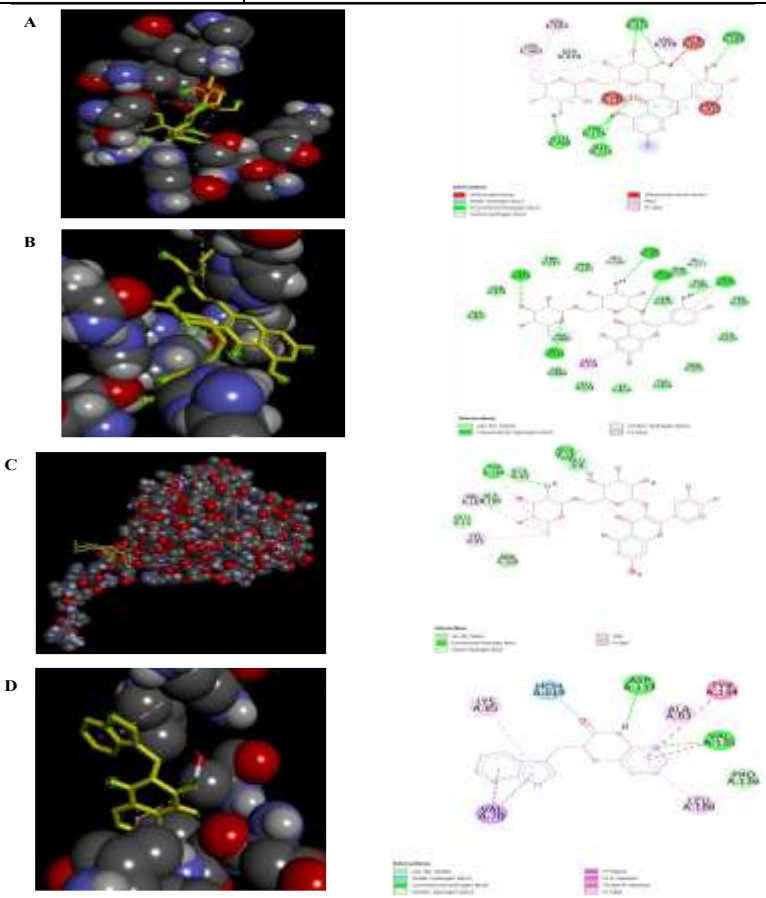


Figure 6 3D/2D molecular docking interaction maps

3.3 Drug Characterization

3.3.1 UV-Visible Spectrophotometry

Rutin demonstrated a λmax of 257 nm in methanol, consistent with the characteristic flavonoid absorption band. The absorbance-concentration relationship followed Beer-Lambert's law, with a correlation coefficient (R^2) of 0.9967, confirming excellent linearity over the tested concentration range (Table 5, Fig. 7).

Table 5. UV absorbance of rutin at various concentrations in methanol

Concentration (µg/mL)	Absorbance (nm)
2	0.263
4	0.404
6	0.536
8	0.677
10	0.812
12	0.952

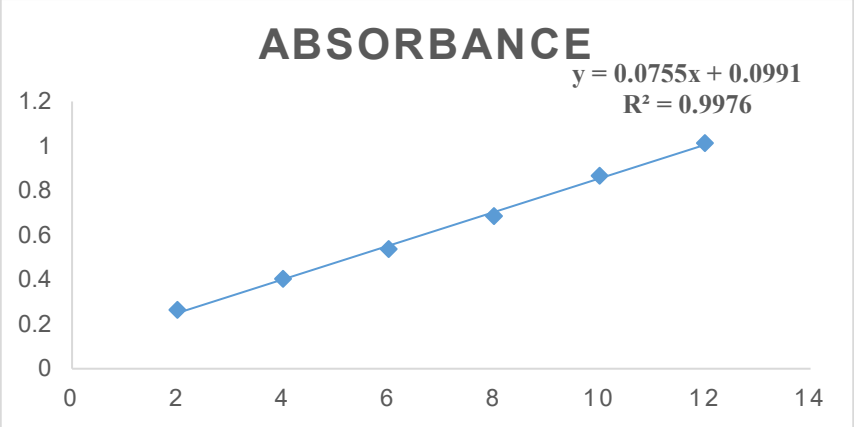


Figure 7 UV calibration curve of rutin

3.3.2 FTIR Spectroscopy

The FTIR spectrum of rutin (Fig. 8) exhibited the following characteristic absorption bands: a broad peak at ~3364 cm^{-1} (O–H stretching of phenolic/alcoholic groups); 2922 cm^{-1} (C–H stretching); 1654 cm^{-1} (C=O stretching of the flavonoid carbonyl group); 1511 cm^{-1} (C=C aromatic ring stretching); 1243 and 1078 cm^{-1} (C–O and C–O–C stretching, indicative of ether linkages and glycosidic bonds). These spectral features confirm the flavonoid glycoside nature of rutin.

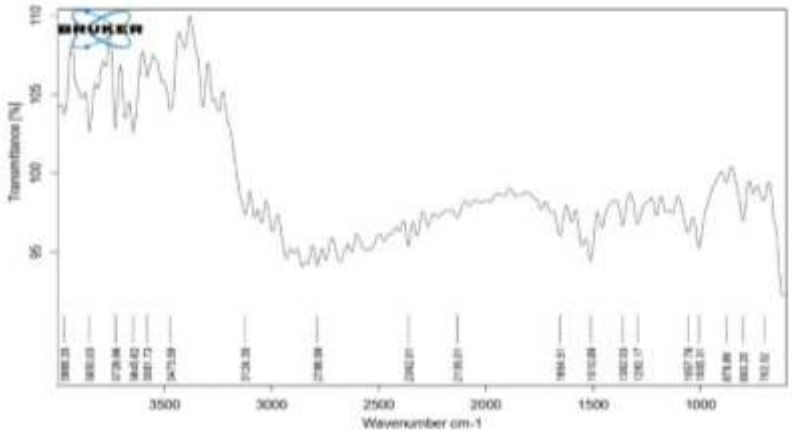


Figure 8 FTIR spectrum of rutin

3.4 Effect on Body Weight

3.4.1 Pre-treatment Period

Body weight was recorded at multiple time points during the 21-day ethanol administration period. The ethanol control group demonstrated a statistically significant reduction in body weight from day 15 onwards compared to the normal control group (†††$P < 0.001$), consistent with ethanol-induced anorexia. Rutin-treated groups, memantine, and the combination group showed significantly higher body weights than the ethanol control group at corresponding time points (***$P < 0.001$; Table 6).

Table 6. Effect of ethanol administration on body weight (g) during the pre-treatment period [mean ± SEM, n = 8]

Group	Day 1	Day 4	Day 8	Day 15	Day 21	Day 23
Normal Control	224.4 ± 0.74	229.3 ± 0.67	234.8 ± 0.67	239.8 ± 0.74	244.8 ± 0.74	247.8 ± 0.64
Ethanol Control	220.1 ± 0.66	223.3 ± 0.54	227.3 ± 0.36	221.5 ± 0.55###	217.0 ± 0.57###	213.8 ± 0.41###
Rutin 50 mg/kg	234.1 ± 2.17	233.9 ± 2.58	239.1 ± 3.15	236.8 ± 3.13***	228.0 ± 2.84	222.4 ± 2.34***
Rutin 100 mg/kg	225.8 ± 0.40	230.3 ± 0.37	234.5 ± 0.33	229.1 ± 0.31***	224.3 ± 0.33	219.6 ± 1.01***
Rutin 200 mg/kg	229.0 ± 0.52	233.5 ± 0.34	238.5 ± 0.29	233.1 ± 0.32***	228.0 ± 0.29	224.5 ± 0.33***
Memantine 20 mg/kg	223.9 ± 0.43	228.4 ± 0.40	233.5 ± 0.29	227.4 ± 1.07***	223.4 ± 0.39	216.3 ± 1.83***

Rutin + Memantine (50+20 mg/kg)	225.0 ± 0.52	229.1 ± 0.36	234.3 ± 0.34	228.6 ± 0.25***	224.0 ± 0.25	222.1 ± 0.58***

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control. Two-way ANOVA, Bonferroni's post hoc test.*

3.4.2 During Treatment Period

Following ethanol withdrawal and 7 days of treatment, rutin (50, 100, 200 mg/kg), memantine (20 mg/kg), and the combination produced a progressive and significant recovery of body weight compared to the ethanol control group (***P < 0.001). The combination group showed the most pronounced weight recovery, significantly superior to memantine alone (@@P < 0.01; Table 7).

Table 7. Effect of treatment on body weight (g) during the post-withdrawal treatment period [mean ± SEM, n = 8]

Group	Day 1	Day 3	Day 5	Day 7
Normal Control	248.5 ± 0.68	252.2 ± 1.29	260.0 ± 2.17	266.2 ± 1.33
Ethanol Control	217.0 ± 1.47	213.7 ± 1.01	208.0 ± 2.19	201.8 ± 1.56###
Rutin 50 mg/kg	223.0 ± 1.17	227.2 ± 0.83	231.0 ± 0.83	235.8 ± 1.19***
Rutin 100 mg/kg	229.2 ± 2.41	233.3 ± 1.67	237.8 ± 1.46	239.3 ± 0.83***
Rutin 200 mg/kg	225.8 ± 0.48	233.3 ± 1.10	240.2 ± 1.58	249.7 ± 1.89***
Memantine 20 mg/kg	221.8 ± 1.05	232.5 ± 3.00	237.3 ± 3.48	246.0 ± 1.89***
Rutin + Memantine (50+20 mg/kg)	227.0 ± 1.88	240.8 ± 2.58	248.7 ± 2.93	257.7 ± 2.32***@@

****P < 0.001 vs ethanol control; @@P < 0.01 vs memantine group. Two-way ANOVA, Bonferroni's post hoc test.*

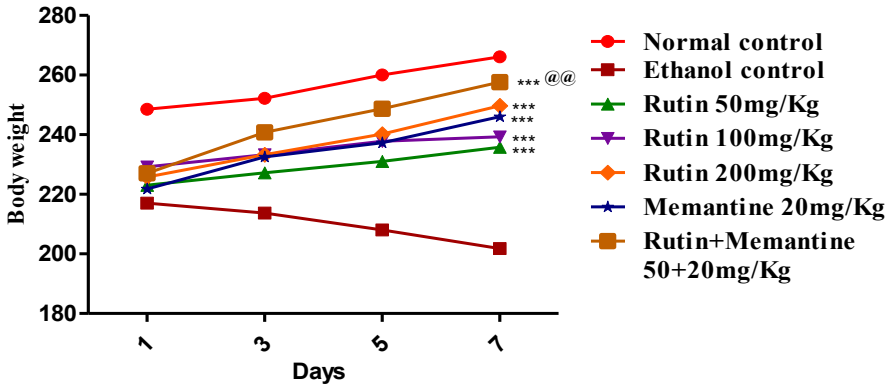


Figure 9 Body weight during treatment

3.5 Blood Ethanol Concentration (BEC)

BEC increased progressively with escalating ethanol concentrations in the diet, confirming successful induction of ethanol dependence. Mean BEC values were 72.74 ± 0.83 mg/dL on day 3 (2.4% ethanol), 139.53 ± 2.41 mg/dL on day 7 (4.8% ethanol), and 323.60 ± 5.90 mg/dL on day 14 (7.2% ethanol) in the ethanol control group. On day 23 (24 hours post-withdrawal), BEC declined to 13.42 ± 0.37 mg/dL, confirming successful ethanol clearance. No significant differences in BEC were observed among ethanol-exposed treatment groups on any measurement day (Table 8).

Table 8. Blood ethanol concentration (mg/dL) on selected measurement days [mean ± SEM, n = 8]

Group	Day 3	Day 7	Day 14	Day 23 (post-withdrawal)
Ethanol Control	72.74 ± 0.83	139.53 ± 2.41	323.60 ± 5.90	13.42 ± 0.37
Rutin 50 mg/kg	76.24 ± 2.37	137.40 ± 1.08	324.17 ± 5.60	13.51 ± 0.18
Rutin 100 mg/kg	75.19 ± 2.47	138.18 ± 2.00	337.19 ± 4.96	14.38 ± 0.42

Rutin 200 mg/kg	73.22 ± 0.78	140.53 ± 2.61	321.76 ± 5.14	15.76 ± 0.30
Memantine 20 mg/kg	89.74 ± 3.19	138.54 ± 2.29	323.27 ± 5.05	16.97 ± 0.17
Rutin + Memantine (50+20 mg/kg)	71.51 ± 2.30	144.58 ± 2.10	320.46 ± 5.91	17.78 ± 0.28

Values analyzed by two-way ANOVA. No significant inter-group differences in BEC.

3.6 Alcohol Withdrawal Symptom Scores

3.6.1 Pre-treatment (Day 23, Before Drug Administration)

On day 23, all ethanol-exposed groups demonstrated significantly elevated withdrawal scores compared to the normal control group at all time points (2, 4, 6, and 8 h), with peak severity at 6 h (***P < 0.001; Table 9). No significant differences in withdrawal scores were observed among the ethanol-exposed groups prior to treatment allocation, confirming comparable baseline AWS severity across all groups.

Table 9. Alcohol withdrawal symptom scores before treatment on day 23 at 2, 4, 6, and 8 h [mean ± SEM, n = 8]

Group	2 h	4 h	6 h	8 h
Normal Control	0.16 ± 0.27	0.33 ± 0.26	0.33 ± 0.27	0.50 ± 0.28
Ethanol Control	1.30 ± 0.33	2.25 ± 0.30	3.25 ± 0.28	4.25 ± 0.25###
Rutin 50 mg/kg	2.75 ± 0.26	4.25 ± 0.22	5.25 ± 0.21	6.12 ± 0.13***
Rutin 100 mg/kg	2.87 ± 0.24	3.62 ± 0.24	4.75 ± 0.16	5.50 ± 0.17***
Rutin 200 mg/kg	2.62 ± 0.25	3.64 ± 0.22	4.36 ± 0.18	5.12 ± 0.19***
Memantine 20 mg/kg	3.25 ± 0.22	4.37 ± 0.21	5.50 ± 0.17	6.50 ± 0.17***
Rutin + Memantine (50+20 mg/kg)	2.50 ± 0.21	3.50 ± 0.19	3.87 ± 0.16	4.50 ± 0.13***

*###P < 0.001 vs normal control; ***P < 0.001 vs normal control. Two-way ANOVA, Bonferroni's post hoc test.*

3.6.2 During Treatment (Days 24–30)

Following 7 days of treatment, withdrawal scores were significantly reduced in all treatment groups compared to the ethanol control group (***P < 0.001). The combination of rutin (50 mg/kg) and memantine (20 mg/kg) produced the most pronounced suppression of withdrawal scores at all time points, significantly superior to memantine monotherapy (@@P < 0.01; Table 10).

Table 10. Alcohol withdrawal symptom scores during treatment at 2, 4, 6, and 8 h [mean ± SEM, n = 6]

Group	2 h	4 h	6 h	8 h
Normal Control	0.63 ± 0.12	0.75 ± 0.12	0.50 ± 0.12	0.50 ± 0.12
Ethanol Control	4.38 ± 0.33	5.25 ± 0.39	5.75 ± 0.38	6.50 ± 0.32###
Rutin 50 mg/kg	6.13 ± 0.30	2.75 ± 0.39	2.88 ± 0.23	2.88 ± 0.23***
Rutin 100 mg/kg	5.50 ± 0.42	2.63 ± 0.34	2.13 ± 0.23	2.38 ± 0.18***
Rutin 200 mg/kg	5.13 ± 0.29	1.88 ± 0.23	1.38 ± 0.18	1.50 ± 0.18***
Memantine 20 mg/kg	6.50 ± 0.34	2.25 ± 0.26	1.50 ± 0.24	1.63 ± 0.17***
Rutin + Memantine (50+20 mg/kg)	4.50 ± 0.36	1.62 ± 0.18	0.75 ± 0.14	0.88 ± 0.11***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine group. Two-way ANOVA, Bonferroni's post hoc test.*

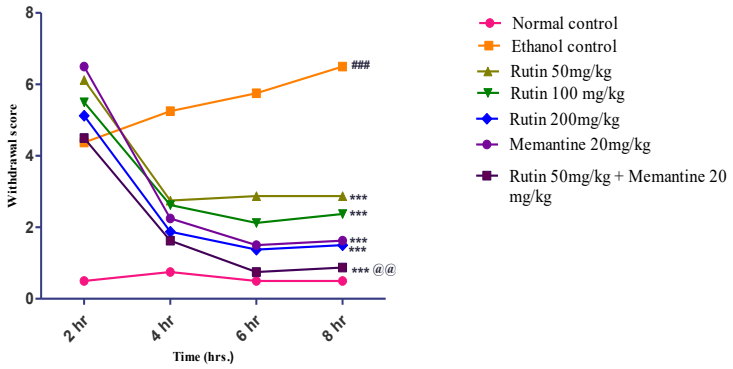


Figure 10 Withdrawal symptom scores during treatment

3.7 Behavioral Assessment of Anxiety

3.7.1 Elevated Plus Maze (EPM)

Open-arm entries: The ethanol control group showed a significant reduction in open-arm entries compared to the normal control group (1.67 ± 0.21 vs. 7.17 ± 0.17; ###P < 0.001), indicative of heightened anxiety. Treatment with rutin (50, 100, 200 mg/kg) and memantine (20 mg/kg) dose-dependently increased open-arm entries. The combination treatment produced near-complete restoration (6.83 ± 0.17), significantly superior to memantine alone (@@P < 0.01; Table 11).

Table 11. Effect of rutin on the number of open-arm entries in the EPM test [mean ± SEM, n = 6]

Group	Open Arm Entries
Normal Control	7.17 ± 0.17
Ethanol Control	1.67 ± 0.21###
Rutin 50 mg/kg	3.33 ± 0.21***
Rutin 100 mg/kg	4.00 ± 0.26***
Rutin 200 mg/kg	5.00 ± 0.26***
Memantine 20 mg/kg	4.67 ± 0.21***
Rutin + Memantine (50+20 mg/kg)	6.83 ± 0.17***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine. One-way ANOVA, Tukey's post hoc test.*

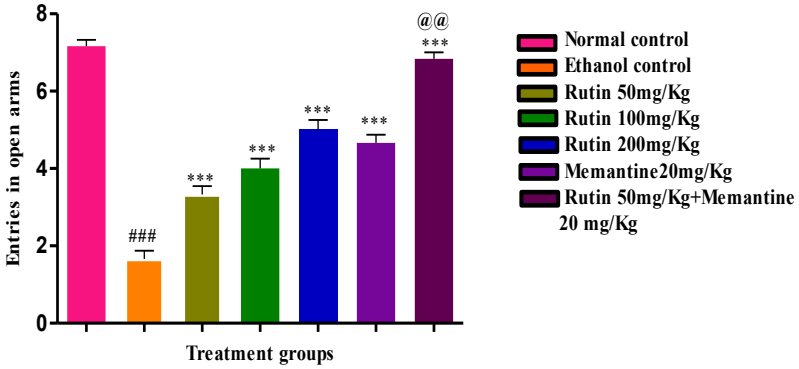


Figure 11 Open-arm entries EPM

Time spent in open arms: Similar to open-arm entries, time spent in the open arms was significantly reduced in the ethanol control group (16.33 ± 1.75 s vs. 71.33 ± 2.28 s; ###P < 0.001). All treatment groups showed significant, dose-dependent increases. The combination group achieved near-normalization (71.00 ± 1.66 s; ***P < 0.001; @@P < 0.01; Table 12).

Table 12. Effect of rutin on time spent in the open arm (seconds) in the EPM test [mean ± SEM, n = 6]

Group	Time in Open Arm (s)
Normal Control	71.33 ± 2.28
Ethanol Control	16.33 ± 1.75###
Rutin 50 mg/kg	29.83 ± 0.75***
Rutin 100 mg/kg	44.16 ± 1.13***
Rutin 200 mg/kg	54.33 ± 1.79***
Memantine 20 mg/kg	50.16 ± 1.81***
Rutin + Memantine (50+20 mg/kg)	71.00 ± 1.66***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

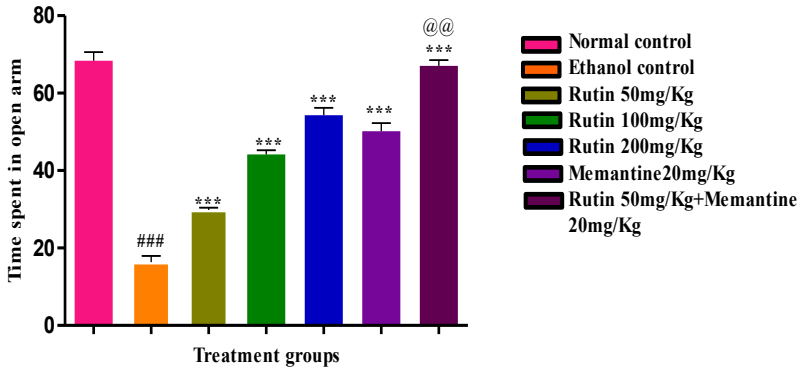


Figure 12 Time in open arm EPM

3.7.2 Light/Dark Box Test (LDT)

Light compartment entries: Ethanol withdrawal significantly reduced light compartment entries (1.50 ± 0.31 vs. 7.50 ± 0.22; ###P < 0.001). Rutin, memantine, and the combination significantly increased light-compartment entries. The combination group reached near-normal values (7.33 ± 0.21; ***P < 0.001; @@P < 0.01; Table 13).

Table 13. Effect of rutin on the number of entries in the light compartment in the LDT [mean ± SEM, n = 6]

Group	Light Compartment Entries
Normal Control	7.50 ± 0.22
Ethanol Control	1.50 ± 0.31###
Rutin 50 mg/kg	3.33 ± 0.42**
Rutin 100 mg/kg	4.33 ± 0.31***
Rutin 200 mg/kg	5.67 ± 0.42***
Memantine 20 mg/kg	4.83 ± 0.31***
Rutin + Memantine (50+20 mg/kg)	7.33 ± 0.21***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

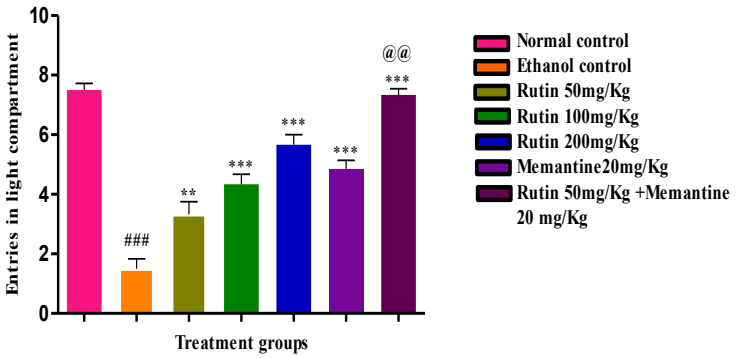


Figure 13 Light compartment entries LDT

Time spent in light compartment: Ethanol withdrawal significantly reduced time in the light compartment (43.50 ± 2.63 s vs. 136.33 ± 2.15 s; ###P < 0.001). All treatment groups showed significant improvements; the combination group restored dwell time to near-normal levels (131.66 ± 3.19 s; ***P < 0.001; @@P < 0.01; Table 14).

Table 14. Effect of rutin on the time spent in the light compartment (seconds) in the LDT [mean ± SEM, n = 6]

Group	Time in Light Compartment (s)
Normal Control	136.33 ± 2.15
Ethanol Control	43.50 ± 2.63###
Rutin 50 mg/kg	60.33 ± 1.97**
Rutin 100 mg/kg	81.00 ± 3.58***
Rutin 200 mg/kg	102.16 ± 3.26***
Memantine 20 mg/kg	90.83 ± 3.06***
Rutin + Memantine (50+20 mg/kg)	131.66 ± 3.19***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

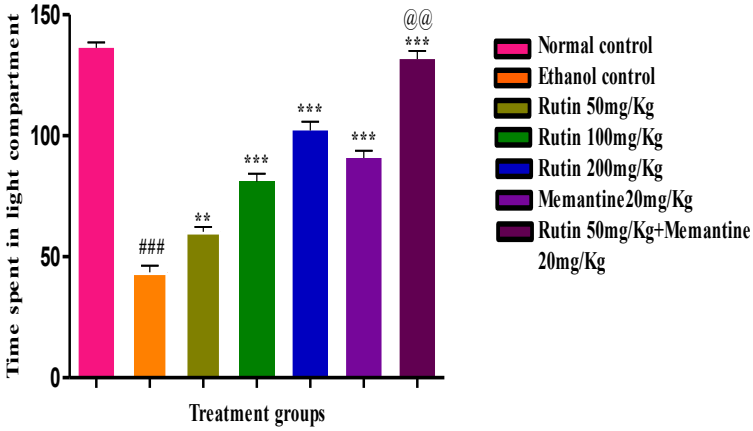


Figure 14 Time in light compartment LDT

Dark compartment entries and time spent in dark compartment (Tables 15 and 16) showed mirror-image patterns, with the ethanol control group demonstrating significantly higher dark-compartment entries and dwell time, all significantly reduced by rutin and combination treatments.

Table 15. Effect of rutin on the number of entries in the dark compartment in the LDT [mean ± SEM, n = 6]

Group	Dark Compartment Entries
Normal Control	4.17 ± 0.31
Ethanol Control	10.83 ± 0.31###
Rutin 50 mg/kg	10.00 ± 0.29**
Rutin 100 mg/kg	8.83 ± 0.48**
Rutin 200 mg/kg	6.33 ± 0.31***
Memantine 20 mg/kg	7.50 ± 0.50***
Rutin + Memantine (50+20 mg/kg)	3.83 ± 0.31***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

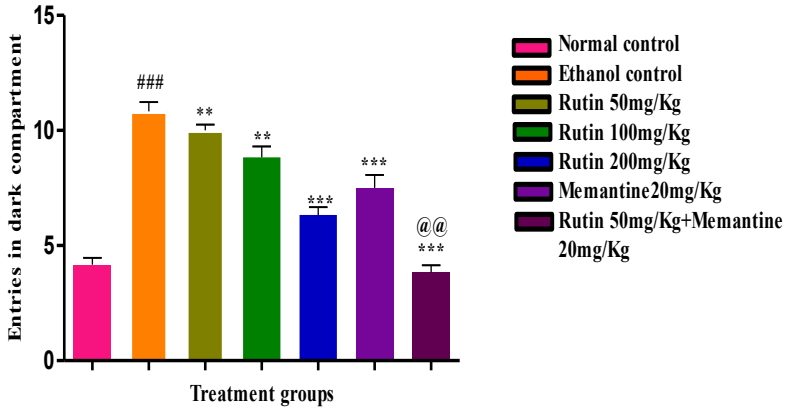


Figure 15 Here — Dark compartment entries LDT

Table 16. Effect of rutin on the time spent in the dark compartment (seconds) in the LDT [mean ± SEM, n = 6]

Group	Time in Dark Compartment (s)
Normal Control	163.17 ± 2.35
Ethanol Control	256.83 ± 2.54###
Rutin 50 mg/kg	238.00 ± 2.00**
Rutin 100 mg/kg	215.50 ± 3.41***
Rutin 200 mg/kg	196.33 ± 2.52***
Memantine 20 mg/kg	205.33 ± 3.61***
Rutin + Memantine (50+20 mg/kg)	166.67 ± 3.37***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

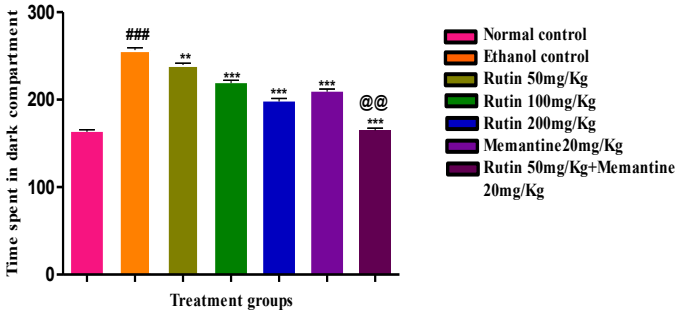


Figure 16 Time in dark compartment LDT

3.7.3 Open Field Test (OFT)

Time spent in the center: Ethanol withdrawal significantly reduced center time (15.50 ± 1.55 s vs. 77.83 ± 2.86 s; ###P < 0.001). Rutin, memantine, and the combination significantly and dose-dependently increased center exploration time. The combination group produced near-normal values (75.66 ± 3.39 s; ***P < 0.001; @@P < 0.01; Table 17).

Table 17. Effect of rutin on time spent in the center zone (seconds) in the OFT [mean ± SEM, n = 6]

Group	Time in Center (s)
Normal Control	77.83 ± 2.86
Ethanol Control	15.50 ± 1.55###
Rutin 50 mg/kg	33.00 ± 2.52**
Rutin 100 mg/kg	43.50 ± 2.56***
Rutin 200 mg/kg	58.16 ± 3.28***
Memantine 20 mg/kg	50.83 ± 3.70***
Rutin + Memantine (50+20 mg/kg)	75.66 ± 3.39***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

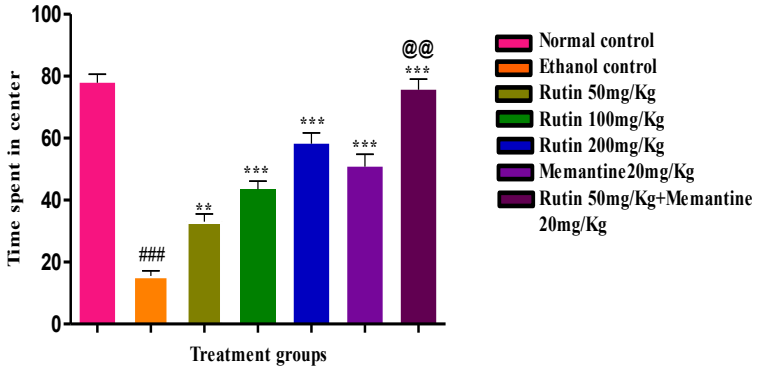


Figure 17 Time in center OFT

Number of crossings: The ethanol control group showed a significant reduction in line crossings (18.00 ± 2.08 vs. 70.83 ± 1.90; ###P < 0.001), reflecting reduced exploratory locomotion. Treatment with rutin and the combination significantly restored crossing frequency (***P < 0.001; @@P < 0.01; Table 18).

Table 18. Effect of rutin on the number of crossings in the OFT [mean ± SEM, n = 6]

Group	Number of Crossings
Normal Control	70.83 ± 1.90

Ethanol Control	18.00 ± 2.08###
Rutin 50 mg/kg	29.83 ± 1.40**
Rutin 100 mg/kg	35.83 ± 2.49***
Rutin 200 mg/kg	47.00 ± 2.83***
Memantine 20 mg/kg	42.50 ± 1.12***
Rutin + Memantine (50+20 mg/kg)	66.33 ± 2.71***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

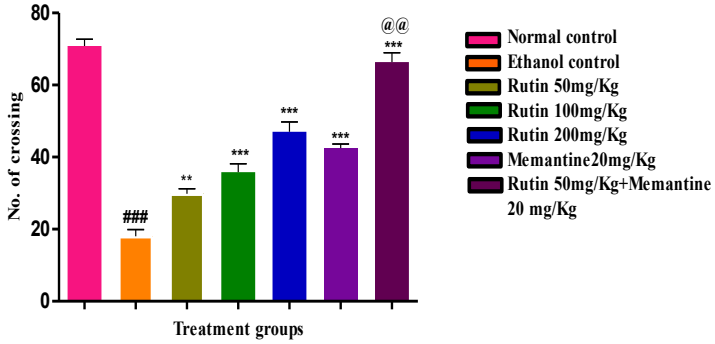


Figure 18 Here — Number of crossings OFT

3.8 Oxidative Stress Parameters

3.8.1 Glutathione (GSH)

Ethanol withdrawal significantly depleted brain GSH levels compared to the normal control group (16.82 ± 1.93 vs. 66.99 ± 2.32 µg/mg; ###P < 0.001). Rutin (50–200 mg/kg), memantine, and their combination significantly restored GSH levels in a dose-dependent manner (***P < 0.001). The combination group achieved near-normal GSH restoration (65.20 ± 3.20 µg/mg; @@P < 0.01 vs memantine; Table 19).

Table 19. Effect of rutin on brain GSH levels (µg/mg) [mean ± SEM, n = 6]

Group	GSH (µg/mg)
Normal Control	66.99 ± 2.32
Ethanol Control	16.82 ± 1.93###
Rutin 50 mg/kg	33.42 ± 2.16***
Rutin 100 mg/kg	44.53 ± 2.58***
Rutin 200 mg/kg	56.93 ± 1.62***
Memantine 20 mg/kg	49.38 ± 2.41***
Rutin + Memantine (50+20 mg/kg)	65.20 ± 3.20***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine. One-way ANOVA, Tukey's post hoc test.*

3.8.2 Superoxide Dismutase (SOD)

Brain SOD activity was significantly reduced in the ethanol control group (2.09 ± 0.26 vs. 6.75 ± 0.27 µmol/mL; ###P < 0.001). Rutin treatment and the combination significantly and dose-dependently elevated SOD activity (***P < 0.001), with the combination group nearly normalizing SOD levels (6.30 ± 0.32 µmol/mL; @@P < 0.01; Table 20).

Table 20. Effect of rutin on brain SOD activity (µmol/mL) [mean ± SEM, n = 6]

Group	SOD (µmol/mL)
Normal Control	6.75 ± 0.27
Ethanol Control	2.09 ± 0.26###
Rutin 50 mg/kg	4.52 ± 0.15***
Rutin 100 mg/kg	5.12 ± 0.10***
Rutin 200 mg/kg	5.62 ± 0.12***
Memantine 20 mg/kg	5.93 ± 0.37***
Rutin + Memantine (50+20 mg/kg)	6.30 ± 0.32***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.8.3 Catalase (CAT)

CAT activity was markedly reduced in the ethanol withdrawal group (21.12 ± 0.79 vs. 70.57 ± 2.83 µmol/mL; ###P < 0.001). All treatment groups demonstrated significant, dose-dependent recovery, with the combination group producing near-complete restoration (69.28 ± 2.31 µmol/mL; ***P < 0.001; @@P < 0.01; Table 21).

Table 21. Effect of rutin on brain CAT activity (µmol/mL) [mean ± SEM, n = 6]

Group	CAT (µmol/mL)
Normal Control	70.57 ± 2.83
Ethanol Control	21.12 ± 0.79###
Rutin 50 mg/kg	34.93 ± 2.98**
Rutin 100 mg/kg	47.64 ± 2.61***
Rutin 200 mg/kg	58.13 ± 2.26***
Memantine 20 mg/kg	52.66 ± 1.15***
Rutin + Memantine (50+20 mg/kg)	69.28 ± 2.31***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.8.4 Malondialdehyde (MDA)

Brain MDA levels were significantly elevated in the ethanol control group (9.92 ± 0.16 vs. 3.03 ± 0.23 nmol/mg; ###P < 0.001). Rutin (50–200 mg/kg), memantine, and the combination significantly reduced MDA in a dose-dependent manner. The combination group restored MDA to near-normal levels (3.31 ± 0.34 nmol/mg; ***P < 0.001; @@P < 0.01; Table 22).

Table 22. Effect of rutin on brain MDA levels (nmol/mg) [mean ± SEM, n = 6]

Group	MDA (nmol/mg)
Normal Control	3.03 ± 0.23
Ethanol Control	9.92 ± 0.16###
Rutin 50 mg/kg	7.64 ± 0.41**
Rutin 100 mg/kg	6.48 ± 0.45***

Rutin 200 mg/kg	4.67 ± 0.53***
Memantine 20 mg/kg	5.30 ± 0.32***
Rutin + Memantine (50+20 mg/kg)	3.31 ± 0.34***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.8.5 Nitric Oxide (NO)

NO levels were significantly elevated in the ethanol withdrawal group (54.67 ± 3.51 vs. 19.83 ± 1.88 µmol/mL; ###P < 0.001). Rutin and the combination significantly and dose-dependently reduced NO levels (***P < 0.001), with the combination group achieving near-normalization (18.83 ± 2.91 µmol/mL; @@P < 0.01; Table 23).

Table 23. Effect of rutin on brain NO levels (µmol/mL) [mean ± SEM, n = 6]

Group	NO (µmol/mL)
Normal Control	19.83 ± 1.88
Ethanol Control	54.67 ± 3.51###
Rutin 50 mg/kg	47.83 ± 2.79**
Rutin 100 mg/kg	40.33 ± 2.43***
Rutin 200 mg/kg	31.33 ± 1.49***
Memantine 20 mg/kg	36.00 ± 1.40***
Rutin + Memantine (50+20 mg/kg)	18.83 ± 2.91***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.9 Neurotransmitter Levels

3.9.1 GABA

Brain GABA levels were significantly reduced in the ethanol control group (104.55 ± 1.08 vs. 247.33 ± 3.38 pmol/mL; ###P < 0.001). Rutin treatment at all doses and the combination significantly restored GABA levels (***P < 0.001), with the combination group achieving near-normalization (232.42 ± 7.57 pmol/mL; @@P < 0.01; Table 24).

Table 24. Effect of rutin on brain GABA levels (pmol/mL) [mean ± SEM, n = 6]

Group	GABA (pmol/mL)
Normal Control	247.33 ± 3.38
Ethanol Control	104.55 ± 1.08###
Rutin 50 mg/kg	125.68 ± 1.06**
Rutin 100 mg/kg	147.80 ± 3.13***
Rutin 200 mg/kg	185.87 ± 5.04***
Memantine 20 mg/kg	158.32 ± 3.17***
Rutin + Memantine (50+20 mg/kg)	232.42 ± 7.57***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.9.2 Serotonin

Serotonin levels were markedly depleted in the ethanol withdrawal group (38.08 ± 3.19 vs. 117.07 ± 3.28 ng/mL; ###P < 0.001). All treatment groups demonstrated significant restoration; the combination group exhibited the greatest recovery (110.56 ± 2.90 ng/mL; ***P < 0.001; @@P < 0.01; Table 25).

Table 25. Effect of rutin on brain serotonin levels (ng/mL) [mean ± SEM, n = 6]

Group	Serotonin (ng/mL)
Normal Control	117.07 ± 3.28
Ethanol Control	38.08 ± 3.19###
Rutin 50 mg/kg	58.10 ± 2.83**
Rutin 100 mg/kg	66.22 ± 4.33***
Rutin 200 mg/kg	85.73 ± 3.66***
Memantine 20 mg/kg	73.52 ± 3.31***
Rutin + Memantine (50+20 mg/kg)	110.56 ± 2.90***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.9.3 Dopamine

Dopamine levels were severely depleted in the ethanol control group (0.79 ± 0.05 vs. 6.70 ± 0.45 ng/mL; ###P < 0.001). Treatment progressively restored dopamine levels. The combination group achieved near-normalization (6.68 ± 0.42 ng/mL; ***P < 0.001; @@P < 0.01; Table 26).

Table 26. Effect of rutin on brain dopamine levels (ng/mL) [mean ± SEM, n = 6]

Group	Dopamine (ng/mL)
Normal Control	6.70 ± 0.45
Ethanol Control	0.79 ± 0.05###
Rutin 50 mg/kg	3.00 ± 0.34**
Rutin 100 mg/kg	3.70 ± 0.22***
Rutin 200 mg/kg	5.36 ± 0.36***
Memantine 20 mg/kg	3.98 ± 0.41***
Rutin + Memantine (50+20 mg/kg)	6.68 ± 0.42***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.9.4 Glutamate

Brain glutamate levels were significantly elevated in the ethanol withdrawal group (4.07 ± 0.17 vs. 0.98 ± 0.19 pmol/mL; ###P < 0.001), consistent with glutamatergic excitotoxicity. All treatment groups significantly reduced glutamate levels, with the combination achieving near-normalization (1.05 ± 0.19 pmol/mL; ***P < 0.001; @@P < 0.01; Table 27).

Table 27. Effect of rutin on brain glutamate levels (pmol/mL) [mean ± SEM, n = 6]

Group	Glutamate (pmol/mL)
Normal Control	0.98 ± 0.19
Ethanol Control	4.07 ± 0.17###

Rutin 50 mg/kg	2.59 ± 0.22***
Rutin 100 mg/kg	2.16 ± 0.17***
Rutin 200 mg/kg	1.69 ± 0.19***
Memantine 20 mg/kg	1.35 ± 0.30***
Rutin + Memantine (50+20 mg/kg)	1.05 ± 0.19***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.9.5 Norepinephrine (NE)

NE levels were significantly elevated in the ethanol control group (168.90 ± 1.84 vs. 69.13 ± 3.69 pmol/mL; ###P < 0.001). Rutin treatment and the combination significantly reduced NE levels. The combination group achieved near-normalization (69.37 ± 3.44 pmol/mL; ***P < 0.001; @@P < 0.01; Table 28).

Table 28. Effect of rutin on brain norepinephrine levels (pmol/mL) [mean ± SEM, n = 6]

Group	Norepinephrine (pmol/mL)
Normal Control	69.13 ± 3.69
Ethanol Control	168.90 ± 1.84###
Rutin 50 mg/kg	148.82 ± 2.13***
Rutin 100 mg/kg	127.23 ± 3.15***
Rutin 200 mg/kg	101.65 ± 3.00***
Memantine 20 mg/kg	120.48 ± 2.74***
Rutin + Memantine (50+20 mg/kg)	69.37 ± 3.44***@@

*###P < 0.001 vs normal control; ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.10 TNF-α Levels

TNF-α concentrations in brain homogenates were significantly elevated in the ethanol control group (347.0 ± 6.73 vs. 141.1 ± 4.45 pg/mL; ###P < 0.001). Rutin (50–200 mg/kg), memantine, and their combination significantly reduced TNF-α levels (***P < 0.001). The combination group nearly completely suppressed neuroinflammation, restoring TNF-α to near-normal levels (139.7 ± 5.05 pg/mL; @@P < 0.01; Table 29).

Table 29. Effect of rutin on brain TNF-α levels (pg/mL) [mean ± SEM, n = 6]

Group	TNF-α (pg/mL)
Normal Control	141.1 ± 4.45
Ethanol Control	347.0 ± 6.73###
Rutin 50 mg/kg	319.3 ± 0.89**
Rutin 100 mg/kg	270.8 ± 4.57***
Rutin 200 mg/kg	205.8 ± 4.87***
Memantine 20 mg/kg	214.5 ± 4.81***
Rutin + Memantine (50+20 mg/kg)	139.7 ± 5.05***@@

*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.11 NMDA Receptor Expression

NMDA receptor protein expression was markedly upregulated in the ethanol control group compared to the normal control group (7.11 ± 0.39 vs. 1.99 ± 0.26 ng/mL; ###P < 0.001), consistent with the neuroadaptive upregulation of glutamatergic signaling during chronic ethanol exposure. Rutin (50–200 mg/kg), memantine, and their combination significantly and dose-dependently downregulated NMDA receptor expression (***P < 0.001). The combination group reduced NMDA expression to near-normal levels (1.92 ± 0.18 ng/mL; @@P < 0.01; Table 30).

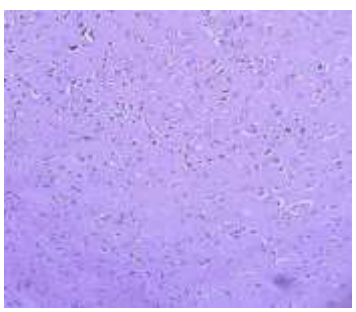
Table 30. Effect of rutin on brain NMDA receptor expression (ng/mL) [mean ± SEM, n = 6]

Group	NMDA Receptor (ng/mL)
Normal Control	1.99 ± 0.26
Ethanol Control	7.11 ± 0.39###
Rutin 50 mg/kg	6.04 ± 0.48**
Rutin 100 mg/kg	4.72 ± 0.45***
Rutin 200 mg/kg	3.60 ± 0.43***
Memantine 20 mg/kg	3.94 ± 0.51***
Rutin + Memantine (50+20 mg/kg)	1.92 ± 0.18***@@

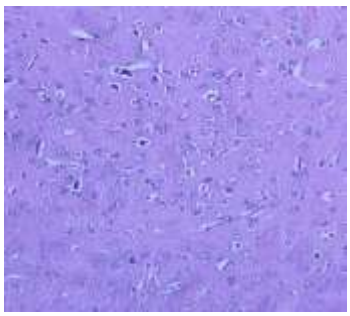
*###P < 0.001 vs normal control; **P < 0.01, ***P < 0.001 vs ethanol control; @@P < 0.01 vs memantine.*

3.12 Histopathological Analysis

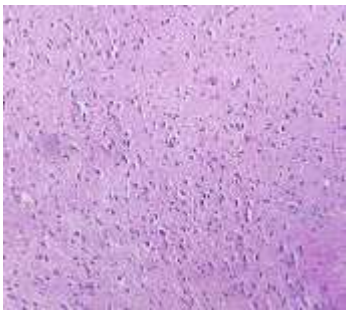
Histopathological examination of hippocampal sections stained with hematoxylin and eosin revealed marked neurodegeneration in the ethanol control group, characterized by diffuse cellular edema, increased pyknotic nuclei, and loss of neuronal architecture in the CA1, CA3, and dentate gyrus subregions. Treatment with rutin produced dose-dependent neuroprotection: Rutin 50 mg/kg showed partial improvement; Rutin 100 mg/kg demonstrated moderate neuroprotection; Rutin 200 mg/kg produced near-normal hippocampal histology. Memantine (20 mg/kg) provided substantial neuroprotection. The combination of rutin (50 mg/kg) and memantine (20 mg/kg) achieved complete neuroprotection, with hippocampal architecture comparable to the normal control group (Fig. 31).



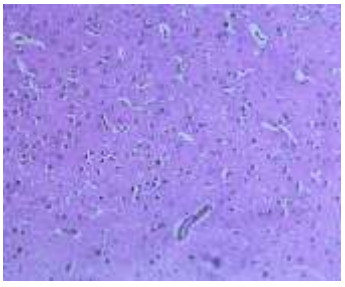
Control Group



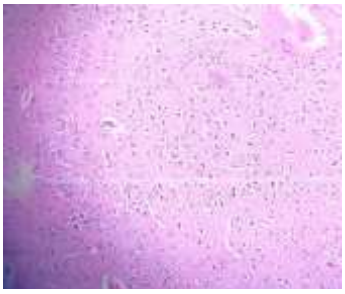
Disease Group



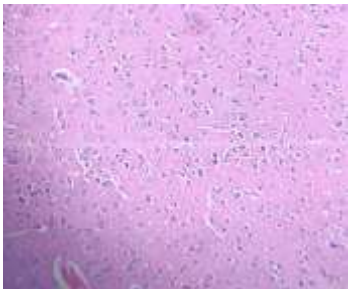
Standard Group



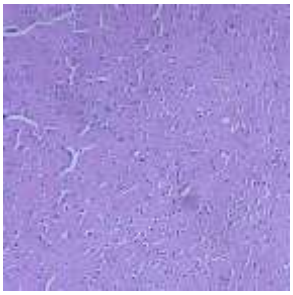
Rutin 50 mg/kg



Rutin 100 mg/kg



Rutin 200 mg/kg



Rutin + Memantine (50+20 mg/kg)

Figure 31 Histopathological H&E sections: (A) Normal Control; (B) Ethanol Control; (C–G) Treatment groups. Scale bar = 50 µm.

4. DISCUSSION

The present study employed an integrated in silico–in vivo strategy to comprehensively characterize the therapeutic potential of rutin in ethanol withdrawal-induced anxiety. The principal findings demonstrate that rutin exerts dose-dependent, multi-target anxiolytic and neuroprotective effects in the rat AWS model, and that its combination with memantine produces synergistic benefits superior to either agent administered alone.

Network pharmacology and molecular docking.

Network pharmacology identified 69 shared targets between rutin and AW-anxiety, highlighting the multi-target nature of rutin's pharmacological action. The PPI network analysis revealed TNF, TP53, EGFR, PTGS2, GSK3B, and ESR1 as central hub proteins, underscoring the convergence of neuroinflammation, oxidative stress signaling, and cell survival pathways in AWS-induced anxiety. KEGG pathway enrichment implicated MAPK/NF-κB, HIF-1, and AGE-RAGE pathways, consistent with oxidative stress-driven neuroinflammation and excitotoxicity in AWS.[27] Molecular docking further validated these predictions: rutin demonstrated particularly high binding affinity for PTGS2 (COX-2; −10.1 kcal/mol), consistent with established inhibition of cyclooxygenase-mediated neuroinflammatory prostaglandin synthesis.[28] Strong binding to TNF (−9.1 kcal/mol) supports suppression of TNF-α-mediated NF-κB activation.[29] Interaction with GSK3B implicates additional modulation of Wnt/β-catenin and MAPK signaling cascades.[30]

Ethanol dependence and BEC.

The progressive BEC escalation over 21 days (72.74 → 323.60 mg/dL) confirmed successful induction of ethanol dependence, and near-complete clearance by day 23 (13.42 mg/dL) validated the integrity of the withdrawal model. The anorexia-driven body weight reduction in the ethanol control group from day 15 onwards is consistent with established effects of chronic alcohol on appetite regulation.[31] More pronounced weight recovery in the combination group further reflects broader systemic ameliorative effects.

Withdrawal symptom scores.

All ethanol-exposed groups demonstrated elevated withdrawal scores on day 23, peaking at 6 hours post-cessation, consistent with the temporal profile of AWS in rodents.[1] Following 7 days of treatment, all active treatment groups showed significant reductions in withdrawal severity, with the combination group achieving the most substantial reduction. This aligns with the complementary mechanisms of rutin (antioxidant, GABAergic, serotonergic) and memantine (NMDA receptor antagonism) in addressing the dual pathological drivers of AWS.

Anxiety-like behavior.

Ethanol withdrawal produced robust anxiety-like behavior across all three behavioral paradigms (EPM, LDT, OFT), consistent with the established hyperexcitability model of AWS.[32] Rutin's dose-dependent anxiolytic effects in the EPM and LDT are congruent with previous reports of flavonoid-mediated anxiolytic activity attributed to positive allosteric modulation of GABA-A receptors and enhancement of serotonergic neurotransmission.[33] Quercetin, the aglycone of rutin, has been shown to bind the benzodiazepine site of GABA-A receptors, augmenting inhibitory tone—an effect that may extend to rutin following metabolic conversion.[34] The near-complete normalization of anxiety-like behavior by the rutin–memantine combination across all three tests indicates that simultaneous enhancement of inhibitory (GABAergic/serotonergic) and attenuation of excitatory (NMDA-glutamatergic) tone provides superior anxiolysis by more completely restoring the inhibitory-excitatory balance disrupted by chronic ethanol.

Oxidative stress.

Chronic ethanol consumption and its withdrawal are well-established triggers of oxidative stress in the brain, characterized by accumulation of reactive oxygen species (ROS), lipid peroxidation (reflected by elevated MDA), reduced antioxidant enzyme activities (GSH, SOD, CAT), and elevated NO through inducible nitric oxide synthase (iNOS) upregulation.[35] Rutin's dose-dependent restoration of all these parameters is attributable to its free radical scavenging capacity, metal chelating activity, and upregulation of endogenous antioxidant defense systems, including via Nrf2/HO-1 pathway activation.[36] The superior antioxidant restoration achieved by the

combination likely reflects complementary actions on both ROS production (NMDA-calcium-mitochondria axis, attenuated by memantine) and ROS scavenging (direct antioxidant activity of rutin).[37]

Neurotransmitter imbalance.
The chronic ethanol-induced compensatory changes in GABAergic and glutamatergic signaling, fully unmasked during withdrawal, are the primary neurochemical substrate for AWS anxiety.[7] The marked GABA depletion and glutamate elevation in the ethanol control group are consistent with this model. Rutin's ability to restore GABA levels is consistent with reports of direct GABA-A receptor modulation by flavonoids[33] as well as indirect enhancement through reduction of neuroinflammation-driven inhibitory interneuron dysfunction.[38] The concurrent depletion of monoaminergic neurotransmitters—serotonin, dopamine, and NE—reflects the disruption of multiple reward, mood-regulation, and stress-response circuits during AWS.[40] Rutin's restoration of serotonin and dopamine is consistent with its known inhibition of monoamine oxidase (MAO) activity and attenuation of neuroinflammation-driven monoaminergic dysfunction.[13]

Neuroinflammation.
TNF-α is a pivotal pro-inflammatory cytokine that drives neuroinflammation through activation of NF-κB signaling during AWS.[29] The pronounced elevation of TNF-α in the ethanol control group, and its near-complete suppression by the combination treatment, is consistent with rutin's established capacity to inhibit NF-κB nuclear translocation and downregulate pro-inflammatory cytokine production.[41] This anti-inflammatory mechanism is further supported by the molecular docking result demonstrating high-affinity binding of rutin to TNF (−9.1 kcal/mol).

NMDA receptor expression.
The upregulation of NMDA receptor expression in the ethanol control group represents the neuroadaptive response to chronic ethanol's inhibitory action on NMDA receptors during the dependence phase, which becomes pathological upon withdrawal, driving glutamatergic hyperactivity.[8] Rutin's dose-dependent downregulation of NMDA receptor expression—in addition to memantine's direct channel-blocking activity—provides a dual mechanism for attenuating glutamatergic excitotoxicity. The combination achieved near-normalization of NMDA expression, consistent with its superior behavioral and biochemical outcomes.

Histopathology.
The severe hippocampal neurodegeneration, edema, and pyknotic neuronal changes observed in the ethanol control group reflect the cumulative neurotoxic effects of chronic ethanol and withdrawal-induced excitotoxicity.[42] Progressive histological improvement with increasing rutin doses, and complete neuroprotection achieved by the combination, collectively validate the biochemical and behavioral findings. The hippocampus is particularly vulnerable to glutamate-mediated excitotoxicity, and its preservation by the combination treatment suggests functional relevance to the observed anxiolytic and cognitive-protective outcomes.

Study limitations.
Several limitations warrant consideration. The study was confined to male Wistar rats, precluding assessment of potential sex differences in AWS pharmacology. The 7-day treatment duration may not reflect the requirements of chronic AWS management in clinical settings. Plasma pharmacokinetic parameters of rutin were not characterized. The bioavailability of rutin is reportedly low following oral administration due to limited absorption and extensive metabolism; future studies should evaluate nanoparticulate or phospholipid-complexed formulations. Finally, clinical translation will require well-designed randomized controlled trials to confirm safety, tolerability, and efficacy in human subjects with AUD.

5. CONCLUSION

The present study provides the first integrated in silico and in vivo evidence for the multi-target anxiolytic and neuroprotective potential of rutin in an ethanol withdrawal rat model of AWS-induced anxiety. Network pharmacology and molecular docking identified PTGS2, TNF, HSP90AB1, and GSK3B as key molecular targets, implicating MAPK/NF-κB signaling and glutamatergic synapse regulation as principal mechanistic axes. In vivo, rutin (50–200 mg/kg) exerted dose-dependent anxiolytic effects across EPM, LDT, and OFT paradigms, simultaneously restoring GABAergic inhibitory tone, suppressing glutamatergic excitotoxicity, reversing monoaminergic depletion, enhancing antioxidant defenses, attenuating neuroinflammation, and downregulating NMDA receptor expression. Critically, the combination of rutin (50 mg/kg) and memantine (20 mg/kg) produced synergistic neuroprotective and anxiolytic effects superior to either agent alone, achieving near-normalization across all behavioral, biochemical, neurochemical, and histological parameters. These findings collectively position rutin as a promising natural, multi-target candidate for the adjunctive management of AWS-induced anxiety. Translational studies, including bioavailability optimization, long-term safety profiling, and clinical trials, are warranted to realize its full therapeutic potential in human AUD management.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
All authors contributed substantially to the conception and design of the study. Material preparation, data collection, data analysis, and interpretation were performed collaboratively by the authors. The first draft of the manuscript was prepared by the corresponding author, and all authors critically reviewed, revised, and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript and accept responsibility for the accuracy and integrity of the work.

Data Availability Statement
All data generated and analyzed during this study are included in this article. Raw data are available from the corresponding author upon reasonable request.

Ethics Statement
All animal experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) in accordance with CPCSEA guidelines (Approval No.: SOPS/IAEC/2019-20/02269). All procedures conformed to the ethical norms and standards for the care and use of laboratory animals.

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