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ORIGINAL ARTICLE

A network pharmacology approach validates resveratrol's in vivo neuroprotective effects against neurotoxic metals-induced cognitive decline

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ABSTRACT

Objectives: To investigate the neuroprotective effects of resveratrol against combined neurotoxicity induced by aluminum (Al), lead (Pb), and arsenic (As) using integrated in-silico and in-vivo approaches.

Methods: Thirty adult male Sprague-Dawley rats (10-12 weeks; 180-250 g) were randomized into three groups (n=10 each): control, metal mixture (Al+As+Pb; 25 mg/kg/day, oral), and metal mixture plus resveratrol (20 mg/kg/day in feed) for 60 days. Cognitive performance was assessed using the Y-maze and Morris water maze (MWM). Gene expression of synaptophysin, post-synaptic-density-protein-95 instead if and CaMKIV in cortex and hippocampus was evaluated by qPCR using the 2^-ΔΔCT method. In-silico target prediction and network pharmacology were performed using Swiss Target Prediction, CTD, and GeneCards; intersecting targets were analyzed via STRING and Cytoscape, followed by GO/KEGG enrichment (DAVID/ShinyGO). Molecular docking (AutoDock Vina) assessed binding of resveratrol with IL6, TNF, IL1-β, synaptophysin, CaMKIV, and PSD-95.

Results: Network pharmacology identified 269 intersecting targets; hub genes included IL6, TNF, and IL1-β. Enrichment analysis highlighted pathways related to inflammation, apoptosis regulation, PI3K-Akt, MAPK, and neurodegeneration. Docking showed strong affinity with CaMKIV (-8.1 kcal/mol) and moderate affinity with IL6 (-6.8), IL1-β (-6.9), and PSD-95 (-6.5). In-vivo, the metal mixture impaired memory in Y-maze and MWM, while resveratrol significantly improved performance. Metal exposure downregulated synaptophysin, PSD-95, and CaMKIV expression in cortex and hippocampus; resveratrol partially to markedly restored expression.

Conclusion: Resveratrol mitigated Al/As/Pb-induced neurotoxicity, improving cognitive performance and synaptic gene expression, potentially through multi-target modulation of inflammatory and neurodegenerative pathways.

Keywords: Metals (MeSH); Neurotoxic metals (Non-MeSH); Resveratrol (MeSH); Neuroprotection (MeSH); Cognitive Dysfunction (MeSH); Cognitive Decline (MeSH); Network Pharmacology (MeSH); Synaptophysin (MeSH); PSD-95 (MeSH); CaMKIV (MeSH).

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INTRODUCTION

Neurotoxic metals such as aluminum (Al), arsenic (As), and lead (Pb) are pervasive environmental contaminants primarily arising from industrial activities and environmental pollution. These metals pose a significant threat to human health, particularly to the central nervous system, where they induce oxidative stress, neuroinflammation, and disruption of critical neural pathways.[1] Chronic exposure has been associated with an increased risk of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, as well as impaired cognitive performance, synaptic dysfunction, and mitochondrial and metabolic disturbances.[2,3]

Accumulating evidence indicates that these metals adversely affect learning and memory by disrupting essential synaptic proteins.[4] Lead exposure has been shown to downregulate postsynaptic density protein-95 (PSD-95),[5] thereby compromising synaptic stability.[6] Arsenic interferes with calcium/calmodulin-dependent protein kinase IV (CaMKIV), impairing memory-related signaling pathways.[7] Additionally, both aluminum and lead reduce synaptophysin SYP expression, contributing to synaptic dysfunction and subsequent cognitive decline.[8]

In vivo experimental findings are increasingly complemented and validated by network pharmacology approaches, which are widely used to elucidate the mechanism-based pharmacological actions of natural compounds.[9] This integrative strategy enables systematic prediction of drug–disease interactions and underlying molecular mechanisms through database integration and molecular docking analyses.[10] In the present study, a network pharmacology framework was applied to identify potential molecular targets of resveratrol and to evaluate its therapeutic relevance against metal mixture-induced neurotoxicity.

Resveratrol, a naturally occurring polyphenol found in grapes, berries, and red wine, has demonstrated considerable neuroprotective potential. Its potent antioxidant and anti-inflammatory properties contribute to the preservation of synaptic integrity, attenuation of oxidative stress, and maintenance of neuronal health.[11] Furthermore, resveratrol has been shown to enhance cognitive function by upregulating key synaptic proteins, including PSD-95, thereby coun-

teracting metal-induced neurotoxicity.[12]

Given the accumulating evidence linking heavy metal exposure to cognitive decline, this study examined the combined neurotoxic effects of three prevalent environmental metals; aluminum, arsenic, and lead, whose cumulative impact on cognitive function is likely to be more pronounced than exposure to individual metals. Although resveratrol is well recognized for its antioxidant and neuroprotective properties, its efficacy against the concurrent neurotoxic challenge posed by multiple metals remains insufficiently explored. To address this gap, the present study evaluated the neuroprotective potential of resveratrol using an integrative approach encompassing network pharmacology, behavioral assessments, and gene expression analysis. By combining in vivo and in silico strategies, this work aims to provide a comprehensive understanding of the mechanisms through which resveratrol may mitigate heavy metal-induced cognitive and molecular impairments.

METHODS

Ethics statement: All experimental procedures were conducted in accordance with the guidelines of Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institutes of Health, USA (Guide for the Care and Use of Laboratory Animals). The study protocols were approved by Institutional Review Board, Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology (NUST), Islamabad (Approval No. IRB-135).

Animals: Adult male Sprague-Dawley rats were obtained from the National Institute of Health (NIH), Islamabad, Pakistan, and acclimatized for one week prior to experimentation. Animals were housed in standard polypropylene cages under controlled environmental conditions (temperature 25±2 °C, 12-h light/dark cycle, and 50–60% relative humidity) with free access to a standard laboratory diet (47% carbohydrates, 30% crude protein, 9% crude fat, 4% crude fiber, and 10% moisture) and distilled water ad libitum.

A total of 30 healthy rats were included and randomly allocated into three experimental groups (n=10 per group). Sample size determination was based on prior neurotoxicity and neuroprotection studies,[13] and was further supported by power analysis using G*Power 3.1, [14] which indicated a minimum of eight animals per group to detect a medium effect size (f=0.4) with 80% power at α=0.05. To accommodate potential attrition, group sizes were increased to ten.

Rats were 10-12 weeks old, weighed 180-250 g, and exhibited no signs of illness or infection during acclimatization. Baseline home-cage observations confirmed normal behavior. Female rats were excluded to avoid hormonal variability that could confound neurobehavioral and biochemical outcomes. Animals showing abnormal behavior, visible illness, or injury were excluded. This standardized selection minimized biological variability and enhanced the reliability and reproducibility of the findings.

Animal model development: To evaluate the neurotoxic effects of heavy metals and the potential neuroprotective role of resveratrol, a preventive treatment model was employed using a combination of Al, As, and Pb. The metal mixture was administered orally at a combined dose of 25 mg/kg/day, dissolved in distilled water, for 60 consecutive days to induce chronic neurotoxicity. Control-animals received a standard laboratory diet and distilled water throughout the study period. Rats in the metal mixture group were exposed to the Al-As-Pb combination at 25 mg/kg/day for 60

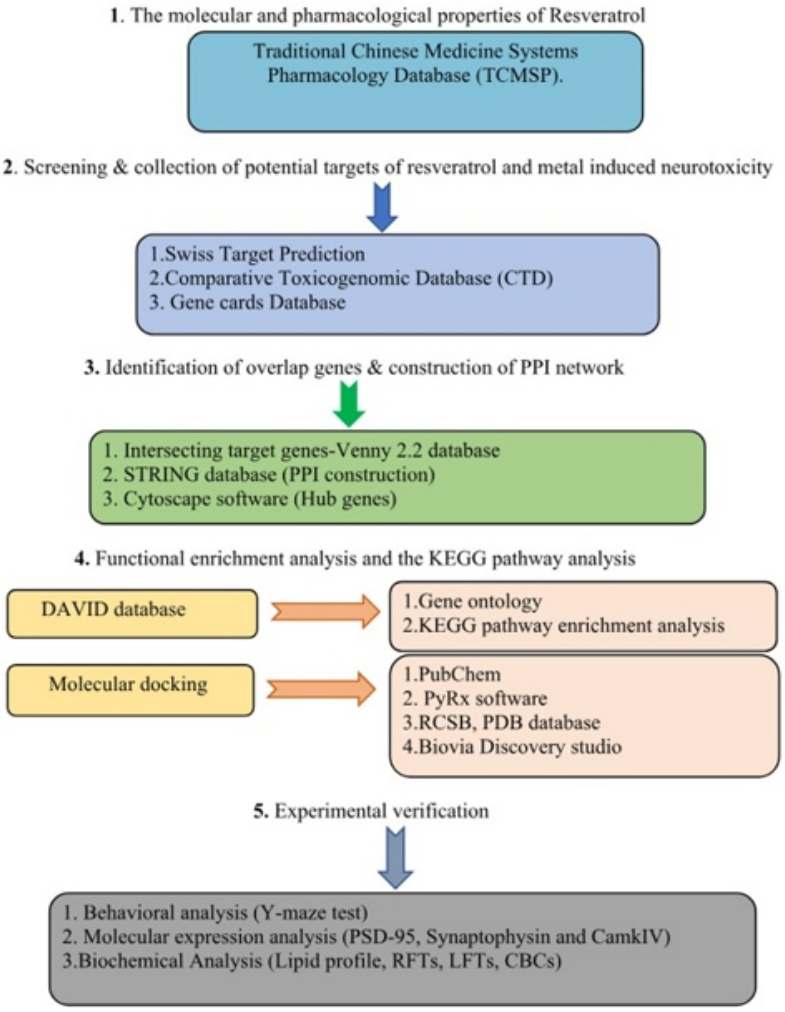

Figure 1: Workflow for network pharmacology analysis.

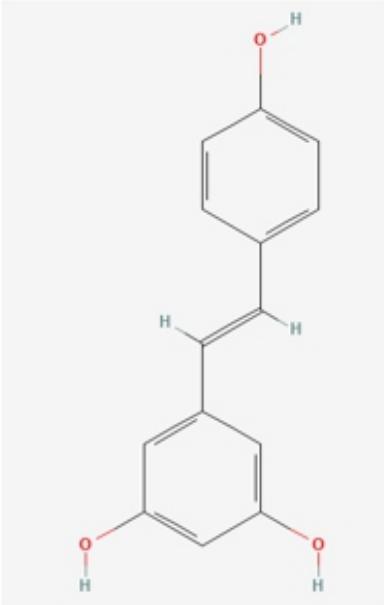

Figure. 2: 2D structure of Resveratrol.

days, whereas the metal mixture plus resveratrol group received the same metal dose along with resveratrol at 20 mg/kg/day administered via the diet. For dietary incorporation, resveratrol was dissolved in 1 mL of ethanol, uniformly mixed with the standard feed, and air-dried at room temperature to allow complete evaporation of ethanol. The prepared feed was stored in airtight zip-lock bags and freshly provided daily.

This regimen ensured continuous dietary intake of resveratrol concurrent with metal exposure, thereby simulating a preventive intervention model. All animals were monitored daily for clinical signs, behavioral changes, and food intake throughout the experimental period.

Animals group allocation in study design: The study was conducted over a 60-day period using 30 healthy adult male Sprague-Dawley rats, which were randomly allocated into three experimental groups (n=10 per group). Simple randomization was applied to ensure unbiased group assignment. Animals were assigned to groups using computer-generated random numbers created with Microsoft Excel's RAND function,[15] which provided equal probability of allocation to the control, metal mixture, or metal mixture plus resveratrol (20 mg/kg) groups, thereby minimizing selection bias and ensuring reproducibility.

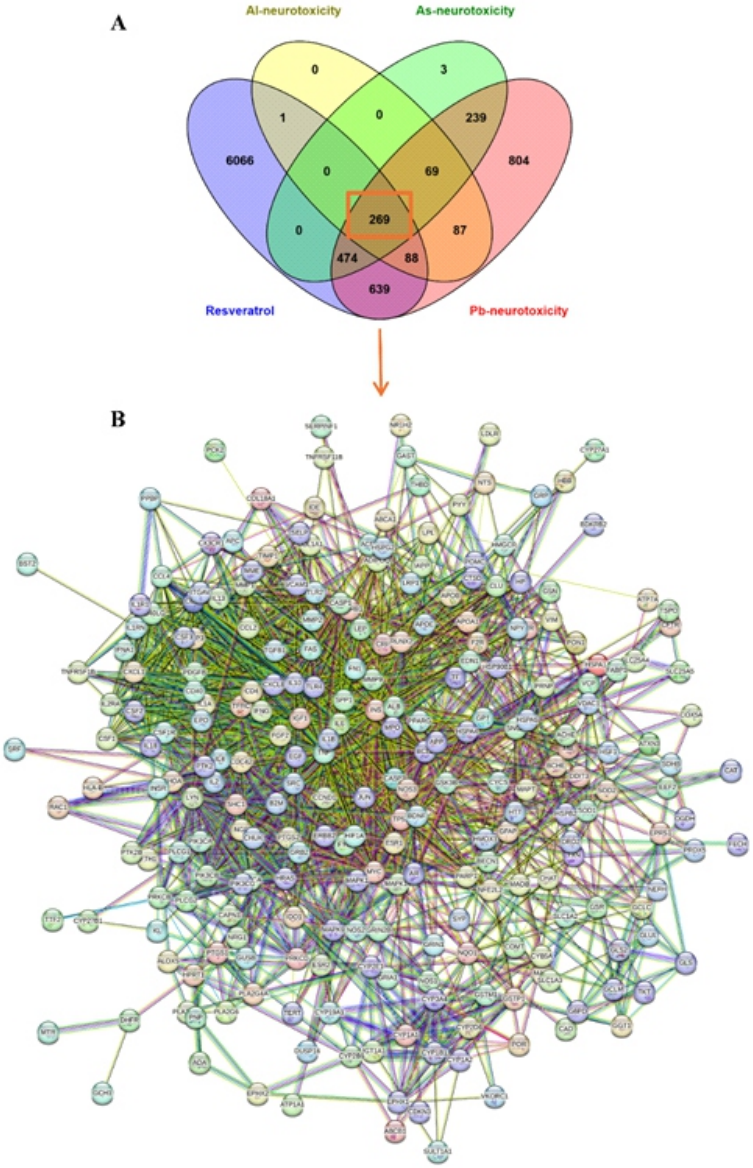

Figure 3: **(A)** Venn diagram of intersected target genes of resveratrol against Al, As and Pb-induced neurotoxicity and their PPI network **(B)**.

Body weight and food intake were monitored throughout the experimental period. Behavioral assessments, including the Y-maze and Morris water maze tests, were conducted after completion of the 60-day treatment protocol. Following behavioral testing, animals were anesthetized and brain tissues were harvested for molecular analyses. In-silico investigations were performed in parallel to identify potential drug–target interactions using protein–protein interaction (PPI) network analysis.

Molecular docking and network pharmacology: The study aimed to identify different signaling pathways and therapeutic targets linked to heavy metal-induced neurotoxicity and evaluate the potential mechanism of resveratrol as a neuroprotective candidate. Figure 1 outlines the complete process of network pharmacology approach carried out in this study.

Identification of drug-likeness properties of resveratrol: Prior to the target prediction, chemical structure and the "SMILES" notation of resveratrol were retrieved from the "PubChem"(https://pubchem.ncbi.nlm.nih.gov/) database [16] using the keyword

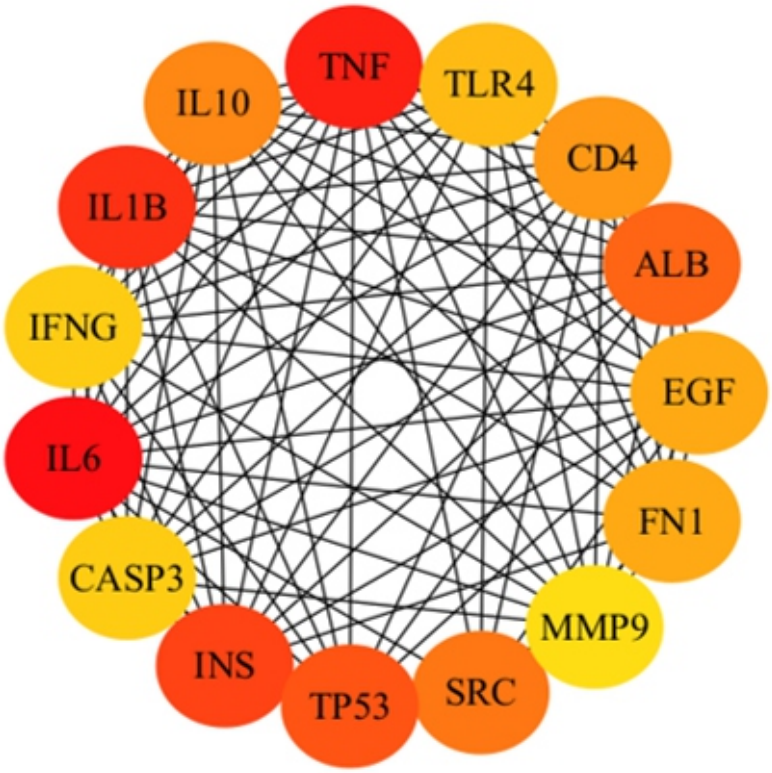

Figure 4: Top 15 hub genes of the PPI network of resveratrol targets against metal-induced (Al, Pb, As) neurotoxicity based on degree. The colors of the above shown nodes reflect the degree of connectivity. The redder/deeper color indicates a higher degree of connectivity.

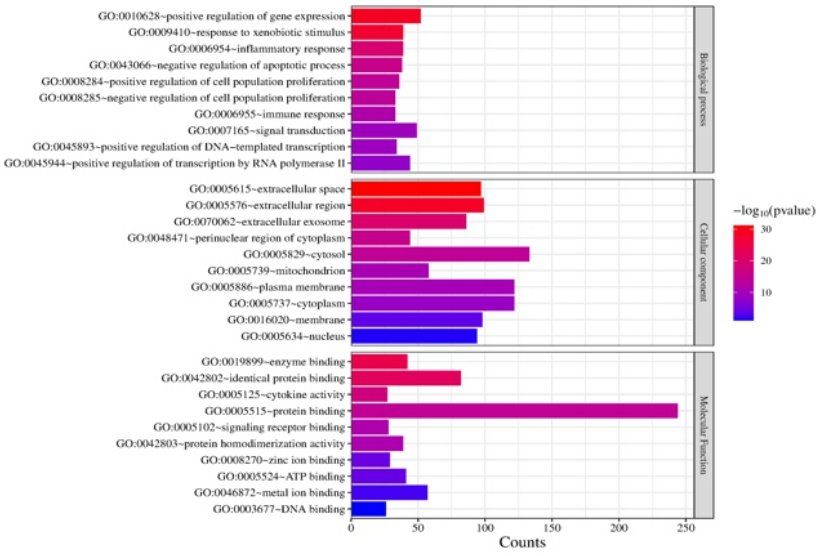

Figure 5: Bar plot of GO (Gene ontology) functional enrichment analysis.

"Resveratrol" in the search box. The molecular and therapeutic characteristic of resveratrol were also obtained from the "Traditional Chinese Medicine Systems Pharmacology Database (TCMSP)" (https://www.tcmsp-e.com/tcmsp.php) database.[17]

Screening and collection of potential targets of Resveratrol and Heavy Metal (Al, As, Pb)-induced neurotoxicity: To identify potential target proteins of resveratrol, data were extracted from three online databases: "Swiss Target Prediction" (http://www.swisstargetprediction.ch/),[18] "Comparative Toxicogenomics Database (CTD)" (http://ctdbase.org/),[19] and the "GeneCards" (https://www.genecards.org/),[23] Following the screening, the targets were obtained from all three databases and were then combined, repeated entries were deleted to develop a comprehensive dataset of resveratrol-related target genes for further analysis. To identify neurotoxicity-associated target genes for Al, As, and Pb, data were downloaded from "GeneCards" (https://www.genecards.org/) database.[20] The search was performed using the terms "Al-induced neurotoxicity," "As-induced neurotoxicity," and "Pb-induced neurotoxicity," with only Homo sapiens-associated proteins included in the final dataset.

Identification of drug-disease common target genes and PPI network construction: To identify shared target genes between resveratrol and Al, As, and Pb-induced neurotoxicity, "Venny 2.0.2" (https://bioinfogp.cnb.csic.es/tools/venny/) software was used. The overlapping genes were uploaded to "STRING (v12.0)" (https://string-db.org/),[21] A "protein-protein interaction (PPI)" network was then generated, specifying Homo sapiens as the species and applying a "confidence threshold" of greater than 0.700. The PPI network was further analyzed using "Cytoscape (v3.10.3) (https://cytoscape.org/)",[22] where the cytoHubba plug-in identified key hub genes based on node degree.

The functional enrichment (gene ontology and KEGG pathway) analysis: To investigate the intersecting genes' biological relevance, "GO" and "KEGG" pathway enrichment analysis were carried out using "DAVID (v6.8) (https://davidbioinformatics.nih.gov/)"[23] with a significance limit of $p < 0.05$. GO keywords were categorized into three sections: biological process (BP), cellular component (CC), and the molecular function (MF). The top 10 GO terms from each section, along with the top 15 KEGG pathways, were identified through enrichment analysis using "ShinyGO".[24] Visualization of the

results was carried out via "Bioinformatics.com.cn".

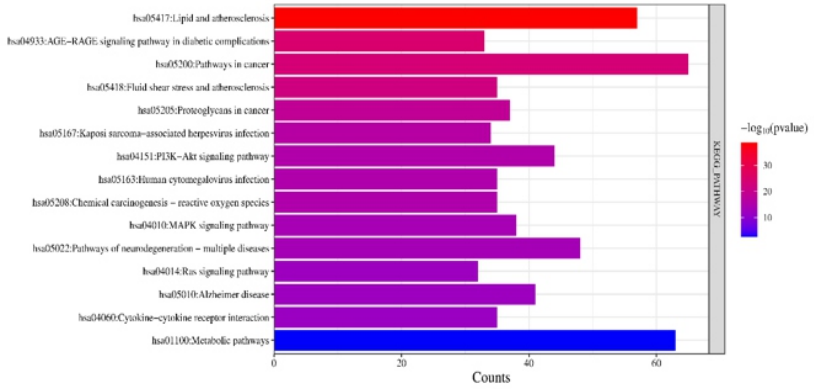

Figure 6: Bar plot of KEG pathway enrichment analysis.

Molecular docking analysis: To validate network pharmacology findings, molecular docking was performed to assess resveratrol's binding affinity with key target proteins related to its protective effects in metals neurotoxicity. "IL-6", "IL1-β" and "TNF", were selected as inflammatory markers, while "SYP", "CaMKIV" and "PSD-95" were chosen for their role in synaptic plasticity. The 3D model of resveratrol was obtained from PubChem and prepared using "Open Babel in PyRx (https://pyrx.sourceforge.io/)". The target proteins were then retrieved from online database "RCSB Protein Data Bank (PDB)" (https://www.rcsb.org/), refined by

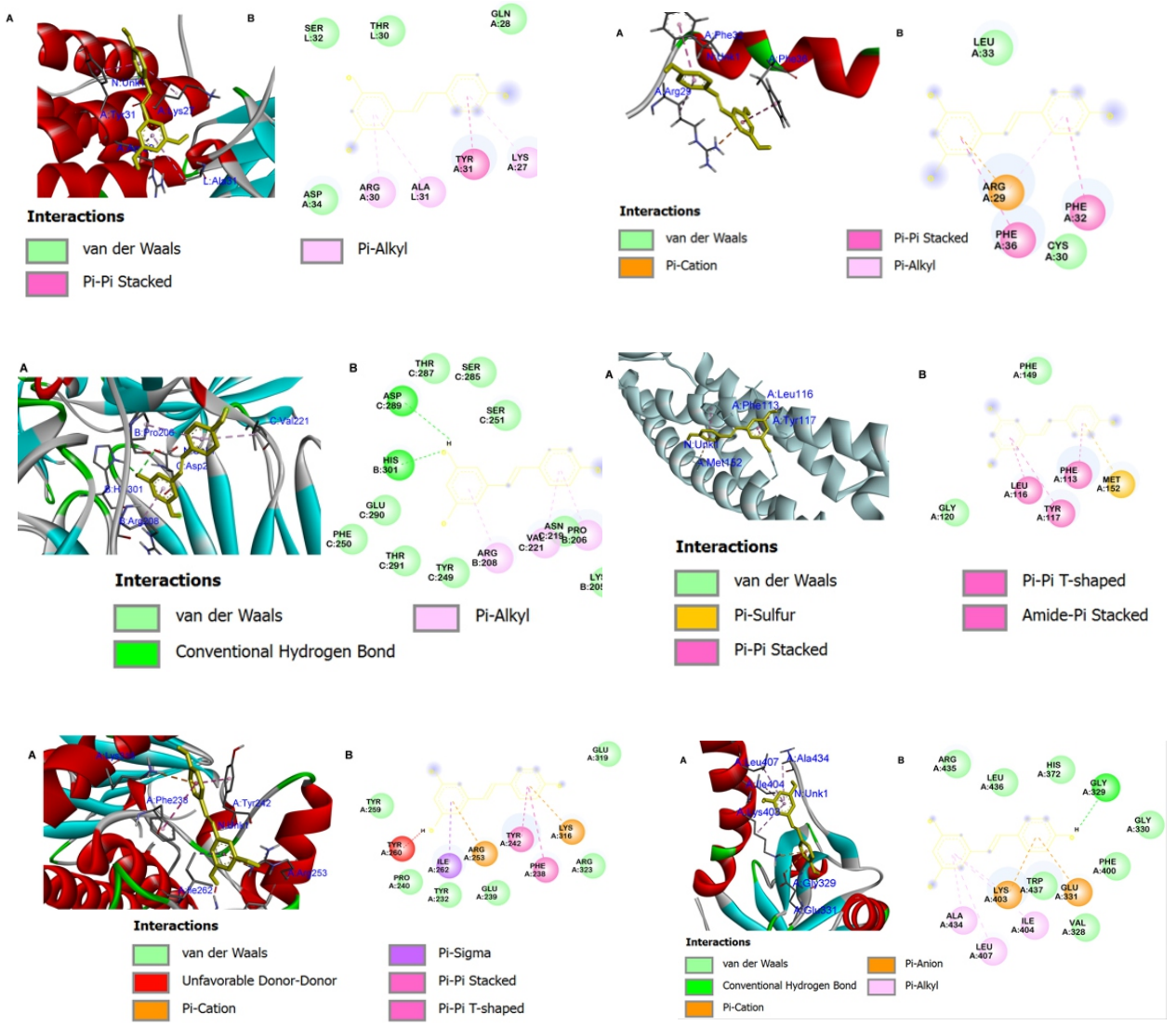

Figure 7: Molecular docking analysis of resveratrol with key target protein. The overall structure, 3D partial view and 2D binding mode of resveratrol with (**A**) IL6, (**B**) TNF, (**C**) IL1-β, (**D**) SYP, (**E**) CamKIV and (**F**) PSD-95.

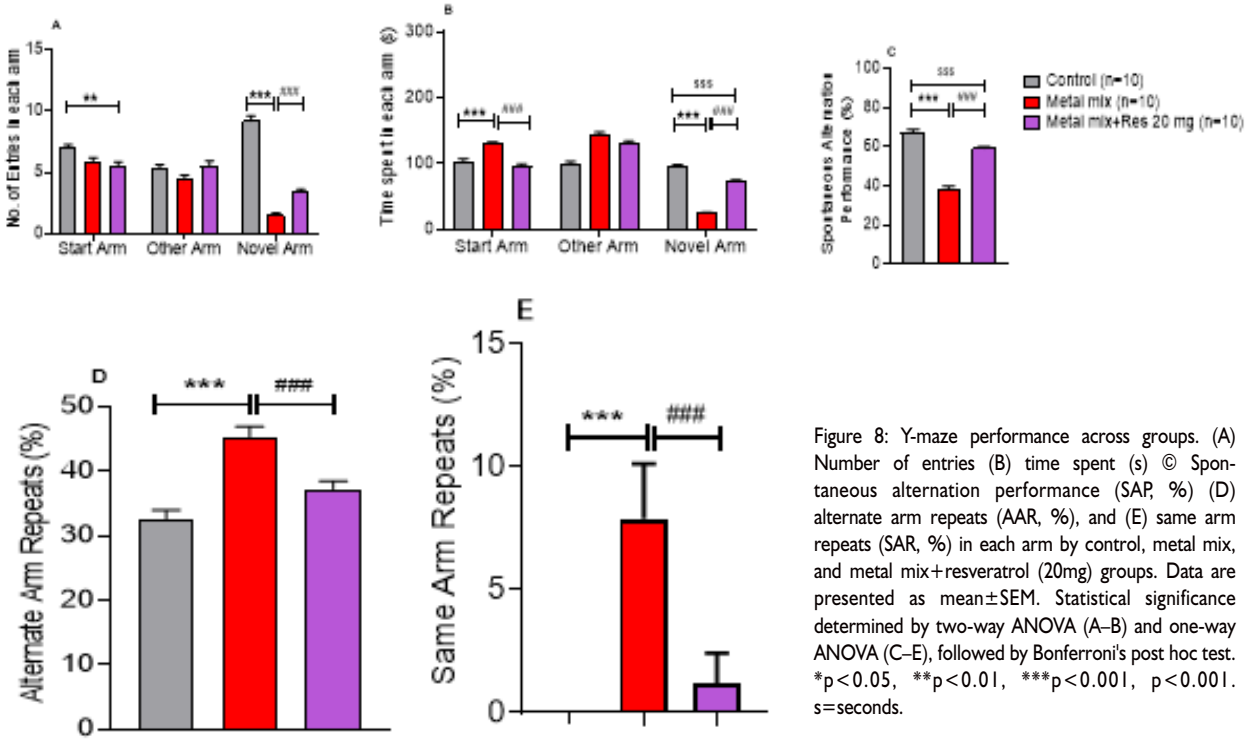

Figure 8: Y-maze performance across groups. (A) Number of entries (B) time spent (s) © Spontaneous alternation performance (SAP, %) (D) alternate arm repeats (AAR, %), and (E) same arm repeats (SAR, %) in each arm by control, metal mix, and metal mix+resveratrol (20mg) groups. Data are presented as mean±SEM. Statistical significance determined by two-way ANOVA (A–B) and one-way ANOVA (C–E), followed by Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001, p<0.001. s=seconds.

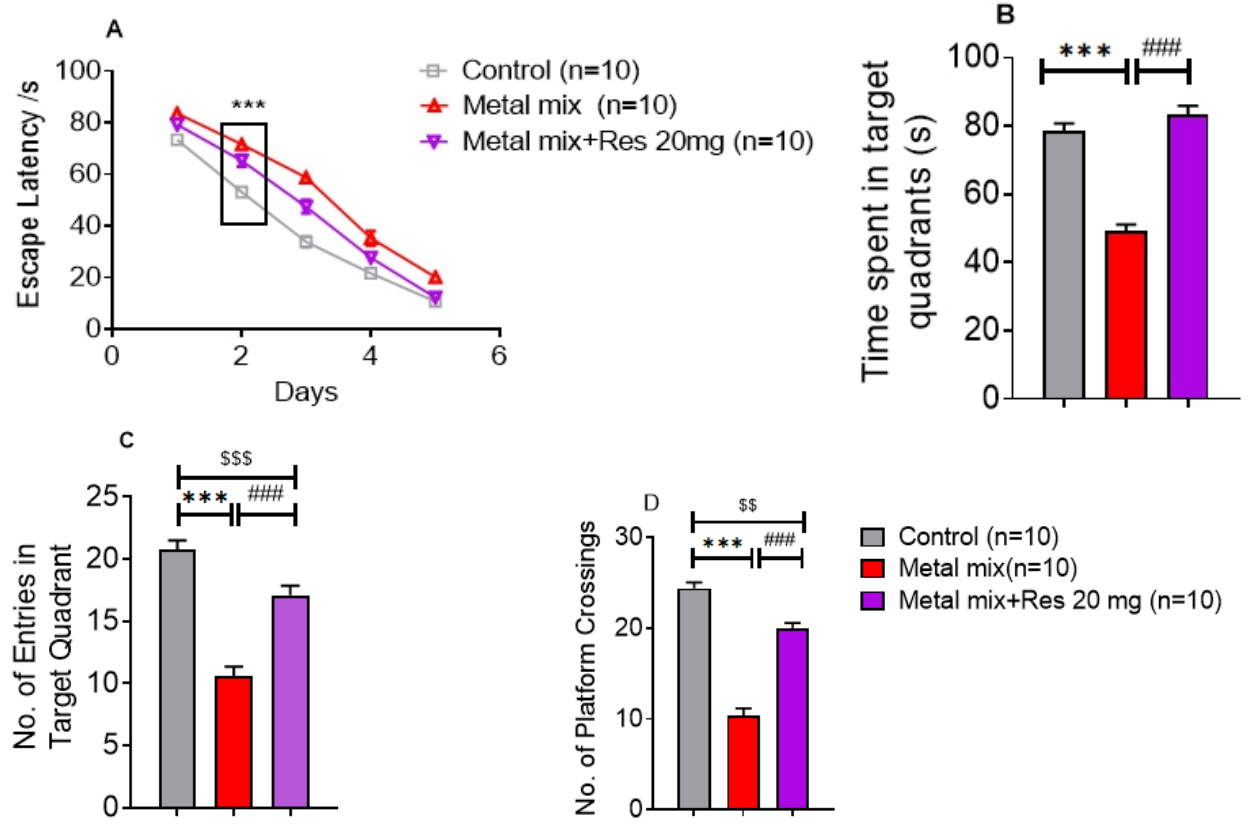

Figure 9: Effect of Resveratrol on learning and memory using Morris water maze test; training. (A)The graph demonstrates the escape latency (s) to assess the reference memory formation and learning among the control, Metal mix, Metal mix+Resveratrol (20mg). The bar charts depict (B) the time spent in the target quadrant (s), by all groups; (C) the number of entries in the target quadrant; (D) shows the number of platform crossings. The error bars are representatives of mean±SEM for One-way ANOVA, followed by Bonferroni's multiple comparison test with *** p<0.001, ###p<0.001, $$$p<0.001 and $$p<0.01. s=seconds.

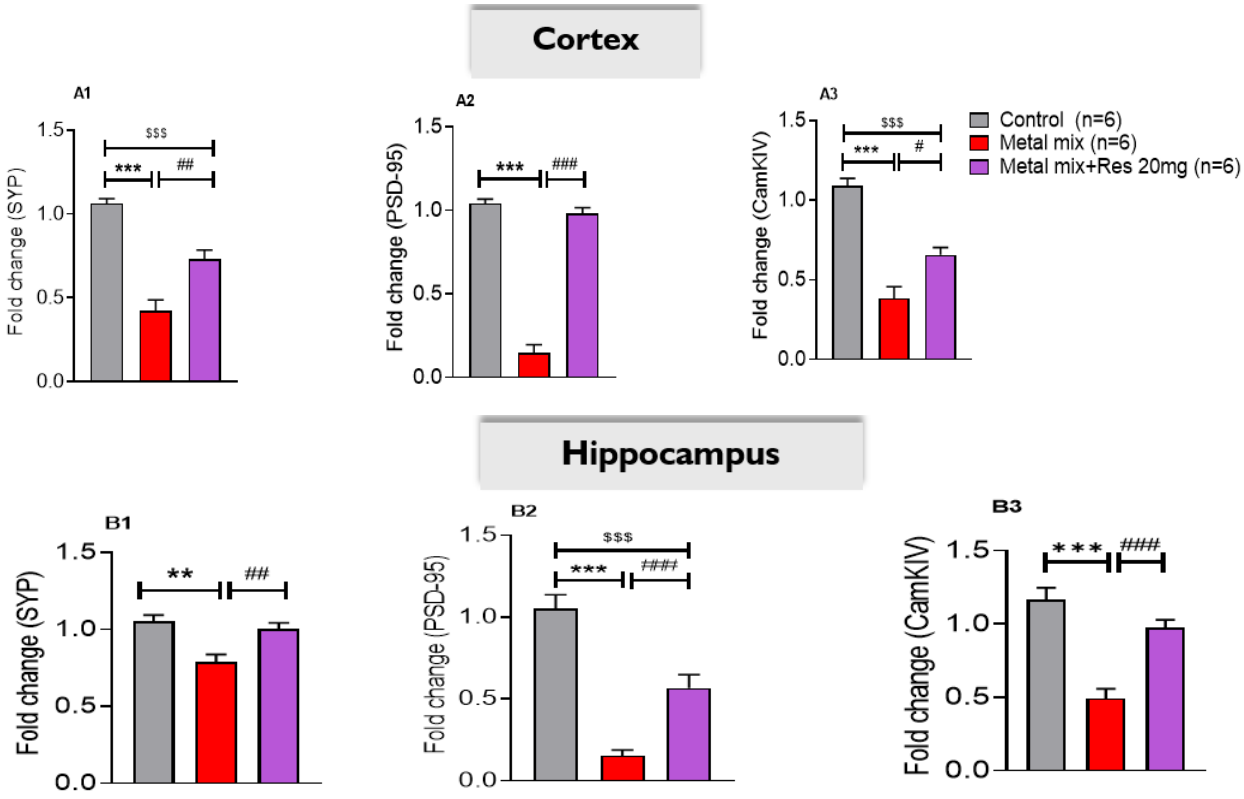

Figure 10: Effect of resveratrol on Synaptophysin (SYP) PSD-95, and CaMKIV expression in the cortex and hippocampus. (A1–A3) qPCR analysis showed significant downregulation of synaptophysin, PSD-95, and CaMKIV in the cortex of the metal mix group, which was reversed by resveratrol (20 mg/kg). (B1–B3) A similar pattern was observed in the hippocampus, where resveratrol significantly restored gene expression, indicating its neuroprotective effect. Data are expressed as mean±SEM; one-way ANOVA with Bonferroni post hoc test (***p<0.001, **p<0.01, ###p<0.001, ##p <0.01, #p <0.05), $$$p <0.001.

removing water molecules and hetatoms, and optimized with polar hydrogen and charge corrections. Docking simulations were conducted using "AutoDock Vina" (https://autodock.scripps.edu/),[25] and BIOVIA Discovery Studio (v25.1.0) was used to visualize receptor-ligand interactions.

Behavioral analysis

Morris water maze test: The Morris water maze test was performed as previously described, with minor modifications.[26] The test was used to assess spatial learning and memory. The apparatus consisted of a circular pool filled with water and a hidden platform submerged below the water surface. Rats underwent five training trials per day for five consecutive days, with each trial initiated from a different starting point while the platform location remained constant. The time required to locate the hidden platform, defined as escape latency, was recorded for each trial.

On day six, a probe trial was conducted with the platform removed. Memory retention was evaluated by recording the number of crossings over the former platform location, entries into the target quadrant, and the time spent in the target quadrant.

Y-Maze Test: The Y-maze test, a hippocampus-dependent paradigm, was used to assess working memory.[27] The apparatus consisted of three identical arms (50×10×20 cm) arranged at 120° angles. During the training phase, rats were placed in the start arm and allowed to explore two arms while access to the third (novel) arm was restricted. In the subsequent probe trial, all three arms were opened to evaluate memory performance based on preference for the novel arm. Outcome measures included time spent in each arm, total arm entries, and spontaneous alternation behavior, defined as consecutive entries into all three arms. An arm entry was recorded when all four limbs of the animal entered an arm.[28]

Gene expression analysis: Total RNA was extracted using the TRIzol reagent, and RNA integrity was confirmed by agarose gel electrophoresis, demonstrating two distinct ribosomal RNA bands.[29] RNA concentration and purity were assessed using NanoDrop spectrophotometry. Complementary DNA (cDNA) synthesis was performed using RNA quantities adjusted according to NanoDrop measurements, following the manufacturer's protocol (Thermo Scientific).

Briefly, RNA samples were incubated with 10 mM oligo(dT) primers and nuclease-free water at 65 °C for 5 minutes, after which 10 mM dNTPs, 5× reverse transcription buffer, and reverse transcriptase enzyme (Thermo Scientific) were added. cDNA synthesis was verified by conventional PCR using glyceraldehyde-3-phosphate

Table I: Real-time PCR primers with sequences

Gene	Sequence
GAPDH (F)	CCTGGCCAAGGTCATCCAT
GAPDH (R)	GTCATGAGCCCTTCCACGAT
Synaptophysin (F)	CATTCAGGCTGCACCAAGTG
Synaptophysin (R)	TGGTAGTGCCCCCTTTAACG
PSD-95 (F)	GGACATTCAGGCGCACAAG
PSD-95 (R)	TCCCGTAGAGGTGGCTGTTG
CaMKIV (F)	CGAAGATGCTCAAAGTCACGG
CaMKIV (R)	ACTCCACCTCGAAGAAATCGC

Table II: Pharmacokinetics and molecular properties of Resveratrol

Name	Resveratrol
Molecular formula	$C_{14}H_{12}O_3$
Molecular weight	228.26
Hdon	3
Hacc	3
Rbon	2
BBB	-0.01
DL	0.11
TPSA	60.69
Lipinski	Yes
Caco-2 permeability	0.80
AlogP	3.01
OB (%)	19.07
FASA	0.49
Gastrointestinal absorption	High
Log Kp (skin permeation)	-5.47cm/s
BBB permeant	Yes

Hdon and Hacc: molecule's capacity for hydrogen bonding, representing the number of potential hydrogen bond donors & acceptors, respectively. Rbon rotatable bonds; BBB for blood-brain barrier permeability; DL drug-likeness; TPSA topological polar surface area reflects molecular polarity; AlogP partition coefficient between octanol & water; OB oral bioavailability; FASA formulation, administration, stability, and availability

dehydrogenase (GAPDH) as the housekeeping gene.

Quantitative real-time PCR was performed using an ABI Prism 7300 system (Applied Biosystems). Each 20 μL reaction mixture contained Maxima SYBR Green Master Mix and gene-specific forward and reverse primers (Table I). Relative gene expression was calculated using livak method $\Delta\Delta CT$.[30]

Statistical analysis: Statistical analyses were conducted using GraphPad Prism version 7.0. Data normality was assessed with the Shapiro-Wilk test ($n = 10$ per group) and further confirmed through visual inspection of histograms and Q-Q plots. As the data met the assumptions of normality, parametric tests were employed. Behavioral outcomes (Morris water maze and Y-maze tests) were analyzed using two-way repeated-measures ANOVA followed by Bonferroni post hoc comparisons. Molecular expression analysis data were assessed using one-way ANOVA. Results are expressed as mean±SEM, with statistical significance defined as $p < 0.05$.

RESULTS

Network pharmacology and molecular docking analysis

Pharmacokinetics and molecular properties of Resveratrol: The "SMILES(C1=CC(=CC=C1/C=C/C2=CC(=CC(=C2)O)O)O)" and chemical structure of resveratrol downloaded from PubChem are shown in Figure 2 and important molecular features such as the molecular weight, molecular formula, oral bioavailability, blood-brain barrier permeability of resveratrol are shown in Table II. The Lipinski Rule of Five (RO5) assesses pharmacokinetic properties based on the molecular properties of a drug. Our results showed that resveratrol complies with RO5, with a bioavailability score of 0.55 and high gastrointestinal absorption, indicating good drug-likeness without any violations.

Target information of Resveratrol and Al, As, and Pb-induced neurotoxicity: A total of 7537 unique targets of resveratrol were identified from 3 databases, including 100 from Swiss Target Prediction, 882 from GeneCards, 7274 from CTD after the removal of duplicate targets. Moreover, 514 Al-induced neurotoxicity targets, 1054 As-induced neurotoxicity targets, and 2669 Pb-induced neurotoxicity targets were attained from the "GeneCards database".

Identification of therapeutic targets of Resveratrol and Al, As, and Pb-

Table III: Topological analysis results of 15 hub genes from PPI network of resveratrol against Al, AS and Pb- induced neurotoxicity

Rank	Gene symbol	Name	Uniprot ID	Degree
1	IL-6	Interleukin-6	P05231	87
2	TNF	Tumor necrosis factor	P01375	81
3	IL1-β	Interleukin-1 beta	P01584	80
4	INS	Insulin	P01308	70
5	Tp53	Tumor protein P53	P04637	68
6	ALB	Albumin	P02768	63
7	SRC	SRC proto-oncogene	P12931	62
8	Il10	Interleukin 10	P22301	61
9	Cd4	Cd4 molecule	P01730	57
10	EGF	Epidermal growth factor	P01133	55
11	Fn1	Fibronectin 1	P02751	55
12	TLR4	Toll-like receptor 4	O00206	54
13	CASP3	Caspase 3	P42574	53
14	IFNG	Interferon-gamma	P01579	53
15	MMP9	Matrix metallopeptidase 9	P14780	51

induced neurotoxicity and PPI network construction: Resveratrol targets overlapped with the metal (Al, As, Pb) induced neurotoxicity targets to obtain potential therapeutic gene targets for resveratrol against neurotoxicity. A total set of 269 intersected targets was then identified (Figure 3(A) and was used to create a PPI network (Figure 3 (B). The resulting network has 269 nodes and 2240 edges with a node degree of 16.7 (average) and a PPI enrichment p-value < 1.0e-16. The connectivity between nodes is good, which indicates that resveratrol may help in mitigating metal-induced neurotoxicity through multi-target regulation.

The core targets were identified based on the degree and their network is shown in Figure 4. The 15 highest-ranked targets were identified based on their weighted degree value was IL6, TNF, IL1-β, INS, TP53, ALB, SRC, IL10, CD4, EGF, FN1, TLR4, CASP3, IFNG and MMP9 respectively and results are shown in Table III.

GO and KEGG pathway analysis: A bar chart was used to visualize the GO enrichment findings, (Figure 5) presents the top 10 functions, with a p-value >0.05 as well as counts related to BP, CC and MF, respectively (Table IV). The GO enrichment finding revealed that target genes of resveratrol were associated with some biological processes such as gene expression regulation, inflammatory response, immune response, negative regulation of apoptotic process, signal transduction and others. The most relevant cellular components include extracellular space, plasma membrane, cytosol, mitochondrion and others. The most pertinent molecular functions are enzyme binding, ATP binding, identical protein binding, DNA binding and others. The KEGG pathway enrichment analysis revealed that the target genes of resveratrol against Al, As, and Pb-induced neurotoxicity were primarily associated with 203 signaling pathways. The top 15 pathways were selected based on p-value >0.05 and were shown in the bar graph (Figure 6) and their p-value and count information were shown in Table IV. The study found that the most important routes of resveratrol against metal-induced neurotoxicity were neurodegeneration, PI3/AKT signaling, cancer, MAPK signaling, lipid and atherosclerosis, and Ras signaling.

Molecular docking studies of resveratrol: The docking interaction of resveratrol with IL-6, TNF, IL1-β, SYP, CaMKIV and PSD-95 yielded binding energies. Lower binding energies indicate higher binding affinity and stronger interaction between receptor and ligand. The results of the docking interaction of resveratrol with these proteins ranged from -5.5-8.2 kcal/mol as shown in Table V. Generally, binding energy values >-5kcal/mol indicate no predicted binding, values <-5kcal/mol indicate moderate predicted binding and values <-7kcal/mol indicate strong predicted binding.[31] Resveratrol showed highest binding affinity with CaMKIV (-8.1 kcal/mol), other proteins showed moderate binding affinity with resveratrol: IL-6 (-6.8kcal/mol), TNF (-5.7kcal/mol), IL1-β (-6.9kcal/mol), SYP (-5.6kcal/mol) and PSD-95 (-6.5) respectively, which indicates that resveratrol was centrally located inside the binding site of these proteins. The molecular docking analysis of resveratrol with key target protein in terms of overall structure, 2D binding mode and 3D partial view of resveratrol with IL6 (A), TNF (B), IL1-β (C), SYP (D), CaMKIV (E) and PSD-95 (F) respectively (Figure 7).

Cognitive enhancing effects of resveratrol

Y-Maze test: To assess the effects of resveratrol on learning and memory in male rats, a Y-maze test was performed. Rats exposed to a metal mix demonstrated impaired short-term spatial memory, evidenced by reduced entries (1.5±0.2) and time spent (24.6±1.2sec) in the novel arm compared to controls (entries: 9.2±0.4; time: 96.7±1.6sec). Resveratrol treatment (20mg) significantly improved performance, with increased novel arm entries (3.4±0.2) and time spent (73.0±1.8sec) relative to the metal mix group (Figure 8A, 8B). The spontaneous

Table IV: Top 10 GO enrichment analysis of intersected target genes of resveratrol against Al, As and Pb-induced neurotoxicity

Variables	GO term ID	Biological process	Count	p-value
Biological Process	GO:0010628	Positive regulation of gene expression	52	1.78E-29
	GO:0007165	Signal transduction	49	6.57E-10
	GO:0045944	Positive regulation of transcription by RNA polymerase II	44	2.35E-08
	GO:0009410	Response to xenobiotic stimulus	39	4.03E-28
	GO:0006954	Inflammatory response	39	3.13E-20
	GO:0043066	Negative regulation of apoptotic process	38	8.29E-17
	GO:0008284	Positive regulation of cell population proliferation	36	5.21E-15
	GO:0045893	Positive regulation of DNA-templated transcription	34	1.39E-09
	GO:0008285	Negative regulation of cell population proliferation	33	1.16E-14
	GO:0006955	Immune response	33	2.40E-12
Cellular Components	GO:0005829	Cytosol	133	4.86E-15
	GO:0005886	Plasma membrane	122	6.27E-11
	GO:0005737	Cytoplasm	122	3.96E-09
	GO:0005576	Extracellular region	99	4.89E-29
	GO:0016020	Membrane	98	2.80E-04
	GO:0005615	Extracellular space	97	6.75E-32
	GO:0005634	Nucleus	94	0.042722
	GO:0070062	Extracellular exosome	86	9.35E-21
	GO:0005739	Mitochondrion	58	2.60E-11
	GO:0048471	Perinuclear region of cytoplasm	44	2.26E-16
Molecular Functions	GO:0005515	Protein binding	244	3.90E-15
	GO:0042802	Identical protein binding	82	9.89E-23
	GO:0046872	Metal ion binding	57	0.004476
	GO:0019899	Enzyme binding	42	6.09E-25
	GO:0005524	ATP binding	41	1.04E-04
	GO:0042803	Protein homodimerization activity	39	6.26E-12
	GO:0008270	Zinc ion binding	29	5.62E-05
	GO:0005102	Signaling receptor binding	28	5.40E-12
	GO:0005125	Cytokine activity	27	1.97E-18
	GO:0003677	DNA binding	26	0.093093

alternation test indicated severe memory impairment in the metal mix group (38.5±1.2) versus controls (66.9±1.8) and resveratrol-treated rats (59.2±0.6; $p<0.001$; Figure 8C). Elevated alternate arm repeats (45.4±1.5) and same arm repeats (7.9±0.7) in the metal mix group reflected cognitive deficits. Resveratrol treatment reduced both parameters (37.3±1.2 and 1.2±0.3, respectively), approaching control levels and indicating improved memory performance (Figures. 8D, 8E).

Morris water maze test: Two-way repeated-measures ANOVA indicated that all groups demonstrated progressive improvement in locating the hidden platform across training days. However, the metal mix group exhibited significantly impaired learning, as evidenced by a higher mean escape latency on the final day (20.1±0.9 sec) compared to the control group (10.7± 0.9 sec; Figure 9A). In contrast, resveratrol treatment significantly improved performance, with reduced latencies of 12.2±0.6 sec, indicating enhanced spatial memory acquisition.

In the probe trial, the control group spent significantly more time in the target quadrant (78.5±2.2 sec) than the metal mix group (49.4±1.7 sec), reflecting superior memory retention. Resveratrol-treated rats showed substantial improvement, spending 83.4±2.5 sec in the target quadrant ($p<0.001$; Figure 9B). Furthermore, resveratrol administration significantly increased the number of entries into the target quadrant (Figure 9C) and platform crossings (Figure 9D), reinforcing its cognitive-enhancing effects.

Molecular expression analysis in hippocampus and cortex after resveratrol treatment: Gene expression analysis revealed significant downregulation of synaptophysin in the cortex of the metal mix group (0.42±0.06) compared to controls (1.06±0.02; $p<0.001$). Resveratrol treatment (20mg/kg) partially restored expression (0.73±0.05; $p<0.01$), indicating its neuroprotective potential (Figure 10A1). Similar patterns were

continued...

Variables	GO term ID	Biological process	Count	p-value
KEGG Pathways	hsa05200	Pathways in cancer	65	0.002411
	hsa01100	Metabolic pathways	63	0.002411
	hsa05417	Lipid and atherosclerosis	57	2.02E-39
	hsa05022	Pathways of neurodegeneration - multiple diseases	48	3.34E-14
	hsa04151	PI3K-Akt signaling pathway	44	4.30E-16
	hsa05010	Alzheimer disease	41	7.13E-13
	hsa04010	MAPK signaling pathway	38	1.72E-14
	hsa05205	Proteoglycans in cancer	37	2.70E-19
	hsa05418	Fluid shear stress and atherosclerosis	35	7.53E-23
	hsa05163	Human cytomegalovirus infection	35	5.69E-16
	hsa05208	Chemical carcinogenesis - reactive oxygen species	35	6.37E-16
	hsa04060	Cytokine-cytokine receptor interaction	35	2.13E-12
	hsa05167	Kaposi sarcoma-associated herpesvirus infection	34	4.10E-17
	hsa04933	AGE-RAGE signaling pathway in diabetic complications	33	1.26E-25
	hsa04014	Ras signaling pathway	32	6.39E-13

DNA: Deoxyribonucelic Acid; RNA: Ribonucelic Acid; ATP: Adenosine Triphosphate; KEGG: Kyoto Encyclopedia of Genes and Genomes; Ras: Rat Sarcoma Virus

Table V: The docking scores of Resveratrol with the key target proteins

Drug	Target	PDB ID	Binding energy (kcal/mol)
Resveratrol	Il-6	4O9H	-6.8
	TNF	7ASY	-5.7
	IL1-β	4DEP	-6.9
	SYP	N/A (AF-P08247-F1-v4)	-5.6
	CaMKIV	2W40	-8.1
	PSD-95	7CQF	-6.5

IL-6: Interleukin-6; TNF: Tumor Necrosis Factor; ILI-β: Interleukin-1 beta; SYP: Synaptophysin; CaMKIV: Calcium/calmodulin-dependent Protein Kinase IV; PSD-95: Post-synaptic Density Protein

observed in the hippocampus, where synaptophysin expression was reduced in the metal mix group (0.78±0.04 vs 1.05±0.03 in controls; $p<0.01$). Resveratrol restored hippocampal expression to near-control levels (1.00±0.03; $p<0.01$; Figure 10B1). PSD-95 expression was significantly reduced in the cortex (0.14±0.04) and hippocampus (0.15±0.03) of the metal mix group compared to controls (cortex: 1.04±0.02; hippocampus: 1.05±0.03; $p<0.001$), indicating metal-induced neuronal damage (Figures 10A2, 10B2). Resveratrol treatment restored PSD-95 levels in both regions (cortex: 0.98±0.03; hippocampus: 0.56±0.08; $p<0.001$), demonstrating its rescuing effect. CaMKIV expression was significantly downregulated in the cortex (0.38±0.07) and hippocampus (0.49±0.06) of the metal mix group compared to controls (cortex: 1.09±0.04; hippocampus: 1.16±0.08; $p<0.001$), indicating neuronal impairment (Figures 10A3, 10B3). Resveratrol treatment improved CaMKIV expression (cortex: 0.65±0.04; hippocampus: 0.97±0.05), suggesting a protective effect.

DISCUSSION

This study provides integrated in vivo and in silico evidence that resveratrol exerts significant neuroprotective effects against combined aluminum-, arsenic-, and lead-induced neurotoxicity. Using a preventive exposure model, resveratrol mitigated metal-induced cognitive impairment, restored synaptic marker expression, and interacted with key molecular targets involved in inflammation and synaptic plasticity. Resveratrol has been widely recognized for its ability to mitigate oxidative damage, restore synaptic protein expression, and regulate metabolic pathways.[32] Resveratrol shows strong neur-oprotective potential but faces pharmacokinetic challenges. While it meets Lipinski's Rule of Five for drug-likeness, its oral bioavailability is low (~0.5%) due to rapid hepatic metabolism, leading to a short half-life (8–14 minutes).[33] Despite this, its longer-lasting metabolites and therapeutic promise have spurred interest in improved delivery strategies to enhance bioavailability.[34] Network pharmacology analysis revealed 269 potential therapeutic targets of resveratrol relevant to Al, As, and Pb-induced neurotoxicity. Key targets included inflammatory mediators such as "interleukin-6 (IL-6)", "tumor necrosis factor-alpha (TNF-α)", "interleukin-1 beta (IL-1β)", as well as apoptotic regulators like tumor protein p53 (TP53). A protein-protein interaction (PPI) network further illustrated that resveratrol's effects are mediated through multiple interconnected pathways, reinforcing its role as a multi-target therapeutic agent.

Molecular docking revealed that resveratrol has the highest binding affinity with CaMKIV (-8.1 kcal/mol), a

key regulator of synaptic plasticity and memory, suggesting its role in enhancing neurocognitive effects.[35] It also showed moderate affinity for pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, supporting its anti-inflammatory effects. Additionally, resveratrol bound moderately to synaptic proteins PSD-95 and synaptophysin, indicating its potential to preserve synaptic integrity and function under heavy metal-induced neurotoxicity as studied earlier.[36] Gene Ontology and KEGG pathway analysis revealed that resveratrol targets genes involved in regulating inflammation, apoptosis, and gene expression. Key pathways include PI3K/Akt and MAPK, which are crucial for neuronal survival and synaptic plasticity.[37] Positive modulation and rescuing effects shown on these pathways suggest resveratrol's potential to counteract heavy metal-induced neurotoxicity which is in line with previous studies.[38]

In this study, heavy metal exposure impaired spatial working memory, evidenced by reduced spontaneous alternation performance (SAP) and increased same-arm returns (SAR) and alternate-arm returns (AAR) in the Y maze test. Resveratrol treatment (20 mg/kg) significantly improved the memory, increasing novel arm entries and time spent, indicating its neuroprotective effect against heavy metal-induced cognitive deficits consistent with previous studies.[28] The Morris water maze test also demonstrated impaired long-term memory in the metal mix group, which was reversed by resveratrol treatment, as evidenced by reduced escape latencies and increased time spent in the target quadrant findings consistent with previous studies.[39]

Exposure to heavy metals has been shown to downregulate critical synaptic genes, including synaptophysin, PSD-95, and CaMKIV, leading to impaired synaptic function and cognitive deficits.[40,41] Resveratrol treatment significantly upregulated the expression of these proteins in both the cortex and hippocampus, restoring them to levels comparable to the control group. These findings suggest that resveratrol exerts neuroprotective effects by preserving synaptic integrity and function.

Our study clearly demonstrates that the neuroprotective action of resveratrol may be attributed to its antioxidative and anti-inflammatory properties, which counteract the oxidative stress and inflammation induced by heavy metal exposure. Remarkably, our study has shown that resveratrol has neuroprotective effects through multiple pathways, suggesting its therapeutic potential in metals induced neurotoxicity. Further studies are required to elaborate its in-depth mechanism in multiple in vivo models.

Limitations of the study

The duration of metal exposure in this study was limited to 60 days; extending the exposure period may provide deeper insight into the long-term neurotoxic effects of combined heavy metals and the sustained therapeutic potential of resveratrol. Future studies should also assess blood–brain barrier permeability and investigate miRNA-mediated regulatory networks to further elucidate underlying mechanisms. In addition, advanced drug-delivery strategies such as nanocarriers, liposomes, and prodrug formulations may enhance the bioavailability and pharmacokinetic profile of resveratrol. To strengthen mechanistic validation of the in-silico findings, molecular dynamics simulations using platforms such as GROningen MAchine for Chemical Simulations (GROMACS) or Assisted Model Building with Energy Refinement (AMBER) are recommended as complementary approaches to molecular docking analyses.

CONCLUSION

The result of this study emphasizes the therapeutic effect of resveratrol in mitigating heavy metal-induced neurotoxicity. By preserving synaptic proteins and key signaling molecules, resveratrol may help maintain synaptic integrity and cognitive function in the face of environmental neurotoxic challenges. However, further clinical investigations are required to assess its efficacy, safety, and long-term benefits in humans.

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AUTHORS' CONTRIBUTION

The Following authors have made substantial contributions to the manuscript as under:

TA: Conception, acquisition, analysis and interpretation of data, drafting the manuscript, critical review, approval of the final version to be published

AZ & SB: Study design, acquisition, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

AT: Study design, drafting the manuscript, approval of the final version to be published

SZ: Acquisition, analysis and interpretation of data, drafting the manuscript, critical review, approval of the final version to be published

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declared no conflict of interest, whether financial or otherwise, that could influence the integrity, objectivity, or validity of their research work.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

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