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AN IN SILICO NETWORK PHARMACOLOGY AND MOLECULAR DYNAMICS SIMULATIONS STUDY OF ENGELETIN IN ISCHEMIC STROKE WITH COMPUTATIONAL PRIORITISATION OF NOS2

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ABSTRACT

Background: Engeletin, a polyphenolic flavonoid, has demonstrated neuroprotective effects in experimental stroke models. However, the molecular interactome underlying its multitarget actions in ischemic stroke remains insufficiently characterized. **Methodology:** An integrated in silico workflow was applied to identify stroke-relevant targets of engeletin, combining target prediction, protein–protein interaction analysis, GO and KEGG pathway enrichment, and engeletin–target–pathway network construction. Network topology metrics were used to prioritize targets for downstream structure-based analyses. Docking was performed on short-listed targets, and selected protein–ligand complexes were further evaluated using molecular dynamics (MD) simulations to assess binding stability. In silico ADME profiling was conducted to contextualize translational considerations. **Results and Discussion:** Nineteen targets were identified through a confidence-driven overlap strategy. Degree-based network filtering short-listed 11 targets for docking. Multi-centrality convergence across protein–protein interaction and engeletin–target–pathway networks prioritized six influential hubs (PTGS2, CASP3, NOS2, MMP9, JAK2, and EGFR) implicated in inflammatory, apoptotic, and vascular regulation. Functional enrichment analyses highlighted interconnected inflammatory–immune, vascular, and metabolic stress pathways, with KEGG pathways interpreted as nominally enriched. Docking and MD analyses differentiated dynamically stable interactions from network-level co-modulated hubs, with engeletin exhibiting the most stable binding to NOS2 (inducible nitric oxide synthase). **Conclusion:** Integration of network pharmacology with structure-based analyses prioritizes NOS2-centered modulation and relaxin-associated vascular signaling as testable mechanisms for future experimental validation of engeletin in ischemic stroke.

INTRODUCTION

Network pharmacology is an interdisciplinary framework that combines systems biology, chemoinformatics, and computational pharmacology to elucidate complex therapeutic mechanisms [1, 2]. Its rise mirrors a broader shift toward hybrid computational-experimental strategies in drug discovery, aimed

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at identifying novel or understudied molecular endpoints often overlooked by traditional single-target approaches [3]. While not a substitute for experimental validation, network-based approaches increasingly shape early-stage discovery by prioritizing targets with greater translational potential and minimizing reliance on broad, resource-intensive preclinical screens that often lack mechanistic specificity [2]. Though originally developed to study synergistic mechanisms in polyherbal formulations, network pharmacology is now increasingly applied to individual phytochemicals with multitarget actions in complex diseases [1]. Such compounds often act on multiple signaling axes, making them particularly relevant to multifactorial disorders such as ischemic stroke, where therapeutic success hinges on modulating interconnected processes including excitotoxicity, oxidative stress, neuroinflammation, blood-brain barrier (BBB) breakdown, and apoptosis [4, 5]. Despite this, there remains a pressing need for safe, multitarget neuroprotective agents that can complement reperfusion therapies and improve long-term outcomes [6].

Phytochemicals, particularly polyphenols with an inherent polypharmacology, exhibit pleiotropic effects on oxidative stress, inflammation, apoptosis, and vascular integrity via diverse signaling pathways [7]. Among such candidates, engeletin, present in Smilax glabra, has recently attracted attention for its therapeutic potential in several inflammatory and neurodegenerative disorders [8]. Emerging preclinical evidence suggests engeletin exerts neuroprotection in stroke by modulating angiogenic and neuroinflammatory pathways [9, 10]. As reviewed by Gomez-Verjan *et al.* (2023), network pharmacology helped elucidate the neuroprotective mechanisms of several bioactive compounds, including cordycepin, salidroside, curcumin, baicalin, resveratrol, cannabidiol, icariin, ginkgolides, and fucose, even in the absence of new *in vivo* validation [7].

Recent studies on ischemic stroke increasingly employ integrated computational pipelines that combine network pharmacology with structure-based validation to interrogate the mechanisms of individual bioactive compounds, including luteolin, irisflorentin, isoliquiritigenin, and marein [11-14]. They employed network-guided target identification followed by molecular docking and, in some cases, molecular dynamics simulations to evaluate compound–target interactions. While collectively underscoring the growing acceptance of network pharmacology-assisted structural analysis in stroke research, these studies differ in methodological emphasis, target selection criteria, and prioritization strategies, reflecting layered approaches that refine multi-target predictions into mechanistically prioritized candidates and strengthen hypothesis generation before experimental validation. Therefore, building on recent computational work on engeletin in ischemic stroke [15], the present study designed a multilayered approach to explicitly emphasize target prioritization through integrated systems-level, network-guided reduction to a compact hub set, followed by molecular dynamics-based stability assessment to rank mechanistically plausible candidates. This highlighted NOS2 as a primary interaction of interest. At the same time, multiple key hubs were identified as network-level co-modulated nodes, thereby establishing a systems-level foundation for subsequent in vivo validation.

MATERIALS AND METHODS

Target Prediction for Engeletin

Putative targets of engeletin were predicted using three online target prediction web servers (accessed between June and August 2022): PharmMapper (http://www.lilab-ecust.cn /pharmmapper/submitfile.html), ChemMapper (http://www.lilab-ecust.cn/chemmapper/index.html), and SwissTargetPrediction (http://www.swisstargetprediction.ch/). PharmMapper employed pharmacophore-based screening (max 1000 hits), and UniProt IDs were retrieved [16]. ChemMapper predictions used SHAFTS-based 3D similarity (score ≥1.2), with redundancies removed [17]. SwissTargetPrediction integrated 2D and 3D similarity algorithms, and the top 100 ranked targets were selected [18]. Analyses were restricted to Homo sapiens proteins, and two refined target sets were generated: a non-redundant union of all predicted targets and another representing shared targets across all three platforms.

Compilation of Stroke-Associated Targets

Stroke-related genes were retrieved from three curated disease-gene association databases (accessed between June and August 2022): DisGeNET (http://www.disgenet.org/), OMIM (Online Mendelian Inheritance in Man; (https://omim.org/), and GeneCards (https://genecards.weizmann.ac.il/v3/), using the keywords “ischemic stroke” and “cerebral ischemia”, restricted to Homo sapiens genes [19-21]. All retrieved entries were standardized to UniProt identifiers. A unified target set was generated by merging outputs from all platforms, while a secondary subset retained only targets consistently identified across all three sources.

Identification of Therapeutic Targets of Engeletin for Stroke

To enhance target specificity, a two-tier Venn strategy was employed. First, engeletin targets shared across all three prediction platforms were intersected with the merged stroke target set. Separately, the complete engeletin target pool was intersected against stroke targets common to all three disease databases. Targets from both intersection sets were combined to yield a unified list of putative therapeutic targets for downstream network and enrichment analyses.

Protein-Protein Interaction (PPI) Network Construction

The finalized therapeutic targets were submitted to the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (https://string-db.org/; accessed during June-August 2022; confidence score ≥0.4; species: Homo sapiens) to construct a protein-protein interaction (PPI) network [22]. The network was then imported into Cytoscape v3.9.1 for topological assessment using the NetworkAnalyzer tool [23]. All proteins exhibiting degree values above the network mean were short-listed for docking analysis. Consistently high rankings across degree, betweenness, and closeness centrality were prioritized as the most influential hub proteins for downstream mechanistic interpretation.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

Functional enrichment analysis of engeletin-associated stroke targets was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.8 (https://david.ncifcrf.gov/tools.jsp; accessed during June–August 2022; species background: Homo sapiens) [24]. All targets were standardized to official gene symbols before submission. A two-tier enrichment strategy was applied to enable comprehensive detection of enriched terms (GO: raw $p \leq 0.01$; KEGG: raw $p \leq 0.05$) while prioritizing statistically robust signals using Benjamini–Hochberg false discovery rate (FDR) correction, with an adjusted p-value threshold of ≤ 0.05. Gene Ontology (GO) enrichment analysis was performed for the Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) categories. GO terms meeting the FDR-adjusted significance threshold (≤ 0.05) were prioritized for downstream interpretation. In contrast, terms with raw p-values ≤ 0.01 that did not remain significant after multiple-testing correction (FDR) were considered nominally enriched, retained for reporting, and treated as exploratory. GO enrichment results were summarised by term name, gene count, raw p-value, and associated significance statistics. KEGG pathway enrichment was performed similarly, whereby pathways meeting the raw p-value threshold were considered nominally enriched, and FDR-adjusted p-values were used to prioritize statistically robust pathway signals. KEGG enrichment outputs were summarised by pathway name, gene count, rich factor (ratio of input genes mapped to a pathway relative to the total number of genes annotated in that pathway), raw p-value, and corresponding FDR.

Engeletin-Target-Pathway (ETP) Network Construction

A tripartite engeletin-target-pathway (ETP) network connecting engeletin, its predicted stroke-relevant targets, and enriched KEGG pathways was constructed using Cytoscape v3.9.1 [23]. Network topology parameters (centrality measures: degree, betweenness, and closeness) were calculated using the NetworkAnalyzer tool. All targets with node degrees above the network average were short-listed for downstream docking analysis, while consistently high rankings across degree, betweenness, and closeness centrality were prioritized as hubs.

Molecular Docking Studies

Molecular docking simulations were performed on all targets collectively short-listed from PPI and ETP network analysis using GLIDE-XP of Schrodinger Maestro v13.0. Crystal structures of human, wild-type proteins (resolution <2.5 Å, cocrystallized with non-peptide ligands) were retrieved from the RCSB PDB. Protein preparation was performed using Protein Preparation Wizard, and ligand geometry was optimized with LigPrep. Receptor grids were generated around native ligands. Each target protein was docked with engeletin, a known standard inhibitor, and its native co-crystallized ligand (re-dock) using the same grid definition and docking parameters. Visual inspection of the re-docked poses in Maestro Viewer confirmed consistency with the docking protocol. Docking scores were interpreted qualitatively, and poses with scores ≤ -5.0 kcal/mol were prioritized as structurally plausible docking poses for further analysis [25, 26].

Molecular Dynamics (MD) Simulations

To evaluate the dynamic stability of selected complexes beyond static docking poses, MD simulations were conducted using the DESMOND module (Schrodinger Suite, v13.0). Selection of protein–ligand complexes was based mainly on dual-network (PPI and ETP) multi-centrality prioritization, favorable docking pose, and established relevance to ischemic stroke pathology. Systems were built in an orthorhombic solvent box with TIP3P

water, neutralized using counterions, and equilibrated under physiological temperature and pressure (300 K, 1 atm). Each simulation was run for 100 ns under NPT (constant Number of particles, Pressure, and Temperature) conditions using the OPLS 2005 force field. Post-run analyses included backbone root-mean-square deviation (RMSD; calculated from Desmond trajectory output files after exclusion of the initial equilibration phase and reported as mean ± standard deviation), RMSF (root-mean-square fluctuation), and time-resolved ligand-protein contact mapping to assess binding persistence. Ligand dynamics were evaluated using Rg (radius of gyration), intramolecular hydrogen bonds, surface area metrics (MolSA - molecular surface area, SASA - solvent-accessible surface area, PSA - polar surface area), and torsional flexibility to infer conformational adaptability.

ADME Assessment of Engeletin

The QikProp module in Schrodinger's Maestro Suite v13.0 was used to predict key pharmacokinetic descriptors of engeletin. These parameters were evaluated to assess the overall ADME profile and drug-likeness. Additionally, the SwissADME tool (Swiss Institute of Bioinformatics; http(s)://www.swissadme.ch; accessed between June and August 2022) was used only to cross-validate BBB permeability [27].

RESULTS AND DISCUSSION

Prediction of Stroke-Relevant Targets of Engeletin

PharmMapper (385 targets), ChemMapper (160 targets), and SwissTargetPrediction (100 targets) were used to predict potential biological targets of engeletin. In parallel, stroke-associated genes were compiled from DisGeNET (116), Online Mendelian Inheritance in Man (OMIM; 1081), and GeneCards (4085). Integration of compound-predicted targets with stroke-associated genes using a two-tier Venn intersection strategy yielded a final panel of 19 stroke-relevant engeletin targets. This overlap-based filtering was applied to reduce likely false positives from individual databases or algorithms, while recognizing that such approaches may preferentially retain targets that are consistently represented across curated resources.

Protein-Protein Interaction Network Analysis of Engeletin Stroke Targets

The STRING protein–protein interaction (PPI) network constructed from the 19 prioritized stroke targets comprised 54 edges (Figure 1), indicating significant interaction enrichment ($p < 1.0 \times 10^{-16}$). Ten targets (CASP3, PTGS2, EGFR, MMP9, ACE, APP, AKR1B1, BACE1, SOD2, and JAK2) with degree values above the network mean were short-listed for subsequent docking analysis. Among these, CASP3, PTGS2, EGFR, and MMP9 consistently ranked highly across degree, betweenness, and closeness centrality measures, identifying them as influential PPI hubs. In interpreting PPI topology, it was recognized that highly connected nodes may partly reflect the density of literature and annotations in curated interaction databases. Nevertheless, targets such as CASP3, PTGS2, and MMP9 also possess well-established mechanistic roles in ischemic neuroinflammation, apoptosis, and BBB disruption and were therefore intentionally retained. Overall, the PPI network revealed a densely interconnected module enriched for regulators of inflammatory signaling, apoptotic execution, and extracellular matrix remodeling, providing a robust topological framework for downstream structure-based prioritization.

Gene Ontology Functional Enrichment Analysis of Engeletin Stroke Targets

GO analysis of the 19 prioritized targets identified enrichment across 10 BP, 5 CC, and 11 MF (raw $p \leq 0.01$). All reported BP terms and a subset of CC and MF terms remained statistically significant after multiple-testing correction (Table 1). In contrast, the remaining CC and MF annotations were interpreted as exploratory. To emphasize biological interconnections relevant to ischemic stroke, enriched terms were interpreted through functional clustering. BP enrichment clustered around stimulus-responsive inflammatory and stress signaling, including responses to lipopolysaccharide, xenobiotic stimuli, drugs, and hypoxia, as well as the positive regulation of inflammatory responses and the regulation of apoptosis.

These processes align with core inflammatory, hypoxic, and injury-response mechanisms activated following cerebral ischemia. CC enrichment supported this context, with statistically robust involvement of membrane rafts & extracellular vesicles, suggesting organization of signaling & intercellular communication at membrane-associated and vesicular compartments. MF enrichment further reinforced these modules, with significant overrepresentation of peptidase activity, enzyme binding & heme binding, consistent with proteolytic regulation and redox-associated enzymatic functions. Collectively, GO enrichment indicates convergence of prioritized targets on interconnected inflammatory, nitric oxide–redox, and stress–apoptosis regulatory modules, providing statistically supported functional context for downstream network and structure-based analyses without implying direct mechanistic confirmation.

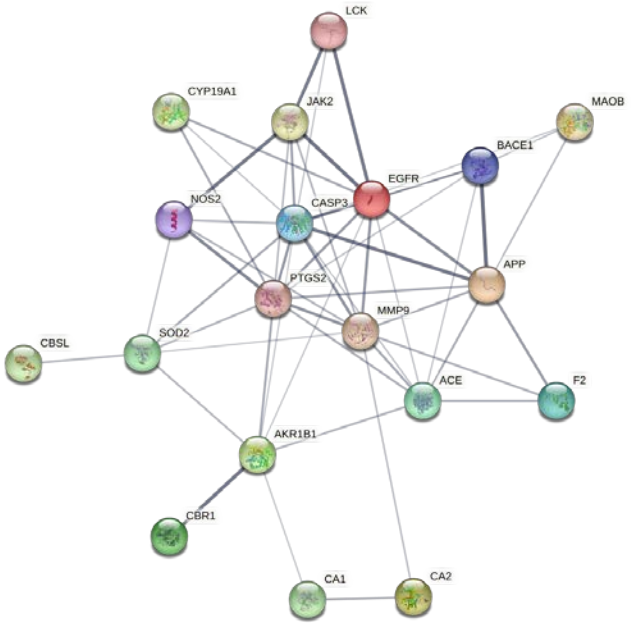


Figure 1: Protein–Protein Interaction Network of Engeletin Stroke-relevant Targets.

Network was generated using the STRING database (confidence score ≥ 0.4; Homo sapiens). Nodes represent proteins and edges indicate known or predicted functional associations, with edge thickness corresponding to interaction confidence.

Table 1: Gene Ontology (GO) Functional Enrichment Analysis.

GO Term		Gene count	%	P-Value	FDR
BP	Response to lipopolysaccharide	7	38.89	2.95×10^{-9}	1.88×10^{-6}
BP	Response to xenobiotic stimulus	6	33.33	1.61×10^{-6}	5.13×10^{-4}
BP	Positive regulation of nitric oxide biosynthetic process	4	22.22	1.03×10^{-5}	2.18×10^{-3}
BP	Response to drug	5	27.78	1.07×10^{-4}	1.48×10^{-2}
BP	Positive regulation of inflammatory response	4	22.22	1.16×10^{-4}	1.48×10^{-2}
BP	Positive regulation of apoptotic process	5	27.78	1.65×10^{-4}	1.75×10^{-2}
BP	Proteolysis	5	27.78	3.83×10^{-4}	2.96×10^{-2}
BP	Response to hypoxia	4	22.22	5.12×10^{-4}	2.96×10^{-2}
BP	Positive regulation of protein phosphorylation	4	22.22	7.66×10^{-4}	3.75×10^{-2}
BP	Negative regulation of apoptotic process	5	27.78	9.87×10^{-4}	4.48×10^{-2}
CC	Membrane raft	6	33.33	1.46×10^{-6}	1.68×10^{-4}
CC	Extracellular exosome	10	55.56	2.18×10^{-5}	1.26×10^{-3}
CC	Endoplasmic reticulum lumen	4	22.22	1.93×10^{-3}	6.12×10^{-2}
CC	Endosome	4	22.22	2.13×10^{-3}	6.12×10^{-2}
CC	Extracellular space	7	38.89	3.51×10^{-3}	8.07×10^{-2}
MF	Peptidase activity	5	27.78	2.58×10^{-6}	3.99×10^{-4}
MF	Heme binding	4	22.22	3.11×10^{-4}	1.81×10^{-2}
MF	Enzyme binding	5	27.78	3.50×10^{-4}	1.81×10^{-2}
MF	Electron carrier activity	3	16.67	1.79×10^{-3}	5.60×10^{-2}
MF	Cyanamide hydratase activity	2	11.11	1.81×10^{-3}	5.60×10^{-2}
MF	Endopeptidase activity	3	16.67	2.75×10^{-3}	6.39×10^{-2}
MF	Identical protein binding	7	38.89	2.89×10^{-3}	6.39×10^{-2}
MF	Protein tyrosine kinase activity	3	16.67	4.59×10^{-3}	7.62×10^{-2}
MF	Transmembrane receptor protein tyrosine kinase activity	3	16.67	4.83×10^{-3}	7.62×10^{-2}
MF	Receptor binding	4	22.22	5.06×10^{-3}	7.62×10^{-2}
MF	Arylesterase activity	2	11.11	5.41×10^{-3}	7.62×10^{-2}

GO terms are classified into Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). Gene count indicates the number of input genes annotated to each term, and percentage represents the proportion of annotated genes. Within each GO category, terms are ordered by ascending Benjamini–Hochberg false discovery rate (FDR). Raw p-values (≤ 0.01) indicate initial enrichment, while FDR values reflect statistical significance after multiple-testing correction. Terms with FDR < 0.05 are considered statistically significant.

Table 2: KEGG Pathway Enrichment Analysis.

S.No	Pathway	Gene Count	Rich factor	P-value	FDR
1	Pathways in cancer	7	0.013	4.93×10^{-4}	6.91×10^{-2}
2	Serotonergic synapse	4	0.035	1.61×10^{-3}	1.13×10^{-1}
3	Alzheimer disease	5	0.013	7.06×10^{-3}	2.80×10^{-1}
4	Lipid and atherosclerosis	4	0.019	9.35×10^{-3}	2.80×10^{-1}
5	Leishmaniasis	3	0.039	1.09×10^{-2}	2.80×10^{-1}
6	PD-L1 expression & checkpoint pathway	3	0.034	1.44×10^{-2}	2.80×10^{-1}
7	Small-cell lung cancer	3	0.033	1.53×10^{-2}	2.80×10^{-1}
8	IL-17 signaling pathway	3	0.032	1.60×10^{-2}	2.80×10^{-1}
9	TNF signaling pathway	3	0.027	2.22×10^{-2}	3.11×10^{-1}
10	Toxoplasmosis	3	0.027	2.22×10^{-2}	3.11×10^{-1}
11	MicroRNAs in cancer	4	0.013	2.49×10^{-2}	3.11×10^{-1}
12	Metabolic pathways	8	0.005	2.80×10^{-2}	3.11×10^{-1}
13	Relaxin signaling pathway	3	0.023	2.89×10^{-2}	3.11×10^{-1}
14	Nitrogen metabolism	2	0.118	3.49×10^{-2}	3.49×10^{-1}
15	Hepatitis B	3	0.019	4.39×10^{-2}	4.09×10^{-1}

Gene count indicates the number of input genes mapped to each pathway & rich factor represents the ratio of mapped genes to the total number of genes annotated in that pathway. Pathways are ordered by ascending Benjamini–Hochberg false discovery rate (FDR). Raw p-values (≤ 0.05) indicate nominal enrichment. None of the enriched pathways remained statistically significant after multiple-testing correction (FDR < 0.05). ***TNF****-tumour necrosis factor;* ***IL-17****-interleukin-17;* ***PD-L1****-programmed death-ligand 1.*

KEGG Pathway Enrichment Analysis of Engeletin Stroke Targets

KEGG analysis identified 15 nominally enriched pathways (raw $p < 0.05$; Table 2), although none remained significant after multiple-testing correction (FDR < 0.05). These pathways were interpreted as hypothesis-generating and context-supportive, rather than definitive associations. Nominal enrichment clustered around three interconnected biological themes relevant to ischemic stroke. First, inflammatory and immune-mediated signaling axes, including TNF and IL-17 pathways, and several infection-associated pathways (leishmaniasis, hepatitis B, toxoplasmosis), collectively reflect activation of innate immune and cytokine-driven cascades linked to glial activation and BBB disruption. Second, vascular and endothelial dysfunction–related pathways, including lipid and atherosclerosis signaling and relaxin signaling. Lipid and atherosclerosis enrichment aligns with endothelial dysfunction and vascular inflammation, while relaxin signaling, although underexplored in stroke, has been implicated in endothelial protection, nitric oxide modulation, and BBB stabilization [28]. Third, metabolic and stress-response pathways, including metabolic pathways, pathways in cancer, Alzheimer's disease, and nitrogen metabolism, likely reflect shared disturbances in mitochondrial function, oxidative stress, apoptosis, and nitric oxide signaling rather than disease-specific overlap. Together, these clusters highlight cross-talk between inflammatory, vascular, and metabolic stress networks and provide pathway-level context that complements network topology and molecular docking findings.

Engeletin-Target-Pathway (ETP) Network Analysis

To complement PPI analysis and reduce potential annotation bias, a tripartite engeletin–target–pathway (ETP) network linking engeletin, its 19 targets, and 15 KEGG pathways was constructed (Figure 2). Six targets (CASP3, PTGS2, NOS2, MMP9, JAK2, and EGFR) exhibited degree values above the average network connectivity and simultaneously demonstrated elevated betweenness & closeness centrality, indicating consistent topological influence across multiple centrality metrics. CASP3 and PTGS2 each connected to nine pathways, reflecting central roles in apoptotic and inflammatory signaling.

NOS2 and MMP9 are linked to seven pathways, consistent with involvement in nitric oxide–redox imbalance and extracellular matrix remodeling. JAK2 and EGFR, although exhibiting comparatively lower pathway connectivity, were retained due to their stable multi-centrality rankings and established roles in inflammatory signaling and neurovascular remodeling. Notably, NOS2 emerged as a prominent hub in the ETP network despite moderate PPI connectivity, highlighting its functional relevance at the pathway level rather than through dense protein–protein interactions alone. By integrating PPI and ETP analyses, hub prioritization was reinforced across complementary network contexts, mitigating excessive reliance on annotation-inflated PPI hubs and strengthening confidence in the biological relevance of the final six prioritized targets (PTGS2, CASP3, MMP9, NOS2, JAK2, and EGFR) selected for downstream structure-based analyses.

Molecular Docking Analysis

To substantiate network-based predictions, 11 topologically important targets short-listed from integrated PPI and engeletin–target–pathway (ETP) network analyses (CASP3, PTGS2, NOS2, MMP9, JAK2, EGFR, ACE, APP, AKR1B1, BACE1, and SOD2) were subjected to molecular docking with engeletin. Across this target panel, Glide XP docking scores ranged from −9.43 to −3.18 kcal/mol. Docking scores below −5.0 kcal/mol were observed for most targets, except for SOD2 and PTGS2. Representative docking poses illustrating key stabilizing interactions, including hydrogen bonding and π–π stacking, are shown in Figure 3, and comprehensive docking scores and residue-level interactions are summarised in Table 3. Targets with lower docking scores were prioritized for further interaction analysis, while recognizing that docking scores are interpreted qualitatively and are not directly comparable across proteins. Engeletin exhibited favorable docking compatibility with AKR1B1 (−9.43 kcal/mol), JAK2 (−8.48 kcal/mol), EGFR (−8.07 kcal/mol), and NOS2 (−7.89 kcal/mol). For each target, docking was performed alongside the corresponding standard inhibitor to provide a within-target comparative context. Notably, for NOS2, EGFR, and APP, engeletin demonstrated docking scores comparable to or lower than those of the respective reference inhibitors, supporting the plausibility of direct engagement within these binding pockets. Among the six network-prioritized influential hubs (CASP3, PTGS2, NOS2, MMP9, JAK2, and EGFR), several targets with favorable docking scores (NOS2, MMP9, CASP3, JAK2, and EGFR) are well-established mediators of ischemic stroke pathophysiology.

NOS2 is rapidly induced post-insult, contributing to oxidative/nitrosative stress, BBB disruption, and neuronal injury. MMP9 facilitates extracellular matrix degradation and edema. CASP3, a central executioner of apoptosis, promotes irreversible neuronal loss, and JAK2 sustains glial activation via the JAK/STAT pathway. EGFR, which has been reported to exert context-dependent effects on BBB integrity and repair processes in ischemic injury [29], exhibited favorable docking compatibility with engeletin, suggesting a possible direct interaction; however, clarification of functional consequences requires validation beyond docking analysis. This is consistent with enrichment (raw $p < 0.05$) of inflammatory, metabolic, and apoptosis-related KEGG pathways. In contrast, a stable docking pose for PTGS2 (COX2) was not obtained. However, given its strong network centrality and established upstream regulatory links to NOS2- and NF-κB-mediated inflammatory signaling [30-33], PTGS2 was retained as a potential, indirectly modulated target rather than excluded solely based on docking outcomes. Additional targets, including APP, BACE1, ACE, and AKR1B1, although not stroke-specific, may contribute secondarily via BBB disruption, amyloidogenic stress, chronic inflammation, or metabolic dysregulation [29, 34]. SOD2, a key mitochondrial antioxidant enzyme, exhibited weak binding, suggesting sparing of protective pathways.

In the present study, molecular docking was employed as a structure-informed interaction-screening step to refine network-derived target prioritization by assessing the plausibility of direct ligand–protein binding. Docking outcomes were therefore used to select representative protein–ligand complexes for subsequent molecular dynamics simulations, rather than as evidence of confirmed biological activity.

Molecular Dynamics (MD) Simulation of Engeletin-Target Complexes

Based on integrated network topology and docking outcomes, three hub complexes: engeletin–NOS2, engeletin–CASP3, and engeletin–MMP9, were subjected to 100 ns molecular dynamics simulations to assess dynamic stability.

Engeletin-NOS2 Complex

Simulations demonstrated a stable association of engeletin with the NOS2 (iNOS) oxygenase domain over the 100 ns trajectory. The protein backbone RMSD stabilized within the first 10 ns. It remained stable throughout the equilibrated phase, with a time-averaged RMSD of 2.71 ± 0.23 Å calculated over the production trajectory, indicating the absence of large-scale conformational drift (Figure 4A). Ligand stability analysis showed a time-averaged ligand RMSD of 1.23 ± 0.49 Å (aligned to the ligand reference) across the equilibrated phase, supporting persistent internal conformational stability of engeletin within the NOS2 binding pocket throughout the simulation.

Protein-ligand interaction fraction analysis revealed that engeletin engages the conserved substrate-binding environment of NOS2 through a network of stabilizing interactions (Figure 4B). Among these, Glu377 emerged as a polar anchoring residue, consistent with its conserved role in mediating substrate and inhibitor interactions within the iNOS oxygenase domain [35]. Additional persistent stabilizing contacts were observed with Tyr347 and Arg388, along with auxiliary interactions involving Tyr347, Thr376, Phe363, Val346, Arg388, and

Asp382, mediated through hydrogen bonding, hydrophobic interactions, and water-bridged contacts. Classical iNOS inhibitors such as 1400W act as slow, tight-binding inhibitors that compete with L-arginine at the substrate pocket via a dominant cationic anchor [36].

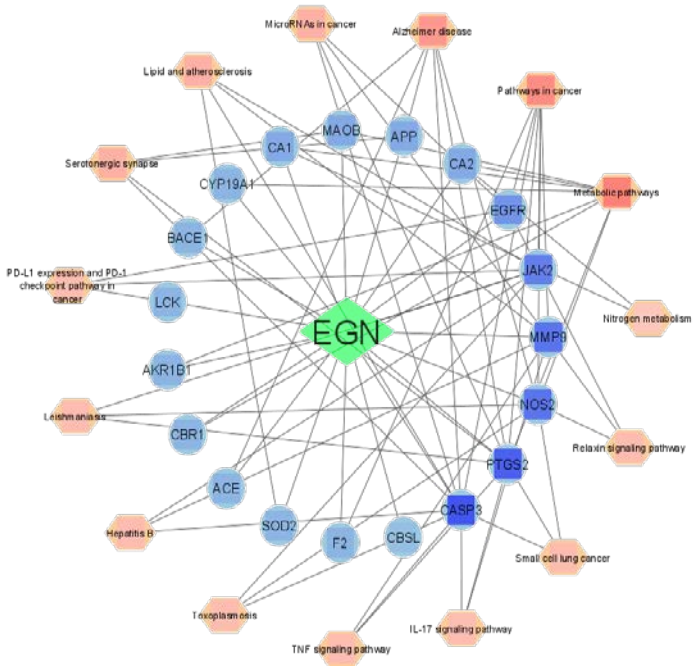
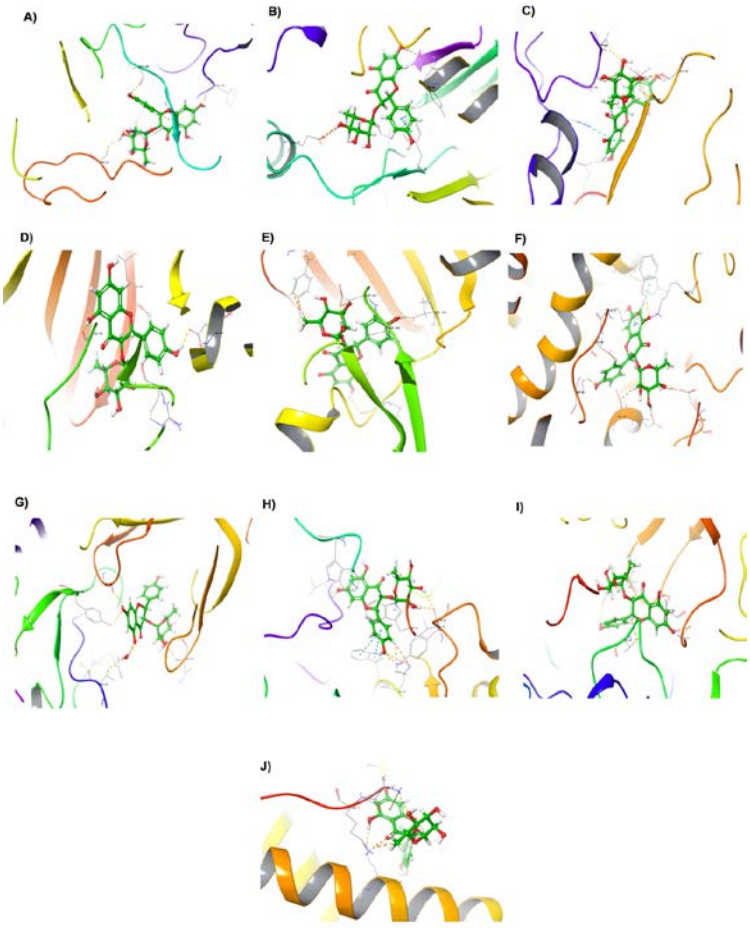


Figure 2: Engeletin–Target–Pathway (ETP) Network of Stroke-relevant Targets.

The tripartite network links engeletin (green diamond), stroke-relevant targets (blue circles), and enriched KEGG pathways (orange hexagons). Node color intensity (light to dark) reflects increasing centrality. Edges indicate target–pathway associations.
Abbreviations: TNF, tumor necrosis factor; IL-17, interleukin-17; PD-L1, programmed death-ligand 1.



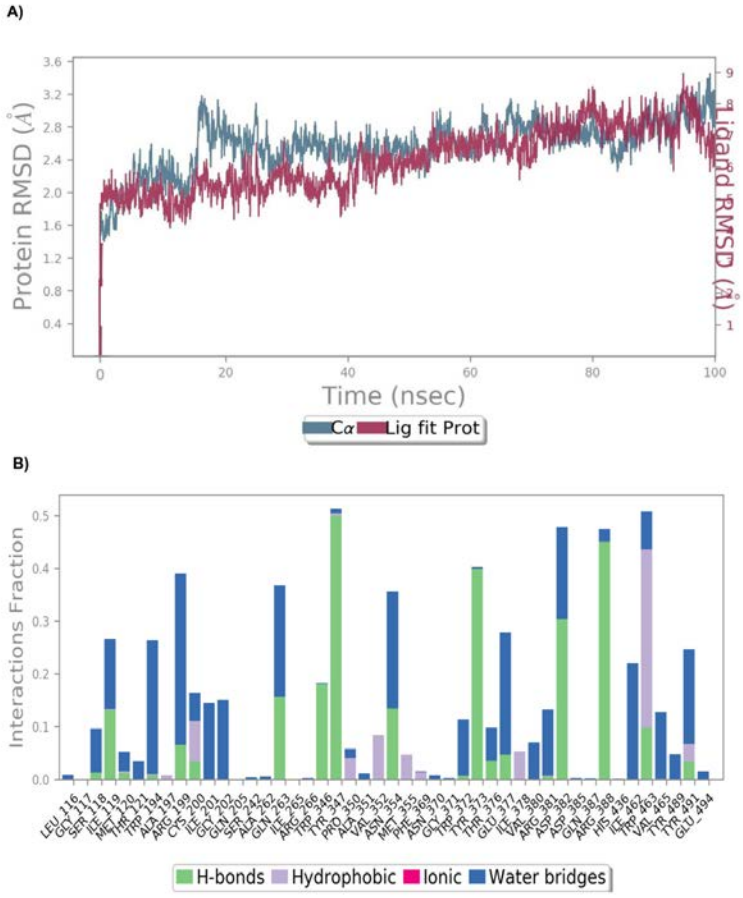
Three-dimensional binding poses of engeletin within the active sites of (A) CASP3, (B) NOS2, (C) MMP9, (D) JAK2, (E) EGFR, (F) ACE, (G) APP, (H) AKR1B1, (I) BACE1, and (J) SOD2. Proteins are shown as ribbon representations, with engeletin displayed as sticks. PTGS2 did not yield a stable docking pose under the applied protocol.

Figure 3: Representative Docking Poses of Engeletin With Short-listed Targets.

Table 3: Molecular Docking Results of Engeletin With Short-listed Targets.

Target Protein	PDB ID	Engeletin Dock-king Score (kcal/mol)	Glide Energy (kcal/mol)	Standard Drug	Std. Drug Docking Score (kcal/mol)	No. & Type of Interactions	Interac-ting Residues
CASP3	3H0E	-5.613	-44.375	Z-DEVD-FMK	-5.835	4 H-bonds	thr62, ser205, arg207, phe250
PTGS2	5IKV	No valid pose	-	Celecoxib	-8.288	-	-
NOS2	3E7G	-7.885	-46.365	Ronopterin	-4.680	1 H-bond, 2 π-π stacking	tyr489, trp194, phe369
MMP9	1GKC	-6.080	-47.425	SB-3CT	-7.400	4 H-bonds, 1 π-π stacking	glu111, gly186, leu188, tyr423
JAK2	3KRR	-8.480	-45.780	Ruxolitinib	-9.143	2 H-bonds, 1 π-π stacking	asn719, gln725, phe778
EGFR	4JQ7	-8.070	-53.717	Erlotinib	-6.060	3 H-bonds	thr766, thr830, asp831
ACE	1UZE	-6.164	-55.431	Enalapril	-7.152	5 H-bonds, 1 π-π stacking, 1 salt bridge	glu162, gln281, ser284, asp377, tyr520, tyr523, lys511
APP	5KQF	-5.340	-46.330	2-PMAP	-5.014	5 H-bonds	gly82, gln121, tyr246
AKR1B1	5OUK	-9.434	-54.464	Epalrestat	-10.020	6 H-bonds, 3 π-π stacking	trp20, val47, tyr48, hie110, leu301, trp111, trp219
BACE1	2FDP	-5.872	-55.537	LY2886721	-6.420	3 H-bonds	asp32, gly34, gln73
SOD2	5T3O	-3.180	-34.959	Pamoic acid	-4.150	2 H-bonds, 1 π-cation	glu42, met0

Docking scores are reported as Glide XP scores (kcal/mol); more negative values indicate stronger predicted binding affinity. Abbreviations: π-π: aromatic stacking; H-bond: hydrogen bond; π-cation: π-cation interaction.



(A) Root mean square deviation (RMSD) of NOS2 backbone and engeletin over the 100 ns simulation (X-axis: time in ns; Y-axis: RMSD in Å). (B) Protein–ligand interaction fraction analysis showing the proportion of simulation time occupied by hydrogen bonds, hydrophobic contacts, ionic interactions, and water bridges.

Figure 4: Molecular Dynamics Simulation of Engeletin-NOS2 Complex.

In contrast, engeletin lacks a canonical amidine or guanidinium motif and therefore does not rely on a single high-affinity ionic interaction. Instead, its stable pocket occupancy appears to be supported by a distributed network of moderate interactions with conserved substrate-site residues, as observed in the MD simulations. This mode of engagement is consistent with substrate-site–directed NOS ligands that modulate active-site dynamics without direct heme chelation or rigid catalytic blockade [37]. Such interaction topology may permit attenuation of pathological nitric oxide signaling while avoiding complete enzymatic shutdown. Analysis of ligand conformational behavior indicated adaptive torsional flexibility without pronounced strain (Figure 5A). Global ligand descriptors, including Rg, MolSA, SASA, and PSA, remained stable across the simulation (Figure 5B), reflecting maintenance of a compact ligand-protein assembly under dynamic conditions. These findings support NOS2 as a structurally plausible target of engeletin and provide a mechanistic basis for prioritizing this interaction for future in vivo validation in ischemic stroke models.

Engeletin-CASP3 and Engeletin-MMP9 Complexes

The engeletin-CASP3 and engeletin-MMP9 complexes exhibited reduced dynamic stability during MD simulations. While protein backbones remained stable, with RMSD values largely within 1.5–2.5 Å, ligand RMSD profiles showed progressive drift and pronounced fluctuations after ~60 ns (Figures 5C and 5D), indicating loss of a well-defined binding pose and weak conformational confinement within the binding sites. Although CASP3 and MMP9 emerged as network hubs and showed favorable docking scores, MD simulations did not support stable direct binding, suggesting that their regulation by engeletin may occur indirectly within the broader inflammatory and apoptotic network.

Unlike CASP3 and MMP9, the engeletin–NOS2 complex retained a stable binding mode throughout the production trajectory, supporting its prioritization as a structurally plausible and dynamically stable engeletin target. In the present target-reduction framework, MD simulations were used as a final, structure-informed prioritization step to distinguish likely direct-interaction candidates (NOS2) from network-level co-modulated hubs (e.g., CASP3 and MMP9). However, MD findings are interpreted as predictive and hypothesis-generating rather than as definitive mechanistic confirmation.

ADME Analysis of Engeletin

In silico ADME profiling indicated that engeletin falls within acceptable ranges for key drug-likeness descriptors (Table 4). It satisfied the Lipinski criteria with minor deviations, demonstrated favorable predicted solubility, and showed moderate human oral absorption. However, low predicted Caco-2 and MDCK permeability values, together with a negative brain–blood partition coefficient, indicate limited passive permeability and restricted penetration across the BBB. These findings were corroborated by SwissADME, which predicted low gastrointestinal absorption and classified engeletin as non-BBB permeant, supporting the inference of limited central nervous system accessibility.

These predictions align with reported pharmacokinetic data for engeletin (rapid absorption, low systemic bioavailability (~1.5%), and a short half-life (~3.7 h)), which may limit systemic exposure despite its promising multitarget activity [38]. Accordingly, future work may require optimization of bioavailability or CNS delivery (e.g., nano- and lipid-based systems, phospholipid complexes, intranasal delivery, or physicochemical optimization), approaches commonly explored to enhance brain access of poorly permeable therapeutic molecules [39]. Moreover, available in vivo studies report no overt toxicity up to 40 mg/kg [8], supporting engeletin as a pharmacologically active but pharmacokinetically constrained scaffold suitable for further optimization rather than exclusion.

Table 4: QikProp Based ADME Profile of Engeletin.

Parameter	Predicted Value	Reference limit
Molecular weight (g/mol)	434.39	130 – 725
H bond donors	5	0 – 6
H bond acceptors	12.5	2 – 20
LogP (octanol/water)	-0.317	-2 – 6.5
Lipinski violations	2	≤ 4
Predicted solubility (logS)	-2.975	-6.5 – 0.5
Caco-2 permeability (nm/sec)	18.840	<25poor, >500excellent
Human oral absorption	2(Moderate)	1 – 3
Brain-blood partition (log BB)	-2.648	-3 – 1.2
MDCK cell permeability (nm/sec)	6.76	<25poor, >500excellent

Reference limits indicate recommended ranges for drug-likeness. Abbreviations: Caco-2- human colorectal adenocarcinoma cell line and MDCK- Madin-Darby Canine Kidney cell.

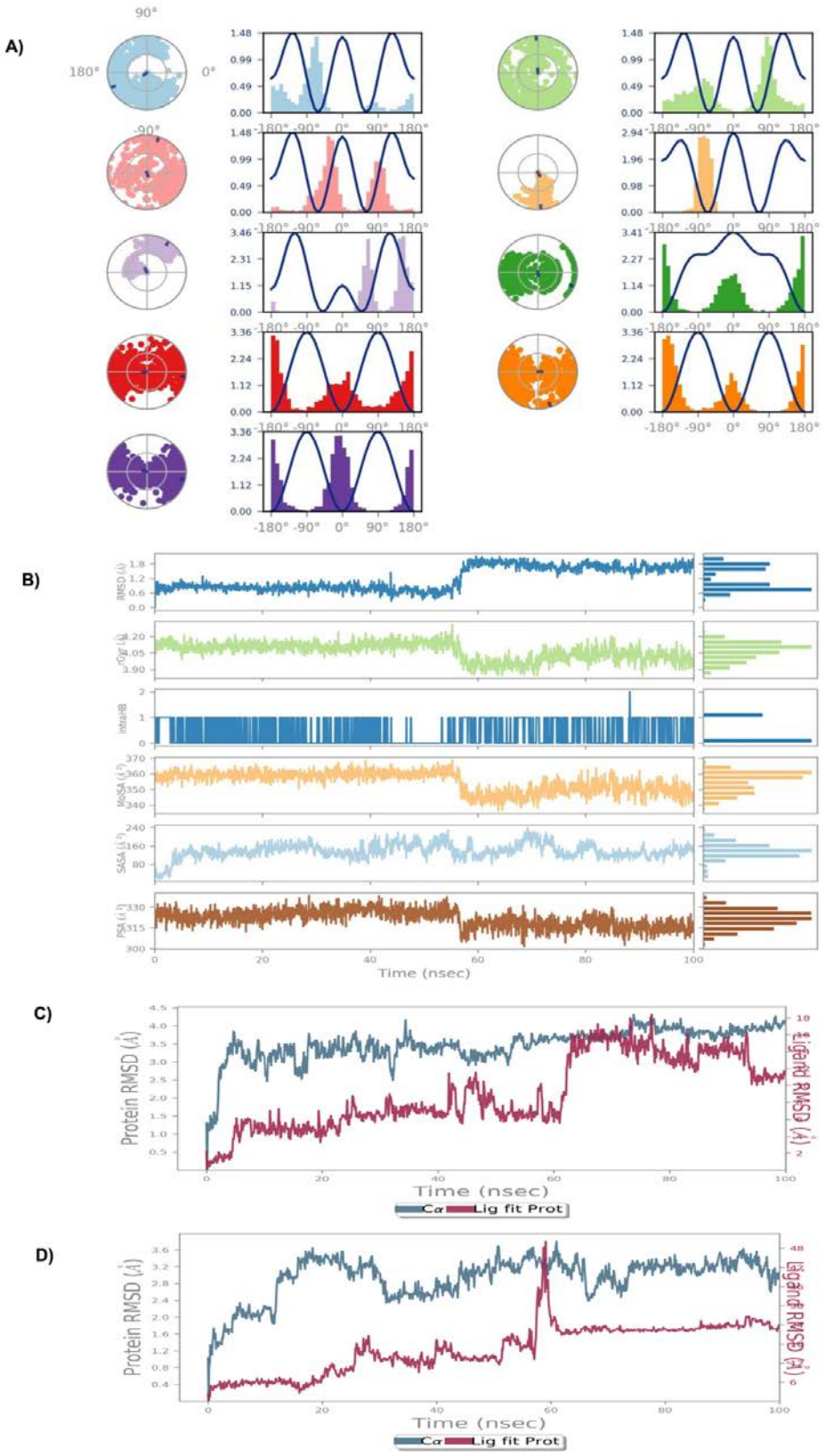


Figure 5: Molecular Dynamics Simulation Outputs for Engeletin–Target Complexes.

(A) *Ligand torsion profile of engeletin in complex with NOS2.* ***(B)*** *Time-resolved ligand properties for the engeletin–NOS2 complex, including solvent-accessible surface area (SASA), polar surface area (PSA), and flexibility metrics.* ***(C–D)*** *Root mean square deviation (RMSD) profiles of protein backbone and ligand for engeletin–CASP3 and engeletin–MMP9 complexes, respectively (X-axis: time in ns; Y-axis: RMSD in Å).*

Integrated Target Prioritization and Mechanistic Interpretation

The current study used a structured, multi-stage workflow to reduce large compound and disease target sets into a compact, mechanistically interpretable panel. Nineteen high-confidence stroke-relevant targets were first defined using a two-tier Venn intersection strategy, then contextualized through enrichment analyses and organized into PPI and ETP networks. Integrated topology across both networks converged on six influential hubs (PTGS2, CASP3, MMP9, NOS2, JAK2, and EGFR), while retaining a consolidated set of 11 topologically important targets. They were subsequently filtered using docking and MD as a structure-informed prioritization layer to distinguish likely direct binders (NOS2) from co-modulated network hubs (CASP3 and MMP9). Network topology and nominally enriched pathway clusters, support a coordinated inflammatory–vascular–metabolic axis underlying engeletin's predicted neuroprotective activity. Notably, the convergence of network centrality with favorable docking and stable MD behavior for NOS2 provides mechanistic coherence between systems-level prioritization and atomistic interaction stability, strengthening its selection as a primary direct-interaction hypothesis. Thus, compared with prior engeletin-stroke network pharmacology study that largely relied on network topology, enrichment, and docking feasibility [15], this work emphasizes an intensified, multilayered reduction strategy and adds MD-based stability assessment to rank interaction plausibility and prioritized NOS2 as a dynamically stable binding partner of engeletin, while characterizing other influential hubs as its indirect or co-modulatory targets within the stroke-associated interactome.

From a therapeutic perspective, this distinction is particularly relevant. Highly selective NOS2 inhibitors, including aminoguanidine and 1400W, were developed to achieve maximal suppression of iNOS-derived nitric oxide and showed benefit in experimental stroke models [40, 41]. However, despite substantial preclinical promise, selective iNOS inhibitors have not translated into approved human therapies [42]. This limitation reflects the multifactorial inflammatory–vascular–metabolic nature of ischemic stroke, in which rigid single-target blockade has shown limited clinical success [5]. In contrast, the present findings suggest that engeletin engages NOS2 within the coordinated inflammatory–vascular network identified in the current analysis, consistent with a modulatory rather than binary inhibition paradigm.

Therefore, engeletin is best framed as a complementary multitarget scaffold that may support adjunctive, post-insult modulation of secondary neuroinflammatory and BBB-disruptive cascades, rather than a direct substitute for selective NOS2 inhibitors or current clinical stroke therapies that primarily target vascular recanalization or platelet aggregation (e.g., thrombolytic or antithrombotic strategies) [43].

Limitations, Validation Perspectives, and Future Scope

Network pharmacology analyses are influenced by database coverage, annotation density, and algorithmic variability, and molecular docking and molecular dynamics simulations provide predictive rather than confirmatory evidence of biological activity. Together with pharmacokinetic constraints noted above, these results should be viewed as hypothesis-generating. Next steps include validating NOS2 modulation (via enzyme inhibition and/or cellular nitric oxide assays) and testing whether PTGS2, CASP3, and MMP9 are primarily influenced via indirect network regulation. Time-resolved studies in established ischemic stroke models will be required to determine whether coordinated modulation translates into BBB preservation and reduced secondary injury in vivo.

CONCLUSION

This study presents an integrated in silico framework to characterize the multitarget neuroprotective potential of engeletin in ischemic stroke. Network pharmacology and topological prioritization converged on PTGS2, CASP3, MMP9, NOS2, JAK2, and EGFR as influential regulatory targets within an interconnected inflammatory, apoptotic, oxidative–redox, and neurovascular signaling landscape. Structure-based docking and molecular dynamics simulations prioritized NOS2 is a structurally plausible candidate for direct interaction. In contrast, other hub targets are more consistent with indirect modulation within broader network-level signaling contexts.

Functional enrichment further highlighted convergence on inflammatory and vascular-associated pathways, including relaxin signaling as an underexplored but potentially relevant neurovascular regulatory axis. Collectively, these findings provide a systems-level, hypothesis-generating foundation for subsequent experimental validation of engeletin in complex inflammatory and vascular signaling environments relevant to ischemic stroke, and should be interpreted as predictive rather than definitive mechanistic confirmation.

FINANCIAL ASSISTANCE
NIL

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Santhrani Thakur designed and supervised the study. Manga Devi Chinta carried out the literature review, data analysis, and manuscript drafting. Both authors discussed the results, contributed to the interpretation, and approved the final version of the manuscript.

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