

Network Pharmacology-Driven Elucidation of Biochanin A's Neuroprotective Mechanism in Cerebral Ischemia: JAK2/STAT3 Signaling Pathway Modulation via p-JAK2, JAK2, p-STAT3, and STAT3 Regulation

Elucidación Mediante Farmacología de Redes del Mecanismo Neuroprotector de la Biocanina A en la Isquemia Cerebral: Modulación de la Vía de Señalización JAK2/STAT3 Mediante la Regulación de p-JAK2, JAK2, p-STAT3 y STAT3

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SUMMARY: Cerebral ischemia resulting from middle cerebral artery occlusion (MCAO) significantly impairs neuronal function and exacerbates cognitive deficits. This study investigates the neuroprotective effects of Biochanin A, a natural isoflavonoid, in a MCAO model using Wistar rats. Fifty rats were divided into five groups: a control group, a MCAO group, and three treatment groups receiving 200 mg/kg and 400 mg/kg of Biochanin A. Cognitive function was assessed using the Morris water maze test, while serum levels of neurotrophic factors (GDNF and BDNF) and inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-10, and IL-4) were measured through enzyme-linked immunosorbent assay (ELISA). Additionally, gene expression of JAK2 and STAT3 was analyzed via real-time PCR. Results indicated that MCAO significantly impaired cognitive function and increased pro-inflammatory cytokines while decreasing neurotrophic factors ($p < 0.05$). In contrast, Biochanin A treatment improved escape latency in the Morris water maze and elevated serum GDNF and BDNF levels ($p < 0.05$). Furthermore, Biochanin A administration significantly modulated the expression of JAK2 and STAT3, promoting neuroprotection through the JAK2/STAT3 signaling pathway. These findings suggest that Biochanin A mitigates cognitive deficits and neuroinflammation following MCAO, potentially by enhancing neurotrophic factor levels and modulating inflammatory responses. Thus, Biochanin A represents a promising therapeutic candidate for ischemic stroke recovery.

KEY WORDS: Biochanin A; Cerebral infraction; Network pharmacology; Cerebral ischemia-reperfusion.

INTRODUCTION

Stroke, a life-threatening condition, remains one of the leading causes of long-term disability and mortality worldwide. The World Health Organization (WHO) estimates that nearly 85 % of all strokes are ischemic in nature, primarily caused by the occlusion of the middle cerebral artery (MCA), which results in a cascade of neuroinflammatory and immune responses that exacerbate brain injury and impair recovery (Salaudeen *et al.*, 2024). Ischemic stroke leads to neuronal death, disrupted cellular signaling, and a wide range of pathological events, including oxidative stress, amyloid production, and tau protein dysfunction. The underlying neuropathophysiological processes associated with ischemic stroke are complex and multifaceted, contributing to the severity and long-term

consequences of the disease. Despite the availability of recombinant tissue plasminogen activator (rt-PA), an effective thrombolytic therapy for ischemic stroke, its clinical application is limited by a narrow therapeutic time window, numerous contraindications, and potential neurotoxicity (Polk *et al.*, 2018). Thus, there is a critical need to explore alternative therapeutic strategies that can mitigate stroke-induced brain injury and promote neurological recovery.

Biochanin A, a natural isoflavonoid derived from the plant *Trifolium pratense* (red clover), has recently emerged as a promising candidate in the treatment of various brain disorders, including ischemic stroke. Flavonoids, such as Biochanin A, are well-known for their antioxidant properties,

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which help to neutralize excessive reactive oxygen species (ROS) that are produced during ischemia (Raheja *et al.*, 2018). ROS are one of the primary causes of neuronal necrosis and apoptosis, and their scavenging is crucial to mitigating neuronal death and improving patient prognosis after stroke. In addition to its antioxidant activity, Biochanin A has been shown to exhibit anti-inflammatory, anti-apoptotic, and neuroprotective effects, suggesting its potential as a therapeutic agent in ischemic brain injury. Numerous preclinical studies have demonstrated that Biochanin A, at high doses (100 - 400 mg/kg), significantly reduces infarct volume and improves neurological function in experimental animal models of cerebral ischemia (El-Sherbeeney *et al.*, 2020). For example, studies comparing Biochanin A with other neuroprotective agents, such as edaravone, have shown that Biochanin A offers superior neuroprotection by reducing cerebral infarct volume and enhancing recovery following stroke. While these findings are promising, the precise mechanisms by which Biochanin A exerts its protective effects in ischemic stroke remain unclear. A deeper understanding of the mechanisms of Biochanin A's action is vital for its potential translation into clinical practice (Singh *et al.*, 2023). Recent research has highlighted the pivotal role of microglia in the pathophysiology of ischemic stroke.

Microglia are the resident immune cells of the central nervous system (CNS) and are typically the first to respond to ischemic injury. Upon activation, microglia migrate toward the infarct core and penumbral regions, where they phagocytose cellular debris and release pro-inflammatory cytokines, further exacerbating neuroinflammation and tissue damage (Prinz *et al.*, 2021). Importantly, activated microglia also trigger the activation of astrocytes, another key cell type in the CNS, which are involved in maintaining the integrity of the blood-brain barrier (BBB) and regulating neuronal homeostasis. The interaction or "cross-talk" between microglia and astrocytes plays a central role in amplifying the neuroinflammatory cascade, thus contributing to the severity of ischemic brain injury. Given the close spatial and temporal relationship between microglia and astrocytes in the ischemic brain, understanding the dynamics of this cross-talk is crucial for identifying novel therapeutic targets to modulate neuroinflammation and promote recovery following stroke (Wheeler *et al.*, 2023).

The JAK2/STAT3 signaling pathway is one such critical pathway implicated in the regulation of neuroinflammation and neuronal survival in ischemic stroke. JAK2 (Janus kinase 2) and STAT3 (signal transducers and activators of transcription 3) are key components of a signaling cascade that regulates numerous

cellular processes, including inflammation, cell survival, and tissue repair (Chen *et al.*, 2023). In response to ischemic injury, the JAK2/STAT3 pathway is activated, leading to the phosphorylation of JAK2 and STAT3. Phosphorylated JAK2 (p-JAK2) activates STAT3, and phosphorylated STAT3 (p-STAT3) forms a dimer that translocates to the nucleus, where it regulates the expression of target genes involved in neuroprotection and angiogenesis. Previous studies have demonstrated that activation of the JAK2/STAT3 pathway enhances cerebral angiogenesis, promotes neuronal survival, and accelerates neurological recovery following ischemic stroke (Tang *et al.*, 2018). Moreover, the JAK2/STAT3 signaling pathway has been shown to mediate the neuroprotective effects of various therapeutic agents, including resveratrol, in ischemic brain injury. As such, the JAK2/STAT3 pathway represents a promising target for therapeutic intervention in ischemic stroke. Given the complex interactions between microglia, astrocytes, and the JAK2/STAT3 pathway, this study aims to elucidate the neuroprotective mechanism of Biochanin A in ischemic stroke through a network pharmacology-driven approach (Zhu *et al.*, 2021). To explore the mechanisms by which Biochanin A protects against ischemic brain injury, we employed a network pharmacology approach to identify potential molecular targets and signaling pathways involved in its neuroprotective action. By using this model, we aimed to better understand how Biochanin A modulates the interaction between microglia and astrocytes, and to assess its potential neuroprotective effects in this context.

MATERIAL AND METHOD

Bioinformatics analyses

Protein-protein interaction (PPI) data were obtained from the STRING database (<https://string-db.org/>), which provides both known and predicted interactions between proteins, including direct and indirect relationships. The data were filtered to include only *Homo sapiens* species and interactions with confidence scores above 0.9. The resulting PPI data were further analyzed and visualized using Cytoscape (version 3.7) to better understand the interaction networks.

Functional enrichment analysis was conducted using the Metascape online database (<http://metascape.org>), focusing on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. GO analysis covered three categories: biological processes (BP), cellular components (CC), and molecular functions (MF). Statistical significance was set at a p-value threshold of < 0.01 to identify the most relevant pathways and biological functions.

From the results of the enrichment analysis, the top 10 core targets and 12 related pathways were selected. These findings were imported into Cytoscape to construct a comprehensive drug-target-pathway-disease network. This network provides a clear visualization of how Biochanin A interacts with specific targets and pathways associated with brain ischemia, offering insights into its potential therapeutic effects (Bajpai *et al.*, 2020; Jia *et al.*, 2023).

Animals and experimental groups

Transient middle cerebral artery occlusion (MCAO)

For the transient middle cerebral artery occlusion (MCAO) procedure, the intraluminal suture occlusion technique was employed as previously described. Initially, the rats were anesthetized with isoflurane, using 5% for induction and 2% for maintenance throughout the procedure. The animal's head was stabilized in a stereotaxic frame, and a small burr hole was drilled into the skull, located 2.0 mm posterior and 2.5 mm lateral to the bregma, to expose the distal portion of the middle cerebral artery (MCA). A 3 cm midline incision was made in the neck to gain access to the right external carotid artery (ECA) and internal carotid artery (ICA). A 3–0 nylon suture with a heat-blunted tip was carefully threaded through the ECA stump into the lumen of the right ICA and advanced to the origin of the MCA. The artery was occluded for 90 minutes, after which the suture was gently retracted to allow reperfusion. Continuous monitoring of blood flow in the MCA was performed using Laser-Doppler flowmetry (DRT4, Moor Instruments Inc., Wilmington, USA) during the entire MCAO procedure. Successful occlusion was confirmed when the MCA blood flow decreased to 20–30 % of the baseline during the ischemic phase and subsequently increased to 60% of baseline upon reperfusion. Rats that exhibited incomplete MCAO were excluded from the study (Yu *et al.*, 2019).

Experimental design and research time line

Fifty male Wistar rats, each weighing 220 ± 40 g, were randomly divided into five groups, with ten rats per group. Before the start of the experiment, the rats underwent a 24-hour acclimatization period to adjust to the environmental conditions, which included temperature, food, and water availability. They were housed in propylene cages at a controlled temperature of 20 ± 5 °C and relative humidity of 42 ± 5 %, with a 12-hour light/dark cycle, and had unrestricted access to standard pellets and tap water. All procedures regarding animal care and euthanasia were approved by the Mongolian Medical of College Inner

Mongolia Minzu University ethics committee and strictly followed established guidelines for laboratory animal welfare.

The control group and the MCAO group received intraperitoneal injections of 0.5 mL phosphate-buffered saline (PBS). The treatment groups, MCAO + 200 and MCAO + 400 Biochanin A, were administered oral doses of 200 mg/kg and 400 mg/kg of Biochanin A, respectively, while the control + 400 Biochanin A group received 400 mg/kg of Biochanin A orally. The non-toxic effective dose was determined using the LD50 method, along with preliminary studies and available literature on Biochanin A. Biochanin A was administered daily at 9 AM for 50 consecutive days.

During the procedures, the rats were anesthetized with 2% isoflurane. A burr hole was drilled into the right side of the skull, 0.8 mm posterior to the bregma and 1.5 mm lateral to the midline. An intracerebroventricular (ICV) injection of 10 mL of SP600125 solution (2 mM in DMSO, ab120065, Abcam) or a 1 % DMSO solution was delivered at a depth of 3.5 mm from the skull using a 10 μ L Hamilton syringe (Zhang & He, 2025).

Morris water maze test

The Morris water maze, a well-established assay for evaluating spatial learning and memory, was performed in this study from day 20 to day 40 after ischemia/reperfusion (IS/R). The maze consisted of a circular pool with a 120 cm diameter and a depth of 50 cm, placed in a quiet, dark environment with clear visual cues. Water temperature was kept at 23 ± 4 °C. A black, circular platform (10 cm in diameter) was submerged 2 cm below the surface in a fixed position at the center of one quadrant for the entire training period. Rats' swimming trajectories were recorded by a video camera connected to a computer, and analysis was carried out with image-processing software. Each rat underwent training twice daily for five consecutive days, starting from one of four randomly assigned starting points around the pool. Escape latency—the time needed for the rat to find the hidden platform—was measured, with a maximum trial duration of 90 seconds. If a rat did not reach the platform within 90 seconds, it was gently guided to the platform and allowed to remain there for 15 seconds, and its escape latency was recorded as 90 seconds. One day after the last training session, the platform was removed, and a 90-second probe trial was conducted to assess memory retention, during which the number of crossings through the area where the platform had been located was recorded (Zhang & Dong, 2024).

Assesment of serum endogenous anti-oxidative enzymes activity

We measured serum antioxidant enzymes using rodent-specific sandwich ELISA kits from Cusabio (China) to quantify SOD, CAT, and GPx levels. The kits used were CSB-EL022397RA for SOD, CSB-E13439r for CAT, and CSB-E12146r for GPx. Assays were performed according to the manufacturers' instructions to ensure accuracy and reliability (Zhao *et al.*, 2018).

Assesment of serum nitric oxide (NO) levels

We used the Griess colorimetric assay to determine serum nitric oxide (NO) levels, a key marker of lipid peroxidation and oxidative stress. In the procedure, 500 μ L of serum was mixed with 6 mg of zinc oxide, thoroughly blended, and then centrifuged at 10,000 g for 15 minutes to separate the components. The collected supernatant was then combined with 500 μ L of Griess reagent. The mixture was incubated at 37 °C for 60 minutes to develop color, after which absorbance was read with a Stat Fax ELISA reader (303-well, Awareness Technology, USA) at 540 nm and 630 nm. This approach provided a dependable measure of serum NO levels, reflecting the oxidative stress status of the subjects (Zhang & Dong, 2024).

Brain tissue thiol, TBARS, and total antioxidant capacity (FRAP) assessments

Thiol determination: To assess thiol levels, a key antioxidant indicator, 100 μ L of brain tissue homogenate was mixed with 20 μ L of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The mixture was incubated at 37 °C for 15 minutes, then centrifuged at 12,000 g for 5 minutes. The absorbance of the supernatant was read at 412 nm with an ELISA reader. Collectively, these measurements reflect the brain's oxidative state and antioxidant defense.

Lipid peroxidation (TBARS): TBARS in brain tissue were quantified by combining 100 μ L of brain homogenate with 100 μ L of TBARS solution and incubating at 37 °C for 30 minutes. The sample was then centrifuged at 12,000 g for 5 minutes, and the supernatant absorbance was measured at 593 nm using an ELISA reader.

Total antioxidant capacity (FRAP): For FRAP analysis, 100 mg of brain tissue was homogenized at 4 °C and diluted with 200 μ L of cold PBS. A 100 μ L aliquot of this homogenate was mixed with 10 μ L of FRAP reagent. After a 15 minutes incubation at 25 °C, the mixture was centrifuged at 12,000 g for 10 minutes, and the absorbance of the supernatant was measured at 593 nm with a Stat Fax

ELISA reader (303-well, Awareness Technology, USA) (Zhang & Dong, 2024).

Serum levels of neurotrophic factors and inflammatory cytokines (GDNF, BDNF, TNF- α , IL-4, IL-1 β , IL-10, and IL-6)

We evaluated the anti-inflammatory effects of PLRE by measuring a panel of cytokines using rodent-specific sandwich ELISA kits from Novus Biologicals (USA). Specifically, we evaluated the anti-inflammatory properties of PLRE by measuring a panel of cytokines using rodent-specific sandwich ELISA kits from Novus Biologicals (USA). Specifically, we quantified the anti-inflammatory cytokines IL-10 (cat. no. R1000) and IL-4 (cat. no. NBP1-91171), along with the pro-inflammatory cytokines IL-1b (cat. no. RLB00), TNF-a (cat. no. NBP2-DY410), and IL-6 (cat. no. M6000B), following the manufacturers' instructions.

Serum samples were prepared by centrifuging whole blood at 10,000 g for 20 minutes. The concentrations of glial cell line-derived neurotrophic factor (GDNF, cat. no. ab244211) and brain-derived neurotrophic factor (BDNF, cat. no. ab108319) in serum were determined using ELISA kits from Abcam (USA), in accordance with the supplier's protocols (Wang *et al.*, 2023).

Brain tissue JAK2 and STAT3 genes expression

Total RNA was isolated using TRIzol reagent in accordance with the manufacturer's protocol (Ambion, China). Subsequently, 5 mg of purified RNA was reverse-transcribed into complementary DNA (cDNA) employing random primers. Quantitative real-time PCR analysis was performed using SYBR Green chemistry (Vazyme, China) on an ABI 7900 real-time PCR platform (CA, USA). The thermal cycling program consisted of an initial incubation at 50 °C for 2 min, followed by enzyme activation at 95 °C for 10 min, and then 40 amplification cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. Transcript levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. The primer sequences used were:
 β -actin: Forward: CCTCTTAATGTCACGCACGAT; Reverse: GCTGCTCGGAGTCCATCTC
JAK2: Forward: GCTGCTGCTGCTGTTGGTAA; Reverse: TGGCTGCTGAGGAGGTACTG
STAT3: Forward: GACCTGGCGGATACCTTCTC; Reverse: CTGAGGCTGCTGGTCTGATA

Relative mRNA expression was determined using the cycle threshold (Ct) values and calculated with the $2^{-\Delta\Delta C_t}$ method to obtain fold changes.

The fold formula change = $2^{-\Delta\Delta Ct}$; $\Delta\Delta Ct = [(Ct \text{ sample} - Ct \text{ GAPDH gene}) - (Ct \text{ sample} - Ct \text{ control})]$ (Gautam *et al.*, 2019).

Western blot

The cerebral cortices of rats from each group were lysed on ice with precooled lysis buffer (Dalian Meilunbio) to prepare tissue homogenates, while cultured TNC-1 astrocytes were lysed in the same manner. Cells were harvested 15 minutes after cleavage using a cell scraper. The tissue homogenates and cell proteins were then centrifuged at 14,000 rpm at 4 °C for 20 minutes, and the supernatant was collected. Protein concentrations were determined using a BCA protein assay kit (Dalian Meilunbio). The protein samples were heated at 95 °C for 10 minutes, loaded onto SDS-PAGE gels, and separated by electrophoresis. Afterward, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5 % skim milk powder for 2 hours. The membranes were then cut and incubated with primary antibodies for p-JAK2 (1:1000), JAK2 (1:1000), p-STAT3 (1:2000), STAT3 (1:1000) (Cell Signaling Technology, cat. nos. 3776, 3230, 9145, and 12,640, respectively), and b-actin (1:5000, Proteintech, cat. no. 66009-1-Ig). After 16 hours of incubation with the primary antibodies, the membranes were incubated with secondary antibodies: goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (1:5000, Proteintech, cat. nos. SA00001-2 and SA00001-1). The specific protein bands were visualized using an ECL chemiluminescence kit (Biosharp Life Sciences) and a gel imaging system (Bio-Rad). All experiments were performed in triplicate (Zhuang *et al.*, 2021).

Brain stereology and histopathology

In the present investigation, brain tissue morphology and neuronal architecture were assessed using methylene blue and Golgi staining techniques. Brain specimens were first fixed in 10 % paraformaldehyde for 24 - 48 hours and subsequently rinsed with phosphate-buffered saline (PBS). The tissues were then dehydrated through ascending concentrations of ethanol, cleared with xylene or a comparable clearing reagent, and embedded in paraffin wax. Paraffin blocks were sectioned at a thickness of 5–10 mm using a rotary microtome (LEICA SM2010RV1.2, Germany). After rehydration, tissue sections were stained with 1 % methylene blue for 10–20 minutes, rinsed in PBS, dehydrated again, cleared, and permanently mounted with a coverslip. For Golgi staining, freshly prepared brain samples were sequentially incubated in potassium dichromate ($K_2Cr_2O_7$) followed by silver nitrate ($AgNO_3$) solutions, maintaining a 1:3 molar ratio for 24–48 hours to enable silver

chromate deposition within neuronal processes. Following incubation, sections were thoroughly washed with deionized water, dehydrated through graded ethanol solutions (70 %, 95 %, and absolute ethanol), cleared in xylene, infiltrated with molten paraffin wax at 56–58 °C for 4–6 hours, sectioned at 4–6 mm, and mounted onto pretreated glass slides using an albumin-based adhesive to preserve neuronal morphology for light microscopic evaluation.

Histopathological examinations were performed using a light microscope at magnifications of $\times 100$ and $\times 400$. Digital images were captured with a calibrated BX61TRF microscopy system (Olympus, Japan) and analyzed using ImageJ software. A quantitative analysis of the relative volume (Vv) of various brain components—including total brain tissue, neuronal parenchyma, blood vessels, ventricles, and ischemic areas—was conducted using the point-counting method. This analysis utilized a Nikon light microscope paired with Kecam imaging hardware (Kecam Technologies) and TopView software (version 3.7), with additional image processing completed in Adobe Photoshop CC (Adobe Systems). For the rat brain gross tissue with a focal ischemic area (indicated by a circle), the orientator method was employed to produce isotropic uniform random sections. Each rat brain was positioned on a circle divided into 10 equal segments (u-clock). A random number between 0 and 10 was generated, and the brain was sectioned into two halves in that direction. The cut surface of one half was oriented vertically on the h-clock, which featured 10 unequal sinus-weighted divisions. A second cut was made by selecting another random number. The cut surface of the remaining half of the brain was aligned parallel to the 0–0 direction of the h-clock, and the second cut was again determined by a random selection (in this case, 3). The entire brain was then sliced into slabs, with the blade directed according to the second cuts, resulting in between 7 and 10 slabs being obtained. A trocar was utilized to extract a circular sample from one of the brain slabs. The diameter of this circular piece and the area of the circle were subsequently calculated. A point probe with 50 points was employed to estimate the volume density of all structures. A total of 50 points were used to calculate the volumetric density of the brain, with the number of points counted for each structure in each field of view and compared to the total number of points in the volume measurement formula: $Vv = \sum P \text{ structure} / \sum P \text{ reference}$ (Fig. 1) (Eronat & Sagır, 2020).

Statistical analysis

The distribution of data was assessed using the Kolmogorov–Smirnov test, establishing a significance threshold at 0.05. All quantitative variables, except for the neurological function scores, exhibited a normal distribution

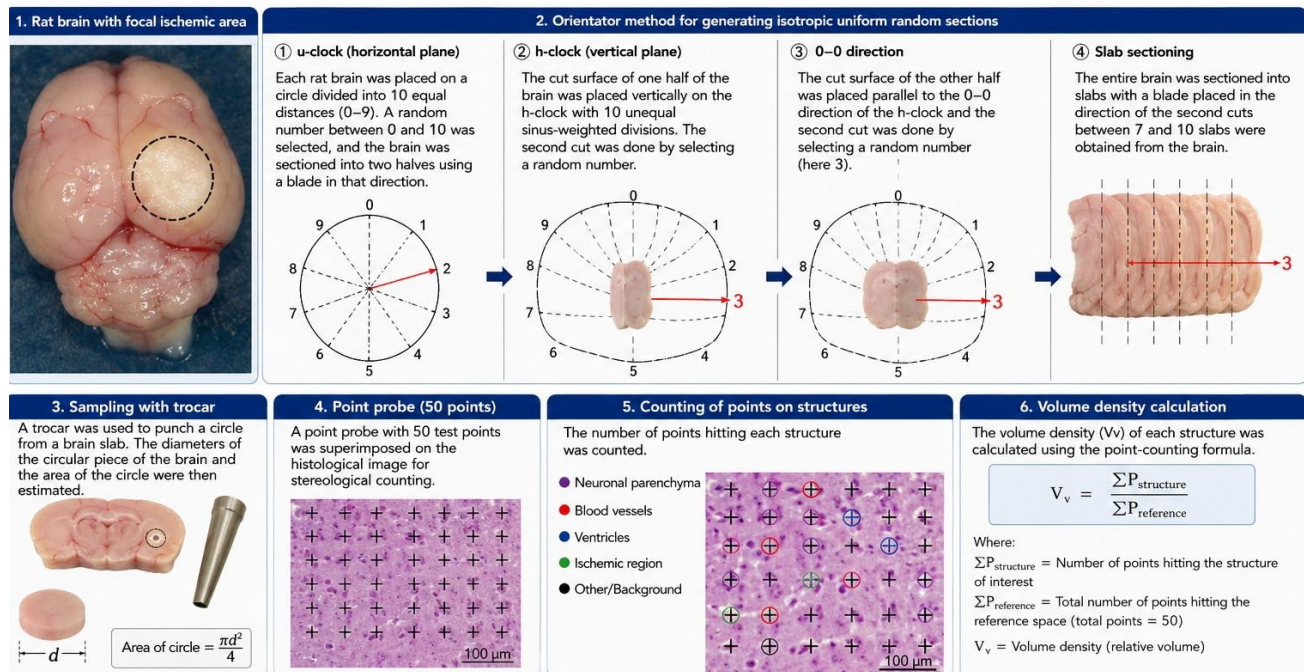


Fig. 1. (1-6) Stereological approach steps for rat cerebral ischemia through quantitative and unbiased estimation of the orientator and Cavalieri methods.

($p > 0.05$). As a result, these data are expressed as mean $\pm$ standard deviation (SD) and were analyzed across groups using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test. Since neurological function scores did not conform to parametric assumptions, they were evaluated using the Kruskal–Wallis test and are reported as median values along with their corresponding minimum and maximum ranges. Statistical significance was set at $p < 0.05$.

RESULTS

Integrated network of Biochanin A and brain ischaemia, functional enrichment analyses, and PPI network construction and analysis

Based on the predictions from PharmMapper and SwissTargetPrediction, a total of 122 targets were identified for Biochanin A, with duplicates removed. By intersecting the prediction results from the OMIM, GeneCards, and DisGeNET databases, we obtained 91 unique targets related to brain ischemia. A target interaction network connecting Biochanin A and brain ischemia was constructed using Cytoscape, comprising 4,372 nodes and 107,457 edges. This network was refined by applying filters based on Degree, Betweenness, Closeness Centrality, LAC, and Neighborhood Connectivity, resulting in a new network with 54 nodes and 675 edges. To investigate the interactions among these 54

common target proteins, a protein-protein interaction (PPI) network was generated from the STRING database and visualized in Cytoscape. The final network included 54 nodes representing 29 protein targets and 321 edges that depict 311 pairs of protein interactions. In this network, nodes colored in warm hues and larger in size indicated more interactions (edges), suggesting a higher degree of importance for further study compared to the cooler-colored, smaller nodes. According to the degree values, the 13 core nodes identified were: ACTB (degree 21), CTNNB1 (degree 29), HSPA4 (degree 14), VCP (degree 17), RPS27A (degree 11), NPM1 (degree 19), PARP1 (degree 15), HSPA8 (degree 17), CDK1 (degree 23), YWHAZ (degree 14), and STAT3 (degree 61). Further analysis of the KEGG pathway involving STAT3 indicated that the chemokine signaling pathway activates the JAK/STAT signaling pathway, with the JAK2/STAT3 signaling pathway playing a crucial role in regulating the inflammatory response. Consequently, this pathway was selected for further experimental validation (Fig. 2).

Biochanin A improved MCAO -induced spatial cognitive deficits

Treatment with Biochanin A alleviated spatial learning and memory impairments induced by MCAO, as assessed by the Morris water maze after 50 days of administration. Across the training period, escape latency

progressively decreased in all groups; however, from day 4 onward, the normal control group performed significantly better than the MCAO group ($p < 0.05$). Rats receiving Biochanin A at doses of 200 or 400 mg/kg following MCAO exhibited significantly longer escape latencies than normal controls on days 4 and 5 ($p < 0.05$), yet their performance did not differ significantly from that of the untreated MCAO group ($p > 0.05$), indicating a dose-related trend toward

improved learning. In the probe trial, animals treated with 400 mg/kg Biochanin A crossed the former platform location significantly more frequently than MCAO rats without treatment ($p < 0.05$), reflecting improved spatial memory retention. Overall, these results suggest that high-dose Biochanin A (400 mg/kg) effectively mitigates both acquisition deficits during training and memory impairment during the retention phase following MCAO (Fig. 3a & 3b).

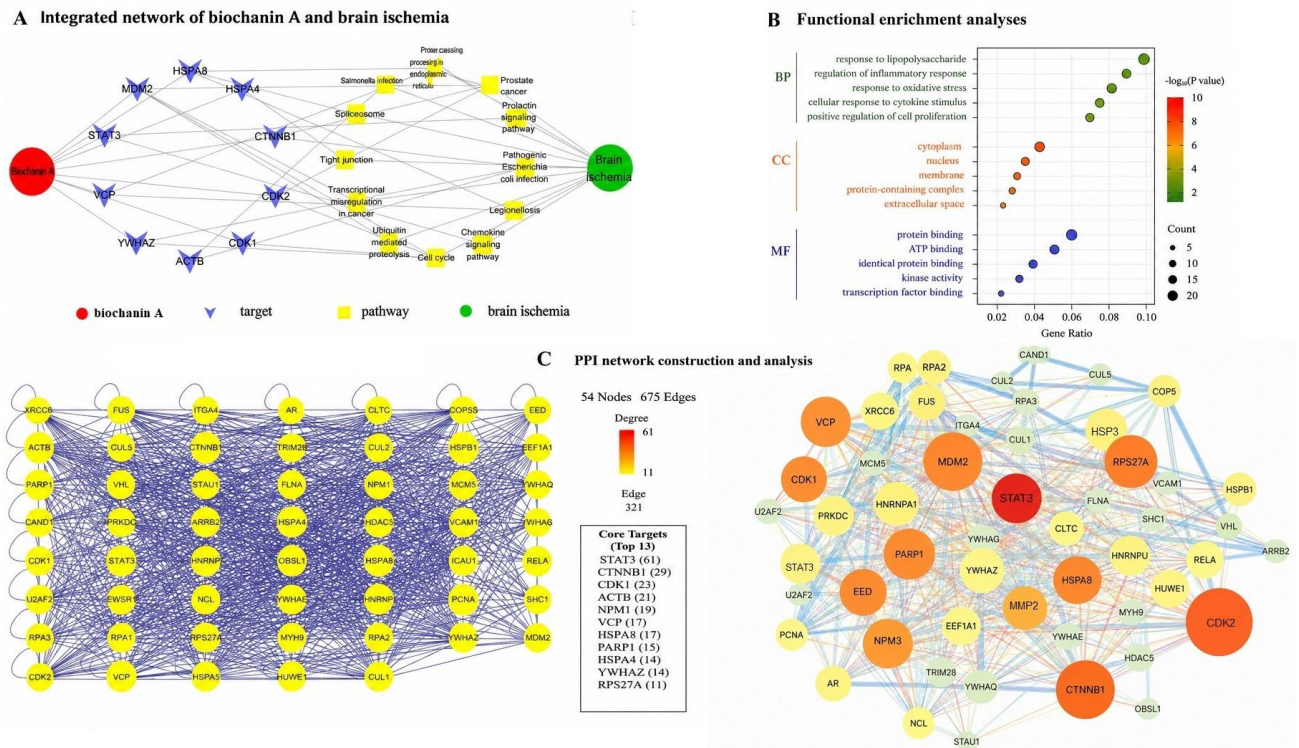
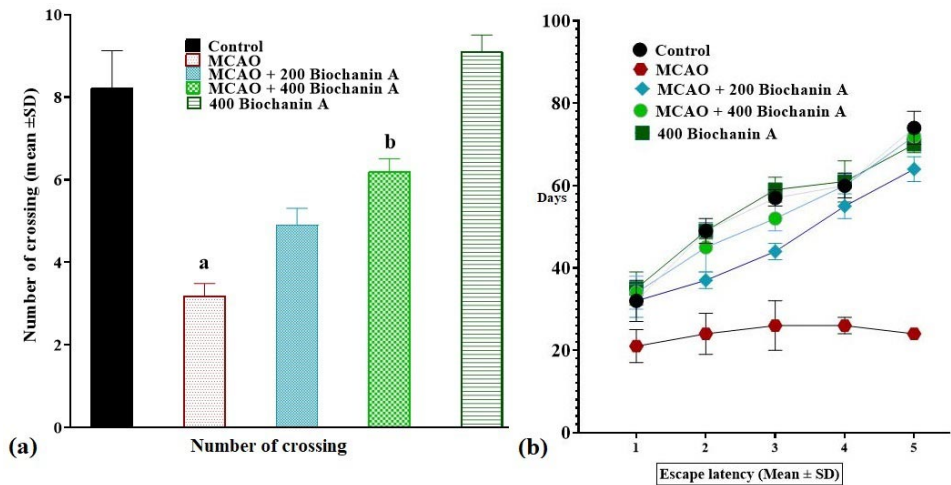


Fig. 2. (A) Chart depicting the drug–target–pathway–disease network. (B) Bubble chart illustrating the enrichment of GO and KEGG pathways. This outlines the screening process for identifying common targets between scutellarin and brain ischemia, where all connections with scores below the threshold are eliminated. Visualization was carried out using Cytoscape (<http://cytoscape.org/>, version 3.70). (C) PPI network showcasing the common target proteins, also visualized using Cytoscape (<http://cytoscape.org/>, version 3.70).



Biochanin A and MCAO effects on serum concentrations of GDNF and BDNF

MCAO markedly inhibited neurotrophic factor production by triggering apoptotic signaling in glial cells, resulting in a significant decrease ($p < 0.05$) in serum GDNF and BDNF levels compared with the control group. In contrast, administration of Biochanin A at both tested doses elevated circulating GDNF and BDNF concentrations, underscoring its pronounced glial-supportive and neuroprotective effects. Importantly, animals treated with 400 mg/kg Biochanin A following MCAO exhibited significantly higher GDNF and BDNF levels than untreated MCAO rats ($p < 0.05$) (Fig. 4a).

Biochanin A and MCAO effects on serum concentrations of TNF- α , IL-4, IL-6, IL-10, and IL-1 β

MCAO induced a robust inflammatory response

characterized by elevated pro-inflammatory cytokine production alongside suppression of systemic anti-inflammatory mediators. Consequently, serum levels of TNF- α , IL-1 β , and IL-6 were significantly increased ($p < 0.05$), whereas IL-10 and IL-4 concentrations were markedly reduced ($p < 0.05$) compared with the control group. In rats treated with 200 mg/kg Biochanin A following MCAO, serum TNF- α and IL-6 levels were reduced relative to untreated MCAO animals; however, these changes did not achieve statistical significance ($p > 0.05$), indicating a trend toward anti-inflammatory activity. Moreover, Biochanin A administration produced a dose-dependent immunomodulatory effect, characterized by progressive suppression of pro-inflammatory cytokines (IL-1 β , TNF- α , and IL-6) and enhancement of anti-inflammatory cytokines (IL-10 and IL-4). These effects were significantly pronounced ($p < 0.05$) in the MCAO + 400 mg/kg Biochanin A group compared with the MCAO group (Fig. 4b).

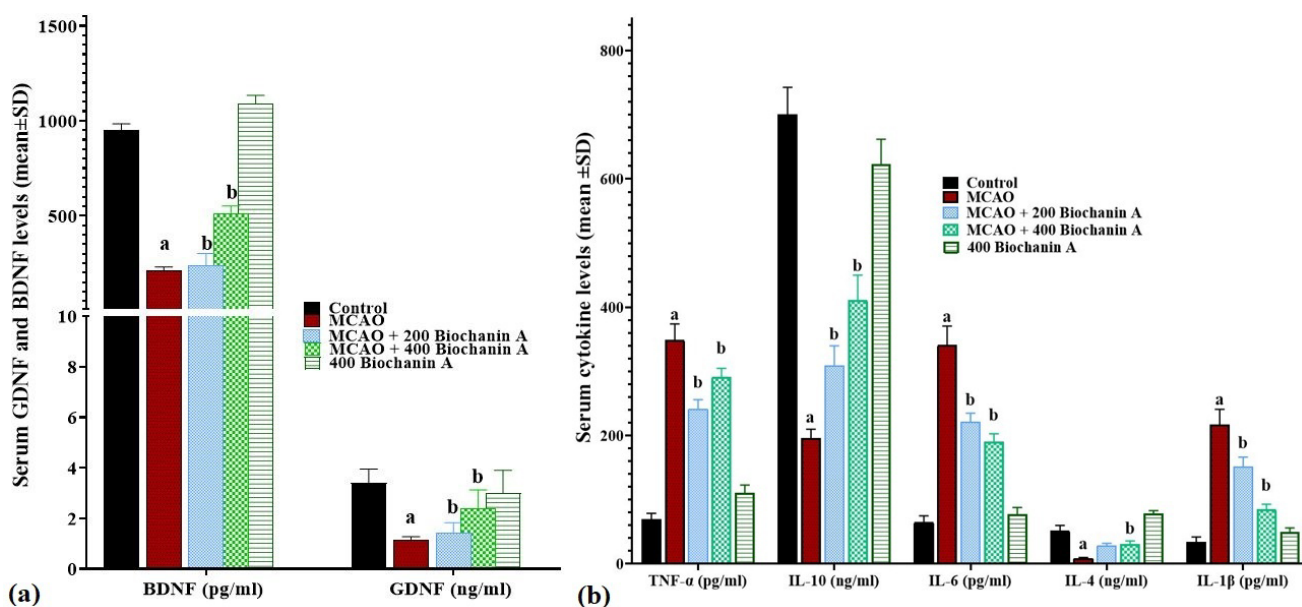


Fig. 4. Serum biomarker profile of (a) neurotrophic factors (GDNF [ng/mL] and BDNF [pg/mL]) and (b) inflammatory mediators (pro-inflammatory: IL-1 β , TNF- α , IL-6 [pg/mL]; anti-inflammatory: IL-10, IL-4 [ng/mL]) across experimental groups (means $\pm$ SD; $n = 10$ /group) in experimental groups. a ($p < 0.05$) MCAO vs. control groups; b ($p < 0.05$) 200 and 400 mg/kg Biochanin A treated vs. MCAO groups.

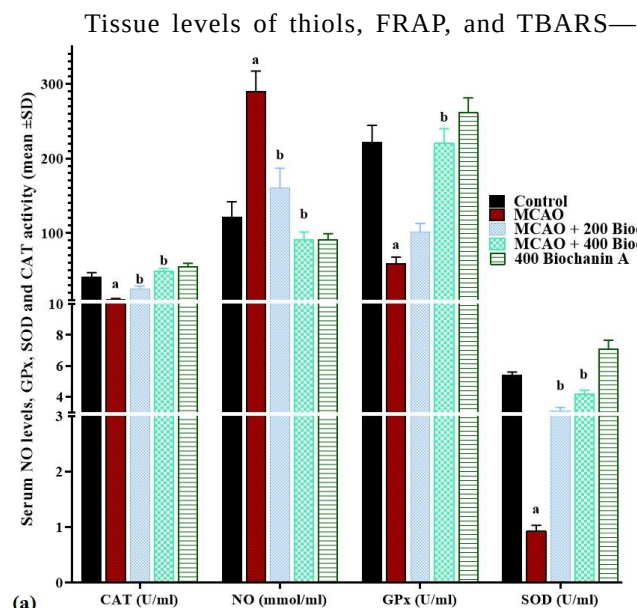
Biochanin A and MCAO effects on serum GPx, CAT, and SOD activity alongside serum NO levels

MCAO promoted excessive free radical generation, as evidenced by a significant elevation in serum nitric oxide (NO) levels compared with the control group. In contrast, Biochanin A administration in both the MCAO + 200 and MCAO + 400

groups attenuated NO production relative to untreated MCAO animals. Treatment with Biochanin A at both tested doses produced a significant reduction in serum NO concentrations ($p < 0.05$), highlighting its potent antioxidant capacity. Furthermore, MCAO markedly suppressed the serum activities of all three endogenous antioxidant enzymes compared with controls. Biochanin A treatment at 200 and 400 mg/kg

effectively restored these enzyme activities, resulting in significant increases ($p < 0.05$) in the MCAO + Biochanin A groups relative to the MCAO group (Fig. 5a).

Biochanin A and MCAO effects on brain thiol, FRAP, and TBARS levels



established markers of total antioxidant capacity and lipid peroxidation—were assessed to evaluate oxidative status. MCAO significantly reduced these parameters ($p < 0.05$) compared with the control group. In contrast, Biochanin A treatment exerted pronounced antioxidant effects, resulting in significantly elevated levels of these markers in the MCAO + 200 and MCAO + 400 Biochanin A groups relative to the MCAO group (Fig. 5b).

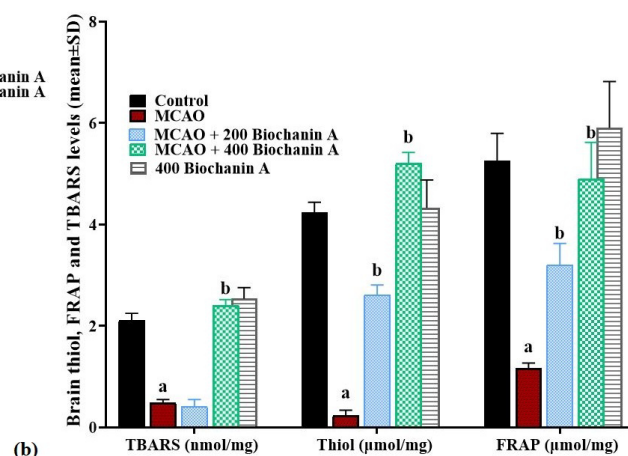


Fig. 5. Oxidative stress modulation profile: (a) Serum nitric oxide (NO) concentration (mmol/mL) and antioxidant enzyme activities (SOD, CAT, GPx; U/mL) (b) Brain tissue oxidative damage markers: TBARS (lipid peroxidation, nmol/mg), thiol content (sulfhydryl groups, μmol/mg), and FRAP assay (antioxidant capacity, μmol/mg) across experimental groups (mean ± SD; n=10). a ($p < 0.05$) MCAO vs. control groups; b ($p < 0.05$) 200 and 400 mg/kg Biochanin A treated vs. MCAO groups.

Biochanin A and MCAO effects on expression of brain JAK2 and STAT3 genes

Analysis of gene expression related to apoptosis, oxidative stress, and metabolic pathways in glial and neuronal cells revealed that MCAO significantly downregulated JAK2 and STAT3 expression in the brain compared with controls ($p < 0.05$). This effect was markedly reversed in the MCAO + 400 mg/kg Biochanin A group, where JAK2 and STAT3 levels were significantly increased relative to the MCAO group ($p < 0.05$), suggesting that Biochanin A modulates inflammatory signaling through the JAK2/STAT3 pathway (Fig. 6a).

Biochanin A and MCAO effects on expression of TNC-1 astrocytes p-JAK2, JAK2, p-STAT3, and STAT3 proteins

Protein expression analysis of apoptosis-, oxidative stress-, and metabolism-related pathways in glial and neuronal cells demonstrated that MCAO significantly decreased the levels of p-JAK2, JAK2, p-STAT3, and STAT3 in TNC-1 astrocytes compared with the control group ($p < 0.05$).

This downregulation was most effectively reversed in the MCAO + 400 mg/kg Biochanin A group, which exhibited significant upregulation of p-JAK2, JAK2, p-STAT3, and STAT3 protein levels relative to the MCAO group ($p < 0.05$), highlighting the involvement of JAK2/STAT3-mediated inflammatory signaling pathways (Fig. 6b & 6c).

Brain stereological and histopathological evaluations

MCAO injury caused significant reductions ($p < 0.05$) in total brain volume (TBV) and neuronal parenchymal tissue volume (NPTV), accompanied by significant increases ($p < 0.05$) in blood vessel volume (BVV) and ischemic foci volume (FIV) compared with controls. These changes were associated with heightened neuroinflammation and structural damage to both neuronal and glial components. Treatment with Biochanin A, particularly at 400 mg/kg (MCAO + 400 Biochanin A group), exhibited significant neuroprotective effects ($p < 0.05$), attenuating inflammatory responses, preserving parenchymal integrity, and partially normalizing vascular alterations, thereby mitigating MCAO-induced volumetric and pathological changes relative to untreated MCAO animals (Fig. 7).

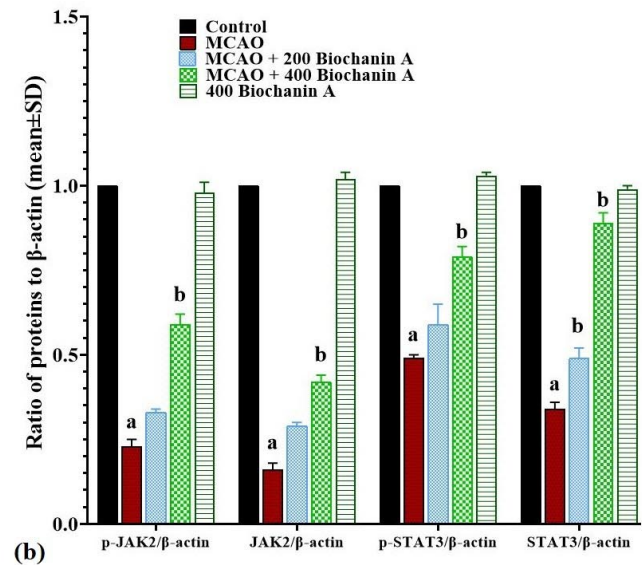
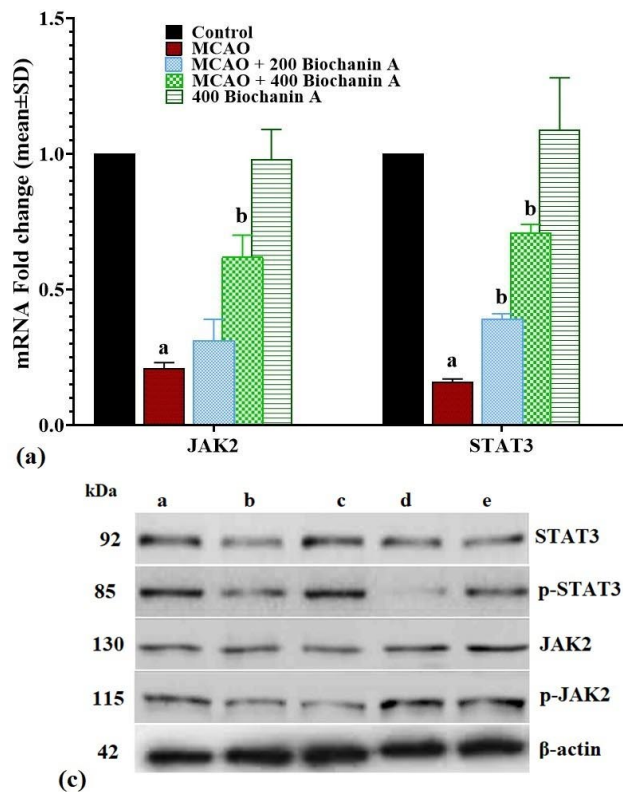


Fig. 6. Molecular mechanisms of ischemia-reperfusion injury and Biochanin A-mediated neuroprotection via JAK2/STAT3 pathway in brain tissue (a) Brain tissue JAK2 and STAT3 genes expression (b) and (c) Brain tissue p-JAK2, JAK2, p-STAT3, and STAT3 proteins expression levels in (a) Control, (b) MCAO, (c) MCAO + 200 Biochanin A, (d) MCAO + 200 Biochanin A, and (e) 400 Biochanin A (mean $\pm$ SD; n=10/group). a (p<0.05) MCAO vs. control groups; b (p<0.05) 200 and 400 mg/kg Biochanin A treated vs. MCAO groups.

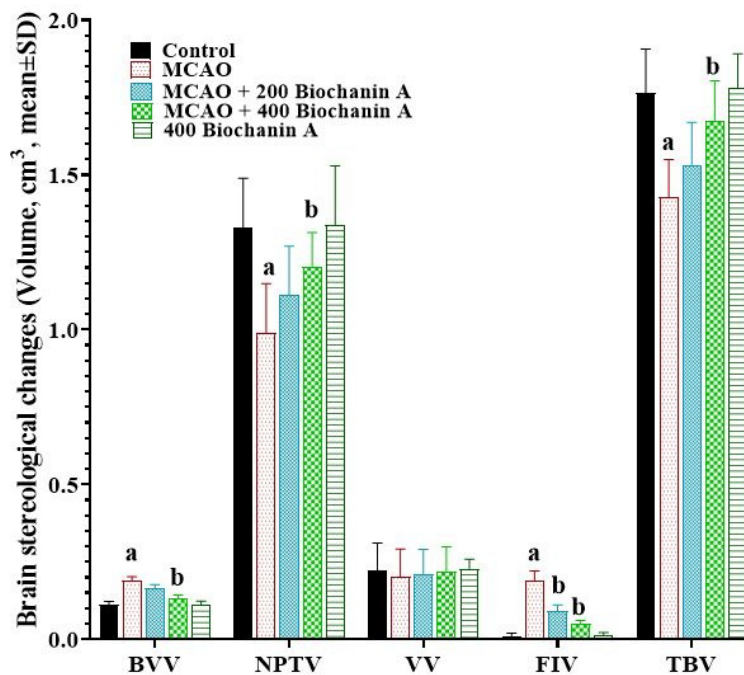


Fig. 7. Stereological changes in brain tissue across experimental groups (mean $\pm$ SD; n=10/group). a (p<0.05) MCAO vs. control groups; b (p<0.05) 200 and 400 mg/kg Biochanin A treated vs. MCAO groups. Total brain (TBV), neuronal parenchyma tissue (NPTV), blood vessels (BVV), and ischemic foci (FIV) volume.

Histopathological analysis revealed that MCAO induced pronounced lymphocytic infiltration, vascular injury, neurons with pyknotic nuclei and vacuolated cytoplasm, apoptotic bodies, and degenerated neurons adjacent to necrotic regions. The MCAO group displayed marked neuronal atrophy and a substantial reduction in normal parenchymal density compared with controls. In contrast, Biochanin A administration at both 200 and 400 mg/kg increased neuronal parenchymal density and substantially decreased apoptotic bodies, neuronal degeneration, and lymphocytic infiltration relative to the MCAO group (Fig. 8, Fig. 9 & Fig. 10).

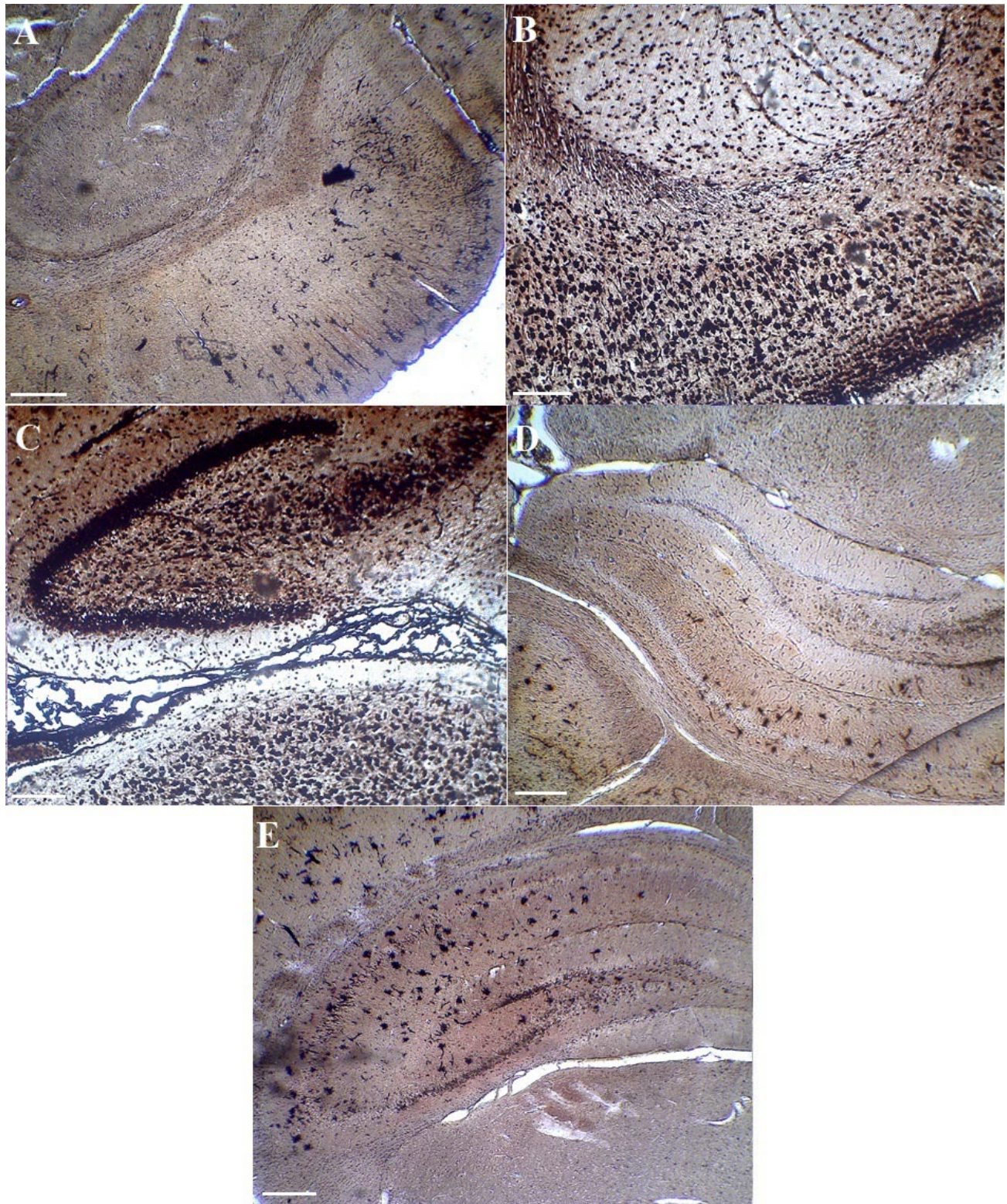


Fig. 8. Histopathological changes in brain tissue in (A) Control, (B) MCAO, (C) MCAO + 200 Biochanin A, (D) MCAO + 200 Biochanin A, and (E) 400 Biochanin A alone (Golgi's staining $\times 100$ with scale bar = 200 μm).

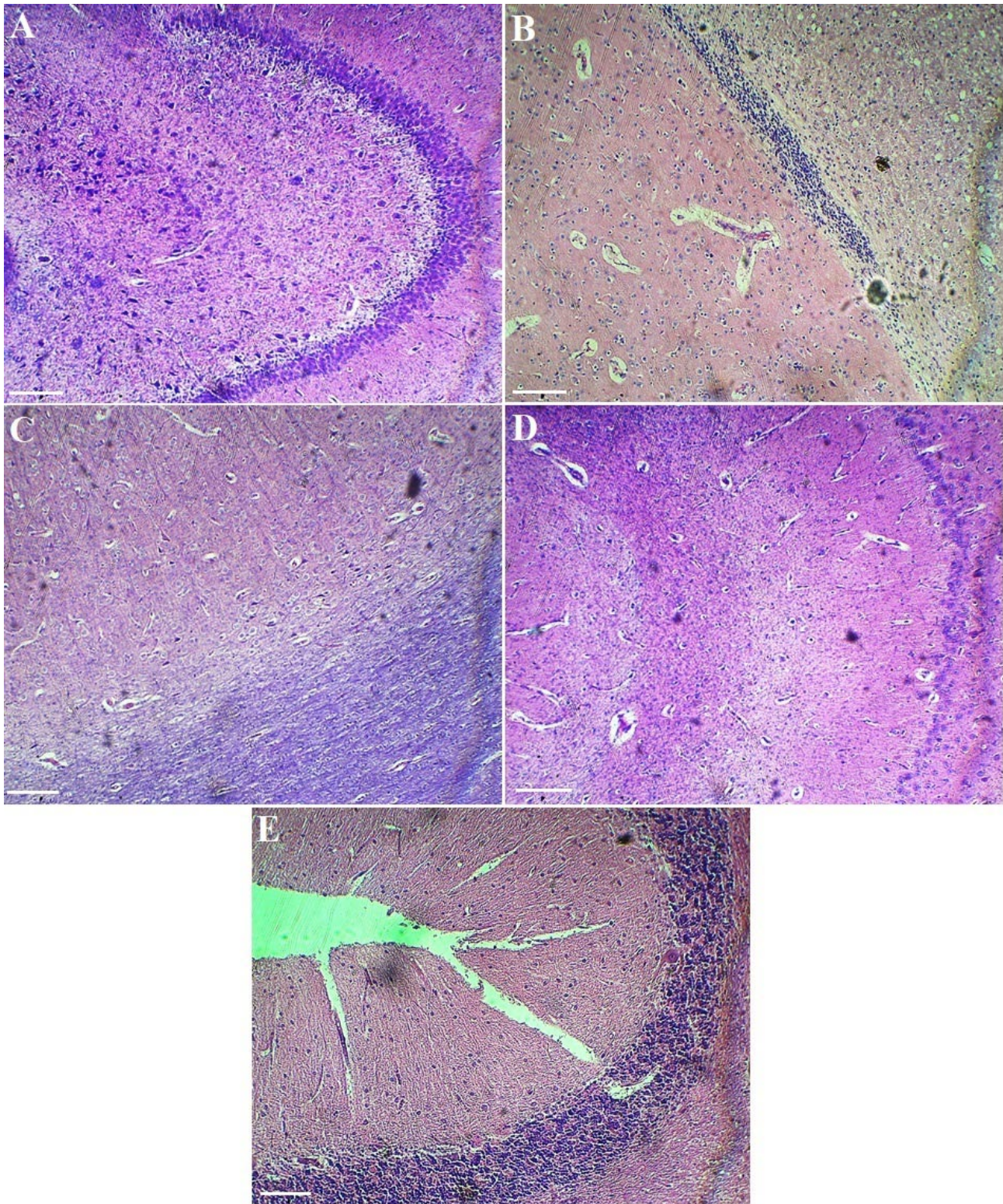


Fig. 9. Histopathological changes in brain tissue in (A) Control, (B) MCAO, (C) MCAO + 200 Biochanin A, (D) MCAO + 200 Biochanin A, and (E) 400 Biochanin A alone (Haematoxyline and eosine staining $\times 100$ with scale bar = 200 μm).

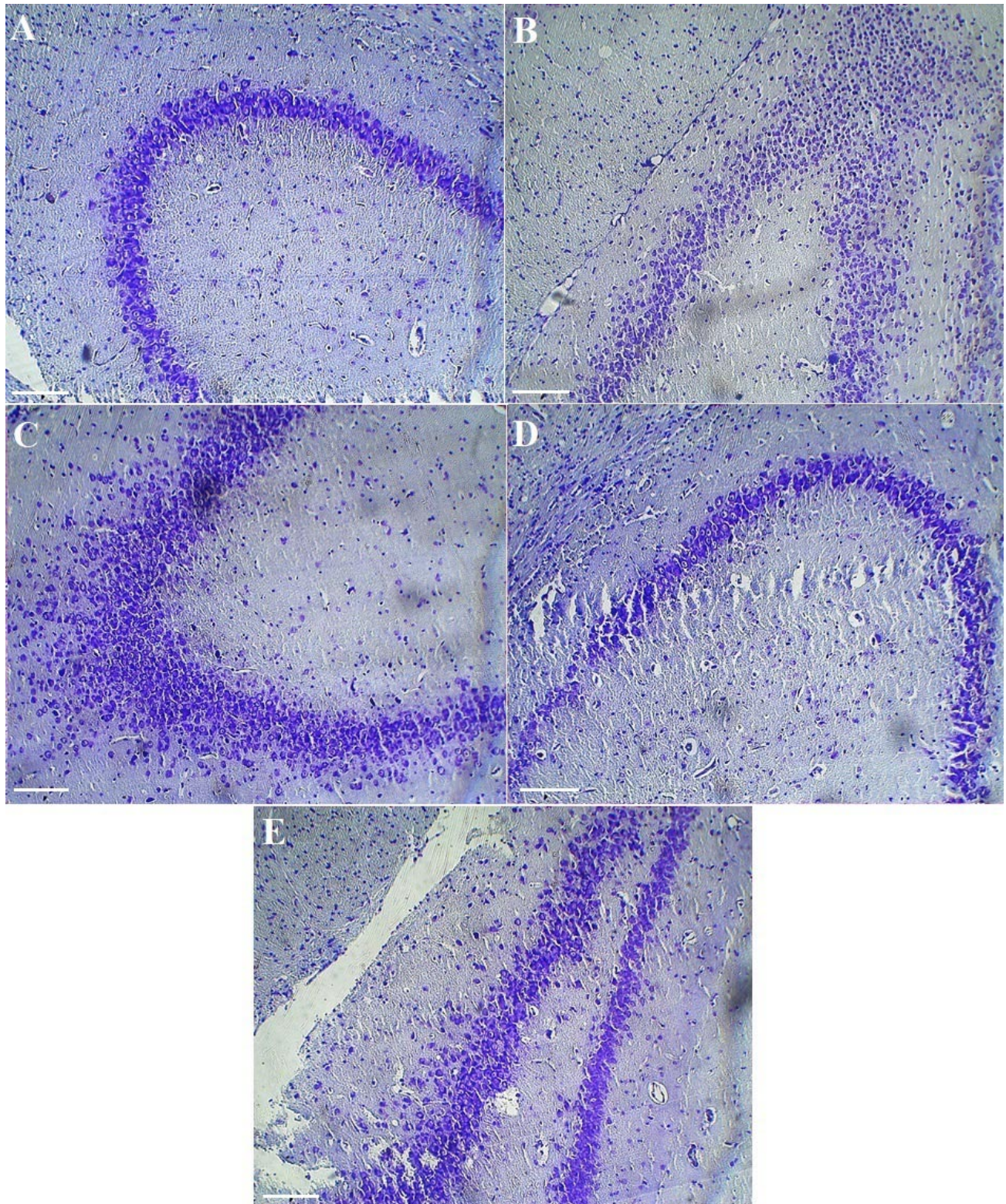


Fig. 10. Histopathological changes in brain tissue in (A) Control, (B) MCAO, (C) MCAO + 200 Biochanin A, (D) MCAO + 200 Biochanin A, and (E) 400 Biochanin A alone (Methylen blue staining $\times 100$ with scale bar = 200 μm).

DISCUSSION

Ischemic stroke is a critical global health issue, recognized as one of the leading causes of long-term disability and mortality. According to the World Health Organization (WHO), nearly 85 % of all strokes are ischemic in nature, primarily resulting from the occlusion of the middle cerebral artery (MCA). This blockage initiates a complex cascade of neuroinflammatory responses that exacerbate brain injury and impair recovery, leading to significant neuronal death, disrupted cellular signaling, and a range of pathological events, including oxidative stress, amyloid production, and tau protein dysfunction (Feigin *et al.*, 2022). The multifaceted nature of ischemic stroke pathophysiology contributes to its severity and long-term consequences, making it a significant public health challenge. Despite advances in medical science, the clinical management of ischemic stroke remains fraught with challenges. Current therapeutic options, such as recombinant tissue plasminogen activator (rt-PA), have proven effective in certain contexts but are limited by a narrow therapeutic time window, numerous contraindications, and potential neurotoxicity (Polk *et al.*, 2018). These limitations highlight the urgent need for alternative therapeutic strategies that can effectively mitigate stroke-induced brain injury and promote neurological recovery. In this context, the exploration of natural compounds with neuroprotective properties, such as Biochanin A, derived from *Trifolium pratense* (red clover), has gained prominence (Raheja *et al.*, 2018).

Biochanin A, a natural isoflavonoid, has emerged as a promising candidate in the treatment of various brain disorders, including ischemic stroke. Its neuroprotective effects can be attributed to several mechanisms, including its antioxidant, anti-inflammatory, and neuroprotective properties. The oxidative stress induced by ischemic conditions leads to the generation of excessive reactive oxygen species (ROS), which are known to cause neuronal necrosis and apoptosis (Tripathi *et al.*, 2024). Biochanin A has been shown to effectively scavenge these ROS, thereby mitigating neuronal death and improving patient prognosis after stroke. In addition to its antioxidant activity, Biochanin A exhibits significant anti-inflammatory effects. The activation of microglia, the resident immune cells of the central nervous system (CNS), plays a pivotal role in the neuroinflammatory response following ischemic injury. Upon activation, microglia migrate toward the infarct core and penumbral regions, where they phagocytose cellular debris and release pro-inflammatory cytokines, exacerbating tissue damage (Guo *et al.*, 2019).

Biochanin A has been found to modulate this microglial activation, reducing the release of pro-

inflammatory cytokines and enhancing the resolution of inflammation. Furthermore, it promotes the activation of astrocytes via Lrg1/TGF- β /Smad2 pathway blockade, which are crucial for maintaining the integrity of the blood-brain barrier (BBB) and regulating neuronal homeostasis (Fu *et al.*, 2023). Numerous preclinical studies have demonstrated that high doses of Biochanin A (ranging from 100 to 400 mg/kg) significantly reduce infarct volume and improve neurological function in experimental animal models of cerebral ischemia. For instance, studies comparing Biochanin A with other neuroprotective agents, such as edaravone, have shown that Biochanin A provides superior neuroprotection by reducing cerebral infarct volume and enhancing recovery following stroke (Zhou *et al.*, 2021). These findings underscore the potential of Biochanin A as a therapeutic agent in ischemic brain injury. However, the precise mechanisms by which Biochanin A exerts its protective effects in ischemic stroke remain unclear, necessitating further investigation to elucidate its action.

The JAK2/STAT3 signaling pathway has emerged as a critical regulator of neuroinflammation and neuronal survival in ischemic stroke. JAK2 and STAT3 are key components of a signaling cascade that regulates numerous cellular processes, including inflammation, cell survival, and tissue repair (Zhu *et al.*, 2021). In response to ischemic injury, the JAK2/STAT3 pathway is activated, leading to the phosphorylation of JAK2 and STAT3. Phosphorylated JAK2 activates STAT3, which translocates to the nucleus and regulates the expression of target genes involved in neuroprotection and angiogenesis. Previous studies have demonstrated that activation of the JAK2/STAT3 pathway enhances cerebral angiogenesis, promotes neuronal survival, and accelerates neurological recovery following ischemic stroke. Moreover, this pathway has been implicated in mediating the neuroprotective effects of various therapeutic agents, including resveratrol (Ahmad *et al.*, 2018). Given the complex interactions between microglia, astrocytes, and the JAK2/STAT3 pathway, our study aimed to elucidate the neuroprotective mechanism of Biochanin A in ischemic stroke through a network pharmacology-driven approach. Focusing on the JAK2/STAT3 signaling pathway, we hypothesized that Biochanin A modulates this pathway to reduce neuroinflammation, oxidative stress, and neuronal apoptosis. Our findings indicate that Biochanin A significantly improves neurological function and reduces infarct volume in MCAO-induced ischemic stroke models. The administration of Biochanin A at doses of 200 mg/kg and 400 mg/kg resulted in a dose-dependent reduction of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-

6, while enhancing the levels of anti-inflammatory cytokines like IL-10 and IL-4. These results suggest that Biochanin A effectively modulates the inflammatory response in the ischemic brain. Furthermore, Biochanin A administration was associated with a significant increase in serum levels of neurotrophic factors, including GDNF and BDNF. These neurotrophic factors play a crucial role in neuronal survival and repair, highlighting the potential of Biochanin A to support neuronal health following ischemic injury (Gong *et al.*, 2025). Biochanin A also exhibited potent antioxidant activity, as evidenced by its ability to reduce serum NO levels and restore the activity of endogenous antioxidant enzymes, including SOD, CAT, and GPx. The reduction of oxidative stress is vital in mitigating neuronal damage and promoting recovery following ischemic events. Our analysis of gene expression revealed that Biochanin A administration significantly upregulated the expression of JAK2 and STAT3 in the brain following MCAO. The activation of the JAK2/STAT3 pathway was further confirmed by the increased levels of phosphorylated JAK2 (p-JAK2) and phosphorylated STAT3 (p-STAT3) in the treated groups. This suggests that Biochanin A not only activates the JAK2/STAT3 signaling pathway but also enhances its downstream effects, promoting neuroprotection and tissue repair.

Interestingly, while the activation of the JAK2/STAT3 pathway (via JAK2/STAT3/PI3K/AKT/mTOR pathway) is generally associated with neuroprotection, some studies have indicated that aberrant activation may contribute to the inflammatory response in ischemic stroke (Hou *et al.*, 2018). This duality highlights the complexity of the JAK2/STAT3 signaling pathway and emphasizes the need for further research to clarify the context-dependent effects of its activation. Our findings suggest that Biochanin A may selectively activate beneficial aspects of the JAK2/STAT3 pathway while mitigating potential adverse effects, thus providing a nuanced approach to therapeutic intervention in ischemic stroke. The promising preclinical results regarding Biochanin A's neuroprotective effects warrant further investigation into its clinical applicability. Understanding the molecular mechanisms underlying its action will be crucial for translating these findings into clinical practice. The network pharmacology approach employed in this study provides a comprehensive framework for identifying the molecular targets and pathways modulated by Biochanin A. By elucidating the interactions between Biochanin A, the JAK2/STAT3 pathway, and the inflammatory response, we can develop targeted therapies that enhance recovery in ischemic stroke patients. Future research should also investigate the potential of combining Biochanin A with existing therapies to enhance therapeutic outcomes.

CONCLUSION

Our study elucidates the neuroprotective mechanism of Biochanin A in the context of cerebral ischemia, highlighting its potential as a therapeutic agent for ischemic stroke. By employing a network pharmacology-driven approach, we demonstrated that Biochanin A significantly improves neurological function and reduces infarct volume in MCAO-induced ischemic models. The modulation of the JAK2/STAT3 signaling pathway was identified as a critical mechanism underlying its protective effects, as evidenced by enhanced expression of neurotrophic factors and a favorable shift in inflammatory cytokine profiles. These findings underscore the importance of Biochanin A in mitigating neuroinflammation and promoting neuronal survival, paving the way for future clinical applications in stroke management. Further investigation into its synergistic effects with existing therapies could enhance recovery outcomes for patients suffering from ischemic stroke.

Ethical Approval. The experimental protocols of this study were approved by the Mongolian Medical of College Inner Mongolia Minzu University ethics committee.

CHARILATU, W. & JIE, X. Elucidación mediante farmacología de redes del mecanismo neuroprotector de la biocanina A en la isquemia cerebral: Modulación de la vía de señalización JAK2/STAT3 mediante la regulación de p-JAK2, JAK2, p-STAT3 y STAT3. *Int. J. Morphol.*, 44(4):1263-1278, 2026.

RESUMEN: La isquemia cerebral resultante de la oclusión de la arteria cerebral media (OACM) deteriora significativamente la función neuronal y exacerba los déficits cognitivos. Este estudio investiga los efectos neuroprotectores de la biocanina A, una isoflavona natural, en un modelo de OACM utilizando ratas Wistar. Cincuenta ratas se dividieron en cinco grupos: un grupo control, un grupo OACM y tres grupos de tratamiento que recibieron 200 mg/kg y 400 mg/kg de biocanina A. La función cognitiva se evaluó mediante la prueba del laberinto acuático de Morris, mientras que los niveles séricos de factores neurotróficos (GDNF y BDNF) y citoquinas inflamatorias (TNF- α , IL-1 β , IL-6, IL-10 e IL-4) se midieron mediante ensayo por inmunoabsorción ligado a enzimas (ELISA). Además, se analizó la expresión génica de JAK2 y STAT3 mediante PCR en tiempo real. Los resultados indicaron que la OACM deterioró significativamente la función cognitiva y aumentó las citoquinas proinflamatorias, al tiempo que disminuyó los factores neurotróficos ($p < 0,05$). Por el contrario, el tratamiento con biocanina A mejoró la latencia de escape en el laberinto acuático de Morris y elevó los niveles séricos de GDNF y BDNF ($p < 0,05$). Asimismo, la administración de biocanina A moduló significativamente la expresión de JAK2 y STAT3, promoviendo la neuroprotección a través de la vía de señalización JAK2/STAT3. Estos hallazgos sugieren que la biocanina A mitiga los déficits cognitivos y la neuroinflamación tras la OACM, posiblemente mediante el aumento de los niveles de factores neurotróficos y la modulación de las respuestas inflamatorias. Por lo tanto, la

biocanina A representa un candidato terapéutico prometedor para la recuperación tras un accidente cerebrovascular isquémico.

PALABRAS CLAVE: Biocanina A; Infarto cerebral; Farmacología de redes; Isquemia-reperusión cerebral.

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