Effect of galangin on oxidative stress, antioxidant defenses and mitochondrial dynamics in a rat model of focal cerebral ischemia

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Abstract. Focal ischemia occurs when a cerebral artery becomes obstructed by an embolus or thrombus, leading to a rapid reduction in cerebral blood flow and significantly increasing the risk of mortality and disability. This condition is of particular concern in developing countries, where its prevalence is on the rise. Galangin, a flavonoid found in *Alpinia officinarum*, shows strong antioxidant, anti-inflammatory and anti-apoptotic properties. Its wide-ranging bioactivity in both *in vitro* and animal studies points to promising therapeutic applications. Given the role of oxidative stress in the pathophysiology of focal ischemia, the present study explored the effects of galangin on oxidative stress markers and antioxidant defenses in an animal model of the disease. A total of 60 healthy male Wistar rats were randomly assigned to six groups: Control, right middle cerebral artery occlusion (Rt. MCAO) + vehicle, Rt.MCAO + piracetam, and Rt.MCAO + galangin at doses of 25, 50 and 100 mg/kg body weight. The results indicated that 7 days of galangin treatment reduces infarct volume, malondialdehyde levels, and the density ratio of mitogen-activated protein kinase, while enhancing catalase, glutathione peroxidase and superoxide dismutase activities, and improving the density ratio of mitofusin 2 protein in the cortex and hippocampus. In conclusion, galangin showed significant *in vivo* potential in mitigating the pathological changes caused by cerebral ischemia, likely due to its antioxidant properties and modulation of mitochondrial dynamics. Additional research is now needed to explore the biochemical and neurological impacts of galangin in focal cerebral ischemia and to fully elucidate its mechanism of action.

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Introduction

Ischemic stroke is a neurological condition that arises from an abrupt interruption of blood flow to the brain. It is the second most common cause of death worldwide, resulting in ~5.5 million deaths each year, according to WHO health statistics. In addition to its high mortality rate, stroke significantly impacts disability, with ~50% of survivors experiencing permanent impairments (1). Currently, the only FDA-approved thrombolytic treatment for stroke is recombinant tissue plasminogen activator (2). However, this therapy is applicable to only ~10% of patients with acute ischemic stroke due to strict eligibility criteria relating to history of hemorrhage, recent surgeries and coagulation disorders, as well as a narrow treatment window of 4.5 h after symptom onset (3). There is therefore a critical need to develop neuroprotective agents that can restore cerebral blood flow (CBF) and mitigate brain damage in ischemic stroke.

Under normal conditions, adult CBF averages ~50 ml/100 g/min, which is essential for maintaining brain function. However, during focal ischemia, CBF can drop below 10 ml/100 g/min, leading to permanent neuronal injury (4). In ischemic stroke, the production of free radicals increases, leading to oxidative stress and brain damage (5). Oxidative stress occurs when there is an imbalance between the generation and removal of reactive oxygen species (ROS) (6). When ROS levels are too high, they can harm the mitochondrial membrane, interfere with the respiratory chain, and damage neuronal DNA, enzymes and cell membranes, eventually causing cell death (7-9). Numerous *in vivo* studies have shown that antioxidants can inhibit ROS-mediated reactions and protect neurons from injury in focal cerebral ischemic stroke (10,11). In addition to oxidative stress, the imbalanced mitochondrial dynamics that occur during stroke can harm the patient. Imbalanced mitochondrial dynamics result in excessive fission, leading to fragmented mitochondria, diminished mitochondrial function, increased ROS levels, and further brain damage. Supporting mitochondrial fusion, especially through the mitofusin 2 (Mfn2) protein, may lessen the harmful effects of stroke by preserving mitochondrial integrity, enhancing energy production, reducing oxidative stress and facilitating recovery (12,13). Mitogen-activated protein kinase (MAPK) pathways are a third component of stroke pathophysiology. These pathways are activated during stroke, influencing inflammation, apoptosis and oxidative stress. Previous studies

have found that suppression of mitochondrial fission through inhibition of the MAPK pathway can protect against brain injury following a stroke (12-14). Further research is needed to understand how antioxidants and modulation of mitochondrial dynamics affect oxidative stress in cerebral ischemia and how this could help in treating patients with stroke. Moreover, treating permanent ischemic stroke is difficult because of the irreversible brain damage caused by extended oxygen deprivation. If blood flow is not restored quickly, neurons in the affected region experience excitotoxicity, oxidative stress and inflammation, resulting in cell death and lasting neurological impairments. Current research is focusing on neuroprotective treatments that target inflammation, apoptosis and mitochondrial dysfunction in an effort to reduce further damage and support recovery. These strategies aim to address the ongoing complex pathological processes that persist after ischemic injury becomes irreversible.

Galangin is a dietary flavonoid, abundant in the rhizome of *Alpinia officinarum* Hance, that has been used in China for centuries as a spice and traditional medicine (15). This compound is noted for its wide range of bioactivities, impacting various cellular processes. These include antioxidant (16,17), anti-inflammatory (18,19), antiulcer (20,21), antidiabetic (22,23), anticoagulant (24) and anti-apoptotic (25) activities detected *in vitro* and in animal models. Despite its known bioactivities, the impact of galangin on stroke-associated brain damage has not been widely studied. In the present study, therefore, the effect of galangin on brain infarct volume, oxidative stress markers, anti-oxidant defenses, and MAPK and Mfn2 pathways was examined in an *in vivo* model of focal ischemic stroke.

Materials and methods

Test substances. Galangin, with a confirmed purity of 98.7% based on high-performance liquid chromatography analysis (PubChem ID: 5281616) was sourced from Biopurify Phytochemicals Ltd. The chemical structure of galangin is presented in Fig. 1. Piracetam (PubChem ID: 4843), which was used as a positive control, was sourced from GlaxoSmithKline (Thailand) Ltd. Dimethyl sulfoxide (DMSO), the vehicle used for both piracetam and galangin, was purchased from Thermo Fisher Scientific, Inc. (cat. no. D/4121/PB15).

Animals. A total of 60 healthy male Wistar rats, each weighing 250-300 g, 8 weeks-old, were sourced from the Northeastern Laboratory Animal Center at Khon Kaen University (Khon Kaen, Thailand). The rats were housed in groups of five in standard metal cages, under a 12/12-h light-dark cycle. Environmental conditions were maintained with relative humidity ranging from 30-60% and a temperature of 23±2˚C. Water and commercial food pellets were provided *ad libitum*. All procedures involving animals were performed following guidelines approved (approval no. IACUC-KKU-105/66) by the Institutional Animal Care and Use Committee at Khon Kaen University (Khon Kaen, Thailand).

Animal treatment. The rats were randomly divided into six groups, with 10 rats per group. Group 1, the control group, underwent a sham operation without further treatment. Group 2, designated as the Rt.MCAO + vehicle group, received an intraperitoneal injection of 1% (v/v) DMSO, which served as the vehicle for the test treatments, administered 7 days after inducing focal cerebral ischemia via right middle cerebral artery occlusion (Rt.MCAO). Group 3, the Rt.MCAO + piracetam group, was treated with an intraperitoneal injection of piracetam at a dose of 250 mg/kg body weight, 7 days following Rt.MCAO, as determined by prior research (12). Groups 4 to 6, the Rt.MCAO + galangin groups, were administered galangin intraperitoneally at doses of 25, 50 and 100 mg/kg body weight, respectively, also 7 days post-Rt.MCAO, with the dosages selected based on preliminary studies and existing literature (10,26). Piracetam was chosen as the positive control because of its demonstrated ability to reduce infarct size, increase CBF and improve neuronal function (27-29). DMSO was used as the vehicle for both piracetam and galangin, as a 1% (v/v) concentration is generally considered safe and non-toxic, making it appropriate for use in biological studies (10,12,30). All animals in the groups received their treatments via intraperitoneal injection once daily for 7 consecutive days following Rt. MCAO and were perfused trans-cardially on the 8th day.

In each group, the brain infarct volume was assessed in 5 rats using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Another 5 rats per group were utilized to analyze malondialdehyde (MDA) levels, catalase (CAT) and glutathione peroxidase (GSH-Px) activities in the cortex and hippocampus, while superoxide dismutase (SOD) activity was measured in the mitochondria of the cortex and hippocampus. Additionally, p38 MAPK and Mfn2 expression levels were measured in the cortex and hippocampus of rats treated with doses of galangin that produced optimal effects on infarct volume and oxidative stress markers. The middle cerebral artery supplies blood to parts of the frontal, temporal and parietal cortices of the brain; therefore, tissue from these cortical areas were collected for molecular analysis. Each test was conducted in duplicate.

Rt.MCAO model. Prior to surgery, all animals underwent an overnight fasting period while having access to water *ad libitum*. Anesthesia was induced in the rats using isoflurane, administered at 5% for induction and maintained at 1-3% during the procedure, delivered in 100% oxygen. The focal ischemic model was induced by permanent occlusion of the right middle cerebral artery using a 4-0 silicone-coated monofilament, following established protocols (31). Each monofilament was carefully inserted into the internal carotid artery until it reached a depth of ~17 mm or until slight resistance was encountered. After the procedure, the incision was sutured, and a 10% povidone iodine solution was applied to the site for postoperative antiseptic care. During subsequent brain removals after the 7-day treatment period, images of the filaments occluding each middle cerebral artery were captured to confirm consistent occlusion across all animals. In the sham operation, rats underwent the same procedure without the insertion of the monofilament.

Assessment of brain infarct volume. Rats were anesthetized with thiopental sodium [80 mg/kg body weight (BW), administered intraperitoneally] before undergoing cardiac perfusion with cold normal saline. The brains were carefully removed

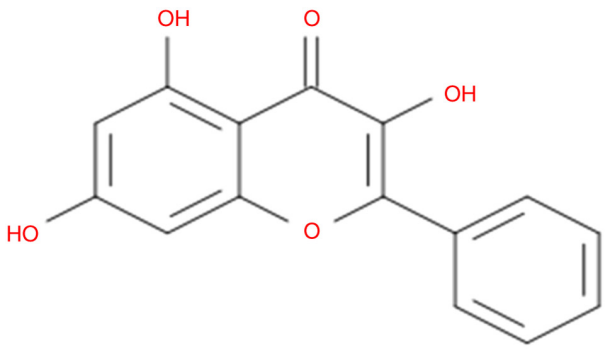

Figure 1. The structural formula of galangin.

from the skull, sectioned into 2-mm-thick coronal slices, and stained with 2% TTC (MilliporeSigma) in normal saline for 30 min at 37˚C. Digital images were captured using a camera, and the infarct volume was measured using ImageJ software (v.1.53e, National Institutes of Health). The infarct volumes were calculated according to a previously described method by the authors (32). The formula was as follows: Infarct volume (%)=[(contralateral hemisphere volume)-(non-infarct ipsilateral hemisphere volume) x100]/(contralateral hemisphere volume).

Isolation of brain mitochondria for biochemical assays. After perfusion, brain tissues from the cerebral cortex and hippocampal regions were isolated and underwent mitochondrial extraction following a protocol detailed in a previous study by the authors (32). Briefly, the cortex and hippocampus regions of the brain were dissected and homogenized in mitochondrial isolation buffer, followed by centrifugation at 1,000 x g for 2 min at 4˚C. The resulting supernatant was collected into a separate tube, while the pellet was resuspended in 0.2 ml of isolation buffer and centrifuged again under the same conditions. The second supernatant was combined with the first, and 0.07 ml of an 80% Percoll solution (MilliporeSigma) was added. A 10% Percoll solution (0.7 ml) was gently layered on top, and the mixture was subjected to centrifugation at 18,500 x g for 10 min. The mitochondrial pellet obtained was further purified by resuspending it in 0.7 ml of washing buffer and centrifuging at 10,000 x g for 5 min. Afterward, the mitochondrial pellet was suspended in washing buffer and stored at -80˚C for later use. SOD activity was subsequently measured using a commercial SOD assay kit (cat. no. 19160; MilliporeSigma). Data are expressed as units/mg of mitochondrial protein.

Protein quantification. After perfusion, the rat brains were quickly extracted and dissected into the cerebral cortex and hippocampus. The protein concentrations in these brain areas were determined using the method outlined by Lowry *et al* (33), with bovine serum albumin (MilliporeSigma) as the standard. In brief, samples were diluted and mixed with freshly prepared Lowry reagent (containing sodium carbonate, copper sulfate and sodium potassium tartrate). After a 10-min incubation at room temperature, Folin-Ciocalteu reagent, diluted 1:1 with distilled water, was added, and the mixture was incubated for 30 min to allow for color development. Absorbance was then measured at 650 nanometers (nm) using a spectrophotometer.

Determination of the MDA level. The lipid peroxidation (LPO) product, MDA, served as an indicator of oxidative stress. Levels of MDA were quantified in all samples using the thiobarbituric acid (TBA; MilliporeSigma) reaction, following the method outlined in a study by Ohkawa *et al* (34). Tissue homogenates were mixed with sodium dodecyl sulfate (SDS), acetic acid and TBA, then heated at 95˚C for 60 min to form the MDA-TBA complex. After cooling, the mixture was extracted with n-butanol/pyridine, centrifuged at a speed of 4,000 x g (10 min, 4˚C), and the organic layer was collected. The absorbance of the product was measured at 532 nm using a spectrophotometer, and MDA levels were calculated by comparison to a standard curve prepared with 1,1,3,3-tetramethoxypropane. Results are expressed as nmol/mg protein.

Determination of CAT activity. CAT activity was assessed following the procedure outlined by Goldblith and Proctor (35). Initially, brain tissue underwent homogenization in ice-cold phosphate buffer to prevent enzymatic degradation. After centrifugation at 10,000 x g for 10 min at 4˚C, the supernatant containing CAT was retrieved. This supernatant was then mixed with phosphate buffer and hydrogen peroxide, and absorbance was recorded at 240 nm using a spectrophotometer. CAT activity is reported as units per milligram of protein (units/mg protein).

Determination of GSH-Px activity. GSH-Px activity was assessed using a GSH-Px assay kit obtained from MilliporeSigma (cat. no. MAK437-1KT). After the rat brain tissue was homogenized and centrifuged at 10,000 x g for 10 min at 4˚C, the supernatant containing GSH-Px was collected. This supernatant was then mixed with phosphate buffer, glutathione reductase, nicotinamide adenine dinucleotide (NADPH) and hydrogen peroxide. The reduction in NADPH absorbance at 340 nm served as an indicator of GSH-Px activity. Enzyme activity was quantified by monitoring the absorbance changes over time. Results are presented as units/mg protein.

Western blot analysis. The expression levels of p38 MAPK and Mfn2 in rat cortices and hippocampi were analyzed via western blotting as outlined in previous studies (12,14). Each of the cortex and hippocampus samples was homogenized in neuronal protein extraction reagent (N-PER™) lysis buffer (Thermo Fisher Scientific, Inc.), and total protein concentrations were determined using the Lowry method. Proteins (30 μg/sample) were separated on a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Hybond-P; GE Healthcare; Cytiva). To prevent non-specific binding, membranes were blocked by incubating them for 1 h at room temperature in 5% non-fat dried milk in TBS-T (0.1% Tween-20 in Tris-buffered saline; pH 7.4). Subsequently, membranes were incubated overnight at 4˚C with primary antibodies: Rabbit monoclonal anti-p38 MAPK (1:500; cat. no. A14401; ABclonal Biotech Co., Ltd.), rabbit monoclonal anti-mitofusin 2 (1:500; cat. no. A12771; ABclonal Biotech Co., Ltd.) and rabbit monoclonal anti-β-actin (1:5,000; cat. no. AC026; ABclonal Biotech

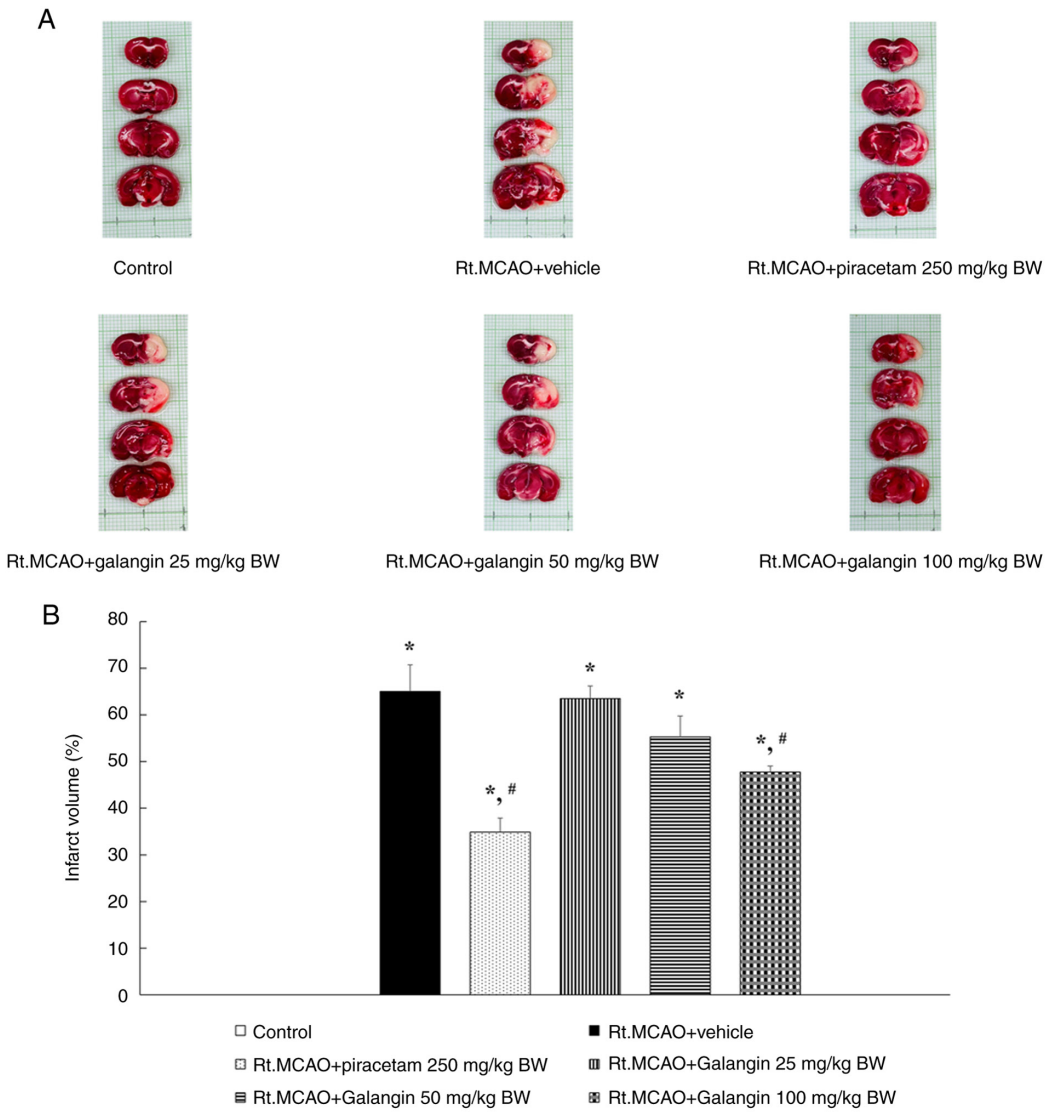

Figure 2. Effects of galangin on brain infarct volume. (A) The effect of galangin on brain infarct volume in rats subjected to permanent Rt.MCAO is demonstrated in the bar graph. Results are expressed as the mean ± SEM (n=5). *P<0.05 compared with the control group; #P<0.05 compared with the Rt.MCAO + vehicle group. (B) Representative images of rat brains stained with 2,3,5-triphenyltetrazolium chloride after permanent Rt.MCAO. The red regions correspond to healthy tissue, while the white regions indicate the infarcted areas. Rt.MCAO, right middle cerebral artery occlusion; BW, body weight.

Co., Ltd.). Following washes with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:2,000; cat. no. AS063; ABclonal Biotech Co., Ltd.) for 1 h at room temperature. Protein bands were visualized using the Clarity™ Western ECL Substrate (cat. no. 170-5060, Bio-Rad Laboratories, Inc.) and imaged with a ChemiDoc™ MP system (Bio-Rad Laboratories, Inc.) using Image Lab software (version 6.0.0 build 25; Bio-Rad Laboratories, Inc.). The density of MAPK and Mfn2 bands was normalized to β-actin, with protein expression levels quantified through ImageJ software v.1.53e (National Institutes of Health).

Statistical analysis. The results are presented as the mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, employing SPSS software v.25 (IBM Corp.). A significance level of P<0.05 was used to determine statistical significance.

Results

Effect of galangin on cerebral damage in rat following Rt.MCAO. Induction of ischemic stroke by Rt.MCAO caused substantial infarction in coronal brain sections. In the present study, TTC staining was used to measure the extent of brain infarction following galangin treatment in rats subjected to permanent Rt.MCAO. Rats administered the vehicle after Rt.MCAO exhibited a significantly larger infarct volume compared with the control group (P<0.05) (Fig. 2). By contrast, treatment with piracetam (250 mg/kg BW) and galangin (100 mg/kg BW) led to a substantial decrease in infarct volumes compared with the Rt.MCAO + vehicle group (P<0.05).

Effect of galangin on MDA levels and endogenous antioxidant enzymes in the cortex and hippocampus following Rt.MCAO. Rats with cerebral ischemia showed a marked increase in MDA levels in both the cerebral cortex and hippocampus compared with controls, indicating elevated lipid peroxidation in the

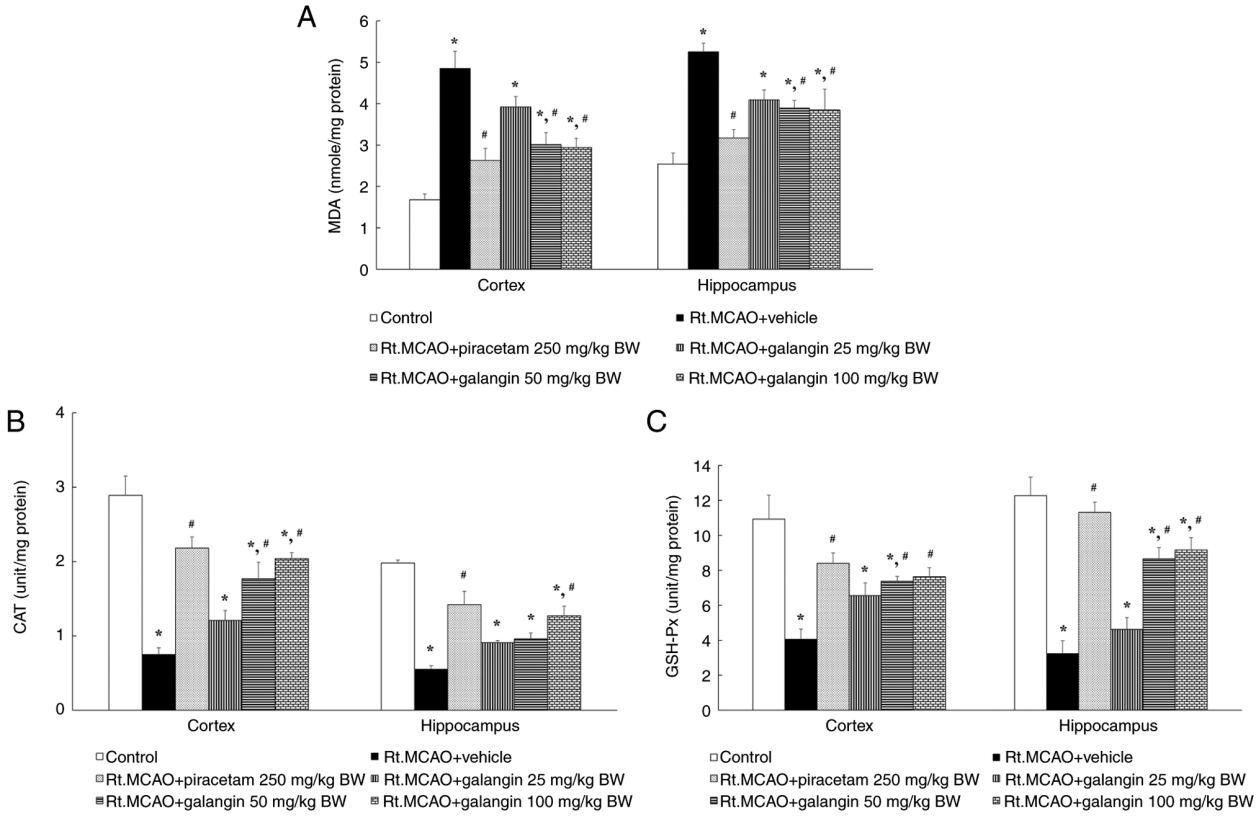

Figure 3. Effect of galangin on MDA levels and endogenous antioxidant enzymes in the cortex and hippocampus following Rt.MCAO. (A) MDA levels, (B) CAT activity and (C) GSH-Px activity. The data are presented as the mean ± SEM (n=5). *P<0.05 compared with the control group; #P<0.05 compared with the Rt.MCAO + vehicle group. Rt.MCAO, right middle cerebral artery occlusion; BW, body weight; MDA, malondialdehyde; CAT, catalase; GSH-Px, glutathione peroxidase.

brain. However, treatment with piracetam (250 mg/kg BW) and galangin (50 and 100 mg/kg BW) significantly decreased lipid peroxidation across all examined regions affected by cerebral ischemia (Fig. 3A). Several antioxidants are known to inhibit ROS-mediated reactions and protect neurons from focal cerebral ischemic stroke-induced injury. In addition to measuring MDA levels, the present study measured the endogenous antioxidant enzymes CAT and GSH-Px. Following cerebral ischemia, the activities of CAT and GSH-Px were significantly reduced in the Rt.MCAO + vehicle group compared with the control group. By contrast, the groups treated with piracetam and galangin (100 mg/kg BW) exhibited a significant increase in CAT and GSH-Px activities across all assessed areas compared with the Rt.MCAO + vehicle group. Galangin at a dose of 50 mg/kg BW induced a significant increase in CAT and GSH-Px activities only in the cortex compared with the Rt.MCAO + vehicle group (Fig. 3B and C).

Effect of galangin on SOD activity in mitochondria from the cortex and hippocampus of ischemic rats. Permanent occlusion of the right middle cerebral artery caused a significant decrease in mitochondrial SOD activity compared with the control group (P<0.05), as revealed in Fig. 4. However, in rats treated with piracetam (250 mg/kg BW) or galangin (100 mg/kg BW), mitochondrial SOD activity was significantly less diminished (P<0.05) compared with the vehicle + Rt.MCAO group in all assessed areas. In rats treated with galangin at 50 mg/kg BW, mitochondrial SOD activity demonstrated a significant difference only in the cortex compared with the vehicle + Rt.MCAO group.

Effect of galangin on western blot analysis of p38 MAPK and Mfn2 expression. Mitochondrial dynamics can mitigate the harmful effects of oxidative stress in cerebral ischemia. Therefore, this assessment examined the effect of galangin on p38 MAPK and Mfn2 protein expression using western blot analysis. Rats in the Rt.MCAO + vehicle group showed a marked increase in the p38 MAPK to β-actin band density ratio and a decrease in the Mfn2 to β-actin band density ratio compared with the control group (Fig. 5A). Treatment with piracetam at a dose of 250 mg/kg BW or galangin at a dose of 100 mg/kg BW significantly reduced the p38 MAPK to β-actin band density ratio compared with the vehicle group (P<0.05; Fig. 5B). Furthermore, the Mfn2 to β-actin band density ratio revealed a significantly smaller reduction in these treated groups compared with the Rt.MCAO + vehicle group (P<0.05; Fig. C).

Discussion

Cerebral ischemia involves a significant reduction or interruption in blood flow to the brain, leading to a decrease in the delivery of oxygen and nutrients to brain cells. This condition triggers a cascade of events that can cause neuronal damage, including excitotoxicity, oxidative stress, inflammation and apoptosis (36). In cases of permanent occlusion, the infarct

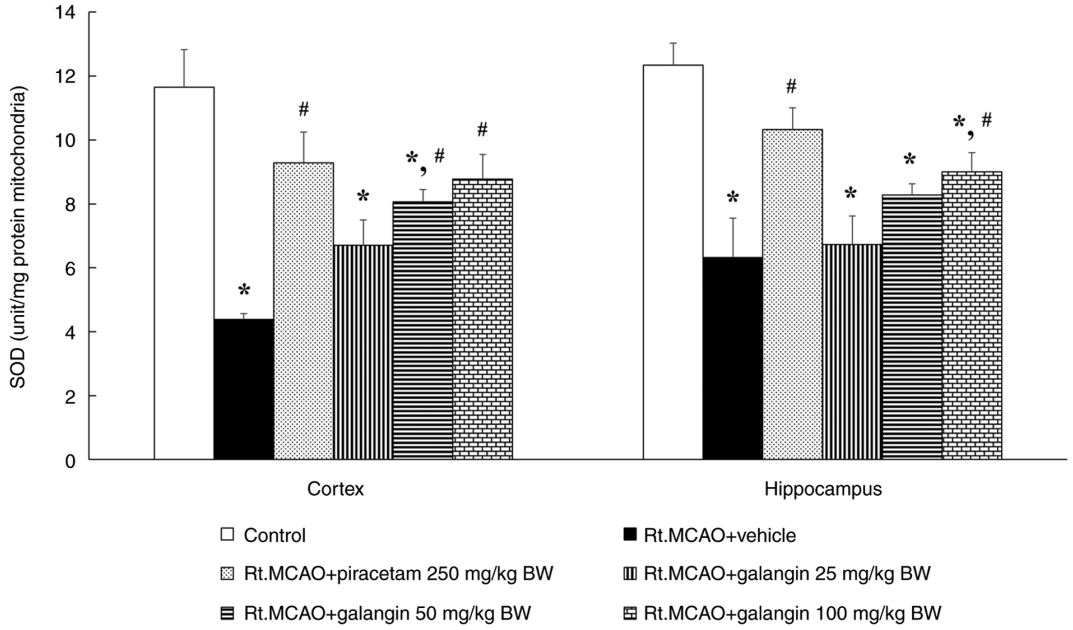

Figure 4. Effects of galangin on SOD activity in mitochondrial fractions from the cortex and hippocampus. The data are shown as the mean ± SEM (n=5). *P<0.05 compared with the control group; #P<0.05 compared with the Rt.MCAO + vehicle group. Rt.MCAO, right middle cerebral artery occlusion; BW, body weight; SOD, superoxide dismutase.

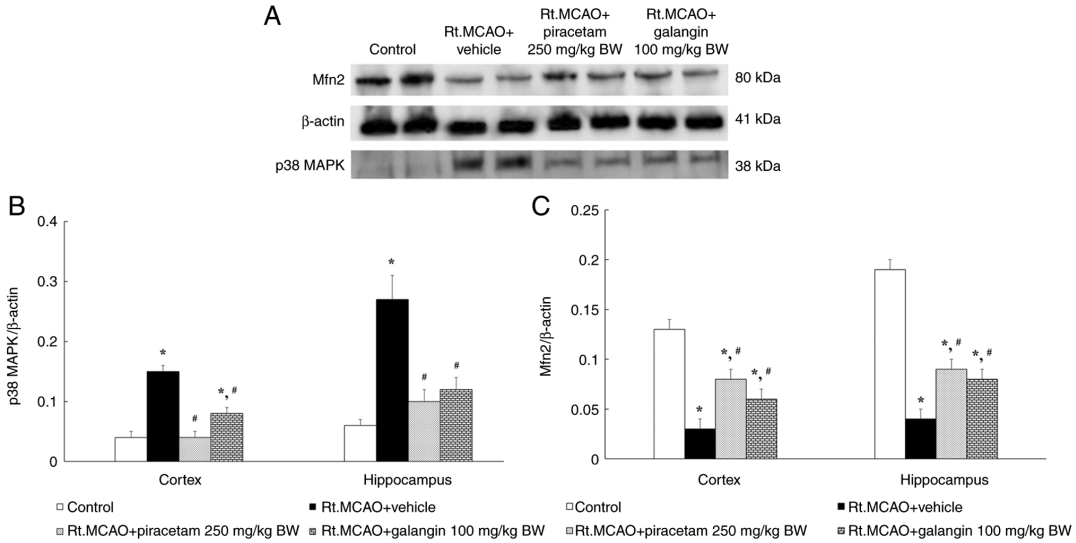

Figure 5. Effect of galangin on the expression of p38 MAPK and Mfn2. (A) Immunoblot image showing p38 MAPK (38 kDa) and Mfn2 (80 kDa) protein levels in the hippocampus, with β-actin (41 kDa) serving as a loading control. (B and C) Quantitative analysis of (B) p38 MAPK and (C) Mfn2 band intensities, normalized to β-actin in the cortex and hippocampus. Data are presented as the mean ± SEM (n=5). *P<0.05 compared with the control; #P<0.05 compared with the Rt.MCAO + vehicle group. MAPK, mitogen-activated protein kinase; Mfn2, mitofusin 2; Rt.MCAO, right middle cerebral artery occlusion; BW, body weight.

becomes detectable within 3 to 12 h (37,38). Initially forming in the core region, the infarct approaches its maximum size, encompassing both the core and penumbra, and continues to expand over the following day. By 7 days post-ischemic stroke, there is a near complete depletion of cellular elements in the affected area (39). This highlights the urgent need for neuroprotective treatments to reduce brain injury. The present study therefore explored the effects of galangin on brain infarct volume in an *in vivo* focal ischemic stroke model. It was demonstrated that permanent Rt.MCAO significantly increased the brain infarct volume. However, administering piracetam at a dose of 250 mg/kg BW or galangin at a dose of 100 mg/kg BW led to a substantial decrease in infarct volumes compared with the Rt.MCAO + vehicle group (P<0.05). Previous studies suggested that galangin may exert a protective effect by enhancing CBF, which could help protect against cerebral damage from ischemic stroke (26,40).

In stroke, reduced oxygen and nutrient availability disrupts the equilibrium between ROS production and the brain's antioxidant defenses. ROS, such as superoxide radicals and hydrogen peroxide, can damage lipids, proteins, DNA and other cellular components (41). The production of ROS, in

stroke, is no longer adequately countered by endogenous enzymatic antioxidants such as CAT, GSH-Px and SOD (42). Lipids can become oxidized, initiating a self-sustaining chain reaction called the lipid peroxidation cascade that generates free radicals (43). Lipid hydroperoxides and aldehydes such as MDA are generated as products of this process. MDA has the capacity to alter the biophysical characteristics of membranes, affecting their fluidity and permeability, ultimately leading to membrane disruption and lysis (44). Given the pivotal role of oxidative stress in ischemic stroke pathology, the effect of galangin on oxidative stress markers, particularly MDA levels, in the cortex and hippocampus of rats subjected to Rt.MCAO was investigated. The results revealed that both the positive control, piracetam, and the experimental treatment, galangin (50 and 100 mg/kg BW), decreased LPO products in the cortex and hippocampus compared with the Rt.MCAO + vehicle group. Previous studies have shown that galangin nanoparticles notably reduce MDA levels in models of acetaminophen-induced liver injury (45) and cardiometabolic disorders (46). In addition, augmenting the activity of antioxidant enzymes has been linked to neuroprotection and enhanced functional recovery post-stroke. Therefore, CAT and GSH-Px activities were evaluated via biochemical assays conducted on the cortex and hippocampus. The results of the present study showed that piracetam and galangin effectively mitigated the Rt.MCAO-induced decline in CAT and GSH-Px activities in both the cortex and hippocampus compared with the Rt.MCAO + vehicle group. CAT breaks down hydrogen peroxide, preventing the formation of highly reactive hydroxyl radicals that could harm cells. Reduced glutathione aids in converting GSH-Px and glutathione-S-transferase into non-toxic products (17). Previous studies have shown that reduced activity of these enzymes may result from free radical damage or enzymatic glycation (47,48). Flavonoids help mitigate oxidative damage by scavenging free radicals, offering protection against various diseases (49). Moreover, galangin can neutralize free radicals by releasing hydrogen atoms from its hydroxyl group.

Because mitochondrial dysfunction and low SOD levels contribute to neuronal death and larger cerebral infarctions, the present study also examined the effect of galangin on mitochondrial SOD activity in the cortex and hippocampus. After Rt.MCAO, rats administered with the vehicle exhibited a significant reduction in SOD activity compared with the control group. Treatment with piracetam or galangin effectively prevented this decline in SOD activity in both the cortex and hippocampus mitochondria compared with the Rt.MCAO + vehicle group. This effect may be attributable to the antioxidant properties of galangin (16,17). Several antioxidants have been reported to inhibit ROS-mediated reactions and protect neurons from injury caused by focal cerebral ischemic stroke (10,11).

During a stroke, mitochondrial dynamics can mitigate the harmful effects of oxidative stress in cerebral ischemia. Therefore, the present study examined the effect of galangin on p38 MAPK and Mfn2 protein expression using western blot analysis. In accordance with expectations, a notable increase was detected in the p38 MAPK to β-actin band density ratio and a decrease was detected in the Mfn2 to β-actin band density ratio in rats with permanent Rt.MCAO compared with the control group. However, administration of piracetam at a dose 250 mg/kg BW or galangin at a dose of 100 mg/kg BW led to a significant decrease in the p38 MAPK to β-actin band density ratio compared with the vehicle-treated group. Furthermore, the reduction in the Mfn2 to β-actin band density ratio was less pronounced in these treatment groups than the Rt.MCAO + vehicle group. After a stroke, disruptions in mitochondrial dynamics skew the balance between mitochondrial fission and fusion, favoring increased fission, which results in neuronal damage. Signaling pathways involving p38 MAPK and dynamin-related protein 1 (DRP1) are associated with various biological mechanisms during cerebral hypoxia/ischemia, such as programmed cell death, oxidative stress, mitochondrial function, calcium signaling and synaptic activity (13,50,51). In rat models of MCAO, inhibiting DRP1 was shown to reduce both cytochrome c release and apoptosis (52). Suppressing DRP1 may help alleviate mitochondrial dysfunction and prevent cell death by reducing mitochondrial fission (51). Additionally, previous studies suggested that inhibition of mitochondrial fission, including the MAPK pathways, could help mitigate brain damage following a stroke (12-14). Therefore, blocking p38 MAPK or DRP1 directly halts neuronal degeneration by decreasing mitochondrial fission, which in turn helps recover mitochondrial membrane potential, restore mitochondrial function, and minimize cell death. Moreover, a 7-day treatment period for galangin based on the pathophysiological changes during the subacute phase of ischemic stroke was selected. This phase involves ATP depletion, ionic imbalance and the generation of reactive species, leading to tissue damage. Both defense mechanisms and inflammatory responses are activated, worsening neuronal injury. Thus, this timeframe was chosen to evaluate the potential antioxidant and mitochondrial effects of galangin in reducing neuronal damage in a focal ischemic stroke model. Recent research indicated that flavonoids play a role in preserving mitochondrial function and protecting against disruptions in mitochondrial dynamics caused by oxidative stress (53). Flavonoids, categorized as secondary metabolites, primarily comprise a benzopyrone ring with phenolic or polyphenolic groups positioned variably (49). These compounds are present in a range of sources including fruits, herbs, stems, cereals, nuts, vegetables, flowers and seeds (54). Flavonoids offer a variety of potential health benefits, such as anticancer, antioxidant, anti-inflammatory and antiviral properties, along with neuroprotective and cardio-protective effects (55-58). Previous research has shown that *Alpinia officinarum* flavonoids can modulate transient receptor potential vanilloid subtype 1 (TRPV1) expression to safeguard the gastric lining (59). Galangin may similarly interact with TRPV1 to inhibit the release of neurotransmitters and inflammatory agents, thereby easing pain and inflammation (59). Galangin also appears to reduce neurogenic pain, particularly that caused by capsaicin, by influencing TRPV1-associated pathways, including those involving NF-κB/TNF-α and COX-2 (60).

The present study has several limitations. First, post-stroke behavioral impairments were not investigated in the current study and there are no data available on the decline in neurological function over time. It would have been useful to assess functional outcomes such as motor, sensory and cognitive abilities in the rat model after the stroke. Second, although DRP1 and phosphorylated MAPK are key regulators of mitochondrial fission and play crucial roles in stroke-induced damage,

the authors were unable to measure DRP1 and phosphorylated MAPK due to funding constraints. This will be addressed in future research. Lastly, the small number of replicates used in the biochemical assays is a different limitation. While three or more replicates would have been preferable, only two were used. Nonetheless, the assays were performed by a skilled researcher, and the standard curves displayed high R^2 values.

Future studies should explore the neuroprotective mechanisms of galangin beyond its antioxidant properties and impact on mitochondrial dynamics, specifically examining its role in inflammation and apoptosis. Research is needed to evaluate its long-term effectiveness and determine the optimal dosing. Investigating the potential of galangin in combination with other therapeutic agents could further enhance its efficacy. Additionally, it would be beneficial to examine how different mitochondrial subtypes respond to treatment. Ultimately, clinical trials are necessary to validate its safety and effectiveness in human populations.

In summary, galangin exhibits notable *in vivo* efficacy in reducing the brain damage induced by cerebral ischemia. This favorable outcome is associated, at least in part, with its antioxidant activity and its effect on mitochondrial dynamics.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

AS handled the funding acquisition, investigation, methodology, writing and editing of the manuscript. NP contributed to the investigation and methodology, reviewed and edited the manuscript. JJ was responsible for conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, validation and both writing the original draft and reviewing and editing the manuscript. JJ and AS confirm the authenticity of all raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experimentation protocols were carefully designed to minimize potential suffering. These protocols were carried out in strict compliance with the approval (approval no. IACUC-KKU-105/66) from the Institutional Animal Care and Use Committee at Khon Kaen University (Khon Kaen, Thailand).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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