In vivo protective effects of 6-gingerol in cerebral ischemia involve preservation of antioxidant defenses and activation of anti-apoptotic pathways

RATCHANIPORN KONGSUI[1] and JINATTA JITTIWAT[2]

[1]Division of Physiology, School of Medical Sciences, University of Phayao, Phayao 56000;
[2]Faculty of Medicine, Mahasarakham University, Mahasarakham 44000, Thailand

Received November 8, 2023; Accepted March 22, 2024

DOI: 10.3892/br.2024.1773

Abstract. Stroke is an important medical problem in developing countries, characterized by a sudden disruption of blood supply to the brain, either through occlusion or hemorrhage. It is a major cause of neurological impairment, resulting in high medical costs. The present study examined the effect of 6-gingerol on morphological changes, antioxidant defenses, and the anti-apoptotic factors p38 mitogen-activated protein kinase (MAPK) and mitofusin (Mfn)2, in a rat model of focal cerebral ischemia. A total of 60 healthy male Wistar rats were randomly allocated into six groups: Control, right middle cerebral artery occlusion (Rt.MCAO) + vehicle, Rt.MCAO + piracetam, and Rt.MCAO + 6-Gin 5, 10 and 20 mg/kg BW groups. The results indicated that 6-gingerol treatment for a duration of 7 days reverses morphological alterations, enhances catalase and glutathione peroxidase activities, reduces Bax, caspase-3 and MAPK expression, and increases Bcl-xL and Mfn2 expression in the cortex and hippocampus. In conclusion, 6-gingerol demonstrated significant *in vivo* effectiveness in mitigating pathological changes induced by cerebral ischemia. This beneficial effect is attributed, at least in part, to preservation of antioxidant defenses and activation of anti-apoptotic pathways.

Introduction

Stroke or 'brain attack' occurs when the blood supply to part of the brain is suddenly interrupted by occlusion or hemorrhage, leading to neuronal dysfunction or neuronal death. It is recognized as a major cause of mortality in Thailand and other developing countries (1,2). Stroke is also a common cause of neurological problems and disability, with very high associated medical costs (3). Recent evidence suggests that cerebral ischemia-induced brain damage is accompanied by increased formation of free oxygen radicals in brain tissue. Excessive production of reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide may cause oxidative stress-induced cell injury (4). Cerebral ischemia also initiates an inflammatory response in the brain, involving the activation of immune cells such as microglia and astrocytes, as well as the release of inflammatory mediators including cytokines, chemokines and adhesion molecules (5). This inflammatory response can exacerbate neuronal damage by promoting additional ROS production and causing the breakdown of the blood-brain barrier, enabling immune cells and harmful substances to enter the brain (6,7). In addition, prolonged cerebral ischemia can trigger apoptotic pathways in neurons, especially in the penumbra area (8). Apoptosis, also known as programmed cell death, is a regulated process involving removal of damaged or unnecessary cells. However, excessive or uncontrolled apoptosis can contribute to neuronal death in ischemic conditions. The apoptotic pathway, which involves Bax, caspase-3 and Bcl-xL, plays a critical role in determining neuronal cell death during a stroke (9).

Targeting the aforementioned factors is a promising strategy for the development of innovative therapeutic interventions. For example, inhibition of Bax activity, inhibition of caspase-3 activity and enhancement of Bcl-xL expression all have neuroprotective effects in preclinical stroke studies (10-12). Mitogen-activated protein kinase (MAPK) is another key factor contributing to the pathogenic process and has been linked to neuronal damage during cerebral ischemia (13). The MAPK signaling pathway plays an important role in cerebral ischemia-induced apoptosis, especially the p38 MAPK pathway (14). Previous studies have shown that inhibition of p38 MAPK reduces apoptosis and improves stroke recovery (14,15). Reduced mitofusin (Mfn) 1 and Mfn2 levels, because they cause excess calcium (Ca^{2+}) to accumulate in mitochondria, facilitate the translocation of Bax to mitochondria, and contribute to neuronal apoptosis too (16). Previous investigations have revealed that Mfn2 plays a protective role in an ischemic stroke model by reducing apoptosis (16-18).

Ginger (*Zingiber officinale*) has been widely used in traditional medicine, and 6-gingerol is considered one of

Correspondence to: Dr Jinatta Jittiwat, Faculty of Medicine, Mahasarakham University, 79/99 Nakornsawan Road, Mahasarakham 44000, Thailand
E-mail: jinatta@gmail.com; jinatta.j@msu.ac.th

Key words: cerebral ischemia, middle cerebral artery, 6-gingerol, antioxidant, anti-apoptotic

its key active constituents. 6-Gingerol exhibits numerous pharmacological activities, including anti-inflammatory, antioxidant (19,20), anticancer (21), gastroprotective and anti-diabetic (22) effects. These properties are attributed to its ability to modulate various molecular targets and signaling pathways, and some research suggests 6-gingerol exerts neuroprotective effects in stroke. A previous study by the authors revealed that 6-gingerol reduces brain damage and infarct volume in the right middle cerebral artery occlusion (Rt.MCAO) model, partly via anti-inflammatory and anti-oxidant pathways (19). To discover if any other mechanisms are contributing to the beneficial effects of 6-gingerol in this model, the present study examined how 6-gingerol affects cell morphology, antioxidant defenses, and the anti-apoptotic factors p38 MAPK and Mfn2.

Materials and methods

Experimental compounds. The test compound, 6-gingerol ($C_{17}H_{26}O_4$; PubChem ID: 442793), was supplied by Chengdu Biopurify Phytochemicals Ltd. (http://www.biopurify.com/about_us.html) with a purity of 98.7%. Piracetam, which served as the positive control, was obtained from GSK plc. The vehicle, dimethyl sulfoxide (DMSO), was obtained from Thermo Fisher Scientific, Inc.

Animals. Male Wistar rats (8 weeks-old; weighing 250-300 g) were obtained from the Northeastern Laboratory Animal Center at Khon Kaen University in Khon Kaen, Thailand. The rats were housed in groups of five within typical metal cages measuring 37.5x48x21 cm^3. They were maintained under standard conditions, following a 12/12-h light/dark cycle, with humidity levels maintained around 30-60%, and temperature set at 23±2˚C. Adequate water and commercial pellets were available to them at all times. All animal-related procedures carried out in the present study received approval (approval no. IACUC-KKU-6/65) from the Institutional Animal Care and Use Committee at Khon Kaen University, Thailand.

Experimental design. A total of 60 healthy male Wistar rats were randomly allocated into six groups (10 rats per group). Group 1 (control group): Rats underwent a placebo surgery and received no treatment. Group 2 (Rt.MCAO + vehicle group): Animals received DMSO, which was used as a vehicle to dissolve the test substance. Group 3 (Rt.MCAO + piracetam group): Animals received piracetam at a dose of 250 mg/kg of body weight (BW), serving as a positive control. Groups 4-6 [Rt. MCAO + 6-gingerol (6-Gin) groups]: Animals received different concentrations of 6-gingerol (5, 10 and 20 mg/kg BW). Following the induction of Rt.MCAO, all groups received their respective treatments intraperitoneally once daily for seven consecutive days. Hematoxylin-eosin (H&E) staining was applied to observe morphological changes of the cortex and the hippocampus in five rats from each group. Biochemical assays were conducted on the cortex and hippocampus of the remaining five animals per group to examine catalase (CAT) and glutathione peroxidase (GSH-Px) activities. In the cortex and hippocampus of rats, the expression levels of Bax, Bcl-xL, caspase-3, MAPK and Mfn2 were assessed. This evaluation was performed in rats treated with doses of 6-gingerol that resulted in optimal changes in oxidative parameters. Piracetam and 6-gingerol doses were selected based on previous research findings by the authors (23,24).

The Rt.MCAO model. Before performing the surgery, all animals underwent an overnight fasting period while being provided with unrestricted access to water. During the operation, the rats were anesthetized with isoflurane, with 5% for induction and 1-3% for maintenance, delivered in 100% oxygen. The focal ischemic model was produced by permanently occluding the right middle cerebral artery using a 4-0 silicone-coated monofilament, as previously described (25). The monofilament was carefully inserted into the internal carotid artery, typically reaching a depth of ~17 mm or until a slight resistance was detected. Following the procedure, the wound was sutured, and a 10% povidone iodine solution was applied to the incision site for postoperative antiseptic care. Later in the present study, when rat brains were being removed following the 7-day treatments, images of the filaments occluding each middle cerebral artery were captured to ensure that occlusion was consistent in every animal. In the sham operation, rats underwent the same procedure as aforementioned, but without the insertion of the monofilament. The criteria for humane endpoints were defined as the inability to move, wound infection following surgery, a weight loss of >20%, dehydration, dyspnea, progressive pain, lack of response to external stimuli and bleeding from any orifice. The authors along with the vet responsible for the present study, monitored the animal health and behavior every day. No animals were lost during the experimental period. The infarct volume was measured in all animals, and this data has recently been published (19).

Histopathological detection with H&E staining. H&E staining involved staining for frozen sections with hematoxylin for 4 min at room temperature (RT), rinsing with running water for 10 min, then staining with eosin for 1 min at RT. Sections were then dehydrated, mounted and examined by light microscopy.

Protein quantification. At the end of the experimental period, rats were anesthetized with thiopental sodium (80 mg/kg BW) via intraperitoneal administration before undergoing cardiac perfusion with a cold normal saline solution. Subsequently, the brains were rapidly removed from the skulls and separated into the cerebral cortex and hippocampus. The concentration of protein in the cortex and hippocampus was determined by the method described by Lowry *et al* (26). Bovine serum albumin (MilliporeSigma) was used as a standard during the process.

Determination of CAT activity. CAT activity was measured using the method of Goldblith and Proctor (27). In brief, brain tissue was homogenized using phosphate buffer on ice to prevent any enzymatic degradation. This homogenate was then centrifuged (10,000 x g, 10 min, 4˚C) and the supernatant containing the CAT was collected. Next, the supernatant was combined with phosphate buffer and hydrogen peroxide

(H_2O_2), and absorbance was measured at 240 nm using a spectrophotometer. The results are expressed as units per milligram of protein (units/mg protein).

Determination of GSH-Px activity. GSH-Px activity was measured using a GSH-Px assay kit from MilliporeSigma (cat. no. MAK437-1KT). Following homogenization of the rat brain tissue and centrifugation (10,000 x g, 10 min, 4˚C) of these homogenates, supernatant containing the GSH-Px was collected. The supernatant was then mixed with phosphate buffer, glutathione reductase, nicotinamide adenine dinucleotide (NADPH), and hydrogen peroxide. Reduction in NADPH absorbance at 340 nm was used as a measure of GSH-Px activity. Enzyme activity was quantified by scrutinizing the temporal evolution of absorbance changes. Data are expressed as units/mg protein.

Western blot analysis. Bax, Bcl-xL, caspase-3, MAPK and Mfn2 expression levels were measured in the rat cortices and hippocampi using the western blot method as previously described (25). Each cortex and hippocampus were homogenized with lysis buffer (Thermo Fisher Scientific, Inc.), and the total protein concentrations were determined using the Lowry method (26). Equal quantities (40 μg of protein) of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and subsequently transferred onto a Hybond-P (PVDF) membrane (GE Healthcare; Cytiva). Non-specific binding sites on the membrane were blocked by incubating with 5% non-fat dried milk in 0.1% Tween-20 in Tris buffered saline (TBS-T), pH 7.4 at room temperature for 1 h. The membrane was then incubated overnight at 4˚C with mouse monoclonal anti-Bax (1:500; cat. no. 14-6999-82; Thermo Fisher Scientific, Inc.), rabbit monoclonal anti-Bcl-xL (1:1,000; cat. no. ab32370; Abcam), rabbit monoclonal anti-caspase-3 (recognizing pro-caspase 3; 1:2,000; cat. no. ab184787; Abcam), 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 Co., Ltd.) antibodies. After washing with 0.1% Tween-20 in Tris-buffered saline, the membrane was incubated with anti-mouse (1:2,000; cat. no. 12-349; MilliporeSigma) or anti-rabbit (1:2,000; cat. no. AS063; Abclonal Biotech Co., Ltd.) secondary antibodies for 1 h at room temperature. The reactivity was visualized with chemiluminescent substrate (Supersignal™ PLUS Chemiluminescent Substrate West Pico; Pierce; Thermo Fisher Scientific, Inc.). A ChemiDoc™ MP imaging system with Image Lab software (Bio-Rad Laboratories, Inc.) was used to capture photographs of the membranes against a white background. The density of Bax, Bcl-xL, caspase-3, MAPK and Mfn2 bands was normalized to β-actin, and protein expression levels were quantified using Image J® software version 1.53e (National Institutes of Health).

Statistical analysis. The data are presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS® software (IBM Corp.). One-way analysis of variance (ANOVA) was conducted, followed by a Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protective effect of 6-gingerol on morphological alterations in the cortex and hippocampus of rats with induced-Rt. MCAO. Previous work in the authors' laboratory (Faculty of Medicine, Mahasarakham University) has quantified Nissl-positive neurons and revealed that 6-gingerol can reverse ischemic stroke-induced neuronal loss. Thus, the present study assessed the potential of 6-gingerol to protect neurons against histopathological changes following Rt. MCAO. The effect of 6-gingerol on Rt.MCAO-induced morphological alterations of the cortex and hippocampus was evaluated qualitatively by H&E staining (Fig. 1). H&E staining of the control group revealed there were intact pyramidal neurons with large nuclei. The Rt.MCAO + vehicle group, by contrast, demonstrated evidence of cell loss, pyknotic nuclei, vacuolation within the cytoplasm, and decreased neuronal density in the cortex and pyramidal layer of the cerebral artery (CA)1 and CA3 hippocampus sub-regions. Treatment with various doses of 6-gingerol reversed these alterations.

Protective effect of 6-gingerol on antioxidant enzymes in the cortex and hippocampus of rats with induced-Rt.MCAO. Lack of oxygen and nutrients leads to an imbalance between the production of ROS and the brain's antioxidant defense mechanisms. Enhancing the activity of these enzymes has been associated with neuroprotection and improved functional recovery following stroke. Therefore, CAT and GSH-Px activities were assessed through biochemical assays performed on the cortex and hippocampus. Rats administered with vehicle following Rt.MCAO exhibited a significant decrease in CAT and GSH-Px activities compared with the control group ($P<0.05$; Figs. 2 and 3). Conversely, the treatments with piracetam or 6-gingerol at doses of 10 and 20 mg/kg BW markedly attenuated Rt.MCAO-induced reduction of CAT and GSH-Px activities compared with the Rt.MCAO + vehicle group ($P<0.05$; Figs. 2 and 3).

Ameliorative effect of 6-gingerol in rats with induced-Rt. MCAO by activating anti-apoptotic pathway. Understanding the relationship between apoptosis and ischemic stroke is essential if apoptosis-modulating drugs capable of reducing brain damage are to be successfully developed. The present study therefore measured Bax, Bcl-xL and caspase-3 protein expression in the cerebral cortex and hippocampus by western blot analysis. Previous experiments demonstrated that a dose of 20 mg/kg BW 6-gingerol yielded favorable modifications in antioxidant enzyme parameters; this dose was chosen to investigate the influence of 6-gingerol on Bax, Bcl-xL and caspase-3. In Fig. 4A, it can be observed that Rt.MCAO rats treated with 250 mg/kg BW piracetam or 20 mg/kg BW 6-gingerol for 7 days demonstrated a reduction in the density ratio of Bax and caspase-3 to the β-actin band compared with the Rt.MCAO + vehicle group ($P<0.05$; Fig. 4B and D). By contrast, the density ratio of Bcl-xL to the β-actin band exhibited a significant increase compared with the Rt.MCAO + vehicle group ($P<0.05$; Fig. 4C).

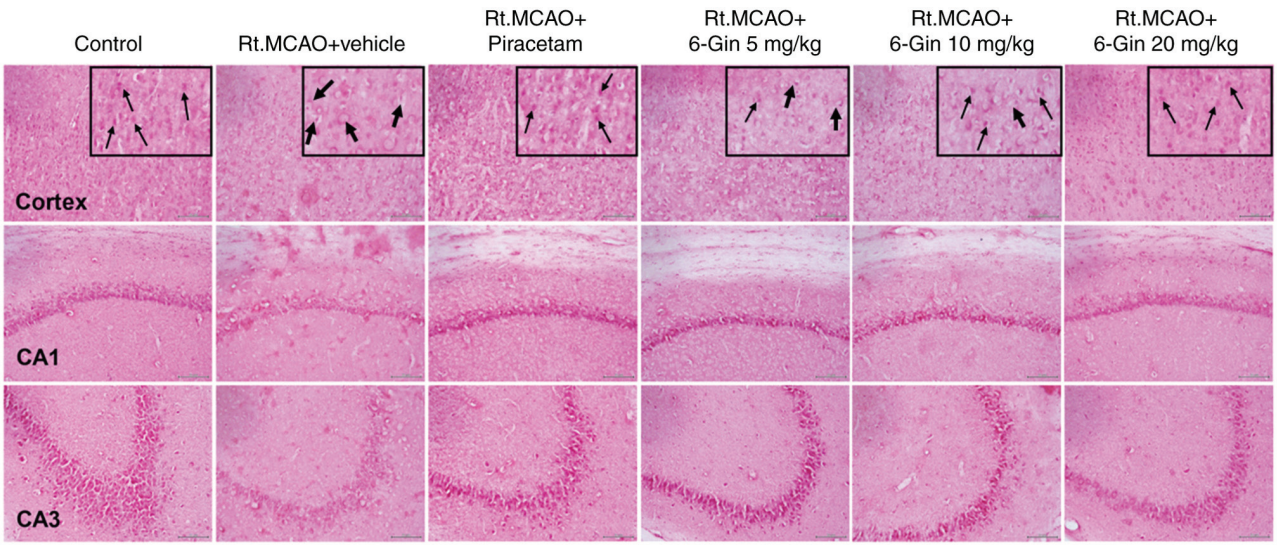

Figure 1. Representative images of hematoxylin and eosin-stained sections of the cortex and hippocampus. Histological changes of rat brain tissue imaged following MCAO with a light microscope (magnification, x20). In the control group, the morphology of most of the neurons were clear with large nuclei (thin arrows). The neurons of Rt. MCAO group rats exhibited features of degenerating neurons with shrinkage, pyknotic nuclei and increased intercellular space (thick arrows). Scale bars, 50 μm. MCAO, middle cerebral artery occlusion; 6-Gin, 6-gingerol; CA, cerebral artery.

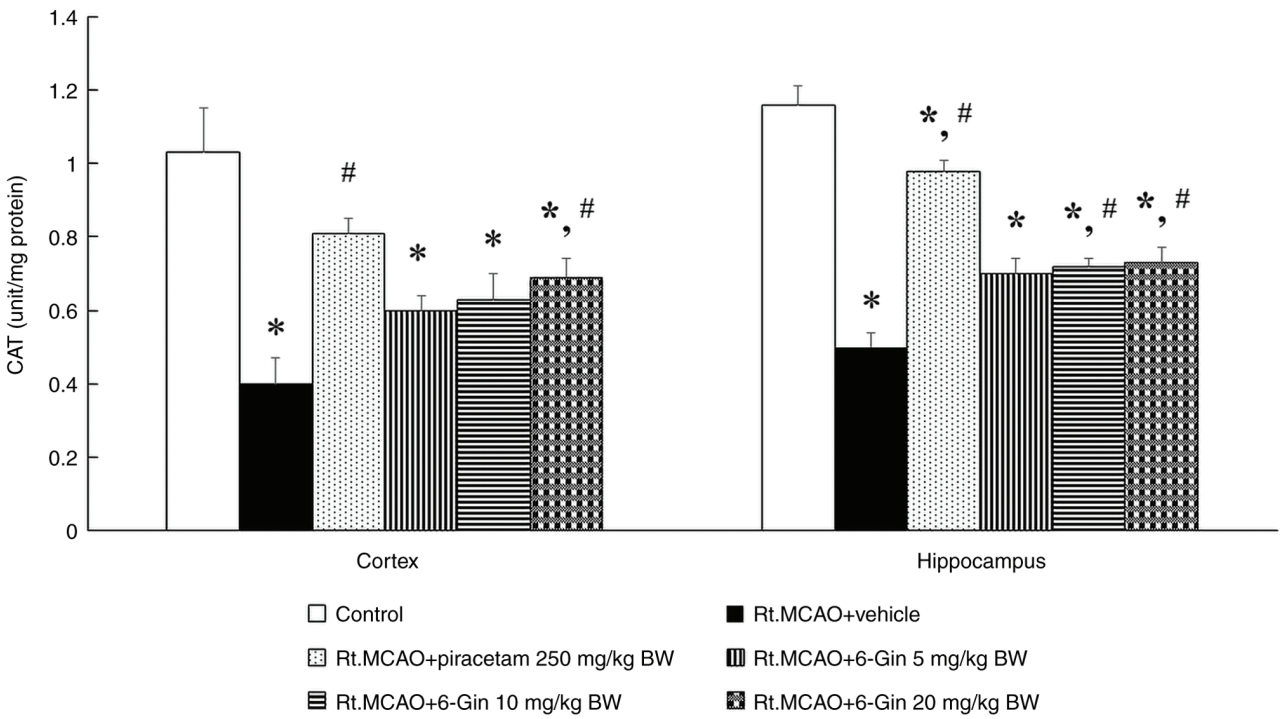

Figure 2. Effect of 6-Gin on CAT activity in the cortex and hippocampus of Rt.MCAO-induced rats. Data are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. the control; #P<0.05 vs. the Rt.MCAO + vehicle group. CAT, catalase; Rt.MCAO, right middle cerebral artery occlusion; 6-Gin, 6-gingerol; BW, body weight.

The effect of 6-gingerol on MAPK and Mfn2 protein expression evaluated using western blot analysis. MAPK signaling and Mfn2 play significant roles in stroke pathophysiology and could be suitable drug targets for mitigating stroke-induced damage, restoring mitochondrial homeostasis, and promoting neuronal survival and recovery. The effect of 6-gingerol on MAPK and Mfn2 protein expression was investigated using western blot analysis. The rats in the Rt.MCAO + vehicle group exhibited a significant increase in the density ratio of MAPK to the β-actin band; while experiencing a decrease in the density ratio of Mfn2 to the β-actin band compared with the control group (P<0.05). Importantly, treatment with piracetam (250 mg/kg BW) or 6-gingerol (20 mg/kg BW) reduced the density ratio of MAPK to the β-actin band compared with the vehicle group (P<0.05; Fig. 5A). Additionally, the density ratio of Mfn2 to the β-actin band exhibited significantly less reduction compared with the rats in the Rt.MCAO + vehicle group (P<0.05; Fig. 5B and C).

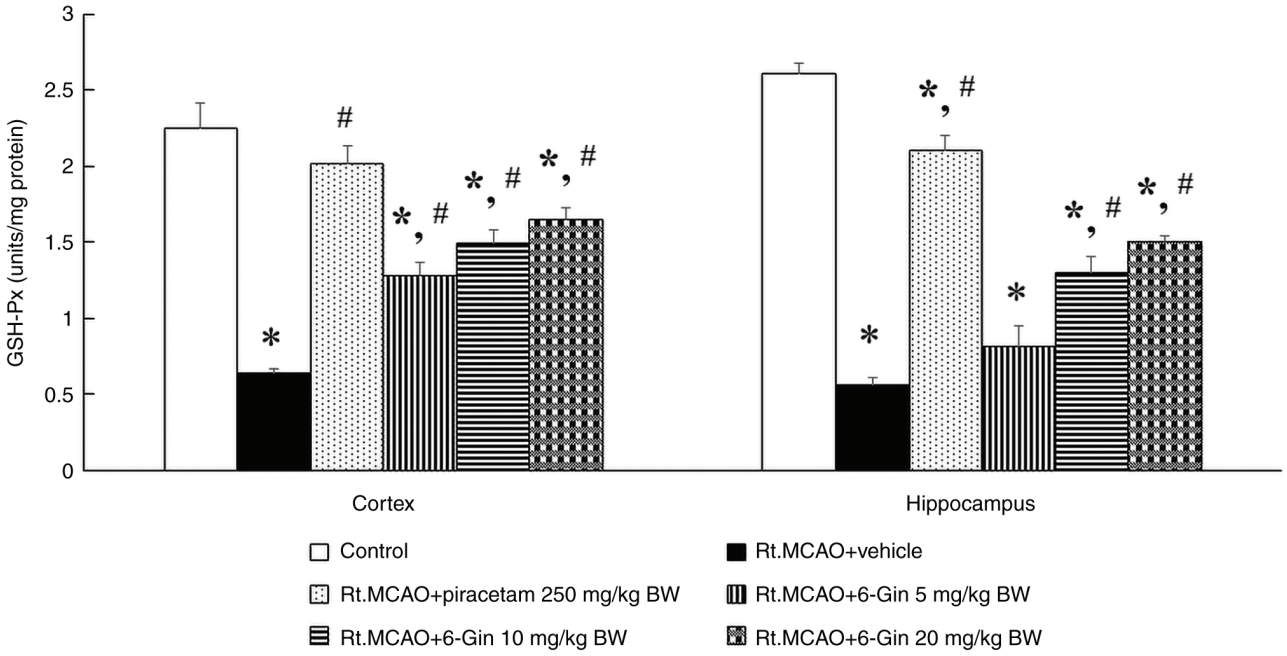

Figure 3. Effect of 6-Gin on GSH-Px activity in the cortex and hippocampus of Rt.MCAO-induced rats. Data are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. the control; #P<0.05 vs. the Rt.MCAO + vehicle group. GSH-Px, glutathione peroxidase; Rt.MCAO, right middle cerebral artery occlusion; 6-Gin, 6-gingerol; BW, body weight.

Discussion

Oxidative stress, resulting from an imbalance between ROS production and antioxidant defenses, plays a pivotal role in stroke pathophysiology. Antioxidants, through their ability to scavenge ROS, may offer neuroprotective effects and mitigate the detrimental consequences of stroke. CAT is an integral component of the cellular antioxidant defense system. It cooperates with other antioxidant enzymes, such as superoxide dismutase and GSH-Px, to maintain cellular redox homeostasis and protect cells from oxidative damage. Hence, the present study examined the influence of 6-gingerol on CAT and GSH-Px enzymes in the cortex and hippocampus. It was revealed that both the positive control, piracetam, and the experimental compound, 6-gingerol, led to increased CAT and GSH-Px activities in both the cortex and hippocampus. These effects were observed when compared with the group treated with vehicle only following Rt.MCAO. Numerous studies have reported that augmenting the levels of these antioxidant enzymes can alleviate brain damage resulting from ischemic stroke (19,23,25,28). Moreover, several studies have demonstrated that 6-gingerol and its analogues have strong antioxidant activity. This is exerted via multiple mechanisms, including the scavenging of free radicals, oxidative stress reduction and enhancement of antioxidant enzyme activity (19,29-32). These properties enable 6-gingerol to protect against oxidative damage in various tissues and organs, such as the brain, kidneys, heart and colon (32).

Apoptosis, or programmed cell death, is a regulated process that plays a role in removing damaged or unnecessary cells. Prolonged cerebral ischemia has the potential to induce the activation of apoptotic pathways in neurons, leading to neuronal death (8). Bcl-xL expression has been observed in both the developing embryonic and adult neurons of the central nervous system (CNS) (33). It plays a crucial role in safeguarding against neuronal apoptosis during brain development and in response to various pathological triggers, such as cerebral ischemia (12). Caspase-3 plays a critical role as a mediator of apoptosis in acute and chronic neurodegenerative conditions, including ischemic stroke (34,35). Additionally, Bax serves as a pro-apoptotic protein localized in the mitochondria, and its expression elevates during the initiation of the intrinsic apoptotic pathway, resulting in mitochondrial damage (36-38). Numerous studies have revealed that apoptosis inhibitors can effectively decrease ischemic neuronal injury (34,36). In comparison with the Rt.MCAO + vehicle group, a significant decrease was observed in the density ratio of Bax and caspase-3 to the β-actin band in Rt.MCAO rats treated with 250 mg/kg BW piracetam or 20 mg/kg BW 6-gingerol during the 7-day study period. Conversely, the density ratio of Bcl-xL to the β-actin band demonstrated a significant increase when compared with the Rt.MCAO + vehicle group in the present study. Accumulating evidence revealed that inhibition of Bax or caspase-3 activity and enhancement of Bcl-xL expression have neuroprotective effects in preclinical studies (34,39-41). The findings of the present study are consistent with a previous cerebral ischemia study showing that gingerol administration elevates Bcl-2 and brain-derived neurotrophic factor (BDNF) levels, while simultaneously reducing Bax and cleaved caspase-3 levels (42). In previous studies, it has also been demonstrated that 6-gingerol exhibits robust antiapoptotic and anti-inflammatory properties (43,44). Additionally, its ability to induce autophagy has been associated with the dissociation of the TRPV1/FAF1 complex (44). In the present study, piracetam was used as a positive control due to its known protective effects. Piracetam enhances cholinergic functions and reduces neuronal inflammation, apoptosis and oxidative stress, thus highlighting its beneficial effects as a therapeutic

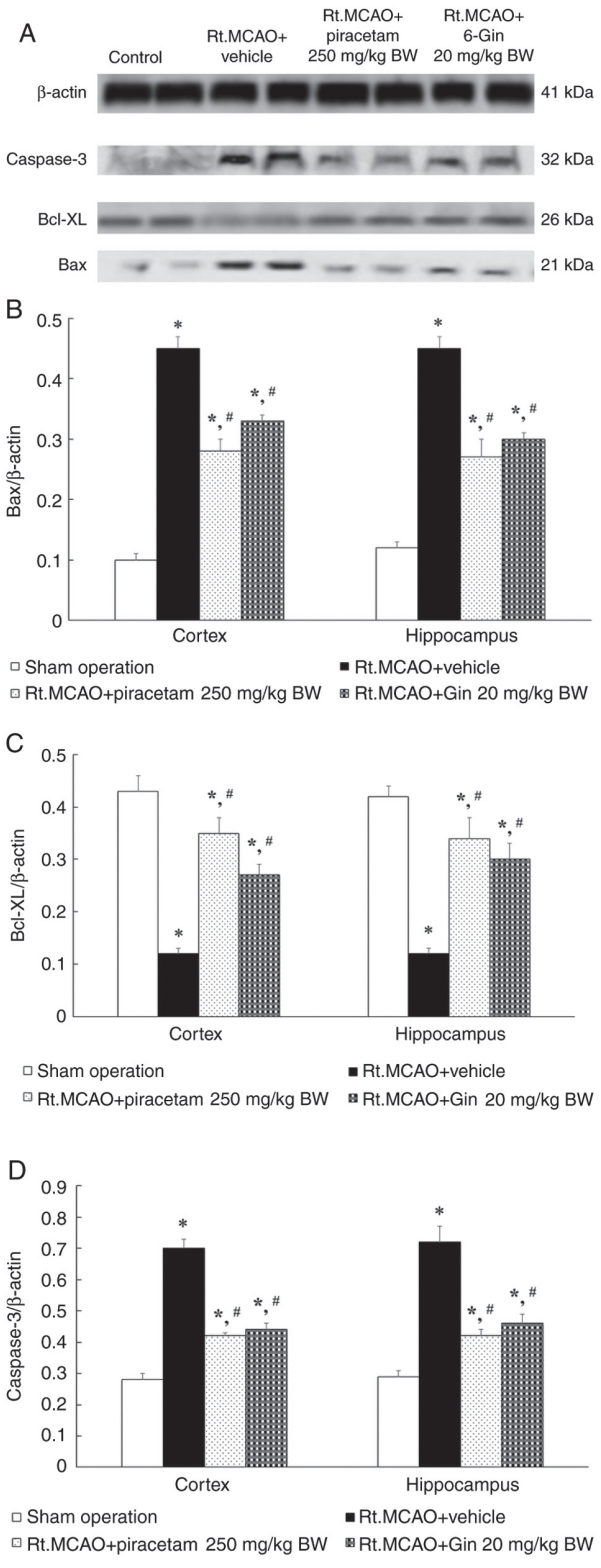

Figure 4. Effect of 6-Gin on the expression of Bax, Bcl-xL and caspase-3. (A) Image of an immunoblot of Bax (21 kDa), Bcl-xL (26 kDa) and caspase-3 (32 kDa) from the cerebral cortex. β-actin (41 kDa) was used as a loading control. (B-D) Quantitative analysis of (B) Bax, (C) Bcl-xL and (D) caspase-3 band density normalized with beta-actin. Data are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. the control; #P<0.05 vs. the Rt.MCAO + vehicle group. 6-Gin, 6-gingerol; Rt.MCAO, right middle cerebral artery occlusion; BW, body weight.

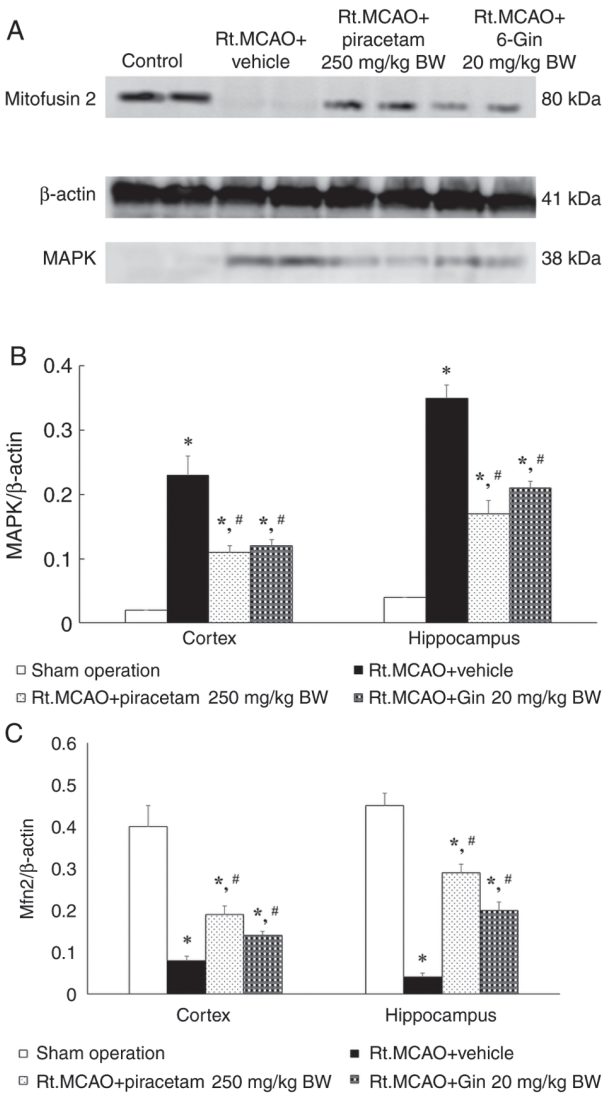

Figure 5. Effect of 6-Gin on the expression of MAPK and Mfn2. (A) Image of an immunoblot of MAPK (38 kDa) and Mfn2 (80 kDa) from the cerebral cortex. β-actin (41 kDa) was used as a loading control. (B and C) Quantitative analysis of (B) MAPK and (C) Mfn2 band density normalized with beta-actin. Data are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. the control; #P<0.05 vs. the Rt.MCAO + vehicle group. Mfn2, mitofusin 2; Rt.MCAO, right middle cerebral artery occlusion; 6-Gin, 6-gingerol; BW, body weight.

agent (45,46). Previous studies have demonstrated that administration of piracetam at doses of 250 or 500 mg/kg via the intraperitoneal (i.p.) route, 6, 9 and 22 h following ischemic insult, leads to a significant reduction in infarct volume (47). Furthermore, piracetam has been revealed to enhance cerebral blood flow (48) and restore the fluidity of the plasma membrane in the brain, thereby promoting improved functioning of neuronal cells (49).

MAPKs are key signaling molecules that contribute to the pathophysiology of stroke through their involvement in neuronal injury, neuroinflammation, blood-brain barrier disruption and oxidative stress (13-15). Hence, the investigation conducted in the present study examined the effect of 6-gingerol on MAPK using the rat model of focal cerebral ischemia. It was demonstrated that rats treated with piracetam at a dosage of 250 mg/kg BW or 6-gingerol at a dosage of 20 mg/kg BW for 7 days following Rt.MCAO, exhibit notable reductions in the density ratios of MAPK to the β-actin band.

Bioinformatics studies have revealed that certain miRNAs play a role in regulating MAPK, a crucial pathway involved in mitigating inflammation and apoptosis in ischemic stroke (13,50,51). The findings of the present study correspond well with a previous sepsis study showing that 6-gingerol treatment diminishes macrophage pyroptosis by inhibiting MAPK signaling pathways (52), and a previous hypoxia study showing that 6-gingerol effectively deactivates the p38 MAPK and JNK pathways (53).

Mitochondrial dysfunction, characterized by impaired oxidative phosphorylation and increased ROS production, has been implicated in the pathogenesis of ischemic stroke (54). Mfn1 and Mfn2 are also involved in the pathophysiology of ischemic stroke (55). In experimental work using the MCAO rat model, it has been observed that reductions in Mfn1 and Mfn2 lead to mitochondrial Bax translocation and subsequent Ca^{2+} overload, resulting in excitotoxicity and neuronal apoptosis (16,56). Furthermore, Mfn2 has been revealed to decrease caspase-3 activity and increase the ratio of Bcl-2/Bax, thereby reducing cellular vulnerability to apoptosis in the context of cerebral ischemic stroke (57). In line with available studies, the current results demonstrated that animals treated with piracetam (250 mg/kg BW) or 6-gingerol (20 mg/kg BW) exhibited significantly less reduction in the density ratio of Mfn2 to the β-actin band compared with rats in the Rt.MCAO + vehicle group. Supplementation with gingerol-enriched ginger has been revealed to alleviate Mfn2 and mitochondrial inner membrane fusion (OPA1) in diabetic rats (58). According to findings presented in a previous study, phenolic compounds such as 6-gingerol and 6-shogaol have pharmacological activities that support the generation of functional mitochondria, thereby promoting mitochondrial biogenesis (59).

DMSO is a commonly used solvent and vehicle in biological and biochemical research. Studies have reported that the lethal dose (LD_{50}) of DMSO in rats, administered via i.p. injection, is 9.9 ml/kg BW (60). Furthermore, another study revealed that significant localized toxic impacts on the liver and kidneys of rats were observed when plant extracts were dissolved in 10% (v/v) DMSO (61). In the present study, 1% (v/v) DMSO solution was selected as the vehicle. This decision was based on literature reviews, which generally consider DMSO safe at low concentrations (62). A total of 1% (v/v) DMSO concentration is frequently employed in numerous biological applications without causing significant toxicity, as supported by previous publications made by the authors (19,25,63).

In addition to determining brain histomorphological changes in rats after a stroke, it is useful to measure functional outcomes such as motor, sensory, and cognitive abilities. The present study did not assess behavioral impairments following stroke. Therefore, a limitation of the present study is the lack of assessment of neurological function to evaluate the degree of damage over time.

The present study suggested that 6-gingerol can ameliorate some of the pathological changes induced by ischemic stroke and does so via antioxidant and anti-apoptotic pathways. In addition to evaluating histomorphological damage to neurons, future research is recommended to investigate the interaction between neurons and glial cells, particularly involving neuroinflammation, in an ischemic stroke model. Glial cells, including microglia, astrocytes and oligodendrocytes, are the primary components of the peri-infarct environment in the CNS and have been implicated in immune regulation following a stroke. Previous studies have demonstrated that glial cells regulate post-stroke neuroinflammation. These cells can modulate signals of neuronal damage, release cytokines, attract immune cells to the site of the stroke, and interact with and affect the condition of other immune cells (64,65). Assessment of the effect of 6-gingerol on neurotrophic factors such as BDNF, nerve growth factor, and glial cell-derived neurotrophic factor will also be necessary to fully elucidate the neuroprotective and neurogenic effects of this compound.

In conclusion, 6-gingerol demonstrates significant *in vivo* effectiveness in mitigating pathological changes induced by cerebral ischemia. This beneficial effect is attributed, in part, to its antioxidant and anti-apoptotic pathways. Further investigations are warranted to explore the neurological and biochemical effects of 6-gingerol in focal cerebral ischemia, as well as to elucidate its complete mechanism of action.

Acknowledgements

The authors are grateful to Dr Tim Cushnie (Faculty of Medicine, Mahasarakham University, Mahasarakham) for language-editing assistance.

Funding

The present study was supported by Mahasarakham University Faculty of Medicine (grant no. MED MSU 01/03/2566).

Availability of data and materials

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

Authors' contributions

RK was involved in data analysis, wrote, reviewed and edited the manuscript. JJ was the project's administrator, designed and conceptualized the present study, acquired funding, curated data, analyzed data, wrote the original draft, and wrote, reviewed and edited the manuscript. RK and JJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols related to animal experimentation were meticulously designed to minimize any potential suffering to the animals involved. These protocols were conducted in strict accordance with the approval granted by the Institutional Animal Care and Use Committee at Khon Kaen University (Thailand), with a designated record number of (approval no. IACUC-KKU-6/65).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Samuthpongtorn C, Jereerat T and Suwanwela NC: Stroke risk factors, subtypes and outcome in elderly Thai patients. BMC Neurol 21: 322, 2021.
2. Gebreyohannes EA, Bhagavathula AS, Abebe TB, Seid MA and Haile KT: In-hospital mortality among ischemic stroke patients in Gondar University Hospital: A retrospective cohort study. Stroke Res Treat 2019: 7275063, 2019.
3. Lucas-Noll J, Clua-Espuny JL, Lleixa-Fortuno M, Gavaldà-Espelta E, Queralt-Tomas L, Panisello-Tafalla A and Carles-Lavila M: The costs associated with stroke care continuum: A systematic review. Health Econ Rev 13: 32, 2023.
4. Checa J and Aran JM: Reactive Oxygen Species: Drivers of Physiological and Pathological Processes. J Inflamm Res 13: 1057-1073, 2020.
5. Zivancevic K, Lovic D, Andjus PR and Radenovic L: Neuroinflammation in Post-Ischemic Brain. In: Cerebral Ischemia. Pluta R (ed). Brisbane, AU, 2021.
6. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X and Zhao L: Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9: 7204-7218, 2017.
7. Wu L, Xiong X, Wu X, Ye Y, Jian Z, Zhi Z and Gu L: Targeting oxidative stress and inflammation to prevent ischemia-reperfusion injury. Front Mol Neurosci 13: 28, 2020.
8. Qin C, Yang S, Chu YH, Zhang H, Pang XW, Chen L, Zhou LQ, Chen M, Tian DS and Wang W: Signaling pathways involved in ischemic stroke: Molecular mechanisms and therapeutic interventions. Signal Transduct Target Ther 7: 215, 2022.
9. Mao R, Zong N, Hu Y, Chen Y and Xu Y: Neuronal death mechanisms and therapeutic strategy in ischemic stroke. Neurosci Bull 38: 1229-1247, 2022.
10. Otsuka S, Itashiki Y, Tani A, Matsuoka T, Takada S, Matsuzaki R, Nakanishi K, Norimatsu K, Tachibe Y, Kitazato R, *et al*: Effects of different remote ischemia perconditioning methods on cerebral infarct volume and neurological impairment in rats. Sci Rep 13: 2158, 2023.
11. Sun Y, Xu Y and Geng L: Caspase-3 inhibitor prevents the apoptosis of brain tissue in rats with acute cerebral infarction. Exp Ther Med 10: 133-138, 2015.
12. Park HA, Broman K and Jonas EA: Oxidative stress battles neuronal Bcl-xL in a fight to the death. Neural Regen Res 16: 12-15, 2021.
13. Gao ZK, Shen XY, Han Y, Guo YS, Li K and Bi X: Pre-ischemic exercise prevents inflammation and apoptosis by inhibiting MAPK pathway in ischemic stroke. Transl Neurosci 13: 495-505, 2022.
14. Zhu Z, Ge M, Li C, Yu L, Gu Y, Hu Y and Cao Z: Effects of p38 MAPK signaling pathway on cognitive function and recovery of neuronal function after hypoxic-ischemic brain injury in newborn rats. J Clin Neurosci 78: 365-370, 2020.
15. Hou K, Xiao ZC and Dai HL: p38 MAPK Endogenous inhibition improves neurological deficits in global cerebral ischemia/reperfusion mice. Neural Plast 2022: 3300327, 2022.
16. Vongsfak J, Pratchayasakul W, Apaijai N, Vaniyapong T, Chattipakorn N and Chattipakorn SC: The alterations in mitochondrial dynamics following cerebral ischemia/reperfusion injury. Antioxidants (Basel) 10: 1384, 2021.
17. Li J, Wu J, Zhou X, Lu Y, Ge Y and Zhang X: Targeting neuronal mitophagy in ischemic stroke: An update. Burns Trauma 11: tkad018, 2023.
18. Li C, Chen C, Qin H, Ao C, Chen J, Tan J and Zeng L: The role of mitochondrial dynamin in stroke. Oxid Med Cell Longev 2022: 2504798, 2022.
19. Kongsui R and Jittiwat J: Ameliorative effects of 6-gingerol in cerebral ischemia are mediated via the activation of antioxidant and anti-inflammatory pathways. Biomed Rep 18: 26, 2023.
20. Almatroodi SA, Alnuqaydan AM, Babiker AY, Almogbel MA, Khan AA and Husain Rahmani A: 6-Gingerol, a bioactive compound of ginger attenuates renal damage in streptozotocin-induced diabetic rats by regulating the oxidative stress and inflammation. Pharmaceutics 13: 317, 2021.
21. Wang S, Zhang C, Yang G and Yang Y: Biological properties of 6-gingerol: A brief review. Nat Prod Commun 9: 1027-1030, 2014.
22. Mao QQ, Xu XY, Cao SY, Gan RY, Corke H, Beta T and Li HB: Bioactive compounds and bioactivities of ginger (Zingiber officinale Roscoe). Foods 8: 185, 2019.
23. Jittiwat J, Suksamrarn A, Tocharus C and Tocharus J: Dihydrocapsaicin effectively mitigates cerebral ischemia-induced pathological changes in vivo, partly via antioxidant and anti-apoptotic pathways. Life Sci 283: 119842, 2021.
24. Adetuyi BO and Farombi EO: 6-Gingerol, an active constituent of ginger, attenuates lipopolysaccharide-induced oxidation, inflammation, cognitive deficits, neuroplasticity, and amyloidogenesis in rat. J Food Biochem 45: e13660, 2021.
25. Jittiwat J: Baihui point laser acupuncture ameliorates cognitive impairment, motor deficit, and neuronal loss partly via antioxidant and anti-inflammatory effects in an animal model of focal ischemic stroke. Evid Based Complement Alternat Med 2019: 1204709, 2019.
26. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275, 1951.
27. Goldblith SA and Proctor BE: Photometric determination of catalase activity. J Biol Chem 187: 705-709, 1950.
28. Davis SM and Pennypacker KR: Targeting antioxidant enzyme expression as a therapeutic strategy for ischemic stroke. Neurochem Int 107: 23-32, 2017.
29. Alharbi KS, Nadeem MS, Afzal O, Alzarea SI, Altamimi ASA, Almalki WH, Mubeen B, Iftikhar S, Shah L and Kazmi I: Gingerol, a natural antioxidant, attenuates hyperglycemia and downstream complications. Metabolites 2: 1274, 2022.
30. Ajayi BO, Adedara IA and Farombi EO: Protective mechanisms of 6-gingerol in dextran sulfate sodium-induced chronic ulcerative colitis in mice. Hum Exp Toxicol 37: 1054-1068, 2018.
31. Ozkur M, Benlier N, Takan I, Vasileiou C, Georgakilas AG, Pavlopoulou A, Cetin Z and Saygili EI: Ginger for healthy ageing: A systematic review on current evidence of its antioxidant, anti-inflammatory, and anticancer properties. Oxid Med Cell Longev 2022: 4748447, 2022.
32. Abolaji AO, Ojo M, Afolabi TT, Arowoogun MD, Nwawolor D and Farombi EO: Protective properties of 6-gingerol-rich fraction from *Zingiber officinale* (Ginger) on chlorpyrifos-induced oxidative damage and inflammation in the brain, ovary and uterus of rats. Chem Biol Interact 270: 15-23, 2017.
33. Bas J, Nguyen T and Gillet G: Involvement of Bcl-xL in neuronal function and development. Int J Mol Sci 22: 3202, 2021.
34. Broughton BR, Reutens DC and Sobey CG: Apoptotic mechanisms after cerebral ischemia. Stroke 40: e331-339, 2009.
35. Glushakova OY, Glushakov AA, Wijesinghe DS, Valadka AB, Hayes RL and Glushakov AV: Prospective clinical biomarkers of caspase-mediated apoptosis associated with neuronal and neurovascular damage following stroke and other severe brain injuries: Implications for chronic neurodegeneration. Brain Circ 3: 87-108, 2017.
36. Bagheri G, Rezaee R, Tsarouhas K, Docea AO, Shahraki J, Shahriari M, Wilks MF, Jahantigh H, Tabrizian K, Moghadam AA, *et al*: Magnesium sulfate ameliorates carbon monoxide-induced cerebral injury in male rats. Mol Med Rep 19: 1032-1039, 2019.
37. Liu G, Wang T, Wang T, Song J and Zhou Z: Effects of apoptosis-related proteins caspase-3, Bax and Bcl-2 on cerebral ischemia rats. Biomed Rep 1: 861-867, 2013.
38. Gupta R and Ghosh S: Putative roles of mitochondrial Voltage-Dependent Anion Channel, Bcl-2 family proteins and c-Jun N-terminal Kinases in ischemic stroke associated apoptosis. Biochim Open 4: 47-55, 2017.
39. Yenari MA: Pathophysiology of acute ischemic stroke. Cleve Clin J Med 71 (Suppl 1): S25-S27, 2004.
40. Shang JL, Cheng Q, Duan SJ, Li L and Jia LY: Cognitive improvement following ischemia/reperfusion injury induced by voluntary running-wheel exercise is associated with LncMALAT1-mediated apoptosis inhibition. Int J Mol Med 41: 2715-2723, 2018.
41. Li Z, Xiao G, Wang H, He S and Zhu Y: A preparation of Ginkgo biloba L. leaves extract inhibits the apoptosis of hippocampal neurons in post-stroke mice via regulating the expression of Bax/Bcl-2 and Caspase-3. J Ethnopharmacol 280: 114481, 2021.
42. Zhai Y, Liu BG, Mo XN, Zou M, Mei XP, Chen W, Huang GD and Wu L: Gingerol ameliorates neuronal damage induced by hypoxia-reoxygenation via the miR-210/brain-derived neurotrophic factor axis. Kaohsiung J Med Sci 38: 367-377, 2022.

43. Alsahli MA, Almatroodi SA, Almatroudi A, Khan AA, Anwar S, Almutary AG, Alrumaihi F and Rahmani AH: 6-Gingerol, a major ingredient of ginger attenuates diethylnitrosamine-induced liver injury in rats through the modulation of oxidative stress and anti-inflammatory activity. Mediators Inflamm 2021: 6661937, 2021.
44. Luo J, Chen J, Yang C, Tan J, Zhao J, Jiang N and Zhao Y: 6-Gingerol protects against cerebral ischemia/reperfusion injury by inhibiting NLRP3 inflammasome and apoptosis via TRPV1/FAF1 complex dissociation-mediated autophagy. Int Immunopharmacol 100: 108146, 2021.
45. Verma DK, Gupta S, Biswas J, Joshi N, Singh A, Gupta P, Tiwari S, Sivarama Raju K, Chaturvedi S, Wahajuddin M and Singh S: New therapeutic activity of metabolic enhancer piracetam in treatment of neurodegenerative disease: Participation of caspase independent death factors, oxidative stress, inflammatory responses and apoptosis. Biochim Biophys Acta Mol Basis Dis 1864: 2078-2096, 2018.
46. Mohammed AS, Al-Hassani AN, Alrawi RA and Tawfeeq RD: The protective effect of taurine, piracetam and vinpocetine on etoposide-induced inflammation and brain injury in the serum of female albino rats. Ecancermedicalscience 17: 1499, 2023.
47. Tortiglione A, Minale M, Pignataro G, Amoroso S, DiRenzo G and Annunziato L: The 2-oxopyrrolidinacetamide piracetam reduces infarct brain volume induced by permanent middle cerebral artery occlusion in male rats. Neuropharmacology 43: 427-433, 2002.
48. Kessler J, Thiel A, Karbe H and Heiss WD: Piracetam improves activated blood flow and facilitates rehabilitation of poststroke aphasic patients. Stroke 31: 2112-2116, 2000.
49. Yang Y, Feng J, Xu F and Wang J: Piracetam inhibits ethanol (EtOH)-induced memory deficit by mediating multiple pathways. Brain Res 1676: 83-90, 2017.
50. Gugliandolo A, Silvestro S, Sindona C, Bramanti P and Mazzon E: MiRNA: Involvement of the MAPK pathway in ischemic stroke. A promising therapeutic target. Medicina (Kaunas) 57: 1053, 2021.
51. Safa A, Abak A, Shoorei H, Taheri M and Ghafouri-Fard S: MicroRNAs as regulators of ERK/MAPK pathway: A comprehensive review. Biomed Pharmacother 132: 110853, 2020.
52. Zhang FL, Zhou BW, Yan ZZ, Zhao J, Zhao BC, Liu WF, Li C and Liu KX: 6-Gingerol attenuates macrophages pyroptosis via the inhibition of MAPK signaling pathways and predicts a good prognosis in sepsis. Cytokine 125: 154854, 2020.
53. Kang C, Kang M, Han Y, Zhang T, Quan W and Gao J: 6-Gingerols (6G) reduces hypoxia-induced PC-12 cells apoptosis and autophagy through regulation of miR-103/BNIP3. Artif Cells Nanomed Biotechnol 47: 1653-1661, 2019.
54. Yang JL, Mukda S and Chen SD: Diverse roles of mitochondria in ischemic stroke. Redox Biol 16: 263-275, 2018.
55. Yang M, He Y, Deng S, Xiao L, Tian M, Xin Y, Lu C, Zhao F and Gong Y: Mitochondrial quality control: A pathophysiological mechanism and therapeutic target for stroke. Front Mol Neurosci 14: 786099, 2022.
56. Martorell-Riera A, Segarra-Mondejar M, Munoz JP, Ginet V, Olloquequi J, Pérez-Clausell J, Palacín M, Reina M, Puyal J, Zorzano A and Soriano FX: Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death. EMBO J 33: 2388-2407, 2014.
57. Jurcau A and Ardelean AI: Oxidative stress in ischemia/reperfusion injuries following acute ischemic stroke. Biomedicines 10: 574, 2022.
58. Wang R, Santos JM, Dufour JM, Stephens ER, Miranda JM, Washburn RL, Hibler T, Kaur G, Lin D and Shen CL: Ginger root extract improves GI health in diabetic rats by improving intestinal integrity and mitochondrial function. Nutrients 14: 4384, 2022.
59. Razak AM, Tan JK, Mohd Said M and Makpol S: Modulating effects of zingiberaceae phenolic compounds on neurotrophic factors and their potential as neuroprotectants in brain disorders and age-associated neurodegenerative disorders: A review. Nutrients 15: 2564, 2023.
60. Kelava T, Javar I and Culo F: Biological actions of drug solvents. Periodicum Biologorum 113: 311-320, 2011.
61. Kurdi SJA, Hamid NFS, Ranneh Y, Meng GY, and Hashim ZB: Dimethyl Sulfoxide and Their Toxicity. Int J Res Biol Pharm 5: 9-18, 2019.
62. Kloverpris H, Fomsgaard A, Handley A, Ackland J, Sullivan M and Goulder P: Dimethyl sulfoxide (DMSO) exposure to human peripheral blood mononuclear cells (PBMCs) abolish T cell responses only in high concentrations and following coincubation for more than two hours. J Immunol Methods 356: 70-78, 2010.
63. Janyou A, Wicha P, Jittiwat J, Suksamrarn A, Tocharus C, and Tocharus J: Dihydrocapsaicin attenuates blood brain barrier and cerebral damage in focal cerebral ischemia/reperfusion via oxidative stress and inflammatory. Sci Rep 7: 10556, 2017.
64. Xu S, Lu J, Shao A, Zhang JH, and Zhang J: Glial cells: Role of the immune response in ischemic stroke. Front Immunol 11: 294, 2020.
65. Amantea D, Micieli G, Tassorelli C, Cuartero MI, Ballesteros I, Certo M, Moro MA, Lizasoain I and Bagetta G: Rational modulation of the innate immune system for neuroprotection in ischemic stroke. Front Neurosci 9: 147, 2015.