Therapeutic effects of a standardized-flavonoid Diospyros kaki L.f. leaf extract on transient focal cerebral ischemia-induced brain injury in mice

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Abstract

This study aimed to investigate the neuroprotective and therapeutic effects of *Diospyros kaki* L.f. leaves (DK) on transient focal cerebral ischemic injury and underlying mechanisms using a middle cerebral artery occlusion (MCAO) model of mice. The animals received the MCAO operation on day 0. The daily administrations of DK (50 and 100 mg/kg, p.o) and edaravone (6 mg/kg, i.v), a reference drug with radical scavenging activity, were started 7 days before (pre-treatment) or immediately after the MCAO operation (post-treatment) and continued during the experimental period. Histochemical, biochemical, and neurological changes were analysed from days 1 to 4, while cognitive performance was evaluated on day 12 after MCAO.

MCAO caused cerebral infarction and neuronal cell loss in the cortex, striatum, and hippocampus in a manner accompanied by spatial cognitive deficits. These neurological and cognitive impairments caused by MCAO were significantly attenuated by pre- and post-ischemic treatments with DK and edaravone, suggesting that DK, like edaravone, has therapeutic potential for cerebral ischemia-induced brain damage. DK and edaravone suppressed MCAO-induced changes in biomarkers for apoptosis (TUNEL positive cell number and cleaved caspase-3 protein expression) and oxidative stress (glutathione and malondialdehyde contents) in the brain. Interestingly, DK, but not edaravone, mitigated an increase in blood-brain permeability and down-regulation of vascular endothelial growth factor (VEGF)-mediated signalling caused by MCAO. These results indicate that DK exerts neuroprotective and therapeutic activity against transient focal cerebral ischemia-induced injury probably by suppressing oxidative stress, apoptotic process, and mechanisms impairing blood-brain barrier integrity in the brain.

Introduction

Ischemic stroke, an acute cerebrovascular disease, is one of the major causes of morbidity and mortality worldwide [1]. Currently, the standard treatments of this disease employ intravenous thrombolysis using aspirin and recombinant tissue plasminogen activator as well as reperfusion by endovascular thrombectomy to avoid brain tissue damage. However, these treatments have small and time-critical therapeutic windows and risks of bleeding [2]. Thus, treatment options available for ischemic stroke are still limited, making it of great necessity to further develop novel agents that can act against ischemic-induced neuronal damage.

Lines of studies exploring therapeutic medications for stroke demonstrated that neuroprotective agents are beneficial to the protection of the brain from ischemic injury by minimizing brain damage, extending the therapeutic time window, and further improving functional outcomes [3–5]. It has been reported that the pathophysiology of cerebral ischemic stroke involves many processes such as cellular excitotoxicity caused by excessive release of excitatory neurotransmitters, free radical generation-mediated oxidative stress, neuroinflammation, and blood-brain barrier (BBB) impairment. These complex physiological processes are interrelated and, thereby, affect various brain functions and contribute to long-term
disabilities [5–7]. Thus, herbal medicines with various chemical components and multi-targeted effects may be beneficial in treating complicated diseases like cerebral ischemia.

_Diospyros kaki_ L.f, called persimmon, belongs to the family Ebenaceae and is widely distributed in China, India, Japan, Korea, and Viet Nam [8]. We previously demonstrated that pre-ischemic treatment with ethanol extract of persimmon leaves (125–500 mg/kg b.w, p.o.) exerted neuroprotective effects on brain injuries in middle cerebral artery occlusion (MCAO) mice by reducing the infarct volume and improving neurofunctional recovery [9]. Our findings were supported by reports that pre-ischemic treatment with a flavonoid-enriched extract from persimmon leaves reduced cerebral ischemia-induced injury in mice [10, 11], suggesting that flavonoid components in persimmon leaves played an important role in the neuroprotective effects of persimmon leaves. However, it remains unclear whether the extract from persimmon leaves exerts therapeutic activity against cerebral ischemia-induced brain damage, and if so, what is the mechanism underlying its actions. In the present study, we investigated the therapeutic effects of a standardized flavonoid extract from _Diospyros kaki_ L.f. leaves (DK) against cerebral ischemic injury in mice and underlying mechanisms using a reference drug, edaravone.

### Materials And Methods

#### Preparation of DK extract

_Diospyros kaki_ L.f leaves used in this study were collected from Lang Son Province, Vietnam, in 2019 and identified by Dr. Pham Thanh Huyen (Department of Medicinal Plant Resources, National Institute of Medicinal Materials (NIMM), Vietnam). A voucher specimen of the herb was deposited in NIMM. A standardized flavonoid extract of this plant was prepared as previously reported [8]. Briefly, 10 kg of the leaves were cleaned, dried at 40-50°C, and cut into small pieces (2–5 mm). The dried persimmon leaves were extracted three times with 70% (v/v) ethanol (EtOH) (10, 8, and 8 ml/g, each) at reflux for 2, 1.5, and 1 h, respectively. The collected extracts were combined and evaporated under reduced pressure until yielding a 40% (v/v) EtOH extract. This ethanol extract was then adsorbed onto a D101 macroporous resin column. The column was washed using 30% (v/v) EtOH until the colour faded and then eluted with 60% (v/v) EtOH. After recovering EtOH using a rotary evaporator, EtOH was further evaporated by water-bathing at 60°C and then dried under vacuum at 50°C, yielding a dry extract (DK extract, 302 g) with moisture less than 5%.

#### Characterization of flavonoid compounds

**HPLC fingerprints**

The separation of flavonoid components in the DK extract was conducted according to the previous report [10] with a minor modification. HPLC analysis was carried out using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of a DGU-20A5R degassing unit, an LC-20AD pump, a SIL-20A HT automatic sampler, a CTO-10AS thermostatic column oven, a dual channel SPD-20A UV-Vis detector, and
a CBM-20A communication bus module. Separation was performed at 25°C with a flow rate of 1.0 ml/min. The mobile phase was 0.01% aqueous TFA acid (A) and acetonitrile (B) in a gradient elution system as follow: 15% B in 0–20 min, 15–20% B in 20–30 min, 20% B in 30–50 min, 20–60% B in 50–70 min, 60–15% B in 70–75 min, and 15% B in 75–90 min. The detection wavelength was set at 210 and 360 nm. The injection volume was 20 µl.

The HPLC chromatograms of DK extract and a standard mixture of 6 flavonoids are shown in Fig. 1A. Based on the HPLC analysis, the DK extract was characterized by 6 characteristic peaks including: (1) isoquercetin, (2) quercetin-3-O-(2''-O-galloyl-β-D-glucopyranoside, (3) trifolin, (4) astragalin, (5) kaempferol-3-O-(2''-O-galloyl-β-D-glucopyranoside, (6) kaempferol-3-O-(2''-O-galloyl-β-D-galactopyranoside.

Total flavonoid content

According to previous reports [10, 11], the total flavonoid content in the DK extract was analysed. Briefly, the DK extract was hydrolysed in an HCl 2 M/methanol solution at 90°C for 2 h. Quercetin and kaempferol contents in hydrolysed DK extract solution were determined using an HPLC (Shimazu 20A) system equipped with a Vertisep C18 column (250 mm × 4.6 mm, 5 µm) (Vertical Chromatography Co., Ltd, Bangkok, Thailand). The content of flavonoids in DK was calculated according to the following equation: 

\[
\text{The content of flavonoids in DK extract} = \text{quercetin content} \times 2.55 + \text{kaempferol content} \times 2.59.
\]

The HPLC chromatograms of the hydrolysed DK extract are shown in Fig. 1B. According to the analysis method, the total content of flavonoids in the DK extract was 21%.

Animals

Six 7-week-old male Swiss mice weighing 30–35 g were obtained from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. The experimental protocols in this study were approved by the Institutional Animal Use and Care Committees of the NIMM, Hanoi, Vietnam. The animals were housed in the laboratory animal room (25 ± 1°C under 65 ± 5% humidity and a 12 h dark-light cycle (from 7:00–19:00)). Commercial laboratory food and tap water were given ad libitum. The animals were acclimatized for at least one week before starting the experiments.

Middle cerebral artery occlusion (MCAO)-and-reperfusion model in mice

Transient focal cerebral ischemia was induced by using MCAO and reperfusion model in mice as previously reported [9, 12]. Briefly, the animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and then the common carotid artery (CCA) was carefully isolated via a midline incision and temporarily occluded using a 5 – 0 silk suture. Two permanent knots were created at the distal part of the external carotid artery (ECA) using 4 – 0 silk sutures. The left internal carotid artery (ICA) was ligated with a vessel clip to avoid bleeding. After creating a small hole on the ECA between two knots, a 12-mm long 6 – 0 nylon suture (Alfresa-pharma Co., Osaka, Japan) with a silicon-coated tip was introduced into the ECA and then inserted into the ICA. The suture was tightly tied around the monofilament to prevent bleeding,
and the reverse-action tweezers were removed. The occluder was inserted into the MCA origin in the circle of Willis (9–10 mm insertion beyond the bifurcation of ECA and CCA). The suture on the ECA was tightly tied to fix the monofilament in position. The temporary suture was removed from the CCA. After 60 minutes of occlusion, the monofilament suture was removed to allow reperfusion.

**Drug administration**

Drug administrations were conducted according to the experimental protocols shown in Fig. 2. Edaravone (5-Methyl-2-phenyl-2,4-dihydro-3H-pyrazol-3-one; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO as a stock solution and diluted in 0.9% saline immediately before injecting via a tail vein of the animals. DK extract was suspended in distilled water. In the pre-ischemic treatment procedure, the animals were administered DK (50 and 100 mg/kg b.w, p.o.) or edaravone (6 mg/kg b.w, i.v) daily for one week before being subjected to MCAO and after reperfusion. In the post-ischemic treatment procedure, DK (50 and 100 mg/kg b.w, p.o.) or edaravone (6 mg/kg b.w, i.v) were immediately administered after reperfusion, and the administrations were continued daily during the experimental period.

**Evaluation of neurological symptoms**

MCAO-and-reperfusion-induced neurological symptoms in the mice, which received daily pre- and post-ischemic treatment with test drugs, were evaluated by scoring according to Menzies et al. [13]. A four level score standard was used: 0 = no apparent deficits; 1 = right forelimb flexion, 2 = decreased grip of the right forelimb while tail pulled, 3 = spontaneous movement in all directions (right circling only if pulled by tail), 4 = spontaneous right circling. The tests were performed daily from day 1 (24 hours after the occlusion) to day 4.

**Modified Y-maze test**

Twelve days after MCAO-and-reperfusion, mice were subjected to the modified Y-maze test. The test was conducted according to previous reports [14, 15]. Briefly, the Y-maze apparatus was executed in an apparatus composed of 3 arms labelled as A, B, and C (Fig. 5A). Each arm with 120° between each other had a length of 40 cm, a height of 18 cm, a width of 12 cm at the top, and 3 cm at the bottom. Different spatial cues surrounded the Y-maze. In the sample trial, 1 h after drug administration, each mouse was individually placed in the centre of the Y-maze with one of the 3 arms closed, and allowed to move freely for 5 minutes. In the test trial conducted 30 min after the sample trial, the mouse was placed again in the Y-maze with all 3 arms opened for 5 min. The arm that was previously closed in the sample test was defined as the new arm. Between each trial, the 3 arms were cleaned using 70% ethanol to avoid olfactory cues. The series of arm entries was monitored via video recording and analysed using ANY-maze software ver. 4.99 (Stoelting™). An arm entry was considered to be complete when the hind paws of the mice were completely inside a given arm. The percentage of time the mouse was in the new arm over total time exploring all 3 arms in the test phase was calculated.

**Histochemical study**
Three days after reperfusion, the MCAO mice were decapitated to estimate the brain infarct volume [10, 16]. The entire brain was rapidly removed from the skull and chilled in ice-cold physiological saline. Ischaemic brain damage was evaluated using 2,3,5-triphenyltetrazolium chloride staining (TTC; Sigma-Aldrich, St. Louis, MO, USA). The coronal tissue was cut into 2-mm thick sections using a tissue slicer. The slices were immersed in a saline solution containing 0.8% TTC at 37°C for 10 min under a light-proof condition. The sections obtained were scanned with a high-resolution digital camera. The area of the infarction was measured using Image J Software (ver. 1.41, NIH; Bethesda, MD, USA). The infarct volume of each brain was quantified as the total volume of the infarct areas in all brain slices and expressed as the percentage of the contralateral hemisphere area.

**Nissl staining**

The animals were fixed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline under pentobarbital anaesthesia 3 days after reperfusion. After perfusion, brains were removed quickly and placed in the same fixative solution. Post-fixed brains were embedded in paraffin. Five-µm thick coronal sections were prepared using a microtome (Thermo Scientific Rotary Microtome Microm HM 340 E, Germany) and stored at 4°C until use.

Nissl staining was conducted as previously described [17, 18]. Paraffin-embedded brain sections on coated slides were deparaffinized and then rehydrated by immersion in the following solutions: xylene, thrice for 5 min each; 100% ethanol, twice for 5 min each; 90% ethanol, 70% ethanol for 3 min; followed by washing with water for 2 min, respectively. The sections were stained using 0.5% cresyl violet acetate (C5042, Sigma, St. Louis, MO, USA) for 10 min and examined using light microscopy at a magnification of 40x (Olympus IX73, Olympus Inc., Tokyo, Japan). The neuron numbers within a 500 x 300-µm² box set in the cerebral cortex, striatum, and hippocampus (CA1 and CA3) were microscopically counted in the contralateral and ipsilateral sides in a blind manner.

**TUNEL staining**

Apoptotic cells in the cortex and striatum were detected by TUNEL staining using an *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan) [19]. After deparaffination and rehydration, the 5-µm thick brain slices were incubated with proteinase K (20 µg/ml) at room temperature for 15 min. The slices were then labelled with FITC-dUTP according to the manufacturer’s instructions. Tissue images were captured using fluorescence microscopy with 20x magnification (Olympus IX73, Olympus Inc., Tokyo, Japan). The number of TUNEL-positive cells was counted within a 1 mm x 1 mm area in a blind manner.

**Determination of blood-brain barrier permeability**

The blood-brain barrier permeability was estimated using Evans blue dye according to a previous report [20] with a minor modification. Briefly, 24 h before decapitation, 1% Evans blue (Acros Organics™, India) was slowly injected into the tail vein of the animals. Three days after the MCAO and reperfusion
operations, brains were rapidly removed from the skull and then the olfactory bulb and cerebellum were dissected. Hemispheres were isolated using a clean razor blade and immediately weighed using an electronic balance. Each hemisphere was homogenized in 500 µl acetone containing 0.5% Na₂SO₄ and then centrifuged at 14000xg for 15 min. Evans blue absorbance was measured using a spectrophotometer (Shimadzu, Japan) at 620 nm wavelength. The concentration of Evans blue was estimated and expressed as µg/g of the brain tissue.

Biochemical study

Three days after the MCAO-and-reperfusion operation, brain tissues were dissected from each mouse under sodium pentobarbital (60 mg/kg, i.p.) anaesthesia. The cortex was quickly separated from the brain and frozen in liquid nitrogen. The tissues were stored at -80°C until use.

Measurement of malondialdehyde (MDA), glutathione (GSH) content in the cortex of MCAO mice

The cortex was weighed and homogenized in a RIPA buffer. Homogenates were centrifuged at 15000 rpm for 15 min at 4°C and the supernatants were used to measure GSH and MDA contents.

GSH content was measured based on the determination of the yellow colour that developed 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Aldrich, Germany) [21]. Briefly, 30 µl homogenate was added to a 230-µl mixture containing 12.5 µl 150 µM DTNB, 17.5 µl 0.2 M Tris HCl, and 200 µl methanol in a 96-well plate. After centrifuging at 3000 rpm for 5 min, the absorbance of the supernatant was measured at 412 nm using a microplate reader (HumanReader HS, Germany).

MDA content in the mouse brain cortex was measured according to Ohkawa et al. [22] with slight modification. Briefly, 50 µL of the cortex homogenate was added to the mixture containing 300 µL distilled water and 300 µL 5.2% thiobarbituric acid dissolved in 50% acetic acid. The mixtures were incubated at 95°C for 60 min. After cooling with tap water, 8 µl 5 M HCl and 0.7 ml n-butanol were added to the mixture. The mixture was vortexed for 2 min and centrifuged at 10,000 rpm for 5 min. The absorbance of the n-butanol layer was measured at 532 nm using a spectrophotometer (Shimadzu, Japan).

Western blot analysis

According to our previous reports [14, 15], western blotting was performed to analyse the expression levels of vascular endothelial growth factor (VEGF), Akt 1/2/3, p-Akt 1/2/3, and cleaved caspase-3 in the cortices of the animals. The cortex tissue was homogenized in a protein lysis buffer and centrifuged at 15000 rpm for 15 min at 4°C to collect supernatant samples. The samples were electrophoresed on a 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and then electrically transferred to a nitrocellulose membrane (Immun-Blot™ PVDF-Bio-Rad Laboratories, United States). The membranes were blocked using 5% skim milk (Sigma-Aldrich, Switzerland) and then probed with the primary antibodies:
VEGF polyclonal antibody (A-20: sc-152, 1:1000 dilution, Santa Cruz Biotechnology, United States), Akt 1/2/3 mouse monoclonal IgG1 (5C10: sc-81434, 1:300 dilution, Santa Cruz Biotechnology, United States), p-Akt 1/2/3 mouse monoclonal IgG2b (B-5: sc-271966, 1:300 dilution, Santa Cruz Biotechnology, United States), cleaved caspase-3 mouse monoclonal IgG1 (31A1067: sc-56053, 1:1000 dilution, Santa Cruz Biotechnology, United States), and β-actin rabbit polyclonal antibody (PA1-183, 1:3000 dilution, Thermo Scientific, United States) at 4°C overnight. After washing 3 times with tris-(hydroxymethyl)-aminomethane buffered saline (TBS) 0.05%, the membranes were incubated with anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology, United States) at room temperature for 1 h. The blots were developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific, United States). The immunoreactive bands were detected by the enhanced chemiluminescence method using ImageQuant TL software and analysed using Image J software (ver. 1.41, NIH; Bethesda, MD, USA).

Data analysis

SigmaPlot 12.0 (SYSTA Software Inc, Richmond, CA, USA) was used for statistical analysis. Obtained data, except neurological scores, were expressed as the mean ± S.E.M. and analysed by a one-way-ANOVA followed by the post hoc Student-Newman-Keuls test for multiple comparisons. Neurological scores were expressed as the median with interquartile ranges and analysed using Kruskal-Wallis and Mann-Whitney U-test. P values < 0.05 were considered significant.

Results

Pre- and post-ischemic treatment of DK attenuated the body weight loss in focal ischemic mice

In the vehicle-treated MCAO group, the body weight of mice was markedly reduced compared with the sham group (p < 0.001, Fig. 3). Pre- and post-ischemic treatment with DK (50–100 mg/kg) and edaravone (6 mg/kg) significantly attenuated the body weight loss compared with vehicle-treated MCAO (p < 0.05, Fig. 3).

Pre- and post-ischemic treatment with DK and edaravone reduced infarct volume in MCAO mice

TTC staining was conducted to assess the cerebral infarct caused by MCAO-induced ischemia. As shown in Fig. 4, approximately 40% of the brain area was infarcted 3 days after the MCAO-and-reperfusion operation. Pre- and post-ischemic treatment with a reference drug, edaravone (6 mg/kg/day, i.v.), significantly reduced the infarct size in the brain of the MCAO model mice (p < 0.001). Similarly, the brain infarct region of the MCAO model animals was dose-dependently reduced by pre- and post-treating daily with DK (50 and 100 mg/kg/day).
Pre- and post-ischemic treatment with DK and edaravone improved neurological symptoms displayed in MCAO mice

As summarized in Table 1, various extents of neurological symptoms were observed in the vehicle-treated MCAO mice from day 1 to day 4 after the MCAO and reperfusion operations (p < 0.05). Pre-ischemic treatment procedures with DK (50 and 100 mg/kg) and edaravone (6 mg/kg) significantly mitigated MCAO-induced ischemic neurological deficits. In the post-ischemic treatment procedure (in which the daily administration of test drugs was started after the MCAO operation), significant improvement of the neurological symptoms was observed in the MCAO groups treated with 100 mg/kg/day DK (p < 0.05) and 6 mg/kg/day edaravone showed significantly improved MCAO neurological deficits induced on days 2–4 and days 2–3, respectively.

Pre- and post-ischemic treatment with DK improves working memory deficits caused by MCAO and reperfusion operations

On day 12 after the MCAO and reperfusion operations, mice were subjected to the modified Y-maze test to assess spatial working memory, a form of short-term memory in mice [23]. As shown in Fig. 5, the time the vehicle-treated ischemic group spent in the new arm was significantly shorter than that of the sham group, indicating spatial working memory impairment. The pre-ischemic treatment with DK (50 and 100 mg/kg) and edaravone (6 mg/kg) significantly ameliorated working memory deficits caused by the MCAO and reperfusion operations (p < 0.05). On the other hand, in the animal groups that received drug treatment daily after the MCAO and reperfusion operations, treatment with 100 mg/kg DK significantly improved the working memory performance. In contrast, 50 mg/kg DK or 6 mg/kg edaravone failed to affect working memory deficits. In addition, no changes in the average performance speed on the maze were observed in all animal groups (p > 0.05).

Post-ischemic treatment of DK ameliorated neuronal cell damage the brain of MCAO mice

In the vehicle-treated MCAO group, Nissl staining revealed that neuronal losses occurred ipsilaterally in the cortex, striatum, and CA1 and CA3 areas of the hippocampus compared with the contralateral brain regions. Atrophic neurons with shrunken cytoplasm and damaged nuclei were also observed. The number of intact neurons counted in each ipsilateral brain region was significantly more in the DK (100 mg/kg) and edaravone (6 mg/kg)-treated groups than in the vehicle-treated MCAO group (p < 0.05). No differences in the number of neurons in the contralateral brain were found between each animal group (p > 0.05) (Fig. 6).

Post-ischemic treatment of DK reduced GSH content and elevated MDA content in the brain cortex of ischemic mice

Oxidative stress in the brain caused by MCAO and reperfusion treatments was assessed by measuring MDA and GSH levels. As shown in Fig. 7, compared with the sham operation group, the MDA level was
significantly elevated ($p < 0.001$) while the GSH level was significantly reduced in the vehicle-treated MCAO group ($p < 0.01$). Post-ischemic treatment of MCAO-and-reperfusion-treated animals with DK (100 mg/kg) and edaravone (6 mg/kg) markedly decreased MDA levels and elevated GSH levels compared with post-ischemic treatment with vehicle alone ($p < 0.05$).

**Post-ischemic treatment with DK improved blood-brain barrier impairment and cerebral edema in the MCAO mice**

As shown in Fig. 8A and B, Evans blue dye extravasation in the ipsilateral hemisphere was markedly increased 3 days after MCAO-and-reperfusion treatment ($p < 0.01$). Post-ischemic treatment with DK (100 mg/kg/day) significantly attenuated an increase in Evans blue dye extravasation in the ischemia hemisphere ($p < 0.05$). Meanwhile, post-ischemic treatment with edaravone (6 mg/kg/day) failed to affect the changes in Evans blue dye extravasation ($p > 0.05$). In addition, brain edema caused by ischemic operation was assessed by measuring the brain weight change (Fig. 8C). Post-ischemic treatment with DK (100 mg/kg/day) and edaravone (6 mg/kg/day) significantly reduced the wet weight of the ischemic hemisphere compared to the one measured in the sham operation group ($p < 0.05$).

**Post-ischemic treatment with DK decreased TUNEL-positive cell number in the brain of the MCAO mice**

There were no differences in the number of TUNEL-positive cells in the contralateral cortex and striatum between the MCAO-and-reperfusion treatment groups ($p > 0.05$). By contrast, a marked increase in the TUNEL positive cell was detected in the ipsilateral side of these brain regions in the vehicle-treated MCAO mice. Post-ischemic treatment with DK (100 mg/kg) and edaravone (6 mg/kg) significantly reduced the number of apoptotic cells in the ipsilateral cortex and striatum compared with post-ischemic treatment with vehicle ($p < 0.01$) (Fig. 9).

**Post-ischemic treatment with DK increased the expression of VEGF, Akt, p-Akt, and cleaved caspase-3 in the cerebral cortex of MCAO mice**

As shown in Fig. 10, the vehicle-treated MCAO mice had a markedly decreased expression level of VEGF in the cortex compared to the sham-operated group ($p < 0.05$). Post-ischemic treatment with edaravone (6 mg/kg) insignificantly affected the VEGF expression. Interestingly, DK post-ischemic treatment significantly reversed MCAO-induced downregulation of VEGF expression in the cortex ($p < 0.05$) (Fig. 10A, B). In addition, expression levels of Akt and p-Akt were remarkably declined by MCAO-and-reperfusion treatment ($p < 0.01$), and post-ischemic treatment with DK but not with edaravone reversed these changes (Fig. 10A, C1-3). No differences in the p-Akt/Akt ratio were found among all animal groups ($p > 0.05$).

Consistent with the results of TUNEL staining, the expression level of cleaved caspase-3 in the cortex was remarkably enhanced in the vehicle-treated MCAO group compared with the sham-operated group ($p <$
However, the enhanced expression of cleaved caspase-3 was significantly reduced in the DK- and edaravone-treated MCAO groups (p < 0.001, Fig. 10D).

**Discussion**

In the present study using an MCAO-and-reperfusion model of mice, we found that cerebral ischemia-induced brain injury and spatial cognitive deficits can be mitigated by post-ischemic treatment with DK via mechanisms mediated by exerting anti-oxidative and anti-apoptotic properties and restoring the blood-brain barrier function. Our findings suggest that DK has the potential to exert neuroprotective and therapeutic activities against cerebral ischemia-induced brain dysfunction.

We first elucidated the protective effects of DK on MCAO-and-reperfusion treatment-induced brain infarction and neurological symptoms using a pre-ischemic drug treatment protocol. The results revealed that all the indices representing transient cerebral ischemia-induced damages; bodyweight loss, brain infarction, neurological impairments, and working memory deficits were attenuated by pre-ischemic treatment with DK (50 and 100 mg/kg/day) in a manner similar to edaravone, a drug clinically used to treat acute ischemic stroke [24]. These findings indicate that DK, like edaravone, can exert a neuroprotective activity against cerebral ischemia and suggest that daily DK intake may be beneficial to cerebral ischemic insults in humans.

One of the most important findings in this study is that post-ischemic treatment with DK dose-dependently mitigated transient ischemia-induced brain infarction, neurological symptoms, and spatial cognitive deficits to a similar extent to that pre-ischemic treatment with the same DK dose attenuated. This finding indicates that DK can exhibit a therapeutic effect on transient cerebral ischemia-induced brain damage, suggesting that DK administration is beneficial for treating cerebral ischemic insults in humans. However, it should be noted that, in the experiments where a low dose of DK (50 mg/kg/day) was employed, the effects of post-ischemic DK treatment on ischemia-induced neurological symptoms and cognitive dysfunction were less potent than that of pre-ischemic treatment with DK. Thus, it is likely that the timing and doses of the DK administration are important to restore the cognitive function impaired by cerebral ischemia, providing information for choosing appropriate DK doses in future clinical studies on DK. Moreover, in contrast to the effects of DK, post-ischemic treatment with edaravone failed to reverse the cognitive deficits. Considering that edaravone possesses a potent radical scavenging activity, this result suggests that the post-ischemic DK treatment improved cognitive performance in transient cerebral ischemia model mice, probably via mechanisms differing from radical scavenging.

To understand the mechanisms underlying the therapeutic effects of DK on ischemia-induced brain damage, we explored histological and neurochemical evidence supporting the effects of post-ischemic DK treatment. As shown in Fig. 6, the present histological study using Nissl staining revealed that the number of neuronal cells in the ipsilateral sides of ischemia-sensitive brain regions, such as the cortex, striatum, and hippocampus (CA1 and CA3 areas) [25], was significantly reduced by the MCAO-and-reperfusion operation as compared to the neuronal cell number in the respective contralateral regions,
supporting at least neurological symptoms and cognitive dysfunction of the MCAO mice. Interestingly, we found that these neuronal cell losses caused in the ipsilateral side after MCAO-and-reperfusion were markedly mitigated by post-ischemic treatment with DK (100 mg/kg/day) as with edaravone (6 mg/kg/day). These results allow us to infer that post-ischemic administration of DK reduced neurological symptoms and cognitive deficits due to neuronal cell loss, probably by suppressing the ischemia and reperfusion-triggered mechanisms involved in neuronal cell death.

Then, considering that oxidative stress reportedly triggers cell death mechanisms and plays a significant role in the pathophysiology of ischemic stroke [7], we investigated the effects of post-ischemic DK treatment on transient ischemia-induced changes in oxidative stress marker levels in the brain. Transient ischemic operation significantly enhanced lipid peroxidation, a marker of lipid membrane damage, and reduced the content of GHS, an antioxidant, in the brain. Moreover, these changes were mitigated by immediate post-ischemic treatment with DK as with a radical scavenging drug, edaravone. The present HPLC analysis detected flavonol glycosides as the DK extract constituents, such as quercetin and kaempferol, with potent antioxidant and radical scavenging activities in in vitro [26, 27]. Taken together, it seems plausible that, as edaravone does, these constituents of DK extract at least partly contribute to the mitigating effects of post-ischemic DK on transient cerebral ischemia-induced brain injury.

In this study, the present MCAO-and-reperfusion treatment severely damaged the BBB integrity and thereby increased brain edema in the ipsilateral but not the contralateral side of the brain, as previously reported [28]. Transient cerebral ischemia impairs the cerebral vasculature, resulting the BBB disruption and neuronal dysfunction via inflammation and apoptosis of endothelial and neuronal cells. Thus, prevention of the BBB damage caused by transient ischemia has been a potential therapeutic target for developing drugs to improve patient outcomes after ischemic stroke [29, 30]. Interestingly, in the present experiments using a post-ischemic treatment protocol, it was found that DK (100 mg/kg/day) was effective in preserving the BBB integrity and reducing brain edema on the ipsilateral side, whereas edaravone (6 mg/kg/day) mitigated brain edema but not BBB damage. Jing Liu et al. [31] previously reported a protective effect of edaravone on BBB damage in ischemic rats. The reason for this conflict on edaravone’s effect between our and their studies is unclear, but it may be due to differences in the experimental conditions, including the dose of edaravone employed. Nevertheless, our findings further support our idea that DK can exhibit therapeutic potential for ischemic stroke and suggest that the effects of DK are, in part, mediated via mitigating transient ischemia-induced BBB damage and brain edema.

Lines of evidence demonstrate that transient ischemia-induced BBB disruption and neuronal cell damage are mainly attributed to endothelial degeneration and pro-apoptotic/anti-apoptotic signalling alterations [31]. To better understand the mechanisms underlying the effects of post-ischemic DK treatment, we focused on VEGF and analysed it as an index of disruption and recovery of BBB after transient ischemia because VEGF plays an essential role in the proliferation and migration of endothelial cells, angiogenesis, and reconstruction of cerebral blood flow [33, 34]. Besides these, the elevation of VEGF levels contributes to neuroprotection [9], reduces neurological deficits during stroke recovery [35] and cognitive performance [36–38]. Our western-blotting analysis clearly showed that the expression level of VEGF was down-
regulated in the cortex 3 days after the ischemia-and-reperfusion operation and that post-ischemic
treatment with DK reversed the reduced expression level of VEGF. Thus, post-ischemic DK treatment may
up-regulate VEGF expression and thereby mitigate transient ischemia-induced BBB disruption, probably
via affecting endothelial cell function.

Then, we analysed the expression levels of cleaved caspase-3, a pro-apoptotic molecule, and Akt and p-
Akt, anti-apoptotic signalling molecules, as indices of brain damage and its recovery by post-ischemic
drug treatment. This analysis was conducted by taking into account the TUNEL staining data that
transient ischemia caused cell apoptosis ipsilaterally in the brain region in a manner reversed by post-
ischemic treatment with DK and edaravone. The results demonstrated that even in a post-ischemic drug
treatment paradigm, DK and edaravone could suppress a transient ischemia-induced increase in the
activated form of caspase-3, an enzyme leading to DNA fragmentation and cellular apoptosis [39]. These
results allow us to hypothesize that post-ischemic DK treatment can exert anti-apoptotic activities,
partially via suppressing cleaved caspase-3-mediated apoptotic mechanisms after transient cerebral
ischemia. This hypothesis can be supported by a study reported by Weijian Bei et al. [40]. They
demonstrated that the in vitro application of standardized flavonoid extract from persimmon leaves
reduced apoptosis of primary cultured cortical neurons. Moreover, considering this study's results
together with those results in a post-ischemic treatment paradigm with edaravone, a radical scavenging
drug also suppressing the cleaved caspase-3 expression and cell apoptosis in the animals receiving the
transient ischemic operation, it seems plausible that the anti-apoptotic activity of DA may be mainly
attributed to the effect of antioxidant constituents of DK. Indeed, there is evidence indicating that GSH, an
endogenous antioxidant, negatively regulates major pathways implicated in apoptotic cell death after
transient cerebral ischemia [33, 34].

Besides DK suppression of caspase-3 activation, the present results also suggest that prosurvival and
anti-apoptotic mechanisms [39] via Akt/pAkt signalling are involved in the mitigating effect of post-
ischemic DK treatment on the brain damage caused by transient cerebral ischemia. Consistent with a
previous report [41], the animals that received the ischemia-and-reperfusion operation showed markedly
reduced expression levels of Akt and p-Akt in the ipsilateral brain regions 3 days after the operation. It is
of interest that post-ischemic DK treatment could reverse the decrease in Akt and p-Akt without altering
the pAkt/Akt ratio, an Akt activation index. The Akt signalling pathway, which is triggered by PI3K-
mediated mechanisms, plays not only a suppressive role in neuronal apoptosis [42] but also a facilitatory
role in the mechanisms of neuronal survival after ischemic stroke [39]. Indeed, this signalling system is
involved in VEGF-induced angiogenesis [43], neuroprotection [44, 45], and neurogenesis [44, 46]. Thus, our
results provide evidence that the VEGF/Akt signalling pathway is also implicated in suppressing transient
ischemia-induced neuronal cell loss by post-ischemic DK treatment.

Finally, it should be noted that in a post-ischemic treatment paradigm, the effect of DK extract
administered orally on the down-regulated expression of VEGF and Akt/pAkt was more potent than that
of edaravone given intravenously. This finding suggests that DK extract can effectively mitigate transient
ischemia-induced brain injuries by acting through the VEGF/Akt signalling pathway. Accumulating
evidence indicates the involvement of interrelated and coordinated events, including neuronal excitotoxicity, mitochondrial dysfunction, cell death processes, neuroinflammation, oxidative stress, and BBB impairment in the pathophysiology of ischemic insults [5, 7]. Therefore, DK extract is more advantageous than edaravone for treating ischemic insult-induced brain damage since DK extract likely exerts therapeutic actions against ischemic insult by affecting the multiple mechanisms mediating oxidative stress, BBB damage, and apoptotic and prosurvival signalling, as we demonstrated (Fig. 11).

**Conclusion**

In the present study using an MCAO-and-reperfusion model mice, we demonstrated that DK extract could exert protective and therapeutic effects on cerebral ischemia-induced brain damage in mice by exhibiting anti-oxidative, anti-apoptotic, and prosurvival properties while preserving the blood-brain barrier integrity. These pharmacological features of DK are beneficial as a therapeutic strategy for ischemic insult.

**Declarations**

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**Author Contributions** LTTN: Investigation, Formal analysis, Writing-original draft, XTL: Conceptualization, Drafting the manuscript, Investigation, HNTP: Interpretation of findings, TNV: Phytochemical analysis, PTN: Investigation, AVTP: Supervision, TBTN: Project administration, KM: Supervision, Writing-Review & Editing.

**Data Availability** All data generated or used during the study are available from the corresponding author by request.

**Conflict of Interest** The authors declare no conflict of interest.

**Ethical Approval** The animal experimental protocols were approved by the NIMM Institutional Animal Use and Care Committees, Vietnam.

**References**


17. Inada C, Le TX, Tsuneyama K et al (2013) Endogenous acetylcholine rescues NMDA-induced long-lasting hippocampal cell damage via stimulation of muscarinic M1 receptors: Elucidation using


Table 1

Table 1 is available in the Supplementary Files section.

Figures

Figure 1
Characterization of flavonoid compounds in DK extract

(A) Representative HPLC chromatography of the DK extract (chromatogram A) and standard mixture (chromatogram B). 1. isoquercetin (RT=38.19 min), 2. quercetin-3-O-(2"-O-galloyl-β-D-glucopyranoside (RT=41.53 min), 3. trifolin (RT=42.94 min), 4. astragalin (RT=46.48), 5. kaempferol-3-O-(2"-O-galloyl-β-D-galactopyranoside) (RT=50.44 min), 6. kaempferol-3-O-(2"-O-galloyl-β-D-glucopyranoside) (RT=53.56) (B). HPLC chromatogram of the hydrolysed DK extract. The DK extract was hydrolysed in a HCl 2M/methanol solution at 90°C for 2 h. Quercetin and kaempferol content in the hydrolysed DK extract solution were determined by HPLC method. Kaempferol (chromatogram 1, RT=28.57 min), quercetin (chromatogram 2, RT=16.57 min), hydrolysed DK extract (chromatogram 3), ethanol as a solvent (chromatogram 4)

Fig. 2

Experimental protocols Swiss albino mice were induced cerebral ischemia using middle carotid artery occlusion (MCAO) model. Neurological score was evaluated for 4 consecutive days (from day 1 to day 4). The modified Y maze test was performed at day 12, (A) Pre-ischemic treatments procedure. DK (50 and 100 mg/kg b.w, p.o.) or edaravone (6 mg/kg b.w, i.v) treatment was started 1 week before being subjected to MCAO and maintained after reperfusion. (B) Post-ischemic treatment procedure (therapeutic treatment). DK (50 and 100 mg/kg b.w, p.o.) or edaravone (6 mg/kg b.w, i.v) were administered immediately after reperfusion. TTC staining, histochemical, and biochemical studies was performed 3 days after reperfusion
Figure 3

Effects of DK on the body weight of mice following cerebral ischemia

(A) Pre-ischemic treatment of DK and (B) post-ischemic treatment of DK. Data were presented as the mean ± S.E.M. (n=9-10). *p<0.05, **p<0.01, ***p<0.001 vs. vehicle-treated ischemic mice, ###p<0.001 vs. sham animal (ANOVA followed by post hoc Student-Newman-Keuls test)
**Figure 4**

**Effects of DK on the infarct volume of MCAO mice**

(A1-2) Representative images of TTC-stained brain sections and quantitative analysis of infarct volume (%) in the mouse brain in the pre-ischemic treatment procedure. (B1-2) Representative images of TTC-stained brain sections and quantitative analysis of infarct volume (%) in the mouse brain in the post-ischemic treatment procedure. The mouse brain tissues were collected 72 h after MCAO-reperfusion. Each data column represents the mean ± S.E.M. (n=6). ***p<0.001 vs. vehicle-treated ischemic mice (ANOVA followed by post hoc Student-Newman-Keuls test)
Figure 5

Effects of DK on spatial working memory of mice following cerebral ischemia in the modified Y maze test

(A) A diagram presenting the modified Y-maze test. Effects of (B1-2) pre- and (C1-2) post-ischemic treatment of DK (50 and 100 mg/kg) on spatial working memory of ischemic mice. Each data column
represents the mean ± S.E.M. (n=9-10). *p<0.05 vs. vehicle-treated ischemic mice, #p<0.05 vs. sham mice (ANOVA followed by post hoc Student-Newman-Keuls test)

Fig. 6

**Figure 6**

**Effects of post-ischemic treatment of DK on the number of neuronal cells in the mouse brain following cerebral ischemia**
(A1-2) Nissl staining of brain section from MCAO mice after 72 h reperfusion. The rectangles show the regions of the cortex, striatum, and hippocampus that were counted. (B-C1) Representative images of Nissl staining on sections of cortex, striatum, and hippocampus. (B-C2) Quantitative analysis of number of neurons. The number of neurons within a 500 x 300-μm^2 box set in the cerebral cortex, striatum, and hippocampus (CA1 and CA3) were microscopically counted on the contralateral and ipsilateral sides. Data represents the mean ± S.E.M. (n=5). *p<0.05, ***p<0.001 vs. vehicle-treated ischemic mice (ANOVA followed by post hoc Student-Newman-Keuls test)

Figure 7

Effects of DK on the GSH and MDA content in mouse brain cortex following cerebral ischemia

The mouse brain tissues were collected 72 h after MCAO-reperfusion. Data represents the mean ± S.E.M. (n=6). *p<0.05, ***p<0.001 vs. vehicle-treated ischemic mice, ##p<0.01, ###p<0.001 vs. sham animals (ANOVA followed by post hoc Student-Newman-Keuls test)
Figure 8

Effects of DK on the blood-brain barrier permeability and cerebral edema of MCAO mice

(A) Representative images of Evans blue extravasation in whole mouse brain 72 h after MCAO-reperfusion. (B) Quantification of Evans blue leakage in ipsilateral and contralateral hemispheres of MCAO mice. (C) Brain weight of MCAO mice. Data represents the mean ± S.E.M. (n=5). *p<0.05 vs. vehicle-treated ischemic mice, ##p<0.01 vs. sham animals (ANOVA followed by post hoc Student-Newman-Keuls test)
Figure 9

**Effects of DK extract on the number of apoptotic cells in the mouse brain following focal cerebral ischemia**

**(A)** Representative images of TUNEL staining on sections of cortex and striatum of mice 72 h following MCAO-reperfusion. **(B)** Number of TUNEL positive cells in the cortex and striatum. Data represents the mean ± S.E.M. (n=5). **p<0.01, ***p<0.001 vs. vehicle-treated ischemic mice (ANOVA followed by post hoc Student-Newman-Keuls test)
Figure 10

Effects of DK extract on the expression levels of VEGF, Akt, P-Akt and cleaved caspase-3 in the mouse brain cortex following cerebral ischemia

(A) Representative western blot image of the expression of VEGF, Akt, P-Akt, and cleaved caspase-3 in cerebral cortex of ischemic mice 72 h following MCAO-reperfusion. The quantification of VEGF (B), Akt, P-Akt (C1-3), and cleaved caspase-3 (D) protein expression. Data represents the mean ± S.E.M. (n=6).

* p<0.05, *** p<0.001 vs. vehicle-treated ischemic mice, # p<0.05, ## p<0.01, ### p<0.001 compared to sham (ANOVA followed by post hoc Student-Newman-Keuls test)
Figure 11

Putative mechanisms underlying the therapeutic effects of standardized-flavonoid *Diospyros kaki* L.f. leaf on cerebral ischemic

Supplementary Files

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- 5Tables.pptx