Neuroprotective effects of *Diospyros kaki* and *Gardenia jasminoides* against ischemic neuronal injury in mice

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Abstract:

This study aims to clarify the neuroprotective effects of ethanol extracts of *Diospyros kaki* (DK) leaves and *Gardenia jasminoides* (GJ) fruit on cerebral ischemia injury using middle cerebral artery occlusion (MCAO) model in mice. *Swiss albino* mice were daily treated with DK extract (125-500 mg/kg b.w) and GJ extract (250-1,000 mg/kg b.w) for 1 week before being subjected to MCAO. The administration of edaravone (6 mg/kg, i.v), which was a reference drug, was started immediately after MCAO. DK and edaravone treatment improved neurological deficits and reduced infarct volume of MCAO mice compared to that of a vehicle-treated one. GJ treatment improved neurological deficits, but did not affect the infarct volume of MCAO mice. These results suggested that the DK and GJ treatment might be beneficial for protecting the neuronal system against cerebral ischemic injury.

Keywords: ***cerebral ischemia, Diospyros kaki, Gardenia jasminoides, middle cerebral artery occlusion, neuroprotection.***

Classification number: ***3.3***

Introduction

Stroke is the major cause of disability and the fourth leading cause of death worldwide. Ischemic stroke accounts for approximately 80% of all strokes. Ischemic injury is associated with vascular leakage, inflammation, tissue injury, and cell death [1]. Cellular changes associated with ischemia include impairment of metabolism, energy failure, free radical production, excitotoxicity, altered calcium homeostasis, and protease activation. All these events affect the brain's functions and contribute to long-term disabilities [2]. The advantage of herbal medicines with diverse chemical components and multi-targeted effects may bring breakthroughs for the complicated and closely related diseases like cerebral ischemia. Therefore, searching for the potential drugs from plants to treat ischemic cerebrovascular diseases will be a worthy direction to explore.

DK is a deciduous tree belonging to the family Ebenaceae. Pharmacological studies have shown that DK leaf flavonoid has extensive pharmacological actions, including dilation of blood vessels, a lipid-reducing effect, a glucose-lowering effect, and antioxidant properties. DK leaf flavonoid can elevate ischemic tolerance by reducing inflammatory reactions and vascular endothelial injury [3]. Moreover, DK leaves possess an antithrombotic activity. A 10,000 D anticoagulant fraction, which was purified from the leaves of DK, inhibited thrombin-catalyzed fibrin formation with a competitive inhibition pattern [4].

GJ is an evergreen flowering plant of the family Rubiaceae. The fruit of GJ is traditionally used due to its homeostatic, antiphlogistic, analgesic, anti-inflammatory, and antipyretic effects. GJ has the obvious effect of preventing and treating atherosclerosis and thrombosis in the cardiovascular system [5]. It also has central sedative, analgesic, anti-diabetes, anti-depression and anti-inflammatory effects [6]. Moreover, Haiyan, et al. [7] reported that GJ extract had the functions of learning and memory improvement and neuroprotective effect on chronic cerebral ischemia in rat models.

In this study, the neuroprotective effects of DK and GJ extracts against cerebral ischemia were investigated by using middle cerebral artery occlusion

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(MCAO) model in mice.

Materials and methods

Extract preparation

DK leaves were collected in Yen Bai province and GJ fruit were collected in Bac Ninh province. These herbs were identified by Dr. Pham Thanh Huyen, Department of Medicinal Plant Resources, National Institute of Medicinal Materials (NIMM).

For the DK extract preparation, the leaves of DK were dried in a hot-air oven and ground. The DK powder (100 g) was extracted with 70% ethanol under reflux for two hours and then filtrated. The extraction was repeated three times. After filtration, the combined extract was concentrated at 50°C under reduced pressure and dried in vacuum oven at 50°C to obtain 18.4 g of DK extract. The total flavonoid content of this DK extract was estimated to be 7.99% using spectrophotometric analysis. For the GJ extract preparation, the fruit of GJ was dried in a hot-air oven and ground. 100 g of GJ powder was extracted with 50% ethanol under reflux for two hours and filtrated. This step was repeated three times and the filtrate was combined, concentrated at 50°C under reduced pressure, and then dried in a vacuum oven at 50°C. The yield of the extraction from the dried fruit was calculated to be 32.4%. The GJ extract was estimated to contain 11.79% of geniposide (HPLC analysis).

Animals

Male *Swiss albino* mice (National Institute of Hygiene and Epidemiology, Hanoi, Vietnam) were purchased at the age of 6-7 weeks old. The animals were housed in the laboratory animal room maintained at 25±1°C with 12-hour light/dark cycle for at least one week before the commencement of the experiments. Animals were given access to food and water *ad libitum*. The behavioural experiments were conducted during the light phase from 9:00 to 18:00.

Middle cerebral artery occlusion

Transient cerebral ischemia in mice was induced as previously reported [8, 9] with slight modifications. Briefly, mice were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.). After disinfecting the fur and skin with 70% ethanol, the midline neck was incised to dissect the left common carotid artery (CCA) from surrounding tissues. The CCA was temporarily occluded by a temporary suture using 5-0 silk. A permanent suture is placed around the external carotid artery (ECA), and another temporary suture is placed on the ECA distal to the bifurcation. The left internal carotid artery (ICA) was clipped using reverse-action tweezers to avoid bleeding. After cutting a small hole into ECA between the permanent and temporary sutures, 12-mm long 6-0 silicon-coated (about 2 mm is coated with silicon) monofilament suture was introduced into the ECA and then inverted into the ICA. The suture was tightly tied around the monofilament to prevent bleeding and the reverse-action tweezers were removed. The occluder was introduced to occlude the origin of the MCA 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 withdrawn to allow reperfusion.

Neurological score

A neurological grading scale was used to assess neurological recovery after MCAO injury according to Menzies, et al. [10]: scale: 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 for 6 days from day 0 (one hour after the reperfusion) and continued until the end of the experiment.

Estimation of brain infarct volume

Six days after reperfusion, the MCAO-subjected mice were killed to estimate the brain infarct volume. Brains were removed quickly from the skulls and chilled in ice-cold saline. The coronal tissue sections (2×5 mm) were obtained using a tissue slicer. The slices were immersed in a saline solution containing 0.8% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) for 10 minutes at 37°C. The area of the infarction was measured using Image J software (ver. 1.41, NIH; Bethesda, MD, USA). The total infarct volume of each brain was calculated by the summation of the infarct areas of all brain slices. The infarct area of each slice was calculated by subtracting the normal ipsilateral areas from the contralateral hemisphere to reduce errors due to cerebral edema and was presented as the percentage of the infarct to the area of the contralateral hemisphere [8, 11].

Drug administration

DK and GJ extracts were suspended in distilled water. The administration period of DK and GJ extracts was started one week before the surgery and continued until the decapitation day (day 6). DK extract at a daily dose of 125, 250, 500 mg/kg b.w or GJ extract at a daily dose of 250, 500, 1,000 mg/kg b.w were per-orally administered to mice. On the operation day, mice were received the DK and GJ extracts one hour before the test. The edaravone (≥ 98% purity) provided by Dr. Nguyen Van Tai, Department of Phytochemistry, NIMM was dissolved in 0.9% saline. The administration of edaravone (6 mg/kg b.w, i.v) was started from the day of operation immediately after the MCAO. Distilled water was

per-orally administered to MCAO vehicle group mice.

Data analysis

Statistical analyses were performed using SigmaPlot 12.0 (SYSTAT Software Inc., Richmond, CA, USA) (statistical analysis software). Data were presented as mean ± S.E.M. or as median (interquartile range). Neurological scores were analysed using Kruskall-Wallis and Mann-Whitney U-test. Infarct volume was analysed using one-way analysis of variance (ANOVA) followed by *post hoc* Student-Newman-Keuls test for multiple comparisons. Differences of $p<0.05$ were considered significant.

Results

The effects of DK and GJ extracts on neurological score

MCAO mice showed neurological deficits after being subjected to MCAO for 60 minutes and reperfusion. The MCAO animals revealed the decrease in motor ability and ability to respond to stimuli on the side of the body contralateral to ischemia.

The animals treated with DK extract showed a significant improvement in neurological deficits induced by MCAO at a dose of 500 mg/kg on day 2, day 4, and day 6 after injury (Table 1). The effect of DK extract on the neurological score is in a dose-dependent manner. Treatment with GJ extract at the dose of 500 mg/kg reduced neurological deficits in MCAO mice observed on day 5 and day 6 after MCAO operation. At the dose of 1,000 mg/kg, the GJ extract treatment also reduced neurological deficits of MCAO mice on day 6 ($p<0.05$). Edaravone-treated MCAO mice showed a significant decrease in neurological deficits compared to the vehicle-treated one on day 2 and day 6 ($p<0.05$) (Table 1).

Table 1. The effects of DK and GJ extracts on the neurological score.

Treatment groups	Dose (mg/kg)	Day(s) after MCAO surgery						
		Day 0	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*
Vehicle	0	4 (3.25; 4)	2.5 (2; 3.75)	2 (2; 3)	2 (1.25; 3.75)	2.5 (1.25; 3)	2 (2; 3)	3 (2; 3)
DK	125	4 (3; 4)	3 (2; 4)	3 (1.5; 3)	3 (1.5; 3)	2 (2; 3)	2 (1.25; 3)	2.5 (0.5; 3)
	250	4 (4; 4)	3 (2; 3)	2 (1; 3)	2 (1; 3)	2 (1; 3)	1 (1; 3)	2 (0; 3)
	500	3 (3; 3.75)	2 (2; 2.75)	1.5* (1; 2)	2 (2; 2)	1* (1; 1.75)	1 (1; 1.75)	2* (1; 2)
GJ	250	4 (4; 4)	2.5 (2; 3)	2.5 (2; 3)	2.5 (1.75; 3)	2 (0.75; 2.25)	2 (0.75; 2.25)	1.5 (0.75; 3)
	500	4 (3; 4)	3 (2; 3)	3 (1.5; 3.5)	3 (1; 3)	2 (1.5; 3)	1* (1; 2)	2* (1.5; 2)
	1000	3.5 (3; 4)	2 (2; 3)	3 (2; 3)	3 (2; 3)	2.5 (1.75; 3)	2 (1.75; 2.25)	2* (1; 2)
Edaravone	6	4 (3; 4)	2 (0; 3)	1* (0; 2)	1 (1; 2)	1 (1; 2)	2 (0; 2)	2* (1; 2)

Data were presented as median (interquartile range). *$p<0.05$ vs. vehicle-treated MCAO mice (Kruskall-Wallis and Mann-Whitney U-test).

The effects of DK and GJ extracts on infarct volume

TTC staining was employed to measure the cerebral infarcts in focal ischemia induced by MCAO. TTC acted as a proton acceptor for many pyridine nucleotide-linked dehydrogenases that, along with the cytochromes, formed an integral part of the inner mitochondrial membrane and made up the electron transport chain. The tetrazolium salt was reduced by the enzymes into a red, lipid-soluble formazan. Viable tissue, therefore, stained deep red while the infarct remained unstained [12].

Figure 1A showed the typical photographs of TTC-stained brain sections of MCAO mice 6 days after reperfusion. As shown in Fig 1B, 6 days after MCAO, the infarct volumes of vehicle-treated MCAO animals were 40% higher in average. The treatment with DK extract dose-dependently and significantly reduced the brain infarction in the mouse brain. The effect of GJ extract on brain infarction in MCAO mice was insignificant while the treatment of edaravone (6 mg/kg) immediately after MCAO produced a significant reduction in brain infarct.

Discussions

The MCAO in rodents has been widely used to evaluate the effects of the potential treatment of cerebral ischemia. This model offers the advantage of inducing reproducible transient or permanent ischemia of the MCA territory in a relatively non-invasive manner. Intraluminal approaches interrupt the blood flow of the entire territory

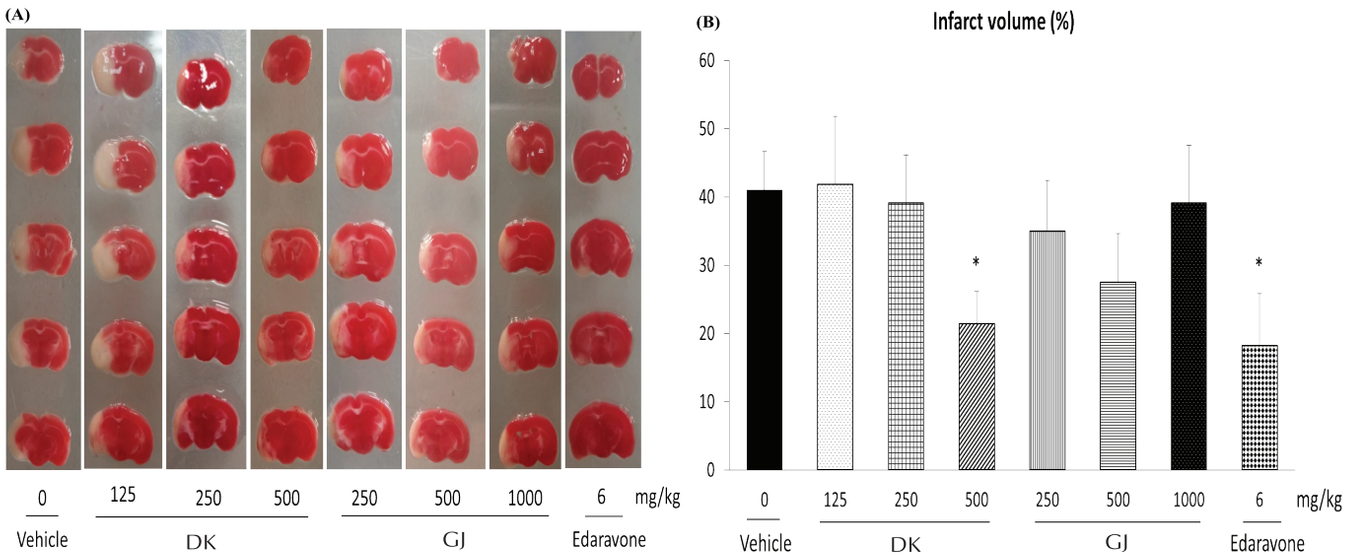

Fig 1. The effects of DK and GJ extracts on the infarct volume of MCAO mice 6 days after reperfusion. (A) Typical images of TTC-stained brain section; **(B)** Quantitative analysis of infarct volume (%) in the brain of MCAO mice. Each data column represents the mean ± S.E.M. (n=6-9). *p<0.05 vs. vehicle-treated MCAO mice (ANOVA followed by post hoc Student-Newman-Keuls test).

of this artery, result in reproducible lesions in the cortex and striatum, and can be either permanent or transient [13]. The reperfusion by removal of the occluding filament at least partially results in the restoration of blood flow after spontaneous or therapeutic lysis of a thromboembolic clot in human. In this study, the 60-minute MCA occlusion after reperfusion induced a remarkable infarct area along with motor function deficits in mice. These results are in agreement with our previous report [8]. Moreover, the administrations of DK and GJ extracts showed potential neuroprotective effects on brain ischemia injury induced by MCAO. These results suggest that the effects of DK and GJ extracts against ischemic brain injury are independent from the suppression of cerebral thrombosis mechanism.

The administration of DK extract significantly and dose-dependently reduced MCAO-induced injury. The effects of DK extract were quite similar to a reference drug, edaravone. Edaravone has been approved for the treatment of acute ischaemic stroke in Japan and is still under clinical investigation in some countries [14]. Edaravone has been reported to reduce brain infarction and oedema after ischemic/reperfusion injury in animal models as well as in stroke patients. The possible mechanism of action of edaravone mainly encompasses the decrease in oxidative stress or lipid peroxidation, pro-inflammatory response, and protection of neurovascular tissues after ischemic stress [15]. It has been reported that flavonoid, which is the main therapeutic constituent in DK leaves, possesses anti-inflammatory and anti-apoptosis activities [16]. If the DK extract contains a total flavonoid content of 7.99%, it can be speculated that the anti-inflammatory and anti-apoptosis activities of DK flavonoids play a role in the neuroprotective action of the DK extract against ischemic neuronal injury.

The administration of GJ extract at the doses of 500 and 1,000 mg/kg showed the improvement of neurological deficit in mice from day 5 and day 6, respectively. This action was different from those of DK extract and edaravone that could be observed from day 2 after ischemic injury. In contrast, these GJ treatments have no effect on the infarct volume of MCAO mice. The plausible explanation for these results is unclear. However, it may infer that the GJ extract may protect peripheral neuron from ischemic injury. This explanation is supported by the fact that the transient cerebral ischemia induces apoptosis in the peripheral neuron [17] and geniposide, which is an active component of GJ extract, possesses anti-apoptosis activity [18].

Conclusions

The present study demonstrated that the administrations of DK and GJ extracts enhanced the recovery of neuronal injury after cerebral ischemia in mice. This finding suggested that the DK and GJ treatment might be beneficial for protecting the neuronal system against ischemic injury.

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REFERENCES

[1] P. Lipton (1999), "Ischemic cell death in brain neurons", *Physiological Reviews*, **79**, pp.1431-1568.

[2] R. Brouns, P.P. De Deyn (2009), "The complexity of neurobiological processes in acute ischemic stroke", *Clinical Neurology and Neurosurgery*, **111**, pp.483-495.

[3] M. Mingsan, Z. Xuexia, B. Ming, W. Linan (2014), "Persimmon leaf flavonoid promotes brain ischemic tolerance", *Neural Regen. Res.*, **8**, pp.2625-2632.

[4] Y.S. Sa, S.J. Kim, H.S. Choi (2005), "The anticoagulant fraction from the leaves of *Diospyros kaki* L. has an antithrombotic activity", *Arch. Pharm. Res.*, **28**, pp.667-674.

[5] T. Hayashi, L. Zongyou (1993), "Stimulating the growth of cultured endothelial cells stimulated by Gardenia", *Chinese Archives of Traditional Chinese Medicine*, **15**, pp.51-53.

[6] H. Liu, Y. Chen, F. Li, H. Zhang (2013), "Fructus Gardenia (*Gardenia jasminoides* J. Ellis), phytochemistry, pharmacology of cardiovascular, and safety with the perspective of new drugs development", *J. Asian Nat. Prod. Res.*, **15**, pp.94-110.

[7] Z. Haiyan, L. Qiong, L. Yan, L. Yang, Y. Ming (2017), "Learning and memory improvement and neuroprotection of *Gardenia jasminoides* (Fructus gardenia) extract on ischemic brain injury rats", *Journal of Ethnopharmacology*, **196**, pp.225-235.

[8] T.X. Le, T.P. Nguyen, N.T.T. Phuong, T.N.H Pham, V.T. Nguyen, et al. (2016), "Neuroprotective effect of *Panax notoginseng* against ischemic neuronal injury in mice", *Journal of Medicinal Materials,* **21(3)**, pp.344-348.

[9] E. Rousselet, J. Kriz, N.G. Seidah (2012), "Mouse Model of Intraluminal MCAO: Cerebral Infarct Evaluation by Cresyl Violet Staining", *J. Vis. Exp.*, **69**, p.e4038.

[10] S.A. Menzies, J.T. Hoff, A.L. Betz (1992), "Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model", *Neurosurgery*, **31(1)**, pp.100-106.

[11] H.Y. Son, H.S. Han, H.W. Jung, Y.K. Park (2009), "*Panax notoginseng* Attenuates the Infarct Volume in Rat Ischemic Brain and the Inflammatory Response of Microglia", *J. Pharmacol. Sci.*, **109**, pp.368-379.

[12] C.N. Joshi, S.K. Jain, P.S. Murthy (2004), "An optimized triphenyltetrazolium chloride method for identification of cerebral infarcts", *Brain Research Protocols*, **13**, pp.11-17.

[13] O. Engel, S. Kolodziej, U. Dirnagl, V. Prinz (2011), "Modeling Stroke in Mice - Middle Cerebral Artery Occlusion with the Filament Model", *J. Vis. Exp.*, **47**, p.2423.

[14] C.X. Wang, A. Shuaib (2007), "Neuroprotective effects of free radical scavengers in stroke", *Drugs & Aging*, **24**, pp.537-546.

[15] K. Kikuchi, N. Miura, Y. Morimoto, T. Ito, S. Tancharoen, et al. (2011), "Beneficial Effects of the Free Radical Scavenger Edaravone (Radicut) in Neurologic Diseases", *J. Neurol. Neurophysiol. S1.*, doi: 10.4172/2155-9562.S4171-4001.

[16] S. Lijun, Z. Jianbao, F. Kun, D. Yan, Z. Liyu, et al. (2014), "Flavonoids from persimmon (*Diospyros kaki*) leaves (FPL) attenuate H_2O_2-induced apoptosis in MC3T3- E1 cells via the NF-kB pathway", *Food & Funct.*, **5(3)**, pp.471-479.

[17] P. Deb, S. Sharma, K.M. Hassan (2010), "Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis", *Pathophysiology*, **17**, pp.197-218.

[18] L.X. Gua, J.H. Liu, Z.N. Xia (2009), "Geniposide inhibits $CoCl_2$-induced PC12 cells death via the mitochondrial pathway", *Chin. Med. J.*, **122**, pp.2886-2892.