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NEUROPROTECTIVE EFFECTS OF TIANMA GOUTENG DECOCTION IN A MIDDLE CEREBRAL ARTERY OCCLUSION MODEL OF ISCHEMIC STROKE

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Summary

This study aimed to evaluate the neuroprotective effects of Tianma Gouteng Decoction (TGD) in a middle cerebral artery occlusion-and-reperfusion (MCAO/R) model. Neurological scores, body weight changes, and cerebral infarct volume were evaluated. In addition, the concentrations of gastrodin, baicalin, 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (THSG), and geniposide in the TGD extract were analyzed by HPLC to characterize its chemical composition. The results indicated that TGD at 13.7 g/kg (raw herbal materials) significantly attenuated MCAO/R-induced cerebral infarction, improved neurological deficits, and attenuated body weight loss compared with the vehicle-treated group. TGD at 6.8 g/kg (raw herbal materials) also conferred neuroprotective effects, with early functional improvement observed 24 h after reperfusion, whereas TGD at 27.4 g/kg (raw herbal materials) did not produce significant protection. The TGD extract contained gastrodin (1.4 mg/mL), baicalin (10.091 mg/mL), THSG (0.0203 mg/mL), and geniposide (9.023 mg/mL), which may collectively contribute to its pharmacological activity. Overall, these findings demonstrate that TGD exerts neuroprotective effects against transient ischemia-induced brain injury, with optimal efficacy observed at 13.7 g/kg (raw herbal materials), suggesting its potential as a promising therapeutic candidate for ischemic stroke.

Keywords*: Tianma Gouteng Decoction; Neuroprotective effects; Ischemic stroke.*

1. Introduction

Ischemic stroke, a common cerebrovascular disease, is the second leading cause of death and a major contributor to long-term disability worldwide. It results from the sudden occlusion of cerebral arteries, leading to insufficient blood supply, oxygen deprivation, and subsequent neuronal injury. Despite advances in acute stroke management, the global burden of ischemic stroke continues to rise due to ageing populations and the increasing prevalence of vascular risk factors [1]. To date, recombinant tissue plasminogen activator (rtPA) remains the only approved pharmacological therapy for acute ischemic stroke. However, its clinical use is limited by a narrow therapeutic time window and the risk of hemorrhagic complications [2]. Consequently, only a limited proportion of patients are eligible for rtPA treatment. These limitations highlight the urgent need for the development of alternative or adjunctive therapeutic strategies that can provide neuroprotection, extend the therapeutic window, and improve functional recovery following ischemic stroke.

Tianma Gouteng Decoction (TGD) is a traditional Chinese medicinal formula widely used to treat hypertension [3]. These conditions are closely associated with cerebrovascular dysfunction and represent important risk factors for ischemic stroke. Because of its long-standing use in the management of hypertension and neurological symptoms, TGD has attracted increasing interest as a potential therapeutic strategy for cerebrovascular diseases. Yuh-Chiang Shen et al. reported that Tianma Gouteng Decoction, formulated according to traditional Chinese herbal medicine and containing 11 medicinal herbs similar to those used in the TGD extract of the present study, improved neurological outcomes, reduced infarct volume, and attenuated neuronal damage in experimental stroke models [4]. These findings suggest that TGD may possess dual pharmacological actions, combining antihypertensive effects with direct neuroprotective activity, thereby highlighting its potential relevance in the management of ischemic stroke. However, despite these promising indications, several limitations remain in the current literature. Different studies have employed modified versions of the formula, varying in herbal composition, dosage ratios, and extraction procedures, leading to inconsistent pharmacological outcomes and limiting reproducibility [4],[5]. In addition, few studies have systematically evaluated the dose–response

relationship of a chemically characterized TGD extract in a standardized experimental ischemia model. The lack of quantitative characterization of major bioactive constituents further limits mechanistic interpretation and quality control.

Therefore, the present study was conducted to evaluate the neuroprotective effects of Tianma Gouteng Decoction (TGD) in a middle cerebral artery occlusion (MCAO) model of ischemic stroke. In addition, several major bioactive constituents of TGD were quantitatively determined.

2. Materials and Methods

2.1. Plant materials

The composition of Tianma Gouteng Decoction (TGD) used in this study followed the formula reported by Liu LF et al. [5]. The TGD formula consisted of the raw herbal materials listed in Table 1. All materials were supplied by Indochina Medicinal Materials Company (Vietnam) and complied with the quality standards of the Chinese Pharmacopoeia and the Vietnamese Pharmacopoeia V.

Table 1. Raw materials used in the Tianma Gouteng Decoction formula

Materials	Origin of materials	Used parts	Amount
Gastrodia elata Blume	Anhui (China)	rhizome	9 g
Haliotis diversicolor Reeve	Hung Yen (Vietnam)	dried shell	18 g
Gardenia jasminoides Ellis	Quang Nam (Vietnam)	ripe fruit	9 g
Scutellaria baicalensis Georgi	Anhui (China)	root	9 g
Achyranthes bidentata Blume	Anhui (China)	root	12 g
Eucommia ulmoides Oliv.	Anhui (China)	bark	9 g
Leonurus japonicus Houtt.	Hung Yen (Vietnam)	whole plant	9 g
Taxillus chinensis (DC.) Danser	Hung Yen (Vietnam)	stems and branches	9 g
Polygonum multiflorum Thunb.	Son La (Vietnam)	stem	9 g
Poria cocos (Schw.) Wolf	Anhui (China)	sclerotium	9 g
Uncaria rhynchophylla (Miq.) Miq. ex Havil.	Hung Yen (Vietnam)	hook-bearing stems	12 g

2.2. Animals

Male *Swiss albino* mice (5–6 weeks old, weighing 35–45 g at the time of surgery) were supplied by the National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). The animals were housed in a controlled environment at 25 ± 1°C with appropriate humidity and a standard 12-h light/dark cycle. Standard laboratory chow provided by the National Institute of Hygiene and Epidemiology and tap water were available ad libitum. The animals were acclimatized for at least one week before starting the experiments.

2.3. Methods

Herbal extract preparation

The raw herbs were cut into small pieces and soaked in cold water for 30 min prior to extraction. Reflux extraction was then performed with water for 1 h, and the procedure was repeated twice. Briefly, 228 g of raw materials (equivalent to two formulations) were placed in a 2-L round-bottom flask and heated at 85°C for 1 h with a water-to-herb ratio of 5:1 (v/w). After heating, the mixture was allowed to cool and filtered three times through cloth and cotton to obtain the first extract. A second extraction was performed under the same conditions to obtain the final TGD extract. The extracts from the first and second extractions were combined and concentrated in a water bath at 60°C for 2 days, yielding 76 mL of condensed extract (corresponding to a raw herb-to-extract ratio of 3:1, w/v). The concentrated extract was stored at 4°C to −20°C until further use.

TGD is clinically administered in humans at a dose equivalent to 114 g of raw herbal materials per day. Based on interspecies dose conversion, the corresponding dose in mice was calculated to be 27.4 g of raw herbal materials per kg body weight. In this study, three dose levels of TGD were evaluated: a high dose of 27.4 g/kg (equivalent dose), a medium dose of 13.7 g/kg, and a low dose of 6.8 g/kg.

Model of transient focal ischemia in mice and drug administration

Middle cerebral artery occlusion and reperfusion (MCAO/R)-induced transient focal cerebral ischemia in mice was performed as previously described [6]. Briefly, mice were deeply anesthetized by intraperitoneal injection of a ketamine (Rotexmedica, Germany) and xylazine (Vemedim) mixture (0.1 mL/10 g body weight). The common carotid artery (CCA) was carefully exposed and temporarily occluded with a 5-0 silk suture. Two permanent knots using 4-0 silk sutures were placed at the distal portion of the external carotid artery (ECA). To prevent bleeding, the internal carotid artery (ICA) was temporarily clamped with a vascular clip. A 12-

mm-long 6-0 nylon monofilament (Alfresa Pharma Co., Osaka, Japan) with a silicone-coated tip was introduced into the ECA through a small incision between the two knots and advanced into the ICA. The filament was secured with a 4-0 silk suture to prevent bleeding and displacement. It was then advanced 9–10 mm beyond the bifurcation of the CCA and ECA to occlude the origin of the middle cerebral artery (MCA) at the circle of Willis and fixed in position. The temporary ligature on the CCA was removed.

The mice were randomly allocated into five groups as follows: Group 1, vehicle-treated MCAO/R mice (n = 8); Group 2, TGD 6.8 g/kg-treated MCAO/R mice (n = 8); Group 3, TGD 13.7 g/kg-treated MCAO/R mice (n = 8); Group 4, TGD 27.4 g/kg-treated MCAO/R mice (n = 7); and Group 5, edaravone 6 mg/kg-treated MCAO/R mice (n = 7).

Edaravone (5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one; Sigma-Aldrich, St. Louis, MO, USA) was solubilized in DMSO as a stock solution and then diluted in physiological saline before tail-vein injection. TGD (6.8, 13.7, and 27.4 g/kg, p.o.) or edaravone (6 mg/kg, i.v.) was administered immediately after reperfusion and continued daily throughout the experimental period (Fig. 1).

2,3,5-triphenyltetrazolium chloride (TTC) staining

The MCAO mice were decapitated three days after reperfusion to determine the cerebral infarct using TTC (Sigma-Aldrich, St. Louis, MO, USA) staining [6]. In brief, the brain was quickly removed from the skull and placed in ice-cold 0.9 % saline. The coronal tissue was cut to a thickness of 2 mm using a tissue slicer and then immersed in a 0.9% saline solution containing 0.8% TTC at 37°C for 10 min under light-proof conditions. The sections were captured with a high-resolution digital camera and analysed using ImageJ software (ver. 1.41, NIH; Bethesda, MD, USA). The area of the infarction was measured using the equation reported by Swanson RA et al. [7] and represented as the percentage of the cerebral infarct volume: Percentage of the cerebral infarct volume = [(contralateral hemispheric volume - ipsilateral non-infarcted volume)/contralateral hemispheric volume]*100.

Neurological scores

The neurological symptom of each mouse was evaluated according to Menzies et al. [8] of a four-level score: 4 = spontaneous right circling; 3 = spontaneous movement in all directions (right circling if pulled by the tail); 2 = decreased grip of the right forelimb while tail pulled; 1 = right forelimb flexion; 0 = no apparent deficits. The test was performed daily from immediately after reperfusion to day 3 by a blinded researcher.

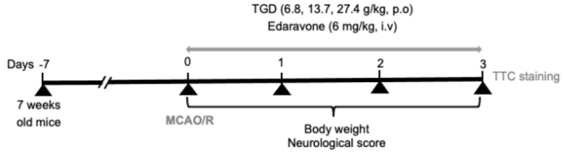


Fig. 1. Experimental protocols.

Determination of gastrodin, baicalin, THSG, and geniposide contents

Reference preparation: Precisely weighed baicalin (ChemFaces - China, Cat. No.: CFN99111) was dissolved in 70% ethanol; gastrodin (ChemFaces - China, Cat. No.: CFN99549) was dissolved in 50% methanol; 2,3,5,4′-tetrahydroxystilbene-2-*O*-*β*-D-glucoside (THSG, ChemFaces - China, Cat. No.: CFN99995) and geniposide (ChemFaces - China, Cat. No.: CFN98261) were dissolved in methanol to prepare standard solutions at concentrations ranging from 0.1-1 mg/mL.

The contents of gastrodin, baicalin, THSG, and geniposide in TGD were determined using a Shimadzu HPLC–UV system. The TGD extract was diluted 10-fold, filtered through a 0.45 μm membrane filter, and a 20 μL aliquot was injected into the system using an autosampler.

- Assay of gastrodin: The mobile phase consisted of solvent A (0.1% phosphoric acid in methanol) and solvent B (0.1% phosphoric acid in water). A linear gradient was applied as follows: 3–6% A from 0–15 min; 6–50% A from 15–45 min; 50–3% A from 45–46 min; and maintained at 3% A from 46–50 min. The column temperature was maintained at 40 °C. The solvent flow rate was 1 mL/min. A spectrophotometric detector was set at 220 nm [9].

- Assay of baicalin and THSG: The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.125% phosphoric acid in water) (20:80, v/v). The flow rate was 1 mL/min. The detection wavelengths were set at 280 nm for baicalin and 320 nm for THSG [9].

- Assay of geniposide: The mobile phase consisted of acetonitrile and water (15:85, v/v). The solvent flow rate was 1 mL/min. The detection wavelength was set at 240 nm [10].

2.4. Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Body weight and cerebral infarct volume were expressed as mean ± standard error of the

mean (SEM), whereas neurological scores were presented as median (first quartile; third quartile). Body weight data was analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc test for pairwise comparisons among groups. Infarct volume and neurological deficit scores were analyzed using the Kruskal–Wallis test, followed by the Mann–Whitney U test for multiple-group comparisons. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. TGD reduced cerebral infarct volume in transient cerebral ischemic mice

Cerebral infarct volume is a critical parameter for evaluating brain injury in the MCAO/R-induced ischemic model. Therefore, TTC staining was performed to assess infarct size following transient cerebral ischemia–reperfusion injury.

As shown in Fig. 2, three days after MCAO, mice in the model group exhibited cerebral infarction involving approximately 30% of the left hemispheric volume. Treatment with edaravone (6 mg/kg/day, i.v.), used as a reference drug, significantly reduced cerebral infarct size compared to the vehicle-treated MCAO/R mice ($p < 0.01$). Treatment with TGD at 6.8 g/kg reduced infarct volume to approximately 12% of the left hemisphere. This reduction was statistically significant compared with the vehicle-treated MCAO/R group ($p < 0.01$). TGD at 13.7 g/kg markedly decreased infarct volume to below 10% of the left hemisphere ($p < 0.001$), representing the greatest reduction among the treatment groups. In contrast, treatment with TGD at 27.4 g/kg did not significantly affect infarct volume, as no statistically significant difference was observed compared with the model group ($p > 0.05$).

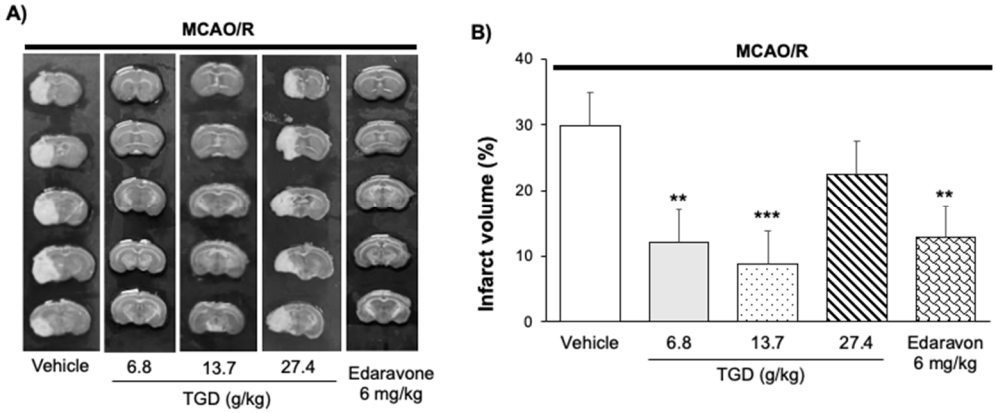


Fig. 2. Effects of TGD on the infarct volume of MCAO/R mice
(A) Representative brain images using TTC staining. (B) Quantitative analysis of infarct volume (%) in the mouse brain. Mouse brain tissue was collected 72 h after MCAO/R. Data were expressed as the mean ± S.E.M. (n=7-8). **p<0.01, ***p<0.001 compared to vehicle-treated MCAO/R mice (One-way-ANOVA followed by post hoc LSD test).

3.2. TGD attenuated the body weight loss and neurological symptoms in MCAO/R-induced ischemic mice

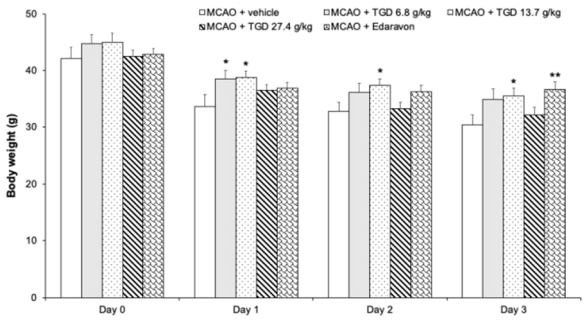


Fig. 3. Effects of TGD on the body weight of mice following cerebral ischemia.

Data were expressed as the mean ± S.E.M. (n=7-8). *p<0.05, **p<0.01 compared to vehicle-treated MCAO/R mice (One-way-ANOVA followed by post hoc LSD test).

As shown in Fig. 3, mice in the vehicle-treated MCAO/R group exhibited a progressive decrease in body weight over time. Treatment with edaravone attenuated body weight loss, with a slight increase observed on day 3 compared with the vehicle-treated MCAO/R group, and the difference was statistically significant ($p < 0.01$). Treatment with TGD at 6.8 g/kg improved body weight on day 1, and this change was statistically significant compared with the vehicle-treated MCAO/R group. However, no significant differences were observed on days 2 and 3 ($p >$

0.05). Treatment with TGD at 13.7 g/kg resulted in significant improvement in body weight compared with the vehicle-treated MCAO/R group on days 1–3 ($p < 0.05$). In contrast, treatment with TGD at 27.4 g/kg did not significantly affect body weight, as no statistically significant differences were observed compared with the vehicle-treated MCAO/R group ($p > 0.05$).

Table 2. Effects of TGD on neurological symptom scores in middle cerebral artery occlusion-and-reperfusion (MCAO/R)-induced cerebral ischemic mice

Operation	Treatment (mg/kg/day)	Neurological symptom scores after the operation			
		0 hr after reperfusion	Day 1	Day 2	Day 3
MCAO/R	vehicle	4 (3; 4)	3 (2; 4)	3 (2; 4)	3 (2; 4)
	TGD 6.8 g/kg	4 (3; 4)	2* (2; 3)	2* (2; 3)	2** (1; 2)
	TGD 13.7 g/kg	3.5 (3; 4)	3 (2; 3)	2** (1; 3)	1.5** (1; 2)
	TGD 27.4 g/kg	4 (3; 4)	3 (2; 3)	2.5 (1; 3)	2 (2; 3)
	edaravone 6 mg/kg	4 (3; 4)	3 (2; 3)	2** (1; 2)	2** (1; 2)

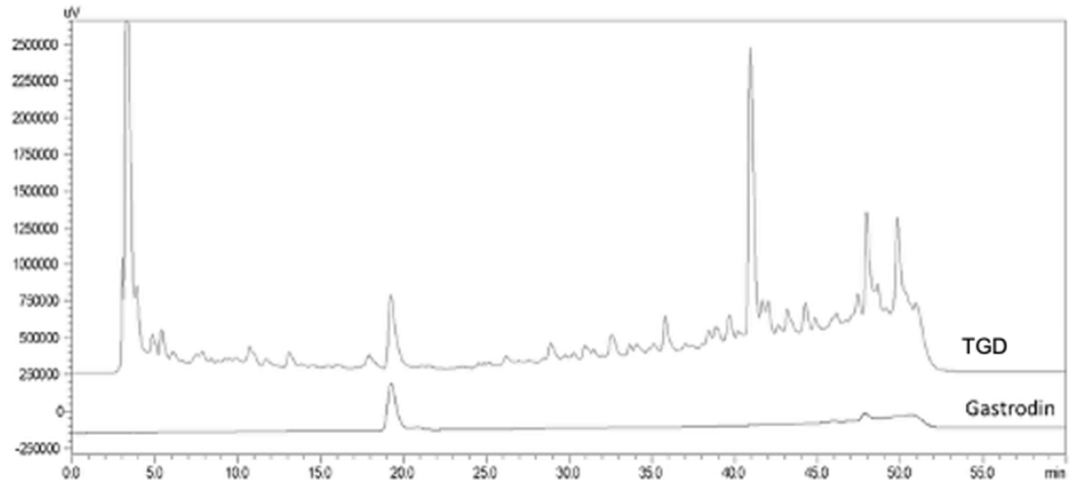
Data were expressed as the median (25 and 75% interquartile range) (n=7-8). *p<0.05, **p<0.01 vs. vehicle - treated MCAO/R mice (Kruskall-Wallis and Mann-Whitney U-test).

As shown in Table 2, various extents of neurological symptoms were observed in the MCAO/R mice from day 0 (0 hr reperfusion) to day 3 after the cerebral ischemia and reperfusion operations. Neurological scores remained high (score 3–4) in the vehicle-treated MCAO/R group from day 0 to day 3 after reperfusion. There were no significant differences in neurological scores between the edaravone-treated MCAO/R group and the vehicle-treated MCAO/R group on days 0 and 1. However, significant improvement in neurological function was observed in the edaravone-treated MCAO/R group on days 2 and 3 compared with the vehicle-treated MCAO/R group ($p < 0.05$).

Significant improvement of neurological scores was observed earlier in the MCAO/R group treated with TGD at 6.8 g/kg, with statistically significant differences compared with the vehicle-treated MCAO/R group on days 1, 2, and 3 ($p < 0.05$). In the MCAO/R group treated with TGD at 13.7 g/kg, neurological function progressively improved over time, reaching statistical significance compared with the vehicle-treated MCAO/R group on days 2 and 3 ($p < 0.05$). Although neurological scores gradually decreased in the MCAO/R group treated with TGD at 27.4 g/kg, no statistically significant differences were observed compared with the vehicle-treated MCAO/R group ($p > 0.05$).

3.3. Determination of gastrodin, baicalin, THSG, and geniposide contents

The four major components in TGD (0.3 g/mL) were analyzed by HPLC, as shown in Fig. 4. The results indicated that the contents of gastrodin, baicalin, THSG, and geniposide were 1.4 mg/mL, 10.091 mg/mL, 0.0203 mg/mL, and 9.023 mg/mL, respectively.



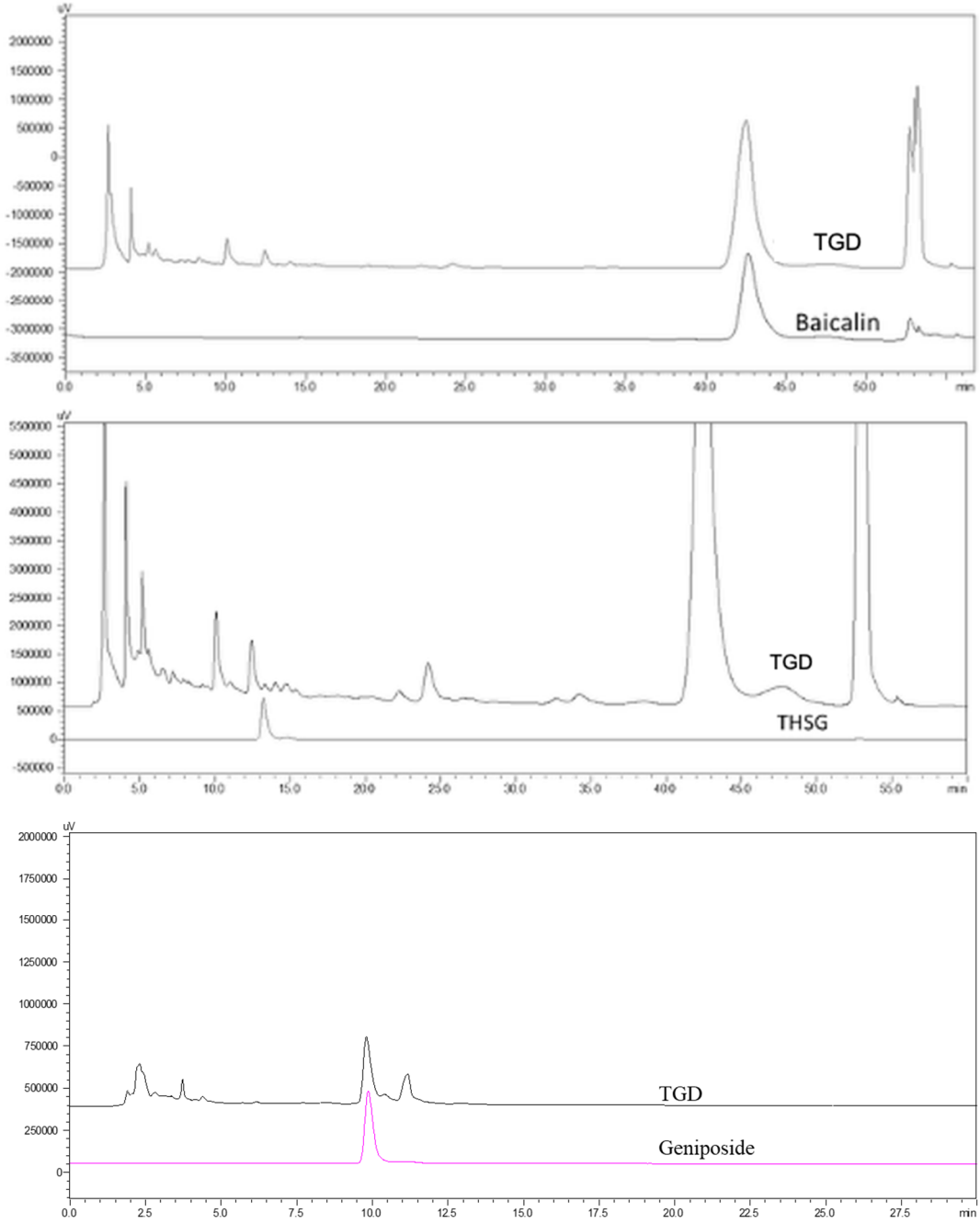


Fig. 4. HPLC chromatograms of TGD

4. Discussion

The present study demonstrated that TGD exerts significant neuroprotective effects in a 60-min MCAO model of transient focal cerebral ischemia. Among the three tested doses, TGD at 13.7 g/kg produced the most pronounced therapeutic effects, as evidenced by improved neurological scores, reduced body weight loss, and a marked reduction in infarct volume. TGD at 6.8 g/kg also showed effects comparable to those of edaravone.

In experimental stroke research, therapeutic efficacy is commonly evaluated using neurological scores, body weight changes, and infarct volume. Neurological scoring reflects motor and sensory dysfunction and provides functional evidence of recovery. In this study, TGD, particularly at 13.7 g/kg, significantly improved neurological outcomes compared with the vehicle-treated MCAO/R group. Body weight monitoring served as an indirect indicator of

general health status; attenuation of body weight loss further supported the functional benefits of TGD treatment. However, infarct volume assessment by TTC staining remains the most objective indicator of structural brain injury. The marked reduction in infarct size observed in the TGD-treated groups confirms its protective effect at the tissue level [11]. However, the highest tested dose (27.4 g/kg) did not produce significant neuroprotective effects. One possible explanation may be related to the traditional therapeutic context of TGD. Clinically, TGD has long been prescribed for the management of hypertension and hypertension-related neurological symptoms. Therefore, its pharmacological effects may involve both antihypertensive activity and direct neuroprotective mechanisms. In the present study, the MCAO model was established in normotensive animals, which may not fully reproduce the complex pathological conditions associated with ischemic stroke in patients with chronic hypertension. Under such conditions, the antihypertensive component of TGD pharmacology may play a limited role, potentially affecting the overall observable therapeutic response at higher doses. In addition, this phenomenon may be related to a hormetic effect, as reported in pharmacological studies of herbal medicines. Hormesis describes a biphasic dose–response relationship in which low or moderate doses produce beneficial effects, whereas higher doses may result in diminished therapeutic responses [12]. Therefore, the lack of neuroprotective efficacy at the highest tested dose may partly reflect a hormetic dose–response pattern in TGD.

Several studies worldwide have reported that the neuroprotective mechanisms of TGD include suppression of ROS overproduction, attenuation of oxidative damage in both *in vitro* and *in vivo* ischemic models, modulation of oxidative stress, and inhibition of the AGE/RAGE signalling pathway [4],[13]. These mechanisms contribute to elucidating the neuroprotective effects of TGD. Given that TGD is a multi-component formula, characterization of its major bioactive constituents is essential for understanding its mechanistic basis. In the present study, we further quantified selected bioactive constituents of TGD to better characterize the formulation and provide additional insight into its potential pharmacological effects. In general, a single herbal medicine may contain dozens of natural bioactive compounds, whereas a traditional prescription may consist of several herbs. Therefore, identifying the active components in such complex formulations remains a significant challenge [14].

The TGD formula consists of 11 medicinal herbs. The four major compounds quantified in the extract were gastrodin (from *Gastrodia elata* Blume (rhizome)), baicalin (from *Scutellaria baicalensis* Georgi (root)), THSG (from *Polygonum multiflorum* Thunb. (caulis)), and geniposide (from *Gardenia jasminoides* Ellis (ripe fruit)). According to our analysis, the TGD extract contained gastrodin (1.4 mg/mL), baicalin (10.091 mg/mL), geniposide (9.023 mg/mL), and a small amount of THSG (0.0203 mg/mL). The formula Tianma Gouteng Keli in the Pharmacopoeia of the People's Republic of China has a composition similar to TGD and specifies baicalin and gastrodin as quality control markers [9]. This finding suggests that gastrodin, baicalin, and geniposide contributed to TGD's actions.

Gastrodin, one of the principal bioactive constituents of TGD, has been widely investigated for its neuroprotective properties. Clinical evidence from randomized controlled trials indicates that gastrodin, when used as an adjuvant therapy, significantly improves neurological outcomes in patients with acute stroke, as reflected by reductions in the National Institutes of Health Stroke Scale (NIHSS) score, a standardized measure of stroke severity. In addition, gastrodin has been associated with decreased biomarkers of neuronal injury and increased levels of brain-derived neurotrophic factor [15]. In experimental animal studies, gastrodin and its derivatives have demonstrated significant neuroprotective effects in models of cerebral ischemia. In a rat MCAO model, a gastrodin derivative (Gas-D) improved neurological deficits, reduced infarct volume, and attenuated neuropathological changes by suppressing inflammatory responses through modulation of the Prx–TLR4 signalling pathway, and by reducing oxidative stress after reperfusion [16]. Recent reviews further indicate that gastrodin exerts multiple neuroprotective mechanisms, including anti-inflammatory and antioxidant effects, regulation of mitochondrial function, inhibition of autophagy and ferroptosis, and modulation of neuronal signalling pathways. In addition, gastrodin exhibits relatively low blood–brain barrier (BBB) permeability under physiological conditions. However, cerebral

ischemia is known to disrupt the integrity of the BBB. Therefore, the BBB disruption observed in MCAO models may facilitate the entry of gastrodin into brain tissue. Despite its limited blood–brain barrier permeability, advances in drug delivery systems may enhance its bioavailability and therapeutic potential in neurological disorders [17]. Overall, these findings highlight the important contribution of gastrodin to TGD's neuroprotective profile in ischemic stroke.

Recent studies have demonstrated the neuroprotective effects of baicalin in ischemic stroke. Baicalin, a major flavonoid derived from *Scutellaria baicalensis*, has been extensively investigated for its protective role in cerebral ischemia. A recent review highlighted that baicalin exerts multifactorial actions, including antioxidant, anti-inflammatory, and anti-apoptotic effects, as well as mitochondrial protection and upregulation of neurotrophic factors, supporting its therapeutic potential in ischemic stroke [18]. In experimental MCAO models, baicalin (100 mg/kg) significantly improved neurological deficits and reduced histopathological damage compared with vehicle-treated animals. Mechanistically, baicalin attenuated ROS overproduction and lipid peroxidation, restored the balance of apoptosis-related proteins (increased Bcl-2 and decreased Bax expression), and suppressed caspase-3 activation, thereby inhibiting neuronal apoptosis [19]. Additionally, recent studies have demonstrated that THSG exerts significant neuroprotective effects against cerebral ischemia/reperfusion injury. In tMCAO/R and OGD/R models, THSG reduced infarct volume, improved neurological outcomes, and attenuated neuronal apoptosis. Mechanistically, THSG enhanced PINK1–PARK2-mediated mitophagy by activating the GluN2B–CaMKII–ERK1/2 signalling pathway, and these effects were abolished by pharmacological inhibition of mitophagy or GluN2B [20]. These mechanistic insights further support the role of baicalin and THSG as a key component contributing to the multi-target effects of TGD. Although the quantitative analysis in the present study indicated that the THSG content in the TGD extract was considerably lower than that of baicalin or geniposide, this compound may still contribute to the overall neuroprotective efficacy of the formula *in vivo*, possibly through synergistic interactions with other bioactive constituents.

In the MCAO model, both the neuroprotective effects and underlying mechanisms of geniposide have been extensively investigated. Geniposide administration significantly improved neurological deficits, reduced brain oedema and infarct volume, and showed no evident toxicity at doses up to 150 mg/kg. Mechanistically, its protective effects were closely associated with attenuation of inflammatory responses, as geniposide suppressed IL-6 and iNOS expression, upregulated A20, and inhibited activation of the TNF receptor–associated factor-6 (TRAF6)/NF-κB signalling pathway [21]. In addition, in both OGD/R-treated BV2 microglial cells and MCAO mice, geniposide further demonstrated anti-apoptotic, anti-inflammatory, and anti-autophagic effects. It modulated Bcl-2/Bax expression, reduced pro-inflammatory cytokines such as TNF-α and IL-1β, and regulated microglial polarization. Notably, geniposide was shown to exert its protective effect by modulating the SOX2/RIPK1 signalling axis, thereby alleviating cerebral ischemic injury *in vivo* [22]. Together, these findings reinforce the notion that geniposide contributes to the integrated neuroprotective mechanisms of TGD.

According to traditional Chinese medicine theory, TGD is traditionally used to calm the liver, extinguish internal wind, clear heat, promote blood circulation, and nourish the liver and kidneys. Within this formula, *Gastrodia elata*, *Uncaria rhynchophylla*, and *Haliotis diversicolor* function as the "Jun" (principal herbs), primarily responsible for calming liver wind and relieving neurological symptoms. *Gardenia jasminoides* and *Scutellaria baicalensis* serve as the "Chen" (minister herbs), clearing heat and suppressing excessive liver fire. Other components, including *Leonurus japonicus*, *Achyranthes bidentata*, *Eucommia ulmoides*, and *Taxillus chinensis*, promote blood circulation and nourish the liver and kidney, while *Polygonum multiflorum* and *Poria cocos* exert calming and sedative effects; these herbs function as the "Zuo" and "Shi" components that support and harmonize the overall therapeutic action of the formula. The traditional formulation principle of "Jun–Chen–Zuo–Shi" may provide a theoretical basis supporting the neuroprotective efficacy of TGD observed in experimental ischemic stroke.

Thus, the neuroprotective effects of TGD observed in the MCAO mouse model may be partially explained by the pharmacological actions of its bioactive constituents. Importantly, the multi-component and multi-target characteristics of TGD may underlie its superior efficacy at the

optimal dose identified in this study. Further studies with longer observation periods are required to determine whether TGD can provide sustained neuroprotective effects and improve long-term neurological outcomes after stroke.

5. Conclusion

Tianma Gouteng Decoction (TGD) exhibited neuroprotective effects in a 60-min MCAO model of transient focal cerebral ischemia. TGD at 13.7 g/kg produced the most pronounced effects, significantly reducing infarct volume, improving neurological deficits, and attenuating body weight loss compared with the vehicle-treated group. Chemical analysis demonstrated that the TGD extract contained gastrodin (1.4 mg/mL), baicalin (10.091 mg/mL), 2,3,5,4′-tetrahydroxystilbene-2-*O*-*β*-D-glucoside (0.0203 mg/mL), and geniposide (9.023 mg/mL), which may contribute to the observed pharmacological effects.

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