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Curcuma longae Radix, *Phellinus linteus* 및 *Scutellariae* Radix 혼합추출물의 산화성 신경세포손상 보호효과

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Protective Effect of an Ethanol Extract Mixture of *Curcuma longae* Radix, *Phellinus linteus*, and *Scutellariae* Radix on Oxidative Neuronal Damage

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ABSTRACT : Previous work demonstrated that an ethanol extract (HS0608) of a mixture of three medicinal plants of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix markedly inhibits Aβ (25-35)-induced neurotoxicity. The present study was performed to further verify the neuroprotective effect of HS0608 on oxidative and ischemic cerebral injury using cultured rat cortical neurons and rats. Exposure of cultured cortical neurons to 100 μM hydrogen peroxide (H_2O_2) induced neuronal apoptotic death. At 10-100 ㎍/㎖, HS0608 inhibited neuronal death, elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), and generation of reactive oxygen species (ROS) induced by H_2O_2 in primary cultures of rat cortical neurons. In vivo, HS0608 prevented cerebral ischemic injury induced by 2-h middle cerebral artery occlusion (MCAO) and 24-h reperfusion. The ischemic infarct and edema were significantly reduced in rats that received HS0608 (200 ㎎/㎏). These results suggest that the anti-oxidative properties of HS0608 may be responsible for its neuroprotective effect against focal cerebral ischemic injury and that HS0608 may have a therapeutic role in neurodegenerative diseases such as stroke.

Key Words : *Curcuma longae* Radix, *Phellinus linteus*, *Scutellariae* Radix, Neuroprotection, H_2O_2, Cultured Cortical Neurons, Cerebral Ischemic Injury

INTRODUCTION

Pharmacological activities of *Curcuma longae* radix, the root of *Curcuma longa*, possessing anti-inflammatory (Guo *et al*., 2008), anti-oxidant (Adaramoye *et al*., 2002) and neuroprotective effects (Rajakrishnan *et al*., 1999) have been extensively studied. Phytochemical analyses have shown that the main constituents of *Curcuma longae* radix are curcumins, curcuminoids, zingiberine, phelandreen, and essential oils (Kapoor, 1990; Srinivasan, 1953). *Phellinus linteus* has been used for its anti-cancer, anti-diabetes and anti-oxidant activities (Ajith and Janardhanan, 2002; Sliva *et al*., 2008). It was defined that *Phellinus linteus* and its active component, hispolon, shows anti-inflammation and analgesic effects via inhibition of nitric oxide and prostaglandin E_2 production and antioxidative activity (Chang *et al*., 2009; Kim *et al*., 2007). *Scutellariae* radix from *Scutellaria baicalensis* Gergi (Labiatae) has been demonstrated to possess antipyretic, antibacterial, and anti-inflammatory properties (Bensky *et al*., 1992; Huang, 1999). Several flavonoids such as baicalin, baicalein and wogonin have been isolated as active components of *Scutellariae* radix (Huang, 1999). *Scutellariae* radix and its flavonoids have also shown neuroprotection against ischemic brain damage, 6-hydroxydopamine-induced Parkinsonism, and Aβ (25-35)-induced amnesia (Mu *et al*., 2009; Wang *et al*., 2004; Zhang *et al*., 2006).

Ischemic stroke results from a transient or permanent reduction

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in cerebral blood flow caused by the occlusion of a cerebral artery via an embolus or local thrombosis (Dirnagl *et al*., 1999). Loss of blood flow results in depletion of metabolic substrates such as oxygen and glucose. Oxidative stress is believed to exacerbate the damage caused by cerebral ischemia (Chan, 2001). Since three medicinal plants of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix exhibit antioxidant and anti-inflammatory activities, we hypothesized that the combination of these three plants might protect neurons against neurodegenerative diseases such as Alzheimer disease and stroke. In a previous report, we demonstrated a significant neuroprotective effect of an ethanolic extract of a mixture of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix, which was named as HS0608, against Aβ (25-35)-induced neuronal cell damage and memory impairment (Kim *et al*., 2009b). In the current study, we investigated the neuroprotective effect of HS0608 against ischemia-induced brain infarction following middle cerebral artery occlusion (MCAO) and reperfusion in rats, as well as the effect of HS0608 on hydrogen peroxide (H_2O_2)-induced neuronal damage in cultured rat cortical neurons, to elucidate the neuroprotective mechanism of HS0608.

MATERIALS AND METHODS

1. Plant materials and extraction

The dried *Curcuma longae* radix and *Scutellariae* radix were purchased at Kyungdong Folk Medicine market, Seoul, Korea, and fruiting bodies of cultured *Phellinus linteus* were supplied from Han Kook Shin Yak, Nonsan, Chungnam. These plants were identified by one of the authors, Dr. KiHwan Bae, Chungnam National University. One hundred g per plant from the three plants were mixed, refluxed in 3 L ethanol at room temperature for 3 h, filtered, and lyophilized to yield an ethanol extract (HS0608, 64 g), which was then stored at −20℃ until required.

2. Experimental animals

Pregnant Sprague-Dawley (SD) rats and male SD rats (Daehan BioLink Co. Ltd., Chungbuk, Korea) were housed in an environmentally controlled room at 22 ± 2℃, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water *ad libitum*. The procedures involving experimental animals complied with the animal care guidelines of the National Institutes of Health and the animal ethics committee of Chungbuk National University.

3. Measurements of H_2O_2-induced neuronal death and intracellular biochemical changes in primary cultures of rat cerebral cortical neurons

Primary cortical neuron cultures were prepared using embryonic day 15 to 16 SD rat fetuses, as previously described (Cho *et al*., 2009). Experiments of H_2O_2-induced neurotoxicity were performed on neurons after 5-6 days in culture as previously described (Cho *et al*., 2009; Kim *et al*., 2009a). Cultured neurons were treated with 100 μM H_2O_2 in a HEPES buffer at 37℃ for 15 min, followed by incubation for 12 h in H_2O_2- and serum-free DMEM medium to produce neuronal death and reactive oxygen species (ROS) generation. HS0608 was dissolved in dimethylsulfoxide (DMSO) at concentration of 100 ㎎/㎖ and further diluted in experimental buffers. The final concentration of DMSO was ≤ 0.1%, which did not affect cell viability. For each experiment, HS0608 was applied 15 min prior to treatment with 100 μM H_2O_2, and then again during the H_2O_2 exposure and post-exposure period.

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay and Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining were performed to measure neuronal death as previously described (Cho *et al*., 2009). Changes in $[Ca^{2+}]_i$ immediately after H_2O_2 treatment were measured with Fluo-4 AM (Molecular Probes), a calcium-sensitive fluorescent dye, using a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg GmbH, Germany) with a 488-nm excitation argon laser and 515-㎚ longpass emission filters (Cho *et al*., 2009). The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes), and a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg GmbH, Germany) with 488-㎚ excitation and 510-㎚ emission filters were used to monitor the generation of ROS in neurons treated with 100 μM H_2O_2 (Cho *et al*., 2009).

4. MCAO/reperfusion-induced focal cerebral ischemia in rats and evaluation of ischemic damage

Before surgery, male SD rats weighing 280-300 g were fasted overnight with free access to water. Focal cerebral ischemia was produced by MCAO for 2 h, followed by reperfusion for 24 h, as previously described (Ban *et al*., 2008). After MCAO/reperfusion, rats were sacrificed by decapitation under anesthesia (diethyl ether), and brains were quickly removed. The infarct and edema volumes of brain tissue were measured using TTC staining, as previously described (Ban *et al*., 2008). HS0608 (100 and 200

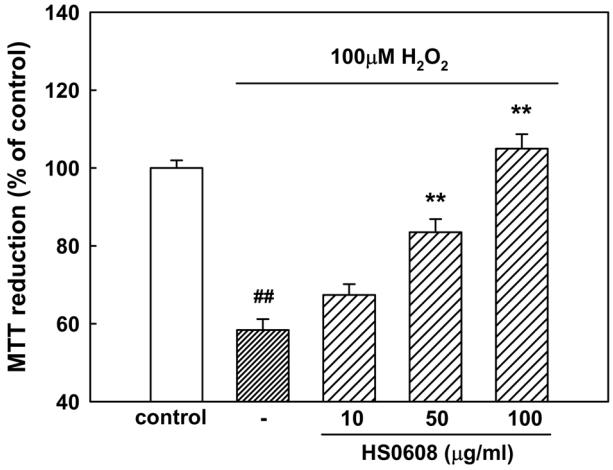

Fig. 1. Inhibitory effect of HS0608 on H_2O_2-induced neuronal cell death in cultured cortical neurons. Neuronal cell death was measured using the MTT assay. The MTT absorbance from untreated cells was normalized to 100%. Results are expressed as mean ± S.E.M. of data obtained from 5 independent experiments. $^{\#\#}P < 0.01$ vs control; $^{**}P < 0.01$ vs 100 μM H_2O_2.

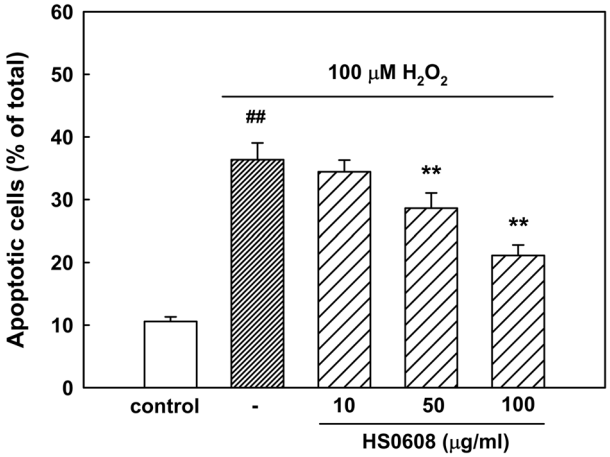

Fig. 2. Inhibitory effect of HS0608 on H_2O_2-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted in 5 to 6 fields per well. The values represent the apoptotic cells as a percentage of the total number of cells expressed as mean ± S.E.M. of data obtained from 4 independent experiments. $^{\#\#}P < 0.01$ vs control, $^{**}P < 0.01$ vs 100 μM H_2O_2.

mg/kg) was administered at three different time points (0.5 h before and 1 h after occlusion, as well as 1 h after reperfusion).

5. Statistical analysis

Data are expressed as mean ± SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's tests. $P < 0.05$ was considered significant.

RESULTS

1. HS0608 inhibits H_2O_2-induced neuronal cell death

The concentration of 100 μM of H_2O_2 was used for determining H_2O_2-induced neuronal cell damage in the present experiments based on our previous result (Park *et al.*, 2006). When cortical neurons were exposed to 100 μM H_2O_2, absorbance in the MTT assay was 58.4 ± 2.8% of that of the untreated controls (Fig. 1), indicating that H_2O_2 caused neuronal cell death. Pretreatment of cortical neurons with 50 and 100 ㎍/㎖ HS0608 reduced the neuronal death induced by 100 μM H_2O_2 (absorbance, 83.5 ± 3.3% and 105.0 ± 3.7% of control, respectively; Fig. 1).

An additional experiment was performed with Hoechst 33342 staining to detect condensed or fragmented DNA, which is indicative of H_2O_2-induced neuronal apoptotic death. Treatment of neurons with 100 μM H_2O_2 produced apoptosis of 36.4 ± 2.7% of the total population of cultured cortical neurons,

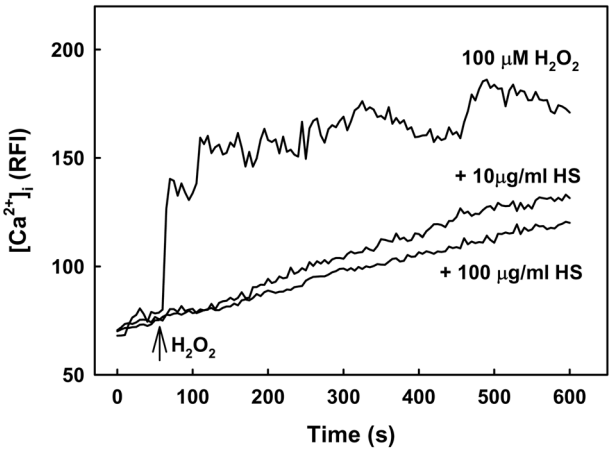

Fig. 3. Inhibitory effect of HS0608 on H_2O_2-induced $[Ca^{2+}]_i$ elevation in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using Fluo-4 AM dye and a confocal laser scanning microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace shows a single cell that is representative of at least 3 independent experiments.

compared with that of 10.6 ± 0.7% in control cultures. The addition of HS0608 (50 and 100 ㎍/㎖) significantly decreased the H_2O_2-induced apoptotic cell death, showing 28.6 ± 2.4 and 21.1 ± 1.7%, respectively (Fig. 2).

2. HS0608 inhibits H_2O_2-induced $[Ca^{2+}]_i$ elevation

Increases in $[Ca^{2+}]_i$ have been associated with H_2O_2-induced

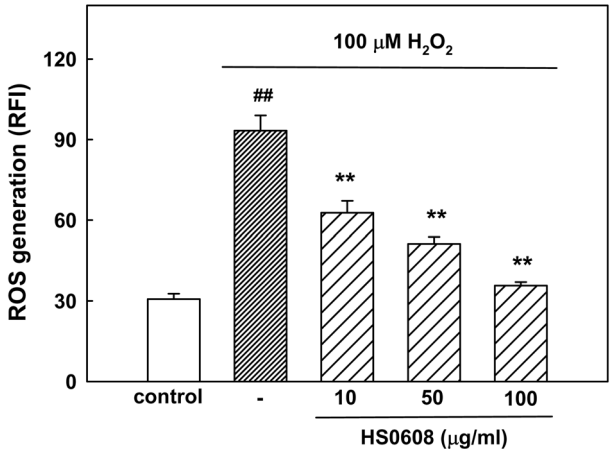

Fig. 4. Inhibitory effect of HS0608 on H_2O_2-induced ROS generation in cultured cortical neurons. ROS was monitored using H_2DCF-DA dye and a confocal laser scanning microscope. Results are expressed as mean ± S.E.M. of RFI obtained from 4 independent experiments. $^{\#\#}P < 0.01$ vs control; $^{**}P < 0.01$ vs 100 μM H_2O_2.

cell death. In our cell cultures, $[Ca^{2+}]_i$ showed initial rapid increase in response to treatment with 100 μM H_2O_2 with subsequently gradual and fluctuant increase for about 10 min (Fig. 3). In contrast, pretreatment with HS0608 (10 and 100 ㎍/㎖) showed significant inhibitions of the increase of $[Ca^{2+}]_i$ induced by 100 μM H_2O_2. HS0608 did not affect basal $[Ca^{2+}]_i$.

3. HS0608 inhibits H_2O_2-induced ROS generation

In H_2DCF-DA-loaded cerebral cortical neurons, 100 μM H_2O_2 increased the fluorescence intensity, indicating that ROS were generated. In neurons treated with 100 μM H_2O_2, the relative fluorescence increased approximately 3-fold to 93.3 ± 5.7 compared with the value in control neurons (30.7 ± 2.0; Fig. 4). The H_2O_2-induced increase in ROS generation was significantly inhibited by HS0608 (10, 50 and 100 ㎍/㎖).

4. HS0608 inhibits MCAO/reperfusion-induced brain infarction

After MCAO/reperfusion, a large ipsilateral cerebral infarction was observed in the rat brain. TTC-stained coronal sections, in which normal brain tissue stains deep red, were used to determine the volume of a cerebral infarction; infarct tissues doese not stain (Fig. 5A). In coronal sections, the infarct size was significantly reduced by HS0608 (200 ㎎/㎏) compared to that of vehicle-treated controls (79.6 ± 13.8 mm^3 for 200 ㎎/㎏ and 330.2 ± 8.4 mm^3 for vehicle) (Fig. 5B). The edema volume increased by MCAO/reperfusion also was significantly reduced

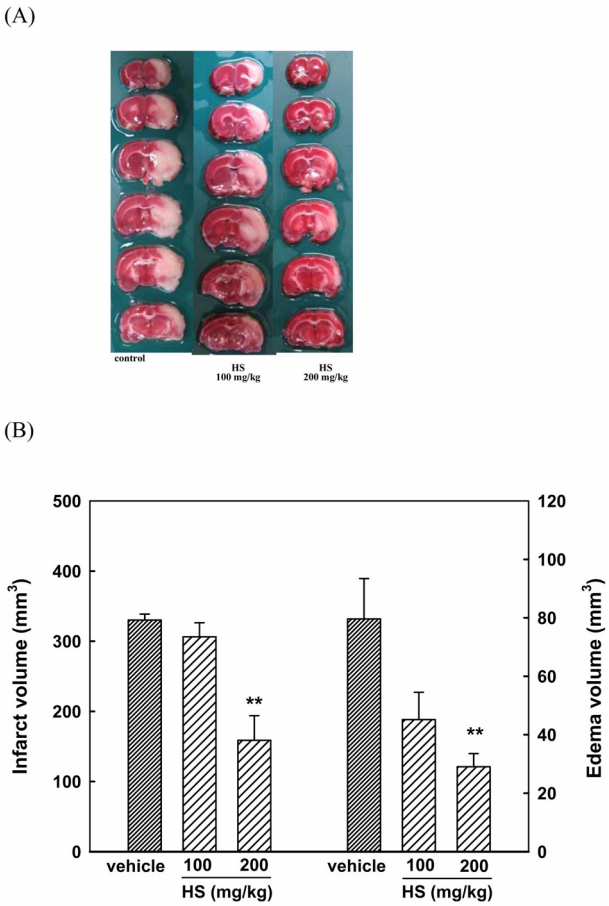

Fig. 5. Protective effect of HS0608 on MCAO/reperfusion-induced infarct and edema formation in rats. (A) A representative rat brain stained with 2% TTC 24 h after reperfusion. (B) The results show infarct and edema volumes caused in the ipsilateral hemisphere and are expressed as mean ± S.E.M. of data obtained from 6 rats. $^{**}P < 0.01$ vs vehicle.

by HS0608 treatment (29.0 ± 4.5 mm^3 for 200 ㎎/㎏), compared to that of vehicle-treated controls (79.6 ± 13.8 mm^3) (Fig. 5B). Animal body temperatures were monitored for 6 h after cerebral reperfusion commenced, and no significant differences were observed between the HS0608 and control groups (data not shown). Thus, the observed neuroprotective effect of HS0608 could not be attributed to hypothermic effects.

DISCUSSION

In vitro H_2O_2 toxicity has been used for studying the neuropathology of oxidative stress in CNS disorders as a well-established model. H_2O_2-induced neurotoxicity in cultured neurons has been demonstrated to involve sustained elevation of $[Ca^{2+}]_i$, NMDA receptor modulation induced by glutamate

release and ROS generation (Duffy and MacVicar, 1996; Mailly *et al*., 1999). The present study also demonstrated that H_2O_2 increased the levels of $[Ca^{2+}]_i$ and ROS generation, resulting in apoptosis of cultured cortical neurons. H_2O_2 exposure causes neuronal cells to exhibit increased permeability to Na^+ ions, resulting in membrane depolarization and subsequent a large influx of Ca^{2+} ions via voltage-dependent Ca^{2+} channels (VDCC) (Wang and Joseph, 2000). H_2O_2 has been demonstrated to inhibit the uptake of glutamate and enhance the release of glutamate, resulting in NMDA receptor overstimulation and a further increase in $[Ca^{2+}]_i$ (Mailly *et al*., 1999; Volterra *et al*., 1994). In the present study, H_2O_2 elicited a significant increase in $[Ca^{2+}]_i$, which was blocked by HS0608. Although these results suggest that HS0608 might prevent H_2O_2-induced Ca^{2+} entry through VDCC- and/or NMDA-receptor-coupled channels to inhibit ROS generation and then neuronal death, its underlying mechanism remains unclear. *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix, the constituents of HS0608, have been reported to possess antioxidant principles, curcumin, hispolon, and baicalein, baicalin and wogonin (Chang *et al*., 2009; Guo *et al*., 2008; Su *et al*., 2008; Zhang *et al*., 2006), repectively, suggesting that inhibition of H_2O_2-induced neuronal death by HS0608 might be due to their ROS scavenging activity. Further study to elucidate the precise mechanism should be performed.

The rat model of MCAO followed by reperfusion, mimics many features of stroke in humans. In particular, the middle cerebral artery, which is the specific occlusion site in this model, is the most commonly affected vessel in both embolic and thrombotic stroke in humans (Longa *et al*., 1989). Infarction and edema are the two main pathophysiological changes observed in the cerebral ischemia (Durukan and Tatlisumak, 2007). Cellular swelling causes brain edema, which is the earliest response to ischemic injury. The severity of the neurological deficit is known to correlate with the size of lesion (Tominaga and Ohnishi, 1989) and significant impairment in neurological function was observed after reperfusion. In addition to the *in vitro* neuroprotective effect of HS0608, we have shown that HS0608 functions *in vivo* as a potent neuroprotectant in transient brain ischemia, effectively decreasing infarct volume and edema in the rat brain. Ion pumps cannot function during ischemia and thus ATP levels become progressively depleted. This results in elevation of $[Ca^{2+}]_i$, which further accelerates ATP depletion. The rise in $[Ca^{2+}]_i$ during ischemia and reperfusion leads to mitochondrial Ca^{2+} accumulation, particularly when oxygen is reintroduced during reperfusion. Reintroduction of oxygen allows generation of ATP; however, damage to the electron transport chain results in increased mitochondrial generation of ROS (Murphy and Steenbergen, 2008). Evidence obtained over the past two decades shows that ROS are involved in brain lesions, including those due to cerebral ischemia-reperfusion. Many reports have demonstrated that antioxidants such as glutathione, superoxide dismutase, tocopherol and $_L$-NAME, as well as Ca^{2+} channel antagonists such as amlodipine and azelnidipine, provide protection against MCAO-induced focal ischemia in rats (Dawson *et al*., 1994; Hurtado *et al*., 2003; Lukic-Panin *et al*., 2007). The neuroprotective effect that HS0608 provides against ischemic brain injury might be attributable to antioxidant activity or inhibition of Ca^{2+} influx.

In terms of neuroprotective activities of the three constituent plants of HS0608 and their active principles, *Curcuma longae* radix and curcumins have been shown to protect neurons against cerebral ischemia and Aβ-induced cognitive deficits and have anti-depressant activity (Frautschy *et al*., 2001; Shukla *et al*., 2008; Wang *et al*., 2005; Yu *et al*., 2002). Recent study has demonstrated that *Phellinus linteus* reduces infarction of ischemic rats (Suzuki *et al*., 2009). Neuroprotective effects of *Scutellariae* radix and its active components, wogonin, baicalein and baicalin, have been widely studied (Heo *et al*., 2009; Mu *et al*., 2009; Wang *et al*., 2004; Zhang *et al*., 2006). It, thus, is presumed that the preparation of HS0608 might reveal synergistic effect of these three plants in protection of ischemia-induced neuronal damage.

In conclusion, we have demonstrated that HS0608 protects neurons from MCAO/reperfusion-induced ischemic brain damage *in vivo* and from H_2O_2-induced neurotoxicity *in vitro*. These results suggest that HS0608 represents promising a agent for the treatment and prevention of neurodegeneration in stoke. Further studies should determine the specific components in three plants of HS0608 that are responsible for preventing the ischemic neuronal death.

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