NEUROPROTECTIVE EFFECT OF SCUTELLARIA BAICALENSIS FLAVONES AGAINST GLOBAL ISCHEMIC MODEL IN RATS

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ABSTRACT
Scutellaria baicalensis Georgi (SB) is the medicinal plant mainly used in traditional Chinese medicine. It has been used for the treatment of various chronic inflammatory syndromes including respiratory disease, fever and gastric ulcer in traditional Eastern medicine and its major components; baicalin, baicalein and wogonin; were reported to have various biological effects. The aim of this study was to isolate the neuroprotective flavones from the root of S. baicalensis (SB) by bioactivity-guided fractionation of S. baicalensis methanol extract (SBME). Neuroprotective effect of isolated flavones, namely was studied on global ischemic model in rat by 4-VO. SBME was fractionated with different solvent and resulting fractions were administered at a dose of 25 mg/kg to the rat and potent neuroprotective fractions were sub-fractionated. At a dose of 10 mg/kg, isolated compounds, wogonin, and baicalein inhibited the hippocampal neuronal cell death by 78.6% and 81.0% respectively. Our study suggested that SB and its isolated flavones have potential neuroprotective effect and these findings may be one of the alternative therapies for the management of stroke and other neurodegenerative diseases.

Keywords: Scutellaria baicalensis; neuroprotection; ischemia; wogonin; baicalein.

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INTRODUCTION
Brain function depends on the continuous delivery of oxygen and glucose through blood flow, and interruption of the cerebral blood supply leads to irretrievable brain damage. Ischemic damage results from a cascade of cellular and molecular events triggered by sudden lack of blood flow and subsequent reperfusion of the ischemic territory. In ischemia produced by occlusion of the middle cerebral artery, the most common type of stroke, the damage is more rapid and severe in the center of the ischemic territory (ischemic core), where flow is lowest [1]. In the ischemic core, the major mechanism of cell death is energy failure - without oxygen and glucose, neurons cannot generate the ATP needed to fuel the ionic pumps that maintain the ionic gradient across the neuronal membrane, mainly the Na$^+$- K$^+$ ATPase 123. Consequently, massive Na$^+$ and Ca$^{2+}$ cytoplasmic accumulation leads to swelling and degeneration of the organelles, loss of membrane integrity and dissolution of the cell (necrotic cell death). Cerebral ischemic stroke is a neurological disease where neuronal cell death is characterized by serial pathophysiological events, so called ischemic cascades, like energy failure, excitotoxicity, oxidative stress, inflammation and apoptosis. These all damaging factors are triggered by either decreased or blocked blood flow that leads to the human death and disability [2, 3]. Two major approaches have been developed for the management of ischemic stroke. First approach is to establish reperfusion by dissolution of the clot with thrombolytic drugs and the second is to treat with neuroprotective agents to interfere with the biochemical cascade of events leading to cell death in the penumbra area [4, 5].

In the recent years, due to the lack of effective and widely applicable pharmacological treatments for ischemic stroke, many people are generating their interests in traditional medicines, mainly of herbal origin [6]. Several natural products have been studied for their potential neuroprotective effects in past few decades [7]. Meanwhile, Scutellaria baicalensis (SB) has been shown to have potential neuroprotective effect in animal model.

SB, one of the popular medicinal plants in traditional Korean medicine, is used for the treatment of high fever, jaundice, ulcer, inflammation and cancer. The main bioactive flavonoids in SB are baicalein, baicalin (baicalein-7-glucuronide), wogonin, wogonoside (wogonin-7-glucuronide), oroxylin A and oroxylin A-7-glucuronide [8]. SB and its flavones have been studied for their various pharmacological activities, including anti-inflammatory [9], antibacterial [10], antiviral [11], antitumor [12], antioxidative [13], neuroprotective [14] and anticonvulsant [15] activities.

In previous studies, neuroprotective effect of SB and its flavonoids has been reported in vitro. However, the neuroprotective effect of the individual flavones and their relative role of neuroprotection in in vivo global ischemic model have not been elucidated yet. In the present study, we isolated two neuroprotective flavones by bioactivity-guided fractionation of SB methanol extract, identified and compared their neuroprotective effects in vivo.

MATERIALS AND METHODS
Plant Material
Radix of SB, in dried form, was purchased from Kyung dong Oriental drug store, Seoul, Korea. It was identified by Dr. Hocheol Kim, Department of Herbal Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul, Korea. Voucher specimen, HP-21001, has been deposited at the Herbarium of the College of Oriental Medicine.
Fractionation and Isolation of Compounds
Dried root of SB (1.5 kg) was extracted with 85% methanol (MeOH) by sonication. The mixture was filtered and the residue re-extracted twice. The filtrate was evaporated under vacuum to give 500 g methanol extract, which was stored at -20 °C until use. 100 g of dried *S. baicalensis* methanol extract (SBME) was suspended in water and successively partitioned with hexane, ethyl acetate (EtOAc), n-butanol (BuOH) and water (H₂O), respectively. The EtOAc fraction and BuOH fraction were further sub-fractionated and E-1 and E-2 sub-fractions of EtOAc were purified and recrystallized. HPLC analysis of each recrystallized sub-fractions were done to isolate, identify and quantify the active flavones (Scheme 1).

Scheme 1: Overall extraction and isolation process of active flavones form SB.

Animals and Drug Treatment
The animal handling procedures were performed in compliance with the animal welfare guidelines issued by the Korean National Institute of Health (KNIH) and the Korean Academy of Medical Sciences. Male Wistar rats (SLC, Japan) weighing 175-180 g were used in the experiment. They were housed under controlled conditions (22 ± 2 °C; lighting 07:00-19:00 with constant humidity). Before the experiment, food was withheld overnight but water was made freely available.

Initially, Hexane, EtOAc, BuOH and H₂O fractions were orally administrated at 25 mg/kg to identify the effective fractions. EtOAc and BuOH fractions, which were effective, were sub-fractionated and each sub-fraction were administered at a dose of 10 mg/kg to predict the most potential sub-fraction. Effective sub-fractions were further recrystallized and purified. Isolated compounds (≥ 95% pure) were administrated at 10 mg/kg and their neuroprotective effect was
compared with 100 mg/kg of SBME. Control (vehicle treated) group was administered the same amount of distilled water after the ischemia induction.

Animals were anesthetized with isoflurane (initiated with 5% and maintained with 1.5% of isoflurane) during the operation period and surgically prepared for 4-VO as previously described by Kim et al., 2001 [16].

Histology
Seven days after ischemia, the animals were anesthetized and their brains were perfusion-fixed with 4% paraformaldehyde after transcardial wash-out with heparinized 0.5% sodium nitrite saline. Fixed brains were cut into 30 µm sections on a sliding microtome (HM 440; Carl Zeiss, Heidelberg, Germany) and the sections were stained with cresyl violet. Neuronal cell density was measured by counting viable cells in a total of 6 frames (1 mm²) of left and right CA1 regions of three coronal sections for each animal. Cell counting was performed by 3 technicians blinded to the experimental conditions.

Statistical Analysis
All data were presented as mean ± S.E.M. Student's t-test was used to make statistical comparisons between different treatment groups. p< 0.05 were considered to be statistically significant.

RESULTS
Isolation of neuroprotective flavones in SB
The neuroprotective flavones against CA1 neuronal cell death were isolated by activity-guided fractionation of SBME. Most effective EtOAc and BuOH fractions were subjected to further sub-fractionation through column chromatography. The sub-fraction E-1 and E-2 from EtOAc fraction, which significantly inhibit the CA1 neuronal cell death, were purified, recrystallized, isolated and identified. Sub-fraction E-1 contained the baicalein whereas E-2 contained wogonin. BuOH fraction contained mixture of compounds and they could not be isolated due to very small quantity. HPLC analysis showed that wogonin and baicalein were the major compounds of SBME and their contents were 0.9 mg and 2.2 mg per 100 mg plant extract, respectively.

Neuroprotective effect of isolated compounds
In the histological examination, dead pyramidal neurons appear to be in shrunken morphology whereas survived neurons possess the intact morphology after ischemic induction. Inhibitions of neuronal cell death by each fraction were observed by counting the neuronal cell density in the CA1 hippocampal region seven days after the ischemia induction (Fig. 1).
Figure 1: Photographs of cresyl violet-stained hippocampal regions treated with fractions of SB. Sham-operated group (A, a), vehicle-treated group (B, b), hexane fraction (C, c), EtOAc fraction (D, d), BuOH fraction (E, e), and water fraction (F, f) treated group.

Neuronal cell density in vehicle-treated group, 38.0 ± 1.7 cells/mm², was highly inhibited as compared to the sham-operated group, 374.1 ± 34.8 cells/mm². Neuronal cells in EtOAc and BuOH fractions treated groups were observed as 265.5 ± 4.0 cells/mm² and 225.5 ± 7.0 cells/mm² respectively (Fig. 2).

![Neuronal cell density graph](image)

Figure 2. Neuronal cell density of hippocampal CA1 region after treatment with different fractions of SBMEs (25 mg/kg). ** p < 0.05, *** p < 0.001 vs the vehicle treated group.

The inhibitions of neuronal cell death by various sub-fractions of EtOAc and BuOH fractions were also observed (Data not shown) and subsequent purification and identification of most effective sub-fractions was done to isolate the neuroprotective flavones baicalein and wogonin. Neuroprotective effect of isolated flavones, against neuronal cell death, was observed by administering the flavones at a dose of 10 mg/kg. The groups treated with isolated compounds significantly inhibited the neuronal cell death as compared to the vehicle-treated group. Neuronal cell density in animals treated with wogonin and baicalein, were 220.0 ± 2.0 cells/mm², 275.1 ± 4.0 cells/mm², respectively (Fig. 3).
DISCUSSION
After ischemia, the CA1 pyramidal neurons in the hippocampus have been shown to undergo selective and delayed cell death, both in experimental animals and in humans. The most vulnerable CA1 neuronal death in ischemia is believed largely due to the apoptosis and sometimes necrosis [17, 18]. Most of the CA1 neurons in penumbra area of hippocampus underwent delayed death at 3 days after induction of ischemia [19].

In this study, the neuroprotective effect of SBME and its individual flavones against CA1 neuronal cell death was found in the order of SBME (92%), baicalein (91%) and wogonin (78.6%) suggesting that baicalein and wogonin are the main neuroprotective flavones in SB against 4-VO ischemic model. Among the component flavones, there was no significant difference in the neuroprotective effects of baicalein and wogonin and wogonoside with that of SBME. HPLC analysis of flavonoid in 100 mg of SBME was found as baicalein (2.2 mg) and wogonin (0.9 mg) respectively. This result was consistent with the previous reports [20, 21] as well. It has been reported that the cellular damage that occurs during cerebral ischemia and reperfusion is at least partly due to oxidative and inflammatory stress [22]. In neurodegenerative diseases including ischemia, reactive oxygen species have a deleterious effect on neuron survival. Therefore, antioxidants have been highlighted in neuroprotective drugs development [23, 24]. Flavonoids isolated from SB have the free radical-scavenging capacity and protective activity against injury [13]. Among the SB flavonoids, baicalein is the most powerful antioxidant. The potent neuroprotective activity of baicalein might be related to its antioxidative activity. It was reported that wogonin possessed neuroprotective activity against kainite-induced excitotoxicity and global ischemic brain damage by inhibiting the inflammatory activation of microglia [25]. Therefore, the protective mechanism of wogonin after global ischemic brain damage might be not only by antioxidative activity but also by anti-inflammatory activation. Consequently, the neuroprotective effect of SB may result from the...
combined effects of antioxidative, anti-inflammatory and other pharmacological effects of each active compound.

In summary, the major compounds in SB which show neuroprotective effect against the hippocampal CA1 neuronal damage following transient global ischemia by 4-VO-induced global ischemia in rats are baicalein and wogonin. When SB is orally administered, both of these flavones may have synergistic role for neuroprotection. SB and its individual components may have the potential in prevention of stroke, vascular dementia and various neurodegenerative disorders.

REFERENCES