

Attenuation of Brain Injury by Water Extract of Goat's-beard (*Aruncus dioicus*) and Its Ethyl Acetate Fraction in a Rat Model of Ischemia-Reperfusion

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Abstract

Ischemic stroke constitutes about 80% of all stroke incidences. It is characterized by brain cell death in a region where cerebral arteries supplying blood are occluded. Under these ischemic conditions, apoptosis is responsible for the cell death, at least in part. Goat's-beard (*Aruncus dioicus* var. *kamtschaticus*) is a perennial plant that grows naturally in the alpine regions of Korea. In the present study, we first determined whether water extract of goat's-beard (HY1646) and some of its fractions prepared by partitioning with organic solvents could improve the viability of human hepatocellular carcinoma cells (HepG2) cultured under hypoxic condition by blocking apoptotic pathways. Based on the *in vitro* findings, we subsequently investigated whether HY1646 and the ethyl acetate fraction (EA) selected from cell culture-based screening could attenuate brain injury in a rat middle cerebral artery occlusion (MCAO) model of ischemia (2 hr), followed by 22 hours of reperfusion. The cell number was sustained close to that initially plated in the presence of HY1646 even after 24 hr of cell culture under hypoxic condition (3% O₂), at which time the cell number reached almost zero in the absence of HY1646. This improvement in cell viability was attributed to the delay in apoptosis, identified by the formation of DNA ladder in gel electrophoresis. Of fractions soluble in hexane, ethyl acetate (EA) and butanol, EA was chosen for the animal experiments because EA demonstrated the best cell viability at the lowest concentration (10 µg/mL). HY1646 (200 mg/kg) and EA (10 and 20 mg/kg) significantly reduced infarct size, an index of brain injury, by 16.6, 40.0 and 61.0%, respectively, as assessed by 2,3,5-triphenyl tetrazolium chloride staining. The findings suggest that prophylactic intake of goat's beard might be beneficial for preventing ischemic stroke.

Key words: apoptosis, *Aruncus dioicus*, cerebral infarction, ethyl acetate fraction, goat's-beard, ischemia-reperfusion, water extract

INTRODUCTION

Brain cells, especially neurons, are vulnerable to ischemia generated by occlusion of cerebral arteries, which eventually results in the cell death accompanied by ischemic stroke (1-4). Restoration of blood flow by thrombolytic drugs within 3 hours of onset of symptoms is the only approved pharmacological treatment for the ischemic stroke; however, this time constraint is difficult to meet clinically (5,6). To overcome the time constraint, identification of neuroprotectants that allow brain cells to remain alive under ischemic conditions is crucial. Most neuroprotectants under development actually failed to demonstrate any benefit during clinical trials (5,7). As neuronal cells in the brain are injured by apoptosis in addition to necrosis during ischemic conditions (8), substances that can inhibit apoptosis could be good candidates as neuroprotectants (9,10). Previously, we developed a HepG2 cell-based system to screen neuroprotectants and successfully identified and characterized

candidate plant extracts, including *Cassia mimosoides* var. *nomame* (11). Another candidate identified is goat's-beard (*Aruncus dioicus* var. *kamtschaticus*), a plant that grows naturally in alpine regions of Korea (12), the leaves of which are edible and contain high antioxidant activities (13,14).

In present study, we tested whether water extract of stems and roots of goat's-beard and ethyl acetate fraction (EA) fractionated by partitioning the extract with ethyl acetate, an organic solvent, could attenuate cerebral infarction in a rat model of cerebral ischemia-reperfusion.

MATERIAL AND METHODS

Extraction

The stems and roots of goat's-beard collected around Hwachun, Gangwon-do, South Korea, were washed, cut and dried. Then, 200 g of them were extracted with 2 L boiling water in a boiling pot (Daewoong DWP-2000, Seoul, Korea) twice for 2 hr each, and the extract was

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subsequently filtered. The filtrate was freeze-dried, with a 16 g yield, which was designated the whole extract (HY1646).

Fractionation

The water extract prepared in the extraction step was partitioned with 1 L hexane three times, and the supernatant recovered was vacuum evaporated to yield 2 g of hexane (HX) fraction. The residual water layer was then partitioned with equal amount of ethyl acetate three times, and the supernatant recovered was vacuum evaporated to yield 10 g of ethyl acetate (EA) fraction. In the final step, 10 g of butanol (Bu) fraction was obtained when partitioned with butanol.

Cell culture conditions for the whole extract (HY1646)

Human hepatocellular carcinoma cells (HepG2) were cultured, as previously described (11). Briefly, the cells were plated at 2.5×10^5 cells/60 mm culture dish and grown in 4 mL of minimum Eagles's medium (MEM) at 37°C for 48 hr under normoxic condition (5% CO₂, balanced with air) in a humidified chamber (Forma Scientific, Inc., Marietta, OH, USA). The cells were cultured either under normoxic (5% CO₂, balanced with air) or hypoxic (1% O₂, 5% CO₂, balanced with N₂) conditions in a humidified chamber (Vision Scientific Co., Bucheon, Korea) for another 48 hr, following exchange of the culture medium with a fresh medium containing 100 µg/mL of HY1646 pre-dissolved in dimethyl sulfoxide (DMSO) (final DMSO concentration: 0.1%), or that containing no HY1646 (Control), respectively. At various time points during cell culture, the viable cells were counted after trypan blue staining (15).

DNA fragmentation assay for the whole extract (HY1646)

DNA fragmentation assay was performed, as previously described (11). Briefly, the cells cultured under hypoxic conditions in the presence (100 µg/mL) (HY1646) or in the absence of HY1646 (Control), as described above, and were then lysed in a lysis buffer comprising 0.5% Triton buffer. Subsequently, DNA was isolated and electrophoresed on a 1.5% agarose gel (16).

Cell culture conditions for the fractions

Human hepatocellular carcinoma cells (HepG2) were cultured, as previously described (11). Briefly, the cells were plated at 2×10^5 cells/12 well-plate culture dish and grown in 0.8 mL of culture medium at 37°C for 48 hr under normoxic condition. The cells were cultured under hypoxic (3% O₂, 5% CO₂, balanced with N₂) in a humidified chamber (Vision Scientific Co.) for another 48 hr, following exchange of the culture medium with a fresh medium containing 0 (Control), 1, 10, 100 and 1,000 µg/mL of the whole extract (HY1646), Bu, EA and HX

fractions, respectively. Forty eight hours after cell culture under hypoxic condition, viable cells were qualitatively assessed with a colorimetric method, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (17).

Animals

Eight-week-old male Sprague Dawley (SD) rats were purchased from Samtaco Inc. (Osan, Korea). Experiments were carried out according to the guidelines for the animal care and use of laboratory animal protocols approved by the Institutional Animal Care and Research Advisory Committee of Catholic University of Daegu. Animals were housed with food and water available *ad libitum* under diurnal lighting conditions and in a temperature-controlled environment until the day of the experiment. The numbers of rats sacrificed for control (vehicle-treated control group), HY1646 (the whole extract-treated group with 200 mg/kg dosage), EA 10 (the ethyl acetate fraction-treated group with 10 mg/kg dosage), and EA 20 (the ethyl acetate fraction-treated group with 20 mg/kg dosage) (n=8) were 17, 8, 8, and 8, respectively.

Focal cerebral ischemia model

The rat experiments were performed as follows and as described previously (11,18); male SD rats weighing 290 to 320 g were anesthetized with enflurane (5% for induction and 3% for the surgical procedure) in a mixture of oxygen/nitrous oxide (20%/80%) and maintained during surgical procedures. Physiological parameters were monitored and maintained in the normal range as shown previously (19). Left common carotid artery was exposed and carefully dissected free of the vagus nerve. The external and internal carotid arteries were also isolated. The external carotid artery was then tied. Ischemia was induced using an occluding intraluminal suture. An uncoated 30 mm long segment of 3-0 nylon monofilament suture (Ethilon, Ethicon, Norderstedt, Germany) with the tip rounded by flame was advanced into the internal carotid artery approximately 19~20 mm from the bifurcation in order to occlude the ostium of middle cerebral artery. Animals were subjected to 2 hr of transient middle cerebral artery occlusion (MCAO), followed by 22 hr of reperfusion. After the 2 hr ischemic period, the suture was removed and the animal was allowed to recover. During surgery, rectal temperature was maintained between $37 \pm 0.5^\circ\text{C}$ with a thermostatically controlled warming plate. In the whole extract-treated and EA fraction-treated group, the whole extract (HY1646) (200 mg/kg) or EA fraction (10 and 20 mg/kg) was intraperitoneally injected twice (20 and 1 hr) before occlusion. In the vehicle-treated control group,

experimental procedures were the same as those in the whole extract-treated group except that normal saline was injected.

Infarct size assessment

Infarct size was assessed, as previously described (11, 18). Animals were euthanized by carbon dioxide overdose and perfused with cold normal saline immediately. The brains were quickly removed and placed in a metallic brain matrix for tissue slicing. Four 3 mm thick slices were immersed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich Co., St. Louis, MO, USA) and incubated at 37°C for 20 min. To assess infarct size, TTC-stained slices were photographed with a digital camera and analyzed by Image-J analysis software (public domain software developed at National Institute of Health, available at <http://rsb.info.nih.gov/ij/>). The ischemic index was determined as the percent of the total ipsilateral hemispheric area that was injured (a lesion formed), and was used to quantify the degree of injury (19).

Statistical analysis

Data is given as means \pm SD. Comparisons between groups were performed using standard statistical methods using SPSS software. The data was analyzed with one-way ANOVA, Kruskal–Wallis one-way ANOVA on ranks, or unpaired *t*-test. Statistical significance was determined at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Effect of HY1646 on cell viability

The HepG2 cells were cultured under hypoxic condition (11,15) in the absence or presence of HY1646 (100 μ g/mL), and the concept of “Ratio” (the number of viable cells at a certain culture time, compared with that plated initially on a 60 mm culture dish, i.e. 2.5×10^5 cells) was adopted to represent the cells that survived under the hypoxic condition (Fig. 1A). In the absence of HY1646 (Control), the number of the viable cells increased slightly until 6 hr of incubation (Ratio = 1.19), which was followed by almost complete cell death at 24 hr of incubation (Ratio = 0.13). In contrast, the number of the viable cells also increased slightly until 12 hr of incubation (Ratio = 1.23), and remained almost the same as that plated initially even at 24 hr of incubation in the presence of HY1646 (HY1646) (Ratio = 0.94). The results clearly show that HY1646 attenuates cell death under the hypoxic condition. In the previous studies, we already demonstrated that timing of almost complete HepG2 cell death under the hypoxic condition coincided with complete consumption of glucose, initially present in the MEM culture medium (20). Therefore, the results

suggest further that HY1646 might prevent cell death under ischemic condition, which occurs in the event of artery occlusion, resulting in low oxygen and glucose concentrations at the affected region. In addition to cell culture under the hypoxic condition, the effect of HY1646 on the cell viability under the normoxic condition was also investigated, under which condition HY1646 (100 μ g/mL) did not affect the cell growth. The result suggests that HY1646 at the concentration of 100 μ g/mL was not detrimental to cell viability; however, efficacy was considered more important than avoiding toxicity for the first trial, so 200 mg/kg of HY1646 was chosen as a concentration to be tested in a rat model of MCAO model instead of 100 mg/kg.

Effect of HY1646 on DNA fragmentation

Since HepG2 cells have previously been reported to die by apoptosis under hypoxic condition (16,21), we also examined whether improvement of the cell survival by HY1646 under the hypoxic condition is due to inhibition of apoptosis (Fig. 1B). To address this question, DNA gel electrophoresis was performed to reveal DNA ladder patterns, as this method has previously been used to assess apoptosis (16,21). In the absence of HY1646, a DNA ladder started to appear at 24 hr of incubation and reached a peak at 30 hr of incubation, time points of which coincided with those of almost complete cell death as shown in Fig. 1B(a). On the other hand, the DNA ladder started to appear at 36 hr of incubation in the presence of HY1646 when the cell death became almost complete, the time of which is later than that in the absence of HY1646 [Fig. 1B(b)]. The result suggests that HY1646 improved the cell viability by delaying apoptosis.

Effect of the fractions on cell viability

Next, we examined which fractions have activity in improving the cell viability under the hypoxic condition, for which we used MTT assay for the quick screening as the viable cells turned into dark color in the assay (Fig. 2). We tested Bu, EA and HX fractions in addition to the whole extract (HY1646) at various concentrations (1, 10, 100 and 1,000 μ g/mL). HY1646, Bu and EA improved the cell viability in the range of 100~1,000, 100~1,000 and 10~100 μ g/mL, respectively, with the maximum effect occurring at 100 μ g/mL for all the three samples, while HX did not improve the cell viability much in the whole range of concentrations tested (1~1,000 μ g/mL). Thus, with a consideration of the lowest concentration necessary for the efficacy, EA at the concentration 10 and 20 mg/kg, equivalent to 10 and 20 μ g/mL, respectively, in cell viability experiments, was

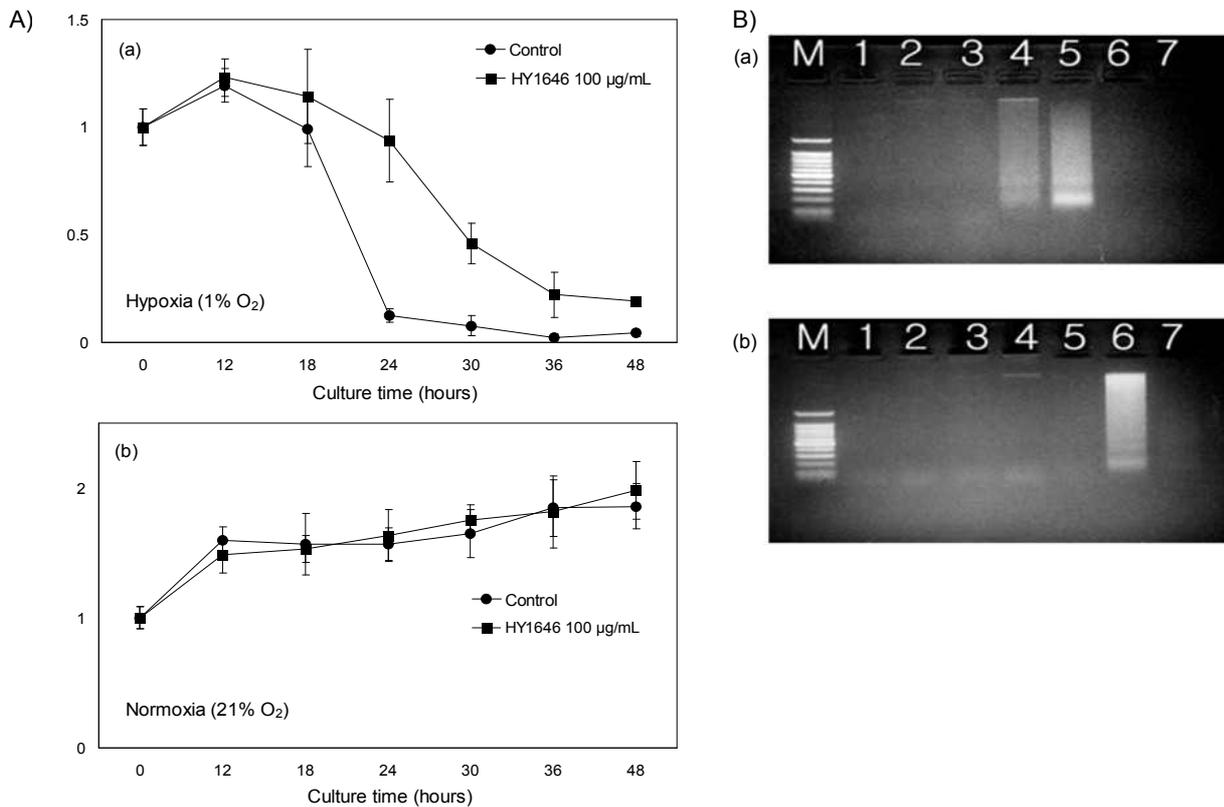


Fig. 1. Effect of the whole extract of goat's-beard (HY1646) on the cell viability and DNA fragmentation under hypoxic conditions. Human hepatocellular carcinoma cells (HepG2) were plated at 2.5×10^5 cells/60 mm culture dish and grown in 4 mL of minimum Eagles's medium (MEM) at 37°C for 48 hr under normoxic condition (5% CO₂, balanced with air) in a humidified chamber (A). The cells were cultured either under hypoxic [A(a)] or normoxic (1% O₂, 5% CO₂, balanced with N₂) [A(b)] condition in a humidified chamber for another 48 hr, following exchange of the culture medium with a fresh medium containing 100 µg/mL of the whole extract (HY1646) pre-dissolved in DMSO (final DMSO concentration: 0.1%), or that containing no HY1646 (Control), respectively. At various time points of cell culture, viable cells were stained with 0.4% trypan blue dye solution and counted using hemocytometer. Error bars represent the standard deviation of at least three samples taken from a single run. The cells cultured under hypoxic conditions in the absence (Control) [B(a)] or in the presence of (HY1646) (100 µg/mL) [B(b)], as described in (A), were collected in the lysis buffer at the indicated times. The lysate was centrifuged to get supernatant, and then DNA was isolated and electrophoresed on a 1.5% agarose gel. Lane M (100 bp DNA marker), Lane 1 (0), Lane 2 (12), Lane 3 (18), Lane 4 (24), Lane 5 (30), Lane 6 (36), Lane 7 (48) hours of culture under hypoxic condition, respectively.

chosen as the fraction and the concentrations to be tested in a rat MCAO model.

Neuroprotective effect of the whole extract and ethyl acetate fraction on infarct size

To demonstrate that cell survival *in vitro* by the whole extract (HY1646) and EA fraction under ischemic conditions correlates with cell survival in the brain under ischemic conditions, too, MCAO rat model with ischemia-reperfusion, as previously described, was adopted (18) (Fig. 3). Two times (20 hr and 1 hr before occlusion) intraperitoneal injection of HY1646 (200 mg/kg), EA (10 and 20 mg/kg) and saline (control), was followed by 2 hr occlusion and subsequent 22 hr reperfusion. To measure infarct size, brain slices with 3 mm thickness were stained with TTC, which causes the damaged region to turn white while the viable region turns red. The representative slides for HY1646 showed that HY1646

seemed to reduce infarct size, compared with that of control (Fig. 3A). To quantify the findings, the ischemic index, which was determined by calculating the percent of the total ipsilateral hemispheric area that was injured (19), was assessed for HY1646 and EA (Fig. 3B), respectively. Administration of HY1646 at 200 mg/kg, and EA, both at 10 and 20 mg/kg, significantly reduced infarct size, compared with that of control (77.5 ± 8.9 , 51.9 ± 18.3 and $36.2 \pm 23.0\%$, respectively, versus $92.8 \pm 2.4\%$; $p < 0.05$).

In the present study, we demonstrated that the water extract of goat's-beard (*Aruncus dioicus* var. *kamtschaticus*) (HY1646) and EA fraction fractionated from HY1646 attenuated brain cell injury in a rat MCAO model. We also demonstrated that HY1646 improved HepG2 cell viability by blocking apoptosis under the ischemic condition. Based on these results and previous

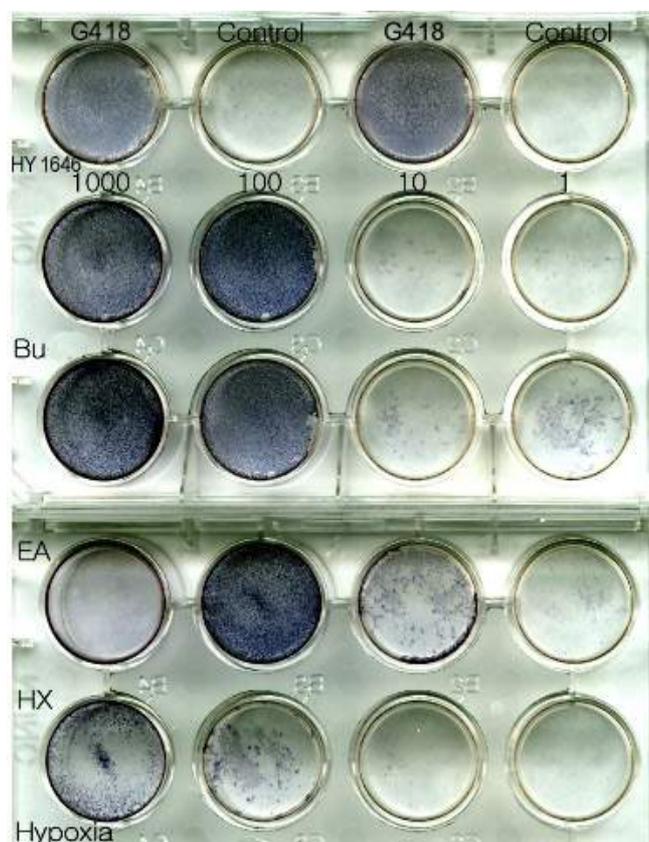


Fig. 2. Effect of the whole extract of goat's-beard (HY1646) and its fractions on the cell viability under hypoxic conditions. Human hepatocellular carcinoma cells (HepG2) were plated at 2×10^5 cells/12 well-plate culture dish and grown in the same ways as described in Fig. 1, with medium containing 0 (control), 1, 10, 100 and 1,000 $\mu\text{g}/\text{mL}$ of the whole extract (HY1646), butanol (Bu), ethyl acetate (EA) and hexane (HX) fractions, respectively. G418 (geneticin) and Control represent positive and negative controls, respectively. Forty eight hours after cell culture under hypoxic condition, viable cells were qualitatively assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

studies revealing that goat's beard has antioxidant activities (14,22), the neuroprotective effect of HY1646 and EA might come from reduction of apoptosis in the brain cells by antioxidative activities of goat's beard. Indeed, some neuronal death during ischemic stroke is caused by apoptosis (23-25), one cause of which is oxidative stress resulting from reactive oxygen species (ROS) produced primarily in mitochondria (26-28). Various dietary antioxidants have been clinically tested for prevention of cell death during ischemic stroke (29), of which citicoline and cytidine 5'-diphosphocholine, which can reduce lipid peroxidation and apoptosis following transient cerebral ischemia, seem to have a benefit, as concluded from a phase III clinical trial (1,30,31). In the same context, HY1646 that is also an antioxidant (14,22) and alleviates streptozotocin-induced diabetic symptoms in a rat

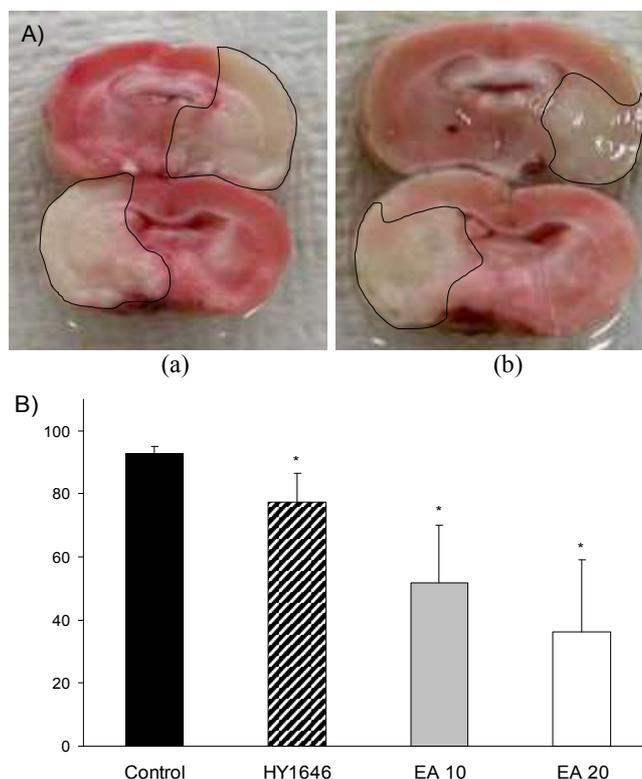


Fig. 3. Effect of the whole extract of goat's-beard (HY1646) and its fractions on infarct size in rats. After ischemia-reperfusion experiments, the brains were quickly removed and placed in a metallic brain matrix for tissue slicing. Four 3 mm thick slices were immersed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) and incubated at 37°C for 20 min. A. Representative TTC-stained slices showing viable (red), ischemic (white) brain tissue. (a) Vehicle-treated control group, (b) The whole extract (HY1646)-treated group. B. The ischemic index, which was calculated by determining the percent of the total ipsilateral hemispheric area that showed a lesion, was used to quantify infarct size. Control, vehicle-treated control group ($n=17$); HY1646, the whole extract-treated group with 200 mg/kg dosage ($n=8$); EA 10, the ethyl acetate fraction (EA)-treated group with 10 mg/kg dosage ($n=8$); EA 20, the ethyl acetate fraction (EA)-treated group with 20 mg/kg dosage ($n=8$). * $p < 0.05$ vs. vehicle-treated control group.

model by inhibiting ROS generating systems (13) might also attenuate ischemic stroke by removing ROS produced during ischemia-reperfusion, resulting in the prevention of apoptosis.

In conclusion, prophylactic intake of goat's beard, might be beneficial for preventing ischemic stroke through inhibition of brain cell apoptosis. This is particularly relevant as cultivation and packaging methods for goat's-beard have already been developed (32,33), and chemical components in it have been analyzed (34).

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NOMENCLATURE

ROS, reaction oxygen species; HepG2, human hepatocellular carcinoma cells; MCAO, middle cerebral artery occlusion; HX, hexane; EA, ethyl acetate; Bu, butanol; WA, water; TTC, 2,3,5-triphenyl tetrazolium chloride; MEM, minimum Eagles's medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, Sprague Dawley.

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