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Methanol Extract of *Cassia mimosoides* var. *nomame* and Its Ethyl Acetate Fraction Attenuate Brain Damage by Inhibition of Apoptosis in a Rat Model of Ischemia-Reperfusion

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Abstract

Ischemic stroke, a major cause of death and disability worldwide, is caused by occlusion of cerebral arteries that, coupled with or without reperfusion, results in prolonged ischemia (hypoxia and hypoglycemia) and, ultimately, brain damage. In this study, we examined whether methanol extract of the whole plant of *Cassia mimosoides* var. *nomame* Makino that grows naturally in Korea, as well as Japan and China, and some of its fractions obtained by partitioning with organic solvents could protect human hepatocellular carcinoma cells (HepG2) under hypoxic condition by inhibiting apoptosis. We also investigated if these extracts could attenuate brain damage in a rat model of 2 hr of ischemia, generated by middle cerebral artery occlusion, and 22 hr of reperfusion. The whole extract (100 μg/mL) maintained the cell number at more than half of that initially plated, even after 24 hr of cell culture under hypoxic condition (3% O_2). In the absence of the whole extract, almost all of the cells were dead by this time point. This improvement of cell viability came from a delay of apoptosis, which was confirmed by observing the timing of the formation of a DNA ladder when assessed by gel electrophoresis. Of fractions soluble in hexane, ethyl acetate (EA), butanol and water, EA extracts were selected for the animal experiments, as they improved cell viability at the lowest concentration (10 μg/mL). The whole extract (200 mg/kg) and EA extract (10 and 20 mg/kg) significantly reduced infarct size, a measure of brain damage, by 34.7, 33.8 and 45.2.0%, respectively, when assessed by 2,3,5-triphenyl tetrazolium chloride staining. The results suggest that intake of *Cassia mimosoides* var. *nomame* Makino might be beneficial for preventing ischemic stroke through inhibition of brain cell apoptosis.

Key words: *Cassia mimosoides* var. *nomame* Makino, methanol extract, ethyl acetate fraction, ischemia-reperfusion, cerebral infarction, apoptosis

INTRODUCTION

Stroke, a major cause of death and disability worldwide, is mainly composed of ischemic and hemorrhagic stroke, of which ischemic stroke is the major form, representing about 80% of the patients (1-3). Ischemic stroke is caused by cerebral infarction, defined as brain cell death due to prolonged ischemia (hypoxia and hypoglycemia) resulting from occlusion of cerebral arteries, coupled with or without reperfusion (4). Reperfusion of occluded arteries by thrombolytic drugs within 3 hours of the onset of symptoms is the only approved pharmacological treatment for the ischemic stroke, the time constraint of which is hard to be clinically met (5,6). Thus, development of neuroprotectants that can complement the reperfusion therapy is crucial (7), although most of clinical trials testing neuroprotectants have failed to demonstrate any benefit (5,8). Under ischemic conditions, brain cells, especially neuronal cells, are damaged by excitotoxicity in minutes, and inflammation and apoptosis in hours (9). Thus, neuroprotectants that can inhibit apoptosis might be beneficial for attenuating cerebral infarction in ischemic stroke (10,11). The extent of DNA fragmentation, as observed by gel electrophoresis, is an indicator for assessing inhibition of apoptosis by a neuroprotectant (12). *Cassia mimosoides* var. *nomame* Makino (CM) grows naturally in Korea, as well as Japan and China, the whole plant of which has been used as a food, especially a tea (13,14). In addition, an extract of CM, its fractions and components isolated from the extract, have various physiological activities (14-16). For example, methanol extract of CM, a fraction soluble in ethyl acetate (EA), and luteolin purified from EA all have high antioxidant and elastase inhibition activities (15). In other studies, both a fraction obtained by ethanol extraction and subsequent column chromatography (14),

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and flavan dimers purified from CM (16) have lipase inhibition activities and concomitant anti-obesity effects.

In our previous studies, we serendipitously found that several classes of antibiotics, including aminoglycosides, G418 (geneticin) in particular, tetracyclines and quinolones, reduced HepG2 cell death in *in vitro* cell culture under ischemic conditions by inhibiting apoptosis (17, 18). Based on these findings, we also showed that G418 attenuated myocardial infarction (17) and cerebral infarction (Lee, unpublished results). These results demonstrated that the HepG2 cell line is a suitable screening system to identify candidates of neuroprotectants in combination with G418 as a positive control, by which methanol extract of the whole plant of CM was selected a candidate for further characterization. In this study, we determined whether the methanol extract of the whole CM plant, and the EA obtained by partitioning the extract with ethyl acetate, an organic solvent, were effective in reducing cerebral infarction in a rat model of cerebral ischemia-reperfusion. Clinical trials for some neuroprotectants have failed due to many undesirable effects outweighing the benefits, despite having some efficacy in clinical trials (8). We therefore seek to determine whether the whole plant of CM proves effective as a neuroprotectant. Because CM is edible, it might be particularly suitable for preventing ischemic stroke.

MATERIALS AND METHODS

Extraction

The whole plants of CM collected in Uiseong area, a county of Gyeongsangbuk-do, South Korea, were washed and dried. Then 500 g of them was put into an ultrasonicator (8210R-DTH, Branson Ultrasonic Corp., Danbury, CT, USA) and extracted in 5 L methanol twice for 24 hr each at room temperature, and the extract was filtered with filter papers (Whatman No. 3, Whatman Inc., Piscataway, NJ, USA). The filtrate was vacuum-dried with a rotary evaporator (NP-1, Tokyo Rikakikai Co. (EYELA), Tokyo, Japan) with a 62 g yield, and is referred to as the whole extract.

Fractionation

Sixteen grams of the whole extract obtained in the extraction step were dispersed in 200 mL water and then partitioned with 200 mL hexane twice, and the supernatant recovered was vacuum evaporated to get 2.9 g of hexane fraction (HX). The residual water layer was then partitioned twice with an equal amount of ethyl acetate, and the supernatant recovered was vacuum evaporated to get 3.5 g of EA. In the next step, 2.6 g of butanol fraction (BU) was obtained when partitioned with butanol. The final residual water layer obtained after butanol extraction was also vacuum evaporated to get 6.8 g of water fraction (WA).

Cell culture conditions for the whole extract

Human hepatocellular carcinoma cells (HepG2) were plated at 2×10^5 cells/12 well-plate culture dish and grown in 0.8 mL of minimum Eagles's medium (MEM) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Gibco BRL) at 37°C for 48 hr under normoxic condition (5% CO_2, balanced with air) in a humidified chamber (Forma Scientific, Inc., Marietta, OH, USA). The cells were cultured either under normoxic (5% CO_2, balanced with air) or hypoxic (3% O_2, 5% CO_2, balanced with N_2) condition 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 or 1,000 μg/mL of the whole extract pre-dissolved in 50% ethanol, or that containing no whole extract (Control), respectively. At 1 and 2 days of cell culture, viable cells were stained with 0.4% trypan blue dye solution and counted using a hemocytometer (19).

DNA fragmentation assay for the whole extract

The HepG2 cells were plated at 1×10^6 cells/60 mm culture dish and grown in 4 mL of culture medium, as described above. Forty-eight hours after cell culture under normoxic condition, the cells were then cultured under hypoxic conditions in the presence (300 μg/mL) or in the absence of the whole extract (Control). The viable cells were counted with trypan blue dye method at various time points during the cell culture, and also lysed in the lysis buffer [0.5% Triton buffer (Sigma-Aldrich Co., St. Louis, MO, USA), 5 mM Tris buffer (pH 7.4) (Boehringer Ingelheim, Ingelheim, Germany) and 20 mM EDTA (Sigma-Aldrich Co.)]. The lysate was centrifuged and the supernatant was treated with protease K (Gibco BRL). DNA was isolated with phenol-chloroform-isoamyl alcohol extraction and precipitated with ethanol. DNA was electrophoresed on a 1.5% agarose gel after RNA was removed by RNase treatment (100 ng/μL) (20).

Cell culture conditions for the fractions

The HepG2 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 conditions (3% O_2, 5% CO_2, balanced with N_2) in a humidified chamber (Vision Scientific Co.) for another 48 hr, following exchange of the culture medium with a fresh medium containing 0 (Negative, negative control), 10, 100 and 1,000

μg/mL of the whole extract (WH), BU, EA, HX and WA fractions, respectively. For a positive control (Positive), 10 μg/mL G418 (geneticin), an antibiotic, was added. 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-di-phenyltetrazolium bromide (MTT) assay, as described previously (21).

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.

Focal cerebral ischemia model

The rat experiments were performed as previously described (22). Briefly, male Sprague-Dawley 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 (23). The 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 2 hr of ischemic period, the suture was removed and the animal was allowed to recover. During surgery, rectal temperature was maintained between 37 ± 0.5^{o}C with a thermostatically controlled warming plate. The whole extract (200 mg/kg) or the EA (5, 10 and 20 mg/kg) was peritoneally injected twice (20 and 1 hr) before occlusion.

Infarct size assessment

Infarct size was assessed, as previously described (22). 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.) and incubated at 37^{o}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 NIH, available at http://rsb.info.nih.gov/ij/). Ischemic index, lesion area determined by the percent of the total ipsilateral hemispheric area, was used to quantify the degree of injury.

Statistical analysis

Data is given as mean ± 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 the whole extract on cell viability

We have successfully applied HepG2 cells cultured under hypoxic condition to screen chemicals, leading to identification of antibiotics that are effective in attenuating stroke and myocardial damage (17). Similarly, the whole extract was also identified to increase HepG2 cell survival under hypoxic condition, with the application of the same screening system. To characterize further, the HepG2 cells were cultured under hypoxic condition (19) in the absence or presence of the whole extract (100 or 1,000 μg/mL), and the concept of "Ratio" (the number of viable cells at a certain culture time, compared with that plated initially in a well of 12 well-plate, i.e. 2×10^5 cells) was adopted to represent the cells that survived under the hypoxic condition (Fig. 1A). In the absence of the whole extract (Control), the cells became almost completely dead at 24 hr (1 day) of incubation. In contrast, more than half of the cells were still alive even at 24 hr of incubation in the presence of both 100 and 1,000 μg/mL of the whole extract (HY2207 100 and HY2207 1000). However, almost half of the cells were still alive at 48 hr (2 days) of incubation for 1,000 μg/mL of the whole extract, whereas almost all the cells became dead for 100 μg/mL of the whole extract. Therefore, administration of higher concentration of the whole extract is desirable, unless toxicity becomes a limiting factor. Nevertheless, the results clearly show that the whole extract attenuates cell death under the hypoxic condition. In a previous study, we have already shown that timing of almost complete HepG2 cell death under the hypoxic

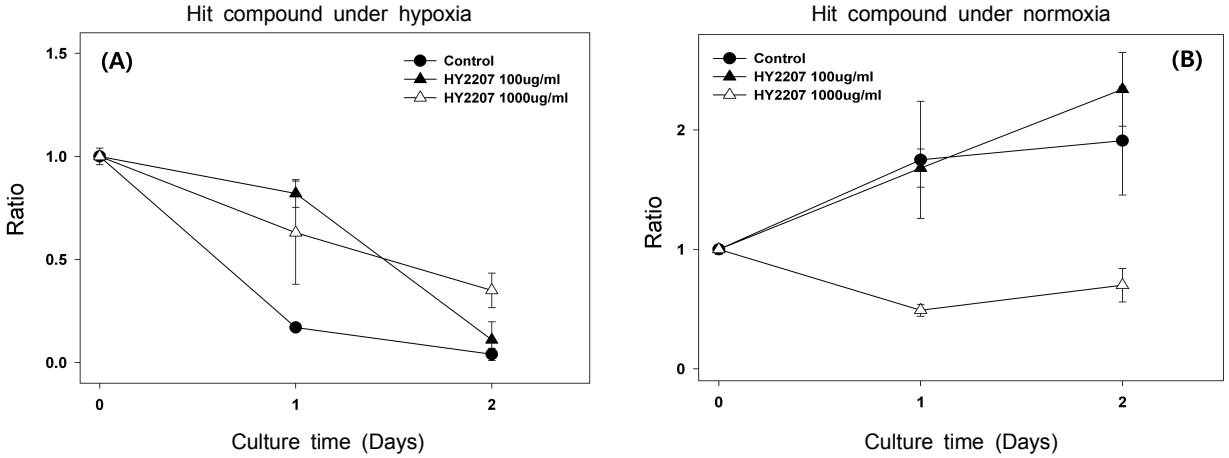

Fig. 1. Effect of the whole extract of *Cassia mimosoides* var. *nomame* Makino (HY2207) on the cell viability. Human hepatocellular carcinoma cells (HepG2) were plated at 2×10^5 cells/12 well-plate culture dish and grown in 0.8 mL of minimum Eagles's medium (MEM) at 37°C for 48 hr under normoxic condition (5% CO_2, balanced with air) in a humidified chamber. The cells were cultured either under hypoxic (3% O_2, 5% CO_2, balanced with N_2) (A) or normoxic (B) condition in a humidified chamber for another 48 hr, following exchange of the culture medium with a fresh medium containing 100 or 1,000 μg/mL of the whole extract (HY2207 100 and HY2207 1000, respectively) pre-dissolved in 50% ethanol, or that containing no whole extract (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.

condition coincided with complete depletion of glucose, initially present in the MEM culture medium (18). Therefore, the results suggest further that the whole extract might prevent brain cell death under ischemic condition, which is the condition leading to low oxygen and glucose concentrations at the affected region in the brain following cerebral artery occlusion. In addition to cell culture under the hypoxic condition, the effect of the whole extract on the cell viability under the normoxic condition was also examined. In normoxic conditions, 100 μg/mL of the whole extract did not have much of an effect on cell proliferation while 1,000 μg/mL of the whole extract killed most of the cells, even at 24 hr of culture. This result suggests that the whole extract at the concentration of 100 μg/mL is not deleterious to cell viability. Thus, a value larger than 100 μg/mL, but smaller than 1,000 μg/mL, should be selected for maximal efficacy and minimal toxicity. Consequently, the concentrations of 300 μg/mL and 200 mg/kg, equivalent to 200 μg/ml in the cell culture, were selected for the subsequent DNA fragmentation assay and a rat MCAO model, respectively.

Effect of the whole extract on DNA fragmentation

Since HepG2 cells have previously been reported to die by apoptosis under hypoxic condition (20,24), we also determined whether improvement of the cell survival by the whole extract under the hypoxic condition arose from inhibition of apoptosis (Fig. 2). To address this question, DNA gel electrophoresis was performed to reveal DNA ladder patterns, as this method has previously been used to assess apoptosis (20,24). Consistent with the results shown above, the HepG2 cells again almost completely died out at 30 hr of incubation in the absence of the whole extract (Fig. 2A). In contrast, more than half of the cells remained alive even at 30 hr of incubation in the presence of 300 μg/mL of the whole extract (HY2207 300). In the absence of the whole extract, DNA ladder started to appear at 24 hr of incubation, the time point of which coincided with that of significant cell death, as shown in Fig. 2B(a). On the other hand, the DNA ladder started to appear at 30 hr and was maintained up to 36 hr of incubation in the presence of 300 μg/mL of the whole extract, although a significant number of the cells still remained alive for the same period, as shown in Fig. 2B(b). The results suggest that the whole extract improved the cell viability by delaying apoptosis.

Effect of the fractions on cell viability

Next, we examined which fractions have higher activity in improving the cell viability under the hypoxic condition. For this experiment, we used the MTT assay for a quick screening because the viable cells turn blue (Fig. 3). We tested BU, EA, HX and WA fractions, in addition to the whole extract (WH), at various concentrations (10, 100 and 1,000 μg/mL). EA and WH improved the cell viability in the range of 10～1,000 and 100～1,000 μg/ mL, respectively, while HX improved the cell viability only at 1,000 μg/mL. The maximum intensity of blue color observed at 1,000 μg/mL of EA and HX is stronger than that for 10 μg/mL of geneticin,

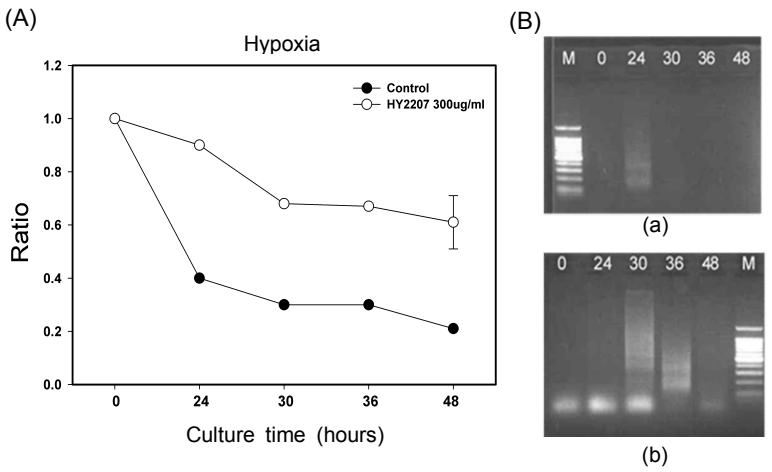

Fig. 2. Effect of the whole extract of *Cassia mimosoides* var. *nomame* Makino (HY2207) on the cell viability and DNA fragmentation under hypoxic conditions. The HepG2 cells were plated at 1×10^6 cells/60 mm culture dish and grown in 4 mL of culture medium, as described in Fig. 1. Forty eight hours after the cell culture under normoxic condition, the cells were cultured under hypoxic conditions in the presence (300 μg/mL) (HY2207 300) or in the absence of the whole extract (Control). The viable cells at various time points of cell culture were counted with trypan blue dye method (A), and also lysed in the lysis buffer. The lysate was centrifuged to get supernatant, and then DNA was isolated and electrophoresed on a 1.5% agarose gel {(B): (a) Control, (b) the whole extract (300 μg/mL)}. Lane M (100bp DNA marker), Lane 1 (0), Lane 2 (20), Lane 3 (30), Lane 4 (36), Lane 5 (48) hours of culture under hypoxic condition.

which was used as a positive control (18), indicating that the ability of EA and HX to improve the cell viability under hypoxic condition is quite strong. However, both BU and WA did not significantly improve the cell viability in the whole range of concentrations tested (1～1,000 μg/mL). Thus, with a consideration of the lowest concentration necessary for the efficacy, EA at concentrations of 5, 10 and 20 mg/kg were chosen as a sample and the concentrations to be tested in a rat MCAO model.

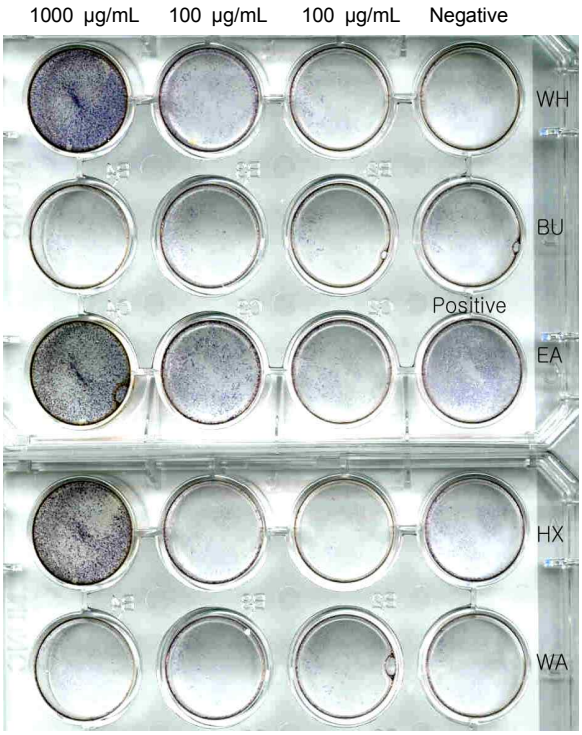

Fig. 3. Effect of the whole extract of *Cassia mimosoides* var. *nomame* Makino (HY2207) and its fractions on the cell viability under hypoxic conditions. Human hepatocellular carcinoma cells (HepG2) were plated at 2×10^5 cells/12 well-plate culture dish and grown in the same ways as described in Fig. 1, with medium containing 0 (Negative), 10, 100 and 1,000 μg/mL of the whole extract (HY2207), butanol (BU), ethyl acetate (EA), hexane (HX) and water (WA) fractions, respectively. For a positive control (Positive), 10 μg/mL G418 (geneticin), an antibiotic, was added. Forty eight hours after cell culture under hypoxic condition, viable cells were qualitatively assessed with MTT assay.

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

Neuroprotectvie effects of the whole extract and the EA were examined using MCAO rat model with ischemia-reperfusion (Fig. 4). Two peritoneal injections (20 hr and 1 hr before occlusion) of the whole extract (200 mg/kg), EA (5, 10 and 20 mg/kg) and saline (0 mg/kg, control), were followed by 2 hr occlusion and subsequent 22 hr reperfusion. To measure infarct size, brain slices with 3 mm thickness were stained with TTC, and the region where brain cells were damaged became white (Fig. 4A(a)). The representative slides for the whole extract showed that the whole extract appeared to reduce infarct size, the area with white color, compared with that of control, as shown in Fig. 4A(b). To quantify the findings, ischemic index, defined as infarct size determined by the percent of the total ipsilateral hemispheric area, was assessed for the whole extract and EA (Fig. 4B), respectively. Administration of the whole extract at 200 mg/kg, and EA at 10 or 20 mg/kg, but not at 5 mg/kg, significantly reduced infarct size, compared with that of control (60.6 ± 27.0, 61.4 ± 6.9, 50.8 ± 7.1 and 70.8 ± 14.7, respectively, versus 92.8 ± 2.4%; $p<0.05$, $p<0.05$, $p<0.01$ and $p>0.05$, respectively). The results suggest the active components are concentrated at least 20 times more in the EA than in the whole extract. Future work is necessary to identify these active components.

In summary, we have shown that the methanol extract

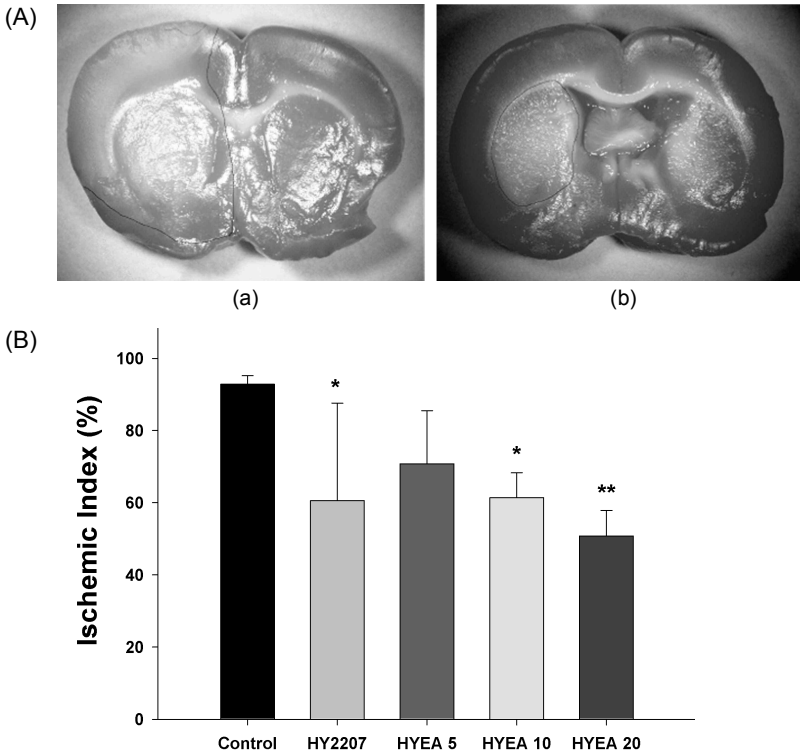

Fig. 4. Effect of the whole extract of *Cassia mimosoides* var. *nomame* Makino (HY2207) and its fractions on infarct size in rats. After ischemia-reperfusion experiments, the brains were quickly removed and placed in a metallic brain matrix for tissue slicing. Four 3 mm thick slices were immersed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) and incubated at 37°C for 20 min. A. Representative TTC-stained slices showing viable (dark, outside black line) and ischemic (white, inside) brain tissue, the boundary of which is marked by black line. (a), Vehicle-treated control group; (b), The whole extract-treated group. B. Ischemic index, lesion area determined by the percent of the total ipsilateral hemispheric area, was used to quantify infarct size. Control, vehicle-treated control group (n=17); HY2207, the whole extract-treated group with 200 mg/kg dosage (n=8); HYEA 5, HYEA 10 and EA 20－the ethyl acetate fraction (EA)-treated groups with 5, 10 and 20 mg/kg dosage (n=8), respectively. $^{*}p<0.05$ and $^{**}p<0.01$, respectively vs. vehicle-treated control group.

of CM and the ethyl acetate fraction (EA) fractionated from the extract, improved HepG2 cell viability by attenuating apoptosis under the ischemic condition, and consequently attenuated brain cell damage in a rat MCAO model. Thus, intake of CM, edible as a whole plant, might be beneficial for preventing ischemic stroke through inhibition of brain cell apoptosis. This is particularly relevant as large scale cultivation conditions for *Cassia mimosoides* var. *nomame* Makino has already been developed (25,26), and chemical constituents in it have been analyzed, at least in part (13,15,16,27).

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NOMENCLATURE

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; CM, *Cassia mimosoides* var. *nomame* Makino; 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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