Neuroprotective Effect of *Metaplexis japonica* against in *vitro* Ischemia Model

[1]Nirmala Jamarkattel-Pandit, [2] Hocheol Kim

[1]School of Health and Allied Sciences, Faculty of Scienece and Technology, Pokhara University, Nepal

[2]Department of Herbal Pharmacology, College of Oriental Medicine, KyungHee University, Seoul, Korea

ABSTRACT

Metaplexis japonica (Apocynaceae) is a perennial herb, extensively used in traditional medicinal system for various diseases. The purpose of the study was to evaluate the protective effect of M. japonica against in vitro ischemia. In the present study, 70% ethanol extract of M. japonica was fractionated with different polarity solvents. For in vitro ischemia, oxygen-glucose deprivation followed by reoxygenation (OGD-R) in cells was used to investigate the effects of M. japonica and its fractions. For oxidative stress model, Hydrogen peroxide (H_2O_2) induced cell death was studied in HT22 cell line. M. japonica and its fractions significantly reduced the HT22 cell damage, which was induced by 4 hrs of OGD followed by 24 hrs of reoxygenation and 24 hrs of H_2O_2, respectively. The effectiveness of ethyl acetate fraction was higher than other fractions/crude extract. Our results suggest that M. japonica could be a neuroprotective agent for the treatment of stroke.

Key words: *Metaplexis japonica, Stroke, Oxygen-glucose deprivation, Neuroprotection*

Corresponding address : Nirmala Jamarkattel-Pandit, PhD, School of Health and Allied Sciences, Pokhara University, Nepal.
E-mail: nirmalajk@hotmail.com

INTRODUCTION

Stroke is a leading cause of death and major disability, which can be due to the ischemic caused by thrombosis or embolism or due to a hemorrhage.[1,2] The major disabilities that results from a stroke are cerebral paralysis, cognitive deficits, speech problems, emotional difficulties, daily living problems, and pain.[3,4] Stroke triggers many processes, including release of excitatory amino acids, reactive oxygen species (ROS) and inflammatory cytokines and then caused brain edema and damage.[5,6] Mainly, two approaches have been developed in ischemic stroke. First approach is to reperfuse by dissolution of the clot with thrombolytic drugs. But these drugs have limitation because these drugs are most effective if administered immediately within 3 hrs of onset. The second approach is to protect the neuronal cell death by interfere with the biochemical cascade of events with neuroprotective agents.[7,8] For the this approach, calcium antagonists, NMDA antagonists, glutamate release inhibitors, free radical scavengers, anti-inflammatory agents and leukocyte adhesion inhibitors are being studied.[7,8] Many neuroprotective agents, despite proving effective in animal models of stroke, they have been failed in clinical trials because of the toxicity, ineffectiveness etc. Recently many researchers have been focused on natural products for the development of neuroprotective agents because the herbal drugs are regarded to have a relatively higher therapeutic window, fewer side-effects and are economic than synthetic drugs.[7] The importance of plant derived material in present day therapy cannot be under estimated. The studies on pharmacological activity of natural compounds/plant extracts present a unique challenge to strike new sources of medicine. Since such useful compounds/plant extracts such as catechins/green tea polyphenols, ginsenoside/*Panax ginseng*, EGb 761/ginkgo biloba, curcumin/*Curcuma longa*, baicalein and wogonin/*Scutallaria baicalensis*, resveratrol/ grape, melatonin etc and many others exhibit a broad range of pharmacological activities including neuroprotection.[7,9]

Therefore, various medicinal plants which are used in Eastern medicine (Ayurveda, Korean and Chinese Traditional medicine), were collected from China, Korea and Nepal and were screened out against oxygen-glucose deprivation followed by reoxygenation (OGD-R), a well accepted *in vitro* ischemia model in rat hippocampus cell line (HT22 cells).[10] Among the plant material studied the extract of *Metaplexis japonica* showed the most effective protective activity against ischemia model. Further, *M. japonica* was extracted with 70% EtOH, then fractionated by different polarity solvent and treated against *in vitro* ischemia model. For oxidative stress model, Hydrogen peroxide (H_2O_2) induced cell death was studied in HT22 cell line. Total phenol, flavonoid and antioxidative activity of *M. japonica* extract and its fractions was also measured to correlate the findings.

MATERIALS AND METHODS

Chemicals and plant materials: Dulbecco's Modified Eagle's Medium (DMEM), lipopolysaccharide (LPS, *Escherichia coli* 0127:138), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), griess reagent, hydrogen peroxide (H_2O_2), were purchased from Sigma-Aldrich (USA); Fetal Bovine Serum (FBS) was from Hyclone (USA), Penicillin and Streptomycin were obtained from Gibco BRL (USA). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck. The plants listed in Table 1 were collected from China, Korea and Nepal. The samples were identified

by Professor Hoyoung Choi and voucher specimens were deposited at the Department of Herbal Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul, Korea.

Extraction of plant materials and screening against in vitro model of ischemia: Hundred gram of each herb were extracted with 70% (v/v) ethanol (EtOH) by using reflux apparatus for 3 hrs at 80°C. The extracts were evaporated on a rotary evaporator under reduced pressure to remove organic solvent and then lyophilized to determine the yield. The name of samples, parts used, and yield of extracts are shown in Table 1. For *in vitro* ischemia model, samples were prepared in DMSO. Final DMSO concentration didn't exceeded 0.1% (v/v) in all treatment. Hippocampus cells (HT22) and microglia cells (BV2) were obtained from Medical department of Kyung Hee University and maintained at 37°C in a humidified atmosphere containing 5% CO_2. HT22 cells were seeded at a density 1.5×10^3 cells/well and BV2 cells were seeded at a density 5×10^4 cells/well then cultured at 37°C in DMEM supplement with 10% heat-inactivated FBS, penicillin (1×10^5 unit/L), streptomycin (100 mg/L) in a 5% CO_2 incubator. For oxygen- glucose deprivation followed by re-oxygenation (OGD-R), HT22 cells were washed with phosphate buffered saline (pH 7.2) then with glucose free DMEM. The cultures were placed in fresh glucose free DMEM and kept in hypoxic chamber (Forma Scientific, Ohio, USA) containing 95% N_2 and 5% CO_2 at 37°C for 4 hrs. At the end of the exposure period, glucose solution was added, and the cells were incubate in the 5% CO_2 incubator at 37°C for an additional 24 hrs. Samples (10 µg/mL) were treated 30 min before and during 4 hrs OGD exposure. Control plate was treated with the equal amounts of serum free glucose DMEM and kept in the 5% CO_2 incubator. Nitric oxide (NO) inhibitory activity of each extract was performed according to our previous experiment.[11] Cells were incubated for 24 hrs with or without 1 µg/mL of LPS, in the absence or presence of extract. The NO concentration was measured using the supernatant from the BV2 cells mixing with equal amount of Griess reagent. The optical density (OD) was measured on plate reader (SPECTRA max, USA) at 540 nm. Standard $NaNO_2$ was used for the calibration curve. Curcumin, a strong antioxidant and anti-inflammatory compound, was used as a reference for NO inhibitory assay.

Extraction and fractionation of M. japonica: The aerial part of *M. japonica* (1.6 kg) was extracted with 16 L, 70% (v/v) ethanol (EtOH) by using reflux apparatus for 3 hrs at 80°C. The extract was filtered and evaporated with a rotary evaporator (Eyela, Japan) under reduced pressure to remove organic solvent until 1 L. For the crude extract (MJC), about 100 ml was lyophilized until dryness. Remaining aqueous solution was successively partitioned with hexane (MJHf), ethyl acetate (MJEf), and butanol (MJBf), respectively.

Determination of total phenol, flavonoid and antioxidative activity of M. japonica extract and its fractions: Total phenolic content, flavonoid and antioxidative activity of *M. japonica* extract and its fractions were determined according to previous experiments.[12] Gallic acid (10 – 500 mg/L) was used for calculation and the results were expressed as mg of gallic acid equivalents (GCE) per g of extract using rutin as a standard. The flavonoid content was calculated from a rutin standard curve and the results were expressed as mg of rutin equivalents per g of extract.

Hydrogen peroxide (H_2O_2) induced cell death: Experiments were carried out 24 hrs after HT22 cells were seeded. For H_2O_2 treatment, the cells were washed with serum free glucose solution. Dilution of H_2O_2 was made from a 30% stock solution into serum free glucose solution just prior to each experiment and 30 µM solution added to the cells. The culture plates were incubated for additional 24 hrs.

Table 1: Name of plants, using parts and extracts yield

S.N.	Sample Name	Collected From	Parts used	Extract yield (%)
1	*Achyranthes bidentata*	Korea	root	41.0
2	*Acorus calamus*	Nepal	fruit	19.2
3	*Alisma canaliculatum*	Korea	root+ stem	21.4
4	*Amomum suvulatum*	Nepal	fruit	13.0
5	*Anethum graveolens*	Korea	seed	3.2
6	*Bambusa textilis*	China	root	0.3
7	*Cibotium barometz*	Korea	root	12.4
8	*Clematis chinensis*	China	root	19.9
9	*Dimocarpus longan*	China	fruit	56.6
10	*Duchesnea indica*	Korea	leaf	14.8
11	*Emblica officinalis*	Nepal	fruit	50.1
12	*Erycibe obtusifolia*	Korea	stem	8.8
13	*Foenicuium vulgare*	Nepal	fruit	10.4
14	*Gleditsia sinensis*	Korea	fruit	21.4
15	*Glycyrrhyza globra*	Nepal	stem	28.2
16	*Leonurus sibiricus*	Korea	seed	5.9
17	*Liquidambar formosana*	Korea	fruit	3.7
18	*Metaplexis japonica*	Korea	leaf	11.9
19	*Nardostachys jatamunsi*	Nepal	root	11.7
20	*Nelumbo nucifera*	Korea	seed	16.9
21	*Patrinia scabiosaefolia*	China	root	17.0
22	*Picrorhiza rhizoma*	Nepal	root	38.5
23	*Piper longum*	Nepal	fruit	13.6
24	*Pseudostellaria heterophylla*	Korea	root	21.3
25	*Rauvolfia serpentina*	Nepal	root	18.0
26	*Sinapis alba*	China	seed	19.9
27	*Spatholobu suberectus*	Korea	stem	66.9
28	*Syzygium aromaticum*	Nepal	fruit	25.5
29	*Terminalia belerica*	Nepal	fruit	60.0
30	*Terminalia chebula*	Nepal	fruit	67.0
31	*Withania somnifera*	Nepal	root	18.0

Stock solution of each test sample was prepared in DMSO, and the solution was diluted in DMEM solution (with glucose/ without glucose) so the final concentration of DMSO did not exceeded 0.1% (v/v). Each test samples with different concentrations were treated 30 min before and during OGD exposure. OGD control cells were treated with equal amount of glucose free DMEM solution containing 0.1% DMSO. Normal cells were treated with the equal amounts of serum free glucose solution containing 0.1% DMSO and kept in the normal incubator. For H_2O_2, samples of different concentrations were treated during H_2O_2 exposure. Normal cells (without H_2O_2) were incubated under the same conditions.

Cell survival was evaluated by the ability to reduce MTT to formazan. MTT was dissolved in DMEM and added to the culture at final concentration of 0.5 mg/ml. After additional 2 hrs of incubation at 37°C, the media was carefully removed and 100 µl DMSO added to each well. The optical density (OD) was measured on plate reader (SPECTRA max Plus^{384}, USA) at 570 nm. Results were expressed as % of the normal cells (untreated cells). All results were presented as mean ± SEM. Significant differences between experimental groups were determined by student't' test. $P < 0.05$ was considered to indicate statistically significance.

RESULTS

Screening of Plant material against in vitro model of ischemia

The rate of cell protection and IC_{50} value for NO scavenging activity of each plant extracts are presented in Table 2. In this study, 31 medicinal plants were collected from China, Korea and Nepal and were examined their neuroprotective effects against *in vitro* ischemia model on neuronal cells. Baicalein was used as a reference for OGD, which showed 30% protection on HT22 cells against OGD-R induced cell death. Out of the 31 plants extract tested, 7 plants extract showed the potent protective activity (20 – 30%), 9 plant showed moderate (10 – 20%) and 15 plants were inactive or showed low activity (< 10%) on the HT22 cell at 10 µg/ mL concentration. Likewise, out of the 31 plants extract tested, 10 plants extract showed the potent protective activity (more than 20%), 7 plant showed moderate (10 – 20%) and 14 plants were inactive or showed low activity (<10%) on the BV2 cell at 10 µg/mL concentration. Among these plant extract, 12 plant extract strongly inhibited the NO (IC_{50} < 10 µg/mL) produced by LPS. The rest of samples showed moderate NO inhibition (IC_{50} > 10 µg/mL) effect against LPS (Table 2). *Protective effect of MJC and its fractions against OGD-R induced cell death in HT22 cells.*

As determined by MTT reduction, the MJC and its fractions (0.1 – 10 µg/ml) had no cytotoxic effects on HT22 cells (data not shown). Four h of OGD followed by 24 hrs of reoxygenation induced 49.9 ± 1.2% of cell death as compared to the normal group. Viable cells increased from 50.1 ± 0.9% in control group to 60.75 ± 2.1% ($p < 0.01$) in MJC (10 µg /ml), 71.9 ± 3.2% ($p < 0.001$), 62.38 ± 2.5% ($p < 0.01$) in MJEf (1 and 10 µg/ml) and 69.6 ± 7.1% ($p < 0.05$), 68.2 ± 3.5% ($p < 0.001$) in MJBf (1 and 10 µg/ml) treated cells respectively, however; MJHf didn't show any significant effect against OGD-R induced cell death (Fig. 1). Protective effect of MJC and its fractions against H_2O_2 induced cell death in HT22 cells.

As determined by MTT reduction, H_2O_2 has dose dependent cytotoxicity effects on HT22 cells. The cells treated with H_2O_2 (30 µM, 24 h) induced 30% of cell death as compared to the normal group. Viable cells increased from 73.2 ± 3.9% in control group to 87.5 ± 4.1% ($p < 0.05$), 94.5 ± 5.2% ($p < 0.01$) and 89.9 ± 4.2% ($p < 0.05$) in MJEf (0.1, 1 and 10 µg /ml), 89.5 ± 6.1% ($p < 0.05$), 92.82 ± 1.4% ($p < 0.001$) in MJBf (1 and 10 µg/ml) treated cells respectively, however; MJC and MJHf didn't show any significant effect against H_2O_2 induced cell death (Fig. 2).

Table 2: Cell protection against OGD-R and LPS, and NO inhibition assay

No.	Cell Protection % [(a)]	Cell Protection % [(b)]	NO inhibition (IC_{50}, µg/mL)
Baicalein	30.0	--	--
1	5.4	7.4	60.5
2	(-)	22.0	11.2
3	17.1	19.8	45.6
4	5.8	22.8	8.4
5	(-)	5.2	34.5
6	(-)	(-)	37.5
7	18.0	7.1	0.1
8	22.4	13.5	60.3
9	9.0	10.3	57.8
10	(-)	(-)	(-)
11	17.0	33.1	8.9
12	(-)	(-)	48.5
13	10.8	24.3	28.4
14	(-)	(-)	(-)
15	11.9	1.4	24.6
16	7.2	1.3	48.0
17	(-)	10.8	6.6
18	34.1	1.9	46.7
19	14.7	44.0	4.3
20	9.1	(-)	80.7
21	26.9	(-)	(-)
22	29.1	20.0	5.6
23	11.8	24.7	6.5
24	(-)	10.9	53.1
25	26.0	24.0	6.2
26	6.9	10.9	54.3
27	8.0	(-)	49.4
28	17.5	24.8	4.8
29	10.6	23.0	<0.1
30	34.9	19.0	<0.1
31	23.9	29.9	4.3

[a] samples at 10 µg/mL protected HT22 cells against OGD induced cell death.

[b] samples at 10 µg/mL protected BV2 cells against LPS induced cell death.

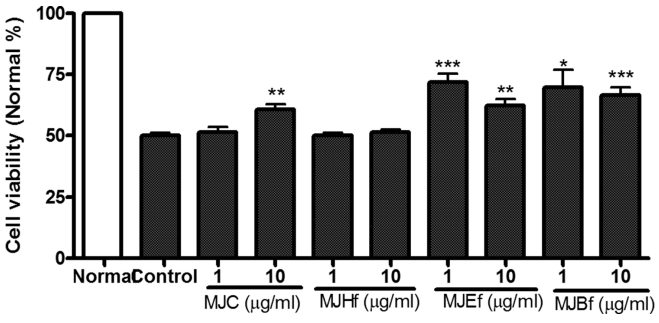

Fig. 1: Protective effect of MJC and its fractions on OGD-induced neuronal injury in HT22 cells.

The HT22 cells were exposed to OGD for 4 hrs followed by 24 hrs of reoxygenation. The cells were treated with different concentrations of samples 30 min before and during OGD exposure. The normal group was set as 100%. The value is the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. control group.

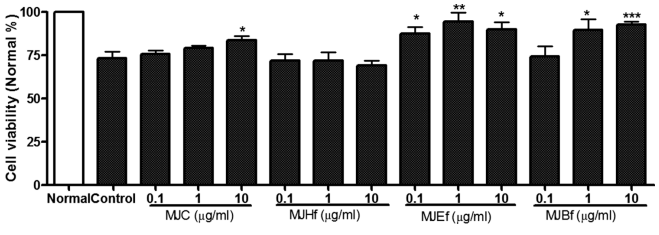

Fig. 2: Protective effect of MJC and its fractions on oxygen-glucose deprivation-induced neuronal injury in HT22 cells.

The HT22 cells were exposed to OGD for 4 h followed by 24 hrs of reoxygenation. The cells were treated with different concentrations (0.1 and 1 g/ml) of samples 30 min before and during OGD exposure. The normal group was set as 100%. The value is express as mean ± SEM. $^{**}p < 0.01$ *vs.* control group.

Table 3: Antioxidative activities, and total phenol and total flavonoid contents in *M. japonica* and its fractions

	[a] DPPH radical scavenging	[b] Total phenol	[c] Total flavonoid
Vit C	5.49 ± 3.45		
EDTA	---		
MJC	> 100	6.78 ± 1.23	53.07 ± 6.71
MJHf	> 100	0.51 ± 0.01	nd
MJEf	49.01 ± 5.67	15.03 ± 2.80	98.45 ± 8.50
MJBf	43.23 ± 2.12	10.45 ± 1.31	57.43 ± 1.19

[a](IC_{50} values in µg/ml), [b] mg GAE/g extract, [c] mg RE/g extract

DISCUSSION

Over the past few decades, many researchers have attempted to develop anti-neurotoxin agents that are capable of preventing the release of glutamate, activation of microglia, oxidative stress, Ca^{2+} influx, apoptosis, and cell cycle arrest.[7,8,13-15] Although many reagents, such as glutamate receptor antagonists, Ca^{2+} channel blockers, anti-inflammatory agents, and NOS inhibitors, have shown neuroprotective effects, their serious side effects have limited their clinical application. Therefore, researchers searched for novel plant extracts/compounds with better neuroprotective effects and fewer side effects. The greater potency of the novel compounds and their effectiveness against ischemia and various neurodegenerative diseases has generated a great deal of excitement among neuroscientists, medicinal chemists, and clinicians.

Thus, to search the effective neuroprotective plants, different plants extract were screened against *in vitro* ischemia model. From the results of screening test, *Metaplexis japonica* was selected for further study. *M. japonica* was further extracted and fractionated by different organic solvents and the neuroprotective effect of MJC and its fractions *in vitro* ischemia model induced by OGD-R was evaluated. For oxidative stress model, H_2O_2 induced cell death was observed in HT22 cells. MJC, MJEf and MJBf significantly reduced the HT22 cell damage, which was induced by 4 hrs of OGD followed by 24 hrs of reoxygenation and 24 hrs of H_2O_2, respectively. Exposure of HT22 cells to 4 h of OGD followed by 24 hrs of reoxygenation decreased in cell viability as compared to normal cells; whereas treatment of MJC and its fractions at 1 and 10 µg/ml concentration increased neuronal cell viability as compared to control group. MJC reduced cell damage by 20% at 10 µg/ml whereas the activity of MJEf and MJBf was found to be higher than that of extract; however MJHf could not show significant effect against OGD-R. The efficacy of MJEf at 1 µg/ml was 44% but at higher concentration it showed lower activity. Likewise, the efficacy of MJBf was 39% and 37% at 1 and 10 µg/ml concentrations, respectively.

The OGD assay is a powerful tool for screening of substances for treatment or prevention of ischemia related diseases. As OGD mimics tissue ischemia, it resulted in the production of ROS, release from glutamate toxicity, and inflammatory cytokines.[1,16] The compounds/plants extract having antioxidative, anti-inflammatory or glutamate antagonist property may protect the neuronal cells OGD-R induced cell death. In order to know the effects of MJC and its fractions against oxidative stress, H_2O_2 was treated to HT22 cells. H_2O_2 is believed to be the major precursor for highly reactive free radicals, and has been reported to induce cell apoptosis in the central nervous system. In the current study, we found that H_2O_2 has dose-dependently and time-dependently toxic to cells (data not shown) whereas treatment of MJC, MJEf and MJBf decreased cell death in a dose-dependent manner (Fig 2). The maximum effect of MJC, MJEf and MJBf

against H_2O_2-induced neurotoxicity was observed at the concentration of 10 µg/ml where as MJHf could not show significant effects. The activity was found in the order of MJEf > MJBf > MJC which is similar with *in vitro* ischemia (OGD) which indicate that protective activity of samples against OGD-R and H_2O_2 induced cell death may related with their antioxidative activity. Further, we examined the effects of MJC and fractions against DPPH free radical scavenging activity, and we found that activity of MJEf and MJBf were higher that MJC, where as MJHf shows very weak activity. According to *in vitro* results (OGD-R, H_2O_2) it is hypothesized that MJC, MJEf and MJBf could prevent ischemia-induced brain damage in *in vivo* system.

CONCLUSION

MJC and its fractions showed a neuroprotective effect *in vitro* ischemia. The effectiveness of MJEf was higher than other fractions/crude extract which indicate that the neuroprotective agent of MJC may partitioned to MJE fraction. Further study is necessary to isolate the neuroprotective compounds from MJC and their mechanisms of action.

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