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Neuroprotective Effects of Cambodian Plant Extracts on Glutamate-induced Cytotoxicity in HT22 Cells

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Abstract – Oxidative stress potentially induces neurotoxicity which is believed to underlie several major age-related diseases of the central nervous system. This study sought to identify the cytoprotective effects of sixty-nine Cambodian plants against glutamate-induced cell death. Cultured HT22 cells were applied as an *in vitro* model, and neurotoxicity was induced in these neuronal cells by exposure to a determined concentration of glutamate. Sixty-nine plant sources, as Cambodia's indigenous species, were purchased from O'reusey Market, Phnom Penh, and extracted with ethanol. These extracts were screened for cytoprotective effects against glutamate-triggered neurotoxicity in HT22 cells at concentrations of 100 and 300 µg/ml. Of these, eight ethanol extracts, bark of *Anacardium occidentale*, bark and sapwood of *Bauhinia pulla*, flowers of *Borassus flabellifer*, stems and leaves of *Coix lacryma-jobi*, bark and sapwood of *Diospyros nitida*, sapwood of *Dipterocarpus obtusifolius*, stems of *Oryza rufipogon*, and fruits of *Phyllanthus emblica*, showed significant cytoprotective effects against glutamate-induced cell damage and degeneration in HT22 cells.
Keywords – HT22 cells, Glutamate, Cambodian plants, Neurotoxicity, Cytoprotective effect

Introduction

Reactive oxygen species (ROS) play a prominent role in cellular functions such as electron transport chains in mitochondria, endocytosis, arachidonic acid (ARA) metabolism, ovulation, and syngamy. Under pathological progression, however, generation of ROS causes harm to the central nervous system (Singh *et al*., 2004), particularly hippocampus (Satoh *et al*., 1998). Dominant cellular ROS over antioxidants underlie the oxidative stress (Ray *et al*., 2012) serving as aetiology of major neurodegenerative diseases like Alzheimer's disease (Liu *et al*., 2012), Parkinson's disease (Thomas and Beal, 2007), Huntington's disease (Singh *et al*., 2004) and amyotrophic lateral sclerosis (Shi *et al*., 2010).

Glutamate, an excitatory neurotransmitter in the central nervous system, is thought to be used by nearly one-third of the synapses (Coyle and Puttfarcken, 1993). High concentration of glutamate results in vulnerability of neuronal cells (Oka *et al*., 1993) implicated in various neurologic diseases (Lau and Tymianski, 2010). Glutamate-induced neurotoxicity is commonly elucidated into two main mechanisms. First one is the excitotoxic mechanism (Martin, 2010) mediated by ionotropic glutamate receptors (Beal, 1992). The mechanism of neuroexcitotoxicity results from calcium fluxes by dint of primarily the N-methyl-D-asparate (NMDA) receptor channel, conducing to an increase of the level of oxygen free radicals, thereby causing neuronal injury (Choi *et al*., 1989). Second mechanism is known extensively as cystine/glutamate antiporter, system x_c^-, functioning as importing cystine into the intracellular *milieu* in exchange for exporting glutamate to the extracellular space (Conrad and Sato, 2011). High concentration of extracellular glutamate potentially interrupts the exchange of cystine/glutamate in the system x_c^-, leading to neural glutathione depletion, oxidative stress and, ultimately, neuron death (Lewerenz *et al*., 2011).

Currently, immortalized mouse hippocampal cell lines (HT22 cells) have been broadly applicable to being an *in vitro* model to observe the mechanism of glutamate-

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stimulated neuronal cell death (Murphy *et al*., 1989; Tan *et al*., 1998; Kulawiak and Szewczyk, 2012). HT22 cells biologically possess no ionotropic glutamate receptor (Maher and Davis, 1996); therefore, the excitotoxic pathway is excluded from a cause of glutamate-triggered neuron injury and degeneration. Several studies have been confirmed that, in cultured HT22 cells, high concentration of glutamate efficaciously inhibits cystine uptake which in turn lowers intracellular glutathione levels, thus inducing oxidative stress and, ultimately, cell death (Murphy *et al*., 1989; Davis and Maher, 1994; Tan *et al*., 1998) through both necrosis and apoptosis (Fukui *et al*., 2009).

Cambodia, a country of tropical climate (World Bank, 2011), consists of two main seasons, dry and rainy seasons (MoE, 2006), conferring a hot-humid temperature plus plenty of fertile soils and silt encouraging life of animals and vegetation. As such, Cambodian medicinal plants have been discovered as many as 515 species of 134 families (Kham, 2004), allowing Cambodians to practically use medicinal plants, coupling with traditional supernatural treatment of diseases, as one of the therapeutic means for ages (Muecke, 1983). Besides, herbal preparations, poultices, and elixirs can be conveniently purchased inexpensively in local markets without prescription (Richman *et al*., 2010). Thus, identification of natural products with a sufficient cytoprotective effect against glutamate-triggered cell death would be beneficial to providing a potential therapeutic application. This paper deals with cytoprotective effects of Cambodian plant extracts against glutamate-induced neurotoxicity in HT22 cells.

Experimental

Materials – Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution and antibiotic-antimycotic solution (containing 10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of amphotericin B/ml) were purchased from Gibco (Invitrogen, Gaithersburg, MD, USA). All other relevant chemicals were obtained from Sigma chemical Co. (St Louis, MO, USA). 96-well tissue culture plates and other tissue culture dishes were obtained from BD Falcon™, USA.

Preparation of Cambodian plants – Sixty-nine Cambodian plants were imported directly from O'reusey Market, Phnom Penh, and extracted with 70% ethanol. The whole process of plant extraction was supported by Pharmacognosy Laboratory, College of Pharmacy, Wonkwang University, Republic of Korea.

Cell culture – Mouse hippocampal HT22 cells were maintained in DMEM supplemented with 10% heat inactivated FBS and 1% antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B) and incubated at 37 °C under humidified atmosphere, 5% CO_2, and 95% air. Cells were subcultured once a day or every 2 days. To determine the cytoprotective effect of Cambodian plant extracts on glutamate-triggered neurotoxicity, cells were seeded in 96-well plates at a density of 10,000 cells per well. The experiment was performed with four groups: the control, glutamate-treated, Trolox pre-treated and sample pre-treated groups. The cells of the control group received neither glutamate nor sample treatments, those of the glutamate-treated group (negative control) were incubated with glutamate at a final concentration of 20 mM for 12 hours, those of the Trolox pre-treated group (positive control) received Trolox treatment at a final concentration of 50 μM, and those of the sample pre-treated group received the sample treatment at final concentrations of 100 and 300 μg/ml. Both Trolox and sample pre-treatments were incubated for 2 hours prior to exposure to glutamate. The samples were dissolved initially in dimethyl sulfoxide (DMSO) as stock solutions and, subsequently, diluted in the culture medium. The final DMSO concentration in each experimental and control well was kept constant at 0.1%, and this final concentration showed no relevant effects of DMSO on cellular growth and survival in the assay. The whole process of treatment lasted for 24 hours.

MTT assay – Identification of cell viability was performed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT (2.5 mg/ml) of 50 μl was added to each well at a final concentration of 0.5 mg/ml, and the mixture was further incubated for 3-4 hours at 37 °C, and in turn the liquid in the wells was removed. Thereafter, DMSO (150 μl) was added to each well, and the absorbance was finally read with a UV Max microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 540 nm. The relative optical density of formazan in the control group (containing cells treated with neither glutamate nor samples) was taken as 100% viability.

Statistical analysis – The data were expressed as mean ± SD of at least three independent experiments. Statistics were performed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results and Discussion

As mentioned earlier, glutamate is potentially able to

Table 1. Protection of ethanol extracts derived from Cambodian plants on glutamate-induced neurotoxicity in HT22 cells

Plant samples	Parts	Protection (%)	
		100 μg/ml	300 μg/ml
Abutilon indicum (L.) Sweet	stem	6.4	12.4
Aegle marmelos (L.) Correa	fruit	41.8	24.1
Amomum krervanh Pierre ex Gagnep.	fruit	-23.5	-18.6
Ampelocissus martinii Planch.	sapwood	-8.3	30.7
Anacardium occidentale L.	bark	14.4	78.8
Anthocephalus chinensis (Lam.) Rich. ex Walp.	stem	-4.9	-40.6
Bauhinia pulla W. G. Craib	bark & sapwood	32.6	85.8
Blumea balsamifera (L.) DC.	stem	-23.7	-73.4
Bombax insigne Wall.	bark	15.7	9.9
Borassus flabellifer L.	root	-4.1	-30.8
Borassus flabellifer L.	flower	37.2	80.5
Brucea javanica (L.) Merr.	stem & leaf	-130.4	-153.5
Cananga latifolia Finet & Gagnep.	bark & sapwood	0.9	-50.9
Capparis sepiaria L.	stem	-9.0	-31.5
Capparis zeylanica L.	bark & sapwood	7.3	-8.3
Cardiospermum halicacabum L.	stem & leaf	-21.9	-37.3
Careya arborea Roxb.	bark	-33.6	-63.6
Carica papaya L.	root	4.7	-23.1
Cassia alata L.	stem	-29.7	-80.6
Cayratia trifolia (L.) Domin	stem	-17.5	26.8
Chrysopogon aciculatus (Retz.) Trin.	stem & leaf	26.5	-31.1
Cissus modeccoides Planch.	stem & leaf	-24.2	-86.4
Clausena excavate Burm. f.	stem	-14.6	-37.8
Coix lacryma-jobi L.	stem & leaf	63.2	70.5
Crateva adansonii DC.	bark	-26.5	-89.9
Cynodon dactylon (L.) Pers.	whole plant	4.2	-118.2
Cyperus rotundus L.	root	5.8	-28.5
Dalbergia hancei Benth.	stem	-13.1	11.1
Dasymaschalon lomentaceum Finet & Gagnep.	stem	-49.1	-16.9
Dillenia hookeri Pierre	stem	-21.8	-48.1
Diospyros ehretioides Wall. ex G. Don	bark & sapwood	6.0	-16.7
Diospyros nitida Merr.	bark & sapwood	59.8	72.3
Diospyros rhodocalyx Kurz	fruit	-11.2	-27.4
Dipterocarpus obtusifolius Teijsm. ex Miq.	sapwood	60.2	85.3
Dracaena angustifolia Roxb.	bark & sapwood	-31.7	-88.8
Dunbaria bella Prain	stem	-48.3	-53.5
Erythrina variegata L.	bark	10.8	-62.4
Euonymus cochinchinensis Pierre	bark & sapwood	-36.5	-53.6
Euphorbia hirta L.	stem & leaf	-74.2	-149.9
Feroniella lucida Swingle	bark	4.4	-141.8
Fibraurea tinctoria Lour.	stem	-7.3	-28.3
Ficus benjamina L.	bark & sapwood	19.1	8.7
Ficus pumila L.	stem	-13.6	-108.7
Ficus racemosa L.	bark & sapwood	33.8	30.2
Gmelina asiatica L.	stem	-36.6	-120.6

Table 1. continued

Plant samples	Parts	Protection (%)	
		100 μg/ml	300 μg/ml
Imperata cylindrica (L.) Raeusch.	root	0.2	-16.1
Leea rubra Blume ex Spreng.	stem	1.1	17.6
Mangifera duperreana Pierre	bark	7.2	10.8
Millingtonia hortensis L. f.	bark & sapwood	-11.9	-39.3
Morinda citrifolia L.	fruit & seed	-1.7	3.8
Morus alba L.	stem	-20.0	-41.6
Nauclea orientalis (L.) L.	bark	35.0	-44.0
Oroxylum indicum (L.) Kurz	bark & sapwood	-30.8	-71.9
Oryza rufipogon Griff.	stem	50.3	70.2
Pandanus humilis Lour.	base	8.2	-45.3
Passiflora foetida L.	stem	11.5	-2.2
Peliosanthes teta Andrews	leaf	-9.9	-29.9
Phyllanthus emblica L.	fruit	42.3	68.2
Physalis angulata L.	stem & leaf	-37.5	-103.6
Piper retrofractum Vahl	fruit	-36.	-123.0
Polyalthia evecta (Pierre) Finet & Gagnep	stem	-79.6	-159.9
Pouzolzia zeylanica (L.) Benn. & R. Br.	stem	-57.2	-104.0
Quisqualis indica L.	whole plant	-40.1	-79.2
Shorea siamensis Miq.	flower	-55.2	20.5
Streptocaulon juventas (Lour.) Merr.	stem	-61.6	-128.5
Strychnos nux-vomica L.	seed	1.5	-14.1
Tinospora crispa (L.) Hook. f. & Thomson	stem	10.4	-26.2
Walsura villosa Wall.	bark	13.3	19.8
Willughbeia cochinchinensis (Pierre) K. Schum.	stem	-22.2	-84.3

induce cell death in HT22 cells (Fukui *et al.,* 2009) underlying such neurodegenerative diseases as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Singh *et al*., 2004). Therefore, it seems like an urgent need to observe a proper medicinal plant so that glutamate-triggered neurotoxicity in HT22 cells can be inhibited. In response to this foremost need, the present study aimed at investigating Cambodian plants in order that cytoprotective effects on glutamate-triggered neurotoxicity in HT22 cells would be identified. Based on the result, all the species of the 69 ethanol extracts at concentrations of 100 and 300 μg/ml, obtained during the course of the study, was shown in Table 1. Amongst 69 EtOH extracts, eight extracts, bark of *Anacardium occidentale*, bark and sapwood of *Bauhinia pulla*, flowers of *Borassus flabellifer*, stems and leaves of *Coix lacryma-jobi*, bark and sapwood of *Diospyros nitida*, sapwood of *Dipterocarpus obtusifolius*, stems of *Oryza rufipogon*, and fruits of *Phyllanthus emblica*, exerted significant cytoprotective effects against glutamate-triggered neurotoxicity in HT22 cells (Fig. 1). To identify neuroprotective effects of the extracts in HT22 cells, Trolox has been widely selected as a positive control so that the cytoprotectiveness can be identified (Satoh *et al*., 2004); therefore, in this research, we used Trolox as a positive control. Based on Fig. 1, Trolox revealed cytoprotective effects at a concentration of 50 μM (73.2%). Imbalance between intracellular oxygen-derived species and antioxidants potentially conduces to oxidative stress (Singh *et al*., 2004; Boelsterli, 2007), underlying major neurological disorders including Alzheimer's disease, Huntington's disease, Parkinson's disease, motor neuron diseases, and spinocerebellar ataxias (Desjardins and Ledoux, 1998). A number of studies have suggested that high concentration of glutamate could block the cystine/glutamate exchange system, thereby inhibiting cystine uptake leading to a decrease of glutathione levels, ultimately oxidative stress and cell death (Murphy *et al*., 1989; Conrad and Sato, 2011). Therefore, based on the discovered neuroprotective effects of eight ethanol extracts of this study, further

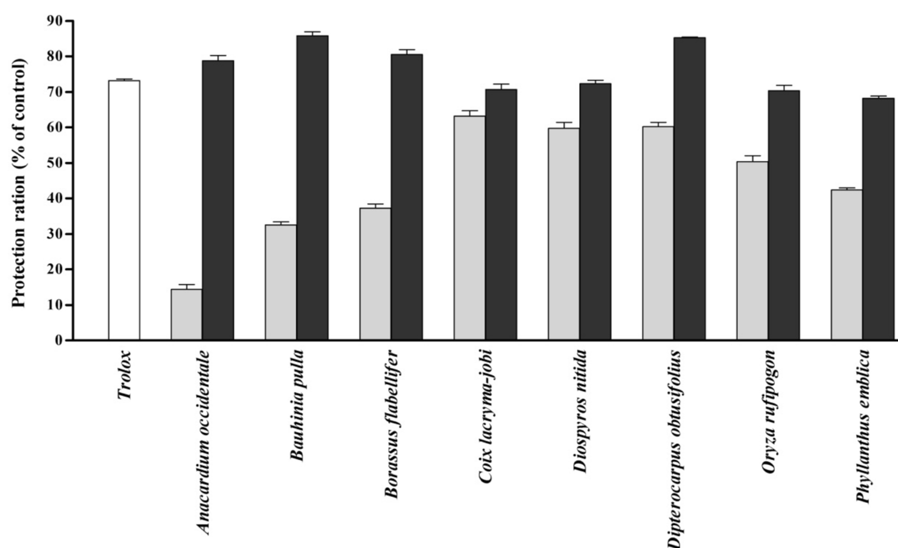

Fig. 1. Neuroprotective effects of eight ethanol extracts against glutamate-induced cytotoxicity in HT22 cells. Cytotoxicity was accessed after incubating for 2 hours with 2 mM of L-glutamine in DMEM. Results were expressed as mean ± SD of three experiments (white bar: Trolox 50 μM, grey bar: 100 μg/ml, black bar: 300 μg/ml).

research with respect to compound isolation for detailing such cytoprotective mechanisms of those extracts should be a necessity in term of pharmacology. Among eight ethanol extracts, *Bauhinia pulla*, *Diospyros nitida*, *Dipterocarpus obtusifolius*, and *Oryza rufipogon* is slightly known of biological mechanisms; however, *Anacardium occidentale* (Doss and Thangavel, 2011), *Borassus flabellifer* (Kommu *et al*., 2011), *Coix lacryma-jobi* (Kuo *et al*., 2002; Huang *et al*., 2009a, b; Chung *et al*., 2011), and *Phyllanthus emblica* (Chatterjee *et al*., 2011a, b), in recent studies, have been found to play a pivotal role in antioxidant activities, but not to be involved in glutamate-induced oxidative stress.

Conclusion

Subsequent to intensive work of extracting as many as sixty-nine Cambodian plants, the obtained 69 ethanol extracts was screened for neuroprotective effects of glutamate-induced cell degradation, and amongst those extracts, ultimately, eight extracts, bark of *A. occidentale*, bark and sapwood of *B. pulla*, flowers of *B. flabellifer*, stems and leaves of *C. lacryma-jobi*, bark and sapwood of *D. nitida*, sapwood of *D. obtusifolius*, stems of *O. rufipogon*, and fruits of *P. emblica*, indicated significant cytoprotective effects against glutamate-triggered cell death in HT22 cells. Nevertheless, some in-depth mechanisms or pathways of glutamate-induced neuronal damage and degeneration pertaining to such eight extracts are needed for ongoing research.

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