Neuroprotective and Anti-inflammatory Effects of Three Fruits of Triphala*, Emblica officinalis*, *Terminalia chebula* and *T. belerica*

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Abstract

As the life expectancy of the human population continues to increase, the possibility of developing neurodegenerative disease has increased considerably. Two of the major and important factors contributing to degenerative brain diseases are oxidative stress and inflammation. Fruits of *Emblica officinalis* (EO), *Terminalia belerica* (TB) and *T. chebula* (TC) are compositions of traditional preparation known as Triphala which is commonly prescribed by many traditional healthcare practitioners as an important health tonic for detoxification, rejuvenation, anti-inflammation and anti-ulcer. In order to elucidate their health related benefits, the neuroprotective and anti-inflammatory effects of methanol extract of the fruits were investigated. In addition, underlying mechanisms of the neuroprotective effect, antioxidative activities, were revealed. The neuroprotective effect was evaluated by using hydrogen peroxide (H_2O_2)-induced neuroblastoma NG108-15 cell death model. The anti-inflammatory effect was investigated using determination of prostaglandin E_2 level in cyclooxygenase 1 (COX-1) and 2 (COX-2) Null Cells. We found that all of the extracts effectively protected the cells from H_2O_2. For antioxidative properties, although all of the extracts did not scavenge H_2O_2 *in vitro* model, they could scavenge hydroxyl radical and showed ion chelating activities. For the anti-inflammatory effect, the extracts of EO and TB showed anti-COX-2 activity. The results from the present study not only support the traditional uses of EO, TB and TC as well as Triphala but also provide benefits in treatment and/or prevention of neurodegenerative diseases or other disorders which oxidative stress and/or inflammation is implicated.

Keywords : Antioxidant,Anti-inflammation,Triphala,*Emblica officinalis*,Neuroprotection,*Terminalia chebula*, *Terminalia belerica*

Introduction

Neurodegenerative disorders have been defined as conditions where there is selective loss of neurons within specific region of the brain. Examples of neurodegenerative disease are Alzheimer's,

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Parkinson's disease, epilepsy, and stroke. Two of the major and important factors contributing to degenerative brain diseases are oxidative stress and inflammation. In neurodegenerative diseases, activated microglia affect neuronal injury and death through production of glutamate, proinflammatory factors, reactive oxygen species (ROS) among others and by mobilization of adaptive immune responses and cell chemotaxis leading to transendothelial migration of immunocytes across the blood-brain barrier and perpetuation of neural damage. At the cellular level, the poly unsaturated fatty acids liberated from the membrane phospholipids are the substrates for the enzymes cyclooxygenases (COX) and the resulting metabolites, the prostaglandins, are involved in various inflammatory disorders (Martel-Pelletier et al., 2003). Drugs targeting the inhibition of these enzymes and oxidative stress, therefore, become a major attraction in controlling the diseases.

It is generally agreed that plant is major source of natural bioactive compounds. In nowadays, a variety of plant products which have potent antioxidative and/or anti-inflammatory properties have been proposed to use for prevent and treatment of neurodegenerative disorders (Frank and Gupta, 2005; Zhao et al., 2005).

Ma-kham-pom *(Emblica officinalis)*, Sa-mor-Thai *(Terminalia chebula*) and Sa-mor-Phe-phek *(T. belerica*) as well as Triphala commonly prescribed by many traditional healthcare practitioners in many countries including Thailand (Paoin, 2008) as an important health tonic for detoxification, rejuvenation and balance especially in the summer season. In addition, they are therapeutic agents for treatment of a variety of conditions such as headache, dyspepsia, constipation, liver conditions, fatigue, infections and assimilation. Recent publications reveal cytoprotective abilities of EO, TC and TB that is fruit extracts of EO exhibited cytoprotective activity against chromium (VI) (Khandelwal et al., 2002; Sai Ram et al., 2002; Sai Ram et al., 2003), indomethacin (Al-Rehaily et al., 2002) and H_2O_2 (Krishnaveni and Mirunalini, 2010; Nampoothiri et al., 2010). Fruit extracts of TC protected human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) cells from UVB- (Gandhi and Nair, 2005) and tertiary butyl hydroperoxide-induced cytotoxicity (Na et al., 2004). Fruit extract of TB showed cytoprotective effect against H_2O_2 (Nampoothiri et al., 2010). Unfortunately, neuroprotective activity of EO, TC and TB is non-existent. According to aforementioned cytoprotective, it is possible that fruit extracts of EO, TC and TB are able to protect neuronal cell from oxidative stress. To test this hypothesis, their protective effects against oxidative stress induced by H_2O_2 in neuronal cell were evaluated.

Some literatures reveal anti-inflammatory activities of fruits extracts of EO and Triphala. Fruit

extracts EO showed anti-inflammatory action in rat (Dang et al., 2010; Muthuraman et al., 2010). Chebulagic acid, a bioactive substance extracted from TC, showed potent COX inhibition activity in *in vitro* purified COX enzyme experiment (Reddy et al., 2009). Triphala exerted a strong anti-inflammatory effect against gouty arthritis on experimental gouty arthritis in mice (Sabina and Rasool, 2008). However, to the best of our knowledge, anti-inflammatory effect on COX activity of EO, TC and TB in cell-based assay has not yet been reported. Therefore, effect on COX-1 and COX-2 activity of EO, TC and TB methanol extracts in COX-1 and COX-2 Null Cell models were examined in this study in order to provide benefits in prevention or treatment of disorders which inflammation is implicated.

Materials and methods

Chemicals

Trolox®, tetrazolium bromide (MTT) and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Dulbecco's modified Eagle Medium, and fetal bovine serums were obtained from Gibco BRL (New York, USA.). All other chemicals used in the experiments were of analytical grade.

Plant material and extraction

The dried fruits of EO, TC and TB which were collected from Nakhon Sawan province (Thailand) in May 2010 were authenticated in the Bangkok Herbarium, Department of Agriculture, Bangkok, Thailand.

Air-dried and powdered fruits were extracted with methanol. The supernatant was collected and evaporated to dryness under reduced pressure in a rotary evaporator. The yields of the methanol extracts of EO, TC and TB were 10±1.1, 14±0.8 and 15±1.8 % respectively.

Determination of total phenolic and tannic acid contents

Total phenolic and tannic contents were determined as reported previously (Kawpoomhae et al., 2010).

Antioxidative activity

DPPH, H_2O_2 and hydroxyl radical scavenging assay as well as metal ion chelating assay were performed as described previously (Kawpoomhae et al., 2010).

Neuroprotective effect

Neuroblastoma NG108-15 cells were donated by Associate Professor Tohda Michihisa, Institute of Natural Medicine, University of Toyama. They were cultured as described by Sukma (Sukma et al., 2003). Cells were grown in DMEM containing 100 μM hypoxanthine, 1 μM aminopterin and 16 μM

thymidine and 10 % fetal bovine serum. Cell cultures were maintained in a humidified incubator with 5% CO_2 – 95% air at 37 ^{o}C

NG108-15 cells (2x10^{3}cells/well) were seeded into 96-well plates and incubated at 37 ^{o}C for 48 h. After plating, they were treated with various concentrations of the samples or Trolox®, a reference substance, for 2 h, and a stock solution of H_2O_2 solution was added to yield a final concentration of 200 μM. Neuronal survival was quantified using MTT, which yields a blue formazan product in living cells but not in dead cells. The resulting colored end product was solubilized in dimethyl sulfoxide (DMSO) and measured using a Microplate Reader (A Packard Bioscience Company, USA) at 550 nm.

Anti-inflammatory activity

Samples were tested for anti-COX-2 and anti-COX-1 activities at 10^{-5} g/ml using radioimmunoassay (RIA). The RIA method used for measuring PGE_2 concentrations in the culture supernatant is based on the competition between PGE_2 in the samples and ^{3}H labeled PGE_2 for anti-PGE_2 antibody binding sites. Aspirin and DMSO were used as positive and negative control, respectively (Kirtikara et al., 1998; Kirtikara et al., 2001).

Statistics

All data are expressed as mean±SD. ANOVA with post-hoc analysis (Turkey's test) was employed using a statistical software package (Openstat®).

Results and Discussion

Content of total phenolic and tannic acid contents in the extracts

Table 1 presents contents of total phenolics and tannic acid which are available in methanol extracts from fruits of EO, TC and TB. The highest total phenolic and tannic acid content was observed in the TB extract.

Table 1 Total phenolic and tannic acid content of test compounds

Extracts	Total phenolic contents (mg of GAE/g)	Tannic acid content (mg/ml)
EO	321.42±6.51*	0.08±0.010*
TB	393.98±3.24	0.13±0.013
TC	210.10±1.27*	0.10±0.010*

All data are expressed as mean±SD. (n=3) of three independent replicates. * p≤ 0.05 compared with the TB fruit extract

Neuroprotective effect

Prior to the neuroprotective study, cytotoxic effect of H_2O_2 on NG 108-15 cells was evaluated. We found that H_2O_2 decrease the cell viability in a concentration dependent manner with an IC_{50} value was around 200 μM. 200 μM H_2O_2 was selected to induce cell death in this study. We found that TC (100-1000 μg/ml), TB (100-1000 μg/ml) and EO (1000 μg/ml) significantly protected the cells (Fig. 1) without cytotoxic effect (Fig. 2). These results are in good agreement with the findings from study carried out by Nampoothiri (2010) which demonstrated that fruit extracts of TB and EO could protect C2C12 mouse myoblast cell lines from H_2O_2-induced cell death (Nampoothiri et al., 2010).

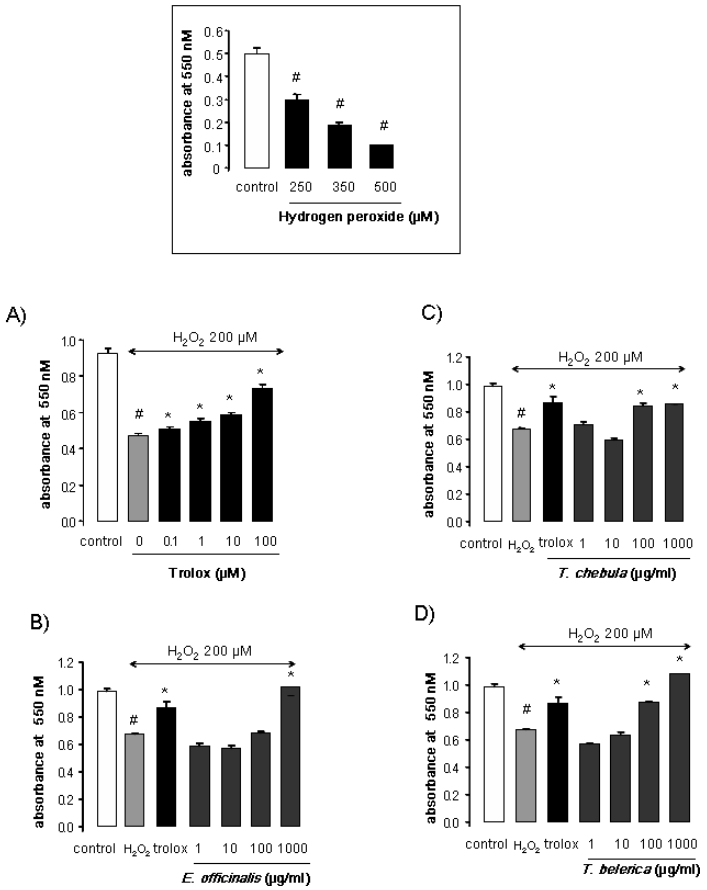

Figure 1 Effect of Test Compounds on H_2O_2-Induced NG108-15 Cell Damage

The cells were treated with H_2O_2 (200 μM) together with various concentrations of test compounds: (A) Trolox, (B) *Emblica officinalis*, (C) *Terminalia chebula* and (D) *T. belerica*. Inset: H_2O_2-induced reduction of viability of the cells. Data are expressed as mean±SD. of three independence study (n = 5).

$p \leq 0.05$ compared with the control group. * $p \leq 0.05$ compared with the H_2O_2-treated control group

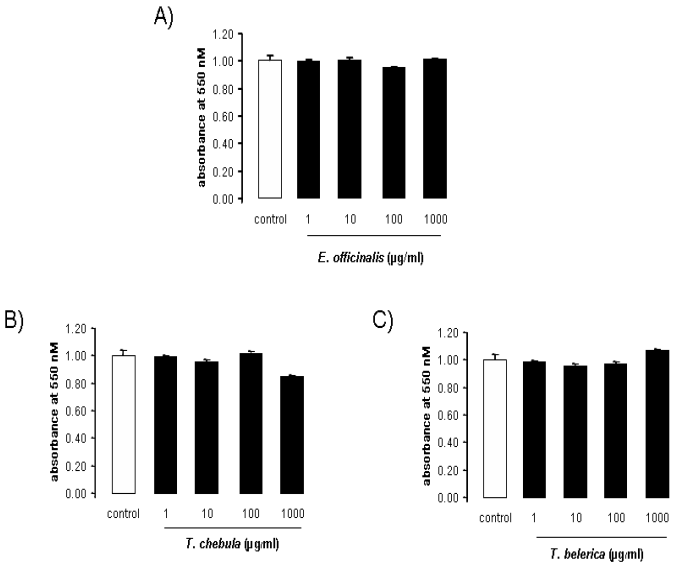

Figure 2 Cytotoxicity Effects of Test Compounds on NG108-15 Cells.

The cells were treated with various concentrations of test compounds (1-1000 µg/ml) (A) *Emblica officinalis*, (B) *Terminalia chebula* and (C) *T. belerica*. Data are expressed as mean±SD. of three independence study (n = 5).

* $p \leq 0.05$ compared with the control group

Antioxidant activities

It is possible that the protective effect of the samples against H_2O_2-induced decrease in cell viability may at least partly result from their antioxidative properties. To test this possibility, free radical scavenging activities and iron chelating effect of the test compounds were investigated. First of all we investigated scavenging effects of the samples on H_2O_2. We found that H_2O_2 scavenging activity of the extracts showed no substantial result compared with the standard butylated hydroxytoluene (BHT) (IC_{50} = 272.91±1.61 µg/ml). Therefore, no figure or IC_{50} values of the extracts were provided. To clarify other possible modes of free radical scavenging abilities, DPPH and hydroxy radical scavenging experiments were carried out. DPPH assay was selected in this study as a model for evaluation of total antioxidant capacity. Hydroxy radical scavenging and ion chelating experiments were performed in response to underlying mechanism of H_2O_2-induced cell death (Kawpoomhae et al., 2010). We found that all of the extracts under investigation could scavenge DPPH and hydroxyl radical. They exhibited an inhibitory effect on hydroxyl radical-induced deoxyribose degradation in both presence and absence of EDTA. In addition, they also showed positive results in metal ion chelating assay (Table 2).

Taken together from the results obtained, it can be suggested that the fruit extracts act as neuroprotective agents against oxidative damage. Their neuroprotective activities may be result from the antioxidative properties.

Table 2 DPPH and hydroxyl radical-scavenging effects, and ion chelating activity of the test compounds

Test compounds	Reference substances	IC_{50}			
		DPPH scavenging activity	Hydroxyl radical scavenging activity		Ion chelating activity
			With EDTA	Without EDTA	
EO		3.33±0.31 μg/ml	1.33±0.11 mg/ml	0.40±0.03 mg/ml	0.47±0.05 mg/ml
TB		1.74±0.21 μg/ml	0.14±0.01 mg/ml	0.21±0.02 mg/ml	0.25±0.02 mg/ml
TC		8.21±0.43 μg/ml	0.82±0.01 mg/ml	1.36±0.09 mg/ml	1.23±0.12 mg/ml
	Trolox®	5.25±0.51 μg/ml			
	Mannitol		11.0 ± 0.8 mM	35.0 ± 1.0 mM	
	EDTA				3.89 ± 1.60 μM

Each IC_{50} value represents mean±SD. of three independent experiments

Anti-inflammatory activity

To evaluate anti-inflammatory effect of TB, EO and TC methanol extracts, we determined anti-COX-2 and anti-COX-1 activities at 10^{-5} g/ml using radioimmunoassay (RIA). Aspirin, reference standard showed anti-COX-1 and anti-COX-2 activity as presented in Table 3. From all of the extracts, only EO and TB fruit extract showed anti-COX-2 activity (Table 3). The anti-inflammatory effect of EO is in good agreement with results from studies carried out by Dang et al. and Mathuraman et al. (Dang et al., 2010; Muthuraman et al., 2010). Apart from those, the anti-inflammatory effect of TB is in accordance with the results from study carried out by Reddy et al. (Reddy et al., 2009). The anti-COX-2 activity of EO and TB extract that we firstly found in the present study not only support their traditional used in wound healing and anti-ulcer but also provide benefit in neuronal disease which inflammation is implicated. However, future experiment focus on inflammation-induced neuronal death is required.

Table 3 Anti-inflammatory activity of test compounds

Test compounds	Concentration (μg/ml)	Anti COX-1 PGE_2	Anti COX-1 %inhibition	Anti COX-2	Anti COX-2
Negative control (DMSO)	-	100.00±29.62	-	100.00±10.03	-
EO	10	60.68±7.66	39.32	50.03±6.56*	46.97
TB	10	65.84±11.56	34.16	73.43±7.79*	26.57
TC	10	64.61±9.97	35.39	83.24±9.65	16.76
Positive control (Aspirin)	10	37.25±9.79*	62.75	56.69±5.34*	43.31

Each value of PGE_2 level represents mean±SD. of three independent experiments

* $p \leq 0.05$ compared with the negative control group (DMSO)

Conclusions

This study reveals the neuroprotective effect of the methanol extracts of fruits from EO, TB and TC. From our findings, it can be suggested that the neuroprotective effect may be due to their antioxidative properties. In addition, we also present anti-COX-2 activity of the extracts of fruits from EO and TB. The results from this study not only support the traditional uses of EO, TB and TC as well as Triphala but also provide benefit in neurodegenerative diseases or other disorders which oxidative stress and/or inflammation is implicated.

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References

Al-Rehaily, A.J., Al-Howiriny, T.A., Al-Sohaibani, M.O., Rafatullah, S. (2002) Gastroprotective effects of 'Amla' *Emblica officinalis* on *in vivo* test models in rats. *Phytomedicine* 9: 515-522.

Dang, G.K., Parekar, R.R., Kamat, S.K., Scindia, A.M., Rege, N.N. (2011) Anti-inflammatory activity of *Phyllanthus emblica*, *Plumbago zeylanica* and *Cyperus rotundus* in acute models of inflammation. *Phytotherapy Research* 25: 904-908.

Frank, B. and Gupta, S. (2005) A review of antioxidants and Alzheimer's disease. *Annals of clinical psychiatry* 17: 269-286.

Gandhi, N.M. and Nair, C.K. (2005) Radiation protection by *Terminalia chebula*: some mechanistic aspects. *Molecular and Cellular Biochemistry* 277: 43-48.

Kawpoomhae, K., Sukma, M., Ngawhirunpat, T., Opanasopit, P., Sripattanaporn, A. (2010) Antioxidant and neuroprotective effects of standardized extracts of *Mangifera indica* leaf. *The Thai Journal of Pharmaceutical Sciences* 34: 32-43.

Khandelwal, S., Shukla, L.J., Shanker, R. (2002) Modulation of acute cadmium toxicity by *Emblica officinalis* fruit in rat. *Indian Journal of Experimental Biology* 40: 564-570.

Kirtikara, K., Laulederkind, S.J., Raghow, R., Kanekura, T., Ballou, L.R. (1998) An accessory role for ceramide in interleukin-1beta induced prostaglandin synthesis. *Molecular and Cellular Biochemistry* 181: 41-48.

Kirtikara, K., Swangkul, S., Ballou, L.R. (2001) The analysis of nonsteroidal anti-inflammatory drug selectivity in prostaglandin G/H synthase (PGHS)-null cells. *Inflammation Research* 50: 327-332.

Krishnaveni, M. and Mirunalini, S. (2010) Therapeutic potential of *Phyllanthus emblica* (amla): the ayurvedic wonder. *Journal of basic and clinical physiology and pharmacology* 21: 93-105.

Martel-Pelletier, J., Lajeunesse, D., Reboul PPelletier, J.P. (2003) Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Annals of the Rheumatic Diseases* 62: 501-509.

Muthuraman, A., Sood, S., Singla, S.K. (2010) The anti-inflammatory potential of phenolic compounds from *Emblica officinalis* L. in rat. *Inflammopharmacology* [Online URL: http://www.springerlink.com/content/m336774103x25520] accessed on October 15, 2010.

Na, M., Bae, K., Kang, S.S., Min, B.S., Yoo, J.K., Kamiryo, Y., Senoo, Y., Yokoo, S., Miwa, N. (2004) Cytoprotective effect on oxidative stress and inhibitory effect on cellular aging of *Terminalia chebula* fruit. *Phytotherapy Research* 18: 737-741.

Nampoothiri, S.V., Prathapan, A., Cherian, O.L., Raghu, K.G., Venugopalan, V.V., Sundaresan, A. (2010) *In vitro* antioxidant and inhibitory potential of *Terminalia bellerica* and *Emblica officinalis* fruits

against LDL oxidation and key enzymes linked to type 2 diabetes. *Food and Chemical Toxicology* 49: 125-131.

Paoin, T. (2008) *Tonmai Yanaruu*. Suviriyasasn, Bangkok.

Reddy, D.B., Reddy, T.C., Jyotsna, G., Sharan, S., Priya, N., Lakshmipathi, V., Reddanna, P. (2009) Chebulagic acid, a COX-LOX dual inhibitor isolated from the fruits of *Terminalia chebula* Retz., induces apoptosis in COLO-205 cell line. *Journal of Ethnopharmacology* 124: 506-512.

Sabina, E.P. and Rasool, M. (2008) An in vivo and in vitro potential of Indian ayurvedic herbal formulation Triphala on experimental gouty arthritis in mice. *Vascular Pharmacology* 48: 14-20.

Sai Ram, M., Neetu, D., Deepti, P., Vandana, M., Ilavazhagan, G., Kumar, D., Selvamurthy, W. (2003) Cytoprotective activity of Amla (*Emblica officinalis*) against chromium (VI) induced oxidative injury in murine macrophages. *Phytotherapy Research* 17: 430-433.

Sai Ram, M., Neetu, D., Yogesh, B., Anju, B., Dipti, P., Pauline, T., Sharma, S.K., Sarada, S.K., Ilavazhagan, G., Kumar, D., Selvamurthy, W. (2002) Cyto-protective and immunomodulating properties of Amla (*Emblica officinalis*) on lymphocytes: an in-vitro study. *Journal of Ethnopharmacology* 81: 5-10.

Sukma, M., Tohda, M., Watanabe, H. (2003) Chronic treatment with imipramine inhibits cell growth and enhances serotonin 2C receptor mRNA expression in NG 108-15 cells. *Journal of Pharmacological Sciences* 92: 433-436.

Zhao, B. (2005) Natural antioxidants for neurodegenerative diseases. *Molecular Neurobiology* 31: 283-293.

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จัดโดย

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