Cytoprotective Efect of *Terminalia chebula* Retz Against Oxygen-Glucose Deprivation on Rat Pheochromocytoma Cell

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ABSTRACT

Terminalia chebula is a medicinal plant cultivated in Asia. The dried, ripened fruit has been used to treat various ailments in the indigenous system of medicine in Asia. This paper aimed to investigate the protective activity of *T. chebula* fruit extract against in vitro ischemia and oxidative damage in cells. Oxygen-glucose deprivation followed by reoxygenation (OGD-R), an in vitro ischemia model and hydrogen peroxide, an oxidative stress model was used to investigate the activity of *T. chebula* in PC12 cells. Cell survival was evaluated by 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Free radical scavenging, lipid peroxidation and nitric oxide inhibition were measured by DPPH, thiobarbituric and Griess reagent, respectively. PC12 cells treated with *T. chebula* 0.01 – 10 µg/ml significantly improved survival of cells against OGD-R and H_2O_2, and inhibited the formation of malondialdehyde. *T. chebula* significantly scavenged the DPPH free radicals and inhibited nitric oxide produced by activated microglia cells. These results suggest that *T. chebula* possesses protective activities against OGD-R and H_2O_2 induced cell injury, and reduces microglia activation by suppressing the production of free radicals, elevating antioxidative and anti-inflammatory activities.

Key words: - *Terminalia chebula; Oxygen-glucose deprivation; PC12 cells; Reactive oxygen species; Protection*

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INTRODUCTION

Free radicals and their related species, which are mainly derived from reactive oxygen species (ROS) and reactive nitrogen species (RNS), have attracted a great deal of attention in recent years. ROS from both endogenous and exogenous sources play an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke.[1,2] Overproduction of ROS in various neurological diseases, especially during ischemia/reperfusion, causes an imbalance between oxidative and antioxidative process which leads to mitochondrial dysfunction, excitotoxicity, lipid peroxidation, and inflammation. Therapeutic efforts aimed to remove ROS or inhibit their formation have been shown to be beneficial in various neuronal diseases including ischemia.[3,4] Natural antioxidants from medicinal or edible plants have recently paid much attention as promising agents for reducing the risk of oxidative stress-induced diseases.[5,6]

Terminalia chebula Retz (Combretaceae) is a medicinal plant highly cultivated in Southeast Asia. Its dried ripened fruit has been used to treat various ailments in the indigenous system of medicine in Asia.[7] *T. chebula* has been reported to exhibit a variety of biological activities; including anticancer, antidiabetic, antimutagenic, antibacterial, antifungal, antiviral and cardioprotective activities.[8-15] It is a laxative and tonic agent containing several biologically active compounds such as gallic acid, ellagic acid, tannic acid, β-sitosterol, ethyl gallate, chebulic acid and mannitol. It is also one of the richest sources of ascorbic acid.[16,17]

Based on the presence of antioxidative and anti-inflammatory compounds in *T. chebula,* we hypothesized that fruit of *T. chebula* can exert protective effects on neuronal cells against ischemia and related diseases. We investigated whether its extract exerts protection against oxygen-glucose deprivation followed by re-oxygenation (OGD-R) and hydrogen peroxide (H_2O_2) induced cell injury on the rat pheochromocytoma cell line (PC12 cells). PC12 cells which display phenotypic characteristics of both adrenal chromaffin cells and sympathetic neurons,[18,19] was used for in vitro models. OGD-R, a well accepted in vitro ischemia model which has been used to screen the active components for neuroprotective potency,[20,21] was used to evaluate the effect of *T. chebula* extract. H_2O_2 induced cell death; a well accepted in vitro oxidative stress model,[22,23] was used to evaluate the effect of *T. chebula* extract in PC12 cells. Further, we investigated the effect of *T. chebula* extract on lipopolysaccharide (LPS) activated microglia cells (BV2 cell), as the activated microglia are thought to be involved in neuronal inflammation by overproduction of various bioactive molecules such as nitric oxide (NO). We also measured the

DPPH free radical scavenging, lipid peroxidation and NO inhibition activity of *T. chebula* extract.

MATERIAL AND METHODS

Plant material and chemicals

The fruits of *T. chebula* were collected from Pokhara, Nepal in September, 2010 and identified by taxonomist Mr. Tikaram Aryal. The voucher specimen (HP203) was deposited in the Materia Medica of School of Health and Allied Sciences, Pokhara University, Nepal. Dulbecco's Modified Eagle's Medium (DMEM), lipopolysaccharide (LPS, *Escherichia coli* 0127:138), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ellagic acid, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO) and Griess reagent were purchased from Sigma chemical Co. (St Louis, OR), Fetal Bovine Serum (FBS), penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY). Other reagent and solvents were HPLC grade unless specified.

Extraction of plant material

T. chebula fruit powder (100 g) was extracted under reflux with 70% (v/v) methanol for 3 h at 80°C. The extract was evaporated with a rotary evaporator under reduced pressure to remove organic solvent and then lyophilized until dryness. The yield of *T. chebula* extract (TCE) was 45.5 g/100 g of crude powder. For the standardized TCE, content of gallic acid and ellagic acid were measured by HPLC method. Stock solution of extract was prepared in DMSO and deionized water, respectively. Final DMSO concentration did not exceed 0.1% (v/v) in all treatments.

Free radical scavenging activity and measurement of total phenol content in TCE

Free radical scavenging activity was measured spectrophotometrically by DPPH assay.[24] Vitamin C was used as a positive control. Phenolic compounds in the TCE were estimated by the colorimetric assay. Briefly, 1 ml of sample was mixed with equal volume of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture. After 90 min of reaction, the optical density (OD) was measured on plate reader (SPECTRA max Plus384, CA, USA) at 725 nm. Gallic acid (10 – 500 mg/l) was used for calculation and the results were expressed as mg of gallic acid equivalents (GCE) per gram of extract.

Cell culture

PC12 cells and BV2 cells were purchased from the Cell-line Bank, Korea, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. PC12 cells were seeded at a density of 1.5 × 103/well and BV2 cells were seeded at a density of 5 × 104 cells/well and cultured at 37°C in DMEM supplement with 10% heat-inactivated FBS, penicillin (1 × 105 U/L) and streptomycin (100 mg/l) in a 5% CO_2 incubator.

Oxygen- glucose deprivation followed by reoxygenation

Experiments were carried out after 24 h cells were seeded. For OGD injury, PC12 cells were first washed with phosphate buffer saline (PBS, pH 7.2) then with glucose free DMEM. The cultures were placed in fresh glucose free DMEM and kept in hypoxic chamber (Forma Science, UK) containing 95% N_2 and 5% CO_2 for 4 h. At the end of the exposure period, glucose solution was added, and the cells were incubated in the normal incubator (5% CO_2 at 37°C) for an additional 24 h. Samples of different concentrations were treated 30 min before and during OGD exposure. Normal group was treated with the same amounts of FBS free glucose DMEM and kept in the normal incubator. Baicalein, a potent antioxidative and neuroprotective compound was used as a positive control.[25,26]

H_2O_2 induced cell injury

For H_2O_2 treatment, PC12 cells were washed with FBS free DMEM. Dilution of H_2O_2 was made from a 30% stock solution into DMEM just prior to each experiment and 200 μM solution added to the cells. The culture plates were incubated for an additional 24 h. Samples of different concentrations were treated 2 h before and during H_2O_2 exposure. Normal group (without H_2O_2) was incubated under the same conditions.

Lipid peroxidation assay

Malondialdehyde (MDA), the most abundant lipid peroxidation product from PC12 cells, was measured using the thiobarbituric acid colorimetric assay. Briefly, 24 h after the treatment of the cells with H_2O_2 (200 μM) in the presence or absence of TCE (0.1 μg/ml and 1 μg/ml), cultures were washed with ice cold PBS, pooled in 0.1 mol/l PBS-5% Triton X-100 buffered solution, and incubated for about 1 h at 37°C. Trichloroacetic acid (350 μl; 20% w/v) was added to 250 μl of cellular lysate and centrifuged at 1000 × g at 4°C for 10 min. Aliquots (450 μl) of supernatant were mixed with equal volume of 0.5% (w/v) thiobarbituric acid. The mixture was boiled at 100°C for 30 min. After cooling MDA formation was measured (OD at 520 nm) and the results were expressed as a percentage of the normal group.

LPS induced microglia activation and the measurement of NO

For LPS induced cell death and nitric oxide inhibitory assay of TCE, BV2 cells were incubated with/without 1 μg/ml of LPS, in the absence or presence of different sample solutions for 24 h. For the measurement of NO produced by activated cells, 90 μl of the supernatant from the BV2 cells was mixed with equal volume of the Griess reagent. The OD was measured at 540 nm. Curcumin, a potent anti-inflammatory compound was used as a positive control.[27]

Cell survival

Cell survival was evaluated by the ability to reduce 2-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. MTT was dissolved in DMEM and

added to the culture at final concentration of 0.5 mg/ml. After additional 2 h of incubation, the media was carefully removed and 100 µl DMSO was added to each well. The OD was measured on plate reader at 570 nm. Results were expressed as a percentage of the normal group.

Statistical analysis

All results were presented as mean ± SEM. Significant differences between experimental groups were determined by Student's t-test. The level of tested significance was $p < 0.05$ and each experiment was performed three times.

RESULTS

DPPH radical scavenging activity and total phenolic compounds in TCE

TCE showed the dose dependent radical scavenging activity with 96% of inhibition against DPPH radical at 100 µg/ml (Table 1). Total phenol content was found to be 787.1 ± 20.8 mg GCE/g of extract. Content of ellagic acid and gallic acid in TCE were 25.5 ± 2.2 mg/g and 29.8 ± 2.8 mg/g of extract, respectively.

Effect of TCE in PC12 cells against OGD-R induced cell death

To examine whether TCE protects against OGD-R induced cell death, PC12 cells were incubated without glucose solution in hypoxia chamber for 4 h followed by 24 h of reoxygenation with glucose solution in normal incubator. Different concentrations of TCE were treated 30 min before and during 4 h OGD. The survival of cells after treatment of TCE was measured by MTT assay (Fig. 1). Cell viability in control group (OGD) was 50.4 ± 1.5% whereas cell viability in TCE treated cells at 0.1 – 10 µg/ml was 62.4 ± 2.2% ($p < 0.05$), 68.0 ± 2.0% ($p < 0.01$) and 54.8 ± 1.2% ($p < 0.05$) respectively as compared to the normal group (set 100%). Baicalein at 0.27 µg/ml (1 µM) treated cells showed 74.0 ± 2.8 % ($p < 0.001$) cell viability against 4 h of OGD followed by 24 h of reoxygenation.

Effect of TCE in PC12 cells against H_2O_2 induced cell death

To determine whether TCE protects cell against oxidative stress death, PC12 cells were exposed with H_2O_2 for 24 h. The toxicity of H_2O_2 in PC12 cells was found to be concentration and time-dependent (data not shown). Cell viability in control group (H_2O_2) was 70.4 ± 1.5% whereas cell viability in TCE treated cells at 0.01 – 1 µg/ml was 80.6 ± 3.6% ($p < 0.05$), 91.4 ± 3.6% ($p < 0.005$) and 86.9 ± 4.4% ($p < 0.01$) respectively, as compared to the normal group (Fig. 2A). Baicalein at 0.27 µg/ml (1 µM) treated cells showed 84.0 ± 2.9% ($p < 0.05$) cell viability against H_2O_2.

Effect of TCE in lipid peroxidation

Thiobarbituric acid colorimetric assay was performed in order to determine whether TCE can inhibit the formation of MDA induced by H_2O_2. The cells treated with H_2O_2 µM) significantly increased MDA levels (237.0 ± 15 compared with normal cells (set 100%). Pretreatment of at 0.1 µg/ml and 1 µg/ml significantly reduced MDA from 237.0 ± 15.2% in control group to 115.8 ± 5.8% 0.001) and 93.7 ± 2.2% ($p < 0.001$) respectively (Fig. 2E

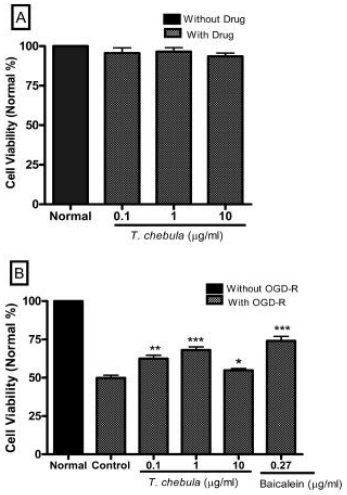

Figure 1: Effect of TCE on normal cell and OGD-R induced cell death.
A) Different concentrations of TCE were treated on normal PC12 cells during 24 h. B) PC12 cells were exposed to OGD for 4 h followed by re-oxygenation for 24 h. Different concentrations of TCE were treated 30 min before and during 4 h OGD. Cells viability was measure by MTT assay. Normal group served as 100%, and data obtained in other groups were calculated as percent of normal accordingly. Data are expressed as the mean ± S.E.M (n=5). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as compared with the control group alone by unpaired t-test.

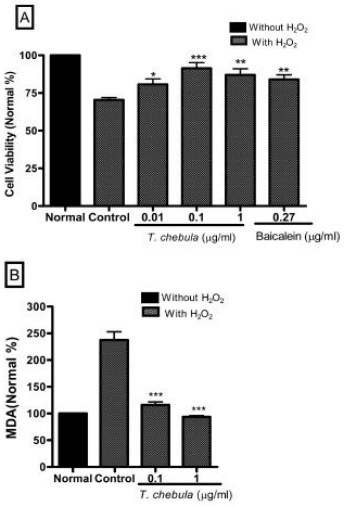

Figure 2: Effect of TCE on H_2O_2-induced cell death and lipid peroxidation assay.
PC12 cells were exposed to 200 µM H_2O_2 for 24 h. Different concentrations of TCE were treated 2 h before and during H_2O_2 exposure. Normal group served as 100%, and data obtained in other groups were calculated as percent of normal accordingly. A) PC12 cells viability was measured by MTT assay. B) Lipid peroxidation assay was measured by thiobarbutic assay. Data are expressed as the mean ± S.E.M (n=5). **$p < 0.01$, and ***$p < 0.001$ as compared with the control group alone by unpaired t-

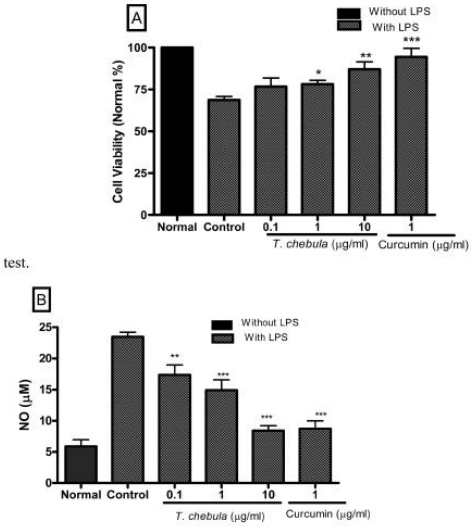

Figure 3: Effect of TCE on LPS-induced cell death and NO inhibition assay.

BV2 cells were exposed to LPS (1 µg/ml) for 24 h. Different concentrations of TCE were treated 2 h before and during LPS exposure. A) Cell viability was measured by MTT assay. Normal group served as 100%, and data obtained in other groups were calculated as percent of normal accordingly. B) Cell-conditioned supernatants were collected and the production of NO in the supernatants was measured using Griess reagent. NO production was calculated by using standard nitrite. Data are expressed as the mean ± S.E.M (n=5). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared with the control group alone and $^{###}p<0.001$ *vs*. normal group by unpaired *t*-test.

Table 1:- DPPH free radical scavenging activity and phenolic compounds in TCE

Sample	DPPH (IC_{50}) (µg/ml)	Total Phenol (mg GAE/g)	Gallic acid (mg/g)	Ellagic acid (mg/g)
Vitamin C	4.7 ± 0.01			
TCE	5.5 ± 1.1	787.1 ± 20.8	29.8 ± 2.8	25.5 ± 2.2

Effect of TCE in LPS induced cell activation and NO inhibition

In order to elucidate the effect of TCE against LPS induced microglia activation, BV2 cells were treated with LPS. Viability of cells after treatment of LPS (1 µg/ml) was found as 68.7 ± 2.0% as compared to normal cells (set 100%) whereas TCE at 0.1 – 10 µg/ml concentration treated cells showed cells viability 76.8 ± 4.9%, 78.2 ± 2.2% ($p < 0.05$) and 87.1 ± 4.3% ($p < 0.01$) respectively (Fig. 3A). The amount of NO released by BV2 cells was calculated as 3.3 ± 0.5 µM in normal cells, 19.2 ± 1.1 µM ($p < 0.001$) in control (LPS) treated cells, and 16.6 ± 0.7 µM, 10.0 ± 0.7 µM ($p < 0.001$) and 7.2 ± 0.9 µM ($p < 0.001$) in TCE (0.1 – 10 µg/ml) treated cells, respectively (Fig. 3B). Curcumin at 1 µg/ml showed 94.3 ± 5.2 % ($p < 0.001$) cell viability and 8.7 ± 1.2 µM ($p < 0.001$) NO in BV2 cells.

DISCUSSION

In the present study, we demonstrated that TCE at 0.1 – 10 µg/ml significantly reduced PC12 cells injury induced by 4 h of OGD followed by 24 h of reoxygenation and 24 h exposure to H_2O_2. H_2O_2 induced MDA production was significantly reduced by TCE at 1 µg/ml. TCE also reduced LPS induced cell death and inhibited the production of NO in a dose dependent manner. To our knowledge, this is the first study of TCE against in vitro model of ischemia.

TCE markedly scavenged nitrogen centre free radical DPPH in a dose dependent manner. The effective concentration of TCE to scavenge 50% of DPPH free radical (EC50) was found as 5.5 ± 1.1 µg/ml which was similar to Vitamin C (Table 1). The ability of TCE to scavenge DPPH free radicals suggests that it is an electron donor and can react with free radicals to convert them to more stable products and terminate radical chain reactions. HPLC analysis showed that the content of phenolic compounds; ellagic acid and gallic acid were 25.5 ± 2.2 mg/g and 29.8 ± 2.8 mg/g of extract, respectively.

The OGD-R cell culture model represents a valid simulation of the conditions in brain ischemia and has been used to imitate ischemic conditions in vivo.[28] This model is widely used for the development of neuroprotective drugs against ischemic injury. Consistent with other studies, the exposure of PC12 cells to 4 h of OGD followed by 24 h of reoxygenation resulted decrease in cell viability as compared to normal cells.[29] Incubation of PC12 cells with TCE (0.1 – 10 µg/ml) 30 min before and during OGD period led to improved cells viability. TCE protected PC12 cells 8.0 – 36.0% against OGD-R and maximum efficacy was found at 1 µg/ml which indicate that TCE can show cytoprotective activity against ischemia. In this study, positive control, baicalein showed 48.1% protective activity at 1 µM (0.27 µg/ml) against 4 h OGD.

As OGD-R mimics tissue ischemia, it results into glutamate toxicity, production of ROS and inflammatory cytokines.[28,30] Once the ROS/RNS are produced within the cells, they have the potential to react with and damage most cellular targets including lipids, proteins and DNA. It has been reported that compounds having antioxidative, anti-inflammatory, anti-glutamate activity can show protective activity against hypoxia induced cell damage.[30-33] The exact protective mechanism of TCE against OGD-R is unknown, however our results showed that TCE is a free radical scavenger and it contains strong antioxidative compounds like gallic acid and ellagic acid.

In order to know the possible mechanism of TCE, we further studied on H_2O_2 induced cell death which has been reported as an in vitro oxidative stress model.[22,23] TCE protected PC12 cells against H_2O_2 induced cell death which was found as 33.0–70.0% at a concentration of 0.01–1 µg/

ml. The maximum efficacy of the extract was found as 70% and even at low concentration the protective rate was 33.0% in comparison to the H_2O_2 treated control cells. A positive control, baicalein at 1 μM (0.27 μg/ml) was found to be 46.3% protective against H_2O_2. This result indicates that TCE is protective against H_2O_2 induced cell death.

H_2O_2 is believed to be the major precursor for highly reactive free radicals, expressed indirectly by the increase in MDA levels, which is one of the most important organic expressions of oxidative stress in various neuronal diseases including ischemia.[34] Oxidative stress induces cellular damage and lipoperoxidation, which may lead to alterations in membranes, producing significant changes in their biophysical.[35] Our results showed that MDA production during H_2O_2 treatment was inhibited by TCE at 0.1 and 1 μg/ml. Many studies have shown that the radical scavengers and inhibitors of lipid peroxidation can ameliorate ischemic cell damage.[2,5,36] Therefore, antioxidant effect through the inhibition of lipid peroxidation and free radical scavenging could be one of the possible protective mechanisms of TCE against ischemia and H_2O_2.

Further, we investigated the effect of TCE on LPS activated microglial cells, as the activated microglia are thought to be involved in neuronal inflammation by overproduction of various bioactive molecules such as NO, ROS and proinflammatory cytokines.[37,38] In the present study, LPS activated BV2 cells and released 19.2 ± 1.1 μM NO in the culture medium, whereas TCE inhibited the accumulation of NO by 16.0 – 76.2% at a concentration of 0.1–10 μg/ml, respectively which means that TCE can inhibit microglia activation. NO is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory responses.[39] Increased inducible nitric oxide synthetase expression and NO generation in hypoxic/ischemic cell injury has been documented as one of the major factors leading to neuronal death and necrosis, in addition to an array of other biochemical aberrations.[30,40] Therefore, anti-inflammatory effect through the inhibition of NO could be another possible protective mechanism of TCE against ischemia.

In summary, TCE showed a protective effect against OGD-R, H_2O_2 and LPS induced cell death. TCE has an antioxidative and anti-inflammatory activity which could be the possible mechanism for the protection of cells against ischemia. Further study is necessary to evaluate the protective effect of TCE on in vivo ischemia. Our results can suggest that *T. chebula* fruit could be useful therapeutic agent for the protection of cells against the ischemia, oxidative stress and microglia-induced secondary damage, which may occur in various neurodegenerative diseases including ischemia.

ACKNOWLEDGMENT

The authors wish to thank to Pokhara University, Nepal and Department of Herbal Medicine, College of Oriental Medicine, Kyung Hee University, South Korea for providing facilities to carry out this work.

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