Dual Effects of Plant Antioxidants on Neuron Cell Viability

Norfaizatul SO (B.Sc.)[1], Zetty Akmal CZ (B.Sc.)[1], Noralisa AK (B.Sc.)[1], Then SM (Ph.D.)[2], Wan Zurinah WN (Ph.D.)[1,2], Musalmah M (Ph.D.)[1*]

1- Department of Biochemistry, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
2- UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Kuala Lumpur, Malaysia
*Corresponding author: Department of biochemistry, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
Tel: +603-92897192, Fax: +603-62938037
Email: musalmah@medic.ukm.my

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Abstract

Background: Many studies have focused on oxidative stress induced damage and hence, the protective effects conferred by antioxidants. An example is neurodegenerative diseases which is thought to occur due to neuronal loss associated with oxidative stress. However, some antioxidants such as vitamin E have been shown to also exert pro-oxidative effects at high concentration.

Objective: In this study the cytotoxicity and neuroprotective potentials of *Chlorella vulgaris* (CV), *Momordica charantia* (MC) and *Piper betle* (PB) were investigated and correlated with the antioxidant potential. Tocotrienol Rich Fraction (TRF) served as positive control since it had been shown previously to have high antioxidant potential as well as to exert neuroprotective and neurocytotoxic effects.

Method: Free radical scavenging activities of hot water extract of CV, aqueous extract of MC, aqueous extract of PB and TRF were determined by using DPPH (1, 1-diphenyl-2-picryl-hydrazyl) assay. Cytotoxicity and neuroprotective effects were measured by using 3 - (4, 5 - dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) against BSO-induced neuron cell death.

Results: Results showed that TRF has the highest radical scavenging activity followed PB> MC> CV. The MTS results showed that TRF (1-50 µg/ml) as positive control, PB (0.001-100µg/ml) and MC (1-500µg/ml) conferred significant protection against BSO-induced cell death. These plants were cytotoxic at high concentrations. However CV extract did not show significant neuroprotective effect against BSO-induced cell death nor cytotoxic effect.

Conclusion: The present findings showed that plant extracts with the higher free radical scavenging activity showed neuroprotective effects at low concentrations but were cytotoxic at higher concentrations.

Keywords: Antioxidant, *Centella asiatica*, *Chlorella vulgaris*, *Momordica charantia*, Neuroprotection, *Piper betle*

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Introduction

Antioxidants have been suggested to be useful in conditions associated with oxidative stress such as atherosclerosis, heart failure, chronic fatigue syndrome, cancer etc. [1]. Oxidative stress which occurs due to the imbalance between production and detoxification of reactive oxygen species (ROS) has also been implicated in many neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [2, 3]. The brain is more susceptible to oxidative stress due to its high oxygen consumption, relatively low antioxidant capacity and high contents of lipid and ferum. Increased oxidative stress will further lead to lipid peroxidation, DNA damage, protein damage and induction of apoptosis [1, 4].

Apart from Vitamin E [5, 6, 7] other plants/plant products which had been shown to exert neuroprotective effects include *Ginkgo biloba* extracts [8, 9, 10], green tea extracts [11, 12, 13] and ginseng extracts [14 - 17]. Most of these studies attribute the neuroprotective effects to the antioxidant properties.

However, recent studies have reported that some antioxidants such as vitamin E also exert pro-oxidant effects [1]. Since not much is known on the cytotoxic effects of plant extracts, we investigated the scavenging properties, neuroprotective and neurocytotoxic effects of a few plants commonly eaten in the South East Asian region and compared it to the effects of TRF (Vitamin E). The plants studied are *Piper betle*, *Momordica charantia*, and *Chlorella vulgaris*.

Piper betle L. (Piperaceae) is a climbing shrub which can be found throughout Southeast Asia. It is used as a masticatory, antiseptic and also to heal ulcers, boils and bruises. The roots, leaves and fruits are carminative, stimulant and used for the treatment of malaria [18]. It has been reported that PB extracts showed significant antioxidant activity tested with various free radical-scavenging systems [19]. *In vivo* studies also showed that PB extracts were able to improve antioxidant status in oxidative stress condition [20, 21].

Momordica charantia L. (Cucurbitaceae) has been widely acclaimed as an important remedy for diabetes mellitus since ancient times. It is also used to treat gastrointestinal complaints, worms, constipation, headaches, skin conditions, viral infections and others [22]. It contains several biologically active constituents that include glycosides (momordicins I and II), steroidal saponins (charantins), alkaloids, fixed oils and proteins (MAP30; Momordica anti - *HIV* protein). The immature fruits are a good source of vitamin C, vitamin A, phosphorus and iron [23]. MC extracts are rich in phenolics and have a strong antioxidant activity and a radical-scavenging action tested in various antioxidant assays [24, 25, 26].

Chlorella vulgaris is a unicellular fresh water micro alga. It is very rich in amino acids, complex carbohydrates, vitamins, minerals, fats, chlorophyll, carotenoids, phytonutrients and the unique Chlorella Growth factor (CGF). Its anti-oxidative effect is due to the high content of beta-carotene along with vitamin C and E, chlorophyll and the trace mineral selenium [27]. Little is known of its antioxidant effects however *in vivo* studies proved that it significantly enhanced antioxidant status in oxidative stress state [28, 29].

To date, the neuroprotective potentials and neurocytotoxic effects of these plant extracts are not known. Hence, this study aims to elucidate these properties and correlate them with the radical scavenging activity.

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Methods

Plant Extracts

Tocotrienol Rich Fraction (TRF) was purchased from Golden Hope, Malaysia. This fraction contains 70% tocotrienol.

Stock of *Chlorella vulgaris* (CV) Beijerinck (Strain 072) was purchased from University of Malaya Algae Culture Collection (UMACC, Malaysia). The CV was grown in Bold's Basal Media (BBM) [30] with 12 hour dark and light cycle. Harvested CV was pelleted before homogenized to break the cell wall. Yield was collected and freeze dried. The aqueous extract of CV was prepared by boiling with distilled water in proportion of 1:10 for 20 minutes. The supernatant was collected, freeze dried and stored at -20^{o}C.

Piper betle (PB) leaves were purchased from Ethnoresources, Malaysia. The aqueous extracts of PB leaves were made in proportion of 1:10 distilled water at 60^{o}C for 1 hour. The extract was filtered with filter paper (Whatman,UK) before freeze dried (Model 35 XL Genesis, Virtis).

Momordica charantia (MC) fruit was obtained from Ethnoresources, Malaysia. The samples were cut into pieces, dried and grinded. The aqueous extract of MC was produced using the same method as PB. The remaining residue was repeatedly extracted before the filtrates were combined, concentrated and lyophilized.

SH-SY5Y neuroblastoma cell culture

Human neuroblastoma SH-SY5Y cells were gifts from Dr Coral Sanfeliu, Institute d'Investigacions Biomèdiques de Barcelona, Spain. The cells were grown in 50% of EMEM pH 7.2 (Flowlab, Australia) and 50% Ham's F-12 pH 7.2 (Sigma, USA) supplemented with 200 mM Non-essential amino acid (NEAA; HyClone), 10 mg/ml gentamicin (PAA, Austria) and 10% Foetal Bovine Serum (FBS) (PAA, Austria). Growing cells were harvested after mild trypsinization and plated at a density of 1.5×10^4 cells per ml on 96-well plates. Cultures were maintained in 5% CO_2 incubator at 37^{o}C. Medium was changed on the second and fifth day after plating. Addition of 10µl of 10µM retinoic acid (Sigma, USA) to the culture changed SH-SY5Y cells to neuron-like cells characteristics which were confirmed microscopically. Experiments were carried out on the seventh day.

Experimental procedure

DPPH radical scavenging activity

The antioxidant activity of the plant extracts, on the basis of the scavenging activity of the stable 1, 1-diphenyl-2-picryhydrazyl (DPPH) free radical, was determined by the method describe by Blois [31] with modifications. Plant extracts were added to 20µg/ml DPPH solution at a ratio of 1:1. After 30 minutes of incubation in the dark, absorbance was measured at 517 nm using microtiter plate reader (VeraMax, Molecular Devices, USA). The percentage of radical scavenging activity was calculated as [(AO-AE)/AO] x 100 (AO = absorbance without extract; AE = absorbance with extract).

Cytotoxicity studies

Plated neuron cells were exposed to various concentrations of TRF (5-50µg/ml) and extracts of PB (25-1000µg/ml), MC (100 - 2000 µg/ml) and CV (200-1000µg/ml) for 24 hours. Cell viability was then determined using the MTS assay.

Neuroprotection studies

Neuron cells plated in 96-wells plate were divided into four groups: control (untreated), incubated with BSO at a concentration which

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induced 50% cell death (IC_{50}) as was determined earlier, pre-treatment and post-treatment with TRF. For the pre-treatment group, cells were incubated with TRF or plant extracts at various concentrations for 24 hours before the addition of BSO. Cells were then incubated for a further 72 hours at 37°C, 5% CO_2. In post-treatment, cells were exposed to BSO for 72 hours followed by incubation with TRF or plant extracts for a further 24 hours at 37°C, 5% CO_2. The untreated group formed the positive control. Cell viability was then analysed using the MTS assay.

Analysis of cell viability

Percentage of viable cells in control, BSO-induced and treated groups were determined by CellTiter 96TM Aqueous Non-Radioactive Cell Proliferation Assay (MTS; Promega, USA). The MTS assay employs 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethophenyl) 2-(4-sulfophenyl)-2H-tetrazolium (MTS) and electron coupling agent phenazine methosulphate (PMS). MTS is converted into a medium soluble formazan product by dehydrogenase enzymes found in metabolically active cells. 20µl MTS solution was added to experiment culture to terminate the exposure of the tested agent. After 2 hours incubation, absorbance was measured at 490nm using microtiter plate reader (VeraMax, Molecular Devices, USA). The quantity of formazan products is proportional to the number of viable cells in the culture.

Statistical analysis

Experiment was done in triplicates and repeated three times. Data of viable cells were calculated as a percentage of controls. Results are presented as Means ± SD. Statistical analysis was performed with ANOVA at the significance level $p<0.05$ (SPSS software).

Results

DPPH radical scavenging activity

Results on DPPH scavenging activity of TRF, PB, MC and CV extracts at varying concentrations ranging from 0 to 1000 µg/ml are shown in Fig. 1. A significant DPPH radical scavenging activity was determined in TRF extract, which act as positive control more than in the BHT (ethanolic standard). TRF was able to reduce the stable free radical DPPH to yellow-coloured, 1-diphenyl-2-picrylhydrazyl. PB extract exhibited 50% reduction of free radical at concentration 300µg/ml. MC and CV showed significant increased in scavenging activity only at high concentrations.

Cytotoxicity studies

Data from the MTS assay revealed that treatment of neuron cultures with TRF, PB and MC at for 24 hours resulted in concentration-dependent reduction in cell viability (Fig. 2). However no significant decrease in neuron cell viability was observed in the group treated with CV at concentration range between 200 to 1000µg/ml. The extracts of PB and MC significantly decreased the number of viable cells when the concentrations used were > 100 µg/ml and > 500µg/ml, respectively.

Neuroprotection studies

Neuroprotective effects of plant extracts against BSO-induced cell death were evaluated by the MTS assay. Neuron cultures were either pre- or post-treated with TRF, PB, MC or CV for 24 hours, followed or preceded by BSO for 72 hours. From the result, post-treatment with PB, MC and TRF (control) showed a significant increase in the number of viable cells compared to the cells exposed to BSO only. This neuroprotective effect was

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concentration-dependent with the greater neuroprotective potential at lower concentrations. Result also showed that post-treatment yielded a significantly better protective ability than pre-treatment. Post-treatment with TRF and PB extracts protected neurons even at ng/ml concentrations (Fig.3) while MC exerts protective effects at 50 - 100 ug/ml. CV extracts did not show any significant protective effect when used either in pre or post-treatment.

Discussion

Oxidative stress has been implicated in the pathology of degenerative diseases. Antioxidants may offer resistance to oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and thus prevent the onset of neurodegeneration. Apart from endogenous antioxidants, our body requires supplement of dietary antioxidants. In this study, we explored selected plant antioxidants for its potential in preventing neurodegeneration and associate it with its capability as antioxidants.

DPPH assay is a widely used method for screening antioxidant activity of plant extracts. The solution progressively reduced to a yellow coloured product, diphenylpicryl hydrazine, with the addition of the extracts in a concentration-dependent manner [32]. The present results clearly indicate that TRF and plant extracts possess free radical scavenging activity at different potencies (Fig 1). At 500µg/ml dose, TRF has the highest free radical scavenging capability followed by PB, MC and CV.

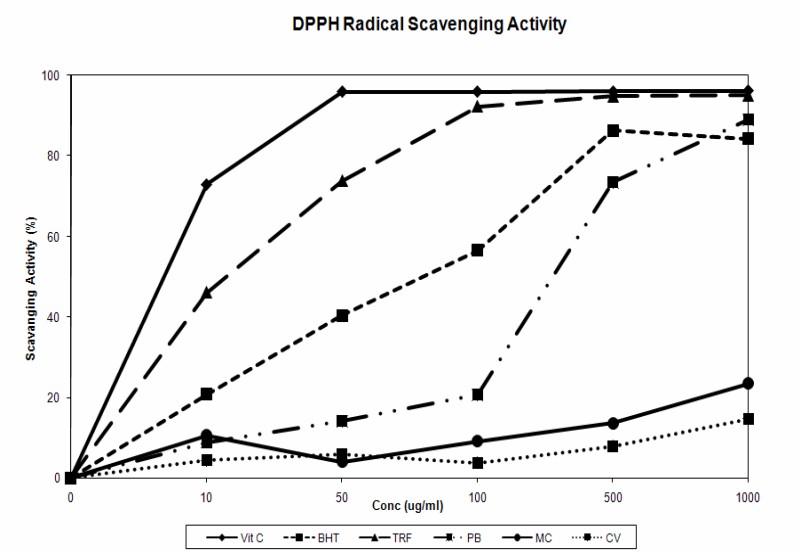


Fig. 1- DPPH radical scavenging activity of TRF, PB, MC and CV. 20µg/ml of DPPH solution was added to TRF, PB, MC and CV at a ratio of 1:1 before 30 minutes incubation at room temperature. Vitamin C and BHT represent DPPH standards, while TRF acts as positive control. TRF exhibit a stronger scavenging activity compared to BHT, followed by PB, MC and CV extracts. Data is presented as means ± SD, *n*=9

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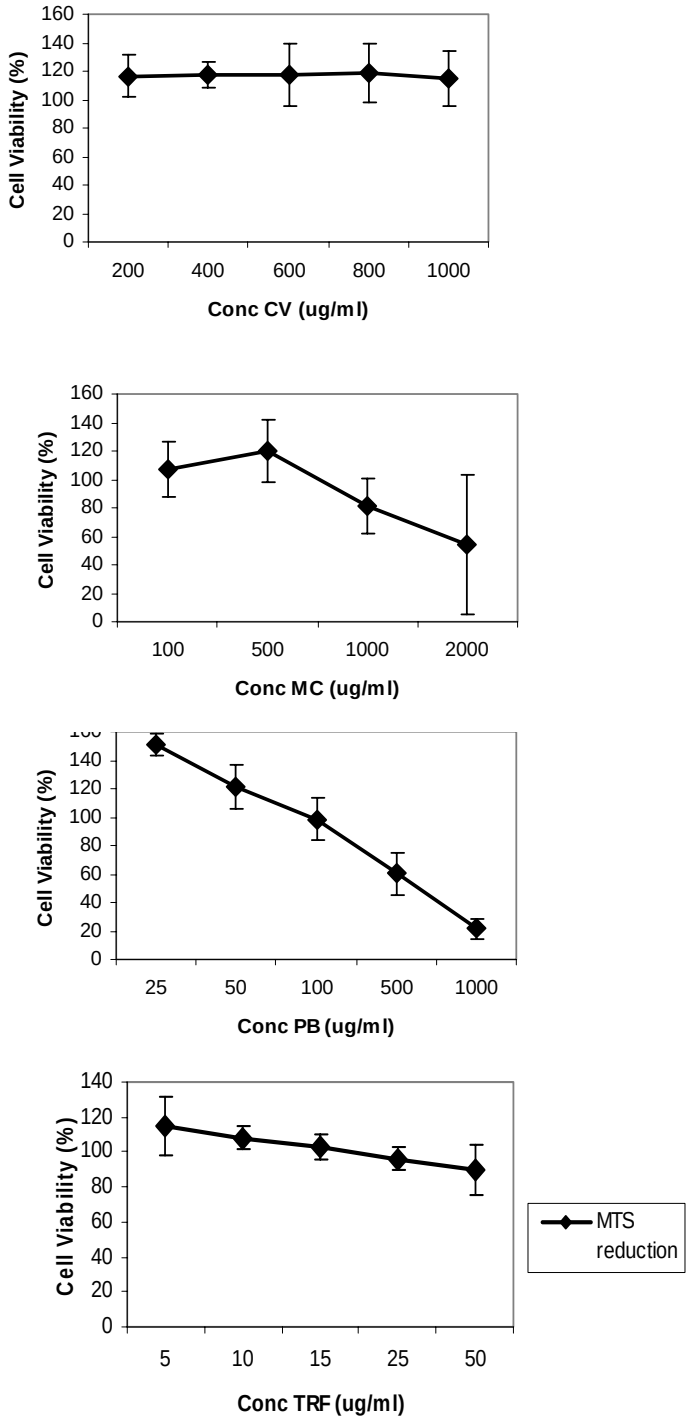


Fig. 2- Cytotoxicity of TRF, PB, MC and CV on neuroblastoma SH-SY5Y cells by MTS Cell Proliferation assay. Percentage of viable cells is proportional to MTS reduction. Cells were incubated at various concentrations of TRF, PB, MC and CV for 24 hours at 37°C. CV was non-toxic to neurons up to 1000 µg/ml. There was a sharp decreased in cell viability when incubated with PB and MC extracts at concentrations ≥ 50 g/ml and ≥500 µg/ml, respectively. TRF was non-toxic up to 50µg/ml. Data is presented as means ± SD, *n*=9. * is significant compared to control ($p<0.05$)

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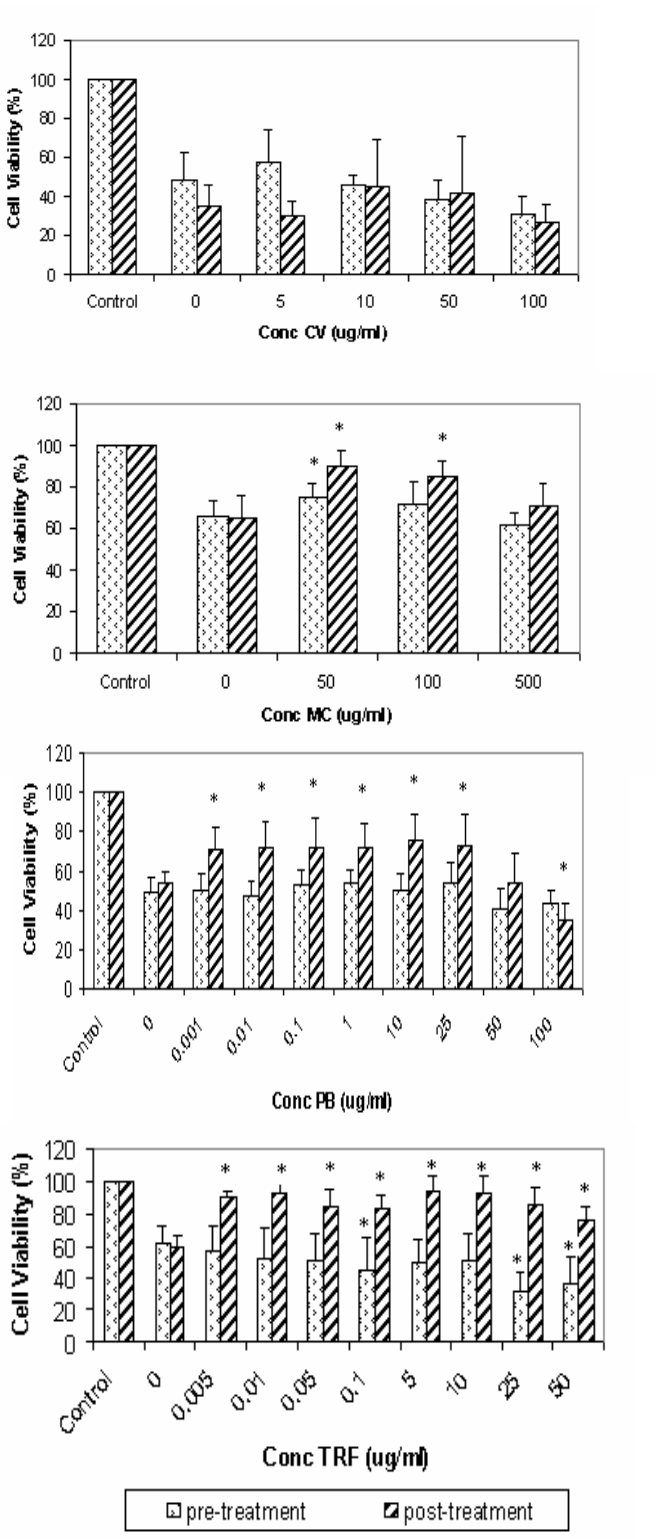


Fig. 3 - Protective effects of TRF, PB, MC and CV against BSO-induced cell death in neuroblastoma SH-SY5Y culture. Cells were pre-treated or post-treated with TRF, PB, MC and CV at different concentrations for 24 hours before or after exposure to BSO at 37°C. Cell viability was determined using the MTS Cell Proliferation assay. Cells post-treated with TRF, PB, and MC showed higher neuroprotection capability compared to pre-treated groups. Neither pre nor post-treatment CV extract showed significant increment in protecting cell viability. Data is presented as means ± SD, *n*=9.* denotes p<0.05 compared to IC_{50} BSO concentration

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The result on TRF is not surprising as tocotrienol and tocopherol are well-known as chain-breaking antioxidants that prevent the propagation of free radical reactions [33, 34].

The beneficial effect of plants as antioxidants is widely known and has been recommended to prevent a variety of degenerative diseases including neurodegeneration. However, little studies have been reported on the cytotoxic effects of these plant extracts on the neurons. Our previous study had shown that tocotrienol is toxic to neurons at concentration >100μM [7]. The present data thus confirmed that the concentration range of TRF used was not toxic. The plant extracts tested revealed that PB was most toxic followed by MC and lastly CV. Interestingly, this was similar to the ranking of antioxidant activity potency of the plant extracts. It appears that plant with higher antioxidant potential may be more likely to act as pro-oxidants and induce cell damage and death.

The present study showed, for the first time, that PB and MC are cytotoxic to neuron cells. It must be stressed that the above findings were determined in *in vitro* model. The *in vivo* situation may be affected by many other confounding factors such as absorption, metabolism and distribution of the active compounds which thus affect its bioavailability. For example, Virdi et al. [35] reported that low doses of MC ingested in rats were non-toxic to nephron and liver cells for up to 2 months. Other studies revealed that oral supplementation of PB extracts to mice (75mg/kg) and rats (200 mg/kg) were not toxic [20, 36]. Although earlier reports using animal model had shown PB not cytotoxic, the dose used in these studies may be too low. Thus the present data indicate the need to establish safe dose for these plant extracts - taking into account the bioavailability factors - as these plants are regularly and commonly consumed.

The present data indicate that although all the plants studied exhibited antioxidant properties, not all could exert the neuroprotection effect against BSO-induced cell death. BSO induced GSH depletion which causes ROS overproduction that leads to cell death. [37]. The result on TRF confirmed the neuroprotective effects of vitamin E as were reported earlier [5, 7, 38, 39, 40]. The data also showed that PB, which exhibited the highest scavenging activity, was also most potent at exerting neuroprotection (Fig.3) followed by MC. However pre-treatment or post-treatment of cells with CV was not able to prevent cell death induced by BSO.

It is interesting to note that although MC and CV which exhibited almost similar antioxidant potential, they were not equally neuroprotective. Although the present data demonstrated that the neuroprotective potential correlated with the antioxidant capacity, this may not be the only mechanism involved in preventing oxidative stress induced neuron cell death. Other mechanisms which may be involve in neuroprotection include induction of gene expression especially genes involved in signal transduction pathways. Thus more studies are needed to elucidate the mechanism of neural protection against oxidative stress.

In conclusion, the present findings showed the plant antioxidants exert neuroprotective and neurocytotoxic effects which correlate with their free radical scavenging potential. The dual effect of the antioxidants are concentration dependent – being neuroprotective at lower concentration, but beoming cytotoxic at high concentrations.

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