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Abstract

Age is the leading risk factor for many of the most prevalent and devastating diseases including neurodegenerative diseases. A number of herbal medicines have been used for centuries to ameliorate the deleterious effects of ageing-related diseases and increase longevity. Oxidative stress is believed to play a role in normal ageing as well as in neurodegenerative processes. Since many of the constituents of herbal extracts are known antioxidants, it is believed that restoring oxidative balance may be one of the underlying mechanisms by which medicinal herbs can protect against ageing and cognitive decline. Based on the premise that astrocytes are key modulators in the progression of oxidative stress associated neurodegenerative diseases, 13 herbal extracts purported to possess anti-ageing properties were tested for their ability to protect U373 human astrocytes from hydrogen peroxide induced cell death. To determine the contribution of antioxidant activity to the cytoprotective ability of extracts, total phenol content and radical scavenging capacities of extracts were examined. *Polygonum multiflorum*, amongst others, was identified as possessing potent antioxidant and cytoprotective properties. Not surprisingly, total phenol content of extracts was strongly correlated with antioxidant capacity. Interestingly, when total phenol content and radical scavenging capacities of extracts were compared to the cytoprotective properties of extracts, only moderately strong correlations were observed. This finding suggests the involvement of multiple protective mechanisms in the beneficial effects of these medicinal herbs.

Keywords

Oxidative stress, Antioxidants, Traditional Chinese Medicine, Cytotoxicity, Cytoprotection, CMMB

Disciplines

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**Cytoprotective properties of traditional Chinese medicinal
herbal extracts in hydrogen peroxide challenged human U373
astroglia cells**

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Abstract

Age is the leading risk factor for many of the most prevalent and devastating diseases including neurodegenerative diseases. A number of herbal medicines have been used for centuries to ameliorate the deleterious effects of ageing-related diseases and increase longevity. Oxidative stress is believed to play a role in normal ageing as well as in neurodegenerative processes. Since many of the constituents of herbal extracts are known antioxidants, it is believed that restoring oxidative balance may be one of the underlying mechanisms by which medicinal herbs can protect against ageing and cognitive decline. Based on the premise that astrocytes are key modulators in the progression of oxidative stress associated neurodegenerative diseases, 13 herbal extracts purported to possess anti-ageing properties were tested for their ability to protect U373 human astrocytes from hydrogen peroxide induced cell death. To determine the contribution of antioxidant activity to the cytoprotective ability of extracts, total phenol content and radical scavenging capacities of extracts were examined. *Polygonum multiflorum*, amongst others, was identified as possessing potent antioxidant and cytoprotective properties. Not surprisingly, total phenol content of extracts was strongly correlated with antioxidant capacity. Interestingly, when total phenol content and radical scavenging capacities of extracts were compared to the cytoprotective properties of extracts, only moderately strong correlations were observed. Total phenol content and antioxidant activity of extracts are moderately correlated with their cytoprotective ability, suggesting the involvement of multiple protective mechanisms in the beneficial effects of medicinal herbs.

Keywords

Oxidative stress

Antioxidants

Traditional Chinese Medicine

Cytotoxicity

Cytoprotection

1. Introduction

For millennia, herbal medicines consisting of whole herbs or plant parts such as leaves, stems, roots and seeds have been used in traditional Chinese medicine (TCM) for the treatment of specific ailments, to maintain and restore body balance and to increase longevity (Suk, 2005). Many of the drugs available in Western medicine (WM) have been directly isolated from plants or semi-synthetic molecules based on the molecular scaffolds of natural products. While WM mainly focuses on the identification and isolation of single active constituents from plants that interact with single therapeutic targets, TCM aims to reverse the underlying “imbalance” between the body and the environment that is thought to cause disease (Cheng, 2000). This often involves the use of complex mixtures of herbs containing multiple chemical groups and compounds with diverse biological and pharmacological actions. It is believed that compounds not only act synergistically with other compounds from the same plant, but also may enhance the activity or counteract the toxicity of compounds from other plants (Howes and Houghton, 2003). A recent study, investigating the antioxidant properties of an extensively used herb in TCM, *Polygonum multiflorum*, found that the radical scavenging abilities of two of its main active components, emodin and quercetin, were lower than crude leaf, stem and root extracts of the herb (Lin et al., 2010). Furthermore, while studies with traditional herbal medicines such as Fuzhisan (Bi et al., 2011) and herbal extracts such as *Panax ginseng* (Lee et al., 2008) have shown success in slowing cognitive decline; results of clinical trials with single “active” compounds, such as curcumin from *Curcuma longa* (Hamaguchi et al., 2011) and huperzine A from *Huperzia*

serrata (Rafii et al., 2011) have not been successful. Alzheimer's disease (AD) is a complex, multifactorial neurodegenerative disease, which starts years or decades before the onset of symptoms. Oxidative stress has long been implicated in the pathogenesis of AD (Markesbery, 1997) and is also believed to play a role in ageing, which is the key risk factor for AD (Finkel and Holbrook, 2000). Since many of the constituents of herbal extracts are known antioxidants, it is believed that restoring oxidative balance may be one of the underlying mechanisms by which medicinal herbs can protect against ageing and cognitive decline (Ho et al., 2010). While there is currently no cure, the purported success of herbal remedies against AD may be due to their inherent multi-compound, multi-targeting nature.

Astrocytes are important determinants of oxidative stress in the brain due to their roles in storage of glutathione (GSH) and delivery of substrates for neuronal GSH synthesis, and their ability to produce a wide variety of pro- and anti-inflammatory mediators and reactive oxygen species (ROS) (Dringen et al., 2000). Although astrocytes are generally regarded as being much better than neurons at defending themselves against oxidative stress (Desagher et al., 1996; Dringen et al., 1999), it has been shown that prolonged inflammatory activation can induce a state of oxidative stress (Gavillet et al., 2008; Malaplate-Armand et al., 2000). Furthermore, induction of oxidative stress in astrocytes might also be detrimental to neurons and therefore present a potential therapeutic target for the prevention of inflammation or oxidative stress related neurodegeneration.

The aim of this study was to investigate thirteen TCM herbal extracts, shown in Table 1, for their ability to protect U373 human astrocytoma cells from

hydrogen peroxide induced cell death. Herbs were selected based on their history of use as cognitive-enhancing and longevity promoting agents or their inclusion in tonic herbal remedies. Tonic herbs are also called “adaptogens” and have been described as *“remarkable natural substances that help the body adapt to stress, support normal metabolic functions, and restore balance. They increase the body’s resistance to physiological, biological, emotional and chronic stress”* (Winston and Maimes, 2007). In order to determine the contribution of antioxidant activity to the cytoprotective ability of extracts, total phenol content (Singleton and Rossi, 1965; Waterhouse, 2002), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (Blois, 1958; Molyneux, 2004) and oxygen radical absorbance capacity (ORAC) (Ganske and Dell, 2006) were also examined, and correlated.

2.1 Materials and Methods

2.1 Materials

The U373-MG human astrocytoma cell line was kindly provided by Dr Peter Locke (The Royal Melbourne Hospital, Australia). All cell culture materials were from Invitrogen (Mulgrave, Australia). Solvents (AR grade), (2,2-diphenyl-1-picryl hydrazyl (DPPH), gallic acid, fluorescein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), Folin-Ciocalteu reagent, sodium carbonate and resazurin were from Sigma-Aldrich (Castle Hill, Australia). Elga Pure Lab Prima 7 water purification unit was the source of water (>18.2 M) (Scoresby, Australia).

2.2 Plant extracts

Commercial herb preparations (Table 1) were provided by LIPA Pharmaceuticals (Minto, Australia). Traceability of each extract to its authenticated starting herb (by a systematic botanist) was carried out at LIPA Pharmaceuticals (QC System).

2.3 Extraction of samples

Sample extraction involved sonication of 5 g of powdered dried material in approximately 50 ml 80% aqueous methanol for 2 x 30 minutes with a 15 minute cooling interval between sonications. The mixture was centrifuged at 4000 g for 5 minutes and the supernatant was filtered using a polyvinylidene fluoride (PVDF) syringe filter. The particle free filtrate was concentrated to dryness in a rotary evaporator at 60°C under vacuum. The residue was

further freeze dried for 12 hours to remove any residual water and stored at 4°C.

2.4 Folin-Ciocalteu Reagent (FCR) assay for the determination of total phenols

The method of Singleton *et al.* and Waterhouse *et al.* using gallic acid as the reference standard, was adapted for the determination of total phenols by the Folin-Ciocalteu reagent (FCR) assay (Singleton, 1981; Singleton and Rossi, 1965; Waterhouse, 2002). All reagents were prepared in 80% aqueous methanol. The gallic acid standard curve was made by diluting a gallic acid stock (3 mM) to form 0.3, 0.6, 0.9 and 1.5 mM working standards. Samples were prepared by dissolving 1 mg of the extract in 10 ml. Serial dilutions (1 in 10 and 1 in 100) of the stock sample solution were also prepared. An 80% aqueous methanol solution was used as the reagent blank. 140 µl of water, 10 µl of Folin-Ciocalteu reagent, 20 µl of sample, standard or blank and 30 µl of sodium carbonate (0.7 M) were added to wells of a 96 well plate. The plate was vortexed briefly and incubated for 30 minutes in the dark prior to measurement of absorbance at 765 nm (POLARstar OPTIMA; BMG). The total phenol content for each herb is reported as the gallic acid equivalent.

2.5 DPPH radical scavenging assay

A method adapted from Blois et al. and Molyneux et al. was used to estimate the DPPH radical scavenging capacity of the 13 extracts compared to a gallic acid standard (Blois, 1958; Molyneux, 2004). All reagents were prepared in 80% aqueous methanol. The gallic acid standard curve was made by diluting a gallic acid stock (6 mM) to form 0.3, 0.6, 1.5, and 3 mM working standards. Samples were prepared by dissolving 100 mg of the extract in 10 ml. Serial dilutions (1 in 10 and 1 in 100) of the stock sample solution were also prepared. An 80% aqueous methanol solution was used as the reagent blank. 180 μ l of the DPPH reagent (250 μ M) was applied to each well of a 96 well plate. In triplicate, 20 μ l of each working standard, sample or blank was added to the DPPH reagent to make a total volume of 200 μ l in each well. To correct for sample absorbance (i.e. absorbance not due to the DPPH), sample blanks were made in triplicate by adding 180 μ l of 80% aqueous methanol to the well and adding 20 μ l of sample. The plate was shaken at 700 rpm for 30 minutes in the dark prior to measuring absorbance at 515 nm (POLARstar OPTIMA; BMG). The sample antioxidant scavenging capacity is reported as the gallic acid equivalent.

2.6 Oxygen Radical Absorbance Capacity assay

The oxygen radical absorbance capacity (ORAC) assay was used to measure the ability of the 13 extracts to protect fluorescein from degradation by peroxy radicals. A modified version of the method described in the BMG LABTECH application note was used (<http://www.bmglabtech.com>). All reagents were prepared in pH 7.4 phosphate buffer (10 mM). A gallic acid

standard curve was made by diluting a gallic acid stock (3 mM) to form 0.3, 0.6, 0.9 and 1.5 mM working standards. Samples were prepared by dissolving 2 mg of extract in 10 ml of 80% aqueous methanol. An 80% aqueous methanol solution was used as the reagent blank. 150 µl of fluorescein (10 nM) and 25 µl of either gallic acid standard, sample or blank were mixed in wells of a 96 well plate. Plates were vortexed briefly and incubated at 37°C for 30 minutes. Following incubation 25 µl of the radical generator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 240 mM) was rapidly added to each well. Fluorescence was determined every 80 seconds for 2 hours, with excitation at 485 nm and emission at 520 nm (POLARstar OPTIMA; BMG). The area under the signal degradation curves of the samples were compared to the gallic acid standard and the results expressed as gallic acid equivalents.

2.7 Cell maintenance

Cells were maintained in Dulbeccos's Modified Eagle Medium (DMEM) containing 25 mM glucose, supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, 2.6 µg/ml Fungizone, 200 mM glutamine and 5% foetal bovine serum (FBS). Cells were grown in 175 cm² tissue culture flasks and incubated at 37°C in 5% CO₂. For experiments, cells were harvested with a solution containing 0.025 mM trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS), plated in 96 well plates at a density of 9 x 10³ cells/well and incubated for 24 hours before treatment.

2.8 Cell based assays

For cell-based experiments, extracts were dissolved in dimethyl sulfoxide (DMSO) to give a stock concentration of 100 mg/ml. Extracts were then diluted in DMEM (1% FBS) to a concentration of 1000 µg/ml and filtered using a PVDF syringe filter before immediate use. To determine the cytotoxicity herbal extracts were diluted in DMEM (1% FBS) to give concentrations from 3.9 – 1000 µg/ml and applied to U373 cells for 24 hours treatment. Cell viability was then determined as described below. GraphPad Prism was used to calculate the 50% lethal concentration (LC₅₀) values where appropriate and enable selection of non-toxic concentrations of herbextracts for assessment of their cytoprotective properties.

2.9 Cell viability assay

For determination of cell viability, media was aspirated from wells and 100 µl of resazurin solution (0.001% resazurin in PBS) was added. Plates were incubated at 37°C, with 5% CO₂ for 45 minutes and then fluorescence was measured at 530nm/590nm (excitation/emission) by a microplate reader (POLARstar OPTIMA; BMG). For every plate, background fluorescence determined in cell-free wells was subtracted from all wells; and values were expressed as a percentage of untreated control cells (Buranrat et al, 2008).

2.10 Treatment of U373 cells with hydrogen peroxide

U373 cells were treated with solutions containing 7.8 - 200 µM hydrogen peroxide in DMEM with 1% FBS for 24 hours. Cell viability was then

assessed by the resazurin reduction assay described above and values expressed as a percentage of untreated control cells.

2.11 Determination of cytoprotective effects of herbal extracts against hydrogen peroxide toxicity

Herbal preparations dissolved in DMEM (1% FBS) were applied to U373 cells for 12 hours incubation. Media was then removed and replaced with 100 μ l of a solution containing 80 μ M hydrogen peroxide in DMEM with 1% FBS. Following 24 hour hydrogen peroxide treatment of cells, cell viability was assessed by the resazurin reduction assay described above. Data are expressed as percentage of untreated control cells and GraphPad Prism was used to calculate the concentration effective at returning cell viability to 50% (EC_{50}) where appropriate.

2.12 Statistics

The mean and standard error of the mean (SEM) values were calculated from two or three (as indicated in the respective figure legend) independent experiments (performed in duplicate) for each analysis. Graphpad Prism was used to calculate the EC_{50} and LC_{50} values using the sigmoidal dose-response function and relationships between antioxidant capacity, cytotoxicity and cytoprotective data were assessed by calculation of Spearman's correlation coefficients.

3. Results

3.1 Effect of herbal extracts on cell viability

U373 cell viability was assessed using the resazurin assay and LC₅₀ values were calculated for extracts that resulted in a decrease in cell viability of 50% or greater. Results are shown in Figure 1A and B, and LC₅₀ values are reported in Table 2. The extracts displayed a large variation in cytotoxicity, with LC₅₀ values ranging from 35.4 ± 1.2 µg/ml for *P.multiflorum* to > 1000 µg/ml for *A. membranaceus*, *C. pilosula* and *R. glutinosa*. *L. barbarum* and *P. ginseng* treatments resulted in an increase in cell viability in response to concentrations up to 250 µg/ml, indicating a pro-proliferative effect of these extracts.

3.2 Effect of hydrogen peroxide treatment on U373 cells

Hydrogen peroxide induced death of U373 cells was investigated in order to establish a cell culture based oxidative stress model for assessment of the cytoprotective properties of herbal extracts. Cell viability was assessed using the resazurin assay and results are shown in Figure 3. A dose-dependent decrease in cell viability was measured, with an LC₅₀ value of 87.0 µM and a 95% confidence interval of 81.1 to 93.3 µM ($R^2 = 0.974$). Therefore, a concentration of 90 µM hydrogen peroxide was selected for investigating the cytoprotective abilities of extracts.

3.3 Cytoprotective effect of herbal extracts

In order to analyse the cytoprotective effects of herbal extracts, U373 cells were supplemented with non-toxic concentrations of extracts for 12 hours, after which extract-containing media was replaced with media containing 90 μ M hydrogen peroxide. After 24 hours hydrogen peroxide treatment, cell viability was assessed using the resazurin assay and EC₅₀ values were calculated for extracts capable of maintaining cell viability to greater than 50% of non-hydrogen peroxide treated control cells. Results are shown in Figures 4 and 5 and EC₅₀ values are reported in Table 2. As with cytotoxicity, a large variation in the cytoprotective ability among herb preparations was observed. *P. multiflorum*, *R. rosea* and *S. chinesis* exhibited the strongest protection against hydrogen peroxide induced cell death, with EC₅₀ values of < 0.2, < 0.5 and 1.4 ± 0.7 μ g/ml, respectively. In contrast, *P. ginseng* was less cytoprotective with an EC₅₀ of 66.6 ± 10.4 μ g/ml; *P. cuspidatum* was not cytoprotective at concentrations from 0.1 to 3.9 μ g/ml. Although in the initial cytotoxicity screening the maximal concentrations selected were non-toxic, in this experiment some reductions in cell viability were measured at the highest concentration for most extracts. This might have been due to the addition of hydrogen peroxide or to the extended incubation time (12 hour pre-incubation with or without extract, followed by 24 hour incubation with or without hydrogen peroxide). DMSO up to concentration of 0.5 % did not show any protection against hydrogen peroxide induced cell death, as well as no reduction of cell viability in the absence of hydrogen peroxide (Fig. 3B).

In addition, the therapeutic index for each extract was calculated by dividing the LC₅₀ for an extract by its determined EC₅₀ (Table 2). A higher therapeutic

index is preferable to a lower one, since having little difference between toxic and therapeutic concentrations of a drug is undesirable and lead to difficulties in effective dosage determination *in vivo*. In this cell based model of oxidative stress induced death, *P. multiflorum*, *S. chinensis* and *L. barbarum* were identified as having the largest therapeutic indices of > 177.0, 83.8 and > 73.0, respectively.

3.4 Total phenol content of herbal extracts

Total phenol content was determined by the Folin-Ciocalteu reagent assay for each extract and normalised against a gallic acid standard curve enabling results to be presented as the gallic acid equivalent (mmol/g GAE). The results are shown in Table 2. *R. rosea*, *P. cuspidatum* and *P. multiflorum* showed the highest phenol contents with values of 5.55 ± 0.55 , 2.57 ± 0.10 and 1.75 ± 0.02 mmol/g GAE, respectively. In contrast, *P. ginseng* had the lowest phenol content with 0.06 ± 0.00 mmol/g GAE.

3.5 Determination of antioxidant capacity of extracts

In order to investigate whether the cytoprotective properties of herbal extracts might be due to their free radical scavenging ability, results from two assays reflecting different antioxidant properties were analyzed. Antioxidant capacity of herbal extracts was determined by DPPH and ORAC assays. The results are summarised in Table 2. The DPPH assay is an electron transfer based assay and measures the reducing capacity of samples, using copper as an oxidant (Huang et al., 2005). Results from this assay were well aligned with

those from the FCR assay identifying *R. rosea*, *P. cuspidatum* and *P. multiflorum* as the most potent extracts with values of 4.20 ± 0.05 , 3.28 ± 0.01 and 2.56 ± 0.02 mmol/g GAE, respectively. The ORAC assay, on the other hand, is a hydrogen atom transfer based assay and measures radical chain-breaking capacity, involving peroxy radicals as the oxidant (Huang et al., 2005). Results from the ORAC assay were also well aligned with the results from the FCR and DPPH assays except that *R. rosea* was less effective in this assay. *P. cuspidatum*, *P. multiflorum* and *S.chinesis* were the most effective with values of 1.30 ± 0.01 , 1.25 ± 0.01 and 1.19 ± 0.05 mmol/g GAE, respectively.

3.6 Relationship between antioxidant activity and cytoprotective ability of herbal extracts

In order to investigate the relationship between total phenol content, antioxidant capacity (determined by the DPPH and ORAC assays), cytoprotective ability and cytotoxicity of extracts, results from the different assays were plotted against each other (Figure 6) and correlation coefficients (r) determined using the non-parametric Spearman's Rank Order Correlation test (Table 3). Total phenol content (FRC assay) showed a strong positive correlation with antioxidant capacities measured by the DPPH ($r = 0.808$; $p < 0.001$) and ORAC ($r = 0.791$; $p = 0.001$) assays. DPPH and ORAC measurements were also strongly and positively correlated ($r = 0.780$; $p = 0.002$).

To test whether there was a relationship between the antioxidant capacity of extracts, and their cytoprotective ability in a cell based oxidative stress model, results from the FCR, DPPH and ORAC assays were compared to the determined EC₅₀ values. Moderate to strong negative correlations were observed between cytoprotection and total phenol content, as determined by the FCR assay ($r = -0.699$; $p = 0.011$); and between cytoprotection and antioxidant activity, as measured by the ORAC assay ($r = -0.643$; $p = 0.024$). As phenol content and ORAC activity of extracts increased, the concentration of extract needed to protect 50% of cells decreased. No significant correlation was observed between cytoprotection and DPPH antioxidant activity.

Correlation between antioxidant activity and the cytotoxicity of extracts was also examined. Strong negative correlations were observed between the LC₅₀ values of each extract and total phenol content ($r = -0.741$; $p = 0.004$), DPPH ($r = -0.791$; $p = 0.001$) and ORAC ($r = -0.897$; $p < 0.001$). A strong positive correlation was observed between LC₅₀ and EC₅₀ values ($r = 0.733$; $p = 0.007$) indicating the extracts that showed the best cytoprotection at low concentrations (low EC₅₀ values) were the most toxic when used at high concentrations.

4. Discussion

Thirteen TCM herbal preparations purported to possess anti-ageing properties were evaluated for their ability to protect U373 cells from hydrogen peroxide induced cell death. Total phenol contents and antioxidant capacities of extracts were also investigated by FCR, ORAC and DPPH radical scavenging assays. *P. multiflorum*, *R. rosea* and *S. chinensis* were amongst the most effective extracts in both the antioxidant and cytoprotection assays. Interestingly, *P. cuspidatum* was the most effective in FCR, DPPH and ORAC antioxidant assays, yet it appeared to have no protective effect towards hydrogen peroxide treated U373 cells. Both *P. cuspidatum* and *P. multiflorum* belong to the Polygonaceae family and have been valued for their anti-inflammatory, anti-pyretic, hypotensive, antioxidant and anticancer properties for centuries (Unschuld, 1986). *P. multiflorum* is especially well known for its reputed anti-ageing benefits and is believed to be longevity promoting when consumed daily for an extended period (Bensky et al., 2004). *P. cuspidatum* elaborates trans-resveratrol, (a stilbene) and emodin, physicon, chrysophanol, and polydatin (hydroxyanthraquinones) while *P. multiflorum* produces 2,3,5,4'-tetrahydroxystilbene-2-O- β -D-glucoside emodine and physicon (Frederich et al., 2011). In the present study, *P. cuspidatum* extract was standardised to contain $\geq 50\%$ trans-resveratrol, and the *P. multiflorum* extract is not standardised. Trans-resveratrol is known to be an active antioxidant and hence, most *P. cuspidatum* commercial extracts are standardised to contain specific levels of trans-resveratrol. Reported concentrations of trans-resveratrol determined in crude extracts of *P. cuspidatum* have ranged from 0.08% to 0.18% (Chu et al., 2005; Zhao et

al., 2005). The *P. cuspidatum* extract used in this study is enriched to contain at least 270 times the amount of *trans*-resveratrol normally encountered in non-standardised extracts of *P. cuspidatum*. The levels of *trans*-resveratrol in samples tested for their cytoprotective effects against hydrogen peroxide treated U373 cells is calculated to be 0.2 - 6.6 μM . Pure *trans*-resveratrol (>99%) was investigated for its cytoprotective ability in hydrogen peroxide challenged C6 glioma cells by Quincozes-Santos and colleagues (2007). Interestingly, they observed that a one hour pre-incubation with 10 μM *trans*-resveratrol could protect against cellular DNA damage induced by a high concentration of hydrogen peroxide (1 mM) during an acute, thirty minute treatment but that this protection was abolished when a lower concentration, six hour treatment (0.1 - 0.5 mM H_2O_2) was administered (Quincozes-Santos et al., 2007). Protection returned however, when cells were pre-incubated with *trans*-resveratrol at concentrations > 50 μM , suggesting that *trans*-resveratrol, and possibly *P. cuspidatum*, may be less effective at protecting against low intensity, chronic oxidative stress than moderate, acute oxidative stress. *Trans*-resveratrol, can also act as a pro-oxidant itself and was shown to cause DNA damage in C6 glioma cells, when used at a concentration of 250 μM for more than 12 hours. These findings might explain the high antioxidant capacity of *P. cuspidatum* (chemical assays), yet not being cytoprotective against extended (24 hour) hydrogen peroxide treatment in U373 cells. Another interesting observation is the reported potent anticancer properties of *trans*-resveratrol and *P. cuspidatum* (Kimura and Okuda, 2001). Both the U373 cells used in this study, as well as the C6 cells used by Quincozes-Santos et al. (2007) are tumour cells and therefore may be

particularly vulnerable to *P. cuspidatum*. *P. multiflorum* extract conversely was equally effective in antioxidant assays and was highly cytoprotective with the highest therapeutic index. *P. multiflorum* was protective against cognitive deficits in mice injected with amyloid β peptide 25-35 (Um et al., 2006) and led to improved learning and memory ability and reduced pathological changes in the brains of senescence accelerated mice (Chan et al., 2003), possibly due to its antioxidant properties.

To investigate the relationship between antioxidant capacity of extracts and cytoprotection, correlation coefficients were calculated. Strong correlation was observed between total phenol content and antioxidant capacity, determined by the DPPH and ORAC assays. Highly linear relationships between total phenol content and antioxidant activity in some medicinal herbs have been reported earlier (Cai et al., 2004; Zheng and Wang, 2001). Plant phenolic acids and flavonoids, have been implicated as natural antioxidants in fruits and vegetables (Kahkonen et al., 1999). However, when total phenol content and ORAC values for extracts were compared to the cytoprotective abilities of extracts, moderate negative correlations were observed. Correlation between DPPH scavenging ability and cytoprotection was not significant. It has been reported that the ORAC method tends to mimic the antioxidant activity of phenolics in biological systems better than the DPPH radical (Cao et al., 1993) since ORAC utilizes biologically relevant free radicals (peroxyl radicals). DPPH is a long-lived, nitrogen radical, bearing no resemblance to the highly reactive peroxyl radicals involved in lipid peroxidation in cells (Huang et al., 2005). Although antioxidant activity of extracts contributes to the cytoprotective effect of extracts, there may be

other non-radical scavenging related mechanisms involved in protection of cells against hydrogen peroxide toxicity. These include anti-apoptotic (Jawan et al., 2003) and anti-inflammatory effects (Li et al., 2003), enhancement of mitochondrial ATP generation (Ko et al., 2006) and induction of Phase II detoxification genes, including glutathione related genes, superoxide dismutase, catalase and heme oxygenase-1 (Lavoie et al., 2009; Li, 1991; Surh, 2008). In addition, the importance of synergism among different compounds has also been demonstrated (Andrews et al., 2007; Li, 1991). An interesting observation was that the extracts that provided the best protection at low concentrations (low EC₅₀ values) were the most toxic at higher concentrations (low LC₅₀ values). This may be due to overall higher assay of constituents (including total phenols) in the active extracts, thus increasing protective efficacy; but also resulting in lower thresholds in terms of toxicity.

In conclusion, total phenol content and antioxidant activity of extracts are correlated with their cytoprotective ability, due to possible involvement of multiple protective mechanisms. Cytoprotection and toxicity were found to be concentration-dependent, indicating low concentrations of synergistic compounds would be a more appropriate treatment option. Extract of *P. multiflorum*, amongst others, was identified as possessing potent cytoprotective properties. Further investigation is required to translate observed cytoprotective effects to protect cells against oxidative stress *in vivo* which may have a bearing on AD and other neurodegenerative and oxidative stress related disorders.

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Figure captions

Figure 1. Cytotoxic effects of Chinese herbal extracts on U373 cells.

Cell viability following treatment with extracts was measured by the resazurin assay and normalized to control, untreated cells. Data shown are mean \pm SEM, n=3.

Figure 2. Effect of hydrogen peroxide treatment on U373 cell viability.

Raw data (A) and log transformed data (B) are shown as percentage of control cells and represented as the mean \pm SEM, n=3. Graphpad Prism was used to fit a sigmoidal dose-response curve to the log transformed data for calculation of LC50 as 87.0 μ M.

Figure 3. Cytoprotective effects of Chinese herbs on U373 cells.

Cell viability following treatment with TCMs was measured by the resazurin assay and normalized to control, untreated cells. Data shown are mean \pm SEM, n=3.

Figure 4. Relationships between antioxidant capacity, cytotoxicity and cytoprotection.

Scatter plots show log total phenols versus DPPH (A) and ORAC (B); log DPPH versus ORAC (C); log total phenols (D), log DPPH (E) and log ORAC (F) versus cytotoxicity; and log total phenols (G), log DPPH (H), log ORAC (I) and cytotoxicity versus cytoprotection.

5. References

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