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Neuroprotective Effect of Peanut Hairy Root Extract Against Oxidative Stress in PC12 Derived Neurons

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Keywords: Arachidin-1, arachidin-3, neuroprotection, resveratrol, stilbenoid

ABSTRACT

Oxidative stress resulting in neurodegeneration in specific areas of the brain leading to disorders such as Parkinson's disease (PD) has been well documented. A specific treatment, however, that can address oxidative stress and thus arrest the progression of PD is unavailable to date. In the present study, using neuronally differentiated rat pheochromocytoma (PC12) cells, a well-established model for studying neuroprotection, we have investigated the protective efficiency of a peanut hairy root extract (PHRE) consisting of resveratrol and other natural resveratrol analogs on hydrogen peroxide (H_2O_2)-induced neurodegeneration. PHRE is a highly defined and stilbenoid enriched product obtained from elicited-peanut hairy root cultures. PC12-derived neurons were treated with 300 µM H_2O_2 to expose them to oxidative stress. The cells underwent marked degeneration as determined by characteristic morphological features of degeneration and lactate dehydrogenase (LDH) assay. Pre-treatment with PHRE containing 15 µM of resveratrol and additional resveratrol analogs attenuated hydrogen peroxide-induced cytotoxicity of PC12 neurons. Even at 20 or 30 µM concentration, however, synthetic/commercial resveratrol alone did not protect the PC12 neurons from the H_2O_2 induced neurodegeneration that matched the level of protection provided by PHRE. Our results suggest that PHRE derived resveratrol along with other constituents in the extract have more potential as neuroprotectant than the synthetic/commercial resveratrol alone to prevent oxidative stress-induced cell death.

INTRODUCTION

The hallmark of Parkinson's disease is the selective and progressive degeneration of the dopaminergic (DA) neurons in the substantia nigra pars compacta (de Lau et al., 2006). Among the many causes including genetic and environmental factors, oxidative stress is reported to be one of the major causes leading to PD (Abou-Sleiman et al., 2006; Jenner and Olanow, 1998). Oxidative stress occurs due to excessive exposure to reactive oxygen species (ROS) which can lead to impaired cellular functions and cell death (Halliwell, 2001). Hydrogen peroxide (H_2O_2) is a common strong oxidant, and is also one of the major reactive oxygen species (ROS) which is used in *in vitro* models to create oxidative stress and toxicity (Stadtman and Berlett, 1991).

The rat pheochromocytoma (PC12) cells can be differentiated into neurons with nerve growth factor (NGF) (Gunning et al., 1981). PC12 cells were used by us to determine if NGF-coupled nanotubes could deliver NGF to these cells and differentiate them into neurons (Xie et al., 2008). PC12-derived neurons have been used routinely as a dopaminergic neuronal model because the differentiated PC12 cells syn-the

size the neurotransmitter dopamine (Fujita et al., 1989) for unraveling the mechanisms underlying neurodegeneration (Shimoke and Chiba, 2001; Venugopalan et al., 2003). PC12-derived neurons have also been used often to screen neuroprotective drugs (Tabakman et al., 2002).

With a significant increase in aging population, there is an urgent need for new drugs to prevent and treat age-related neurodegenerative disorders. Recently, plant derived compounds have been shown to delay or alter the age-related degenerative changes in the brain and hence may provide a new generation of drugs (Ng et al., 2006). One of the promising plant-derived natural compounds is resveratrol, which has diverse functions (Bastianetto and Quirion, 2004; Baur and Sinclair, 2006).

Research on resveratrol started when epidemiological studies showed that there was low incidence of cardiovascular disease in the French population because of their consumption of red wine (Siemann and Creasy, 1992). The beneficial constituents of the red wine were identified as resveratrol and other polyphenols. In addition to preventing cardiovascular diseases, resveratrol has also been reported to protect against cancers, prevent or treat neurodegenerative disorders (Anekonda, 2006; Dore, 2005) and promote anti-aging effects in several organisms (Constant, 1997). For example resveratrol protected PC12 cells and endothelial cells against β-amyloid and oxidized low-density lipoprotein (oxLDL)-induced oxidative injury and apoptosis (Jang and Surh, 2003; Ou et al., 2006).

Animal experiments show that resveratrol protects rats from β-amyloid induced neurotoxicity by reducing iNOS expression and lipid peroxidation (Huang et al., 2011). Resveratrol provides protection against kainic acid induced seizures and oxidative stress in rats (Gupta et al., 2002). Resveratrol has also been shown to protect dopaminergic neurons in midbrain slice cultures against 1-methyl-4-phenyl pyridinium (MPP^+), sodium azide, and thrombin (a microglia activating agent) induced neurodegeneration (Alvira et al., 2007; Okawara et al., 2007). Most studies have used synthetic/commercial forms of resveratrol. Plants, such as peanuts and grapes, that synthesize resveratrol also have the capacity to make derivatives and other analogs in addition to the parent compound.

Hairy roots cultures produced via genetic transformation by *Agrobacterium rhizogenes* are recognized as bioproduction platforms for bioactive specialized metabolites. Because of their genetic and biochemical stability, these biological systems provide an alternative route for sustainable production of high value compounds. Several recent reviews have addressed the multiple applications of hairy roots for mass production of targeted plant metabolites, studying their biosynthetic pathways and identification of novel bioactive compounds from different plant species (Georgiev et al., 2012; Talano et al., 2012). To increase the levels of selected bioactive compounds, hairy root cultures have been treated with biotic and abiotic elicitors (Goel et al., 2011). Indeed, we showed that hairy root cultures of peanut can be treated with abiotic elicitors to induce the biosynthesis of stilbenoids thereby providing a sustainable and reproducible bioproduction system for these polyphenolic compounds (Condori et al., 2010; Medina-Bolivar et al., 2007; Medina-Bolivar et al., 2010).

Resveratrol and several prenylated stilbenoids such as arachidin-1 and arachidin-3 can be produced in this tissue culture bioproduction system (Medina-Bolivar et al., 2010) (Figure 1). Because the constituents are secreted into the culture medium, these polyphenols can be easily purified from the medium with organic solvents or non-polar resins to provide stilbenoid-enriched extracts (Abbott et al., 2010).

A recent report suggested that resveratrol in combination with other plant-derived molecules might be more potent in beneficial effects than resveratrol alone (Radhakrishnan et al., 2011). Importantly, arachidin-1 purified from peanut hairy root cultures has shown higher antioxidant activity than resveratrol *in vitro* (Abbott et al., 2010). Thus a combination of resveratrol and several prenylated stilbenoids such as arachidin-1 and arachidin-3 in the peanut hairy root extract (PHRE) may be more potent than resveratrol alone in providing neuroprotection from oxidative stress.

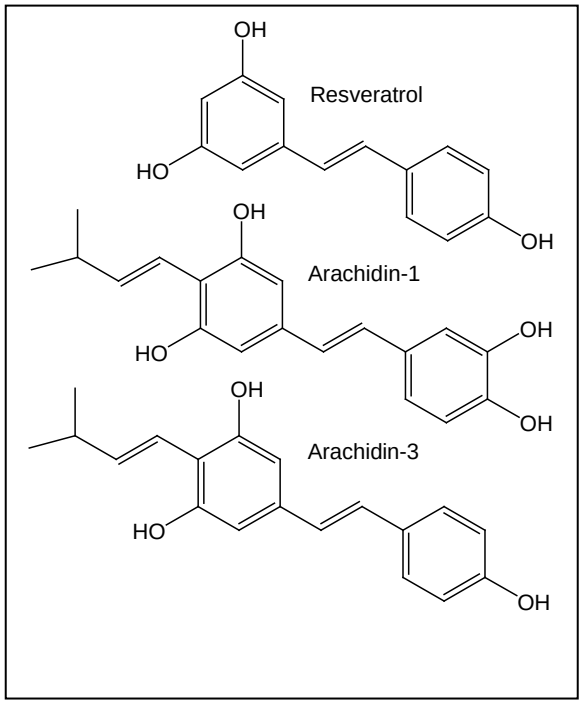

Figure 1. Selected stilbenoids identified in the peanut hairy root extract (PHRE).
Stilbenoids were identified in *trans* configuration as shown.

Therefore, our objective was to determine the neuroprotective efficacy of the PHRE, a natural product which contains resveratrol and other stilbenoids, in comparison to resveratrol alone against oxidative stress in PC12 derived neurons.

MATERIALS AND METHODS

Culture and differentiation of PC12 cells. PC12 cells were obtained from American Type Culture Collection (ATCC), USA. Stock cultures were established and maintained at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with F12 (DMEM/F12; Invitrogen) supplemented with 5% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis), 10% horse serum (HS) (Sigma-Aldrich) as described else-where (Xie et al., 2008). For the experiments in this study, PC12 cells from the stock were plated onto poly-D-lysine and collagen coated 35 mm sterile plastic dishes at a density of 10,000 cells/dish. The cells were maintained in serum-free neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 1 mM glutamax (Invitrogen) 1 mM glutamine (Invitrogen) and antibiotic solution (100 µ/mL penicillin, 100 µg/mL streptomycin, 250 ng/mL amphotericin B, Sigma-Aldrich) in humidified incubator at 5% CO_2 and 37 ^{o}C. NGF (nerve growth factor (75 ng/mL) (Almone Labs, Jerusalem, Israel) was added to cultures to allow cells to differentiate and grow neurites for 8-10 days with a medium change every third day.

Preparation of peanut hairy root extract (PHRE). Hairy roots of peanut cv. Andru II line 2 were cultured in Gamborg's B5 medium (Gamborg et al., 1968) and treated for 24 h with 10 mM sodium acetate to induce the biosynthesis of stilbenoids as described before (Medina-Bolivar et al., 2007). Stilbenoids were extracted from the culture medium with ethyl acetate and concentrated to dryness under a nitrogen stream.

The amount of *trans*-resveratrol in the extract was determined by reverse phase HPLC as detailed before (Condori et al., 2009). *Trans*-resveratrol, which constituted approximately 10% of the total dry weight of the extract, represented one of the major constituents of the extract. Thus, the concentrations of the PHRE in the present experiments were adjusted for the resveratrol content and are expressed as PHRE that contains a defined amount (µM) of resveratrol. In addition to resveratrol, several other stilbenoids, such as arachidin-1 and arachidin-3, were present in the extract.

The dry mass of the PHRE was reconstituted in dimethyl sulfoxide (DMSO) and neurobasal medium. The synthetic/commercially available resveratrol (Sigma-Aldrich) was also used to compare the effects with that of PHRE that had resveratrol as a major constituent along other stilbenoids.

Culture treatments. Once PC12 cells were well differentiated, the medium was replaced with the neurobasal medium without B27 supplement and NGF. The PC12 neurons were pretreated with PHRE containing a 15 µM concentration of resveratrol (based on results from a dose response experiments) or with commercially available resveratrol (20 to 30 µM, Sigma-Aldrich) for 3 h, whereas control cultures only had the medium changed, and vehicle controls were treated with 0.25% (2.5µL/mL) of DMSO. One set of control and all experimental cultures were challenged with exposure to 300 µM H_2O_2 for 4h.

After treatment, the culture medium was collected, centrifuged for 10 min at 16,080 x g (Heraeus Bofuge) at 4 ºC to pellet cell debris. Aliquots of the supernatant were stored at -80ºC for use in a lactate dehydrogenase (LDH) assay, a biomarker used to quantify cell death.

Lactate dehydrogenase (LDH) assay. The LDH assay was done in triplicates using the cytotoxicity detection kit (Roache Diagnostics Corp., Indianapolis, IN) and following instructions provided by the manufacturer for microplate assays (Prabhu et al., 2010). The relative percentages of LDH released in experimental cultures in proportion to that from control cultures was calculated by [A] test/[A] control x 100, where [A] control is the absorbance of the control sample and [A] test is the absorbance of the experimental sample.

Trypan blue staining. After removing culture medium, the cultures were rinsed gently with sterile pre-warmed phosphate buffered saline (PBS, pH 7.4, 0.01 M) and then 0.4% trypan blue stain solution (Invitrogen, Life Technologies, Grand Island, NY) was added to the culture dishes for a 4-5 min incubation period that was followed by removal of the staining solution. Culture dishes were washed with phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde. The fixed cells were observed under a light microscope and imaged. Since trypan blue is excluded from live cells, cells which stained blue were counted as dead cells.

Statistical analysis. The differences between the values of experimental and control treatments were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for individual comparisons. P value <0.05 was considered significant. The means ± SD are from four independent experiments, each experiment consisting of six to eight repeats for each treatment condition.

RESULTS

Morphological observations. When the PC12 cells were maintained in neurobasal medium supplemented with B27 and NGF, PC12 cells differentiated into neurons. When these PC12 cell-derived neurons were observed under the inverted microscope (Olympus IX71), they exhibited phase bright soma with intact membrane and long smooth neurites, profusely branched to form an extensive network among the neurons (Figure 2A). After a 4 h exposure to 300 µM concentration of H_2O_2, however, these PC12 cell-derived neurons showed significant changes in morphology displaying distorted and broken cell membrane, shrinkage and vacuolization. Several cells detached from the substratum and floated in the medium. Furthermore, those that were still attached showed disrupted neuritic network (Figure 2B). Neurons derived from PC12 cells that were pre-treated with PHRE showed a significant resistance against 300 µM H_2O_2-induced degenerative changes and exhibited an intact cell membrane with phase bright soma and retained a neuritic network in almost 75% of the cells (Figure 2C). PC12 neurons treated with PHRE alone did not cause any morphological changes indicating that PHRE by itself is neuron compatible.

Cell death determined by trypan blue staining. When treated with 300 µM of H_2O_2, approximately 70-80% of the PC12 neurons were stained blue with trypan blue, indicating that most of the neurons died (Figure 3B). In contrast, there was very faint or no stain in control cells indicating that most of the neurons were alive and excluded the dye (Figure 3A).

PC12 neurons pre-treated with PHRE and then subjected to oxidative stress, however, showed only a few trypan blue stained cells suggesting a resistance of cells to injury caused by 300 µM H_2O_2 induced oxidative stress (Figure 3C). PC12 neurons pre-treated with PHRE and then subjected to oxidative stress, however, showed only a few trypan blue stained cells, suggesting a resistance of cells to injury from 300 µM H_2O_2 induced oxidative stress (Figure 3C).

Cell death from lactate dehydrogenase (LDH) release. Compared with the control cultures, LDH release was significantly increased in PC12 neuron cultures exposed to 300 µM H_2O_2, indicating a loss of membrane integrity following oxidative stress (Figure 4). As compared with cultures treated only with H_2O_2 , a significant 40 to 50% decrease in LDH levels was released from PC12 cultures pretreated with PHRE and then treated with 300 µM H_2O_2 ($P<0.001$).

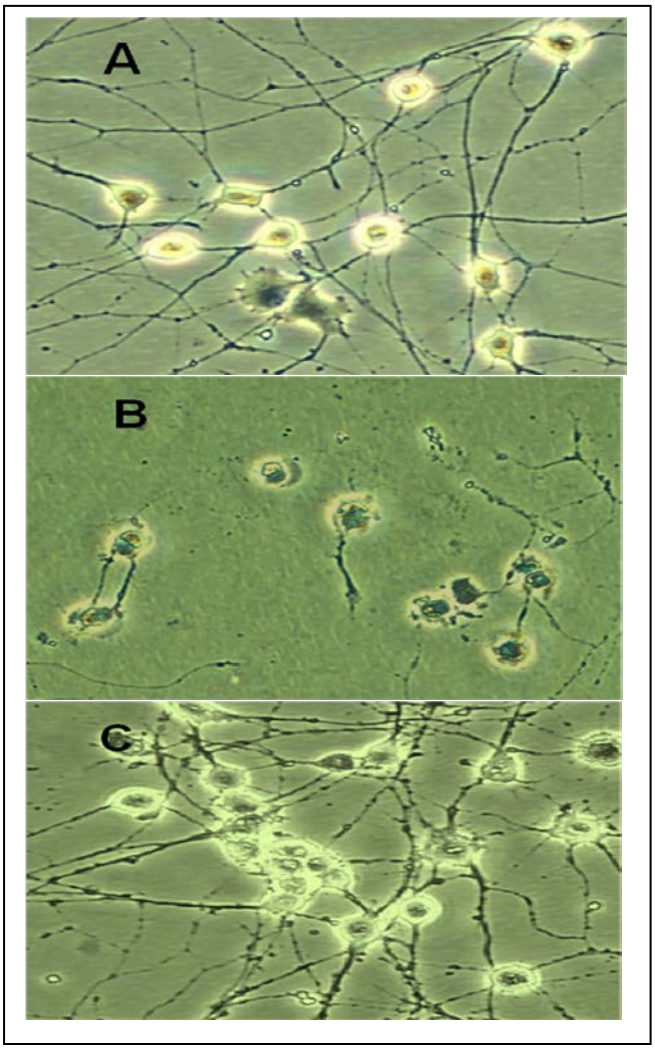

Figure 2. Differentiated PC12 cells under control conditions (A), after exposure to 300 mM H_2O_2 for 4 h (B), and after pretreatment with PHRE containing 15 mM resveratrol for 3 h before exposure to 300 μM H_2O_2 for 4 h.
PHRE exposure provided significant protection from cell death and helped retain intact neurite network, (200X).

LDH levels in PC12 neuron cultures exposed to 0.25% DMSO and 15 μM PHRE alone were not significantly different from LDH levels of control cultures, indicating DMSO and PHRE did not have any toxic effect (data not shown). An investigation of the neuroprotective efficacy of the PHRE and the commercial/synthetic resveratrol showed a significant 20 to 30% difference in the levels of released LDH between the two groups (Figure 4). Thus, PHRE provided better protection to PC12 neurons against oxidative stress from exposure to H_2O_2 compared with protection provided by resveratrol.

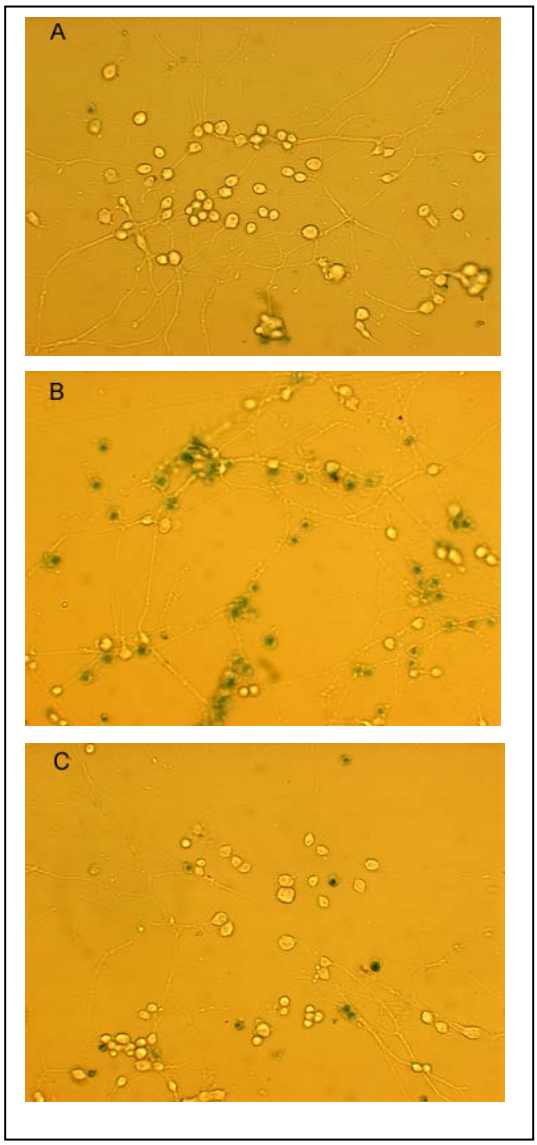

Figure 3. Differentiated PC12 cells under (A) control conditions, showing live neurons with no or very few cells retaining trypan blue statin, (B) exposed to 300 mM H_2O_2 showing dead neurons (blue stain), (C) pretreated with PHRE containing 15 mM resveratrol before being subjected to oxidative stress with 300 U of H_2O_2.
PHRE pre-treated cells were protected from cell death caused by exposure to H_2O_2.

DISCUSSION

The present study demonstrated that PHRE containing resveratrol and other stilbenoids provided resistance to cultured PC12 neurons against H_2O_2-induced cytotoxicity. Interestingly, PHRE was more effective than the synthetic/commercial resveratrol used in this study. A three hour pretreatment with

PHRE containing 15 µM of resveratrol significantly reduced cell death caused by exposure to 300 mM H_2O_2 for four hours, whereas, 20 µM of synthetic/commercial resveratrol did not show a similar neuroprotective effect.

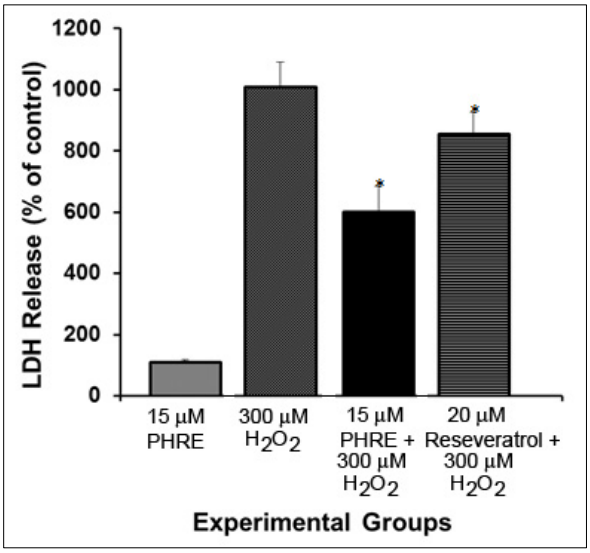

Figure 4. LDH released from PC12 neurons with indicated treatments.
Values are means ± SD. Analysis of variance showed a significant difference between the groups. df (4, 17), *p<0.001. Synthetic resveratrol was used.

At the end of H_2O_2 treatment, many PC12 neurons detached and floated in the medium indicating the loss of membrane integrity of the neurons. In addition some attached neurons showed vacuolation, disrupted neurites and degeneration. The neurodegenerative features were obvious within the treatment duration of four hours. H_2O_2 readily enters cells and interferes in many metabolic reactions, generating highly reactive hydroxyl radicals that further attack other cellular components, leading to neuronal dysfunction and degeneration (Shang et al., 2006).

With the membrane compromised, dying neurons would have released cytoplasmic content to the culture medium. As a result, the LDH values obtained from H_2O_2 treated cultures were significantly higher as compared with the control cultures. Also, vital staining using trypan blue showed darkly stained PC12 neurons in H_2O_2 treated cultures. Conversely, only a faint stain of trypan blue was observed control cultures, and only a few of the PC12 neurons showed dark, stained cells in cultures pretreated with PHRE, indicating reduced cell death.

Several studies have demonstrated that plants and plant constituents have antioxidant properties (Ng et al., 2006). An ethanolic extract of *Valeriana officinalis* and *Hypericum perforatum* are neuroprotective, acting as antioxidants in preventing apoptosis of rat hippocampal neurons in culture when incubated with Aβ25-35 (Malva et al., 2004; Silva et al., 2004). The methanol extracts of *Areca catechu* var. dulcissima, *Paeonia suffruticosa*, *Alpinia officinarum*, *Glycyrrhiza uralensis* and *Cinnamomum cassia* strongly enhanced viability against H_2O_2-induced oxidative damage in V79-4 cells (Lee et al., 2003). No previous reports on the protective effects of hairy root culture derived extracts, such as PHRE, against oxidative injury are known.

The current study demonstrated neuroprotective effects of PHRE against H_2O_2-induced neurotoxicity in PC12 cells derived dopaminergic neurons. Using commercial/synthetic resveratrol, earlier studies demonstrated neuroprotective effects against H_2O_2-induced oxidative stress and the resulting cell damage (Jang and Surh, 2001; Vieira de Almeida et al., 2007). In our experiments, however, the effects of PHRE against H_2O_2-induced PC12 neuron death were much stronger than that of commercial/synthetic resveratrol.

Although the molecular mechanisms for the neuroprotective effect of PHRE have not yet determined, the inhibition of H_2O_2-mediated neural degeneration by PHRE could be attributed to the antioxidative properties of the constituent stilbenoids, such as resveratrol and arachidin-1 (Abbott et al., 2010). Thus, an additive effect from these natural resveratrol analogs present in the extract could be providing neuroprotection.

Recent studies with the prenylated stilbenoids arachidin-1 and arachidin-3 purified from peanut hairy root cultures have shown that these compounds can modulate the cannabinoid receptors at micromolar levels. Indeed, in preliminary studies in our laboratory, arachidin-3 acted as a competitive cannabinoid receptor 1 (CB1R) antagonist, whereas arachidin-1 antagonized CB1R agonists by both competitive and non-competitive mechanisms.

Furthermore, *in silico* studies, using cannabinoid receptor 2 as a model, demonstrated the prenylated unit of arachidin-1 and arachidin-3 was important for the affinity to the cannabinoid receptor (Brents et al., 2012). Compounds shown to modulate cannabinoid receptors could provide neuroprotective effects, indicating these prenylated stilbenoids present in PHRE need further study (Fowler et al., 2010).

Ongoing experiments are being used to characterize the different constituents of the PHRE by liquid chromatography-tandem mass spectrometry and novel compounds are being purified using high performance counter current chromatography for structure elucidation by NMR. Investigating the neuroprotectiveness of each of the constituents of PHRE alone and in combination will help in understanding why PHRE has better neuroprotective property than resveratrol alone. The current results underscore the importance of further studies on the individual as well as combined effects of the components of PHRE.

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