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Original Article

NEUROPROTECTIVE EFFECT OF *ARTOCARPUS LAKOOCHA*EXTRACTAND OXYRESVERATROL AGAINST HYDROGEN PEROXIDE-INDUCED TOXICITY IN SH-SY5Y CELLS

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ABSTRACT

Objective: *Artocarpuslakoocha* Roxb. Is a traditional medical plant native to Southeast Asia and used as a dried aqueous extract so-called puaghaad. Its role (and its major ingredient, oxyresveratrol) as an antioxidant neuroprotectant were explored.

Methods: Differentiated SH-SY5Y neuroblastoma cells in 96-well plates were challenged with 200 µM H_2O_2 for 4h and either Trolox (100 µM), oxyresveratrol (5-100 µM), or puaghaad (1.2-25 µg/ml) applied 2h before H_2O_2 or for 20 h after H_2O_2 washout. Cell viability, mitochondrial function, intracellular ROS, and lipid peroxidation were assessed.

Results: Continuous presence of both H_2O_2 and antioxidant reduced mitochondrial function by ~50% but only by 30% with antioxidant. Sustained 24 h H_2O_2 showed no recoveries with antioxidants. Cell viability was modestly restored when antioxidants accompanied H_2O_2 for 4 h and both washed for another 20 h, but little recovery of mitochondrial function even though antioxidants removed ROS and prevent lipid peroxidation. Antioxidants added for 20 h after H_2O_2 marginally improve mitochondria and modestly restore cell viability, but lipid peroxidation was completely reversed.

Conclusion: These results show that mitochondrial protection was illusive, yet both tested compounds, puaghaad and oxyresveratrol, improved cell viability and especially ROS levels and lipid peroxidation. The potency oxyresveratrol on theredox-sensitive expression of antioxidant enzymes and its pharmacokinetics suggests that oral puaghaad could provide effective protection in transient neurodegenerative disease.

Keywords: Neuroprotection, Oxidative stress, Hydrogen peroxide, Oxyresveratrol, *Artocarpuslakoocha*

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INTRODUCTION

Oxidative stress is one of the causal factorsin neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), ischemia, and especially amyotrophic lateral sclerosis (ALS) which are characterized by excessive ROSs(superoxide, peroxide, hydrogen peroxide and hydroxyl radicals) generated by superoxide dismutase (SOD)[1, 2]. These ROSs disrupt cell function by peroxidation of lipids and proteins and DNA/RNA oxidation[3-6]. Furthermore, high rates of oxidative metabolism in neuronsandthat have limited antioxidantre sources most of which come from astrocytes renders neurons particularly vulnerable[7]. Modest and transient rises in H_2O_2 is an important inter-and intracellular physiological signalling mechanism [8]. Nevertheless, H_2O_2 is intrinsically stable, but at higher sustained levels it generates highly reactive $OH^{\cdot}$radicals via the Fenton reaction which oxidizes almost any cell constituent leading to cellular dysfunction [9, 10].

Several animal studies show convincing protective effects of antioxidants against ischemia-induced damage including the cell/brain permeanttocoferol analogue, Trolox[11] and the SOD-mimetic (Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrinpentachloride) reduced lipid peroxidation and DNA fragmentation[12].

Many medicinal plants and their ingredients have also prevent the formation of these oxidized products and in animal studies show brain protection which includes amelioration of pathologies found in AD and other neurodegenerative pathologies[13]. *Artocarpuslakoocha* Roxb(monkey fruit)("ma haad" in Thai) is indigenous to South/Southeast Asia, including Thailand where it has been used for hundreds of years as an antihelmintic [15]. It is formulated as a powdered aqueous dried extract and known as puaghaad. Recently, it was shown to beantiviral [14], antiglycation and antioxidant [15]. Oxyresveratrol comprises ahigh content of puaghaad and is likewise neuro protective *in vitro* and animal studies[16-21],commensurate with its permeability of the blood-brain barrier (BBB)[22].

Nevertheless, puaghaad is a complex mix of potentially bioactive compounds which collectively may modulate the oxyresveratrol action. Therefore, we sought compare actions of puaghaad against oxyresveratrol in neuroblastoma cells usingH_2O_2 as the oxidative challenge.

MATERIALS AND METHODS

Materials

The following materials were purchased from Sigma (St. Louis, MO): Dulbecco's modified Eagle's medium (DMEM)/F12, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH),2,4,6-tripyridyl-s-trizine (TPTZ), retinoic acid, trichloroacetic acid, thiobarbituric acid,′,7′ - dichlorofluorescein diacetate (DCFH-DA). Fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were purchased from Gibco (Grand Island, NY).Hydrogen peroxide (H_2O_2)was purchased from Merck KGaA (Darmstadt, Germany). 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assay kit was purchased from Roche Diagnostics (Mannheim, Germany).

Hardwood aqueous extract of *A. lakoocha*so called puaghaad (PH) was obtained from Origin Plant Co., Ltd (Bangkok, Thailand).

Puag-haadoxyresveratrol content

A 20 µl sample of puaghaad (1mg/ml in DMSO) was injected into a Shimadzu LC-20AT liquid chromatograph equipped with a SPD-20A UV detector, a Ultra HPLC column (250 x 4.60 mm) with C18 column packing, 5 µm particle size; isocratic elution by methanol/H_2O (35:65, flow rate 0.8 ml/min. Peaks were integrated at 254 nm. Puaghaad was assigned by retention time of oxyresveratrol 17 min and calibrated using 2.5–250 µg/ml oxyresveratrol.

SH-SY5Y cell culture preparation

SH-SY5Y cells were cultured in DMEM/F-12 containing L-glutamine, 10% FBS, 0.01% penicillin-streptomycin. Cells (3-10 passage) stored in liquid N_2 and plated out at 1×10^6 cells/cm^2 into 75 cm^3 flasks and grown to confluence at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged<30 times to ensure cell uniformity and reproducibility then re-seeded into 96-well plates at 20,000 cells/well for 24 h at 37 °C. They were differentiated with low serum culture medium (2% FBS) containing retinoic acid (10µM) for 6 dbefore use. The medium (100µl) was refreshed at days 3 and 6[23].

H_2O_2-induced oxidative stress in SH-SY5Y cells

The experiments used H_2O_2 (200µM) as the oxidizing agent and cell protection examined using Trolox (100µM), oxyresveratrol (5-100µM) and puaghaad (1.25-25 µg/ml) all added in 100% DMSO (final DMSO concentration<0.01%). Three protocols were used where H_2O_2 was present for 4 or 24h, and the cell protectants present before, during, or after H_2O_2. Protocols are shown in fig. 2-4.

Cell viability measured by lactate dehydrogenase (LDH) activity

After treatment, 50 µl of the medium was aspirated and place into a new 96-well plate and 0.3mmol NADH and 3mmolpyruvates added. The absorbance was then measured at 340 nm at 0, 5, 10, 15 and 20 min[23].

Determination of mitochondrial metabolic activity

According to the kit instructions, XTT solution was added to the remaining culture cells and the orange formazan measured by absorbance at 460 nm produced by the mitochondrial enzyme. This gives an estimate of mitochondrial respiratory chain function.

Determination of intracellular ROS

Other differentiated SH-SY5Y neuroblastoma cells in a 96-well black plate were treated with DCFH-DA(5µM)for 30 min. The culture medium was removed, and cells washed with PBS prior. DCFH-DA penetrates cells, then hydrolyzed by intracellular esterases to DCFH which is oxidized to fluorescent diclorofluorescein in the presence of ROS. The fluorescence was excited at 485 nm excitation and emission measured at 530 nm in a microplate reader[23].

Lipid peroxidation by thiobarbituric acid reactive substances (TBARs) assay

For other cells, the end of treatment, 100 µl of TBARs reagent containing 0.4% TBA, 1.4% TCA, and 8% HCl (1:2:1) were added to each well and cells resuspended. The mixtures were incubated at 90 °C for 1 h, cooled to room temperature, and fluorescence was measured at excitation at 535 nm and emission at 595 nm wavelengths[24].

DPPH scavenging assay

Free radical scavenging activity was measured by mixing 0.2mmol DPPH assay and test compounds at various concentrations, incubated at room temperature for 30 min in the dark, and absorbance measured at 515 nm wavelength[24].

Reduction by ferric reducing antioxidant power (FRAP) assay

This assay depends on Fe^{3+}reduction to Fe^{2+}. FRAP reagent (comprising 10:1:1 of 3mmol acetate buffer (pH 3.6), 10mmol TPTZ/40mmolHCl, 20mmol $FeCl_3$,) was mixed with the test compounds and absorbance measured at 595 nM[24].

Statistical analysis

Data from all assays are expressed as the mean±SEM (standard error of themean). The data were analyzed by analysis of variance (ANOVA)and the Prism program (GraphPad Software Inc). Differences were considered to be significant when p value ≤ 0.05.

RESULTS AND DISCUSSION

Oxyresveratrol content in puag-haad

Hardwood aqueous extract of *A. lakoocha*(puaghaad) used in this study contained 64.68% oxyresveratrol by HPLC (fig. 1).

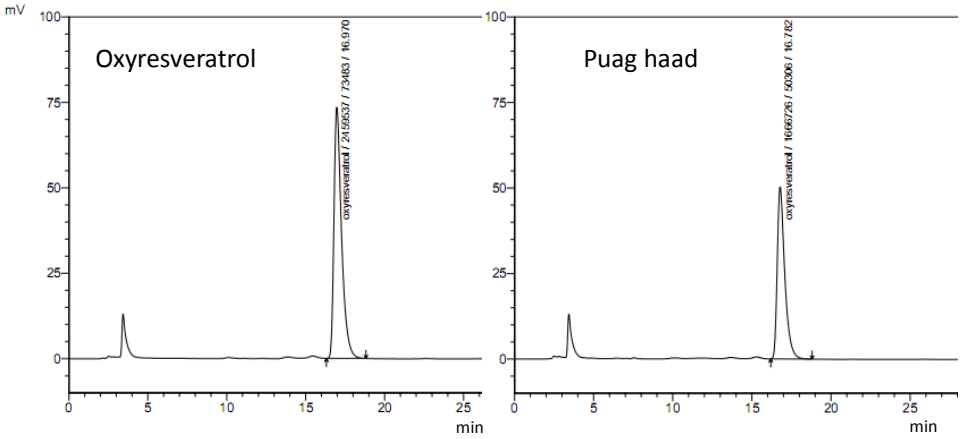

Fig.1: HPLCchromatogram of oxyresveratrol and *A. lakoocha*extract (puaghaad) at RT=17 min with detector response at 254 nm

Acute and chronic H_2O_2 action

Differentiated SH-SY5Y neuroblastomacells were exposed to 200 µM H_2O_2 for 4 h to emulate acuteoxidative stress, and 24h representing a chronic challenge (fig.2A).

Acutely, H_2O_2clearly depressed formazan production (XTT assay) indicative of compromised mitochondrial energy feed through the electron transport chain and oxidative phosphorylation (fig.2C).This depression was manifest at 4h and continued through to 24h.

With the continued presence of Trolox, oxyresveratrol, orpuaghaad (containing 100 µM oxyresveratrol by analysis) could partially rescue mitochondrial function (at 4 h) but failed to do so after24hof chronic H_2O_2 treatment (fig.2C).

Cell viability measured by LDH release into the medium appeared preserved after 4h of H_2O_2, but succumbed to 24 h of exposure (fig.2B). None of the anti-oxidant treatments was able to rescue the cells. H_2O_2-induced oxidative stress reduced glutathione level [25], induced DNA damage and apoptosis in cultured cells [26]. H_2O_2 action is accompanied by mitochondrial depolarization, aggregation, and cytochrome C release [27], and increased MAPK and p38 [28] in other cells and SH-SY5Y cells[29].

Our proposition that electron chain transport disrupted is confirmed by inhibition of NADH-Co Qreductase and ATP synthase [30]. All these factors are associated with apoptosis confirmed in our experiments with 24h exposures but most authors confined protocols to short treatments and unable to assess long-term consequences.

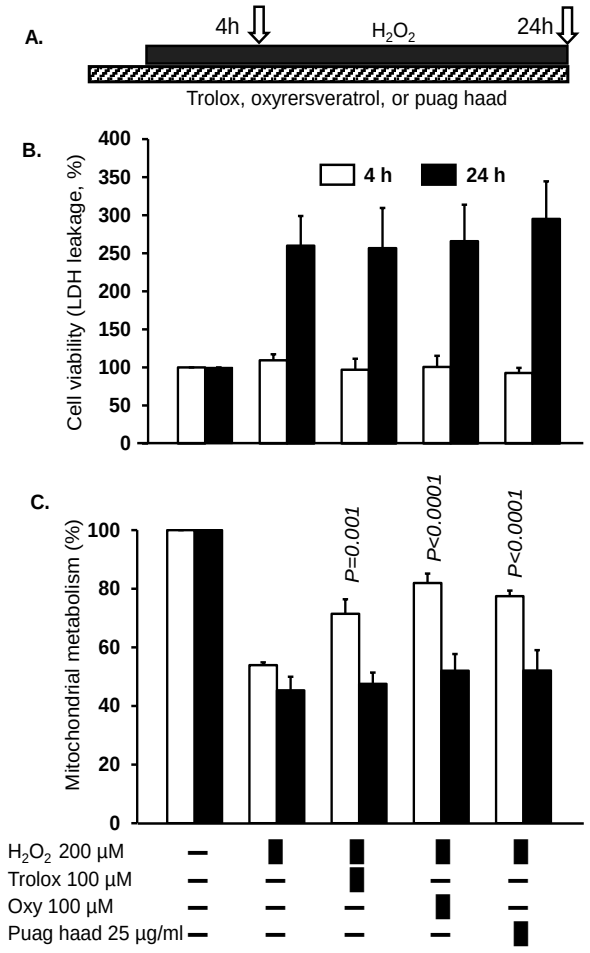

Fig.2:(A) Protocol showing interventions, either nothing, trolox, oxyresveratrol (Oxy), or puaghaad added to cultured SH-SY5Y cells for 2h followed by addition of H_2O_2 for a further 24 h.The arrows denote measurement times. (B) Changes in cell integrity using LDH leakage into the culture medium. (C) Effects on cell mitochondrial metabolism indicated by the XTT assay. Values are expressed as means±SEM of 5 (B) or 6 samples(C). The *p*-values refer to differences compared to H_2O_2 treatmentalone

No neuroprotectionby oxyresveratrol and puaghaad

To show whether the late (24h) cellular demise is already set in train by the early H_2O_2 challenge, we used a protocol where both the challenge and interventions were removed after 4h (fig.3A).Now, cell viability was poor even though at 4h exposure to H_2O_2 (fig.2B) it had been fully intact. This implies that following 4h pathological changes had already begun which only became manifest after a further 20 h. Trolox, oxyresveratrol, and puaghaadcould partly prevent this demise, although dose-response of both tested compounds were not clearly demonstrated (fig.3B).

The 4 h H_2O_2exposure left mitochondrial function depressed (fig.2C) and with 20 h of washout, this dysfunctional state had persisted (fig.3C).Furthermore, this state could not be rescued by antioxidant treatments.

Oxidative stress

To verify that oxidative species were present 20 h after 4 h in H_2O_2 treatment, we measured ROSs by probing with DCFH. There was clearly elevated oxidizing species present that was completely neutralized by Trolox, and dose-dependently by oxyresveratrol and puaghaad (fig. 3D). Since H_2O_2 is highly diffusible across the plasma membrane, it would have rapidly washed out of cells. But clearly, oxidizing species remained such as OOH^-, O^{-2-}, and $ONOO^-$whose charge would keep them trapped in the cytosol, or that sustained damage has activated peroxisomal enzymes [31], and disrupted the mitochondrial electron transport chain generating O^{-2-}[32].

Important victims of ROSs are polyunsaturated fatty acids which form multiple reactive intermediates and fragments including dialdehydes. H_2O_2 modestly increased levels of reactive malonyldialdehyde and Trolox, oxyresveratrol, and puaghaad suppressed this lipid peroxidation levels lower than the baseline (fig. 3E). This suggests that control cells are already oxidatively stressed/auto-oxidized, commensurate with low antioxidant capacity and high glucose concentrations of serum-free media.

All concentrations of oxyresveratrol and puaghaad were equally effective as Trolox.

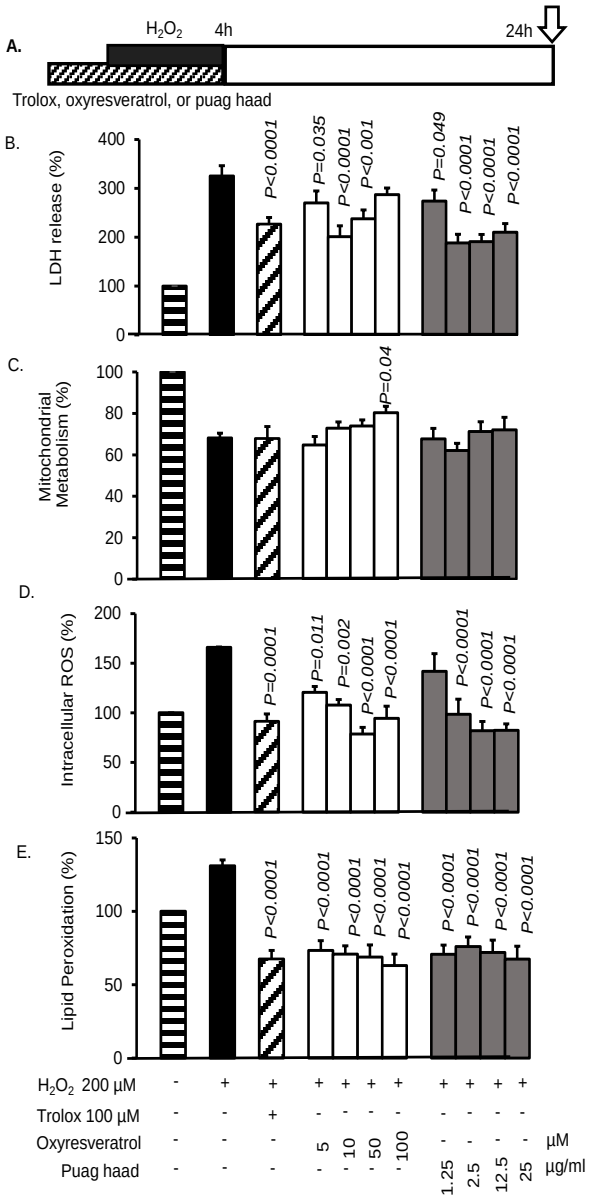

Fig.3: (A) Protocol showing SH-SY5Y cells treated with Trolox, oxyresveratrol or puaghaad (2h) followed by H_2O_2 for another 4h. Then antioxidants and H_2O_2 were washed out for another 20 h and the following measures made: (B) Cell viability measured as LDH activity, (C) Mitochondrial function, (D) Intracellular ROS generation, (E) Cell lipid peroxidation by malonyldialdehyde production. Values are expressed as means±SEM of 6 experiments. The*p*-values compared to H_2O_2 alone (black bar)

Post-treatment with antioxidants

In the next series of experiments, antioxidants were added during 20 h washout period following the 4 h H_2O_2 challenge (fig.4A). For both LDH leakage and mitochondrial hypofunction, none of the anti-oxidant interventions had much influence on recovery (fig.4B,C). But for malonyldialdehydes all three anti-oxidants were highly effective (fig.4D).

Mitochondrial function showed little restoration byany protocolespecially for Trolox which was designed for mitochondrial function. However, our protocol had 20% oxygen and 20mmol glucose present to feed into an uncoupled electron transport chain thereby generatingexcessive superoxide. Even in normally perfused brain oxygen and substrate would be more limiting. All the antioxidants were effective reducing agents for peroxidated lipids irrespective of protocol and concentration. Trolox easily partitions into membranes while resveratrol at least bindsonto membranes and their extracellular and cytosolic concentrations have less influence on thefunction as reflected by oxyresveratrol action irrespectiveof concentration. Thus, the antioxidants are well placed to efficiently scavenge peroxidated fragments before running riot in the cytosol.

For polyphenols, oral oxyresveratrol bioavailability is relatively high achieving 2 µM in rat plasma[33]which in humans would need an oral dose ~500mg by allometric scaling. This is within the range of our lowest dose (5 µM)that produced antioxidation. While blood-brain barrier permeability to oxyresveratrol is very low, this is greatly increased during ischemic brain damage achieving ~1 µM[22]and fostering repair[18]. However, 5 µM would have little impact compared to the totalplasma antioxidant capacity of ~0.5mmol[34].Furthermore, it is below the IC50 by the DPPH assay (table 1). At these lower concentrations, a more plausible mechanism of oxyresveratrol antioxidant action is through increased expression of endogenous antioxidant enzymes via their transcription factors, Nrf-2 [35], and FOXO3a [36]. Ultimately, these enzymes are trafficked to sources of particular ROSs where they are most effective. In contrast, Trolox appears to need higher concentrations than used here thus likely to be a directly acting antioxidant against H2O2 and other ROSs [37].

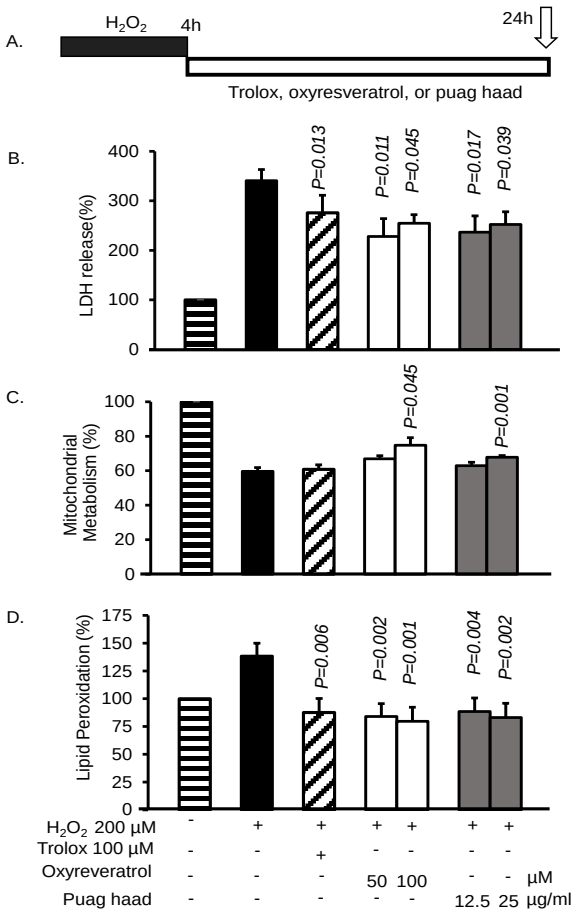

Fig.4: (A) Cells were treated with 200 µM H_2O_2 for 4 h and the medium changed for trolox, oxyresveratrolor puaghaadfor 20 h. (B–D) the measurements were as in fig. 3. The *p*-values compared with H_2O_2 without anti-oxidant post treatment

Table 1: Antioxidant activities of oxyresveratrol and puaghaad

Compounds	DPPH scavenging capacity (IC_{50})	FRAPFe^{2+}concentration
Oxyresveratrol	38.1 µM	11.2 µM (at 100 µM oxyresveratrol)
Puaghaad	9.3 µg/ml	12.8 µM (at 37.78 µg/mlpuaghaad)
Trolox	55.3 µM	8.2 µM (at 100 µM Trolox)

Note: 37.78 µg/mlpuaghaad containsoxyresveratrol equivalent to 100 µM.

CONCLUSION

These results suggest that both oxyresveratroland oxyresveratrol-containing puaghaad provides protection of SH-SY5Y neuroblastoma cells against H_2O_2 oxidant toxicity and that oxyresveratrol pharmacokinetics suggests that it may offer protection against neuroprotective diseases.

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AUTHOR CONTRIBUTION

Hasriadi conducted and analyzed most of the results.

Matusorn Wong-On developed and analyzed oxyresveratrol content in puaghaad.

PhakhamonLapphanichayakool developed a test method of H_2O_2-induced neuronal toxicity.

Nanteetip Limpeanchob supervised, evaluated, and summarized overall results.

CONFLICT OF INTERESTS

Declared none

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