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Phloroglucinol ACTS2 As A Neuroprotective Agent in Rotenone Induced Parkinsonism in SH-SY5Y Cells

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorders characterized by impaired motor functions. SH-SY5Y is a dopaminergic neuronal cell line which has been used as an invitro model for neurotoxicity experiments. Phloroglucinol, a compound from Ecklonia cava belongs to the Rutaceae family. The present study was aimed to evaluate the neuroprotective effect of phloroglucinol against rotenone (Neurotoxin) induced SH-SY5Y cells which is the model for Parkinsonism. Cell viability was assessed by MTT assay, ROS production by DCFH-DA, nuclear damage by DAPI and the activity of Catalase (CAT), superoxide dismutase (SOD), level of reduced glutathione (GSH) and thiobarbituric acid reactive substance (TBARS) were done by standard protocols. The present study revealed the neuroprotective effect of Phloroglucinol by increasing the cell viability while rotenone decreased the same. Rotenone exhibited increased ROS generation and nuclear damage whereas Phloroglucinol diminished the same. SH-SY5Y cells exhibited increased activities of SOD and CAT, and decreased the levels of lipid peroxides and increased the level of GSH, when treated with Phloroglucinol. These results suggest that the phloroglucinol suppressed cell death in rotenone induced SH-SY5Y cells and prevents the oxidative stress. Thus, phloroglucinol may serve as a potent therapeutic agent by virtue of its multiple pharmacological properties in the treatment of neurodegenerative disorders including PD.

Key words: *Phloroglucinol, SH-SY5Y cells, Rotenone, GSH, Antioxidant potential.*

INTRODUCTION

Parkinson's disease is the second most common neurodegenerative movement disorder with a prevalence of 9 million people worldwide by 2030 [1]. It was first described by British surgeon James Parkinson and it is characterized by progressive loss of dopaminergic neurons in the substantia nigra. PD shows both motor and non- motor symptoms such as resting tremor, bradykinesia, rigidity of skeletal muscle, postural instability, stooped posture and freezing of gait and non- motor symptoms including cognitive and behavioral problems and patients may also suffer from sleeping disorder or autonomic dysfunction[2].

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Presently, there is no successful treatment available to cure Parkinson's disease. But some drugs and clinical trials such as surgery, exercise and medication can help to control or minimize the symptoms. Drugs mainly used for PD such as Carbidopa, selegiline, rasagiline and L-DOPA has many side effects. Our work was planned to investigate the effect of plant based compounds on Parkinson's model. So, we choose Phloroglucinol has our treatment model. It is a polyphenol that is a component of phlorotannins which is present in *E.cava* of the Laminariaceae family [3]. It is a naturally occurring secondary plant metabolite currently used for treatment of gastro-intestinal disorders. In combination with tri-O-methyl derivative of phloroglucinol (1, 3, 5-trimethoxy benzene) it is commercialized in France and some other countries as an anti-spasmodic [4]. During recent years several studies have reported that phloroglucinol exerts a number of pharmacological activities such antithrombotic and profibrinolytic activities[5].

Rotenone is widely used as a pesticide and insecticide. It is highly lipophilic in nature and it is an inhibitor of mitochondrial complex I. In nigrostriatal dopaminergic pathway, the pathological features of rotenone were examined in PD pathogenesis, including the loss of dopaminergic neurons in substantial nigra and enhanced oxidative stress and neuroinflammation [6,7]. Furthermore, rotenone induced the formation of α- synuclein cytoplasmic inclusions in DA neurons (Lewy pathology), DJ-1 acidification and translocation, proteasomal dysfunction and nigral iron accumulation[8]. Sherer *et al*.[6] reported that the low concentration of rotenone to cells produce 50% inhibition of complex I and it is similar to the levels of complex-I deficiency described in PD brain and tissue. Hence, we preferred rotenone has our induction model for PD.

SH-SY5Y neuroblastoma cell lines are widely used as a cellular model of Parkinson's disease. In 1970, SH-SY5Y cell lines were subcloned from SK-N-SH (Parental neuroblastoma cell lines) and it was produced from a bone marrow biopsy that contains neuroblastoma cells of a four years old female[9]. Based on the literature survey, SH-SY5Y cell lines are mainly used for these three human diseases such as neuroblastoma, Alzheimer's disease (AD) and Parkinson's disease (PD). The sensible activity of dopamine-β- hydroxylase, insignificant level of choline acetyl- transferase, acetyl cholinesterase and butyryl cholinesterase[10] basal nor adrenaline (NA) release[11] and tyrosine hydroxylase (TH) activity[12] are the initial characterization of SH-SY5Y cell lines. TH is the enzyme that is involved in catecholamine synthesis pathway and it converts tyrosine to L-dopa. L-dopa is a gold standard drug and it is used to increase dopamine concentrations in the treatment of Parkinson's disease[13]and dopamine (DA) is converted to nor-adrenaline by the enzyme dopamine- β- hydroxylase[14]. Hence, the SH-SY5Y cell line represents an appropriate model for PD. Therefore, we aim to explore the neuroprotective effect of Phloroglucinol on SH-SY5Y cell lines.

MATERIAL AND METHODS

Chemicals

Phloroglucinol, rotenone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Dulbecco's modified eagle's medium (DMEM), Dimethyl sulfoxide (DMSO), 2,7-diacetyl dichlorofluorescein (DCFH-DA), 4', 6-diamidino-2-phenylindole (DAPI), Phosphate buffer solution (PBS), Malondialdehyde (MDA), Thiobarbitric acid (TBA), Trichloro acetic acid (TCA), Hydrochloric acid (HCL), p-nitroblue tetrazolium chloride (NBT), Ethylene diamine tetra acetic acid (EDTA), Sodium phosphate buffer, hydrogen peroxide and 5,5- dithiobis (2-nitrobenzoic acid) were purchased from Sigma Aldrich.

Cell Culture

Human neuroblastoma SH-SY5Y cells were obtained from National center for cell science, Pune. Cells were maintained in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin at 37ºC in a humidified

incubator with 5% CO_2. The cell culture medium was changed every 2-3days. Dimethyl sulfoxide (DMSO) concentration was maintained at 0.1% for all cell culture assays. The experimental groups were as follows: Untreated control, Induction model (treated with Rotenone), Phloroglucinol treatment alone and Phloroglucinol co-treated with rotenone at different concentration.

MTT assay

Cell viability was determined by MTT assay by the method Mossman *et al.*[15]. The cells were cultured on 96 well plates at a density of 1×10^5 cells/well in DMEM medium. The cells were washed, then the cells were treated with rotenone (20µg) and concurrently phloroglucinol was added at different concentrations (5µg, 15µg, 30µg, 60µg and 90µg) for 24 hrs. After the exposure of rotenone and phloroglucinol, the medium was removed and replaced with 10µl of MTT. The plates were further incubated for 4 hr in a humidified atmosphere at 37ºC and containing 5% CO_2. The MTT was discarded from the cells. The Formazan crystals were dissolved in 100µl of DMSO and the absorbance was measured at 570nm using micro plate reader.

ROS

Intracellular ROS measurement was performed using DCFH-DA as described by Ling *et al.*[16] . The SH-SY5Y cells were cultured in 12 well plates at a density of 1×10^5 cells/well and cells were treated with rotenone and exposed to phloroglucinol and incubated for 24hrs. The medium was removed and 10µM of DCFDA was the added. The cells were incubated at 37 $^{\circ}$C for 30mins. The fluorescence intensity of dichlro-fluorescein (DCF) was measured at (485nm and 530nm) wavelength.

Nuclear damage

The DAPI staining was used to observe the changes in nuclear morphology of SH-SY5Y cells by the method of Elumalai *et al.*[17] Cells were seeded at a density of 1×10^5 cells/well. The cells were treated with rotenone and exposed to phloroglucinol and incubated at 37 $^{\circ}$C. After the incubation, the media was removed and washed twice with phosphate buffer saline (PBS), then add 0.5 µg/ml DAPI fluorescence dye and incubated for 1 hr at 37 $^{\circ}$C. the fluorescence were observed under a fluorescent microscope (excitation 359 nm, emission 461 nm).

TBARS

Lipid peroxidation was measured by Malondialdehyde (MDA) method according to Hammouda *et al.*[18]. The level of TBARS indicates lipid peroxidation. The rotenone and Phloroglucinol treated cells were mixed with TBA-TCA-HCL reaction mixture and boiled at 15 mins. Then, the samples were centrifuged at 3000rpm for 10mins. The absorbance of the resultant pink product was measured at 532nm.

SOD activity assay

Superoxide dismutase (SOD) activity was examined by the method based on the inhibition of the formation of (NADH-PMS-NBT) complex[19]. The reaction mixture consist of 50µl of the supernatant, 13 mM L-Methionine, 75 µM P- nitroblue tetrazolium chloride (NBT), 100 µl Ethylene diamine tetra acetic acid (EDTA) and 2 µl riboflavin in a 50 mM potassium phosphate buffer (pH 7.8). The absorbance was measured at 560 nm. The activity of SOD was determined and expressed as unit/min/mg protein.

Catalase Activity assays

Catalase activity was determined by the decomposition of hydrogen peroxide by the method of Luck, (1974). The reaction mixture consist of 5ml of distilled water, 2.5ml of 25mM sodium phosphate buffer (pH 7.0), 0.1ml supernatant and 0.1ml 25mM hydrogen peroxide. A decrease in absorbance due to the hydrogen peroxide degradation was observed at 240nm for 1min. The activity of catalase was determined and expressed as unit/min/mg protein.

Estimation of Glutathione

The total GSH content was measured by the method of Ellman[21]. This method is based on the development of a yellow colour, when 5,5 –dithiobis (2- nitrobenzoic acid) was added to compound containing sulfhydryl groups. The optical density was read at 412nm. The amount of GSH was expressed as nmol/mg protein.

Data Analysis

Statistical analysis was performed by one- way anova using the software package SPSS version 23 and results were expressed as mean ± SD for each group. P < 0.05 was considered as significant.

RESULTS AND DISCUSSION

Rotenone is a well-known neurotoxin that mimics the parkinsonian state. In mitochondria, rotenone obstructs the oxidative phosphorylation process and leads to formation of free radicals. We investigated the neuroprotective effects of phloroglucinol on rotenone induced Parkinson's disease in *invitro* by using SH-SY5Y cells.

Phloroglucinol defend SH-SY5Y cells against rotenone

The MTT assay was performed to ensure the cytotoxic effect of rotenone in SH-SY5Y, a neuroblastoma cell line. The cells were treated with various concentrations of phloroglucinol (5µg, 15 µg, 30 µg, 60 µg and 90 µg) and simultaneously administered with rotenone (20 µg) for 24 hours. Treatment with rotenone shows decreased cell viability **(Fig 1 and 2)**. Conversely, the maximal protection was provided by the phloroglucinol at 60 µg. Based on the results obtained from the cell viability this effective concentration of phloroglucinol was utilized in the subsequent experiments for evaluating the protective effect from rotenone toxicity. The phloroglucinol treated cells suppressed rotenone effects on cell viability. Thus our study was compatible with Dhanalakshmi *et al*.[22] who reported that vanillin could counteract cell death induced by rotenone by preserving mitochondrial functions by its antioxidant potential.

Phloroglucinol attenuates rotenone induced ROS generation

The over production of free radicals includes oxide, hydroxyl, hydroperoxyl, peroxy, alkokyl radicals and the other non-free radicals are formed during cell metabolism in human body. These reactive molecules are recognized as reactive oxygen species. The ROS are toxic to the living cells[23] and it can cause oxidative damage to the biological molecules[24]. This oxidative stress can cause cell damage and is strongly implicated in pathogenesis of PD. Mitochondrial dysfunctions are allied with many neurological disorders and it increases the free radical production[25] **Fig 3** shows the level of intracellular ROS formed and was enumerated by fluorescence with DCFH-DA. Rotenone treatment (20µg) increased the green fluorescence, due to the high level of ROS production and the phloroglucinol (60 µg) were simultaneously administered with rotenone revealed a reduction in green colour intensity, due to the decreased level of ROS formation. Phloroglucinol alone treated cells diminished rotenone induced ROS production. Fluoresence of the oxidizied DCFH to DCF is proportional to the concentration of ROS in the samples. Ryu[26] reported that the phloroglucinol inhibits the increased level of ROS production caused by 6-OHDA induced SH-SY5Y cells. In addition, Kang *et al*.[3],demonstrated that Phloroglucinol from *Ecklonia cava* a species of brown algae has a ROS scavenging activity and inhibited Hydrogen peroxide induced apoptosis.

Phloroglucinol shields rotenone induced Nuclear damage

In DAPI staining method, treatment with rotenone (20 µg) was found to show nuclear damage. Jagathesan *et al*.[27] reported that exposure of rotenone induced morphological changes (nuclear damage) in SH-SY5Y cells. Damage was found to be low with Phloroglucinol (60 µg) cotreated with rotenone, when compared to rotenone induced cells. **(Fig 4)** There is no significant changes observed in SH-SY5Y cells treated with Phloroglucinol alone.

Phloroglucinol reduces the lipid peroxidation induced by Rotenone

In biological system, the presence of lipid peroxidation shows the increased level of TBARS. In contrast, the significant reduction in the TBARS level indicates the high level of antioxidant scavenging activity[28] **Fig 5** shows that the lipid peroxidation in the rotenone induced cells was found to be increased without enough antioxidant activity.

Phloroglucinol when simultaneously administered with rotenone was found to be decrease and the phloroglucinol alone treated cells diminished the lipid peroxidation. So and Cho[29] reported that phloroglucinol has a scavenging effect against free radicals and increases the pr-otection against lipid peroxidation.

Determination of enzyme activity (SOD and CAT) and GSH

SOD is one of the most important members of the enzymatic antioxidant defense system and played important roles, in the maintenance of cellular redox homeostatis[30]. A significant decrease in the activity of SOD was observed in the rotenone treatment **(Fig 26)**. The activity of SOD was found to be increased in Phloroglucinol administered with rotenone. There is no significant changes observed in SH-SY5Y cells treated with Phloroglucinol alone. Tamilselvam *et al*.[31], reported that the activity of SOD was found to be decreased in SH-SY5Y cells treated with rotenone.

Catalase plays a significant role in effective augmentation of antioxidant defense mechanism in cells. The enzymatic antioxidant such as catalase is used to neutralizing the effect of neurotoxins. Catalase protects cells from oxidative damage by non reactive oxygen species and it prevents the generation of hydroxyl radicals The rotenone treated cells showed a decrease in the activity of catalase and the phloroglucinol and rotenone combination showed increased activity of catalase as compared to rotenone induced group **(Fig 7)**. The phloroglucinol alone shows similar results as control. Kang *et al*.[3], reported that phloroglucinol enhanced the catalase activity suggesting that the scavenging of ROS may be related to the increased antioxidant activity. Ryu *et al*.[26] demonstrated that the phloroglucinol can increase the activity of antioxidant enzyme such as catalase, which was reduced in 6-OHDA treated cells.

The naturally occurring antioxidants in plant can provide some defence against the oxidants. GSH is an important factor in aetiology of Parkinson's disease. Enzymatic and non enzymatic antioxidant such as reduced glutathione (GSH)[32] is mainly used to repair the oxidative damage caused by intracellular ROS or directly scavenging oxygen radicals. Moreover, GSH depletion is the indicator of oxidative stress during PD progression. **Fig 8** shows a reduction in GSH levels in rotenone treatment. Phloroglucinol and rotenone combination showed a significant increase in level of GSH as compared to rotenone group. Kavitha *et al*.[33], reported decreased level of GSH in SK-N-SH cell incubated with rotenone treatment.

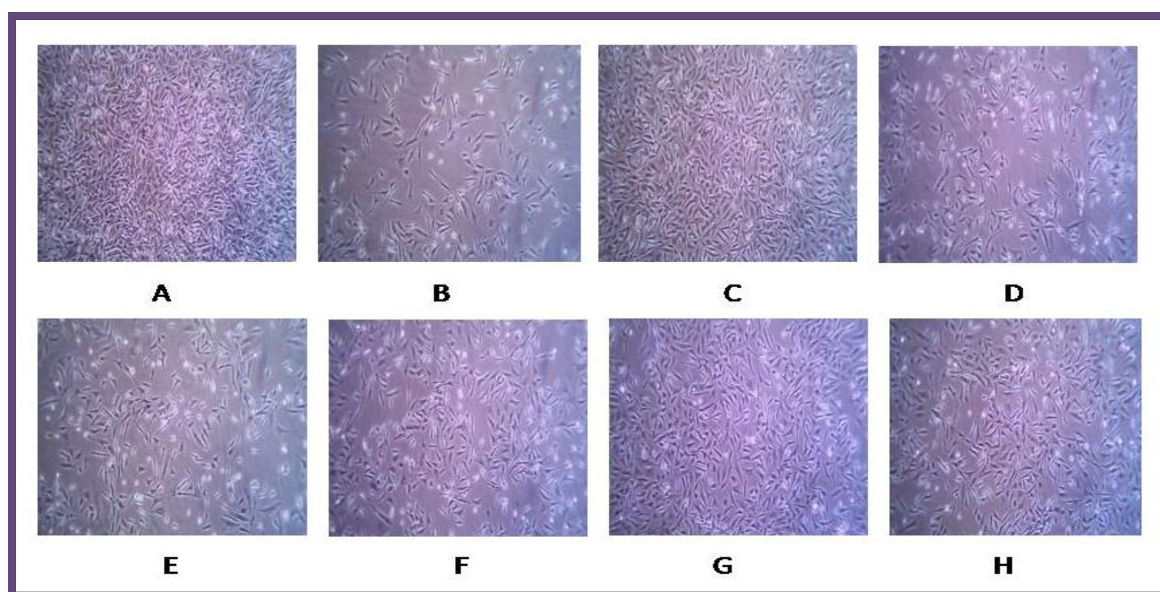
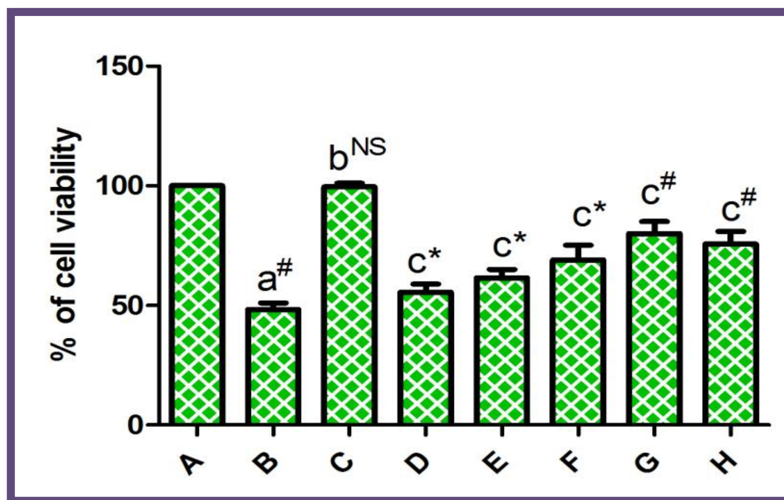


Fig 1: and Fig 2: Shows cell viability and Percentage of cell viability assay



(A) Control, (B) Rotenone 20 µg, (C) Phloroglucinol 90 µg, (D) Rot 20 µg + Phlo 5 µg, (E) Rot 20 µg + Phlo 15 µg, (F) Rot 20 µg + Phlo 30 µg, (G) Rot 20 µg + Phlo 60 µg and (H) Rot 20 µg + Phlo 90 µg. The protective effect of various concentrations of Phloroglucinol with rotenone induced cell death was determined by MTT assay. Values are expressed as mean ± SD. The maximum inhibition of cell viability was obtained at 60 µg concentration. Statistical significance: # P< 0.01; *P< 0.05; NS- non significant. Whereas, a – Comparision between Vechicle control and Rotenone, b – Comparision between Vechicle control and Phloroglucinol, c – Comparision between Rotenone and the combinations of Rot (20 µg) + Phlo (5, 15, 30,60 and 90 µg). Magnification: 10X

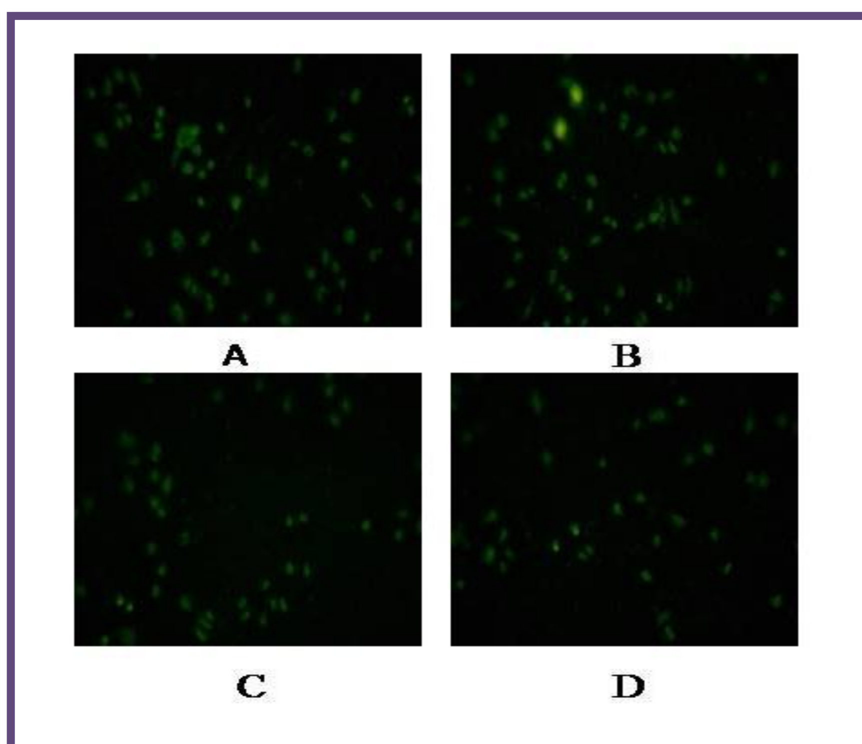


Fig. 3: shows Reactive Oxygen Species by DCFH-DA

(A) – Control, (B) – Rotenone 20 µg/ml, (C) – Phloroglucinol 90µg/ml and (D) – Rot 20 µg /ml + Phlo 60 µg/ml. Effects of Phloroglucinol on rotenone induced Ros generation by DCFDA staning. (B) Rotenone 20 µg/ml induced cell shows increased ROS production

when compared to control cell. Then C and D Phloroglucinol and Rot + Phlo treated cells shows decrease the ROS production when compared to rotenone. Magnification: 10X.

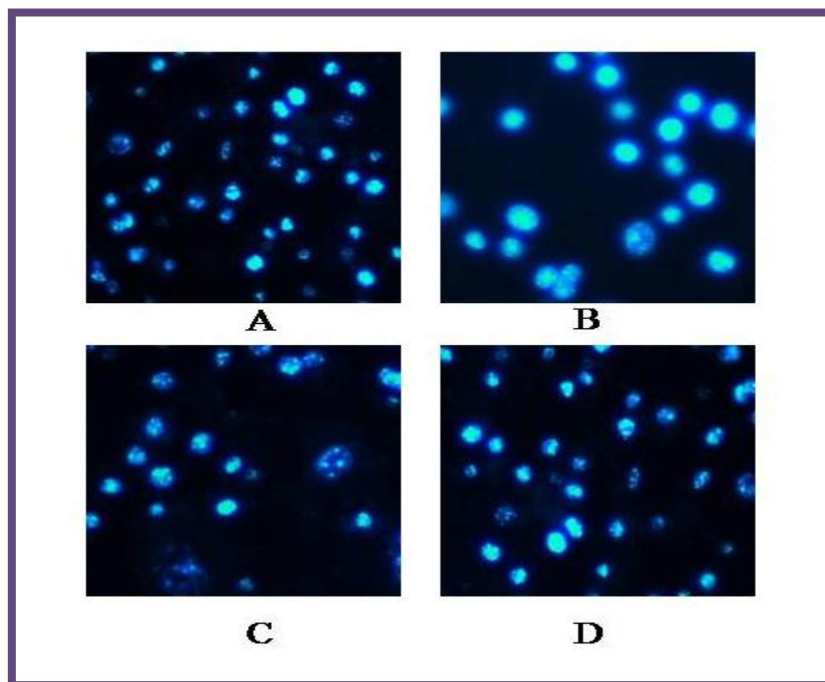


Fig. 4: shows Nuclear damage by DAPI Staining

(A) – Control, B – Rotenone 20 µg/ml, (C) – Phloroglucinol 90µg/ml and (D) – Rotenone 20 µg /ml + Phloroglucinol 60 µg/ml. Effect of phloroglucinol on rotenone induced nuclear damage was determined by DAPI staining. (B) Rotenone induced cells show increased nuclear damage when compared to control. C and D Phloroglucinol and Rot + Phlo treated cells shows decreased morphological changes when compared to rotenone. Magnification: 20X

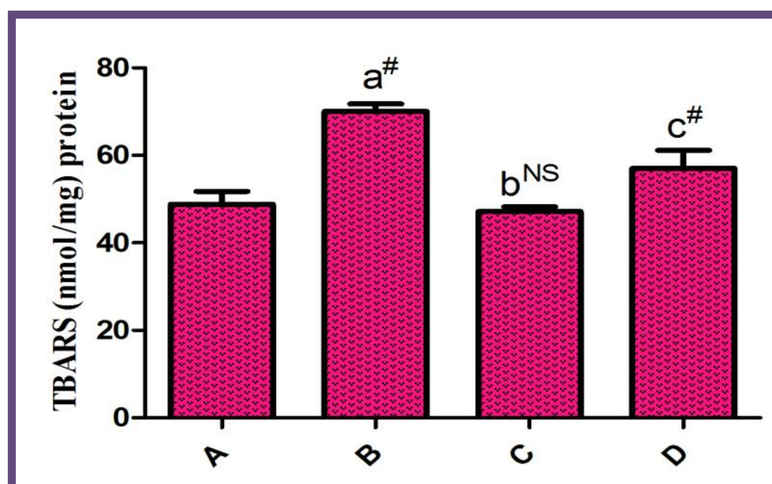


Fig. 5: shows level of Thiobarbituric acid reactive substances (TBARS)

(A) – Control, (B) – Rotenone 20 µg/ml, (C) – Phloroglucinol 90µg/ml and (D) – Rotenone 20 µg /ml + Phloroglucinol 60 µg/ml. Statistical significance: NS – Non significant; # P< 0.01. Rotenone treatments enhance the level of TBARS as compared to control cells,

while phloroglucinol treated cells shows decreased lavel of TBARS as compared to rotenone. a – Comparision between control group and Rotenone treated group, b – Comparision between control group and Phloroglucinol treated group and c – Comparision between Rotenone treated group and Rot+Phlo treated group.

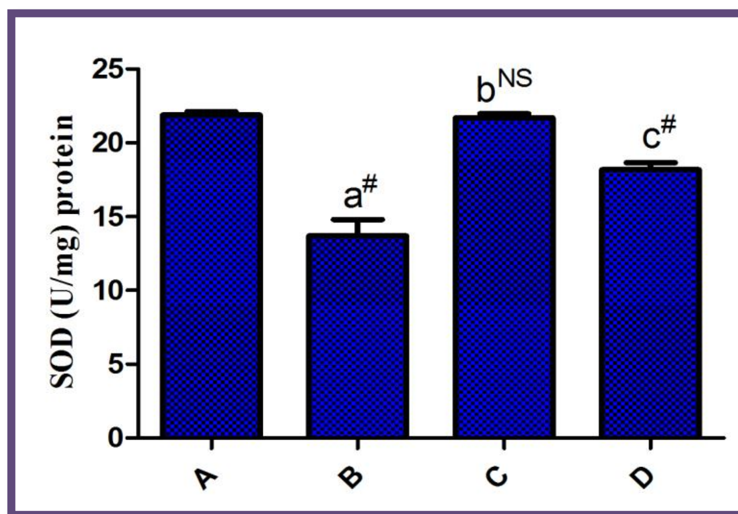


Fig. 6: shows Activity of Super Oxide Dismutase

(A) – Control, (B) – Rotenone 20 µg/ml, (C) – Phloroglucinol 90µg/ml and (D) – Rotenone 20 µg /ml + Phloroglucinol 60 µg/ml. Statistical significance: NS – Non signification; # P< 0.01. Rotenone treatment significantly decreases the activity of SOD as compared to control cells, while phloroglucinol treated cells shows Increases the activity of SOD as compared to rotenone group. a – Comparision between control group and Rotenone treated group, b – Comparision between control group and Phloroglucinol treated group and c – Comparision between Rotenone treated group and Rot+Phlo treated group.

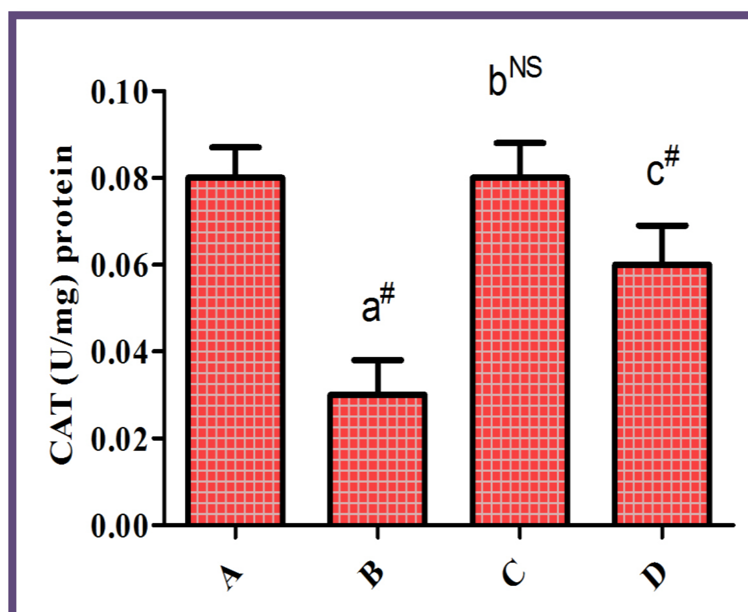


Fig. 7: shows Activity of Catalase

(A) – Control, (B) – Rotenone 20 µg/ml, (C) – Phloroglucinol 90µg/ml and (D) – Rotenone 20 µg /ml + Phloroglucinol 60 µg/ml. Statistical significance: NS – Non significance; # P< 0.01. Rotenone treatment significantly decreases the activity of CAT as compared to control cells, while phloroglucinol treated cells shows Increased the activity of CAT as compared to rotenone group. a – Comparision between control group and Rotenone treated group, b – Comparision between control group and Phloroglucinol treated group and c – Comparision between Rotenone treated group and Rot+Phlo treated group.

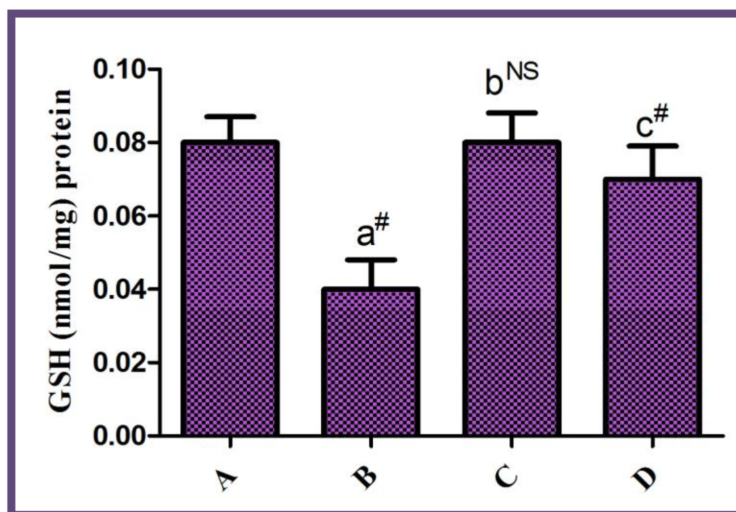


Fig. 8: Shows Level of Reduced Glutathione (GSH)

(A) – Control, (B) – Rotenone 20 µg/ml, (C) – Phloroglucinol 90µg/ml and (D) – Rotenone 20 µg /ml + Phloroglucinol 60 µg/ml. Statistical significance: NS – Non significance; # P< 0.01. Rotenone treatment decreases the level of GSH as compared to control cells, while phloroglucinol treated cells shows increased the level of GSH as compared to rotenone. a – Comparision between control group and Rotenone treated group, b – Comparision between control group and Phloroglucinol treated group and c – Comparision between Rotenone treated group and Rot+Phlo treated group.

CONCLUSION

In conclusion, Phloroglucinol a polyphenol compound exhibited protective effects on rotenone induced oxidative stress and cell death in SH-SY5Y cells. It can also prevent the oxidative damage caused by ROS and Nuclear damage. Antioxidant supplement is a promising approach to halting the neurodegeneration process. Hence, our previous study demonstrated that phloroglucinol possessed potent anti oxidant activity. These finding indicates that phloroglucinol may be a potential agent for the treatment of neurodegenerative disorders such as PD. Rotenone induced *invitro* model of PD is considered as a excellent model, which resembles numerous pathological features of clinical studies, but further extensive studies on *in vivo* model of PD is warranted to find out the therapeutic potential of Phloroglucinol.

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