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## Effect of Quercetin and Hesperidin on Rotenone Induced SH-SY5Y Cells

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Received: 17 September 2017    Revised: 25 January 2018    Accepted: 30 January 2018

### ABSTRACT

To investigate the effect of quercetin and hesperidin on rotenone induced SH-SY5Y cell line model of Parkinson's disease. SH-SY5Y cell line was procured from NCCS Pune. SH-SY5Y cells were maintained below standard conditions SH-SY5Y cells were treated with quercetin and hesperidin. Cell viability, ROS production, mitochondrial membrane potential, nuclear condensation and antioxidant levels were investigated by standard procedures. Quercetin and hesperidin significantly increased the cell viability of SH-SY5Y cells. It decrease the ROS production and maintained mitochondrial membrane potential and barred nuclear damage. It increased the activities of SOD and catalase. Quercetin and hesperidin dose dependently protected dopaminergic neurons beside neurotoxicity. SH-SY5Y cell lines when co-treated with quercetin and hesperidin sheltered them from oxidative damage. Thus both compounds proved to be therapeutic agents against Parkinson's disease.

**Keyword:** Parkinson's disease, SH-SY5Y, Quercetin, Hesperidin, Rotenone.

### INTRODUCTION

Parkinson's disease (PD) is an age-related progressive neurodegenerative disorder with a prevalence of over the age of 50. In recent times, major advances have been ready in the experimental studies of PD, particularly through the use of cell models. The development of a constant dopaminergic (DAergic) neuronal cell model is predominantly required for studying the pathogenesis of PD and increasing therapeutic strategies. Now, pheochromocytoma (PC12) cells for used for Parkinson disease cell model [1], neuronal tumor cell lines represented with human neuroblastoma (SHSY5Y) cells [2] and primary mesencephalic neurons [3]. These cells take off many aspects of the DAergic

neuron death observed in PD when treated by neurotoxins such as 1-methyl-4-phenylpyridinium (MPP+), 6-hydroxydopamine (6-OHDA) and rotenone. Neurotoxins, such as environmental pesticides and herbicides that produce PD like symptoms *in vivo* are generally used to study PD in different neuronal cell line [4-5]. Particularly the SH-SY5Y cell line provides an limitless supply of cells of human origin with the similar biochemical characteristics to human DAergic neurons. In the present study SH-SY5Y neuroblastoma cells were used since these cells share different biochemical and functional characteristics of innate neurons [6]. It is supposed that the pesticide, rotenone induces symptoms of

Parkinson's disease in neurons through disrupting adenosine triphosphate (ATP) supply [7]. The cellular components such as lipids, proteins and DNA was damaged by the oxidative products. The pesticide rotenone can freely cross cellular membranes and accumulate in mitochondria, where it inhibits complex-I by impairing oxidative phosphorylation [9]. This happens when rotenone forms a complex with members of the mitochondrial electron transport chain (ETC), purposely at complex I, resulting in limited ATP production [10]. Many factors are speculated to control in the mechanism of cell death of nigrostriatal dopaminergic neurons in PD, including oxidative stress and cytotoxicity of reactive oxygen species (ROS), trouble of intracellular calcium homeostasis exogenous and endogenous toxins mitochondrial dysfunction [11], redox cycling and reduced NADPH before being oxidized by an electron acceptor to produce superoxide [12]. One of the probable ways to prevent the cell death induced by oxidative stress is dietary or pharmacological intake of antioxidants [13]. Therefore it was considered to investigate the anti-Parkinson effect of quercetin, hesperidin on rotenone induced PD in SH-SY5Y cells.

## MATERIALS AND METHODS

### Chemicals

Quercetin, Hesperidin, Rotenone, thiobarbituric acid (TBA), Phenazine methosulfate (PMS), Nitroblue tetrazolium (NBT), MTT, 2,7-diacetyl dichlorofluorescein (DCFH-DA), Dulbecco's modified Eagle's medium (DMEM), rodmine 123 and 4'-6-diamidino-2-phenylindole (DAPI) were purchased from sigma chemicals were of analytical grade.

### Cell viability Assay

SH-SY5Y cells were seeded in a 96 well plate at a thickness of  $1 \times 10^3$  cells per well. After adding up, cells were treated with  $20 \mu\text{M}$  rotenone for 24 hrs. After that cells were treated with quercetin and hesperidin it was incubated with  $0.5 \text{ mg/ml}$  MTT for 4 h at  $37^\circ\text{C}$ . Subsequent target of the MTT solution the same volume of DMSO was supplementary into each well to dissolve the coloring agent, absorbance was examine in a microtiter plate reader at  $490 \text{ nm}$ , cell viability was expressed as a percentage of the absorbance from control cells according to the method of Liu et al [14].

### ROS Production

Intracellular ROS generation induced by rotenone in SH-SY5Y cells were quantified using fluorescent dye, 2,7-dichlorofluorescein diacetate (DCFH-DA). Over night grown SH-SY5Y cells were used for the study. Rotenone was added to all the plates not including control for 24hrs. Quercetin and hesperidin at a concentration of  $100 \mu\text{M/ml}$  was treated in stress induced SH-SY5Y cell line in a 24 well plates for 24 hrs. After exposure, the cells were washed by centrifugation and loaded with  $20 \mu\text{M}$  DCFH-DA in Muller Hinton broth for 30min at  $37^\circ\text{C}$ . After that, control and treated cells were washed with Muller Hinton broth and fluorescence was recorded using a fluorescence microplate reader at  $37^\circ\text{C}$  in the method of Wang H and James [15].

### Mitochondrial membrane potential

Mitochondrial membrane potential were handling with quercetin and hesperidin in SH-SY5Y cells were measured by rhodamine-123 using a fluorescence spectrophotometer. Briefly cells were treated with rotenone, quercetin and hesperidin for 24h and then incubated with rhodamine-123 in a final concentration  $10 \mu\text{M}$  respectively for 30 min at  $37^\circ\text{C}$ , after washing twice with buffered saline, fluorescence was recorded at  $488 \text{ nm}$  excitation and  $523 \text{ nm}$  emission wave lengths each field of cells was photographed using a fluorescence microscopy [16].

### Nuclear Condensation by DAPI

Nuclear damage and cell death in treated neuro cells by DAPI staining was performed as described by Morikawa and Mitsoshiro [17]. Briefly; the cells were seeded into 24 well plates and treated with quercetin and hesperidin ( $100 \mu\text{M/ml}$ ) for 24 hrs. Control and tested cells were rinsed with phosphate buffered saline (PBS), fixed with ice-cold 10% trichloroacetic acid and further washed with cold ethanol. The cells were treated with Triton-X (10%v/v) and stained with  $1 \mu\text{g/ml}$  4'-6-diamidino-2-phenylindole (DAPI) for 3 min. To reduce the surroundings the stained cells were washed with PBS, cover-slipped with 90% glycerol and observed under a fluorescence microscope and nuclear condensation was observed.

### Antioxidant activity

#### Superoxide dismutase activity assay

The superoxide dismutase activity was measured by the extent inhibition of amino blue tetrazolium formazan improvement in the mixture of nicotinamide adenine dinucleotide, phenazine methosulphate and nitroblue tetrazolium [NADH-PMS-NBT], according to the method of Kakkar et al [18]. One unit of enzyme which caused 50% inhibition of NBT reduction/mg protein was calculated.

#### Catalase activity assay:

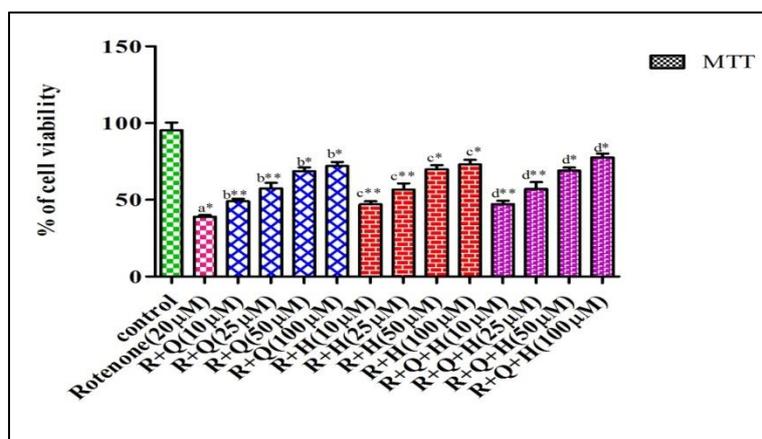
The catalase activity was measured by the method of Aebi9 in which the rate of breakdown of H<sub>2</sub>O<sub>2</sub> was determined spectrophotometrically at 240 nm. The enzyme activity was expressed as x10<sup>-1</sup> k/mg protein.

Where K correspond to the rate constant of the first order reaction of catalase. Protein concentration was resolved by the method of Lowry [20].

## RESULTS

### Cell viability (MTT)

The cell viability were analyzed by MTT assay. In the current study on rotenone the viability of SH-SY5Y cell was reduced and shows the cytotoxicity of rotenone. Quercetin and hesperidin treated cells, viability was improved in dose depended manner when compared with rotenone treated SH-SY5Y cell. The combination of quercetin, hesperidin and rotenone treated cell viability was improved compared with rotenone treated SH-SY5Y cells.

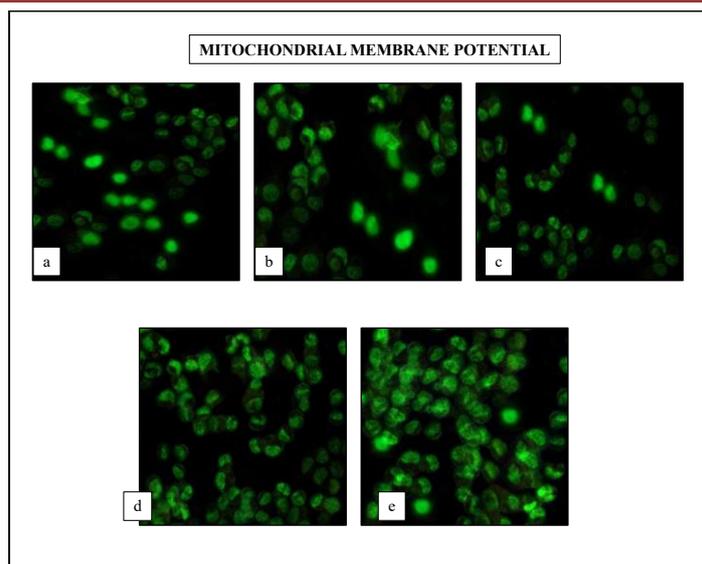


**Fig. 1: Measurement of percentage of viable cells by MTT assay: The values were expressed as Mean  $\pm$ SD. a - assessment between control cells and rotenone (R) induced cells. b - assessment between rotenone and rotenone + quercetin (Q), c - assessment between rotenone and rotenone + hesperidin (H), d - assessment between rotenone and rotenone + quercetin + hesperidin. Significance \* $p < 0.001$ , \*\* $p < 0.01$ ,  $n = 3$ .**

### Mitochondrial Membrane

The mitochondrial membrane potential (MMP) was reduced when SH-SY5Y cells were treated with rotenone compared with control. Mitochondrial membrane potential is considered by determining the green fluorescence in the presence of Rhodamine-123. Cells treated with 20  $\mu$ M rotenone resulted in significant changes take place in mitochondrial membrane potential.

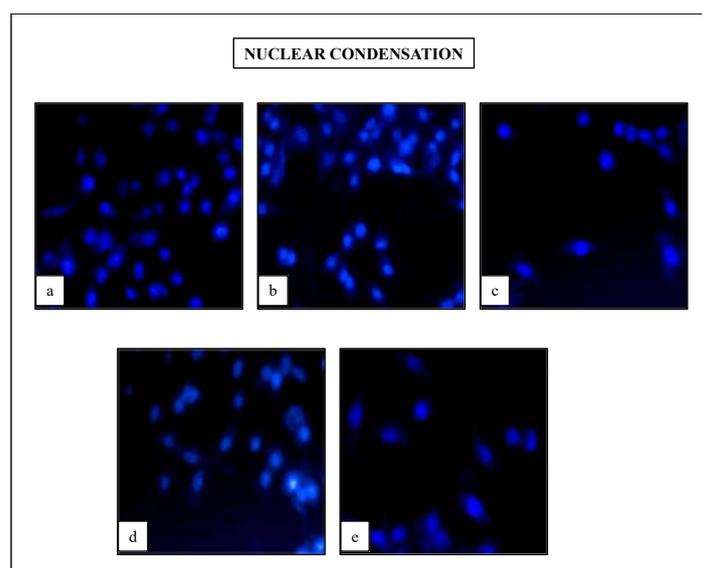
The average green fluorescence ratio was increased as a result of rotenone treatment as compared to untreated control. Co treatment with quercetin and hesperidin to rotenone treated cells displayed much higher green fluorescence, representing a polarized state of mitochondrial membrane as compared to rotenone treatment alone.



**Fig. 2:** Shows the determination of mitochondrial membrane potential (MMP): a-vehicle control, b- rotenone (20 $\mu$ M) , c- rotenone+quercetin (100 $\mu$ M), d- rotenone+hesperidin (100 $\mu$ M), e- rotenone(20 $\mu$ M) + quercetin (100 $\mu$ M)+ hesperidin (100 $\mu$ M). Magnification-40X  
**Nuclear condensation**

The DNA damage induced by rotenone were detected by DAPI staining method. Fig.3 show the control, induced and treated cells stained with DAPI. On rotenone induction the DNA damage was found to be high and this was indicated by high intensity of DAPI fluorescent dye. On quercetin and hesperidin combination the intensity of DAPI was reduced which indicate that the DNA damage was reduced by

combination treatment. Thus the present study shows that quercetin and hesperidin protect the SH-SY5Y cells from DNA damage induced by rotenone. Measuring DNA fragmentation condensation in conjunction with measuring caspase activation is considered the most reliable method for identifying apoptotic cells [21].

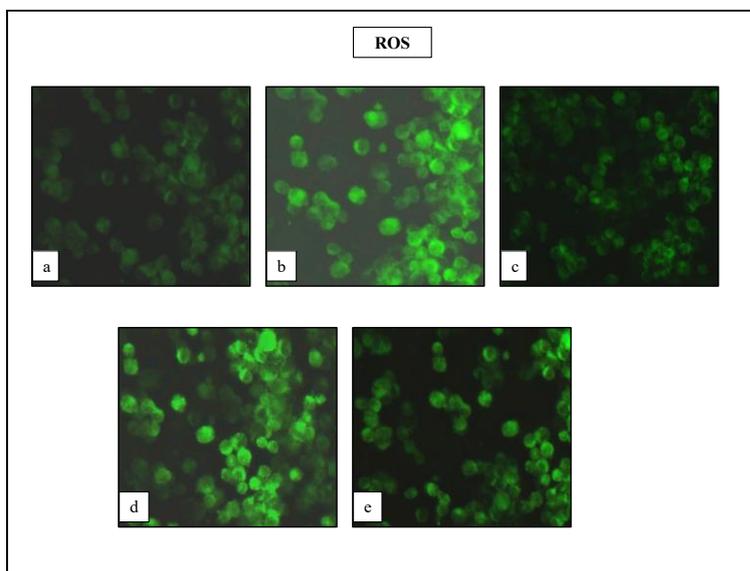


**Fig 3:** Shows the nuclear condensation : a-vehicle control, b- rotenone (20 $\mu$ M), c- rotenone+quercetin (100 $\mu$ M), d- rotenone+hesperidin (100 $\mu$ M), e- rotenone(20 $\mu$ M) + quercetin (100 $\mu$ M)+ hesperidin (100 $\mu$ M). Magnification- 40X.

### Reactive oxygen species

The ROS generated within the cell was measured by DCFH-DA. Cell pigment indicator which is non-fluorescence until deduction of the acetate group by intracellular esterase and oxidation occur within the cell. On rotenone treatment on SH-SY5Y cells the ROS was found

to be generated within the cells and this was indicated by the strength of the fluorescence of DCFH. The generated ROS in the cells was reduced by combination of quercetin and hesperidin treatment and maximum reduction was seen at 100 $\mu$ M concentration.

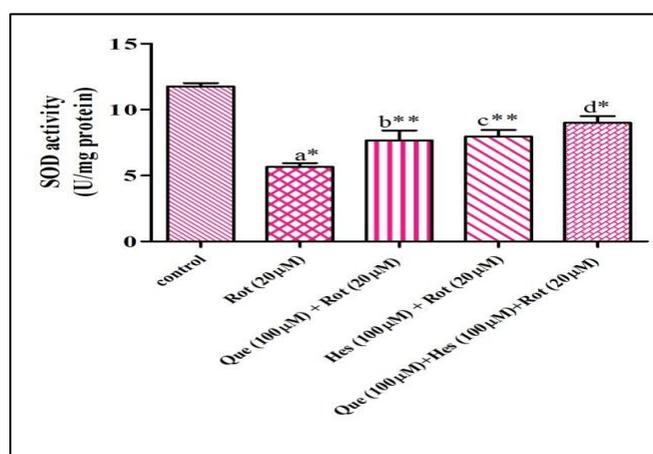


**Fig 4:** Shows the determination of reactive oxygen species : a-vehicle control, b- rotenone (20 $\mu$ M), c- rotenone+quercetin (100 $\mu$ M), d- rotenone+hesperidin (100 $\mu$ M), e- rotenone(20 $\mu$ M) + quercetin (100 $\mu$ M)+ hesperidin (100 $\mu$ M). Magnification- 40X.

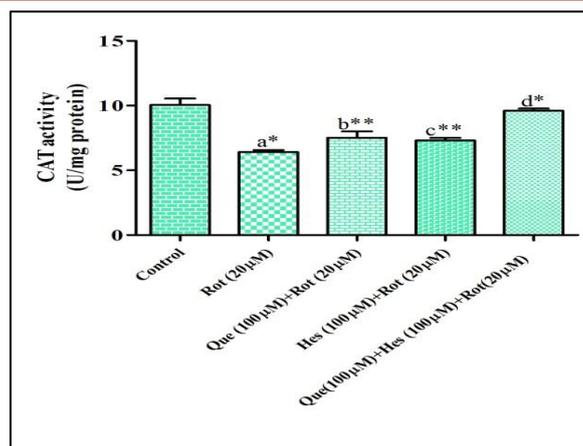
### Activity of SOD (Superoxide dismutase) and Catalase (CAT)

Intracellular levels of antioxidant enzymes such as SOD and CAT in SH-SY5Y cells following exposure were measured by spectrophotometric analysis. The results are presented in figures 5 (a)&(b) SH-SY5Y cells exposed rotenone induced cells showed a significantly decrease in the particular activities of SOD and CAT when

compared to cells treated with vehicle control ( $p < 0.001$ ). Cells exposed to quercetin and hesperidin showed significantly ( $p < 0.01$ ) improved activity when compared to rotenone cells. SOD and catalase activity were significantly ( $p < 0.001$ ) increased in combination group SH-SY5Y cells when compared to cells treated with rotenone only.



(a)



(b)

**Fig 5:** The values were expressed as Mean  $\pm$ SD. As a - comparison between control cells and rotenone (R) induced cells. b- comparison between rotenone and rotenone + quercetin (Q), c - comparison between rotenone and rotenone + hesperidin (H), d - comparison between rotenone and rotenone + quercetin + hesperidin. Singnificance \* $p$ <0.001, \*\* $p$ <0.01,  $n$ =3.

## DISCUSSION

Keiko Imamura et al. [22] demonstrated that D-beta-hydroxybutarate (bHB) has a neuroprotective effect against mitochondrial inhibitors on differentiated SH-SY5Y dopaminergic neuroblastoma cells. bHB supports the mitochondrial respiration system and inhibits the initiation of apoptosis in the early stage. Chance et al., recommended the rotenone binds to mitochondrial complex-I and inhibits NADH dehydrogenase and reduce cell respiration and the mitochondrial dysfunction associated with pathogenesis of Parkinson's disease. Betarbet et al., Alam and Schmidt [24-25] reported that rotenone is regarded to provide good *invitro* and *invivo* models for PD. Keabetsure Seoposengure et al. [26] suggested that the plant extracts limited the depletion of intera-cellular glutathione content caused by rotenone exposure and demonstrated potent anti-apoptotic effects. *Invitro* assays were working to assess cytotoxicity, interacellular redox state ROS and intracellular glutathione content, MMP and casepase-3-activity of the plant extracts, these parameter were preferred as oxidative stress and mitochondrial dysfunction appear to play key roles of PD manifestation reported by Thoman et al [27]. Differentiated and undifferentiated SH-SY5Y cells have gained broad acceptance as models of DAergic neurons for PD research. SH-SY5Y cells are more suitable PD cell models than others. These cells have DAergic neuronal properties and have related susceptibility to neurotoxins

and neuroprotective agents as primary neurons. Differentiation serves to make SH-SY5Y cells more equivalent to DAergic neurons and thus, a practical model for investigation of the pathogenesis of PD and evolution of therapies reported by XIE Hong-rong et al. [28] Chandramani et al., suggested that the neuroprotective potential of mono-therapy and combination therapy of NAC and IGF-1 on proteasome dysfunction induced neurotoxicity in human neuroblastoma SH-SY5Y cells by exploring the affiliation between oxidative stress, ER stress, autophagy and apoptotic cell death [29]. Proteasome inhibition in SH-SY5Y cells triggers apoptotic cell death. A drug combination therapy comprised of NAC and IGF-1 entirely alleviates cytotoxicity associated with proteasome dysfunction. The neuroprotective effect relies on modulation of multiple pathways association with apoptosis, which consist of reducing ROS, GSH depletion, lowering ER stress and preventing autophagy. Thus a combination therapy employing a neurotrophic factor and a free radical scavenger may afford a novel and shows potential therapeutic approach for up dysfunction mediated neuro cell dysfunction [30].

## CONCLUSION

Quercetin and hesperidin dose dependently protected dopaminergic neurons against neurotoxicity. SH-SY5Y cell lines when co-treated with quercetin and hesperidin protected them from oxidative damage. Thus both

compounds proved to be therapeutic agents against Parkinson's disease.

#### ACKNOWLEDGEMENT

We thank to the Tamil Nadu government for the financial support for this study in the form of scholarship.

#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**Cite this article as:**

Srimathi Priyanga K, Vijayalakshmi K. Effect of Quercetin and Hesperidin on Rotenone Induced SH-SY5Y Cells. *J Pharm Chem Biol Sci* 2017; 5(4): 429-436.