ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

NEUROPROTECTIVE EFFECT OF HESPERIDIN ON 6-OHDA INDUCED PARKINSONISM IN SHSY5Y CELLS

Priya Nagappan*[1], Vijayalakshmi Krishnamurthy[2], Khadira Sereen[3]

[1]*PG & Research Department of Biochemistry, St. Joseph's College of Arts & Science, Cuddalore, India*

[2]*PG & Research Department of Biochemistry, Bharathi Women's College, Chennai, Tamil Nadu, India*

[3]*Research Scholar, PG & Research Department of Biochemistry, Bharathi Women's College, Chennai, Tamil Nadu, India*

**Corresponding author: priyan7shrine@gmail.com*

ABSTRACT

Parkinson's disease (PD) occurs due to oxidative stress and it is the second most common neurodegenerative disorder after Alzheimer's disease. 6-OHDA (6-hydroxydopamine) is a widely used neurotoxin for investigating the pathology of PD both *in vitro* and *in vivo* conditions. Studies have shown that the neurotoxicity of 6-OHDA may be related to its ability to generate reactive oxygen species (ROS) leading to neuronal damage. The current study was carried out to investigate the neuroprotective effects of hesperidin, a citrus fruit flavanol, against 6-OHDA induced neurotoxin in human neuroblastoma SHSY5Y cells. We assessed cell viability by MTT assay, ROS production by DCFH-DA and Nuclear change by DAPI. Cell death was determined in normal, 6-OHDA and hesperidin treated cells. The cell death in 6-OHDA induced SHSY5Y cells was accompanied by the loss of neurons, increased ROS generation, the depletion of GSH, enhanced activities of enzymatic antioxidants which were attenuated in the presence of hesperidin. Our data suggests that hesperidin exerts its neuroprotective effect against 6-OHDA due to its antioxidant properties in a neuroblastoma SHSY5Y cell lines.

Keywords: Parkinson's disease, SHSY5Y cell line, ROS production, Oxidative stress

1. INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by a selective loss of dopaminergic neurons in the substantia nigra. Many factors are speculated to operate in the mechanism of cell death of nigrostriatal dopaminergic neurons in PD, including oxidative stress and cytotoxicity of reactive oxygen species (ROS), disturbances of intracellular calcium homeostasis, exogenous and endogenous toxins and mitochondrial dysfunction. An endogenous neurotransmitter dopamine (DA) is thought to be a major source of oxidative stress to these neural cells [1-2]. DA contains unstable catechol moiety, and it can oxidize spontaneously to form ROS, free radicals, and quinones [3-5]. In the human substantia nigra, the DA oxidation products may further polymerize to form another neurotoxin, neuromelanin [6]. These oxidation products can damage cellular components such as lipids, proteins and DNA [7]. One of the plausible ways to prevent the cell death induced by oxidative stress is dietary or pharmacological intake of antioxidants. One family of naturally occurring compounds possessing free radical scavenging properties is the flavonoids and it is found in citrus fruits. Hesperidin is reported to exert a wide range of pharmacological effects such as antioxidant, anti-inflammatory, antihypercholesterolemic and anticarcinogenic properties [8]. It has also been demonstrated that hesperidin can protect neurons against various types of insults associated with many neurodegenerative disease [9].

Current pharmacological therapies for PD are inadequate, and alternative strategies such as stem cell therapy, neurotransplantation and deep brain stimulation are still in infant stage. There has been considerable interest in the development of neuroprotective drugs from natural origins as a therapeutic strategy for PD [10]. Citrus fruits and their products are important sources of health-promoting constituents and are widely consumed around the world [11].

In this study, we investigated the protective effect of hesperidin on 6-hydroxydopamine induced cellular model for PD by analysing its effect on hesperidin-mediated oxidative stress generation in human neuroblastoma SHSY5Y cells.

2. MATERIAL & METHODS

6-OHDA, hesperidin, thiobarbituric acid (TBA), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2, 7-diacetyl dichlorofluorescein (DCFH-DA), Dulbecco's modified Eagle's medium (DMEM), EDTA and trypsin were purchased from Sigma Chemicals. All other chemicals used were of analytical grade.

2.1. Cell culture

Catecholaminergic neuroblastoma SHSY5Y cells were grown in DMEM supplemented with 10% FBS, 100U/ml penicillin, and 100 g/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The cells were plated at an appropriate density according to each experimental scale.

2.2. Cell viability by MTT assay

Cell viability was performed by the method [12]. Cells were grown in DMEM, 100µl of 80µM 6-OHDA was induced to all the plates. Cells were maintained at 37°C in CO_2 incubator in a saturated humid atmosphere containing 5% CO_2 and 95% air. Cells were incubated with different concentrations of hesperidin (25µM, 50µM, 80µM, 100µM and 125µM) for 24hrs and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed to detect IC_{50}. The effective dose of hesperidin was used to identify potential neuroprotective effect against 6-OHDA toxicity.

2.3. ROS Production

Intracellular ROS levels were measured using the dichlorodihydrofluorescein diacetate (DCFH-DA) assay [13]. DCFH-DA is a lipophilic cell permeable compound that is deacetylated in the cytoplasm to DCF by cellular esterases. DCF is then oxidized by radicals such as hydroxyl, peroxyl, alkoxyl, nitrate and carbonate to a fluorescent molecule (excitation 530 nm, emission 485 nm). Overnight grown SHSY5Y cells were used for the study. 6-OHDA was induced to all the plates except control for 24 hrs. Hesperidin at a concentration of (80 and 100µM /ml) was treated in stress induced SHSY5Y cell line in a 24 well plates for 24hrs. After exposure, the cells were washed by centrifugation and loaded with 20 µM DCFH-DA in Muller Hinton broth for 30 min at 37°C. Thereafter, control and treated cells were washed with Cation adjusted Muller Hinton broth and fluorescence was recorded every 5 min for 30 mins (excitation 485 nm, emission 535 nm) using a plate reader at 37°C. ROS level was calculated as mean slope per min and normalized to the unexposed control.

2.4. Nuclear condensation by DAPI stain

To determine the level of nuclear damage based cell death in drug treated neuro cells, DAPI staining was performed as described by the method [14]. Briefly, the cells were seeded into 24 well plates and treated with hesperidin (80 and 100µM /ml) for 24 hrs. Control and treated cells were rinsed with phosphate buffered saline (PBS), fixed with ice-cold 10% trichloroacetic acid, and further washed with cold 70, 80, 90% absolute ethanol. The cells were treated with Triton-X (10 % v/v) and stained with 1 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) for 3 min. To reduce the background, the stained cells were washed with PBS, cover-slipped with 90% glycerol and observed under a fluorescence microscope (Labomed- Carl zeiss Lens with blue filter Olympus India) and nuclear condensation was observed.

2.5. Antioxidant Activities

2.5.1. Thio Barbituric Acid Reactive Substances

SHSY5Y neuroblastoma cells were suspended in 130mM Kcl and 5mM PBS containing .1ml of 0.1M dithiothreitol (DTT) and centrifuged at 20, 000g for 15min (4°C). The supernatant was taken for biochemical estimation. The level of TBARS was carried out by the method [15]. The pink coloured chromogen formed by the reaction of 2-TBA with breakdown products of lipid peroxidation was measured.

2.5.2. Enzymatic antioxidants activities

2.5.2.1. SOD Activity assay

Superoxide dismutase (SOD) activity was assayed by the method based on the inhibition of the formation of (NADH-PMS-NBT) complex by the method [16].

2.5.2.2. CAT Activity assay

CAT activity was assayed by the decomposition of hydrogen peroxide by the method [17]. H_2O_2 degradation was monitored at 240nm for 1min.

2.5.2.3. Glutathione Peroxidase Activity assay

The activity of glutathione peroxidase (GPx) was assayed spectrophotometrically by the method [18]. Briefly a known amount of enzyme preparation was allowed to treat with hydrogen peroxidase (H_2O_2) and GSH for a

specified time period. The GSH content remaining after the reaction was measured.

2.5.3. Non-enzymatic antioxidant activities

Estimation of Glutathione: The total GSH content was measured by the method [19]. This method was based on the development of a yellow colour, when 5, 5-dithiobis (2-nitro benzoic acid) was added to compound containing sulfhydryl groups.

2.6. Statistical Analysis

All the results presented were as mean±SD and all statistical analysis was performed using SPSS 20.0 statistical software. Significant differences among the treatment groups were analyzed by variance (One way ANOVA) followed by least significant difference (LSD) test. Results were considered to be statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

The present study was to test the hypothesis that 6-OHDA induces cell death through a series of events involving oxidatively damaged DNA in SHSY5Y neuronal-like cell line as a model. The human neuroblastoma SHSY5Y cell line, a well characterized neuronal-like model, extensively used for *invitro* neurotoxicity testing, was chosen for this study because it could be induced to differentiate into the adrenergic phenotype [20].

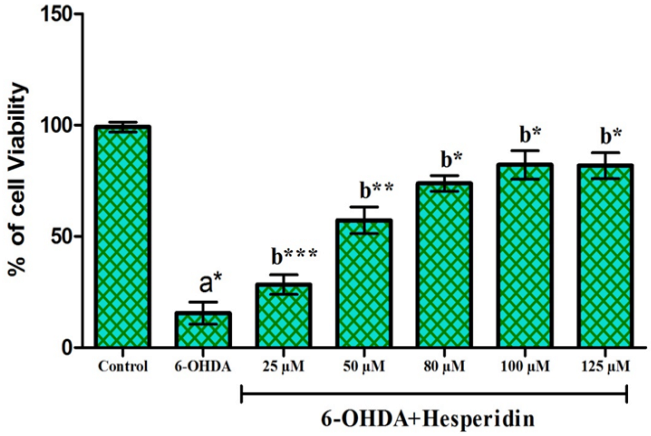

Fig. 1: Cell viability by MTT assay#

The purpose of this study was to elucidate the role of hesperidin in combating oxidative stress SHSY5Y cells. All data provide strong evidence that hesperidin could protect SHSY5Y cells against oxidative stress induced by 6-OHDA. The survival rates of SHSY5Y cells treated with 80µM and 100µM hesperidin was increased when compared with cells treated with 25 µM and 50 µM. This results show that the high concentrations of hesperidin against 6-OHDA could be exerting a direct free radical scavenging effect. SHSY5Y cells induced by 6-OHDA lead to ROS production as shown in the experiments with the fluorescent dye H_2DCFDA. Therefore hesperidin was able to reduce ROS production induced by 6-OHDA treatment.

The effect of hesperidin on SHSY5Y cells were recorded using the MTT reduction assay. SHSY5Y cells were exposed to varying concentrations (25µM, 50µM, 80µM, 100µM and 125µM) for 24 hrs in which the concentration of 80µM, and 100µM was found to effective for survival of 50%. So SHSY5Y cells are found to survive as the hesperidin concentration increases and it was found to be dose dependent. Natural compounds are found to exhibit neuroprotective properties based on their ability to quench reactive oxygen species and thereby protect cellular damage.

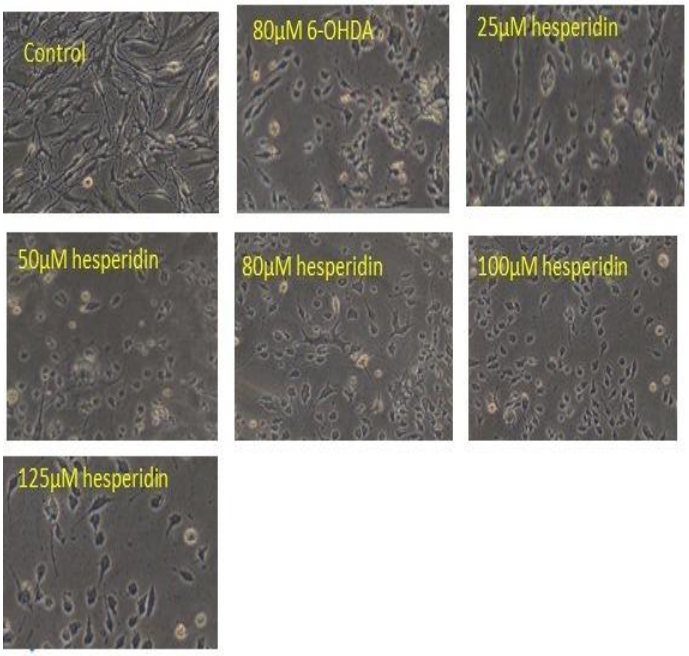

Fig. 2: Depiction of cell viability by MTT assay

The formation of ROS was measured by using a fluorescent probe, 2, 7-Dichlorodihydrofluorescein diacetate (DCFH-DA) that can penetrate into the intracellular matrix of cells, where it is oxidized by ROS to form fluorescent dichlorofluorescein (DCF). SHSY5Y cells exposed to 6-OHDA showed a significant increase in DCF fluorescence whereas there is no significant changes in ROS formation in SHSY5Y cells treated with hesperidin. DCFH-DA is a cell-permeate indicator for

reactive oxygen species (a thiol-reactive tracer) which is non-fluorescent until removal of the acetate groups by intracellular esterase's, and oxidation occurs within the cell.

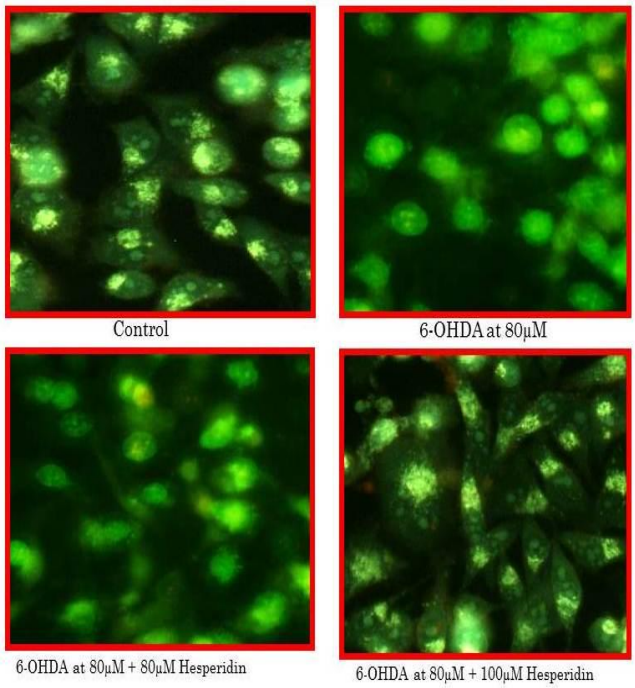

Fig. 3: ROS Production by DCHF-DA assay

Cytoplasmic enzymes hydrolyze the acetate groups from this membrane-permeant probe and the chloromethyl moieties become conjugated to intracellular thiols. In the presence of intracellular peroxides, the esterase-mediated deacylation of DCFH-DA to DCFH occurs within the cells, and the non-fluorescent DCFH is subsequently oxidized, resulting in the formation of the highly fluorescent DCFH. DCFH has been reported to be sensitive to oxidation by peroxynitrite, hydrogen peroxide (in combination with cellular peroxidases), peroxidases alone, and hydroxyl radicals and it is unsuitable for the measurement of NO, hypochlorous acid, or superoxide radicals in biological systems. A dose dependent reduction in the fluorescence intensity was observed with the cells treated with the hesperidin. Treatment with hesperidin showed dramatical loss in green fluorescence by DCF which suggests that ROS generation was inhibited in cells treated with hesperidin whereas in 6-OHDA treated cells it was found that the fluorescence was higher as discussed in results.

DAPI staining shows the level of nuclear damage in 6-OHDA and drug treated cells. Decreased cell death was found at higher concentration of 100µM hesperidin whereas in 6-OHDA treated cells cell death was found to be more.

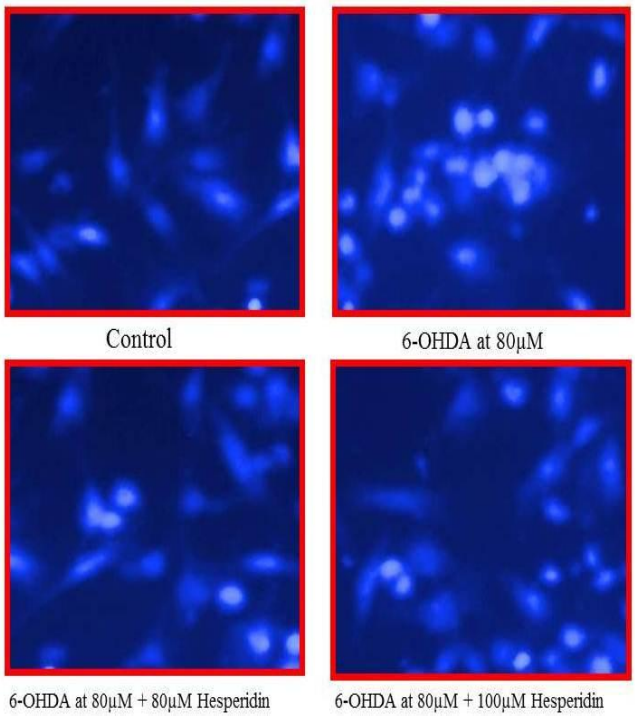

Fig. 4: Nuclear condensation by DAPI

The mitochondria are the principle intracellular sources of ROS and also the major targets of oxidative stress [21]. Additionally, other potential mechanisms such as the ability of hesperidin to enhance glutathione content could be involved in its protective effect on 6-OHDA induced oxidative stress. When cells were incubated with 6-OHDA there was an increased level of TBARS indicating overproduction of free radicals along with glutathione depletion, which is reported by previous studies [22]. Our results showed that the decrease in the enzymatic activities of SOD, GPx and CAT in neuroblastoma cells incubated with 6-OHDA alone is likely due to the response towards increased ROS generation following 6-OHDA treatment. Complex I inhibition induces SOD activity in the brain tissue [23]. Decrease in the activity of GPx in 6-OHDA alone treated cells indicated that there was an increase in the concentration of lipid peroxides and/or H_2O_2. Reduced GSH levels may also account for the decrease in GPx activity. The antioxidant activities of SOD, CAT, GPx and GSH have been significantly increased with hesperidin and the level of TBARS has been decreased when compared to the 6-OHDA treated SHSY5Y cell lines.

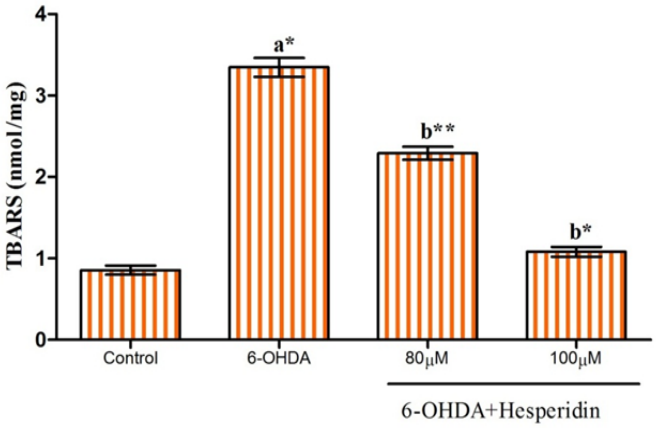

Fig. 4: Effect of hesperidin on TBARS level in SHSY5Y cell lines#

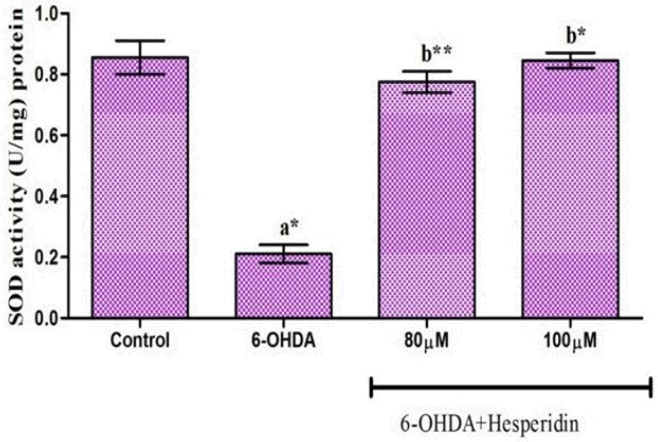

Fig. 5: Effect of hesperidin on Superoxide dismutase activity in SHSY5Y cell lines#

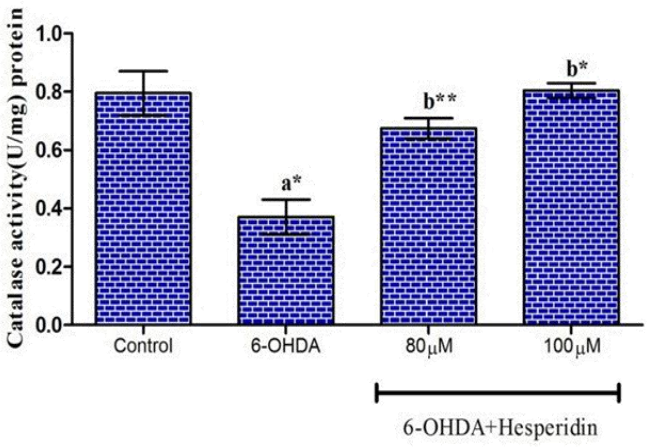

Fig. 6: Effect of hesperidin on Catalase activity in SHSY5Y cell lines#

Hesperidin therapy could significantly attenuate ROS formation by reducing the levels of TBARS and restored in cultured PC12 cells [24]. Another study has shown antioxidant enzyme activity and GSH to physiological levels in the brain [25] that hesperidin treatment could reduce stress-induced anxiety, impaired locomotor activity, and mitochondrial dysfunction in mice by modulating the nitrergic pathway [26]. Hesperidin has also protected cortical neurons from ROS-mediated injury by the activation of prosurvival Akt and ERK1/2 signaling pathways [27].

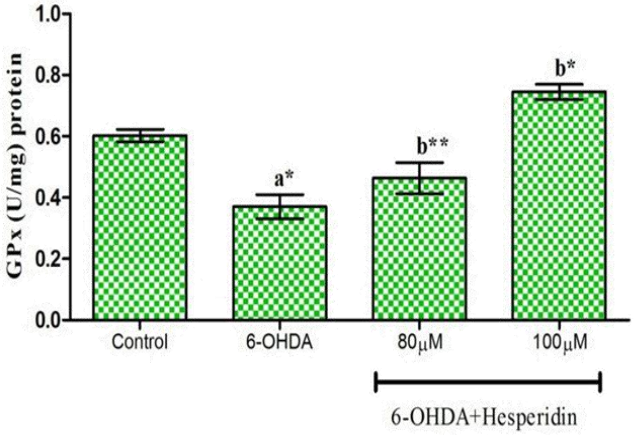

Fig. 7: Effect of hesperidin on Glutathione peroxidase activity on SHSY5Y cell lines#

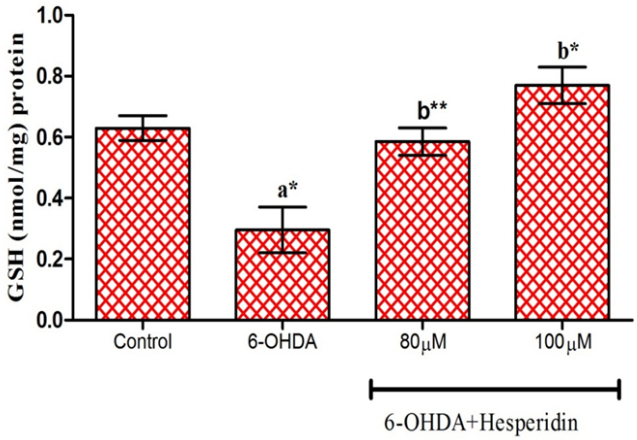

Fig. 8: Effect of hesperidin on Reduced Glutathione in SHSY5Y cell lines#

*# Statistical significance *p<0.001 and **p<0.01*
a=comparison between 6-OHDA and control
b= comparison between hesperidin treated cells and 6-OHDA cells

Tunisian *Rosmarinus officinalis* extracts have a relaxing effect, through their high antioxidant activity, their capacity of enhancing neurite outgrowth and cell differentiation of PC12 cells and their aptitude for regulation of cholinergic system [28].

These pathways are involved in the inhibition of the release of proapoptotic proteins such as apoptosis signal

regulation kinases1 (ASK1), BAD, and Caspases 3 and 9, suggesting that the neuroprotective effects of hesperidin may be due to its effect on a yet identified receptors [28]. Moreover, hesperidin can also protect against amyloid-beta-(Aβ) associated neurotoxicity, and glutamate-induced excitotoxicitys. A more recent study showed that hesperidin treatment could reduce cerebral damage induced by stroke in the rat brain which is due to the reduction of free radicals and associated neuro inflammation [29]. Hesperidin also shows better results in neurotransmitter levels like dopamine, nor-epinephrine, epinephrine and serotonin in combination with L-Dopa on 6-OHDA treated animal model [30]. The results of the present study suggests that hesperidin attenuates neuronal damage induced by 6-OHDA by reducing oxidative stress. These findings may have important implications in the use of hesperidin for the prevention of Parkinson's disease.

4. CONCLUSION

The present study confirms that the cell viability of hesperidin was in a dose dependent manner. Antioxidant potential shows elevated level in the cells treated with hesperidin at 100μM /ml. ROS production and nuclear damage was also significantly decreased in SHSY5Y cells treated with hesperidin at 100μM /ml. However, further research involving various animal models and clinical trials is needed to validate hesperidin as new therapeutic agent.

5. REFERENCES

1. Olanow CWA. *Trends Neuroscience,* 1993; **16**:439-444.
2. OffenD, Ziv I, Panet H, Wasserman L, et al. *Cell Mol Neurobiol.,* 1997; **17**:289-304.
3. Cohen G, Heikkila RE. *Journal of Biol Chem.,* 1974, **249**:2447-2452.
4. Graham DG. *Mol Pharmacol.,* 1978;**14**:633-43.
5. Hastings TG, Zigmond MJ. *Journal of Neurochemistry*, 1994; **63**:1126-1132.
6. Jellinger K, Kienzl E, Rumpelmair G, Riederer P, et al. *Journal of Neurochemistry*, 1993; **59**:1168-1171.
7. Halliwell B, Gutteridge JM. *Federation of European Biochemical Society Letter*, 1992; **307(1)**:108-112.
8. Chen MC, Ye YI, Guang JI, Jian-Wen LIU. *Journal of Agricultural and Food Chemistry*, 2010; **58(6)**:3330-3335.
9. Cho J. *Archives of Pharmacal Research*, 2006; **29(8):** 699-706.
10. Fukui M, Choi HJ, Zhu BT. *Free Radical Biology and Medicine*, 2010; **49(5)**:800-813.
11. Benavente-Garcisa O, Castillo J, Marin FR, et al. *Journal of Agricultural and Food Chemistry,* 1997; **45**: 4505-4515.
12. Mosmann T. *Journal of Immunol Methods*., 1983; **65(1-2)**:55-63.
13. Possel H, Noack H, Augustin W, Keilhoff G. *FEBS Lett.,* 1997; **416**:175-178.
14. Sandra F, Matsuda M, Yoshida H, Hirata M. *Carcinogenesis,* 2002; **23**:2031-2041.
15. Ohkawa H, Ohishi N, Yagi K. *Anal. Biochem;* 1979; **95**:351-358.
16. Misra, Fridovich. *Acc Chem Res.,* 1972; **5**:321.
17. Sinha, Asra K. *Analytical Biochemistry*, 1972; **47(2):**389-394.
18. Rotruck JT, Pope AL, Ganther HE, Swanson AB, et al. *Science*, 1973; **179**:588-590.
19. Moron MS, DePierre JW, Manerwik KB. *Biochemica Biophysica Act.,* 1979; **582**:67-68.
20. Pahlman S, Ruusala AI, Abrahamsson L, Odelstad L, Nilsson K. *Cell Differ.*,1983; **12(3)**:165-170.
21. Fiskum G, Rosenthal RE, Vereczki V, et al. *Journal of Bioenergetics and Biomembranes,* 2004; **36(4)**:347-352.
22. Molina-Jim enez MF, Sanchez-Reus MI, Andres D, Cascales M, et al. *Brain Research,* 2004; **1009(1-2):**9-16.
23. Cassarino DS, Fall CP, Swerdlow RH, et al. *Biochimica et Biophysica Acta*, 1997; **1362(1)**:77-86.
24. Heo HJ, Kim DO, Shin SC, Kim MJ, et al. *Journal of Agricultural and Food Chemistry*, 2004; **52(6)**:1520-1525.
25. Kamisli S, Ciftci O, Kaya K, et al. *Toxicology and Industrial Health*., 2013.
26. Viswanatha GL, Shylaja H, Sandeep Rao, et al. *ISRN Pharmacology*, 2012; 8
27. Omri EIA, Han J, Hashizume R, Abdrabbah MB, et al. 2009; **19(1):**117-120.
28. Vauzour D, Vafeiadou K, Rice-Evans C, Williams RJ, et al. *Journal of Neurochemistry,* 2007; **103(4)**:1355-1367.
29. Raza SS, Khan MM, Ahmed A, et al. *Brain Research*, 2011; **1420**:93-105.
30. Priya N, Vijayalakshmi K. *International Journal of Pharm Tech Research*, 2016; **9(4)**:266-273.