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Anti-Parkinson Effect of Hesperidin in Combination with L-DOPA on 6-OHDA Induced Parkinsonism in Wistar Rats - A Neurochemical, Histopathological and Immunohistochemical Analysis

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Abstract: Parkinson's disease (PD) is a neurodegenerative disorder in the nigrostriatal pathway of animals and humans and is responsible for most of the movement disorders, including rigidity. The present study was aimed to determine the effect of hesperidin in combination with L-Dopa on 6-hydroxydopamine (6-OHDA) induced rat model of PD. Animals were divided into 5 groups: GroupI served as normal. GroupII was induced with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline). GroupIII: 6 hydroxydopamine +50mg/kg b.w hesperidin. GroupIV: 6-hydroxydopamine + 50mg/kg b.w hesperidin+100mg/kg b.w of L-Dopa. GroupV: 6-hydroxydopamine+100mg/kg b.w L-Dopa.After treatment, the effect of these factors was determined by biochemical, histopathological and for immunohistochemistry evaluation. Neurotransmitter levels like dopamine, nor-epinephrine, epinephrine and serotonin shows better results in group IV than other treated groups. Similarly, histopathological and immunohistochemistry observation has also shown better results in group IV than other treated groups. Thus it may be concluded that effect of hesperidin in combination with L-Dopa on 6-OHDA treated animal model has an Anti-Parkinson effect. Further investigation is required to understand the exact etiology of clinical parkinsonism.
Keywords : Parkinson disease, Dopamine, 6-OHDA, hesperidin, L-DOPA.

Introduction

Parkinson disease (PD) has a lifetime risk of 2% making it the second most common neurodegenerative disease after Alzheimer's disease, affecting approximately 1%of the population older than 50 years **(1).** Parkinson's disease is a chronic neurodegenerative disease characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The development of PD in patients becomes clinically apparent with severe motor symptoms, including uncontrolled resting tremor, bradykinesia, rigidity and postural imbalance **(2).**In most cases, these symptoms appear when 70-80% of SNpc dopaminergic neurons are lost **(3).**

PD occurs from the following process in the brain. PD develops as cells are destroyed in certain parts of the brain stem, particularly the crescent-shaped cell mass known as substantia nigra. PD is a slowly progressive disorder that affects movement muscle control and balance. Nerve cells in the substantia nigra send out fibres to tissue located in both sides of the brain. There the cells release essential neurotransmitters that help control movement and co-ordination. These cells release dopamine, an essential neurotransmitter (a chemical messenger in the brain). Loss of dopamine in the corpus striatum is the primary defect in Parkinson's disease. Dopamine deficiency is the hallmark feature in PD. Dopamine also appears to be important for efficient

information processing and deficiencies may also be responsible for the problems in memory and concentration that occur in PD patients.

Hesperidin occurs in greatest concentration in green fruit and its concentration in the fruit increases during storage **(4).** Its distribution in the epicarp, mesocarp, endocarp and juice of citrus fruits has been reported **(5).**In seeds, the hesperidin content increases after germination suggesting that there is a net production of this compound in the developing seedling, which is partly stimulated by light**(6).**Hesperidin occurs in crystalline, feather-like aggregates or sphaerocrytalline masses in the cells **(7).** Hesperidin has antioxidant against DPPH, superoxide radical, nitric oxide radical, hydroxyl radical and hydrogen peroxide in an *invitro* condition **(8).**

The aim of the present study is to explore the effect of hesperidin in 6-OHDA induced neurotoxicity, utilizing histopathological changes, immunohistochemical markers and biochemical studies.

Materials and Methods

6-OHDA, ascorbic acid, Hesperidin were purchased from sigma Aldrich. All other chemicals used were of analytical grade.

Experimental Animals

Adult male wistar rats (145-150g) were used for the study. Animals were purchased from Tamilnadu Veterinary and Animal Science University, Madhavaram, Chennai and were housed under controlled temperature provided with food and water ad libitum. The protocol was approved by the institutional animal ethics committee of the Saveetha University, Chennai. (IAEC NO: SU/BRULAC/RD/008/2013).

Experimental Protocol

The animals were divided into 5 groups, each containing 6 animals.

Group I: Animals were treated normally and served as control.

Group II: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum once and were maintained for development of Parkinson's disease for 45 days.

Group III: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum once and maintained for development of Parkinson's disease for 21 days. On 22nd day, Hesperidin (50 mg/kg b.w) dissolved in distilled water were given for next 24 days.

Group IV: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum once and were maintained for development of Parkinson's disease for 21 days. On 22nd day, Hesperidin (50mg/kg b.w) and L-Dopa (100mg/kg b.w) dissolved in distilled water were given for next 24 days.

Group V: Rats were infused with 6-hydroxydopamine (8µg/2µl in 0.1% ascorbic acid-saline) in right striatum for once and were maintained for development of Parkinson's disease for 21 days. On 22nd day (L-DOPA 100mg/kg b.w) was given for next 24days.

6-OHDA Induced Lesions

All animals in the experimental groups were anaesthetized with Ketamine (100mg/kg b.w) (i.p) and Xylazine (10 mg/kg b.w) (SC). Each animal was mounted on a stereotaxic apparatus, skin overlying the skull was cut to expose it, and the co-ordinates for the striatum were measured accurately (anterio-posterior 0.5 mm, lateral 2.5 mm, dorso-ventral from dura) with the tooth bar set at 0 mm. Thereafter, all animals in experimental groups were lesioned by injecting (8µg/2µl in 0.1% ascorbic acid-saline) in the right striatum, while the Group I served as a control. The injections were made manually, with the help of a Hamilton syringe, through the burr holes made on the skull surface in experimental groups. The injection rate was 1µl/min and the needle was kept in place for an additional 1 min before being slowly retracted.

Post-Operative Care

Recovery from anaesthesia took approximately 4-5hrs. The animals were kept in a well-ventilated room at 25±3^{0}C in individual cages until they gained full consciousness; they were then housed together in groups of 6 animals per cage. Food and water mixed with 1ml of Ibuprofen was kept inside the cages for two days, allowing animals easy access, without physical trauma due to overhead injury. Animals were then treated normally with food; water and the bedding of the cages were changed twice per week, as usual.

Brain Tissue Collection

The animals were sacrificed and striatal portion of the brain was separated and homogenised separately in ice-cold phosphate buffer (pH7.5) at a concentration of 15% (W/V). This would release soluble protein leaving only membrane and non-vascular matter in a sediment form. Homogenised samples were then centrifuged at 5000rpm for 10min. Aliquots were taken for biochemical studies. Tissue homogenate was stored at -20°C until the use for further analysis.

Neurotransmitters Level

The levels of dopamine, nor-epinephrine, epinephrine and serotonin was estimated by the method of Kari **(9).**

Histopathological Analysis

Tissue sections of the striatum and mid brain of the rats were obtained from all groups of rats and were fixed in 10% formalin. The fixed tissues were processed, embedded in paraffin wax and sectioned. The sections were stained with haemotoxylin and eosin, observed under light microscope. The tissue sections were examined for histopathologicalchanges and it was compared with control tissues.

Immunological Studies

The tissue sections were deparaffinised in xylene I and xylene II at 60°C for 20minutes each and hydrated through a graded series of alcohol, and the slides were incubated in a citrate buffer (pH 6.0) for three cycles of 5minutes each in a microwave oven for antigen retrieval. The sections were then allowed to cool at room temperature and then rinsed with TBS (Tris buffered saline), and treated with 0.3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. Non-specific binding was blocked with 3% BSA at room temperature for 1hr. The sections were then incubated with diluted primary antibody TH (1:500). The slides were washed with TBS and then incubated with anti-rabbit labelled secondary antibody at a dilution (1:500) for 1hour at room temperature. The peroxidase activity was visualized by treating the slides with 3, 3'-diaminobenzidine tetra hydrochloride. The slides were counterstained with Meyers haematoxylin. Negative controls were incubated with TBS instead of primary antibodies.

Statistical Analysis

All the data was expressed as Mean± SD. Difference in means was estimated by means of ANNOVA followed by Dunne's post hoc test. Results were considered significant at *$p<0.05$.

Results

Restoration of the levels of dopamine, nor-epinephrine, epinephrine and serotonin demonstrates the protective role of hesperidin (50mg/kg b.w) in combination with the L-DOPA (100mg/kg b.w) in 6-OHDA induced neurotoxicity.

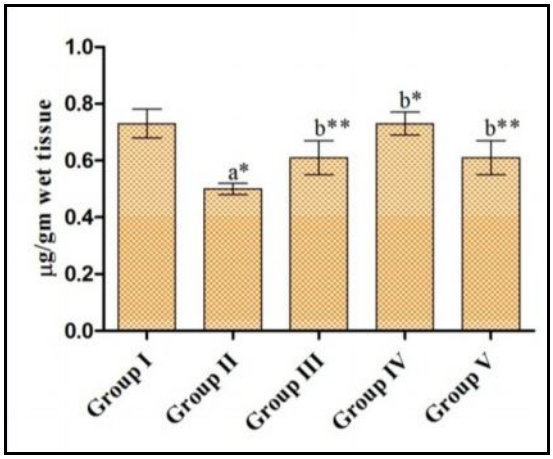

Figure 1

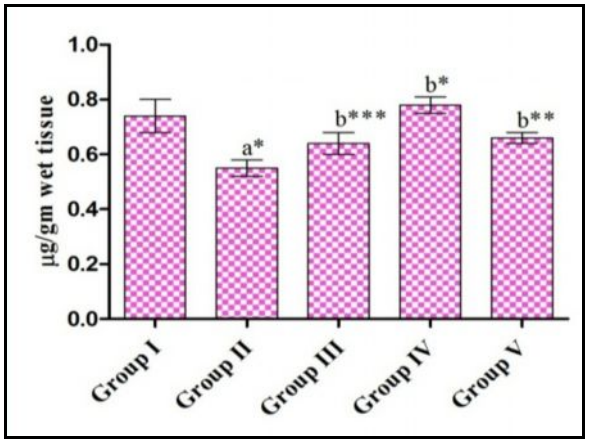

Figure 2

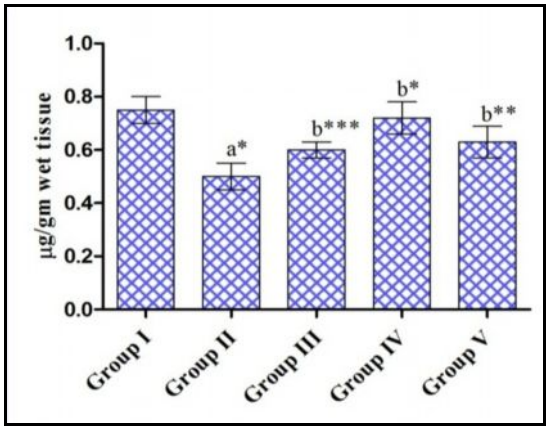

Figure 3

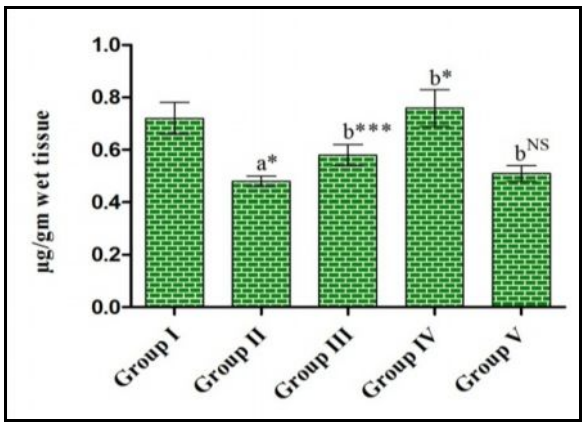

Figure 4

Each value is expressed as mean±SD for six animals in each group. Group I: Control animals, Group II: 6-OHDA lesioned animals (Parkinson disease bearing animals), Group III: Lesion + Hesperidin (50mg/kg b.w), Group IV: Lesion Hesperidin (50mg/kg b.w) +L-DOPA (100mg/kg b.w), Group V: Lesion+L-DOPA (100mg/kg b.w). Statistical significance: *p<0.001, **p<0.01, ***p<0.05 and NS - Non significant.

Comparison: **a-** as compared with group I; **b-** as compared with group II.

Dopamine levels of striatal tissue of experimental animals is shown in **figure 1.** Dopamine level shows significant (p<0.001) reduction in 6-OHDA (Group II) lesioned group when compared with the normal animals (Group I). Its level was significantly (p<0.001) enhanced with the administration of hesperidin + L-DOPA treatment as compared to 6-OHDA group. Animals treated with hesperidin (Group III) and L-DOPA (Group V) showed less significant (p<0.01) changes when compared with lesioned group (Group II).

Nor-epinephrine levels of striatal tissue of experimental animals is shown in **figure2.** Nor-epinephrine levels shows highly significant (p<0.001) reduction in 6-OHDA (Group II) lesioned group when compared with the control animals (Group I). Its level was significantly (p<0.001) enhanced with the hesperidin +-L-DOPA treatment as compared to lesioned group (Group II). Group III shows less significant changes (p<0.05) as compared to that of lesioned group II. Group V shows significant changes at p<0.01 as compared to that of lesioned groupII.

Epinephrine levels of striatal tissue of experimental animals is shown in **figure 3** Epinephrine level was significantly (p<0.001) reduced in 6-OHDA (Group II) lesioned group. Its level was significantly (p<0.001) enhanced with the hesperidin +-L-DOPA treatment as compared to lesioned group (Group II). Group III showed lower significant changes (p<0.05) as compared to that of lesioned group II. Group V showed significant changes at p<0.01 as compared to that of lesioned group II.

Serotonin levels of striatal tissue of experimental animals is shown in **figure 4.** Serotonin level was significantly ($p<0.001$) reduced in 6-OHDA (Group II) lesioned group. Its level was significantly ($p<0.001$) enhanced with hesperidin +L-DOPA treatment as compared to lesioned group (Group II). Group III and Group V showed less significant changes ($p<0.05$) when compared with 6-OHDA lesioned group. Group V showed non-significantchange when compared to Group II.

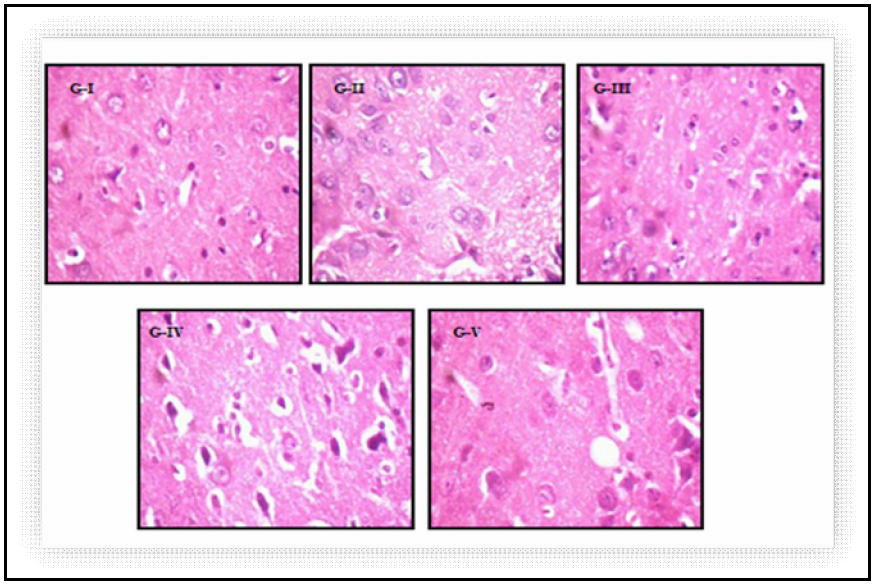

Figure 5: Histopathological studies of striatum viewed under light microscope in control and experimental animals. Hematoxylin/Eosin staining of paraffin section (100X H&E).The striatal region of control animal reveals the normal architecture (Group-I), whereas 6-OHDA induced Parkinson animal shows (Group-II) degenerative changes of the neuronal cells with cytoplasmic vacuolation. Hesperidin (Group-III) treated animals show slight reduction in degeneration of cells and vacuolation. Hesperidin+L-Dopa treated animals (Group-IV) show much reduction of degeneration of cells, which tends towards normalcy. L-dopa (Group –V) treated animal shows the normal architecture as that of control.

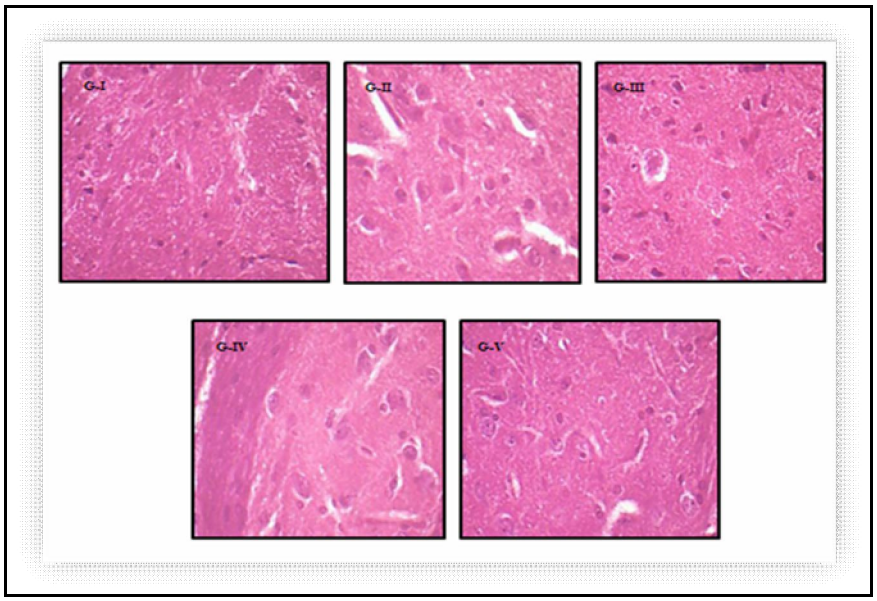

Figure 6: Histopathological studies of mid brain viewed under light microscope in control and experimental animals. Hematoxylin/Eosin staining of paraffin section (100X H&E).Mid brain region of animal reveals the normal architecture (Group-I) whereas 6-OHDA induced Parkinson animal shows (Group-II) degeneration of cells and large cytoplasmic vacuolation. Hesperidin treated animals (Group-III) showed slight reduction in degeneration and vacuolation. Hesperidin and L-Dopa treated animals(Group-IV) showed much reduction of degeneration of cells, which resembles the control. L-Dopa (Group-V) treated animals shows the normal architecture as that of control.

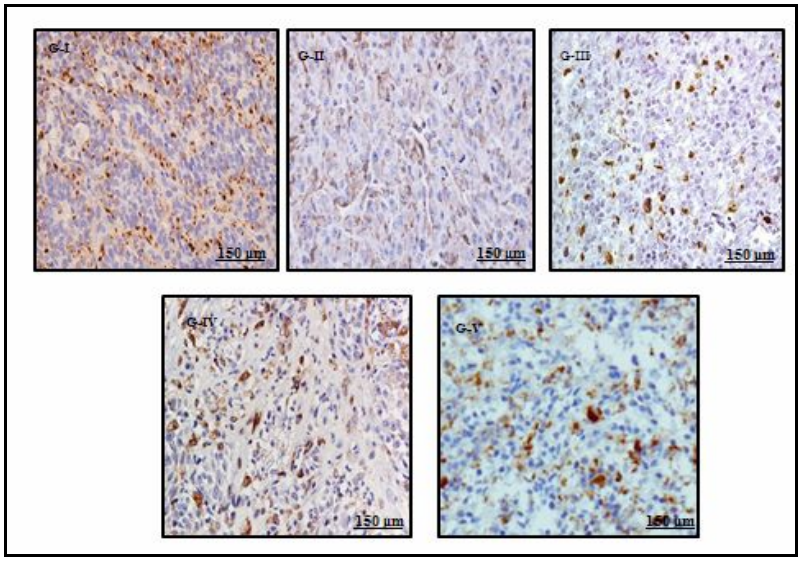

Figure 7: Observation of tyrosine hydroxylase activity in striatum

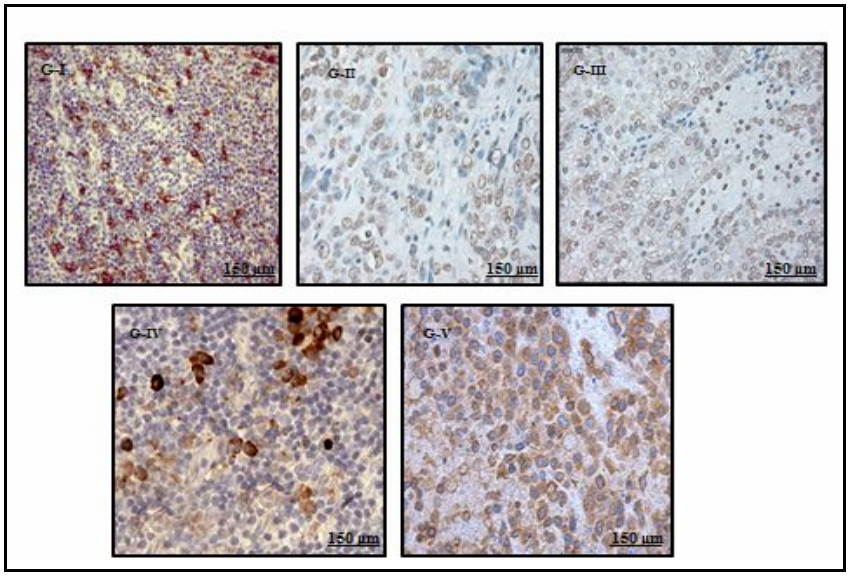

Figure 8: Observation of tyrosine hydroxylase activity in mid brain

TH immunostaining pattern in the striatum and mid brain are shown in **Figure 7**and **8.** Tyrosine hydroxylase (TH) immunoreactivity was studied in the cell body region of striatum and mid brain. The bodies and fibers of dopaminergic cell were intensely stained which is evident by immuno positive observation. A decrease in number of cells was noticed in rats induced with 6-OHDA administration. In contrast, the administration of hesperidin (50mg/kg b.w), hesperidin (50mg/kg b.w) + L-Dopa (100mg/kg b.w), L-Dopa (100mg/kg b.w) resulted in a comparative increase in the number of dopaminergic neurons.

Discussion

Endogenous administration of 6-OHDA is found to induce neuronal damage, which closely resembles parkinsonism. After administration of 6-OHDA into the right striatum, 6-OHDA is selectively taken up by dopaminergic neurons due to its affinity for the dopamine transporter and this result in selective toxicity to dopaminergic neurons.

Biogenic amines such as epinephrine, nor-epinephrine, dopamine and serotonin (5-Hydroxytryptamine, 5-HT) act as a neurotransmitters in different areas of rat brain. The relation between their metabolism in the CNS and cerebral function varies in different brain regions under different conditions **(10).**

Histopathology and immunohistochemistry of striatum and mid brain, where degeneration of neurons and cytoplasmic vacuolation occurs by the induction of MPTP induced Parkinson animal model and Asiaticoside treatment group resembles the normal architecture**(11).** The Methanolic extract of Stereospermum suaveolens in 6-OHDA induced Parkinson disease shows the histology of striatal tissue which showed degeneration of neurons and cytoplasmic vacuolation in 6-OHDA induced group and treatment group showed less degeneration as that of control **(12).** Immunohistochemistry of striatum of animals revealed the less number of cells showing positive for tyrosine hydroxylase activity on 6-OHDA induced in right striatum of Parkinson animal model, where as it was reverted back to normal by the high expression of tyrosine hydroxylase in the curcumin treated groups **(13).**

Expression of tyrosine hydroxylase in the mid brain was negligible in lesion group and it was restored by Ginkgo biloba in a dose dependent manner**(14).** Similarly our findings also correlate with their results.

The results of the present study clearly shows the effect of hesperidin in combination with L-DOPA in this PD model. It was found that treatment with hesperidin in combination with L-DOPA for three weeks after a 6-OHDA injection can improve performance in neurotransmitters level, histopathological and immunohistochemistry studies. 6-OHDA is one of the most common neurotoxins used in experiments in order to mimic Parkinsonism in rodents. The possible underlying mechanism of neurotoxicity induced by 6-OHDA has been reported to be related to the oxidative stress caused by the production of hydroxyl radicals during auto-oxidation **(15, 16, 17)** and the inhibition of complex I **(18)** resulting in excessive oxidative stress leading to neuronal death.

We found that the intrastriatal administration of 6-OHDA leads to degeneration of cytoplasmic vacuolation in the striatal and mid brain region. The increased oxidative stress is considered as a cardinal feature of 6-OHA neurotoxicity. Degeneration of cytoplasmic vacuolation was found to be decreased in the treatment of hesperidin in combination with L-Dopa induced by 6-OHDA lesions. Histopathology of striatal tissue and mid brain showed degeneration in 6-OHDA induced rats and no other organs were shown to be affected. This suggest that 6-OHDA injected into the right striatum affect only the specific target area of interest and not the peripheral organs as reported by **(19).**

In this study, tyrosine immunoreactive neurons of striatum and mid brain were reduced after 6-OHDA treatment. The same was reversed by hesperidin and much more better by hesperidin in combination with L-Dopa. Behavioural and serum levels like TG, glucose and protein levels has been showed to be improved by the treatment of hesperidin **(20).**Hesperidin and in combination with L-Dopa has also shown better in gene expression studies of Parkinson animal **(21).**Hesperidin also has also been docked with some upregulated proteins such as alpha synuclein, UCHL-1, MAO-A and COMT inhibitors**(22).**

Conclusion

It was concluded that hesperidin in combination with L-Dopa exerts Anti-Parkinson effect in this PD model via increasing the neurotransmitters level resulting in the promotion of neuron survival. Consequently, the use of hesperidin in combination with L-Dopa as an adjuvant therapeutic agent for the treatment of neuroprotective effect in PD should be considered. The study highlights the depletion of nigrostriatal neurons in 6-OHDA treated animals which could not be observed if 6-OHDA insulted animals were treated with hesperidin in combination with L-DOPA.

References

1. Ebadi M, Hiramatsu M. Glutathione and metallothionein in oxidative stress of Parkinson's disease. In: Poli G, Cadenas E, Packer L, editors. Free Radicals in Brain Pathophysiology. New York: Marcel Dekker; 2000. p. 427‑65.
2. Samii A, Nut JG, Ransom BR. Parkinson's disease. Lancet 2004; 363:1783‑93.
3. Cookson MR. The biochemistry of Parkinson's disease. Annu Rev Biochem 2005; 74:29‑52.
4. Higby RH (1941). The chemical nature of hesperidin and its experimental medicinal use as a source of vitamin P-a review. Journal of the American Pharmaceutical Association, 30 (12): 629-635.

5. Kawaguchi K, Mizuno T, Aida K, Uchino K (1997). Hesperidin as an inhibitor of lipases from porcine pancreas and pseudomonas. Biosci Biotech Biochem, 61(1): 102-104.
6. Barthe, Gary A, Jourdan PS, Mclntosh Ca, Mansell RL (1988). Radioimmunoassay for the quantitative determination of hesperidin and analysis of its distribution in citrus sinensis. Phytochemistry, 27(1): 249-254.
7. Evans WC (1996). Trease and Evans. Pharmacognosy.Saunders; London.
8. Priya Nagappan and Vijayalakshmi Krishnamurthy (2014). IC_{50}value of hesperidin against free radicals: An *invitro* study. The Journal of free radicals and antioxidants. Photon 140: 271-277.
9. Kari HP, Davidson PP, Herbert HH, Kochbar MH (1978). Effects of ketamine on brain monoamine levels in rats. Res Comm Chem Path Pharmacol, 20: 475-488.
10. Krnjevic K (1974). Chemical nature of synaptic transmission in vertebrates. Physiol. Rev, 54(2): 418-540.
11. Sampath Uvarajan, Vanisree Arambakkam Gopinath (2012). Neuro-shielding effect of Asiaticoside against MPTP induced variations in experimental mice. International journal of engineering science and technology, 4(3): 960-965.
12. Shalavadi MH, Chandrashekhar VM, Avinash SP, Sowmya C, Ramkishan A (2012). Neuroprotective activity of Stereospermum suaveolens DC against 6-OHDA induced Parkinson's disease model. Indian Journal of Pharmacology, 44(6): 737-743.
13. Gulrana Khuwaja, Mohd Moshahid Khan, Tauheed Ishrat, Ajmal Ahmad, Syed Shadab Raza, Mohammad Ashafaq, Hayate Javed, Badruzzaman Khan M, Andleeb Khan, Kumar Viabhav, Mohammed M.Safhi, Fakhrul Islam (2011). Neuroprotective effects of curcumin on 6-hydroxydopamine-induced Parkinsonism in rats: Behavioural, neurochemical and immuno histochemical studies. Brain Research, 1368: 254-263.
14. Muzamil Ahmad, Sofiyan Saleem, Abdullah Shafique Ahmad, Seema Yousuf, Mubeen Ahmad Ansari, M Badruzzaman khan, Tauheed Ishrat, Rajnish Kumar Chaturvedi, Ashok Kumar Agrawal, Eakhrul Islam, (2005). *Ginkgo biloba* affords dose-dependent protection against 6-hydroxydopamine-induced parkisnonism in rats: neurobehavioural, neurochemical and immunohistochemical evidences. Journal of Neurochemsitry, 93: (94-104).
15. Cohen G, Heikkila RE, Allis B, Cabbat F, Dembiec D, MacNamee D, Mytillineou C, Winston B (1976). Destruction of sympathetic nerve terminals by 6 hydroxydopamine: Protection by 1-phenyl-3-(2 thiazolyl)-2- thiourea, diethyldithiocarbamate, methimazole, cysteamine, ethanol and n butanol. Journal ofPharmacology and Experimental Therapeutics, 199(2): 336–352.
16. Kumar R, Agarwal AK, Seth PK (1995). Free radical generated neurotoxicity of 6-hydroxydopamine. Journal ofNeurochemistry, 64(4): 1703–1707.
17. Soto-Otero R, Mendez-Alvarez E, Hermida-Ameijeiras A, Munoz-Patino AM, Labandeira-Garcia JL (2000). Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. Journal of Neurochemistry, 74(4): 1605–1612.
18. Glinka YY, Youdim MB (1995). Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine. Eur. J. Pharmacol, 292(3-4): 329–332.
19. Nagarajan Habagrahara, Cheng Jun Siew, Kumar Ponnusamy (2013). Effect of quercitin and desferrioxamine on 6-hydroxydopamine induced neurotoxicity in striatum of rats. The journal of toxicology sciences, 38(1): 25-33.
20. Priya Nagappan and Vijayalakshmi Krishnamurthy (2014). Investigation on the neuroprotective effects of hesperidin on behavioural activities in 6-OHDA induced Parkinson model. International journal of pharma and Biosciences .Oct; 5(4): (B)570-577.
21. Priya Nagappan and Vijayalakshmi Krishnamurthy (2015). Hesperidin a bioflavonoid modulates the expression levels of SNCA and Parkin in 6-hydroxydopamine induced neurotoxicity in rats. International journal of Pharmacy. 5(1): 219-224.
22. Priya Nagappan and Vijayalakshmi Krishnamurthy (2015). Structural prediction and comaparative molecular docking studies of hesperidin and L-Dopa on A-synuclein, MAO-B, COMT and UCHL-1 inhibitors. International journal of pharmaceutical and clinical research. 7(3): 221-225.

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