EFFECT OF STACHYTRAPHETA INDICA ON HSP 60 IN CONTROLLING NEURODEGENERATION ON ROTENONE INDUCED PARKINSON’S DISEASE (PD) IN ZEBRA FISH MODELS

Dr. Muralidhar S. Talkad1*, Juganta Das2, Pedenla Lama3, Surabhi Chetia4, Dipanjan Dasgupta5

1,2,3,4 Post Graduate Department of Biotechnology, R&D Centre, Dr. C.D. Sagar Centre for Life Sciences, Dayananda Sagar Institute, Kumaraswamy Layout, Bangalore-560078, India.
5 C. R. Park, Hill View (East), S. B. Gorai Road, Asansol - 713304, Burdwan, West Bengal

ABSTRACT

Parkinson’s disease (PD) is quiet peculiar owing to its sudden and rapid occurrence. Several environmental toxins have been known to trigger PD. Therefore, such Neurotoxin-based models (particularly MPTP / Rotenone) have become preferred and important models in studying the degeneration of cholinergic and dopaminergic neurons. In this study we established Neuroprotective activity methanolic extract of Stachytarpheta indica on Rotenone induced Parkinson’s condition in Zebra fish. In addition oxidative stress enzyme GSH (0.189 ± 0.0135 u/l) in liver of induced group, when compared to plant extract protection against oxidative stress on Zebra fish (0.327±0.0119 u/l). The plant extract treated group showed the acetylcholine esterase enzyme activity (3.460 ± 0.0208 µmols/min), it is closer to the Normal control group (2.940 ± 0.0025 µmols/min) as compared to the Parkinson’s induced group (4.882±0.0051 µmols/min). Since the inhibition or reduced activity of Acetylcholine esterase enzyme will curtail the progress of neurodegeneration in Parkinson’s and Alzheimer’s disease alike. The plant extract also shows acetyl choline inhibitory activity. The S.indica plant extract were shown Dopamine content 4.17% and reduce the increased activity of Acetyl choline esterase and re-establish the mitochondrial succinate dehydrogenase activity there by reducing neurodegeneration, it was confirmed from the expression of small Heat Shock
Proteins was studied, and the *S.indica* treated group showed the expression of HSP 60 around 60 KDa, hence it is proven for the establishment of neuronal homeostasis.

**KEYWORDS:** Neuroprotection, *stachytarpheta indica*, Parkinson’s disease (PD), Flavonoids, Rotenone, HSP60.

**INTRODUCTION**

Heat shock proteins (HSPs), known as molecular chaperone to assist protein folding, have recently become a research focus in Parkinson's disease (PD) because the pathogenesis of this disease is highlighted by the intracellular protein misfolding and inclusion body formation. The present review will focus on the functions of different HSPs and their protective roles in PD. It is postulated that HSPs may serve as protein folding machinery and work together with ubiquitin-proteasome system (UPS) to assist in decomposing aberrant proteins.

Failure of UPS is thought to play a key role in the pathogenesis of PD. In addition, HSPs may possess anti-apoptotic effects and keep the homeostasis of dopaminergic neurons against stress conditions. The critical role of HSPs and recent discovery of some novel HSPs inducers suggest that HSPs may be potential therapeutic targets for PD and other neurodegenerative disorders.[1]

Studying the Parkinson’s disease in a vertebrate is quintessential owing to the fact that understanding the disease is necessary for healing it. Several models have been seen in studying these disease, one of the newest model is the Zebra fish or *Danio riero*. [2] According to studies, 70 % of protein-coding human genes are related to zebrafish genes, and 84 % of the genes known to be associated with human disease have a counterpart in the zebrafish genome. [3]

These findings highlight the importance of the zebrafish model in human disease research. The similarity also exists in the LRRK 2 and PINK 1 genes which are important in the study of Parkinson’s disease.[4]

Genes provide validation and significant data only after several processes are recognised and understood by tracing the gene products or by trailing the changes in the normalcy of biological processes that is supposed to exist.[4]
Enzymes are sources for diagnosing several conditions in this case Acetyl Cholinesterase and Mitochondrial Succinate Dehydrogenase enzymes have been instrumental in providing the information for diagnosis and assessment of the disease.

Other enzymes like Amino Transferases and Glutathione Transferases have been used to study the effect of the drug in the organism. Apart from enzymes, stress related physiology pointing out certain proteins called as heat shock proteins which are primarily housekeeping proteins.\[5, 6\]

These proteins play a major role in re-establishing physiological dynamic equilibrium after or as soon as there is some stress or stimulus that causes chaos in the cellular niche.

Eukaryotic Heat Shock Proteins are lesser studied and their understanding is under darker veils. Unveiling their functions will certainly promissable for providing new therapeutic target for various disease including Neurodegenerative diseases. In neurodegenerative diseases, HSP 70, 60 and larger proteins actively take part in formation of protein deposits in the extra cellular fluids which promotes degeneration to a greater level.\[6\]

Parkinson’s disease (PD) is quiet peculiar owing to its sudden and rapid occurrence. Indeed several environmental toxins have been known to trigger PD. Therefore, such Neurotoxin-based models (particularly MPTP / Rotenone) have become preferred and important models in studying the degeneration of cholinergic and dopaminergic neurons.

It is postulated that Hsp70 itself or cooperating with other factors can protect the neurons from cytotoxicity caused by aberrant proteins. The crosstalk between the Hsp70 and UPS may provide a clue for the intrinsic mechanism of protein aggregation and degradation. Moreover, Hsp70 exerts anti-apoptotic activity by blocking the function of several key proapoptotic factors.\[7\]

Recently, several studies have demonstrated that Hsp70 may play a role in neuroprotection against rotenone-mediated apoptosis in human dopaminergic cell line SH-SY5Y \textit{in vitro} and against MPTP-induced nigral injury \textit{in vivo} by inhibiting the proapoptotic factors as well as activating the survival pathway.\[8, 9\]
Mitochondrial dysfunction is probably the leading cause of increased oxidative stress and apoptosis in PD. Dopaminergic neurons are more vulnerable to oxidative stress than other neurons because of the special substrate dopamine.[10] In general, apoptotic process can be divided into the three phases: induction (or triggering), transduction of signal, and execution. Theoretically, HSPs may modulate any of these apoptotic phases to rescue the cells.[11, 12]

Numerous studies implicate that at least two components of cellular proteins are associated with PD: the ubiquitin proteasomal system (UPS) and the HSPs.[8, 13]. Transcriptional analysis of multiple brain regions in PD indicates the impairment of multiple electron transport chain complexes and the dysfunction of UPS in PD, along with a robust induction of several forms of HSPs.[14]

Inclusion bodies called Lewy bodies with aberrant misfolding and aggregative proteins are common pathological hallmark in PD, indicating that abnormality of protein homeostasis may contribute to the pathogenesis of the disease.[8]

The S.indica plant extract was found to control and reduce the increased activity of Acetyl choline esterase and re-establish the mitochondrial succinate dehydrogenase activity there by reducing neuro degeneration. The reduced neurodegeneration in the extract treated group was confirmed from histopathological studies of the Zebra fish. The reduced neurodegeneration in the extract treated group was confirmed from the expression of small Heat Shock Proteins which is proved to be important for the neuronal homeostasis

In this study we establish Neuroprotective activity of Stachytarpheta indica methanolic extract on Rotenone induced Parkinson’s condition in Zebra fish. The major focus of the study on Acetylcholine esterase [E.C 3.1.1.7] and mitochondrial succinate dehydrogenase [EC 1.3.5.1] which are necessary to monitor neurodegeneration, in addition GSH was performed to assess the Stachytarpheta indica plant extract neuroprotection against oxidative damage on Zebra fish.

MATERIAL AND METHODS

Collection of plant materials

The plant material Stachytrapheta indica was obtained from Kunigal Taluk of Tumkur District. The aerial parts were washed, dried and deposited in the P.G Department of Biotechnology, Dayananda Sagar College, Dr C.D Sagar centre for life sciences, DSI.
Procurement & Maintenance of zebra fish (*Danio rerio*)
Zebra fish were purchased (20 pairs) from Mermaid Aquarium in Jayanagar 3rd Block, Bangalore 560011. The fish were stored in a 20L aquarium at ideal conditions in the Lab. Fish was fed with shrimp flakes and commercially available feed once every 36 hours. They were kept undisturbed for one week and fed regularly.\cite{15}

Purchase of Chemicals
Chemicals and reagents like Ellman’s reagent (5, 5’-dithio-bis-[2-nitrobenzoic acid]), DCPIP (2, 6-Dichloroindophenol sodium salt hydrate) and Rotenone were obtained from Sigma Aldrich. L-Glutathione reduced and DNPH (2, 4-Dinitrophenylhydrazine) were obtained from Merck Millipore.

Preparation of plant extract
Methanolic extract of *Stachytrapheta indica* was prepared using 25gms of powdered plant aerial parts in a 250ml Soxhlet at 67°C

Preparation of Rotenone Stock solution
Rotenone is an organic compound and is soluble in acetone. A Solution of 50µg/ml was prepared. The solution was stored at 4°C and subsequent dilutions were prepared in distilled water to induce Parkinson’s disease in the fish.

Induction of Parkinson’s disease
Fish were exposed to various concentrations of Rotenone from 0.5, 1, 1.5, 2, 2.5, 3 pg/ml (pico-grams/millilitre) for a duration of 48 to 72 hours.

Homogenisation of organs and tissues for enzyme assays
The fish were dissected (post ensured euthanization) along the dorsal and ventral sides to expose the Central Nervous System with intact brain and spinal cord, homogenised in a pestle and mortar with 5ml of 0.1M phosphate buffer, after centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was carefully separated and used in enzyme assays. Liver was also prepared in the same way for enzyme assay.\cite{16,17}

Parameters for assessing PD related cognitive impairments in Zebra fish
Latency to travel from one point to another: Catalepsy diminishes the speed of fishes due to rigidity of muscular movements. In the present study, after induction of catalepsy, rigidity of fins was observed due to which there was a difficulty in swimming experienced by the fishes.
Keeping this into consideration, time taken by fish to travel from first vertical line of examination tank to last line was measured at every time interval during the study period.

**Isolation of mitochondria**
The central nervous system homogenate was subjected to differential centrifugation using 0.2 M mannitol, 50 mM sucrose, 1 mM EDTA (ethylene diamine tetra acetate), 10 mM HEPES-NaOH, pH 7.4. The homogenate was centrifuged at 600g for 10 min and supernatant was centrifuged at 7000g and 10,000g for 10 minutes at 4°C and mitochondria was pelleted out in 0.1M phosphate buffer at pH 7.2 for further use.\(^{[18]}\)

**Acetylcholine esterase enzyme assay**
The acetyl choline esterase was assayed using Ellman’s method. Acetylcholine esterase activity was determined by using a reaction mixture comprising of 200µl of Acetyl choline esterase enzyme (E.C. 3.1.1.7) the activity was determined after 5 minutes of incubation using a spectrophotometer at 412nm wave length.\(^{[17]}\)

**Mitochondrial Succinate dehydrogenase assay**
The succinate dehydrogenase activity was determined by suspending 100µl of isolated mitochondria followed by 8µM of DCPIP (dichlorophenolindophenol) in 5ml of Phosphate buffer pH 7.4. The optical density was measured in a spectrophotometer at 595nm. The value was plotted on the standard DCPIP curve and the enzyme activity was determined.\(^{[19]}\)

**Assay of total Glutathione (GSH)**
Measurement of glutathione was done by the addition 50µl of 20mM 1chloro-2,4dinitrobenzene; the increment in absorbance is monitored at 340nm. The activity was calculated by using the molar extinction coefficient.\(^{[20]}\)

**Preparation of sample for SDS page**
Parkinsonian induced and *Stachytrapheta indica* treated fish; Parkinson’s induced fish, along with the normal fish were euthanized and dissected to retain only the brain and spinal cord. This was then homogenised with 1ml 0.1M pH 7.2 Tris Buffer and centrifuged at 15,000 rpm for 15 minutes and the samples were subjected to SDS PAGE. Bangalore GeNei™ SDS PAGE kit was used with a marker with molecular weights ranging from 3.5kDa to 205kDa.
RESULTS AND DISCUSSION

*S.indica* methanolic extract was subjected to HPLC and the reports suggested that the plant extract has 4.17% of Dopamine, 16.19% Catechins and 18.43% of Rutin. The plant extract is rich in flavonoids and dopamine.

Alkaloids and flavonoids like rutin and catechins are good antioxidant and help in easing oxidative stress. L-DOPA the drug used in the treatment of Parkinson’s disease has dopamine as the chief component and the plant extract has a good concentration of dopamine i.e., 4.19%. Therefore, *S.indica* is a good plant that can act as an herbal medicine in the treatment of Parkinson’s conditions and other neurodegenerative disorders.[21]

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DOPAMINE</td>
<td>4.17</td>
</tr>
<tr>
<td>2. CATECHIN</td>
<td>16.19</td>
</tr>
<tr>
<td>3. RUTIN</td>
<td>18.43</td>
</tr>
</tbody>
</table>

1 is Dopamine  
2 is Catechin  
3 is Rutin

<table>
<thead>
<tr>
<th>Test Group</th>
<th>AChE µmols/min X ± SEM</th>
<th>SDH µmols/min X ±SEM</th>
<th>GSH U/L X ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>2.940±0.0025</td>
<td>4.821±0.0041</td>
<td>0.386±0.0094</td>
</tr>
<tr>
<td>PD Induced</td>
<td>4.882±0.0051</td>
<td>1.983±0.026</td>
<td>0.189±0.0135</td>
</tr>
<tr>
<td><em>S. indica</em> Treated</td>
<td>3.460±0.0208</td>
<td>3.643±0.098</td>
<td>0.327±0.0119</td>
</tr>
</tbody>
</table>

Table 1: Estimation of dopamine and flavonoid content in *Starchitaperta indica* plant extract by HPLC
A second advantage of the plant extract is that due to the presence of both antioxidants like Alkaloids and Flavonoids with Dopamine it is a consorted medicine to cure Neurodegeneration and in turn cure Parkinson’s disease the drug commonly administered in the treatment L-DOPA (L-3,4dihydroxyphenylalanine) is a precursor of neurotransmitters, since it could be S. indica plant extract proved to be effective even in chronic conditions when compared to L-DOPA in rotenone induced Parkinson’s disease.\textsuperscript{[22]}

L-DOPA has a major side effect i.e., the dyskinesia that is associated with the drug administration.\textsuperscript{[20,22]} A noteworthy observation during the administration of S. indica plant extract and L-DOPA is that there was no Dyskinesia in S. indica extract treated fish but the fish treated with L-DOPA showed dyskinesia to a considerable degree.\textsuperscript{[21]}

The acetyl choline assay revealed that the plant extract can reduce the enzyme activity. The Parkinson’s induced and treated with S.indica plant extract shows that the enzyme activity is $(3.460 \pm 0.0208)$ $\mu$mols/min as compared to the Normal control group $(2.940 \pm 0.0025)$ and the Parkinson’s induced group $(4.882 \pm 0.0051)$ $\mu$mols/min.

Researchers have revealed that inhibition or reduced activity of Acetylcholine esterase enzyme will curtail the progress of neurodegeneration in Parkinson’s and Alzheimer’s
diseases were same. The S.indica plant extract also showed acetyl choline esterase inhibitory activity.

The activity of mitochondrial succinate dehydrogenase enzyme was monitored in comparison to Normal control, Parkinson induced, Parkinson induced and treated with S.indica plant extract. The activity of the enzyme in the control group was found out to be (4.821 ± 0.0041) µmols/min. In Parkinson’s disease induced condition, the value was found to be (1.983 ± 0.026) µmols/min, in case of S.indica extract treated group the enzyme activity was seen to considerably attaining normal status i.e., (3.643 ± 0.098) µmols/min and showed promising recovery of the fish.

One of the chief contributors to neurodegenerative diseases is the mitochondrial malfunction which results in the generation of Oxygen free radicals leading to oxidative stress, the malfunction in the succinate dehydrogenase enzyme; if the enzyme malfunctions, there are two risks, at first a deficit in the amount of ATP generated due to the failed production of FADH₂ and secondly serious risks of oxidative stress which in case of the nerve cell can lead to neurodegeneration leading to Parkinson’s or Dementia like conditions. Due to the presence of antioxidants and the antioxidant activity of the S.indica plant extract there will be a promissable beneficial effects in mitochondrial activity as well.

Recent studies further reveal that some compounds may contribute specific biochemical effects that are beyond their antioxidant and radical-scavenging properties, for example, involvement in alterations of members of the “vitagene” system, such as heme oxygenase-1 (HO-1), heat shock protein (Hsp) 70, thioredoxin, and sirtuins. These effects may have an impact on the onset and progression of neurodegenerative diseases and aging. The understanding of these metabolic and signalling effects of polyphenols has proven plant metabolites for novel nutritional interventions.

In four botanical phenolic compounds: resveratrol from grapes, curcumin from turmeric, apocynin from Picrorhizakurroa, and epigallocatechin (EGC)-gallate from green tea were discussed their potential beneficial effects in the prevention and treatment of neurodegenerative diseases, with an emphasis on AD, PD, and stroke.

Administration of acetyl salicylic acid to rats alters HSP-72 expression mechanism in a way that it becomes more efficient in response to in vitro heat shock. Among NSAIDs acetyl
salicylic acid remains as a valuable tool because of the variety of benefit prophylactic and therapeutic effects. Nevertheless, the molecular bases for these responses have not been known.[30]

Mouse models of type 1 and type 2 diabetes to determine the relationship of changes in sensory neuron mitochondrial bioenergetics to the onset of and recovery from DPN, improved mitochondrial function following KU-32 therapy required Hsp70, since the drug was ineffective in diabetic Hsp70 knockout mice.[6]

A widespread increase in pink1 mRNA expression, mild oxidative stress induced a clear decline in tyrosine hydroxylase 2 (th2), but not tyrosine hydroxylase 1 (th1) expression, in the brain of wild-type larvae. The drug L-Glutathione Reduced (LGR) has been associated with anti-oxidative and possible neuroprotective properties. The pink1 gene is a sensitive marker of oxidative stress in zebrafish, and LGR effectively normalizes the consequences of mild oxidative stress, suggesting that the neuroprotective effects of pink1 and LGR may be significant and useful in drug development.[4]

Although much evidence exists for a positive effect of HSPs in animal and cellular models of PD, some limitations must be overcome before a therapeutic use for these proteins is established: (1) the reported findings primarily concern animal models; (2) the mode of action differs in different types of genetic Parkinsonism, and (3) the effect of increased synthesis induction of HSPs is unpredictable because quantitative control of the amount of HSPs useful for a therapeutic response is, at present, difficult to determine.

HSPs have two main cellular functions aimed at promoting the ubiquitin proteasomal system (UPS) function and inhibiting the apoptotic activity. However, the detailed molecular mechanisms underlying their biological functions are still unclear. It is believed that HSPs, UPS, mitochondria and other organelles may work co-ordinately to keep the cell in a stable and well-operated state. HSPs are particularly important in PD and other neurodegenerative disorders because aberrant protein aggregation and neuron degeneration are the common pathophysiology of these disorders.

Numerous studies in vitro and in vivo model of PD have demonstrated that increase in the expression of HSPs especially Hsp70 by gene transfer or HSPs inducers can reduce the aberrant protein misfolding and inhibit the proapoptotic pathway to attenuate dopaminergic
neuron degeneration. Thus such intervention provides a promising therapy for PD. Advance in the research of HSPs targets will shed a light on the feasibility of clinical application of HSPs in PD. The future study can be focused on finding the mechanisms of aberrant protein aggregation and searching for the selective HSPs inducers for the treatment of PD.\textsuperscript{[31]}

**CONCLUSION**

The \textit{S.indica} plant has tremendous potential in treatment of Neurodegenerative diseases. Due to the presence of antioxidants, the plant as a drug also has several associated beneficial effects. Further research is required to establish the activity at a drug receptor level and identify specific compounds which are responsible for the plants medicinal property.

The HSPs are certainly molecules of interest in association with PD, and the findings reported to date suggest future possibilities for regulating the production of these molecules to achieve a predictable and dose-dependent effect to elucidate the roles of HSPs in PD.

**ACKNOWLEDGEMENTS**

The authors are awfully thankful to Dr. Premchandra Sagar, Vice Chairman and C.E.O, DSI, Dr. Krishne Gowda, Director, Dayananda Sagar College of Biological Sciences, Dr. C.D. Sagar Centre for Life Sciences, Bangalore-560078, India, for their colossal guidance and support for this project.

**REFERENCES**


cells induced in vivo by acetyl salicylic acid can be reproduced in vitro when combined with H2O2. PLoS One, 2013 Jun 6; 8(6).