Effect of *Centella asiatica* leaf extract on the transgenic Drosophila model of Parkinson's disease

Beaulah Angel P[1,2], Manjula K R[1*] and Shubha[3]

[1]Department of Biotechnology, REVA University, Bengaluru, Karnataka, India
[*1]Department of Biotechnology, REVA University, Bengaluru, Karnataka, India
[2]Department of Biotechnology and Genetics, M. S. Ramaiah College of Arts, Science and Commerce Autonomous, Bengaluru, Karnataka, India
[3]Department of Botany, Government First Grade College, Vijaynagar, Bengaluru, Karnataka, India

Abstract:

Parkinson's disease (PD) is a progressive neurodegenerative disease that causes motor and cognitive deficits due to α-synuclein aggregation and dopaminergic neuron loss. One of the most important factors in PD pathogenesis is oxidative stress. Parkinson disease transgenic Drosohphila model is used to examine the neuroprotective potential of *Centella asiatica*, a medicinal plant with antioxidant qualities. TH-GAL4 females were crossed with UAS-SNCA males to create transgenic PD model flies that express human α-synuclein in dopaminergic neurons. For 21 days, the flies were treated to leaf extract from *C.asiatica* at doses of 10, 50, and 100 µl/ml. To evaluate motor and sensory abilities, behavioural tests such as climbing, vortex-induced seizure, phototaxis, and olfactory response tests were conducted. Oxidative stress indicators, such as catalase activity and lipid peroxidation (LPO) levels, were assessed using biochemical methods. When compared to untreated PD flies, flies treated with *C. asiatica* extract shown notable improvements in locomotor performance, seizure recovery, and sensitivity to sight and smell. Reduced lipid peroxidation and catalase activity suggests improved ROS scavenging and less oxidative stress in treated groups. The concentration of 100 µl/ml had the strongest protective effect. The results indicate that through enhancing motor and sensory abilities and lowering oxidative stress, C. asiatica extract has neuroprotective benefits in PD model flies. These findings suggets for more research into the mechanisms and clinical uses of *C.asiatica* and demonstrate its potential as a treatment for neurodegenerative illnesses like Parkinson's disease.

Keywords: *Centella asiatica*, Neurodegenerative disorder, Oxidative stress, Parkinson's disease

Introduction

The most common age related neurodegenerative disease affecting millions of aged individuals is Parkinsons disease. The main signs of parkinson disease are movement related, like slowness of movement, absence of regular unconscious movements, muscle stiffnss and tremor while at rest.

Parkinsons disease is characterized by the formation of lewy bodies due to accumulation of intraneuronal aggregates in the brain leading to the death of dopaminergic neurons (1).

The development of this condition is largely attributed to oxidative stress, which is caused by an excess of reactive oxygen species (ROS) and nitrogen oxygen species (NOS).

Oxidative stress causes inflammation in brain tissue which activates glial and microglial cells. This process results in the generation of a number of inflammatory mediators such as ROS, chemokines and cytokines. These mediators cause mitochondrial malfunction and an increase in intra cellular calcium ions, which leads to the over expression of certain genes as alpha- synuclein (α-Syn) and other genes as Lewy bodies (LB) in striated neurons. The accumulation of these proteins in neurons causes toxicity leading to neuronal dysfunction and neuronal cell apoptosis. (2)

The effective treatment for such disorder is basically use of levodopa or dopaminergic agonists, which reduce the motor symptoms and not the course of the disease and persistent usage of these drugs, may result in severe adverse effects like tiredness and other motor issues (3).Therefore it is important to develop a novel neuroprotective substance which aims at slowing or stopping the progression of Parkinson's Disease. Studies on humans remain challenging and thus animal models are explored by researchers that may replicate various features of Parkinson's disease. The Drosophila model is one of the animal models that can mimic Parkinson's disease symptoms in order to investigate the disease cause and find potential treatment medications. (4)

The transgenic fly is made to stimulate the symptoms of Parkinson's disease by allowing the expression of SNCA gene. SNCA codes for α-Synuclein protein and over expression of SNCA in neurons leads to neuronal dysfunction. Drosophila lacks an SNCA homolog but when human SNCA is over expressed using GAL4/ UAS system, it expresses the main symptoms of Parkinson's disease, including age-dependent neuronal loss, locomotor impairment and Lewy body aggregation (5).

Numerous research conducted over the past few years have demonstrated the anti Parkinson disease potential in plants due to the presence of phytochemicals like alkaloids, glycosides, terpenoids and flavanoids. These phytochemicals antioxdative, anti-inflammatory, dopamine boosting properties gave them anti-PD qualities (6).

The traditional plant *Centella asiatica*, commonly referred to as Gotu kola in India has been widely used in ayurvedic and traditional Chinese medicine. Despite being recommended for a number of ailments, the plants possible neuroprotective benefits have been investigated. In addition to earlier research, more recent investigations have found that *C. asiatica* has antioxidant properties that lessen the effects of oxidative stress both in vitro and in vivo. The phytochemicals present in *C. asiatica* and their mode of action that are specifically engaged in neroprotection still require more investigation (7).

Materials and Methods

Preparation of Leaf extract:

Fresh samples of *Centella asiatica* was collected from Tumkur area. The plant samples were identified and authenticated in GKVK (Accession no. UASB 5719). The collected plant sample was cleaned with tap water and briefly rinsed with distilled water before being it to air dry. Air-dried samples were ground into a fine powder. In order to extract the bioactive components, soxhlet extraction was performed using ethanol as solvent for per weighed plant powder (20g/250ml).

Drosophila stock:

Wild type human synuclein gene is expressed in transgenic fly lines under UAS control in neuronsThe following fly lines, w [1118]; P {w[+mC]=UAS-SNCA.J}1/CyO and TH-GAL4 were obtained from Indian Institute of Science, Bangalore. The expression of human α synuclein in the dopamergic cells of neurons is done by crossing TH-GAL4 females with UAS (Upstream Activation Sequence)- SNCA.J strains males and the resultant progeny expresses human α synuclein gene (8).

Drosophila culture and crosses:

The flies were raised on common Drosophila media consisting of corn meal, agar, sugar and yeast at 25^{o}C(24±1) . Crosses were made using virgin females of TH-GAL4 and mated with UAS-SNCA males. The resultant progeny flies were referred as Parkinson's disease (PD) flies because the offspring from the cross will express human α synuclein in the neurons (9). The PD flies were exposed to different concentrations of *Centella asiatica* extract mixed in the culture medium. The plant extract was added into the medium at final concentrations of 10µl/ml, 50µl/ml and 100µl/ml. The UAS-SNCA-CyO was used as control. The PD flies were also exposed to 10^{-3}M of L-dopamine.

Climbing assay:

An empty glass vial was taken to carry out the climbing assay. Ten centimetres above the vial's bottom, a horizontal line was drawn and 20 flies were transferred into the vial using a small funnel. The flies were allowed to get adapted for 10 min at room temperature. The vial was plugged and tapped using a foam pad. Allow the vial to stand vertically and immediately start the stop watch.Count the number of flies that reach the marked line at 10cm in 10 seconds as the flies climb the tube. Determine the percenatge of flies that climbed to a height of 10cm in 10 seconds, out of the total flies examined. The same is repeated thrice and the average percentage of flies that climb to the 10cm height is calculated (10).

Vortex assay:

The adult flies were anaesthetized by cold anesthesia method. Ten flies should be placed in a new, food-free vial and allow to restore from the stress of anaesthesia for an hour at 24 or 25°C. The

vials bottom with flies is fixed on a bench top vortexer, then vortex the vial for 10 seconds at its naximum speed to induce seizures. Examine for clear signs of seizures in flies such as paralysis, wing flapping and body stiffness. The amount of time taken for each fly to return to its normal posture and agility is known as seizure recovery (11).

Phototaxis:

The phototaxis assay uses a Y-shaped glass tube called the Y-maze with a 12 mm internal diameter for all arms. The base arm, approximately 13 cm long, serves as the fly inlet, while the two 25 cm longer arms act as the light and dark arms. The base arm is wrapped in black paper or tape to block external light. A sleeve is placed over the dark arm during the assay, while the light arm remains uncovered. All Y-maze openings are sealed with cotton plugs to prevent fly escape.

Anesthetize flies and collect 20 flies per food vial, preparing at least five vials for each genotype. Maintain the vials at 24°C with a 12-hour light/dark cycle for 24 hours before the phototaxis assay. Plug all openings of a clean Y-maze with cotton and place it near a lamp (initially off). Transfer dark-conditioned flies into the Y-maze's upward-facing base arm using a funnel, then replug the arm. Position the Y-maze horizontally, with the light arm near the lamp and one arm covered with a light-proof sleeve to create a dark arm. Turn on the lamp and, after 30 seconds, count flies in the light arm (positively phototactic), while those in the base and dark arms are non-phototactic. Repeat for at least five replicates per genotype (10).

Olfactory response:

Using a gentle hair brush, remove the early third instar larvae from the media bottle and put them in water at 25°C to manually remove any food residue. The larvae are transferred into the agar plate (1% agar). The agar plate is marked with a start zone at the centre of the plate where in the larvas are place initially. Two other zones named as odorant 1(O1) and Odorant 2 (O2) are marked towards the sides of the agar plate. The remaining area in the petri plate is named as C. To give the white larvae with a proper contrast, the agar plate is placed on a black surface. Two circular filter discs are then inserted in the O1 and O2 odorant zones, respectively, using fine forceps. Dispense 20µl of test odorant (Ethyl acetate) on one of the filter disc and the other disc contains 20µl of distilled water. 20 larvae from each control and treated groups are taken and transferred to the control (start zone) of the testing plate.

Count the larvae in zones O1, O2, and C after two minutes. By using the below formula determine the olfactory Response Index (R.I.)

$$R.I = \frac{O_1 + O_2}{O_1 + O_2 + C} \times 100$$

(O_1, O_2 and C represent the number of larvae in corresponding zones)(10)

Biochemical assay:

Tissue sample preparation:
After homogenising the fly heads from the treated and control groups in ice-cold PBS (sodium phosphate, 0.1M, pH 7.0), the fly heads were cold centrifuged at 2500g for 5 minutes.The supernatant was then utilized for biochemical assays.

Catalase activity:

Catalase activity was measured using a slightly modified version of Haddadi's procedure. Following the addition of the homogenised sample (100µl) to the reaction mixture, which also contained 200µl (8mM H202) and 50µl of PBS (50mM pH 7.0), the OD was measured at 240 nm (12).

Lipid Peroxidation (LPO) assay

The MDA content was determined using TMP as a standard. In brief 200µl of 8.1% SDS, 1.5ml of 20% acetic acid and 1.5ml of 0.8% aqueous TBA solution were added to 100µl of homogenate to make a total volume of 4ml. After 60 minutes of heating over a water bath, the solution was cooled and 1ml of d/w was added. After adding, 5ml of butanol and pyridine (15:1), the mixture was thoroughly agitated. After that, the mixture was centrifuged for 10 minutes at 4000rpm. At 532nm, the absorbance was measured (13).

Results

The compounds present in leaves of *C.asiatica* were extracted by soxhlet extraction method by using ethanol as solvent. The UAS-SNCA males were crossed with TH-GAL4 males and the resultant progeny were referred to as PD flies which expresses α-synuclein gene. The PD flies and the control flies were exposed to 10, 50,100µl/ml of *C.asiatica* leaf extract for 21days and considered as treated groups and PD flies without plant extract treatment was untreated group. After exposure to the extracts, climbing assay was performed to check the locomotor effect. The Supplementation of the plant extract helped improved the locomotor ability in the treated group. The protective ability of the plant extract treated group was higher compared to that of untreated (PD) group (fig 1). When the flies were subjected to the vortex assay, the treated group of flies showed faster recovery rate than the untreated PD flies. The fastest recovery was seen in flies treated with concentration of 100 µg/ml (fig 2). The phototaxis assay shows that the treated group of flies could moves towards the light when compared to untreated group with maximum number of flies in light arm are seen in flies treated with a concentration of 100 µg/ml (fig 3). In Olfactory assay the treated group of flies moved towards the odorant 1 (ethyl acetate) and there was an increase in RI value when compared to that of the PD flies without treatment (fig 4). The fly heads -of both treated and untreated group was taken to prepare the homogenate and perform the biochemical assays. The catalase activity in the plant extract treated group was significantly less when compared to the untreated PD flies showing the ROS scavenging was more in treated group (fig 5). The LPO assay showed a decreased activity in plant extract treated flies when compared to that of the untreated group (fig 6).

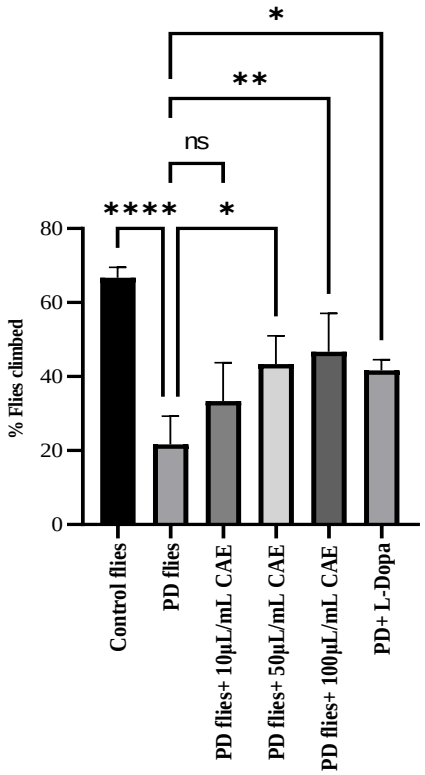

Fig 1:

Effects of *Centella asiatica* leaf extract on the climbing ability.

Plant extracts treated flies showed improvement in the climbing ability. Data are presented as the mean ±SE (n= 3). * Significant at $p < 0.05$; * Significant at $p < 0.001$.**

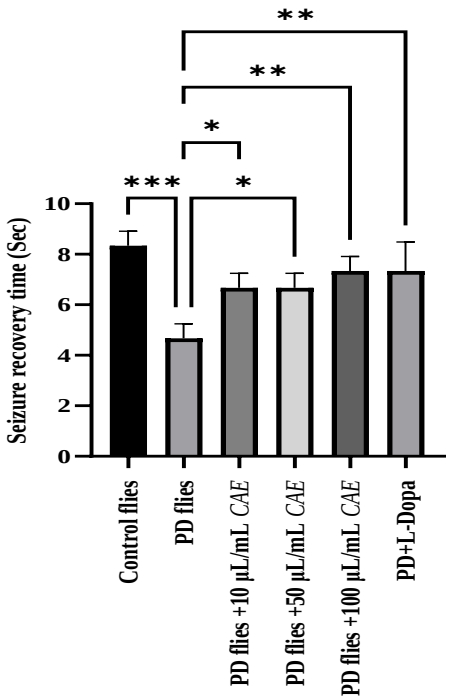

Fig 2:

Effects of *Centella asiatica* leaf extract on the vortex assay. The rate of seizure recovery time improved with the plant extract treated group when compared to that of the recovery time of flies in untreated flies. Data are presented as the mean ±SE (n= 3). * Significant at $p < 0.05$; * Significant at $p < 0.001$.**

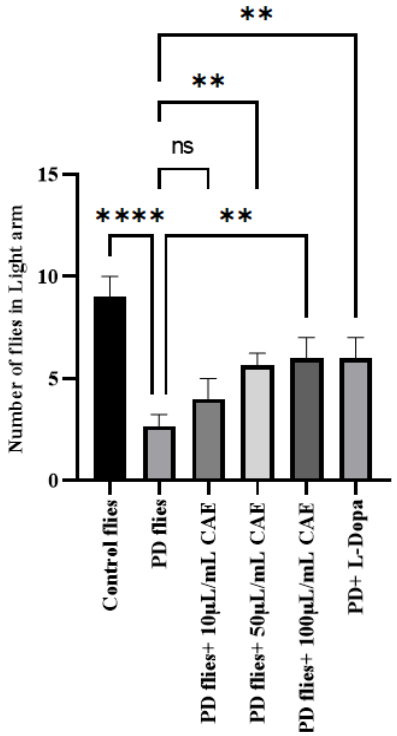

Fig 3

Effects of *Centella asiatica leaf* extract on the phototaxis assay. The number of flies in the light arm of Y maize was more with the plant extract treated group when compared to that of the flies in untreated flies. Data are presented as the mean ±SE (n= 3). * Significant at $p < 0.05$; * Significant at $p < 0.001$.**

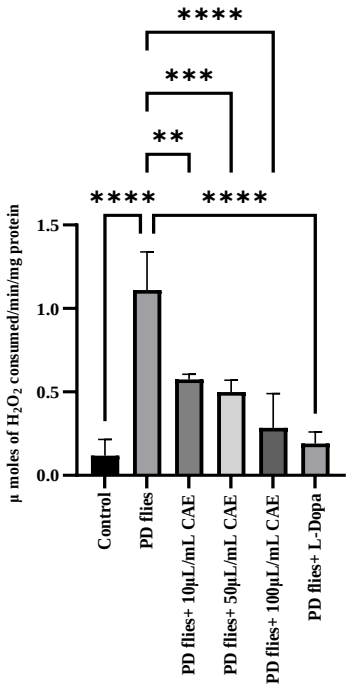

Fig 4

Effect of *Centella asiatica leaf* extract on catalase activity. The amount of catalase was more in untreated group. The Plant extract treated group showed decreased the amount of catalase in the treated flies group. Data are presented as the mean ±SE (n= 3). * Significant at $p < 0.05$; * Significant at $p < 0.001$.**

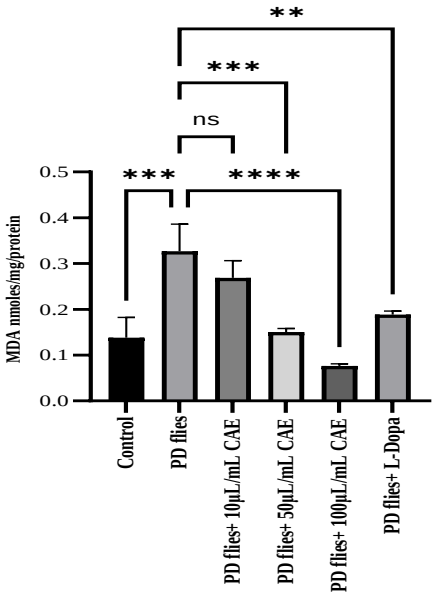

Fig 5

Effect of *Centella asiatica* leaf extract on Lipid peroxidation activity. The amount of MDA was more in the untreated group. The Plant extract treatment reduced the amount of MDA in the treated flies' group. Data are presented as the mean ±SE (n= 3). * Significant at $p < 0.05$; * Significant at $p < 0.001$.**

Discussion

The results of the current investigation shows that *C.asiatica* leaf extract effectively reduces the PD symptoms shown by the transgenic flies. A dose related protection against oxidative stress was shown by *C.asiatica* extract at 10,50 and 100µg/ml. The most effective model for examining the mechanism behind different neurodegenerative diseases in humans is fruit flies(14). Our current study uses a transgenic Drosophila model that expresses human α synuclein gene.When compared to the untreated PD flies, the flies treated with 100µg/ml of plant extract had a greater capacity for climbing. The flies treated with plant extract recovered more quickly than the untreated flies when they were put through the vortex assay.The vortex assay was done to mimic epilepsy in flies as epilepsy is one of the most common neurological condition affecting approximately 500 million people worldwide. Using a vortex when mechanical shock is supplied, the untreated PD group which expresses α synuclein gene displays a behavioural seizure phenotype (10). The organism's capacity to see light through its photoreceptors influence phototaxis. The behaviour of phototaxis, which reflects how organisms move in reaction to light, has been extensively investigated in variety of species. The compound eyes of Drosophila are where the majority of the photoreceptors are found. Drosophila like the majority of insects respond favorably to light and hence migrate in the direction of the light source. Fly phototaxis is impacted by defects which affect the way photoreceptors in the eyes operate. Through the visualisation of deep pseudopupils, external eye morphology and eye pigmentation,structural abnormalities in the photoreceptors neurons can be evaluated (15). Investigations into how photoreceptor neuron function is more difficult. Therefore, the phototaxis test is a simple and popular technique for investigating the normal development and functioning of optical neurons in fly models of neurodegenerative diseases.The treated group of flies moved more towards the light source than the untreated group as the PD flies without plant extract treatment caused damage to the optical neurons. When PD flies are treated with plant extract they could move towards the light source. One of the most crucial stimulus for the animal brain is the ability to detect and distinguish between various volatile chemical forms, which are usually referred to as odours.When the treated and untreated PD flies were subjected to olfactory assay, the plant extract treated PD flies were able to sense the odour of ethyl acetate when compared to that of the untreated PD flies suggesting that olfactory sensory neurons are affected in the PD flies while the treated flies recovered the sense of odour.Further, the flies exposed to plant extract, showed a significance reduction in the lipid peroxidation. Our studys finding correspond with those of Haleagrahara and Ponnuswamy (17) in which treatment of *C. asiatica* extract reduced the lipid peroxidation in rats. Lipid Peroxidation represents a reliable marker for free radical generation. Treatment of the flies with plant extract reduced the catalase content when compared to that of the untreated group. The studies reveal that supplementation of plant extract increases the resistance ability of the flies against oxidative stress damage as seen in neurodegenerative disease (18, 19).

Conclusion

Natural bioactive components found in plant extract exhibited improvements in the climbing ability, faster recovery from seizures, recognition of light and odour in the treated flies of PD model. The antioxidant activity was also well established with decrease in the activity of catalase and lipid peroxidation. The current investigation has shown that incorporating plant extract to the diet has increased antioxidant enzymes and had a good impact on the locomotor, visual and olfcatory ability of flies. The fruit flies should be used more widely since they offer an interesting approach for metabolic research. Additional research on the molecular basis of the neuroprotective activity of these plants are being planned.

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