Protective activity of ferulic acid on rotenone-induced Neurodegeneration in Zebra-fish model

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Abstract
Back ground- Parkinson disease is acknowledged as progressive disorder that cause degeneration of a neuron which occur as a result of abnormal cluster of a protein termed as alpha-synuclein that leads to a diminishing effect to the level of brain dopamine level in basal ganglia. Methods and Materials determine the behavioral parameter, dopamine and catalase level and mitochondrial function of Rotenone induced zebra fishes we carried out an experiment on 40 fishes induced with Rotenone (Parkinson inducer) and 10 normal fishes. Result -this article provides an inclusive, general and practical experimental procedure on zebra fish using Ferulic acid as our test compound and it act as anti-Parkinson phytochemical. Conclusion-we noticed that while the fish treated with Rotenone shows unusual feature like erratic movement and being in cataleptic state after some time and decrease on their Dopamine, Catalase and Mitochondrial function level, on the other hand the fish that was priorly treated with Ferulic acid followed by a direct exposure to Rotenone shows an increasing level of bio chemical parameter.

Keywords: Alpha-synuclein, abnormal cluster, Brain dopamine level, Behavioral parameter, Biochemical parameter, Catalepsy.

Introduction

Parkinson's disease is named by the British doctor James Parkinson in 1817 who wrote the first book about the disease. According to his definition, Parkinson is called the shaking palsy or paralysis agitans (1). Neuronal loss in the substantia nigra, which result in striatal dopamine deficiency, and intracellular inclusions containing bunch of α-synuclein are the neuropathological target of Parkinson disease (2). Parkinson disease is a unique clinical and neuropath logical entity which is manifested clinically by bradykinesia, resting tremor, rigidity and postural reflex impairment (3).

The etiology of Parkinson disease is not known but genetic and environmental factors have its own implication (4). Abnormal mutation in *alpha-synuclein* (*SNCA*), *parkin* (*PARK2*), *PTEN-induced putative kinase 1* (*PINK1*), *DJ-1* (*PARK7*), and *Leucine-rich repeat kinase 2* (*LRRK2*) are the main genetical factor for the disease (5). An increase in the level of genetically cell aging process as a result of the combined effect of triggering factor like environmental toxins result in idiopathic

Parkinson. When the level of the toxic substances, becomes beyond the scavenging capacity of the cell in quantity or in time of subjection, it causes damage to organelles and to membranes that leads to the aggregation of Lewy bodies by an autoimmune reaction to damaged filaments and to cell death mainly in the pigmented neurons of the brainstem (6).

Ferulic acid is an ever-present plant constituent that result from metabolism of phenylalanine and tyrosine. It is found primarily from seeds and leaves both in its free form and covalently joined to lignin and other biopolymers (7). Ferulic acid is a phenolic acid which has low toxicity; it can be easily absorbed and metabolized in the human body. FA plays essential role in providing the sharpness to the cell wall and formation of other important organic compounds like coniferyl alcohol, vanillin, synaptic, and ferulic acid and cur cumin. FA manifests wide variety of biological activities such as antioxidant, anti-inflammatory, antimicrobial, antiallergic, hepatoprotective, anticarcinogenic, antithrombotic, increase sperm viability, antiviral and vasodilatory actions, metal chelation, and modulation of enzyme activity, activation of transcriptional factors, gene expression and signal transduction (8). FA is used as a potential treatment for many disorders including Alzheimer’s disease, cancer, cardiovascular diseases, diabetes mellitus and skin disease.

Figure 1. Zebra fish

The zebrafish (*Danio rerio*) is a flexible model organism that has been used in biomedical research for several years to study a wide range of biological phenomena. They are readily available, hardy, easy, and inexpensive to carry on in the laboratory, have a short life cycle, and have excellent fecundity. Due to its optical clarity and reproducible capabilities, it has become one of the most important models of human genetic diseases

Why zebra fish

Zebra fish is known as the best model for identifying the neuro- degenerative disease like Parkinson. It is the best and selective type of animal due to its easy cost, availability, transparency as well as similarity to mammals. Zebra fish also shows mental, emotional and motor reaction. It is a tropical fresh water fish which is mainly available in aquarium can be used to study the development of human disease. Therefore, zebra fish is preferred as a model because

. It used as alternative animal model, no need to take ethical committee permission, no specific animal house and easy to handle, Transparency makes it one of the best models

Materials and Methods

Study design

Zebrafish were procured from local aquarium shop, Vijayawada. Adult wild-type AB strains of zebrafish (3-5 cm) of both the sexes (4-6-months old) were used. The fishes were habituated to the laboratory conditions for at least 14 days and housed in a 50-L tank filled with un-chlorinated aquarium water at temperature of 28 ± 2°C with constant filtration and aeration. Density of five fishes per liter was maintained. Animals were kept on 14:10 h light/dark cycle and were fed twice a day with aquarium food supplemented with brine shrimp eggs.

Experimental design

Fish were divided in to four groups (number of fish taken per group were =4) labeled as group one as normal (only fish treated with vehicle), group two as toxic (fish treated with rotenone 10 µg), group three as standard (fish treated with dopamine and the anti-Parkinson inducer rotenone and group four as test group (fish treated with ferulic acid and rotenone solution).

Fishes are divided into four groups, each group contain 4 fishes (n=4)

Group I (Normal group) - Normal fishes

Group II (Toxic group) - Fishes are treated with Rotenone (10ug/ml)

Group III (Standard group) – Fishes treated with Rotenone (10ug/ml) and Dopamine (10 ug/ml)

Group IV (Test group) - Fishes treated with Rotenone (10ug/ml) and Ferulic acid (10 ug/ml)

Three test group of fishes were treated individually and subjected to the solution of Dopamine, Rotenone, Ferulic acid at a concentration of 10µg respectively and the remaining control group is treated with vehicle (distilled water). Once this exposure was given, fishes are kept in a separate beaker accordingly for 30 minutes. After this subjection of the then the fish were shifted to another beaker containing fresh water where they were incubated for 15 minutes, then the fish from all treatment groups were individually shifted to examination tank to examine different parameters like locomotors, time spent at the bottom of the tank and complete cataleptic time where they were habituated for 5minute.The examination tank contain afresh aerated water. It consists of a 5-liter tank (30×15×10 m) length x height x weight with number of vertical lines which divided the water the water filled portion of the tank into two halves. These vertical lines were used to find out the speed of the fish by measuring the time taken by fish to travel from the first vertical lines to last and the horizontal line that indicate the time spent in the upper and lower half of the tank by fish. All behavioral evaluation were done by trial-and-error method measured and visual observation was done. (9)

In addition to this, visual observations were made throughout our experiments and unpredictable pattern like vertical swimming, sideway swimming, and upside-down swimming.

Estimation of Behavioral parameters

The following behavioral parameters like lomotor activity, complete cataleptic time and time spent near to the bottom of the tank

Estimation of locomotors of zebra fish

Locomotor is the total distance to travel by the fish from one fixed point to another in examination tank.

In this total distance that the fish moved to travel from first vertical line to the last was calculated and the total distance the fish travel under examination was noted down.

Time spent near the bottom of the tank

The time spent by the fish below the horizontal line which was drawn on the examination tank was measured at different time intervals. The stressful behavior of the fish under our observation was noted down. (10)

Complete cataleptic time

It is the time at which the fish did not move at all, loss sensation of movement.

Estimation of biochemical parameters

Zebrafish were sacrificed by putting them in freezing water, and then brains of
fishes were
Isolateed an pooled in order to get adequate volume of fraction. Synaptosomal fraction fr om fish brain was isolated. The Bio chemical parameters which are evaluated in our experiment are mitochondrial function, dopamine and catalase levels.

Estimation of dopamine level

Procedure: To the 0.2 ml of aqueous phase, 0.05 ml 0.4 M HCl and 0.1 ml of Sodium acetate buffer were added followed by 0.1 ml iodine solution (0.1 M in ethanol) for oxidation. The reaction was terminated after 2 min by addition of 0.1 ml Na SO solution. 0.1 ml Acetic acid is added after 1.5 min. The solution was then heated to 100°C for 6 min on water bath when the sample again reached room temperature; excitation and emission spectra were obtained by reading the spectrofluorometer. The readings were taken at 330-375 nm.(11)

Estimation of Catalase

Catalase activity was measured by taking 0.1 ml of supernatant was decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 440 nm. Activity of catalase was expressed as units/mg protein. A unit is defined as the velocity constant per second. (12)

Table 1. Procedure for estimation of Catalase level

Reagent	Rotenone	Rotenone +Ferulic acid	Rotenone +Dopamine
Phosphate buffer	1,9ml	1.9ml	1.9ml
Sample	0.1ml	0.1ml	0.1ml
H2O2	1ml	1ml	1ml

The reaction occurs immediately after the addition of H_2O_2 solutions is mixed well. Its absorbance is read at wave length 440nm.

Estimation of Mitochondrial function

The mitochondrial function of normal group was considered as 100 % viable.

Liquots of 400 µL containing synaptosomal fractions of zebra fish brain were incubated in the presence of rotenone (1 mM) at 28 ± 2°Cfor 120 min. Eight micro liters of MTT (5 mg/mL) was added and mixtures were incubated at 37°C for 12 h. Determination of formazan was made by estimation of optical density at a wavelength of 570 nm on Jasco V-530 UV visible spectrophotometer. Results were expressed in terms of percentage of MTT reduction (% viability) with respect to control value where the control value considered as 100 % viable). Percent viability of the group treated with dopamine, rotenone and our test compound ferulic acid were calculated with respect to the control group. (13 &14)

Statistical Analysis

Graph-pad prism 5 was used for all statistical calculations. Data are expressed as mean ± SEM. One-way ANOVA was applied using Dunnett's test as post hoc test. A significant difference was attributed to value less than *###P < 0.001*.

Results

Effect of Ferulic acid on locomotor activity (travel from one particular point to another in Sec)

Rotenone -treated group fishes show decrease in number of movements (9) and travel from one particular point to another in cm (151±0.45) when compared to normal control group of fishes, whereas dopamine-treated fishes increase in number of movements (33) and travel from one particular point to another in cm (560±0.12) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly increase number of movements (13) and travel from one particular point to another in cm (285±0.85)compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases number of movements and travel from one particular point to another in cm. Results were shown in table 2 and figure 2.

Table 2. Effect of Ferulic acid on locomotor activity

Treatment Groups	Number of movements	travel from one particular point to another in cm Mean±SEM) After 30 Min
Group I Normal group	19	345.4±0.248**
Group II Rotenone	9	151±0.45***
Group III Dopamine group	33	560±0.12
Group IV Ferulic acid	13	285±0.85###

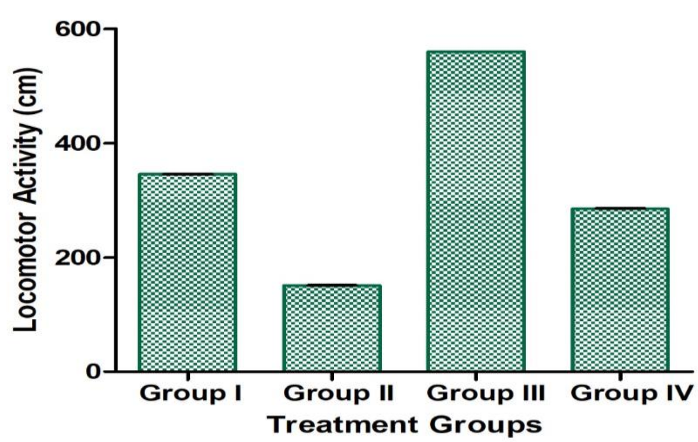

Figure 2. Effect of ferulic acid on locomotor action

Values are shown as mean ± SEM and n=4. Rotenone(***p<0.01) treated group fish's exhibits decrease in number of movements and distance travelled from one particular place to another when compared to normal group and ferulic acid (###p<0.001) treated fishes show significantly increase the number of movements and distance travelled from one particular place to another when compared to rotenone group.

locomotor activity (Number of movements and travel from one particular point to another in Sec)

Figure 3. Recording of locomotor and number of movements of fishes

Effect of Ferulic acid on complete cataleptic time

Rotenone -treated group fishes show increase in complete cataleptic time (120±1.24) when compared to normal control group of animals, whereas dopamine-treated fishes decrease in complete cataleptic time (78 ±0.65) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly decrease in complete cataleptic time (90±0.75) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly decrease complete cataleptic time. Results were shown in table 3 and figure 4.

Table 3: Effect of Ferulic acid on complete cataleptic time(sec)

Treatment Groups	Complete cataleptic time in sec (Mean±SEM)
Group I Normal group	0±0**
Group II Rotenone	120±1.24***
Group III Dopamine group	78 ±0.65
Group IV Ferulic acid	90±0.75###

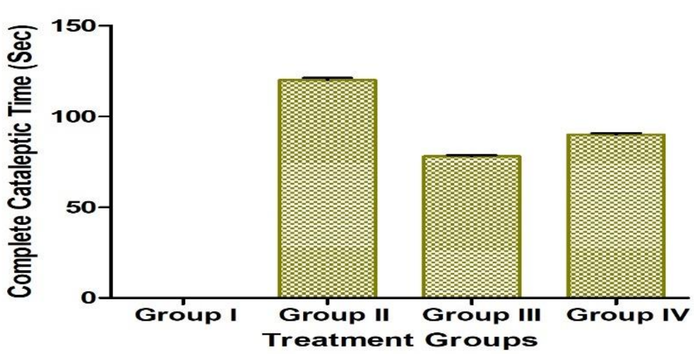

Figure 4. Effect of Ferulic acid on complete cataleptic time(sec).

Values are shown as mean ± SEM and n=4. Rotenone(***p<0.01) treated group fish's exhibits increase in complete cataleptic time(sec) when compared to normal group and ferulic acid (###p<0.001) treated fishes show significantly decrease complete cataleptic time(sec) when compared to rotenone group.

Effect of Ferulic acid on time spent near the bottom of the tank (Sec)

This parameter gives the index of anxiety in fishes. Rotenone -treated group fishes show increase in time spent near the bottom of the tank (184±0.25) when compared to normal control group of animals, whereas dopamine-treated fishes decrease in time spent near the bottom of the tank (172±0.14) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly decrease in time spent near the bottom of the tank (142±0.45) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly decrease time spent near the bottom of the tank and significant reversal of the anxious behavior at 30 m as compared to rotenone. Results were shown in table 4 and figure 5.

Table 4. Effect of Ferulic acid on time spent near the bottom of the tank (Sec)

Treatment Groups	Time spent near the bottom of the tank (Sec) (Mean±SEM)
Group I Normal group	26±0.96**
Group I Rotenone	184±0.25***
Group I Dopamine group	172±0.14
Group I Ferulic acid	142±0.45###

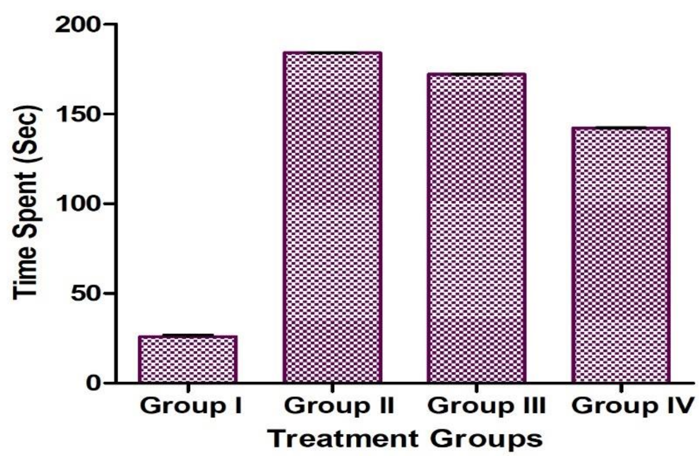

Figure 5: Effect of Ferulic acid on time spent near the bottom of the tank (Sec).

Values are shown as mean ± SEM and n=4. Rotenone(***p<0.01) treated group fish's exhibits increase in time spent near the bottom of the tank (Sec) when compared to normal group and ferulic acid (###p<0.001) treated fishes show significantly decrease time spent near the bottom of the tank (Sec) when compared to rotenone group.

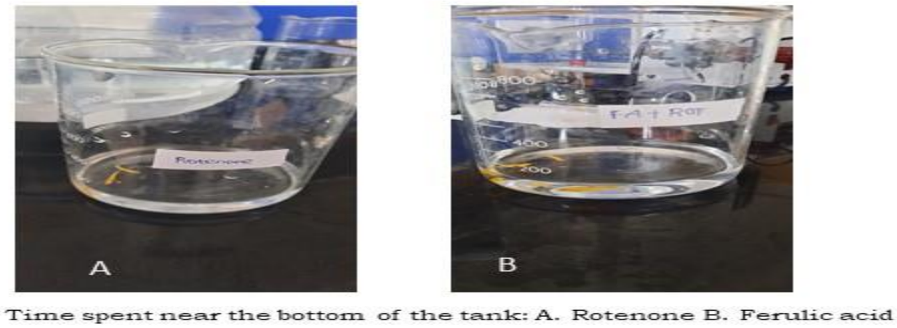

Figure 6. Recording of time spent near the bottom of the tank (Sec)

Bio chemical parameters

Estimation of mitochondrial function

In MTT assay, Rotenone (10 ug/ml) -treated group exhibits decrease in % cell viability (15±0.45) when compared to normal control group of animals, whereas dopamine-treated group exhibits increase in % cell viability (85±0.68) when compared to Rotenone -treated group and Ferulic acid (10 ug/ml) treated group significantly increase in % cell viability (37±0.35) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases the % cell viability. Results were shown in table 5 and figure 7

Table 5: Effect of Ferulic acid on mitochondrial function

Treatment Groups	% cell viability
Group I Normal group	100**
Group II Rotenone	15±0.45***
Group III Dopamine group	85±0.68
Group IV Ferulic acid	37±0.35###

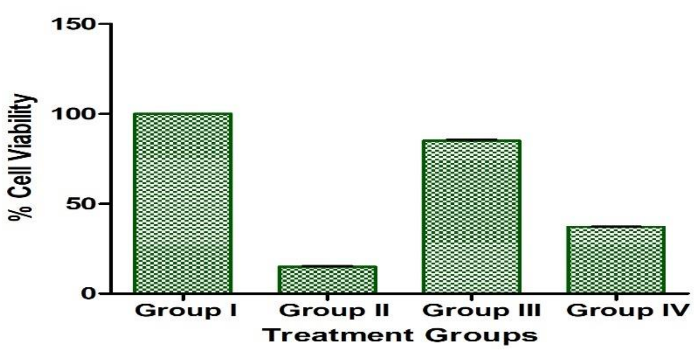

Figure 7. Effect of Ferulic acid on mitochondrial function.

Values are shown as mean ± SEM and n=4. Rotenone(***p<0.01) treated group fish's exhibits decrease in %cell viability when compared to normal group and ferulic acid (###p<0.001) treated fishes show significantly increase %cell viability when compared to rotenone group.

Estimation of Dopamine levels

Rotenone -treated group fishes show decrease in dopamine levels (2.04±0.12) when compared to normal control group of animals, whereas dopamine-treated fishes increase in dopamine levels (41.3±0.78) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly increase in dopamine levels (38.4±0.78) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases the dopamine levels. Results were shown in table 6 and figure 8.

Table 6. Effect of Ferulic acid on Dopamine levels

Treatment Groups	Dopamine levels mg/tissue
Group I Normal group	100**
Group II Rotenone	2.04±0.12**
Group III Dopamine group	41.3±0.78
Group IV Ferulic acid	38.4±0.78###

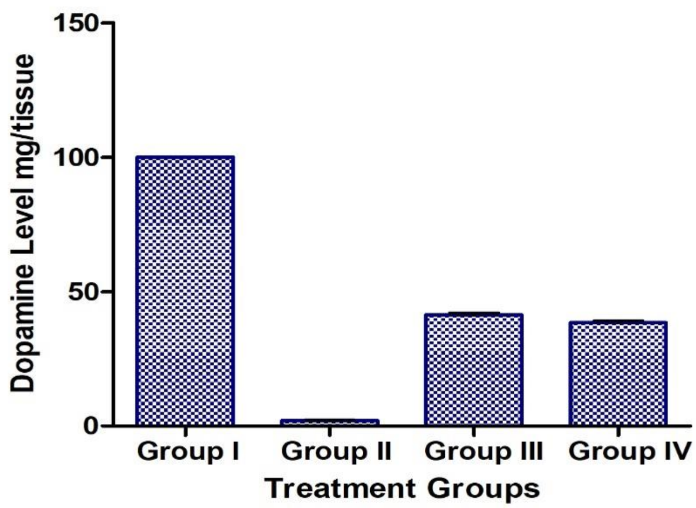

Figure 8. Effect of Ferulic acid on Dopamine levels.

Values are shown as mean ± SEM and n=4. Rotenone(***p<0.01) treated group fish's exhibits decrease in dopamine levels when compared to normal group and ferulic acid (###p<0.001) treated fishes show significantly increase dopamine levels when compared to rotenone group.

Estimation of catalase levels

Rotenone -treated group fishes show decrease in catalase levels (10.12±0.48) when compared to normal control group of animals, whereas dopamine-treated fishes increase in catalase levels (34.60±0.28) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly increase in catalase levels (27.29±0.89) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases the catalase levels. Results were shown in table 7 and figure9.

Table 7. Effect of Ferulic acid on catalase levels

Treatment Groups	Catalase levels units/mg protein
Group I Normal group	100**
Group II Rotenone	10.12±0.48***
Group III Dopamine group	34.60±0.28
Group IV Ferulic acid	27.29±0.89###

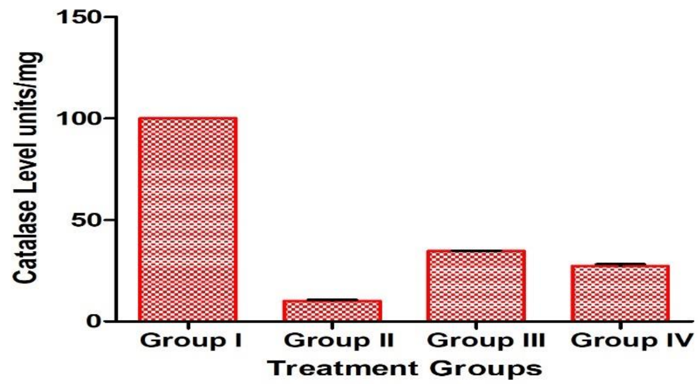

Figure 9. Effect of Ferulic acid on catalase levels.

Values are shown as mean ± SEM and n=4. Rotenone(***p<0.01) treated group fish's exhibits decrease in catalase levels when compared to normal group and ferulic acid (###p<0.001) treated fishes show significantly increase catalase levels when compared to rotenone group.

Discussion

Parkinson disease observed as a result of a reduction of dopamine level due to an abnormal over accumulation of alpha – synuclein. Triggering factors like environmental and hereditary can also leads to a diminishing effect to brain dopamine level. Therefore, along with dopamine mimicking agent, antioxidant like ferulic acid is vital to prevent the formation of reactive oxygen. According to today investigational studies carried out on ferulic acid the incidence of Parkinson disease can be decreased by its antioxidant action which the formation of free radical, and act as anti-apoptotic agent. On the basis of this multiple activity ferulic acid will help for the treatment of Parkinson disease progression.

Parkinson is a progressive neurodegenerative disorder that affect more than 10 million of people across the globe. There is no standard treatment for Parkinson disease. Treatment for each individual with PD is based on symptoms.

There are medications available to treat the symptoms of Parkinson disease. Although none of them yet reverse the effect of the disease completely.

Through our experiment we have tried to resolve such problems and come up with an alternative means of treatment by checking the protective activity of a phytochemical, which is ferulic acid on rotenone induced PD on zebra-fish model.

Rotenone -treated group fishes show decrease in number of movements (9) and travel from one particular point to another in cm (151±0.45) when compared to normal control group of fishes, whereas dopamine-treated fishes increase in number of movements (33) and travel from one particular point to another in cm (560±0.12) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly increase number of movements (13) and travel from one particular point to another in cm (285±0.85)compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases number of movements and travel from one particular point to another in cm.

Rotenone -treated group fishes show increase in complete cataleptic time (120±1.24) when compared to normal control group of animals, whereas dopamine-treated fishes decrease in complete cataleptic time (78 ±0.65) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly decrease in complete cataleptic time (90±0.75) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly decrease complete cataleptic time.

This parameter gives the index of anxiety in fishes. Rotenone -treated group fishes show increase in time spent near the bottom of the tank (184±0.25) when compared to normal control group of animals, whereas dopamine-treated fishes decrease in time spent near the bottom of the tank (172±0.14) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly decrease in time spent near the bottom of the tank (142±0.45) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly decrease time spent near the bottom of the tank and significant reversal of the anxious behavior at 30 m as compared to rotenone.

In MTT assay, Rotenone (10 ug/ml) -treated group exhibits decrease in % cell viability (15±0.45) when compared to normal control group of animals, whereas dopamine-treated group exhibits increase in % cell viability (85±0.68) when compared to Rotenone -treated group and Ferulic acid (10 ug/ml) treated group significantly increase in % cell viability (37±0.35) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases the % cell viability.

Rotenone -treated group fishes show decrease in dopamine levels (2.04±0.12) when compared to normal control group of animals, whereas dopamine-treated fishes increase in dopamine levels (41.3±0.78) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly increase in dopamine levels (38.4±0.78) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases the dopamine levels.

Rotenone -treated group fishes show decrease in catalase levels (10.12±0.48) when compared to normal control group of animals, whereas dopamine-treated fishes increase in catalase levels (34.60±0.28) when compared to Rotenone -treated fishes and Ferulic acid (10 ug/ml) treated fishes significantly increase in catalase levels (27.29±0.89) compared to Rotenone -treated fishes. Based on these results Ferulic acid significantly increases the catalase levels.

Conclusion

Based on the results and discussion, ferulic acid reverse the Rotenone induced Parkinson’s disease by increasing locomotor activity and decrease the formation of catalepsy in zebra fishes and also enhance the dopamine, catalase and mitochondrial functions in zebra fishes brain tissues.

Therefore, ferulic acid is found to be effective in reversing the condition of rotenone induced Parkinson on zebra fish model.

Further studies need to development of molecular mechanism of ferulic acid for the treatment of Parkinson's disease.

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Conflicts of interest

The authors declare that they have no competing interests.

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