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Myricetin attenuates neurodegeneration and cognitive impairment in Parkinsonism

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1. ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disease due to dopaminergic neuron degeneration. It mostly affects the aged population, leads to memory decline and loss of motor coordination. The present study investigates the neuroprotective role of myricetin a flavonol isolated from the brown seaweed *Turbinaria ornata* in rotenone induced *Drosophila* model of PD. Rotenone administration led to dopaminergic neuronal degeneration, dopamine depletion, impaired muscular coordination, gait disturbances, memory decline oxidative stress and apoptosis. Ingestion of myricetin by *Drosophila* significantly prevented rotenone induced neuronal degeneration. These results confirm that myricetin exerts neuroprotective effect in experimental PD.

2. INTRODUCTION

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disease, which occurs due to dopaminergic neuronal degeneration in the Substantia Nigra pars compacta (SNc) and depletion of dopamine (DA) in the striatum (ST). Lewy body inclusions, declined memory and the motor manifestations include bradykinesia, rigidity, tremor, postural and gait abnormalities are prime features of PD (1-2). Although the progression of PD intensifies with age and the etiology is unclear, various reports confirmed that increased oxidative stress, mitochondrial dysfunction, protein aggregation, apoptosis and proteasomal dysfinction are associated with pathological process of PD (3). The multifactorial etiology of PD merely allows present treatment strategies and drugs for PD to alleviate symptomatic relief alone. PD models of neurotoxins are having significant role in investigating the disease pathology and the potential of neuroprotective agents. Rotenone, a highly lipophilic and environmental toxin, which inhibits complex I of ETC, easily crosses all biological membranes and induces mortality, oxidative stress and mitochondrial dysfunction in DAgeric neurons. Strategies of drug discovery for PD from nature resources especially the phytochemicals may render ample neuroprotective effect to prevent the onset or to halt the progression of PD (4). Seaweeds have been reported to have curative properties in disease management in metabolic and life style oriented diseases like cancer, obesity, diabetes etc. They were also reported to have antihypertensive, antihyperlipidemic, antioxidant, anticoagulant, anti-inflammatory, immunomodulatory, neuroprotective, antiviral, antifungal, antibacterial and wound healing properties *in vivo*. Active compounds include sulphated polysaccharides, phlorotannins, flavonoids, carotenoids and minerals, have been proved in degenerative metabolic diseases (5). In this study, Myricetin a flavonol isolated from the brown seaweed *Turbinaria ornata* was investigated for its neuroprotection in rotenone induced Parkinsonism in *Drosophila melanogaster*.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Myricetin was isolated from *Turbinaria ornate.* Rotenone, thiobarbituric acid (TBA), reduced glutathione and 3,5-dithio-bis-nitrobenzoic acid (DTNB), xanthine, xanthine oxidase, bovine serum albumin (BSA) and PBST were purchased from Sigma, Bangalore, India. Tyrosine hydroxylase antibody, Anti-Bax, Bcl-2, caspases-3, -9, Cytochrome c (cyt-c), and anti- β-actin antibodies were obtained from Cell signaling Technology (USA). All other reagents were of analytical grade and were procured locally.

3.2. Collection of algal samples and isolation of Myricetin

The marine brown alga *Turbinaria ornata* was collected by hand picking from intertidal waters of the Mandapam coast (Longitude 78^0 8'E, Latitude 90^0 17' N) in the Gulf of Mannar during the early hours in the month of May. The algal material was identified and authenticated in BSI Coimbatore, Tamilnadu (BSI/SRC/5/23/2015/Tech./1304). A voucher specimen was maintained in our research laboratory. Collected algal material was washed with sea water and then with fresh water to remove sand, salts and epiphytes. The dried *Turbinaria ornata* seaweed materials (50g) were milled and extracted using 500 ml of various solvents such as ethanol, methanol and water for 24 h by using a Soxhlet apparatus. The methanolic extract with more bioactive compounds was lyophilized using freeze dryer (Lark, Penguin Classic Plus, India) and used for further extraction and charecterisation. The flavonoids were determined by reversed-phase HPLC based on the earlier method (6). Flavonoid standards were morin and myricetin, an aliquot of sample extracts was loaded on the HPLC equipped with a reverse phase column. Solvents comprise deionized water (solvent A) and acetonitrile (solvent B) were used. The pH of water was adjusted to 2.5. with trifluoroacetic acid. The flavonoid compounds were detected at 350nm. The column was equilibrated with 85% deionized water and 15% acetonitrile. Then the ratio of deionized water was increased to 85% in 50 min followed by reducing acetonitrile to 15% in 55 min. This ratio was maintained to 60 min for the subsequent analysis with flow rate at 0.6. mL/min. The methanolic fraction (40g) with most bioactive compounds was subjected to silica gel column chromatography (75 µm, 6.0.cm x 40cm) and eluted with methanol of increasing polarities with water (25 to 100% Methanol). The volume of each collected fraction was 50 ml to give 4 fractions (A, B, C, and D). Appreciable antioxidant activity was found in fraction

C and D. Among the two, The most active D fraction obtained (12.2.1mg) was then subjected to silica gel column chromatography (Silica gel 60, 230-400 mesh, 1.0. cm i.d. x 20 cm) and eluted with n-hexane:EtOAc, EtOAc, acetone and methanol. Compound 1 was yielded (4.2.mg) from n-hexane: EtOAc (3:7) fraction which is yellow crystals. The isolated compound was recorded on NMR-400 MHz and chemical shifts were recorded as δ values. The result was compared with the reference chart and possible functional groups present in the plant were determined (data not shown) as per the earlier report (7).

3.3. Drosophila melanogaster treatment strategies

Neuroprotective investigations were conducted out in the CS strain of male *Drosophila melanogaster* (8-10 days old flies). They were housed in polypropylene vials with drosophila culture medium, renewed every three days. Flies were under 12/12h: light /dark cycle at around 20°C (8). The culture medium was as follows; Agar 2.0.g, maize powder 17.0.g, sucrose 15.0.g, yeast tablets 6.0.g and water 360 ml were taken in a 1 litre glass beaker and heated for 1 h with constant stirring. After cooking, 1.0.g nepagin and 1 ml propionic acid (myricetin and rotenone were added to the respective experimental groups) were added and in the corn meal medium which was transferred to vials for Drosophila culture. Then, the flies were transferred to these vials and cultured.

3.4. Experimental design

The flies were exposed to varying concentrations (250μM, 500 μM, 750 μM and 1000 μM) of rotenone (ROT) for 7 days in order to assess the lethality responses to choose the effective dose for LD_{50} (9). On the basis of reduction in mortality, the preventive dose was fixed as per the earlier findings out of different concentrations (78.6.mM) 0.0.25%, (157mM) 0.0.5% and (314mM) 0.1.%) against 500μM (LD50) rotenone in 7 days treatment. 0.1.% (314 mM) myricetin shows significant reduction in mortality and was selected as the active dose (10). After segregation of 50 flies per vial into four groups (three vials in each group and a separate set of experimental groups of flies for histochemical studies). Group I control flies fed with corn meal media. Group II flies received ingestion of rotenone (500 μM effective dose for LD_{50}) through the media for seven consecutive days. Group III flies were pretreated with 314 mM of myricetin (0.1.% effective dose) for three hours before they transferred to vials with rotenone (500μM) for 7 consecutive days. Group IV flies were treated with 314 mM of myricetin alone for seven days through the corn meal media. On the end of experiment (8^{th} day), climbing assay, neuromuscular coordination assay and T maze assay were executed. The flies in the each group were decapitated; the heads were homogenized in phosphate buffer (pH-7.0.) and centrifuged at 5000rpm for 20 min at 4°C. The supernatant was used to estimate the levels of dopamine, biochemical estimation and western blot analysis. The brains of the other set of experimental group flies were prepared for immunohistochemical studies.

3.5. Neuromuscular coordination assessment

The crucial manifestations of Parkinson's disease are slow movement, difficulties with gait and balance, and tremor. Neuromuscular coordination was assessed on movement variables, such as a limb's position, speed, control and coordination. To assess the motor performance, muscular coordination, cognitive and memory impairment the following assessment methods were employed as per the earlier studies with slight modifications (11-12). This type of assessment strategy in PD treatment is significant in exhibiting the neuronal degeneration and the effectiveness of the therapy and brain recovery process (13).

3.5.1. Climbing assay

Climbing assay was done as per the protocols of the previous study (14). Briefly, in climbing assay, experimental flies were placed in an empty glass tube (25cm height and 2cm diameter). After a 10 min rest period, the flies were tapped to the bottom of the glass tube, and the number of flies able to climb in 60 s was recorded at each interval of time. The assay was replicated three times at 5 min intervals. The scores are the mean of the numbers of flies at the top and at the bottom, expressed as percentages of the total number of flies.

3.5.2. The styrofoam bead test and leg print test

Experimental group flies were fixed on microscope slides in the ''legs-up'' position using natural adhesive solution. The styrofoam bead. Flies were tested for their ability to hold, maintain and rotate the styrofoam bead. Microscope slides were coated with soot using a kerosene lamp. Experimental group flies were anaesthetized by chilling them on ice, their wings were trimmed to prevent them from flying and on recovery placed on the carbon soot coated slide and allowed to walk. Tracks were photographed (12).

3.6. Cognitive and memory assessment

3.6.1. T-maze Aversive Phototaxis Suppression Assay (TAPS assay)

These assays were done as per the previous studies with modifications that dealt with positive phototaxic behavior in flies to train them to associate light with aversive stimuli (filter paper with neem leaf

paste coating). The experimental flies with good memory and learning may avoid entering the lighted area with aversive taste since they are familiar with the bitter taste during training sessions. Flies with declined learning capacities might have fail to avoid the entry. Further the APS assay was used to measure short term memory function of flies by subjecting already trained flies to the same test 6 hours post conditioning to test their ability in remembering the learned task.

The flies of each experimental group were placed in an empty polystyrene vial with water moistened filter paper for six hours before the assay it was ensured that the flies were starved which made them more perceptive to aversive taste. T-maze apparatus (shown in Figure 4) consists of the center column with two independent chambers, a “dark” chamber and a “lighted” chamber of 20 ml glass boiling tubes fitted at each side of a connecting tube. The tubes were wrapped with dark colored adhesive tape and the other tube with the light source and this tube served as the “lighted” chamber. The neem paste was applied to the filter paper and placed in the light chamber. T-maze was assembled by screwing the lighted and dark chambers on each side of the center column, with the trap door in the middle closed (Figure 4a). The dark chamber was unscrewed from the T-maze and a single fly was transferred into this chamber and immediately the dark tube was screwed back to the maze. The lights in the experimental room were turned off and a red lamp was ignited The fly was allowed to acclimatize in the dark chamber for 30 seconds and slowly light source was turned on to illuminate the lighted chamber. The trap door that separates the two chambers was opened. The fly walked to the lighted chamber within 10 seconds, was considered positively phototactic, for Aversive Phototaxis Suppression (APS) assay the flies which were positively phototaxis were tapped back to the dark chamber, after one minute the fly was allowed into the quinine coated lighted chamber. After one minute, the fly was tapped back to the dark chamber this was repeated for several times. The test trials were conducted and in each test trial, the fly which avoids entering the lighted vial was recorded as “Pass”, and was considered as “learning ability”. The Pass rate over six consecutive trials was recorded.

3.6.2. Short-Term Memory assessment

After the T-maze aversive phototaxis suppression assay the experimental group flies were transferred to their respective vials. After six hours the flies were again subjected to the T maze chamber analysis in the same way as before, and the number of times the flies were avoided to enter the light chamber was recorded as the indicator of short term memory (15-16).

3.7. Analysis of dopamine concentrations by HPLC

The flies from each group were homogenized in 500µL of 0.1. M phosphate buffers (ice cold, pH, 7.4.) containing 1 mM EDTA. Followed by centrifugation at 2,500×g for 10 min, the supernatant of the homogenization was filtered and injected into the HPLC column detected at 280nm with UV detectors. The flow rate of a mobile phase consisting of 0.2.% aqueous trifluoroacetic acid and methanol (70:30 v/v) was maintained flow rate of 1 ml/ min (17).

3.8. Biochemical analysis

3.8.1. Estimation of TBARS (Lipid peroxidation)

0.2. ml homogenate was pipetted in an eppendorf tube, 0.4. ml of TCA and 0.4. ml of TBA was added to it. The reaction mixture from the vial was transferred to the tube and centrifuged at 3500 rpm for 15 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535 nm. The rate of lipidperoxidation was expressed as nmol TBARS formed/g tissue (18).

3.8.2. Estimation of total protein

Protein concentration in the whole body fly homogenate was determined after trichloroacetic acid precipitation using bovine serum albumin as the standard. 0.5. ml of fly homogenate was mixed with 0.5. ml of 10% TCA and centrifuged for 10 mins. The precipitate was dissolved in 1.0. ml of 0.1. N NaOH. From this, an aliquot was taken, and 4.5. ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 minutes. 0.5. ml of Folin’s phenol reagent was added and the blue colour developed was read after 20 minutes at 640 nm. A standard curve was obtained with standard bovine serum albumin and was used to assay the tissue protein level for enzyme activity (19).

3.8.3. Estimation of superoxide dismutase (SOD)

The reaction mixture of total volume 1.0. ml consisted of 0.5. M phosphate buffer pH 7.4., 0.1. ml post mitochondrial supernatant (PMS), 25µl xanthine, 3µl NBT. It was incubated for 15 min at room temperature and reaction was initiated by the addition of 15µl xanthine oxidase. The rate of reaction was measured by enzyme required to inhibit 50% formation of formazan, a reduction product of NBT at 550 nm (20).

3.8.4. Estimation of catalase

The assay mixture been composed of 0.0.5 M phosphate buffer pH 7.0., 0.0.19 M hydrogen peroxide and

0.0.5 ml post mitochondrial supernatant (PMS) in a total volume of 3.0.ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of µ moles of H_2O_2 consumed/min per mg protein (21).

3.8.5. Estimation of reduced glutathione (GSH)

0.5. ml of fly homogenate was precipitated with 0.5. ml of sulfosalicylic acid (4%). The samples were kept at 4 ° C for 1 hour. To this 0.2. ml DTNB (40 mg/10 ml of phosphate buffer, 0.1. M, pH 7.4.) and 0.3. ml 0.1. M phosphate buffer (pH 7.4.) was added and centrifuged at 1500x/g for 5 min. The yellow color developed was read immediately at 412 nm. The results were expressed as µg of GSH/g tissue (22).

3.8.6. Estimation of glutathione peroxidase (GPx)

Determination of Glutathione peroxidase (GPx) activity measured in accordance with the procedure described by the previous study. The reaction mixture consisted of 1.4.4 ml phosphate buffer, 0.1. ml of EDTA, 0.1. ml of sodium azide 0.0.5 ml of GR (1 eu/ ml), 0.1. ml of glutathione (1 mM), 0.1. ml of NADPH (0.2. mM), 0.0.1 ml of hydrogen peroxide (0.2.5 mM) and 0.1. ml of post mitochondrial supernatant (PMS) (10% w/v) in a final volume of 2.0. ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated by the amount of glutathione utilized per minute per mg protein (23).

3.9. Immunohistochemistry

Whole brain staining was carried out to analyze the dopaminergic neuron clusters. Flies were anesthetized under chilling and were decapitated with forceps. Each Head from the experimental groups was transferred to 1.5. ml microcentrifuge tubes containing fixative (4% paraformaldehyde, 0.3.% Triton X-100) and was stayed on ice for 3 hrs. Cuticle and trachea were removed and brains were dissected under stereomicroscope, fixed at room temperature for 30 min and incubated with 0.3.% PBST (0.3.% Triton X-100, 1×PBS) three times on a rotator, each time 20 min; then blocked for 1 hr at room temperature with blocking buffer (5% normal goat serum, 1×PBS, 0.1.% Triton X-100), then incubated with anti TH (1:200 dilute in blocking buffer) for 36 hrs at 4°C on a rotator. The primary antibody was removed and the brain was washed with 0.3.% PBST three times on a rotator, each time 20 min, then incubated in goat anti-rabbit (1:200 dilutions in blocking buffer). The brain was washed with 0.3.% PBST three times on a rotator, each time 20 min, and finally mounted. Mounted brain was analyzed under a confocal microscope (OLYMPUS-OLS4100). All the sections from the various groups were incubated under the same conditions with similar antibody concentration to make the immunoreactivity comparable among the different experimental groups (24).

3.10. Western blot analysis

Total volume of 30 µg of isolated protein from Drosophila homogenate was loaded per lane. The separated proteins were blotted onto a PVDF membrane by semi-dry transfer (BIO-RAD). After blocking with 5% non fat milk in TBS, The membranes were then incubated with various antibodies: Bcl-2, Bax, caspases-3 and 9, cytochrome c and β-actin. The following dilutions were used for Bcl-2 and Bax (1:500), cytochrome c, caspases-3 and 9 (1:1000), and β-actin (1:2000). After primary antibody incubation, the membranes were incubated with secondary antibody at a concentration of 1:2000. Then the membranes were washed with Tris-buffered saline and 0.0.5 % Tween-20 thrice for 10 min interval. After extensive washes in TBST, the bands were visualized by treating the membranes with 3, 3'-diaminobenzidine tetrahydrochloride (25).

3.11. Statistical analysis

All numerical results were expressed as mean ± standard deviation (SD) and analyzed using the statistical package for the social sciences (SPSS) IBM, SPSS version 16. One-way analysis of variance (ANOVA) was conducted for statistical comparison of study whereby $p<0.0.5$ being the criterion for statistical significance. The significant means were further subjected to Duncan's post hoc test.

4. RESULTS

4.1. Rotenone lethal dose study (LD_{50})

Among the different doses, 500µM of rotenone induced 50 % of mortality and was fixed as the effective dose to induce Parkinsonism in *Drosphila melanogaster* (Figure 1).

4.2. Climbing assay of experimental groups

The rotenone ingested flies consumed more time to climb the glass tube and only about 38% of flies managed to reached the top as compared to control flies in which 92% of flies reached the top. 91% of flies in myricetin alone treated group have reached top. Flies in myricetin pre-treated and rotenone ingested shown appreciable reduction in time consumption and about 77% of flies climbed to top compared to rotenone ingested flies (Figure 2).

4.3. Styrofoam bead test of experimental groups

Only 9 % of rotenone ingested flies managed to hold the bead compared to control flies and myricetin

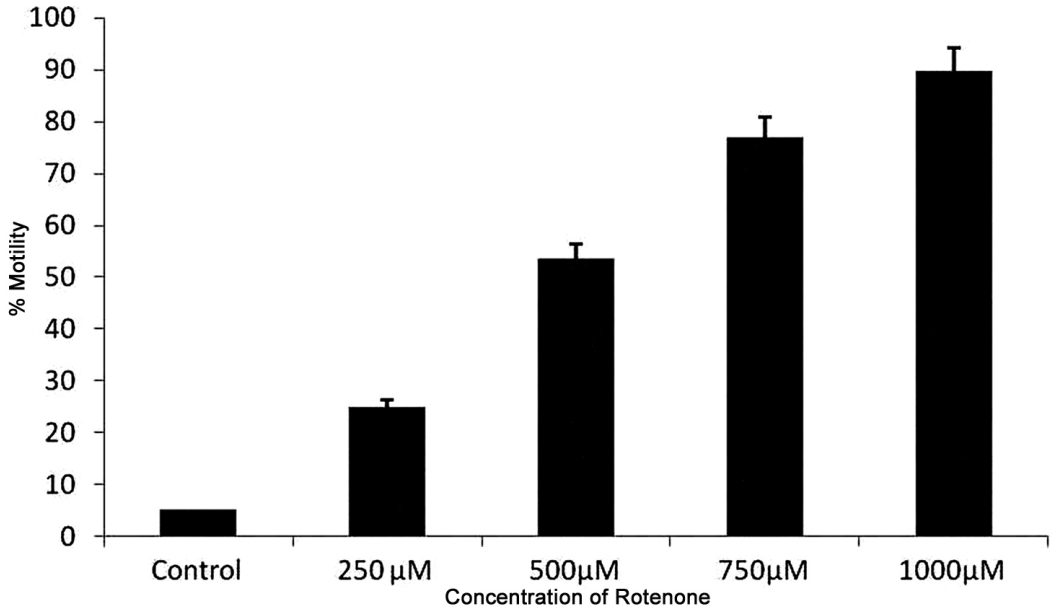

Figure 1. Shows the dose-dependent effect of rotenone (250, 500, 750 and 1000 µM) induced 50 % percentage of mortality in experimental flies (LD_{50} obtained at 500 µM rotenone concentration).

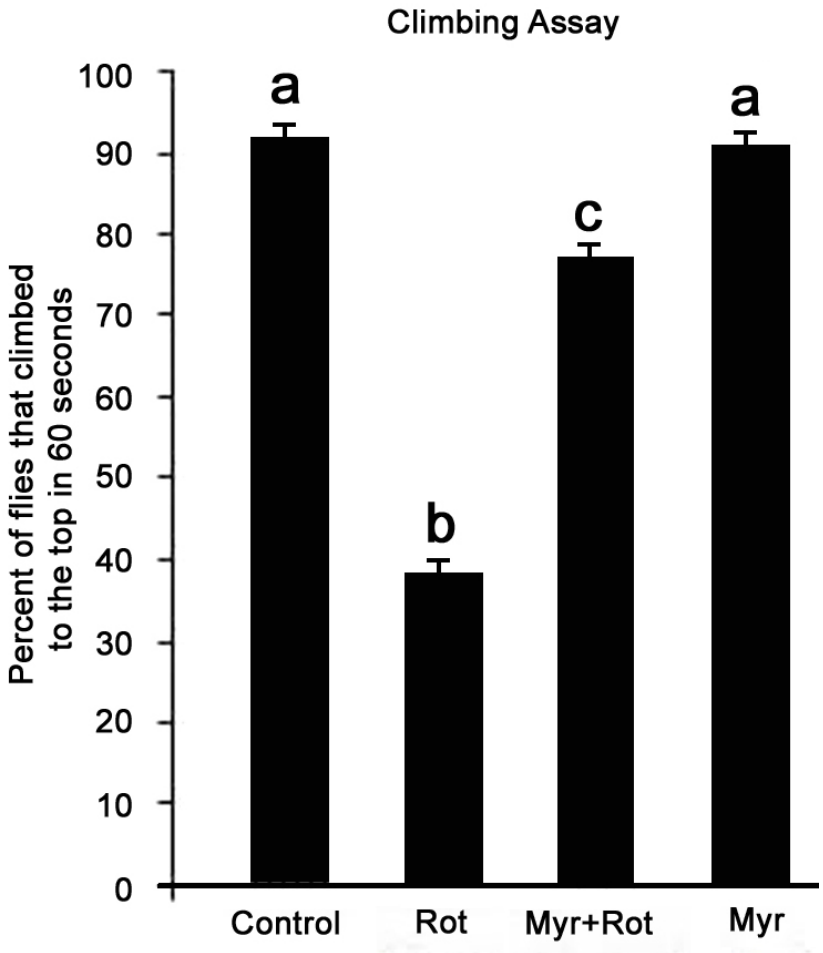

Figure 2. Shows the Climbing assay the time taken to climb 25 cm long glass tube in control and experimental groups in 60s (Values are given as mean ± S.D for 3 vials of drosophila 50 flies each group. (Values not sharing a common superscript letter differs significantly at p<0.0.5 (DMRT))

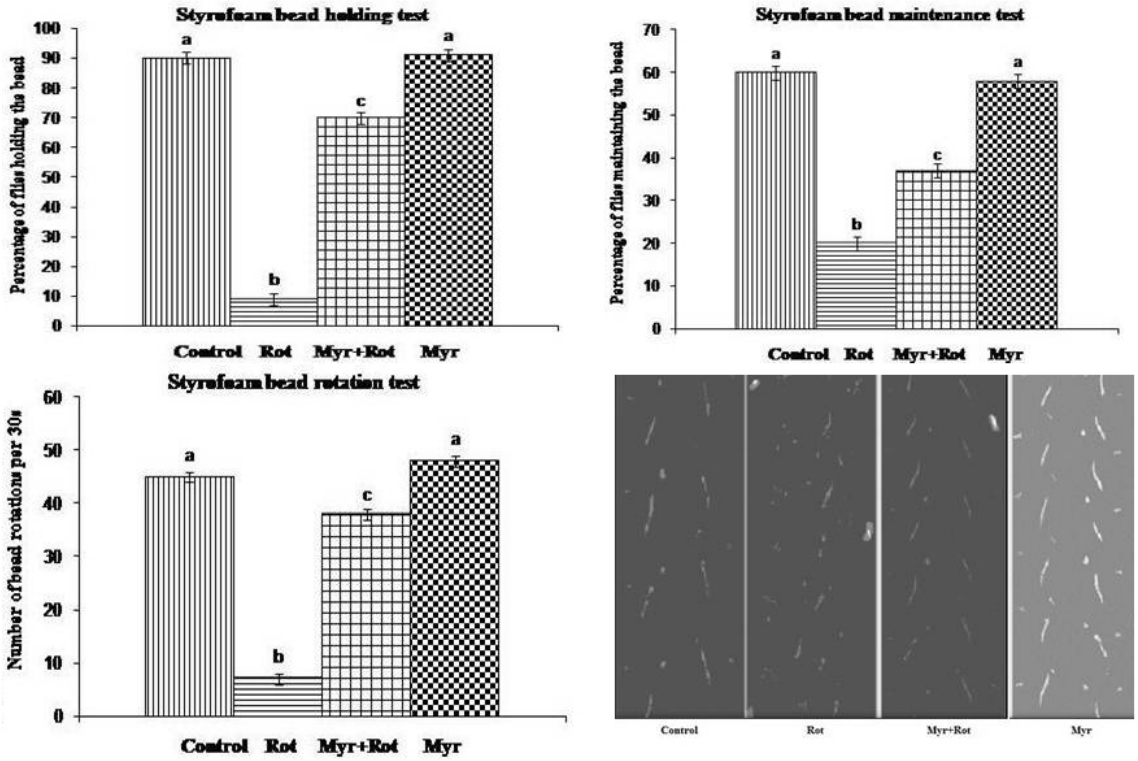

Figure 3. Shows styrofoam bead test for neuro-muscular coordination "A, B, C" (Values not sharing a common superscript letter differ significantly at p<0.0.5 (DMRT)) and foot print test for gait assessments "D".

alone treated groups in which about 90% and 91% of flies hold the bead respectively. Myricetin pre-treated and rotenone ingested flies showed appreciable frequency, about 70% of flies hold the bead as compared to rotenone ingested flies.

In Styrofoam bead maintenance test of experimental flies, about 20 % of rotenone ingested flies maintained the bead as compared to control flies and myricetin alone treated groups in which about 60% and 45% of flies maintained the bead respectively. About 37% of flies in the Myricetin pretreated and rotenone ingested group maintained the bead compared to rotenone treated flies.

Rotenone ingestion reduced the Styrofoam bead rotation ability of flies, and they have exhibited an average of 7 rotations per 30 seconds compared to control and myricetin alone treated flies which showed average bead rotations of 45 and 48 times respectively. Myricetin pretreatment reduces the toxicity of rotenone and those flies have shown 38 rotations per 30 seconds.

The 'foot-print' test for walking pattern was done with experimental group flies. They were allowed to walk on a kerosene lamp carbon-soot coated glass slide and their crawling tracks were examined with the help of the foot prints on the glass side. The direction of climbing was towards the top of each slide. The leg prints made by the first second and third leg of the left hemisegment were marked, control, myricetin alone treated and myricetin and rotenone treated flies shows a proper gait. In rotenone treated flies, the legs are held closer to the body and the foot-print depicts the shuffling gait as in the Parkinson's disease and the legs were being unusually placed with respect to the body (Figure 3).

4.4. T Maze assay

After several training sessions, the experimental group flies were allowed to enter the T maze apparatus with light and dark chamber, where the light chamber contains filter paper coated with fresh neem paste to induce aversion whenever the flies entered the light chamber. About 35 % of Control flies and 37% of myricetin alone treated flies shows aversion to enter the light chamber since they have learned that light chamber contains bitter neem paste and restricted themselves from entering the light chamber; they remembered the same even after six hours in short term memory analysis also and restricted them from entering the light chamber. In rotenone ingested flies only 5 % flies showed restriction and the rest of the flies shown no restrictions and enters the light chamber at every analysis, they repeated the same even after six hours in short term memory

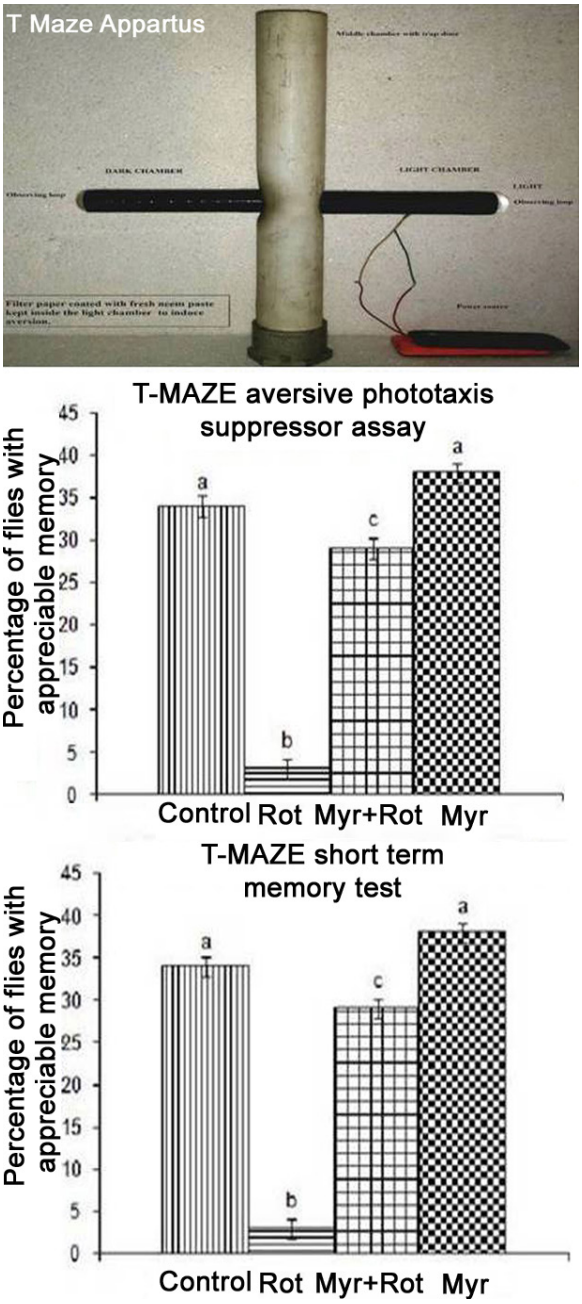

Figure 4. Shows T-Maze apparatus and T-Maze test for learning ability and short term memory. (Values not sharing a common superscript letter differ significantly at p<0.0.5 (DMRT)).

analysis; clearly indicates that rotenone reduced learning ability and developed the memory loss in those flies. About 30 % of myricetin pretreated and rotenone ingested flies shows aversion to enter the light chamber since they learned that light chamber contains bitter neem paste and restricted themselves from entering the light chamber; they remembered the same even after six hours in short term memory analysis also compared to the rotenone treated group (Figure 4).

4.5. Effect of Myricetin and rotenone on dopamine level

Rotenone treated Drosophila homogenate samples shows decreased concentration of about 8.2.0pg of dopamine compared to the control and myricetin alone treated flies (19.9.0 pg and 19.4.5 pg respectively). Myricetin pretreatment maintains the dopamine concentration (12.5.3 pg) against the rotenone toxicity which is similar to control flies (Figure 5).

4.6. Effect of myricetin on lipid peroxidation and enzymatic antioxidants

Rotenone ingested Drosophila homogenate samples shows increased TBARS level, SOD, Catalase, GPx activities; and decreased GSH level compared to the control flies and myricetin alone treated flies. Myricetin prevents the impact of rotenone in induced free radicals and oxidative stress and keeps the TBARS and GSH levels and activities of SOD, Catalase, GPx near normal (Figure 6).

4.7. Effect of Myricetin on rotenone induced dopaminergic neuron clusters degeneration

Rotenone treated flies brain section shows reduced dopaminergic clusters compared to the brain sections of control and myricetin alone treated flies. Myricetin reduces the loss of dopaminergic neuron clusters against the impact of rotenone Clearly shows the preventive effect myricetin on rotenone induced neurodegeneration of DA (dopaminergic neurons) neurons (Figure 7- A, B, C, and D).

4.8. Effect of Myricetin on apoptosis

Rotenone treatment (500 μM) leads to decreased expression levels of anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2), but it increased the expression levels of pro-apoptotic Bcl-2-associated X protein (Bax). Myricetin pretreatment (0.1.%) retains the normal expression of Bcl-2 and Bax. Rotenone ingestion increased expression levels of cyt c, caspases 3 and 9. myricetin pretreatment significantly suppressed the enhanced cyt c, caspases 3 and 9 expressions and prevents the apoptosis due to rotenone toxicity (Figure 8).

5. DISCUSSION

Rotenone administration (500μM) leads to oxidative stress and apoptosis, loss of dopaminergic clusters in Drosophila brain and results impaired cognition and muscular coordination in the flies which are evident in our study. Lack of muscular coordination is the important pathologic features in PD, due to degeneration of pigmented DA neurons in SN (60-70% of the neuronal loss in SN and striatal DA content

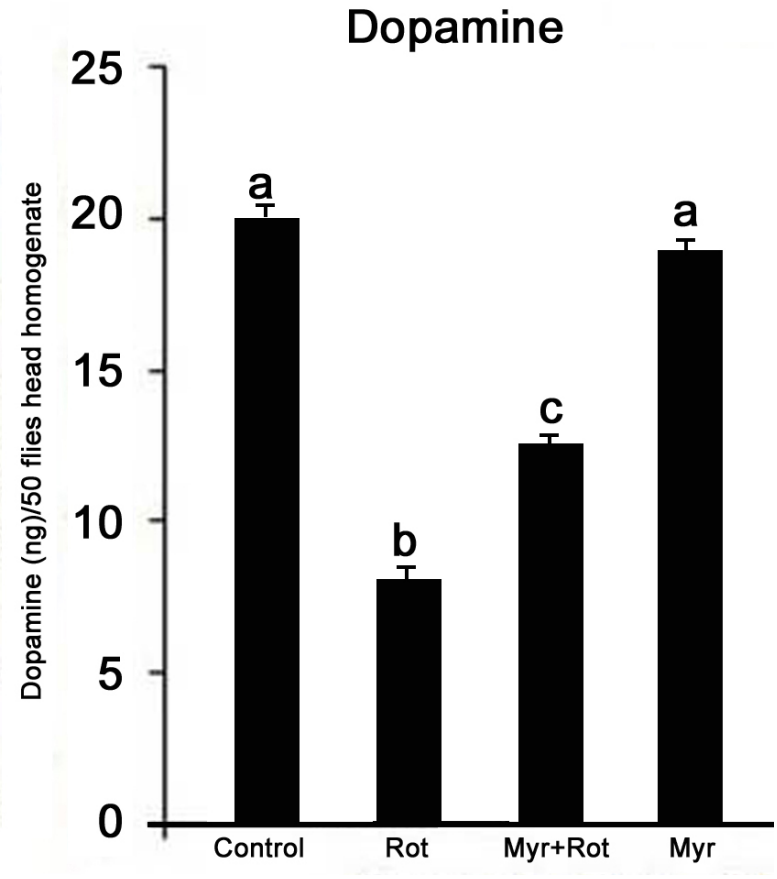

Figure 5. Elucidates dopamine levels in the control and experimental groups. (Values are given as mean ± S.D for 3 vials of drosophila 50 flies each group. Values not sharing a common superscript letter differ significantly at p<0.0.5 (DMRT)).

is reduced by 80%), that triggers the symptoms such as akinesia, rigidity, resting tremor, gait and postural abnormalities (26). Deficits in muscular coordination are shown by the percentage of flies climbing to the top in the 25cm glass tube. Rotenone treatment hindered the muscular coordination and allowed only few flies to climb to the top as compared to control flies. The pharmacological properties of Myricetin (27) prevented the rotenone toxicity and maintained the muscular coordination in flies by enhancing the survival of dopaminergic clusters and levels of dopamine.

Abilities of flies to hold, maintain and rotate a styrofoam bead are the sign of normal dopamine metabolism and survival of dopaminergic clusters. Rotenone treated flies exhibit reduced ability to hold, to maintain the bead; they rotated the bead slowly and the frequency of rotations was meager. Control flies and Myricetin treated flies shows appreciable performance in styrofoam bead test as they were holding and maintaining the bead for a longer time. They have exhibited faster and regular rotations. Myricetin pretreated and rotenone ingested flies holds the bead and maintained it for a long time as compared to the rotenone alone treated group flies. The 'Foot print' test for walking pattern was done with experimental group flies with trimmed wings. They were allowed to climb on a kerosene lamp carbon-soot coated glass slide and their crawling tracks were examined with the help of the leg prints on the glass side. The direction of climbing was towards the top of each slide. Control and Myricetin alone treated flies shows a proper gait. In rotenone ingested flies, the legs are held closer to the body and the foot-print depicts the consequence of a shuffling gait as in the Parkinson's disease and the legs were being unusually placed with respect to the body. Myricetin pretreated and rotenone ingested flies showed appreciable performance in Styrofoam bead test compared to rotenone ingested flies clearly indicates the protective role of myricetin against rotenone induced impairment in neuro-muscular motor coordination. Our results are well supported by the previous report (12).

PD patients are at an amplified risk of rising dementia and cognitive deficits in PD are associated with worse quality of life (28-29). The effect of myricetin in the learning ability and short term memory in experimental flies was assessed with the T-maze apparatus with light chamber contains neem paste

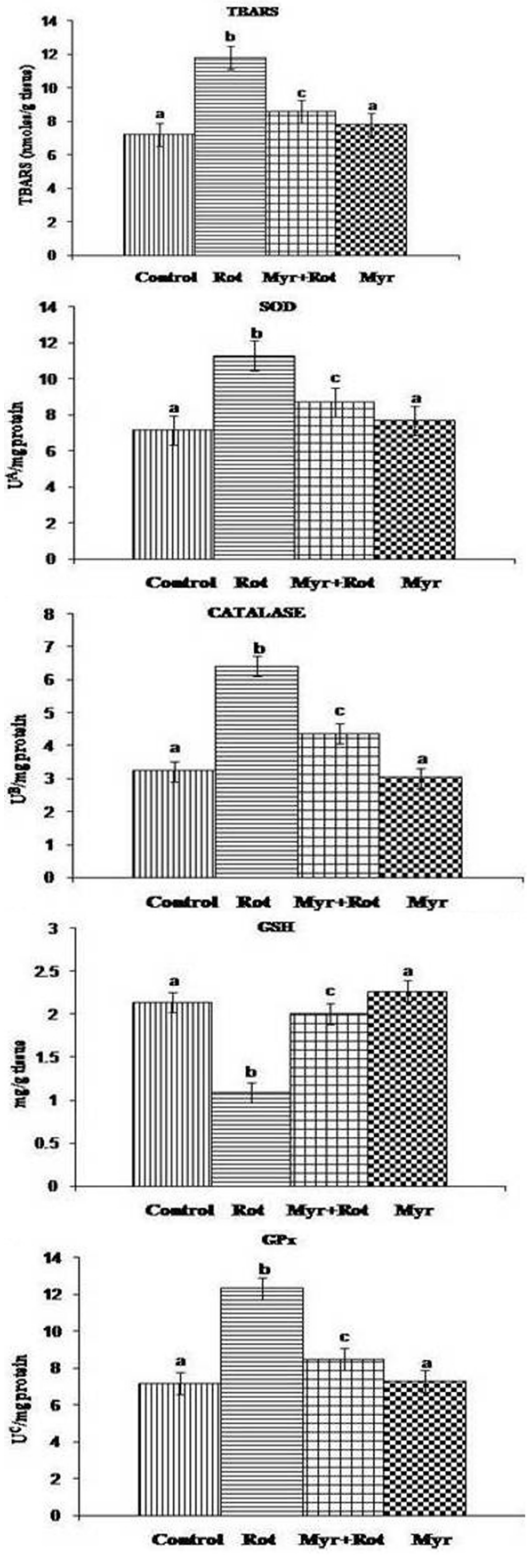

Figure 6. Effect of myricetin on rotenone induced oxidative stress in control and experimental group. (Values are given as mean ± S.D for 3 vials of drosophila 50 flies each group. Values not sharing a common superscript letter differ significantly at p<0.0.5 (DMRT). A = enzyme required for 50% inhibition of NBT reduction. B = μ moles of H_2O_2 utilized /min. C = μ moles of glutathione utilized/min).

coated filter paper which creates aversion in flies and make them to avoid entering the light chamber and a dark chamber. Rotenone treated flies never restrict themselves in entering the light chamber, since rotenone inhibits their learning ability due to neuronal degeneration, whereas Myricetin pretreated and rotenone ingested flies showed appreciable aversion towards the neem paste contained light chamber, shows the positive role of myricetin in upholding the learning ability against rotenone. Myricetin alone treated flies were similar to control flies shows aversion towards the light chamber due to the bitter taste of neem. Similarly in short term memory analysis, the flies treated with myricetin remembered the bitter taste of neem paste in the light chamber even after six hours and made them to restrict entering the light chamber, thus myricetin prevents the memory loss against the rotenone induced memory impairments out of neurodegeneration, our results are similar to the previous report (15).

Postmortem examination of brains of PD patients confirmed the impact of oxidative stress and decreased levels of reduced glutathione with enhanced lipid peroxidation and modifications of proteins were found. Reactive oxygen species (ROS) are generated in normal metabolism of dopamine and mitochondrial respiration. Rotenone inhibited complex I, which is the site of electron leakage that produces ROS (30). The toxic effect of rotenone on the electron transport chain could cause an increase in free radicals, leads to oxidative stress and ultimately resulting in severe damage of the DAgic neurons. The level of increased TBARS, decreased GSH levels and increased activities of SOD and catalase were observed in rotenone treated group flies as compared to control group flies. However, a decrease in TBARS, increase in GSH level and significant reduction in the activities of SOD and catalase were observed in myricetin pretreated and rotenone ingested flies is the proof of antioxidant property of myricetin against rotenone neurotoxicity. Our results are well supported by previous findings in different experimental model of PD (25).

The experimental group flies brains were immunostained with TH through whole mount assay. TH-positive neurons in *Drosophila* brains were shown and counted. The number of TH positive neurons suggested that myricetin prevented dopaminergic neuronal loss caused by rotenone. To further confirm these findings, dopamine levels in the brain of flies were determined using HPLC analysis. Myricetin significantly prevents the DA neurons and thereby shows increased dopamine concentration of compared to the rotenone administered flies, which corroborates with earlier study (31). Myricetin blocked the loss of $\Delta\psi m$ and thereby it protects cells via inhibition of the mitochondria dependent apoptosis pathway. The protective effect of myricetin against H_2O_2-induced

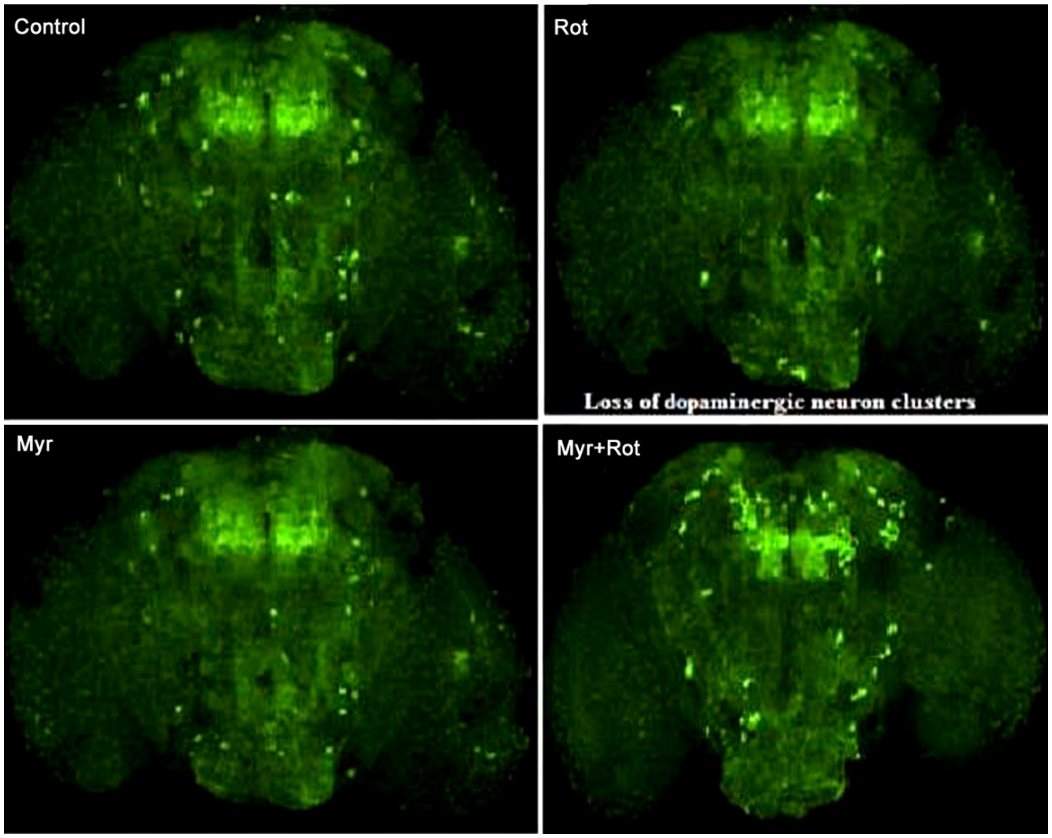

Figure 7. Tyrosine hydroxylase immunolabeling showing dopaminergic neuron clusters in experimental fruit fly groups brain "A,B,C" and "D" after 7 days of exposure to 500 µm rotenone.

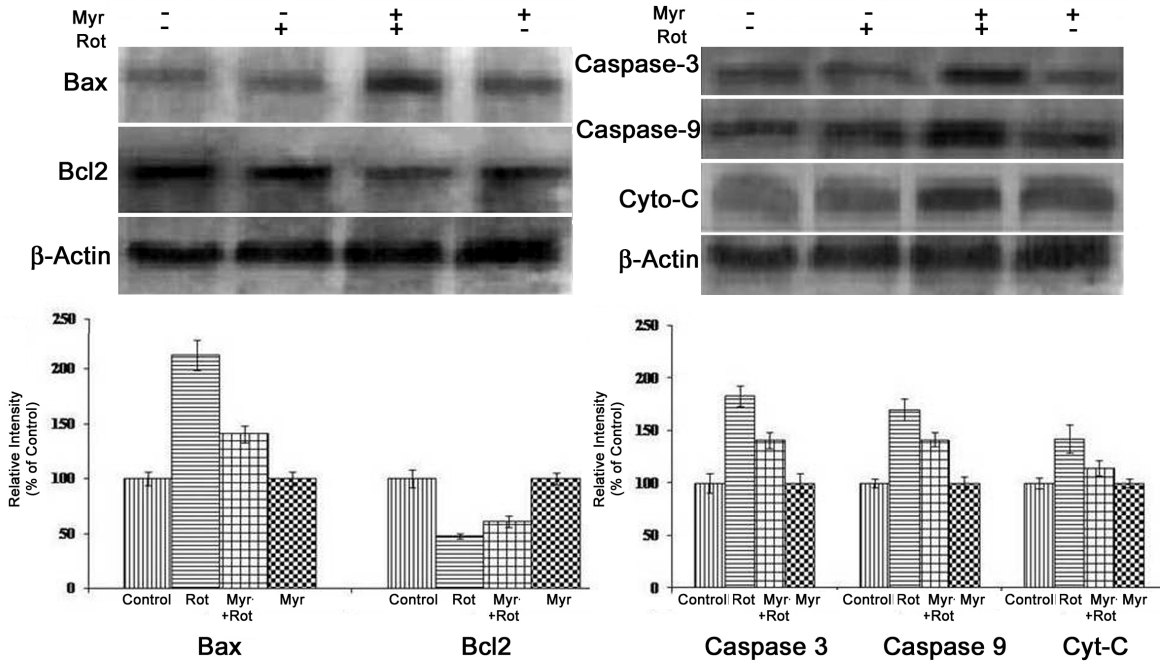

Figure 8. Effect of myricetin on rotenone induced changes in the expressions of apoptotic protein markers (Bax, caspase 3, 9 and cyto-c) in control and experimental flies. Protein expressions by using β-actin as an internal control. Data are shown as mean ± SD p< 0.0.5 compared to the control flies, cp< 0.0.5 compared to the rotenone treated flies (DMRT).

cytotoxicity in a earlier study showed that myricetin increased Akt phosphorylation, a significant cell signaling molecule as compared to the decreased Akt phosphorylation with H_2O_2 treatment (32). In group II flies rotenone treatment decreased the expression levels of anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2), however it increased the expression levels of pro-apoptotic Bcl-2-associated X protein (Bax). Myricetin pretreatment retains the customary expression of Bcl-2 and Bax against the rotenone induction of apoptosis. Treatment with rotenone increased expression levels of apoptotic caspases cyt c, caspases 3 and 9. Myricetin pretreatment significantly suppressed the enhanced cyt c, caspases 3 and 9 expressions and inhibits the apoptosis in DA neurons due to rotenone. Mitochondrial membrane depolarization due to rotenone toxicity leads to decline in mitochondrial membrane potential and induce apoptosis. Myricetin is appreciably having attenuation ability on rotenone induced abnormalities in mitochondrial membrane potential and expression of apoptotic proteins. Mitochondria act as imperative machinery for signals during apoptosis, and the loss of mitochondrial integrity can be provoked or repressed by many regulators of apoptosis (33). Oxidative stress due to rotenone induces caspase activation through cytochrome c release. Oxidative stress increased the expressions of Bax, active caspase-9, and -3, which are pro-apoptotic factors, but decreased the expression of Bcl-2, which is an anti-apoptotic factor Myricetin inhibited the release of mitochondrial cytochrome c. In the apoptotic process, Bcl-2 put off the opening of the mitochondrial membrane pores, whereas Bax persuade the opening of membrane pores. Therefore, the reduction in loss of $\Delta\psi m$ by myricetin might be the result of Bcl-2 up-regulation, and Bax down-regulation (34).

6. CONCLUSION

In the present study, a flavonol myricetin isolated from *Turbinaria ornata*, brown seaweed with the richest source of bioactive compounds was investigated for its neuroprotective activity in *Drosophila melanogaster* PD model. Our study results show that myricetin inhibits DA death and promotes the muscular coordination and psychological well being of fruit flies which is clearly elucidated clearly in the learning ability assays in which myricetin upholds the memory in fruit flies. DA neurodegeneration amelioration and apopotosis prevention due to the neuroprotective activity of myricetin and helps maintaining the dopamine level against rotenone to alleviate the Parkinsonic symptoms.

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