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Protective effect of tangeritin in transgenic *Drosophila* model of Parkinson's disease

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1. ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder caused due to the loss of dopaminergic neurons in substantia nigra region of midbrain. The disease is characterized by the accumulation of alpha-synuclein into depositions known as lewy bodies. Till date there is no cure for PD but the limited number of medications may provide temporary relief from the PD symptoms. Flavonoids are a group of polyphenols found in plants. The health benefits of flavonoids have been universally accepted. Tangeritin is a pentamethoxy flavone found in the peels of Mandarin oranges (*Citrus reticulata*). The present study was conducted to study the effect of tangeritin on the symptoms of PD exhibited by the PD model transgenic flies (*Drosophila melanogaster)*. Tangeritin at a final concentration of 5, 10 and 20 microM was added to the diet and the flies were allowed to feed on it for 24 days. At the same time other set of PD flies were allowed to feed on a diet having 10^{-3} M of L-Dopa. The effect of tangeritin was studied on the activity pattern, climbing ability, dopamine content, oxidative stress markers (lipid peroxidation, reduced glutathione, glutathione-S-transferase, protein carbonyl content and monoamine oxidase activity) and on the histopathology of the brain of PD model flies. The study showed that the exposure of PD flies to different doses of tangeritin showed a marked delay in the loss of climbing ability and increase in the dopamine content. Tangeritin also showed a reduction in various oxidative stress markers. Hence it is concluded that tangeritin showed a marked reduction in the PD symptoms and thus could be of great importance for further research in treating PD.

2. INTRODUCTION

Parkinson's disease is the second most common neurodegenerative disease after Alzheimer's disease (1). PD is characterised by resting tremor, bradykinesia, rigidity and postural instability and pathologically by the loss of dopaminergic neurons in the substantia nigra region of brain (2). The disease is also marked by the presence of ubiquinated protein deposits in the cytoplasm of neurons known as lewy bodies (3). It is estimated that around 6.3. million individuals are suffering from PD worldwide and the number is increasing per day (4). PD affects around 0.3.% of entire population and upto 1% among 60 years and above (1). Till date PD cannot be cured but the available medicines can control the signs and symptoms for as long as possible. Owing to the limited availability of clinical treatments, alternative and preventive therapeutics are the need of the hour which can control the occurrence and progression of PD. Flavonoids are a broad polyphenol family found in plants. They protect the plant against reactive oxygen species and also play a role in floral pigmentation (5). The health benefits of flavonoids

are universally accepted. They not only possess high antioxidant properties but also exhibit a variety of other protective effects. Recent researches have shown that dietary flavonoids could be used to target the pathological manifestations of neurological disorders owing to their ability to cross the blood brain barrier (6). Tangeritin (5,6,7,8,4'-pentamethoxy flavone) is a polymethoxylated flavone found in the peels of Mandarin oranges (*Citrus reticulata*). Tangeritin shows a range of biological activities which includes enhancing gap junction intercellular communication (7), neuroprotection (8), antimetastatic (9), apoptotic (10) and anticancerous properties (11-13). The development of different models has rapidly enhanced the understanding of the pathology of PD. These models prove quite beneficial in the elaborate understanding of the mechanism of neurogeneration and also in making possible improved methods of neuroprotection (14). The present study aims to highlight the effect of tangeritin through the dietary supplementation in the transgenic *Drosophila* model of PD.

3. MATERIAL AND METHODS

3.1. Fly strain

Transgenic fly lines that expresses wild-type human synuclein (h-alpha S) under UAS control in neurons "w(asterisk); P{w(+mC) = UAS-Hsap/SNCA.F}"5B and GAL4 "w(asterisk); P{w(+mC) = GAL4-elavL}"3 were obtained from Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The males of UAS- (Upstream Activation Sequence-) Hsap/SNCA.F strains are crossed with the virgin females of GAL4-elav.L (vice-versa). The progeny will express the human alpha S in the neurons (15).

3.2. *Drosophila* culture and crosses

The flies were cultured on standard *Drosophila* food containing agar, corn meal, sugar, and yeast at 25°C (24 ± 1) (16). Crosses were set up as described in our earlier published work (17). The PD flies were exposed to 5, 10 and 20 µM of tangeritin (Sigma, USA) mixed in diet at final concentration. The PD flies were also exposed to 10^{-3} M of L-dopamine. The UAS-Hsap/SNC.F acted as a control. The control flies were also separately exposed to different doses of tangeritin.

3.3. *Drosophila* climbing assay

The climbing assay was performed as described by Pendleton *et al* (2002) (18).Ten flies were placed in an empty glass vial (10.5. cm × 2.5. cm). A horizontal line was drawn 8 cm above the bottom of the vial. The flies were acclimatized at room temperature for 10 min. 10 trials for each group were performed. All behavioral studies were performed at 25°C under standard lightning conditions.

3.4. *Drosophila* activity pattern

Drosophila Activity Monitor (DAM) (TriTek, USA) was used to monitor and analyse the activity of flies (males) in all exposed groups from the 12th day onwards. The activity was recorded every hour for a total of 384 h and the data was analyzed by Actogram J software. The results were presented as chi-square periodogram (19).

3.5. Histological evaluation of the *Drosophila* brain

The fly heads were removed and kept in 10% buffered neutral formalin for 24 h. Then, the fixed heads from each control and PD flies were embedded in paraffin and processed for light microscopy by staining individual sections with heamatoxylin and eosin (20).

3.6. Lipid peroxidation assay

The assay was performed according to the method described by Ohkawa *et al* (1978) (21). The reaction mixture was made by adding of 5µL of 10mM butyl-hydroxytoluene (BHT), 200 µL of 0.6.7% thiobarbituric acid, 600 µL of 1% O-phosphoric acid, 105 µL of distilled water and 90 µL of supernatant. The resultant mixture was incubated at 90ºC for 45 min and the OD was measured at 535nm. The results were expressed as nmol of TBARS formed/h/g tissue.

3.7. Estimation of protein carbonyl content

The protein carbonyl content was estimated according to the protocol described by Hawkins *et al* (22). The larvae homogenate was diluted to a protein concentration of approx 1mg/ml. About 250 µL of diluted homogenate was taken in eppendorf centrifuge tubes separately. To it 250 µL of 10mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5.M HCl) was added, vortexed and kept in dark for 20 min. About 125 µL of 50% (w/v) trichloroacetic acid (TCA) was added, mixed thoroughly and incubated at -20°C for 15min. The tubes were then centrifuged at 4°C for 10 min at 9000 rpm. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol: ethyl acetate (1:1). Finally the pellets were re-dissolved in 1mL of 6M guanidine hydrochloride and the absorbance was read at 370nm.

3.8. Estimation of glutathione-S-transferase (GST) activity

The method given by Habig *et al* (23) was used to determine GST activity. The reaction mixture contained 500µL of 0.1. M phosphate buffer, 150 µL of 10mM CDNB, 200µL of 10mM reduced glutathione and 50µL of supernatant. The OD was taken at 340 nm and the enzyme activity was expressed as µM of CDNB conjugates/min/mg protein.

3.9. Estimation of glutathione (GSH) content

The method given by Jollow *et al* (24) was used to estimate the glutathione (GSH) content colorimetrically using Ellman's reagent (DTNB). The supernatant was precipitated with 4% sulphosalicyclic acid (4%) in the ratio of 1:1. The samples were kept at 4ºC for 1 h and then subjected to centrifugation at 5000 rpm for 10 min

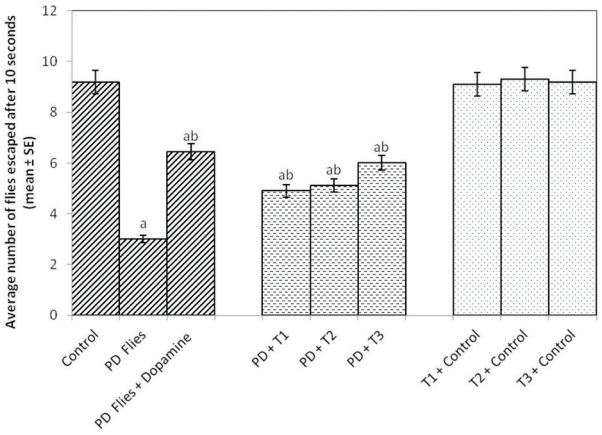

Figure 1. Effect of tangeritin on the climbing ability. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for climbing ability. The values are the mean of 5 assays. (a significant with respect to control, p<0.05; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5μM of Tangeritin; T2=10μM of Tangeritin; T3=20μM of Tangeritin; Dopamine: 10-3M).

at 4ºC. The assay mixture consisted of 550 μL of 0.1.M phosphate buffer, 100μL of supernatant and 100μL of DTNB. The OD was read at 412nm and the results were expressed as μM of GSH/gram tissue.

3.10. Estimation of monoamine oxidase (MAO)

The method described by Pine *et al* (25) was used to estimate the monoamine oxidase activity. The assay mixture consisted of 400 μL of 0.1.M phosphate buffer (pH 7.4.), 1300μL of distilled water, 100μL of benzylamine hydrochloride and 200μL of brain homogenate. The assay mixture was incubated for 30 min at room temperature and then 1mL of 10% perchloric acid was added and centrifuged at 1500g for 10 min. The OD was taken at 280nm.

3.11. Estimation of dopamine content

The method given by Schlumpf *et al.* (26) was used to estimate the dopamine content. A total of 20 heads of PD flies were homogenized in 500μL of HCl-butanol (0.8.5 ml of 37% HCl in 1L n-butanol). The suspension was centrifuged at 3000 rpm for 5 min. To the supernatant 250 μL of heptane and 100μL of 0.1.M HCl was added and centrifuged at 3000rpm for 5 min. The lower aqueous layer was used for the assay. The assay mixture consisted of 100 μL of supernatant, 50 μL of 0.4.M HCl, 100 μL of iodine solution. After incubation of 2 min 100 μL of sodium sulphite and 100 μL of 10M acetic acid was added and boiled at 100ºC for 6 min. The samples were read at 375nm after cooling at room temperature.

4. RESULTS

The results of the present study reveal the potential of tangeritin in reducing the PD symptoms. The first assay to be performed was the climbing assay. The climbing response of the control flies did not show any change for 24 days of exposure but the response of PD flies decreased significantly from 12th day. Hence, the duration of exposure was selected for 24 days and the climbing assay was performed after 24 days of exposure to various doses of tangeritin. A significant 3.06 folds loss in the climbing ability was observed in PD flies as compared to control (p<0.0.5; Figure 1). The exposure of 5, 10 and 20μM of tangeritin resulted in 1.63, 1.70 and 1.99 folds delay in the loss of climbing ability respectively (p<0.0.5; Figure 1).

The exposure of control flies only to tangeritin showed no significant change in the climbing activity. The activity pattern of flies was studied using the data collected by the *Drosophila* Activity Monitor (DAM). The data was analyzed by chi-square periodogram. The data obtained showed a greater number of significant peaks for control (Figures 2 a and b) as compared to PD flies (Figures 2 c and d). The exposure of 5, 10 and 20 μM of tangeritin showed a dose dependent delay in loss of activity (Figures 2 g-l). The individual exposure of tangeritin to control flies showed no change in the activity pattern of flies (Figures 2 m-r). The exposure of PD flies to 10^{-3} M dopamine also showed a delay in the loss of climbing activity (Figures 2 e and f).

The histological evaluation of the brain section of both control and PD flies exposed to various doses of tangeritin showed no major changes in the gross morphology (Figure 3 a and b). The results obtained for lipid peroxidation are shown in Figure 4. The brain homogenate of PD flies showed 2.41 folds increase in lipid peroxidation as compared to control (p<0.0.5; Figure 4). The exposure to 5, 10 and 20μM of tangeritin showed 1.38, 1.61 and 1.93 folds decrease in the lipid peroxidation respectively (p<0.0.5; Figure 4).

The result obtained for PC content is shown in Figure 5. The protein carbonyl content showed a significant 3.20 folds increase in PD flies as compared to control (p<0.0.5; Figure 5). The exposure of 5,10 and 20 μM of tangeritin results into 1.28, 1.45 and 1.77 folds decrease in the PC content respectively as compared to PD flies (p<0.0.5; Figure 5).

The results of GST are shown in Figure 6. The level of GST was found to increase by 2.03 folds in PD flies as compared to control (p<0.0.5; Figure 6). A significant 1.11, 1.36 and 1.51 folds decrease in GST activity was observed respectively on exposure of 5, 10 and 20 μM of tangeritin (p<0.0.5; Figure 6).

The result of GSH is shown in Figure 7. The levels of GSH was observed to be 1.65 folds less in PD flies as compared to control (p<0.0.5; Figure 7). A significant 1.16, 1.28 and 1.43 folds increase in GSH

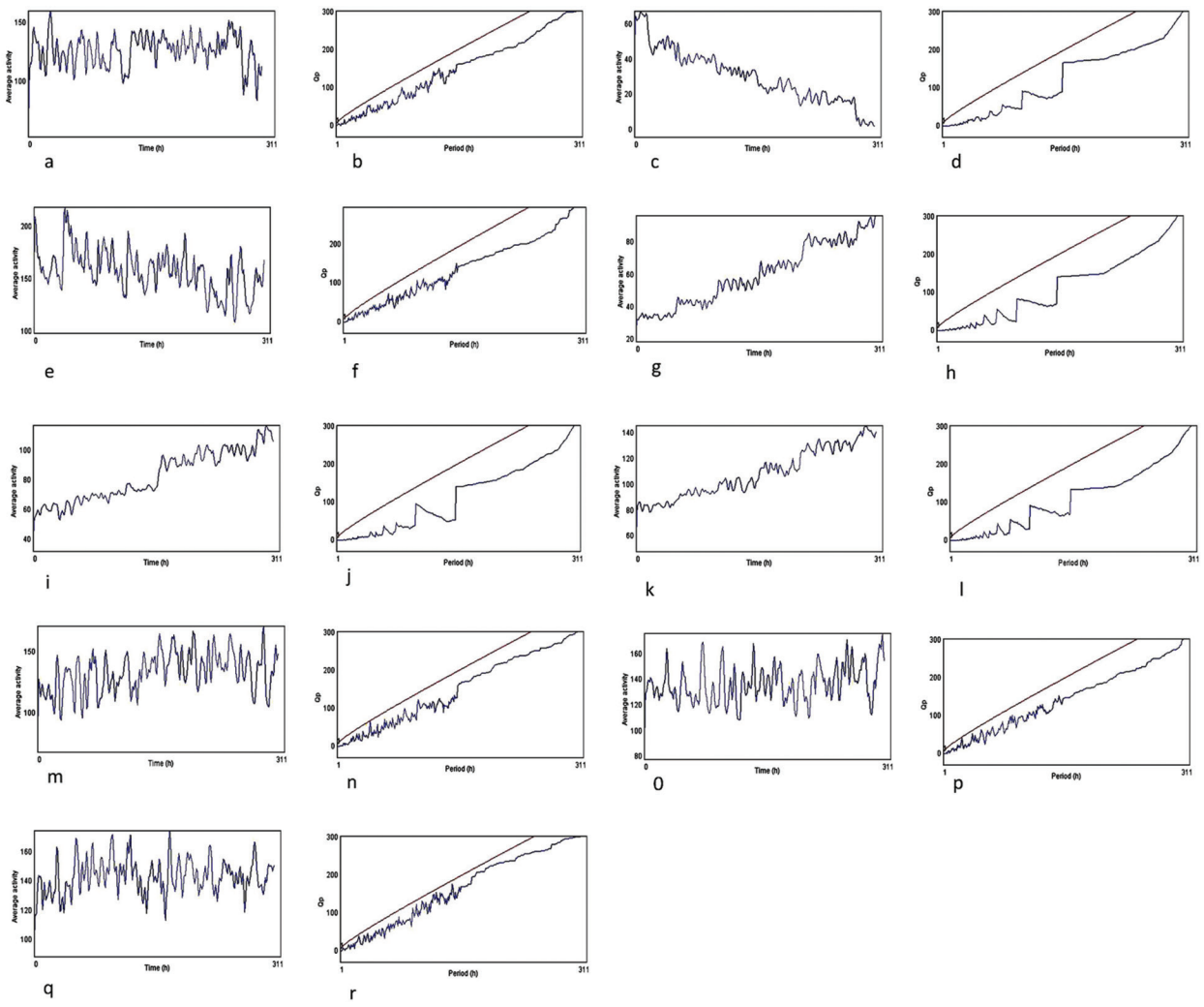

Figure 2. (a) and (b) show the average activity pattern and chi-square periodogram, respectively, for the control flies (Number of flies = 20). (c) and (d) show the average activity pattern and chi-square periodogram, respectively, for the PD flies (Number of flies = 20). (e) and (f) show the average activity pattern and chi-square periodogram, respectively, for the PD flies exposed to 10-3 molar dopamine in diet (Number of flies = 20). (g) and (h) show the average activity pattern and chi-square periodogram, respectively, for the PD flies exposed to 5 micro molar of tangeritin in diet (Number of flies = 20). (i) and (j) show the average activity pattern and chi-square periodogram, respectively, for the PD flies exposed to 10 micro molar of tangeritin in diet (Number of flies = 20). (k) and (l) show the average activity pattern and chi-square periodogram, respectively, for the PD flies exposed to 20 micro molar of tangeritin in diet (Number of flies = 20). (m) and (n) show the average activity pattern and chi-square periodogram, respectively, for the control flies exposed to 5 micro molar of tangeritin in diet (Number of flies = 20). (o) and (p) show the average activity pattern and chi-square periodogram, respectively, for the control flies exposed to 10 micro molar of tangeritin in diet (Number of flies = 20). (q) and (r) show the average activity pattern and chi-square periodogram, respectively, for the control flies exposed to 20 micro molar of tangeritin in diet (Number of flies = 20).

levels was seen upon exposure of tangeritin ($p<0.0.5$; Figure 7). The result of monoamine oxidase activity is given in Figure 8. The activity of monoamine oxidase was found to be increased by 1.57 folds in PD flies as compared to control ($p<0.0.5$; Figure 8). The exposure of flies to the various doses of tangeritin showed 1.13, 1.31 and 1.42 folds decrease in the activity of monoamine oxidase respectively ($p<0.0.5$; Figure 8).

The result of dopamine content in the brain homogenate of flies is shown in Figure 9. A significant 2.11 folds decrease in dopamine content was seen in PD flies as compared to control ($p<0.0.5$; Figure 9). Upon exposure to 5, 10 and 20μM of tangeritin the level of dopamine was found to increase significantly by 1.33,1.55 and 1.70 folds respectively ($p<0.0.5$; Figure 9).

5. DISCUSSION

The results of the present study suggest that the exposure of tangeritin is potent in reducing the PD symptoms in the transgenic *Drosophila* model of PD. PD is caused due to the selective loss of neurons in the substantia nigra pars compacta of the mid brain. These

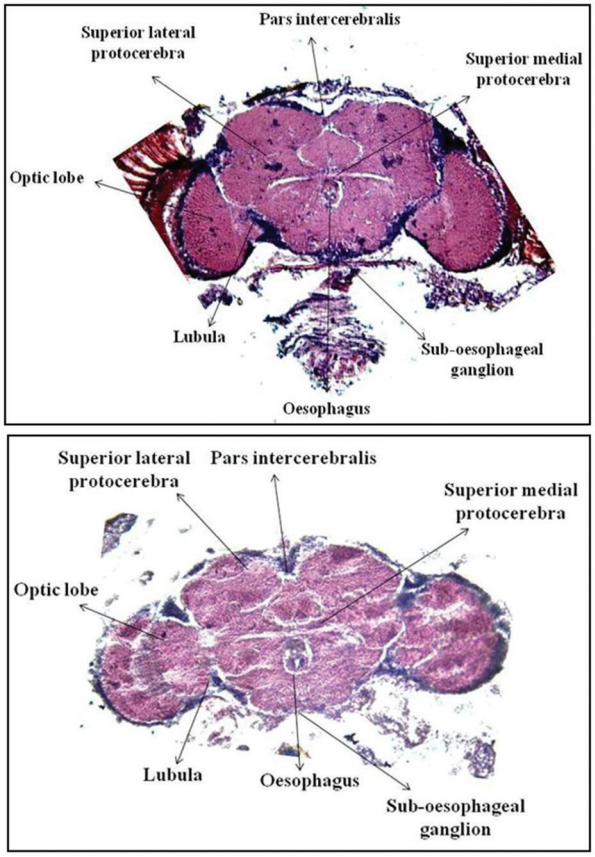

Figure 3. Microscopic illustrations of the brain of *Drosophila* melanogaster stained by heamatoxylin and eosin (a- control fly; b- PD fly).

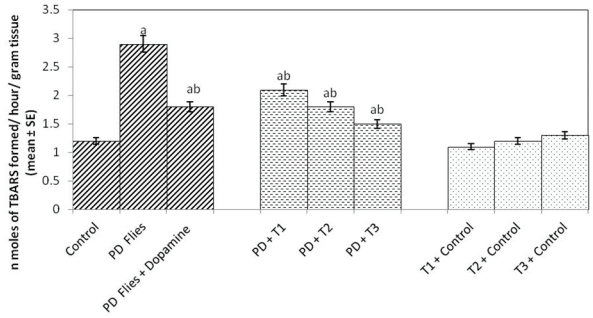

Figure 4. Effect of tangeritin on the lipid peroxidation in the brains of flies. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for lipid peroxidation. The values are the mean of 5 assays. (a significant with respect to control, $p<0.05$; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5 µM of Tangeritin; T2=10 µM of Tangeritin; T3=20 µM of Tangeritin; Dopamine: 10^{-3}M).

neurons contain dopamine and their projecting nerve fibres reach the striatum (27). Since the voluntary movements are controlled by the neurons, the degeneration in these neurons leads to the common symptoms observed in the PD patients (28). Alpha-synuclein (α-synuclein) is a

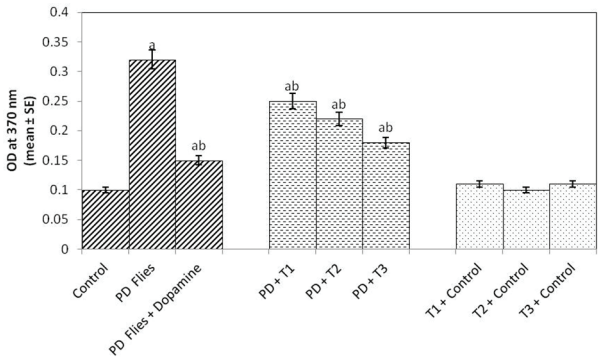

Figure 5. Effect of tangeritin on protein carbonyl content measured in the brains of flies. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for protein carbonyl content. The values are the mean of 5 assays. (a significant with respect to control, $p<0.05$; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5 µM of Tangeritin; T2=10 µM of Tangeritin; T3=20 µM of Tangeritin; Dopamine:10^{-3}M).

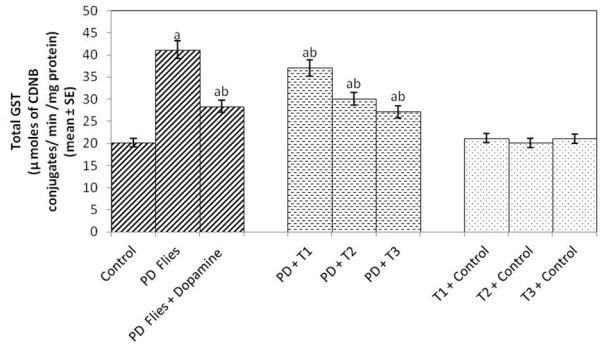

Figure 6. Effect of tangeritin on the GST content in the brains of flies. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for GST content. The values are the mean of 5 assays. (a significant with respect to control, $p<0.05$; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5 µM of Tangeritin; T2=10 µM of Tangeritin; T3=20 µM of Tangeritin; Dopamine: 10^{-3} M).

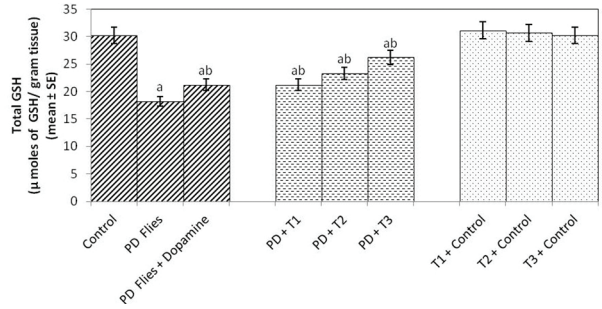

Figure 7. Effect of tangeritin on the GSH content in the brains of flies. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for GSH content. The values are the mean of 5 assays. (a significant with respect to control, $p<0.05$; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5µM of Tangeritin; T2=10 µM of Tangeritin; T3=20µM of Tangeritin; Dopamine: 10^{-3}M).

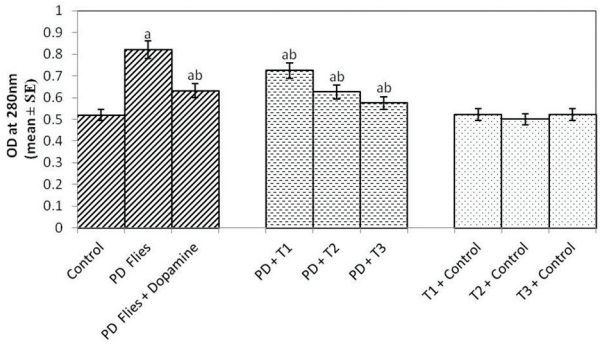

Figure 8. Effect of tangeritin on monoamine oxidase (MAO) activity measured in the brains of flies. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for MAO activity. The values are the mean of 5 assays. (a significant with respect to control, $p<0.05$; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5 µM of Tangeritin; T2=10 µM of Tangeritin; T3=20 µM of Tangeritin; Dopamine: 10^{-3}M).

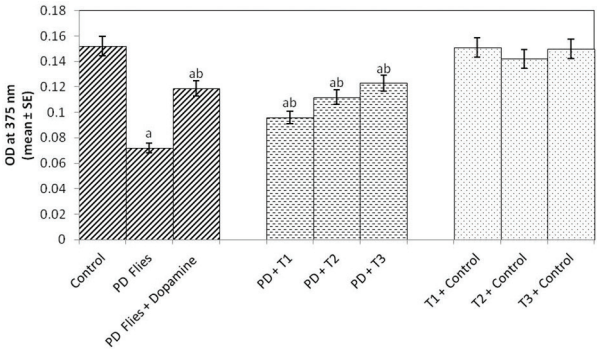

Figure 9. Effect of tangeritin on dopamine content measured in the brains of flies. The flies were allowed to feed on the diet supplemented with tangeritin for 24 days and then assayed for dopamine. The values are the mean of 5 assays. (a significant with respect to control, $p<0.05$; b significant with respect to PD model flies; PD=Parkinson's disease; T1=5 µM of Tangeritin; T2=10 µM of Tangeritin; T3=20 µM of Tangeritin; Dopamine: 10^{-3}M).

small soluble protein which is expressed primarily at the presynaptic terminals and its dysfunction seems to be a common feature in all forms of PD (29). Studies have shown that the accumulation of α-synuclein leads to toxicity and oxidative stress (30,31). Oxidative stress is thought to be a major cause of cellular dysfunction and death in both idiopathic and genetic cases of PD (27,32).

Oxidative stress is a condition which arises due to an imbalance between the cellular antioxidant activity and the formation of reactive oxygen species (ROS). The nigral dopaminergic neurons also contain iron. This iron catalyzes the Fenton reaction resulting in the production of superoxide radical and hydrogen peroxide and thus adds on to the oxidative stress (33). Tangeritin is a pentamethoxy flavone found in citrus peels (34). The ability of tangeritin in crossing the blood brain barrier and protecting the striato-nigral integrity and functionality has already been shown in rat model of PD (8). The climbing assay showed a time dependent loss in the climbing ability of the PD flies. As stated by Feany and Bender (2000) (15), the progressive degeneration and loss of dopaminergic neurons leads to reduced climbing ability with time. The exposure of PD flies to various flavonoids have shown to delay the loss of climbing activity (35,36). The exposure of tangeritin also showed a delay in the loss of climbing ability confirming its protective effect on the neurons. Similar result was observed in activity pattern and periodogram of flies exposed to various doses of tangeritin.

Dopamine is a neurotransmitter used for passing message between the striatum and substantia nigra. With the progression of disease the level of dopamine decreases significantly due to the loss of dopaminergic neurons (37). The exposure of tangeritin showed a marked increase in the dopamine content in the brain of PD flies suggesting the protective nature of tangeritin on the neurons. The effect of tangeritin on various oxidative stress markers also showed promising results. Lipid peroxidation is a measure of oxidative stress resulting from the production of reactive oxygen species (ROS) that leads to the damage of cell membranes, proteins and DNA (38). Malondialdehyde (MDA) is a major reactive aldehyde formed due to the peroxidation of biological membranes (39). Earlier studies have shown an increased level of lipid peroxidation is observed in the PD patients (40). The elevated levels of TBARS in PD flies compared to control flies indicates the increased lipid peroxidation in the brain of PD flies which may be due to the increased amounts of free radicals formed due to aggregation of alpha-synuclein. Protein carbonyl content is an indicator of protein oxidation (41).

A significant increase in the levels of protein carbonyl content was observed which is in agreement with a previous study done on PD patients (42). The other oxidative stress markers like GST and GSH also showed the protective effect of tangeritin. GST is a family of enzymes involved in detoxification. The increased level of GST found in PD flies may be due to the production of reactive oxygen species. This result is in accordance to the previous studies conducted by our lab (43). The reduced level of GSH is the earliest known biochemical indicator of neurodegeneration. The decrease in the GSH levels in substantia nigra is a characteristic of PD flies (44-46). Monoamine oxidase (MAO) catalyzes the oxidation of monoamines (47,48). MAO breaks dopamine leading to its deficiency in PD (49). The results in our present study show increased activity of monoamine oxidase in PD flies as compared to control. The effect of tangeritin in neutralizing the different oxidative stress markers may be due to its high antioxidant properties (50). Tangeritin has also been proven to interfere with the NO synthase activity (51).

Nitric oxide reacts with free radicals and generates peroxynitrites that directly oxidise ROS (52). Moreover, the exposure of tangeritin elevates the levels of GSH which also acts as an antioxidant in the detoxification of products from ROS promoted lipid peroxidation (53). The use of *Drosophila* for modelling human brain diseases offers many advantages in the investigation of cellular and molecular mechanisms of the disease. The availability of time and tissue specific promoters, short life span, large number of offsprings, many genetic techniques and a wide range of mutants makes *Drosophila* an ideal model organism. In the present study the *Drosophila* model of PD expressing human wild type α synuclein is used (15). The present model mimics the motor impairments associated with PD and is of great value in testing compounds for neuroprotective properties. Thus, the present study concludes that the exposure to various doses of tangeritin delays the symptoms of PD and this could play a pivotal role in designing an alternative and better treatment of PD in near future.

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