

Protective effect of luteolin on the transgenic *Drosophila* model of Parkinson's disease

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In the present study we have studied the effect of 25, 50, 75 and 100 μM of luteolin on the transgenic *Drosophila* expressing human alpha synuclein. The doses of luteolin were established in diet and the PD flies were allowed to feed on it for 24 days. After 24 days of exposure the flies were assayed for climbing assay, oxidative stress markers, caspase-3 & 9 activity and dopamine content. The immunohistochemistry was also performed on the brain sections for the activity of tyrosine hydroxylase. The exposure of luteolin showed a dose dependent delay in the loss of climbing ability and activity, reduction in oxidative stress markers, caspase-3&9 activities and results in an increase in the dopamine content. The results obtained for the immunohistochemistry also supports the protective role of luteolin against the damage of the dopaminergic neurons.

Keywords: Luteolin. Parkinson's disease. *Drosophila*. Oxidative stress.

INTRODUCTION

Parkinson's disease (PD) is a common progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons due to the over expression of alpha synuclein and formation of the Lewy bodies (Shaltiel-Karyo *et al.*, 2012). The loss of the dopaminergic neurons has been implicated in causing severe motor symptoms (Jenner, Olanow, 2006). Lewy bodies are spherical protein inclusions found in the cytoplasm of surviving neurons and consisted of a dense core surrounded by a halo of radiating fibrils of α -synuclein (Meng *et al.*, 2010). The formation of Lewy bodies has been linked with the production of reactive oxygen species (ROS) leading to a state of oxidative stress producing superoxide radical anions, hydrogen peroxide and hydroxyl radicals (Thomas, Beal, 2007; Cookson, 2005; Auluck *et al.*, 2002). There are enough evidences showing an increase of oxidative stress during the progression of neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Marsh, Thompson, 2006; Filomeni *et al.*, 2012; Behl, Holsboer, 1998).

Oxidative stress has also been implicated in neuronal loss associated with cognitive decline. The potential role of ROS and reactive nitrogen species (RNS) in the pathophysiology of neurodegenerative disorder is relevant as nitric oxide (NO^{\cdot}) and superoxide ($\text{O}_2^{\cdot-}$) may be generated in the brain and their formation is often co-localised in specific neurons (Youdim *et al.*, 2002). Recently, there has been a considerable attention for establishing a therapeutic and dietary strategy to combat oxidative stress during the progression of neurodegenerative diseases. In this context a number of dietary antioxidants especially flavonoids have been screened for their biological actions (Singh, Rajini, 2004; Seidl *et al.*, 2014). As there is no preventive therapy for the neurodegenerative diseases till date, hence any agent that can slow /or prevent aggregation and fibrillation of α -synuclein or reduced the oxidative stress could act as a possible therapeutic agent. Luteolin is a polyphenolic compound found in celery, green pepper, perilla leaf and camomile tea (Chen *et al.*, 2008). It has been reported to possess anti-mutagenic, anti-tumorigenic, antioxidant and anti-inflammatory properties but the investigations exploring its nature on the neurodegenerative disorders are warranted. The present study deals with the effect of luteolin on the PD model flies expressing human alpha synuclein in the brain.

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MATERIAL AND METHODS

Drosophila stocks

Transgenic fly lines that express wild-type human synuclein (h- α S) under UAS control in neurons “[w[*];P{w[+mC]=UAS-Hsap/SNCA.F}”5B and GAL4 “[w[*];P{w[+mC]=GAL4-elavL}”3] were obtained from Bloomington Drosophila Stock Centre (Indiana University, Bloomington, IN). When the males of UAS (Upstream Activation Sequence)-Hsap/SNCA.F strains are crossed with the females of GAL4-elav. L (vice-versa), the progeny will express human α S in the neurons (Feany, Bender, 2000).

Drosophila culture and crosses

The flies were cultured on standard *Drosophila* food containing agar, corn meal, sugar and yeast at 25 °C (24 ± 1) (Siddique *et al.*, 2014). Crosses were set up as described in our earlier published work (Siddique *et al.*, 2012). The PD flies were allowed to feed separately on different doses of luteolin (Sigma, USA) mixed in the diet. Luteolin was added in the diet at final concentration of 25, 50, 75 and 100 μ M. The PD flies were also exposed to 10⁻³ M of L-dopamine. The UASHsap/ SNC.F act as a control. The control flies were allowed separately to feed on the selected doses of luteolin. Fly heads from each group were isolated (50 heads/group; five replicates/group) and the homogenate was prepared in 0.1 M phosphate buffer for the biochemical parameters.

Antioxidative assays

Two assays were used for estimating the antioxidative potential of luteolin for the doses selected in our study

Superoxide anion scavenging assay

The inhibition of Nitroblue tetrazolium (NBT) reduction by phenazine methosulphate (PMS) generated O₂^{•-} was used to determine the superoxide anion scavenging activity of the luteolin (Sghaier *et al.*, 2011) The reaction mixture consist of 75 μ L of each concentration of luteolin, 750 μ L of Tris HCl (100 mM; pH 7.4); 187 μ L of NBT (300 μ M), 187 μ L of NADH (936 μ M). The reaction was initiated by adding phenazine methosulphate (PMS) (120 μ M). The reaction mixture was incubated at 25 °C for 5 min and the OD was read at 560 nm and the degree of scavenging was calculated by the following equation:

$$\text{Scavenging (\%)} = \left[\frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right] \times 100$$

Diphenyl-picrylhydrazyl (DPPH) free radical scavenging

For estimating free radical scavenging potential of the luteolin DPPH method as described by Wongsawatkul *et al.* (2008) was used in the present study. When DPPH (a stable purple color) react with an antioxidant, it is reduced to yield a light yellow coloured diphenyl picrylhydrazine. Color change was spectrophotometrically measured. The reaction mixture consisting of 500 μ L of luteolin and 250 μ L of DPPH (0.3 mM). The reaction mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 min. The OD was read at 518 nm and the radical scavenging activity was calculated by the following equation:

$$\% \text{ Radical scavenging} = \frac{(1 - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Drosophila climbing assay

The climbing assay was performed as described by Pendleton *et al.* (2002). 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. After the flies had acclimated for 10 min at room temperature, both controls and treated groups were assayed at random to a total of 10 trails for each. The mean values were calculated and then averaged, and a group mean and standard error were obtained. All behavioral studies were performed at 25 °C under standard lighting conditions.

Drosophila activity pattern

From the 12th day the activity of flies (males) in all treated groups were analyzed by using Drosophila Activity Monitor (TriTek, USA). The activity was recorded every hour for a total of 287 h and the data was analyzed by Actogram J software. The results were presented as chi-square periodogram (Rasato, Kyriacou, 2006).

Estimation of glutathione (GSH) content

The GSH content was estimated colorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow *et al.* (1974). The supernatant was precipitated with 4% sulphosalicyclic acid in the ratio

of 1:1. The samples were kept at 4 °C for 1 hr and then subjected to centrifugation at 4200g for 10 min at 4 °C. The assay mixture consisted of 550 µL of 0.1 M phosphate buffer, 100 µL of supernatant and 100 µL of DTNB. The OD was read at 412 nm and the results were expressed as µ moles of GSH/gram tissue.

Estimation of glutathione-S-transferase (GST) activity

The GST activity was determined by the method of Habig *et al.* (1974). The reaction mixture consisted of 500 µL of 0.1 M phosphate buffer, 150 µL of 10 mM CDNB, 200 µL of 10 mM reduced glutathione and 50 µL of supernatant. The OD was taken at 340 nm and the enzyme activity was expressed as µ moles of CDNB conjugates/min/mg protein.

Lipid peroxidation assay (LPO)

LPO was measured according to the method described by Ohkawa, Nobuko, Yagi (1978). The reaction mixture consisted of 5 µL of 10 mM butyl-hydroxytoluene (BHT), 200 µL of 0.67% 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 90 °C for 45 min and the OD was measured at 535 nm. The results were expressed as µmol of TBARS formed/h/g tissue.

Estimation of Protein Carbonyl content (PCC)

The PCC was estimated according to the protocol described by Hawkins, Morgan, Davies (2009). The brain homogenate was diluted to a protein concentration of approx 1 mg/mL. About 250 µL of each diluted homogenate was taken in eppendorf centrifuge tubes separately. To it 250 µL of 10 mM 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 15 min. The tubes were then centrifuged at 4 °C for 10 min at 8200 g. 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 6 M guanidine hydrochloride and the absorbance was read at 370 nm.

Assay for caspase-3 (Drice) and caspase-9 (Dronc) activities

For estimating the activities of caspase 3 and 9

the assay was performed according to the manufacturer protocol with some modification (Bio-Vision, CA, USA). The assay was based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after specific action of caspase-3 and caspase-9 on tetrapeptide substrates, DEVD-pNA and IETD-pNA, respectively. The assay mixture consisted of 50 µL of fly head homogenate from each group and 50 µL of chilled cell lysis buffer incubated on ice for 10 min. After incubation, 50 µL of two times reaction buffer (containing 10mM DTT) with 200 µM substrate (DEVD-pNA for Drice, and IETD-pNA for Dronc) was added and incubated at 37 °C for 1.5 h. The reaction was quantified at 405nm.

Dopamine content

Dopamine content was measured as per method described by Schlumpf *et al.* (1974). Fifty heads of flies from each group were taken in 500 µL of HCl-butanol (0.85 mL of 37% HCl in 1 litre n-butanol). After homogenization the samples were centrifuged at 2200 g for 5 min. After collecting the supernatant, 250 µL of heptane and 100 µL of 0.1 M HCl were added. The samples were vortexed and centrifuged at 2200g for 5 min. The upper organic phase was discarded and the lower aqueous phase was kept for dopamine assay. To 100 µL of aqueous phase, 50 µL 0.4 M HCl, 100 µL of sodium acetate buffer (pH 6.9), 100 µL of iodine solution was added and kept for two min. The reaction was stopped by adding of 100 µL of sodium sulphite solution. After two min, 100 µL of acetic acid (10 M) was added and then the mixture was heated at 100 °C for 6 min. The OD was taken at 375 nm after cooling the samples at room temperature.

Immunohistochemistry

The fly heads were isolated and paraffin sections were prepared according to the procedure described by Palladino *et al.* (2000). The sections were deparaffinized and rehydrated. The slides were blocked in 8% Bovine Serum Albumin (BSA) for 2.5hr. Then the slides were washed with phosphate buffer saline (pH 7.2) containing 2% BSA for 5 minutes. After washing the slides were incubated with primary antibody (anti-tyrosine hydroxylase, Merck) in a humidified chamber for 12 hr at 4 °C. The slides were then washed with PBS containing 2% BSA for 5 min and incubated with secondary antibody (Goat anti-Rabbit alkaline phosphatase, Santacruz, Biotechnology, USA) at room temperature for 2 h. The final wash was given by PBS containing 2% BSA for 5 min. BCIP-NBT was used as a chromogenic substrate

which interacts with secondary antibody to produce blue coloured product. The slides were then mounted in DPX and observed under the microscope.

Statistical analysis

The statistical analysis was done by performing one-way ANOVA post hoc test using SPSS Ver. 16.

RESULTS

The result obtained for superoxide anion scavenging activity is shown in Figure 1S. A clear dose dependent significant increase in the superoxide anion scavenging activity of luteolin was observed at 25, 50, 75 and 100 μM compared to control. The results obtained for free radical scavenging also showed similar results. A clear dose dependent significant increase in the free radical scavenging activity of luteolin was observed at 25, 50, 75 and 100 μM compared to control (Figure 2S). The results obtained for the average activity pattern are shown in Figure (3S-13S). The activity of flies was recorded for 287hrs and then with the help of software programme was present in the form of average activity pattern and chi-square periodogram. The PD flies showed a decrease in the average activity (Figure 4S) compared to control flies (Figure 3S) which is evident by the chi-square periodogram. The numbers of peaks are more near the midline. The exposure of PD flies to 25, 50, 75 and 100 μM of luteolin showed a dose dependent delay in the loss of activity as is evident from chi-square periodogram (Figure 5S-8S). The control flies showed no change in the activity when exposed to 25, 50, 75 and 100 μM of luteolin (Figure 9S-12S). The PD flies exposed to 10^{-3} M of dopamine also showed a delay in the loss of activity compared to unexposed PD flies (Figure 13S).

The results obtained for the climbing ability of PD flies showed a significant 4.5 fold of decrease compared to control flies (Figure 1; $p < 0.05$). The exposure of PD flies to 25, 50, 75 and 100 μM of luteolin showed a dose dependent significant delay in the loss of climbing ability of 1.52, 1.85, 2.19 and 2.67 folds compared to the PD flies (Figure 1; $p < 0.05$). The PD flies exposed to 10^{-3} M of dopamine showed a 3.04 fold significant delay in the loss of climbing ability compared to the PD flies (Figure 1; $p < 0.05$). The GSH content of PD flies showed a significant decrease of 1.98 fold compared to the control flies (Figure 2; $p < 0.05$). The PD flies exposed to 25, 50, 75 and 100 μM of luteolin showed a dose dependent 1.10, 1.31, 1.48 and 1.64 folds of significant increase in the GSH content compared to unexposed PD flies (Figure 2; $p < 0.05$). The exposure

of PD flies to 10^{-3} M of dopamine showed a 1.32 fold of significant increase in the GSH content compared to PD flies (Figure 2; $p < 0.05$). PD flies showed a 2.53 fold of increase in the GST activity compared to control flies (Figure 3; $p < 0.05$). The PD flies exposed to 25, 50, 75 and 100 μM of luteolin showed a 1.11, 1.19, 1.25 and 1.45 folds of significant decrease in the GST activity compared to unexposed PD flies (Figure 3; $p < 0.05$). The PD flies exposed to 10^{-3} M of dopamine showed a 1.67 fold of significant decrease in the GST activity compared to unexposed PD flies (Figure 3; $p < 0.05$). The PD flies showed a 5.16 fold significant increase in LPO compared to control flies (Figure 4; $p < 0.05$). The exposure of PD flies to 25, 50, 75 and 100 μM of luteolin showed a dose dependent 1.21, 1.47, 1.77 and 2.13 folds of significant decrease respectively in the LPO (Figure 4; $p < 0.05$). The PD flies exposed to 10^{-3} M of dopamine showed a 1.93 fold of significant decrease in LPO compared to unexposed PD flies (Figure 4; $p < 0.05$). The PD flies showed 3.81 fold of significant increase in PC content compared to control flies (Figure 5; $p < 0.05$). The PD flies exposed to 25, 50, 75 and 100 μM of luteolin showed a dose dependent significant decrease of 1.16, 1.40, 1.61 and 2.1 folds respectively compared to unexposed PD flies (Figure 5; $p < 0.05$). The PD flies exposed to 10^{-3} M of dopamine showed a significant decrease of 2.0 fold in the PC content compared to unexposed PD flies (Figure 5; $p < 0.05$). The PD flies showed a 4.33 fold significant increase in the activity of caspase-9 compared to control flies (Figure 6; $p < 0.05$). The PD flies exposed to 25, 50, 75 and 100 μM of luteolin showed a dose dependent significant decrease of 1.3, 1.5, 1.95 and 2.29 folds respectively compared to unexposed PD flies (Figure 6; $p < 0.05$). The PD flies exposed to 10^{-3} M of dopamine showed a 2.43 fold of significant decrease in the caspase-9 compared to unexposed PD flies (Figure 6; $p < 0.05$). Similarly, PD flies showed a significant increase of 2.90 fold in the activity of caspase-3, compared to control flies (Figure 7; $p < 0.05$). The exposure of 25, 50, 75 and 100 μM of luteolin showed a significant decrease of 1.23, 1.39, 1.60 and 1.88 folds respectively compared to unexposed PD flies (Figure 7; $p < 0.05$). The exposure of PD flies to 10^{-3} M of dopamine showed a 1.88 fold of significant decrease compared to PD flies (Figure 7; $p < 0.05$). A significant decrease of 2.7 fold was observed in the dopamine content in PD flies compared to control flies (Figure 8; $p < 0.05$). The PD flies exposed to 25, 50, 75 and 100 μM of luteolin showed a significant increase of 1.2, 1.3, 1.5 and 1.7 folds respectively in the dopamine content (Figure 8; $p < 0.05$). The PD flies exposed to 10^{-3} M of dopamine showed a significant increase of 1.9 fold in the dopamine content compared to PD flies (Figure 8;

$p < 0.05$). The results obtained for immunohistochemistry are depicted in Figure 9(A-C). The less activity of tyrosine hydroxylase was observed in PD flies (Figure 9A) compared to control flies (Figure 9B). The exposure to various doses of luteolin showed a higher activity of tyrosine hydroxylase compared to unexposed PD flies (Figure 9C).

DISCUSSION

The results of the present study reveal that luteolin not only reduced the oxidative stress but also delayed the loss of climbing ability of the PD model flies. PD

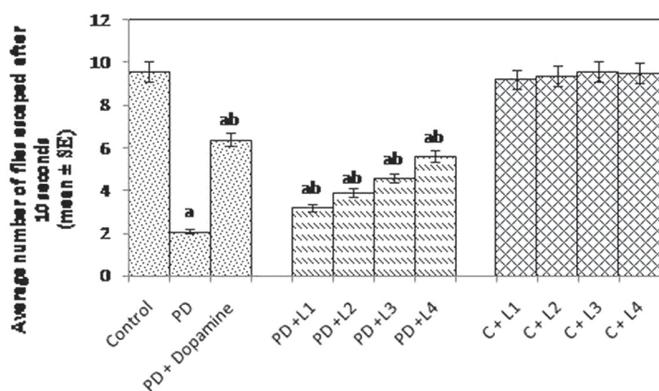


FIGURE 1 - Effect of luteolin on the climbing ability. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4=100 μ M; PD = PD flies; Dopamine=10⁻³ M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for climbing ability. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

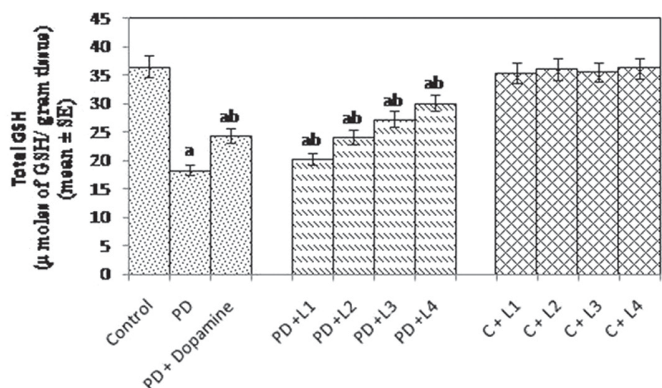


FIGURE 2 - Effect of luteolin on the glutathione (GSH) content in the brains of flies. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4= 100 μ M; PD = PD flies; Dopamine=10⁻³ M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for GSH content. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

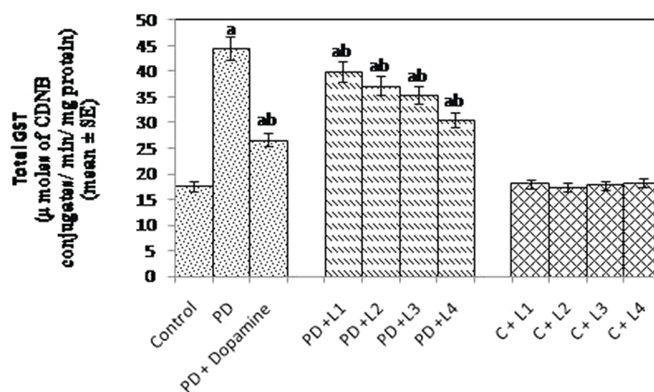


FIGURE 3 - Effect of luteolin on the glutathione-S-transferase (GST) activity in the brains of flies. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4= 100 μ M; PD = PD flies; Dopamine=10⁻³ M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for GST activity. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

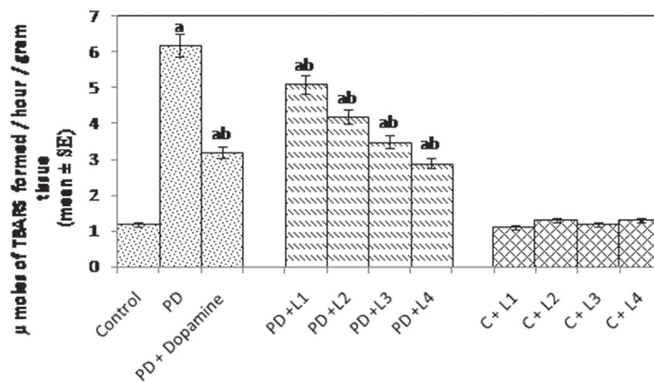


FIGURE 4 - Effect of luteolin on the lipid peroxidation in the brains of flies. [L1= 25 μ M; L2 = 50 μ M; L3 = 75 μ M; L4 = 100 μ M; PD = PD flies; Dopamine = 10⁻³ M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for lipid peroxidation. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

flies exposed to luteolin also exhibit delay in the loss of climbing ability in a dose dependent manner. The results obtained for activity pattern also suggest that the exposure of PD flies to luteolin showed a dose dependent delay in the loss of activity. There are evidences which supports that dietary derived flavonoids have the potential to improve human memory and neuro-cognitive performance via their ability to protect vulnerable neurons, enhancing neuronal function and stimulate the neuronal regeneration (Spencer, 2008). Luteolin has been reported to improved cognitive impairments in rats and mice (Liu *et al.*, 2013; Jang, Dilger, Johnson, 2010; Xu *et al.*, 2010).

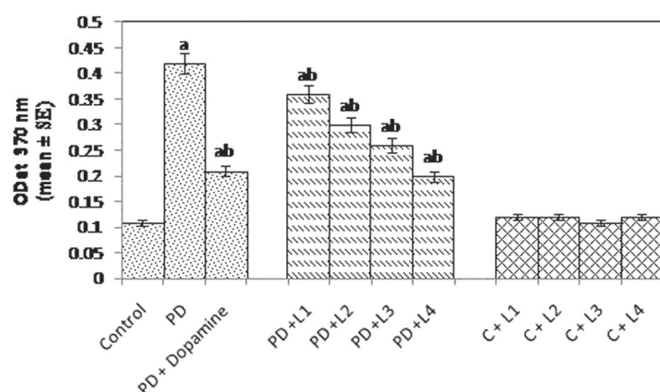


FIGURE 5 - Effect of luteolin on the protein carbonyl content measured in the brains of flies. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4= 100 μ M; PD = PD flies; Dopamine=10-3 M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for lipid peroxidation. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

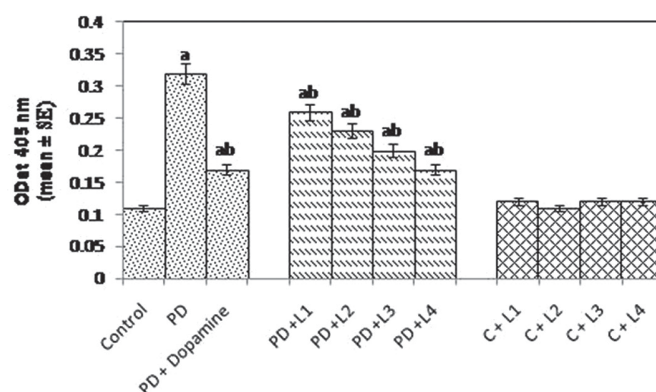


FIGURE 7 - Effect of luteolin on caspase-3 activity in the brains of flies. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4= 100 μ M; PD = PD flies; Dopamine=10-3 M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for caspase-3 activity. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

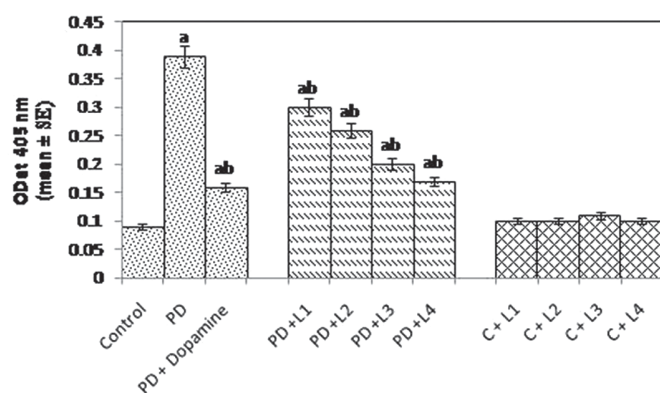


FIGURE 6 - Effect of luteolin on caspase-9 activity in the brains of flies. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4= 100 μ M; PD = PD flies; Dopamine=10-3 M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for caspase-9 activity. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

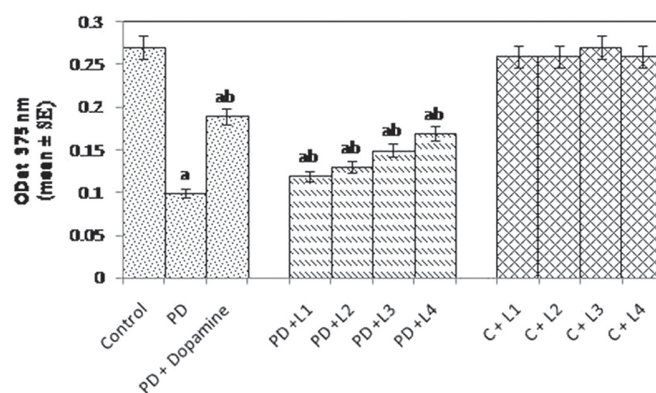


FIGURE 8 - Effect of luteolin on dopamine content measured in the brains of flies. [L1= 25 μ M; L2= 50 μ M; L3 = 75 μ M; L4= 100 μ M; PD = PD flies; Dopamine=10-3 M]. The flies were allowed to feed on the diet supplemented with luteolin for 24 days and then assayed for dopamine content. The values are the mean of 5 assays. [a - significant at $p < 0.05$ compared to control; b - significant at $p < 0.05$ compared to PD flies].

Oxidative stress is the key component of the pathology of neurodegenerative disorders (Aruoma, Bahorun, Jen, 2003). During the past several years there has been a tremendous progress in the understanding the causative factors for various neurodegenerative disorders but there have been no therapies available that benefits patient suffering from these diseases (Solanki *et al.*, 2015). It is well accepted that the accumulation of intracellular damage by ROS might orchestrate the progressive loss of control over biological homeostasis (Cencioni *et al.*, 2013).

GSH is a tripeptide present in the majority of cells, is responsible for hydrophilic xenobiotics conjugation

sulphydryl group of glutathione is essential for its antioxidant activity against the ROS in cells (Durgo *et al.*, 2007). GSH is one of the essential compounds for maintaining cell integrity against ROS and as being non enzymatic free radical scavenger, it participates in the detoxification of ROS and reduces H_2O_2 (Waseem, Parvez, 2013). A decrease in the GSH content has been reported in PD patients due to the neuronal loss. This decrease in GSH would reduce the capacity of the neurons to detoxify not only hydrogen peroxide but also lipid hydroperoxides and increase the risk of free radical formation and lipid peroxidation (Sofic *et al.*, 1992). There are evidences that some flavonoids can enhance the intracellular basal level

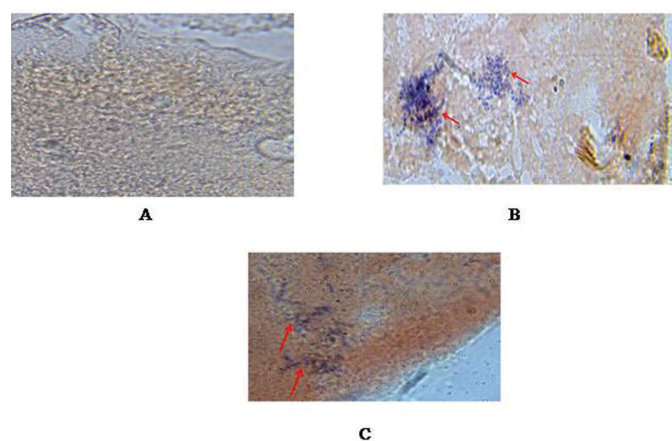


FIGURE 9 - Immunohistochemistry performed on the brain sections using anti-tyrosine hydroxylase (primary antibody) and alkaline phosphatase (secondary antibody). BCIP-NBT solution was used as a substrate (100X) [A-PD fly; B-Control; C-PD fly exposed to 100 μ M of luteolin].

of GSH, thereby promoting the tolerance against free radicals (Cipak *et al.*, 2003). The exposure of luteolin to PD flies showed a dose dependent increase in the GSH content.

Free radicals may also attack unsaturated lipids in a cell, resulting in the chain reaction leading to the breakdown products such as malondialdehyde (MDA). The measurement of MDA concentration is a common method for the determination of LPO. The exposure of luteolin to the PD flies showed a dose dependent decrease in the LPO. GST family of genes has been implicated in multiple neurodegenerative disorders (Allen *et al.*, 2012). The altered level or functions of GST has been associated with the oxidative stress in a way that contributes the progression of the disease (Allen *et al.*, 2012). In our study the PD flies were associated with an increased activity of GST. The exposure of luteolin to PD flies showed a dose dependent decrease in the activity of GST. The increase in the GST activity has also been reported to rescue the dopaminergic neurons in *Drosophila* (Whitworth *et al.*, 2005). Protein oxidation has been implicated in the pathogenesis of various human diseases (Dalle-Donne *et al.*, 2013). Oxidative damage to protein modifies its symmetrical arrangements via aggregation, fragmentation and formation of cross linkages in the polypeptide chain which further extends the generation of superoxide radical (Waseem, Parvez, 2013; Chen *et al.*, 2005). In our study the PD flies showed a higher content of PC compared to control flies and the exposure of PD flies to luteolin showed a dose dependent decrease in the PC content. In our study the activity of caspase-3 and 9 were found to be elevated in the brain of PD flies. The flies exposed to

luteolin showed a dose dependent decrease in the activity of caspase 3 and 9. Apoptotic neuronal death plays an important PD (Guo *et al.*, 2013). In number of studies besides showing the antioxidative properties luteolin has shown anti-apoptotic properties (Guo *et al.*, 2013; Zhu *et al.*, 2011; Lin *et al.*, 2010; Kim, Chin, Cho, 2017).

Many plant polyphenolic compounds possess anti-oxidative as well as other biological activities (Yang *et al.*, 1997). We have estimated the potential of luteolin in scavenging the ROS by performing superoxide anion scavenging and DPPH assay. Both the assays showed that luteolin have a significant potential in scavenging the ROS at the doses used in our present study. The results obtained for the immunostaining for tyrosine hydroxylase also showed the presence of dopaminergic neurons in the PD flies exposed to various doses of luteolin. The results clearly demonstrates that the luteolin effectively provide protection to the neurons by reducing the oxidative stress.

The exposure of luteolin showed a dose dependent increase in the dopamine content in the brains of PD flies. Luteolin showed the protection of dopaminergic neurons by inhibiting the microglial activation and thereby preventing the generation of tumor necrosis factor- α , nitric oxide and superoxide (Chen *et al.*, 2008). A decrease in dopamine content is associated with the progressive degeneration of dopaminergic neurons (Rekha *et al.*, 2013). Tyrosine hydroxylase is the rate limiting enzyme for the formation of dopamine. In our present study its expression was used to identify surviving dopaminergic neurons (Jackson-Lewis *et al.*, 1995). Tyrosine hydroxylase immuno-reactivity describes the number of survival neurons. PD patients generally show a decrease in the activity of tyrosine hydroxylase, being more pronounced in the nigrostriatal system (Rausch *et al.*, 1988). Considering our results in the present study the localization of tyrosine hydroxylase in PD flies exposed to luteolin further strengthens and supports the protective role of luteolin in the flies expressing human alpha synuclein. Using *Drosophila* as a study model, one can easily analyze the pharmacological properties of plants/ plant derived components by performing several assays. The pharmacological properties of various natural plant products using *Drosophila* as a model have been extensively reviewed by Panchal and Tiwari (2017). Our earlier studies using the same study model on flavonoids/ plant extracts (Fatima *et al.*, 2017; Siddique *et al.*, 2016; Siddique, Naz, Jyoti, 2014) have also shown the promising results in reducing the PD symptoms. Hence, it is concluded that the luteolin is potent in reducing the PD symptoms in transgenic *Drosophila* expressing human alpha synuclein in the neurons.

CONCLUSION

It is concluded that luteolin is potent in reducing the PD symptoms in transgenic *Drosophila* expressing human alpha synuclein in the neurons.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY FIGURES

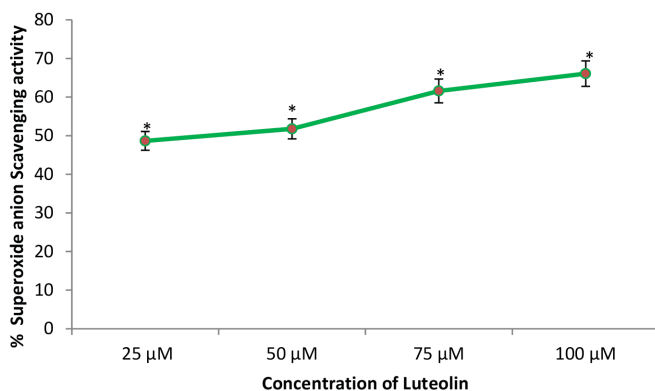


FIGURE 1S - Super oxide anion scavenging activity of luteolin. The values are the mean of five assays [*significant with respect to control, $p < 0.05$].

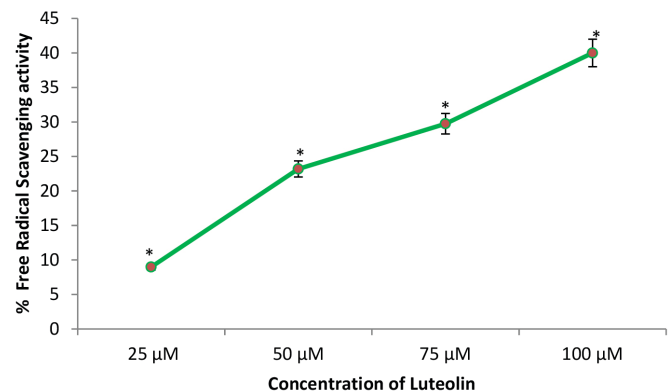
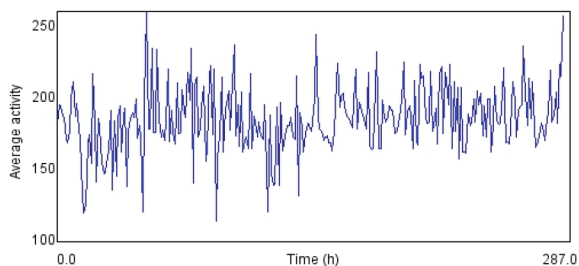
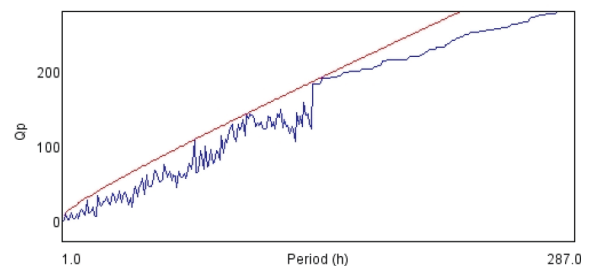


FIGURE 2S - Free radical scavenging activity of luteolin. The values are the mean of five assays [*significant with respect to control, $p < 0.05$].

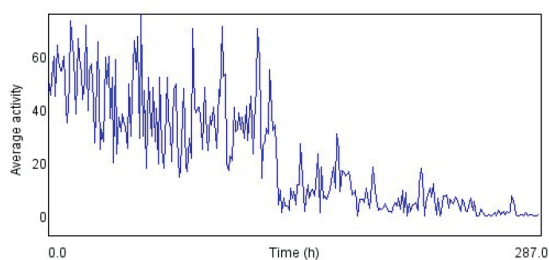


(a)

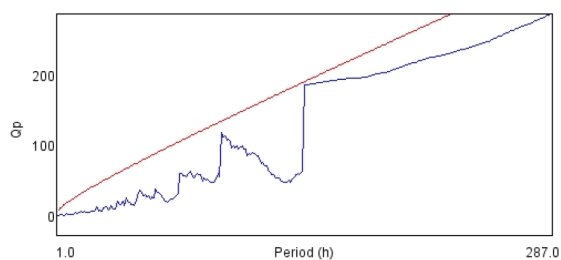


(b)

FIGURE 3S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for control flies (N=20).

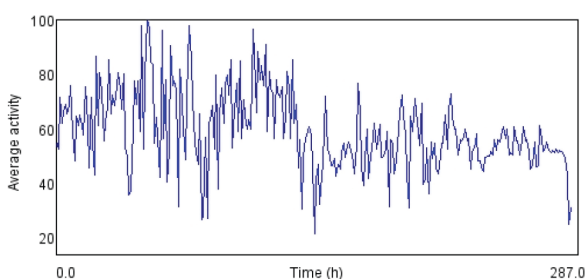


(a)

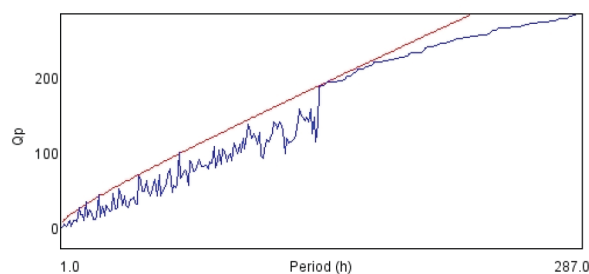


(b)

FIGURE 4S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for PD flies (N=20).

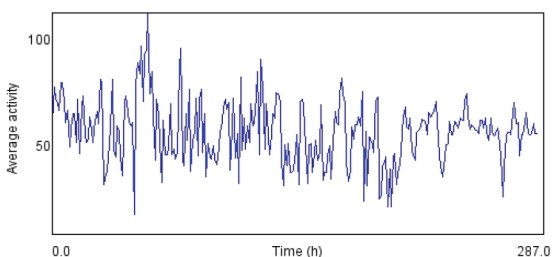


(a)

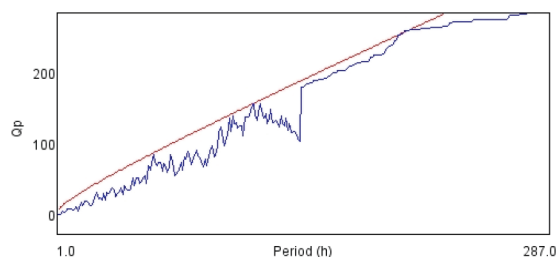


(b)

FIGURE 5S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for PD flies exposed to luteolin (25 μ M).

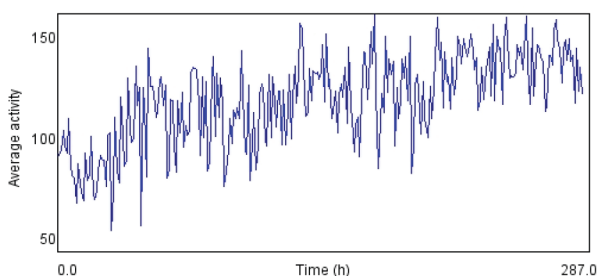


(a)

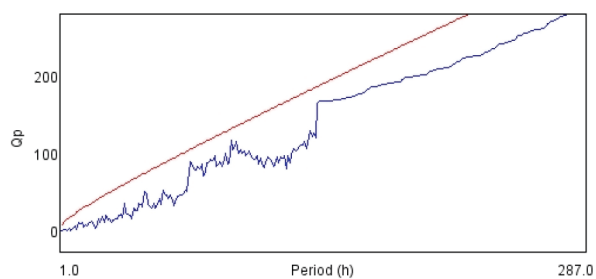


(b)

FIGURE 6S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for PD flies exposed to luteolin (50 μ M).

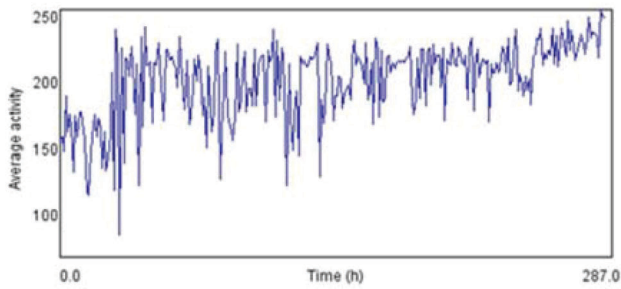


(a)

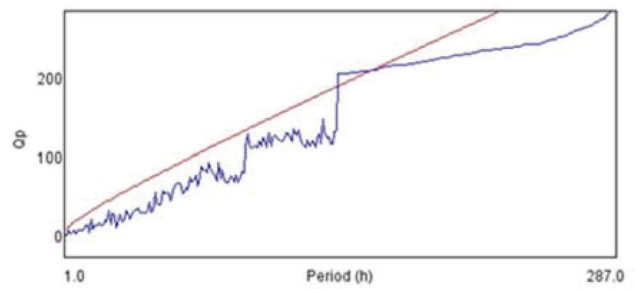


(b)

FIGURE 7S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for PD flies exposed to luteolin (75 μ M).

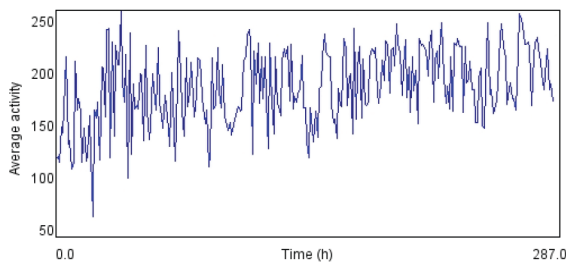


(a)

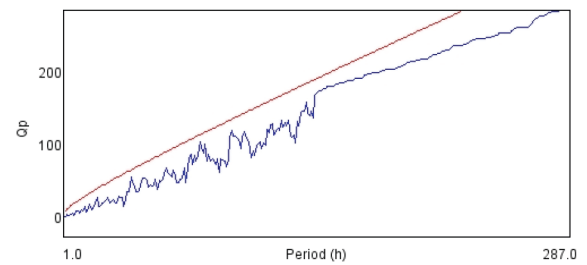


(b)

FIGURE 8S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for PD flies exposed to luteolin (100 μM).

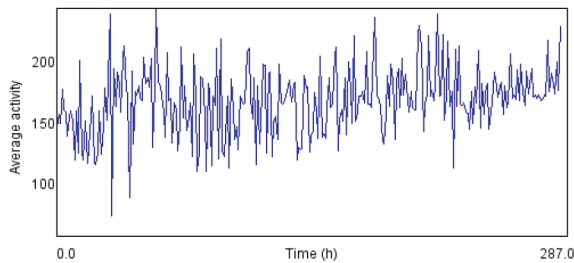


(a)

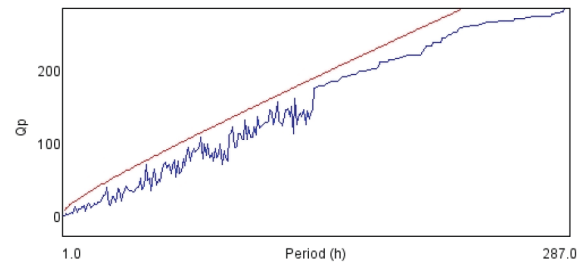


(b)

FIGURE 9S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for Control flies exposed to luteolin (25 μM).

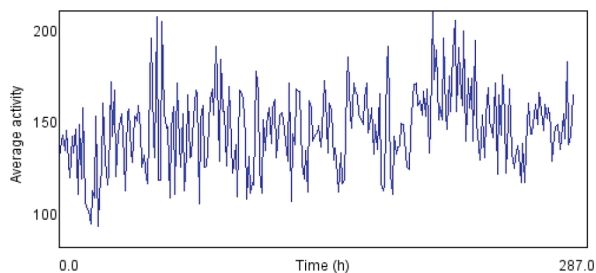


(a)

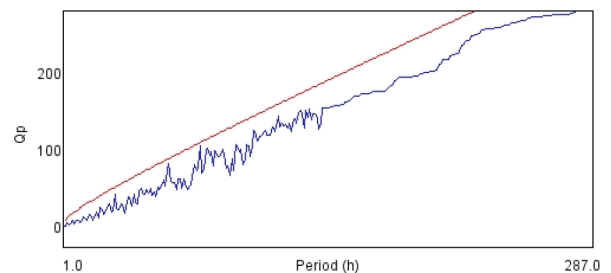


(b)

FIGURE 10S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for Control flies exposed to luteolin (50 μM).



(a)



(b)

FIGURE 11S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for Control flies exposed to luteolin (75 μM).

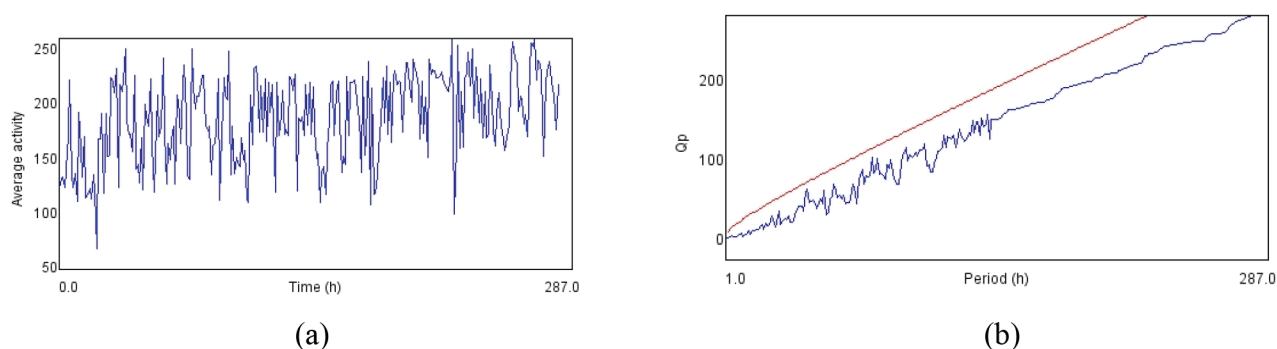


FIGURE 12S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for Control flies exposed to luteolin (100 μ M).

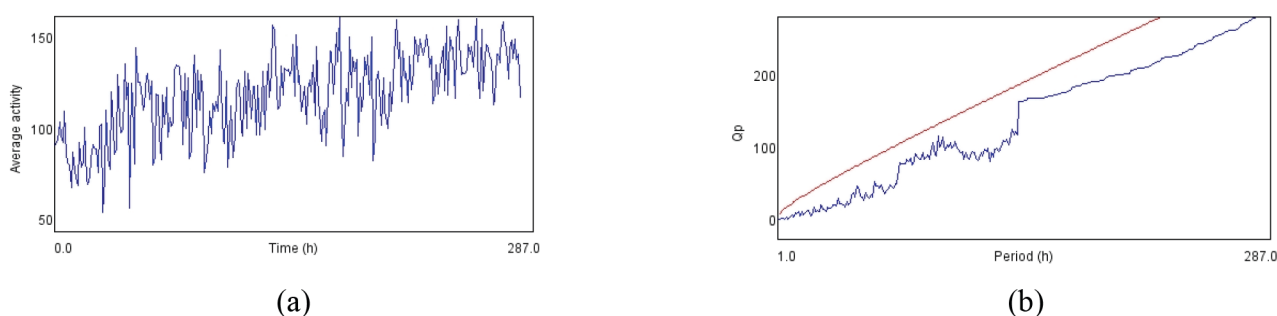


FIGURE 13S - (a) and (b) shows the average activity pattern and chi-square periodogram respectively for PD flies exposed to dopamine (10^{-3} M).

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