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RESEARCH ARTICLE

EFFICACY OF KAEMPFEROL IN ATTENUATING OXIDATIVE STRESS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Oxidative stress is a state of imbalance between free radicals and antioxidants in our body. The excessive oxidative stress leads to increase the amount of ROS that damages the cellular levels, which can be measures through various oxidative markers. The major oxidative markers are measurement of ROS, lipid peroxidation (LPO) and apoptosis levels, measuring these markers helps to assess pathological risk, monitor as well as treatment effects to lead a better health. Medicinal plants have emerged as the premier source of potent exogenous antioxidants which neutralize ROS and activates the internal protective pathways. These plant-derived compounds lower the concentrations of oxidative markers. In this study, we assessed the protective efficacy of Kaempferol, a flavonoid isolated from *Selaginella tenera* in two concentrations, against Paraquat-induced oxidative damage in *Drosophila melanogaster*. The present study demonstrates that Kaempferol supplementation significantly mitigates Paraquat-induced mortality, extending the survival rateunder stress condition. High dose of Kae fed flies increases the oxidative stress resistance ability by 1.35 folds than control flies.Biochemical analysis revealed that Kaempferol treatment leds to a marked decrease in intracellular ROS by 1.52 foldsthan PQ induced control flies. A robust reduction in malondialdehyde (MDA) levels by 1.6foldsin Kae dose II groups than PQ induced control groups, indicating the suppression of lipid peroxidation in the cell membranes. The reduction of ROS accumulation the cell facilitates reduction in apoptotic markers, suggesting the stabilization of mitochondrial integrity. The percentage of viable cells were found to be less in OS induced control groups with high percentage of early aswell as late apoptosis. Kae fed flies were significantly increasing survival rate by reducing ROS, lipid peroxidation activities and prevents the early and late apoptosis. By restoring the balance between pro-oxidants and endogenous antioxidant defenses, Kaempferol proves to be a high-potential compound.

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INTRODUCTION

The Reactive oxygen species (ROS) are generated as a part of the regular metabolism of a cell.Excessive ROS causes mitochondrial dysfunction, lipid peroxidation, and DNA damage. It regulates many biological processes such as signal transduction pathways when present in transient amounts, high levels of ROS can cause severe damage to DNA, protein and lipids (1).ROS production may lead to cellular dysfunction eventually leads to cell death. Oxidative stress creates an excess of ROS that directly damages critical cellular structures. ROS produced during oxidative stress initiates the signaling cascades, further leads to apoptosis (2). Oxidative stress triggers apoptosis when damage exceeds cellular repair capacity. Hence measuring oxidative stress is pivotal for evaluating, diagnosing, and monitoring the progression of various chronic diseases. It can be measures through various oxidative markers.

Oxidative markers are measurable indicators for damages of DNA, lipids or proteins due to body's oxidative stress which damages cells and contributing to early aging and various diseases like diabetes, cardiovascular issues, or neurodegenerative disorders. The major oxidative markers are measurement of ROS, LPO and apoptosis levels. This also helps to measure the damage caused by an imbalance between free radicals and antioxidant defences. ROS measurement helps to quantify oxidative stress, to understand the impact of antioxidants and efficacy of therapeutic compounds. Lipid peroxidation is a free radical driven reaction which causes tissue membrane damage by reaction of oxygen with polyunsaturated fatty acids (3). The oxidative stress and apoptosis are closely linked physiological phenomena, oxidative stress riggers the mitochondrial dysfunction and releasing apoptotic factors like cytochrome c, leading to caspase activation and cell death (4). Measuring these markers helps to assess pathological risk, monitor as well as treatment effects to lead a better health. *Drosophila* model has been used

extensively for toxicological studies (5,6and 7), drug discovery and targeting pathway genes of human diseases (8, 9). This alternative models has gained prominence for studying oxidative stress (10). Since environmental stressors can produce reactive oxygen species (ROS), overproduction of ROS can cause cellular dysfunction leading to cell death or appoptosis (11). Various stressors present in the environment including pesticides are capable of reacting with DNA and causing DNA damage (12).It has been proved that pesticides induce toxicity in *D. melanogaster* (13, 14). Oxidative stress typically induced by using various agent like Paraquat (PQ), Hydrogen peroxide, Acrylamide etc. Paraquat (PQ) (1,1′-dimethyl-4-4′-bipyridinium dichloride) is the most widely using herbicide. Paraquat induction generates large amount of ROS that intern causes oxidative destruction of lipids, leads to Lipid peroxidation.Natural bioactive substances have a strong antioxidant capacity, which may help reduce OS and even repair the damage caused by ROS(15). Pre-treatment with *Morus indica* significantly increasesthe survival rate of *D.melanogaster* (16). To evaluate the efficacy of antioxidant compounds for protecting against oxidative damage, LPO is the best tool that measures by TBARS (thiobarbituric acid reactive substances) assay. Oxidative stress markers in *Drosophila melanogaster* (fruit fly) models are widely used to evaluate the antioxidant efficacy of plant extracts and bioactive compounds. One such antioxidant potential bioactive compound of *Selaginella tenera* is Kempherol. In the present study the level of oxidative stress markers such as ROS, LPO and rate of apoptosis were measured in Kaempferolfed flies with PQ induced *D.melanogaster* under stress and non-stress conditions.

MATERIALS AND METHODS

Chemicals: All the chemicals used were of analytical grade. Propionic acid, acetic acid, sucrose and ethanol obtained from Rankem, Mumbai. Agar-agar was obtained from Himedia, Mumbai. Paraquat dichloride (PQ),TBA, n-butanol, Tetramethoxy Propane, Cell culturemedium- DMEM- high Glucose, D-PBS, FITC (Fluorescein isothiocyanate), Annexin V (BD Biosciences), PI (Propidium Iodide), DCF-DA all chemicals were procured from Himedia, Mumbai.

Culturing of *Drosophila:* *Drosophila melanogaster*, wild stocks of strain Oregan K were obtained from *Drosophila* Stock Center, Department of Zoology, Manasagangothri, University of Mysore, Mysore, Karnataka. The flies were cultured in a standard wheat cream agar media seeded with yeast granules and maintained at 22 ± 1^{o} C of relative humidity 70 – 80%. For all the experiments synchronized flies were used from isofemale line stocks.

Isolation of Kempherol: Kaempferol was isolated from the plant *Selagenella tenera* by following the standard procedure as described in the previous paper (17). Based on LC-MS and HPLC analysis a flavonoid compound Kaempferol was separated and isolated compound was employed for all the analyses.

Oxidative resistance stress analysis (OS assay): To know the stress resistance capacity of the bioactive compound, flies were subjected to oxidative stress conditions. Paraquat dichloride has been employed as OS molecule (18). Kaempferolsupplimentedmale flies of different age groups (20, 40, 60days) were used for OS induction. The flies were starved in empty vials of size 9 x 3 cm for 2 hours. Then, the treated flies were exposed to 20 mM PQ in 5% sucrose solution through a soaked filter paper. In this experiment, 50 flies (10 each vial) were kept in each batch. The mortality rate was recorded for every six hours once. The survival rates were calculated in both extract treated with control and OS induction until all flies died.

Biochemical analysis: All the biochemical analysis were measured in the Kaempferol (Kae) supplemented flies with two different concentrations (Dose I- 1mg/ml and Dose II-5mg/ml). 40 days aged Kae fed flies were used for OS induction. The flies were starved in empty vials of size 9 x 3 cm for 2 hours. Then, flies were exposed to 20 mM PQ in 5% sucrose solution through a soaked filter paper for 2 hours duration. Survival was determined after 24 hours and survived flies were used for all biochemical assays. The Kae treated flies with PQ induced were considered as stressed group and the Kae treated without PQ induced flies were considered as non-stressed group. About 50 flies were maintained in each batch (10 flies/vial) and each assay was repeated thrice.

Reactive oxygen species (ROS) assay: The quantification ofROS was analysedby DCF-DA spectrofluorometric method(19). The total oxidative stress in cells were measured using the intracellular DCF fluorescence as an indicator. ROS was measured in 40 days aged male flies. The flies were homogenized in 1 mL of Tris-HCl buffer (0.1M, pH 7.4) and centrifuged at 5,000 rpm for 15 min at 4°C. The reaction mixture comprised of 85 µL of Tris-HCl buffer (0.1M, pH 7.4), 100 µL of tissue homogenate and 15 µL of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). The reaction mixture was incubated for 1 h under dark condition at room temperature on a gel rocker. The breakdown of DCF-DA to the fluorescent product DCF was measured using fluroscence spectophotometer with excitation wavelength of 488 nm and emission of 525 nm. The values were plotted against the standard DCF graph and expressed as µmol DCF formed/min/mg protein.

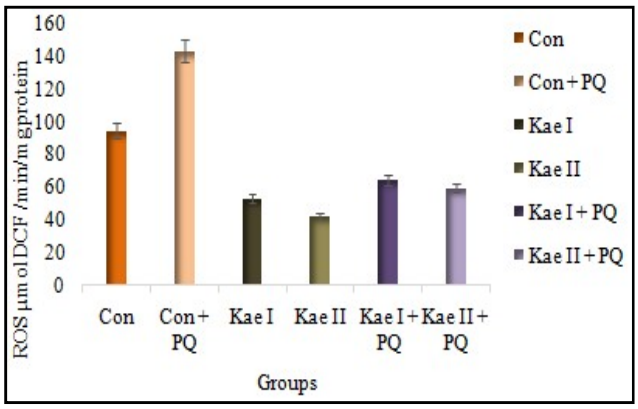

Fig. 1. Effect of Kae on ROS level in 40 days age grouped flies of *D.melanogaster*under stress and non-stress conditions.

Lipid Peroxidation Assay (LPO): Lipid peroxidation was quantified using the thiobarbituric acid (TBA) assay to measure malondialdehyde (MDA) formation according to the standard protocol (20). The activity was carried out in 40 days aged Kae supplemented male flies. The reaction mixture consists of 0.2 µl of homogenate sample, 0.2 µl of SDS (8.1 % w/v), 1.5 ml of acetic acid (pH 3.5, 20%), 1.5 ml of TBA

(0.8% w/v) and reaction mixture was made up to 4 ml with distilled water, mixed well. The mixture was incubated in a water bath at 90° C for 60 min and then cooled in ice-bath. After cooling, samples were mixed with 3 ml of n-butanol and centrifuged at 5000 rpm for 10 min. Further, supernatant was carefully transformed to another test tube and the absorbance was measured at 532nm. LPO was quantified as malondialdehyde (MDA) equivalents using 1,1,3,3-tetramethoxypropane as the standard (molar extinction coefficient value is 15600 M^{-1} cm^{-1}). Blank solution was prepared by mixing all the reagents except sample homogenate.

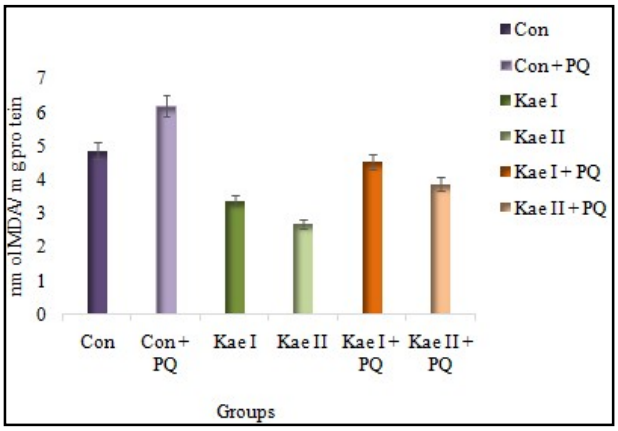

Fig. 2. Effect of Kae on LPO activity in 40 days age grouped flies of *D.melanogaster*under stress and non-stress conditions

Apoptosis assay: Apoptosis assay was quantified by flow cytometry method (21). It was performed in accordance with the Annexin V-FITC staining technique. 40 days aged group male flies were employed to perform the apoptosis analysis. The study was made in Kae fed and control flies. Cultured flies were collected in a 6-well plate at a density of 0.5 x 10^6 cells/2ml and incubated in a CO_2 incubator overnight at 37°C.

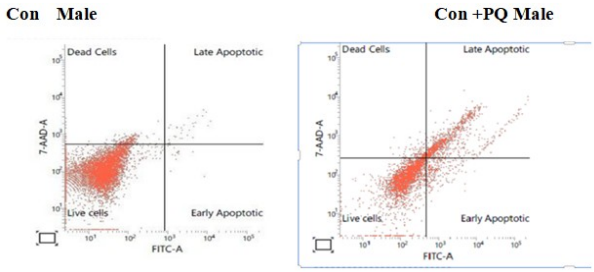

Fig 3. Quadrangular plot representing the Annexin V/PI expression in Control flies of *D.melanogaster*upon culturing with and without PQ induction.

The spent media was aspirated and cells were treated with 2 ml of culture media and incubated the cells for 48hours. At the end, the media was removed from all the wells and washed with PBS, then 200µl of trypsin-EDTA solution was added and incubated at 37°C for 3-4 minutes. Further, 2 ml culture media was added and the cells were harvested directly into 12 x 75 mm polystyrenetubes, centrifuged for five minutes at 300xg at 25°C. Later supernatant was decanted carefully and cells were washed twice with PBS and once PBS was completely decanted, 5 µl of FITC AnnexinV was added. Cells were gently mixed using vortex mixture and incubated for 15 min at RT (25°C) in the dark condition and added 5 µl of Propanium Iodide (PI) and 400 µl of 1X Binding buffer was added to each tube and mixed gently. Further, the results were analyzed by flow cytometry after addition of PI.

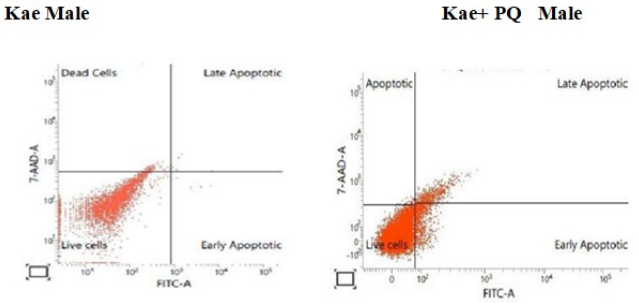

Fig 4. Quadrangular plot representing the Annexin V/PI expression in Kae supplemented flies of *D.melanogaster*upon culturing with and without PQ induction

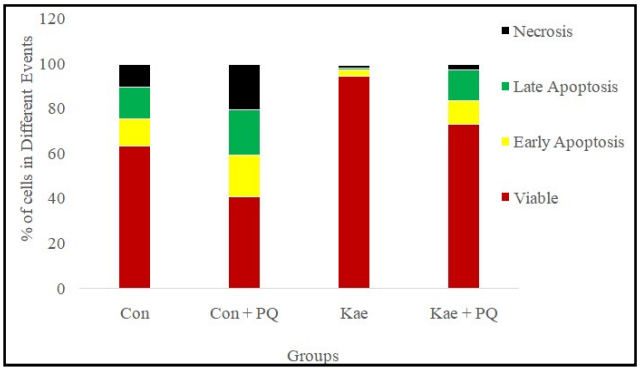

Fig. 5. Result of Apoptosis in Control and Kae treated flies of *D.melanogaster* under stress and non-stress condition -% of Viable cell, , Early apoptosis, Late apoptosis and necrotic cells.

Statistical analysis: The data obtained from the oxidative stress test and all the biochemical assay were expressed as mean ± SE and subjected to statistical analysis using SPSS software version 16.0. To know the level of significance among the different groups, data were subjected to one-way ANOVA, followed by DMRT with $p<0.05$ considered as statistically significant.

RESULTS

Oxidative stress assay: The result of oxidative resistance test was represented in Table 1.

Table 1. PQ Stress resistance in Control and Kae supplemented *D. melanogaster* at different age group flies

	PQ Stress resistance Assay in Male flies		
Groups	20 days	40 days	60 days
Con +PQ	28.56±0.26(a)	30.70±0.41(a)	26.70±0.23(a)
Kae I+PQ	33.63±0.29(b)	35.47±0.41(b)	32.56±0.32(b)
Kae II+PQ	37.54±0.33(c)	41.60±0.28(c)	35.60±0.25(c)
F value	224.16	210.95	273.62

Note: The groups with mean values having different letter in the parenthesis are significantly different at the 5 %level according to DMRT

The oxidative stress resistance was analysed in different age grouped Kae fed flies with PQ induction. The survival resistance in Kae fed batches were gradually increased from 20 days of PQ exposed flies, peaked at 40 days aged flies, and

eventually decreased at 60 days of age grouped flies. The mortality in control group after PQ induction begins at 12hrsin 20 days grouped flies, while mortality begins at 6 hrs in 40 days and 60 days group. The early mortality was noticed on 60 days in control grouped flies. However Kae fed flies exhibits delayed in their mortality, that begins at 18hrs in 20 and 40 days batches, 12 hrs in 60 days flies batch. Dose-II of Kae showed better resistance ability than Dose-I batch. Further, it was observed that 40days aged Dose-II of Kae fed batch flies survived till 60 hrs. after PQ induction. The highest mean survival was noticed in 40 days grouped flies after PQ induction, where mean resistance was 35.47±0.41 and 41.60±0.28 in Dose-I and Dose-II of Kae fed batches respectively. All the analysed group showed statistically significant differences with each other (F value 210.95).

ROS assay: The quantification of ROS levels were made in both PQ induced control flies and PQ induced Kae fed flies (Kae I and Kae II doses). ROS were measured in 40-day-old male flies and expressed as ROS µmol DCF /min/mg protein. The result were compiled in Fig.1.A progressive increase in ROS levels was observed in control and with PQ induced groups. The ROS levels were found to be high in control male flies induced by PQ with the mean ROS level was 143.08±0.19. The higher ROS levels in PQ induced flies suggest the increased level of oxidative stress. The supplimented with Kae compounds (Kae I and Kae II) showed significantly reduction in endogenous ROS production. Kae II supplemented flies showedprogressive decreased ROS levels in both with PQ and without PQ batches, the mean ROS levels were found to be 41.89±0.28 and59.16±0.27respectively. The amount of ROS in all the analysed groups showed statistically significant difference with each other.

Lipid peroxidation assay: Lipid peroxidation was measured through MDA activity per mg protein. The result of the analyzed groups for LPO activities are illustrated in Fig 2.LPO activities were significantly more in control with PQ induced batch. The mean LPO activity in control with PQ was found to be 6.19±0.05 nmole MDA/mg of protein, while it was 4.86±0.01 nmole MDA/mg of protein in control group without PQ. The higher LPO levels in PQ induced flies suggest increased level of oxidative stress. Further it was noticed that the bioactive compound Kaesupplemented batches had less LPO activity. However, the LPO activity was decreased in Kae fed batches compared to control group both under stress and non stress state (Fig. 2). Interestingly, Dose-II of Kae fed groups with PQbatches had least LPO activity compare to control with PQ. The least LPO activity was noticed in Kae II without and with PQ, the mean LPO activity were found to be 2.69±0.02 and 3.87±0.01 respectively.

Appoptosis assay: The data on Apoptosis by Flow cytometry was represented in Fig. 3 and 4. The analysis was performed using 40 days aged male grouped flies. It was measured in Kae supplimented flies of dose 5mg/ml with and without PQ in comparison with control flies and PQ induced flies. The results were compelled into four groups: Viable cells, early apoptosis, late apoptosis and necrosis. The graph represents the distribution of cell in each quadrangular graph. Each dot plot represents the cell of an individual X and Y axis of each plot shows the shape and size of cell which determine the early late or necrosis in apoptosis. The result revealed that the percentage of viable cells were 63.24% in control group, while it was progressively reduced in PQ induced groups (40.65%). However the percentage of viable cells were significantly high in Kae fed groups under stress and non stress groups, viable cells was 94.59% in Kae fed group, and 73.26% in Kae with PQ induced group.

The percentage of early and late apoptosis were also found to be high in control and control with PQ group. The highest increase of late apoptosis was observed in control grouped flies with PQ (20.13 %). However the drastic decrease of late apoptosis was observed in Kae fed grouped flies (0.87 %). The percentage of early and late apoptotic cell was more when compared to control with and without PQ induction grouped flies. The cells fed with Kae reached early apoptosis was very less, about 2.87%, while it was 10.56% in control with PQ induction group. The cells reached late apoptosis was 14.02% in control, 20.13% in control with PQ induction, 13.56% in Kae with PQ induction and 0.87% in Kae supplemented groups. Another measured parameter was necrosis occur in pathological defective cells. The maximum cell necrosis was observed in control with PQ (20.50%), while it was 10.23% in control group without PQ induction. Kae supplemented groups showed least cell necrosis (1.07%). The bar graph in Fig.5represents the apoptosis activity of Kae supplemented *D. melanogaster* in control and PQ treated group. Each graph represents the activity of early apoptosis, late apoptosis viable cells, necrosis.

DISCUSSION

Oxidative stress markers serve as essential biological indicators that measure the critical imbalance between the production of reactive oxygen species (ROS) and the body's protective antioxidant defenses.Antioxidants play a vital role in maintaining cellular health by neutralizing reactive oxygen species (ROS) and mitigating oxidative stress and increases the survival rates.H_2O_2-induced oxidative stress was decreased by Amaranth leaf extract, acrylamide induced oxidative stress was reduced by *Phyllanthes* species and paraquat induced oxidative stress *was reduced by Withania somnifera* extract.(22, 23 and 24). Various bioactive compounds activate the NRF2/ARE signaling pathway, which leads to the production of antioxidant enzymes that help mitigate oxidative damage. Antioxidant*Curcumin* of turmeric, protects against oxidative damage by activating Nrf2 signaling (25). Sulforaphane**of** broccoli plays a strong role in reducing oxidative stress (26).In the present study, it was noticed that both the dosage treatment batch of Kae fed flies survived longer duration, acquired greater resistance ability against oxidative stress. High dose of Kae fed flies increases the oxidative stress resistance ability by 1.35 folds than control flies. The resistance ability in Kae fed flies was exhibits in dose dependent manner. Measuring oxidative stress through biomarkers in Kae fed flies helps to understand the potentiality of the of bioactive compound Kae against stress and how it mitigates ROS-induced damages. In the present analysis three major oxidative makers such as ROS levels, LPO activity and apoptosis been measured in Kae fed flies under stress status. Elevated ROS levels, measured using fluorescent a fluorescence-based probes DCFH-DA or DHE converts into a fluorescent molecule, dichlorofluorescein (DCF), upon reacting with free radicals like ROS and RNS in the presence of a catalyst (27). ROS levels has been estimated in different strains of *D.melanogaster* and $Sod1^{n1}$ mutant line, they shown that $Sod1^{n1}$ flies displayed the highest ROS levels in normoxia and after hypoxia (28).

Long lived lines of *D.melanogaster* had lower ROS levels under oxidative stress (29). The level of fluorescence is directly proportional to the amount of free radicals present in the sample.In the current study the ROS levels were increased by 1.52 folds in the control flies after PQ induction. However, it was drastically reduced in Kae fed grouped flies 2.24 folds.

Lipid peroxidation (LPO) is a critical biomarker of oxidative stress in plant extracts fed *D. melanogaster* flies. LPO was measured through MDA activity that was a byproduct of lipid peroxidation. The extractofhigh antioxidant plants *Taraxacum officinale*, *Cassia siamea*, *Amaranth* are shown to reduce LPO levels in flies exposed to stressors like paraquat, hydrogen peroxide, or high-fat diets (30, 31, and 22). In the present study LPO levels were increased by 1.3 foldsin the control flies after PQ induction. In contrast to this, bioactive compound Kae (Dose-II) fed group of OS-induced flies decreases the LPO activity by 1.60 fold than PQ induced control groups. It was decreased by 1.6 fold OS induced kae (Dose-II) fed batch. This result confirms that Kae have ability to reduce oxidative stress by decreasing LPO. Dopamine prevents lipid peroxidation in the PD line of *D.melanogaster* (32). The up-regulation antioxidant Shathavari-IV inhibits lipid peroxidation and reduces oxidative stress (33). The present result is similar to the findings of earlier studies conducted on Shathavari-IV and Dopamine (32& 33). Apoptosis is a conserved cell death pathway that helps to create new structures and eliminate unnecessary tissues or extra cells (34).Oxidative stress plays a pivotal role in apoptosis (4). Gupta have studied the apoptosis in the third instar larvae of *D. melanogaster* by inducing Chlorpyrifos and shown that high concentration of Chlorpyrifos leads to apoptotic mode of cell death (10). Further, it has been shown that antioxidant property of Shr-IV of *Asparagus racemosus* protect *Drosophila melanogaster* flies from apoptotic mechanism and extend life span revealed by Kiran, 2022 (35). In the current study apoptosis was carried out using Kae fed flies of concentration 5mg/ml through flow cytometry. The percentage of viable cells were found to be less in OS induced control groups with high percentage of early well as late apoptosis. Contrastingly, Kae fed flies exhibits greater percentage of viable cells with substantial reduction of apoptosis (both early and late). PQ treatment causes an increase in MDA levels and the decreases in the activities of antioxidase and antioxidants such as SOD, GSH-Px and CAT (36). Further it has been reported that PQ exposure leads to apoptosis and programmed necrosis via the oxidative stress (36). It was noticed that PQ induces the oxidative stress, that proves through oxidative markers. It exhibits higher ROS, LPO and apoptotic levels, inturn that could be mitigated by Kae. The action of Kae was found to be dose dependent manner.

CONCLUSION

In conclusion, the studies summarizes that the supplementation of Kaempferol, a natural flavonoid from *Selaginella tenera*, acts as a potent antioxidant against Paraquat-induced oxidative stress effectively lower the concentrations of oxidative markers in *Drosophila melanogaster*, significantly increasing survival rate by reducing ROS, lipid peroxidation, and apoptosis. These results suggest Kaempferol as a promising bioactive compound for mitigating oxidative damage and highlight its potential for therapeutic development in age-related and chronic diseases.

Key points

- *Selaginella tenera*serves as a rich natural source of Kaempferol, a bioactive flavonoid exhibiting superior antioxidant capacity.
- Kaempferol directly scavenges ROS and suppresses destructive lipid peroxidation.
- Kaempferol prevents cellular apoptosis and enhancses survival rate in *Drosophila melanogaster*

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Conflict of Interest

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