Neuroprotective Activity of Pyrazolone Derivatives Against Paraquat-induced Oxidative Stress and Locomotor Impairment in *Drosophila melanogaster*

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ABSTRACT

Objectives: In the present study, we have demonstrated the antioxidant and neuroprotective effects of certain substituted hydrazine pyrazolones.

Methods: The compounds were synthesized and characterized by spectral data by reported methods. The antioxidant ability of the compounds was confirmed through in vitro antioxidant (DPPH scavenging, ABTS radical scavenging, total antioxidant capacity, and ferric reducing activity) studies. In vivo neuroprotective activity of the test, compounds were determined in Drosophila melanogaster Oregon K (OK) adult male flies. Oxidative stress was induced by using paraquat (PQ). Edaravone (EDA) was used as a standard for studies.

Results: Compound C4 was efficient in the modulation of lipid peroxidation marker levels such as malondialdehyde (MDA) and hydroperoxide (HP). Glutathione (GSH) levels were elevated in C4 treated flies significantly. The modulatory effect on enzyme antioxidants superoxide dismutase (SOD) and catalase (CAT) was observed in compound pretreated flies. Pretreatment of compounds through dietary feeding and co-exposure to PQ showed a lower death rate in flies. The neuroprotective efficacy of the compounds was confirmed through a negative geotaxis assay.

Conclusion: Flies treated with compounds and PQ co-exposure showed improvement in motor activities, suggesting the neuroprotective potential of pyrazolone derivatives. This might be due to their antioxidant ability while the precise mechanism of action needs further investigations. Among the tested compounds, C4 showed significant antioxidant and neuroprotective activity.

Key Words: Neuroprotection, Drosophila, Oxidative stress, Pyrazolone, Paraquat, Antioxidant

INTRODUCTION

Oxidative stress could play a vital role in many neurological disorders, such as epilepsy, Alzheimer’s, Parkinson’s disease, stroke, cerebral ischemia, multiple sclerosis, Huntington’s chorea, tardive dyskinesia, amyotrophic lateral sclerosis, and other diseases.¹ Paraquat (PQ) has been used as a broad-spectrum herbicide which is extremely toxic and leads to multiple organ dysfunction in humans. This pesticide was used as a model factor inducing oxidative stress in vivo mainly hydrogen peroxide and hydroxyl radical.²³ It has been shown that PQ causes damage to the number of dopaminergic neurons due to its ability to penetrate the blood-brain barrier (BBB).⁴ *Drosophila melanogaster* was used in this study because it has been an excellent model for gerontological research due to its relatively short life span and also appears to show many of the manifestations of cellular senescence observed in mammals. Oxidative stress plays an important role in governing the life span of the fly.⁵ Pyrazolone scaffold possesses...
a broad spectrum of applications including anti-inflammatory, antioxidant, neuroprotective, anticonvulsant, and antidepressant activity.6,7

Many commercially available pyrazolone derivatives are in use for the treatment of various neurological diseases. Edaravone (EDA), a pyrazolone derivative was also used in the treatment of acute ischemic stroke and is used as a standard drug in this study.8,9 This work was attributed to the evaluation of neuroprotective efficacy of some reported edaravone derivatives in the D.melanogaster Oregon-K(OK) flies model system through the antioxidant mechanism and negative geotaxis assay.

MATERIALS AND METHODS

The analytical grade chemicals and the reagents required for the study were procured from Sigma and Himedia Pvt Ltd., India.

Synthesis of pyrazolone derivatives

The compounds (4E)-4-[2-(4-fluorophenyl) hydrazinylidene]-5-methyl-2,4-dihydro-3H-pyrazol-3-one (C1), (4E)-4-[2-(3-chloro-4-fluorophenyl)hydrazinylidene]-5-methyl-2,4-dihydro-3H-pyrazol-3-one (C2) and 3-Methyl-5-Pyrazolone (C4) were synthesized and characterized by reported methods.10,11 The structures of the compounds were further characterized by recording FT-IR, 1H and 13C NMR, and LC-MS data (Data not given). The melting point was recorded on a melting point apparatus taken in an open capillary tube and was uncorrected. The purity of the compound was confirmed by thin-layer chromatography using Merck silica gel 60 F254 coated aluminium plates. The IR spectrum was recorded on a Shimadzu-FT-IR Infrared spectrometer in KBr (Vmax in cm−1) (Shimadzu, Kyoto, Japan). The 1H-NMR (400 MHz) spectrum was recorded on a Bruker AMX 400 spectrometer (Bruker Optik, Ettlingen, Germany), with 5 mm PABBBO BB-1H TUBES at IISC, Bengaluru, Karnataka, India. The 13C-NMR (100 MHz) spectrum was recorded on a Bruker Ascend for approximately 0.03 M solutions in DMSO-d6, at IISC, Bengaluru. The mass spectrum was obtained using Agilent 1200 series LC and Micromass ZQ spectrometer and a Shimadzu LCMS-8030 mass spectrometer (Shimadzu Corporation, Kyoto, Japan), operating at 70 eV at Mangalore University, Managalagangothri, Karnataka, India. The elemental analysis was carried out using a CHN-SO analyser.

In vitro antioxidant studies

All the compounds were determined for in vitro antioxidant efficacy at 100 μg/ml concentration. DPPH radical scavenging assay was carried and data compared with vitamin C (Ascorbic acid -AA) and IC50 for synthesized compounds were reported.12 ABTS radical scavenging activity was performed using Trolox as standard. Results are expressed as Trolox equivalents in mean ± standard deviation.13 Total Antioxidant Capacity assay was performed using ascorbic acid as standard and results were expressed as mg Ascorbic Acid Equivalent (AAE) of the test compound in mean ± standard deviation.14 Ferric reducing antioxidant power assay was performed using ascorbic acid as a reference standard and results were expressed as AAE of the test compound in mean ± standard deviation.15

Drosophilaculture and compound treatment

D. melanogaster (Oregon K) adult males (8-10 days old) were obtained from Drosophila stock centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore, Karnataka, India. The flies were maintained at constant temperature and humidity (22 ± 1°C and 60-70 %), fed on a standard wheat cream agar medium seeded with yeast.16,17 The test compounds C1, C2, C4, and standard EDA were dissolved in 1% dimethyl sulfoxide (DMSO). The compounds were introduced into the medium at semisolid state, mixed well and was allowed to solidify. 50 adult males were introduced into the vials containing media for further studies. All the compounds were evaluated for their toxicity by the lethality test. The male flies were fed on a medium containing C1, C2, and C4 at 50, 100 and 200 μg/ml concentrations. Lethality due to compounds at all concentrations and solvent was monitored by counting dead flies every 24h up to 7 days, and data was expressed in terms of percentage mortality. The experiment was conducted in triplicates.17

Paraquat exposure studies

In a preliminary study, flies were exposed to paraquat at concentrations of 10, 15, 20 and 25 mM for 96 h to determine lethality concentration due to paraquat at experimental concentration. The results were expressed as percent mortality. However, C1, C2, C4 and 15 mM concentrations of paraquat was employed to assess the antioxidant effects of test compounds against paraquat-induced lethality. In this assay, PQ exposed flies were fed with C1, C2, C4 and EDA (200 μg/ml) in the diet (co-exposure). Lethality of the flies was monitored for 96 h and data was expressed in terms of percent survival.17

Study design for compound treatment

1. Group I: Wild type flies fed with conventional media with 200μL 1% DMSO
2. Group II: Wild type flies fed with media containing paraquat
3. Group III: Wild type flies + 200 μg/ml C1 + Paraquat
4. Group IV: Wild type flies + 200 μg/ml C2 + Paraquat
5. Group V: Wild type flies + 200 μg/ml C4 + Paraquat
6. Group VI: Wild type flies + 200 μg/ml EDA + Paraquat
**Paraquat resistance test**
Two days after emergence from the pupae, as per the group- ing, 50 male flies were fed with the standard food with 1% DMSO in the control group. Flies in the treatment groups were fed with the food containing test compounds C1, C2, C4, and EDA respectively for seven days. Flies from all the groups along with paraquat group except control group were starved for 6 h to ensure that no food remained in the digestive tract so that none of the compounds would alter the uptake of paraquat. Later, these flies were transferred to vials containing only filter paper soaked with 15 mM paraquat in a 5% sucrose solution. The survival of the flies was determined 24 h and 48 h after the paraquat exposure. Survived flies were used for homogenization for biochemical assays. Each assay was repeated thrice.\(^{17,18}\)

**Biochemical investigations**
Whole-body homogenates of flies were prepared in 0.1 M Sodium-phosphate buffer (pH7.4) after performing the study. 30 male flies from the batch I and II were used for this purpose. After homogenizing, the samples were centrifuged. The supernatant was filtered through a nylon mesh (pore size, 10μm) and used for biochemical assays.\(^{17}\)

**Assay for Lipid Peroxidation**
Lipid peroxidation was measured by employing thiobarbituric acid (TBA). Absorption was measured at 532 nm against the blank and malondialdehyde (MDA) equivalents were measured from the calibration curve of MDA.\(^{19}\) The results were expressed as nMol malondialdehyde per mg protein.

**Assay for Hydroperoxide**
Hydroperoxide generation was determined in the homogenate of flies using FOX reagent. The absorbance was taken at 560 nm. Hydrogen peroxide content was estimated from the calibration curve of hydroperoxide and results were expressed as nMol HP/mg protein.\(^{20}\)

**Estimation of Reduced Glutathione**
Reduced glutathione (GSH) content was estimated based on the reported method. The reduced glutathione was estimated from the calibration curve of reduced glutathione standard and the results were expressed as mg GSH per mg protein.\(^{21}\)

**Antioxidant enzyme assays**
Catalase activity in the homogenate was estimated through standard reported methods. The results were expressed as μMol hydrogen peroxide per mg protein. SOD activity was monitored for 3 min at 406 nm, expressed as the amount of protein required to inhibit 50% of quercetin auto-oxidation.\(^{22,23}\)

**Locomotor assay**
The locomotor capacity was evaluated by following the negative geotaxis behaviour of drosophila flies of different groups as mentioned in the study design described by Coulom and Birman with some modifications. Twenty flies per group were anaesthetized and placed in a vertical glass column (length, 25 cm; diameter, 1.5 cm) sealed at one end. After a brief recovery period, flies were gently tapped to the bottom of the column. Following 1 min, the number of flies that reached 6 cm of the column (passed) and flies that remained below this mark (failed) were noted. The assays were repeated three times for each fly. Results were represented as the percentage of flies passed the test (mean ± SD) obtained from three independent experiments.\(^{17,18}\)

**Statistical analysis**
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnet’s test. The values were expressed as Mean ± S.D computed from all the three replicates. Prism software (Ver. 5.0) and Microsoft Excel was used for statistical analysis.

### RESULTS

#### Structures of compounds
The synthesized compounds formed were recrystallized and analytically characterized through FTIR, \(^{1}\)H NMR, \(^{13}\)C NMR, and LCMS. The data is reported previously.\(^{10,11}\)

#### In vitro antioxidant studies

##### DPPH radical scavenging activity
All the compounds showed moderate to very good antioxidant activity in comparison with standard EDA. The compound C4 exhibited good DPPH scavenging activity than EDA. Compounds C1 and C2 showed less activity than C4. The percentage of inhibition data is depicted in figure 1A. IC\(_{50}\) values for all the compounds were calculated. IC\(_{50}\) for C4 is significant in comparison with the standard data is illustrated in table 2.

##### ABTS radical scavenging assay and total antioxidant capacity assay
All the compounds were screened for ABTS and total antioxidant capacity assay. Among the tested compounds, C4 showed good activity which is significant compared to EDA. All other compounds showed moderate antioxidant activity. The data is given in figures 1B and 1C respectively.

##### Ferric reducing antioxidant power assay
Compounds were assayed for ferric reducing antioxidant power assay and data are expressed as ascorbic acid equiva-
lents. Among the compounds, C4 showed significant activity in comparison with EDA. The compound C1 showed the least activity among the tested compounds. The data is illustrated in figure 1D.

Neuroprotection studies in Drosophila flies

Lethality and Paraquat exposure studies
PQ induced lethality test was conducted to determine the nonlethal concentration of compounds. The mortality rate of flies treated with 10, 15, 20, and 25 mM concentration of PQ for 96 h in the interval of 24 h is depicted in figure 2A. Exposure of adult flies to PQ resulted in a concentration-dependent lethality during a 96 h experimental period. Mortality occurred between 72 h and 96 h among paraquat exposed flies. The cumulative percent mortality at the tested concentrations is shown in figure 2A. From the results obtained, 15 mM concentration of PQ is selected for further studies. Flies were pretreated with compounds at different concentrations viz., 50, 100, and 200 μg/ml. Lethality was determined by counting dead flies every 24 h for 7 days. The data were compared with normal media-fed flies. Compounds were found to be safe under-tested concentrations.

Paraquat resistance studies
The flies were pretreated with the compounds C1, C2, C4, and EDR for 7 days. Compounds pretreated D. melanogaster showed increased survival rate after paraquat exposure in contrast with the controls; the difference was significant (p < 0.001) in comparison with EDA pretreated flies with C4. Other compounds showed moderate protective ability. Data are expressed as survival percentage and reported in figure 2B.

Locomotor assay (Negative geotaxis assay)
Paraquat induced neurotoxicity was evidenced through locomotor deficits in normal flies fed with common media containing PQ. Flies with locomotor deficits prefer to remain at the bottom of the glass column and do not appear to coordinate their legs in a normal fashion. Compound C4 was able to substantially save the flies from worsening locomotive dysfunctions in comparison with EDA pretreated and compound unfed flies. C1 and C2 pretreated flies showed moderate activity in comparison with C4. Data are illustrated in figure 3.

In vivo antioxidant and oxidative stress marker assays
Lipid peroxidation Malondialdehyde (MDA) and Hydroperoxide (HP)
There was a significant elevation in the level of malondialdehyde (MDA) and hydroperoxide (HP) in the case of PQ treated flies. C1, C2, C4, and EDA supplemented diet for 7 days showed a significant diminution in MDA (Figure 4A) and HP levels (Figure 4B). The endogenous levels of oxidative stress markers (MDA and HP) were reduced significantly in C4 pretreated flies concerning EDA pretreated flies. Compound C4 showed good antioxidant activity in comparison with C1 and C2.

Reduced Glutathione
The levels of reduced GSH were significantly enhanced in whole-body homogenates among flies fed with compounds. Among the compounds, C4 was successful in ameliorating the activity of GSH in the flies when compared with EDA pretreated ones. In the case of PQ pretreated flies, the GSH level was decreased significantly in comparison with normal flies. Compound C4 was also successful in elevating GSH levels in comparison with PQ pretreated flies. C1 and C2 pretreated flies showed moderate activity. The data is illustrated in figure 5A.

Activities of Antioxidant Enzymes CAT and SOD
Compounds pretreated flies showed moderate activity to restore antioxidant enzyme levels viz., superoxide dismutase (Figure 5B), and catalase (Figure 5C). Among the tested compounds, C4 showed significant activity in comparison with EDA pretreated flies. However, these compounds were not successful in bringing SOD and CAT levels to basal levels in comparison with normal flies.

DISCUSSION
Oxidative stress is induced by an imbalanced redox state, involving the either excessive generation of reactive oxygen species (ROS) or dysfunction of the antioxidant system, thereby leading to neurodegenerative diseases like Alzheimer’s and Parkinson’s. Antioxidant therapy has been suggested for the prevention and treatment of neurodegenerative diseases. The capacity of antioxidants to nullify the oxidative stress-mediated damage is supposed to contribute to their therapeutic potential in preventing or slowing down neurodegeneration. Several studies have demonstrated that the antioxidant compounds protect neuronal cells by neutralizing the excessive free radicals and/or by enhancing the antioxidant defences.

For elucidating the neuroprotective activity, we synthesized the pyrazolone derivatives (reported) which are structurally identical with standard drug Edaravone. It is a member of the substituted 2-pyrazolin-5-one and IUPAC name is 3-methyl-1-phenyl-2-pyrazolin-5-one. It was originally developed as a potent free radical scavenger and neuroprotective agent in several diseases in vivo, including stroke, spinal cord injury, traumatic brain injury, neurodegenerative diseases, and brain tumors. Due to its antioxidant and neuroprotective effi-
cacy, Edaravone was selected as a standard compound for in vivo antioxidant and neuroprotective studies.

The compounds were evaluated for in vitro antioxidant property through DPPH scavenging, ABTS assay, ferric reducing power ability and total antioxidant capacity assay. The compounds showed good to moderate antioxidant activity. Among all the test compounds, C4 exhibited good in vitro antioxidant activity. The good activity of the compound might be due to methyl substitution on 5-Pyrazolone. It was reported that methyl substitution enhanced antioxidant efficacy in pyrazolone. Our data is well correlated with previously reported work.29

Further, the selected compounds were evaluated for in vivo antioxidant and neuroprotective studies in Drosophila melanogaster OK flies. Oxidative stress plays an important role in governing the life span of the fly.30

The antioxidant ability of the synthetic compounds was estimated by inducing oxidative stress in the flies using paraquat (1,1-dimethyl-4-4-bipyridinium dichloride). It is a quaternary nitrogen herbicide and highly toxic substance. The toxicity of paraquat is due to the generation of the superoxide anion which can lead to the synthesis of toxic reactive oxygen species (ROS) such as hydroxyl radicals, hydrogen peroxide and leads to the cellular apoptosis.31 In this study, we observed the lethality of D. melanogaster due to paraquat treatment as reported earlier. However, the significant reduction in mortality rate with the compound treatment in PQ exposed flies indicates the protective effect of the compounds. This might be due to their free radical scavenging activity which neutralizes ROS generated by paraquat.

Further, pretreatment of flies with compounds C1, C2 and C4, exhibited in vitro antioxidant property. The dose of compounds for pretreatment was determined through toxicity studies (study design was explained in methods). In this experiment, 1% Dimethyl sulfoxide (DMSO) was taken as an aprotic solvent that can solubilize a wide variety of poorly soluble polar and nonpolar molecules. This is coupled with its apparent low toxicity at concentrations <10% which has led to its ubiquitous use and widespread application.32 Our results confirmed no effect of solvent on drosophila at tested doses comparable to the normal flies.

Antioxidant efficacy of the compounds was determined by estimating the oxidative stress markers and antioxidant enzymes in the whole-body homogenate of flies. Paraquat exposed flies showed significant induction of oxidative stress as evidenced by the elevation in the levels of MDA, HP, and depletion of GSH. This supports the fact that it is a potent oxidative stress inducer, greatly increases the ROS production, and inhibits the regeneration of reducing equivalents, compounds necessary for the antioxidant activity. It was observed that there was a statistically significant reduction in the levels of MDA and HP in C4 pre-treated flies. Other compounds showed moderate but insignificant activity. The level of GSH was significantly elevated in case of C4 compound treated flies. C4 was successful in modulating the activity of GSH by bringing the levels to near basal, which could be attributed to lipid peroxidation inhibition capacity of the compound. The results were analyzed statistically and found significant at the P<0.05 level.

The antioxidant enzymes such as SOD and CAT levels were induced by PQ in the fly which was upturned in compound fed flies. The compound C4 was successful in modulating the activities by bringing antioxidant enzymes SOD and CAT to near basal levels comparable with normal flies by restoring redox condition. In paraquat sublethal dose exposed flies, locomotor defects were noted due to the selective degeneration of dopaminergic neurons. Drosophila is being widely used as a model to understand the mechanisms involved in neurodegenerative disorders, to model environmental toxin-induced parkinsonism and a convenient system to screen therapeutic agents for their neuroprotective effects, before their testing in mammalian model.33 It is known that nigrostriatal dopaminergic neurons are very sensitive to the toxicity of paraquat.34,35 Our results showed that flies exposed to PQ exhibited locomotion deficits and stayed at the bottom of the jar in the negative geotaxis assay, as they could not coordinate their legs while climbing. The flies pretreated with C4 compounds before PQ exposure showed significant improvement in locomotor activity. Other compounds showed less activity in comparison with C4.

In general, a pyrazolone molecule containing labile hydrogen as -NH-C=O group in the ring exhibit the tautomerism phenomenon. This makes the molecule to act as a good antioxidant scaffold. But the compounds C1 and C2 have similar pyrazolone moiety without a tautomerism phenomenon. This could be due to the presence of an intramolecular hydrogen bond formed between the oxygen atom of the carbonyl group of pyrazolone ring and the hydrogen atom attached to the nitrogen of exocyclic azo group. The formed N-H----O=C bond hinders the tautomerism phenomenon owing to the formation of a six-membered ring, which might be giving stability to the system. It is worth noting that a report has been cited for the existence of arylhydrazonotautomer, both in solid form as well as in solution for a similar analogue.36-38 This might be the reason for the poor antioxidant activity of the compounds C1 and C2. This kind of bonding was not observed in compound C4 and edaravone molecule. Hence, they showed potent antioxidant activity.

This is the study that first reported on the neuroprotective potential of tested compounds in the Drosophila model against PQ induced oxidative stress. This work contributes a new scaffold towards the development of a potential neuroprotective agent. Further investigations are required to unravel the
mechanisms of its action at the cellular and higher organism levels.

**CONCLUSION**

Our results suggest that pyrazolone derivative prophylaxis has the propensity to protect against neurotoxic exposure might be due to its antioxidant potential. In this model, the protective activity of C4 was comparable with EDA known neuroprotective agent. Our data further confirm the utility value of the drosophila system as a primary model to rapidly screen potential compounds for their antioxidant and neuroprotection properties before their testing in mammalian models.

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**Conflict of interest**

The authors declare that there are no conflicts of interest.

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Table I: Structures of pyrazolone derivatives used in this study

| Compounds | Structures |
|-----------|------------|
| C1        | ![Structure C1](image1) |
| C2        | ![Structure C2](image2) |
| C4        | ![Structure C4](image3) |
| Edaravone | ![Structure Edaravone](image4) |

Table II: IC<sub>50</sub> values of compounds C1, C2, C4 and EDA in DPPH radical scavenging assay

| Treatment   | IC<sub>50</sub> (µg/mL)  |
|-------------|-------------------------|
| EDA         | 110.59 ± 1.43            |
| Compound C1 | 288.85 ± 2.34            |
| Compound C2 | 371.57 ± 4.23            |
| Compound C4 | 79.41 ± 1.764            |

Figure 1: Represents in vitro antioxidant assays such as 1A: DPPH scavenging activity; 1B: ABTS assay; 1C: TAC assay; 1D: FRAP assay of compounds C1, C2, C4 and EDA. Values were presented as Mean ± SD.

Figure 2: A: PQ mortality test; B: Paraquat co-exposure survival study. Values were expressed as Mean ± SD (in triplicates).
Figure 3: Modulation of paraquat-induced locomotor (expressed as percent flies escaped) deficits among adult male Drosophila melanogaster by compounds and standard EDA treatments (n = 50 flies per replicate, three such replications used for assay). Values were expressed as Mean ± SD; *** p<0.001, ** p<0.01 compared to PQ.

Figure 4: A: Modulatory effect of C1, C2, C4 and EDA treatments on paraquat (PQ) induced oxidative stress measured as malondialdehyde levels, B: Hydroperoxide in whole-body homogenates of adult D. melanogaster. Values were expressed as Mean ± SD; *** p<0.001, ** p<0.01 compared to PQ.

Figure 5: A: Ameliorative effect of C1, C2, and C4 treatments on PQ induced oxidative stress measured as reduced glutathione levels, B: SOD; C: CAT assay in whole-body homogenates of adult D. melanogaster. Values were expressed as Mean ± SD; *** p<0.001,** p<0.01, * p<0.05 compared to PQ.