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In vivo antioxidative stress measurement of a 1, 2, 4- Triazole derivative in *Drosophila melanogaster* oregon K flies

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ABSTRACT

*Anti oxidative efficacy of a 4-amino-3-(4-chlorobenzyl)-1H-1,2,4-triazole-5(4H)-thione (**C**) is evaluated against electron beam irradiation (EBR) and Paraquat (Pq) induced oxidative stress in Drosophila melanogaster- Oregon K male flies. It is evaluated through biochemical estimation of oxidative stress markers like malondialdehyde (MDA), glutathione (GSH), antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) in the homogenate of the flies. Elevation in the level of MDA and depletion in the level of glutathione was observed in homogenate of flies due to stress induced by both irradiation and Pq. But significant protective effect is observed with compound treated flies. The triazole derivative emerges as promising molecule with significant antioxidative stress property.*

Key words: Antioxidant; 1,2,4-Triazole derivatives; *Drosophila melanogaster*; Electron beam radiation, Paraquat.

INTRODUCTION

Antioxidants are substances, when present in small quantities prevent the oxidation of cellular organelles by minimizing the damaging effects of ROS and RNS (Reactive Nitrogen species) or oxidative stress. Under normal healthy conditions, a balance is maintained between oxidative stress and antioxidant requirements. The endogenous antioxidant defense comes mainly from three different types of systems, viz., antioxidant enzymes e.g. catalase (CAT), superoxide dismutase (SOD), metal sequestering proteins e.g. ferritin and low molecular weight molecules like vitamin C, vitamin E etc. However under pathological conditions or during radiation injury, stress and pollution etc. the balance is lost and excessive supplementation of antioxidants is necessary.

The increased incidence of neurodegenerative diseases like Parkinson's diseases, Alzheimer's disease, lateral sclerosis, senile dementia etc may be attributed to a pro-oxidative environment caused by smoking, alcohol abuse, ultra-violet ray radiations, air pollution, pressure packed life style as well as inappropriate nutrition. The dependence of disease severity by an imbalance between oxidants and natural defenses suggests that oxidative stress plays a pivotal role in the progression of neurodegenerative diseases and could serve as a useful target for treatment [1].

A large number of heterocyclic compounds containing the 1,2,4-triazole ring are associated with diverse pharmacological properties, such as anticonvulsant, antifungal, antimicrobial, antihypertensive, analgesic, antiviral, anti-inflammatory, antioxidant, antitumor and anti-HIV activity [2]. Radioprotective property has been reported for sulphydryl groups containing compounds like cysteine, cysteamine and 4-amino-5-mercapto-3-methyl-1, 2, 4-triazole [3].

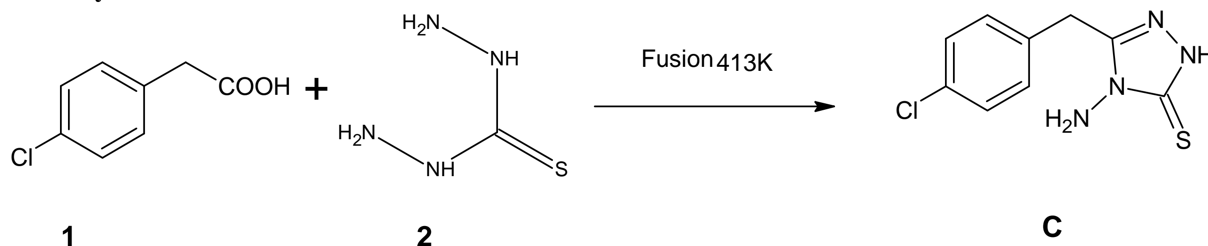
In this contest considering the requirement of novel antioxidant molecule and wide spectrum of biological properties of 1,2,4-triazole derivatives, series of compounds were synthesized, characterized and studied for *in vitro* DPPH scavenging assay [4]. Among the tested compounds, 4-amino-3-(4-chlorobenzyl)-1*H*-1,2,4-triazole-5(4H)-thione (**C**) exhibited good activity. Hence it was selected for further *in vivo* antistress studies in *Drosophila melanogaster* OK male flies. *Drosophila melanogaster* has become a model system for the study of development and behavior [5]. In the year 1910, Thomas Hunt Morgan identified the white gene, and since then it was considered as a model organism [6].

In our study two types of stress inducers were used, one was electron beam radiation and the other Paraqaut (Pq), an insecticide which is a known neurotoxin.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich without further purification. Melting points were determined by open capillary method and are uncorrected. The structure of **C** was further characterized by recording IR, ^{1}H NMR, ^{13}C NMR and LC mass spectral data.IR spectrum was recorded by Shimadzu 8400S FTIR spectrometer using KBr pellets. Purity of the compound was checked on precoated TLC plates using silica gel plates and visualised using UV chamber. Elemental analysis was carried out by using VARIO EL-III (Elementar Analysensysteme GmBH). ^{1}H NMR spectrum was recorded in DMSO-d6 at 400MHz and ^{13}C NMR spectrum was recorded in DMSO-d6 at 100MHz.

Chemistry:



Scheme1: Reaction scheme for the synthesis of 4-amino-3-(4-chlorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (C)

Table 1: Analytical data of the compound C

% Yield	Mol. form.	M.P. (K)	Mol. Mass	Elemental analysis (in %)					
				C		N		H	
				Obs.	Cal.	Obs.	Cal.	Obs.	Cal.
82	$C_9H_9ClN_4S$	449-451	241	44.87	44.91	23.22	23.28	3.69	3.74

Spectral details:

The IR spectrum of the compound **C** showed absorption bands at 3242 and 3161cm^{-1}, due to NH asymmetric and symmetric stretch respectively. Absorption bands at 3086 and 3039 cm^{-1} were due to asymmetric and symmetric NH_2 stretch respectively. Another sharp band at 2934 cm^{-1} was seen, which was assigned for aromatic C-H stretch. A sharp and strong absorption band at 1558 was due to NH bend and 1490cm^{-1} due to C=C ring stretch. The C=N group of the molecule showed an absorption band at 1294 cm^{-1}. A band at 765 cm^{-1} was assigned for C-Cl stretch and 663 cm^{-1} for C=S stretch.

The ^{1}H NMR spectrum of compound **C** exhibited a singlet at δ 4.041 ppm integrating for two methylene protons of benzyl group. A singlet seen at δ 5.551 ppm was assigned to NH_2 of triazole moiety. The four aromatic protons were resonated in the region δ 7.301- 7.394 ppm. They appeared as two doublets at δ 7.394-7.373 with J=8.4 Hz (ortho to Cl) and at δ 7.322-7.301 with J=8.4 Hz (-meta to Cl). A singlet peak at δ 13.551 ppm integrating for a proton was due to NH- SH tautomeric proton of triazole moiety.

In ^{13}C NMR spectrum of the compound **C** the peak at δ 29.52ppm was assigned for carbon of CH_2 group attached to benzene ring. The signals due to aromatic carbons appeared in the region 128.12 to134.42 ppm. The resonating peak for C=N was observed at δ 151 ppm. The carbon of C=S resonated at δ 166.1 ppm. Mass spectrum showed a molecular ion peak at m/z 241.33(M^+) corresponding to the molecular formula $C_9H_9ClN_4S$. Elemental analysis also gave satisfactory results for the compound.

Procedure for the green synthesis of the compound C

Thiocarbohydrazide was prepared by Taguchi method [7]. The well triturated equimolar mixture of thiocarbohydrazide (**2**) and 4-chloro phenyl acetic acid (**1**) was fused in a round bottom flask for one hour on oil bath at 413K. Then it was cooled to room temperature and washed with 5% sodium bicarbonate solution to remove unreacted acid and again washed with water to obtain the product as a white solid with yield 82%. The dried compound was recrystallized from methanol (m.p.449K).

Spectral details of the compound C:

IR(KBr)cm^{-1} :3242, 3161(NH), 3086, 3039 (NH_2), 2934 (Ar. C-H str.), 1558(NH bend), 1490(C=C ring str.), 1294 (CN str.), 765 (C-Cl str.), 663 (C=S); ^1H NMR (400 MHz, DMSO-d_6): δ ppm,13.55(1H, s, NH-SH), 7.39 (2H, *d*, aromatic, J = 8.4 Hz), 7.32 (2H, *d*, aromatic, J = 8.4 Hz), 5.55(2H, s, of NH_2), 4.04 (2H, s, of CH_2); ^{13}C NMR (100 MHz, DMSO- d_6): δ ppm, 29.52 (CH_2), 128.12, 128.36, 130.75, 131.48, 134.42, 134.88 (Aromatic carbons), 151 (C=N), 166.10 (C=S); Anal. cal. for $C_9H_9ClN_4S$: C, 44.91; H, 3.77; N, 23.28. Found: C, 44.87; H, 3.69; N, 23.22; LCMS (ESI-MS) *m/z* 241.33(M^+).

***In vitro* Antioxidant assay**

DPPH scavenging assay

The ability of the compound **C** to act as hydrogen donor was measured *in vitro* by stable free radical as 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^•$) as described by Blois [8]. Antioxidant reacts with DPPH (deep violet color, a stable free radical), converts it to 1,1-diphenyl-2-picrylhydrazine (colourless, nonradical $DPPH_2$). The degree of discoloration indicates the scavenging potential of the test compound. The reaction mixture contained 1 mL of $DPPH^•$ solution (0.3 mM in methanol) and 1mL of the test compounds or glutathione (GHS) as standard at different concentrations. The mixture was shaken and allowed to stand at room temperature for 20 min. and the absorbance was measured at 517 nm using Systronics UV spectrophotometer-169 and calculated as the percentage of inhibition using the equation,

% Inhibition = (Control – Test) / Control x100

Drosophila culture

Drosophila melanogaster (Oregon K) adult males (5-7 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 22 ± 1°C and 70-80% relative humidity, fed on a standard wheat cream agar medium seeded with yeast. The medium was prepared according to standard protocol of the media (100 mL) containing 10g wheat flour, 10g jaggery, 1g agar agar and 0.75 mL propionic acid (antifungal agent) few granules of yeast were added. After 24 hours flies were transferred to fresh media bottles to avoid sticking of flies to media. Whenever required, the flies were exposed to the fumes of diethyl ether in a small airtight glass container for 1 min for observation under stereozoom and for other studies [9].

Preparation of Compound C for Feeding the Flies

The compound **C** was dissolved in 0.5% dimethyl sulfoxide (DMSO). The control used was 0.5% DMSO. The compounds were introduced into the medium at semisolid state and mixed well and were allowed to solidify. Adult males (50 no.) were introduced into the vials containing media.

The toxicity of 0.5% DMSO was checked in the medium by rearing the flies in media with and without 0.5% DMSO. As there was no mortality in the flies reared on medium containing 0.5% DMSO, for all experimental purposes wheat cream agar medium was used with 0.5% DMSO.

Safety evaluation of compound C

To confirm the safety level of the compound **C** before carrying out *in vivo* antioxidant assay it was necessary to find the lethality of the flies to a tested concentration of the compound C. Flies fed with compound at 150 and 300 µg /mL for 7 days and tested for its toxic effect. In each vial 4 mL of control food or food containing the compounds were added. The vials were closed with cotton stoppers. Lethality due to compound was monitored by counting dead flies on 7th day. The result revealed that the compound **C** was safer at 300 µg /mL concentration. Hence this concentration was used for further biochemical investigations.

***In vivo* study**

Study Design

GPA: Normal flies fed with media

GPB: Normal flies fed with media + Irradiated

GP C: Normal flies fed with media + Pq

GP D: Normal flies fed with media + Compound
GP E: Normal flies fed with media+ Compound+ Irradiated
GP F: Normal flies fed with media + Compound + Pq

Stress induction
Irradiation
The male flies of 5-7 days old were taken for studies. They were irradiated using electron beam with 3 Gy dose in polypropylene tubes of 65x25mm size and width 2 mm thick. This was done at Microtron Accelerator centre at Mangalore University, Mangalore, Karnataka, India. Flies were introduced into fresh vials containing standard wheat cream agar medium after irradiation and they were starved for 6 h in order to empty the digestive tract. Survived flies were used for homogenization for biochemical assays. Each assay was repeated thrice [10].

Paraquat Exposure
50 adult flies per replicate (three replicates) were exposed to **C** or Pq or a combination of **C** + Pq for a period of 7 days. Then they were starved for 6 h in order to empty the digestive tract so that none of the compounds would alter the uptake of Pq. Then the flies were transferred to vials containing only filter paper soaked with 15 mM Pq in 5% sucrose solution. Survival was determined after 24 and 48 h. Survived flies were used for homogenization to carry out biochemical assays. Each assay was repeated thrice [11].

Whole body homogenate preparation:
Whole body homogenate of flies was prepared using 0.1M Sodium-phosphate buffer (pH 7.4). After homogenizing, the samples were centrifuged at 2500 x g for 12 min at 4^0C. The supernatant was filtered through nylon mesh (pore size, 10μm) and used for biochemical assays.

Protein estimation
Protein estimation of fly homogenate was carried out using Lowry method [12].This is a biochemical assay, which determines the total level of protein in the homogenate. The total protein concentration is exhibited by a colour change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques.

MDA Assay
Lipid peroxidation was estimated by employing thiobarbituric acid reactive species (TBARS), using Malanoldialdehyde, (MDA) as standard. The reaction mixture contained 500 μl fly homogenate, 1.5 mL acetic acid (pH 3.5, 20%), 1.5 mL of TBA (0.8% w/v), 200 mL sodium lauryl sulphate (SDS) (8% w/v). The mixture was boiled for 45 min, cooled and adducts formed were extracted into 3mL of 1-butanol and centrifuged to remove the precipitate. The absorbance of the supernatant solution was measured at 535nm and quantified as Malanoldialdehyde equivalents using 1,1,3,3- tetramethoxy propane as the standard [13].

Estimation of reduced glutathione
Reduced glutathione (GSH) content was estimated based on a fluorimetric method [14] employing o-phthalaldehyde (OPT). An aliquot of homogenate (stable reduced glutathione with 0.1 M formic acid, 5200 X g for 10 min) was allowed to react with OPT (1 mg/mL in methanol) at room temperature for 30 min and fluorescence measured at excitation of 345 nm and emission at 425 nm.

Antioxidant Enzymes assay
SOD assay
SOD activity was determined by monitoring the inhibition of quercetin auto oxidation. Total volume of 1 mL reaction mixture containing 3-5 µg protein; 0.016M sodium phosphate buffer, pH 7.8; N,N,N,N Tetramethylethylenediamine(TEMED), 8mM and ethylene diamine tetra acetic acid (EDTA), 0.08mM and reaction was started by adding 0.15% Quercetin dissolved in dimethyl formamide. Reaction was monitored for 3 min at 406 nm, expressed as amount of protein required to inhibit 50% of Quercetin auto oxidation [15].

Catalase assay
Catalase activity was determined according to a reported method [16]. To 1mL reaction mixture containing 8.8mM H_2O_2 (3%), 0.1mM sodium–phosphate buffer of pH 7.0. The reaction was initiated by adding an aliquot (equivalent to 10 µg protein). The decrease in the concentration of H_2O_2 was monitored for 3 min at 240 nm.

RESULTS AND DISCUSSION

The DPPH assay

The DPPH assay of the compound **C** at two concentrations showed good radical scavenging activity, 38.5% (25µg/mL) and 55.3% (50µg/mL) in comparison to standard Glutathione which has an activity of 46.7%(25µg/mL) and 61.7%(50µg/mL). The data is given in **Fig. 1**

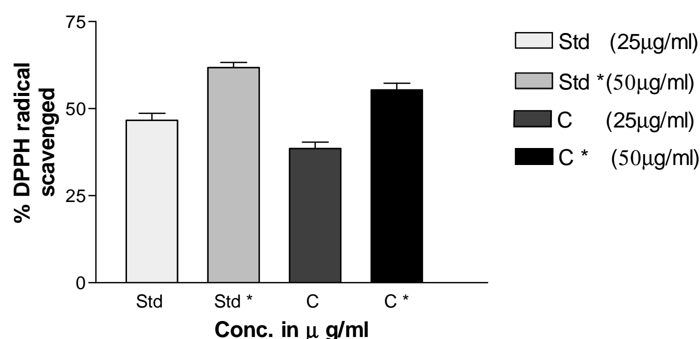


Fig.1: *In vitro* DPPH radical scavenging activity of C

In vivo studies on oxidative stress markers and enzyme antioxidants

The study was focused on the effect of **C** on endogenous levels of oxidative markers in whole body homogenate of flies. The aim was to elucidate the *in vivo* antioxidative stress and neuroprotective efficacy of the synthetic compound **C**. *Drosophila melanogaster* Oregon K flie*s* were used in this study.

The compound was evaluated for safety studies at different concentrations. The result revealed that the compound **C** is safe and non lethal at 300µg/mL. The data is given in **Fig. 2**

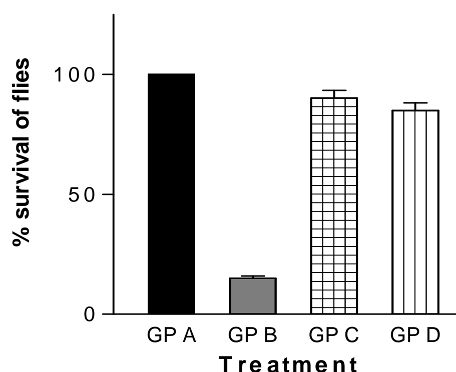


Fig.2: Safety evaluation of compound C for Pq exposure in adult male *Drosophila melanogaster*. (n = 50 flies per replicate, three such replication used for assay)

Malanoldialdehyde (MDA) assay of the compound C

Radiation exposure of biological system results in radiolytic cleavage of water to produce hydroxyl (•OH) and hydrogen (•H) free radicals. The •OH free radical can initiate lipid peroxidation and damages protein, DNA and causes the cell death. Hence the agent that protects such alterations can also prevent radiation damage [17].

Pq (1,1-dimethyl-4,4-bipyridynium dichloride) is a quaternary nitrogen herbicide and highly toxic substance for humans and animals; many cases of acute poisoning and death have been reported [18]. The toxicity of Pq is due to the generation of the superoxide anion which can lead to the synthesis of more toxic reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide [19].

In the present investigation, the flies were exposed to electron beam irradiation at 3Gy and also to 15 mM Pq to induce oxidative stress. The treatment was carried as mentioned in the study design. It was found that there was significant increase in the level of MDA in homogenate of irradiated / Pq treated flies. The increased MDA level is one of the stress markers. This could be due to the attack of free radicals on the fatty acid component of membrane lipids [20]. The level of MDA was elevated significantly in case of normal irradiated flies (67.9 µ mol MDA /mg protein) and Pq treated flies (71.32 µ mol MDA /mg protein) in comparison with normal flies (23.0 µ mol MDA /mg

protein). In case of normal flies and compound **C** fed flies (24.10 μ mol MDA /mg protein) the level of MDA was found to be at basal level. This shows that compound is not inducing any stress to the flies. In case of **C** pretreated and irradiated/ Pq treated flies showed significant decrease (38.17 and 40.11 μ mol MDA /mg protein respectively) in the level of MDA in comparison with irradiated/Pq treated flies. The protective effect of the compound **C** against lipid peroxidation in irradiated/Pq treated flies could be due to the free radical scavenging ability of NH group present in the molecule. The compound showed good antistress ability by decreasing the MDA level in case of irradiated flies than in Pq treated flies. The data is illustrated in the **Fig.3**

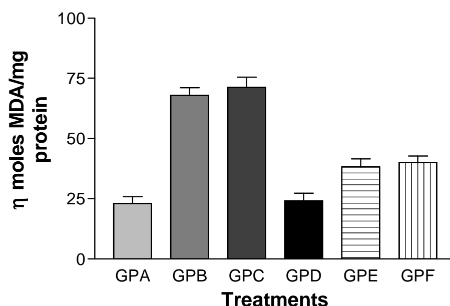


Fig.3: Modulatory effect of C treatment against oxidative stress measured as MDA

Superoxide dismutase (SOD) and Catalase (CAT) assay

The antioxidant enzymes capable of scavenging ROS are SOD and CAT [21].The induction of the phase II enzyme system is an important event of the cellular response during which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they cause damage to cellular macromolecules [22]. In the present study it was observed that there was a decrease in activities of SOD (176.35 and 170.0 units/ mg protein in homogenates of irradiated/Pq treated flies respectively in comparison with normal flies, where it is 187.7 units/ mg protein) and CAT (0.16 and 0.17 μ mol H_2O_2/min/mg protein in homogenates of irradiated/Pq treated flies respectively in comparison with the normal flies, where it is 0.19 μ mol H_2O_2 /min/mg protein). This decrease might be due to radiation/Pq induced free radicals which in turn can impair the antioxidant defense mechanisms. The flies fed with compound **C** restored the enzyme activity to near basal level. The data for SOD and CAT assay are illustrated in the **Fig.4** and **Fig.5** respectively.

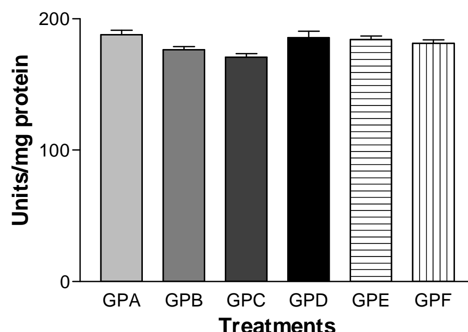


Fig. 4: Modulatory effect of C against oxidative stress measured as SOD activity

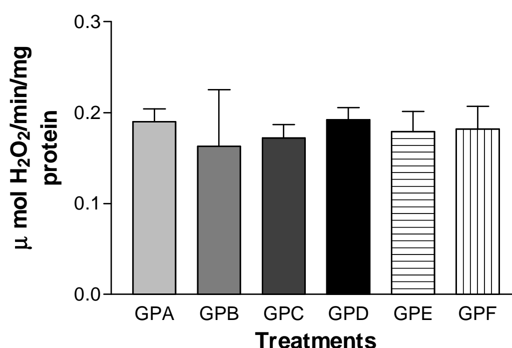


Fig. 5: Modulatory effect of C treatment against oxidative stress measured as Catalase activity

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Reduced glutathione (GSH) Assay

Glutathione (GSH) is a tripeptide that contains an unusual peptide linkage between the amine group of cysteine (which is attached by normal peptide linkage to a glycine) and the carboxyl group of the glutamate side-chain. It is a natural antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteine by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG), also called L(-)-Glutathione. Glutathione is found almost exclusively in its reduced form, as the enzyme that converts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity [23]. In this experiment, the level of GSH was decreased in homogenates of irradiated / Pq treated flies (10.80 and 11.340 µ mol GSH /mg protein respectively). This reduction in the level of GSH could be due to the utilization of GSH by enhanced amount of ROS [24]. The level of GSH was enhanced in case of compound **C** treated flies; even after inducing Oxidative stress by irradiation/ Pq (14.130 and 13.020 µ mol GSH /mg protein respectively). This result indicates the protective efficacy of 1,2,4-Triazole derivative **C.** The result is shown in **Fig.6.**

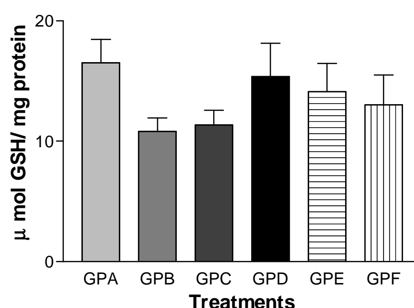

Fig.6: Modulatory effect of C treatment against oxidative stress measured as GSH level

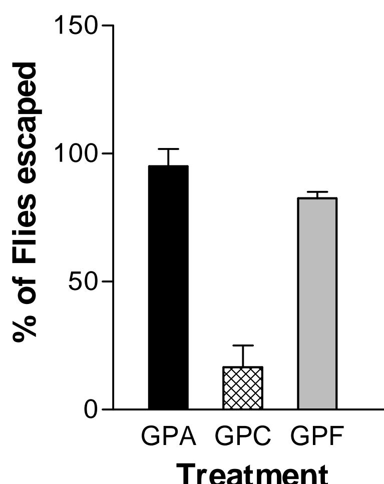

Figure 7 : Modulation of Pq induced locomotor (expressed as percent flies escaped) dysfunction among adult male *Drosophila melanogaster* by C treatment in different combinations

Negative Geotaxis Assay

Pq exposed toxicity effects nigrostriatal dopaminergic neurons and glial cells [25, 26]. It was reported that repeated exposure of *Drosophila* to sub lethal doses of Pq causes selective loss of dopaminergic neurons, including locomotor defects resulting in Parkinson's disease [27]. To determine neuroprotective efficacy of the compounds **C**, geotaxis assay was carried out [28]. In this study Pq treated flies exhibited severe locomotor impairments with 84% of flies stayed at the bottom of the glass column. But by co-treatment with **C,** 82% of flies could escape, exhibiting significantly improved performance. In general, flies co-exposed to **C**, along with Pq appeared to be more active than only Pq treated flies. The data is presented in **Fig. 7.**

Statistical Analysis

Statistical analysis was performed using one way analysis of variance (ANOVA). The values are Mean ± S.D computed from all the triplicates. The data was analyzed by one- way ANOVA followed by post hoc 'Tukey' test to

compare control and treatment groups. $P < 0.05$ was considered as statically significant. Assay was done in triplicate with 50 flies each per replicate. Prism software (Ver. 3.0) was used for all statistical analysis.

CONCLUSION

In the present study, 4-Amino-3-(4-chlorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione (**C**) was chosen to determine antioxidative stress against two stress inducers namely electron beam radiation and using Pq. The induction of stress was confirmed through MDA assay. There was a significant reduction in the level of MDA, restoration of GSH level and modulatory effect on enzyme antioxidants level in the compound **C** pre treated and stress induced flies. The compound **C** is exhibiting good activity against radiation induced stress markers and enzyme antioxidants than Pq induced stress. This result shows that same antioxidant molecule is exhibiting variable level of activities against different sources of stress.

Abbreviations

C 4-Amino-3-(4-chlorobenzyl)-1*H*-1,2,4-triazole-5(4*H*)-thione
Gy Gray
TLC Thin layer chromatography
EBR Electron beam radiation
Pq Paraquat
ROS Reactive oxygen species
DMSO Dimethyl sulfoxide
DPPH 1,1-diphenyl-2-picryl-hydrazyl
DTNB 5,5'-dithiobis nitro benzoic acid
GSH Reduced Glutathione
MDA Malanoldialdehyde
SOD Superoxide dismutase
CAT Catalase

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