

Neuroprotective and Antioxidant Efficacy of Hydroalcoholic Extracts of Pomegranate Peel

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Abstract : Pomegranate (*Punica granatum*) peel was extracted with water: ethanol (1:1) at room temperature and the extract was evaluated for antioxidant potential against AAPH induced lipid peroxidation *in vitro* in mice synaptosomes. In addition, its neuroprotective efficacy against rotenone-induced mortality, motor deficits and oxidative stress in *Drosophila melanogaster* was also assessed. Extract showed high radical scavenging activity (RSA) as determined by DPPH and ABTS methods. Reconstituted aqueous extract showed inhibitory effect against AAPH induced lipid peroxidation in rat brain homogenate and synaptosomes. The extract also showed inhibition of acetylcholinesterase (AChE) activity in the brain homogenate ($IC_{50} = 56.2 \mu\text{g/ml}$). The extract provided significant protection against rotenone, as determined by percentage mortality and escape tests. The experiments conducted on head and body of the flies indicate the protection offered by extracts, as determined by content of thiols and hydroperoxides and the levels of Catalase, superoxide dismutase (SOD), thioredoxin reductase (TRx), glutathione-S-transferase (GST) and acetylcholine esterase (AChE).

INTRODUCTION:

The rind of pomegranate (*Punica granatum*) is used as medicine or traditional remedy against diarrhea, dysentery and intestinal parasites. Different parts of pomegranate have been known as a reservoir of bioactive compounds with potential biological activities. Pomegranate, especially the leaves, peels of pomegranate, decreased the dyslipidemia of obesity and cardiovascular risk factors (Lei et al., 2007). Anti-parasitic, anti-microbial and antioxidant activities of pomegranate leaves extracts were reported (Egharevba et al., 2010; El-Shennawy et al., 2010 and Wang et al., 2013).

Several studies were reported the ability of pomegranate PPE to fight obesity (Al-Muammar and Fozia Khan, 2012), cancer and other human diseases (Lansky and Newman, 2007). (Singh, et al., 2002). The antioxidant activity, Phenolics in

pomegranate leaves are thought to contribute in their health benefits (Lan et al., 2009). To our knowledge, a lot literature for anti-inflammatory, anti-cholinesterase inhibitory and cytotoxic activities of *P. granatum* peel.

In the present study, an attempt has been made to study the antioxidant profile and neuromodulatory properties of ethanolic (or ethanol water) extract of pomegranate peel powder by using various *in vitro* methods, rat brain synaptosomes and adult *Drosophila melanogaster*. Modulatory efficacy of PPE against Rotenone induced oxidative stress markers in the head and body regions of *Drosophila melanogaster*. The results of the studies presented in this investigation report would be useful in utilizing PPE in the various products at appropriate amount and also will be helpful in biomedical sciences (Parkinson's Diseases).

2. MATERIALS AND METHODS

Collection of and processing of raw material:

Pomegranate fruits were purchased from the local supermarket and were cut, rind, mesocarp, and capillary membranes were manually separated from the edible part manually. Rind was cut into square shape of approximately 1cm X 1cm size and was sun dried. Dried samples were ground to moderate coarse powder using mixer-grinder. The powdered samples were packed in polythene covers and stored in desiccators at room temperature for future experimentations.

Chemicals

20,70-Dichlorofluorescein (DCF), 20,70-dichlorofluorescein diacetate (DCFH-DA), thiobarbituric acid (TBA), 1,1,3,3-tetramethoxy propane (TMP), rotenone (ROT), Rhodamine 123 (Rh123) and other fine chemicals were procured from M/s Sigma Chemical Co., St. Louis, USA. 2,4-

Dinitro phenyl hydrazine (DNPH), nicotinamide adenine dinucleotide reduced (NADPH), 1,1-dithio nitro-bi-benzoic acid (DTNB), 1-chloro 2,4-dinitro benzene (CDNB) and all other chemicals were of analytical grade and procured from Sisco Research Laboratory Chemicals, India.

Extraction

Extraction of peel was carried out in solvents, like water, ethanol, and ethanol: water (1:1) as described by Sharma et al. (2014). 1g of rind powder was subjected to overnight extraction at room temperature in 10 ml of respective solvent/solvent systems with intermittent shaking, centrifuged at 3000 rpm for 5 mins and filtered using Whatman filter 2 (Singh et al., 2002).

Radical scavenging activity (RSA) by DPPH assay:

0.2 ml methanol and 5 ml of 100 μ M methanolic DPPH (100 μ M) was used as control. Different aliquots (0.1 and 0.2 ml) of various extracts were taken for analysis. 5 ml of DPPH was added to all the samples, vortexed and incubated at room temperature for 20 minutes under dark. Absorbance was read at 517nm using UV spectrometer, UV-1800 and recorded (ResatApak et al., 2008). The percentage of antioxidant activity was determined using following formula:

$$\%RSA = \frac{(\text{absorbance of control} - \text{absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

RSA by ABTS assay

ABTS⁺ radical cation was produced by reacting 7mM aqueous ABTS with 2.6 mM potassium persulfate in equal quantity and keeping it in the dark at room temperature for 16 hrs. Blue-green ABTS⁺ was formed at the end of this period. The solution was diluted with ethanol to an absorbance of 0.70 \pm 0.02 at 734 nm and 405nm, the wavelength of maximum absorbance in the visible region, by Shimadzu UV spectrometer UV-1800 (Roberta Re et al., 1999). As described above, 0.1 and 0.2 ml PPE was taken in separate tubes and to these, 5ml ABTS was added and incubated at room temperature for (?). The OD was taken at 734nm and the percentage RSA was determined using following formula.

$$\%RSA = \frac{(\text{absorbance of control} - \text{absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

Estimation of total polyphenols

Aliquots of different volumes of standard tannic acid solution and PPE in a total volume of 1.0 ml were taken in different test tubes and made upto 10ml with water. Aliquots of samples (0.1ml, 0.2ml, 0.4ml) were also taken and made upto 10ml using water. 0.5ml of Folin Ceocalteau reagent was added to all the tubes followed by the addition of 1.0ml of saturated sodium carbonate solution. The reaction mixture was mixed and incubated at room temperature for 30 minutes. A blank was prepared without tannic acid solution. The absorbance was read at 760nm and the total polyphenol content of the samples were estimated and expressed as Tannic Acid equivalents.

Animals and Care

Adult (8–9 weeks old) Swiss albino male mice (35.1 \pm 3.2 g) were drawn from the stock colony of Institute Animal Facility and were housed (4 Nos./ cage) in rectangular polypropylene cages in a controlled atmosphere with a 12 h light/dark cycle. These were provided commercial chow diet and water *ad libitum*. All the experiments were conducted strictly in accordance with approved guidelines by the Institute Animal Ethical Committee (IAEC, Registration number: 49/1999/CPCSEA) regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Government of India, India. Handling and care of animals was carried out strictly according to the standard guidelines laid by the IAEC.

Effect of PPE against AAPH -induced lipid peroxidation in striatum

Mice were sacrificed using general anesthesia and brain was dissected to separate and collect striata (St) at 4°C. These were homogenized (10% w/v) in ice cold phosphate buffered saline (PBS, 0.1M, pH 7.4, 0.8% NaCl) and centrifuged at 4,000rpm for 10 min at 4°C to obtain post nuclear supernatant. The supernatant was separated and recentrifuged (10,000 rpmX10 min. at 4°C) to obtain the supernatant and crude synaptosomal pellet. The synaptosomes were washed in HEPES buffer and resuspended in the same. Synaptosomes and homogenates were freshly prepared and used for experiments. St homogenates / synaptosomes were challenged with varying concentrations of AAPH (50, 100, 200 and 500 μ M) *in vitro* (30hr, 37°C) in Krebs-Ringer bicarbonate (KRB) solution to obtain a concentration-related response. To measure the efficacy of PPE, St-homogenates/ synaptosomes were treated with

different concentrations (50, 100 and 200 µg/ml) of PPE were incubated with or without AAPH (200µM) for 1hr. The extent of malondialdehyde was measured by quantifying thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979).

Effect on Acetylcholinesterase

AChE inhibitory activities were measured using Ellman's method (Akkol et al., 2012) with modifications. 50 µl of 0.1 M sodium phosphate buffer (pH 8.0), 25 µl of AChE (or BuChE) solution, 1, 5, 10, 25, 50 µl PPE and 125 µl of DTNB were added in a 96-well microplate and incubated for 15 min at 25°C. The reaction was initiated with the addition of 25 µl of acetylthiocholine iodide and its hydrolysis iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, at 412 nm. The concentration of the extracts which caused 50% inhibition of AChE (IC₅₀) was calculated by nonlinear regression analysis. The % inhibition was calculated from $(1 - S/E) * 100$, where E and S were the respective enzyme activities without and with the test sample, respectively. Ethidium bromide was used as positive control.

Neuroprotective efficacy of PPE in *Drosophila*

Drosophila culture and husbandry

D. melanogaster, wild (Oregon K) derived from the fly laboratory of our institute which were maintained under standard conditions were used throughout the study. Flies were maintained at 22±1°C and 70–80% relative humidity and fed on a standard wheat flour–agar diet with yeast granules as the protein source (Hosamani and Muralidhara, 2009). Age synchronized adult (9–10 days old) male flies (50/replicate; 3 replicates/group) were introduced into glass vials with 2ml medium containing the test compounds.

Reconstitution of PPE: 5 ml PPE was evaporated to dryness (0.5g dry extract) and was resuspended in 2ml water (250mg/ ml) for using in the *Drosophila* experiments.

Treatment protocol

In a preliminary study, adult male flies were maintained on enriched medium containing 0.05, 0.1, 0.2 and 0.4% PPE to assess its modulatory effect on the endogenous levels of oxidative markers in head and body regions of flies (n = 50/ replicate; 2

replicates per group). The propensity of PPE to modulate ROT-induced mortality, locomotor dysfunction, oxidative stress and neurotoxicity was determined in independent experiments employing a co-exposure paradigm. The concentration of ROT (500 µM) was selected based on findings from our lab and other studies (Girsih and Muralidhara, 2012; Hosamani et al., 2010; Saini et al., 2010). Flies were monitored regularly for the incidence of mortality and locomotor deficits (negative geotaxis assay).

Measurement of locomotor deficits

The motor function was assessed using a negative geotaxis assay (Feany and Bender, 2000). Flies were introduced into a graduated flat bottom glass tube (l 25 cm; d 2 cm). The tube was gently tapped to the bottom of the tube and observed for 60 sec for the climbing activity (25 flies/trial; 3 trials/replicate). Locomotor behavior was expressed as percent flies escaped beyond a minimum distance of 10 cm in 20 s.

Preparation of homogenates from head and body regions of *Drosophila*

After the treatment protocol, the flies were anaesthetized and head were separated with a sharp edge immediately. Both head and body regions were homogenized in Phosphate buffer (0.1M, pH 7.4) and centrifuged at 4000 rpm for 10 min at 4°C. The supernatants were used for the assays. Amount of protein in the fractions was estimated (Lowry et al., 1951) by incubating an aliquot of the sample with Folin–Ciocalteu's reagent in an alkaline medium (30 min) and measuring the optical density at 750 nm using a UV–visible Spectrophotometer (Shimadzu). The amount of protein was quantified using a bovine serum albumin standard curve.

Measurements of hydroperoxides (HP)

HP were determined based on ferrous ions mediated oxidation of xylenol orange (Wolff, 1994). An aliquot (20 mg protein cytosol/mitochondria) was allowed to react with FOX1 reagent (xylenol orange 100mM, ammonium ferrous sulphate 250 mM, sorbitol 100 mM) prepared in 25 mM H₂SO₄ (30 min) at room temperature. The color developed was quantified at 560 nm ($\epsilon = 2.2 * 10^5/M/cm$) and expressed as nmol hydroperoxides (HP)/mg protein.

Measurement of thiols

Thiols were estimated (Ellman 1976) by incubating an aliquot of the sample (0.05mg protein) with DTNB (0.02mM) in Phosphate buffer (0.1M, pH 8.2) for 20 min and measuring the absorbance at 412 nm. Thiol levels were calculated using the MEC 13.6/mM/cm and expressed as nmol thiols/ mg protein.

Thioredoxin reductase (TR) assay

TR activity (Luthman and Holmgren, 1982) was measured by monitoring the reduction of DTNB at 412 nm, in potassium phosphate buffer (0.1 M, pH 7.0, containing EDTA 10mM, NADPH 0.2mM). The activity was expressed as nmol substrate reduced/min/mg protein ($\epsilon = 13.6/\text{mM}/\text{cm}$).

Glutathione reductase (GR) assay

GR activity was measured (Carlberg et al., 1985) by the addition of cytosolic aliquot (0.2 mg protein) to phosphate buffer (0.2 M, pH 7.0 containing EDTA 2mM, oxidized glutathione 20mM and NADPH 2mM). The decrease in the absorbance at 340 nm due to oxidation of NADPH was monitored for 3 min and the activity was expressed as nmol NADPH oxidized/min/ mg protein ($\epsilon = 6.22/\text{mM}/\text{cm}$).

Glutathione-S-transferase (GST) assay

Glutathione-S-transferase (GST) activity was quantified (Guthenberg et al., 1985) by monitoring the conjugation of glutathione to CDNB at 340 nm. The reaction was started by adding a cytosolic aliquot (0.01 mg protein) to the phosphate buffer (0.1 M, pH containing EDTA 0.5mM, CDNB 0.075 mM, GSH 0.05 mM). The increase in the optical density at 340 nm was recorded over 3 min and the activity expressed as nmol conjugate formed/min/mg protein ($\epsilon = 9.6/\text{mM}/\text{cm}$).

Acetylcholinesterase (AChE) assay

AChE activity was estimated according to Ellman et al. (1961). The reaction was initiated by the addition of acetylthiocholine iodide (ATCI, 1.95 mM) to the phosphate buffer (0.1 M, pH 8.0) containing DTNB (2.5 mM) and cytosolic aliquot (0.01 mg protein). The change in absorbance was monitored at 412 nm for 3 min. The enzyme activity was expressed as nmol substrate hydrolysed/min/mg protein.

AChE inhibitory activities were measured using Ellman's method, as previously reported (Akkol et al., 2012) with modifications. In this study, 50 μl of

0.1 M sodium phosphate buffer (pH 8.0), 25 μl of AChE (or BuChE) solution, 1,5,10,25,50 μl PPE and 125 μl of DTNB were added in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 25 μl of acetylthiocholine iodide. The hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm. The concentration of the extracts which caused 50% inhibition of the AChE activity (IC₅₀) was calculated by nonlinear regression analysis. The percentage of inhibition was calculated from $(1 - S/E) * 100$, where E and S were the respective enzyme activities without and with the test sample, respectively. Ethidium bromide was used as positive control.

Catalase assay

Catalase activity was estimated (Aebi, 1984) by adding an aliquot of cytosol (0.25 mg protein) to phosphate buffer (0.1 M, pH7.4, containing H₂O₂ – 10 mM). The decomposition of H₂O₂ was monitored at 240 nm and activity was expressed as nmol substrate/min/mg protein ($\epsilon = 44.2/\text{mM}/\text{cm}$).

Superoxide dismutase (SOD) assay

SOD activity was measured indirectly by monitoring the inhibition of quercetin autooxidation (Kostyukand Potapovich, 1989). Reaction mixture consisted of phosphate buffer (0.016 M, pH 7.8, containing TEMED 4 mM and EDTA 0.04 mM) quercetin (100 mM) was added and the rate of its autooxidation was monitored at 406 nm for 3 min. The ability of the test sample to inhibit quercetin oxidation by 50% was defined as one unit of the enzyme and activity was expressed as units/mg protein.

Results and discussion

EXTRACTION AND ANTIOXIDANT PROFILING of PPE

Extracts were made using different solvents like water, ethanol, water: ethanol (1:1) and 0.1ml,0.2ml aliquots of the extract was taken and the %RSA of each were determined using DPPH assay But no significant difference was seen in the %RSA .

Table No. 1

Extracts	%RSA (1:200)	
	0.1ml	0.2ml
Water	30.61	60.61
Ethanol	18.51	36.59
Water: Ethanol	27	50.80

Extraction continued with water: ethanol (1:1) ratio

2. AAPH-INDUCED IN VITRO LIPID PEROXIDATION

2 (a). DIETARY ANTIOXIDANTS

Antioxidant-rich fractions were extracted from pomegranate (*Punica granatum*) peels and seeds using ethyl acetate, methanol, and water. The extracts were screened for their potential as antioxidants using various in vitro models. (Singh, *et al.*,2002). *The antioxidant activity of pomegranate peel extracts* was evaluated by ABTS and DPPH methods

2(c). Lipid peroxidation

2,2'-Azobis(2-amidinopropane) (AAPH)

PPE is inhibiting its activity at different conc. at different levels.

3. ACETYLCHOLINEESTERASE INHIBITION

3(a). Acetylcholinesterase inhibition

Alzheimer’s disease (AD) is the most common form of neurodegenerative disease and characterized by memory dysfunction. Reduction of acetylcholine levels in the brain is the most notable biochemical change in AD (Loizzo *et al.*, 2009). Therefore, AD can be treated by use of agents which restore the level of acetylcholine through inhibition of both two major form of cholinesterase: acetylcholinesterase(AChE) and butrylcholinesterase (BChE) (Jaen *et al.*, 1996). Tacrine, rivastigmine and galanthamine are used as cholinesterase inhibitors for treatment of AD. However, these compounds have been reported to have their adverse effects such as, hepatotoxicity and gastrointestinal disturbances (Lee *et al.*, 2011; Schulz, 2003; Melzer, 1998). Therefore, safe and active AChE inhibitors particularly from natural products have been gaining more attention in recent decades.

4. NEUROPROTECTIVE EFFICACY

4(c). Parkinson's disease

1.1 EXTRACTION AND ANTIOXIDANT PROFILING

3.1.2 Determination of RSA by DPPH Assay

3.1.3 Extraction effect of shaking and without shaking

Table no. 2

S.NO.	VOL. of PPE extract (ml)	%activity
With Shaking		
1.	0.1	41.41
2.	0.2	68.78
Without shaking		
1.	0.1	41.41
2.	0.2	70.22

RESULT: As there was no significant difference in the activity of extract performed with or without shaken the extraction was further continued without shaking which is energy saving procedure.

3.2 ABTS assay

Table.3 ABTS (1:500)

S.NO.	VOL. of PPE extract (ml)	% activity
PPE		
1.	.1	37.15
2.	.2	77.44

RESULT: At 1:500 dilutions 0.1 ml of PPE shows 37% RSA

AAPH-INDUCED LIPID PEROXIDATION IN VITRO

The propensity of PPE to inhibit AAPH induced lipid peroxidation in brain homogenates and synaptosomes was determined. AAPH exposure (100-1000µM) resulted in a concentration-related formation of MDA (100µM-18%, 200µM-51%, 500µM-130% and 1000µM-215%, data not shown). PPE *per se* not only reduced the formation of MDA, but also significantly inhibited the AAPH (200µM)-induced lipid peroxide formation in the homogenates (PPE 12.5µg-35%, Fig 1) as well as in synaptosomes (PPE 12.5µg-55%, Fig 2).

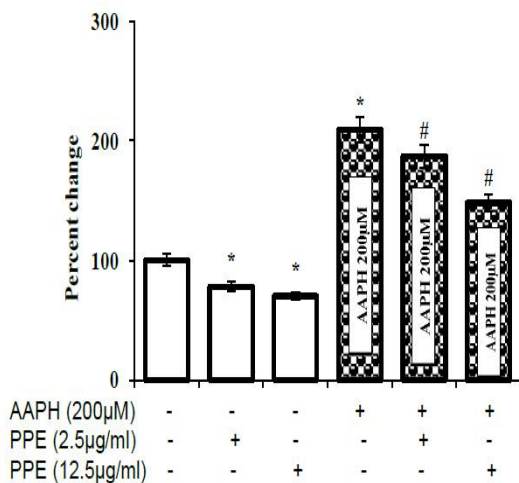


Fig 1. Inhibitory effect of ethanolic extract pomegranate peel extract (PPE) on malondialdehyde levels *in vitro* in mice brain homogenate. Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey’s test. * P<0.05, as compared to saline control ; # P<0.05, as compared to AAPH alone.

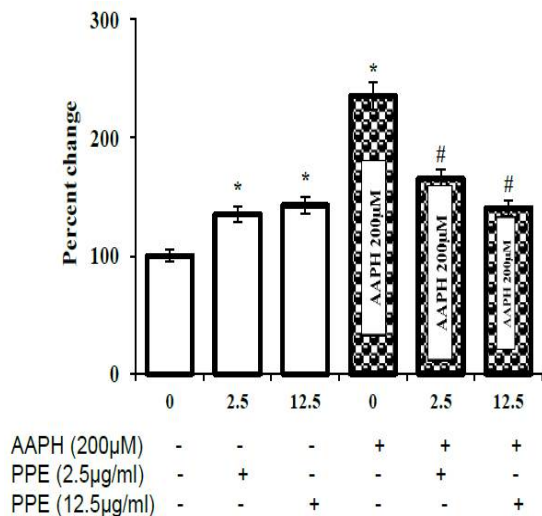


Fig 2. Inhibitory effect of ethanolic extract pomegranate peel extract (PPE) on malondialdehyde levels *in vitro* in mice brain homogenate. Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey’s test. * P<0.05, as compared to saline control ; # P<0.05, as compared to AAPH alone.

3.3 . Result Analysis: PPE exhibit a concentration dependent anti-AChE with an IC50 value of 56.82 ± 0.73 µg/ml (Ethidium bromide 0.5 ± 0.03mM).

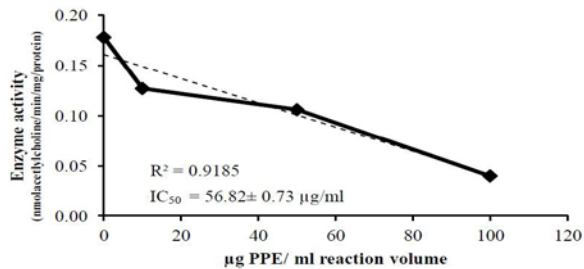


Fig 1. Acetylcholinesterase inhibition by PPE. Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey’s test.

Neuroprotective efficacy of PPE against Rotenone (ROT) in Drosophila

ROT exposure (500µM) induced lethality was evident from day 3 onwards and increased with duration of exposure and terminally (by day 6) the cumulative mortality was 55%. Coexposure with PPE resulted in significant protection against ROT-induced mortality as depicted in Fig 1. While PPE at lowest concentration (PPE1) provided only a moderate (15%) protection, robust protection was evident at higher concentrations (PPE2 –60%; PPE3 - 70%; PPE4 –95%).

Protection against Rotenone (ROT) –induced locomotor deficits

ROT (500µM) treated flies exhibited severe (70%) impairment of locomotor activity by day 6 as evident by the decreased number of flies escaping the 10cm height of the glass column. Interestingly, co-exposure with PPE resulted in marked improvement in locomotor deficits and the effect was concentration-dependent suggesting robust protection offered by PPE(Fig 1).

Efficacy of PPE to attenuate ROT induced oxidative stress

While ROT exposure enhanced the levels in HP in head (20%) and body regions (43%), PPE significantly diminished the levels in a concentration-dependent manner (Fig 1). Though ROT did not affect TSH levels in head and body regions, coexposure to PPE elevated TSH levels at higher concentrations tested.

ROT affected the CAT activity levels were reduced significantly among the head (80%) and body regions (20%) of flies while coexposure with PPE attenuated

the ROT effect. Further, ROT caused significant diminution (61%) in the activity levels of SOD in the body region which was attenuated by PPE at lower concentration while higher concentrations of PPE did not alter the ROT-effect.

While the activity levels of TR were reduced with ROT exposure in head (20%) and body region (16%), PPE supplements restored the activity levels in a concentration-dependent manner (Fig 2). GST activity levels were differentially affected in head and body regions of ROT flies. There was a significant decreased (18%) activity levels in head regions while the levels were increased (16%) in body regions (Fig 3).

Modulatory efficacy of PPE against ROT induced cholinergic function

The activity levels of AChE were significantly elevated in head (22%, Fig 3) and body (13%) regions among ROT flies, while the levels were restored to normalcy by PPE.

DISCUSSION

Free radicals are produced in the body, primarily as a result of aerobic metabolism and their accumulation over the time, causes neurodegeneration in specific areas of brain leading into Alzheimer's and Parkinson's disease as well as aging (Halliwell, 2006). Therefore, a putative therapy that attenuates both redox balance and antioxidant defenses may serve as an ideal option to attenuate oxidative stress mediated neurodegenerative conditions. The *Drosophila* model in the recent past has emerged as an effective primary screen for several phytochemicals. Since, ROT is a well-known redox disruptor; It was chosen as a model in flies in our studies. Recently, the role of oxidative stress and dopamine as the key molecules responsible for the specific loss of dopaminergic neurons in PD in both familial and sporadic forms of the disease has been demonstrated (Sakka et al., 2003; **Bayersdorfer et al., 2010**). In our study, ROT induced lethality as well as significant locomotor deficits in accordance with previous findings from this lab (Hosamani and Muralidhara, 2009). Though the precise mechanism of ROT- induced lethality is not well established, it is however attributed to the specific sensitivity of dopaminergic neurons to oxidative damage. Previous findings with L-DOPA and melatonin clearly suggest that ROT- induced neurotoxicity involves oxidative stress mechanisms (Coulom and Birman, 2004). Additional support to this hypothesis stems from

several observations made in the brain samples of PD patients (Beal, 2005). In this study, exposure of flies to ROT caused a severe oxidative insult as evident by the elevated levels of oxidative markers (HP) and significant alterations in the activity levels of antioxidant enzymes. This finding is consistent with previous observations of our laboratory in both rodent and fly models (**Girish and Muralidhara, 2012**; Hosamani et al., 2010; **Shinomol et al., 2011**). ROS and HP not only act as endogenous oxidants but serve as second messengers activating different enzyme cascades including protein phosphatase cascades directed towards inflammatory gene expression (**O'Loghlen et al., 2003**, Hao et al., 2006, Stone et al., 2006).

PPE was shown to be a potent antioxidant as evident by the decreased levels of ROT -induced oxidative markers and restoration of activity levels of antioxidant enzymes in both head and body of flies. Hence the significant protection rendered by PPE against ROT -induced lethality, locomotor deficits as well as the oxidative markers strongly suggests its primary role in modulating a severe oxidative burst *in vivo*. In the present model, PPE not only caused significant elevation in the activity levels of endogenous TR enzyme, but effectively restored the ROT-induced reduction in the activity levels in both head and body suggesting its efficacy to modulate the non-glutathione redox system *in vivo*. TR is a selenium dependent enzyme and is well known to protect cells against oxidative injury (Brown and Arthur, 2001). Slight elevation in the TR levels is associated with hormetic effects of phytochemicals (Calabrese et al., 2008). TR is one of the four vitagenes which plays a significant role in maintenance of redox balance in the cell. There exists an active cooperation in mammalian system between glutathione and thioredoxin systems, which provides regulation of cellular redox in various processes (**Cho et al., 2003**). Both the antioxidant systems are known to provide reducing equivalents for a variety of redox processes which are directly linked to the regulation of the cellular redox state (Sun et al., 2001). Hence It could be speculated that enhanced TR activity may play a predominant role in the neuroprotective effect of PPE supplements in the present model. However, further studies are required to substantiate this specific effect of PPE in cell models.

4. CONCLUSION

The findings of this study clearly suggest that PPE supplements not only significantly modulate the endogenous levels of oxidative markers among flies,

but also offset ROT-induced oxidative stress and mitochondrial dysfunction. Furthermore, improved motor function among flies provided with PPE-enriched diet provide clear evidence of its neuromodulatory propensity. Although we cannot

precisely attribute these protective effects to any specific bioactives from PPE, our data nevertheless suggest the need to further understand the mechanisms underlying the neuromodulatory property of PPE.

5. Results

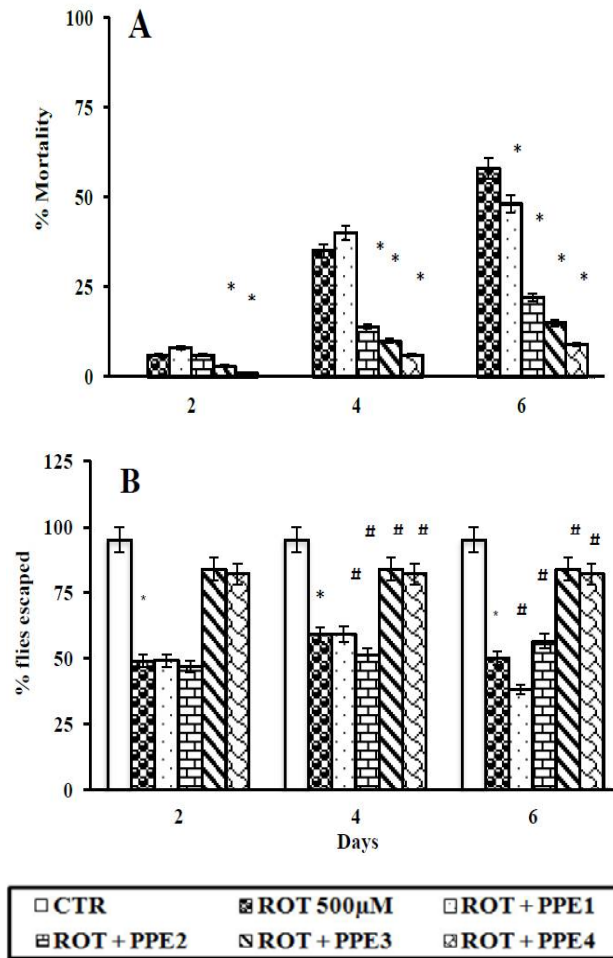


Fig 1. Protective efficacy of Pomegranate peel extract (PPE) against ROT-induced mortality (A) and motor deficits (B) in adult *Drosophila melanogaster*. Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. * P<0.05, as compared to control ; # P<0.05, as compared to ROT alone. ROT 500µM- Rotenone; PPE- Pomegranate Peel Extract; PPE1-0.05%; PPE2-0.1%; PPE3-0.2%; PPE4-0.4%

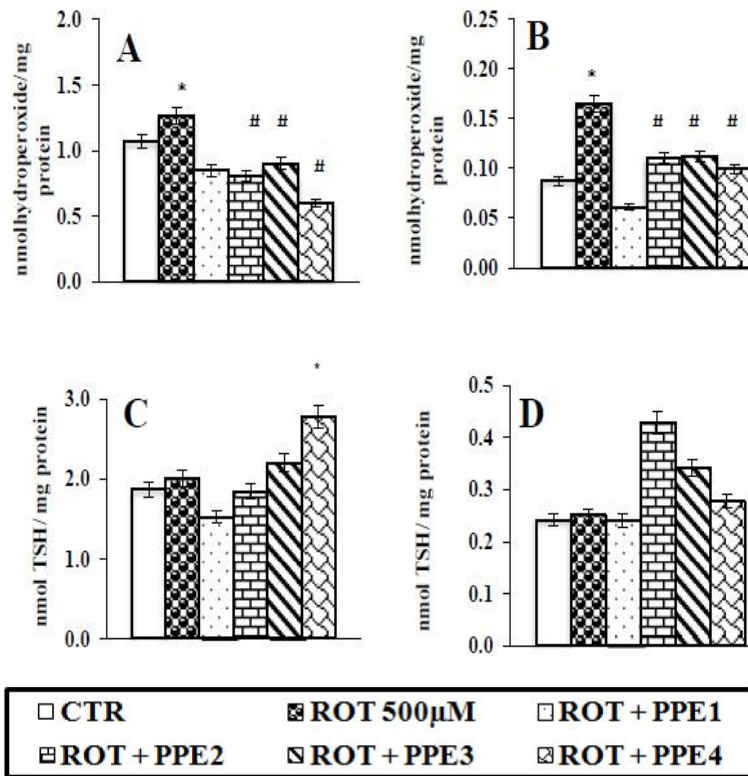


Fig 2. Protective efficacy of Pomegranate peel extract (PPE) against ROT-induced alterations in Hydroperoxides levels (A-head; B-body) and Thiols content (C-head; D-body) head and body regions of adult *Drosophila melanogaster*. Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey's test. * $P < 0.05$, as compared to control ; # $P < 0.05$, as compared to ROT alone.

ROT 500µM- Rotenone; PPE- Pomegranate Peel Extract; PPE1-0.05%; PPE2-0.1%; PPE3-0.2%; PPE4-0.4%.

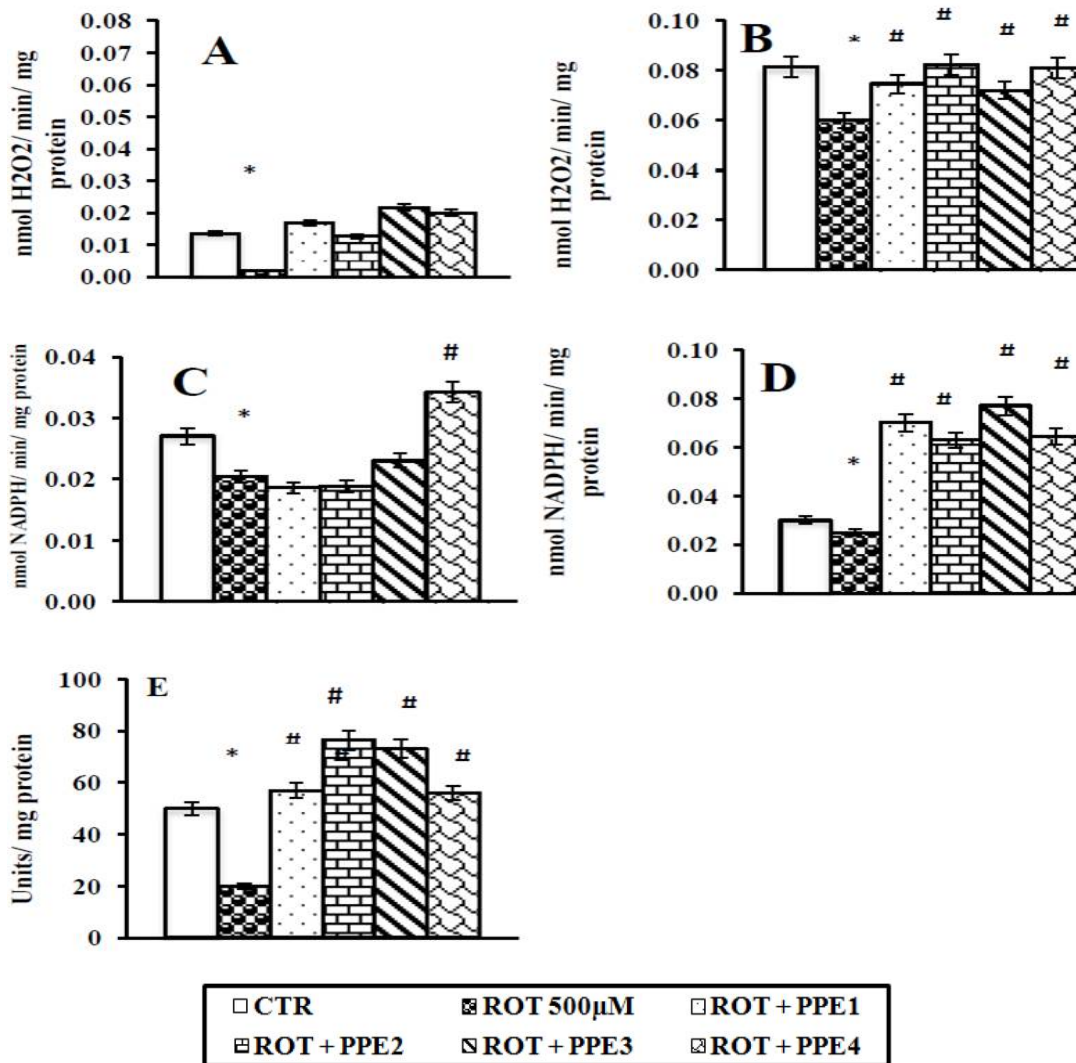


Fig 3. Protective efficacy of Pomegranate peel extract (PPE) on the perturbations in the activity levels of catalase (A-head; B-body), Superoxide dismutase (E-body), Thioredoxin reductase (C-head; D-body) among adult *Drosophila melanogaster* exposed to rotenone. Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey’s test. * P<0.05, as compared to control ; # P<0.05, as compared to ROT alone. ROT 500µM- Rotenone; PPE- Pomegranate Peel Extract; PPE1-0.05%; PPE2-0.1%; PPE3-0.2%; PPE4-0.4%

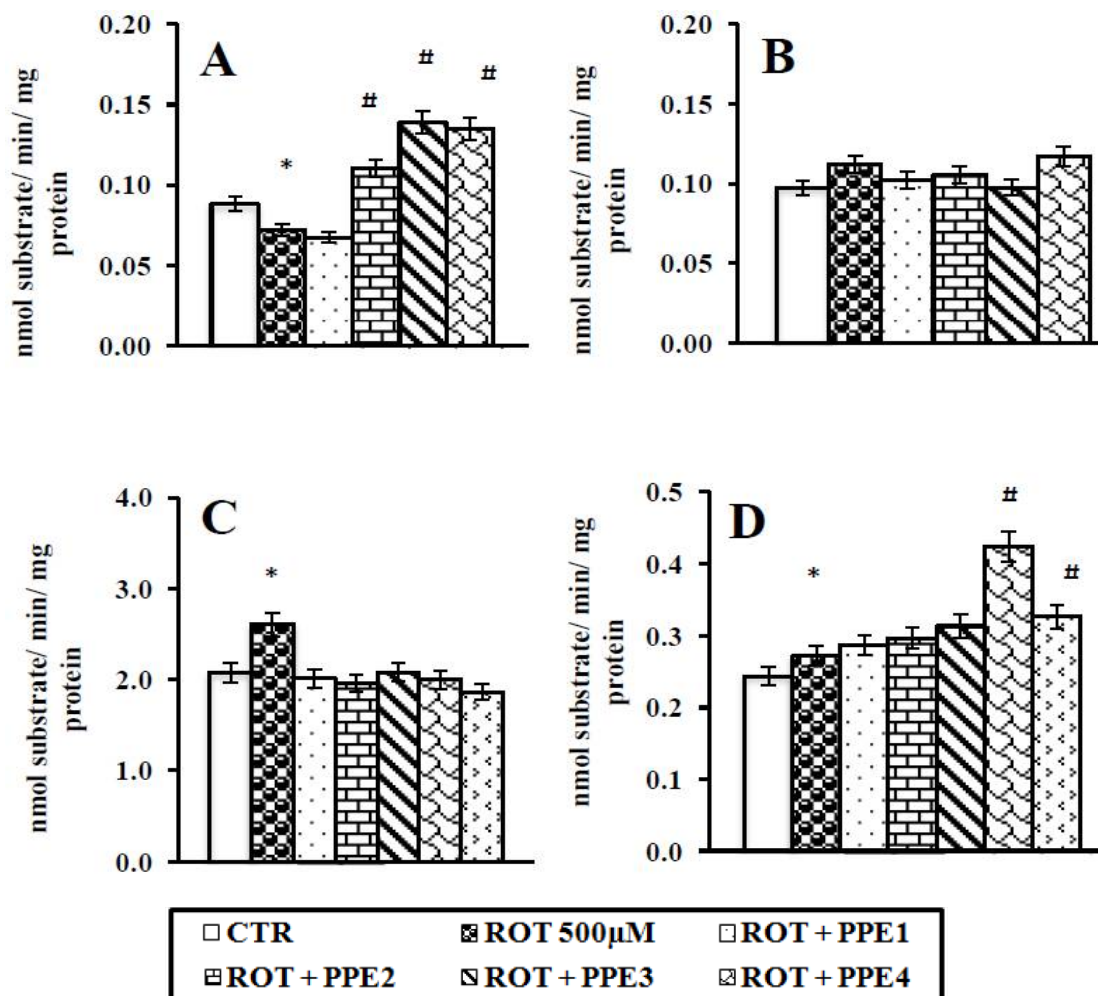


Fig 4. Modulatory propensity of Pomegranate peel extract (PPE) on the alteration in the activity levels of GST and (Glutathione-S-transferase) (A-head; B-body), AChE (acetylcholinesterase) (C-head; D-body) regions of adult *Drosophila melanogaster* exposed to ROT(500µM). Data analyzed by non-parametric one-way ANOVA followed by post hoc Tukey’s test. * P<0.05, as compared to control; # P<0.05, as compared to ROT alone. ROT 500µM- Rotenone; PPE- Pomegranate Peel Extract; PPE1-0.05%; PPE2-0.1%; PPE3-0.2%; PPE4-0.4

SUMMARY AND CONCLUSIONS

In the present investigation, an attempt was made to evaluate the biological properties of pomegranate peel extract (PPE). PPE prepared by using water: ethanol (1:1) at room temperature shows inhibitory effect against AAPH induced lipid peroxidation in rat brain homogenate and synaptosomes. The extract also shows inhibition of acetylcholinesterase activity in the brain homogenate. (IC50 =56.2µg/ml). In *D. melanogaster* model, the extract provides significant protection against rotenone as determined by % mortality and % escape tests. The experiment carried out separately for head and body of the flies also confirms the protective effect of PPE as determined

by content of Thiols and hydroperoxides the levels of Catalase, SOD,TRx ,GST and AChE.

Conflict of interest

The authors declare that there are no conflicts of interest.

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