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Research Article

# PROTECTIVE ROLE OF FENUGREEK LEAF EXTRACT AND QUERCETIN AGAINST PETROL EXHAUST NANOPARTICLE INDUCED LIPID PEROXIDATION AND OXIDATIVE STRESS IN RAT ERYTHROCYTES *IN VITRO*

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**ABSTRACT**

**Objective and Methods:** Inhaled nanoparticles of airborne origin enter the bloodstream and react with the circulatory system leading to cardiovascular and systemic events. Fenugreek (*Trigonella foecum-graecum)* and quercetin are known for their antioxidant and antitoxic potentials. To understand this mechanism we studied in detail the interaction of petrol exhaust nanoparticles (PEPs) treated with or without aqueous fenugreek leaf extract (EXT) or quercetin (Q) in rat erythrocytes (red blood cell [RBC]) *in vitro*.

**Results:** Incubation of RBCs with PEP alone (1, 10 and 100 µg/ml) for 60 minutes caused highest hemolytic activity (~up to 46%). Erythrocyte susceptibility to lipid peroxidation (*in vitro*) measured by malondialdehyde content (MDA) showed significant dose-dependent increase in MDA. The superoxide dismutase (SOD), and catalase (CAT) activities were found to significantly decrease ($p<0.05$) in a dose dependent manner when compared to the control. The glutathione S transferase (GST) and glutathione (GSH) activities were found to increase significantly ($p<0.05$) in the PEP treated groups. Prior treatment with the EXT or Q showed replenishment of antioxidant levels and brought the values to near normal, hence proving the protective effect of EXT or Q.

**Conclusion:** The phytochemicals in the EXT could play a key role in protecting the RBC cells against the PEP induced toxicity. Thus, the study demonstrates PEP induced erythrocyte toxicity in a dose-dependent manner and the alleviative effects of the EXT and Q.

**Keywords:** Petrol exhaust particles, Red blood cells, Quercetin, Fenugreek, Oxidative stress.

## INTRODUCTION

Petrol and diesel exhaust particles (PEPs and DEPs) are the major components of particulate matter ($PM_{2.5}$) (PM with diameter <2.5 µm) and also are the main contributors to PM related pollution [1]. Experimental acute studies have demonstrated that exposure to $PM_{2.5}$ was found associated with endothelial dysfunction, myocardial infarction, decrease in fibrinolysis, arterial stiffness, myocardial ischemia, increased thrombus formation, and platelet activation [2]. Studies also showed oxidative stress, asthma, airway inflammation and acute cardiovascular diseases related to PM exposure. Studies by Mills *et al*., 2009 showed that inhaled $PM_{2.5}$ caused airway related oxidative stress and hence up-regulation of inflammatory genes [3]. Researchers have presented various hypotheses to explain in detail the mechanism involved in the adverse effects of PM-related air pollution. Brook *et al*., 2010 stated that the autonomic nervous system could be a key target for these air pollutants hence leading to toxicity [2]. Our previous *in vitro* studies in human A549 and RAW 264.7 macrophages exposed to different concentrations of PEPs demonstrated that they caused cytotoxicity to both cells by the mechanism of oxidative stress, DNA damage and inflammation [4].

Studies also showed that airborne particles crossed cell barriers, entered the circulation and hence interacted directly with the vascular tissues and circulatory cells (platelets or erythrocytes), leading to adverse effects at different body parts [5,6]. Hence, investigation of PEP toxicity on red blood cells (RBCs) is needed in order to understand the underlying mechanisms of PM-related cardiovascular effects. Delfino *et al*., 2009 showed that the traffic-related air pollutants caused decrease in RBC antioxidant activity [7]. Decreased antioxidant activity of mouse, rat and human erythrocyte exposed to different concentrations of DEPs were seen along with lipid peroxidaton, oxidative stress and hemolysis [8]. Different researchers reported decrease in erythrocyte numbers is related to ultrafine particle (UFP) or $PM_{10}$ (PM with diameter <10.0 µm) exposure in rats or humans [9,10]. Cells exhibit various disruptions and structural alterations in the antioxidant status on exposure to different environmental pollutants and toxic chemicals [11]. Fenugreek *(Trigonella foenum-graecum)* and quercetin were found to act as potent antioxidants [12]. Fenugreek leaves are abundant sources of quercetin, gallic acid, kaempferol, luteolin and gallic acid. Studies demonstrated the antioxidant activity of the plant extract [13]. The anti-oxidant activity of the fenugreek extract was found attributed to the presence of flavonoids in it [14]. The protective role of fenugreek extract against cyclophosphamide toxicity was demonstrated by Rehman *et al*., 2006 [15]. Studies by Kaviarasan *et al*., 2004 showed that fenugreek extract protected RBCs from both lipid peroxidation and hemolysis [16]. Quercetin a widely known flavonoid is found in various vegetables and fruits. The antioxidant effects of fenugreek and quercetin include chelating ions and scavenging free radicals. The protective effect of quercetin against oxidative stress induced in human retinal pigment epithelium cells were examined *in vitro* [17]. Studies by Yetuk *et al*., 2014 also proved the protective role of quercetin and catechin in human erythrocytes against sodium benzoate induced oxidative stress and lipid peroxidation [18]. Dimethoate toxicity was cleared by the alleviate effects of quercetin in human peripheral blood lymphocytes [11].

According to literature survey, there are no reports on the protective effects of EXT or Q against PEP induced *in vitro* toxicity in erythrocytes and hence explored here for the first time. For these reasons the aim of the present study was, (1) To evaluate dose-dependent hemolytic activity of PEPs on rat erythrocytes, (2) to evaluate the erythrocyte (RBC) antioxidant status on exposure to different concentrations of PEPs, including the malondialdehyde content (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) and glutathione-

S-transferase (GST) levels and, (3) to evaluate the protective effects of quercetin (Q) and aqueous fenugreek leaf extract (EXT) against PEP induced erythrocyte toxicity.

## METHODS

### Materials

Quercetin was supplied by Sigma-Aldrich (India). All fine grade chemicals were purchased from Sigma, USA. Other reagents of analytical grade were purchased from Merck or Himedia India Ltd.

### Particles

PEPs were collected and characterized according to our previous study [4]. The PEPs were suspended in sterile saline (Nacl 0.9%) containing tween 80 (0.01%) and sonicated for 15 minutes before incubation with RBCs to minimize aggregation. Normal saline containing tween 80 (0.01%) served as a control.

### Blood collection

This research and the protocols employed in the current study were reviewed and approved by our institutional animal ethical committee (CPCSEA). Male Wistar rats (150-200 g) were housed in polypropylene cages in temperature (30±2°C) and light controlled rooms (12 hrs light and 12 hrs dark cycle). They were given standard pellet diet and water ad libitum. For a collection of blood, rats were anaesthetized with 45 mg/kg b.wt of sodium pentobarbital intra-peritoneally. Following this, blood was collected in 4% ethylenediaminetetraacetic acid tubes by puncturing the retro-orbital plexus [19].

### Hemolysis and treatment of erythrocytes

Hemolysis was performed according to the study described by Lu *et al*., 2009 [20]. The collected rat blood was mixed by a gentle tube inversion and centrifuged (200 g for 10 minutes). The plasma and buffy coats were discarded, and the RBCs were washed thrice with cold saline (0.9%) before centrifugation again at 200 g for 10 minutes. The suspension contained 5% RBCs in saline. The control groups were incubated with 0.9% NaCl for 1 hr at 37°C (pH 7.4). The experimental groups were divided as follows: PEP treated group, EXT + PEP group, Q + PEP group and EXT or Q alone treated groups. The experimental group RBCs were exposed to 1 (P1), 10 (P2) and 100 (P3) µg/ml of PEPs in the presence or absence of Q (2.5 µg/ml) and EXT (8.13 µg/ml) for 1 hr at 37°C. Different concentrations of PEPs with or without Q or EXT were added to the 96 well plates. 75 µL of RBCs were added to each well and mixed by pipetting. This was followed by incubation for 30 minutes at 37°C. The plates were then centrifuged for 5 minutes and the contents were transferred in to a different plate. The % hemolysis was calculated according to the procedure described by Nemmar *et al*., 2012 [8]. Cell mixtures were incubated at 24 hrs at −20°C. The mixtures were then centrifuged, and the supernatants were separated. The activities of GST, GSH, SOD, CAT and MDA were measured spectrophotometrically.

### LPO in erythrocytes

LPO was measured in RBCs as the MDA content assayed by thiobarbituric acid tests [21]. The absorbance was measured spectrophotometrically. The specific activity was represented as nmol/mg protein.

### Antioxidant enzyme status in erythrocytes

The SOD, CAT, GST and GSH enzyme activities were estimated in RBC lysate according to the McCord and Fridovich method (1969) [22]. SOD was determined according to the procedures described by Marklund and Marklund (1974) [23]. Results were expressed as U/mg protein. CAT was determined by the method of Aebi (1984) [24] and the activity of CAT was expressed as µmoles of $H_2O_2$ utilized/minutes/g protein. The reduced GSH levels were estimated by the method of Ellman (1959) [25] and the amount of GSH is expressed as µg/mg protein. GST activity was determined using the GST Kit (Sigma, India). The absorbance was read at 340 nm spectrophotometrically, and the activity was expressed in µmoles/minutes.

### Statistical analysis

All experiments were performed in triplicates. The results were expressed as mean±standard deviation. Data were analyzed by standard statistical analysis one-way ANOVA for multiple comparisons to determine the significance between different groups. The results were considered statistically significant if p value was 0.05 or less.

## RESULTS

### Effect of PEPs on erythrocyte hemolysis

Dose-dependent hemolytic effects (~up to 46%) were observed in the PEP treated RBCs when compared to the control groups. Hemolytic effects were partially reversed significantly ($p<0.05$) in the EXT + PEP and Q + PEP treated groups when compared with the PEP treated groups (Fig. 1a).

### Measurement of MDA levels

Significant elevations ($p<0.05$) in MDA levels were found in rat erythrocytes after 1 hr incubation with 1 (P1), 10 (P2) and 100 (P3) µg/ml PEPs when compared to control. In rat erythrocytes, significant elevation was found to be 1.15, 1.27 and 1.34 times higher than the control levels. Whereas, MDA levels were reduced significantly ($p<0.05$) in the EXT + P2, EXT + P3, Q + P2 and Q + P3 treated groups (Fig. 1b) respectively when compared to the PEP treated groups. The protective effect of the EXT or Q was not statistically evident in the EXT + P1 and Q + P1 group when compared to the PEP treated groups in rat erythrocytes.

### Measurement of SOD activity

Decrease in enzyme activity of SOD was found in rat erythrocytes exposed to different concentrations of PEPs. Compared with the control, significant SOD reductions of 59.69% and 71.37% in rat erythrocytes were observed after 1 hr incubation with 10 (P2) and 100 µg/ml (P3)

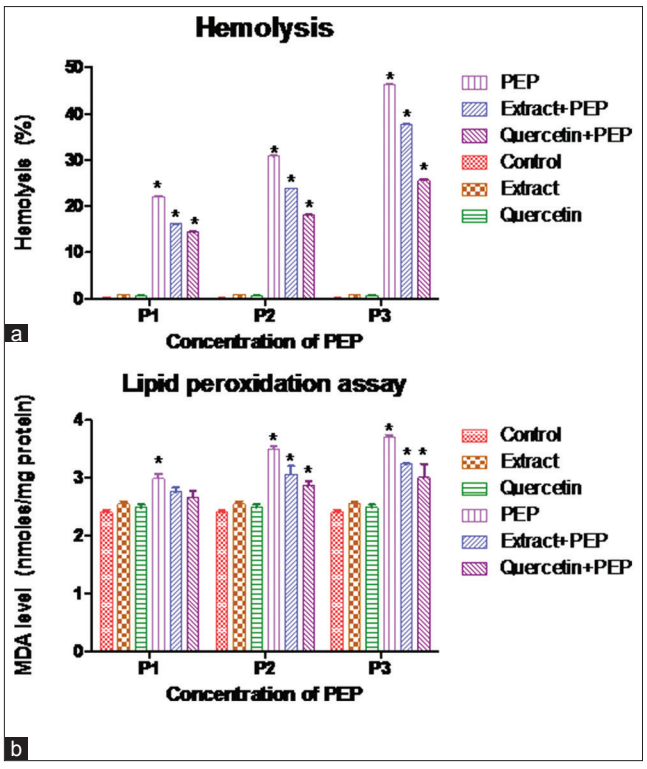


**Fig. 1: Effects of petrol exhaust particles on the, (a) Hemolytic action and (b) malondialdehyde (MDA) levels measured in the incubation medium of erythrocytes isolated from rat blood. Data are expressed as mean±standard deviation. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple range tests *p<0.05**

PEPs. The SOD levels were significantly increased in the EXT + P2 and Q + P2 groups ($p<0.05$) (Fig. 2a). Although, SOD levels were increased in the EXT + P1/P3 and Q + P1/P3 groups, the results were not statistically significant ($p>0.05$).

**Measurement of CAT activity**

The CAT activity decreased significantly in the PEP treated groups in a dose-dependent manner when compared to the control groups. Significant CAT reductions of 32.9% and 43.19% were observed in rat erythrocytes after 1 hr incubation with 10 (P2) and 100 µg/ml (P3) PEPs. EXT or Q supply along with PEP reversed this change particularly for EXT + P3, Q + P2 and Q + P3 groups ($p<0.05$). CAT activity in the EXT + P1, EXT + P2 and Q + P1 groups were higher than PEP exposed RBCs, but was not found to be statistically significant when compared with the control, EXT or Q groups ($p>0.05$) (Fig. 2b).

**Measurement of GST activity**

PEP treated rat erythrocytes resulted in 3.09 and 3.57 times significant increase ($p<0.05$) in GST levels compared to the control, when treated with 10 (P2) and 100 µg/ml (P3) PEPs. However, the GST levels were significantly decreased in RBCs in the EXT + P2, Q + P2 and Q + P3 groups compared to the PEP treated RBCs ($p<0.05$) (Fig. 3a). The fenugreek leaf extract had no protective effect on the P3 group.

**Measurement of GSH activity**

As depicted in (Fig. 3b), PEP treated rat erythrocytes resulted in significant decrease ($p<0.05$) in GSH activities compared to the control, when treated with 10 (P2) and 100 µg/ml (P3) PEPs. Whereas, P1 dose had no significant change in enzyme activity compared with the control. EXT or Q supply to the PEP treated groups significantly increased GSH activity in RBCs compared to the PEP treated groups.

## DISCUSSION

In this study PEP induced dose-dependent RBC hemolysis and RBC related oxidative stress was explored. Studies explained the relationship between increased concentrations of PM and increased cardiovascular mortality and morbidity [2]. Exposures to UFPs, of which PEPs are significant contributors, in industrial and urban areas have increased significantly because of sources of anthropogenic origin such as incinerators, internal combustion engines and power plants [26]. Studies by Nemmar *et al*., 2002 [5] explained that UFPs can enter the circulation via the respiratory epithelium and hence interact and exert toxicity to circulating cells like the RBC's and to the vascular endothelium. Previous studies also showed that UFPs penetrate deeply into the pulmonary tract and enhance inflammatory response, thus damaging the interstitium and epithelial cells [27]. However, to the finest of our acquaintance, the effect of PEPs on rat erythrocytes has not been reported so far. Our results showed that PEPs exert statistically dose-dependent hemolytic activity ~up to 46% on the rat erythrocytes.

Studies showed that DEPs exerted hemolytic activity on human, mouse and rat erythrocytes [8]. Previous studies by Seaton *et al*., 1999 [28] and Rückerl *et al*., 2007 [9] showed that PM pollution exposure caused a significant decrease in RBC numbers in rats and humans. PM causes systemic inflammation and adhesion of RBCs to endothelial cells and hence subsequent direct toxicity on circulating RBCs. The effect of $TiO_2$ nanoparticles on rabbit erythrocyte hemolysis was previously explored [29]. High-resolution transmission electron microscopy analysis of mouse and rat RBCs showed that either DEPs got adsorbed onto the RBCs or internalized by RBCs [8].

Similarly, it was shown that inhaled $TiO_2$ nanoparticles were internalized by erythrocytes and blood capillaries [30]. Recent studies showed that silica nanoparticles got adsorbed onto the erythrocyte surfaces without any disturbance to the RBC morphology or membrane. Erythrocytes exhibit various mechanisms to protect themselves from oxidative stress. This includes the presence of antioxidants such as SOD, CAT, GST and GSH. Any variation in these enzyme activities leads to oxidative stress that further leads to attenuation of the antioxidant defence system [31,32]. In spite of the developed antioxidant system

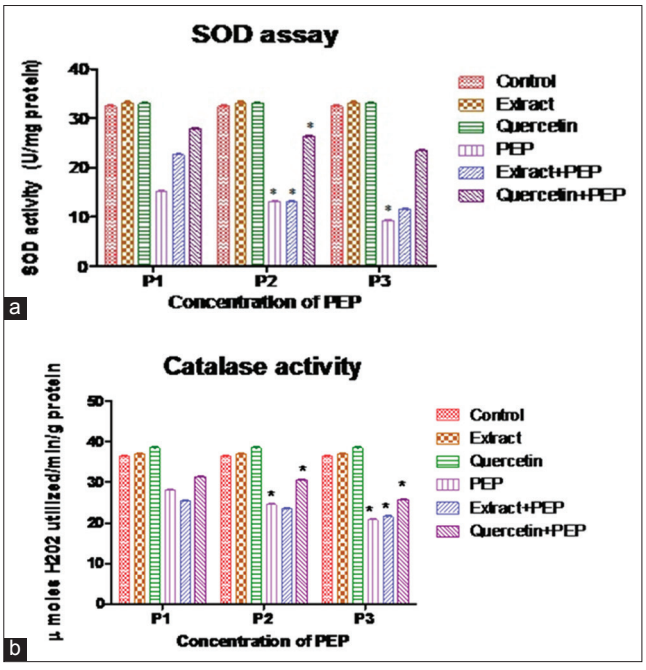


**Fig. 2: Effects of petrol exhaust particles on, (a) Superoxide dismutase levels (SOD) and (b) catalase (CAT) activity measured in the incubation medium of erythrocytes isolated from rat blood. Data are expressed as mean±standard deviation. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple range tests *p<0.05**

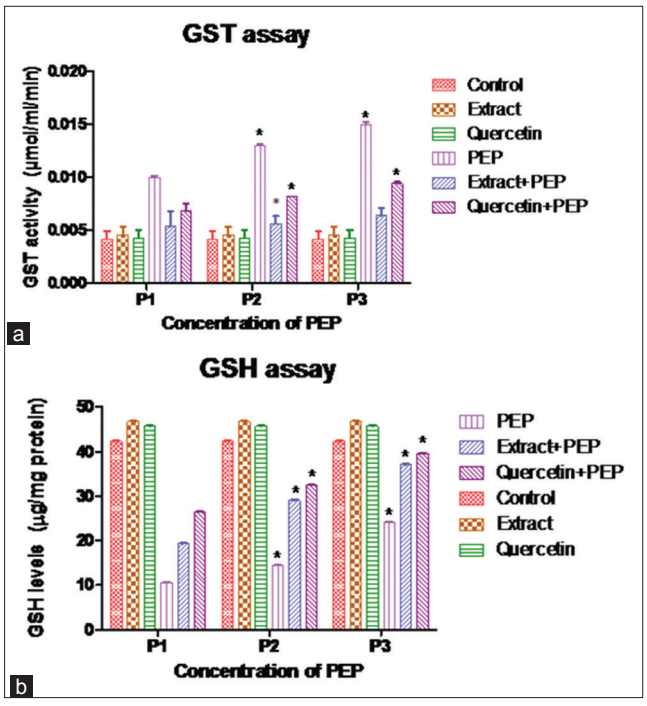


**Fig. 3: Effects of petrol exhaust particles on (a) Glutathione-S-transferase levels (GST) and (b) glutathione leavels (GSH) measured in the incubation medium of erythrocytes isolated from rat blood. Data are expressed as mean±standard deviation. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple range tests *p<0.05**

RBCs become oxidatively damaged on exposure to environmental air pollutants.

MDA measurement is frequently used as a reliable technique for the evaluation of lipid peroxidation [21]. In this study, we quantified MDA to determine rat erythrocyte lipid susceptibility *in vitro*. Similar to the hemolysis results, rat RBCs exhibited dose-dependent statistical increase in MDA levels. This clearly indicates that the PEPs lead to RBC related oxidative stress with plasma membrane as the primary site of damage, and thus the peroxidation of plasma membrane lipids caused hemolysis and cross-linking of lipids and protein molecules to various extends [33]. The increased hemolysis suggests insufficiency of the cytosolic enzymes to react with ROS generated in the plasma membrane. Besides evaluating the % hemolysis and MDA content, we measured levels of antioxidants such as SOD, CAT and GSH, as an overall index of oxidative stress. Our findings suggested a statistically significant dose dependent decrease in these antioxidants (SOD and CAT) in rat RBCs exposed to PEPs. The main mechanism behind the consumption of these antioxidants is the breakdown or decomposition of ROS or free radicals (generated by PEP intoxication) [32]. Furthermore, the direct inhibition of SOD, CAT and GSH activities by PEPs could contribute to this phenomenon.

Our data's also showed that rat erythrocytes supplied with either Q or EXT prior to PEP intoxication protected cells from hemolytic oxidative stress and damage. Previous studies in humans and animals suggest that phytochemicals may decrease the development of cardiovascular diseases [34,35], cancer and cerebrovascular diseases. Both fenugreek and quercetin are rich sources of antioxidants [12]. These antioxidants protect the RBC membrane from oxidative stress [36] and hence prevent oxidative damage by scavenging ROS, such as superoxide anions and hydroxyl radicals.

Quercetin is vital flavonoid that has health benefits because of its antioxidant and anti-toxic function. The protective effects of quercetin, myricetin, epicatechin and catechin were investigated against N-nitrosodibutylamine and nitrosopiperidine induced toxicity in HepG2 cells [37]. Studies revealed that quercetin was capable of protecting cells from oxidative stress and cellular senescence in a dose-dependent manner [17]. Quercetin is a vital chelating agent and free radical scavenger that chelates iron that is accountable for the formation of reactive oxygen species [38]. Studies by Boots *et al*., 2008 [39] revealed that quercetin decreased tumor necrosis factor-α production in a dose-dependent manner in human volunteers induced with lipopolysaccharide. Previous studies demonstrated that quercetin reduced oxidative damage in rats induced with 4 nitro-3 phenylphenol and hence caused a reversion of SOD, GSH-peroxidase activity, MDA and cell viability to near normal levels [40]. Studies showed that quercetin blocked the production of hypodiploid cells, DNA ladders and formation of intracellular peroxides by its antioxidant activity. Quercetin significantly decreased lipid peroxidation (LPO), prevented DNA damage and GSH depletion in HepG2 cells [41]. Similarly, the oxidative stress caused by biphenyls was inhibited by the action of quercetin [42].

Studies reported that plant extracts play a vital role in preventing toxicity [43]. Fenugreek (*T. foenum-graecum* L.) is a traditionally used medicinal, leguminous plant. Fenugreek is an essential source of various important flavonoids. Studies by Ravikumar and Anuradha, 1999 [44] demonstrated that the dietary fenugreek was found to increase the level of circulating antioxidants like beta-carotene, GSH and alpha-tocopherol. The flavonoids in the extract may be accountable for its activity. Studies showed that the activity of fenugreek extract inhibited urotoxicity in rats by repairing the antioxidant status [45].

In the current study Q and EXT inhibited inflammation and oxidative stress. These data's on the protective ability of Q and EXT against inflammation, and oxidative stress are consistent with previous literatures. Studies revealed that the chemical structure of quercetin plays an important role in its free radical scavenging activity. The O-dihyroxyl group present in the B-ring and C-ring is primarily responsible for quercetin's activity [46].

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