CARDIOPROTECTIVE POTENTIAL OF QUERCETIN AGAINST DIESEL OR PETROL EXHAUST NANOPARTICLE INDUCED TOXICITY: A PROSPECTIVE *IN VITRO* PHARMACOLOGICAL STUDY IN H9C2 CELLS

MOHAN DURGA[1]*, THIYAGARAJAN DEVASENA[2]

[1]Department of Biochemistry and Bioinformatics, Dr MGR Janaki College of Arts and Science for Women, Chennai, Tamil Nadu, India.
[2]Centre for Nanoscience and Technology, Anna University, Chennai, Tamil Nadu, India. Email: durgam2k7@gmail.com

ABSTRACT

Objective: Phytochemicals are known to elicit potential antioxidant activity. This study examined the cardioprotective effects of quercetin against oxidative damage to rat cardiomyocyte cells (H9c2) after treatment with Diesel Exhaust Nanoparticles (DEPs) or Petrol Exhaust Nanoparticles (PEPs).

Methods: Cardiomyocyte cells were exposed to DEPs or PEPs alone and in a combination with quercetin for 24 h.

Results: Results showed that quercetin had no lethal effect on H9c2 cells up to a concentration of 1.0 µg/ml. Exposure to DEPs (4.0 µg/ml) or PEPs (10.0 µg/ml) induced cytotoxicity, oxidative stress, and inflammation ($p<0.05$). It also provoked lipid peroxidation by an increase in MDA and a decrease in SOD activity and glutathione activity ($p<0.05$). Simultaneous addition of quercetin restored these parameters to near normal.

Conclusion: These results thus specify that quercetin plays a protective role in cardiac cells exposed to DEPs and PEPs.

Keywords: Diesel exhaust nanoparticles, Petrol exhaust nanoparticles, H9c2, Antioxidants, Quercetin, Inflammation.

INTRODUCTION

Flavonoids are a group of natural, polyphenolic compounds found widely in fruits, flowers, vegetables, stem, wine, and tea. Nearly 400 different flavonoids have been discovered till date. Quercetin (3, 5, 7, 3, 4 - pentoxy flavones) is one of the best illustrated flavonoids with potential antioxidant properties [1]. Quercetin was found in its glycosylated form in apples, onions, broccoli, and French beans [2]. Studies revealed that quercetin intake showed decreased frequency of neoplastic and cardiovascular diseases [3-5]. Studies by Tieppo *et al.*, 2007, reported that quercetin increased the genomic stability and hence enhanced the antioxidant defense system in rat models [6]. Studies also showed that quercetin inhibited the expression of nitric oxide (NO) synthase, cyclooxygenase, lipid peroxidation (LPO), and xanthine oxidase [7]. Farombi and Onyema, 2006, indicated that dietary antioxidants such as quercetin, Vitamin E, and Vitamin C played a protective role against oxidative stress induced by monosodium glutamate in the brain, liver, and kidney of rats [8]. Studies showed that quercetin prevented different disorders caused by environmental contaminants [9].

Environmental contaminants such as diesel exhaust nanoparticles (DEPs) and petrol exhaust nanoparticles (PEPs), pose a great threat to human health because of their small size and cause dangerous effects such as asthma, bronchitis, various allergies, and lung cancer [10]. As a component of particulate matter (PM), DEPs were seen to exhibit lethal effects on cardiac cells both *in vivo* and *in vitro*. *In vitro* studies showed that different cell types took up DEPs and induced inflammation and oxidative stress in various cell types [11]. DEPs evoked cardiac failure in rats [12]. Other studies showed that DEPs induced reactive oxygen species (ROS) formation and hence oxidative stress and inflammation in the lung and respiratory tract of rats [13]. Studies also showed that DEPs induced toxicity by the production of hydroxyl radicals [14].

Reproductive toxicity evoked by DEPs in rats was treated by quercetin [15]. Other studies showed that quercetin exhibited ameliorating effects on reproductive toxicity induced by 3-methyl-4-nitrophenol (PNMC) (a vital component of DEPs) in chicken embryos [16]. Available data's on PEP toxicity are insufficient and hence need to be studied in detail.

To the finest of our acquaintance, there are no data's in the available literature reporting the protective effect of quercetin on DEP/PEP-induced cardiotoxicity. The current study was thus, undertaken to investigate the anti-toxic and antioxidant effects of quercetin in DEP/PEP-induced cardiotoxicity *in vitro*.

METHODS

Chemicals

Quercetin (98.5%) was purchased from Sigma-Aldrich Pvt. Ltd., Bengaluru, India. All other analytical grade chemicals were purchased from HiMedia Laboratories, Mumbai, India.

Collection and characterization of nanoparticles

The collection and characterization of nanoparticles were done according to our previous study [17].

Cell culture and MTT assay

H9c2 cells (Rat cardiomyocyte cell line) were used for the determination of cytotoxic end-points. The cell lines were purchased from National Centre for Cell Science, Pune. Cytotoxicity determination (IC_{50}) of DEPs and PEPs was performed by MTT assay according to our previous study [17]. The different concentrations of Quercetin (Q) were added to the cells in 100 µL of medium and incubated for 24 h. After 24 h of incubation, 10 µL MTT was added to each well and incubated further for 4 h. 100 ml of DMSO solution was added to each well for the formazan crystal solubilization. The wells were then quantified by measuring absorbance of the solution at 570 nm using a microplate reader (BIO-RAD, USA). The IC_{50} value was calculated from the absorbance.

Experimental design for cardioprotective activity

The cells were incubated in medium containing DEP or PEP with or without prior treatment with quercetin. 1/25th IC_{50} value of DEP

(4.0 µg/ml) and PEP (10.0 µg/ml) was taken. 1/10th and 1/25th IC_{50} of Q (2.5 µg/ml and 1.0 µg/ml) (Q1 and Q 2) were chosen for the study. The maximum concentrations of DEPs and PEPs were taken based on the relevant concentration of PM2.5 in urban air [18]. The control received vehicle only. After 24 h exposure, the medium and cultured cells were used to determine NO, Total Protein (TP), Lactate Dehydrogenase (LDH), Superoxide Dismutase (SOD), Glutathione (GSH), Malondialdehyde (MDA), intracellular Hydrogen peroxide (H_2O_2), and cytokines (TNF α, IL-6, and IL-8).

Measurement of NO, TP, and LDH in cell culture supernatant fluids
H9c2 cells were seeded into 6-well plates at a density of 5000 cells/well in 100 µL of medium. Cells were allowed to proliferate attach and cover around 80% of the plate surface area before exposure to different treatment groups for 24 h. After 24 h of exposure, the culture supernatants were collected to determine the levels of NO (Biovision Inc), TP (Bio-rad, USA), and LDH (Sigma-Aldrich, India). The TP levels were measured using a Coomassie Brilliant Blue protein assay kit. The LDH activity was determined spectrophotometrically in the presence of lactate by observing the reduction of NAD+ at 340 nm, according to the manufacturer's protocol. The NO levels were determined using a nitric acid reductase kit according to the manufacturer's protocol.

Measurement of intracellular SOD, GSH, and MDA
H9c2 cells were seeded into 6-well plates at a density of 5000 cells/well in 100 µL of medium and allowed to proliferate, attach, and cover around 80% of the plate surface area before the treatment for 24 h. After 24 h treatment, the cells were washed with ice-cold phosphate buffered saline (PBS), followed by trypsinization and immediate disruption by continuous frozen-thaw process (3 times). The cell lysates were collected, centrifuged, and stored at −20°C for the determination of intracellular SOD, GSH, and MDA (Sigma-Aldrich, India) using commercial kits following the manufacturer's instructions.

Measurement of intracellular H_2O_2 formation
H9c2 cells were seeded into 6-well plates at a density of 5000 cells/well in 100 µL of culture medium and allowed to proliferate attach and cover around 80% of the plate surface area before exposure to different treatment groups. The intracellular H_2O_2 was determined by the chemiluminescence (CL) method using horseradish peroxidase (HRP). After 1 h treatment, the cells were collected, rinsed with ice-cold PBS and followed by trypsinization. For the radical measurement, the cell lysates were centrifuged, washed, and suspended in two different Eppendorf's containing 400 µL PBS. To this, 8 µL of HRP and 4 µL luminol were added. CL was measured at 25°C for each second from the 0th to 9th s with a Luminometer apparatus (Zylux, U.S.).

Measurement of cytokine secretion in H9c2 cell lines
H9c2 cells were seeded into 6-well plates at a density of 5000 cells/well in 100 µL of culture medium and allowed to proliferate attach and cover around 80% of the plate surface area before exposure to different treatment groups. DEPs and PEPs were suspended in sterile normal saline (NaCl 0.9%) containing Tween 80 (0.01%). The setup was incubated with DEPs and PEPs up to 24 h at 37°C in a humidified atmosphere containing 5% CO_2. After 12 and 24 h, the quantitative measurement of pro-inflammatory cytokines (IL-8, TNF-α, and IL-6) was performed in the supernatants. Supernatants were collected by centrifugation of culture medium at 2500 rpm for 20 min at 18°C and assayed for and TNF-α, IL-6, and IL-8 using commercial ELISA assay kits (ebiosciences, USA), according to the manufacturer's recommendations. The samples and standards were all run in triplicates and the data's were analyzed.

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

RESULTS

From the MTT assay, the IC_{50} values for DEP, PEP, and Q treated H9c2 cells were found to be 100 µg/ml, 250 µg/ml, and 25.80 µg/ml, respectively (Fig. 1). The levels of LDH, TP, and NO increased significantly in DEP/PEP treated H9c2 cells while the DEP+Q or PEP+Q treated groups showed a significant decrease (p<0.05) in all the above cytotoxicity parameters (Fig. 2a-c).

The activities of cell antioxidants SOD and GSH decreased significantly (p<0.05) in DEP/PEP treated groups, while the DEP+Q and PEP+Q groups showed a significant increase in these cells antioxidants when compared to the DEP or PEP treated groups alone (p<0.05; Fig. 3a and b). The levels of MDA and H_2O_2 in DEP/PEP treated H9c2 cells showed significant increase when compared to the control (p<0.05) and the levels were near normal in DEP+Q and PEP+Q treated H9c2 cells (p<0.05; Fig. 3c and d).

DEP or PEP treated H9c2 cells showed a significant increase (p<0.05) in the levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) when compared with the normal control cells. The DEP+Q or PEP+Q groups showed a significant decrease in these biomarkers when compared to the DEP or PEP treated cells (Fig. 4a-c).

DISCUSSION

The overall outcome of the present study proposes that exposure to DEPs (4.0 µg/ml) or PEPs (10.0 µg/ml) induces significant cytotoxicity, oxidative stress, and inflammation (p<0.05) in H9c2 cells. This is apparent from (a) increase in levels of LDH, TP, and NO, indicators of cytotoxicity, (b) decrease in the activity of detoxifying enzymes, SOD and GSH, (c) increase in the levels of MDA and H_2O_2, and (d) increase in the levels of pro-inflammatory cytokines IL-6, IL-8, and TNF-α.

Environmental pollution is identified as a universal problem. Both DEPs and PEPs are major contributors of environmental air pollution. They evoke oxidative stress and thus influence the antioxidant mechanism of the cell by either the inhibition or enhancement of a range of antioxidant enzymatic or non-enzymatic functions. In the current study, the cardio-protective effect of quercetin (Q) was examined in DEP/PEP induced toxicity.

Cells exhibit structural alterations and disturbances in cell function and antioxidant defense organization following exposure to environmental pollutants and toxic chemicals. Increase in levels of cytotoxicity marker enzymes (LDH, TP, and NO) in cell culture supernatants was found in the present study. They are key indicators of cytotoxicity and cell membrane damage. Cell death or cellular toxicity involves complex mechanisms that entail detailed evaluation.

LDH is distributed extensively in the cytoplasm of cells, yeasts, mammalian tissues, and bacteria. LDH is released from the cytosol by cell lysis. LDH release in regarded as a late episode in apoptosis and
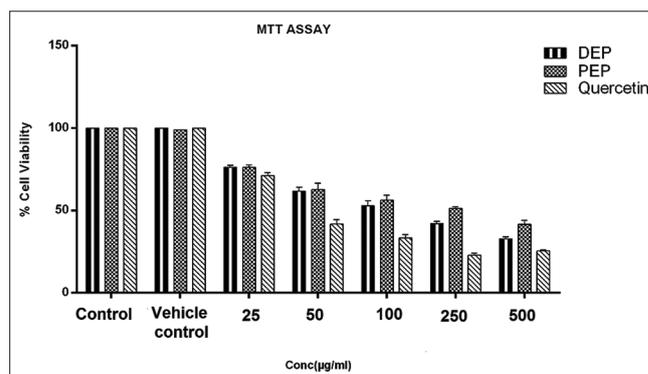

Fig. 1: Cell viability of DEPs, PEPs, and Q exposed to H9c2 cells for 24 h was determined by MTT assay. Data are expressed as percentage of the control at the concentration of 25, 50, 100, 250, and 500 µg/ml, respectively
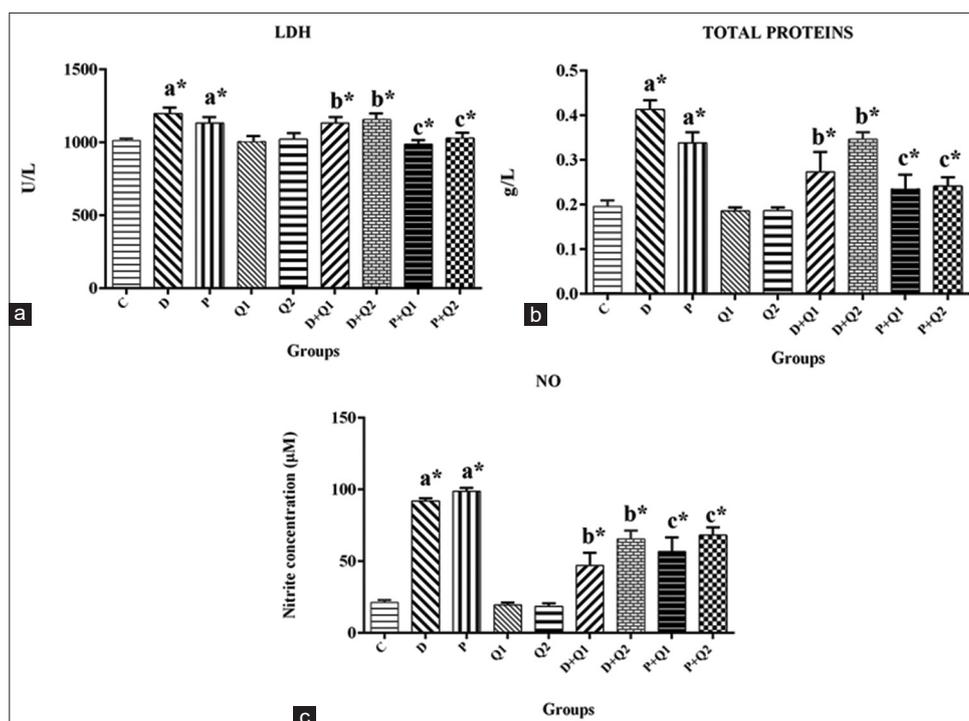
Fig. 2: (a-c) Levels of LDH, TP, and NO in H9c2 cells treated with DEP (4.0 µg/ml), PEP (10.0 µg/ml), Q1 (2.50 µg/ml), and Q2 (1.0 µg/ml) for 24 h. Values are represented as mean±SD of three repeated experiments; (a) as compared with Group I (control); (b) as compared with Group II (DEP treated); and (c) as compared with Group III (PEP treated; *p<0.05)
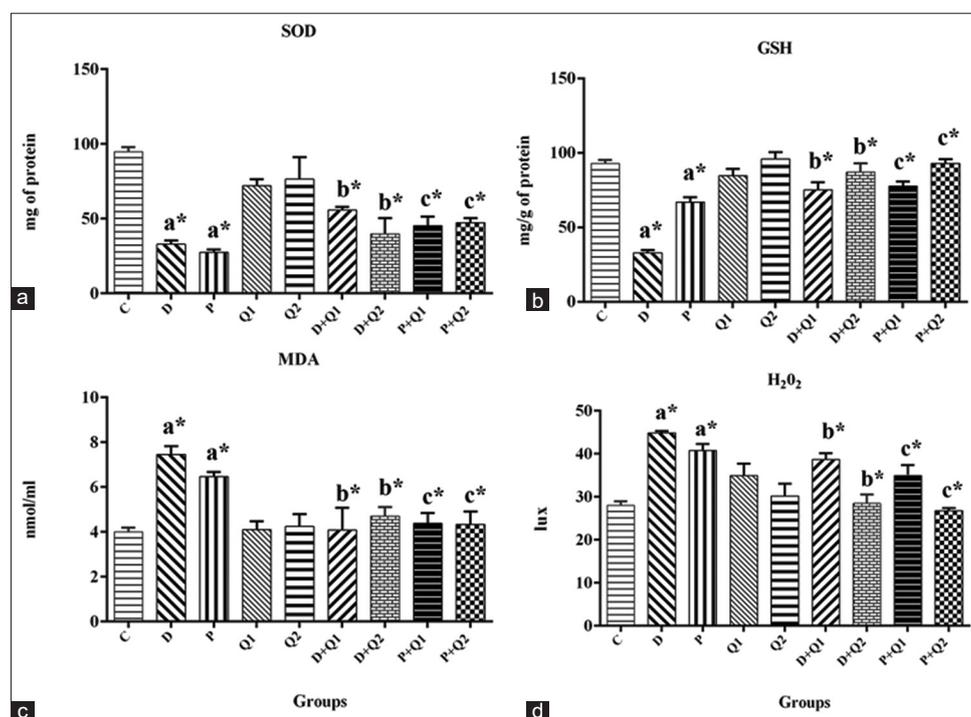

Fig. 3: (a-d) Levels of SOD, GSH, MDA, and H2O2 in H9c2 cells treated with DEP (4.0 µg/ml), PEP (10.0 µg/ml), Q1 (2.50 µg/ml), and Q2 (1.0 µg/ml) for 24 h. Values are represented as mean±SD of three repeated experiments; (a) as compared with Group I (control); (b) as compared with Group II (DEP treated); and (c and d) as compared with Group III (PEP treated; *p<0.05)

as an early event in necrosis. An exact temporal evaluation of toxic responses is needed as apoptosis may change into necrosis and cell viability is lost without any noticeable cell morphological alterations or response to assays determining the late events hence resulting in toxicity being unnoticed [19]. Therefore, by assessing the LDH activity alterations in cellular factors such as cell damage and impermeability

can be determined. In the present study, membrane damage was evaluated by release of enzyme LDH, since cell membrane could be a possible site for the interaction of exhaust nanoparticles.

Protein plays an important role in the metabolic activities of the cell. It is regarded as the main nutrient used by cells to develop tissues. In the
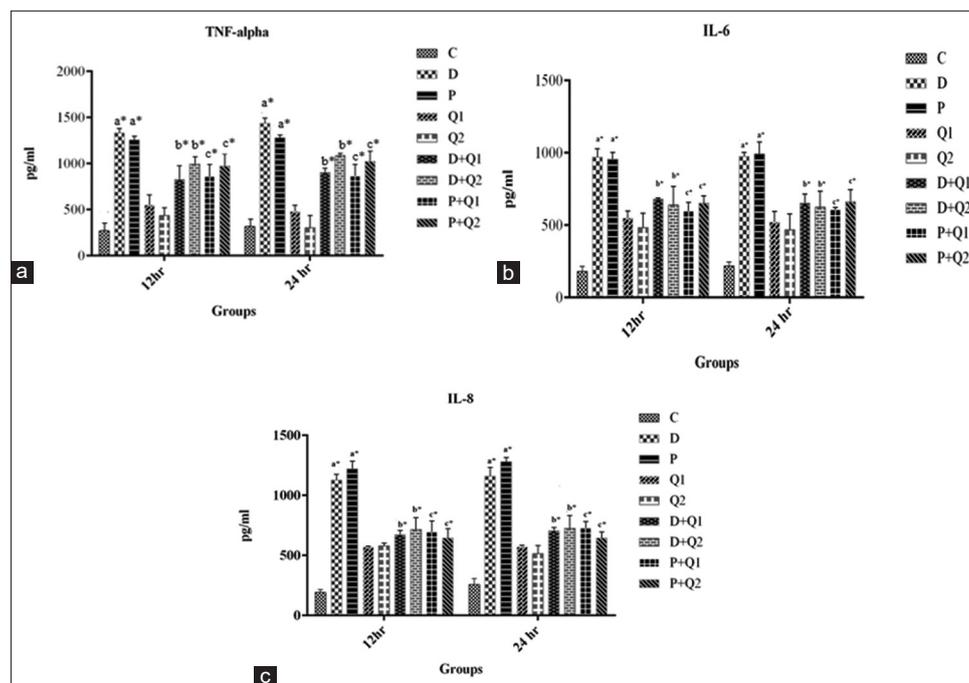
Fig. 4: (a-c) Levels of TNF-α, IL-6, and IL-8 in H9c2 cells treated with DEP (4.0 μg/ml), PEP (10.0 μg/ml), Q1 (2.50 μg/ml), and Q2 (1.0 μg/ml) for 24 h. Values are represented as mean±SD of three repeated experiments; (a) as compared with Group I (control); (b) as compared with Group II (DEP treated); and (c) as compared with Group III (PEP treated; *p<0.05)

current study, it was seen that an increase in the TP content depends on the concentration of toxins in the cell culture medium. The increased protein content possibly suggests stress produced by the exhaust nanoparticles [20].

NO is a potential signaling molecule that acts as a pro-inflammatory mediator under abnormal conditions such as oxidative stress [21]. NO is produced by various cells involved in inflammation and immunity. High levels of cytokines induce the production of NO in cells. At elevated concentrations of NO, generated by NO synthase – 2 (NOS-2), "NO" is oxidized to Reactive Nitrogen Oxide Species (RNOS), that induces most of the inflammatory responses. NO can be oxidized and complexed with lipids and proteins contributing to local tissue or cell injury [22]. Many key enzymes in the mitochondria are repressed by RNOS, leading to the exhaustion of cellular energy and ATP. Such interactions of NO, explain its vital action in the inflammatory and immune cells [23]. Thus, results of the present study revealed considerable cytotoxicity and cell membrane damage contributed by the exhaust nanoparticles. The increase in LDH, TP, and NO was significantly decreased in combination with quercetin as compared with the DEP or PEP treatment alone (p<0.05; Fig. 2a-c).

The decrease in SOD and GSH activities in H9c2 cells is due to the inactivation of the antioxidant enzymes as the superoxide anions are shown to decrease the enzyme activity [24]. The decrease in GSH along with the depletion of SOD may affect the cells ability to scavenge hydroxyl radicals and superoxide anions. The decrease in GSH content by DEP/PEP intoxication can also be due to the DEP/PEP-SH binding, depletion of GSH, or due to GSH reductase levels [25]. The antioxidants defend the cell membrane from oxidative stress and thus prevent oxidative damage [26]. Oxidative stress is explained as the imbalance situation involving decreased antioxidants or increased oxidants [27].

The human cells are constantly damaged by ROS, which arise by exposure to toxins in environment. Under normal conditions the body protects cells from oxidative stress by various non-enzymatic and enzymatic antioxidants [28]. Among antioxidants catalase, decomposes H_2O_2 to non-toxic products whereas, the decomposition of superoxide to H_2O_2 and oxygen is the major function of SOD. Thus, the depletion in

activities of SOD and GSH in cells signifies its decrease in the cells as a result of DEP/PEP intoxication.

Oxidative stress due to increase in mitochondrial β-oxidation, microsomal ω-oxidation, and peroxisomal β-oxidation leads to LPO which, in turn, causes the release of MDA and hence cellular damage [29,30]. Increased MDA levels in the current study indicated damage to cell membrane by LPO. LPO was seen as the main molecular mechanism involved in DEP/PEP-induced toxicity. This status became evident by significant rise in MDA levels when H9c2 cells were incubated with different concentrations of DEP and PEP for 24 h. The increased exposure of cells to free radicals/ROS resulted in the oxidative degradation of lipids. Cell damage arises when the free radicals take electrons from membrane lipids. At present, LPO is regarded as the major molecular mechanism engaged in oxidative cellular damage and toxicity processes leading to cell death. The cell membrane primarily composed of polyunsaturated fatty acids act as the primary target for attack by ROS, hence directing to oxidative cell damage. Breakdown of phospholipids occurs mostly in the phospholipid membrane, mitochondria, peroxisomes, and microsome. The products of LPO were seen involved in nephrotoxicity, hepatotoxicity, and neurotoxicity [27]. Oxidative stress caused by H_2O_2 (free radicals) is an important feature of several acute and chronic infections and is currently considered as one of the main reasons leading to the adverse health impacts of airborne PM.

Earlier studies on DEP showed that inflammatory markers influence systemic cells by a TNF-α dependent mechanism with an increase in chemokines and cytokines [31]. Recent work on DEPs showed an increase in IL-8 and IL-6 with no TNF-α release in human epithelial cells [32]. Studies also showed that PM such as PM2.5 was found to provoke pro-inflammatory cytokines and thus contribute to oxidative stress and inflammation [33]. Animal and human experiments suggest that flavonoids are potent antioxidants that reduce the threat of cardiovascular diseases, cancers, and cerebrovascular diseases [34]. Quercetin is an important flavonoid that has potential effects on health due to its anti-toxic and anti-oxidant function. The alleviate effects of catechin, myricetin, and quercetin were investigated against N-nitrosopiperidine and N-nitrosodibutylamine induced damage in cell

lines [35]. Studies showed that quercetin was able to defend cells from cellular senescence and oxidative damage *in vitro* in a dose-dependent manner [36]. Quercetin is a potent free-radical scavenger and chelating agent that chelates the iron metal that is responsible for the production of ROS [37].

Studies showed that flavonoids play a key role in combating toxicity [38]. Dose-dependent studies on quercetin revealed that it inhibited TNF-α formation in lipopolysaccharide induced human volunteers [39]. Other studies showed that, quercetin inhibited oxidative stress in 4 nitro, 3 phenylphenol (PNMPP)-induced rats and hence allowed reversion of GSH-Px, SOD activity, cell viability, and MDA to normal levels [7]. Studies by Chow *et al.*, 2005, revealed that quercetin blocked the formation of DNA ladders, hypodiploid cells and intracellular peroxides induced by H_2O_2, through its antioxidant activity [40]. Quercetin was found to significantly decrease LPO, prevent GSH depletion, and DNA damage in HEP G2 cells [41]. Similarly, the oxidative damage induced by biphenyls was prevented by the activity of quercetin [42].

In the present study, quercetin inhibited oxidative stress and inflammation. These data's on the protective ability of quercetin against oxidative stress and inflammation are consistent with previous literatures. Studies explained that quercetin's chemical structure plays a key role in its free-radical scavenging activity. The O-dihyroxyl group in the C-ring and B-ring is mainly responsible for its activity [43]. The antioxidant activity of quercetin is associated not only with its structure but also with its ability to penetrate and interact with lipid bilayers. Therefore, it can prevent the cytotoxicity of DEPs or PEPs by maintaining normal cell morphology.

CONCLUSION

The effects of DEP, PEP, Q, DEP+Q and PEP+Q on cytotoxicity, oxidative stress, and inflammation were evaluated *in vitro* in H9c2 cells. Different concentrations of DEP and PEP induced oxidative stress by increasing MDA and altering anti-oxidant enzyme levels in H9c2 cells. We scrutinized that LDH, NO, TP, MDA, antioxidant, and cytokine levels were ameliorated in DEP/PEP+Q treated groups. There are no adequate data's on the protective ability of quercetin on DEP/PEP induced toxicity *in vitro*. Therefore, this study aimed to explore the protective ability of quercetin against DEP/PEP induced *in vitro* toxicity.

AUTHORS' CONTRIBUTIONS

Durga M – Manuscript preparation, analysis of results and statistical applications, literature review.

Devasena T – Overall work guidance.

CONFLICTS OF INTEREST

The authors hereby declare that we have no conflicts of interest.

AUTHORS' FUNDING

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