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Original Article

# Protective role of poncirin against polyethylene microplastics instigated cardiac toxicity via regulating Nrf2/keap1 pathway

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## Abstract

Polyethylene microplastics (PEMPs) are one of the most toxic pollutants in our surroundings that induce damage to various organs including heart. Poncirin (PON) is a natural flavonoid that shows diverse pharmacological activities. This study was aimed to assess the alleviative potential of PON against PEMPs provoked cardiac damage in rats. Twenty-four rats were segregated into 4 groups including control, PEMPs (1.5 mg/kg) treated group, PEMPs (1.5 mg/kg) + PON (5mg/kg) exposed group and PON (5mg/kg) alone treated group. It was revealed that PEMPs exposure notably decreased the expression of Nrf2 and its associated antioxidant genes while upregulating the expression of Keap-1. Besides, PEMPs intoxication reduced the activities of superoxide dismutase (SOD), catalase (CAT), heme-oxygenase-1 (HO-1), peroxidase (GPx), glutathione reductase (GSR), and glutathione (GSH) content while increasing reactive oxygen species (ROS) and malondialdehyde (MDA) levels. Additionally, exposure to PEMPs resulted in upregulation of lactate dehydrogenase (LDH), creatine kinase MB (CK-MB), troponin I and phosphokinase (CPK). Besides, PEMPs administration escalated the levels of TNF- α IL-6, NF-κB, TNF- α, IL-1β, and COX-2 activity. Moreover, the administration of PEMPs escalated the levels of Caspase-3 and Bax, while downregulating the levels of Bcl-2. Additionally, PEMPs exposure disrupted the architecture of cardiac tissues. Nonetheless, PON supplementation remarkably protected the cardiac tissues by regulating the aforementioned damages.

**Keywords**: Polyethylene microplastics, Poncirin, Cardiac damage, Oxidative stress, Inflammation

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## Introduction

Since the 1950s, plastic production has exhibited exponential growth reaching approximately 368 million metric tons annually (Ackerman and Levin, 2023). Owing to its durability and low costs, plastics are considered as ubiquitous materials in various industrial sectors and have become an intrinsic component of municipal waste. An estimation suggests that 19-23 million metric tons of plastics waste is dumped into aquatic environment which affects the living beings (Borrelle et al., 2020). Plastics are considered as "boundary threats" particularly when they are fragmented into micro (≤5 mm) or nano-plastics due to elevated temperature, mechanical abrasion, sunlight, and wave action (Shi et al., 2023). Furthermore, Microplastics (MPs) pose detrimental impacts on the health of aquatic as well as terrestrial organisms by instigating inflammation, reproductive impairments, oxidative stress, metabolic disruptions as well as genetic abnormalities (Mani et al., 2015).

PEMPs are highly prevalent in aquatic as well as terrestrial ecosystems. It was reported that PEMPs exposure induced various behavioral, cytotoxic, developmental as well as mutagenic disruptions (Malafaia et al., 2020). Moreover, PEMPs can pass through blood barriers and accumulate in body organs which instigate various impairments in energy metabolism as well as morphometric alterations in hepatic tissues (Anbumani and Kakkar, 2018). It was reported that exposure to PEMPs prompted various histological impairments in renal tissues of rats (Ahmad et al., 2023). Furthermore, the administration of PEMPs provoked testicular abnormalities by disrupting hormonal, steroidogenic and morphological parameters in rats (Ijaz et al., 2022) as well as significantly altered genetic, epigenetic and hematological, and parameters in mammals (Farag et al., 2023).

Natural products are getting global attention owing to their potential biological properties against various disorders (Sorokina and Steinbeck, 2020). Poncirin is a plant-based flavonoid that demonstrates antimicrobial, antioxidant, anticancer and anti-inflammatory potentials (Patel, 2021; Li et al., 2022). Therefore, this investigation was aimed to determine the palliative potential of poncirin against PEMPs provoked heart damage in rats.

## Material and Methods

### Chemicals

Both PEMPs and PON were procured from Sigma Aldrich (USA).

### Animals

Twenty-four albino rats were randomly distributed into four groups and kept in the Animal house of University of Agriculture, Faisalabad. The animals were provided optimum laboratory conditions such as humidity (55±5%), temperature (22-25 ºC) and 12 hours of dark and light cycles. Rats were given free access to tap water and standard feed chaw. The rats were handled according to the guidelines of "European Union for Animal Care and Experimentation".

### Experimental design

Twenty-four rats were apportioned into four different groups (6 rats in each). These groups included control, PEMPs (1.5 mg/kg), PEMPs (1.5 mg/kg) + PON (5mg/kg) and PON (5mg/kg) alone. The dose of PEMPs was selected according to the previous study conducted by Ijaz et al. (2022), while the dose of PON was selected according to the study of Ullah et al. (2022). After the completion of the experiment, the rats were anaesthetized, slaughtered and blood was collected from trunk in EDTA and gel activator vials. The blood was centrifuged (3000 rpm for 15 minutes) to isolate the plasma. The heart was surgically excised from thoracic cavity and divided into two equal parts. One half was fixed in formalin (10%) for histo-morphological evaluations whereas, other half was packed in zipper bags and stored at –20 ºC for biochemical evaluations.

### Biochemical assessment

The assessment of CAT activity was conducted by following the methodology described by Chance and Maehly (1955). The procedure outlined by Kakkar et al. (1984) was employed to quantify the activity of SOD. GPx activity was determined in accordance with the method of Rotruck et al. (1973), while the GSR activity was determined by following the protocol of Carlberg and Mannervik (1975). The levels of MDA and ROS were assessed by using the technique elucidated by

Ohkawa (1979) and Hayashi et al. (2007) respectively. The technique described by Magee et al. (1999) was used to determine HO-1 activity.

**The expression of apoptotic markers and Nrf2/keap1 pathway**

The expressions of Nrf2/keap1 and its associated genes as well as apoptotic markers (Bcl-2, Caspase-3 and Bax) expressions were determined by using qRT-PCR. Total RNA was isolated by using TRIzol reagent and subsequently converted into cDNA. Variations in the expressions of abovementioned biomarkers were measured by $2^{-\Delta\Delta CT}$ while β-actin was considered as an internal control (Livak and Schmittgen, 2001). The primer sequences of targeted genes are shown in table 1 as described by Hamza et al. (2023) and Ijaz et al. (2022).

**The cardiac injury markers**

The levels of CK-MB (MBS2515061), CPK (E4608-100), LDH (CSB-E11324r), and troponin I (CSB-E08594r) were assessed by using standard ELISA kits by following the guidelines provided in the manual book of product.

**Inflammatory indices**

Inflammatory markers, including the levels of NF-κB (CSB-E13148r), IL-6 (CSB-E04640r), IL-1β (CSB-E08055r), TNF-α (CSB-E07379r), and COX (CSB-E13399r) activity were assessed by using rat ELISA kits. The procedure was carried out by following the guidelines of Abcam, USA.

**Table-1. Primers sequences for the real-time quantitative reverse transcription polymerase (RT-qPCR)**

| Gene | Primers 5′ -> 3′ | Accession number |
|---|---|---|
| Nrf-2 | F: ACCTTGAACACAGATTTCGGTG | NM_031789.1 |
| | R: TGTGTTCAGTGAAATGCCGGA | |
| Keap-1 | F: ACCGAACCTTCAGTTACACACT | NM_057152.1 |
| | R: ACCACTTTGTGGGCCATGAA | |
| CAT | F: TGCAGATGTGAAGCGCTTCAA | NM_012520.2 |
| | R: TGGGAGTTGTACTGGTCCAGAA | |
| SOD | F: AGGAGAAACTGACAGCTGTGTCT | NM_017051.2 |
| | R: AAGATAGTAAGCGTGCTCCCAC | |
| GPx | F: TGCTCATTGAGAATGTCGCGTC | NM_030826.4 |
| | R: ACCATTCACCTCGCACTTCTCA | |
| GSR | F: ACCAAGTCCCACATCGAAGTC | NM_053906.2 |
| | R: ATCACTGGTTATCCCCAGGCT | |
| GST | F: TCGACATGTATGCAGAAGGAGT | NM_031509.2 |
| | R: CTAGGTAAACATCAGCCCTGCT | |
| HO-1 | F: AGGCTTTAAGCTGGTGATGGC | NM_012580.2 |
| | R: ACGCTTTACGTAGTGCTGTGT | |
| Bax | F: GCACTAAAGTGCCCGAGCTG | NM_017059.2 |
| | R: CCAGATGGTGAGTGAGGCAG | |
| Bcl-2 | F: ACTGAGTACCTGAACCGGCA | NM_016993.1 |
| | R: CCCAGGTATGCACCCAGAGT | |
| Caspase-3 | F: GTACAGAGCTGGACTGCGGT | NM_012922.2 |
| | R: TCAGCATGGCGCAAAGTGAC | |
| β-actin | F: AGGAGATTACTGCCCTGGCT | NM_031144 |
| | R: CATTTGCGGTGCACGATGGA | |

**Histopathological assessment**

The tissues (6 samples/group) were fixed in formalin (10%) following the process of dehydration in 80, 90, and 100% ethanol. Samples were embedded in paraffin wax. The paraffin blocks were sliced into 4-5µm of thick pieces by using rotatory microtome. These pieces were fixed on glass slide and stained with hematoxylin/eosin and examined by using a light microscope at 400X. The microphotographs of all the groups were captured by MoticTM camera to assess the impacts of PEMPs and PON on cardiac histology.

**Statistical analysis**

The findings were shown as Means ± SEM. One-way ANOVA followed by Tukey's test were applied to compare the differences among groups. The level of significance was set at $p < 0.05$.

## Results

**Impacts of PON and PEMPs on the expression of Nrf2/keap1 pathways**

The expressions of Nrf2 and keap1 are shown in figure 1. It was revealed that PEMPs exposure notably ($p < 0.05$) lowered the expression of Nrf2 and its cytoprotective genes GPx, HO-1, SOD, GSR, GST and CAT while upregulating Keap-1 expressions in contrast to control group. However, PON + PEMPs treatment considerably ($p < 0.05$) upregulated the expressions Nrf2 and its cytoprotective genes while reducing Keap-1 expressions. However, no significant differences were observed in the expressions of PON alone treated group and the control group.

**Impacts of PON and PEMPs on biochemical parameters**

The impacts of PON on the biochemical parameters (antioxidant and oxidants) are illustrated in table 2. It was observed that PEMPs intoxication notably ($p < 0.05$) decreased CAT, SOD, HO-1, GPx, GSR activities and GSH contents while escalating the ROS and MDA levels when compared with the control group. However, the co-treatment with PON + PEMPs remarkably ($p < 0.05$) augmented the abovementioned antioxidant enzymes activities while downregulating ROS and MDA levels. Nonetheless, the PON alone supplemented group showed no discrepancies as compared to the control group.

**Table-2. The effects of PEMPs and PON on biochemical profile of albino rats**

| Parameters | Groups | | | |
|---|---|---|---|---|
| | Control | PEMPs | PEMPs + PON | PON |
| **CAT (U/mg protein)** | 9.78±0.72$^{a}$ | 4.55±0.27$^{c}$ | 8.05±0.25$^{b}$ | 9.83±0.73$^{a}$ |
| **SOD (U/mg protein)** | 8.05±0.50$^{a}$ | 3.05±0.25$^{c}$ | 5.91±0.63$^{b}$ | 8.13±0.51$^{a}$ |
| **GSR (nM NADPH oxidized/min/mg tissue** | 6.12±0.21$^{a}$ | 1.78±0.19$^{c}$ | 4.26±0.27$^{b}$ | 6.31±0.40$^{a}$ |
| **GPx (U/mg protein)** | 31.41±2.23$^{a}$ | 9.16±0.48$^{c}$ | 26.43±0.98$^{b}$ | 31.86±2.61$^{a}$ |
| **GSH (nM/min/mg protein)** | 18.58±0.72$^{a}$ | 6.63±0.30$^{c}$ | 14.41±0.83$^{b}$ | 18.67±0.82$^{a}$ |
| **GST (nM/min/mg protein)** | 24.67±0.80$^{a}$ | 6.59±0.72$^{c}$ | 19.48±1.89$^{b}$ | 25.21±1.43$^{a}$ |
| **HO-1 (U/mg protein)** | 329.37±10.78$^{a}$ | 91.38±6.06$^{c}$ | 238.97±13.66$^{b}$ | 333.87±9.09$^{a}$ |
| **MDA (nmol/mg protein)** | 0.62±0.11$^{c}$ | 6.54±0.22$^{a}$ | 1.51±0.20$^{b}$ | 0.59±0.12$^{c}$ |
| **ROS (nmol/g)** | 1.39±0.15$^{c}$ | 7.31±0.23$^{a}$ | 2.71±0.16$^{b}$ | 1.35±0.16$^{c}$ |

Different superscripts in the same row are showing the variations among different groups at $P < 0.05$.

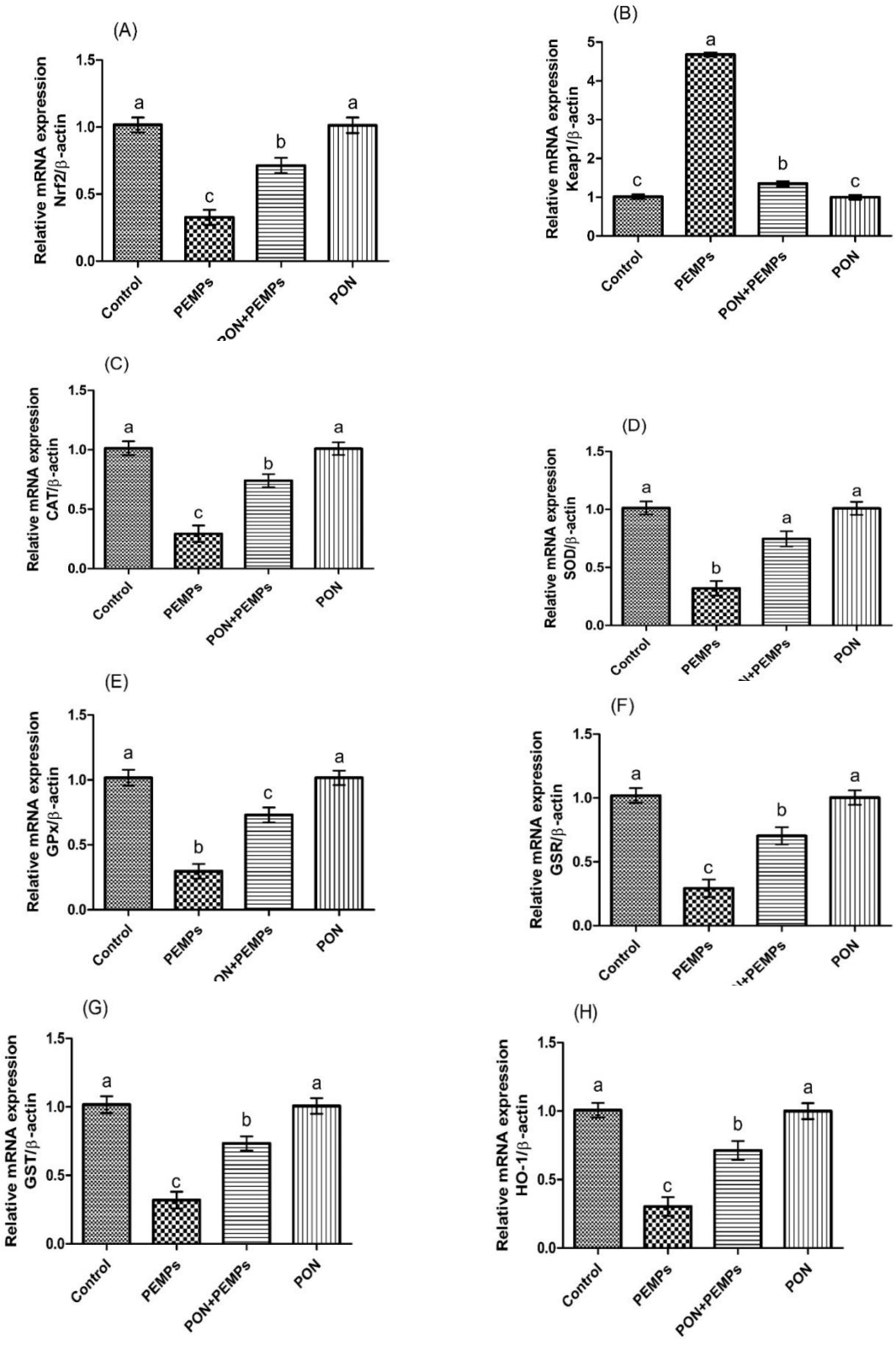



**Figure-1**: **Impacts of PON and PEMPs on the expression of following parameters (A) Nrf2 (B) Keap1 (C) CAT (D) SOD (E) GPx (F) GSR (G) GST (H) HO-1**

**Impacts of PON and PEMPs on Cardiac injury biomarkers**

It was revealed that PEMPs exposure substantially ($p < 0.05$) elevated CK-MB, troponin-I, LDH, and CPK levels in comparison to the control group. However, PON + PEMPs markedly ($p < 0.05$) reduced the levels of abovementioned cardiac injury biomarkers. Furthermore, the mean values of PON alone supplemented group were comparable to the control group (Table 3).

**Impacts of PON and PEMPs on Inflammatory indices**

It was noted that PEMPs administrated group showed remarkable ($p < 0.05$) escalation in NF-κB, TNF-α, IL-6, IL-1β levels and COX-2 activity as compared to the control rats. Moreover, the administration of PON + PEMPs notably ($p < 0.05$) reduced the levels of abovementioned inflammatory biomarkers. Nonetheless, PON alone supplemented group showed mean values close to the control group (Table 4).

**Impacts of PON and PEMPs on Apoptotic parameters**

It was noted that PEMPs exposure substantially ($p < 0.05$) increased the expressions of caspase-3 and Bax while decreasing the expressions of Bcl-2 as compared to the control group. However, the co-treatment with PON + PEMPs markedly ($p < 0.05$) reduced the expressions of caspase-3 and Bax, while upregulating the Bcl-2 expressions. Furthermore, PON alone supplemented group showed insignificant differences as compared to the control group (Figure 2).

**Impacts of PON and PEMPs on histomorphology of cardiac tissues**

The histological examination revealed that PEMPs exposure significantly ($p < 0.05$) increased various histological abnormalities such as cellular edema, endothelial degeneration, hypertrophy, myocardial ischemia, interstitial fibrosis, and degeneration of cardiomyocytes. However, the supplementation of PON + PEMPs remarkably restored the aforementioned histological disruptions. Furthermore, PON alone treated group, and the control group exhibited normal morphology of heart tissues (Figure 3).

**Table-3. The effects of PEMPs and PON on cardiac injury markers of albino rats**

| Parameters | Groups | | | |
|---|---|---|---|---|
| | Control | PEMPs | PON + PEMPs | PON |
| **Troponin(pg/ml)** | $0.48\pm0.23^{c}$ | $5.30\pm0.31^{a}$ | $1.34\pm0.20^{b}$ | $0.46\pm0.23^{c}$ |
| **LDH (mg/dl)** | $13.85\pm1.69^{c}$ | $66.28\pm3.06^{a}$ | $21.94\pm1.28^{b}$ | $13.74\pm1.65^{c}$ |
| **CPK (mcg/L)** | $154.76\pm8.24^{c}$ | $417.2\pm18.0^{a}$ | $222.57\pm11.01^{b}$ | $151.40\pm7.07^{c}$ |
| **CK-MB (ng/mL)** | $25.37\pm1.89^{c}$ | $76.10\pm3.26^{a}$ | $48.26\pm1.64^{b}$ | $25.22\pm1.37^{c}$ |

Different superscripts in the same row are showing the variations among different groups at $P < 0.05$.

**Table-4. The effects of PEMPs and PON on Inflammatory indices of albino rats**

| Parameters | Groups | | | |
|---|---|---|---|---|
| | Control | PEMPs | PON + PEMPs | PON |
| **NF-kB (ng/g tissue)** | 16.82±1.93c | 74.48±1.80a | 25.58±1.83b | 15.36±2.57c |
| **TNFα (ng/g tissue)** | 7.98±0.79c | 68.79±1.56a | 15.51±0.74b | 7.95±0.75c |
| **IL-1ß (ng/g tissue)** | 23.51±0.92c | 91.44±1.96a | 38.33±2.70b | 23.43±0.97c |
| **IL-6 (ng/g tissue)** | 10.44±1.02c | 72.62±2.58a | 25.95±1.47b | 10.39±1.02c |
| **COX-2 (ng/g tissue)** | 17.48±0.98c | 83.26±3.10a | 32.27 ±2.21b | 17.45±0.99c |

Different superscripts in the same row are showing the variations among different groups at $P < 0.05$.

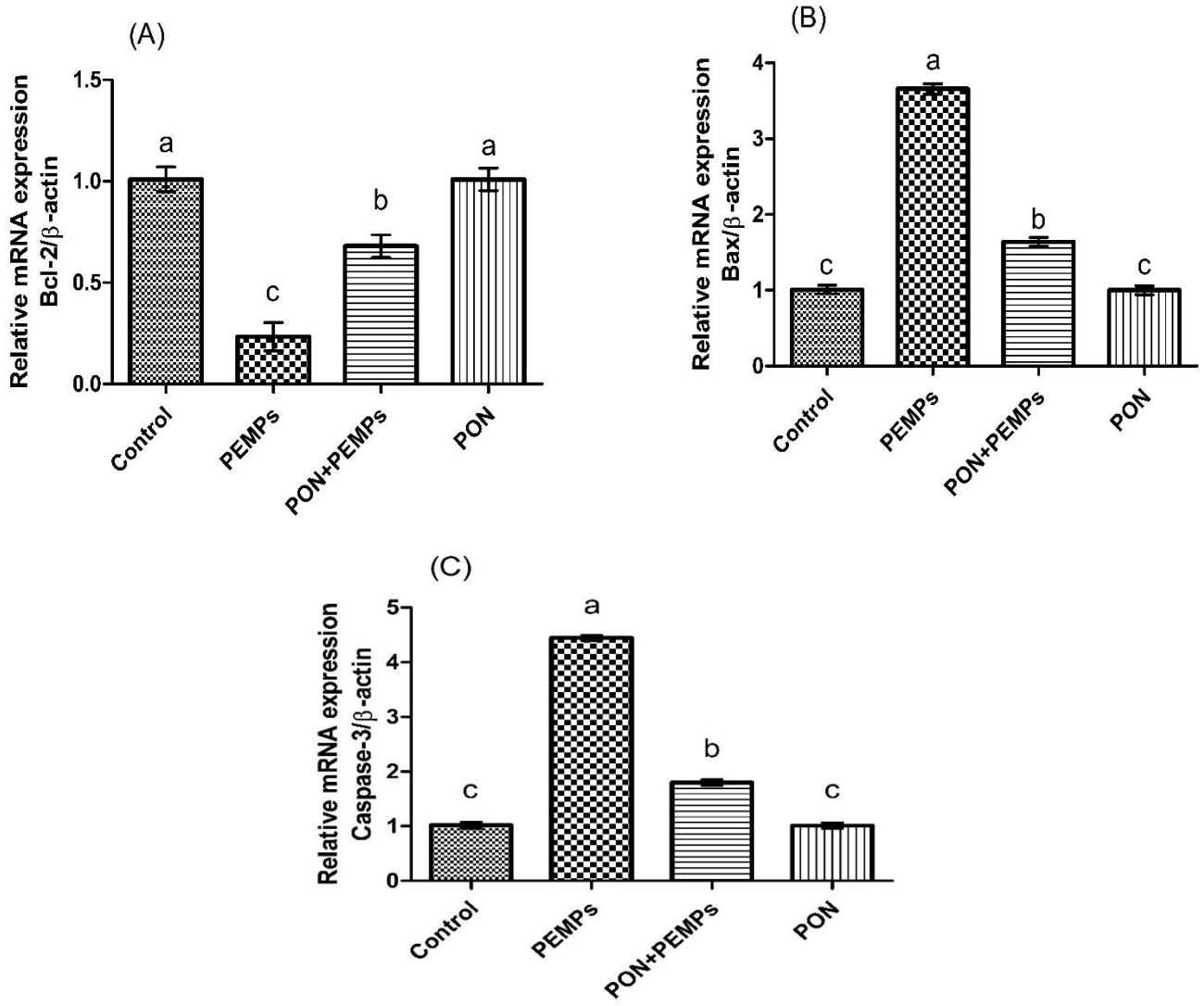
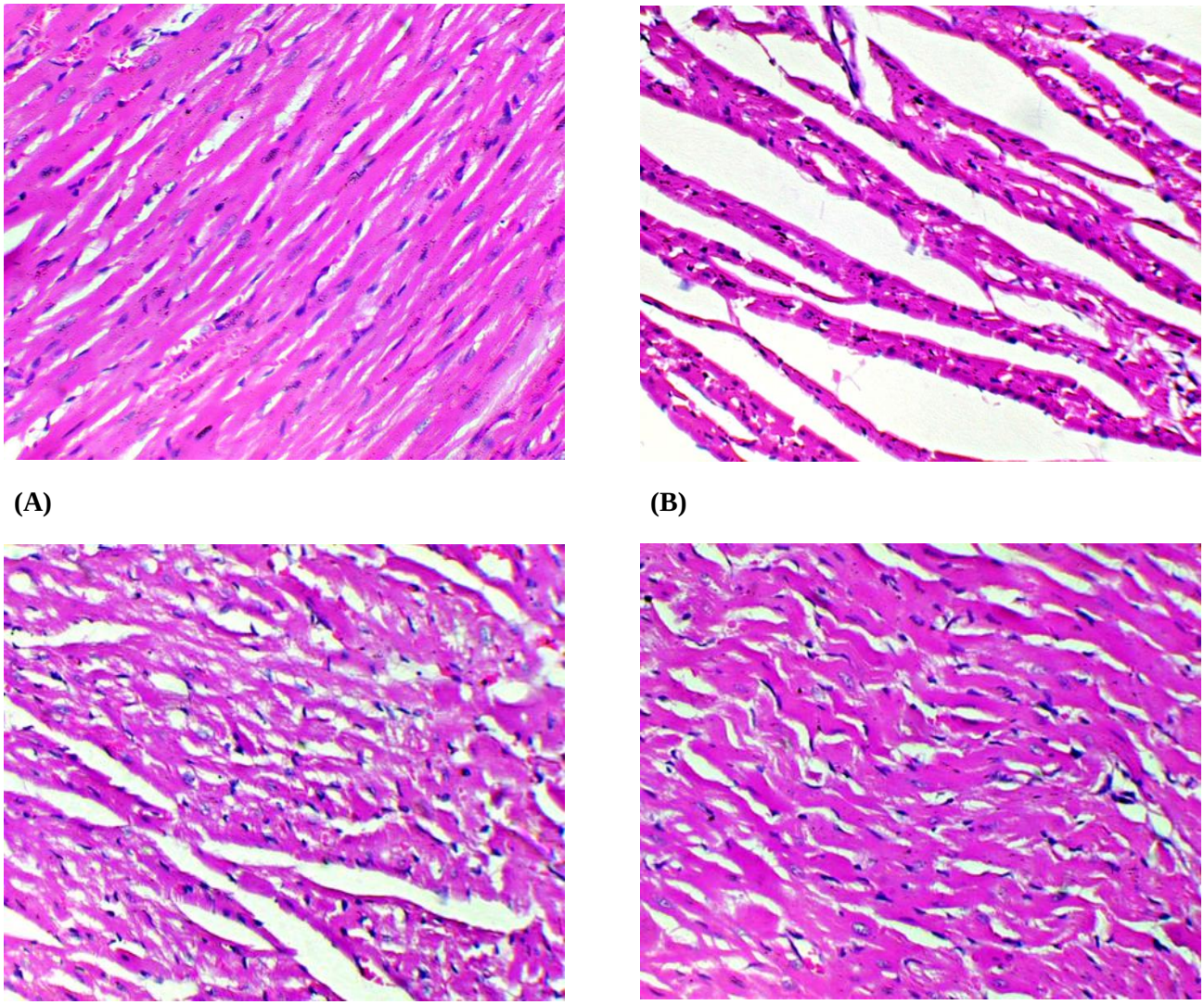



**Figure-2 is showing the impacts of PON and PEMPs on the expression of following parameters (A) Bcl-2 (B) Bax (C) Caspase-3**



**(A)** **(B)**

**(C)** **(D)**

**Figure-3. Microphotographs of histological investigation by H and E stain at 400x.**
**(A): Control group demonstrated normal histology of cardiac muscles.**
**(B): PEMPs intoxicated group exhibited cardiac damages including inflammation, interstitial edema as well as fibrosis.**
**(C) Co-administrated (PEMPs + PON) treated group revealed remarkable recovery in contrast to PEMPs intoxicated group.**
**(D): Alone PON supplemented group exhibited normal histology as in control group.**

## Discussion

The current study was performed to investigate the efficacy of PON against PEMPs prompted cardiac impairments via regulating biochemical parameters, cardiac injury biomarkers, inflammatory indices, apoptotic markers, and histopathological parameters. Polyethylene is accountable for 36% of total plastic production in the world and considered as a top-notch type of plastic which ultimately pollutes the aquatic environment (Bardají et al., 2020). Furthermore, numerous investigations have revealed that administration of PEMPs demonstrates pro-oxidant potentials by escalating the levels of ROS (Palaniappan et al., 2022).

Nrf2 is an important transcription factor that regulates various antioxidative as well as anti-inflammatory genes expressions whereas keap1 is a cytosolic repressor protein which regulates Nrf2 activity (Lu et al., 2016). Nrf-2/Keap-1 is an indigenous intracellular pathway which protects the cell against adverse effects of oxidative stress (Bartolini et al., 2018). Our findings revealed that PEMPs intoxication reduced Nrf2 expressions which subsequently reduced the expression of antioxidative cytoprotective genes while escalating the expression of keap1. Nrf2 gets separated from its repressor (keap1) and enters the nuclear region where it binds with AREs and initiates the transcription of cytoprotective genes (Buendia et al., 2016). Therefore, the Nrf-2/Keap-1 pathway ensures the cellular integrity against cellular oxidative stress produced by ROS (Long et al., 2022). Nonetheless, supplementation of PON + PEMPs remarkably enhanced Nrf2 expression while downregulating keap1 expression ultimately counteracting the generation of ROS.

The present study revealed that PEMPs exposure decreased the activities of antioxidant enzymes while upregulating the levels of ROS as well as MDA. The human body harbors a sophisticated antioxidant system comprising of endogenous enzymatic and non-enzymatic antioxidants which combat the free radicals thereby mitigating their deleterious effects on various body tissues (Ighodaro and Akinloye, 2018). CAT is an antioxidative enzyme which reduces hydrogen peroxide into $H_2O$ and $O_2$ (Bratovcic, 2020). GPx stimulates the breakdown of $H_2O_2$ as well as lipid peroxides into water molecules and alcohols respectively (Sarıkaya and Doğan, 2020). It is documented that OS instigates myocardial injury therefore, the use of antioxidant may prevent the onset of cardiac failure (Aimo et al., 2020). ROS are unstable molecules that are responsible for the onset of cellular oxidative stress. The balance between ROS and antioxidants enzymes is crucial to regulate cellular homeostasis (Zhang et al., 2022). However, our study revealed that PON + PEMPs exposure notably escalated the aforementioned antioxidant enzymes activities while reducing ROS and MDA levels. Flavonoids demonstrate ROS scavenging potential owing to the presence of hydroxyl groups as well as conjugation between aromatic rings. Therefore, PON is a flavonoid and shares a similar structure with other flavonoids which is attributed to its ROS scavenging ability (Shen et al., 2022).

CK-MB and LDH are important biomarkers of cardiac damage and referred to as "Gold standard signal" against various cardiac impairments while the creatinine kinase plays pivotal role in cellular energy metabolism (Yucel, 2022). It is reported that troponin I exhibits high specificity to diagnose various myocardial damages at early stages (Afsar et al., 2017). Our investigation demonstrated that PEMPs intoxication escalated CK-MB, CPK, LDH, and troponin I level. Furthermore, the elevated levels of the abovementioned cardiac injury biomarkers demonstrate various damages to cardiomyocyte as well as myocardial endothelium resulting from ROS which in turn release these biomarkers from cardiomyocytes into blood stream (Omole et al., 2018). However, administration of PON + PEMPs remarkably reduced the levels of aforementioned cardiac injury biomarkers.

The present investigation revealed that PEMPs intoxication upregulated the levels of inflammatory markers, including NF-κB, IL-1β, IL-6, TNF-α, and COX-2 activity. Inflammation is a non-specific immune response against different types of organ damage in the body (Oronsky et al., 2022). Elevated levels of inflammatory cytokines in cardiac tissues are early signs of cardiac impairments (Yang et al., 2017). The activation of NF-κB triggers the modulation of other inflammatory cytokines such as TNF-α, IL-1β, COX-2, and IL-6 (Liu et al., 2017). It was reported that excessive generation of ROS triggers the activation of NF-κB, and the use of antioxidant may be a novel therapeutic against chronic inflammatory responses (Cinar et al., 2022). In line with the previous observation, the supplementation of PON downregulated the levels of

abovementioned inflammatory cytokines which ultimately prevented the onset of cardiac inflammation.

Apoptosis is a programmed cell death that is involved in the development of various pathological events. Dong et al. (2019) elucidated that dysregulated apoptosis is the underlying cause of various morphological impairments in heart. Our findings demonstrated that administration of PEMPs augmented the expressions of Caspase-3 and Bax while reducing the expressions of Bcl-2. Bax is a pro-apoptotic biomarker, and its activation triggers the formation of a pore in mitochondrial membrane. This pore subsequently liberates cytochrome C from mitochondrial membrane which plays a pivotal role in mediating apoptotic pathways (Radhiga et al., 2012). In contrast, Bcl-2 exerts antagonistic effects to Bax by inhibiting the process of apoptosis therefore safeguarding the structural and functional integrity of mitochondria in cardiomyocytes (Deavall et al., 2012). Nevertheless, the treatment with PON + PEMPs markedly reduced the expressions of pro-apoptotic markers while escalating the expressions of anti-apoptotic biomarkers which suggests that PON has anti-apoptotic potential that can conserve the functional as well as structural integrity of heart.

The current investigation revealed that PEMPs intoxication instigated various architectural damages such as cardiac edema, vascular congestion, myocardial fibrosis, dilated cardiomyopathy, endocardial thickening, and myocardial infraction. Furthermore, Kaludercic and Di Lisa (2020) revealed that excessive generation of ROS induced histopathological alterations in cardiac tissues of rats as evidenced by biochemical and histopathological analysis of heart tissues. However, supplementation of PON + PEMPs significantly restored the aforementioned histopathological alterations in cardiac tissues of rats via inhibiting intracellular ROS.

## Conclusion

The present investigation revealed that PEMPs intoxication reduced the expression of Nrf2 and cytoprotective genes while upregulating OS levels. Besides, the levels of cardiac injury, and inflammatory parameters were escalated following the PEMPs intoxication. Furthermore, PEMPs administration disrupted the balance between pro-apoptotic and anti-apoptotic markers. Moreover, PEMPs administration prompted various architectural impairments in the cardiac tissues. Nonetheless, supplementation of PON + PEMPs alleviated cardiac damage via regulating antioxidant, inflammatory, apoptotic, and histopathological dysfunctions. However, human based trials should be conducted to further evaluate the potential of PON against PEMPs instigated cardiac injuries.

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### Contribution of Authors

Rasheed Y & Ehsan N: Designed the experiment and wrote the manuscript
Hayat MF, Ashraf A & Khan HA: Conducted the experiment and wrote the manuscript
Khatoon A & Ijaz MU: Performed the statistical analysis and wrote the manuscript
Raouf YS, Samadi A & Chtita S: Validated the study and reviewed the literature
All authors read and approved the final version of manuscript.

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