



# Attenuative effects of poncirin against polyethylene microplastics-prompted hepatotoxicity in rats

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## ABSTRACT

Polyethylene microplastics (PE-MPs) are of significant concern due to their widespread use, pervasive persistence in the environment that induce multiple organ damage especially in the liver. Poncirin (PON) is a naturally present flavone with conspicuous pharmacological properties. The current investigation was formulated to ascertain the palliative role of PON against PE-MPs-provoked hepatic dysfunction. Twenty-four male albino rats were randomly divided into four groups: control, PE-MPs-treated (1.5 mg/kg), PE-MPs + PON co-treated (1.5 mg/kg and 20 mg/kg), and PON-treated (20 mg/kg). PE-MPs intoxication markedly lowered the expressions of antioxidant genes and Nrf-2, besides escalating Keap-1 expression. It also decreased antioxidants i.e., glutathione (GSH), glutathione S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione reductase (GSR) activities, while remarkably upsurged reactive oxygen species (ROS) along with malondialdehyde (MDA) contents. Additionally, a notable escalation was observed in the levels of hepatic serum markers i.e., alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate aminotransferase (AST). Furthermore, PE-MPs exposure increased the levels of inflammatory biomarkers, including tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), nuclear factor kappa B (NF- $\kappa$ B), interleukin-1 $\beta$  (IL-1 $\beta$ ) levels and cyclooxygenase-2 (COX-2) activities. PE-MPs intoxication augmented the expressions of Caspase-3 and Bax along with decreasing the expression of Bcl-2. Nevertheless, PON treatment notably abated PE-MPs prompted liver injuries owing to its hepatoprotective efficacy. Thus, it may be inferred that PON could be a potential therapeutic option for treating hepatic damage caused by PE-MPs.

## 1. Introduction

Plastics play a crucial role in daily life due to their durability, versatility, affordability as well as convenience. However, their widespread accumulation in the environment has garnered significant concerns (Hou et al., 2021). According to reports of 2017, global plastic production has surged up to 3.48 x 10<sup>8</sup> tons (Plastics Europe, 2018). Microplastics (MPs) are ubiquitous environmental pollutants that are produced by the fragmentation of large plastic products into tiny plastic particles (diameter < 5 mm). MPs enter into the food chain due to their small size and low-density. Humans and other living organisms are exposed to microplastics (MPs) through dermal contact, inhalation, and ingestion. Numerous human food items, including beer, drinking water,

honey, seafood, sugar, and table salt, have been found to contain MPs (Cox et al., 2019). MPs exposure has been reported to retard growth, instigate hormonal disturbance, oxidative stress (OS), immunological dysfunction and genotoxicity (Choi et al., 2018; Ehsan et al., 2023; Ahmad et al., 2023).

One of the most popular forms of plastic is polyethylene (PE). PE is used in the production of composite materials, packaging, and in various other industries, including agriculture (Beg et al., 2016). Polyethylene microplastics (PE-MPs) can cause systematic toxicity in kidney, colon, heart, lung and liver, along with reproductive abnormalities (Kim et al., 2021). PE-MPs also can induce hepatotoxicity by interfering with the lipids metabolism in liver cells (Leal Filho et al., 2019). Exposure to PE-MPs leads to the excessive production of reactive oxygen species (ROS)

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**Table 1**  
Primers sequences for RT-qPCR.

Gene	Primers 5' → 3'	Accession number
Nrf-2	F: ACCTTGAACACAGATTTCGGTG R: TGTGTTTCAGTGAAATGCCGGA	NM_031789.1
Keap-1	F: ACCGAACCTTCAGTTACACACT R: ACCACTTTGTGGGCCATGAA	NM_057152.1
CAT	F: TGCAGATGTGAAGCGCTTCAA R: TGGGAGTTGACTGGTCCAGAA	NM_012520.2
SOD	F: AGGAGAAACTGACAGCTGTGTCT R: AAGATAGTAAGCGTGTCCCAC	NM_017051.2
GPx	F: TGCTCATTGAGAAATGTGCGGTC R: ACCATTACCTCGCACTTCTCA	NM_030826.4
GSR	F: ACCAAGTCCCACATCGAAGTC R: ATCACTGGTTATCCCCAGGCT	NM_053906.2
HO-1	F: AGGCTTTAAGCTGTGTATGGC R: ACGCTTTACGTAGTGTGTGT	NM_012580.2
Bax	F: GGCCTTTTGTCTACAGGGTT R: AGCTCCATGTTGTTGTCCAG	NM_017059.2
Bcl-2	F: ACAACATCGCTCTGTGGAT R: TCAGAGACAGCCAGGAGAA	NM_016993.1
Caspase-3	F: ATCCATGGAAGCAAGTCGAT R: CCTTTGCTGTGATCTTCCT	NM_012922.2
β-actin	F: TACAGCTTACCACCACAGC R: GGAACCGCTCATTGCCGATA	NM_031144

that results in OS, which leads to metabolism disturbance and DNA damage (Rai et al., 2021). PE-MP exposure may also alter signaling pathways leading to autophagy and apoptosis (Zhao et al., 2020).

Flavonoids are a class of polyphenolic substances that exhibit remarkable biological properties, such as anti-inflammatory properties and the potential to eliminate free radicals and inhibit hydrolases and oxidases. Due to these tremendous therapeutic properties, flavonoids are used to treat multiple ailments (Pourmora et al., 2006). Poncirin (PON) is found in *Poncirus trifoliata* plant and various citrus fruits such as oranges, tangerines, mandarins, grapefruits, tangelos, and chinotto. PON shows multiple biological characteristics i.e., free radical scavenging, anti-oxidant as well as anti-inflammatory properties (Ullah et al., 2020). Therefore, this research aimed to check the putative efficacy of PON in alleviating liver damage caused by PE-MPs.

## 2. Materials and methods

### 2.1. Chemicals

PON (Cas No. 14941–08-3) and PE-MPs (Cas No. 9002–88-4) were retrieved from Merck (United States of America).

### 2.2. Experimental animals

The current experiment was conducted on 24 male albino rats weighing  $200 \pm 20$  g. Animals were confined in animal care facility of University of Agriculture Faisalabad, Pakistan under controlled environmental conditions (Humidity  $45 \pm 5$  %, temperature  $23\text{--}26$  °C, and 12 h. day/dark light cycles). The animals were granted complete access to pelleted food as well as water. The animals were treated and handled according to the guidelines of EU Directive 2010/63/EU for animal experiments.

### 2.3. Experimental layout

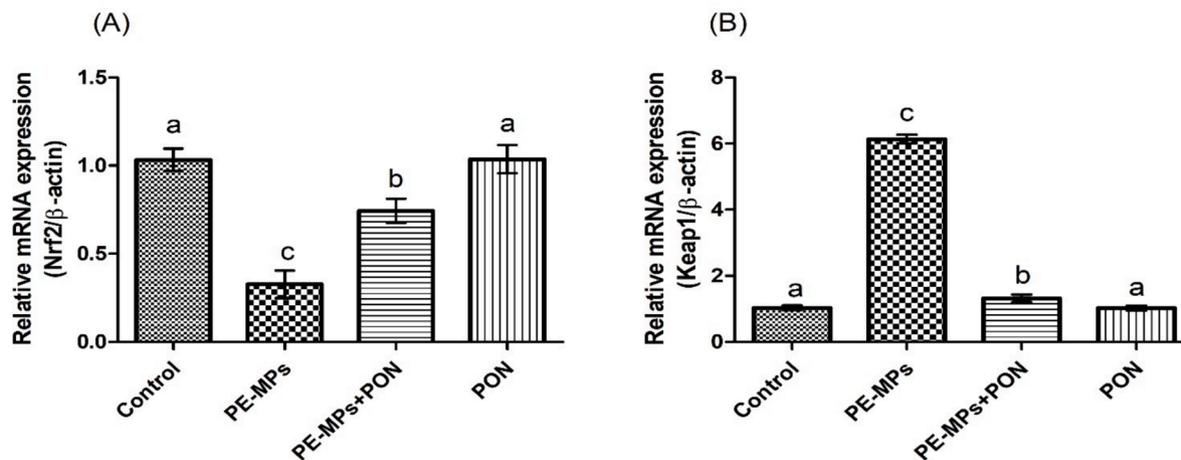
The rats were categorised into four groups of equal size, with each group including six rats; control, a group exposed to PE-MPs (1.5 mg/kg), a group co-treated with PE-MPs and PON (1.5 mg/kg + 20 mg/kg) respectively and the last group supplemented with only PON (20 mg/kg). The dose of PE-MPs and PON were administered according to the previous studies of Ijaz et al. (2022) and Kang and Kim (2016), respectively. Following a 30-day treatment period, the rats were anesthetized with ketamine and xylazine and decapitated, and their cardiac blood was taken using heparinized syringes. The collected blood samples were then placed at a temperature of  $-20$ °C for biochemical testing. The liver was carefully excised, rinsed with a saline solution and stored in a zippered bag at a temperature of  $-80$  °C for further analysis.

### 2.4. Evaluation of antioxidant profile

Chance and Maehly's (1955) protocol was followed to assess CAT activity. The methodology of Kakkar et al. (1984) was employed for the analysis of SOD activity. GPx activity was quantified via following the protocols of Lawrence and Burk (1976). GSR activity was appraised with the approach of Carlberg and Mannervik (1975). The method of Couri and Abdel-Rahmans (1979) was applied to quantify GST activity. GSH activity was ascertained using the method of Sedlak and Lindsay (1968), while the activity of HO-1 was quantified by using the technique outlined by Magee et al. (1999). The contents of ROS were accessed using the method of Hayashi et al. (2007). Moreover, the level of MDA was appraised via following the method of Ohkawa et al. (1978).

### 2.5. qRT-Polymerase chain reaction (qRT-PCR)

The expressions of Nrf-2/Keap-1, apoptotic markers (Caspase-3, Bax and Bcl-2) and antioxidant genes were appraised by qRT-PCR. The total RNA of the cell was obtained with the help of TRI-zol reagent. RNA was



**Fig. 1.** Protective role of PON on (a) Nrf-2, (b) Keap-1 expression. Values are depicted as mean  $\pm$  standard error of mean. Different superscripts on bars are presenting significant variation.

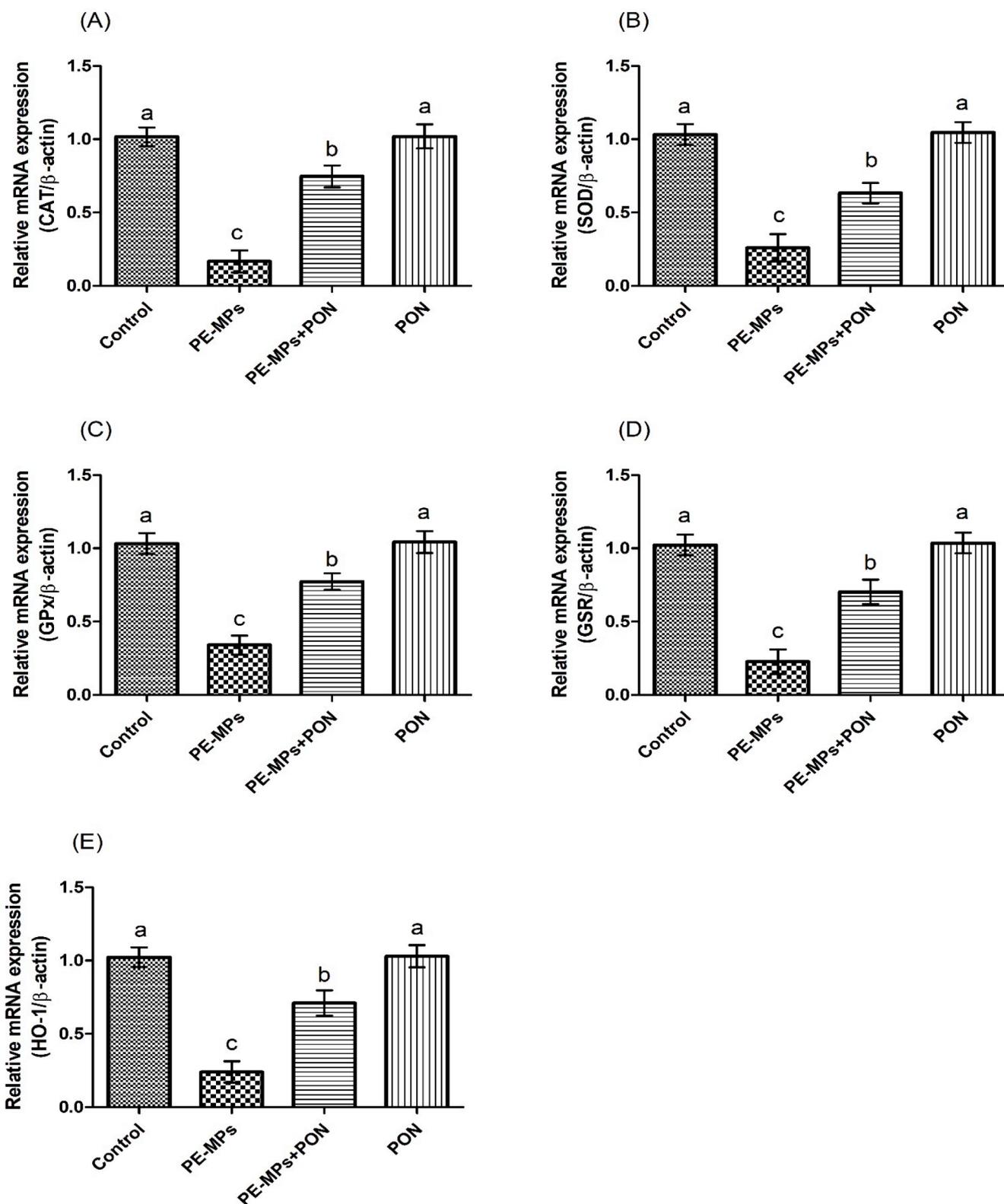


Fig. 2. Protective role of PON on (a) CAT, (b) SOD, (c) GPx, (d) GSR, (e) HO-1 expression. Values are depicted as mean  $\pm$  standard error of mean. Different subscripts on bars are presenting significant variation.

transcribed to produce cDNA via using Fast Quant reverse transcription kit (Takara, China). Alterations in the expressions of these parameters were determined by  $2^{-\Delta\Delta CT}$ , employing  $\beta$ -actin as internal control. Table 1 depicted the primer of the genes (Ijaz et al., 2022).

### 2.6. Assessment of hepatic serum indices

The levels of hepatic markers i.e., AST (ab263883), ALP (ab287823) and ALT (ab285264) were analyzed with the help of commercially available ELISA kits (Wiesbaden, Germany). The company's guidelines

**Table 2**  
Protective role of PON on antioxidant and oxidative stress profile.

PARAMETERS	GROUPS			
	Control	PE-MPs	PE-MPs + PON	PON
CAT (Umg <sup>-1</sup> protein)	14.14 ± 1.08 <sup>a</sup>	6.96 ± 0.20 <sup>c</sup>	11.58 ± 0.93 <sup>b</sup>	14.95 ± 1.138 <sup>a</sup>
SOD (Umg <sup>-1</sup> protein)	10.77 ± 1.02 <sup>a</sup>	4.23 ± 0.35 <sup>c</sup>	8.01 ± 0.48 <sup>b</sup>	11.06 ± 1.22 <sup>a</sup>
GPx (Umg <sup>-1</sup> protein)	24.80 ± 1.35 <sup>a</sup>	8.27 ± 0.99 <sup>b</sup>	22.49 ± 1.02 <sup>a</sup>	25.74 ± 1.58 <sup>a</sup>
GSR (nM NADPH oxidized/min/mg tissue)	8.41 ± 0.34 <sup>a</sup>	2.28 ± 0.36 <sup>c</sup>	4.86 ± 0.50 <sup>b</sup>	8.750 ± 0.72 <sup>a</sup>
GST (nM/min/mg protein)	35.54 ± 2.71 <sup>a</sup>	9.86 ± 1.69 <sup>c</sup>	27.52 ± 1.32 <sup>b</sup>	34.32 ± 2.86 <sup>a</sup>
GSH (µM/g tissue)	17.27 ± 1.26 <sup>b</sup>	6.62 ± 0.62 <sup>c</sup>	25.25 ± 2.00 <sup>a</sup>	16.72 ± 1.02 <sup>b</sup>
HO-1 (pmoles bilirubin/mg protein/h)	285.60 ± 11.44 <sup>a</sup>	53.72 ± 10.25 <sup>c</sup>	214.67 ± 12.75 <sup>b</sup>	293.62 ± 14.33 <sup>a</sup>
ROS (Umg <sup>-1</sup> tissue)	1.36 ± 0.35 <sup>b</sup>	9.41 ± 1.09 <sup>a</sup>	2.49 ± 0.42 <sup>b</sup>	1.28 ± 0.37 <sup>b</sup>
MDA (nmol/mg protein)	0.54 ± 0.29 <sup>b</sup>	3.38 ± 0.47 <sup>a</sup>	1.38 ± 0.27 <sup>b</sup>	0.47 ± 0.33 <sup>b</sup>

The values with different superscript are significantly distinct from other groups.

**Table 3**  
Protective role of PON on liver function markers.

PARAMETERS	GROUPS			
	Control	PE-MPs	PE-MPs + PON	PON
ALT (U/L)	54.92 ± 2.12 <sup>c</sup>	92.63 ± 2.59 <sup>a</sup>	67.79 ± 2.44 <sup>b</sup>	53.82 ± 2.38 <sup>c</sup>
AST (U/L)	75.75 ± 2.65 <sup>c</sup>	249.83 ± 10.53 <sup>a</sup>	159.24 ± 8.27 <sup>b</sup>	73.70 ± 1.39 <sup>c</sup>
ALP (U/L)	98.41 ± 3.79 <sup>c</sup>	414.58 ± 12.43 <sup>a</sup>	187.78 ± 9.76 <sup>b</sup>	97.38 ± 3.54 <sup>c</sup>

The values with dissimilar letters are notably distinct from other groups.

were followed to access these parameters.

### 2.7. Evaluation of inflammatory indices

NF-κB (CSB-E13148r), IL-1β (CSB-E08055r), IL-6 (CSB-E04640r), TNF-α (CSB-E07379r) levels and COX-2 activity (CSB-E13399r) was evaluated via ELISA kit (YL Biotech Co. Ltd., Shanghai, China). The analyses were performed as per the directions of manufacturers.

### 2.8. Statistical analysis

The data were reported as the mean value ± SEM. A one-way ANOVA followed by Tukey's test was used for group comparisons. The level of statistical significance was set at  $p < 0.05$ .

## 3. Results

### 3.1. Efficacy of PON on the expression of Nrf-2/Keap-1

The group intoxicated with PE-MPs lead to a remarkable ( $p < 0.05$ ) decline in the expression of Nrf-2 and antioxidant genes, whereas increasing the expression of Keap-1, in relation to the control. Nonetheless, PON + PE-MPs co-treatment regulated the expressions of these parameters in contrast to PE-MPs treated rats. Moreover, the rats subjected to PON only exhibited these expressions close to control animals (Figs. 1,2).

### 3.2. Efficacy of PON on biochemical indices

A substantial ( $p < 0.05$ ) down-regulation in the activities of antioxidant activities (GSH, GST, CAT, GPx, HO-1, SOD, and GSR), while an escalation in the levels of ROS and MDA was observed in rats administered with PE-MPs, relative to control group. Nevertheless, the co-supplementation of PON + PE-MPs substantially augmented the activities of antioxidant enzymes, whereas MDA and ROS contents were significantly decreased, as compared to the PE-MPs treated rats. In addition to this, no marked variations were noted in the level of these biomarkers in PON only supplemented and control animals (Table 2).

### 3.3. Efficacy of PON on hepatic function markers

The levels of ALT, ALP and AST were markedly ( $p < 0.05$ ) escalated in rats exposed to PE-MPs, as matched with the control. While PON + PE-MPs treatment reduced these levels relative to animals subjected to PE-MPs. In addition to this, only PON treated group displayed the levels of these markers almost in line with control (Table 3).

### 3.4. Efficacy of PON on apoptotic markers

PE-MPs treatment led to a remarkable ( $p < 0.05$ ) increased in the expressions of Caspase-3 and Bax, besides down-regulating the expression of Bcl-2, as matched with control group. Nevertheless, concurrent treatment of PON + PE-MPs remarkably regulated these expressions, relative to PE-MPs administered rats. Additionally, the rats supplemented with PON only exhibited the aforementioned parameters comparable to the control (Fig. 3).

### 3.5. Efficacy of PON on inflammatory indices

PE-MPs markedly ( $p < 0.05$ ) elevated the levels of inflammatory biomarkers, as compared to the control. However, PON + PE-MPs supplementation lowered the levels of inflammatory indices, compared to the PE-MPs treated rats. However, the levels of inflammatory indices in PON only treated animals were presented to be almost similar to the control (Table 4).

## 4. Discussion

Liver is the main detoxifying organ in the body which is involved in metabolism, but becomes susceptible to OS when exposed to toxins. PE-MPs intoxication results in production of ROS in the liver which leads to hepatic damage (Vethaak and Legler, 2021). PE-MPs treatment damages the hepatocytes via disturbing the metabolism, microtubule separation and protein denaturation. PON belongs to a diverse group of phytonutrients (flavonoids) and exhibits versatile biological characteristics such as, anti-inflammatory, antioxidant and immunoregulatory (Ullah et al., 2020). Thus, this research was formulated to access the therapeutic efficacy of PON against PE-MPs-provoked hepatic damages in albino rats by determining the liver function enzymes, biochemical and inflammatory indices.

PE-MPs treatment reduced the expressions of antioxidant genes and Nrf-2, whereas increasing Keap-1 expression. Yamamoto et al. (2018) stated that Nrf-2 performs crucial function in the regulation of OS. While Keap-1 acts as the inhibitor of Nrf-2 and controls its stability (Pintard et al., 2004). Nrf-2 separates from Keap-1 during ROS production through some physical modifications. Following the separation, Nrf-2 is relocated to the nucleus, where it engages with small MAF (musculoaponeurotic fibrosarcoma) proteins. Therefore, Nrf-2 has an indispensable part in monitoring the stimulation of antioxidant genes (Hawkes et al., 2014). Nevertheless, PON administration elevated the expressions of Nrf-2 and antioxidant genes, while lowering Keap-1 expression. Therefore, it is assumed that PON can regulate the expressions of Nrf-2 and Keap-1.

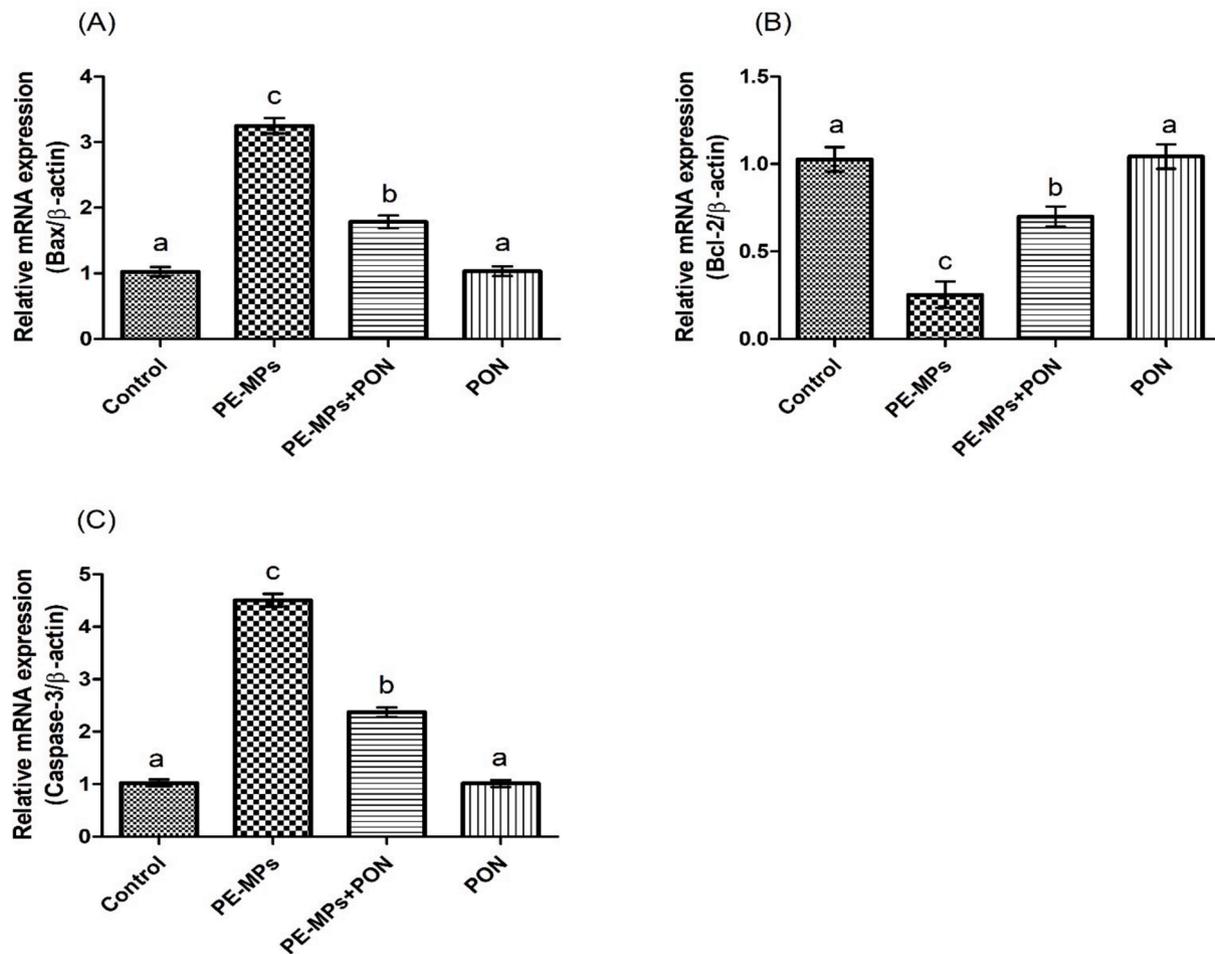


Fig. 3. Protective role of PON on (a) Bax, (b) Bcl-2 and (c) Caspase-3 expression. Values are depicted as mean ± standard error of mean. Significant variation is depicted by different superscripts each bar.

Table 4  
Protective role of PON on inflammatory indices.

PARAMETERS	GROUPS			
	Control	PE-MPs	PE-MPs + PON	PON
NF-κB (ngg <sup>-1</sup> tissue)	23.89 ± 1.55 <sup>c</sup>	84.31 ± 2.06 <sup>a</sup>	33.82 ± 2.13 <sup>a</sup>	22.96 ± 1.26 <sup>c</sup>
TNF-α (ngg <sup>-1</sup> tissue)	16.70 ± 1.66 <sup>c</sup>	65.59 ± 1.82 <sup>a</sup>	28.07 ± 2.30 <sup>a</sup>	15.71 ± 1.54 <sup>c</sup>
IL-1β (ngg <sup>-1</sup> tissue)	7.48 ± 1.00 <sup>c</sup>	58.39 ± 3.88 <sup>a</sup>	17.76 ± 1.68 <sup>b</sup>	7.35 ± 1.10 <sup>c</sup>
IL-6 (ngg <sup>-1</sup> tissue)	4.32 ± 1.85 <sup>bc</sup>	34.39 ± 2.30 <sup>a</sup>	9.92 ± 2.52 <sup>b</sup>	4.19 ± 1.98 <sup>c</sup>
COX-2 (ngg <sup>-1</sup> tissue)	16.12 ± 2.53 <sup>c</sup>	94.99 ± 3.25 <sup>a</sup>	25.99 ± 2.20 <sup>b</sup>	15.52 ± 2.79 <sup>c</sup>

The values with dissimilar letters are significantly different from other groups.

The results of our study indicated that PE-MPs administration decreased the activities of antioxidants i.e., SOD, GSR, GSH, GST, CAT, HO-1 and GPx, while markedly escalating ROS and MDA contents in the liver. These antioxidants are the 1st wall of protection of the cell which protects the cellular proteins, lipids and DNA against OS damage (Bansal et al. 2005). GSR sustains GSH level by converting glutathione disulfide (GSSG) into GSH (Zhu et al., 2012). GST plays a critical role in the detoxification of toxins to safeguard the cells from oxygen free radicals (Hayes et al., 2005). However, the decrease in the activities of antioxidant enzymes leads to excessive ROS generation, which weakens the

anti-oxidative capacity of the cell to fight against free radicals and ultimately results in OS. MDA is the byproduct of lipid peroxidation (LP), which is used as a biomarker for the detection of LP and ROS induced cellular damages (He et al., 2012). Nevertheless, the supplementation of PON + PE-MPs augmented the antioxidants activities, while reducing the ROS and MDA concentrations. These attenuative effects of PON in restoring the antioxidants can be due to its free radical scavenging nature.

PE-MPs intoxication dramatically elevated the levels of hepatic markers, including, ALP, ALT and AST. The effect of any toxicant on the function of liver can be analyzed by estimating the level of these function enzymes. These aminotransferases are present in the mitochondria of hepatic cells and their release into the blood indicates hepatic injuries. OS increases the mitochondrial permeability, allowing the leakage of these hepatic markers into blood which leads to hepatotoxicity (Nagai et al., 2016). Additionally, multiple studies proposed that OS is the major reason behind this aberrant increase in hepatic function markers (Knudsen et al., 2016). However, the supplementation of PON with PE-MPs remarkably mitigated PE-MPs prompted hepatotoxicity by substantially lowering the levels of these hepatic enzymes owing to the ROS scavenging and hepatoprotective features of PON.

The results of our study demonstrated that PE-MPs administration elevated the expressions of Bax and Caspase-3, besides downregulating Bcl-2 expression. ROS triggered apoptosis and OS are the ultimate causes of hepatotoxicity (Danial and Korsmeyer 2004). The overall process of apoptosis is controlled by the proteins belonging to Caspase and Bcl-2 families. Bax promotes the apoptotic cell death, besides Bcl-2 performs

an antagonistic role to Bax and protects the cells from apoptosis. The increase in the expression of Bax along with the reduction in Bcl-2 prompt efflux of cytochrome C from the mitochondrial membrane into the cytosol. The increased contents of cytochrome C content inside the cytosol leads to the activation of Caspase-3. Caspase-3 cleaves the cellular proteins, therefore altering the structural makeup of cells and eventually triggering apoptotic cell death (Végran et al., 2011). However, concurrent treatment of PON + PE-MPs markedly upregulated the expression of Bcl-2, besides reducing the expressions of Caspase-3 and Bax, as a result of anti-apoptotic potential of PON.

PE-MPs treatment noticeably elevated the levels of inflammatory markers. The activation of inflammation modulator, NF- $\kappa$ B, due to any cellular activity such as excessive OS, stimulates the production of IL-6, TNF- $\alpha$ , IL-1 $\beta$  (Nakajima and Kitamura 2013). The generation of these markers leads to acute inflammatory response and several other anomalies linked with abnormal ROS accumulation. COX-2 is another inflammatory marker which is reported to instigate inflammation (Caglayan et al., 2018). Nevertheless, PON supplementation down-regulated these levels due to its anti-inflammatory characteristics. This anti-inflammatory potency of PON might be due to the methoxylation of 5'- or 7' OH groups and non-methoxylation of 3' OH groups on A and B rings of PON respectively (Yang et al. 2020).

## 5. Conclusion

In summary, our investigation demonstrated that PE-MPs administration instigated OS and LP in hepatic tissues that potently disturbed the levels of oxidative stress markers, liver function enzymes, inflammatory indices. It also disturbed the expressions of Nrf-2/Keap-1 and apoptotic markers. Nevertheless, PON administration effectively recovered all these impairments due to hepatoprotective, antioxidant, anti-inflammatory as well as anti-apoptotic potential. Thus, it is assumed that PON might be applied as a therapeutic compound in future to treat hepatic damage. Due to its therapeutic potential, PON could be integrated into current treatment protocols, providing a novel approach to treat patients suffering from hepatic damage. However, the current study was performed on model animals, therefore, we recommend clinical trials of this compound to check its efficacy on human beings.

## CRedit authorship contribution statement

**Naila Ghafoor:** Writing – original draft, Methodology, Investigation, Conceptualization. **Tooba Mehar:** Writing – original draft, Methodology, Investigation, Conceptualization. **Moazama Batool:** Visualization, Validation, Software, Data curation. **Muhammad Zaid Salar:** Writing – review & editing, Validation, Methodology, Investigation. **Mohammad Z. Ahmed:** Writing – review & editing, Resources, Funding acquisition, Data curation. **Usman Atique:** Writing – review & editing, Formal analysis, Data curation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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