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Ginkgetin alleviates polystyrene microplastics-instigated liver injury in rats through Nrf-2/Keap-1 pathway activation

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ABSTRACT

Polystyrene microplastics (PS-MPs) are potential environmental toxicants that are reported to instigate oxidative stress (OS) in the liver. Ginkgetin (GK) is a natural biflavonoid with potential therapeutic activities. This experiment was executed to access the putative effect of GK against PS-MPs provoked hepatotoxicity. Four groups were formed from 48 rats including control, PS-MPs (0.01 mg/kg) group, PS-MPs (0.01 mg/kg) + GK (25 mg/kg) co-treated group and GK (25 mg/kg) alone group. The exposure of PS-MPs markedly decreased the expressions of antioxidant genes and Nrf-2, besides escalating Keap-1 expression. It also decreased the activities of 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), while increasing reactive oxygen species (ROS) and malondialdehyde (MDA) contents. Additionally, a notable escalation in hepatic serum markers i.e., alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate aminotransferase (AST) level was observed. Furthermore, PS-MPs exposure escalated the levels of inflammatory markers, i.e., tumor necrosis factor-α (TNF-α), interleukin- 6 (IL-6), nuclear factor-kappa B (NF-kB), interleukin-β (IL-1β) level and cyclooxygenase-2 (COX-2) activity. PS-MPs treatment augmented Caspase-3 and Bax expressions and decreased Bcl-2 expression. Nevertheless, GK treatment notably abated PS-MPs prompted liver injuries owing to its hepatoprotective efficacy.

1. Introduction

Our environment is highly contaminated with different types of pollutants, but the pollution caused by plastics is of utmost concern (Li et al., 2016). When plastic or its products enter into the environment, hydrolytic forces and UV radiation break them into small fragments of less than 5 mm size, these small particles are called microplastics (Suhrhoff and Scholz-Böttcher, 2016). Large polymers of styrene are degraded into tiny particles known as polystyrene microplastics (PS-MPs) (Cox et al., 2019). Cheap prices, high durability and long-term applications have made PS-MPs the first choice of material used in lunch boxes, cleaning supplies, disposable teacups, and various sorts of insulatory materials (Hou et al., 2021). Humans are directly exposed to these microplastics via inhalation, drinking contaminated water and consuming food containing MPs such as table salt, beer, shellfish, honey, and sugar (Romeo et al., 2015). According to a study, the average per capita intake of MPs in the USA is 74,000–121,000 particles (Cox et al., 2019). PS-MPs exert negative impacts on the normal physiological processes of the body which ultimately damage various body organs including liver, stomach, testicular, kidney, brain, and heart (Leal Filho et al., 2019).

Polystyrene microplastics (PS-MPs) could instigate OS that results in lipid peroxidation (LP) and causes oxidative injury in the vital organs including gut, intestines, kidney, testes, and liver (Ijaz et al., 2021; Pan et al., 2021; Goodman et al., 2022). These cellular changes ultimately result in serious metabolic disorders (Deng et al., 2017). Liver is a vital body part that is responsible for the detoxification of harmful substances, at the same time it is highly vulnerable to the damages induced

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by PS-MPs and its metabolites (Zhao et al., 2020). PS-MPs prompt hazardous damages in humans including disruption in immune responses, induced oxidative stress (OS) and disruptions in IL-6, TNF-α levels as well as malondialdehyde (MDA) content (Dong et al., 2020).

Flavonoids are diverse naturally present polyphenolic entities occurring in plants as secondary metabolites (Panche et al., 2016). These metabolites are widely extracted from vegetables, beverages, cherries, apples, and cloves (Park et al., 2014). A plethora of research showed that these compounds act as strong antioxidant against free radicals (Ishtiaq et al., 2022). Ginkgetin (GK), a flavonoid, is reported in the leaves of *Ginkgo biloba*. GK is reported to alleviate testicular and renal toxicity due to its antioxidant as well as antiinflammatory activities (Akbar and Ijaz, 2024; Ehsan et al., 2023). Considering the aforementioned therapeutic roles of GK, this study was performed to reveal the remedial potentials of GK on PS-MPs triggered liver damages in albino rats.

2. Materials and methods

2.1. Chemicals

PS-MPs (Purity ≥ 96 %, CAS # 9003–53–6) and GK (Purity 98 %, CAS # 481–46–9) were brought from Merck (US).

2.2. Animals

The experiment was conducted on 48 male albino rats weighing 200 ± 20 g. Animals were confined in animal care facility of University of Agriculture Faisalabad, Pakistan at controlled environmental conditions (Humidity 45 ± 5 %, temperature 23–26 °C, and 12 h. day/dark light cycles). The animals were provided with unrestricted food access with standard pallets along with water. Rats were maintained in line with the animal ethics guidelines of the European Union.

2.3. Experimental design

Forty-eight rats were allocated in 4 groups (n = 12) and kept in steel cages. The dose administrated to rats was as follows: control group, PS-MPs administrated group (0.01 mg/kg), PS-MPs + GK co-treated group (0.01 mg/kg and 25 mg/kg respectively) and GK only administered group (25 mg/kg). These doses were administered according to our previous study (Akbar and Ijaz, 2024). The rats were acclimatized in the laboratory environment for seven days, following that the doses of PS-MPs and GK were administered for 30 days. Upon completion of the treatment, all rats were sedated and decapitated on the last day. Blood was drawn into heparinized tubes. In order to obtain serum, blood was centrifuged for about 15 min at 3000 rpm, which was then kept for further analysis at −20 °C. The liver was excised and preserved at −80 °C for biochemical testing.

2.4. Biochemical profile

Chance and Maehly's (1955) protocol was executed to assess CAT activity. The methodology of Kakkar et al. (1984) was employed for the analysis of SOD activity. GPx activity was quantified via the methodology of Jollow et al. (1974). GSR activity was appraised with the approach of Carlberg and Mannervik (1975). Habig et al. (1974) strategy was applied to quantify GST activity. The method demonstrated by Sedlak and Lindsay (1968) was applied to ascertain GSH activity, while the HO-1 activity was quantified by examining bilirubin biosynthesis through the technique outlined by Magee et al. (1999). By implementing the approach of Hayashi et al. (2007) the ROS content was assessed. The level of MDA was appraised through 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) as well as antioxidant genes were appraised by qRT-PCR. The total RNA of the cell was obtained with the help of TRI-zol reagent. RNA was transcribed to produce cDNA via employing Fast Quant reverse transcription kit from Takara located in China. Alterations in the expressions of these parameters were determined by $2^{-\Delta\Delta CT}$, considering β-actin as inner control (Ijaz et al., 2022). Table 1 depicted the gene primers.

2.6. Assay of liver enzymes

The levels of liver serum markers i.e., AST (ab263883), ALP (ab287823) and ALT (ab285264) were analyzed with the help of commercially available ELISA kits purchased from Wiesbaden (Germany) as per the instructor's guidelines.

2.7. Estimation 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

Results were depicted as Mean ± SEM. One-way ANOVA and Tukey's test was applied to assess the whole data. The significance level was set at $p < 0.05$.

3. Results

3.1. Protective role of GK on the expression of Nrf-2/Keap-1

PS-MPs inebriation resulted in a remarkable ($p < 0.05$) decline in the expression of Nrf-2 and antioxidant genes, whereas upsurging Keap-1 expression in relation to control. Alternatively, GK + PS-MPs co-treatment regulated the expressions of these parameters. Moreover, the rats subjected to GK only, exhibited these expressions approximately nearby to control animals (Figs. 1, 2).

Table 1
Primers sequences for RT-qPCR.

Gene	Primers 5′ −> 3′	Accession number
Nrf-2	F: ACCTTGAACACAGATTTCGGTG R: TGTGTTCAGTGAAATGCCGGA	NM_031789.1
Keap-1	F: ACCGAACCTTCAGTTACACACT R: ACCACTTTGTGGGCCATGAA	NM_057152.1
CAT	F: TGCAGATGTGAAGCGCTTCAA R: TGGGAGTTGTACTGGTCCAGAA	NM_012520.2
SOD	F: AGGAGAAACTGACAGCTGTGTCT R: AAGATAGTAAGCGTGCTCCCAC	NM_017051.2
GPx	F: TGCTCATTGAGAATGTCGCGTC R: ACCATTCACCTCGCACTTCTCA	NM_030826.4
GSR	F: ACCAAGTCCCACATCGAAGTC R: ATCACTGGTTATCCCCAGGCT	NM_053906.2
GST	F: TCGACATGTATGCAGAAGGAGT R: CTAGGTAAACATCAGCCCTGCT	NM_031509.2
HO-1	F: AGGCTTTAAGCTGGTGATGGC R: ACGCTTTACGTAGTGCTGTGT	NM_012580.2
Bax	F: GGCCTTTTTGCTACAGGGTT R: AGCTCCATGTTGTTGTCCAG	NM_017059.2
Bcl-2	F: ACAACATCGCTCTGTGGAT R: TCAGAGACAGCCAGGAGAA	NM_016993.1
Caspase-3	F: ATCCATGGAAGCAAGTCGAT R: CCTTTTGCTGTGATCTTCCT	NM_012922.2
β-actin	F: TACAGCTTCACCACCACAGC R: GGAACCGCTCATTGCCGATA	NM_031144

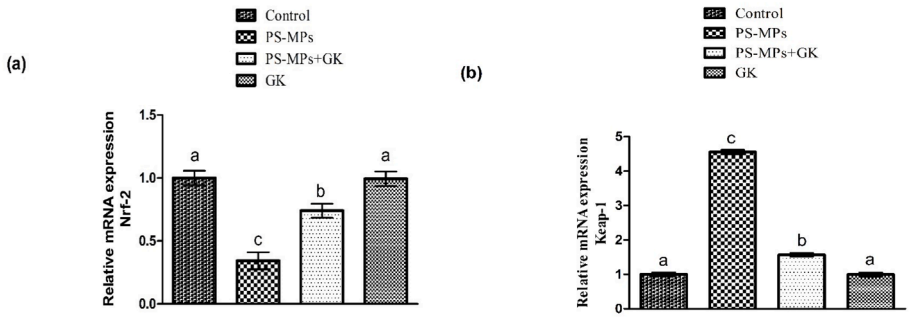

Fig. 1. Protective role of GK on **(a)** Nrf-2, **(b)** Keap-1 expression. Bars are shown on the basis of mean ± standard error of mean. Different superscripts on bars presenting significant variation.

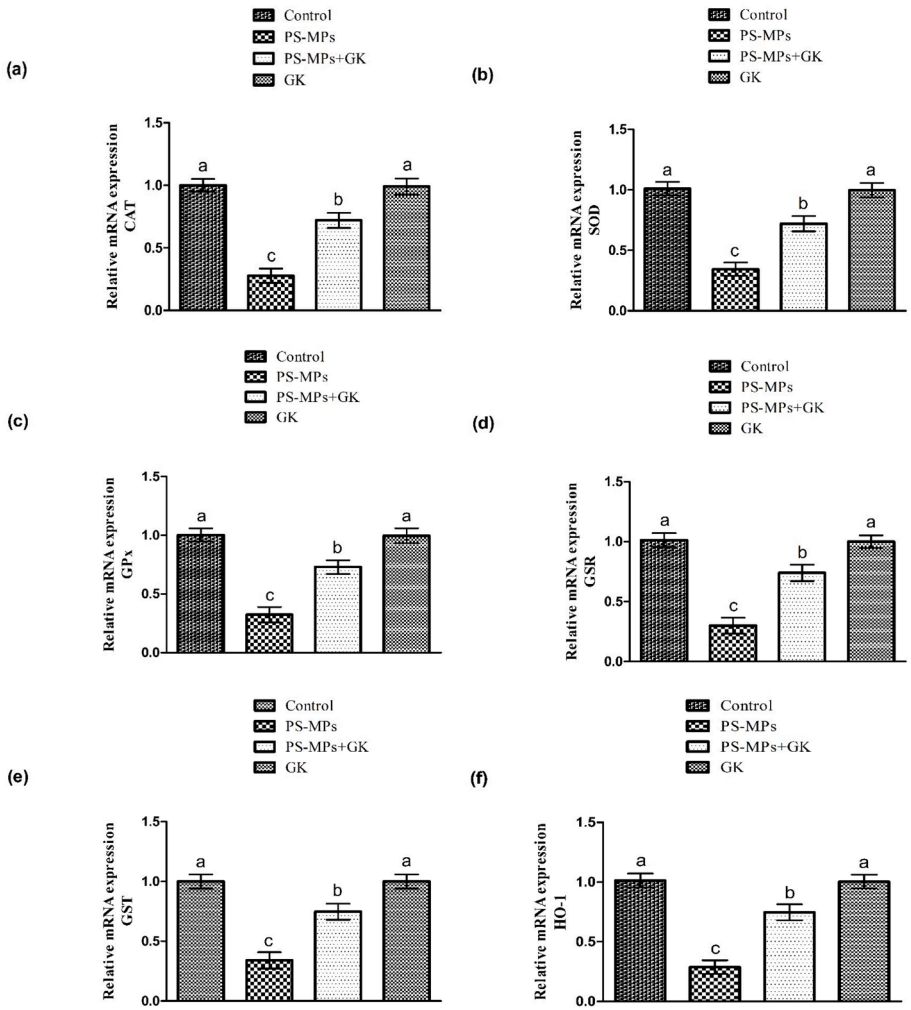

Fig. 2. Protective role of GK on **(a)** CAT, **(b)** SOD, **(c)** GPx, **(d)** GSR, **(e)** GST along with **(f)** HO-1 expression. Bars are based on mean ± standard error of mean. Different superscripts on bars presenting significant variation.

3.2. Protective role of GK on biochemical indicators

A substantial ($p < 0.05$) down-regulation in antioxidants activities, while an escalation in ROS and MDA levels was identified in rats administered with PS-MPs as compared to the control group. Nevertheless, supplementation of GK + PS-MPs substantially augmented the activity of antioxidant enzymes, whereas MDA and ROS contents were significantly decreased relative to PS-MPs treated rats. In addition to this, no marked variations were noted in the level of these biomarkers in GK only supplemented animals, in comparison with the control (Table 2).

3.3. Protective role of GK on liver enzymes

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

Table 2
Protective role of GK on oxidative stress markers.

Parameters	GROUPS			
	Control	PS-MPs	PS-MPs + GK	GK
CAT (Umg^{-1} protein)	9.59 ± 0.43^{a}	5.03 ± 0.14^{c}	8.41 ± 0.25^{b}	9.62 ± 0.44^{a}
SOD (Umg^{-1} protein)	7.66 ± 0.24^{a}	4.30 ± 0.15^{c}	6.88 ± 0.16^{b}	7.67 ± 0.33^{a}
GPx (Umg^{-1} protein)	17.71 ± 0.46^{a}	8.03 ± 0.36^{c}	14.51 ± 0.49^{b}	17.76 ± 0.47^{a}
GSR (nM NADPH oxidized/min/mg tissue)	6.52 ± 0.12^{a}	2.40 ± 0.16^{c}	5.58 ± 0.18^{b}	6.55 ± 0.13^{a}
GST (nM/min/mg protein)	23.47 ± 1.18^{a}	11.22 ± 0.52^{c}	19.89 ± 0.23^{b}	23.55 ± 1.19^{a}
GSH (μM/g tissue)	14.96 ± 0.47^{a}	6.96 ± 0.09^{c}	11.85 ± 0.32^{b}	14.98 ± 0.44^{a}
HO-1(pmoles bilirubin/ mg protein/h)	282.06 ± 8.11^{a}	61.30 ± 3.02^{c}	123.05 ± 7.22^{b}	291.16 ± 7.65^{a}
ROS (Umg^{-1} tissue)	1.21 ± 0.11^{a}	7.99 ± 0.34^{c}	2.36 ± 0.10^{b}	1.19 ± 0.11^{a}
MDA (nmol/mg protein)	0.45 ± 0.12^{a}	3.34 ± 0.16^{c}	1.47 ± 0.14^{b}	0.44 ± 0.13^{a}

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

Table 3
Protective role of GK on liver function markers.

Parameters	GROUPS			
	Control	PS-MPs	PS-MPs + GK	GK
ALT (U/L)	43.41 ± 1.08^{a}	91.41 ± 1.03^{c}	56.97 ± 1.41^{b}	42.77 ± 0.89^{a}
AST (U/L)	82.70 ± 1.31^{a}	173.41 ± 1.95^{c}	95.93 ± 0.89^{b}	81.80 ± 1.19^{a}
ALP (U/L)	118.23 ± 1.88^{a}	347.64 ± 3.93^{c}	189.57 ± 3.06^{b}	116.74 ± 1.59^{a}

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

3.4. Protective role of GK on apoptotic markers

PS-MPs treatment led to a remarkable ($p < 0.05$) increment in Caspase-3 and Bax expressions, besides decreased the expression of Bcl-2 as compared with the control group. Nevertheless, GK + PS-MPs supplementation notably ($p < 0.05$) regulated these expressions relative to PS-MPs administered rats. Additionally, in rats supplemented with GK only, the aforementioned parameters were comparable to the control (Fig. 3).

3.5. Protective role of GK on inflammatory indices

PS-MPs markedly ($p < 0.05$) elevated the levels of inflammatory markers, as compared to the control. However, in GK + PS-MPs supplemented animals, these levels were noticeably lowered, as compared to the group of rats treated with PS-MPs. However, inflammatory indices levels in GK only animals were presented to be almost similar to the control (Table 4).

4. Discussion

PS-MPs can aggregate in the hepatocytes and disrupt liver functioning, leading to hepatotoxicity (Sharma et al., 2012). GK, a polyphenolic, dietary flavonoid, extracted from the leaves and nuts of *Ginkgo biloba*. It has been found that, GK display numerous pharmacological effects i.e., anti-oxidative, neuroprotective, anti-apoptotic, anti-tumor and anti-inflammatory (Baek et al., 2016). Therefore, this investigation

Table 4
Protective role of GK on inflammatory indices.

Parameters	GROUPS			
	Control	PS-MPs	PS-MPs + GK	GK
NF-κB (ngg^{-1} tissue)	16.49 ± 0.66^{a}	64.02 ± 1.78^{c}	27.34 ± 1.34^{b}	16.38 ± 0.62^{a}
TNF-α (ngg^{-1} tissue)	8.55 ± 0.28^{a}	25.80 ± 0.83^{c}	14.40 ± 0.54^{b}	8.53 ± 0.29^{a}
IL-1β (ngg^{-1} tissue)	24.48 ± 0.49^{a}	85.73 ± 0.97^{c}	33.22 ± 0.77^{b}	24.26 ± 0.32^{a}
IL-6 (ngg^{-1} tissue)	5.48 ± 0.42^{a}	28.37 ± 0.74^{c}	9.63 ± 0.41^{b}	5.39 ± 0.49^{a}
COX-2 (ngg^{-1} tissue)	22.97 ± 0.84^{a}	67.93 ± 1.11^{c}	36.83 ± 0.88^{b}	22.91 ± 0.81^{a}

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

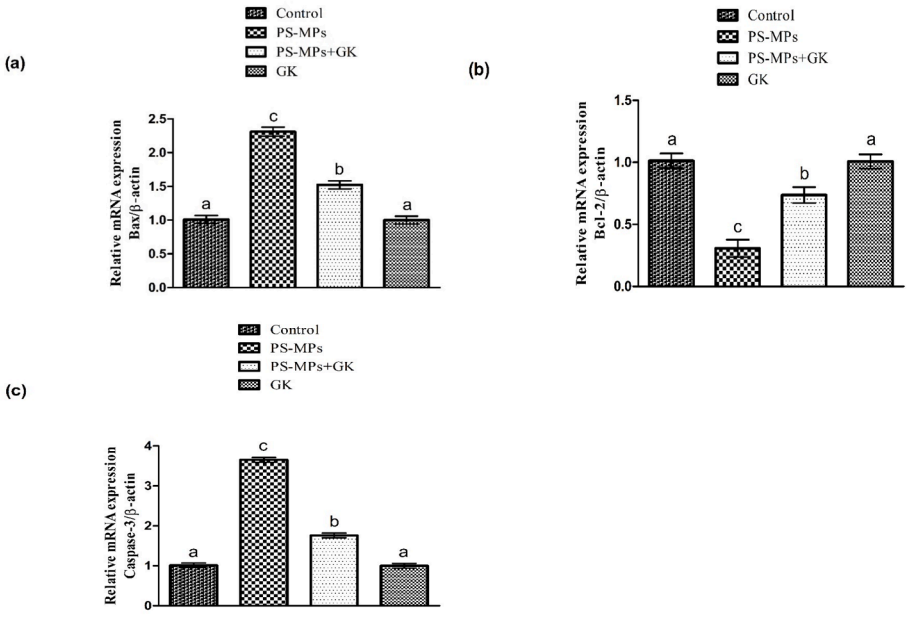

Fig. 3. Protective role of GK on **(a)** Bax, **(b)** Bcl-2 as well as **(c)** Caspase-3 expression. Bars are based on mean ± standard error of mean. Different superscripts on bars displaying significant difference.

was intended to appraise the hepatoprotective role of GK on PS-MPs provoked hepatic damages in male albino rats.

PS-MPs treatment reduced the expresions of antioxidant genes and Nrf-2, while increasing Keap-1 expression. 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 induction of antioxidant genes (Hawkes et al., 2014). Nevertheless, GK administration elevated Nrf-2 as well as anti-oxidant genes expressions, whereas lowering Keap-1 expression. Therefore, it is assumed that GK could modulate the expression of Nrf-2/Keap-1.

The results of our study indicated that PS-MPs administration potentially decreased antioxidants activities 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 that protects the proteins, lipids and DNA against OS damage via lowering ROS generation (Selamoglu Talas et al., 2009). GSR sustains GSH level by converting glutathione disulfide into GSH. GST plays a critical role in detoxification of toxins to safeguard the cells from damages prompted by oxygen free radicals (Hayes et al., 2005). Hence, this pronounced decrease in the activities of enzymatic antioxidants leads to excessive ROS generation, which weakens the anti-oxidative capacity to fight against free radicals and ultimately prompts OS. MDA is the byproduct of LP, which is indirectly used as a biomarker for the detection of LP and ROS induced cellular damages (Agarwal and Sengupta, 2020). However, the supplementation of GK + PS-MPs potentially augmented the antioxidants activities, while considerably reducing the ROS and MDA concentrations. These attenuative effects of GK are due to its free radical scavenging nature.

PS-MPs intoxication significantly elevated the levels of hepatic biomarkers, 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 hepatic mitochondria and their release indicate hepatic injuries. OS increases the mitochondrial permeability, allowing the leakage of these hepatic markers into blood and thereby elevating their levels that lead to hepatotoxicity (Nagai et al., 2016). Additionally, multiple studies proposed that OS is the major culprit behind the increase in hepatic function markers (Knudsen et al., 2016). However, according to our results, supplementation of GK with PS-MPs remarkably mitigated PS-MPs prompted hepatotoxicity by substantially lowering hepatic enzymes levels owing to its ROS scavenging and hepatoprotective features.

The results of our study showed that PS-MPs administration upsurged Bax and Caspase-3 expressions, besides downregulating Bcl-2. ROS triggered apoptosis and OS are the ultimate causes of hepatotoxicity (Zhang et al., 2003). The overall procedure of apoptosis is controlled by the proteins belonging to Caspase and Bcl-2 families. Bax boosts the apoptosis, besides Bcl-2 performs an antagonistic role to Bax and protects the cells from programmed cell death. The increase in Bax expression and the reduction in Bcl-2 prompt efflux of cyt-C into the cytosol from the membrane of mitochondria. Activation of Caspase-3 is due to increased cyt-C content inside the cytosol. Caspase-3 cleaves the cellular proteins, altering the structural makeup of cells and eventually triggering cell death due to apoptosis (Wang et al., 2013). However, co-treatment of GK + PS-MPs markedly upregulated the Bcl-2 expression, besides reducing the expressions of Caspase-3 and Bax, as a result of its anti-apoptotic potentials.

PS-MPs treatment noticeably elevated the levels of inflammatory markers. Excessive OS leads to the stimulation of inflammation modulator, NF-kB, which further activates the production of IL-6, TNF-α, IL-1β (Janssen-Heininger et al., 2016). The generation of these biomarkers specifies acute inflammatory response and several other anomalies linked with abnormal ROS accumulation. COX-2 is another markers of inflammation and has been reported to instigate inflammation (Kim et al., 2019). Nevertheless, GK supplementation down-regulated these levels due to its anti-inflammatory characteristics. This anti-inflammatory potency of GK 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 GK respectively (Wei et al., 2013).

5. Conclusion

In summary, our investigation demonstrated that PS-MPs administration instigated OS and LP that potently disturbed the levels of oxidative stress markers, liver function enzymes, inflammatory markers along with the expressions of apoptotic markers and induced micro-morphological anomalies in the hepatic tissues. Nevertheless, GK administration effectively recovered all these disruptions due to hepatoprotective, anti-inflammatory, antioxidant, as well as anti-apoptotic potential. Due to its therapeutic potential, GK could be integrated into current treatment protocols, providing a novel approach to treat patients suffering from hepatic injury. 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, Investigation, Data curation, Conceptualization. **Kainat Fatima:** Writing – original draft, Methodology, Investigation, Conceptualization. **Moazma Batool:** Writing – review & editing, Software, Formal analysis, Data curation. **Muhammad Imran:** Visualization, Validation, Methodology, Data curation. **Shaik Althaf Hussain:** Writing – original draft, Project administration, Funding acquisition. **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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