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RESEARCH ARTICLE

Ameliorative Effects of Rhamnetin against Polystyrene Microplastics-induced Nephrotoxicity in Rats

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A B S T R A C T

The present study was intended to ascertain the attenuative role of rhamnetin (RHM) against polystyrene microplastics (PS-MPs) prompted nephrotoxicity in rats. 24 male albino rats (6-8 weeks old) were arbitrarily separated into 4 groups viz. control, PS-MPS (0.01mg/kg) treated group, RHM+PS-MPs (0.01mg/kg + 50mg/kg) co-treated group and only RHM (50mg/kg) treated group. The experiment was executed for 30 days. At the last day, all the rats were slaughtered to analyze the various kidney parameters. Our findings demonstrated that PS-MPs exposure significantly lowered the enzymatic antioxidant activities (CAT, GPx, GSR, SOD, GST & GSH), while it augmented the level of oxidative stress markers (ROS and MDA). PS-MPs administration significantly up-regulated the level of KIM-1, NGAL, serum urea & creatinine, whereas reduced the creatinine clearance. Additionally, PS-MPs significantly elevated the inflammatory markers level such as TNF-α (tumor necrosis factor alpha), IL-1β (Interleukin-1 beta), NF-κB (nuclear factor kappa-B), IL-6 (Interleukin-6) and COX-2 (cyclooxygenase-2) activity along with histological abnormalities. However, RHM co-treatment with PS-MPs exhibited protective effect against PS-MPs provoked nephrotoxicity and restored all the anomalies. These findings have proposed that RHM is a potent flavone that can mitigate the kidney damages caused by PS-MPs.

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INTRODUCTION

Plastics' pervasive environmental accumulation and bioaccumulation have drawn a lot of attention globally due to the tremendous expansion of their usage (Chen *et al.,* 2022). In the environment the deterioration of plastic wastes is continuously carried out by external factors resulting in micro or nano-sized particles, referred to as microplastics (MPs<5 mm) and nanoparticles (NPs<100 nm). These small particles can readily enter the organism through numerous mechanisms and have negative health effects (Kik *et al.,* 2020). PS-MPs are among the most prevalent MPs found in surface waters, soil, marine ecosystems, and sewage (Wang *et al.,* 2021). Polystyrene (PS) is a kind of aromatic hydrocarbon that can be produced by polymerizing styrene monomers. PS is used in the production of styrofoam and various other plastic products such as home appliances, toys, vehicle components, food packaging, medical equipment and electronic products due to its flexibility and inert nature. Studies have also revealed that styrene monomers can be transferred into food items from PS containers that potentially cause health problems. PS-MPs have been spotted in seafood, air and salt as well as water. Small size, low density and large surface area helps the entrance of PSMPs into the biota and so, they accumulate in the food chains. PS-MP exposure to human and animals occurs by inhalation, ingestion and topical contact (Lee *et al.,* 2021).

PS-MPs are generally considered to penetrate the tissues of many organs in the body, including kidney, brain, as well as liver (Shi *et al.,* 2022). Kidneys are complicated organs that are involved in blood filtration, PS-MPs has the potential to cause renal toxicity (Fan *et al.,* 2022). Exposure to PS-MPs alter biochemical reactions in renal tissues that lead to nephrotoxicity. PS-MPs can disrupt zonula occludens-2 proteins & 1-antitrypsin, disrupt the integrity of renal barrier and leads to acute renal injury. This indicates that exposure to PS-MPs in the environment may raise the risk of kidney ailments (Chen *et al.,* 2022).

Currently, flavonoids are important bioactive compounds, recognized for their possible health advantages.

They have been used to treat a variety of chronic ailments, including cancer, antiviral, inflammatory, cardiovascular, and neurodegenerative disorders. It is generally believed that the main dietary components are actually antioxidant nutrients found in vegetables and fruits (Ullah *et al.,* 2020). Rhamnetin (RHM) is a flavonoid, reported in the seeds, flowers & leaves of many plants, i.e., *Hippophae rhamnoides L*. and *Ginkgo biloba L*. (Pang *et al.,* 2019). It is reported that RHM has the potential to reduce testicular damage in rats induced by PS-MPs (Hamza *et al.,* 2023). Therefore, the present study was formulated to estimate the ameliorative role of RHM against PS-MPs prompted renal damage in rats.

MATERIALS AND METHODS

Chemicals: PS-MPs (CAT No. 9003-53-6) and RHM (CAT 90-19-7) were acquired from Merck (USA).

Experimental animals: Male albino rats having weight 150–200, g were purchased and placed in a research center at the University of Agriculture, Faisalabad. In a standard laboratory setting, the animals were confined in steel cages under 25±2°C, 12/12h light/dark cycles, and humidity of 54±10%. Food pellets and water were freely available to the rats. Before conducting trials, the animals were acclimatized for a week.

Experimental design: The experimental protocols were accepted by the UAF Animal Handling and Protection Committee and compliance was made with their ethical guidelines. 24 rats were allocated at random into four groups (n = 6) and given 30 days of treatment.

1. Control group; rats were provided with food and water on a daily basis.
2. PS-MPs group; rats received PS-MPs dosage ($0.01 mgkg^{-1}$).
3. PS-MPs + RHM group; rats received PS-MPs ($0.01 mgkg^{-1}$) along with RHM ($50 mgkg^{-1}$).
4. RHM group; rats were administered RHM orally ($50 mgkg^{-1}$).

After thirty days, rats were anesthetized with ketamine and slaughtered. Kidneys were excised after dissection; one kidney was stored in zipper bag at -80ºC for biochemical observation. The 2nd kidney was kept for histological evaluation in formalin solution (10%).

Assessment of biochemical profile: Chance and Maehly's (1955) method was followed for evaluating the activity of catalase (CAT). The approach of Kakkar *et al.* (1984) was followed to measure superoxide dismutase (SOD) activity. GPx activity was assessed according to the method of Rotruck *et al.* (1973). The Carlberg and Mannervik (1975) methodology was followed to measure glutathione reductase (GSR) activity. The approach of Younis *et al.* (2016) was employed for estimating glutathione-S-transferase (GST) activity. Glutathione (GSH) concentration was measured in compliance with the technique of Jollow *et al.* (1974). Reactive oxygen species (ROS) level was assessed via the technique demonstrated by Hayashi *et al.* (2007). The method proposed by Ohkawa *et al.* (1979) was applied to determine the malondialdehyde (MDA) level.

Assessment of kidney function marker: Using the lab protocols given with the Randox standardized laboratory kits (Crumlin Company, Antrim, UK), the levels of blood urea (Cat No. ab83362), creatinine (Cat No. ab65340) as well as creatinine clearance (Cat No. ab65340) were analyzed. NGAL Quantikine ELISA Kits & KIM-1 Quantikine ELISA Kits (R and D Systems company Ltd. Changning, China) were employed for assessing serum NGAL & urinary KIM-1 as per directions provided by the manufacturer.

Inflammatory markers assessment: Inflammatory markers were assessed using commercially available kits. TNF-α (CSB-E07379r), NF-κB (CSB-E13148r), IL-6 (CSB-E04640r), IL-1β (CSB-E08055r) levels and COX-2 (CSB-E13399r) activity were assessed by ELISA kits (Cusabio Technology Llc, Houston, TX, USA).

Histopathological analysis: Renal samples were kept in 10% formalin, then the tissue samples were dehydrated using rising concentrations of ethanol (80, 90 and 100%) and fixed in paraffin wax. Each sample was cut into thin slices (4-5 µm). The sections were stained using the Hematoxylin & Eosin stain. Microphotography was done at a magnification of 400X using a light microscope (Nikon-187842, Japan). Histopathological analysis of renal tissues was performed in accordance to the approach of Ibrahim *et al.* (2018).

Statistical analysis: The data was shown as Mean ± SEM. Statistical evaluation of variations in the concentrations of the studied markers was carried out by one-way ANOVA. To compare the results of different data groups, the Tukey's multiple comparisons test was applied. Statistics with $P<0.05$ difference were taken as significant.

RESULTS

Ameliorative effect of RHM on anti-oxidant enzymes: The activities of CAT, GPx, GSR, SOD, GST & GSH were significantly ($P<0.05$) reduced after PS-MPs intoxication relative to the control animals. Contrarily, RHM administration with PS-MPs remarkably escalated anti-oxidant enzymes activities in the co-treated group as compared to the PS-MPs exposed group. Moreover, anti-oxidant enzymes activities in RHM alone administrated group were close to control animals (Table 1).

Ameliorative effect of RHM on oxidative stress parameters: PS-MPs intoxication significantly ($P<0.05$) escalated ROS & MDA levels in PS-MPs exposed group in contrast to control animals. Nevertheless, the supplementation of RHM + PS-MPs notably lowered ROS & MDA levels relative to PS-MPs exposed animals. Furthermore, these parameters in RHM alone administrated rats were close to control animals (Table 2).

Ameliorative effect of RHM on renal markers: The intoxication of PS-MPs led to a significant increase in urea, creatinine, KIM-1 & NGAL levels, besides, the level of creatinine clearance was lowered as compared to control animals. The supplementation of RHM + PS-MPs recovered the levels of these markers. Moreover, in RHM alone supplemented group these markers were close to control (Fig. 1).

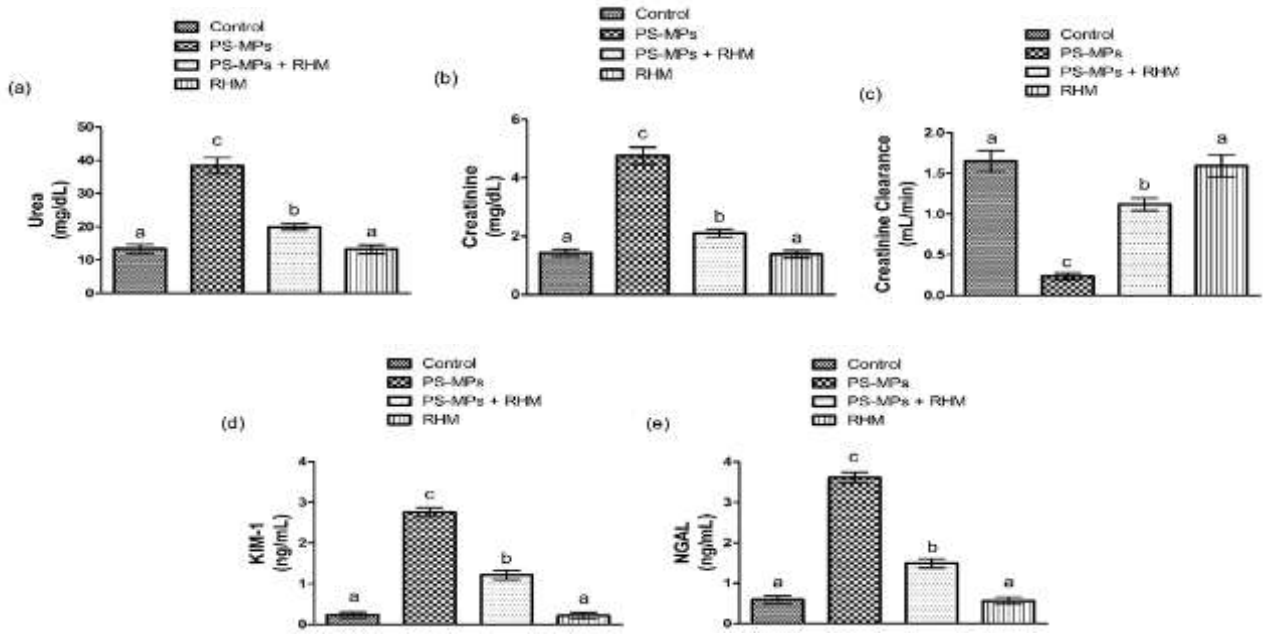

Fig. 1: Effect of PS-MPs and RHM on the level of renal function markers a) urea, b) creatinine, c) creatinine clearance, d) KIM-1 and c) NGAL.

Table 1: Protective effect of RHM on antioxidant enzyme

Groups	CAT (Umg^{-1} protein)	SOD (Umg^{-1} protein)	GSH (μM/g tissue)	GST (nM/min/mg protein)	GPx (Umg^{-1} protein)	GSR (nM NADPH oxidized/min/mg tissue)
Control	9.05 ± 0.16[a]	6.49±0.12[a]	18.25±0.27[a]	22.96±0.77[a]	19.41±0.31[a]	4.37±0.20[a]
PS-MPs	4.23±0.16[c]	3.04±0.13[c]	7.66±0.26[c]	10.26±0.2[b]	7.96±0.10[c]	1.23±0.10[c]
RHM + PS-MPs	8.31±0.20[b]	5.58±0.19[b]	13.59±0.71[b]	21.65±0.48[a]	15.36±0.16[b]	3.37±0.15[b]
RHM	9.17±0.26[a]	6.57±0.13[a]	18.13±0.52[a]	23.09±0.71[a]	19.49±0.37[a]	4.46±0.21[a]

Values that don't share a superscript in the same column are significantly different.

Table 2: Protective effect of RHM on oxidative stress markers.

Groups	ROS (U/mg tissue)	MDA (nmol/mg protein)
Control	1.60±0.03[c]	0.72±0.3[c]
PS-MPs	7.81±0.21[a]	2.78±0.11[a]
RHM + PS-MPs	2.18±0.09[b]	1.26±0.06[b]
RHM	1.54±0.04[c]	0.68±0.04[c]

Values that don't share a superscript in the same column are significantly different.

Table 3: Protective effect of RHM on inflammatory markers.

Groups	NF-κB (ng/g tissue)	TNF-α (ng/g tissue)	IL-1β (ng/g tissue)	IL-6 (ng/g tissue)	COX-2 (ng/g tissue)
Control	14.47±0.49[c]	7.41±0.2[c]	27.98±1.37[c]	7.81±0.49[c]	24.65±0.93[c]
PS-MPs	69.15±2.07[a]	23.78±1.14[a]	92.62±0.95[a]	27.07±0.49[a]	76.22±1.67[a]
RHM + PS-MPs	24.17±1.25[b]	11.59±0.67[b]	32.69±0.76[b]	10.34±0.63[b]	30.04±1.28[b]
RHM	14.25±0.43[c]	7.36±0.39[c]	27.37±1.41[c]	7.78±0.58[c]	24.23±1.15[c]

Values that don't share a superscript in the same column are significantly different.

Ameliorative effect of RHM on inflammatory markers: Inflammatory indices, i.e., NF-κB, IL-6, TNF-α, IL-1β & COX-2 activity was significantly ($P<0.05$) elevated in PS-MPs exposed animals as compared to control. The supplementation of RHM + PS-MPs notably decreased inflammatory markers relative to PS-MPs exposed animals. Moreover, these markers in RHM alone treated animals were close to the control animals (Table 3).

Ameliorative effect of RHM on histology: PS-MPs intoxication significantly ($P<0.05$) led to kidney damage. PS-MPs prompted histopathological anomalies in renal tissues i.e., direct damage to tubular structure, widespread necrosis, tubular cell desquamation, dilatation of proximal tubules and vacuolization as compared to the control group. Nevertheless, the administration of RHM + PS-MPs remarkably recovered these abnormalities. Moreover, the histological profile was almost similar in only RHM administrated and control animals (Fig. 2).

DISCUSSION

Polystyrene microplastics (PS-MPs) are potential toxicants formed by the breakdown of bigger plastic particles found in nature and have gained a lot of attention due to their adverse effects on the health of animals & humans (Yin *et al.,* 2021). Therefore, the purpose of this study was to ascertain the mitigating role of rhamnetin (RHM) on PS-MPs-prompted nephrotoxicity in rats. PS-MPs intoxication disrupted the antioxidant enzyme balance, significantly elevated MDA and ROS levels, while decreasing CAT, GPx, GSR, SOD, GST & GSH activities and eventually inducing oxidative stress. These findings are in line with an earlier research of Banerjee and Shelver (2021), who indicated that PS-MPs lead to oxidative stress in aquatic species & mammals. Excessive MDA production is an indication of lipid peroxidation that is caused by an upsurge in free radicals (Ijaz *et al.,* 2022). LP is a measure of both oxidative stress and the activities

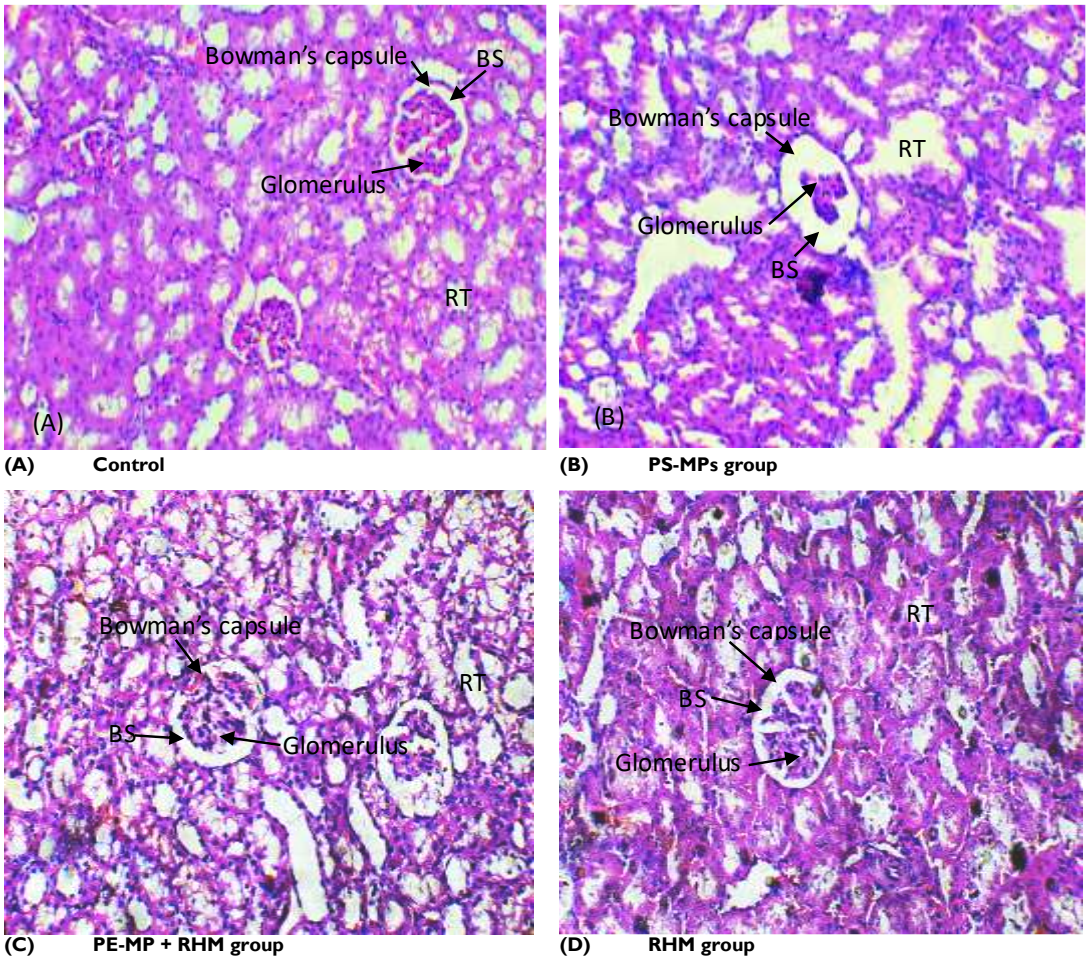

Fig. 2: Effect of RHM on PS-MPs induced histological abnormalities in kidney (400X). (A) Control group (B) PS-MPs exposed group (C) PS-MPs + RHM co-treated group (D) only RHM administrated group. Bowman's capsule, BS: Bowman space, Glomerulus, RT: Renal tubules.

of antioxidant enzymes (Ijaz *et al.,* 2021). CAT is a major antioxidant enzyme that plays an important function in H_2O_2 catabolism (Tuzet *et al.,* 2019). According to Nieskens *et al.* (2018) SOD protects from free radicals, as it convert them into less dangerous substance. GSR converts glutathione disulfide into glutathione hydrochloride (GSH) by lowering the concentrations of hydrogen peroxide and other peroxides thus protects mammalian cells from oxidative stress (Deponte, 2013). Thus, it is essential to maintain the regular activities of these enzymes to reduce oxidative stress and renal damage. Plant based foods can be used to supplement these anti-oxidants to minimize the oxidative stress (Nahid *et al.,* 2017). It is indicated that the supplementation of RHM + PS-MPs decreased the detrimental impacts of PS-MPs in rat kidney tissue by lowering oxidative stress. Our findings show that RHM treatment increased anti-oxidant enzyme activity, while decreasing MDA and ROS levels. RHM's therapeutic potential may be ascribed to its free radical foraging properties.

According to the present research, PS-MPs exposure prompted a notable increase in creatinine & urea levels as well as lowered creatinine clearance. Creatinine & urea are both widely used in clinical settings to assess the renal function (Ramsey *et al.,* 2018). Urea is produced during protein breakdown, besides creatinine is a nitrogen based substance that is made from phosphocreatine & creatine and is removed by glomerular filtration (GF) (Sepulveda, 2019). Decreased creatinine clearance & high creatinine as well as urea are the signs of oxidative damage in renal tissues (Shirani *et al.,* 2019) that indicate reduced GF rate. However, co-treatment with RHM, reduced creatinine & urea levels by increasing GF rate as shown by elevated creatinine clearance.

Our results revealed, KIM-1 & NGAL levels were increased after PS-MPs treatment. KIM-1 & NGAL have been identified as signs of renal injury (Lei *et al.,* 2018). A transmembrane protein KIM-1 is a primary diagnostic indicator of AKI. It is not reported in healthy kidney tissue, besides it can be observed in the initial phases of renal toxicity (Song *et al.,* 2020). Following renal injury, NGAL typically releases into blood in higher amounts that is eliminated by the urine (Khawaja *et al.,* 2019). However, RHM therapy reduced KIM-1 & NGAL levels in co-treated rats, indicating that RHM has the ability to restore kidney damage.

Our findings showed that after PS-MPs intoxication inflammatory indices were increased in comparison to control group. NF-κB activation escalated IL-1β, TNF-α & IL-6 production through gene up-regulation and leads to renal damage (Sun and Karin, 2008). Furthermore, COX-2 is a key inflammatory cytokine that causes renal inflammation (Gandhi *et al.,* 2017). It is indicated that PS-MPs administered group had elevated COX-2 activity, which is an indication of the inflammatory condition. However, RHM therapy in RHM + PS-MPs administrated animals inhibits NF-κB activation and decreased the levels

of inflammatory markers that may be credited to RHM's anti-inflammatory nature. Therefore, it is determined that RHM's anti-inflammatory property may be employed to lower the levels of inflammatory markers.

Histopathological analysis revealed that PS-MPs caused renal damage, which was further confirmed by the aberrant urine profile. After PS-MPs treatment, there was a significant inflammatory response, loss of cellular differentiation, tubular, interstitial & glomerular impairments as well as enlarged tubules and tubular necrosis. PS-MPs intoxication raises the production of ROS and induced LP in the kidney tissue by lowering antioxidants (Wang *et al.,* 2022), resulting in morphological damage that leads to notable tubular, glomerular and interstitial abnormalities in the kidneys of PS-MPs exposed animals. However, RHM treatment notably alleviated these injuries of renal tissues due to its renoprotective nature.

Conclusions: The current study concluded that RHM plays a protective role against ROS, which actually mediates PS-MPs instigated renal damage. Following RHM co-treatment, antioxidant enzymes activity and renal markers were recovered. While oxidative stress markers, inflammatory markers and histopathological anomalies were considerably reduced. The antioxidant as well as anti-inflammatory nature of RHM may be responsible for its nephroprotective activities.

Authors contribution: MA, NE and MUI planned the study, AH and RA accomplished the trial. AH assisted in statistical analysis. MA, HAK and MUI wrote the manuscript. All authors approved the manuscript before the submission.

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