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RESEARCH ARTICLE

Attenuative Effects of Ginkgetin Against Polystyrene Microplastics-Induced Renal Toxicity in Rats

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ABSTRACT

The present study was designed to evaluate the palliative potential of ginkgetin (GK) against polystyrene microplastics (PS-MPs) instigated renal damage in rats. 24 rats were separated into 4 groups i.e., control, PS-MPs-intoxicated (0.01mgkg^{-1}), PS-MPs (0.01mgkg^{-1}) and GK (25mgkg^{-1}) administrated and GK (25mgkg^{-1}) only treated group. Results of the experiment showed that the activities of anti-oxidant enzymes i.e., glutathione S-transferase (GST), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GSR) & catalase (CAT) were significantly decreased, while the levels of ROS and MDA were increased following PS-MPs treatment. Furthermore, PS-MPs intoxication increased the levels of kidney function markers such as creatinine, urea, NGAL and KIM-1, while a significant reduction was observed in creatinine clearance. Moreover, PS-MPs significantly increased the TNF-α, NF-κB, IL-6, IL-1β levels and COX-2 activity. Furthermore, it reduced the Bcl-2 level, while increased the levels of Caspase-9, Bax and Caspase-3 and induced histological damages in the renal tissues. However, the treatment of GK significantly regulated the biochemical, renal, inflammatory, apoptotic markers and attenuated the PS-MPs induced histopathological damages due to its reno-protective, anti-apoptotic, anti-oxidant and anti- inflammatory nature.

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INTRODUCTION

Plastics pollution in the environment is a serious global problem and it is adversely affecting the animal and human health (Fackelmann and Sommer, 2019). The quantity of plastics manufactured globally every year has expanded dramatically. Since 1950, plastics production has increased from two million tons to approximately four hundred million tons in 2021 and it is anticipated that its generation will be raised up to 1,480 million tons, by 2050 (Plastic Europe, 2022). Weathering and fragmentation of plastics waste eventually leads to the generation of smaller plastic particles known as microplastics (MPs). MPs are plastic fragments with diameter of less than 5mm. Polystyrene, a type of plastics, is typically applied due to its inexpensive cost and exceptional physical characteristics. PS-MPs are the most common type of MPs (Wagner *et al.,* 2014), which are reported in food packaging materials, cups, plates, beauty products i.e., facial cleanser, scrubbers and toothpastes (Kentin, 2018). PS-MPs internalize more readily into the biota, due to their low density, high relative surface area and small size; as a result, they accumulate in organic food chains. PS-MPs enter into animal body via, ingestion, skin contact and inhalation (Campbell *et al.,* 2017). PS-MPs intoxication induces hepatotoxicity, gastrointestinal toxicity, cardiotoxicity as well as neurotoxicity (Deng *et al.,* 2017), and testicular damage (Hamza *et al.,* 2023).

Kidneys are vital organs as they control body fluids, electrolyte balance, acid-base balance, and homeostasis. However, various endogenous and exogenous environmental toxins can damage the kidneys and lead to renal failure (Hertzberg *et al.,* 2017). PS-MPs stimulate ROS production, which promotes the immunological and oxidative mechanism that leads to irregular expression of protein (Zeng *et al.,* 2019). Additionally, PS-MPs in rodents instigate inflammation and apoptosis, leading to

disturbed mitochondrial activities as well as significant histological alteration in renal tissues (Wang *et al.,* 2021).

Numerous researchers during the last few years have concentrated on the importance of natural anti-oxidants to treat OS-related disorders. Flavonoids are polyphenolic compounds that multiple therapeutic potentials (Kumar and Pandey, 2013). Flavonoids are secondary metabolites found in plants and display various therapeutic properties with little side effects. Ginkgetin (GK) ($C_{32}H_{22}O_{10}$) is also a natural flavonoid that is present in *Ginkgo biloba*. GK is described to show anti-oxidant, anti-bacterial and anti-inflammatory activities (Tian *et al.,* 2019). It was reported that GK has tendency to cure neurological injury prompted by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) via regulating iron homeostasis (Wang *et al.,* 2015). The current study was planned to evaluate the curative effects of GK against PS-MPs induced kidney damage in albino rats.

MATERIALS AND METHODS

Chemicals: Polystyrene microplastics (PS-MPs) (CAS Number: 9003-53-6, purity ≥ 96%) & ginkgetin (GK) (CAS Number: 481-46-9**,** purity 98%) both were acquired from Sigma Aldrich, Darmstadt (Germany). All other chemical were of analytical grades.

Animals: 24 albino rats, (210 ± 10g) were housed at 12-h night/12-h day cycle, temperature 22-25°C and humidity 55-70%. The rats were housed in steel enclosures in the University of Agriculture, Faisalabad, Pakistan. Commercial diet and tap water were given to the rats. Rats were handled as per the guidelines of European Union of Animal Care and Experimentation (CEE Council 86/609).

Experimental layout: Adult rats were allocated into 4 groups (n=6): control, PS-MPs exposed (0.01mgkg^{-1}), PS-MPs + GK co-administered (0.01 mgkg^{-1} +25 mgkg^{-1}), and GK treated (25mgkg^{-1}) groups. The trail was performed for 30 days. On the 31st, the animals were sedated, beheaded and blood was collected in sterile containers for detailed analysis and the kidneys were excised. The right kidney was stored at −80º C in zipper bag for further analysis, left kidney was kept in formalin (10%) for histological analysis.

Assessment of anti-oxidants activity: Catalase (CAT) activity was estimated by using the approach outlined by Chance and Maehly (1955). The activity of glutathione reductase (GSR) was appraised by using the approach of Carlberg and Mannervik (1975), whereas the activity of glutathione peroxidase (GPx) was measured by Rotruck *et al.* (1973) procedure. The activity of superoxide dismutase (SOD) was appraised via the practice of Nishikimi *et al.* (1972). Glutathione (GSH) content and glutathione S-transferase (GST) were evaluated via the protocols demonstrated by Jollow *et al.* (1974) and Habig *et al.* (1974) respectively.

Assessment of MDA and ROS: The protocol of Hayashi *et al.* (2007) was used to estimate ROS level, while MDA level was evaluated by using commercially available kit (ab118970) (MA, Abcam, USA).

Assessment of renal functional marker: The levels of creatinine clearance (Cat No. ab65340), urea (Cat No. ab83362) and creatinine (Cat No. ab65340) were appraised by using Randox standardized kits (Antrim, UK). KIM-1 and NGAL levels were evaluated via KIM-1 Quantikine ELISA Kits and NGAL Quantikine ELISA Kits (R and D Systems Company Ltd. Changning, China) in compliance with the company's guidelines.

Assessment of inflammatory indices: The levels of IL-1β (CSB-E08055r), TNF-α (CSB-E07379r), NF-κB (CSB-E13148r), IL-6 (CSB-E04640r) and COX-2 (CSB-E13399r) activity were determined by using the ELISA kits (Cusabio Technology Llc, Houston, TX, USA).

Evaluation of apoptotic markers: Caspase-9 (CSB-E08863r), Bax (CSB-EL002573RA), Bcl-2 (CSB-E08854r) and Caspase-3 (CSB-E08857r) levels were assessed with the help of ELISA kits (Houston, TX, USA) in compliance with the company's guidelines.

Histopathological examination: For histological examination, renal tissues were immersed in formalin (10%). Then the tissues were dehydrated using the rising grades of alcohol and inserted in paraffin wax. 5µm slices were made using 820 rotatory microtome (American Optical–Rotary Microtome/ Model 820) and stained by using hematoxylin-eosin. Lastly, these slides were examined microscopically (Nikon, Japan), connected with an automatic micro-photographic system.

Statistical analysis: Data were expressed as Mean ± SEM. One-way ANOVA and Turkey's test were applied by using Minitab software. The level of significance was considered as $P<0.05$.

RESULTS

Protective effects of GK on antioxidants activity: PS-MPs administration led to a significant ($P<0.05$) reduction in antioxidants i.e., GPx, GST, SOD, GSR, GSH and CAT activities. Nevertheless, GK + PS-MPs co-administration significantly increased the activities of antioxidants in GK + PS-MPs supplemented group as compared to PS-MPs exposed animals. Moreover, the supplementation of GK showed the activity of antioxidants comparable to control rats (Table 1).

Protective effects of GK on MDA and ROS: PS-MPs administration significantly increased ROS and MDA levels in PS-MPs treated animals with respect to control. On the other hand, GK + PS-MPs supplementation considerably decreased MDA and ROS levels as compared to PS-MPs group. Additionally, GK alone supplemented animals expressed these levels near to control (Table 2).

Protective effects of GK on renal markers: PS-MPs administration prompted a substantial ($P<0.05$) increase in creatinine, urea, serum NGAL and urinary KIM-1 levels as compared to control animals while creatinine clearance was decreased. The outcomes of our study illustrated that GK supplementation along with PS-MPs significantly lowered creatinine, urobilinogen, urea, serum NGAL and urinary

Table 1: Protective effect of GK on antioxidants activity

Groups	CAT (U/mg protein)	SOD (U/mg protein)	GSH (µM/g tissue)	GST(nM/min/mg protein)	GPx (U/mg protein)	GSR (nM NADPH oxidized/min/mg tissue)
Control	9.31 ± 0.13[a]	7.67 ± 0.33[a]	15.47±0.75[a]	26.77±1.04[a]	19.54±0.49[a]	6.64±0.32[a]
PS-MPs	4.41±0.17[c]	3.56±0.23[c]	5.93±0.25[c]	11.24±0.77[c]	8.90±0.39[c]	2.21±0.23[c]
PS-MPs + GK	8.36±0.32[b]	6.54 ± 0.21[b]	11.51±0.74[b]	22.59±0.73[b]	15.73±0.83[b]	5.40±0.35[b]
GK	9.34±0.14[a]	7.69±0.33[a]	15.68±0.72[a]	26.83±1.04[a]	19.61±0.50[a]	6.67±0.33[a]

Values with dissimilar superscripts are significantly different from other groups.

Table 2: Curative effect of GK on ROS and MDA

Groups	ROS (U/mg tissue)	MDA (nmol/mg protein)
Control	1.34±0.11[a]	0.72±0.06[a]
PS-MPs	7.11±0.36[c]	2.57±0.15[c]
PS-MPs + GK	2.13±0.16[b]	1.36±0.12[b]
GK	1.31±0.10[a]	0.69±0.06[a]

Values with dissimilar superscripts are significantly different from other groups.

Table 3: Protective effect of GK on renal markers

Groups	Urea (mg/dL)	Creatinine (mg/dL)	Creatinine clearance (mL/min)	Urinary KIM-1 (ng/day)	NGAL (mg/ml)
Control	12.92±0.74[a]	1.24±0.04[a]	1.93±0.05[a]	0.21±0.11[a]	0.65±0.11[a]
PS-MPs	61.56±1.09[c]	7.29±0.32[c]	0.44±0.07[c]	2.81±0.09[c]	3.65±0.08[c]
PS-MPs + GK	24.93±0.93[b]	2.31±0.07[b]	1.54±0.07[b]	1.30±0.07[b]	1.45±0.06[b]
GK	12.88±0.74[a]	1.22±0.05[a]	1.95±0.05[a]	0.18±0.09[a]	0.62±0.11[a]

Values with different superscripts are significantly different from other groups.

Table 4: Protective effect of GK on inflammatory indices

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	14.84±0.75[a]	8.48±0.14[a]	21.82±0.94[a]	7.33±0.32[a]	22.23±1.00[a]
PS-MPs	67.23±1.24[c]	28.37±0.69[c]	88.87±0.74[c]	29.48±1.22[c]	74.44±1.38[c]
PS-MPs + GK	27.97±1.47[b]	13.15±0.63[b]	37.45±1.25[b]	9.85±0.93[b]	33.02±1.43[b]
GK	14.81±0.76[a]	8.45±0.14[a]	21.79±0.95[a]	7.31±0.33[a]	22.19±0.99[a]

Values with different superscripts are significantly different from other groups.

Table 5: Protective effect of GK on apoptotic markers

Groups	Bax (pg/mL)	Bcl-2 (ng/mL)	Caspase-3 (pg/mL)	Caspase-9 (pg/mL)
Control	1.64±0.18[a]	18.41±0.69[a]	1.64±0.13[a]	2.29±0.13[a]
PS-MPs	8.74±0.24[c]	4.11±0.14[c]	13.89±0.77[c]	11.64±0.78[c]
PS-MPs + GK	3.17±0.14[b]	13.08±0.86[b]	2.86±0.15[b]	3.71±0.17[b]
GK	1.62±0.18[a]	18.46±0.74[a]	1.62±0.12[a]	2.28±0.19[a]

Values with dissimilar superscripts are significantly different from other groups.

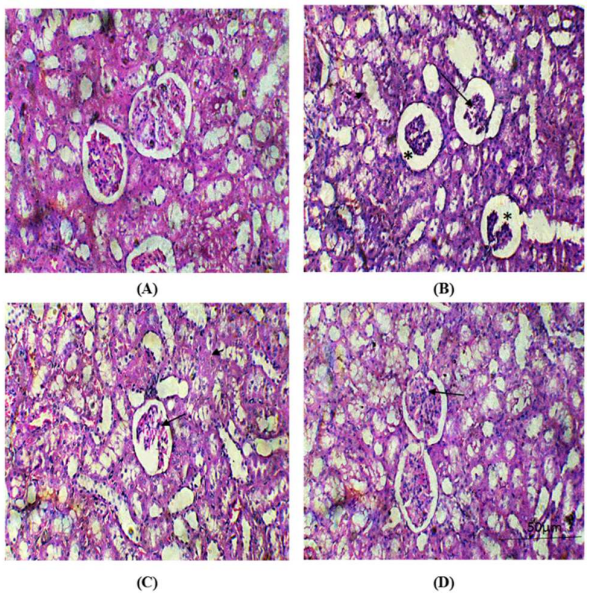

Fig. 1: A) Control group demonstrating regular histology. B) PS-MPs group showing shrinkage of glomerulus (arrow), increased bowman's space (*) and tubular necrosis (arrow head); C) PS-MPs + GK group showed restoration of glomerulus (arrow) and renal parenchyma (arrow head); D) GK group showing normal glomerulus (arrow) and renal parenchyma. Abbreviations: PS-MPs: Polystyrene microplastics; GK: Ginkgetin.

KIM-1 levels, besides a remarkable increase was observed in creatinine clearance and albumin levels with respect to PS-MPs administered animals. Furthermore, only GK administered animals showed these levels close to the control group (Table 3).

Protective effects of GK on inflammatory indices: PS-MPs exposure exhibited a substantial ($P<0.05$) escalation in TNF-α, IL-6, IL-1β, NF-κB levels and COX-2 activity. However, these levels were significantly lowered in co-treated rats with respect to PS-MPs exposed animals. Moreover, GK only administered animals showed these levels comparable to the control (Table 4).

Protective effects of GK on apoptotic markers: PS-MPs administration significantly escalated Caspase-3 and Bax levels in PS-MPs administered group with respect to control. Besides, Bcl-2 level was significantly reduced in PS-MPs group. However, GK + PS-MPs supplementation significantly decreased Caspase-3 and Bax levels, whereas significantly ($P<0.05$) increasing the Bcl-2 level as compared to PS-MPs treated animals. Additionally, in GK only administered rats these levels were close to control (Table 5).

Protective effects of GK on kidney histology: PS-MPs treatment induced significant histopathological damages in the renal tissues i.e., degenerated glomerulus, increased Bowman's spaces, direct damage to renal tubules and vacuolization than the control rats. Nevertheless, the supplementation of GK + PS-MPs significantly reduced all

the PS-MPs induced renal damages with respect to PS-MPs group. Furthermore, in only GK administered animals the histological profile was similar to the control animals (Fig. 1).

DISCUSSION

Plastic pollution is an emerging problem of the environment that is adversely affecting the human and animals. Plastic exposure is linked to multiple health effects including cancer, brain damage, fertility issues, heart damage as well as pulmonary toxicity (Campanale *et al.,* 2020). PS-MPs are the abundant chemical pollutant that pollutes the environment. Animals are exposed to PS-MPs via ingestion and dermal contact. Additionally, PS-MPs have also been indicated in salt, honey and beer. PS-MPs induce oxidative damage in different body organs i.e., heart, liver, brain, as well as kidney (Liu *et al.,* 2022). Ginkgetin (GK) is an important flavone that is present in a diverse range of plant species i.e., *Ginkgo biloba, Agava angustifolia,* and *Glyphoglossus yunnanensis*. GK *displays diverse* therapeutic activities i.e., antioxidant, anti-tumor and anti-inflammatory (Tian *et al.,* 2019). The supplementation of GK protects from neurological injury instigated by MPTP via regulating iron homeostasis (Wang *et al*., 2015). The present study was designed to assess the curative role of GK against PS-MPs caused renal toxicity in rats.

In PS-MPs administered group, the activities of antioxidant enzymes (SOD, GSR, CAT, GSH, GST and GPx,) were reduced, whereas MDA and ROS levels were increased. These antioxidant enzymes act as first wall of defense that safeguard the cellular macromolecules i.e., proteins, lipids and DNA, via lowering ROS generation. CAT is an essential enzyme that converts H_2O_2 into oxygen and water (Selamoglu, 2014). SOD helps in the transformation of O^{-2} into H_2O_2. GSR turns the glutathione disulfide to the GSH and it safeguards the cells of animals by lowering the H_2O_2 and other peroxides levels from oxidative damage. GSH effectively inhibits the generation of ROS and helps in transformation of peroxides into hydroxyl compounds that is comparatively less dangerous. GST is involved in the detoxification of hazardous substances. MDA is an end-product of LP and its level show the impairment prompted by LP as well as ROS (Yu *et al.,* 2018). The antioxidant defense mechanism of the body get disrupted when surplus amount of ROS are produced, which leads to OS. In addition to endogenous anti-oxidants, these enzymes could also be taken from various plants species to combat the OS (Nahid *et al.,* 2017). However, GK treatment significantly increased SOD, GSR, CAT, GST, GSH and GPx activities, while significantly decreasing the MDA and ROS levels due to its anti-oxidant property. Arora *et al.* (2000), reported that a double bond between C_2 and C_3 in the pyrone ring of GK and a keto-function at C_4 might be responsible for its anti-oxidant property.

In PS-MPs-intoxicated rats, urea and creatinine levels were significantly increased, while a significant decrease in creatinine clearance level was observed. Renal toxicity induced by PS-MPs is characterized by the increased serum creatinine and blood urea levels (Farooqui *et al.,* 2017). An upsurge in creatinine level is the sign of impaired kidney function because creatinine is a metabolic product and that is completely eliminated via glomerular filtration (GF). Additionally, increased levels of urea, and reduction in creatinine clearance level are the indications that the kidneys have suffered significant oxidative damage (Khan *et al.,* 2010). Nevertheless, the supplementation of GK + PS-MPs decreased urea and creatinine levels possibly by elevating the GF rate, which is further confirmed by the escalated creatinine clearance due to its ROS scavenging nature.

PS-MPs-intoxicated rats showed a profound increase in serum NGAL and urinary KIM-1 levels. NGAL as well as KIM-1 are the two important markers that indicate severe renal damage (Lei *et al.,* 2018). KIM-1 is a protein (transmembrane), absent in normal renal tissues however, it emerges during the earlier phases of kidney damages. NGAL is a protein (cytosolic) and it is discharged into the blood and urine followed by nephrotoxicity that results in renal ischemia and parenchymal injury. Nevertheless, GK + PS-MPs administration significantly reduced the NGAL and KIM-1 levels, which indicates its nephro-protective nature.

The exposure of PS-MPs significantly increased TNF-α, IL-6, IL-1β, NF-κB levels and COX-2 activity in PS-MPs intoxicated animals. NF-κB stimulates inflammatory cytokines i.e., TNF-α, IL-1β, COX-2 and IL-6 that are associated with severe inflammation. COX-2 is also a major inflammatory indicator, which has significant role in inflammatory response (Gandhi *et al.,* 2017). Nevertheless, GK administration significantly decreased the levels of inflammatory markers. This protective activity of GK might be attributed to its anti-inflammatory property. During and Larondelle (2013) stated that the ameliorative role of flavones against inflammation was due to the methoxylation of 5- or 7-hydroxyl groups on the A-ring or non-methoxylation of the 3'-hydroxyl groups on the B-ring.

Exposure to PS-MPs increased Caspase-3, Bax and Caspase-9 levels, while decreasing the Bcl-2 level. Proteins from the Caspase and Bcl-2 families are mainly crucial for apoptosis. Bcl-2 prevents the cells from undergoing apoptosis by acting as an anti-apoptotic protein. Contrarily, Bax encourages the death of cell (Hou *et al.,* 2021). A reduction in Bcl-2 and an increase in Bax change mitochondrial membrane permeability and subsequently prompts the release of cytochrome C into cytoplasm. The elevated level of cytochrome C instigates Caspase-9 that further activates Caspase-3, which chops up the cellular protein and results in apoptotic death. However, the supplementation of GK + PS-MPs resulted in a significant decrease in Caspase-3, Bax and Caspase-9 levels, while increasing the Bcl-2 level that might be due to its anti-apoptotic activity.

PS-MPs exposure induced significant histopathological damages in the renal tissues including degenerated glomerulus, increased Bowman's spaces, direct damage to renal tubules and vacuolization. Our results are further endorsed by Wang *et al.* (2022) who revealed that PS-MPs exposure increases ROS formation and induces LP in renal tissue by decreasing the activities of anti-oxidants and leading to histopathological damages in renal tissues. However, the supplementation of GK mitigated all these renal damages that might be credited to anti-apoptotic, antioxidant and anti-inflammatory activities of GK.

Conclusions: The present study revealed that GK supplementation significantly alleviated the PS-MPs-induced renal damage. GK treatment considerably recovered anti-oxidants activities, MDA and ROS levels, the levels of renal function, apoptotic, inflammatory indices as well as histological anomalies that might be attributed to its reno-protective, anti-apoptotic, antioxidant and anti-inflammatory activities.

Author's Contribution: NE, SA and MUI formulated the study, AH and MB performed the experiment. KAA and UA helped in statistical analysis. SA, AH and MUI wrote the manuscript. The authors have authorized the manuscript for submission.

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