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RESEARCH ARTICLE

Nephroprotective Potential of Zapotin Against Perfluorooctane Sulfonate-Induced Kidney Damage in Male Rats: A Biochemical, Inflammatory, Apoptotic and Histopathological Evaluation

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A B S T R A C T

Perfluorooctane sulfonate (PFOS) is a stable fluorinated organic pollutant that induces adverse effects on kidneys. Its toxicity is primarily linked with oxidative imbalance, inflammation, and apoptosis in renal tissues. The current investigation was aimed to explore the protective efficacy of zapotin (ZAP), a plant-derived flavonoid, against PFOS-induced nephrotoxicity in male albino rats. The animals were subjected to four treatments, each having 12 rats as follows: Control, PFOS (10mg/kg), PFOS + ZAP (10mg/kg + 20mg/kg), and ZAP (20mg/kg). Our findings showed that PFOS-exposure lowered the enzymatic activity of antioxidants, including glutathione S-transferase (GST), superoxidase dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GSR), catalase (CAT) and glutathione (GSH) content, while the levels of MDA and ROS were escalated. Furthermore, the levels of nuclear factor-kappa B (NF-κB), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and cyclooxygenase-2 (COX-2), were significantly elevated. PFOS-intoxication upsurged creatinine, NGAL, urinary proteins, KIM-1, and blood urea nitrogen (BUN) levels, at the same time, it reduced creatinine clearance. PFOS intoxication also increased the levels of apoptotic markers Bax, caspase-3, and caspase-9, accompanied by a reduction in the levels of anti-apoptotic marker Bcl-2. Besides, PFOS exposure instigated severe histological damage in the renal tissues. Nonetheless, ZAP markedly restored the above-mentioned alterations that may be due to its reno-protective potential.

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INTRODUCTION

Fluorinated organic compounds (FOCs) constitute a group of resilient hydrocarbon derivatives that contain fluorine and hydrogen (Rossen, 2023). Perfluoroalkyl substances (PFAS) comprise a broad group of synthetic fluorinated compounds, among them perfluorooctane sulfonate (PFOS) is one of the most ubiquitous and persistent contaminants in the environment (Shi *et al.,* 2024). Owing to its resistance to degradation and bioaccumulative nature, PFOS has become widely distributed in soil, sediments, and in aquatic bodies, posing an increasing adverse health concerns (Suman and Kwak, 2025). PFOS is extensively used in a wide range of consumer and industrial applications, including surfactants, paper coatings, adhesives, flame retardants, and cosmetic products (Zhang *et al.,* 2024). Dermal contact, inhalation, and ingestion are the primary routes of PFOS exposure (Poothong *et al.,* 2020). Ingesting contaminated food serves as a key route of exposure to PFOS in animals (Trudel *et al.,* 2008; Wilhelm *et al.,* 2008; Fromme *et al.,* 2009). PFOS intoxication in animals has been reported to induce notable oxidative stress and disruption of normal physiological functions due to its bioaccumulative nature (Peritore *et al.,* 2023).

Studies on animal models have revealed PFOS exposure resulted in endocrine disruption (Coperchini *et al.,* 2017), neurotoxicity (Negri *et al.,* 2017), and impaired reproductive developments (Xu *et al.,* 2017). PFOS has been reported in the serum of companion animals, such as cats and dogs (Wang *et al.,* 2018; Weiss *et al.,* 2021; Rock *et al.,* 2023). Evidence suggests that pet food serves as a primary source of PFOS exposure in dogs and cats (Brake *et al.,* 2023). Research indicates that cats diagnosed with thyroid, liver,

respiratory, and renal disorders exhibit elevated blood serum levels of PFOS (Bost *et al.,* 2016). Controlled feeding studies have demonstrated that livestock are susceptible to PFOS exposure through contaminated feed. In sheep, dietary exposure to PFOS resulted in its accumulation in liver, whereas replacement of contaminated forage with clean feed decreased hepatic PFOS concentration, highlighting diet as a primary route of PFOS exposure in livestock (Zafeiraki *et al.,* 2016). PFOS can easily accumulate in body tissues and pose certain health concerns due to its interaction with a variety of serum transport proteins and hepatic proteins (Chaparro-Ortega *et al.,* 2018). Experimental studies have shown that PFOS exposure induces a broad spectrum of health effects, i.e., hepatotoxicity, neurotoxicity, cardiotoxicity, and nephrotoxicity (Sen *et al.,* 2022). Owing to high blood perfusion rate and extensive surface area relative to tissue mass, the kidney is particularly susceptible to toxicants (Vervaet *et al.,* 2017). PFOS deposits mainly in kidneys and liver via enterohepatic circulation and it is eliminated via renal pathway without biotransformation. PFOS instigates oxidative stress (OS), a key contributor behind the inception of cellular inflammation and apoptosis (Gai *et al.,* 2019). Excessive generation of ROS activates redox signaling pathways particularly NF-κB, resulting in the elevated production of inflammatory cytokines. Moreover, inflammation and OS disrupt mitochondrial integrity thereby altering the balance between Bax and Bcl-2 and activate caspase-dependent apoptotic pathway (Hou *et al.,* 2021). These factors together play a significant role in renal injury (Greiber *et al.,* 2002).

Flavonoids are a diverse group of polyphenolic compounds that have demonstrated a broad range of applications in the cosmetic, nutraceutical, and pharmaceutical industries (Jomova *et al.,* 2025). Natural compounds have demonstrated tremendous therapeutic potential against various disorders. Plant-based flavonoids are widely used as antioxidants against various health issues (Cui *et al.,* 2025). Zapotin (ZAP), a member of the family polymethoxyflavones extracted from *Casimiroa edulis*, has demonstrated promising pharmacological activities (Toton *et al.,* 2016). Previous studies have reported that ZAP possesses antimicrobial (El-Seideek *et al.,* 2016), anticancer (Maiti *et al.,* 2007), antioxidant (Awaad *et al.,* 2006), anti-inflammatory (Elkady *et al.,* 2017) and antiviral activities (Esposito *et al.,* 2011). However, its protective potential against PFOS-induced nephrotoxicity has not yet been investigated. Therefore, the current study was designed to assess the nephroprotective potential of ZAP against PFOS-mediated renal injury by analyzing the inflammatory markers, antioxidant profile, and kidney function biomarkers, along with histopathological alterations in albino rats.

MATERIALS AND METHODS

Chemicals: PFOS (Cas No. 1763-23-1); Purity: >95%) and ZAP (Cas No. 14813-19-5; Purity: >95%) were procured from Biosynth Carbosynth (London, UK).

Animals: The male albino rats (weight: 230-250g) aged between 6 to 8 weeks were utilized for the experimental trial in the vivarium of the University of Agriculture (UAF). The rats were accommodated in animal cages under standard laboratory conditions, with a temperature of 20-22°C. Animals had uninterrupted access to food and water throughout the experiment. Upon completion of one-week acclimatization period, the rats were allocated randomly into four groups (n=12/group). The study was approved by the institutional committee of the University of Agriculture, Faisalabad, Pakistan (Approval No DGS. 20757-60). All the experimental procedures were performed as per the institutional guidelines for the use and care of laboratory animals.

Experimental layout: After a one-week acclimatization period, the animals were divided into four experimental groups, as follows: Control, PFOS (10mg/kg), PFOS+ZAP (10mg/kg + 20mg/kg), and ZAP (20mg/kg). After 8 weeks of dose regimen, the animals were anesthetized, decapitated, and the trunk blood was collected using sterile syringes. Serum was isolated from the blood and kept at -20°C for biochemical analysis. One kidney was cryopreserved at -80°C in zipper bags, while the remaining one was stored in a 10% formalin solution for histopathological analysis.

Estimation of biochemical parameters: The activities of CAT and SOD were determined as per previously explained methodologies by Aebi, (1984) and Kakkar *et al.* (1984) respectively. The assessment of GPx activity was performed via the approach of Lawrence and Burk, (1976). GSR activity was quantified by using the techniques of Carlberg and Mannervik, (1975). GST activity was determined in line with the methodology explained by Habig *et al.* (1974). The methodology of Moron *et al.* (1979) was followed to determine the GSH content. The levels of oxidative stress markers, including ROS and MDA, were estimated as per the methodologies of Hayashi *et al.* (2007) and Ohkawa *et al.* (1979), respectively.

Assessment of kidney markers: The kidney parameters, including KIM-1 (MBS564137), creatinine clearance, NGAL (MBS260195), BUN (MBS2611086), along with creatinine (MBS3809095), were determined using ELISA kits of MyBioSource, USA. Moreover, the urinary proteins (E02U0025) were determined by using ELISA kit as per the manual provided by the manufacturer (Shanghai BlueGene Biotech CO., LTD China).

Estimation of inflammatory markers: The levels of TNF-α (CSB-E11987r), COX−2 (CSB-E13399r), IL-6 (CSB-E04640r) and IL-1β (CSB- E08055r) were estimated by using ELISA kits as per the manual provided by the manufacturer (Cusabio, USA).

Estimation of apoptotic markers: The levels of apoptotic markers i.e., Bcl-2 (MBS704498), Bax (MBS2024395), Caspase-9 (MBS264306) and Caspase-3 (MBS763727) were estimated by using ELISA Kits according to the manual provided by the manufacturer (MyBioSource, USA).

Histological investigation: For histopathological evaluation, kidney tissue samples were fixed in 10% formalin and subsequently dehydrated in graded ethanol series ranging from 70% to 100%. After clearing in xylene, the specimens were embedded in paraffin wax. Microtome was used to cut thin 4µm sections from the blocks. Sections were subsequently stained with hematoxylin and eosin

(H&E) prior to evaluation under a light microscope at 400X magnification to identify structural changes.

Statistical analysis: The results of these experimental protocols were compared using statistical analysis by one way ANOVA followed by Tukey's post hoc test for group difference and were represented as the mean ± standard error (SE). The analyses were found to be statistically significant at ($P<0.05$).

RESULTS

Effects of PFOS and ZAP on oxidative and antioxidative parameters: PFOS exposure noticeably ($P<0.05$) increased MDA and ROS while reducing the activities of antioxidant enzymes, as compared to the control. This reveals the ability of PFOS to disturb the antioxidant defense system of the body. However, PFOS+ZAP concurrent administration remarkedly escalated the activities of antioxidant enzymes, while suppressing MDA and ROS concentrations. Nonetheless, only ZAP supplemented groups exhibited no significant variations as compared to the control group (Table 1).

Table 1: The effect of PFOS and ZAP on oxidative and antioxidant markers

Parameters	Groups			
	Control	PFOS	PFOS+ZAP	ZAP
CAT (U/mg protein)	16.79±1.12^{a}	6.48±0.46^{b}	17.24±0.55^{a}	17.55±1.18^{a}
GSR (nm NADPH oxidized/min/mg tissue)	12.62±0.67^{a}	3.13±0.78^{b}	11.73±0.63^{a}	13.97±0.73^{a}
SOD (U/mg protein)	14.49±0.57^{a}	4.61±0.38^{b}	13.72±0.69^{a}	15.23±0.74^{a}
GPx (U/mg protein)	25.77±0.96^{a}	11.39±0.59^{b}	25.81±0.59^{a}	27.08±0.99^{a}
GSH (nmol/mg protein)	23.71±1.07^{a}	8.39±0.51^{b}	23.62±0.65^{a}	25.11±0.86^{a}
GST (nmol/min/mg protein)	35.62±1.19^{a}	12.83±0.90^{b}	33.71±1.22^{a}	36.94±1.19^{a}
MDA (nmol/mg protien)	0.59±0.34^{b}	4.87±0.50^{a}	1.15±0.35^{b}	0.42±0.28^{b}
ROS (µmol/H_2O_2 equivalents/ mg protein)	1.35±0.45^{b}	8.23±0.66^{a}	2.01±0.49^{b}	1.04±0.58^{b}

^{abc} Various superscripts above Mean ± SE demonstrate significant differences between the groups.

Effects of PFOS and ZAP on renal injury markers: ZAP treated group and control group exhibited similar levels of kidney markers. However, in contrast to the control group, PFOS exposure significantly ($P<0.05$) augmented the levels of KIM-1, creatinine, BUN, urinary proteins and NGAL while downregulating the level of creatinine clearance. However, the levels of KIM-1, creatinine, urea and NGAL were dramatically ($P<0.05$) lowered by PFOS+ZAP cotreatment (Table 2).

Table 2: The effect of PFOS and ZAP on renal injury markers

Parameters	Groups			
	Control	PFOS	PFOS+ZAP	ZAP
BUN (mg/dL)	17.58±1.44^{b}	44.22±2.35^{a}	19.96±1.07^{b}	16.54±1.40^{b}
Creatinine (mg/dL)	0.67±0.32^{b}	7.11±0.53^{a}	1.59±0.44^{b}	0.44±0.30^{b}
Creatinine Clearance (mL/min)	1.77±0.23^{a}	0.34±0.15^{b}	1.52±0.21^{a}	1.97±0.0.14^{a}
Urinary proteins (mg/mL)	0.87±0.50^{b}	8.57±0.72^{a}	2.28±0.58^{b}	0.70±0.43^{b}
KIM-1 (ng/mL)	0.24±0.12^{b}	6.69±0.35^{a}	1.06±0.27^{b}	0.14±0.09^{b}
NGAL (ng/mL)	1.96±0.37^{b}	13.91±0.70^{a}	3.37±0.24^{b}	1.79±0.31^{b}

^{abc} Various superscripts above Mean ± SE demonstrate significant differences between the groups.

Effects of PFOS and ZAP on inflammatory markers: The findings of the present study showed that the control group and only ZAP treated group showed no significant variations in the levels of inflammatory markers. Compared with the control group, the PFOS-treated group showed a significant increase in the levels of inflammatory markers. Nevertheless, these markers were noticeably ($P<0.05$) downregulated in the co-treated group (Table 3).

Table 3: The effect of PFOS and ZAP on inflammatory markers

Parameters	Groups			
	Control	PFOS	PFOS+ ZAP	ZAP
NF-κB (ng/g tissue)	23.40±0.65^{bc}	74.98±0.97^{a}	26.75±0.87^{b}	22.57±0.97^{c}
TNF-α (ng/g tissue)	21.01±0.76^{bc}	81.89±1.01^{a}	29.47±1.07^{b}	19.87±1.27^{c}
IL-1ß (ng/g tissue)	17.28±0.62^{b}	69.03±1.44^{a}	20.93±0.91^{b}	16.75±0.84^{b}
IL-6 (ng/g tissue)	13.86±0.91^{c}	54.32±0.72^{a}	18.59±0.68^{b}	13.04±1.39^{c}
COX-2 (ng/g tissue)	32.14±0.83^{b}	87.29±1.25^{a}	35.81±0.73^{b}	31.67±0.84^{b}

^{abc} Various superscripts above Mean ± SE demonstrate significant differences between the groups.

Table 4: The effect of PFOS and ZAP on apoptotic markers

Parameters	Groups			
	Control	PFOS	PFOS+ ZAP	ZAP
Bax (ng/mL)	6.14±0.43^{c}	16.74±0.60^{a}	8.62±0.38^{b}	5.33±0.58^{c}
Bcl-2 (ng/mL)	14.45±0.71^{a}	3.81±0.49^{b}	13.86±0.55^{a}	15.27±0.62^{a}
Caspase-3 (ng/mL)	2.86±0.36^{b}	13.63±0.76^{a}	4.23±0.87^{b}	2.16±0.57^{b}
Caspase-9 (ng/mL)	4.16±0.68^{b}	18.64±1.07^{a}	6.95±0.74^{b}	4.34±0.60^{b}

^{abc} Various superscripts above Mean ± SE demonstrate significant differences between the groups.

Effects of PFOS and ZAP on apoptotic biomarkers: Both the control and ZAP treated group had similar levels of pro-apoptotic (caspase-9, Bax, and caspase-3) and anti-apoptotic (Bcl-2) markers. Moreover, compared to control, PFOS intoxication substantially ($P<0.05$) elevated the levels of pro-apoptotic indices, whereas the level of anti-apoptotic marker was decreased. Further, cotreated group showed remarkably ($P<0.05$) lowered levels of pro-apoptotic indices and escalated level of Bcl-2 than PFOS intoxicated animals (Table 4).

Effects of PFOS and ZAP on kidney histology: Compared to the control group, PFOS exposure caused glomerular shrinkage, proximal tubule dilation, expansion of Bowman's space, and tubular degradation. However, ZAP administration mitigated these histopathological defects compared to the PFOS-treated group. Furthermore, the group treated with ZAP alone showed no notable histological or structural changes relative to the control group (Fig. 1).

DISCUSSION

Since the middle of 20th century, PFOS has been used extensively in textiles and in many industrial processes as surfactants, a water and oil repellent in firefighting foams, and a flame-retardant agent (Stoffels *et al.,* 2023). It is reported that PFOS enters the body and accumulates in kidneys, liver, breast milk, and blood serum (Gaballah *et al.,* 2020; Rericha *et al.,* 2023). ZAP (5,6,2′,6′-tetramethoxyflavone), initially isolated from *Casimiroa edulis,* exhibits notable anti-analgesic anti-inflammatory, and antioxidant activities (Toton *et al.,* 2016). According to our findings, ZAP substantially protected the renal tissues in albino rats primarily by mitigating OS and related damages.

Our findings reported that PFOS inebriation reduced antioxidants activities while augmenting the levels of

MDA and ROS. OS is considered as the major culprit behind cellular as well as renal toxicity while antioxidant enzymes are regarded as "first line of defense" against various toxicities (Mani *et al.,* 2022). CAT is an important anti-oxidant enzyme which breaks hydrogen peroxide (H_2O_2) into oxygen and water (Tuzet *et al*., 2019), protecting cells from oxidative damage (Singhal *et al*., 2013). Moreover, SOD is involved in the conversion of superoxide to H_2O_2 as well as oxygen (Asadi *et al*.. 2017). GPx takes part in the reduction of the levels of H_2O_2 and LP to reduce OS (Wang *et al*., 2021). GSR regulates GSH concentration which modulates the stimulation of GPx (Ali *et al*., 2020). Excessive generation of ROS directly interacts with the lipid of the plasma membrane, which provokes a cascade of reactions called "lipid peroxidation" (Chen *et al.,* 2024). Our study is aligning with the prior literature which shows that OS plays integral roles in PFOS-induced renal damage (Wen *et al.,* 2021). However, PFOS+ZAP co-treatment remarkably upregulated antioxidant enzyme activities while reducing ROS and MDA levels. Jiang *et al.* (2014) reported that flavonoids contain phenolic compounds in their structural arrangement, which are responsible for their electron-donating properties that ultimately scavenge free radicals.

Kidneys act as key physiological regulators, responsible for sustaining internal homeostasis via precise control of fluid balance, electrolyte composition, and metabolic waste elimination (Blantz *et al.,* 2007). The present findings revealed that PFOS administration led to a decrease in creatinine clearance along with changes in urinary proteins, BUN, creatinine, KIM-1 and NGAL levels. It has been reported earlier that elevated levels of creatinine and urea are early signs of acute kidney injury (Ronco, 2008). KIM-1 is a transmembrane protein that demonstrates elevated levels during chronic and acute kidney injuries (Ichimura *et al*., 2012). NGAL is a secretory protein which is found in tubular epithelial cells and its high levels were observed during acute renal damage (Ronco 2008). Our findings are consistent with previous literature demonstrating that PFOS exposure causes multi-organ damage including renal damage (Durham *et al.,* 2023) In the current investigation, co-supplementation of ZAP enhanced creatinine clearance while reducing the levels of creatinine, BUN, urinary proteins, KIM-1 and NGAL in renal tissues of the rats.

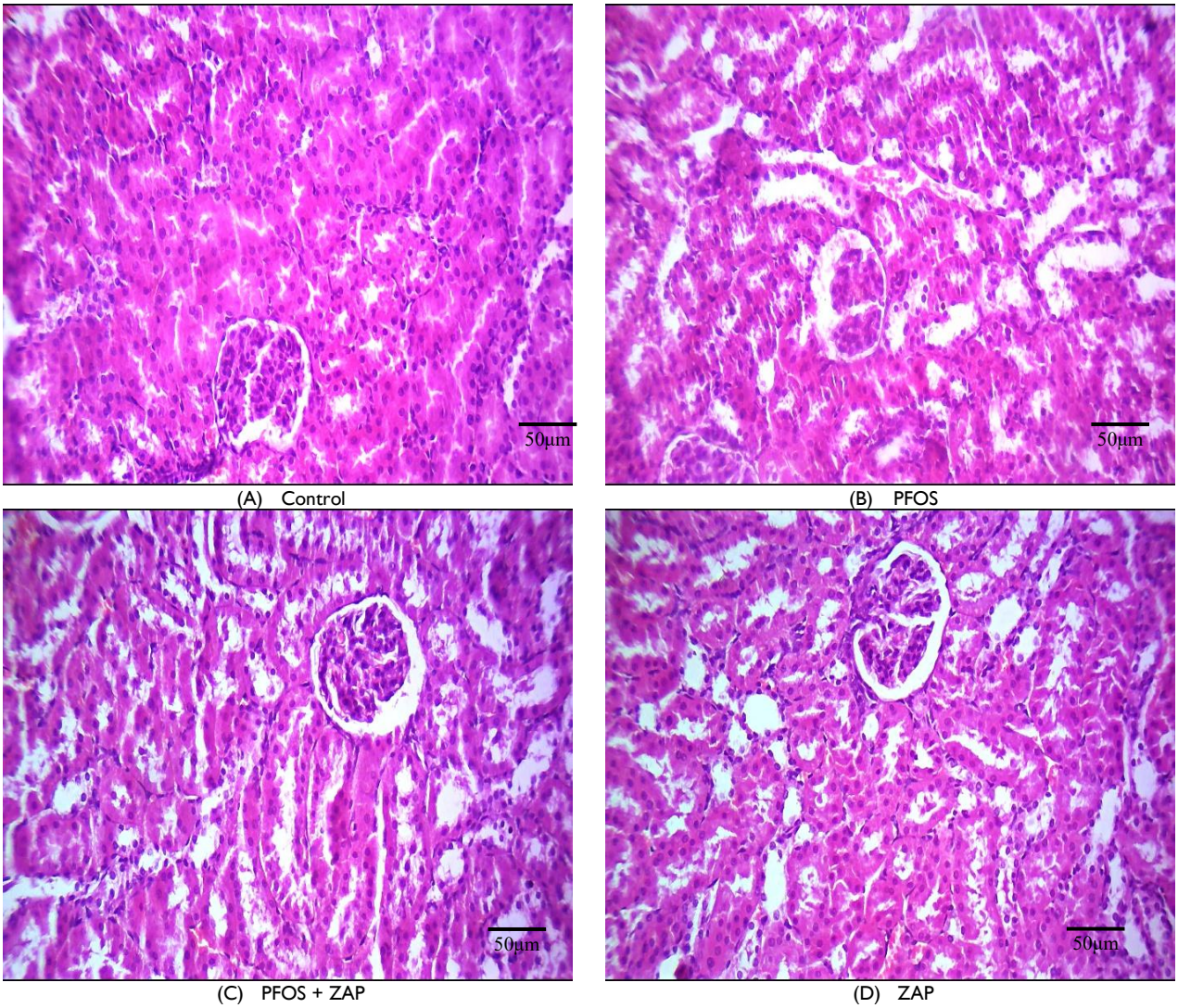

Fig. 1: The histological changes in renal tissues following administration of PFOS and ZAP. (A) Control and (D) ZAP groups are showing normal renal histology. (B) PFOS groups show remarkable damage, i.e., compromised tubular structure, dilated proximal tubules and elevated bowman's space. (C) PFOS + ZAP group showing significant reduction in histological damage.

In the current study, PFOS exposure escalated the levels of inflammatory indices such as IL-1β, NF-κB, COX-2, IL-6, and TNF-α. Inflammation is a physiological process which is specialized to shield the body against pathogens, toxins and external stimuli (Chovatiya and Medzhitov, 2014). Interestingly, these stimuli are initially detected by the immune cells present in kidneys (Jang and Rabb, 2015). Subsequently, these intrarenal cells release different inflammatory cytokines (IL-6, IL-1β) which penetrate the nearby blood vessels and recruit immune cells (Rabb *et al.,* 2016). NF-κB is a regulatory factor of inflammation, which provokes the transcription of other inflammatory cytokines (Khan and Khan, 2018). The escalated levels of these inflammatory cytokines indicate severe renal inflammation (Amdur *et al.,* 2016). The increased inflammatory mediators following PFOS exposure can be attributed to excessive ROS generation. Upregulated intracellular ROS promotes the phosphorylation and degradation of inhibitor of κB, thereby facilitating the nuclear translocation of NF-*κ*B where it stimulates the transcription of pro-inflammatory cytokines, including IL-1β, COX-2, IL-6, and TNF-α. The activation of this signaling cascade promotes inflammation. Inflammation leads to the production of ROS that result in tubular damage as well as renal failure (Qian, 2017). Nonetheless, co-treatment of ZAP remarkably downgraded the levels of inflammatory indices and ROS thereby reducing the inflammation in renal tissues.

Our findings demonstrated that PFOS intoxication significantly increased the levels of pro-apoptotic markers, whereas the level of the anti-apoptotic marker Bcl-2 was reduced. Excessive ROS production due to PFOS exposure causes lipid peroxidation and mitochondrial dysfunction, thereby disrupting mitochondrial membrane (Tsujimoto and Shimizu, 2007). During programmed cell death, the migration of activated Bax proteins to the outer mitochondrial membrane increases permeability, leaking cytochrome-c into the cytosol. Decreased levels of the anti-apoptotic protein Bcl-2 further drive this cytosolic leakage, inducing an enzymatic cascade where caspase-9 sequentially activates caspase-3 (Hou *et al.,* 2021). Our investigation revealed that exposure to PFOS triggered renal cell death by significantly boosting the levels of caspase-3, caspase-9 and Bax while concurrently suppressing the level of Bcl-2. These findings are consistent with previous evidence showing that PFOS exposure disrupts the Bax/Bcl-2 balance, promotes activation of the caspase cascade, and thereby contributes to apoptosis in renal tissues (Lee *et al.,* 2022). Conversely, administering ZAP successfully counteracted these variations and returned the marker levels to baseline, showcasing its protective and anti-apoptotic properties.

In the present investigation, PFOS exposure instigated various histopathological impairments including renal hemorrhagic changes, atrophy of glomeruli, intratubular inflammation, vasodilation and fibrosis in renal tissues. It is reported that environmental toxicants can cross intestinal barriers and enter systemic circulation that eventually leads to their accumulation in renal and other body tissues (Sarkar and Sil, 2021). Priyadarshanee *et al.* (2022) studied that these toxicants disrupted the actual architecture of kidney tissues by escalating the levels of ROS. Our findings are consistent with Zuo *et al.* (2025) who documented interstitial inflammatory invasion, renal cylindrical epithelial destruction, and glomerular atrophy in the kidney after PFOS exposure. However, the co-treatment of ZAP showed efficient protection against the structural alterations induced by PFOS. The ability of ZAP to scavenge free radicals as well as its anti-inflammatory properties may be responsible for its protective effects.

Conclusions: This study showed that exposure to perfluorooctane sulfonate (PFOS) caused clear damage in the renal tissues of albino rats, characterized by disturbances in biochemical profile, increased oxidative stress, and noticeable changes in tissue structure. However, when ZAP was given along with PFOS, these harmful effects were mitigated to a considerable extent. The co-treated animals showed better antioxidant activity, improved biochemical profile, and healthier tissue appearance compared to those exposed to PFOS alone. These findings suggest that ZAP has tendency to protect against PFOS-induced toxicity, possibly by reducing oxidative damage, apoptosis and inflammation. Based on these results, ZAP may have potential as a protective agent against PFOS-related renal toxicity, however further, mechanistic and clinical studies are required to explore its exact mode of action and possible applications.

Authors contribution: AT along with MUI designed the research approach after which they conducted the experiment. MUI supervised the research. AT performed statistical analyses. AT wrote the complete manuscript. All contributing authors have authorized the submission of the final manuscript version.

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