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RESEARCH ARTICLE

Isorhoifolin Attenuates Cadmium-Induced Renal Toxicity in Rats Via Regulating Biochemical and Histological Profiles

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ABSTRACT

Cadmium (Cd) is a hazardous environmental pollutant that has the potential to instigate multi-organ toxicities including, renal toxicity. Isorhoifolin (IRF) is a flavonoid glycoside that possess potential therapeutic properties due to its anti-oxidant nature. Hence, the present research was designed to assess the attenuative potential of IRF against Cd provoked kidney damage. 48 male albino rats (*Rattus norvegicus*) were randomly divided into 4 equal groups (12 rats/group), including the control, Cd intoxicated (5mg/kg) group, Cd + IRF (5mg/kg + 10mg/kg) co-treated group, and IRF (10mg/kg) treated group. The trial was completed in 28 days and then the animals were euthanized, decapitated and further analysis were performed. The results showed that Cd intoxication lowered the activities of antioxidant enzymes and increased the levels of oxidative stress (OS) markers (ROS and MDA). Moreover, Cd intoxication reduced the level of creatinine clearance and increased the levels of NGAL, urea, KIM-1 and creatinine. Furthermore, Cd treatment elevated the levels of inflammatory markers including NF-κB, IL-1β, TNF-α, IL-6 and COX-2. Cd intoxication also increased the levels of Caspase-9, Bax and Caspase-3, while decreasing Bcl-2 levels. Additionally, Cd induced various disruptions in the histological architecture of the renal tissues. Nonetheless, IRF administration protected the renal tissues from Cd prompted impairments due to its anti-oxidant, anti-apoptotic and reno-protective nature.

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INTRODUCTION

Cadmium (Cd) is a non-essential trace element which is generally used in nickel-cadmium (Ni-Cd) batteries, pigments, metal coating alloys and chemical stabilizers (Edwards and Prozialeck, 2009). It is a heavy metal that has versatile uses in agricultural and industrial sectors (Ma *et al.,* 2019). Cd is regarded as one of the main pollutants of the biosphere that produces severe impacts on humans, plants, animals and other living organisms (Unsal *et al.,* 2020). Humans as well as other living organisms are exposed to Cd via polluted air, food, and water (Almeer *et al.,* 2019). Cd can accumulate in various parts of the body including hepatic, cardiac, renal, reproductive and skeletal tissues and it causes detrimental effects that may lead to hepatotoxicity, nephrotoxicity, cardiotoxicity, and reproductive toxicity (Renu *et al.,* 2021).

Multiple investigations have reported that kidney is highly vulnerable to Cd induced damages as it can accumulate in the proximal tubules of nephrons (Satarug *et al.,* 2024). Cd exposure damages kidney via prompting inflammation and production of reactive free radicals i.e., reactive oxygen species (ROS). These free radicals further damage the cells and result in apoptosis, autophagy and endoplasmic reticulum (ER) stress (Shi *et al.,* 2024; Hamza *et al.,* 2025). Extended exposure to Cd can consequently cause renal failure by causing damage in the histology of renal tissues and reducing glomerular activity (Dkhil *et al.,* 2014; Ma *et al.,* 2023).

Plant based compounds, including flavonoids are widely used in the fields of medicine and pharmacology due to their widespread presence and potential therapeutic roles against various disorders (Kumar *et al.,* 2018). Isorhoifolin (IRF) or apigenin-7-*O*-rutinoside is a flavonoid glycoside

initially obtained from *Parquetina nigrescens*, which is also known as Ewe Ogbo or Ovie ukpakoma (Wu *et al.,* 2022). Multiple studies have reported that IRF is an effective therapeutic agent against diabetes, osteoporosis and Alzheimer's diseases (Bansal *et al.,* 2012; Wu *et al.,* 2022). Previous *in vivo* investigations have reported that IRF possess significant anti-oxidant, hypolipidemic, ROS scavenging and anti-inflammatory effects against multiple health conditions (Bansal *et al.,* 2012; Wang *et al.,* 2024). Additionally, IRF is also reported to improve the abundance of gut microbiota and mitigate inflammatory bowel disease in ulcerative colitis-induced murine models (Wang *et al.,* 2024). Moreover, IRF was also used as a promising agent in the treatment of chronic venous hypertension in animal models (de Souza *et al.,* 2018). However, no prior study has documented the potential protective effects of IRF against renal damages. Therefore, the current research was formulated to examine the potential effects of IRF against Cd-induced renal injury via estimating oxidative stress (OS), inflammatory and apoptotic parameters.

MATERIALS AND METHODS

Chemicals: Cd (CAS Number: 7440-43-9; Purity: > 99 %) and IRF (CAS Number: 552-57-8; Purity: ≥90%) were procured from Merck (Darmstadt, Germany).

Animals: The experiment was performed on forty-eight male albino rats (age: 8-10 weeks and body weight: 240±20g). The research trial was conducted in the animal house of University of Agriculture Faisalabad (UAF). The rats were kept in standard environmental conditions i.e., 24-26ºC temperature, 12h light/dark cycle and 50-55% humidity. The rats were given free access to water and palleted feed. Moreover, guidelines of European Union (Directive 2010/630/EU) regarding the use of animals in scientific studies were followed during the experiment.

Experimental layout: 48 rats were randomly distributed into the following 4 groups (12 rats/group):

1. First group was considered as control group
2. Cd intoxicated group: Received 5mg/kg of Cd via oral gavage
3. Cd + IRF co-treated group: Received 5mg/kg of Cd and 10mg/kg of IRF via oral gavage
4. IRF-treated group: Received 10mg/kg of IRF via oral gavage

The doses of Cd and IRF were selected on the basis of previously available literature (Paysant *et al.,* 2008; Hamza *et al.,* 2025). After 28 days, the experimental animals were euthanized, decapitated and blood was taken into heparinized tubes. The blood was centrifuged at 1000rpm for 15 minutes and resulting samples were stored at -20℃ for further analysis. Moreover, kidneys were excised from the body and washed immediately with saline solution. One kidney was kept in formalin (10%) for histopathological analysis. Meanwhile, the second kidney of each rat was stored in zipper bags (-80℃) for further biochemical analyses.

Estimation of oxidant/antioxidant parameters: The levels of MDA and ROS were estimated via following methodology of Ohkawa *et al.* (1979) and Hayashi *et al.* (2007), respectively. GSH contents were estimated by following the protocol described by Moron *et al.* (1979). Moreover, the activities of GSR, CAT, GST, GPx and SOD were assessed by following the methodologies of Carlberg and Mannevirk (1975), Kakkar *et al.* (1984), Younis *et al.* (2016), Lawrence and Burk (1976) and Aebi (1984), respectively.

Estimation of serum kidney markers: The serum levels of renal biomarkers, including, KIM-1, urea, creatinine, NGAL, and creatinine clearance were estimated by using ELISA kits. Rat KIM-1 (Cat. No. MBS564137), urea (Cat. No. MBS2600001), creatinine (Cat. No. MBS3809095), and NGAL (Cat. No. MBS260195) ELISA kits were used for the estimation of renal parameters. All the assays were performed by following the instructions provided by the manufacturer (MyBioSource, San Diego, USA).

Estimation of inflammatory markers: The levels of inflammatory markers were quantified using Cusabio (USA) ELISA kits by following the instructions provided by the manufacturer. Rat NF-κB (Cat. No. CSB-E13148r), COX-2 (Cat. No. CSB-E13399r), TNF-α (Cat. No. CSB-E11987r), IL-1β (Cat. No. CSB-E08055r), and IL-6 (Cat. No. CSB-E04640r) ELISA kits were used to analyze the inflammatory parameters.

Estimation of apoptotic markers: The levels of apoptotic markers, including proapoptotic (Caspase-9, Caspase-3, Bax) and antiapoptotic (Bcl-2), were quantified by using rat ELISA kits via following the instructions provided by the manufacturer (Elabscience Biotechnology Inc., Texas, USA). Rat Caspase-3 (Cat. No. E-EL-R0160), Bax (Cat. No. E-EL-R0098), Caspase-9 (Cat. No. E-EL-R0163) and Bcl-2 (Cat. No. E-EL-R0096) ELISA kits were used for the estimation of apoptotic markers.

Histological investigation: Kidneys were stored in formalin (10%) solution for 24 hours and then dehydrated in ascending grades of alcohol (80%, 90%, 100%). Following dehydration, the renal tissues were kept in paraffin wax and cut into 4-5µm thick slices. Then, the samples were mounted on the slides. Subsequently, the slides were stained with H&E stain and the alteration in the renal histology were observed by using a light microscope at 400X (de Menezes *et al.,* 2019).

Statistical analyses: Data were presented as Mean ± SE. The analysis of the data was performed using one-way ANOVA followed by Tukey's post hoc test. Minitab (V17) was used for the analysis and the variations with $P<0.05$ were considered significant.

RESULTS

Effects of Cd and IRF on oxidant/antioxidant parameters: The activities of antioxidant enzymes and the levels of OS markers, MDA and ROS, in the control and IRF only treated animals were approximately similar. However, Cd intoxication remarkably ($P<0.05$) elevated the levels of OS markers and reduced the activities of antioxidant enzymes, as compared to the control animals. Nevertheless, IRF and Cd co-treatment markedly ($P<0.05$) elevated the

activities of antioxidant enzymes and lowered the levels of OS markers as compared to Cd treated animals (Table 1).

Effects of Cd and IRF on kidney serum markers: The levels of renal markers in IRF administered and the control group were almost similar. Nonetheless, Cd exposure markedly ($P<0.05$) increased the levels of KIM-1, urea, NGAL and creatinine, while reducing the levels of creatinine clearance, as compared to the control. However, IRF + Cd co-treatment significantly ($P<0.05$) reduced the levels of above-mentioned parameters (Table 2).

Effects of Cd and IRF on inflammatory markers: The results demonstrated the levels of inflammatory markers in the control group and IRF group were almost similar. However, Cd intoxication noticeably ($P<0.05$) elevated the levels of inflammatory markers, as compared to the control. Nevertheless, the co-treatment of Cd and IRF substantially ($P<0.05$) lowered the levels of inflammatory parameters IRF (Table 3).

Effects of Cd and IRF on apoptotic markers: The levels of apoptotic markers in the control and IRF treated rats were approximately similar. Besides, Cd treatment significantly ($P<0.05$) increased the levels of Caspase-3, Caspase-9 and Bax, while Bcl-2 levels were reduced as compared to the control. However, Bax, Caspase-9 and Caspase-3 levels were substantially ($P<0.05$) reduced, and Bcl-2 levels were increased in Cd + IRF co-treated animals, as compared to Cd exposed group (Table 4).

Effects of Cd and IRF on histology of renal tissues: The control and IRF-treated animals showed the normal renal histological architecture. However, Cd intoxication resulted in marked damage in the renal histology as evidenced by glomerulus shrinkage, compromised tubular structure, necrosis, dilated proximal tubules and elevated bowman's space. Besides, co-treatment of IRF and Cd remarkably restored the histological structure of renal tissues (Fig. 1).

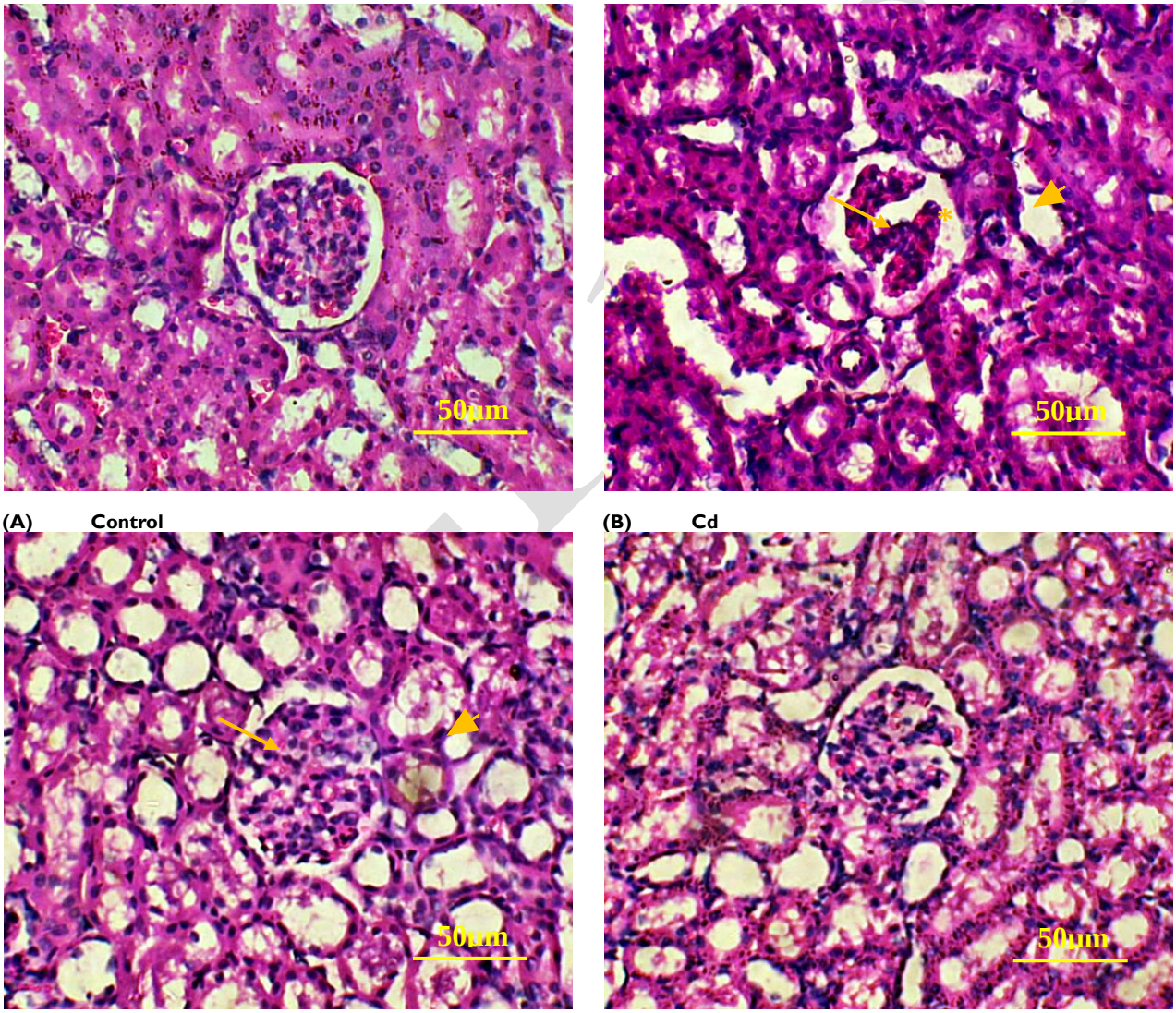

Fig. 1: Microphotographs of the renal tissues. (A) Control and (D) IRF are showing normal histological architecture. (B) Cd treatment resulted in increased tubular necrosis (arrowhead), bowman's space (*) and shrinkage of glomerulus (arrow). (C) Cd + IRF co-treatment improved the histological architecture of the renal tissues and recovered glomerulus (arrow) and renal parenchyma (arrowhead).

Table 1: Impact of Cd and IRF on biochemical parameters

Parameters	Groups			
	Control	Cd	Cd+IRF	IRF
CAT (Umg^{-1} protein)	15.24±0.60^{a}	7.20±0.29^{c}	12.09±0.89^{b}	15.93±0.84^{a}
SOD (Umg^{-1} protein)	12.32±0.53^{a}	5.96±0.35^{c}	9.59±0.66^{b}	12.83±0.76^{a}
GPx (Umg^{-1} protein)	33.57±0.99^{a}	11.05±0.72^{c}	26.22±0.92^{b}	34.05±1.22^{a}
GSR (nM NADPH oxidized/min/mg tissue)	10.38±0.93^{a}	4.09±0.66^{c}	7.94±0.31^{b}	10.66±1.06^{a}
GST(nM/min/mg protein)	38.85±1.30^{a}	14.45±0.74^{c}	30.07±1.14^{b}	39.77±1.69^{a}
GSH (µM/g tissue)	25.78±0.90^{a}	8.97±0.47^{c}	15.96±0.81^{b}	26.24±0.94^{a}
ROS (Umg^{-1} tissue)	1.77±0.16^{c}	7.61±0.48^{a}	2.81±0.22^{b}	1.67±0.19^{c}
MDA (nmol/mg protein)	0.83±0.15^{c}	2.81±0.47^{a}	1.6±0.27^{b}	0.76±0.16^{c}

Values with dissimilar superscripts are significantly different.

Table 2: Impact of Cd and IRF on renal markers

Parameters	Groups			
	Control	Cd	Cd+ IRF	IRF
KIM-1(ng/ml)	0.18±0.11^{c}	4.14±0.25^{a}	0.97±0.17^{b}	0.16±0.10^{c}
NGAL (ng/ml)	0.81±0.19^{c}	6.62±0.23^{a}	2.49±0.15^{b}	0.73±0.21^{c}
Urea (mg/dL)	15.74±0.94^{c}	46.35±2.87^{a}	25.59±1.06^{b}	15.67±0.93^{c}
Creatinine (mg/dL)	0.97±0.15^{c}	8.01±0.55^{a}	1.88±0.09^{b}	0.91±0.13^{c}
Creatinine clearance (ml/min)	2.51±0.23^{a}	0.56±0.20^{c}	1.34±0.14^{b}	2.58±0.24^{a}

Values with dissimilar superscripts are significantly different.

Table 3: Impact of Cd and IRF on inflammatory indices

Parameters	Groups			
	Control	Cd	Cd+ IRF	IRF
NF-κB (ngg^{-1} tissue)	22.09±1.44^{c}	83.43±1.03^{a}	37.80±0.96^{b}	20.89±1.42^{c}
TNF-α (ngg^{-1} tissue)	17.88±0.77^{c}	67.05±1.70^{a}	27.36±0.62^{b}	17.67±0.49^{c}
IL-1β (ngg^{-1} tissue)	12.02±0.89^{c}	54.76±1.42^{a}	20.10±0.76^{b}	11.14±1.03^{c}
IL-6 (ngg^{-1} tissue)	8.17±0.90^{c}	69.12±1.68^{a}	15.98±1.09^{b}	7.63±0.50^{c}
COX-2 (ngg^{-1} tissue)	25.47±1.16^{c}	87.37±1.43^{a}	36.73±1.62^{b}	24.31±1.03^{c}

Values with dissimilar superscripts are significantly different.

Table 4: Impact of Cd and IRF on apoptotic indices

Parameters	Groups			
	Control	Cd	Cd+ IRF	IRF
Bax (pg/mL)	1.42±0.13^{c}	3.32±0.18^{a}	1.88±0.16^{b}	1.37±0.12^{c}
Caspase-3 (pg/mL)	2.15±0.25^{c}	7.65±0.45^{a}	3.50±0.21^{b}	2.07±0.27^{c}
Caspase-9 (pg/mL)	3.41±0.12^{c}	13.50±1.19^{a}	5.25±0.26^{b}	3.37±0.13^{c}
Bcl-2 (ng/mL)	19.49±1.13^{a}	6.92±0.97^{c}	15.01±0.93^{b}	20.08±1.49^{a}

Values with dissimilar superscripts are significantly different.

DISCUSSION

Cd is a detrimental environmental contaminant that exerts notable health risks to humans and other living organisms. Exposure to Cd is one of the major factors that induce hepatotoxicity, cardiotoxicity and renal toxicity (Ijaz *et al.,* 2023; Alruhaimi *et al.,* 2024; Cirovic *et al.,* 2024). Oxidative stress (OS) is one of the primary factors that cause renal injury. Cd exposure results in OS via disturbing the production and removal of ROS from the body that consequently damages proteins present in the cell (Yan *et al.,* 2021). IRF is a plant based flavonoid with potential therapeutic effects (Bansal *et al.,* 2012). A previous investigation has reported that IRF enhanced the activities of antioxidant enzymes including CAT, SOD and GSH and mitigated diabetes-induced damages in animal models (Bansal *et al.,* 2012). In addition to anti-oxidant mode of action, IRF also possess anti-inflammatory properties that are evidenced by the reduced levels of IL-6, TNF-α and IL-1β in the mice suffering from ulcerative colitis (Wang *et al.,* 2024). Moreover, IRF is reported to prevent venular enlargement, functional capillary density and reduced endothelium interaction in the animals (de Souza *et al.,* 2018). Hence, this research was formulated to estimate the palliative role of IRF against Cd provoked kidney damage.

The outcomes of our research showed that Cd inebriation escalated the levels of MDA and ROS and reduced the activities of the antioxidant enzymes. Cd disrupts the normal levels of ROS and antioxidant enzymes. This results in abundant ROS production which destroys the biomolecules present in the cell and leads to OS (Abdellatief *et al.,* 2017). Antioxidants protect the body from ROS induced damages. Therefore, the consumption of antioxidant rich foods not only bring a positive impact on the health but also protects the body from chronic diseases (Dembinska-Kiec *et al.,* 2008). CAT, GPx, GSH, GSR and SOD are important antioxidant enzymes that protect the body from oxidative damage. SOD converts superoxide (O^{-}_{2}) to oxygen (O_2) and Hydrogen peroxide (H_2O_2) (Asadi *et al.,* 2017). CAT breaks H_2O_2 into water and oxygen (Tuzet *et al.,* 2019). Moreover, GSR modulates GSH contents, which further regulates the stimulation of GPx (Ali *et al.,* 2020), an enzyme that reduces the concentration of H_2O_2 and lowers lipid peroxidation (LP) (Wang *et al.,* 2021). Moreover, LP produces MDA as its by-product and it is a marker of OS and LP (Agarwal and Sengupta, 2020). The results of the current study are supported by Fang *et al.* (2021), who asserted that Cd induces renal damage via triggering OS and histopathological damages. Nonetheless, IRF supplementation lowered the levels of OS markers and enhanced the activities of antioxidant enzymes due to its free radical scavenging properties. The antioxidant effects of IRF may be attributed to the presence of hydroxy group at C-3′ position and rutinoside group at C-7 in its chemical structure.

The current research demonstrated that Cd inebriation lowered creatinine clearance levels while increasing the levels of urea and creatinine. In the blood, the escalated levels of urea and creatinine along with significantly reduced renal functions are the evidence of Cd-induced renal damage (Barnett *et al.,* 2018). The metabolism of amino acids produces urea as its by-product. Furthermore, muscle metabolism results in the breakdown of phosphocreatine as well as creatine which in turn produces creatinine. Creatinine is normally eradicated from body via glomerular filtration (Sepulveda, 2019). The reduced creatinine clearance and elevated urea as well as creatinine levels are the markers of acute kidney injury (Hu *et al.,* 2024). Nevertheless, IRF administration reduced the levels of urea and creatinine and elevated creatinine clearance levels due to its nephroprotective characteristics.

NGAL and KIM-1 are the markers of kidney damage (Sun *et al.,* 2017). They are not normally present in blood, but their escalated levels have been found in the individuals affected by renal damage (Khawaja *et al.,* 2019). The present research illustrated that Cd treatment increased the levels of KIM-1 and NGAL. OS is the key mediator of renal impairments, and the levels of OS markers are associated with the concentrations of NGAL and KIM-1 (Assadi and Sharbaf, 2019). Therefore, the escalated levels of OS markers lead to an increase in these renal markers which further cause renal disorders. However, IRF supplementation reduced NGAL and KIM-1 concentrations owing to their nephroprotective properties. The results of the current investigation showed that Cd intoxication resulted in a substantial elevation in the levels of inflammatory markers. The activation of NF-κB as a

result of excessive ROS substantially increases the production of proinflammatory cytokines (Stender *et al.*, 2012). The escalation in the levels of these inflammatory cytokines results in inflammation and various other disorders (Pan *et al.*, 2021). Furthermore, COX-2 is an important inflammatory mediator that induces inflammation (Gandhi *et al.*, 2017). Therefore, Cd induced inflammation, as reflected by increased levels of inflammatory cytokines in the renal tissues, may lead to renal dysfunction. However, IRF administration reduced the levels of inflammatory cytokines and protected the renal tissues from inflammation.

The outcomes of our study demonstrated that Cd exposure reduced the level of Bcl-2 and escalated the levels of Caspase-3, Caspase-9 and Bax. Bcl-2 and Bax are important apoptotic proteins. Bcl-2 is an antiapoptotic protein while Bax is a proapoptotic protein (Li *et al.*, 2015; Hou *et al.*, 2021). The disrupted equilibrium of Bcl-2/Bax triggers the liberation of cytochrome C in the cytoplasm of the cell that consequently activates Caspase-3 (Siddiqui *et al.*, 2015). Additionally, Caspase-3 disintegrates the cellular proteins and provokes apoptosis (Hou *et al.*, 2021). Prolonged and extensive occurrence of apoptosis leads to fibrosis, atrophy as well as organ failure (Mao *et al.*, 2010). The outcomes of our current investigation are in line with the research of Huang *et al*. (2022), who stated that Cd-intoxication provoked apoptosis in the renal tissues via caspase-dependent pathway. However, IRF and Cd co-administration reduced the levels of proapoptotic markers, while increasing the level of anti-apoptotic marker. Therefore, the remedial potential of IRF may be due to its anti-apoptotic properties.

The histological outcomes of our study showed that Cd administration instigated significant damages in kidney such as necrosis, glomerular, tubular and interstitial damages, as well as expanded tubules. Reportedly, Cd-induced OS is the ultimate cause of renal damage (Thévenod, 2010). Furthermore, Cd inebriation instigates significant structural changes in the renal tissues including glomerular, and tubular damages (Fang *et al.*, 2021). The histopathological outcomes of our research are in line with the study of Barregard *et al.* (2022), who reported that Cd-treatment provoked tubular atrophy as well as glomerular damage in the renal tissues. Nonetheless, IRF treatment reduced Cd induced histological damage due to its antioxidative properties.

Conclusions: Taken together, our findings demonstrated the nephroprotective effects of IRF against Cd provoked renal damage. Cd intoxication resulted in a marked reduction in the activities of antioxidants. Additionally, Cd increased the levels of apoptotic, oxidative stress and inflammatory markers. Moreover, Cd exposure disrupted the levels of renal serum markers and provoked histological changes in renal tissues. Nonetheless, IRF treatment reduced the Cd-triggered disruptions and protected the renal tissues due to its antioxidant characteristics. However, the current research was carried out on animal models. Therefore, further clinical trials are required to validate the current findings.

Authors contribution: MUI, HQ and MZS designed the experiment. KA and MZS conducted the research. KAA and AI performed statistical analyses. MUI, HQ, MZS, KA, KAA and AI wrote and reviewed the final manuscript version. All authors approved the final submission of the manuscript.

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