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STUDY ON THE EFFECTS OF CASTICIN AGAINST GENTAMICIN-INDUCED DYSREGULATION OF OXIDATIVE STRESS, MITOCHONDRIA AND SERUM CHEMISTRY IN RATS

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ABSTRACT

Gentamicin (GM) is an important aminoglycoside antibiotic to treat different infections caused by gram-negative bacteria. Mitochondrial dysfunction is considered as a key factor in the pathogenesis of renal disorders, and an important consequence of GM-induced nephrotoxicity that results in structural and functional alteration. Casticin (CAS) is a potential phytochemical having various pharmacological properties. The current investigation was formulated to ascertain the protective effects of CAS on GM induced mitochondrial dysfunction in kidney of rats. For this study, mature Sprague Dawley rats (n=48), weighing 200 ± 20g were used and divided into four groups (n=12) using a completely randomized design (CRD); Group 1 (control group), Group 2 (GM dose, 80 $mgkg^{-1}$ b. wt i.p), Group 3 (80 mg/kg GM (i.p) and 50 $mgkg^{-1}$ b.wt of CAS orally) and Group 4 (CAS 50 $mgkg^{-1}$ b.wt orally). All rats were treated for ten days continuously. Our finding showed that GM administration significantly increased the concentration of urea and creatinine; however, creatinine clearance was reduced. GM treatment increased the level of mitochondrial reactive oxygen species (ROS) and lipid peroxidation, while the activity of glutathione, catalase, superoxide dismutase and glutathione peroxidase were decreased. Mitochondrial tricarboxylic acid (TCA) cycle enzymes (succinate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase and alpha-ketoglutarate dehydrogenase) activities were decreased after GM exposure. In addition, mitochondrial electron transport chain (ETC) enzymes, i.e., NADH dehydrogenase, succinate-dehydrogenase, succinic-coenzyme Q and cytochrome c-oxidase activities were reduced followed by GM administration. GM administration decreased mitochondrial membrane potential ($\Delta\Psi m$) while significantly induced histological damage. However, treatment of CAS abrogated the damaging effects of GM in isolated renal mitochondria. Therefore, the present study demonstrated that CAS exhibits palliative effects against GM-induced renal mitochondrial impairment in the rats.

Key words: Gentamicin, Mitochondrial dysfunction, Kidney, Casticin, Antioxidant enzymes

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INTRODUCTION

Aminoglycosides are antibiotics, generally used to treat serious infections. However, they may cause adverse effects, including nephrotoxicity, allergic skin reactions, ototoxicity and neuromuscular blockade (Trevor *et al.,* 2015; Bakry *et al*., 2021). Gentamicin (GM) is an important aminoglycoside used to treat various bacterial infections caused by gram-negative bacteria (Akhtar *et al*., 2020; Merdana *et al.,* 2021). GM induced nephrotoxicity involves different factors such as oxidative stress (Christo *et al.,* 2011), lipid peroxidation (Govindappa *et al.,* 2019) and decreased efficacy of various antioxidant enzymes (Abdel-raheem *et al.,* 2009). GM elevates the concentration of ROS (Yaman *et al.,* 2010), hydrogen peroxide and hydroxyl radicals (Tavafi, 2012). Nephrotoxicity induced by GM is also exhibited by increased blood nitrogen, urea, creatinine and decreased glomerular filtration rate (Tavafi, 2012).

Mitochondria are a potential source of energy for cells that plays a vital role in different biological mechanisms in the body (Tao *et al.,* 2018). Mitochondria

are major site for the production of reactive nitrogen species (RNS) and ROS in cells (Bhargava *et al*., 2017). High production of RNS and ROS induces oxidative stress that leads to mitochondrial dysfunction. In chemicals-induced toxicity, mitochondrial dysfunction is one of the key underlying reasons behind renal damage (Liu *et al.,* 2021; Mannan *et al*., 2021). Mitochondrial dysfunction occurs due to the decline in endogenous enzymatic and non-enzymatic antioxidants. A decrease in these enzymatic antioxidants occurs due to the protein denaturation and peroxidation (Adil *et al.,* 2016).

Casticin (CAS) (3′,5-dihydroxy-3,4′,6,7-tetramethooxyflavone), also known as vitexcarpin, is a major part of the Chinese traditional herb *Fructus viticis* for decades (Lee *et al*., 2019). CAS shows various pharmacological properties such as anti-asthmatic (Koh *et al*., 2011), anti-neoplastic (Ramchandani *et al*., 2020), antioxidant (Ijaz *et al*., 2020), immunomodulatory (Mesaik *et al*., 2009), liver fibrosis attenuation (Zhou *et al.,* 2017) and lung injury protection (Wang *et al.,* 2016). As it is evident that oxidative stress and decreased antioxidant potential are two primary factors contributing to GM-induced toxicity therefore, considering the antioxidant properties of CAS, the goal of recent investigation was to explore the possible capacity of CAS against GM induced oxidative stress, serum chemistry and mitochondrial damage in the renal tissues of rat.

MATERIALS AND METHODS

Experimental design: In this experimental trial, male Sprague Dawley rats (body weight 200 ± 20 g 8-10 weeks) were purchased from breeding and rearing center and housed in a standard specific facility with a 12 h light and dark cycle, 22 ± 1 °C temperature, food and water in the animal house of University of Agriculture, Faisalabad. The study was conducted in the year of 2021. Ethical Committee of University of Agriculture, Faisalabad provided approval of this research study (13809-12/18-04-2022).

Forty eight male Sprague Dawley rats were randomly distributed into four groups (n= 12) and given the treatment as follows: Group 1 (control group), Group 2 (GM dose, 80 $mgkg^{-1}$ b. wt i.p), Group 3 (80 mg/kg GM (i.p) and 50 $mgkg^{-1}$ b.wt of CAS orally) and Group 4 (CAS dose, 50 $mgkg^{-1}$ b.wt orally). The research design used in this research was a completely randomized design (CRD). The dosages of GM and CAS were selected according to the previous studies conducted by Ibraheem *et al*. (2014) and Ijaz *et al*. (2020), respectively. Urine was collected from each animal. After ten days, all rats were anesthetized by pentobarbital and then decapitated and blood was collected in heparinized tubes and kidneys were excised.

Isolation of renal mitochondria: Renal mitochondria were separated according to Mingatto *et al.* (1996). Kidney tissues were homogenized using medium I (70 mM sucrose, mannitol (250 mM), Tris-Hydrochloric acid (50 mM), 10 mM HEPES potassium hydroxide, 1 mM EDTA, 120 mMKCl and pH 7.4). Centrifugation (at 755×g) was carried out for 5 min. The obtained homogenate further went through centrifugation process at 13300×g for almost 15 min. Medium labeled II (50 mM Tris-Hydrochloric acid, 250 mM mannitol, 70 mM sucrose, 10 mM HEPES, at 7.4 pH) was used for the suspension of subsequent pellets and same buffer was used to clean it twice. Then further centrifugation was carried out in 13300× g for 15 minutes. Resulting pellets of mitochondria were stored in corresponding buffer for further assay.

Measurement of serum creatinine, urea and creatinine clearance: Renal functions were assessed by the determination of serum creatinine (Thermo Fisher: EIACUN), urea (Thermo Fisher: EIABUN) and creatinine clearance levels (Thermo Fisher: EIACUN) according to guidelines provided with standard laboratory kit.

Assessment of renal oxidative stress and anti-oxidative capacity: The catalase (CAT) activity was evaluated by following the method reported by Aebi (1984). The process of Kakkar *et al*. (1984) was followed to examine superoxide dismutase (SOD) activity. Glutathione peroxidase (GPx) activity was determined by following the procedures of Paglia and Valentine (1967). Glutathione (GSH) activity was determined by following procedures of Ellman (1959). The level of ROS was quantified by ELISA kit (Shanghai Enzyme-Linked Biotechnology Co. Ltd., Shanghai, China). Level of TBARS were evaluated by LPO. TBARS was assessed by the procedure reported by Ohkawa *et al*. (1979)

Measurement of TCA cycle enzymes: Activity of isocitrate dehydrogenase (ICDH) was evaluated by following the method of Bernt and Bergmeyer's (1974). Succinate-dehydrogenase (SDH) activity was evaluated using method reported by Slater and Borner (1952). Malate dehydrogenase (MDH) activity was determined by following procedures of Mehler *et al*. (1948). alpha-ketoglutarate dehydrogenase (α-KGDH) activity was evaluated by following the procedure reported by Reed and Mukherjee (1969).

Mitochondrial ETC complex activities: Mitochondrial ETC complex assay kit (Suzhou-Comin Biotechnology Ltd. China) was utilized to evaluate respiratory chain complexes activities in kidney mitochondria.

Determination of Mitochondrial membrane potential (MMP) change: Rhodamine 123 (Rh 123) mitochondrial specific cationic florescent probe was used for the

measurement of mitochondrial membrane potential. Briefly, incubation of mitochondrial suspension in cylindrical tube was shaken with Rhodamine 123 (1.5 μM) for ten minutes at 37ºC. For the estimation of fluorescence, the Elmer LS-50B Luminescence fluorescence spectrophotometer was applied at wavelength of emission (490 nm) and excitation (535 nm) (Baracca et al. 2003).

Tissue Histology: The renal tissues were fixed in 10% formalin solution for 48 hours. Then, specimens were dehydrated in ascending grades of alcohol and embedded in paraffin wax. 4-5 μm thick slices were cut by using rotatory microtome and finally stained with hematoxylin-eosin stain (dissolved in 70% alcohol). Finally, these slides were observed under a light microscope (Nikon, 187842, Japan) at 400X and microphotography was performed by Leica LB microscope connected to Olympus Optical Co. LTD, Japan. Image J2x software was used to analyze the photographs.

Data analysis: Analysis of the results was done by one-way analysis of variance (ANOVA) and the treatments means were compared by Tukey's test with the aid of Minitab software. Quantitative data were articulated as the mean ± SEM. We considered the value of $p < 0.05$ to be statistically significant.

RESULTS

CAS effect on GM induced toxicity in renal serum markers: GM administration resulted in a substantial ($p < 0.05$) elevation in serum creatinine and urea concentration; on the other hand, creatinine clearance was substantially ($p < 0.05$) reduced in GM intoxicated animals as compared to control animals. Co-treatment of GM plus CAS led to significant reduction in creatinine and urea concentration and increased the level of creatinine clearance in comparison to GM treated rats. However, CAS (alone) treated animals showed the concentration of urea, creatinine and level of creatinine clearance near to the control animals (Table 1).

Effect of CAS on antioxidant enzymes, ROS and TBARS in GM-exposed rats: The obtained results showed that mitochondrial CAT, SOD, GPx and GSH activities were lessened and TBARS and ROS concentration were increased in GM exposed groups as compared to control rats. Co-administration of CAS plus GM increased the anti-oxidant enzymes (GPx, SOD, CAT and GSH) activities, on the other hand significantly ($p < 0.05$) decreased the concentration of ROS and TBARS as compared to the GM receiving group. However, CAS (alone) treated animals demonstrated the activities of CAT, SOD, GSH, GPx, TBARS and ROS near to control animals (Figure 1).

CAS effects on TCA cycle enzymes: GM administration showed a considerable ($p < 0.05$) reduction in enzymatic activities of TCA cycle (ICDH, α-KGDH, MDH and SDH) as compared with normal animals (Figure 2). Co-administration of CAS express substantial ($p < 0.05$) elevation in enzymatic activities of TCA cycle as compared with GM intoxicated animals. CAS treatment showed the enzymatic activities of TCA cycle enzymes were near to control group.

CAS effect on mitochondrial ETC complexes: A substantial ($p < 0.05$) reduction in the activities of mitochondrial ETC complex (I-IV) in GM administrated rats as compared to normal animals (Figure 3). Co-administration of CAS plus GM substantially ($p < 0.05$) increase the activities of ETC complexes (I-IV) of mitochondrial ETC as compared to GM treated animals. CAS treated group demonstrated normal functioning of complexes (I-IV) in ETC as compared to normal rats.

CAS effect on mitochondrial membrane potential (ΔΨm): Animals treated with GM displayed a significant ($p < 0.05$) decrease in ΔΨm as compared to normal rats. But, the co-treatment of CAS + GM significantly increase ΔΨm as compared to GM administrated rats. CAS treated group demonstrated normal functioning of ΔΨm as compared to normal rats (Figure 4).

Table 1. Effect of GM and CAS on the level of serum urea, creatinine and creatinine clearance in renal tissues of rats.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Creatinine Clearance (ml/min)
Control	17.65 ± 0.69^{c}	2.62 ± 0.22^{b}	1.93 ± 0.08^{a}
GM	38.99 ± 0.92^{a}	5.55 ± 0.16^{a}	0.54 ± 0.06^{c}
GM+ CAS	24.51 ± 0.55^{b}	3.18 ± 0.13^{b}	1.36 ± 0.07^{b}
CAS	16.58 ± 0.43^{c}	2.71 ± 0.12^{b}	1.91 ± 0.07^{a}

Means that do not share similar letters in a column are significantly different from each other

Effect of CAS and GM on histopathology: Kidney of rats in the control group showed normal renal tubules and glomeruli. Most of the renal glomeruli were normal without mesangial cells having cell proliferation or vascular congestion. The shape of the renal tubules ranged from round to oval ducts lined with polygonal or

cuboidal epithelial cells. While the kidneys in the GM-treated group showed distortion of the normal structure of the kidney where the glomerular tuft was atrophied, disruption of Bowman's capsule and vacuolation in the epithelium of renal tubules. However, co-treated group displayed mild to moderate vacuolation in the epithelium of tubules. The glomeruli were also normal in size and presented a mild level of tufts and distortion in the capillaries. Whereas, only CAS administration showed normal renal tubules and normal histological architecture as in the control group (Fig.5).

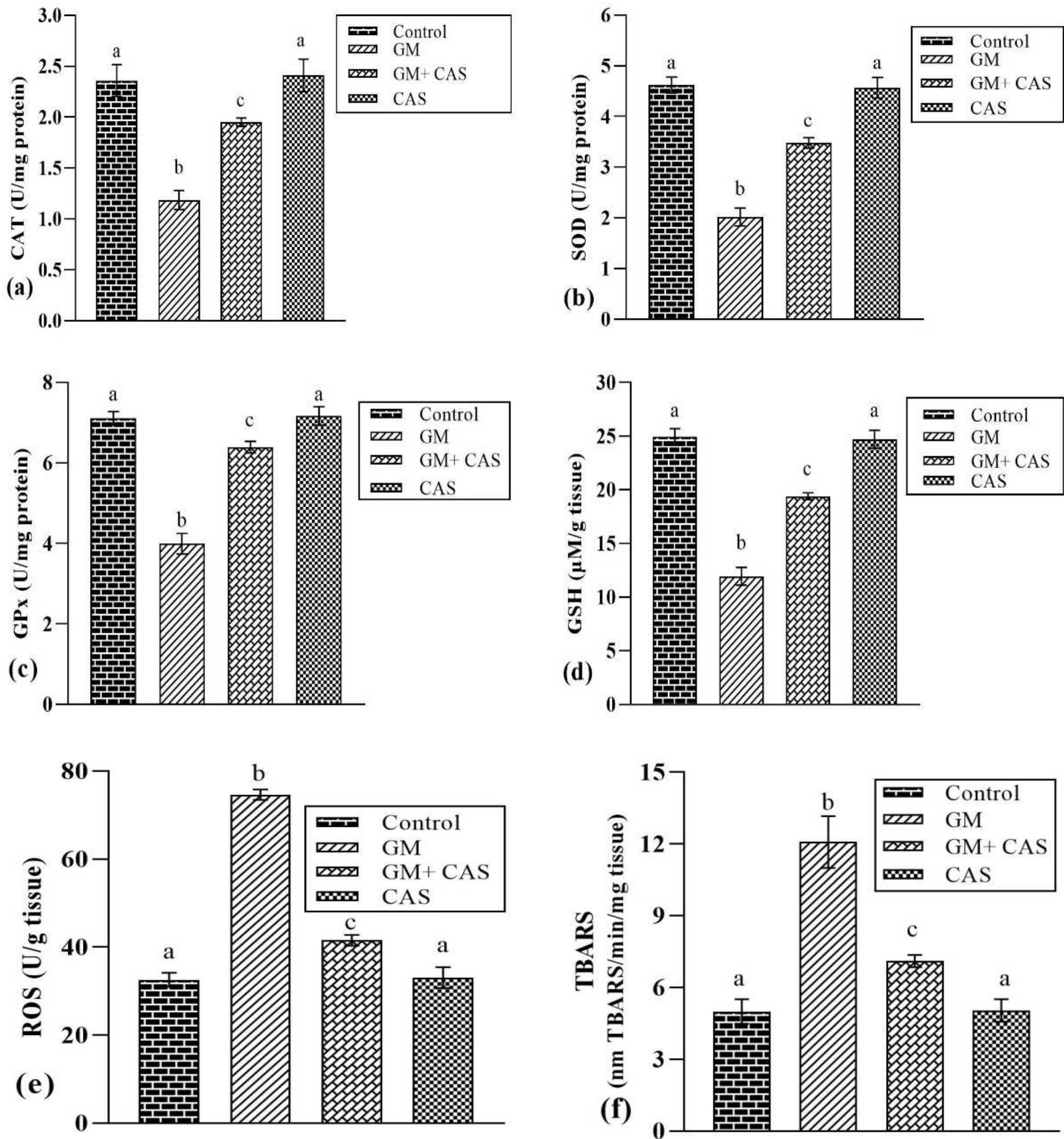

Figure 1. Effect of CAS on the mitochondrial antioxidant enzymes; (a) CAT, (b) SOD, (c) GPx, (d) GSH, (e) ROS and (f) TBARS in GM-treated rats. Bars sharing different superscripts are significantly ($p < 0.05$) different from each other.

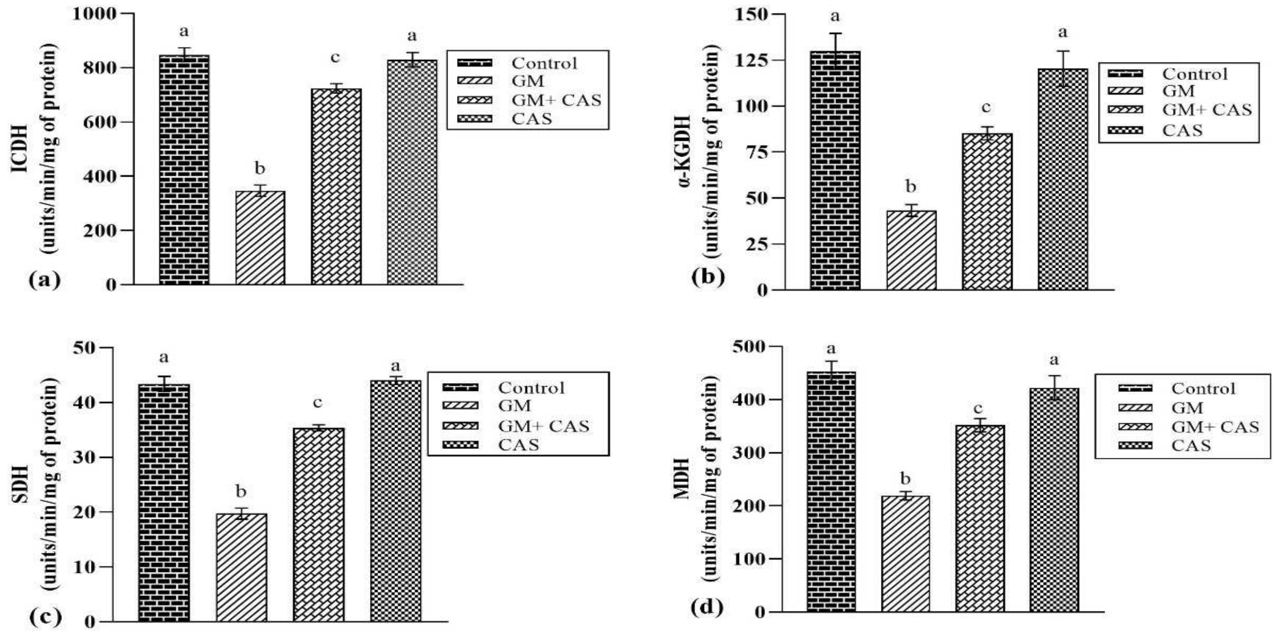

Figure 2. Effect of CAS on the TCA cycle enzymes activities; (a) ICDH, (b) a-KGDH, (c) SDH (d) MDH in the renal mitochondria of GM-administered rats. Bars sharing different superscripts are significantly ($p < 0.05$) different from each other.

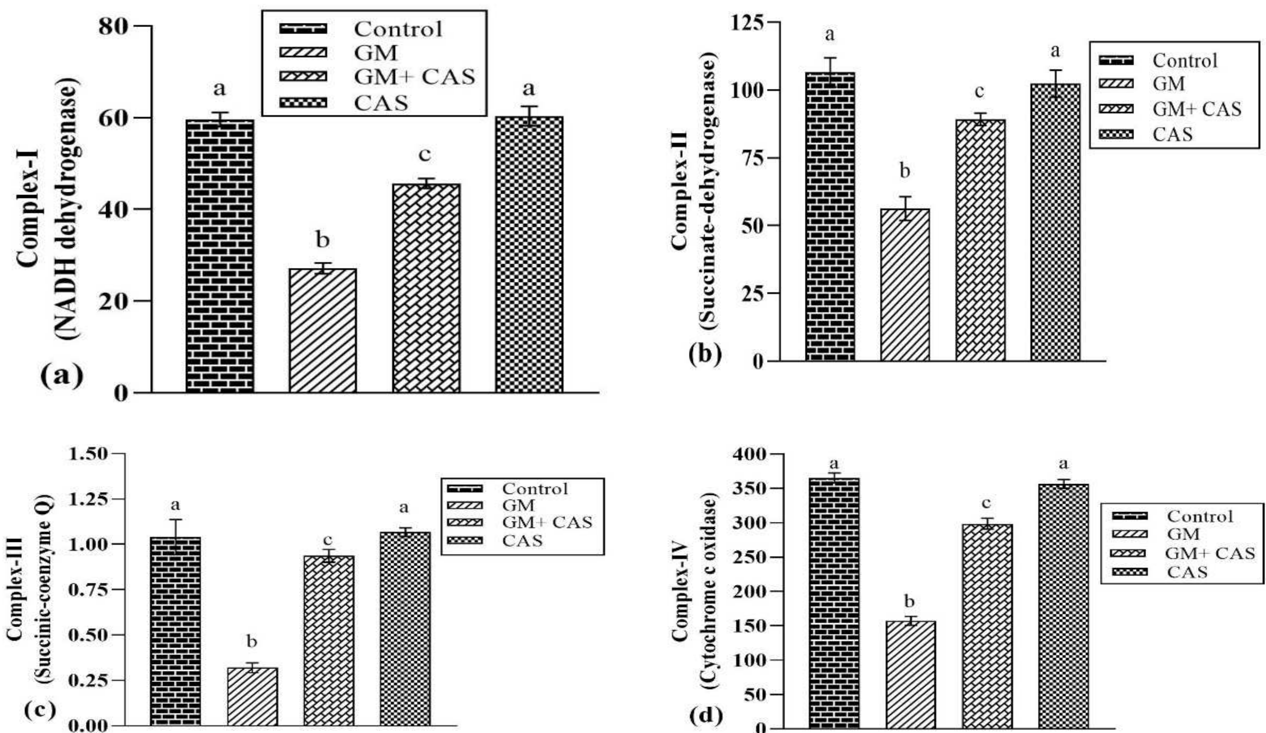

Figure 3. Effect of CAS on the activities of ETC complexes (a) Complex-I (b) Complex-II (c) Complex-III (d) Complex-IV in renal mitochondria of GM-treated rats. Bars sharing different superscripts are significantly ($p < 0.05$) different from each other.

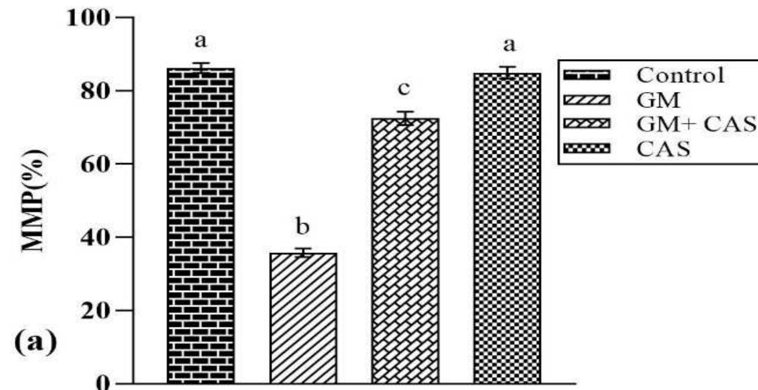

Figure 4. Effect of GM and CAS on ΔΨm of GM intoxicated rats. Bars sharing different superscripts are significantly ($p < 0.05$) different from each other.

(a) Control

(b) GM

(c) GM+CAS

(d) CAS

Figure 5. Histopathological examination of different groups of renal tissues. (A) Renal section of control group rats, displaying normal histological structure of glomeruli and renal tubules; (B) Renal section of GM (80 $mgkg^{-1}$) administered rats, representing significant degenerative changes, granular deposits in their lumens and desquamation of the kidney epithelium; (C) A microphotograph of CAS (50 $mgkg^{-1}$) + CP (80 $mgkg^{-1}$) treated rats displaying reduced degenerative alterations in renal epithelium and renal tubules; (D) Renal section showing normal histological structure of glomeruli and renal tubules as in control rats treated with CAS (50 mg/kg) alone.

DISCUSSION

In the present study, we aimed to investigate the effect of casticin, against gentamicin-induced nephrotoxicity, in order to gain new insights into the treatment modality of gentamicin-induced nephrotoxicity. The results of our study showed that the level of serum urea and creatinine were elevated after GM administration, while the extent of creatinine clearance was reduced. The reduced glomerular filtration rate (GFR) is reflected by increased level of creatinine and urea in plasma/serum (Higgins, 2016). It has been revealed that the GM induces tubular necrosis, elevates renal vascular resistance and reduces the glomerular ultrafiltration coefficient, which results in decreased GFR (Plajer *et al*., 2015). Previous studies exposed that GM can induce renal toxicity in rats by increasing serum creatinine and urea (Erseçkin *et al*., 2020). Change in kidney functional markers may be attributed to elevated levels of oxidative stress in kidney (Khan *et al*., 2010). However, our results show a significant reduction in creatinine and urea levels as well as elevated creatinine clearance after CAS treatment. CAS administration recovered these markers to the normal level.

In our investigation, it was found that GM administration decreased the antioxidant enzymes activities (GPx, SOD and CAT and) as well as GSH content and increased the TBARS and ROS levels in renal mitochondria. Imbalance in ROS and antioxidant defense systems leads to oxidative stress, ultimately resulting in oxidative damage (Ratliff *et al*., 2016; Asala *et al*., 2022). SOD, CAT and GPx are the key enzymes involved in ROS elimination. SOD converts O^{-2} into hydrogen peroxide (H_2O_2), which is the primary step of antioxidant pathway. In the presence of GSH, CAT and GPx catalyzes the conversion of H_2O_2 into H_2O and O_2 (Schieber and Chandel, 2014). Observations have shown that GM induces damage in renal cells by producing the ROS and disturbing the composition of lipids in the cell membrane by oxidative degradation of lipids (Udupa, and Prakash, 2019). The previous observations demonstrated that GM disrupts the redox balance and produces free radicals, which increases lipid peroxidation (Yilmaz *et al*., 2018). However, the treatment of CAS remarkably reduced the TBARS and ROS by restoring the antioxidant status. This may be attributed to the antioxidant potential to the CAS. According to Chan *et al.* (2018), the unique structure of casticin with three rings as well as orthocatechol moiety on ring B and two hydroxyl groups confer it antioxidant potential. Our findings are in conformity with the study conducted by Ijaz *et al.* (2020) in which CAS restored the antioxidant enzymes level in the liver of rats.

Mitochondria are known as the most important organelles involved in energy production that are also vulnerable to OS (Guo *et al.,* 2013). In the current experiment, GM treatment declined the enzymatic activities of TCA cycle. Reduction in the enzymatic activities of TCA cycle might be due to GM-induced membrane destabilization. Mitochondrial enzymes (ICDH, SDH, αKGDH and MDH) catalyze the oxidation of substrate by TCA cycle producing reducing-equivalents. By the process of oxidative phosphorylation, these reducing equivalents are routed via ETC for the production of ATP (Chandramohan *et al.,* 2015). NADPH is the important producer of ICDH enzyme in TCA cycle that plays a significant role in the protective mechanism against damage persuaded by oxidative stress (Vedi *et al*., 2014). Decrease in the activity of ICDH causes the reduction in mitochondrial glutathione, resulting in significantly low resistance of other TCA cycle enzymes against OS (Jo *et al.,* 2001). In mitochondria, SDH is one of the most essential enzymes that helps to control the production of ATP. It is key enzyme of thiol (SH) group and is highly susceptible to the free radicals (Murugesan *et al.,* 2013). Decreased activities of these enzymes alter mitochondrial substrate oxidation, resulting in decreased substratum oxidation that effect the transfering of reducing-equivalents, as a result it depletes cellular energy (Morimoto *et al*., 2000). However, in the present study, co-treatment of GM with CAS reversed the enzyme's activity. Our results are in agreement with the study conducted by Ijaz *et al.* (2021) in which morin, another member of flavonoid family improved the activities of TCA cycle enzyme. Decreased production of ROS might be responsible for the re-establishment of enzymatic status.

Our investigation demonstrated that GM administration decreased the activities of mitochondrial ETC complex (I-IV). ETC is considered as the key site of ROS generation under normal conditions (Tan *et al*., 1998). Previous data revealed that accumulation of intracellular ROS subsequently damage mitochondrial ETC (Druck *et al*., 2019). Mitochondrial dysfunction is an important factor in renal dysfunction (Che *et al.,* 2014). GM induces mitochondrial dysfunction by inhibiting complex I-IV enzyme activities (Adil *et al.,* 2016). On the other hand, CAS co-treatment effectively reversed ETC complexes activities. Our results are in line with study conducted by Ijaz *et al.* (2022) in which nobiletin, another member of flavonoid family improved the activities of ETC complexes.

In the current study, GM administration led to the mitochondrial membrane potential collapse. Elevated level of ROS can disturb various mitochondrial functions i.e., ΔΨm collapse, mitochondria swelling and dehydrogenase activity reduction (Georgieva et al., 2017). The ΔΨm plays a key role in maintaining the mitochondrial homeostasis (Zorova *et al.,* 2018). It has been stated that the depolarization of ΔΨm linked with the inhibition of ETC as it decreased the proton efflux across mitochondrial inner membrane (Forkink *et al.,*

2014). Our findings apprised that CAS has the potential to reverse GM induced ΔΨm loss via elevating the activities of ETC complexes.

In the present study, GM-induced degeneration of epithelial cells, as well as tubular and glomeruli atrophy and disruption of Bowman's capsule in the renal proximal tubules when compared with the control group, indicating the GM-induced nephrotoxicity. Histomorphological changes could reflect direct impairment to renal tissues. Our results are in line with Fauzi *et al.* (2020), who reported that GM administration caused histomorphological changes in renal tissues such as glomerulus were necrosis and Bowman's space becomes more tenuous. Increased production of free radical mediated by GM can be responsible for the induction of necrosis in the kidney (Kapic *et al.,* 2014). However, CAS co-treated group displayed mild to moderate vacuolation in the epithelium of tubules. CAS co-treatment with GM substantially restored the above-mentioned histopathological damages to renal tissues. It might be due to the antioxidant capability of CAS that significantly decreased oxidative stress followed by a decrease in pathological alterations.

Conclusion: In light of the above results, we concluded that CAS has curative effects against GM-induced toxic effects on functional kidney markers, mitochondrial anti-oxidant status, ETC complexes, TCA cycle, mitochondrial membrane potential and histological damage. CAS has the potential to regulate mitochondrial functions in kidneys via decreasing the levels of ROS and TBARS, as well as it can potentially increase the activities of ETC complexes and TCA cycle enzymes. A diet containing CAS could prove beneficial to mitigate the mitochondrial damage of renal tissues.

Authors' contribution: SS, MUI and HN designed the current investigation. SS, MUI and SM performed the experiments. RZA, SA and RH helped in interpretation and curation of data. SS, MUI and MUI drafted the manuscript. All the authors meticulously read and unanimously approved article.

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