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Protective effect of Boerhaavia diffusa on cisplatin induced nephrotoxicity- Role of apoptosis and oxidative stress

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Abstract:

Objective: To study the preventive effect of Boerhaaviadiffusa (BD) extract on cisplatin induced nephrotoxicity in animal model.

Materials and Methods: The wistar rats were divided into four groups for different treatment allocation. The rats were given rodent chow,Boerhaaviadiffusa (BD) extract (200 mg/Kg body weight), Cisplatin injection (7mg/kg BW) and BD with cisplatin injection for 15 days. Subsequently, blood samples and renal tissues were collected before sacrificing animals. The samples were used for routine biochemical analysis and tissues were used for gene expression, immunohistochemistry and histopathology studies.

Results: Cisplatin-injected rats significantly declined renal function, induced oxidative stressand apoptosis. The apoptotic gene such as bax and caspase-3 were significantly upregulated and Bcl-2 was downregulated in cisplatin-treated rats when compared to control rats. Pre-treatment with BD extract improved renal function by decreasing oxidative stress and improved anti-oxidant enzymes such as glutathione peroxidase (GPx) and Catalase. Further BD extract decreased perivascular inflammation, necrosis and nuclear dropout in

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histopathological analysis.BD extract also increased the expression of anti-apoptotic genes and decreased apoptotic gene expression in cisplatin-treated rats.

Conclusions: Boerhaavia diffusais beneficial in alleviating the nephrotoxicity induced by cisplatin. The effect is mediated mainly by decreasing oxidative stress, inflammation and its anti-apoptotic effects.

Keywords: Cisplatin, nephrotoxicity, renal function, Boerhaaviadiffusa, apoptosis, oxidative stress

1. Introduction

Cisplatin (Cis-diamminedichloroplatinum) is a chemotherapeutic agent with potent anti-tumor activity used to treat various types of cancers(1).It accumulates in the proximal tubules of kidney and induces acute kidney injury (AKI) which is the most frequent complication seen in 20-40% of the populations of cisplatin-treated patients(2).It remains the standard drug for various types of cancer due to the limited availability of chemotherapeutic agents with similar effects. It is evident from various studies, cisplatin is transported via cationic transporter-2 in the renal tubular cells and accumulates in the kidney which is associated with nephrotoxicity and acute kidney failure (AKI) (3). Though reno-protective interventions are given during treatment still persistent renal damage in the patients.

The mechanism of cisplatin-induced renal damage is due to mitochondrial dysfunction and reactive oxygen species (ROS) (4). It has been shown that increased oxidative stress, apoptosis and inflammation by cisplatin in the proximal tubule leads to nephrotoxicity. The increasedproduction of ROS and inflammatory mediators reduces antioxidant enzymes which induces apoptosis in the renal cell(5). Increased ROS also increases the levels of malondialdehyde (MDA), the lipid peroxidation product involved in cell damage.MDA is generated from the peroxidation of polyunsaturated fatty acids of the membrane and reacts with protein, lipids and DNA which leads to cell damage (6). Therefore, numerous natural plant products with phytochemical activities have been used for its nephroprotective effect(7). *Boerhaaviadiffusa* L. (Nyctaginaceae) is aherbaceous plant of the family. It is known as "*punarnava"* in Sanskrit, which means that it 'renews the body'. It was said that based on ayurveda it removes waste from the body, reduces edema and improves renal function. It provides relief from joint pains and inflammation. In India it used as green vegetables in various part of the state. It is evident from the literature that

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Boerhaaviadiffusa has phytochemical compound such as polyphenol, tannins, flavonoids, steroids, saponin and terpins(8). In vivo,studies have shownthe protective effect of BD in nephrotoxicity induced by gentamycin, mercuric chloride (9)and cardiotoxicity by arsenic trioxide(10). Pre-treated with aqueous ethanolic extracts of BD improved the liver damage inacetaminophen-induced toxicity in animal models(11). Studies have shown the efficacy of roots of BD on nephrotoxicity, but limited studies have shown the effect of BD leaves on renal function. However, the mechanism of action of leaves of BD on nephrotoxicity has not been evaluated. Thus,the present study is designed to explore the effect of Boerhaaviadiffusaon cisplatin induced nephrotoxicity in animal model.

Materials and Methods

2.1. Reagents

The chemicals were purchased from SRL, Hi-media and Sigma Aldrich (USA), SRL (India). The primary antibodies for bax, caspase-3 and bcl-2 and ImmPRESS® UniversalPLUS Polymer Kit were procured form Abclonal (everon life science, USA). Primers and SYBER green master mix were purchased from Sigma,USA

Gene	Primer
Bax	Forward 5’-AGGTCTTTTTCCGAGTGGCAGC-3’
	Reverse 5’-CCGGAGGAAGTCCAATGTCC-3’
Bcl-2	Forward 5’- GGAGGGCACTTCCTGAG -3’
	Reverse 5’ - GCCTGGCATCACGACT-3’
Caspase 3	Forward 5’- CCTCAGAGAGACATTCATGG-3’
	Reverse 5’ - GCAGTAGTCGCCTCTGAAGA-3’
GAPDH	Forward 5’- CGGCCGAGGGCCCACTAAAG-3’
	Reverse 5’ - TGCTCAGTGTTGGGGGCTGAGT-3’

Preparation of Boerhaaviadiffusa extract

Fresh leaves of Boerhaaviadiffusawere purchased from the local market. The leaves were cleaned, washed and ground into a paste. The paste was mixed with 80% methanol and kept in a shaker at room temperature. The solvent was removed and evaporated in a rotary vacuum evaporator. The residue was lyophilized and stored at 40^{o} C for further use.

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Animals

Animals and interventions

The study was done in the Department of Biochemistry, AIIMS, Jodhpur after obtaining Institutional animal ethical committee approval (JNVU/IAEC/2020/14). Four-month-old male Wistar rats were housed in plastic polycarbonate cages. The animals were acclimatization for one week with rodent chow and water available ad libitum. After acclimatization for one week, the animals were randomized into four groups based on their body weight. The cisplatin grouprats wereinjected with cisplatin (7 mg/kg, i.p) on day 11 to establish the acute kidney injury and continued for five days. The extracts of Boerhaaviadiffusa (BD)were administered by oral gavage at a dose of 200 mg/kg body weight for the respective groups from day one till the end of the experiment. Rats in group 1 were given only rodent chow for 15 days. Rats in group 2 were given BD (200mg/kg body weight) and rodent chow. Rats in group 4 were given BD (200mg/kg body weight) for 15 daysand cisplatin7 mg/kgi.p. At the end of the experiment, blood samples were collected. The samples were separated and stored at -40^{o} for further analysis. Biochemical parameters and oxidative stress markers were analyzed.The renal tissue samples were stored in RNAlater®for the expression studies. The part of the renal tissues is stored in phosphate-buffered formalin for histopathological and immunohistochemical analysis.

Estimation of biochemical parameters and anti-oxidant status

Blood glucose, lipid profile, kidney function and liver function were assessedin Beckman coulter Clinical Chemistry Analyser USA). Whole blood-reducedglutathione, Glutathione peroxidase and catalase were estimated by the method ofBeutler,(1963), Wendel (1981)and Aebi (1984).et al respectively.

Histopathology and immunohistochemistry assay

The renal tissueswerecut and fixed in 10% neutral buffered formalin containing 3.7% formaldehyde, 33mM NaH_2PO_4 and 46mM anhydrous Na_2HPO_4 for 24 hours. The tissues were processed in an automated histopathology tissue processor embedded in molten paraffin. The kidney sections were sectioned and stained with Ehrlich'shematoxylin and 1% eosin solution. solution. The sections were finally mounted using the permanent mounting medium Distrene Plasticiser Xylene (DPX). The sections were scored for 0 to 1 as per the necrosis.

The immunohistochemical analysis was done after antigen retrieval and rehydration for 10 mins. The sections were treated with a blocking buffer. After blocking the sections were

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incubated with different primary antibodies (bcl-2, anti-bax and anti-cas-3) and secondary anti-body. It was washed with buffer and stained with DAPI and rinsed in PBS and mounted with DPX. The sections were visualized under light microscopy.

RNA isolation and cDNA synthesis

The total RNA was isolated from the kidneyusing kit as per the manufacturer protocol and the concentration was quantified by Nanodrop (Thermoscientific, USA). The RNA was converted to cDNA by using a High capacity cDNA reverse transcription Kit (Thermo Fischer, USA).The reactions were prepared as per the manufactured protocol and run the reverse transcriptase cycle in quantitative PCR. The bcl2, Bax and caspase gene expression were studied using specific primers by real-time PCR. The PCR reactions mixture was prepared and amplification was carried at 95 °C for 10 min for initial denaturation, 95 °C for 15s for denaturation and 60 °C for 60s for extension. The $\triangle$Ct value and $2^{-\triangle\triangle Ct}$ value was calculated to determine the fold differences in gene expression. The Ct of the gene of interest was normalized with GAPDH and mRNA levels and compared with the control. The products were analyzed using a melting curve analysis.

Statistical analysis

All of the data were expressed as mean ± SD. The normality of the test and statistical analysis was done using SPSS 22.0 software. The differences between the groups were analyzed by One-way ANOVA andpost hoc Tukey test. $P < 0.05$ was considered statistically significant.

Results:

Table 1: Effect of Boerhaaviadiffusaon food intake, body weight and relative organ weights in cisplatin treated rat

Parameter	Chow	Chow + BD	Cisplatin	Cisplatin + BD
Initial Body weight (g)	186 ± 6.5	195.5 ± 12.9	196.5 ± 8.3	187.7 ± 6.5
Final Body weight (g)	253.8 ± 14.8	257.2 ± 1.7	174.8 ± 3.1	251.8 ± 19.2

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Relative weight of Kidney tissue (g/100g)	0.76 ± 0.1	0.74 ± 0.1	0.64 ± 0.2[a]	0.71 ± 0.1[b]

Data represent the mean ± SD. (n=10) P<0.05, ^{a} *in comparison with control,* ^{b} *in comparison with Cisplatin group. Differences between the groups were analyzed using one way ANOVA with Tukey post-Hoc method.*

Table 2: Effect of Boerhaaviadiffusa on glucose and lipid profile in cisplatin induced nephrotoxicity

Parameters	**Chow**	**Chow + BD**	**Cisplatin**	**Cisplatin + BD**
Fasting glucose (mg/dl)	74.7 ± 2.8	76.8 ± 5.0	79.6 ± 5.5	78.2 ± 5.9
TC (mg/dl)	48 ± 1.9	46.5 ± 3.2	50.7 ± 3.5	48.5 ± 2.1
TAG (mg/dl)	80.5 ± 2.3	76.3 ± 2.3	83.2 ± 6.0	78.1 ± 2.6
HDL-c (mg/dl)	30.7 ± 1.3	30.3 ± 1.3	27.3 ± 1.1	28.7 ± 1.3
VLDL-c (mg/dl)	16.1 ± 0.5	15.3 ± 0.5	16.6 ± 1.2	15.6 ± 0.5

Data represent the mean ± SD. (n=10) P<0.05 Differences between the groups were analysed using one way ANOVA with Tukey post-Hoc method.

TC *- Total cholesterol,* ***TAG*** *- Triacylglycerol,* ***HDL-c*** *- High-density lipoprotein cholesterol,* ***VLDL-c*** *- Very-low-density lipoprotein cholesterol*

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Table 1 showed the body weight and relative weight of renal tissues in all the rats. There was no significant difference between the initial and final body weight of the rats. The relative kidney weight is significantly decreased in cisplatin-treated rats. The renal tissue weight was improved when treated with BD extract.There was no significant difference between blood glucose and lipid profile in all the groups (Table 2).

Table 3: Effect of Boerhaaviadiffusa on renal and liver profile in cisplatin-induced nephrotoxicity

Parameters	Chow	Chow + BD	Cisplatin	Cisplatin + BD
Urea (mg/dL)	25.5 ± 1.8	28 ± 2.9	230.3 ± 16.2 [a]	38.67 ± 1.8 [b]
Creatinine (mg/dL)	0.49 ± 0.1	0.5 ± 0.2	2.23 ± 0.30 [a]	0.45 ± 0.02 [b]
AST (IU/L)	84.6 ± 4.8	85.14 ± 5.3	88.1 ± 4.25	87.5 ± 1.6
ALT (IU/L)	42 ± 1.8	39.2 ± 0.9	48.5 ± 1.31	47.4 ± 1.6
Total Protein (mg/dL)	6.6 ± 0.1	6.7 ± 0.09	6.9 ± 0.06	6.8 ± 0.08
Albumin (mg/dL)	3.5 ± 0.1	3.0 ± 0.05	2.8 ± 0.07	2.9 ± 0.02
Uric acid (mg/dL)	0.55 ± 0.01	0.53 ± 0.06	0.67 ± 0.11	0.65 ± 0.11

Data represent the mean ± SD. (n=10) P<0.05, [a] in comparison with control, [b] in comparison with cisplatin treated rats. Differences between the groups were analyzed using one way ANOVA with Tukey post-Hoc method. ***AST****– Aspartate aminotransferase,* ***ALT*** *– Alanine aminotransferase*

To evaluate the effect of Boerhaaviadiffusa on preventing kidney dysfunction induced by cisplatin, the serum urea, serum creatinine and serum uric acid levels were recorded in all

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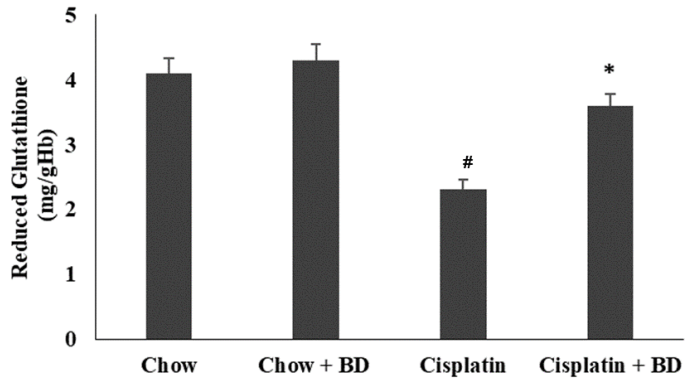
four groups of study (Table 3). Cisplatin administration (7mg/kg) resulted in a significant elevation of serum urea and creatinine as compared to the chow-fed normal control group. This observation reveals the high degree of renal dysfunction caused due to cisplatin administration which was significantly reduced in the cisplatin and Boerhaaviadiffusa administered group denoting the nephroprotective effect of this herb. The liver function test was evaluated by recording the serum concentrations of AST, ALT, total protein and albumin. No significant change was observed in all the parameters of all the four groups.

A)



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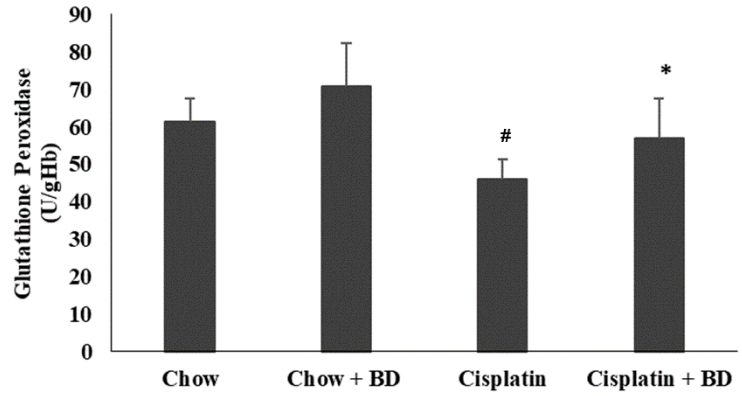

C)

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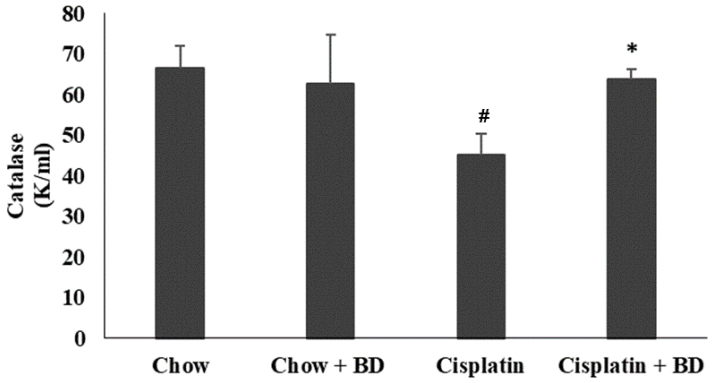

Figure 1*: Effect of Boerhaaviadiffusa on blood antioxidant status in cisplatin induced nephrotoxicity. A) Reduced Gluthione, B) Glutathione Peroxidase, C) Catalase. Values are expressed as mean ± S.D. n=6/group. Differences between the groups were analyzed by ANOVA. #p<0.05 in comparison to Cisplatin group *****p<0.05 in comparison to Cisplatin group*

Figure1 shows the effect of Boerhaaviadiffusa on blood antioxidant status in cisplatin-induced nephrotoxicity. The anti-oxidant enzymes such as glutathione peroxidase and catalase were significantly increased in the group treated with cisplatin and BD and decreased Malondialdehyde level. Thus, improved anti-oxidant status and decreased oxidative stress show the anti-oxidant properties of BD extract.

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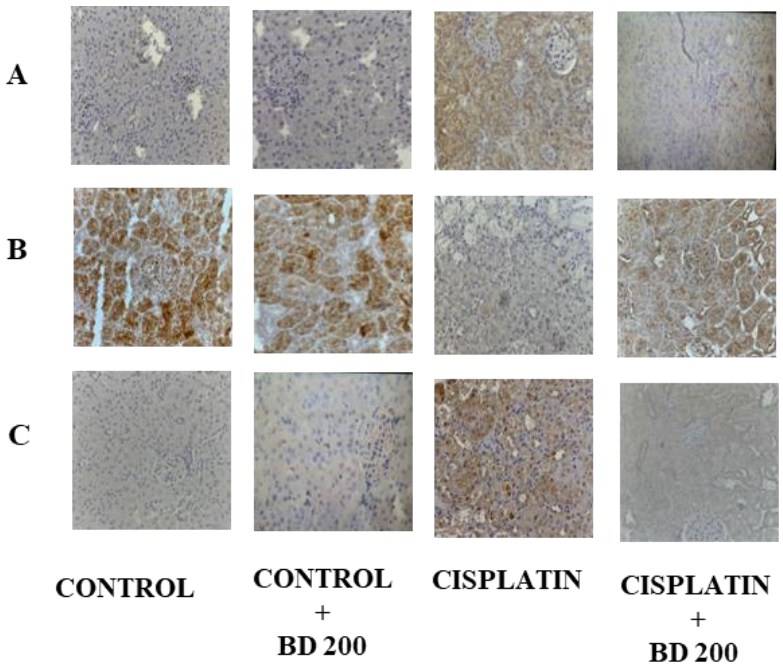

Figure 2: *Representative images for the immunohistochemical detection of apoptosis in renal tissues (X 400). Image A represents Bax, Image B represents Bcl-2, Image C represents caspase -3. BD extract (200mg/kg BW) treatment upregulated the Bcl-2 gene expression and downregulated the Bax and caspase-3 in cisplatin treated group. BD: Boerhaaviadiffusa, BW: Body weight.*

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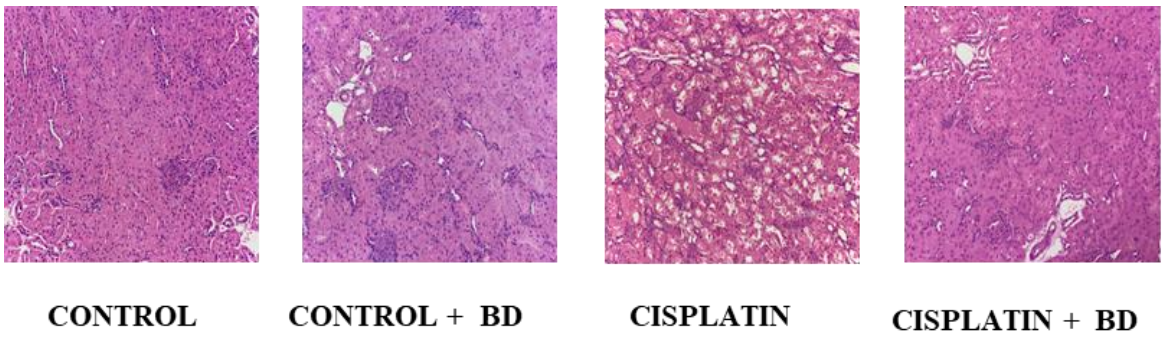

Figure 3: *Effect of Boerhaaviadiffusa on histopathological changes in kidney in the experimental groups: Rats were treated with cisplatin, BD and cisplatin with BD for 15 days. Images were captured using phase contrast microscope under 20X resolution. Cisplatin treated kidney showed perivascular inflammation, cast formation, cytoplasmic vacuolizationof proximal tubules and nuclear dropout, Interstitial congestion and apical blebbing in epithelial lining. These changes were significantly reduced by BD extract treatment. BD: Boerhaaviadiffusa.*

Immunohistochemical analysis of the kidney showed the downregulation of Bcl-2 and upregulation of Bax and caspase-3 (Figure 2). Figure 3 showed the results of the histopathological analysis of the kidney. Renal tissues of cisplatin-injected rats showed infiltration of inflammatory cells, perivascular inflammation, necrosis and nuclear dropout. Inflammation was absent in the kidney tissue of the cisplatin + BD extract-treated group. This further confirms the anti-inflammatory effect of BD extract.

A)

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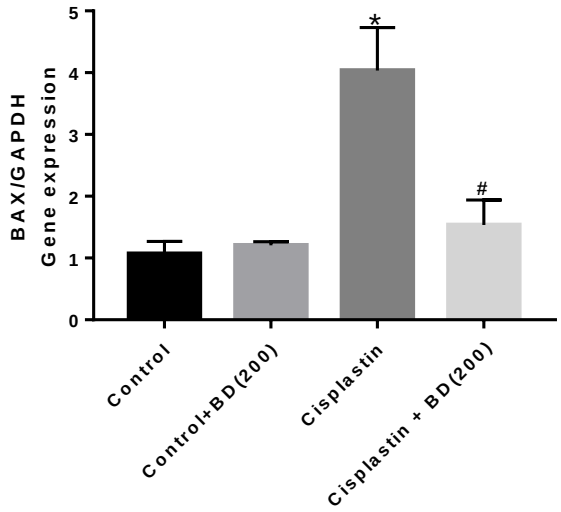
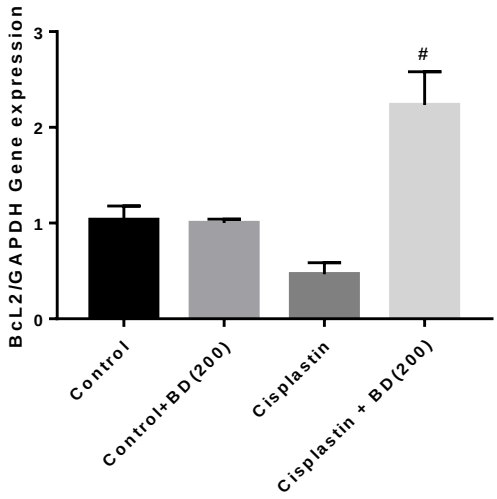


B)



C)

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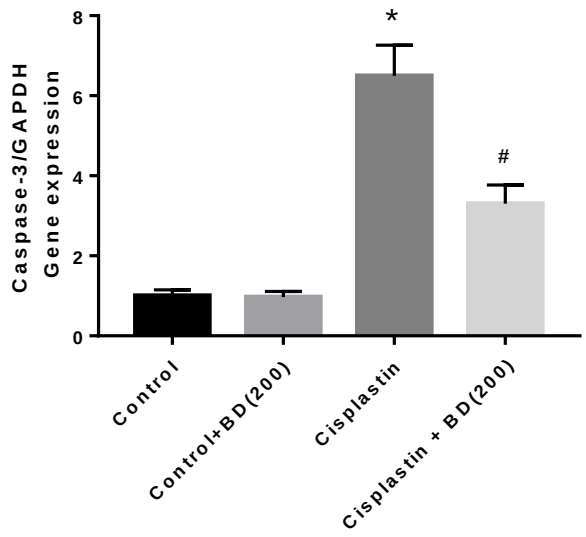

Figure 4: Effect of Boerhaaviadiffusa on gene expression of A) Bax, B) Bcl2 and C) casp-3 in the kidney of cisplatin induced nephrotoxicity

Cisplatin injected showed significant upregulation of bax and caspase-3 with a fold change of 4.5 and 6.5 respectively. Further, it downregulated the anti-apoptotic gene Bcl-2 (0.5). Pre-treatment with BD extract increased the expression of the anti-apoptotic gene (bcl-2) and decreased apoptotic gene expressionbax and caspase-3 significantly.

Discussion

The major limitation of cisplatin-based chemotherapy is nephrotoxicity in cancer patients. It has been shown that cisplatin is taken up by the kidney through organic cation transporter-2 and accumulated in renal tubular cells(12). This leads to toxicity in renal tubules and nephronswhichis associated with acute kidney injury and renal failure. The mechanism of toxicity is multifacetedand associated with oxidative stress, inflammation, apoptosis and mitochondrial dysfunctions (Pabla and Dong, 2008). The toxicity mechanism is due to the generation of free radicals such as reactive oxygen and superoxide anion radical and decreased anti-oxidant status such as glutathione reductase, catalase, glutathione peroxidase and superoxide dismutase. The accumulation of free radicals leads to oxidative stress and the release of inflammatory mediators such as TNF-alpha, IL-6 and hs-CRP in renal cells(14). The oxidative stress, inflammationand apoptosis cause nephrotoxicity in cisplatin-

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treated rats (15). It will be beneficial for cancer patients if any plant compound is used as an adjuvant along with cisplatin therapy to prevent nephrotoxicity. Studies have shown that *Boerhaaviadiffusa (BD)*has a nephroprotective role and is beneficial in preventing renal damage.

In the present study, Cisplatin induces the nephrotoxicity in kidney by increasing blood urea nitrogen (BUN) and creatinine levels. Supplementation with Boerhaaviadiffusaprotected the kidney from the toxic side effectsof cisplatin in the rats. Our data indicate that the pretreatment of ratswith Boerhaaviadiffusasignificantly improved renal functions by decreasing the BUN and creatinine levels when compared to control rats. This was consistent with the previous report (16).

The cisplatin treatment also significantly increased malondialdehydewhich in turn decreased anti-oxidant enzymes such asGlutathione peroxidase and catalase. The administration of BD extracts improved the anti-oxidant enzymes and decreased oxidative stress. Similarly, the administration of aqueous plant extracts improved the renal function and anti-oxidant status in gentamycin-induced toxicity in rats(17). Another study has shown that administration of Aqueous root extract of B. diffusa (200-400 mg/kg/day)decreased renal toxicityin acetaminophen treated rats. Karwasra et al have shown that administration of Boerhaaviadiffusa root extract in cisplatin-treated rat suppressed renal MDA and improved anti-oxidant defense(18). The same author also highlighted the anti-inflammatory role of BD extracts by decreasing the circulating levels of inflammatory markers such as TNF-a, IL-1 and IL-6(18).

Several reports have shown that cisplatin treatment activates the apoptotic pathway via its interactions with DNA and oxidative stress(19). It is known that activation of Bax and caspase-9 induces apoptosis in the kidney. Studies have shown that caspase activation and cisplatin-induced apoptosis. Cispatin may causethe mitochondrial release of cytochrome c and caspase-9 andcaspase-3 activation. To observe the role of cisplatin-mediated apoptosis, the apoptotic markers such as cas-9, bcl-2 and Bax were examined in the renal tissues of rat cisplatin-treated rats. Cisplatin-induced apoptotic cell death in renal tissue as evidenced by increased caspase 3, bcl-2 and decreased Bax expression. It is consistent with a previous report where it was shown that increased caspases expression and decreased Bcl-2 expression indicated apoptosis in cisplatin-treated rats. The result of the present study depicts similar apoptotic damage in renal tissues of cisplatin-treatedrats as shown in H &E staining. Renal tissues of cisplatin-injected rats showed infiltration of inflammatory cells,

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perivascular inflammation, necrosis and nuclear dropout in immunohistochemical analysis.Thecurrent study demonstrated that BD extracts downregulated the casp-3, bcl-2 expression and upregulated expression and apoptotic damage of the kidney. The Renal dysfunction was attenuated by treatment with BD extracts in line with improved oxidative stress and apoptotic damage. This could be due to the anti-oxidant and anti-inflammatory properties of the plant extracts. Studies have shown that it has hypoglycemic, anti-inflammatory, diuretic, hepatoprotective, antimicrobial, antioxidant and antifibrinolytic(20).These activities have been attributed to its phytochemical activities such as glycosides, steroids, flavonoids, alkaloids, phenolic glycosides.A previous report has shown that the methanolic extract of BD has the highest polyphenol and flavonoid content. Another study has shown a similar trend in the total phenolic (24.5 mg QE/g) and flavonoid contents (79.8 mgGAE/g) in the methanol extract of B. diffusa(21). Sinan et al LC/MS/High-Resolution Mass Spectrometry (HRMS) analysis has shown that BD extracts contain a significant amount of flavonoids such as kaempferol 3-O-glucoside, Quercetin, ferulic acid (22). The beneficial effect exerted by BD extract could also be attributed to the presence of these phenolicacids in the extract. Flavonoids, especially quercetin, have been studied in various diseases due to their potent antioxidant properties and anti-inflammatory properties.

Conclusion

Boerhaaviadiffusahas many phytochemical constituents which are beneficial in alleviating the nephrotoxicity induced by cisplatin. The effect is mediated mainly by decreasing oxidative stress, inflammation and its anti-apoptotic effects.

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Ethical Approval. The present study was approved by an animal ethics committee, JNVU, Jodhpur.

Conflict of interest:The authors report that there is no conflict of interest.

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