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## Ameliorating effect of *Berberis lycium* root bark extracts against cisplatin-induced nephropathy in rat

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### Abstract

The aim of this study was to investigate the sub-acute nephroprotective effect of *Berberis lycium* root bark extracts against cisplatin-induced nephrotoxicity. Aqueous and methanol extracts (200 and 400 mg/kg) were co-administered with cisplatin (4 mg/kg) for 19 days. Results showed that cisplatin significantly ( $p < 0.05$ ) raised the serum creatinine, uric acid, urea level as well as tissue MDA level as compared to control group. It decreased GST activity, tGSH and catalase activity in rats. Both extracts significantly reduced the serum creatinine, urea and uric acid levels. Moreover, GST, CAT activity and tGSH content were significantly ( $p < 0.05$ ) increased and MDA level was decreased. Histopathological examination showed that both extracts efficiently reversed the morphological changes and damage induced by cisplatin. It is concluded that both extracts of *B. lycium* root bark possess nephroprotective effect against cisplatin-induced nephrotoxicity.

### Introduction

Nephropathy is a major concern of cisplatin use. Dose-dependent nephropathy of cisplatin is well-documented and woefully limits its use despite its efficacy in reducing tumor burdens. Nephrotoxicity develops in approximately 28-36% of patients receiving cisplatin (Miller et al., 2010) and results in irreversible damage and effects different segments of the nephron, primarily proximal tubules in the S3 segment. Acute and chronic glomerular toxicities occur leading to decreased glomerular filtration rate as a result of decreased glomerular blood flow (Gaedeke et al., 1996). The primary target of cisplatin is mitochondria which cause induction of oxidative stress leading to the loss of mitochondrial protein and sulfhydryl group, inhibition of calcium uptake and reduction in membrane potential of mitochondria (Saad et al., 2004). Due to increase in metabolic activity and malfunction of mitochondria, cancer cells exhibit the high level of reactive oxygen species (ROS). It has been proven that tumor necrosis factor  $\alpha$ ,

interleukin-1  $\beta$  and ROS play role in nephropathy (Zunino et al., 1989). Various studies have suggested that suppressed renal GSH levels and anti-oxidants enzyme activity and enhanced lipid peroxidation leads to free radical mediated oxidative damage and nephropathy in cisplatin-induced damage (Husain et al., 1996). ROS causes DNA damage and activates P53 gene. ROS production leads to the sequel of activation of signaling pathways and protein kinases which play role in phosphorylation and activation of p53 (Pinzani et al., 1994). Of many pathways proposed responsible for cisplatin-induced nephropathy oxidative stress is the most frequent and prevalent which acts as a trigger and as a result lead to renal damage and renal cell death (dos Santos et al., 2012).

Medicinal plants *Orthosiphon stamineus* (Madhukar et al., 2009), *Ficus religiosa* (Hashmi et al., 2013), *Carica papaya* (Talete et al., 2012), *Petalium murex* (Shelke et al., 2009), *Digera muricata* (Khan., 2009) and *Rhazya strictas* Decne (Ali, et al 2014) showed nephroprotective activity.



This study was done to evaluate and assess the nephro-protective effects of *Berberis lycium* root bark extracts on cisplatin-induced nephrotoxicity.

## Materials and Methods

### Chemicals and reagents

All the chemicals and reagents used in the study were of analytical grade obtained from Sigma-Aldrich Co. Ltd., USA, Merck, Germany and mdi Europa GmbH, Germany.

### Animals

Female Sprague Dawley rats weighing 100-120 g were used in the study. The rats were obtained from Punjab University, University College of Pharmacy (PUCP), Allama Iqbal Campus, Lahore, Pakistan. Rats were housed in steel cages in the animal house. The animals were kept in a separate room under proper light and ventilation. The rats were acclimatized to the laboratory conditions for one week. Rats were kept under controlled conditions of 25°C ± 2 temperature, 60% relative humidity, 12/12 hours of dark/night cycle. Rats were provided with standard diet and water *ad libitum*.

### Preparation of extracts

*B. lycium* root bark was collected from Murree Valley, Pakistan. The plant was authenticated from the Sultan Herbarium of Botany, Government College University, Lahore and voucher number, (GC.Herb.Bot.2902) was issued. Barks were dried under shade and grinded. Maceration was done by soaking 500 g of powder in 1000 mL of methanol. The powder was macerated for 4 days at room temperature with constant stirring. The mixture filtered to get extract and concentrated by a rotary evaporator at 45°C. The condensed extract was placed in an oven to get dried. The same procedure was followed for aqueous extract preparation by using water instead of methanol.

### Experimental design

For the sub-acute study, a total of 60 rats were used. Rats were divided into ten groups comprising of 6 rats in each as follows:

Group I: Normal control, water daily for 19 days; Group II: Toxic control, water daily for 19 days and cisplatin (4 mg/kg single i.p) was administered on day 14; Group III: aqueous extract (200 mg/kg) daily for 19 days by oral gavage; Group IV: aqueous extract (400 mg/kg) daily for 19 days by oral gavage; Group V: aqueous extract (200 mg/kg) daily for 19 days and cisplatin (4 mg/kg single i.p) on 14<sup>th</sup> day; Group VI: aqueous extract (400 mg/kg) daily for 19 days and cisplatin (4 mg/kg single i.p) was administered on day 14; Group VII: methanol extract (200 mg/kg) daily for

19 days by oral gavage; Group VIII: methanol extract (400 mg/kg) daily for 19 days by oral gavage; Group IX: methanol extract (200 mg/kg) daily for 19 days and cisplatin (4 mg/kg single i.p) was administered on day 14; Group X: methanol extract (400 mg/kg) daily for 19 days and cisplatin (4 mg/kg single i.p) was administered on 14<sup>th</sup> day.

On day 20, blood was collected through cardiac puncture under anesthesia for biochemical determinations. The kidneys were excised out and promptly rinsed with chilled normal saline to wash excess blood.

### Biochemical analysis

Separation of blood serum was done by centrifugation at 4,000 rpm for 10-12 min and further used for the assessment of renal biomarkers. The kidneys were blotted dry and weighed. Kidneys were pooled in 0.02 M phosphate buffer (pH 7.4) finely chopped with scissors and homogenized with a homogenizer. The kidney homogenate was centrifuged at 12,500 × g at 4°C for 15 min in a refrigerated centrifuge. The resultant supernatant postmitochondrial supernatant was transferred in Eppendorf tubes and stored at -80°C until further study.

### Estimation of creatinine

Rat plasma creatinine was estimated calorimetrically at 505 nm by a UV-2500 Pharmaspec, Shimadzu spectrophotometer using a creatinine standard from mdi Europa GmbH, Germany Diagnostic Kit.

### Estimation of uric acid

Uric acid in serum was estimated by enzymatic endpoint method at 520 nm by a UV-2500 Pharmaspec, Shimadzu spectrophotometer using mdiEuropa GmbH, Germany Diagnostic kit.

### Determination of urea

The enzymatic kinetic method was used for estimation of serum urea level at 600 nm by a UV-2500 Pharmaspec, Shimadzu spectrophotometer using a Human Gesellschaft fur Biochemical Germany Diagnostic Kit.

### Protein quantification

Protein estimation of kidney tissues was carried (Lowry et al., 1951) using bovine albumin as a standard.

### Glutathione-S-transferase (GST) assay

GST activity was measured (Habig et al., 1974) with slight modification. To all test tubes 100 mM phosphate buffer pH 6.5, 30 mM GSH and 30 mM CDNB were added. The reaction was initiated by addition of PMS. Blank test tube contained phosphate buffer pH 6.5 instead of PMS and vortexed. Change in the absorbance was measured at 340 nm on a spectrophotometer. After auto zeroing, reading was measured immediately at 0 min and then after every 1 min, up to 3 min. GST

activity was expressed in  $\mu\text{mol}/\text{minute}/\text{mg}$  protein.

#### Total glutathione (tGSH) analysis

Total GSH content in samples was determined (Sedlak et al., 1968) with slight modification. Kidneys were homogenized in 67 mM phosphate buffer (pH 7.4). Immediately 25% trichloroacetic acid was added to precipitate the homogenate, which was then centrifuged at 4,200 rpm at 4°C for 40 min. To supernatant 200 mM Tris HCl buffer containing 0.2 M EDTA (pH 7.5), 10 mM DTNB and 7.9 mL of methanol were added respectively. Blank contains 67 mM phosphate buffer (pH 7.4) instead of homogenate. Test tubes were vortexed and then incubated for 30 min at 37°C in an oven. After incubation absorbance of each was read at 412 nm on a spectrophotometer.

#### MDA analysis

MDA was measured (Ohkawa et al., 1979) using thiobarbituric assay with slight modification. Kidney samples were homogenized in 1.2% KCl to make homogenate (10%). To homogenate 8% sodium lauryl sulfate, 20% acetic acid, 0.8% TBA and DW were added. Test tubes were incubated for 1 hour at 98°C in an oven, cooled at room temperature. After cooling the tubes butanol: pyridine (15:1) were added, vortexed for 1 min and centrifuged for 30 min at 4,000 rpm. The supernatant was carefully transferred to the cuvette and the absorbance was read at 532. MDA (mmole/mg tissue) in the sample was interpolated from the standard curve of MDA.

#### Catalase analysis

Catalase in the homogenate was measured (Sinha et al., 1972) with slight modifications. The reaction mixture consists of tissue homogenate, phosphate buffer (0.01 M, pH 7) and 0.2 M  $\text{H}_2\text{O}_2$ . The reaction mixture was

then immediately added to dichromate/acetic acid. Test tubes were heated in a boiling water bath for 10 min to give permanent green color. Test tubes were cooled at room temperature and absorbance was read at 570 nm. Catalase activity in the homogenate was interpolated from the standard curve.

#### Histological slides

Sections of the kidneys were fixed in 10% formalin, embedded in paraffin and cut at 5  $\mu\text{m}$  thickness. Kidney sections were then processed and stained with hematoxylin and eosin dye for histological evaluation of renal injury, according to standard protocols. All sections were examined under light microscope. The slides were coded to prevent observer bias during evaluation. Criteria were set to score specimen. The criteria for kidney damage included degeneration in renal tubules, sloughing in renal tubular cells, cellular swelling, peritubular congestion and hydropic degeneration. Each specimen was scored using a scale from none to severe.

#### Statistical analysis

The analysis was done by using paired t-test and one-way ANOVA followed by post-hoc Dunnett's test to make the comparison between control and cisplatin-treated group and control group and different groups respectively. All data are expressed mean standard deviation (Mean  $\pm$  SD).  $p < 0.05$  was considered statistically significant.

## Results

Results showed a statistically significant difference in the level of renal biomarkers in control and experiment groups among the groups (Table I). The levels of serum

Table I

### Effect *B. lycium* extracts on renal biomarkers in cisplatin-induced nephrotoxicity in rat

Groups	Creatinine (mg/dL)	Uric Acid (mg/dL)	Urea (mg/dL)
Control	0.6 $\pm$ 0.0	1.5 $\pm$ 0.0	21 $\pm$ 3.2
Cisplatin (4 mg/kg)	2.2 $\pm$ 0.2 <sup>a</sup>	4.5 $\pm$ 0.1 <sup>a</sup>	61.5 $\pm$ 2.2 <sup>a</sup>
Aqueous extract (200 mg/kg)	0.6 $\pm$ 0.0	2.3 $\pm$ 0.4	16 $\pm$ 1.0
Aqueous extract (400 mg/kg)	0.6 $\pm$ 0.0	2.3 $\pm$ 0.4	18.4 $\pm$ 1.2
Methanol extract (200 mg/kg)	0.5 $\pm$ 0.0	1.8 $\pm$ 0.2	19 $\pm$ 1.5
Methanol extract (400 mg/kg)	0.5 $\pm$ 0.0	1.9 $\pm$ 0.1	21 $\pm$ 1.5
Cisplatin (4 mg/kg) + Aqueous extract (200 mg/kg)	0.6 $\pm$ 0.0 <sup>b</sup>	2.4 $\pm$ 0.0 <sup>b</sup>	18.1 $\pm$ 1.0 <sup>b</sup>
Cisplatin (4 mg/kg) + Aqueous extract (400 mg/kg)	0.6 $\pm$ 0.0 <sup>b</sup>	2.2 $\pm$ 0.4 <sup>b</sup>	26.7 $\pm$ 0.5 <sup>b</sup>
Cisplatin (4 mg/kg) + Methanol extract (200 mg/kg)	0.6 $\pm$ 0.0 <sup>b</sup>	2.2 $\pm$ 0.1 <sup>b</sup>	35 $\pm$ 1.2 <sup>b</sup>
Cisplatin (4 mg/kg) + Methanol extract (400 mg/kg)	0.5 $\pm$ 0.0 <sup>b</sup>	1.7 $\pm$ 0.2 <sup>b</sup>	25 $\pm$ 2.1 <sup>b</sup>

Values are expressed as the mean  $\pm$  SD. n = 6, <sup>a</sup> $p < 0.05$  represents statistical different from control; <sup>b</sup> $p < 0.05$  represents statistical different from CIS control group. Data was analyzed by using one-way ANOVA followed by Dunnett's test

**Table II**  
**Effect of *B. lycium* extract on protein content, GSH, GST, MDA and CAT in cisplatin-induced rat**

Groups	Protein content mg/mL	t GSH mmole/mg	GST $\mu\text{mol}/\text{min}/\text{mg}$ protein	MDA $\mu\text{mol}/\text{mg}$ tissue	CAT $\mu\text{mol}/\text{min}/\text{mg}$ protein
Control	64 $\pm$ 0.1	0.07 $\pm$ 0.03	0.03 $\pm$ 0.00	46.7 $\pm$ 1.7	86 $\pm$ 2.5
Cisplatin (4 mg/kg)	21 $\pm$ 1.0 <sup>a</sup>	0.02 $\pm$ 0.00 <sup>a</sup>	0.02 $\pm$ 0.00 <sup>a</sup>	115.6 $\pm$ 3.1 <sup>a</sup>	41.9 $\pm$ 2.6 <sup>a</sup>
Aqueous extract (200 mg/kg)	66 $\pm$ 1.5	0.09 $\pm$ 0.01 <sup>b</sup>	0.03 $\pm$ 0.00	46.0 $\pm$ 5.0	91.7 $\pm$ 5.1 <sup>b</sup>
Aqueous extract (400 mg/kg)	70.4 $\pm$ 4.4	0.09 $\pm$ 0.00 <sup>b</sup>	0.03 $\pm$ 0.00	45.4 $\pm$ 4.1	109 $\pm$ 2.5 <sup>b</sup>
Methanol extract (200 mg/kg)	66.4 $\pm$ 1.5 <sup>b</sup>	0.09 $\pm$ 0.00 <sup>b</sup>	0.03 $\pm$ 0.00	44.9 $\pm$ 2.6	117 $\pm$ 2.0 <sup>b</sup>
Methanol extract (400 mg/kg)	72.3 $\pm$ 3.3 <sup>b</sup>	0.09 $\pm$ 0.00 <sup>b</sup>	0.04 $\pm$ 0.01 <sup>b</sup>	41.9 $\pm$ 3.5	117 $\pm$ 3.0 <sup>b</sup>
Cisplatin (4 mg/kg) + Aqueous extract (200 mg/kg)	61.4 $\pm$ 1.2 <sup>b</sup>	0.06 $\pm$ 0.00 <sup>b</sup>	0.04 $\pm$ 0.00 <sup>b</sup>	64.7 $\pm$ 3.0 <sup>b</sup>	79 $\pm$ 4.1 <sup>b</sup>
Cisplatin (4 mg/kg) + Aqueous extract (400 mg/kg)	65.4 $\pm$ 0.9 <sup>b</sup>	0.07 $\pm$ 0.00 <sup>b</sup>	0.04 $\pm$ 0.00 <sup>b</sup>	52.3 $\pm$ 3.5 <sup>b</sup>	94 $\pm$ 3.1 <sup>b</sup>
Cisplatin (4 mg/kg) + Methanol extract (200 mg/kg)	63.6 $\pm$ 1.2 <sup>b</sup>	0.08 $\pm$ 0.00 <sup>b</sup>	0.04 $\pm$ 0.00 <sup>b</sup>	46.7 $\pm$ 2.3 <sup>b</sup>	94.3 $\pm$ 2.5 <sup>b</sup>
Cisplatin (4 mg/kg) + Methanol extract (400 mg/kg)	68.3 $\pm$ 3.0 <sup>b</sup>	0.09 $\pm$ 0.00 <sup>b</sup>	0.04 $\pm$ 0.00 <sup>b</sup>	47.7 $\pm$ 1.6 <sup>b</sup>	97 $\pm$ 4.0 <sup>b</sup>

Values are expressed as the mean  $\pm$  SD. n = 6, <sup>a</sup>p<0.05 represents statistical different from control; <sup>b</sup>p<0.05 represents statistical different from toxic control group. Data was analyzed by using one-way ANOVA followed by Dunnett's test

creatinine, uric acid and urea were significantly ( $p < 0.05$ ) increased in cisplatin-treated rats (toxic control) as compared to the control rats. However, rats co-administered with aqueous and methanol extract of *B. lycium* root bark (200 and 400 mg/kg) along with cisplatin showed significantly decreased ( $p < 0.05$ ) levels of serum creatinine, uric acid and urea as compared to toxic control group. Results also indicated that methanol extract of *B. lycium* root bark was more effective to reverse cisplatin nephrotoxicity than aqueous extract. Moreover, no significant difference was observed in these serum markers with extracts alone.

Sub-acute study results showed that cisplatin significantly ( $p < 0.05$ ) lower the protein content, tissue GSH level, GST activity, CAT activity and enhanced MDA levels in renal tissue as compared to control group (Table II). Co-administration of aqueous and methanolic extracts with cisplatin showed significant ( $p < 0.05$ ) elevation of tissue GSH level, GST activity and CAT activity. It was revealed that methanol extract showed the pronounced effect to reverse the MDA level and raise GST activity as compared to aqueous extract. Oral administration of both extracts alone showed insignificant change in protein content and MDA level as compared to normal control. However, both extracts showed the significant increase in GSH level and CAT activity when administered alone.

Histopathological examination showed that the control (normal) kidney of rat had normal glomerulus, proximal and distal tubules (Figure 1). All the vessels and interstitium revealed unchanged basement membrane and mesangium. In cisplatin-treated group (4 mg/kg), severe and widespread necrosis with dilatation were observed along with vacuolar degeneration,

epithelial desquamation, and intraluminal cast formation in the proximal convoluted tubules. A spectrum of changes was found from condensation of chromatin to tubular necrosis. In methanol extract-treated groups at dose of 200 and 400 mg/kg, no significant changes in the histology of kidney tissues were observed. The kidney tissue appeared normal in shape and structure. Histological examination of cisplatin plus extract 200 mg/kg-treated group revealed slight degenerative changes of S3 segment and single cell necrosis was a rare event. However, cellular swelling and hydropic degeneration were seen. The kidney tissue of cisplatin plus extract 400 mg/kg-treated group depicted mild interstitial and tubular congestion.

## Discussion

Aqueous and methanol extract of *B. lycium* evinced mitigation of cisplatin-induced nephrotoxicity. Methanol extract had more potential to extenuate the renal damage and toxic effects. In *in vivo* sub-acute study, both extract significantly ( $p < 0.05$ ) decreased renal biomarkers i.e., serum creatinine, uric acid and urea levels. The protein content GST, GSH and CAT activity was significantly increased and MDA level was decreased followed by treatment with aqueous and methanol extract. The histopathological evaluation revealed that aqueous and methanol extract subdue the damage in renal tissue caused by cisplatin. *Berberis lycium* Royale has been reported to possess antioxidant properties (Sabir et al., 2013). The inherent nephrotoxicity of cisplatin has impelled focus on the investigation of new and possible protective strategies as current measures are not satisfactory. Of many

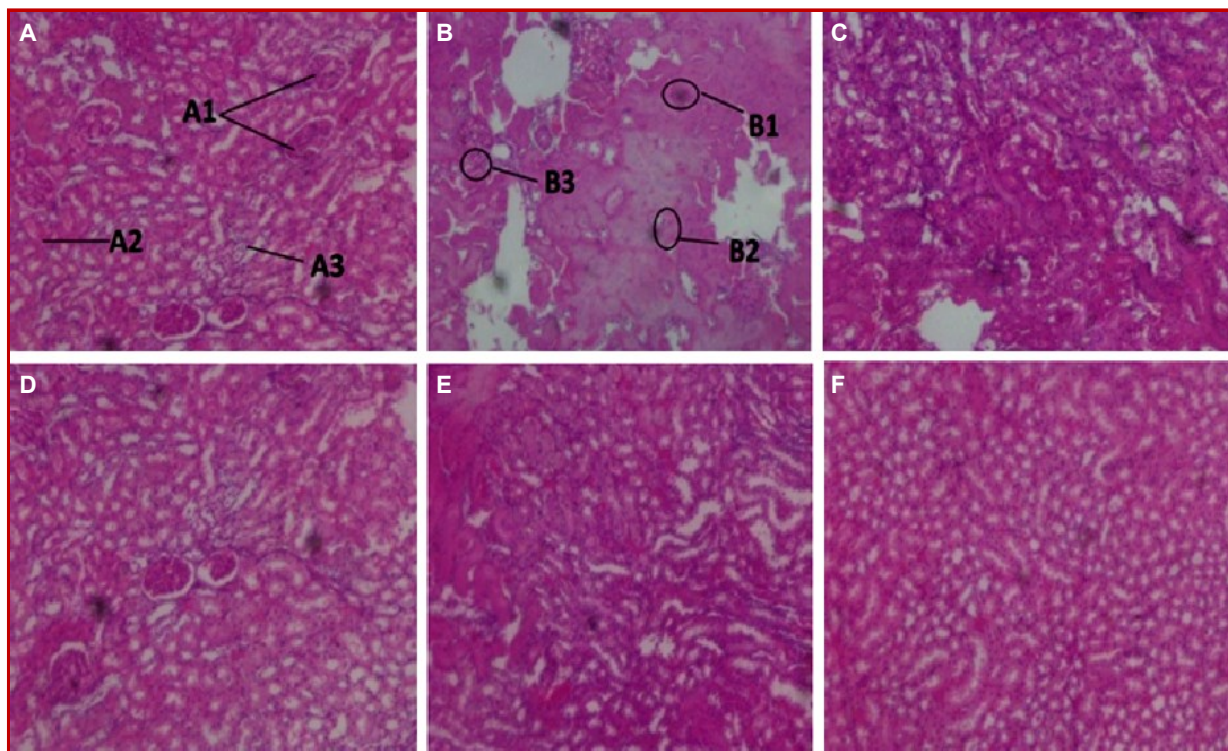


Figure 5: Light photomicrograph of kidney tissue of rats treated with methanol extract of *Berberis lycium* in sub-acute study. Haematoxylin and eosin, X 400 A: Control kidney, A1: Glomerulus, A2: Proximal tubule, A3: Distal tubule, B: Cisplatin 4 mg/kg, B1: Peritubular congestion, B2: Coagulative necrosis, B3: Degeneration, C: AE-200, D: AE-400, E: CIS + AQ200, F: CIS + AE400

pathways proposed responsible for cisplatin-induced nephrotoxicity, the oxidative stress is the most frequent and prevalent cause. It acts as a trigger and leads to renal damage and cell death (dos Santos et al., 2012). The main focus of this study is to find the protective strategies through the assessment of oxidative stress biomarkers (GST, tGSH, MDA and CAT) and renal function assessment in serum (creatinine, uric acid and urea levels) on the most exploited mechanism of nephrotoxicity i.e., oxidative stress leading to nephropathy.

Creatinine is directly related to the glomerular filtration rate (GFR) as it is freely filtered through the glomerulus. An increase in serum creatinine is a direct indicator of renal dysfunction (Lardiniois and Rouxhet, 1996). Uric acid is a metabolite of purines, nucleic acids and nucleoproteins. Renal dysfunction leads to an increase in uric acid level (Obermayr et al., 2008). Urea is a normal metabolic waste product of protein that is excreted by the kidneys. It is an indicator of aqueous kidney disease and sub-acute kidney injury. It occurs due to accumulation of waste materials and urea build up in the body when kidneys are unable to eliminate them (Bokemeyer et al., 1996).

Glutathione transferase (GSTs) are known to take part in detoxification of many potentially carcinogenic compounds (Riaz et al., 2016; Latif et al., 2017) The majority of its substrates are either products of oxidative stress

or xenobiotics (Friedberg et al., 1979). Reduce glutathione (GSH) is highly abundant in all cell compartments. It has a reactive -SH group which has reductive property. GSH has a fundamental role in counteracting oxidative stress as it detoxify ROS by acting as a coenzyme or cofactor (Gürer et al., 1998). It can also act as an anti-oxidant by non-enzymatically interacting with the ROS with its -SH group.

Catalase is an anti-oxidant enzyme with high specific activity in all aerobic cells. It is a metalloprotein and accomplishes its anti-oxidant function by enzymatically detoxifying the hydrogen peroxide (Ahamed and Siddiqui, 2007). It catalyzes the rapid decomposition of hydrogen peroxide (Johansson and Borg, 1988; Lardiniois and Rouxhet, 1996). Malondialdehyde is the principal and most widely studied byproduct of lipid oxidation. It is a very toxic aldehyde and is a major marker of lipid peroxidation (Del Rio et al., 2005). The literature showed that most medicinal plants protect the kidney by scavenging the ROS (Mishra et al., 2014). Tocotrienol showed renal protection against damage through reduction of lipid peroxidation. Moreover, it also improved the index of  $\text{NO}_2^-/\text{NO}_3^-$  generation (Khan et al., 2011).

Aqueous and methanol extracts of *B. lycium* provide protection against cisplatin-induced nephrotoxicity in rats. The results indicate that *B. lycium* significantly

reduced the depletion of GSH levels in renal tissue treated with cisplatin and thus provided protection to kidneys. In rats treated with aqueous and methanol extract along with cisplatin, the protection against nephrotoxicity was dependent on dose. GSH depletion leads to mitochondrial dysfunction in rat renal tissue when treated to cisplatin (Kameyama and Gemba, 1991). Mitochondrial dysfunction caused by GSH depletion is followed by lipid peroxidation (Jin-Gang and Lindup, 1993). The results of the current study showed the marked increase in MDA levels in cisplatin treated rats which were tremendously decreased on treatment with *B. lycium* root extract. The reduction in MDA levels in PMS of rat kidney treated with graded dose of aqueous and methanol extracts indicate dose-dependent nephroprotection by *B. lycium* extract from the cisplatin-induced renal damage. The restoration of GST and CAT activity with *B. lycium* aqueous and methanol extract pretreatment suggest that it can rescue these enzymes even after cisplatin treatment. The activity of CAT and GSH is decreased after cisplatin administration and hence decreased the ability of kidney to scavenge toxic H<sub>2</sub>O<sub>2</sub> and peroxides and ultimately lead to increase in MDA levels. Thus, the nephrotoxicity associated with cisplatin is due to depletion of GSH and other antioxidant enzymes which is reverted with treatment and pretreatment with *B. lycium* root bark extract. Thus, the use of this extract having anti-oxidant property supports the rationale for its use to ameliorate cisplatin-induced nephrotoxicity.

## Conclusion

A single dose of cisplatin leads to inhibition of renal anti-oxidant enzyme activity, increase in plasma creatinine, uric acid and urea level and marked decrease in GSH, GST and CAT activity and a remarkable increase in MDA levels. However, the administration of root bark extracts of *B. lycium* restored the anti-oxidant enzyme activity and decreased plasma creatinine, uric acid and urea level and lead to decrease in lipid peroxidation.

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## Ethical Issue

The animals were handled according to the Animal Ethics Committee guidelines, University College of Pharmacy, Punjab University. The committee reviewed and approved the experiment protocol and issued (approval voucher no: AEC/PUCP/1051) for this research protocol.

## Conflict of Interest

Authors declare no conflict of interest

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