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PHARMACOLOGICAL ACTIVITY OF BETA VULGARIS L. (BEET ROOT) FOR THE MANAGEMENT OF NEPHROTOXICITY INDUCED BY CYCLOPHOSPHAMIDE

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ABSTRACT

This study investigates the therapeutic efficacy of Beta Vulgaris L. leaves extract in mitigating Cyclophosphamide-induced nephrotoxicity in experimental rats. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, steroids, amino acids, proteins, and carbohydrates in both methanolic and aqueous extracts. Cyclophosphamide-induced elevation in renal function markers including blood urea nitrogen, creatinine, urea, and uric acid levels was significantly attenuated by Beta Vulgaris L. extract treatment, indicating improved renal function and effective clearance of waste products. Notably, oral administration of the plant extract resulted in a significant reduction in these markers, suggesting its potential therapeutic utility in renal diseases. In conclusion, Beta Vulgaris L. leaves extract demonstrates promising nephroprotective effects against Cyclophosphamide-induced nephrotoxicity, possibly attributed to its rich phytochemical composition. These findings underscore its potential as a natural remedy for renal disorders, warranting further exploration for clinical application.

KEYWORDS: Beta Vulgaris L., Nephrotoxicity. Cyclophosphamide, Renal function markers.

INTRODUCTION

Nephrotoxicity poses a significant clinical challenge, characterized by structural and functional impairments of the kidneys, often leading to renal failure and associated morbidity

and mortality. Various factors such as exposure to medications, environmental toxins, and metabolic disorders contribute to the development of nephrotoxicity. Among these, Cyclophosphamide, an alkylating agent widely used in the treatment of malignancies and autoimmune diseases, stands out for its potent nephrotoxic effects.[1]

Cyclophosphamide-induced nephrotoxicity is primarily attributed to its metabolites, which undergo bioactivation in the liver, leading to the formation of toxic intermediates that damage renal tissues.[2] This damage is characterized by tubular epithelial cell injury, glomerular dysfunction, and impaired renal function, ultimately resulting in renal failure if left untreated.[3]

In recent years, there has been a growing interest in exploring the therapeutic potential of medicinal plants and their bioactive constituents in the management of renal disorders. Beta Vulgaris L., commonly known as beetroot, is one such plant known for its diverse pharmacological properties, including antioxidant, anti-inflammatory, and tissue-protective effects.[4] Previous studies have reported the beneficial effects of Beta Vulgaris L. extract in various disease conditions, including cardiovascular diseases, diabetes, and inflammation.[5]

However, the potential nephroprotective effects of Beta Vulgaris L. extract against Cyclophosphamide-induced nephrotoxicity remain largely unexplored. Therefore, this study aims to investigate the therapeutic potential of Beta Vulgaris L. leaves extract in attenuating Cyclophosphamide-induced renal damage in experimental rats. By comprehensively evaluating histopathological changes and renal function markers, we seek to elucidate the underlying mechanisms of Beta Vulgaris L. extract-mediated nephroprotection.

Mechanisms of Cyclophosphamide-induced nephrotoxicity: Cyclophosphamide undergoes hepatic bioactivation to form toxic metabolites, including acrolein and phosphoramide mustard, which are implicated in renal injury.[6] These metabolites induce oxidative stress, inflammation, and apoptosis in renal cells, leading to tubular epithelial cell damage and glomerular dysfunction.[7] Role of oxidative stress and inflammation: Oxidative stress and inflammation play pivotal roles in the pathogenesis of Cyclophosphamide-induced nephrotoxicity. Increased production of reactive oxygen species (ROS) and pro-inflammatory cytokines exacerbates renal damage and impairs renal function.[8] Herbal remedies for nephroprotection: Herbal medicines have garnered attention for their potential nephroprotective effects against drug-induced renal injury. Several plant extracts and

phytochemicals exhibit antioxidant, anti-inflammatory, and cytoprotective properties, thereby mitigating renal damage.[9] Among these, Beta Vulgaris L. extract has shown promising pharmacological activities, including antioxidant and anti-inflammatory effects.[10] Nephroprotective potential of Beta Vulgaris L. extract: Beta Vulgaris L., commonly known as beetroot, contains various bioactive compounds, including betalains, flavonoids, and polyphenols, which contribute to its pharmacological properties.[11] Studies have reported the nephroprotective effects of Beta Vulgaris L. extract in various experimental models of renal injury, highlighting its potential as a natural remedy for renal disorders.[12] Evaluation of renal function markers: Assessment of renal function markers, including blood urea nitrogen (BUN), serum creatinine, urea, and uric acid levels, is crucial for monitoring renal function and evaluating the efficacy of nephroprotective interventions.[13]

We concluded from the literature survey that Cyclophosphamide-induced nephrotoxicity poses significant challenges in clinical practice, necessitating the exploration of novel therapeutic interventions. Herbal remedies, particularly Beta Vulgaris L. extract, hold promise for mitigating renal damage and preserving renal function. Further research is warranted to elucidate the molecular mechanisms underlying the nephroprotective effects of Beta Vulgaris L. extract and its potential clinical applications in managing drug-induced nephrotoxicity.

The findings of this study hold promise for the development of novel therapeutic interventions for Cyclophosphamide-induced nephrotoxicity and other renal disorders, contributing to the advancement of evidence-based herbal medicine in nephrology.

MATERIAL AND METHODOLOGY

Successive solvent extraction

Successive extraction of *Beta vulgaris L.*(Beet root) involves sequential extraction using solvents with petroleum ether, methanol and water increasing polarity.

Phytochemical test

Chemical Test for Carbohydrates

- **Molish test:** Aqueous solution of drug (carbohydrate) was mixed with few drops of Molish reagent (alpha naphthol) and Concentrated H2SO4 was added from sidewall of test tube Formation of purple coloured ring at junction indicates presence of carbohydrates.

- **Barford test:** Two ml of Barford reagent (Cupric acetate, acetic acid and water) was mixed to 1ml aqueous solution of drug and boiled. Formation of brick red precipitate indicates the presence of carbohydrate.
- **Fehling solution test:** Equal volume of Fehling A and Fehling B solutions were mixed (1 ml each) and 2 ml of aqueous solution of drug was added followed by boiling for 5-10 minutes on water bath. Formation of reddish-brown colour precipitate due to formation of cuprous oxide indicates the presence of carbohydrate.
- **Benedict's test:** To the 4 ml of aqueous solution of drug, 1 ml of Benedict solution was added and heated till to boiling. Formation of green, yellow, orange, red or brown colour in order of increasing concentration of carbohydrate in the test solution, due to formation of cuprous oxide.

Chemical Test for Proteins and Amino Acids

- **Biuret test:** Aqueous solution of protein in hot water was mixed with few drops of Biuret reagent (KOH, CuSO4 and sodium potassium tartrate) which turned blue reagent to violet.
- **Millons test:** In few drops of Millons reagent took 1ml of drug solution .White precipitate was produced, which turned into red colour after heating for 5 minutes on water bath.
- **Ninhydrin test:** When drug (protein) solution was heated with Ninhydrin molecules, characteristic deep blue and pale-yellow colour indicates the presence of proteins.
- **Vanillin HCl test:** Vanillin HCl was mixed with alcoholic solution of drug, formation of pink colour indicates the presence of flavonoids.

Chemical tests for flavonoid glycosides

- **Ammonia test:** When filter paper was dipped into alcoholic solution of drug, which leads to formation of yellow spot on the filter paper, indicates the presence of flavonoid.
- **Shinoda test:** When Mg turning and dilute HCl was mixed with alcoholic solution of drug formation of red colour indicate the presence of flavonoids. When Zinc turning, dilute HCl and alcoholic extract of drug combined with each other formation of deep blue to magenta colour indicates the presence of flavonoids.

Chemical tests for glycosides

- **Borntragor's Test:** 1 gm of drug was mixed with 5-10 ml of dilute HCl, boiled on water bath for 10 minutes and filtered. Filtrate was extracted with CCl4/benzene. Then equal amount of ammonia solution was added to the filtrate and shaked. Formation of pink or red colour in ammonical layer indicates the presence of glycoside.
- **Libermann Bruchard test:** Alcoholic extract of drug was extracted with chloroform. Then added few drops of acetic anhydride followed by concentrated sulphuric acid from side wall of the test tube to chloroform extract. Formation of violet blue coloured ring at the junction of two liquid indicates the presence of glycosides.
- **Keller Killiani test:** Alcoholic extract of drug was mixed with equal volume of water and 0.5ml of strong lead acetate solution, shaked and filtered. Then filtrate was extracted with equal amount of chloroform. Chloroform extract was evaporated to dryness and residue was dissolved in 3 ml of glacial acetic acid followed by addition of few drops of ferric chloride solution. The resultant solution was mixed with 2 ml of concentrated sulphuric acid. Reddish brown layer is formed, which turns bluish green after standing due to presence of digitoxose.

Chemical tests for lipids

➢ **Sudan IV test: -** When Sudan IV was added to a mixture of lipids and water, the dye moved into the lipid layer and made it red.

Chemical Test for Alkaloids

- **Dragendorff's Test:** Drug solution + Dragendorff's reagent (Potassium Bismuth Iodide), formation of Orangish red colour.
- **Mayer's Test:** Drug solution + few drops of Mayer's reagent (Potassium mercury iodide), formation of creamy-white precipitant.
- **Hager's Test:** Drug solution + few drops of Hagers reagent (Saturated aqueous solution of Picric acid), formation of crystalline yellow precipitate.
- **Wagner's Test:** Drug solution + few drops of Wagner's reagent (dilute Iodine solution), formation of reddish-brown precipitate.
- **Tannic Acid Test:** Drug solution + few drops of tannic acid solution, formation of buffy coloured precipitate.

Chemical tests of resins

- **Solubility test:** Resins are insoluble in water, rarely soluble in light petroleum (except Colophony and Dammar) soluble in alcohol, ether, acetone, chloroform, fixed oils and volatile oils etc.
- **Turbidity test:** When resinous drug was extracted with alcohol and water is added in excess to form turbidity, because these are insoluble in aqueous solutions.

Ex vivo enzymatic assay

Estimation of superoxide dismutase (SOD)

Superoxide dismutase activity in the kidney was estimated. The kidney homogenate was prepared by homogenizing. 1 gm of kidney tissue with 10 ml of ice-cold isotonic buffer homogenate at 3000 rpm for 5 min then was centrifuged at 1600 g for 10 min at 2-8 ºC. After that, 800 µl of ice-cold chloroform/ethanol (37.5/62.5 v/v) was added to 500 µl of homogenate (supernatant) so as to inactivate Mn and Fe SOD. The solution was allowed to shake for 30 sec. the mixture was then centrifuged at 2500 rpm for 10 min at 2-8 ºC. The upper layer of homogenate was used for SOD estimation. 3.82 gm of pyrogallol was dissolved in 10 ml HCl (10mM) and tissue immediate for the assay.

Calculation

Results were expressed in units per gm of tissue or per mg of protein for tissue homogenate and units per gm Hb for erythrocyte hemolysate.

All the experiments were carried out in an air-conditioned room at 25°C. Protein content was estimated according to the method of Lowry.

$$\mathbf{A_{420}\ nm/min = (A_{420}\ nm/min\ at\ 5:30\ min - A_{420}\ nm/min\ at\ 0:30\ min) / 5}$$

$$\mathbf{\%\ Inhibition = [(-A_{420}\ nm/min^{control}) - A_{420}\ nm/min^{test}) / A_{420}\ nm/min^{control})] \times 100}$$

One unit of SOD inhibited the rate of increase in absorbance at 420nm by 50% under the condition of assay. The % inhibition of the test sample was correlated with the results of standard SOD activity.

Estimation Of lipid peroxidation (LPO)

Rats were sacrificed and decapitated, the kidney was rapidly dissected. kidney were washed with cold phosphate buffer saline (PBS). Kidney were minced separately into small pieces.

The small pieces (10% w/v) were allowed to homogenize in ice-cold Teflon homogenizer at 67 rpm up and down strokes over a 3 min period. GSH levels were determined using the supernatant of the kidney homogenate, which was centrifuged at 8500 rpm for 25 min at 2º C.

Calculation and Statistical analysis

Calculate based on 1.56 × 105 as the molar extinction coefficient for malondialdehyde. Use the proteins precipitated in the samples (after decantation) for protein measurement and express the results as MDA mol/gm of protein.

Estimation of glutathione (GSH)

Rats were sacrificed and decapitated, the kidney was rapidly dissected. Kidney were washed with cold phosphate- buffered saline (PBS). Kidney were minced separately into small pieces. The minced of kidney (10% w/v) were allowed to homogenize in ice-cold Tris buffer (10mM/L Tris, pH 7.4) using an ice-cold Teflon homogenizer at 67 g up and down strokes over a 3 min period. GSH levels were determined using the supernatant of the kidney homogenate, which was centrifuged at 8500 g for 25 min at 2ºC.

Calculation and statistical analysis

The results were expressed in µg/mg of protein. All experiments were carried out in an air-conditioned room at 25ºC.

GSH (µg/mg of protein) = (Abs_{412}nm-0.14) X dilution factor

Protein estimation

Tissue protein content was estimated in aliquots of diluted membrane fraction using a colorimetric reaction with Folin's phenol reagent. The colour developed was measured at 640nm. Values expressed as mg protein/g wet tissue.

Procedure for protein estimation

Diluted membrane fraction aliquots (0.1ml) was added to 0.8ml of 0.1M NaOH and 5ml of Lowry C reagent, and allowed to stand for 15 min. then 0.5 ml of 1N folins phenol reagent was added to the reaction mixture and mixed well. The abs was measured at 640 nm in UV spectrophotometer and compared with the readings obtained from standard solutions.

***In vivo* studies**

Experimental animals

Wistar rats (8-12 weeks old; 160–200 g) were used and housed in plastic cages under normal laboratory conditions (12 h light/dark cycle: 25±2ºC) for an acclimatization period of 7 days prior to the experiments. All the animals were given food and water *ad libitum*. The bioassay was conducted in accordance with the internationally acceptable guidelines for evaluating the safety and efficacy of herbal medicines (OECD, 2008).Animal study was performed in Pharmacology Laboratory, Malhotra College of Pharmacy, Bhopal, India. The procedures were reviewed and approved by the Institutional Animal Ethics Committee (Proposal No.08).

Toxicity study

Adult male Wistar rats weighing (160-200) were used for acute toxicity studies. The rats were divided into control and test group containing 6 animals in each. The rats were administered intraperitonially(ip) with MEBV and AEBV at a dose of 1000 mg/kg (high dose) and 200 mg/kg (low dose). Normal control rats received the same amount of vehicle (saline) only. Rats were observed carefully for 24 hours after extract administration and then for the next 7 days. End of experimental period the rats were observed for a sign of toxicity, mortality and morphological behaviour. Toxicity was evaluated based on the previous study.

Cyclophosphamide Induced Nephrotoxicity

Six rats were kept as normal control group (Group 1 below), while rats were made Nephrotoxicity by ip induction of Cyclophosphamide(150 mg/kg body weight) daily for 7 days. Rats that developed Nephrotoxicity were recruited for the study. Nephrotoxic rats were randomly divided into 6 groups and treated.

1. **Group I** - Control- normal saline (normal water)
2. **Group II** - Negative control- Cyclophosphamide
3. **Group III** – CP+ Methanolic extract (100mg)
4. **Group IV** – CP + Methanolic extract (200mg)
5. **Group V** – CP + Aqueous extract (100 mg)
6. **Group VI** – CP + Aqueous extract (200 mg)

The animal was divided into 6 groups with six animals in each. Group I was kept as normal control (administered vehicle only). In group II, Nephrotoxicity was induced in rats by intraperitoneal injection of Cyclophosphamide at 150 mg/kg for 7 days. Group III was given CP at the dose of 150 mg/kg body weight (i.p.) along with methanolic extract at the dose of

100 mg/kg body weight (i.p.). Group IV was given CP at the dose of 150 mg/kg body weight (i.p.) along with methanolic extract at the dose of 200 mg/kg body weight (i.p.). Group V was given CP at the dose of 150 mg/kg body weight (i.p.) along with aqueous extract at the dose of 100 mg/kg body weight (i.p). Group VI was given CP at the dose of 150 mg/kg body weight (i.p.) along with aqueous extract at the dose of 200 mg/kg body weight (i.p).Blood sample were collected on the 7 days and evaluated for Nephrotoxicological parameters like BUN, Creatinine level etc.

Blood urea nitrogen

The blood urea nitrogen Enzymatic Kit is a microplate based colorimetric assay for the determination of urea in serum samples produced from blood. Blood urea nitrogen (BUN) is an important marker for normal kidney and liver function. BUN is a very useful tool for preclinical investigation of experimental drug formulations and BUN levels are commonly used to monitor and attenuate the toxic effects of experimental drug formulations in rodents. Blood Urea Nitrogen Enzymatic Kit uses an enzyme-based assay to determine urea in liquid samples such as serum. The test is based on a highly proven method for urea determination. The Blood Urea Nitrogen Enzymatic Kit contains sufficient materials to test 42 samples in duplicate.

The assay utilizes urease, a metabolic enzyme, to specifically detect urea in serum. The Blood Urea Nitrogen Enzymatic Kit provides rapid, accurate, proven results even in complex liquid mixtures.

Serum creatinine

The methods most widely used today are based on the jaffe reaction. This reaction occurs between creatinine and the picrate ion formed in alkaline medium (sodium picrate); a red-orange solution develops.

Statistical analysis

The results were evaluated as mean ± SEM for nephrological source, oxidative stress parameters and. One way analysis of variance (ANOVA) followed with Tukey's test was used to determine significant differences. The statistical analysis was performed by using GraphPad software.

RESULTS

Determination of Percentage Yield

Yield of Extraction: The crude extracts so obtained after the maceration extraction process; extract was further concentrated on water bath evaporation the solvents completely to obtain the actual yield of extraction. To obtain the percentage yield of extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant. The yield of extract obtained from *Beta vulgaris L.(*Beet root) using hydroalcoholic as solvent is depicted in the table.

Table 1: Percentage Yield of *Beta vulgaris L*. (Beet root).

S. No.	Solvent	Extraction time (hours)	Yield (gram)	Yield (%)	Colour extract
1.	Petroleum ether	20	7.0	2.3%	Yellowish-brown
2.	Methanol	20	21.0	7%	Greenish
3.	Water	20	6.0	2%	Brown

Note: 300 gm of crude drug was taken for extraction.

Phytochemical screening of extract

A small portion of the dried extracts were subjected to the phytochemical test using (Kokate, 1994) methods to test for alkaloids, glycosides, tannins, saponins, flavonoids and steroids separately for extract of all samples. Small amount of extract is suitably resuspended into the sterile distilled water to make the concentration of 1 mg per ml. The outcomes of the results are discussed separately in the table.

Table 2: Result of Phytochemical Screening of hydroalcoholic extract of *Beta vulgaris* L. (Beet root).

S. No.	Phytoconstituent	Identification Test	MeOH Extract	Aqueous Extract
1.	Alkaloids	Mayer Test	+ve	+ve
		Wagner's Test	+ve	+ve
		Dandruff's Test	+ve	+ve
		Hager's Test	+ve	+ve
2.	Glycosides	Legal Test	-ve	+ve
		Killer Killani Test	-ve	-ve
		Raymond's Test	-ve	+ve
3.	Tannins	Vanillin-HCl Test	-ve	-ve
		Gelatin Test	-ve	-ve
		Ferric Chloride Test	+ve	+ve
4.	Flavonoids	Shinoda Test	-ve	+ve
		Lead Acetate Test	-ve	+ve

5.	Steroids	Salkowski Test	+ve	+ve
		Libberman Test	-ve	+ve
6.	Amino Acids	Ninhydrin Test	+ve	+ve
		Cysteine Test	+ve	+ve
		Biuret Test	-ve	+ve
7.	Proteins	Precipitate Test	+ve	+ve
		Biuret Test	+ve	+ve
		Ninhydrin Test	-ve	+ve
8.	Carbohydrates	Molish Test	+ve	+ve
		Benedict Test	-ve	+ve
		Fehling's Test	+ve	+ve

+ve= Present, -ve= Absent

Table 3: Observation table of *In vivo* Nephroprotective activity of *Beta vulgaris L.*(Beet root).

S. No.	Treatment/ Group	BUN (mg/dl) Mean ± SEM	Creatinine (mg/dl) Mean ± SEM
1.	Control	15.64±0.969	0.638±0.043
2.	Cyclophosphamide	26.81±3.180	1.68±0.296
3.	CP+ MeOH extract (100mg)	19.45±1.568	1.003±0.126
4.	CP+ MeOH extract (200mg)	16.16±1.837	0.728±0.070
5.	CP + Aqueous extract (100 mg)	22.31±3.156	1.44±0.358
6.	CP + Aqueous extract (200 mg)	19.46±2.435	0.961±0.127

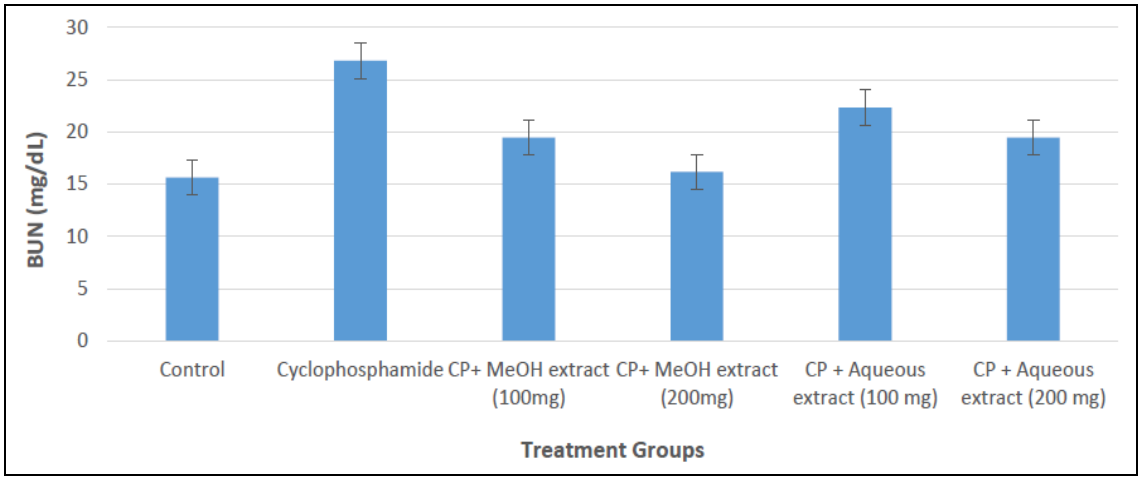

Fig. 1: Observation of In vivo Nephroprotective activity of Beta vulgaris L.(Beet root).

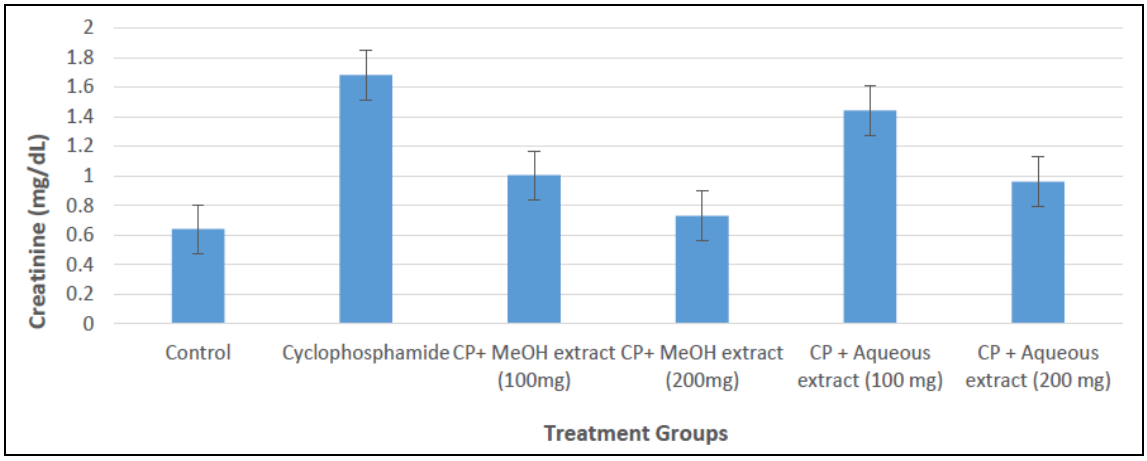

Fig. 2: Observation of In vivo Nephroprotective activity of Beta vulgaris L.(Beet root).

Table 4: Observation of In vivo Nephroprotective activity of Beta vulgaris L.(Beet root).

S. No.	Treatment/ Group	SOD (U/gm)	LPO(nM MDA/gm)	GSH (nmol/gm)
1.	Control	48.95 ± 3.535	16.58 ± 1.649	2.32±0.336
2.	Cyclophosphamide	17.45 ± 2.985	45.09 ± 4.483	0.74±0.155
3.	CP+ MeOH extract (100mg)	35.13 ± 4.822	31.75 ± 3.590	1.49±0.137
4.	CP+ MeOH extract (200mg)	44.28 ± 4.096	19.98 ± 2.729	2.003±0.115
5.	CP + Aqueous extract (100 mg)	29.48 ± 2.727	37.57 ± 4.551	1.19±0.147
6.	CP + Aqueous extract (200 mg)	40.06 ± 4.187	26.06 ± 3.761	1.80±0.175

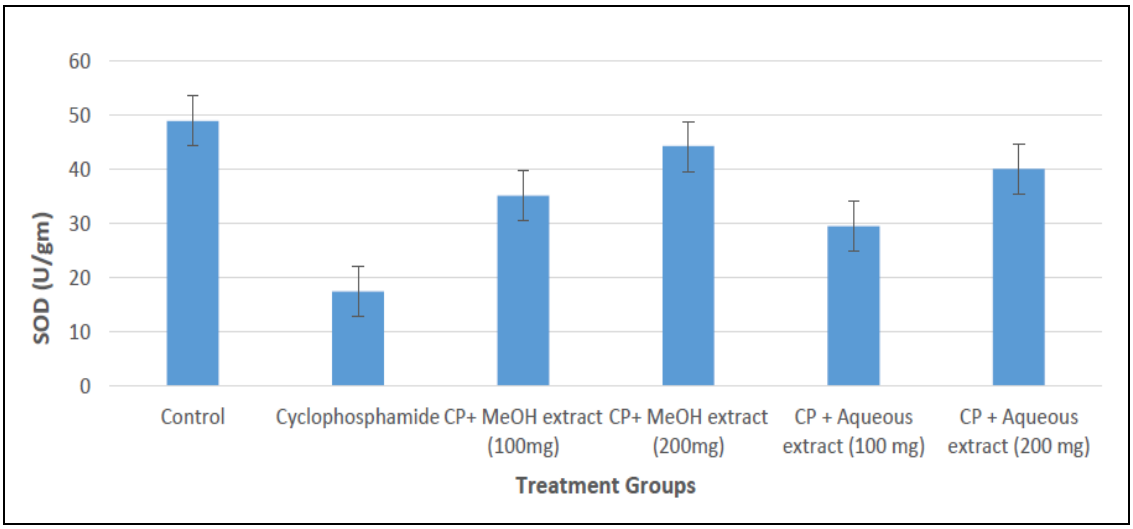

Fig. 3: Observation of In vivo Nephroprotective activity of Beta vulgaris L.(Beet root).

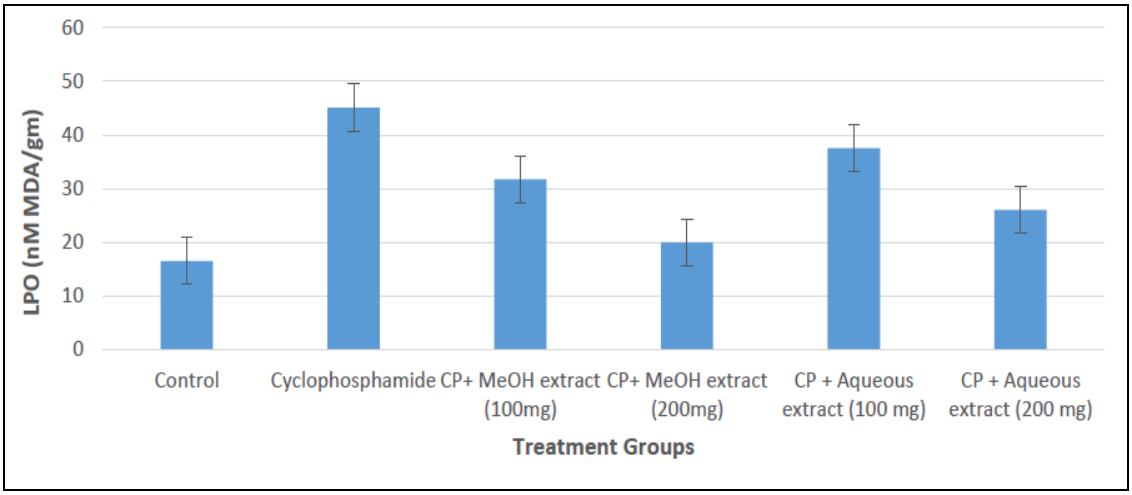

Fig. 4: Observation of In vivo Nephroprotective activity of Beta vulgaris L.(Beet root).

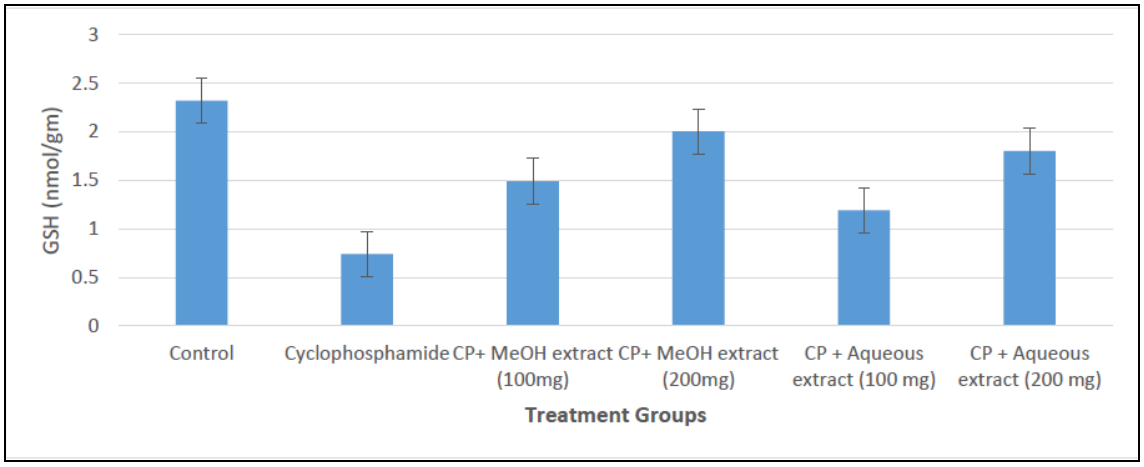

Fig. 5: Observation of In vivo Nephroprotective activity of Beta vulgaris L.(Beet root).

SUMMARY

The preliminary phytochemical screening of Beta Vulgaris L. leaves extract revealed the presence of various bioactive compounds such as alkaloids, flavonoids, tannins, steroids, amino acids, proteins, and carbohydrates in both methanolic and aqueous extracts. However, groups treated with Cyclophosphamide along with either methanolic or aqueous extracts showed milder tubular epithelial changes, with the higher dose of aqueous extract inducing tubular epithelial cell regeneration. Renal function markers including blood urea nitrogen (BUN), creatinine, urea, and uric acid levels were significantly elevated in the Cyclophosphamide-treated animals compared to the normal group. Treatment with both methanolic and aqueous extracts significantly reduced these markers, indicating improved renal function and effective clearance of urea and creatinine. Oral administration of the plant extract notably decreased BUN, urea, creatinine, and uric acid levels in the blood. Elevated

levels of urea and creatinine in serum were indicative of nephrotoxicity, which was mitigated by treatment with Beta Vulgaris L. leaves extract.

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CONFLICT OF INTEREST

No authors declared Conflict of Interest.

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