"Preclinical evaluation of phloretin on cisplatin induced nephrotoxicity in peri-menopausal mouse model"

Praful G Rajpurohit [1], Dr. Rajendra Bapna[2], Ragini Bundela[2], Dr. Karunakar Shukla[3]

1PG Scholar, College of Pharmacy, Dr. A.P. J. Abdul Kalam University, Indore.

2Associate Professor, College of Pharmacy, Dr. A.P. J. Abdul Kalam University, Indore.

3Professor and Principal, College of Pharmacy, Dr. A.P. J. Abdul Kalam University, Indore.

.

ABSTRACT

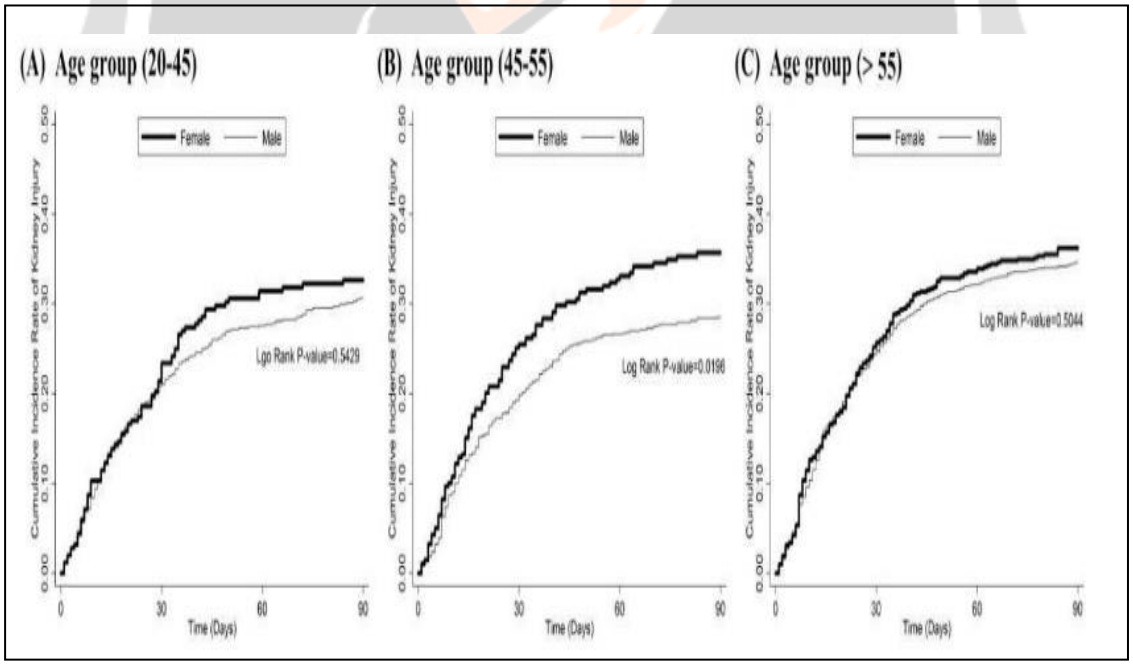
Cisplatin is an effective on cancer like prostate cancer, bladder cancer, overian cancer,lung cancer that causes remarkable toxicity to the kidney, particularly to glomerular filter, by generating reactive oxygen species. From literature it is evident that women are more prone to Cisplatin induced nephrotoxicity, and the women >45 yrs of age aremore prone to cisplatin induced nephrotoxicity. More specifically women of 45-55 agegroup (peri-menopausal age) are more prone for cisplatin induced nephrotoxicity. It hasalso become evident that inflammation provoked by injury to renal epithelial cells serves to amplify kidney injury and dysfunction.In the Cisplatin induced nephrotoxicity study in perimenopausal mouse model, Cisplatin treated group significantly increased level of serum creatinine, BUN, ALP and decreased the level of albumin. Cisplatin treated mice the decrease the level of GSH, SOD and increased the level of LPO. Cisplatin treated animals shows the damaged to the kidney. Phloretin shows the significant protective effect in kidney function parameters (CRE, BUN and albumin in serum), oxidative stress parameters and microscopic tissue injury in histopathology studies. While the phloretin treatment with low and high-dose (20–60 mg/kg) was possessed the significant protective effects against the cisplatin-induced renal damage, more importantly it also increased survival and delayed mortality in cisplatin treated perimenopausal mice model. The observed results of the current research work clearlyexhibited the clinical potential of phloretin as cotreatment with cisplatin for cancer treatment in perimenopausal women to reduce cisplatin induced nephrotoxicity.

Keywords: Phloretin, Cisplatin, Nephrotoxicity, Peri-menopause

INTRODUCTION

Acute kidney injury (AKI) is a clinical condition characterized by a rapid decline in renal function as well as the accumulation of waste products such as urea. (Ozkok and Edelstein, 2014). The kidneys are the most imperative organs of the humanbody. They are very essential for the maintenance of a variety of body fluids volume, regulations of fluid osmolality, balancing of acid-base concentrations, maintaining concentrations of various electrolytes, and removal or excretion of toxins from the bodythrough the urine. Cisplatin is a platinum-based drug that has been widely using it as achemotherapeutic agent to treat diseases such as bladder cancer, prostate cancer, cervixcancer, and lung cancer. Nonetheless, the cisplatin was associated with the various severe side effects such as nephrotoxicity, ototoxicity, neurotoxicity, and in some casesocular toxicity (Zhao *et al.*, 2020)**.** It is also reported that CDDP-induced nephrotoxicityis gender-relat

Fig no-1. The cumulative incidence rate of kidney injury(Chen *et al.*, 2017).



Pathophysiological event in cisplatin nephrotoxicity:

When tubular cells are exposed to cisplatin, signalling pathways that promote cell death (MAPK, ROS, and so on) or cytoprotection (p21) are activated. Meanwhile, cisplatin causes tubular cells to produce TNF-a, which causes a strong inflammatory response,

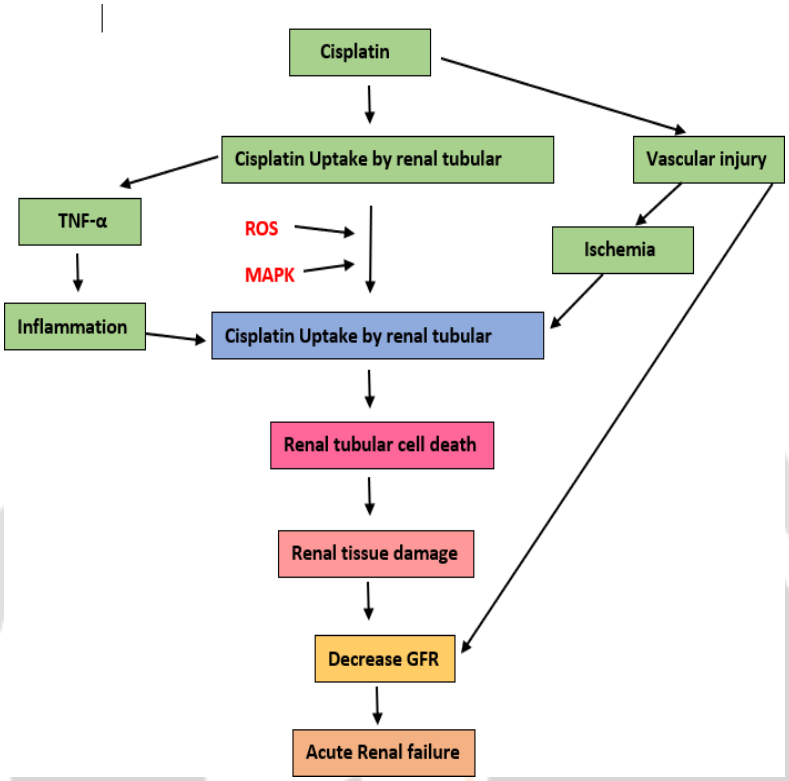


Fig no. 2 Pathophysiological events in cisplatin Nephrotoxicity (Pabla and Dong, 2008)**.**

causing tubular cell damage and death. Cisplatin may potentially cause **MATERIALS AND METHODS**

Total no. of animal in study:

Total number of swiss albino mice used in the study were 54.

Drugs:

Phloretin (PHL) BLD Pharmatech (India) Pvt Ltd. Cat. no.- BD147786-5g, CAS No.60-82-2, MW-274.27 Purity-98%, Cisplatin (Cizcan).

Kits:

Creatinine kit (ERBA, India), Urea/ BUN Kit (ERBA, India), Albumin kit (ERBA, India), Alkaline Phosphate (ERBA, India), Total protein kit (ERBA, India**).**

Chemicals:

5-5 -dithiol bis (2-nitrobenzoic acid) and Thiobarbituric acid (Loba Chemicals Pvt. Ltd.), Sodium chloride, Phosphoric acid, n-butanol, nitro blue tetrazolium, N-N- dimethyl acetamide (Sigma), hydroxylamine, glacial

acetic acid, sodium carbonate, triton-X-100, ethylene diamine tetra acetic acid, formaldehyde, ketamine(themis medicare limited, batch no. KMI002) and xylazine(brilliant bio-pharma pvt, batch no. XLB2004), spirit alcohol, povidone iodine, 0.9% saline solution, 10 %formalin, neomycin powder (antibiotic).

Glassware:

Test tube, mesuring cylinder, graduated glass pippite, beaker, conical flask.

Equipment's:

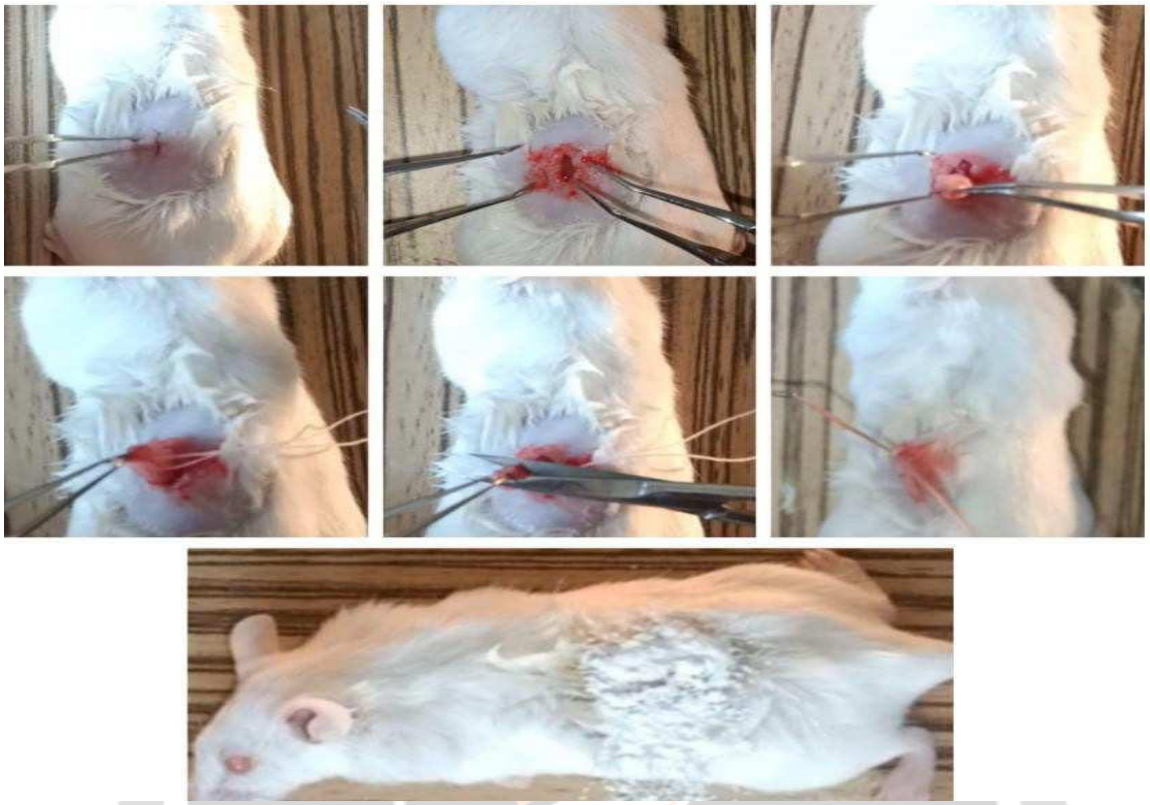
Micropipette (SUPERFIT XL, BioEra, Pune), Cooling Centrifuge (C30BL, Remi, India), Digital microscope (Motic, DMWB2-223, Leica Biosystem USA), 96 well microplate (Nulclon Surface, Denmark), Homogenizer, UV spectrophotometer(Shimadzu), Vortex mixer , microplate reader (BioTek), mercury thermometer. Common Surgical equipments like scissors, forceps, etc, surgical cotton, lamp, curve needle, surgical thread, insulin syringe, petri plate, lamp etc.

Other requirement- Oral feeding needle, Eppendorf tube, falcon tube, petri plate, aluminum foil, tissue paper, weight balance(Tapson), topical powder, depilatory creametc.

Ovariectomy procedure:

1. Animal was weighed.
2. The animal was administered Ketamine (80 mg/kg) & xylazine (6 mg/kg),to anesthetize it.
3. The hair was removed from dorsal side of the animal, between the rib cageand hip surgical area using a depilatory cream.
4. The skin surface was wiped with 70% alcohol followed by povidone-iodine solution.
5. A midline incision was made, approximately 1cm in length, in the mid-dorsum of mice.
6. The skin was gently separated from the underlying muscle using forceps.
7. A left ovary was located by visualizing a white spot under the muscle on the flanks of the animal.
8. A small incision was made, approximately 0.5cm in length, directly overthe white spots (ovary).
9. The white fat pad was grasped using fine forceps and the ovary was gentlypull out of the incision.
10. Ovary was tied with the suture and cut it and then re-positioned the tube inside.

11. The muscle and skin was sutured to close the incision made.
12. After closing the incision povidone iodine and neomycin powder wasapplied on it.



Step of ovariectomy surgery

Drug solution preparation:

Preparation of phloretin suspension:

Phloretin (20 and 60 mg/kg) was used as a test drug for the treatment of nephrotoxicity. Phloretin was suspended in 0.5% CMC solution & given orally with the help of oral feeding needle for 7 days**.**

Preparation of cisplatin:

Removed Cisplatin from vial and diluted for desired concentration using 0.9% saline solution and administered in a dose 5 mg/kg IP for 5 consecutive days. The concentration of cisplatin in stock vial was 50mg/50 ml i.e. 1 mg/ml, so the working solution of cisplatin to administered to the animals was prepared of 0.5 mg/ml.

Experimental groups:

Swiss albino mice (female) was selected for our study, mice were randomly divided into six groups. Three of the group (G1, G2 and G3) were commonly shared with the another study where the protocol used was similar to evaluate the neproprotective effectof another test drug (Glycyrrhetinic acid) during the same time frame and facility

Parameter evaluation:

Physical parameter Body weight:

The body weight of mice was recorded daily through out the experimentation phase using the effect of treatment intervention on body weight, percent change in body weight was evaluated, using basal (before initiation of treatment) and the terminal (at the day of sacrification) body weights of each mice. The daily recorded body weights are used to calculate the daily dose volume to be administered to the respective animals.

Feed and water consumption:

The mice were housed as 4 mice/cage and the pre-weighed 20g of pelleted food were provided to each cage. Next day the food remaining in each cage was observed and weighed. The feed intake percage per day is derived from the values of amount of food kept in each cage on previous day and the food remained on next day. Thereafter, the values of feed intake per cage/day were used to calculate daily feed intake by each animals. Similarly water intake for each animal is calculated from the daily known quantity of water (150 ml), provided for each cage, throughout the study period.

Urine Collection:

There was plan to collect urine to assess the urinary createnine, BUN and albumin. However, due to Cisplatin administration animals were very dull, and need to take extra care for watering and feeding, due to that urine collection was not possible in this study.

Blood collection and serum separation:

Mice was first anesthetize by anesthesia then gently scruffed to made to eye bulge. Glass capillary was inserted into the outer corner of the eye and then blood was allowed to flow through capillary action and finally collected in eppendorf tube. Afterward the blood was kept for 20 min at Room temp to allow it to clot and blood was centrifuged at 2000 RPM for 5 min, serum was separated and stored at -20℃ for furthered biochemical analysis.

Sacrification of animal:

At the end of the treatment protocol (Treatment day 8) all animals were sacrificed by cervical dislocation. The abdominal cavity was opened to collect the Kidneys. The collected kidneys were isolated, washed with ice-cold saline to remove residual blood, weighed, and after gross examination cut one kidney in two parts. The one part was homogenized in ice-cold KCL (to make 10% homogenate), second part, was stored in 10% formalin solution for histological examination and another whole kidney was homogenized in ice-cold PBS (to make 10% homogenate), for oxidative stress parameters.

Estimation of Serum parameter:

Creatinine Estimation:

Principle: In alkaline medium, creatinine reacts with picric acid to form an orange coloured complex and the rate of change in this absorbance is measured at 505 nm. By predetermined time interval. This method has optimized the concentration of picric acid and sodium hydroxide as well as the reaction time to avoid CLIF (creatinine as interfering factor) interference in the sample.

Statistical analysis:

The data obtained from estimations of various parameters was tabulated as per the respective treatment group. The mean and standard error of mean were calculated for respective groups. The data was evaluated for statistical comparision using GraphPad Prism 8.0.2 software. One-way analysis of variance (ANOVA) and Two-way analysis variance was applied whereever required for statistical analysis, the post-hoc test used is Dunnette' multiple comparison test.

Estimation of lipid peroxidation :

During prostaglandin biosynthesis in cells. MDA reacts with amino groups on proteins and other biomolecules to form a variety of adducts, including adducts with DNA bases that are mutagenic and possibly carcinogenic. Increased levels of lipid peroxidation products, by measurement of MDA, have been associated with various conditions and pathological states of diseases

1% w/v phosphoric acid:- 100mg H_3PO_4 in 10ml distilled water.

0.6% w/v thiobarbituric acid:- 60mg thiobarbituric acid in 10ml distilled water

RESULT AND DISCUSSION

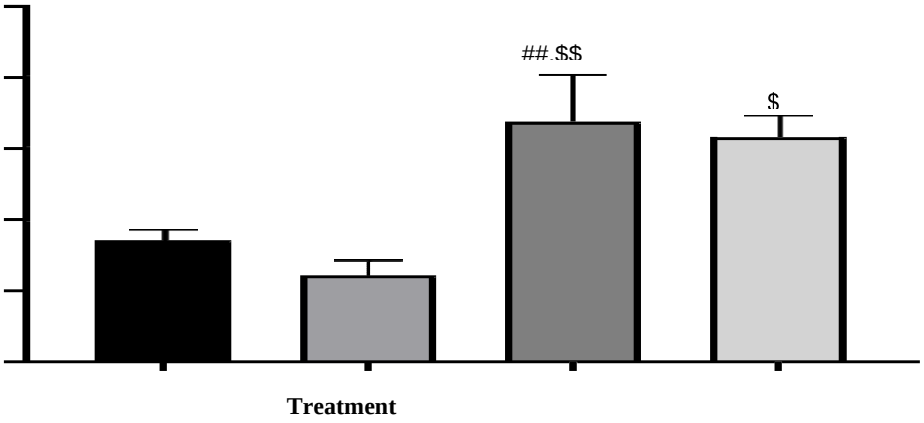
Effect of phloretin treatment on cisplatin- induced nephrotoxicity in perimenopausal mouse:

Serum/Urine parameters:

Cisplatin treatment in ovariectomized mice and normal mice caused marked impairment of renal functions as evident from the decrease serum albumin and increasing serum creatinine, BUN, Serum ALP levels as compared to the sham group.

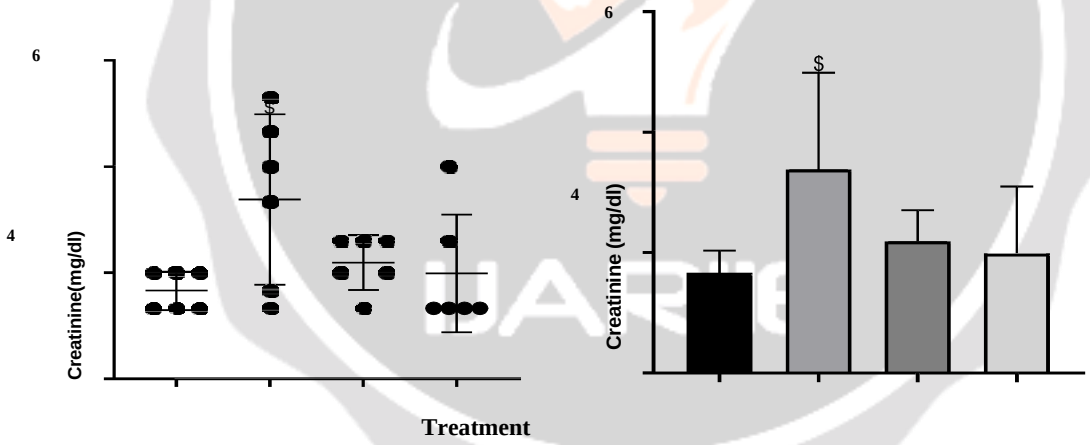
Serum Creatinine Estimation:

Fig no. 9 Effect of cisplatin on serum creatinine in cisplatin treated perimenopausal mouse model



Data represented as mean±SEM and evaluated using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The p value <0.05 is considered as statistically significant. ##p<0.01 is compared with OVX group; $$p<0.01 is comparedwith SHAM group; $p<0.05 is compared with SHAM group. The values in theparenthesis are dose in mg/kg. OVX- Ovariectomy; CIS- Cisplatin.

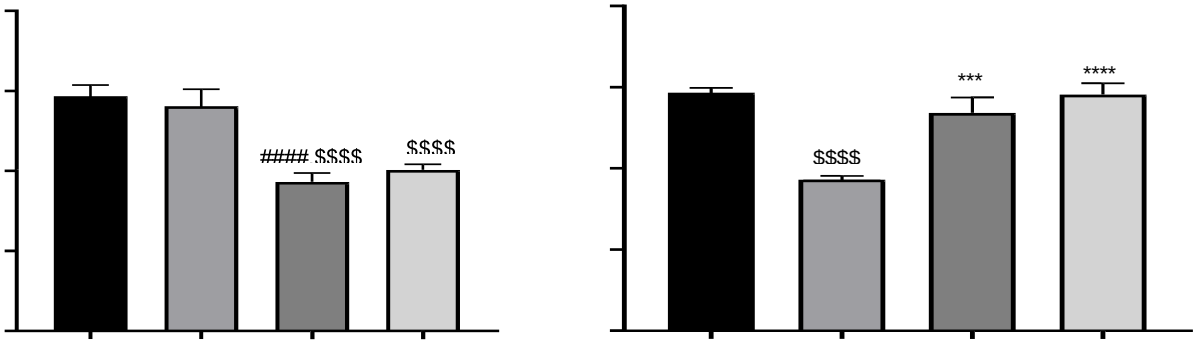
Effect of treatment of phloretin on serum creatinine in cisplatin treated perimenopausal mouse model



The creatinine level was significantly increase in the OVX+CIS treated group (upto 3.380±0.6550mg/dl) as compared to SHAM group (1.665±0.1498 mg/dl). In the PHL 20 mg/kg and PHL 60 mg/kg treated group (2.188±0.2095 mg/dl and 1.987±0.4529 mg/dl)

Serum Albumin Estimation:

Effect of cisplatin on serum albumin in cisplatin treated perimenopausal mouse model

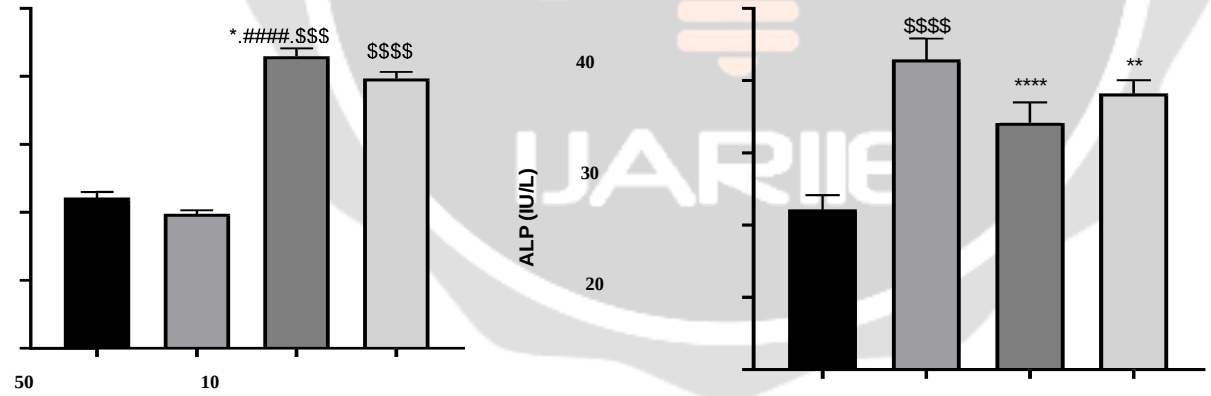


Data represented as mean±SEM and evaluated using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The p value <0.05 is considered as statistically significant. #### p<0.0001 is compared with OVX group; $$$$p<0.0001 is compared with SHAM group; $$$$p<0.0001 is compared with SHAM group. The values in the parenthesis are dose in mg/kg. OVX- Ovariectomy; CIS- Cisplatin

Effect of treatment of phloretin on serum albumin in cisplatin treated perimenopausal mouse model

Serum ALP Estimation:

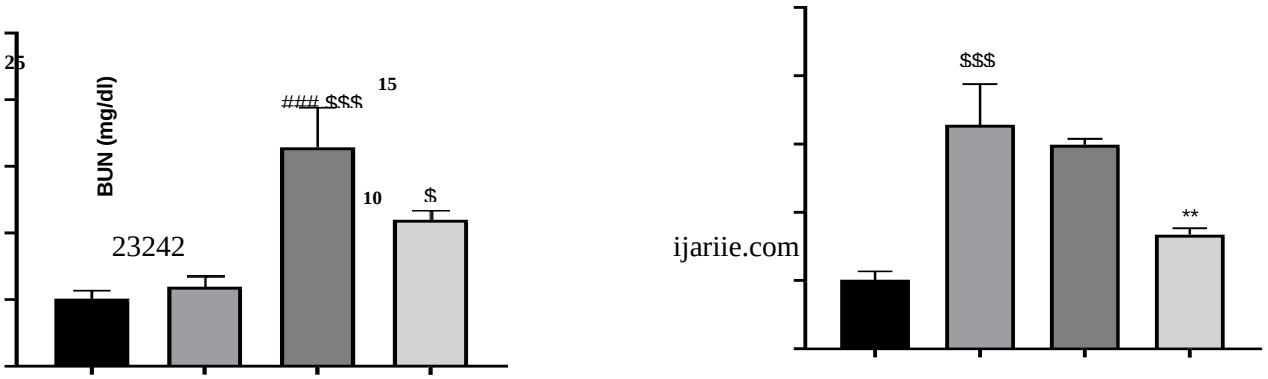
Effect of cisplatin on serum ALP in cisplatin treated perimenopausal mouse model



Data were represent as mean±SEM and evaluated using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The p value <0.05 is considered as statistically significant. ****p<0.0001 is compared with OVX+CIS group; **p<0.01 is compared with OVX+CIS group; $$$$p<0.0001 is compared with SHAM group. The value in the parenthesis are dose in mg/kg. OVX- Ovariectomy; CIS- Cisplatin, PHL- Phloretin.

Serum BUN Estimation:

Effect of cisplatin on serum BUN in cisplatin treated perimenopausalmouse model



20

Physical parameter

Body weight:

Fig no. 17 Body weigh

SHAM

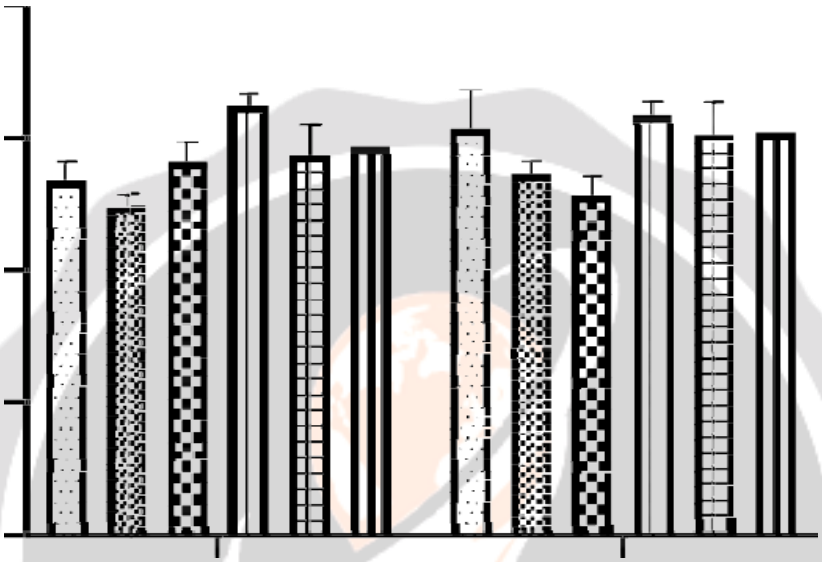
OVX

OVX+ CIS (5)

CIS (5)

PHL 20

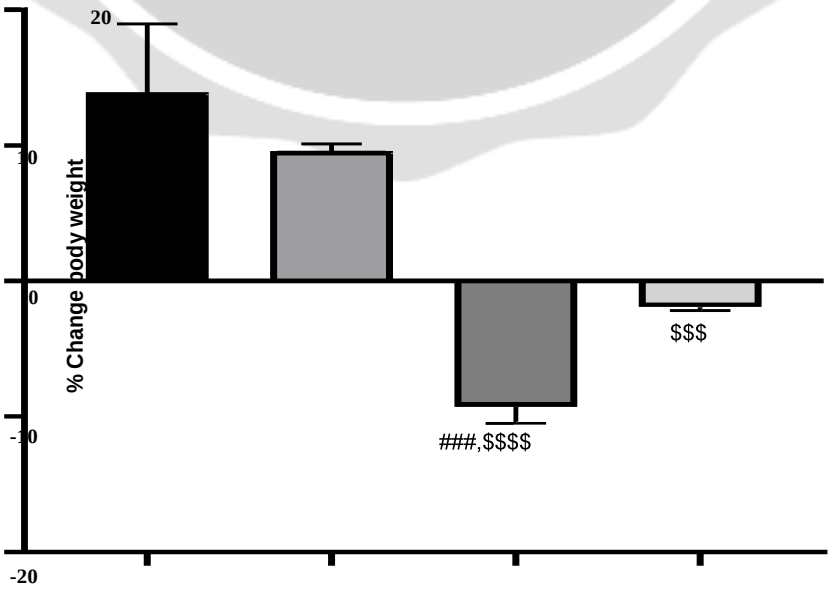
PHL 60



0 Day **7 Day**

Percentage (%) Change Body Weight:

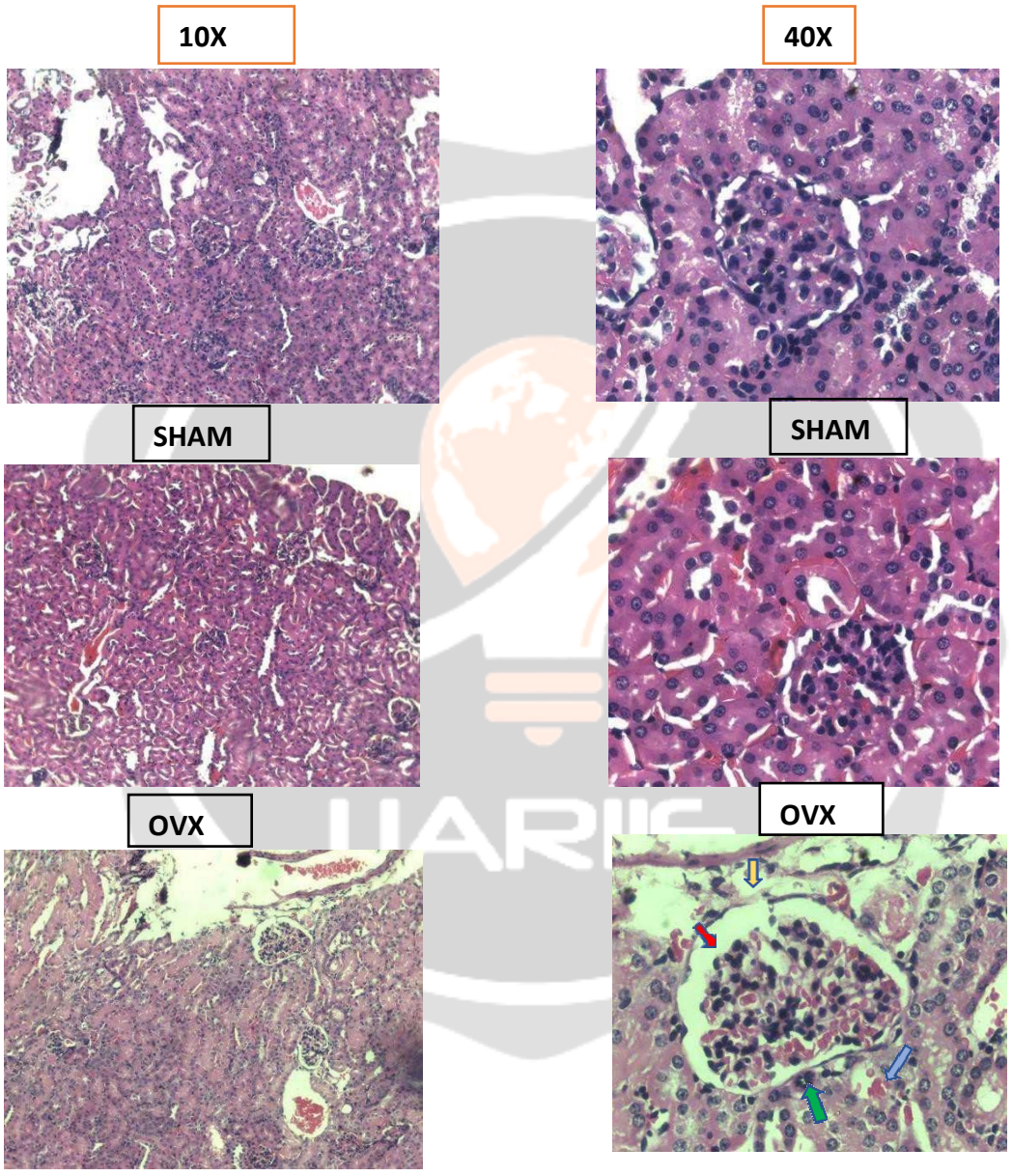
Fig no. 18 Effect of cisplatin on % change body weight in cisplatin treated perimenopausal mouse model

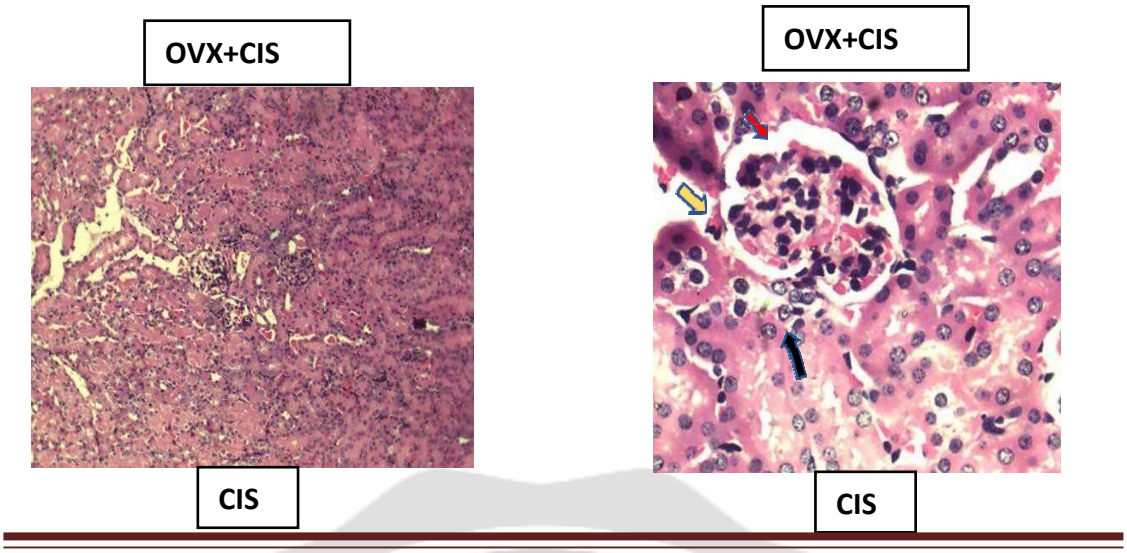


Treatment

Histopathology Assessment:

Fig no. 34 Effect of cisplatin on histopathological structure in mouse model of perimenopause

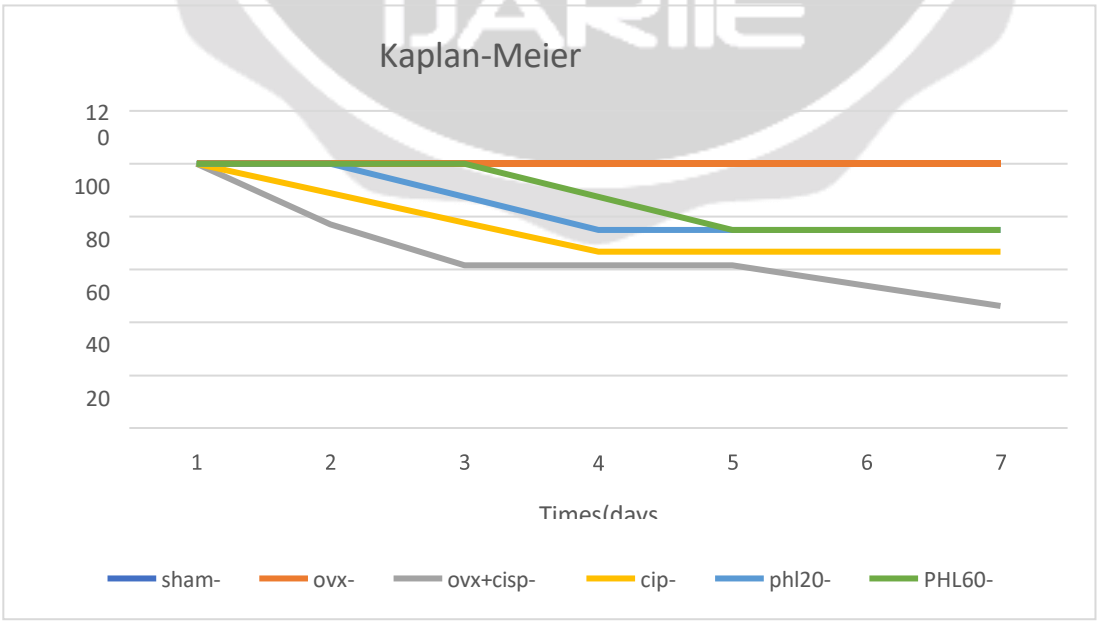




Kaplan-Meier Estimator (Mortality graph):

Table % Survived rate (Kaplan- Meier Estimator)

GROUP			DAYS				
	1	2	3	4	5	6	7
SHAM-7	100	100	100	100	100	100	100
OVX-8	100	100	100	100	100	100	100
OVX+CIS-13	100	76.92308	61.53846	61.53846	61.53846	53.84615	46.15385
CIS-9	100	88.88889	77.77778	66.66667	66.66667	66.66667	66.66667
PHL 20-8	100	100	87.5	75	75	75	75
PHL 60-8	100	100	100	87.5	75	75	75



Effect of phloretin on survival rate (kaplan-meier estimator)

Kaplan-Meier Estimator showed the no. of survived animal in respective days. the SHAM and OVX group animasl were showed the 100% survived rate i.e. no any deathwas occurred during the experimentation.

The OVX+CIS group showed the 54% of the mortality during experimentation and CIS group showed 33% survived rate.

Phlretin 20 mg/kg and 60 mg/kg showed 75% survived rate mean. Thus, study revealedthat the Phloretin treated group showed 29% higher survival also delay the onset of mortality as compared to OVX+CIS treated group. The mortality onset was day 3 andday 4 in the Phloretin 20mg/kg and 60 mg/kg group respectively.

	Serum				**KIDNEY**	**KIDNEY**
Group	**Creatinine**	**BUN**	**ALP**	**Albumin**	**Kidney weight**	**Histopath**
SHAM	N	N	N	N	N	--------
OVX	↓ ## (-28%)	↓ ### (+18%)	↓ #### (-11%)	↑ #### (-4%)	↑#### (-7%)	---------
OVX+CIS(5)	↑ $$ (+97%)	↑ $$$ (+224%)	↑ $$$$ (+93%)	↓ $$$$ (-36%)	$$$$ (-45%)	+++++
CIS(5)	↓ &↑ $ (+85%)	↓ & ↑ $ (+117%)	↓ * & $$$$ (+79%)	↑ & $$$$ (-31%)	↓ & ↑$$$$ (-39%)	++++
OVX+CIS+PHL 20	↓ (-35%)	↓ (-8%)	↓ **** (-20%)	↑ *** (+44%)	↑ * (+21%)	++
OVX+CIS+PHL 60	↓ (-41%)	↓ ** (-49%)	↓ ** (-11%)	↑ **** (+56%)	↑ (+14%)	++

group	Tissue homogenate		
	LPO	SOD	GSH
SHAM	N ##	N ##	(+34%)
OVX+CIS(5)	$$ (+141%)	$$ -(73%)	(-26%)
OVX+CIS+PHL20		(+30%)	(-49%)
OVX+CIS+PHL60	* (+46%)	(+48%)	(+58%)

Effect of phloretin on cisplatin treated perimenopausal mouse model

BIBLIOGRAPHY

01. Casanova, A.G., Hernández-Sánchez, M.T., López-Hernández, F.J., Martínez-Salgado, C., Prieto, M., Vicente-Vicente, L. and Morales, A.I., 2020. Systematic review and meta-analysis of the efficacy of clinically tested protectants of cisplatin nephrotoxicity. *European journal of clinical pharmacology*, *76*(1), pp.23-33.
02. Chen, W. Y. *et al.* (2017) 'Cisplatin nephrotoxicity might have a sex difference.an analysis based on women's sex hormone changes', *Journal of Cancer*, 8(19), pp. 3939–3944. doi: 10.7150/jca.20083.
03. Cornelison, T. L. and Reed, E. (1993) 'Nephrotoxicity and hydration management for cisplatin, carboplatin, and ormaplatin', *Gynecologic Oncology*,pp. 147–158. doi: 10.1006/gyno.1993.1184.
04. Cui, D. *et al.* (2020) 'Phloretin ameliorates hyperuricemia-induced chronic renal dysfunction through inhibiting NLRP3 inflammasome and uric acidreabsorption', *Phytomedicine*, 66, p. 153111. doi: 10.1016/j.phymed.2019.153111.
05. Karasawa, T. and Steyger, P. S. (2015) 'An integrated view of cisplatin-induced nephrotoxicity and ototoxicity', *Toxicology Letters*, 237(3), pp. 219–227. doi: 10.1016/j.toxlet.2015.06.012.
06. De Oliveira, M. R. (2016) 'Phloretin-induced cytoprotective effects on mammalian cells: A mechanistic view and future directions', *BioFactors*, 42(1),pp. 13–40. doi:

10.1002/biof.1256.

07. Ozkok, A. and Edelstein, C. L. (2014) 'Pathophysiology of cisplatin-induced acute kidney injury', *BioMed Research International*, 2014. doi: 10.1155/2014/967826.
08. Pabla, N. and Dong, Z. (2008) 'Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies', *Kidney International*, 73(9), pp. 994–1007. doi: 10.1038/sj.ki.5002786.
09. Peres, L. A. lbert. B. and da Cunha, A. D. anta. (2013) 'Acute nephrotoxicity of cisplatin: molecular mechanisms', *Jornal brasileiro de nefrologia : 'orgão oficial de Sociedades Brasileira e Latino-Americana de Nefrologia*, 35(4), pp. 332–340. doi: 10.5935/0101-2800.20130052.
10. Santos, N. A. G. *et al.* (2007) 'Cisplatin-induced nephrotoxicity is associated
11. Tanaka, H. *et al.* (1986) 'Histopathological study of human cisplatin nephropathy', *Toxicologic Pathology*, 14(2), pp. 247–257. doi: 10.1177/019262338601400215.
12. Ueki, M. *et al.* (2013) 'Curcumin ameliorates cisplatin-induced nephrotoxicity by inhibiting renal inflammation in mice', *Journal of Bioscience and Bioengineering*, 115(5), pp. 547–551. doi: 10.1016/j.jbiosc.2012.11.007.
13. Wang, Y. *et al.* (2020) 'Enhanced oral bioavailability and bioefficacy of phloretin using mixed polymeric modified self-nanoemulsions', *Food Science and Nutrition*, 8(7), pp. 3545–3558. doi: 10.1002/fsn3.1637.
14. Yao, X. *et al.* (2007) 'Cisplatin nephrotoxicity: A review', *American Journal of the Medical Sciences*, 334(2), pp. 115–124. doi: 10.1097/MAJ.0b013e31812dfe1e.
15. Zhang, L. *et al.* (2019) 'Establishment and evaluation of a novel mouse model of peri/postmenopausal depression', *Heliyon*, 5(2), p. e01195. doi: 10.1016/j.heliyon.2019.e01195.
16. Zhao, Y. yuan *et al.* (2020) 'Studies on pharmacokinetic properties and absorption mechanism of phloretin: In vivo and in vitro', *Biomedicine and Pharmacotherapy*, 132(October), p. 110809. doi: 10.1016/j.biopha.2020.110809.
17. Aebi, H. (1984) '[13] Catalase in Vitro', *Methods in Enzymology*, 105(C), pp. 121–126. doi: 10.1016/S0076-6879(84)05016-3.
18. Beydilli, H. *et al.* (2015) 'The effects of thymoquinone on nitric oxide and superoxide dismutase levels in a rat model of diazinon-induced brain

damage', *Studies on Ethno-Medicine*, 9(2), pp. 191–195. doi: 10.1080/09735070.2015.11905434.

19. Goyal, A. K. *et al.* (2016) 'Ameliorating reactive oxygen species-induced in vitro lipid peroxidation in liver, carbohydrate and DNA damage by Dendrocalamus hamiltonii different leaf extracts', *Chiang Mai Journal of Science*, 43(1), pp. 80–88.