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# EVALUATION OF HERBAL FORMULATION NEERI (NS-RF) FOR PROTECTIVE EFFECTS AGAINST HEAVY METAL INDUCED NEPHROTOXICITY IN RATS

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**ABSTRACT**

The present study is aimed to investigate the protective effects of Neeri: NS-RF (an herbal formulation developed for improving renal functions) on heavy metal (lead acetate) induced nephrotoxicity in Wistar albino rats. Lead acetate is considered as a major nephrotoxicity inducing agent causing direct damage through multiple pathways including oxidative stress. Lead acetate (8mg/kg *i.p.* for 6 weeks) induced significant oxidative stress and nephrotoxicity in rats; indicated by increased levels of serum creatinine, serum urea, urinary protein, urinary glucose; and reduced levels of serum albumin, serum total proteins, urinary creatinine; and also the structural damage in kidney. Co-treatment with NS-RF (1640 and 3280mg/kg, p.o. for 6 weeks) significantly prevent the altered serum and urinary biochemical parameters and histological renal tubular damages by lead acetate. Conclusively, NS-RF is a potent nephro-protective formulation protecting kidneys from nephrotoxins including oxidative damage induced by lead acetate.

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## INTRODUCTION

Lead is a heavy metal distributed into the environment from natural and human actions, resulting from either agricultural or industrial pollution. Humans get exposed to lead through canned food, drinking water, paints, smoking etc. and by inhalation in case of occupational setting.[1,2] Lead poisoning is also known as saturnism, plumbism or painter's colic.[3] Children and women are found to be most vulnerable to lead poisoning especially in developing countries.[4] Both industrial exposure (lead smelters, accumulator battery industry) and non-industrial exposure (leaded gasoline, lead based paints) of lead leads to various toxicities like renal toxicity,[3,5] reproductive toxicity, [6,7] neurotoxicity, [8,9] hepatotoxicity,[10] carcinogenicity,[11] hypertension,[12] immune system impairment.[13] Among these, nephrotoxicity is the major toxicity induced by lead.[14] Nephrons are the structural and functional unit of kidney which is an important excretory organ and is directly affected by heavy metals through blood circulation. The potent nephrotoxic effect of lead includes proximal tubular dysfunction, development of nuclear inclusion bodies, interstitial fibrosis, necrotic tubule and renal failure.[15] In spite of tremendous advances in modern medicine, traditional herbal medicines have been practiced with utmost advantage for patients."Neeri" a polyherbal formulation developed by Aimil Pharmaceuticals India Ltd., consists of number of herbs like *Bergenia ligulata, Vernonia cinerea, Boerhaavia diffusa, Crataeva nurvala, Rheum emodi, Tribulus terrestris* etc. and used for various kidney disorders. Individually many of these herbs are scientifically screened and reported to possess rejuvenative, nephroprotective, antiurolithiatic potential along with diuretic, anti-inflammatory and anti-microbial activities.

The present study is focused to evaluate the NR-SF for claimed nephroprotective potential by using lead acetate induced nephrotoxicity model in rats.

## MATERIALS AND METHODS

### Experimental Animals

Healthy adult albino Wistar rats (150-250g) of either sex, aged 4-5 months, procured from Panacea Biotec Ltd, Lalru (India), were housed in polypropylene cages. They were kept in standard laboratory conditions (temperature 25±2 ºC with 12/12h light/dark cycle). Animals were fed with standard rat pellet diet (Sanjay Biological Museum, Amritsar) and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) [Protocol No. IAEC/2012/III/0014(PCL-M)] of CT Institute of Pharmaceutical Sciences, Jalandhar (Punjab), India under the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment & Forests, New Delhi. After one week of acclimatization to laboratory, the animals were used for experimental work.

### Chemicals

Lead acetate was purchased from Merck Specialties Pvt. Ltd. (India) and Vitamin E from Hi Media Laboratories Pvt. Ltd. (India). Thiobarbituric acid (TBA) was purchased from Avarice Laboratories Pvt Ltd. (India) and Trichloroacetic acid (TCA) from Thermo Fisher Scientific Pvt Ltd. (India). Assay kits for estimation of creatinine, urea, albumin, total protein and glucose were purchased from ERBA Diagnostic Mannheim GmbH, (Germany). All chemicals used were of analytical grade.

### Herbal Formulation

The herbal formulation Neeri (NS-RF) is an innovative polyherbal formulation especially developed, for improving renal functions and treating various renal disorders, by Aimil Pharmaceutical India Ltd. (New Delhi) India and is obtained as gift sample for conducting this research.

### Preliminary Phytochemical Screening

The formulation was subjected to preliminary phytochemical screening for the presence of alkaloids, carbohydrates, flavonoids, glycosides, proteins, tannins, terpenoids, saponins etc. as per the standard procedures.[16]

### Induction of nephrotoxicity

Nephrotoxicity was induced in rats by single daily dose of lead acetate (8mg/kg, *i.p.*) for 6 weeks. The solution of lead acetate was prepared freshly in distilled water.

### Experimental Protocol

The 25 albino Wistar rats of either sex were divided into five groups, each containing five animals. Group I served as normal control and received normal saline. Group II served as negative control group and received lead acetate (8mg/kg i.p.) once daily, for 6 weeks. Group III served as standard pre-treated with Vitamin E (100mg/kg; in olive oil) orally for 5 days and then concurrently lead acetate was administered once daily with Vitamin E (100mg/kg) for 6 weeks. Group IV and V served as test group and received pre-treatment with NS-RF (1640mg/kg, 3280mg/kg respectively) orally for 5 days, then lead acetate once daily for 6 weeks with concurrent administration of NS-RF (1640 & 3280 mg/kg) upto 6 weeks respectively.

### Biochemical Estimation to Assess Nephroprotective Potential

### Urine Analysis

After 6 weeks of experimental period, rats were individually placed in metabolic cages for 24 hours and urine samples were collected. The urine samples were analysed for quantitative estimation of creatinine, glucose and protein using autoanalyzer.

**Serum Analysis**

After 6 weeks of experimental period, blood samples were collected by retro-orbital sinus puncture and serum was separated by centrifuging at 3000 rpm for 15minutes using micro-centrifuge. The serum samples were then quantitatively analysed for creatinine, urea, albumin and total protein levels using autoanalyser. Lipid peroxidation was determined by the method described by Stocks and Dormandy (1971).[17]

**Histopathological Analysis**

For histopathological evaluation of kidneys, rats were sacrificed by cervical dislocation at the end of experiment. Kidneys were removed, washed with cold saline and preserved in 10% formalin in buffered form and subsequently dehydrated in graded alcohol. After embedding in paraffin 4µM sections were cut, stained with hematoxylin and eosin. Now the slides were examined at the magnification of 450X using a laboratory light microscope attached with camera.[18]

**Statistical Analysis**

Tall data were expressed as mean±SEM (standard error of mean). Statistical differences between groups were analysed using One-Way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison tests using GraphPad Prism 5. $P<0.05$ was considered to be significant, $P<0.01$ considered to be more significant and $P<0.001$ considered to be highly significant.

**RESULT AND DISCUSSION**

The continuous exposure to the heavy metal i.e. lead has been well-known to induce nephrotoxicity and acute high doses also involved in impairment of proximal and distal tubular function. Continued exposure can cause toxic stress on kidneys and often leads to irreversible damages to nephron.[19] As a measure of renal functions, serum creatinine, serum urea, serum protein, serum albumin, urine creatinine, urine protein, urine glucose are often regarded as reliable markers.[20-24]

In the present study, lead induced nephrotoxicity was established by once daily intraperitoneal injection of lead acetate (8mg/kg) for 6 weeks. This toxicity is characterized by marked ($p<0.001$) elevations in levels of serum creatinine (1.70±0.09), serum urea (51±1.39), urinary glucose (18.30±0.52), urinary proteins (2.25±0.17) and decrease in levels of serum total protein (4.18±0.12), serum albumin (2.10±0.11) and urinary creatinine (9.14±0.15) in comparison to normal group (Table 1 and 2).

**Table 1: Effect of NS-RF on Serum Biochemistry of Rats.**

| Groups | Creatinine (mg/dl) | Urea (mg/dl) | Total Protein (g/dl) | Albumin (g/dl) |
|---|---|---|---|---|
| Normal | 0.58±0.06*** | 28±1.03*** | 6.70±0.27*** | 5.0±0.28*** |
| Lead acetate (8mg/kg) | 1.70±0.09 | 51±1.39 | 4.18±0.12 | 2.10±0.11 |
| Vitamin E (100mg/kg) | 1.03±0.17** | 45±1.06** | 4.74±0.19 | 3.04±0.25* |
| NS-RF (1640mg/kg) | 1.04±0.09** | 32±1.23*** | 5.40±0.15*** | 3.46±0.22** |
| NS-RF (3280mg/kg) | 0.75±0.13*** | 29±0.96*** | 5.92±0.14*** | 4.30±0.20*** |

Values are expressed as mean±SEM. (n=5). *indicates significance at $P<0.05$, **indicates significance at $P<0.01$ and *** indicates significance at $P<0.001$as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test.

**Table 2: Effect of NS-RF on Urine Biochemistry of Rats.**

| Groups | Protein (g/dl) | Creatinine (mg/dl) | Glucose (mg/dl) |
|---|---|---|---|
| Normal | 0.08±0.01*** | 15.34±0.40*** | 0.20±0.045*** |
| Lead acetate (8mg/kg) | 2.25±0.17 | 9.14±0.15 | 18.30±0.52 |
| Vitamin E (100mg/kg) | 1.77±0.04** | 9.98±0.15 | 15.34±0.41* |
| NS-RF (1640mg/kg) | 0.36±0.01*** | 11.52±0.40*** | 12.14±1.27*** |
| NS-RF (3280mg/kg) | 0.23±0.01*** | 13.70±0.30*** | 7.40±0.57*** |

Values are expressed as mean±SEM. (n=5). *indicates significance at $P<0.05$, **indicates significance at $P<0.01$ and *** indicates significance at $P<0.001$as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test.

However, these changes were found to be attenuated by pretreatment with once daily oral doses of Neeri (NS-RF) for 5 days followed by co-treatment with lead acetate for 6 weeks. NS-RF (3280 mg/kg/day p.o.) very significantly ($p<0.001$) prevented elevation in serum concentration of creatinine (Fig. 1), urea (Fig. 2) and reduction in the concentrations of total serum protein (Fig. 3), serum albumin (Fig. 4) and also prevented elevation of urinary protein (Fig. 5), urinary glucose (Fig. 7) and reduction of urinary creatinine (Fig. 6), maintaining their values within near normal range and quite superior to that with standard anti-oxidant, Vitamin E (100mg/kg/day) treated group.

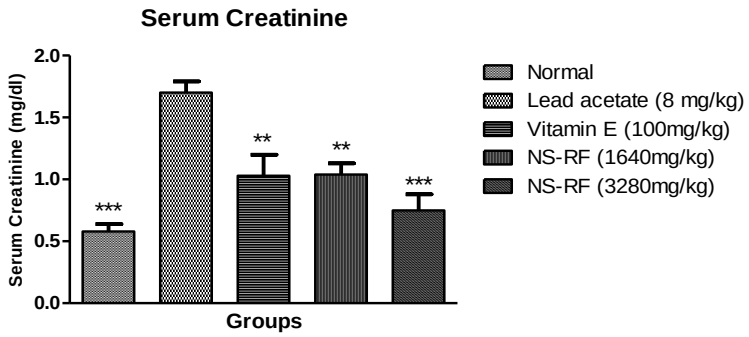


**Fig. 1: Effect of NS-RF on serum creatinine level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the increased serum creatinine level with the values being near to normal range.**

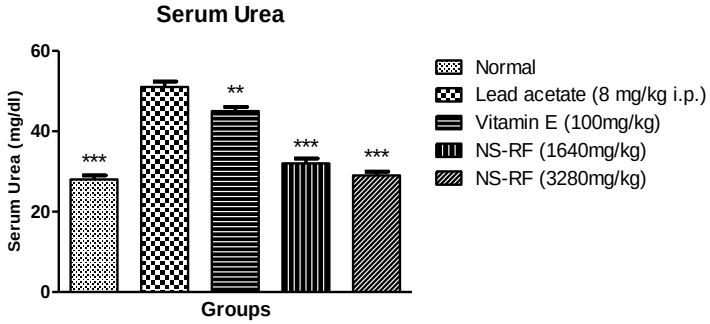


**Fig. 2: Effect of NS-RF on serum urea level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the increased serum urea level with the values being near to normal range.**

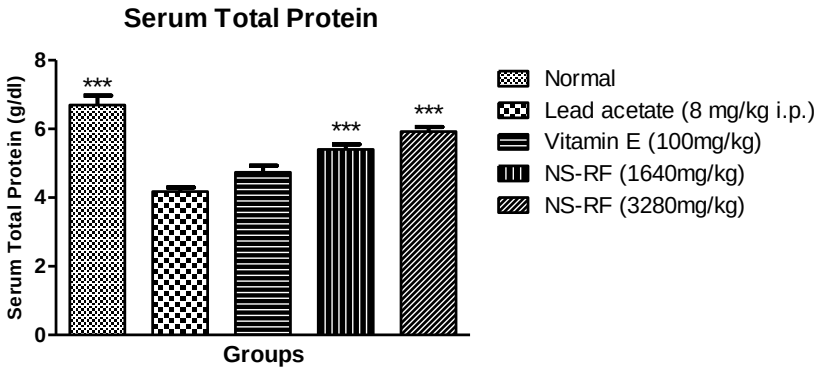


**Fig. 3: Effect of NS-RF on serum total protein level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the decreased serum total protein level with the values being near to normal range.**

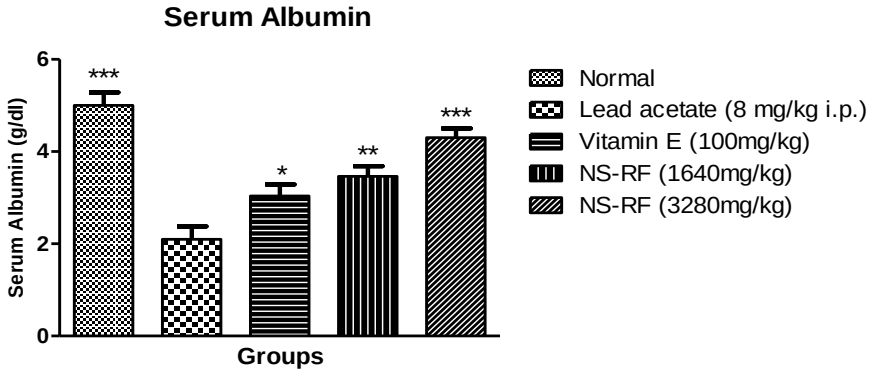


**Fig. 4: Effect of NS-RF on serum albumin level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the decreased serum albumin level with the values being near to normal range.**

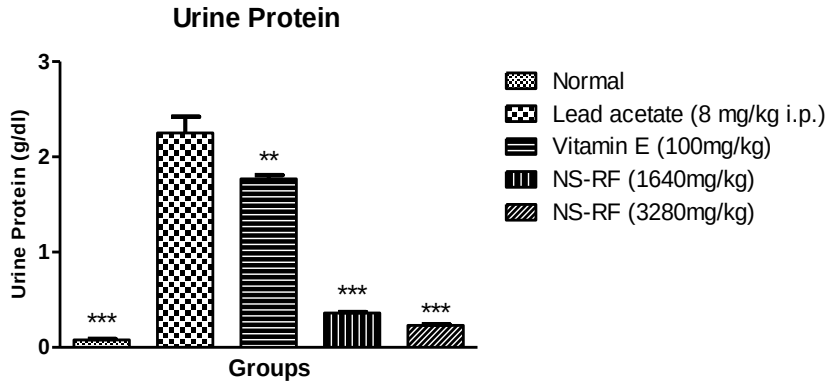


**Fig. 5: Effect of NS-RF on urinary protein level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the increased urinary protein level with the values being near to normal range.**

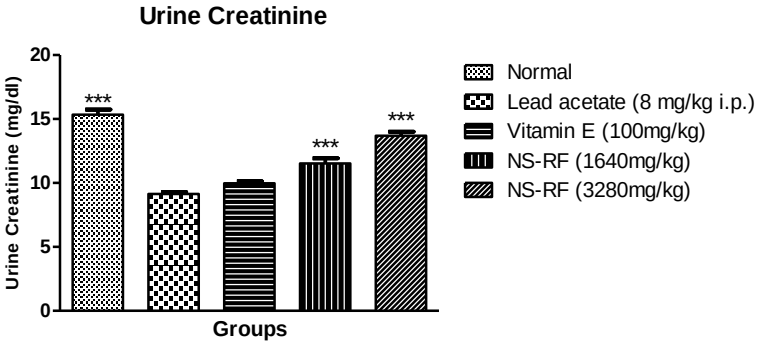


**Fig. 6: Effect of NS-RF on urinary creatinine level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the decreased urinary creatinine level with the values being near to normal range.**

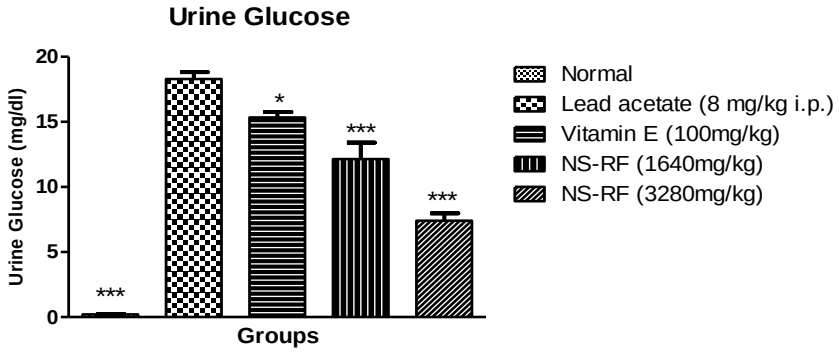


**Fig. 7: Effect of NS-RF on urinary glucose level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the increased urinary glucose level with the values being near to normal range.**

All these results showed that polyherbal formulation NS-RF has nephroprotective effect and preliminary phytochemical screening showed that it contained multiple active principals like flavonoids, alkaloids, glycosides, carbohydrates, proteins, tannins, steroids and amino acids. The exact mechanism(s) involved for neproprotective effect is not very clear, though it might be due to presence of various phytoconstituents like alkaloids, flavonoids, tannins, steroids etc. as these are already reported to have nephroprotective action. Tannins are claimed to have cleansing and astringent effect, thus may have renotoning activity. *Solanum nigrum (Makoi)*, containing alkaloidal glycosides, solamargine and solasonine, has been claimed to exert significant protective effect on kidney cells from toxic effects due to gentamicin. The observed cytoprotection is due to increase in activity of free-radical scavenging enzymes, counteracting free radicals.[26] The same cytoprotection may be considered in-this case of lead induced nephrotoxicity.

Lead causes nephrotoxicity via several mechanisms and oxidative stress is one which has been suggested to have significant role. It has also been reported that oxidative stress may induce injury of proximal convoluted tubule of nephron.[27] The antioxidant level were significantly decreased and TBARS content increased in lead nephrotoxicity (Table 3) suggesting an increased oxidative stress and lipid peroxidation. However, highly significantly lower lipid peroxidation levels recorded in lead+NS-RF (0.209±0.0144 and0.098±0.0143 in 1640mg/kg & 3280mg/kg treated groups respectively) is attributable to free radical scavenging ability of NS-RF (Fig. 8), that prevents membrane lipid per-oxidation and also subsequent depletion of antioxidant enzymes and the activity being quite comparable to that with standard anti-oxidant, vitamin E (0.188±0.044), due to lead induced nephrotoxicity (0.338±0.0173).

**Table 3: Effect of NS-RF on Anti-Oxidant Profile (based on TBARS assay).**

| Groups | Lipid peroxidation (Absorbance at 532nm) |
|---|---|
| Normal | 0.002±0.003*** |
| Lead acetate (8mg/kg) | 0.338±0.0173 |
| Vitamin E (100mg/kg) | 0.188±0.044*** |
| NS-RF (1640mg/kg) | 0.209±0.0144** |
| NS-RF (3280mg/kg) | 0.098±0.0143*** |

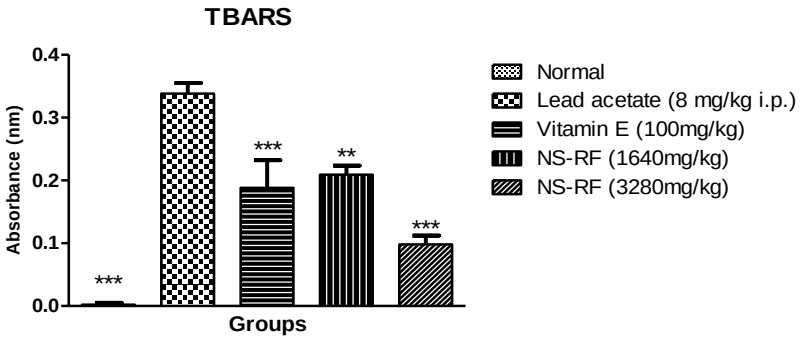


**Fig. 8: Effect of NS-RF on TBARS level. *indicates significance at P<0.05, **indicates significance at P<0.01 and *** indicates significance at P<0.001as compared to lead acetate treated group, using one way ANOVA followed by Dunnett's multiple comparison test. NS-RF (3280 mg/kg) treated group showed significant prevention in the increased TBARS level.**

Nephroprotective potential of NS-RF can be due to its free radical scavenging antioxidant property, taking into account that flavonoids and many other compounds like alkaloids, tannins, steroids etc. isolated from various nephroprotective medicinal plants have been reported to inhibit xenobiotics induced nephrotoxicity in experimental animal models, due to their potent free radical scavenging effects.[28] It is also possible for various active principles contained in NS-RF to be responsible for nephroprotective effect. The aqueous extract of *Crataeva nurvala* (Varuna) extract was effective in significantly altering the kidney dysfunction by decreasing the concentration of blood urea nitrogen, creatinine and lipid peroxidation in cisplatin induced nephrotoxicity thus indicating the anti-oxidative property of Varuna.[29] *Tribulus terrestris* is reported to have protective effect on cadmium induced renal and hepatic damage. It significantly decreases the blood urea nitrogen and serum creatinine levels.[30]

This is further supported by histopathological examination of kidney. The kidney sections of normal group *i.e.* group I (Fig. 9 A) showed normal tubular brush border, intact glomerulus and Bowman's capsule while the kidney of group II (Fig. 9 B) *i.e.* lead acetate treated group showed the deranged and necrosed tubular cells. However, the kidney of NS-RF (1640 and 3280 mg/kg) treated group (Fig. 9 D and E respectively) showed normalized tubular cells which are comparably better than vitamin E treated group (Fig. 9 C). Also Neeri (NRT-RF) has been proved to show antiurolithiatic activity in rats.[31]

In light of the above text, the test drug formulation NS-RF exhibits significant nephroprotective and antioxidant potential. This might be due to presence of multiple active constituents as the said formulation is a polyherbal formulation. These constituents are claimed to be potent anti-inflammatory and anti-microbial, thus protecting nephrons from conditions like nephritis and probability of infections. Further since the beginning of time, treatment using herbal formulations has been a favorite tool of naturopathically inspired practitioners and approximately 75% of the global population, including that from most of the developing world, depends on herbal medicines for their basic healthcare needs as herbal medicines are not only inexpensive but are better compatible with human body and have fewer side effects.[32]

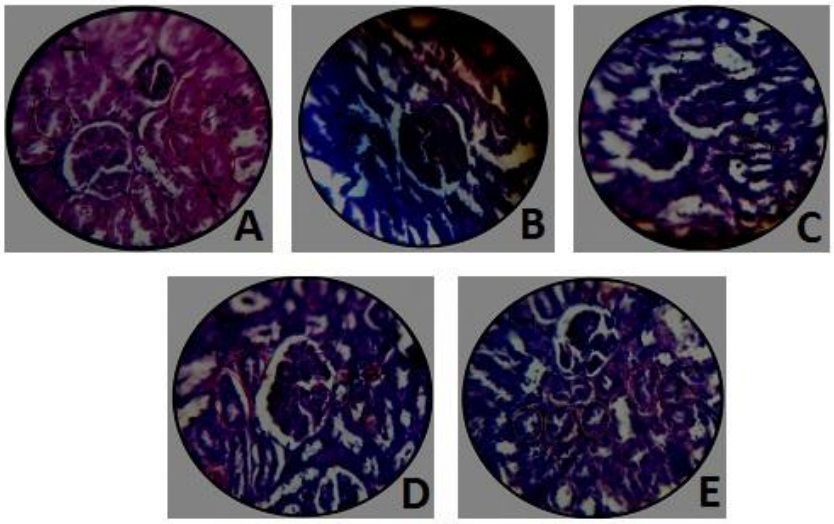


**Fig. 9 Photomicrograph of histology of rat kidney tissues examined at 450X magnification.**

(A) Normal group (I) showing normal proximal convoluted tubule (PCT), distal convoluted tubule (DCT).
(B) Negative Control (Group II, Lead acetate 8mg/kg, *i.p.*) showing necrosed PCT and DCT with deranged cell structure.
(C) Positive Control (Group III), Vitmain E (100mg/kg) + lead acetate (8mg/kg) showing dilated PCT and DCT cells
(D) Test (Group IV) NS-RF (1640mg/kg) + lead acetate (8mg/kg) showing dilated PCT and DCT membrane lining.
(E) Test (Group V) NS-RF (3280mg/kg) + lead acetate (8mg/kg) indicating prevention of necrosis with near normal PCT and DCT as compared to negative control.

Present study has been conducted for 6 weeks only, as per the IAEC approved protocol but the observations suggested that although NS-RF showed highly significant nephroprotective activity with the biochemical values being quite near to those of normal group, still 6 weeks experimentation is not sufficient and restoration in case of nephrotoxicity is not up to the normal values in this much time of experimentation. Thus, it is also suggested to conduct such studies for at least 3 months in order to observe if the restoration is up to normal values or less than that.

This study is the first scientific report that scrutinizes nephroprotective potential of NS-RF and also validates its use against dysuria and urinary disorders.

## CONCLUSION

Neeri (NS-RF) showed nephroprotective effect in animal studies. The observed effect of NS-RF may be due to multiple active constituents that may prevent lipid peroxidation-induced renal damage and direct deleterious effects caused by lead acetate on nephron. In case of nephrotoxicity, NS-RF treatment significantly prevents alterations and maintains the various serum and urinary biochemical parameters towards normal range. Further, NS-RF (3280mg/kg) has been found to possess pronounced anti-oxidant activity quite comparable with that of the standard anti-oxidant drug, Vitamin E and hence provides nephroprotective activity against oxidative stress induced by lead acetate (as assessed with TBARS assay). Further, significantly higher nephroprotectrive activity of NS-RF has been observed as compared to the standard anti-oxidant drug Vitamin E,which signifies that nephroprotective activity of NS-RF is not only due to anti-oxidant activity but also due to comprehensive, multiple nephroprotective mechanisms against lead acetate induced oxidative stress and direct deleterious effect on nephrons. It might be due to additive/synergistic interaction of different herbal drug components present in NS-RF having multiple nephroprotective, diuretic and antioxidant potential. The present study suggests that NS-RF has a comprehensive protective effect against lead acetate induced nephrotoxicity in rats and it simultaneously improves kidney biochemical parameters probably by increasing the functional capacity of nephrons.

## ACKNOWLEDGEMENT

Authors are thankful to Aimil Pharmaceutical India Ltd., New Delhi for providing free gift samples of polyherbal formulation NS-RF developed for improving renal functions to conduct this study and also for providing its own library, internet & testing facilities that were quite helpful in procuring relevant literature.

## Conflict of Interest

Authors declared no conflict of interest.

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