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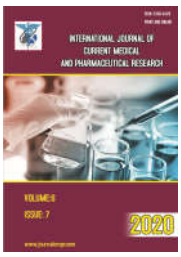

Research Article

EFFECT OF AQUEOUS EXTRACT OF *Piper betle* LEAVES ON DRUG INDUCED NEPHROTOXICITY IN ALBINO RATS

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ABSTRACT

Background: Gentamicin, an aminoglycoside antibacterial agent, is nephrotoxic as it can cause tubular necrosis if not properly monitored and its toxicity remains a major problem in clinical use. The drug is often used for inducing nephrotoxicity in experimental animal models. The study was undertaken to evaluate the effect of aqueous extract of *Piper betle* against gentamicin induced nephrotoxicity in albino rats.
Methods: A total of 25 albino rats were divided into five groups of five each. Group I (Normal control) received 0.1 ml normal saline intramuscularly (day 1–5) and 2% gum acacia orally (day 1–12). Group II (Toxic control), Group III (Standard control), Group IV (Test I), Group V (Test II) received injection gentamicin 80 mg/kg intramuscularly (day 1– 5). Group III (Standard control) received N-acetylcysteine 40 mg/kg orally (day 1–12). Group IV and Group V received aqueous extract of *Piper betle* 200 mg/kg and 400 mg/kg respectively per orally (day 1-12). Serum urea, creatinine and total protein levels were estimated and histopathology of renal tissues were compared among the groups.
Results: The renal biomarkers (serum urea, creatinine, total protein) significantly improved in *Piper betle* treated groups. The histopathological study also supported the findings.
Conclusion: Aqueous extract of *Piper betle* revealed protective effect against gentamicin induced nephrotoxicity.

INTRODUCTION

The kidneys play a central role in homeostatic control of volume status, blood pressure, plasma electrolyte composition, acid-base balance, excretion of nitrogenous and other waste products.[1] Drug introduced in the body has to undergo certain metabolism. In the process, alteration in renal function is one of the most commonly encountered effect as the kidneys are exposed to drug and its metabolites during the excretion process.[2] Gentamicin is a systemic aminoglycoside group of antibiotic. It is active mainly against aerobic gram-negative and gram positive bacterial infection and is a potent nephrotoxic agent. Gentamicin induced nephrotoxicity is characterized by direct tubular necrosis apparently related to its preferential accumulation in the renal convoluted tubules and its effect on biological membrane. Studies show that nephrotoxicity is caused by inhibition of an intracellular lysosomal phospholipase A_2 in the renal brush border. This leads to lysosomal distension, rupture and release of acid hydrolases, and free aminoglycosides into the cytosol.[3] Reactive oxygen species are considered to be important mediators in gentamicin induced nephrotoxicity. Abnormal production of reactive oxygen species directly damages macromolecules and induces cellular injury via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. Hence, the administration of several compounds with antioxidant activity has been successfully used to prevent or ameliorate gentamicin induced nephrotoxicity.[4] Since thousands of years, plants have been utilized traditionally to treat many diseases before researchers realised its potentials in medicine. One such plant is *Piper betle,* belongs to piperaceae family, which is extensively found in the tropical and subtropical countries. In Ayurveda, *Piper betle* juice is commonly used as an adjuvant and combined with different other medicines. In the Sushruta samhita, betel leaves have been described as aromatic, sharp, hot, acrid and valuable for voice. It is also a laxative and appetizer.[5] The plant has been found to be useful in anorexia, dyspepsia, colic, flatulence, skin diseases, leprosy, haemorrhages, chronic ulcer, tumours, haemorrhoids, epilepsy,

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convulsion, fractures and swelling. It has also been used in the treatment of filariasis, cough, asthma, orchitis, arthritis, mastitis, eczema and cut wounds.[6] The plant contains proteins, carbohydrates, fats, minerals, fibers and other compounds like tannins, essential oils, phenols, alkaloids, vitamin C, niacin, thiamine and riboflavin. Aqueous extract of *Piper betle* leaves shows strong antioxidant property by virtue of its flavonoids and tannin compounds.[7]

Even though the herb has been used for the treatment of various ailments, there is still paucity of scientific data to support the various uses. Considering the role of reactive oxygen species to cellular injury and the antioxidant property of the aqueous extract, the present study has been undertaken to evaluate nephroprotective properties of aqueous extract of *Piper betle* in suitable experimental model in albino rats.

METHODS

Approval of Institutional Animal Ethics Committee (IAEC)

The study was conducted in the Department of Pharmacology, Regional Institute of Medical Sciences, Imphal after getting approval of the Institutional Animal Ethics committee, RIMS, Imphal (No.1596/GO/a/12/CPCSEA).

Set up

Department of Pharmacology and Department of Pathology, Regional Institute of Medical Sciences (RIMS), Imphal, Manipur from September 2016 to August 2018.

Sample size

25 healthy wistar albino rats.

Requirements

Albino rats, polypropylene cages, feeding tubes, distilled water, soxhlet apparatus, plant extract, mixer grinder, evaporating dish, gum acacia, gentamicin injection (80 mg) – Abbott healthcare pvt. Ltd., Haryana, N-acetylcysteine tablet (600 mg) – Tab. Mucinac, Cipla Ltd. (Batch no. E1631), urea, creatinine and total protein estimation kit- Meril diagnostics Pvt. Ltd, Gujarat, diethyl ether- Merck Specialities Pvt. Ltd. Mumbai, semi-auto analyzer- Robonik (India) Pvt. Ltd, Navi Mumbai, diethyl ether, haematoxylin and Eosin stain.

Inclusion Criteria

Healthy adult wistar albino rats with baseline serum levels of urea, creatinine and total protein within the range of 10-42 mg/dl, 0.4-1.3 mg/dl and 6-8.2 g/dl respectively.

Exclusion Criteria

Albino rats with higher or lower baseline serum levels of the above blood parameters, pregnant and lactating albino rats.

Preparation of aqueous extract

The fresh leaves of *Piper betle* was purchased from Lamphel market, Imphal, Manipur in the month of October, 2016. The plant was identified and authenticated by Professor P. K. Singh, Department of Life Sciences, Manipur University, (Voucher no. MUMP 0761). The leaves were cleansed with water, air dried under shade and made into coarse powder by mixer grinder. Aqueous extract of *Piper betle* (AEPB) was prepared by soxhlet extraction method described by Verma SCL and Agrawal SL.[8] 50 grams of the coarse powder was extracted with distilled water. The dried extract was scraped out, weighed and stored in airtight container. The yield was 17%.

Acute oral toxicity study

For acute oral toxicity study, Limit test was done in female albino rats according to OECD guideline 423.[9] Healthy adult rats were acclimatized for 5 days at room temperature and natural light:dark cycle. Limit test with 2000 mg/kg of AEPB was carried out in six animals (three animals per step). After administration of the extract, food was withheld for 4 hours. Animals were observed once during the first 30 minutes for 4 hours then daily for 14 days. All 6 rats were healthy and there was no mortality observed at the dose of 2000 mg/kg till 14 days. So a working dose of 200 mg/kg ($1/10^{th}$ of maximum test dose) and 400 mg/kg ($1/5^{th}$ of maximum test dose) of the extract were selected for the study.

Phytochemical studies

Qualitative phytochemical analysis of AEPB were done using standard techniques.

Outcome Measure

The following parameters were compared in control and test groups to see the effect of the plant extract

1. Serum levels of urea, creatinine and total protein.
2. Body weight difference (Final body weight – Initial body weight).
3. Kidney to final body weight ratio of rats (kidney-somatic index).
4. Histopathological changes of the kidneys.

Experimental animals

Healthy albino rats of either sex (3-6 months) weighing 100-210 g were obtained from the Animal House, RIMS, Imphal. These animals were acclimatized to the laboratory conditions for 7 days before the experiment. The animals were housed in the departmental animal room in groups in polypropylene cages at room temperature with natural light and dark cycle. They were housed in groups of 5 animals per cage and maintained on a standard animal diet with water *ad libitum.*

Experimental design

Animals were categorized and treated as follows

Table 1 Allotment of animals to different groups and their treatment

Groups	Drugs given orally: Gum acacia/NAC/AEPB	Drugs given i.m: Inj. Gentamicin/Normal saline
Group I (Normal control)	2% gum acacia (day 1^{st} - 12^{th})	Normal saline (day 1^{st} – 5^{th})
Group II (Toxic control)	2% gum acacia (day 1^{st} - 12^{th})	Gentamicin 80 mg/kg (day 1^{st} – 5^{th})
Group III (Standard control)	NAC 40 mg/kg (day 1^{st} - 12^{th})	Gentamicin 80 mg/kg (day 1^{st} – 5^{th})
Group IV (Test I)	AEPB 200 mg/kg (day 1^{st} - 12^{th})	Gentamicin 80 mg/kg (day 1^{st} – 5^{th})
Group V (Test II)	AEPB 400 mg/kg (day 1^{st} - 12^{th})	Gentamicin 80 mg/kg (day 1^{st} – 5^{th})

AEPB/NAC was dissolved in 2% gum acacia to make suspension. Normal saline was given at 0.2 ml i.m. 2% gum acacia, NAC (N-acetylcysteine) and AEPB (aqueous extract of *Piper betle*) suspension were given at 1 ml/100 gm body weight.

AEPB and NAC, given to all groups were made as suspension in 2% gum acacia and a volume of 1 ml/100 g body weight was administered by feeding tube.

Blood collection

First blood samples were drawn before any drug was given to the animals to assess the baseline biochemical parameters and blood samples were collected again on the 13^{th} day after 24

hours of last dose of AEPB. Animals were anaesthetised with ether. Blood was collected from the retro-orbital venous sinus.[10] About 2 ml of blood from each animal was collected in a plain vacutainer from all groups. These blood samples were kept at room temperature for 30 minutes to coagulate. The blood was then centrifuged at 3000 r.p.m. for 10 min. The serum separated was kept in refrigerator at maintained temperature of 4°C for biochemical estimation of above-mentioned parameters.

Biochemical estimations

Serum biochemical parameters were assessed using commercially available test kits in semi-auto analyzer as per the instructions described in the kits. Serum creatinine, serum urea and total protein were estimated by using Jaffe's method, urease-glutamate dehydrogenase method and biuret method respectively.[11]

Determination of relative kidney-somatic index (kidney to final body weight ratio)

Kidney-somatic index = (wt. of 2 kidneys ÷ wt. of the rat at the end of experiment) x 10^{-3}

Histopathological examination

Animals were sacrificed with high dose of ether. A middle abdomino-thoracic incision was performed. The kidney tissue samples were dissected out and washed in ice cold saline, dried on filter paper, weighed immediately and fixed in 10% formalin for 24 hours.[12] Then, the paraffin sections were prepared (automatic tissue processor, auto-technique) and cut into 5 µm thick sections in a rotary microtome.[13] The sections were stained with haematoxylin and eosin (H & E)[14] and observed under light microscope for histopathological comparison among different groups of rats. Histopathology of the parameters[15] like glomerular congestion, glomerular atrophy, tubular degeneration, tubular necrosis, tubular dilatation, hyaline protein casts, interstitial leucocytic infiltration were assessed. The above parameters were scored as none (-), mild (+), moderate (++), and severe (+++).

Disposal of animal carcasses

The animal carcasses were buried deep into the ground covered with lime and disinfectants after the experiment by laboratory attendant.[16]

Data management and Statistical analysis

Datas were checked for consistency and completeness. After that, datas were analysed using SPSS version 21 IBM. Findings in the different treated groups were analysed statistically by One way analysis of variance (ANOVA) followed by Bonferroni test and *p* value <0.05 was considered as significant.

RESULTS

Acute toxicity study

No sign of toxicity or mortality was observed up to 2000 mg/kg after 14 days of extract treatment.

Phytochemical screening

Phytochemical analysis of aqueous extract of *Piper betle* (AEPB) revealed presence of active constituents like tannins, flavonoid, protein, amino acids, reducing and non-reducing sugars, saponins, steroids, alkaloids, triterpenoids, terpenoids and the absence of gums, glycosides and hydroxyl anthraquinone.

Body weight, kidney weight and kidney-somatic index

Gentamicin treatment significantly ($p<0.05$) altered the body weight, kidney weight and kidney somatic index as compared to normal control rats. AEPB produced significant ($p<0.05$) improvement in gentamicin induced body weight changes and kidney-somatic index. The extract also reduced ($p<0.05$) the increased kidney weight in the gentamicin treated rats.

Table 2 changes in body weight, kidney weight and kidney-somatic index of different groups

Groups	Initial body wt. (g)	Final body wt. (g)	Wt. Difference (Final-initial) (g)	Wt. Of kidneys (g)	Kidney-somatic index($\times 10^{-3}$)
Normal	164.41±7.00	190.15±5.96	25.74±2.56	1.26±0.04	6.64±0.05
Toxic	177.51±8.45	132.90±7.37	-44.61±7.58*	1.54±0.04*	11.62±0.09*
Standard	173.27±9.40	159.40±8.77	-13.87±2.71*†	1.43±0.03	8.98±0.05*†
Test I	163.90±8.29	131.88±6.97	-32.02±2.03*‡‡	1.38±0.03††	10.46±0.05*†‡
Test II	176.37±8.46	153.82±7.87	-23.18±2.76*††	1.49±0.04**	9.66±0.05*†‡§
ANOVA					
F	0.657	9.40	42.441	5.938	63.425
df	6	6	6	6	6
p	>0.05	<0.001	<0.001	<0.001	<0.001

Values are expressed as mean ± SEM, n=5, One way ANOVA (SPSS21), *P<0.001 when compared to normal, **P<0.01 when compared to normal, †P<0.001 when compared to toxic, ††P<0.05 when compared to toxic, ‡P<0.001 when compared to standard, ‡‡P<0.01 when compared to standard, §P<0.001 when compared to Test I. "-" symbol indicates reduction in weight.

Biochemical parameters of gentamicin induced nephrotoxicity

Gentamicin administered groups revealed significantly elevated serum urea, creatinine and reduced serum total protein levels when compared to normal group. AEPB and NAC treated rats showed significant ($P<0.05$) reduction in serum levels of urea, creatinine and elevation in serum total protein.

Table 3 Effect of AEPB on serum urea, creatinine and total protein in gentamicin induced nephrotoxicity

Groups	Serum urea (mg/dl)	Serum creatinine (mg/dl)	Serum total protein (g/dl)
Normal	22.56±1.79	0.78±0.03	7.69±0.20
Toxic	61.54±2.34*	2.68±0.09*	4.35±0.19*
Standard	35.17±0.95*†	1.38±0.09*†	6.54±0.20**†
Test I	51.93±0.51*††‡	2.16±0.08*††‡	5.03±0.18*‡
Test II	46.91±1.14*†‡	1.69±0.07*†§	5.91±0.12*†§
ANOVA			
F	88.03	62.98	38.42
df	6	6	6
P	<0.001	<0.001	<0.001

Values are expressed as mean ± SEM, n=5, One way ANOVA (SPSS21), *P<0.001 when compared to normal, **P<0.01 when compared to normal, †P<0.001 when compared to toxic, ††P<0.01 when compared to toxic, ‡P<0.001 when compared to standard, §P<0.01 when compared to Test I.

Kidney histology

Gross examination

The size, shape, colour, contour and surface of the intact kidney were normal without any significant change in the normal control, toxic control, NAC and extract treated groups (Figure 1).

Photomicrograph

The processed histological slides were examined and renal histological parameters of glomerular atrophy, tubular necrosis, tubular dilatation, hyaline cast, interstitial lymphocytic infiltration, blood vessel congestion were observed, and scoring was done as none (-), mild (+), moderate (++) and severe (+++).[17] (Table 4 and Figure 2).

Table 4 Histopathological scoring for severity of renal tissue damage

Histological parameters	Normal	Toxic	Standard	Test I	Test II
Glomerular atrophy	-	++	-	-	-
Tubular necrosis	-	+++	-	-	-
Tubular dilatation	-	+++	+	+	+
Hyaline cast	-	+++	-	++	+
Interstitial lymphocytic infiltration	-	++	-	+	-
Blood vessel congestion	-	++	-	+	-

Figure 1 gross kidney specimen of rats.

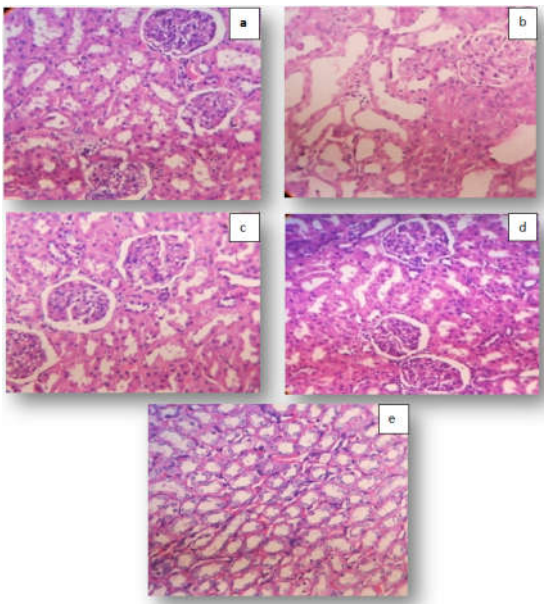

Figure 2 Photomicrograph of kidney tissues a) Normal control, b) Toxic control, c) Standard control, d) Test I, e) Test II. Stain: H and E, Magnification: 400 X.

DISCUSSION

A large number of animal models have been developed to mimic the clinical conditions of renal injury. Rats have been chosen for this experiment because of close resemblance of anatomy of viscera, physiology and biochemistry to that of human. Gentamicin is commonly used antibacterial for the treatment of life threatening gram negative infections. Because of its nephrotoxic potential, gentamicin is often used for drug induced nephrotoxicity in experimental animals.[17,18]

In our study, gentamicin administered rats showed significant decrease in body weight and increase in kidney weight. The reduction in body weight could be due to increased catabolism seen in renal tubular injury, acidosis induced impaired dehydration and anorexia. Increased kidney weight could be due to inflammation, oedema and oxidative stress after gentamicin administration.[19] Treatment with aqueous extract of *Piper betle* (AEPB) significantly reduced kidney weight and kidney-somatic index.

Creatinine is a non-protein waste product of creatine phosphate metabolism of muscles, filtered by the glomerulus of the kidney and it is considered as one of the most important biomarkers of kidney injury. The level of creatinine increases in the circulation if there is decrease in renal filtration process. The elevation of serum creatinine concentration is therefore an indication of reduced glomerular filtration rate (GFR) and renal dysfunction.[20]

Urea is a waste product of metabolism of protein. It is dissolved into blood, transported and excreted by the kidneys. Serum urea levels are found to be increased in renal parenchymal tissue injury. Therefore, serum urea is also considered as an important biomarker of glomerular filtration. Serum total protein also correlates well with the metabolic status of the body. In gentamicin induced acute renal toxicity, there is a state of increased state of catabolism which is reflected by decreased serum total protein level.

In our study, it was observed that AEPB and NAC treatment significantly ameliorate the elevated levels of serum creatinine and urea, and increased serum total protein level significantly. Higher dose of AEPB (400 mg/kg) showed more favourable changes in biomarkers than the lower dose (200 mg/kg).

Histopathological study and comparison of renal section is considered gold standard parameter in evaluating nephrotoxic studies and to assess the preventive interventions taken up.[21] Nephrotoxic features seen in gentamicin administered rats are consistent with other similar studies.[22] Treatment with AEPB and NAC markedly reduced pathological changes of the kidney as evidenced by less tubular dilatation, absence of tubular necrosis, reduced glomerular atrophy and reduced hyaline casts. Moreover, treatment with AEPB significantly reduced gentamicin-induced increase in the kidney-somatic index. Considering our observations and the fact that nephrotoxicity induced by gentamicin largely involves high renal oxidative stress, the phytochemicals with anti-oxidant properties like tannins, flavoinoids and alkaloids of AEPB might have contributed in the alleviation of gentamicin induced renal structural and functional abnormalities.

Our study had some limitations. In-vivo antioxidant activity studies like reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) activity and catalase (CAT) activity in renal tissues were not

assessed. Other biomarkers of nephrotoxicity like clusterin, kidney injury molecule-1, cystatin-c, serum albumin and serum uric acid levels were not done. Urine volume measurement and urine analysis for micro proteinuria, leucocytes count, RBC count, urine urea and creatinine levels were also not measured. However, the kidney biomarkers assessed and kidney histopathology provided substantial evidence of nephroprotection offered by the aqueous extract of *Piper betle*.

CONCLUSION

In gentamicin induced nephrotoxic groups, aqueous extract of *Piper betle* significantly reduced the levels of serum urea, serum creatinine and significantly increased the level of serum total protein and improved the histopathological features. The study suggested that, aqueous extract of *Piper betle* possesses a good nephroprotective property. Biochemical and histopathological studies confirmed the nephroprotective role. However, further studies are needed to elucidate the exact mechanism of action of nephroprotection offered by its phytoconstituents and its clinical application to prevent or cure renal injury, dysfunction and diseases.

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