

# *Hibiscus plantinifolius* Ameliorates Renal Oxidative Damage Induced by Gentamicin in Rats: By Targeting Membrane Bound enzymes

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## ABSTRACT

**Objective:** Present study evaluates the effect of *Hibiscus plantinifolius* leaves on kidney function in rats using gentamicin-induced nephrotoxic animal model. **Material and Methods:** *Hibiscus plantinifolius* leaves powder was extracted with 95% methanol and performed phytochemical screening. Antioxidant activity was determined by Superoxide anion scavenging, Hydrogen peroxide-scavenging, 1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity, Fe<sup>2+</sup>ascorbate induced lipid peroxidation methods. Acute toxicity was studied as per OECD 423 guidelines. A total 30 male rats weighing about 150-180 gm were taken and after acute toxicity studies three different doses (75, 150 and 300 mg/kg bd.wt) were selected. The rats were divided into five groups; first group was kept as control (Sodium CMC), Second as toxic control (Gentamicin 100 mg/kg bd.wt.) and remain groups as test I, test II and test III given selected doses of methanolic extract of *Hibiscus plantinifolius* leaves. After 15 days of treatment, serum was separated and subjected for estimation of creatinine, BUN, uric acid, urea, total proteins, minerals (Na<sup>+</sup> and K<sup>+</sup>), collected urine for estimation of minerals (Na<sup>+</sup> and K<sup>+</sup>), kidney homogenate supernatant used for determination of antioxidants like SOD, GSH, MDA membrane and bound enzyme function such as H<sup>+</sup>ATPase, Na<sup>+</sup>/K<sup>+</sup>ATPase, Ca<sup>2+</sup>ATPase, Mg<sup>2+</sup>ATPase, Total ATPase. Further histopathological studies were also performed. **Results:** Phytochemical screening revealed that presence of flavonoids, carbohydrates, glycosides, fixed oils, fats, tannins, phenolic compounds and alkaloids. Extract was showed significant neutralize free radicals of DPPH, OH<sup>\*</sup>, SO<sup>\*</sup>, Fe<sup>2+</sup> LPO \* with IC<sub>50</sub> 350 µg/ml, 900 µg/ml, 780 µg/ml and 580 µg/ml respectively. Acute toxicity studies results revealed extract has no toxicity (LD<sub>50</sub> > 5000 mg/kg). MEHP showed a significant (\*P < 0.05) decrease creatinine, BUN, uric acid, urea, total proteins, Na<sup>+</sup> (\*\*P < 0.01), K<sup>+</sup> (\*\*P < 0.01), significant increase urinary Na<sup>+</sup> (\*\*P < 0.01), K<sup>+</sup> (\*\*P < 0.01), significant (\*P < 0.05) increase H<sup>+</sup>ATPase, Na<sup>+</sup>/K<sup>+</sup>ATPase, Ca<sup>2+</sup>ATPase, Mg<sup>2+</sup>ATPase, Total ATPase when compared to gentamicin treated rats. **Conclusion:** In the current work, Methanolic extract of *Hibiscus plantinifolius* leaves demonstrated nephroprotective activity against gentamicin induced nephrotoxicity. The mechanism of protection may be due to augmentation of cellular antioxidants and also improve membrane bound enzyme activity.

**Key words:** Methanolic extract of *Hibiscus plantinifolius* (MEHP), Gentamicin, Membrane bound enzymes, Antioxidants.

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## INTRODUCTION

Aminoglycosides are exhibited dose dependent non-oliguric acute renal failure in 10–25% of therapeutic courses manifested by decrease concentric urine, tubular proteinuria, lysosomal enzymuria, mild glucosuria, alterations in electrolyte levels, decrease

ammonium excretion, depression of glomerular filtration rate, and increased serum creatinine and blood urea nitrogen (BUN).<sup>1-3</sup> It was strongly suggested that the nephrotoxicity is an indication of altered mitochondrial function as a result of oxidative stress.<sup>4</sup>



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Indeed, gentamicin has been shown to enhance the generation of superoxide anion and hydrogen peroxide by renal cortical mitochondria.<sup>5</sup> Scientific approaches have been attempted to protect gentamicin-induced nephrotoxicity. Amongst them, antioxidant agents were shown to consistently ameliorate and protect rats against this toxicity.<sup>6</sup> In fact, extracts of several plants endowed with free radical scavenging activity have been shown to produce reliable reduction of gentamicin-induced nephrotoxicity.<sup>7-11</sup>

*Hibiscus plantinifolius* Linn (Malvaceae) known as Maple leaved mallow is an ayurvedic plants with important medicinal properties. It is an evergreen tall tree, leaves are alternate, simple, stipulate, petiolate and ovate to lanceolate, often with a toothed or lobed margin. In ayurvedic literature of India, supported that parts of plant have been recommended as remedy for various ailments like hyperlipidemic, diabetes, hypertension and liver disorders as antidotes to poisoning chemicals, Hibiscus petals are useful for thicker hair growth and to prevent premature graying, hair loss and scalp disorders,<sup>12</sup> antioxidants, anti-diabetic and hypolipidemic activity were also reported.<sup>13</sup> A number of active principles from this plant have been identified which include taraxeryl acetate, beta sitosterol, campestral, stigma sterol, cholesterol, ergosterol, lipids, citric, tartaric and oxalic acids, fructose, glucose, sucrose, flavonoids and flavonoid glycosides.

Despite their nephrotoxicity, aminoglycosides are still considered to be important agents for the treatment of life-threatening infections due to their bactericidal efficacy, synergism with  $\beta$ -lactam agents, low cost, limited bacterial resistance and a post antibiotic effect.<sup>14</sup> There is thus a need for intervention that could improve their safety profile thereby increasing the quality of life of patients treated with aminoglycosides. To the best of our knowledge there were no any scientific reports available in support of its traditional claim of *Hibiscus plantinifolius* on nephroprotective potential. Therefore, by taking into lime light the traditional use of *Hibiscus plantinifolius*, present study was designed to demonstrate the effect of methanolic extract *Hibiscus plantinifolius* leaves against gentamicin induced renal damage in experimental animals.

## MATERIALS AND METHODS

### Wistar albino rats

The rats were maintained under natural lighting conditions (12 h light and 12 h dark cycle) with temperature of 22-25°C and relative humidity of approximately 50%. The rats were fed on pellet diet and water *ad libitum*.

All rats were handled according to CPCSEA and the protocol was approved by institute animal ethical committee (439/PO/01/a/CPCSEA).

### Plant Materials

Fresh leaves of *Hibiscus plantinifolius* was collected in Vaddeswaram, Guntur district, Andhra Pradesh, India and authenticated by the botanist, Mrs. P. Prasanna Kumari, Head of the Department of Botany, D.N.R (A) College, Bhimavaram, Andhra Pradesh, India.

### Preparation of extracts

*Hibiscus plantifolius* leaves were collected, shade dried and powdered. The powdered plant material was macerated with methanol for seven days, filtrate was concentrated by condensed distillation and obtained extract was subjected to phytochemical and pharmacological investigation.

### Phytochemical investigation of the plant extract

Phytoconstituents estimation carried out by prescribed chemical procedure.<sup>15,16</sup>

### Acute oral toxicity

The acute oral toxicity was carried out as per the OECD, 423 guideline.

### In-vitro antioxidant

Superoxide anion scavenging activity, Hydrogen peroxide-scavenging activity, 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity, Fe<sup>2+</sup>ascorbate induced lipid peroxidation.<sup>17,18,19,20,21</sup>

### Experimental design for the nephroprotective activity of methanolic extract of *Hibiscus plantinifolius* leaves against gentamicin induced nephrotoxic wistar albino rats.

Wistar rats were divided into 5 groups of six each (n=6).

**Group-I:** Normal control rats (1ml/kg of 1% Sd. CMC) for 15 days.

**Group-II:** Gentamicin control rats (100 mg/kg/day/ i.p) for 15 days

**Group III:** MEHP (75 mg/kg/day p.o) + Gentamicin (100 mg/kg/day/i.p) for 15 days

**Group-IV:** MEHP (150 mg/kg/day p.o) + Gentamicin (100 mg/kg/day/i.p) for 15 days.

**Group-V:** MEHP (300 mg/kg/day p.o) + Gentamicin (100 mg/kg/day/i.p) for 15 days.

### Sample collection

Twenty-four hours later the last treatment, treated rats were slightly anesthetized with diethyl ether and blood was collected into polyethylene tubes by punching the vein plexus of the retro-orbital sinus.<sup>22</sup> The samples

were left at room temperature for 30 min for coagulation and then centrifuged at 3000 rpm for 15 min at 4°C cooling centrifuge to separate serum. Serum was used for estimation of creatinine, Urea, uric acid, BUN, Na<sup>+</sup> and K<sup>+</sup>. After that animals were placed in metabolic cage, for collection of urine which was subjected for the estimation of Na<sup>+</sup> and K<sup>+</sup>.

Rats were sacrificed by cervical dislocation under ether anesthesia. The abdominal cavity was immediately opened and both kidneys were removed, homogenate, supernatant subjected for estimation of antioxidants such as SOD, GSH, catalase, oxidative molecule MDA, membrane bound enzymes H<sup>+</sup> ATPase, Na<sup>+</sup>/K<sup>+</sup> ATPase, Ca<sup>2+</sup> ATPase, Mg<sup>2+</sup> ATPase, Total ATPase and also other kidney was fixed in 10% formalin for histopathological examination.<sup>23</sup>

### Statistical Analysis:

The values were expressed as Mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. \*\*\*P <0.001, \*\*P<0.01, \*P <0.05 considered as significant.

## RESULTS

### Phytochemical investigation

The phytochemical analysis of MEHP leaves showed the presence of different groups of phytochemicals such as carbohydrates, glycosides, fixed oils, fats, tannins, phenols, flavonoids, alkaloids.

### In vitro antioxidant activity

Methanolic extract of *Hibiscus plantinifolius* leaves showed significant dose dependent free radical scavenging activity against SO<sup>\*</sup>, OH<sup>\*</sup>, DPPH<sup>\*</sup> and Fe<sup>2+</sup> LPO<sup>\*</sup> free radicals with IC<sub>50</sub> of 780 µg/ml, 900 µg/ml, 300 µg/ml and 580 µg/ml, respectively (Figure 1).

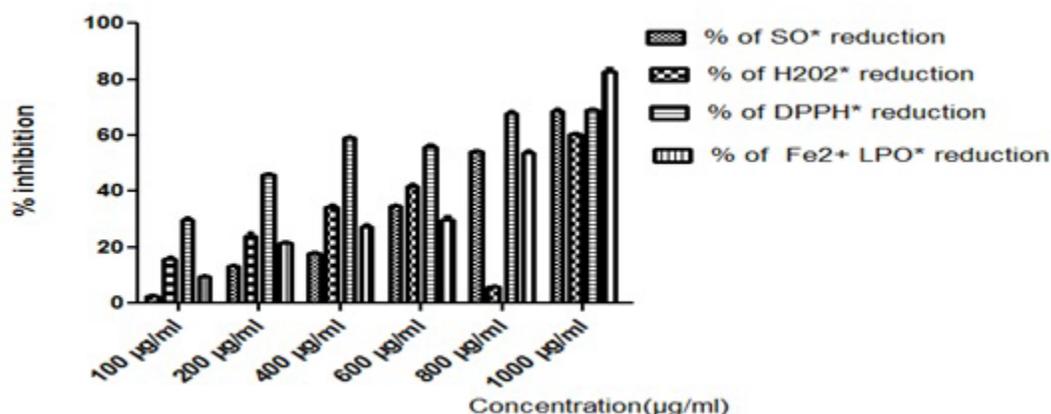


Figure 1: In vitro antioxidant activities of methanolic extract of *Hibiscus plantinifolius* leaves.

### Acute toxicity

The acute toxicity test result of this study documented that the methanolic extract of *Hibiscus plantinifolius* leaves was safe by oral route at a dose of 2000 mg/kg. After 72 hrs of treatment, there were no significant changes in behavior such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, and coma and in appearance of the rats. There was no mortality within 14 days of observations and lethal dose (LD<sub>50</sub>) is considered to be greater than 2000 mg/kg.

### Effect of methanolic extract of *Hibiscus plantinifolius* leaves on serum parameters and urine volume in gentamicin induced nephrotoxicity.

In gentamicin control rats, there was a significant increase serum creatinine (P<0.001\*\*\*), urea (P<0.001\*\*\*), BUN (P<0.001\*\*\*), uric acid (P<0.01\*\*), decrease total protein (P<0.001\*\*\*) and urine volume (P<0.001\*\*\*) as compared to normal control rats. Upon administration of MEHP of 300 mg/kg, significant decrease serum creatinine (P<0.05\*), urea (P<0.05\*), BUN (P<0.05\*), uric acid (P<0.05\*) and increase total protein (P<0.05\*) urine volume (P<0.05\*) when compare with gentamicin treated rats (Table 1).

### Effect of methanolic extract of *Hibiscus plantinifolius* leaves on serum and urine electrolytes in gentamicin induced nephrotoxicity.

In Gentamicin treated rats, there was significant increase serum Na<sup>+</sup> (P<0.05\*) decrease K<sup>+</sup> levels (P<0.05\*), decrease urinary Na<sup>+</sup> levels (P<0.001\*\*\*) increase urinary K<sup>+</sup> (P<0.001\*\*\*) levels as compared to control rats. In contrast, treatment with extract attenuated this alters, while significant decrease Na<sup>+</sup> (P<0.05\*), increase K<sup>+</sup> levels (P<0.05\*) and increase urinary Na<sup>+</sup> (P<0.05\*), decreased K<sup>+</sup> (P <0.05\*) as compared with gentamicin treated rats (Table 2).

**Table 1: Effect of methanolic extract of *Hibiscus plantinifolius* leaves on serum parameters and urine volume in gentamicin induced nephrotoxicity.**

Drug Treatment	Creatinine (mg %)	Urea (mg/dl)	BUN (mg/dl)	Uric acid (mg/dl)	Total protein (g/lit)	Urine volume (ml)
Normal control rats	0.85±0.53	42.8±0.58	19.9±0.58	3.2±0.25	7.1±0.35	25.1±0.47
Gentamicin control rats	3.3±0.64 <sup>a***</sup>	63.2±0.26 <sup>a**</sup>	29.5±0.38 <sup>a**</sup>	9.3±0.56 <sup>a***</sup>	3.5±0.43 <sup>a**</sup>	15.3±0.58 <sup>a***</sup>
MEHP 75mg/kg	2.7±0.56	55.6±0.36	26.4±0.45	8.5±0.34	4.8±0.59	19.1±0.68
MEHP 150mg/kg	2.1±0.45	50.4±0.35	23.5±0.34	6.6±0.65	5.0±0.83	20.2±0.94
MEHP 300mg/kg	1.6±0.37 <sup>b*</sup>	47.6±0.2 <sup>b*</sup>	22.2±0.47 <sup>b*</sup>	4.8±0.34 <sup>b*</sup>	6.0±0.59 <sup>b*</sup>	22.0±0.27 <sup>b**</sup>

P<0.001<sup>a\*\*\*</sup>, P<0.01<sup>a\*\*</sup>, P<0.05<sup>b\*</sup> a) Compare to Normal control rats, b) compare to Gentamicin control rats

**Table 2: Effect of methanolic extract of *Hibiscus plantinifolius* leaves on serum and urine electrolytes in gentamicin induced nephrotoxicity.**

Drug Treatment	Serum Ions (mMol/lit)		Urine Ions (mMol/lit)	
	Na <sup>+</sup>	K <sup>+</sup>	Na <sup>+</sup>	K <sup>+</sup>
Normal control rats	140.6±0.26	3.6±0.48	180±0.37	106±0.56
Gentamicin control rats	159.3±0.35 <sup>a**</sup>	3.0±0.49 <sup>a**</sup>	34±0.82 <sup>a***</sup>	278±0.38 <sup>a***</sup>
MEHP 75mg/kg	153.4±0.24	3.1±0.43	65±0.65 <sup>b***</sup>	223±0.56
MEHP 150mg/kg	148.7±0.47	3.3±0.46	92±0.56 <sup>b***</sup>	192±0.67
MEHP 300mg/kg	145.4±0.36 <sup>b**</sup>	3.4±0.45 <sup>b**</sup>	150±0.76 <sup>b**</sup>	136±0.12 <sup>b**</sup>

P<0.001<sup>a\*\*\*</sup>, P<0.01<sup>a\*\*</sup>, P<0.05<sup>b\*</sup> a) Compare to Normal control rats, b) compare to Gentamicin control rats

**Table 3: Effect of methanolic extract of *Hibiscus plantinifolius* leaves on antioxidant enzyme in gentamicin induced nephrotoxicity.**

Drug Treatment	SOD (µg/mg of protein)	GSH (µg/mg of protein)	MDA (ng/ mg of protein)
Normal control rats	9.2±0.45	6.3±0.37	76.2±0.49
Gentamicin control rats	4.1±0.23 <sup>a***</sup>	2.5±0.95 <sup>a***</sup>	141.0±0.58 <sup>a***</sup>
MEHP 75mg/kg	5.3±0.67	3.6±0.57	121.1±0.89
MEHP 150mg/kg	6.8±0.68	4.5±0.94	108.3±0.78
MEHP 300mg/kg	8.7±0.47 <sup>b*</sup>	5.1±0.37 <sup>b*</sup>	87.3±0.46 <sup>b*</sup>

P<0.001<sup>a\*\*\*</sup>, P<0.01<sup>a\*\*</sup>, P<0.05<sup>b\*</sup> .a) Compare to Normal control rats, b) compare to Gentamicin control rats

### Effect of methanolic extract of *Hibiscus plantinifolius* leaves on antioxidant enzyme in gentamicin induced nephrotoxicity.

In Gentamicin control rats, there was a significant decrease in tissue SOD (\*\*P<0.01), GSH (\*\*P<0.01) compared to control rats. In contrast, treatment with MEHP (300 mg/kg bd.wt.) showed a significant (\*P<0.05) increase in tissue SOD and GSH levels as compared to gentamicin control rats (Table 3).

### Effect of methanolic extract of *Hibiscus plantinifolius* leaves on membrane bound enzymes in gentamicin induced nephrotoxicity.

In Gentamicin control rats, the membrane bound enzymes such as H<sup>+</sup> ATPase, Na<sup>+</sup> K<sup>+</sup> ATPase, Ca<sup>+</sup> ATPase, Mg<sup>2+</sup> ATPase, Total ATPase activity was signif-

icant (\*\*P<0.001) decrease compared to control rats. Extract treatment was significant (\*P<0.05) increase membrane bounded enzyme activity was H<sup>+</sup> ATPase, Na<sup>+</sup> K<sup>+</sup> ATPase, Ca<sup>+</sup> ATPase, Mg<sup>2+</sup> ATPase and Total ATPase as compared to gentamicin control rats (Table 4).

### Histopathology studies

The Kidney of the control rats were showed normal glomerular apparatus, renal parenchyma on another hand gentamycin rats showed marked tubular glomerular necrosis, that was recovered by extract treated rats (Figure 2).

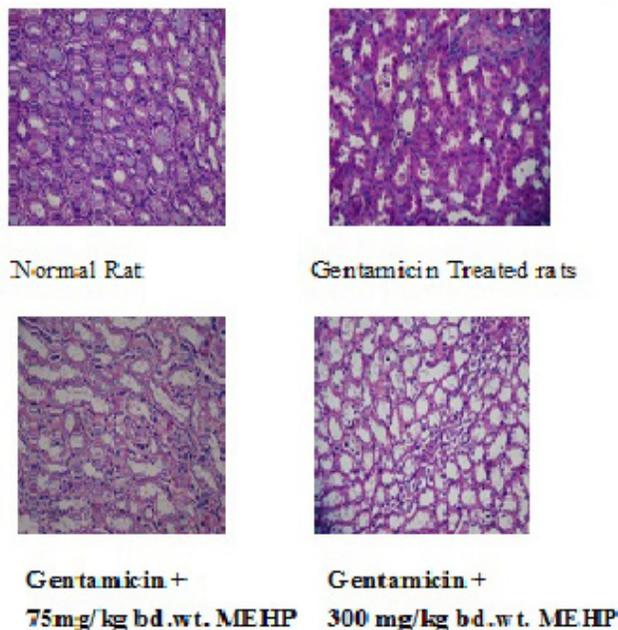
### DISCUSSION

Although the use of aminoglycosides is associated with detrimental nephrotoxicity, the antibiotics still constitute

**Table 4: Effect of methanolic extract of *Hibiscus plantinifolius* leaves on membrane bound enzymes in gentamicin induced nephrotoxicity.**

Drug Treatment	H <sup>+</sup> - ATPase	Na <sup>+</sup> /K <sup>+</sup> - ATPase	Ca <sup>2+</sup> - ATPase	Mg <sup>2+</sup> - ATPase	Total - ATPase
	μ.moles of Pi liberated/mg protein/min				
Normal control rats	180±0.47	185±0.26	140±0.53	206±1.2	232±0.67
Gentamicin control rats	52±0.38 <sup>****</sup>	58±0.46 <sup>****</sup>	43±0.76 <sup>****</sup>	74±0.96 <sup>***</sup>	75±0.45 <sup>****</sup>
MEHP 75mg/kg	71±0.42	76±0.37	57±0.46	86±0.83	94±0.56
MEHP 150mg/kg	94±0.47	105±0.44	88±0.74	107±0.78	123±0.84
MEHP 300mg/kg	128±0.42 <sup>b</sup>	134±0.35 <sup>b</sup>	105±0.63 <sup>b</sup>	149±0.86 <sup>b</sup>	167±0.34 <sup>b</sup>

P<0.001<sup>\*\*\*\*</sup>, P<0.01<sup>\*\*\*</sup>, P<0.05<sup>b</sup>. a) Compare to Normal control rats, b) compare to Gentamicin control rats

**Figure 2: Kidney histological studies.**

the only effective therapeutic alternative against microorganisms' refractory to other antibiotics. Present investigation, provides evidence for the potential nephroprotective property of methanolic extract of *Hibiscus plantinifolius* leaves against gentamicin induced nephrotoxicity.

Gentamicin has been shown to enhance the generation of superoxide anion and hydrogen peroxide by renal cortical mitochondria, which subsequently leads to generation of the extremely reactive hydroxyl free radical.<sup>24,25</sup> In line with previous observations, the present study also demonstrated that gentamicin induced oxidative stress as a result of depletion of the renal antioxidant enzymes like glutathione, catalase, and SOD. Exhaustion of these renal oxidative defense mechanisms along with enhanced reactive oxygen species generation could aggravate the oxidative damage in gentamicin treated rats.<sup>26,27,28</sup> Consistent with this notion, the results of this

study revealed that gentamicin treated rats showed accelerated lipid peroxidation in the renal tissue as reflected by an increase in MDA. Serum creatinine concentration is a more potent indicator than urea in the first phases of kidney disease and urea concentrations begin to increase only after parenchymal injury.<sup>29</sup> Accordingly, gentamicin treated rats were exhibited a marked elevation serum creatinine uric acid, urea, BUN, which reflect a significant functional impairment of kidney in gentamicin rats. In other hand gentamicin treated rats membrane bound enzyme activity was significant decrease and imbalance ionic composition observed in the present study is in line with previous reports.<sup>30</sup>

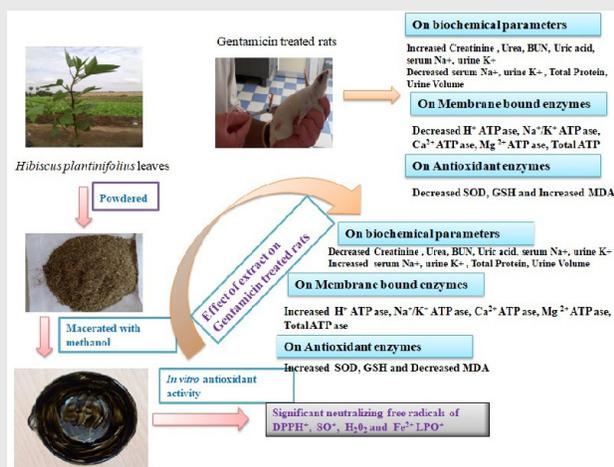
In this study, methanolic extract of *Hibiscus plantinifolius* leaves conferred nephroprotection was demonstrated by its ability to exhibit significant decrease serum biomarkers for renal damage increase renal membrane bound enzymes function as well as elevation of the renal antioxidant defense system. Indeed, the *in vitro* free radical scavenging assay indicated that extract to be endowed with free radical inhibitory activity. The effect could be attributed to the protective phytoconstituents of plant extract, which in turn might lead to variation in antioxidant effect to improve the renal hemodynamics. The histopathological results were paralleled by the serum, antioxidant and lipid peroxidation findings. Gentamicin treated rats were revealed extensive and marked renal tubular necrosis, in line with several reports indicating the aforementioned changes.<sup>33,34</sup> The extensive histopathological changes in the renal tubules of rats treated with gentamicin were mitigated by concomitant treatment with *Hibiscus plantinifolius* leaves, at least in part due to its antioxidant properties. The histopathological results in connection with protective effects of plant extracts against gentamicin induced nephrotoxicity were in agreement with published reports.<sup>35,36</sup> A growing line of evidence has demonstrated that flavonoids and phenolic groups possess antioxidant effect in animal models. Studies have also reported that flavonoids such as rutin, quercetin, luteolin produced

significant antioxidant activity. Hence it was suggested that the antioxidant of MEHP may related to flavonoid contents. In the present study, one could see a very good correlation between *in vivo* and *in vitro* studies and thus it is plausible to suggest that the attenuation of gentamicin-induced oxidative stress by methanolic extract of *Hibiscus plantinifolius* leaves could be attributed to its antioxidant property. This assertion is in concordance with earlier studies demonstrating that gentamicin-induced nephrotoxicity is ablated by substances endowed with antioxidant properties.<sup>31, 32</sup>

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## PICTORIAL ABSTRACT



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## SUMMARY

- Gentamicin was exhibiting significant nephrotoxicity by estimation of serum, urine biochemical parameters, tissue membrane bound enzymes as well of tissue antioxidants and also compared to normal rats.
- In vitro* studies are revealed that the Methanolic extract of *Hibiscus plantinifolius* (MEHP) shown significant free radicals neutralizing capacity against stable free radicals.
- Treatment of MEHP significantly normalize the serum and urine biochemical parameters in gentamicin treated rats.
- also reduced gentamicin induced nephrotoxicity in rats.
- MEHP also enhanced liver antioxidant levels in gentamicin induced nephrotoxicity in rats.
- MEHP restored liver membrane bound enzymes function in gentamicin induced nephrotoxicity in rats.
- MEHP was exhibiting potent nephroprotective activity against gentamicin by improve membrane bound enzymes as well as increased antioxidant activity.

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