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# EVALUATION OF NEPHROPROTECTIVE ACTIVITY OF AQUEOUS EXTRACT OF *HEMIGRAPHIS COLORATA* (BLUME) LEAVES

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## ABSTRACT

The present study was designed to investigate the nephroprotective effect of *Hemigraphis colorata* (Blume) leaf extract both *in-vitro* and *in-vivo*. The *in-vitro* study examined the cell viability by using HEK 293 cells by MTT assay and investigated the role of *Hemigraphis colorata* in modulating this effect. This study also evaluated the *in-vivo* effect of *Hemigraphis colorata* against gentamicin induced nephrotoxicity in rats by studying the biochemical parameters and histopathological changes. The estimation of biochemical parameters like serum creatinine, serum urea and blood urea nitrogen were done using the serum. Gentamicin administration showed nephrotoxicity by a significant elevation in BUN, creatinine, urea and urine volume than normal range along with histopathological abnormalities. Treatment with *Hemigraphis colorata* normalized almost all the parameters of gentamicin induced nephrotoxicity in rats. The above findings were confirmed by histopathological examinations. Results of the present study suggests that *Hemigraphis colorata* has a significant effect on protecting kidneys against gentamicin induced nephrotoxicity. This protective effect could be attributed to the presence of flavonoids and phenolic compounds such as ferulic and gallic acid which is present in the plant. Therefore, *Hemigraphis colorata* has the potential to be developed as a good nephroprotective.

**Keywords: *Hemigraphis colorata*, Gentamicin, Nephrotoxicity, Antioxidant, MTT Assay**

## INTRODUCTION

Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to a drug or toxin that causes damage to the kidneys. The kidneys are bean-shaped organs, with medial concavity and lateral convexity, weighing anywhere from 150 to 200 g in males and about 120 to 135 g in females. Each kidney is about the size of a closed fist **[1]**. They are located retroperitoneally on the posterior abdominal wall and are found between the transverse processes of T12 and L3. The primary function of the kidney is to make urine and purify the blood. The kidney participates in whole-body homeostasis, regulating acid base balance, electrolyte concentrations, extracellular fluid volume, and blood pressure **[2]**.

Nephrotoxicity is defined as rapid deterioration in the kidney function due to toxic effect of medications and chemicals. Nephrotoxins are substances displaying nephrotoxicity **[3]**. Different mechanisms lead to nephrotoxicity, including renal tubular toxicity, inflammation, glomerular damage, crystal nephropathy, and thrombotic microangiopathy. The traditional markers of nephrotoxicity and renal dysfunction are blood urea, serum creatinine, blood urea nitrogen **[4]**. The nephrotoxic effect of most drugs is more profound in patients already suffering from kidney failure **[5]**. Aminoglycoside causes nephrotoxicity, which particularly affects the proximal tubule epithelial cells due to selective endocytosis and accumulation of aminoglycosides via the multi-ligand receptor megalin **[6]**. Toxic agents and drugs cause potential damage to the tubular transport system through the induction of oxidative stress which leads to tubular mitochondrial damage. Causes of nephrotoxicity includes antibiotics, analgesics, heavy metals, contrast agents, anti-cancer drugs, solvents, herbicides and pesticides, overproduction of uric acid **[7-10]**.

*Hemigraphis colorata*, a purple-coloured perennial herb, belonging to family Acanthaceae. *Hemigraphis colorata* is a creeping herb native to Indonesia and Malaysia, distributed in tropical and subtropical countries like Asia, America, the Caribbean and many islands of the Indian and Pacific Ocean. The plant is rich in both primary and secondary metabolites comprising of phenols, saponins, flavonoids, terpenoids, coumarins, carboxylic acid, xanthoproteins, tannins, proteins, alkaloids, steroids and sterol. Several studies suggest that the main chemical constituent present in the *H. colorata* is β-Carotene. It is also rich in gallic acid and ferulic acid both of which contribute to its nephroprotective activity. The plant has immense antioxidant potential

because of these phytoconstituents **[11]**. In many parts of South-India the leaves of *H. colorata* are used in treating wounds because of its incredible potency to heal wounds. The plant is also known to be used as a remedy in treatment of bloody dysentery, bacterial infection, fungal infection and haemorrhoids. The paste of these plant leaves have been used in treating fresh cuts, the juice of the fresh leaves is used as a coagulants in order to prevent blood lose. The plant *Hemigraphis colorata* is also known to be used for treating anaemia, gall stones, excessive menstruation and as a contraceptive **[12, 13]**. The present study was carried out to investigate the nephroprotective activity of *Hemigraphis colorata* (Blume) leaves by MTT assay and by using the gentamicin induced nephrotoxicity model in Wistar rats.

## MATERIALS AND METHODS

### Experimental Animals

In house laboratory bred healthy Wistar Albino rats weighing 150-200 g were included for the study. Animals were housed in polypropylene cages. Animals were fed normal standard diet and ad. Libitum under controlled environmental condition of temperature (24-28°C, relative humidity and natural light/dark cycle 12:12).

### Collection And Preparation of Plant Extracts

The fresh leaves of *Hemigraphis colorata* were collected from Thiruvalla, Pathanamthitta district, Kerala. Plant extracts were prepared by Soxhlet method. The plant was washed, cleaned to remove dirt and shade dried. The dried leaves of *Hemigraphis colorata* (Blume) was pulverized and stored in an air tight container. 15 g of dried finely powdered leaves were extracted in 225 ml distilled water for 6 hours till extraction was completed. The crude extract was obtained by evaporation **[14]**.

### *IN-VITRO* STUDIES

### MTT Assay

HEK 293 (Human Embryonic Kidney cells) cell lines was purchased from NCCS Pune was maintained in Dulbecco's Modified Eagles Media. The cell line was cultured in 25 $cm^2$ tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100 µg/ml) and Amphotericin B (2.5 µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% $CO_2$ incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

### Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and the cells suspended in 10% growth medium, 100 µl cell suspension ($5 \times 10^{-4}$ cells/ well) was seeded in 96 well

tissue culture plate and incubated at 37°C in a humidified 5% $CO_2$ incubator.

**Preparation of compound stock**

1 mg of the sample (aqueous extract) was weighed and completely dissolved in 1 ml 0.1% DMSO using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. Gentamicin (20 µM) was added to induce toxicity.

**Cytotoxicity Evaluation**

After attaining sufficient growth, Gentamicin (20µM) was added to induce toxicity and incubated for one-hour, prepared extract in 5 % DMEM were five times serially diluted by two-fold dilution (25 µg, 12.5 µg, 6.25 µg, 3.1 µg, 1.5 µg in 500 µl of 5% DMEM) and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 37° C in a humidified 5% $CO_2$ incubator.

**Cytotoxicity Assay by MTT Method**

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30 µl of reconstituted MTT solution was added to all the test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5 % $CO_2$ incubator for 4 hours. After the incubation period, the supernatant was removed and 100 µl of MTT solubilization solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm **[15]**.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

***IN-VIVO* STUDIES**

**Acute Toxicity Studies**

The acute toxicity study was carried out as per the procedure given in Organisation for Economic Co-Operation and Development (OECD) guideline 425. Five albino rats (150-200g) were used in the study. The animals were fasted overnight and provided only with water. Test dose [2000mg/kg] was given for one animal. If the animal die, conduct main test to determine the LD50. As the animal survived, four additional animals were dosed and were observed for 14 days for toxic symptoms like tremors, convulsions, behavioural changes, locomotion, and mortality. Cage side observations were also observed. The LD50 is greater than 2000 mg/kg if three or more animals survive. If an animal unexpectedly dies late in the study, it is appropriate to stop dosing and observe other animals to see whether they will also die during a similar

observation period. Late deaths should be counted the same as other deaths **[16]**.

**Gentamicin Induced Nephrotoxicity in Rats**

Wistar rats were divided into five groups of six rats each. Standard and aqueous extract was administered orally by gavage every day, 1 hour before gentamicin injection. Then 24 hours after the last injection, rats were sacrificed with 4 doses of 50 mg/kg pentobarbital (i.p) and blood samples were collected by cardiac puncture. Both kidneys were isolated and weighed from all the groups.

**Table 1: Gentamicin induced nephrotoxicity in rats**

| GROUP | GROUP NAME | TREATMENT |
|---|---|---|
| Group 1 | Normal control | Normal Saline p.o (12 days) |
| Group 2 | Disease control | Gentamicin 100 mg/kg i.p (12 days) |
| Group 3 | Standard | Silymarin 100 mg/kg p.o (12 days) + Gentamicin 100 mg/kg i.p (12 days) |
| Group 4 | Test low dose [200 mg/kg] | *Hemigraphis colorata* p.o (12 days) + Gentamicin 100 mg/kg i.p (12 days) |
| Group 5 | Test high dose [400 mg/kg] | *Hemigraphis colorata* p.o (12 days) + Gentamicin 100 mg/kg i.p (12 days) |

**Collection of urine**

Urine specimens were collected by keeping each rat in separate metabolic cages and urine volume was measured.

**Biochemical analysis**

On the 13th day, blood sample was collected from each animal through cardiac puncture under anaesthetic conditions. Blood samples were centrifuged for 10 min at 7000 rpm using micro-centrifuge to separate the serum for the evaluation of serum creatinine, serum urea and blood urea nitrogen. The levels were estimated using standard kits.

**Histopathological Examination**

The kidney specimens obtained from the control and treated groups were fixed in 10 % neutral formalin for 24 hours. The sections were taken (5 µm thickness) using microtome, processed in alcohol xylene series, and stained with alum-haematoxylin and eosin. The sections were examined microscopically for the evaluation of histopathological architecture.

**Statistical Analysis**

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using computer based fitting programme (Graph Pad Instat, GraphPad Prism). Results were expressed as mean $\pm$ SEM from 6 rats in each group **[17]**.

**RESULTS**

**Extraction of plant material and calculation of percentage yield**

The aqueous extract of dried powder of *Hemigraphis colorata* was prepared by the method of soxhlation. Dried finely powdered leaves were extracted in distilled water till extraction was completed. The crude extract was obtained by evaporation. Percentage yield of the extract was found to be 9 % w/w yield.

**Table 2: Evaluation of acute toxicity of aqueous extract of leaves of *Hemigraphis colorata***

| S. No. | PARAMETERS | RESULTS |
|---|---|---|
| 1 | Motor activity | Normal |
| 2 | Tremors | Absent |
| 3 | Convulsion | Absent |
| 4 | Straub reaction | Absent |
| 5 | Pilo erection | Absent |
| 6 | Loss of light reflex | Absent |
| 7 | Sedation | Absent |
| 8 | Muscle relaxation | Absent |
| 9 | Hypnosis | Absent |
| 10 | Analgesia | Absent |
| 11 | Ptosis | Absent |
| 12 | Lacrimation | Absent |
| 13 | Diarrhoea | Absent |
| 14 | Changes in skin colour | No change |

**Dose of 2000 mg/kg neither produced mortality nor any signs of morbidity**

## *IN-VITRO* STUDIES

**Table 3: Effect of Silymarin and *Hemigraphis colorata* on cell viability of HEK 293 cell lines**

| GROUPS | PERCENTAGE VIABILITY OF STANDARD (Silymarin) | PERCENTAGE VIABILITY OF TEST (H.colorata) |
|---|---|---|
| Control | 100** | 100** |
| Disease control | 40.077 ± 0.5769 | 40.077 ± 0.5769 |
| Samples(µg/ml) | | |
| 1.5 | 54.770 ± 0.5508** | 49.587 ± 0.4145** |
| 3.1 | 60.140 ± 0.2001** | 52.947 ± 0.1576** |
| 6.25 | 64.863 ± 0.3102** | 59.023 ± 0.2153** |
| 12.5 | 62.563 ± 0.2281** | 58.177 ± 0.3245** |
| 25 | 57.847 ± 0.1919** | 56.673 ± 0.3503** |

**Values are expressed as mean ± SEM, N =3. Data analysed by using one way ANOVA followed by Dunnett's multiple comparison test. **p< 0.01 denote value significantly differ from toxic control.**

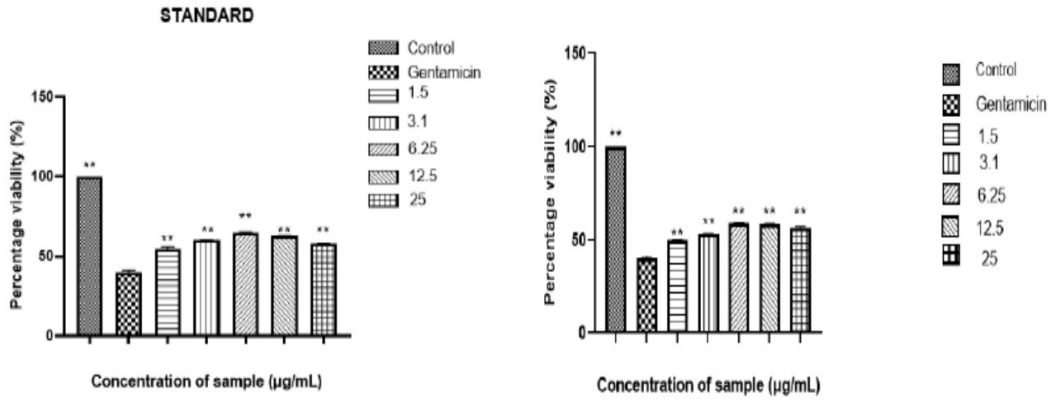


**Figure 1: Percentage viability of standard (silymarin) and sample of *H. colorata* respectively using HEK 293 cell lines– MTT assay**

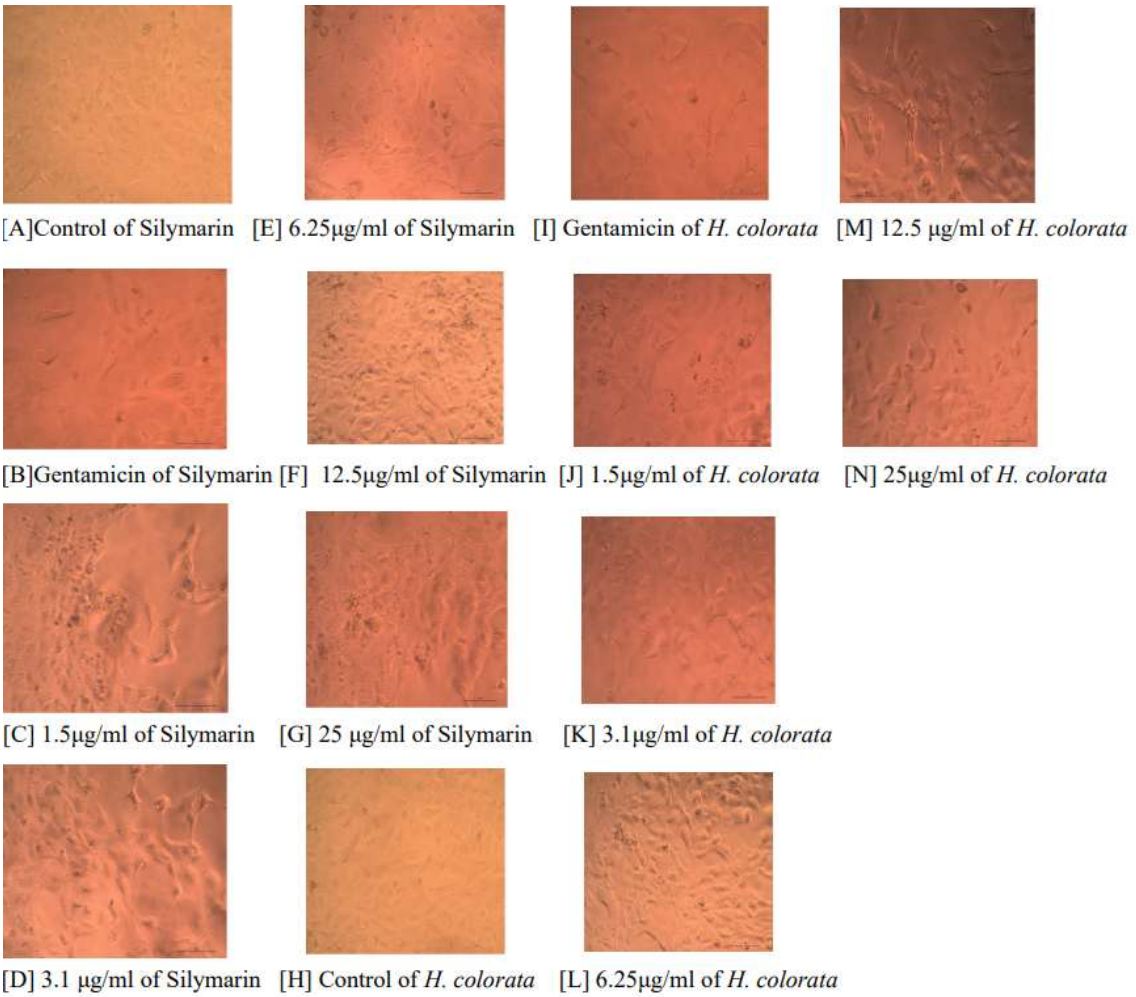


**Figure 2: Microscopic images of effect of silymarin and *Hemigraphis colorata* aqueous extract on cell viability of HEK 293 cell line. Images from A – G shows that of standard (silymarin) and from H – N shows that of test (*H. colorata*).**

## *IN-VIVO*: GENTAMICIN INDUCED NEPHROTOXICITY

**Normal control** **Disease control** **Standard** **Test low dose** **Test high dose**

**Figure 3: Images of kidneys of different experimental groups**

**Table 4: Effect of Treatment groups on Blood Urea Nitrogen (BUN), Creatinine and Urea**

| GROUPS | BUN | CREATININE | UREA |
|---|---|---|---|
| Normal control | 24.623 ± 0.3043 | 0.5383 ± 0.01701 | 17.250 ± 0.5082 |
| Gentamicin | 51.702 ± 0.4004** | 2.042 ± 0.05431** | 58.765 ± 0.8333** |
| Standard group | 30.117 ± 0.4930**## | 1.298 ± 0.08799**## | 25.427 ± 0.5085**## |
| Test low dose | 34.835 ± 0.5687**## | 1.467 ± 0.03383**## | 37.593 ± 0.3887**## |
| Test high dose | 32.732 ± 0.5550**## | 1.345 ± 0.06922**## | 33.793 ± 0.4126**## |

**Values are expressed as mean ± SEM, N = 6; Data analysed by one way ANOVA followed by Dunnett's multiple comparison test: ## p<0.01 denote value significantly differ from toxic control, ** p<0.01 value significantly differ from normal control**

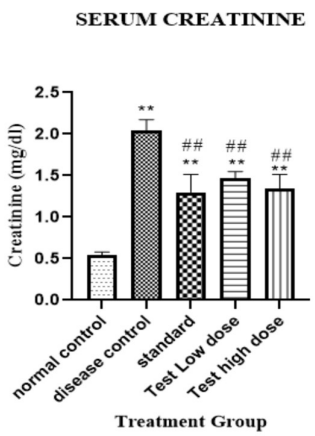


**Figure 4: Effect of Treatment groups on Creatinine**

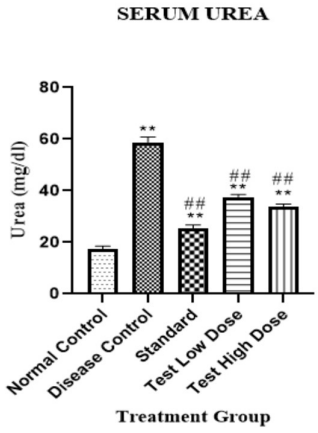


**Figure 5: Effect of Treatment groups on Urea**

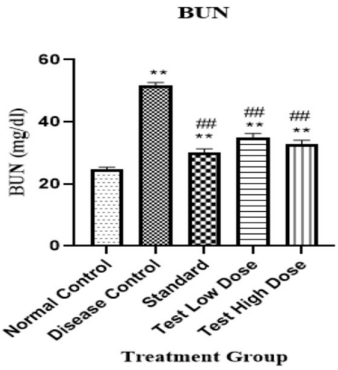


**Figure 6: Effect of Treatment groups on BUN**

**Table 5: Effect of Treatment groups on Urine Volume**

| GROUPS | URINE VOLUME |
|---|---|
| Normal control | 1.217 ± 0.09667 |
| Gentamicin (Disease control) | 3.742 ± 0.07162** |
| Standard group | 1.757 ± 0.05149**## |
| Test low dose | 2.748 ± 0.04578**## |
| Test high dose | 2.207 ± 0.07360**## |

**Values are expressed as mean ± SEM, N = 6; ## p<0.01 denote value significantly differ from toxic control, ** p<0.01 value significantly differ from control**

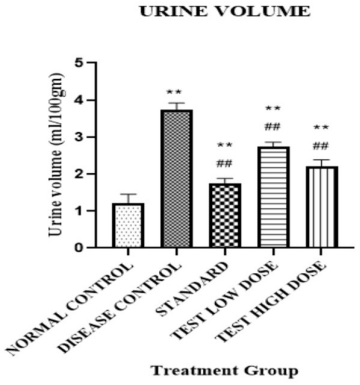


**Figure 7: Effect of Treatment groups on Urine Volume**

## HISTOPATHOLOGICAL EXAMINATION

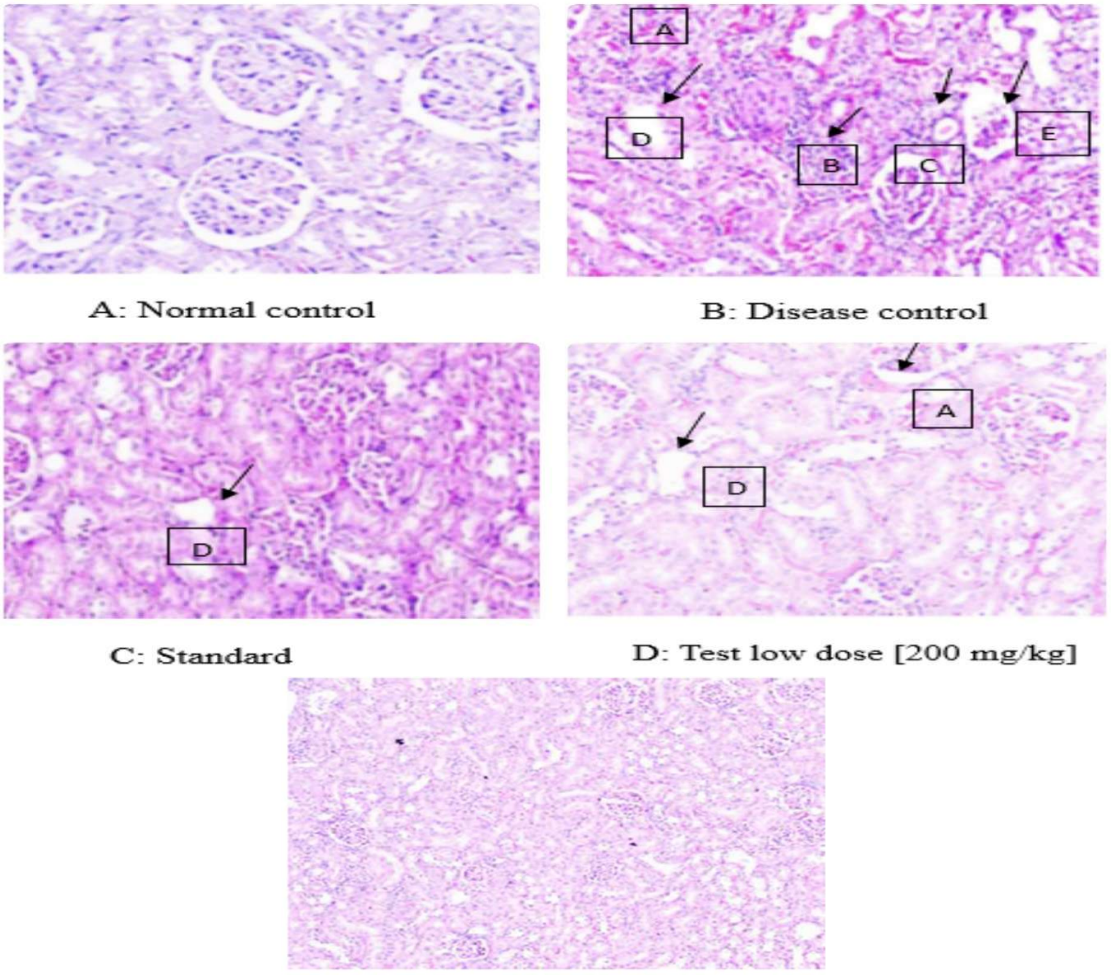


**Figure 8: Histopathological images of kidneys of various experimental groups**
**A – Haemorrhage, B – cell infiltration, C – Hyaline cast, D – Tubular dilation, E – Glomerular congestion**

## DISCUSSION

The present study aimed to investigate the nephroprotective activity of aqueous extract of *Hemigraphis colorata* (Blume) leaves in wistar albino rats using gentamicin induced nephrotoxicity model and by MTT assay. *Hemigraphis colorata* is rich in both primary and secondary metabolites comprising of phenols, saponins, flavonoids, terpenoids, coumarins, carboxylic acid, xanthoproteins, tannins, proteins, alkaloids, steroids, and sterol. Several studies suggest that the main chemical constituent present in the *H. colorata* is β-Carotene. It is also rich in gallic acid and ferulic acid both of which may contribute to its nephroprotective activity. Gallic acid as an antioxidant is capable of mitigating the tissue damage caused by oxidative stress and improving renal function. Ferulic acid has reduced the inflammation in nephrotoxicity induced by

GM, by decreasing oxidative stress. Flavonoids play an important role in preventing and managing CKD and renal fibrosis. Alkaloids can provide protection in acute kidney injury through various mechanisms including antioxidant pathways, improvement of mitochondrial damage, reduction of cell death, induction of autophagy, and inhibition of inflammation. The plant has immense anti-oxidant potential because of these phytoconstituents.

Acute oral toxicity testing of aqueous extract of *Hemigraphis colorata* (Blume) leaves are conducted by OECD 425 Guidelines (Up and Down Procedure). Wistar rats weighing around 150-200 grams were used for the test. The animals were individually observed for initial 30 minutes and periodically during the first day. They were observed regularly for 14 days for any signs of delayed toxicity. No late deaths were seen. The extract was found to be having good safety margins. It did not show lethal effects on rats up to doses of 2000 mg/kg. Hence, submaximal doses, 200 mg/kg and 400 mg/kg of doses were selected for the study.

In *in-vitro* study, cell viability using HEK 293 cells – MTT assay was done. The control group shown 100 % viability when compared to other groups. Gentamicin group showed least viability as compared with the control group. The samples of five different concentrations (1.5, 3.1, 6.25, 12.5, 25 µg/ml) have shown statistically significant viability. From this we can conclude that, all the sample groups have more nephroprotective activity than disease control (Gentamicin). In case of standard group silymarin, it showed an increase in percentage viability with each of the five different concentrations when compared with the concentrations of sample. In *in-vivo* study, parameters evaluated are blood urea nitrogen, creatinine, urea and urine volume. In this study it was found that the values of BUN, creatinine, urea and urine volume were highest for gentamicin group and lowest for normal control group. Both test high and low dose groups showed nephroprotective activity, but the test high dose group showed more nephroprotective activity.

From histopathological examinations, it is seen that the normal control group showed normal cell architecture, no tubular dilation, no hyaline or tubular cast, no infiltration, no glomerular congestion. Gentamicin control group showed mononuclear cell infiltration, tubular dilation, hyaline cast, glomerular congestion, and haemorrhage. Standard group showed normal glomerulus, no infiltration, no blood vessel congestion, no haemorrhage, no hyaline cast, only showed slight tubular dilation. Both the test groups showed significant nephroprotective effect which is evident through histopathological examinations.

Eventually, we conclude that administration of aqueous extract of *Hemigraphis colorata* leaves potentially protect the kidneys against gentamicin induced nephrotoxicity as well as in MTT assay conducted and all these findings suggest that the plant extract is having a good nephroprotective activity.

## CONCLUSION

The current study concludes that the aqueous extract of *Hemigraphis colorata* leaves exhibited significant nephroprotection in both *in-vitro* and *in-vivo* studies. The *in-vitro* studies showed increased percentage of cell viability in *Hemigraphis colorata* treated groups when compared to gentamicin treated groups. The *in-vivo* model, Gentamicin induced nephrotoxicity in rats the *Hemigraphis colorata* showed significant effect on biochemical and histopathological changes that support the nephroprotective activity. The protective effect may be due to the presence of phytochemical constituents like flavonoids and phenolic compounds, which also possess strong antioxidant activities. Hence this study proves that *Hemigraphis colorata* possess strong nephroprotective activity and might be a promising adjuvant to help manage kidney diseases.

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