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ORIGINAL ARTICLE **OPEN ACCESS**

HPLC analysis and Screening of aqueous Methanolic extract of *Callestimon viminalis* leaves against Gentamicin induced Nephrotoxicity

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ABSTRACT

The current study was conducted to evaluate nephroprotective potential and to determine chemical components of aqueous methanolic extract of Callestimon viminalis at the dose of 250 and 500 mg/kg for 8 days against gentamicin induced kidney damage in mice. Plant extract significantly ($p<0.001$) improved physical parameters (body weight change and kidney weight change) and declined gentamicin induced serum creatinine, urea, blood urea nitrogen (BUN) and uric acid. The histopathological investigations also confirmed the nephroprotective action of the extract. Furthermore, HPLC analysis indicated the presence of Quercetin, Gallic acid, Chlorogenic acid, p-Coumeric acid, m-Coumeric acid, Vitamin C, Trans-4-hydroxy 3-methoxy Cinamic acid. These phytochemicls may be responsible for the nephroprotective activity of aqueous methanolic extract of Callestimon viminalis against gentamicin induced kidney damage in mice.

Key words: *Nephroprotective; Callestimon viminalis; Quercetin; HPLC analysis*

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INTRODUCTION

Nephrotoxicity (renal disease) or dysfunction emerges out as a direct or indirect result of exposure to environmental and industrial chemicals or any drug [1]. Due to kidney disorder, about 20,000 deaths are found every year and it becomes the 10th leading causes of death across the world [2, 3]. Nephrotoxicity is majorly caused by drugs, accounting for twenty percent (20%) of all cases of acute renal failure [4, 5].
According to previous studies it has been reported that after 48 hours of gentamicin treatment, cellular protein synthesis is reduced, which is enhanced to 50% after further 24 hours of treatment [6]. It has been reported that hydrogen peroxide, hydroxyl radical and superoxide anion production is enhanced from the mitochondria with gentamicin treatment. According to previous research, these hydrogen peroxide, hydroxyl radical and superoxide anion affect many biological process and leads to acute renal failure [7].
The use of herbs for their medicinal effect has been a common practice by the physicians since ages [8]. World Health Organization acknowledges the use of herbal medicines in more than 80% developing countries of the world [9]. At least 25% of commercial drugs are plant derived [10, 11].
*Callestimon viminalis*is an evergreen shrub grown in plain areas of the Punjab. It is multi-truncked plant with low branches and pendulous growth. *Callistemon viminalis*, native to Australia and nowadays is broadly cultivated plant to Asia, Europe and America [12]. Traditionally this plant is used to treat gastro-enteritis, diarrhea, kidney disorders, skin infections (acute eczema) [13], as diuretic and for the treatment of prostatic hypertrophy [14].

MATERIAL AND METHOD

Collection of plant

Callestimon viminalis leaves were collected in the month of April from Faisalabad, Punjab, Pakistan. Identification of the plant was done by Dr. Mansoor Hameed, Department of Botany, University of Agriculture, Faisalabad, Pakistan.

Preparation of plant extract

After collection *Callestimon viminalis* leaves were thoroughly washed with tap water and dried under shade at room temperature. Dried leaves were powdered and soaked in aqueous methanolic solvent (70:30) for 7 days with 3 to 4 times daily shaking. The plant extract was filtered and evaporated with the help of rotary evaporator to get the solid residue at temperature below 50°C which was stored in amber color glass bottle at 4°C (15).

Experimental Animal

Swiss albino mice were procured from National Institute of Health, Islamabad. All mice body weight was ranging from 20 to 25g and housed in animal house of College of Pharmacy, G.C.U. Faisalabad. An ambient temperature of 25ºC ± 2°C with relative humidity 50% ± 15% and 12 hour in light and dark cycles were maintained in animal house (16). Study was conducted after approval by the Institutional Animal Ethical Committee.

Experimental protocol

Experimental mice were randomly divided into four groups having six animals in each group.

Group I served as normal control; received normal saline, for 8 days.

Group II served as toxic control that received gentamicin (100 mg/kg/day, I.p.), for 8 days.

Group III and IV served as test control which received gentamicin + methanol: water extract of *Callestimon viminalis* leaves at a dose of 250 mg/kg/day and 500 mg/kg/day respectively for 8 consecutive days (16) as shown below in Figure 01.

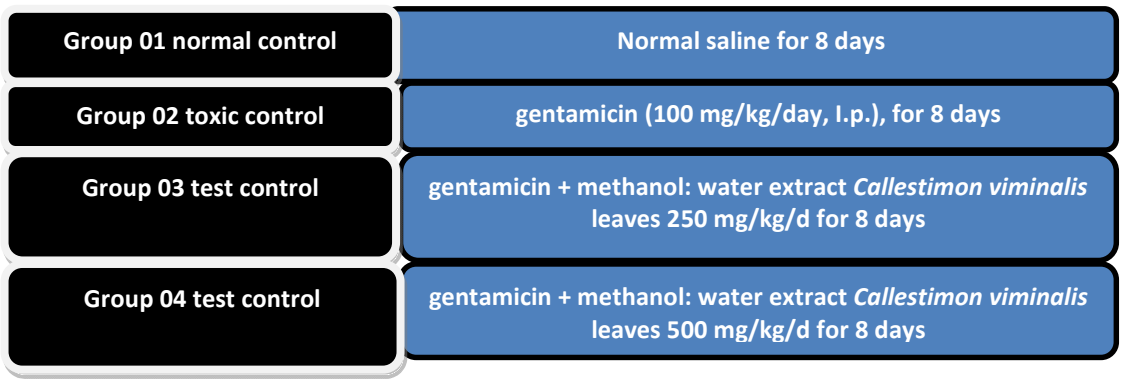

Figure 01: Experimental design

Collection of Samples

After 24 hours of the last administered dose, the experimental animals were anesthetized, sacrificed and blood samples were collected and centrifuged at 6000RPM for 10 minutes at 25°C.

Screening of nephroprotective activity

Callestimon viminalis leaves were screened for their nephroprotective activity against gentamicin induced renal toxicity by evaluating physical parameters (body weight change and kidney weight change), biochemical parameters (urea, blood urea nitrogen, serum creatinine and uric acid) and supported by histopathalogical examination.

Preliminary phytochemical screening

Aqueous methanolic extract of *Callestimon viminalis* leaves were subjected to different tests for the identification of chemical components present in the plant like terpenoids, alkaloids, saponins, tannins, flavonoids and anthraquinon [17].

Phytochemistry by HPLC

High Performance Liquid Chromatography (HPLC) of aqueous methanolic (70:30) extract of *Callestimon viminalis* leaves was carried out with the help of gradient HPLC attached with UV-visible detector. Shim-Pack CLC-ODS (C-18) column (25cm × 4.6mm, 5 µm) was used for HPLC, with mobile phase as; A (H2O: Acetic Acid-94:6, pH=2.27) and B (acetonitryl (ACN) 100%). Mobile phase was run in isocratic mode with a flow rate of 1 ml/minand each run time for 10 min. Monitoring of different peaks for different compounds was done at 280 nm on UV-visible detector and comparison of the peaks of sample with standard and retention time was noted for each sample [18, 19].

RESULTS

The average body weight change of normal control animals was increased while treatment with gentamicin significantly decreased the average body weight of mice in toxic control group. *Callestimon viminalis* leaves extract at 250 and 500mg/kg/d to the treatment control groups has significant positive effect on the body weight by showing significant increase with the value (P<0.01) as compared to the toxic control. Average kidney weight of experimental animals in toxic control group showed significant increase in comparison with normal control group with the value of 0.59±0.009 to 0.71±0.009. Oral administration of *Callestimon viminalis* leaves extract showed significant decrease in kidney weight at both doses i.e. 250 and 500mg/kg/d having values of 0.61±0.005 and 0.63±0.007 respectively (Table 01).

Table 01: Effect of aqueous methanolic extract of *Callestimon viminalis* leaves at 250 mg/kg and 500 mg/kg on physical parameters

	Physical Parameters			
	Initial body weight	**Final body weight**	**Body weight change**	**Kidney weight**
	g	**g**	**g**	**g**
Normal control	21.75±0.479	22.80±0.594	1.05±0.126	0.59±0.009
Gentamicin (100 mg/kg/d)	21.25±0.479	18.80±0.485	2.45±0.119	0.71±0.009
Gentamicin (100 mg/kg/d) + **Plant Extract** (250 mg/kg/d)	23.64±0.168	23.14±0.278	-0.5±0.134 [b]	0.61±0.005 [b]
Gentamicin (100 mg/kg/d) + **Plant Extract** (500 mg/kg/d)	24.45 ±0.614	23.70±0.689	-0.65±0.096 [b]	0.63±0.007 [b]

Mean ± S.E is used to show the results; The results are compared by one way ANOVA (analysis of Variance); Significant [a]p<0.01, [b]p<0.001

Callistimon viminalis aqueous methanolic extract was also screened against gentamicin induced toxicity. 8 days treatment with 100mg/kg/d gentamicin intraperitoneal significantly increased the urea, BUN, uric acid and creatinine levels. Hydro-alchohlic plant extract was administered orally through naso-gastric tube at 250mg/kg/d and 500mg/kg/d along with intraperitoneal injection of gentamicin (100mg/kg/d) showed highly significant (P<0.01) results by reducing the creatinine, serum urea, BUN and uric acid level towards the normal value.

In normal control average creatinine level was 0.71 ± 0.009 mg/dl which was increased up to 1.59±0.017 mg/dl in animals that were treated with gentamicin at a dose of 100mg/kg. *Callestimon viminalis* leaves extract at a dose of 250 and 500mg/kg/d brought the serum creatinine to the value of 0.7 2± 0.015mg/dl and 0.81 ± 0.017mg/dl respectively. The average value of serum urea in normal group was 39.10 ± 0.937mg/dl which was increased in gentamicin treated animals to 64.9±1.192mg/dl. *Callestimon viminalis* leaves extract was showed significant decrease in serum urea level with the value of 41.60 ± 1.243 mg/dl at 250mg/kg and 48.08 ± 0.699mg/dl at 500mg/kg as shown in Table 02. Plant leaves at 250 and 500mg/kg/d decreased blood urea nitrogen level up to 19.44 ± 0.581mg/dl and 22.46 ± 0.327mg/dl respectively in comparison with toxic control group having value up to 30.33±0.557mg/dl (Table 02). Average uric acid level in normal control was 3.97 ± 0.066mg/dl and toxic control showed average value of uric acid up to5.00 ± 0.014mg/dl which was decreased to 4.00±0.082mg/dl at 250mg/kg extract dose while 500mg/kg showed 4.06±0.078mg/dl as shown in Table 02 and graph 01.

Table 02: Effect of aqueous methanolic extract of *Callestimon viminalis* leaves at dose of 250 mg/kg and 500 mg/kg on Biochemical Parameters

	Biochemical Parameters			
	Creatinine	**Urea**	**BUN**	**Uric acid**
	mg/dl	**mg/dl**	**mg/dl**	**mg/dl**
Normal control	0.71±0.009	39.10± 0.937	18.27±0.438	3.97±0.066
Gentamicin(100 mg/kg/d)	1.59±0.017	64.90±1.192	30.33±0.557	5.00±0.014
Gentamicin(100 mg/kg/d) + **Extract** (250mg/kg/d)	0.72±0.015[b]	41.60±1.243 [b]	19.44±0.581 [b]	4.00±0.082 [b]
Gentamicin(100 mg/kg/d) + **Extract** (500mg/kg/d)	0.81±0.017 [b]	48.08 ±0.699 [b]	22.46 ±0.327 [b]	4.06±0.078 [b]

Mean ± S.E is used to show the results; The results are compared by one way ANOVA (analysis of Variance); Significant [a]p<0.01, [b]p<0.001

Figure 02: *Callestimon viminalis* leaves methanol water extract on serum creatinine, urea, blood urea nitrogen (BUN) and serum uric acid level of normal control group, toxic control group and two treatment control groups.

NC: Normal control**; TxC:** Toxic control (Gentamicin 100mg/kg/d); **GM+CV250:** Gentamicin 100mg/kg/d plus *Callestimon viminalis* leaves extract 250mg/kg/d; **GM+CV500:** Gentamicin 100mg/kg/d plus *Callestimon viminalis* leaves extract 500mg/kg/d**.**

Histopathalogical Examination:

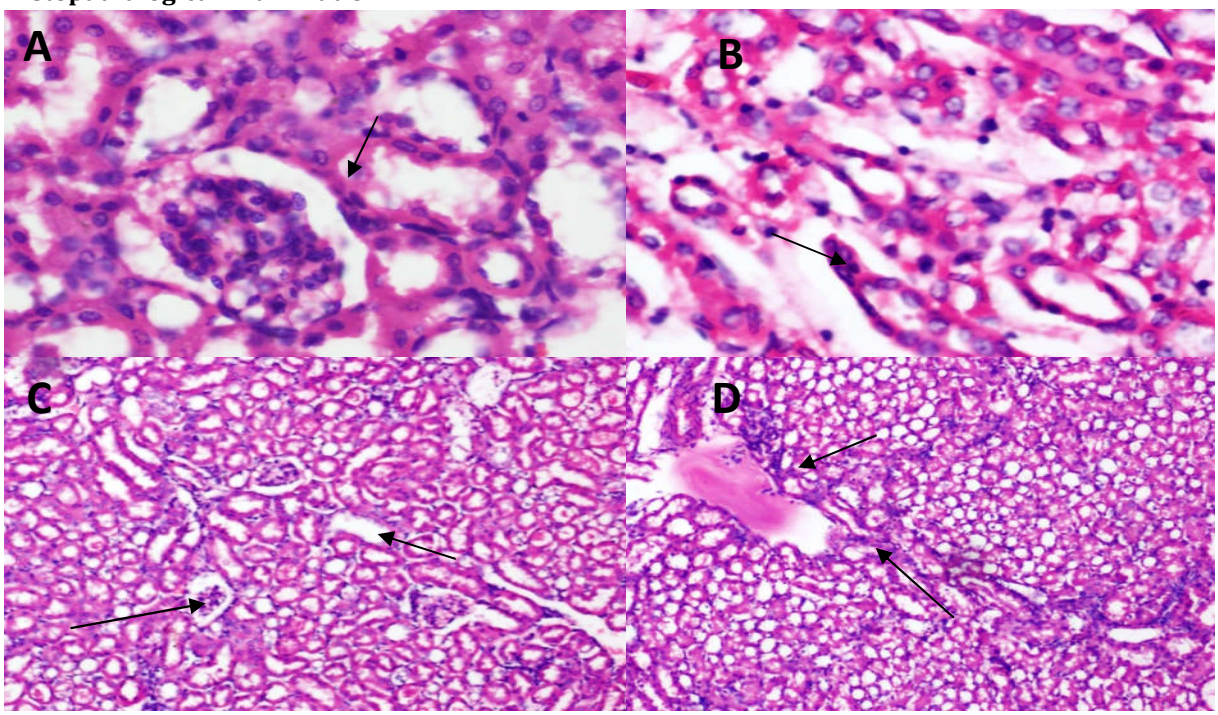

Figure 03: Histopathological studies of kidney sections. **A:** Normal control **B:** Toxic Control Gentamycin (TXC) **C:***Callestimon viminalis* methanol water extract 250mg/kg/d. **D:** *Callestimon viminalis* methanol water extract 500mg/kg/d.

Table 03: Histopathalogical effects of aqueous methanolic extract of *Callestimon viminalis* leaves at 250 and 500 mg/kg/d on physical parameters

	Tubular degeneration (TD)	**Tubulointerstitial inflammation (TIN)**	**Tubular necrosis (TN)**
Normal Control	NIL	NIL	NIL
Toxic control	MODERATE	SEVERE	MODERATE
Gentamicin(100mg/kg/day)**+ Plant Extract**(250mg/kg/day)	NIL	NIL	NIL
Gentamicin(100mg/kg/day) + **Plant Extract**(500mg/kg/day)	NIL	MODERATE	NIL

Preliminary Phytochemical Analysis

Preliminary phytochemical analysis of hydro-alcoholic extract of both plants confirmed the presence of chemical constituent in the plant as shown in Table 04.

Table 04: Preliminary Phytochemistry of Aqueous methanolic extract of *Callestimon viminalis* leaves

Phytochemical Constituents	**Aqueous methanolic extract of *Callestimon viminalis* leaves**
Tannins	**++**
Alkaloids	**+**
Flavanoids	**+**
Saponins	**++**
Anthraquinone	**+**
Terpinoids	**+**

HPLC Analysis

HPLC analysis of *Callestimon viminalis* aqueous methanolic extract showed the presence of different constituents with different retention time as shown in Table 05.

Table 05: Phytochemical constituents present in *Callestimon viminalis* extract by HPLC analysis

Compound name	Retention time	Area [mV.s]	Area [%]	Quantity [ppm]
Quercitin	2.820	1877.511	8.3	99.48
Gallic acid	5.020	2017.675	8.9	72.61
Chlorogenic acid	15.327	468.252	2.1	36.5
P-Coumeric acid	17.440	3034.997	13.4	39.44
M-Coumeric acid	20.440	573.927	2.5	6.87
Vitamin C	23.253	583.654	2.6	11.66
Trans-4-hydroxy 3-methoxy Cinamic acid	25.573	395.605	1.7	13.82

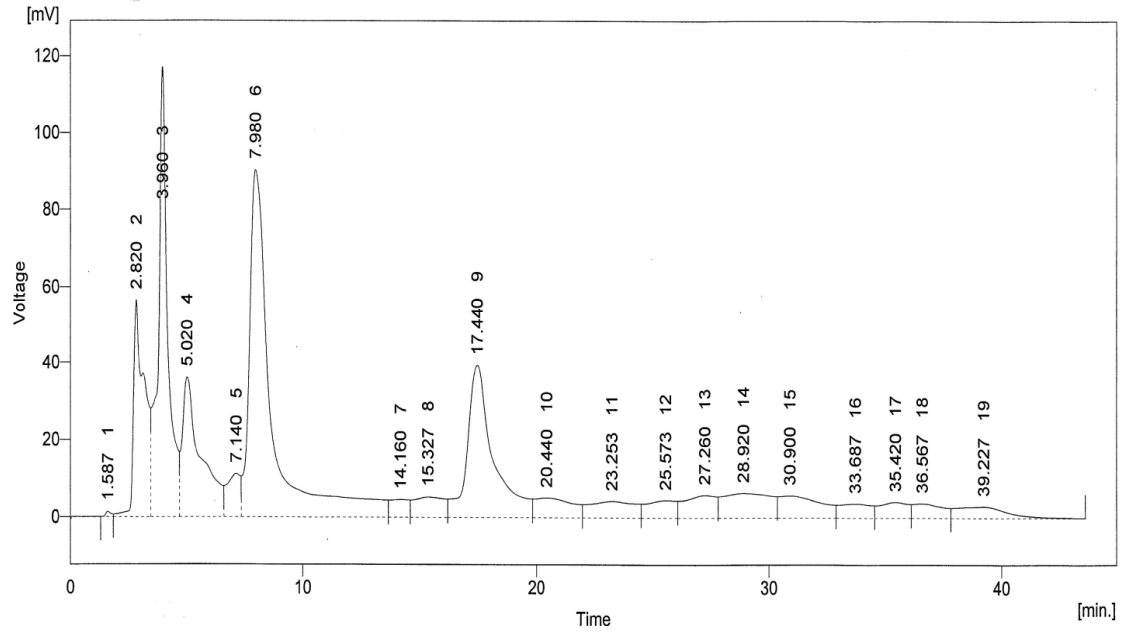

Figure 04: HPLC graph of *Callestimon viminalis* was showing different peaks at different retention time representing the presence of different compounds.

DISCUSSION

Kidney damage induced by gentamicin is mostly used model for the evaluation of nephroprotective drugs. Nephrotoxicity brings forth lowered glomerular filtration rate (GFR) and nitrogenous wastes accumulation. One of the most studied mechanisms behind the nephrotoxicity is the damage to the kidney tubules by the overproduction of free radicals [1]. Gentamicin provokes alteration in the glomerular ultrastructure which directly decrease the glomerular filtration rate (GFR) [4, 5] that leads to the elevation of biochemical parameters including serum creatinine, urea, BUN and uric acid (6). Gentamicin induces kidney tubular injury and loss of tubular cells becoming them unable to reabsorb water. It results in dehydration due to which decrease body weight was reported that is potent indicator of toxicity [7].

In previous studies it has been reported that histopathalogical examination of kidneys after administration of gentamicin showed cellular swelling of renal cells, as first manifestation of toxicity and swelling of endothelium lining of glomerular tufts and tubular vacuolization is the reflection of these reversible changes in kidneys [7, 20].

In the present study, normal control showed normal renal parenchyma with normal tubules and glomeruli and toxic control gentamycin shown renal parenchyma with tubular atrophy, thinning and edema but renal parenchyma after *Callestimon viminalis* methanol water extract 250mg/kg/d had shown normal tubules and glomeruli while at 500mg/kg/d renal parenchyma depicted the foci of mild to moderate lymphocytic infiltrate as shown in figure 03 (A, B, C and D).

Callestimon viminalis leaves extract at a dose of 250 and 500mg/kg/d reduced the serum creatinine to the value of 0.7 2± 0.015mg/dl and 0.81 ± 0.017mg/dl respectively and serum urea reduced to 41.60 ± 1.243 mg/dl at 250mg/kg and 48.08 ± 0.699mg/dl at 500mg/kg as shown in Table 02. Plant leaves at 250 and 500mg/kg/d also decreased blood urea nitrogen level up to 19.44 ± 0.581mg/dl and 22.46 ± 0.327mg/dl

respectively and average uric acid level decreased to 4.00±0.082mg/dl at 250mg/kg while 500mg/kg showed 4.06±0.078mg/dl as shown in Table 02 and graph 01.

Previous analysis of *Callestimon viminalis* extract reported the presence of α-pinene, 1,8-cineole [21], Viminadione A, viminadione B, linalool, α-terpeneol [22], α-phellandrene [23] eucalyptol, α-pinene, phellandrene and D-limonene. HPLC analysis of aqueous methanolic extract of plant leaves in the current study indicated the presence of *Quercetin, Gallic acid, Chlorogenic acid, p- Coumeric acid, M-Coumeric acid, Vitamin C, Trans-4-hydroxy 3-methoxy cinamic acid* as shown in Table 05 and Figure 04.

From different studies it has been proven that flavonoids, quercetin and vitamin C are potent antioxidants that prevent the cells from inflammation and damage from free radicals and also powerful nephroprotective agents [24-30]. Gallic acid showed ameliorative effect against renal toxicity [31, 32].Chlorogenic acid is a phenolic compound possessing powerful antioxidant activity and its role in protection of kidney cells against oxidative stress has been proposed [33]. *P- Coumeric acid, M-Coumeric acid* also possessed radical scavenging activity [34].

CONCLUSION

So, it may be concluded that aqueous methanolic extract of *Callestimon viminalis*(at doses of 250 and 500 mg/kg/d) possess significant nephroprotective activity against gentamicin induced kidney damage in mice which may due to the presence of Quercetin, Gallic acid, Chlorogenic acid, p-Coumeric acid, m-Coumeric acid, Vitamin C, Trans-4-hydroxy 3-methoxy Cinamic acid, confirmed by HPLC analysis.

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