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Renoprotective effects of stevia (*Stevia rebaudiana* Bertoni), amlodipine, valsartan, and losartan in gentamycin-induced nephrotoxicity in the rat model: Biochemical, hematological and histological approaches

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ABSTRACT

The current study investigated the renoprotective effects of stevia, angiotensin-II type 1 receptor (AT_1) blocker and calcium (Ca^{2+}) channel blocker in gentamycin-induced nephrotoxicity in rat models. Six groups of male Sprague-Dawley rats of eight weeks old were taken for the experiment: sham control, nephrotoxicity, treatment with amlodipine (4 mg/kg/day); stevia (200 mg/kg/day); losartan (15 mg/kg/day) and valsartan (5 mg/kg/day), accordingly. The blood sample was taken for the assessment of renal and hepatic-functional variables like serum creatinine, blood urea, BUN and SGPT, SGOT, and total serum bilirubin. Hematological parameters were also examined. Histological examination has been done on kidneys and liver. Alterations of the body weight and the organ's weight were documented. Treatment with stevia and valsartan significantly decreased serum creatinine levels. A reduction of liver enzymes, and total serum bilirubin levels were observed in all the treatment groups. Treatment with valsartan and amlodipine, remarkably and stevia, mildly reduced the renal tissue damage, inflammation, and tubular necrosis. However, the present study demonstrated that losartan treatment aggravated kidney damage by increasing protein cast, calcification, tubular necrosis, and injury. This comparison indicated that both stevia and valsartan have beneficial renoprotective effect and valsartan offers a better treatment option in renal damage over losartan.

1. Introduction

Worldwide, the prevalence of kidney disease and the metabolic syndrome is becoming a significant medical concern and public health burden. Stevia, *Stevia rebaudiana* Bertoni, a sweet herb indigenous to South America, confirmed promising results as the remedy of diabetes, hypertension, sexual dysfunction and other metabolic disorders [1,2]. Antioxidant and anti-inflammatory are the common effects of stevia, which help to lessen the cardiovascular and metabolic disorders [3]. Stevioside and rebaudioside are the main active constituents of stevia and are responsible for antidiabetic, antihypertensive and antioxidant activity [4]. Since cardiovascular disease and diabetes are closely associated with chronic kidney disease (CKD), it has up raised the interest to investigate the renoprotective effect of stevia in CKD.

A strong and consistent relationship has been established between lipid peroxidation products of oxidative stress and nephrotoxicity by a

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin-II type 1 receptor (AT1) blockers; AT_1, angiotensin-II type 1 receptor; AT_2, angiotensin-II type 2 receptor; BUN, blood urea nitrogen; CCB, calcium (Ca^{2+}) channel blocker; CKD, chronic kidney disease; EDTA, ethylene diamine tetra acetate; Hb, hemoglobin; HCT, hematocrit; HDL, high density lipoprotein; LDL, low density lipoprotein; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cells; RBS, random blood sugar; RDW-CV, red blood cell distribution width-CV; RDW-SD, red blood cell distribution width-SD; ROS, reactive oxygen species; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; TG, triglycerides

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number of studies [5,6]. Reactive oxygen species (ROS) produced by antineoplastic agents facilitate cytotoxicity, for example - cisplatin-induced nephro- and neurotoxicity, doxorubicin-induced cardiotoxicity, which in turn reduce the efficacy of these drugs in treating cancer [7]. Antioxidants play significant beneficial role in ameliorating cytotoxicity such as grape seed extract improved homodynamic balance in scarboplatin and thalidomide-induced neuro- and nephrotoxicity [5]. Extract of Indian gooseberry leaves demonstrated antioxidant and renoprotective effect in cisplatin-induced nephrotoxicity [8]. Moreover, contrast media-induced organ toxicity specially nephrotoxicity is one of the major concerns nowadays [6,9]. Again, natural products with antioxidant and free radical scavenging ability have the potential beneficial effect in the prevention and treatment of organ toxicity.

Potential nephroprotective effects of Renin-Angiotensin System (RAS) inhibitors in drug induced nephrotoxicity have been documented in several studies [10]. ARB, such as losartan and valsartan are unique due to their high affinity for AT_1 receptors and less dissociation from the binding sites [11]. Losartan was reported to show an antioxidant effect and decreased renin production by inhibiting AT_1 induced vasoconstriction and showed a nephroprotective effect in chronic cyclosporins induced nephrotoxicity [12].

Amlodipine, a calcium (Ca^{2+}) channel blocker, showed antithrombotic, and antiatherosclerotic effects, were not dependent on modulation of L-Type calcium channels [13]. Relaxation of arteriolar smooth muscle and decreasing the peripheral vascular resistance can reduce the blood pressure due to the action of CCB [14]. The protective effects of CCBs against gentamycin-induced nephrotoxicity is assumed to be mediated through the inhibition of intracellular calcium release [10,15–17]. However, insufficient information is available about the effect of CCB in gentamycin-induced nephrotoxicity.

In clinical practice, Gentamycin – an aminoglycoside antibiotic, is the drug of choice for gram-negative bacterial infections due to its therapeutic efficacy against bacterial strains resistant to other antibiotics [18]. Nephrotoxicity is the major complication of this drug. Only 10–15% of cases were found to treat with gentamycin with renal dysfunction [19]. In recent years, once daily dosage regimen, effective observation and monitoring of the patients for gentamycin-induced risks factors have contributed to increase the clinical uses of this drug [18].

Gentamycin-induced nephrotoxicity is widely used in rodent model which produces proximal tubular necrosis similar to human subjects [10,20]. In last two decades, various parameters of aminoglycoside-induced nephrotoxicity have been researched [21]. Reactive oxygen species (ROS) play the leading role in nephrotoxicity caused by the extensive accumulations of gentamycin in the kidney tissue [9,10,15]. Again, gentamycin increased intracellular calcium concentration causing mesengial cell contraction, in turn, can induce the renin-angiotensin system, resulting in the formation of many vasoconstrictor substances like angiotensin II, endothelin I, and thromboxane A_2 [10,15,16].

Gentamycin demonstrated relevant and reversible acute renal injury and produce nephrotoxicity biomarkers similar to other nephrotoxic agents in animals [22]. Moreover, previous results demonstrated that gentamycin did not potentiate renal damage caused by streptozotocin or the disease diabetes; rather reduced renal dysfunction and less tubular injury were observed in streptozotocin-induced diabetic rats [23].

A large-scale of population globally uses herbs and natural products for the treatment of different diseases. Stevia is widely used as a natural sweetener having a beneficial effect on diabetes and hypertension. Previous study demonstrated that stevia ameliorated cisplatin-induced nephrotoxicity through reduction of inflammation and oxidative stress [24]. However, the outcomes of the treatment of stevia on gentamycin-induced nephrotoxicity are yet to be published. Furthermore, initial results of our clinical study showed that stevia improved the biochemical parameters like serum creatinine and serum uric acid levels in CKD patients [25].

Therefore, the present study explored the potential renoprotective effect of stevia on gentamycin-induced nephrotoxicity in rat models through biochemical, hematological and histological examination. This study also compared the effect of stevia, amlodipine, losartan, and valsartan in the progression and treatment of renal damage in these animal models.

2. Materials and methods

2.1. Experimental animal care & experiment design

Eight weeks old thirty-six male Sprague-Dawley rats (180–200 g m) were assigned in this study. These rats were adapted in standard laboratory settings for one week prior to the study. Room temperature of (22 ± 3 °C), and a humidity of 50 ± 10% with a 12 -h light/dark cycles was maintained. Standard pellet diet and drinking water *ad libitum* was provided to the rats. In this study, the selected animals were handled in absolute compliance and in accordance with the guidelines for the care and use of Laboratory Animals by the National Institute of Health and the study was approved by the Biosafety, Biosecurity and Ethics Committee of Jahangirnagar University, Savar, Dhaka, Bangladesh [Ethic approval no: BBEC, JU/M 2019 (1)6]. This project was jointly carried out in the Division of Pharmacology of Jadavpur University, Kolkata, India and Pharmacology Laboratory of Jahangirnagar University, Savar, Bangladesh.

The dose of stevia was selected from the study of Yesmine et al. [26], which reported that stevia 200 mg/kg/day once daily (p.o) demonstrated beneficial effect in the prevention of vascular and gastrointestinal damage in diabetic rats. Moreover, other studies showed that stevia given at 100 and 200 mg/kg (iv/ip) produced an antihypertensive effect [27,28]. Stevioside given at 100 and 200 mg/kg (ip) caused slow and persistent lowering of blood pressure in hypertensive rats [27]. Therefore, stevia 200 mg/kg/day might provide an optimum dose to achieve an effective plasma concentration in rats. The dose of losartan was chosen according to the study of Álvarez et al. [29], which showed that losartan (15 mg/kg/day) prevented oxidative stress and decreased lipid peroxidation in hypertension in rats. A study by Gasparo et al. [30], reported that valsartan (5 or 50 mg/kg) or in combination with enalapril, demonstrated protective effect on renal function. This study also demonstrated that non-hypotensive doses of valsartan and enalapril combination increased survival of spontaneously hypertensive rats with endothelial dysfunction. Therefore, valsartan 5 mg/kg/day was chosen in our study to observe the renoprotective effect.

Nephrotoxicity was induced by gentamycin (100 mg/kg body weight/day; i.p.) for 8 days. The animals were randomly divided into six groups; and six rats in each group-(i) sham control without nephrotoxicity (CON); (ii) standard group with nephrotoxicity followed by 0.09% NaCl solution (i.p.), (STD) once daily; (iii) nephrotoxicity treated with stevia (200 mg/kg/day; p.o.) (STV); (iv) nephrotoxicity treated with losartan (15 mg/kg/day, p.o.) (LOS); (v) nephrotoxicity treated with valsartan (5 mg/kg/day, p.o.) (VAS); (vi) nephrotoxicity treated with amlodipine (4 mg/kg/day, p.o.) (AML). All the treatments were carried out for 30 days, starting at 4 days before the gentamycin injection. Weekly measurement of body weight of all the animals was done during the experimental period.

2.2. Chemicals and reagents

The stevia plant material was collected from Burudi Gram, Purulia, West Bengal, India. It was processed and HPLC-verified in Raipur Rani, Panchkula (HR), India. Stevia powder contains Rebaudioside A-82% and Stevioside-18%. Amlodipine (Amlodipine besylate) (CAS No.: 111470–99-6), Losartan (Losartan potassium) (CAS No.: 124750–99-8), Valsartan (CAS No.: 137862–53–4), Gentamycin sulfate (CAS No.: 1405–41-0), and the source was Sigma-Aldrich Co., Germany.

2.3. Blood collection and serum separation

After the 30 days treatment period, an overdose of sodium pentobarbital (60 mg/kg; i.p.) was used to euthanize the experimental animals. Blood samples were collected via posterior venacava into EDTA (ethylene diamine tetra acetate) tubes for hematological tests and mixed properly to avoid clotting and tubes without EDTA for biochemical analysis, and reserved on ice. Then transfer into sample tubes for biochemical analysis. To obtain serum, blood was kept for 30 min to coagulate and centrifuged at 3000 rpm for 15 min using a bench top centrifuge (MSE minor, England). The supernatant serum samples were collected using dry Pasteur pipettes, and then it was stored in the freezer at -80^{0}C for further examination [31].

2.4. Biochemical & Hematological Analysis

The separated serum samples were analyzed to determine the condition of the kidney and liver. Blood urea, serum creatinine, serum albumin, total protein, random blood sugar, total serum bilirubin, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high-density lipoprotein (HDL), and liver enzymes such as Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT), were examined by using Dimension RxL Max integrated Chemistry's system (USA) automated biochemistry analyzer.

The automated SYSMEX 6-part Diff hematology analyzer (Model: XN-550) was used for the total Red Blood Cell, White Blood Cell, and platelet count, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV). Wright stain was used for blood smear preparation, and microscopic examination was done at 40X magnifications. An automated analyzer was used to find out the total and the differential count of WBC [32]. For plasma total protein analysis, the plasma liquid was fallen on the prism of a refractometer (Atago T2-NE, Japan) and was measured the concentration value of the scale.

2.5. Histological examination

Kidneys and livers of the experimental animals were prepared for the examination of gross lesions. 10% buffered formalin solution was used for the fixation of the organs and the tissue fixation was done in 48 -h duration and embedded with paraffin wax. The samples were trimmed about 4–5 µm thickness of tissue sections using a sectioning rotary microtome (Thermo Fisher Scientific, Model: HM 325, UK), and sectioned tissues were kept directly into the water bath (45 °C), and after that mounting was done. The mounted glass slides were preserved on a hot plate (54 °C) for whole night. Finally, Hematoxylin and Eosin (H&E) staining protocol was followed for mounted slides [33]. Photomicrographs were taken with ZEISS Axio Microscope, Germany (Camera: Leica) at 10X, 20X and 40X magnification.

2.5.1. Histological assessment of kidney tissues

Toxicological lesions of kidney tissue, such as tubular necrosis, medullary congestion, and the presence of protein and granular cast, degeneration, inflammation, and tubular injury were examined and scored. To do the assessment of kidney sections, a blind manner process was followed by an expert histologist. Tubular necrosis and protein cast were graded as follows: for no damage (0); mild (1), (unicellular, patchy, isolated damage); moderate (2), (< 25% damage); severe (3), (damage, 25%–50%); and very severe (4), (> 50% damage), based on the percentage of tissues affected [33,34].

2.5.2. Histological assessment of liver tissues

Toxicological lesions of liver tissues such as inflammation, portal area expansion, activated kupffer cells, fibrosis, ductular proliferation, congestion in blood vessels, and sinusoidal dilatation were examined and scored. Liver tissue lesions were scored as no lesions (0); mild (1), (1%–30% lesions); moderate (2), (> 30%-70% lesions) and severe (3), (> 70% lesions), based on the percentages of the tissues affected [32].

2.6. Statistics

All data were expressed as mean (± SEM) (Standard Error Mean). Drug-dose model, organ's weight, and hematological parameters were analyzed by Independent sample *t*-test. Two-way repeated measures ANOVA following Tukey B test was used to analyze the body weight of the experimental animals. One-way ANOVA was used to analyze the biochemical parameters. Multiparametric and semi-quantitative analysis has been done for histological scoring of kidney and liver tissue. Statistical analysis was performed using the SPSS software (Statistical Package for the Social Sciences, version 23.0, SPSS Inc, Chicago, III, USA). A *p*-value less than 0.05 was considered significant; highly significant < .01; very highly significant < .001.

3. Result

3.1. Determination of nephrotoxicity dose model

To establish the nephrotoxicity dose model, the animals were divided into three different groups: healthy control (CON), group-1 (treated with 100 mg gentamycin/kg/day; i.p.), and group-2 (treated with 150 mg gentamycin/kg/day; i.p). Blood Urea Nitrogen (BUN), serum creatinine and uric acid levels were determined. Among the parameters, a significant level of increase has been observed compared to the healthy control (Table 1).

3.2. Estimation of the weight of Body and Organs

Weekly measurement of body weight demonstrated that all experimental rats continuously gained weight (Table 2). Insignificant results were found for the body weight of the experimental groups during the four-week study period. Follow up comparisons indicated the difference was significant ($p < .01$) and increase the body weight over time found in the Two-way repeated measures ANOVA following Tukey B test (Table 2). Terminal weights of liver ($^*p < .001$) were found to be significantly elevated in the untreated disease (STD) group compared to the control (CON) rats (Table 3). Treatment with amlodipine reduced the liver weight (Table 3). A significant decrease in heart weight in the losartan treated group was found (Table 3).

Table 1
Comparison of the biochemical parameters of the nephrotoxicity dose model of animals.

Parameters	CON	Group-1	Group-2
Blood urea nitrogen (mg/dL)	22.43(± 1.44)	44.80(± 2.62) (p = .264)	73.13(± 1.04) (p = .230)
Serum creatinine (mg/dL)	0.79(± 0.01)	2.49(± 0.18) **(p = .002)** *	4.20(± 0.24) **(p = .006)** $^\#$
Uric acid (mg/ml)	1.06(± 0.03)	1.48(± 0.06) (p = .553)	1.75(± 0.07) (p = .061)

Results are expressed as Mean (± SEM). Independent Sample *t*-test was used to analyze the data. Here, N = 6 for all groups. Here, CON = Healthy Control, Group-1 represents the experimental animals treated with 100 mg of gentamycin and Group-2 represent the experimental animals treated with 150 mg of gentamycin. Here, $^*p < .001$ when compared to the healthy control with Group-1; $^\#p < .001$ when compared to the healthy control with Group-2.

Table 2
Comparison of the Body Weight of Sprague Dawley rats during the four-week treatment period.

Treatment Groups	Day	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
CON	1	199.833	14.928	169.346	230.321
	2	225.500	14.946	194.976	256.024
	3	232.500	15.438	200.971	264.029
	4	238.833	15.089	208.018	269.648
	5	235.000	13.985	206.439	263.561
STD	1	201.667	14.928	171.179	232.154
	2	197.000	14.946	166.476	227.524
	3	205.833	15.438	174.304	237.362
	4	222.500	15.089	191.685	253.315
	5	211.333	13.985	182.772	239.894
STV	1	195.333	14.928	164.846	225.821
	2	219.667	14.946	189.143	250.190
	3	228.333	15.438	196.804	259.862
	4	239.167	15.089	208.352	269.982
	5	239.333	13.985	210.772	267.894
AML	1	198.667	14.928	168.179	229.154
	2	219.167	14.946	188.643	249.690
	3	223.833	15.438	192.304	255.362
	4	234.000	15.089	203.185	264.815
	5	236.500	13.985	207.939	265.061
VAS	1	195.000	14.928	164.513	225.487
	2	221.000	14.946	190.476	251.524
	3	234.500	15.438	202.971	266.029
	4	244.333	15.089	213.518	275.148
	5	236.333	13.985	207.772	264.894
LOS	1	213.000	14.928	182.513	243.487
	2	206.333	14.946	175.810	236.857
	3	196.167	15.438	164.638	227.696
	4	200.500	15.089	169.685	231.315
	5	205.833	13.985	177.272	234.394

Data was analyzed by Two-way repeated measures ANOVA following Tukey B test. Here, N = 6 for all groups. Here, CON = Healthy Control; STD = Gentamycin-induced disease control; AML = Gentamycin-induced disease control treated with amlodipine; LOS = Gentamycin-induced disease control treated with Losartan; VAS = Gentamycin-induced disease control treated with Valsartan; STV = Gentamycin-induced disease control treated with Stevia. Significant value was considered as $^{*}p < .01$.

3.3. Determination of biochemical & hematological parameters

Significant result has been observed in blood urea ($p < .000$) and serum creatinine ($p < .000$). Again, the value of serum albumin ($p < .003$), total cholesterol ($p < .004$), HDL ($p < .009$), and LDL ($p < .050$) was increased significantly among the treatment groups. Furthermore, all the treatment groups showed the significant decrease in the value of SGPT ($p < .000$), SGOT ($p < .000$), and serum total bilirubin ($p < .000$) (Table 4). On the other hand, LOS significantly increased serum creatinine and blood urea levels compared to the untreated STD group (Table 5). However, serum total bilirubin level increased significantly in the STD group compared to that of CON group ($^{*}p < .02$) (Table 5). Treatment with valsartan markedly reduced the bilirubin level ($^{\#}p < .05$). However, it could not normalize the value (Table 5). The STD group demonstrated an increase in SGPT and SGOT levels compared to the CON group. Treatment with amlodipine ($^{\#}p < .04$), losartan ($^{\#}p < .02$) and stevia ($^{\#}p < .04$) significantly decreased SGOT level after the four-week treatment period. Serum cholesterol and LDL levels were found to be elevated in the STD group (Table 5). Treatment with valsartan and stevia reduced these values (Table 5).

A significant increase in hematocrit (HCT) level was observed ($^{*}p < .001$) in the STD group compared to the CON (Table 5). The untreated STD group showed an increase in basophil and lymphocyte levels (Table 5). Among the treatment groups, stevia ($^{\#}p < .009$) and amlodipine ($^{\#}p < .035$) demonstrated a significant increase in the basophil level (Table 5).

3.4. Examination of histological features

Histological features of kidney showed the comparison of organ toxicity among the groups for the tubular necrosis, glomerular congestion, blood vessel congestion, interstitial edema, inflammatory cells, and deposition of protein cast. All the figures are presented in the Fig. 1. Tubular necrosis was graded as based on the percentage of tissues affected [33,34]. In the liver tissue inflammation, fibrosis, portal expansion, ductular proliferation, congestion in blood vessels, activated kupffer cells, and sinusoidal dilatation were found as toxicological lesions and presented in Fig. 2 and scoring was done by the percentages of the tissues affected [32].

Section of rat kidney of the CON group had a normal morphological structure with the appearance of the tubules and glomeruli (Fig. 1 and Table 6). The STD Kidney showed congestion of blood vessels, tubular necrosis, deposition of protein casts in the tubules, interstitial inflammation, presence of inflammatory cells, and calcification at renal medulla and cortex. Chronic inflammation and inflammatory cells in the renal pelvis area were also observed in the STD rat model. Treatment with stevia reduced chronic inflammation and presence of inflammatory cells and tubular injury (Fig. 1 and Table 6). In addition, treatment with valsartan reduced nephrotoxicity by decreasing chronic inflammation, presence of inflammatory cells, tubular necrosis, protein cast, calcification and tubular injury. Treatment with amlodipine also improved renal tissue damage over the four-week treatment period (Fig. 1 and Table 6).

Histological features of liver tissue demonstrated normal arrangement of hepatic cells, central veins and normal blood sinusoids in the CON groups (Fig. 2 and Table 6). The portal expansion with inflammation and fibrous expansion in the portal tract and ductular proliferation, congestion in the portal vein was observed in the STD rats (Fig. 2 and Table 6). Administration of stevia, losartan and valsartan reduced portal expansion with inflammation and fibrosis (Fig. 2 and Table 6).

4. Discussion

Significant increase of serum creatinine, uric acid and blood urea

Table 3
Comparison of the organ's weight of the experimental rats at the end of the treatment period.

Weight of the organs (mg/kg body weight)	CON	STD	STV	AML	VAS	LOS
Kidney	1.25 ± 0.06	1.41 ± 0.09	1.23 ± 0.06	1.32 ± 0.07	1.37 ± 0.09	1.65 ± 0.06
Liver	6.15 ± 0.19	**6.21 ± 0.60***	6.94 ± 0.41	5.84 ± 0.30	6.41 ± 0.29	**6.94 ± 0.23**$^{\#}$
Lungs	0.96 ± 0.13	0.98 ± 0.06	1.18 ± 0.04	1.03 ± 0.15	1.15 ± 0.03	1.18 ± 0.03
Heart	0.70 ± 0.04	0.62 ± 0.03	0.70 ± 0.03	0.70 ± 0.04	0.64 ± 0.02	**0.57 ± 0.01**$^{\#}$

Data are expressed as Mean (± SEM). Independent Sample *t*-test was used for the analysis. N = 6 for all groups. Here, CON = Healthy Control; STD = Gentamycin-induced disease control; AML = Gentamycin-induced disease control treated with amlodipine; LOS = Gentamycin-induced disease control treated with Losartan; VAS = Gentamycin-induced disease control treated with Valsartan; STV = Gentamycin-induced disease control treated with Stevia. Here, $^{*}p < .001$, compared to the healthy control and $^{\#}p < .001$, compared to gentamycin-induced disease control group.

Table 4
Comparison of the biochemical parameters of gentamycin-induced nephrotoxic rats in treatment groups.

Parameters	Different Groups	Sum of Squares	Df	Mean Square	F	Sig.
Blood Urea	Between Groups	1743.701	5	348.740	7.758	**.000**
	Within Groups	1348.542	30	44.951		
	Total	3092.243	35			
Serum Creatinine	Between Groups	1.954	5	.391	6.579	**.000**
	Within Groups	1.782	30	.059		
	Total	3.735	35			
RBS	Between Groups	1.390	5	.278	2.230	.077
	Within Groups	3.740	30	.125		
	Total	5.130	35			
Total Protein	Between Groups	.182	5	.036	.721	.613
	Within Groups	1.517	30	.051		
	Total	1.699	35			
Serum Albumin	Between Groups	1.327	5	.265	4.592	**.003**
	Within Groups	1.733	30	.058		
	Total	3.060	35			
Total Cholesterol	Between Groups	2736.889	5	547.378	4.422	**.004**
	Within Groups	3713.333	30	123.778		
	Total	6450.222	35			
TG	Between Groups	97.222	5	19.444	.634	.676
	Within Groups	920.667	30	30.689		
	Total	1017.889	35			
HDL	Between Groups	202.222	5	40.444	3.784	**.009**
	Within Groups	320.667	30	10.689		
	Total	522.889	35			
LDL	Between Groups	1100.333	5	220.067	2.528	**.050**
	Within Groups	2611.667	30	87.056		
	Total	3712.000	35			
SGPT	Between Groups	8104.667	5	1620.933	38.861	**.000**
	Within Groups	1251.333	30	41.711		
	Total	9356.000	35			
SGOT	Between Groups	11140.333	5	2228.067	12.955	**.000**
	Within Groups	5159.667	30	171.989		
	Total	16300.000	35			
Total Serum Bilirubin	Between Groups	2.470	5	.494	38.041	**.000**
	Within Groups	.390	30	.013		
	Total	2.860	35			

One-way ANOVA has been done for the data analysis. Here, N = 6 for all groups. RBS = Random Blood Sugar, TG = Triglycerides, HDL = High Density Lipoprotein, LDL = Low Density Lipoprotein, SGPT = Serum glutamic pyruvic transaminase, SGOT = Serum glutamic oxaloacetic transaminase. *The mean difference is significant at the 0.05 level.

nitrogen levels over a five-day administration of 100 mg gentamycin/kg/day in the inductive group of rats represented that nephrotoxicity was established in these experimental animals. No mortality was noted in this group. Although, administration of 150 mg gentamycin/kg/day demonstrated more pronounced renal damage than 100 mg gentamycin/kg/day as represented by highly elevated blood urea nitrogen and serum creatinine levels, fifty percent of the experimental animals died after 5th day of experimental period.

The current study expressed the continuous increase of body weight in the gentamycin-induced untreated group which might be due to edema caused by dysfunction in reabsorption process and reduced GFR [35], and tubular necrosis. The nephrotoxicity can be assessed by measuring body weight and the biochemical markers like serum creatinine level and blood urea nitrogen. The elevated serum creatinine levels are the most powerful indicator in the first phases of kidney disease. The concentration of blood urea was started to increase only after parenchymal injury [36]. In this study, administration of gentamycin led to a significant increase in blood urea and serum creatinine levels, which corroborated with previous results reported by others [37,38]. In this case, treatment with stevia, valsartan, and amlodipine showed significant protective effects against renal failure by reducing the serum creatinine level and blood urea.

Gentamycin distinctly raised intracellular Ca^{2+} levels and activate both calcium influx from the external source and Ca^{2+} release from the internal stores causing renal mesengial cellular contraction [39]. Accordingly, Ca^{2+} channel blocker was considered to have the beneficial effects on gentamycin-induced nephrotoxicity [40]. Previous studies suggested that the hypotensive mechanism of stevia might be due to interference of the Ca^{2+} influx [41]. Our results indicated that treatment with amlodipine had a positive effect on progression and development of nephrotoxicity caused by gentamycin. Toba et al. demonstrated that amlodipine and manidipine inhibited excessive expression of NADPH oxidase in angiotensin-II, which was stimulated by endothelial cells and reduced superoxide generation [42]. Furthermore, ACE inhibitors and ARBs were indicated to slow down the progression and development of diabetic glomerulopathy and chronic renal dysfunction [43]. For this reason, these two groups of drugs are reported as the first-line treatment for the patients of diabetes, hypertension and CKD [43]. However, our results demonstrated that losartan treated group failed to improve or prevent renal failure and nephrotoxicity.

AT_1 receptor is the member of G protein-coupled receptor superfamily containing 359 amino acids [44]. The AT_1 receptor blockers used in this study were losartan and valsartan. Losartan has the structure of an imidazole derivative with a biphenyl-tetrazole side chain, while valsartan has a tetrazole-biphenyl-valine derivative with only one heterocyclic structure [45]. It was found that the tetrazole ring and carboxylic acid group of valsartan possibly bind with Lys199 of TM5 and Ser109 of TM3 and Asn295 of TM7 of AT_1 receptor. Repeatedly, the hydroxymethyl group and tetrazole ring of losartan probably bind with Asn295 of TM7 and Ser109 of TM3 of AT_1 receptor. Therefore, Bhuiyan et al., (2009) mentioned about the larger number of binding sites of valsartan of the AT_1 receptor than losartan in their study [46]. Furthermore, Fogari et al., (2010) demonstrated that valsartan is an immediately active drug and excreted unchanged through urine, whereas

Table 5
Comparison of the biochemical & hematological parameters of gentamycin-induced nephrotoxic rats in treatment groups.

Different Parameters	CON	STD	STV	AML	VAS	LOS
Blood urea (mg/dL)	19.50 ± 0.76	22.00 ± 1.03	22.83 ± 2.13	20.16 ± 0.98	19.66 ± 1.02	**39.33 ± 6.05**$^{\#}$
Serum creatinine (mg/dL)	1.06 ± 0.03	1.08 ± 0.05	1.20 ± 0.10	1.03 ± 0.06	0.97 ± 0.02	**1.44 ± 0.15**$^{\#}$
RBS (mmol/L)	2.85 ± 0.10	2.96 ± 0.12	2.58 ± 0.16	2.88 ± 0.13	2.51 ± 0.09	2.41 ± 0.21
Total Protein (g/dL)	6.40 ± 0.10	6.40 ± 0.06	6.33 ± 0.07	6.25 ± 0.09	6.20 ± 0.05	6.23 ± 0.14
Serum Albumin (g/dL)	2.96 ± 0.08	3.18 ± 0.10	3.20 ± 0.10	3.01 ± 0.11	2.73 ± 0.05	3.31 ± 0.11
Total Cholesterol (mg/dL)	68.50 ± 2.29	83.16 ± 1.88	85.83 ± 9.23	73.33 ± 2.20	62.33 ± 2.66	82.50 ± 4.20
TG (mg/dL)	42.16 ± 1.70	44.50 ± 1.94	43.16 ± 1.95	42.16 ± 1.83	40.33 ± 1.72	45.33 ± 3.77
HDL (mg/dL)	50.33 ± 1.14	51.83 ± 1.77	53.50 ± 1.23	49.83 ± 1.10	45.83 ± 1.66	50.33 ± 0.88
LDL (mg/dL)	11.00 ± 1.96	21.83 ± 1.74	22.66 ± 7.66	14.83 ± 1.13	9.33 ± 1.96	22.33 ± 4.05
SGPT (per Liter)	47.16 ± 3.75	92.16 ± 1.70	86.33 ± 2.48	86.50 ± 2.56	81.50 ± 2.40	86.33 ± 2.48
SGOT (per Liter)	178.16 ± 5.33	234.50 ± 10.17	**191.66 ± 3.09**$^{\#}$	**198.66 ± 3.12**$^{\#}$	189.66 ± 4.08	**193.33 ± 1.97**$^{\#}$
Serum total bilirubin (mg/dl)	0.29 ± 0.02	**1.17 ± 0.06***	0.87 ± .04	0.89 ± 0.06	**0.87 ± 0.03**$^{\#}$	0.88 ± 0.04
Hb (gm/dL)	12.46 ± 0.13	10.71 ± 0.27	13.06 ± 0.28	12.36 ± 0.21	12.33 ± 0.14	12.44 ± 0.13
RBC (million/cumm)	6.51 ± 0.07	5.85 ± 0.08	6.77 ± 0.14	6.64 ± 0.15	6.24 ± 0.10	6.32 ± 0.22
HCT (%)	38.80 ± 0.31	**42.76 ± 0.90***	41.41 ± 0.72	39.20 ± 1.30	36.83 ± 0.48	40.40 ± 0.63
MCV (fL)	57.98 ± 0.44	59.85 ± 1.16	59.50 ± 0.46	50.93 ± 8.36	56.60 ± 0.76	60.10 ± 1.02
MCH (pg)	18.73 ± 0.15	18.33 ± 0.32	18.35 ± 0.27	18.60 ± 0.28	18.41 ± 0.27	18.80 ± 0.14
MCHC (gm/dL)	32.38 ± 0.32	33.08 ± 0.52	32.93 ± 0.14	29.18 ± 3.20	33.73 ± 0.31	31.73 ± .218
RDW-SD (fL)	33.10 ± 0.25	31.28 ± 0.55	33.75 ± 0.43	33.25 ± 0.55	34.16 ± 0.83	35.05 ± 1.06
RDW-CV (%)	17.63 ± 0.33	17.26 ± 0.21	17.40 ± 0.19	**17.05 ± 0.09**$^{\#}$	17.76 ± 0.38	17.58 ± 0.32
WBC (X 10^3)	5.51 ± 0.28	4.65 ± 0.59	4.88 ± 0.56	4.86 ± 0.32	5.20 ± 0.68	4.68 ± 0.40
Neutrophils (%)	15.50 ± 0.6	7.66 ± 0.84	10.83 ± 1.16	8.33 ± 1.17	10.16 ± 0.9	6.33 ± 0.84
Lymphocytes (%)	81.00 ± 0.51	89.16 ± 1.1	85.16 ± 1.10	86.66 ± 1.3	85.83 ± 0.9	91.00 ± 0.85
Monocytes (%)	2.16 ± 0.30	1.16 ± 0.16	1.33 ± 0.21	**1.83 ± 0.40**$^{\#}$	1.66 ± 0.21	1.16 ± 0.16
Eosinophils (%)	1.33 ± 0.21	1.16 ± 0.16	1.16 ± 0.16	**1.00 ± 0.00**$^{\#}$	1.50 ± 0.22	**1.00 ± 0.00**$^{\#}$
Basophils (%)	0.26 ± 0.07	1.14 ± 0.13	**1.50 ± 0.22**$^{\#}$	**2.21 ± 0.51**$^{\#}$	1.04 ± 0.07	0.99 ± 0.26
Platelet (X 10^3)	620.50 ± 21.6	546.66 ± 54.2	493.33 ± 65.1	493.16 ± 57.4	528.50 ± 44	537.33 ± 4

Values are expressed as Mean (± SEM). Independent sample *t*-test has been followed. N = 6 for all groups. Here, CON= Healthy Control; STD = Gentamycin-induced disease control; AML = Gentamycin-induced disease control treated with amlodipine; LOS = Gentamycin-induced disease control treated with Losartan; VAS = Gentamycin-induced disease control treated with Valsartan; STV = Gentamycin-induced disease control treated with Stevia. Here, RBC = total count of Red Blood Cells, Hb = Hemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volume, MCH = Mean Corpuscular Hemoglobin, MCHC = Mean Corpuscular Hemoglobin Concentration, RDW SD = Red Blood Cell Distribution Width SD, RDW-CV = Red Blood Cell Distribution Width-CV. $^{*}p < .001$ when compared to the healthy control and $^{\#}p < .001$ when compared to gentamycin induced disease control.

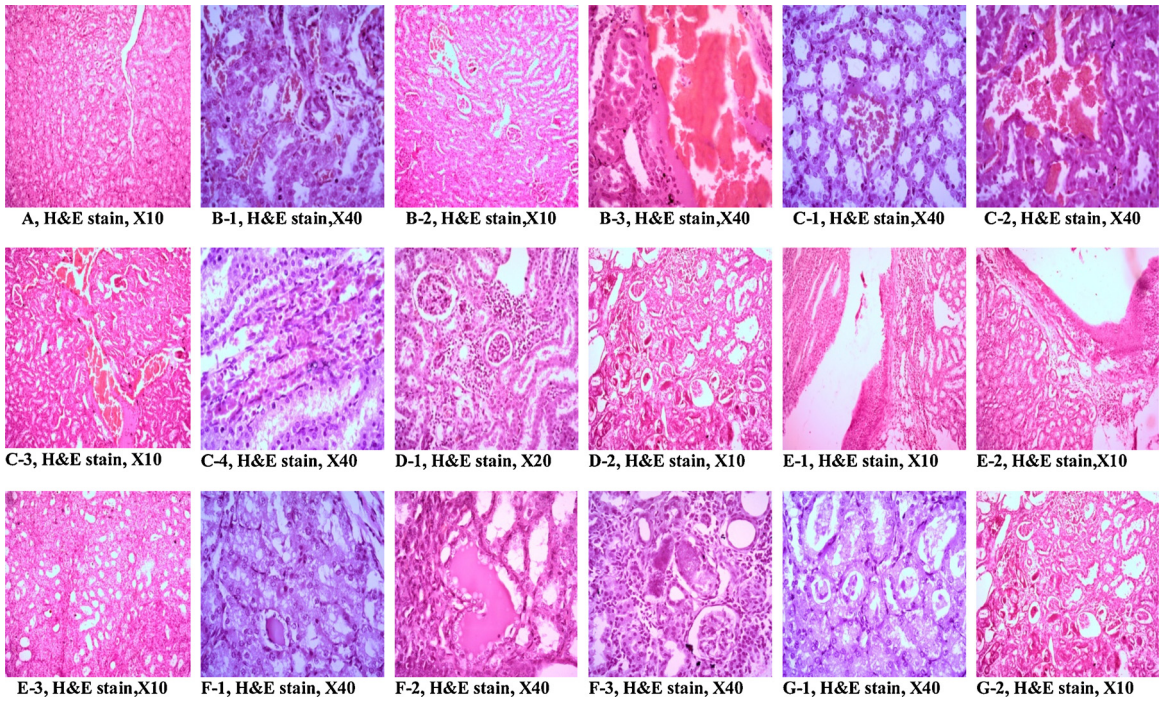

Fig. 1. Photomicrographs of Kidney sections of rats at the end of the 4th week at different magnification (H&E stain, X10, X20, X40).
In this Figure section, **A** represents no change of the renal cortex of CON group; **B (1, & 3)** represents the glomerular & peritubular congestion in the renal cortex of STV, & VAS group; **B-2** represents the glomerular & peritubular congestion in the renal medulla of AML rats; **C-1** represents the congestion in the blood vessels in the renal medulla of STD rats and **C (2 & 3)** shows in the renal cortex of AML & VAS rats and **C-4** expresses in the renal medulla of STV rats; Interstitial inflammation or edema has been found in the renal cortex in figure **D-1** of STV rats and **D-2** of LOS rats. **E (1 & 2)** expressed chronic inflammation & inflammatory cells in the renal pelvis of STD and AML rats and **E-3** shows in the renal medulla of LOS rats. **F (1 & 2)** shows the tubular necrosis, protein cast & calcification in the renal medulla of STD and STV rats and **F-3** in the renal cortex of LOS rats. **G (1 & 2)** represents the tubular injury in the renal cortex of STD and LOS group.

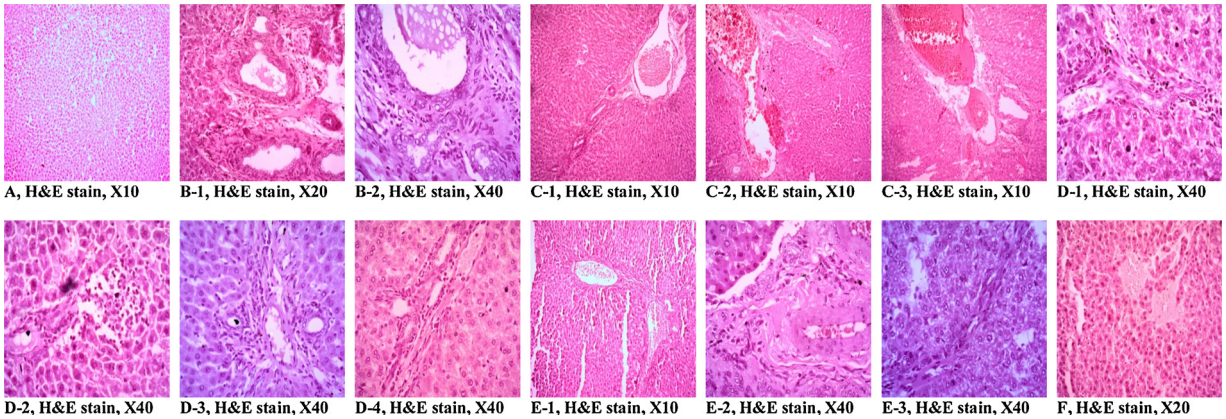

Fig. 2. Photomicrographs of Liver sections of rats at the end of the 4th week at different magnification (H&E stain, X10, X20, X40).
In this Figure section, **A** represents no change of the liver tissue in CON rats. Portal expansion with inflammation and fibrosis and ductular proliferation has been observed in **B (1 & 2)** of STD and VAS rats. Blood vessel congestion in the portal area was observed in **C (1, 2 & 3)** of STD and STV and AML rat model. Mild inflammation in the portal area was found in **D (1, 2, 3, & 4)** of STD, STV, AML and LOS rats. Mild fibrosis & inflammation were observed in the portal area in **E (1, 2 & 3)** of STD and STV and AML rats. Sinusoidal Dilatation has been observed in **F** of STV experimental rat models.

Table 6
Histological scoring of kidney and liver tissues of rat models.

Histological Features of Kidney	CON	STD	STV	AML	VAS	LOS
Glomerular Congestion	0	2	1	1	1	1
Peritubular Congestion	0	2	1	1	1	1
Congestion in Blood Vessels	0	3	2	1	1	2
Interstitial inflammation with oedema	0	2	1	0	0	3
Chronic Inflammation	0	2	1	1	0	2
Inflammatory Cells	0	2	1	1	0	2
Tubular Necrosis	0	4	1	0	0	4
Protein Cast	0	2	1	0	0	3
Calcification	0	1	1	0	0	2
Tubular Injury	0	3	1	0	0	4
Histological Features of Liver						
Portal expansion with inflammation	0	2	0	0	2	0
Fibrosis	0	2	0	0	2	0
Ductular proliferation	0	2	0	0	2	0
Blood vessel congestion	0	3	3	3	1	0
Mild Inflammation	0	1	1	1	1	1
Mild Fibrosis	0	1	1	1	1	1
Sinusoidal Dilatation	0	0	3	0	0	0

Preliminary observation of the histological slides of kidneys and liver of all groups of rats. Multiparametric, semi-quantitative analysis has been done for histological scoring of kidney and liver tissue. Tubular necrosis and protein cast were graded as follows: for no damage (0); mild (1), (unicellular, patchy, isolated damage); moderate (2), (< 25% damage); severe (3), (damage, 25%–50%); and very severe (4), (> 50% damage), based on the percentage of tissues affected [33,34]. The lesions scoring for liver tissues were scored as no lesions (0); mild (1), (1%–30% lesions); moderate (2), (> 30%-70% lesions) and severe (3), (> 70% lesions), based on the percentages of the tissues affected [32]. Here, CON = Healthy Control; STD = Gentamycin induced disease control; AML = Gentamycin induced disease control treated with amlodipine; LOS = Gentamycin induced disease control treated with Losartan; VAS = Gentamycin induced disease control treated with Valsartan; STV = Gentamycin induced disease control treated with Stevia.

losartan converted into more-active metabolite [45]. Moreover, the observed renoprotective effect of valsartan might be due to its greater capacity to activate renal angiotensin II type 2 (AT_2) receptors than that of losartan. In the current study, valsartan also demonstrated a significant beneficial effect on renal damage over losartan.

Different studies on animal models established that the production and accumulation of free radicals are major causes of gentamycin induced nephrotoxicity [19]. Elewa HA et al., (2016) demonstrated that gentamycin-induced rats showed glomeruli atrophy and hypertrophy in animals, tubular necrosis with cystic luminal dilatation at the cortex [47]. The control group of the study showed normal histological structure of the glomeruli and renal tubules in the cortex and in the medulla [48]. Gentamycin induced renal damage was observed by glomerular and peritubular congestion, interstitial inflammation, edema, protein cast, calcification, tubular necrosis and injury. Treatment with stevia and amlodipine partially and valsartan mildly decreased renal damage. Previous results reported that losartan markedly ameliorated gentamycin-induced renal tubular damage in histological examination and scoring [47]. Besides, a few studies showed that losartan aggravated renal damage caused by gentamycin [48]. In the current study, losartan treatment provoked renal damage in the renal medulla and cortex. Although both losartan and valsartan are highly effective controlling blood pressure, losartan has smaller number of hydrogen bonds and lesser affinity to bind to AT_1 receptor and activate AT_2 receptors compared to valsartan. AT_2 receptor activation results in small vessel dilatation and which possibly prevent further renal and cardiac damage.

SGPT, SGOT, and total serum bilirubin are the specific indicators of impairment of hepatic cell membrane and hepatocellular necrosis. Gentamycin increased oxidative stress and lipid peroxidation developing a greater level of SGOT [49]. In the present study, amlodipine, losartan and stevia improved the histological damage of the liver, such as, portal expansion and inflammation, fibrosis, ductular proliferation, and blood vessel congestion. In oppose with the results found in the kidney tissue, liver histological slides demonstrated that only losartan showed an improvement in all areas of damage to hepatocytes except mild inflammation and fibrosis. Treatment with stevia, amlodipine and losartan reduced SGPT, SGOT, and serum bilirubin levels. In brief, valsartan and stevia demonstrated the beneficial modulatory and renoprotective effects in gentamycin-induced nephrotoxicity. Our published data demonstrated the multimodal mechanism of stevia, via calcium channel antagonism, M_2 muscarinic receptor activation, and increased nitric oxide secretion [50].

5. Conclusion

In conclusion, the comparison of the renoprotective effect of amlodipine, losartan, valsartan and stevia in gentamycin-induced renal damage in Sprague-Dawley rats have been done. Our results indicated that valsartan is the treatment of choice in nephrotoxicity and amlodipine has a beneficial protective effect on the renal system comparable to that of ARBs. Stevia showed a beneficial effect in reducing serum biochemical parameters and tissue damages. Our preliminary clinical study in CKD patients also supported this outcome [25]. The

mechanisms of renoprotective effects of stevia might be multimodal, via the reduction of inflammation through other neuro-hormonal pathways. Further study is needed to establish these valuable effects and illuminate the precise mechanism of renoprotective effects of these drugs.

Conflict of interest

None.

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