Nephroprotective potentials of *Citrus Aurantium*: A prospective pharmacological study on experimental models

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Abstract: *Citrus aurantium* is traditionally used in various kidney problems like burning of urine, urinary hesitancy and renal colic. The main objective of the present work was to evaluate the protective role of *Citrus aurantium* against gentamicin induced renal damage, due to its free radical scavenging properties to present experimental facts for their traditional use. 200 mg/kg/day of ethanolic extract of the plant employed in combination with the toxic doses of gentamicin for twenty-one days. The group GC-au (animals treated with co-administration of *Citrus aurantium* and gentamicin) protected renal damage expected with gentamicin, assessed by known functional and morphological parameters, significantly different from group G (animals treated with gentamicin). All the renal functioning parameters including; Blood urea nitrogen, Serum creatinine, Serum uric acid, Creatinine clearance, Serum electrolytes, Body weight, Urinary volume, Enzyme excretions, Urinary protein excretions and histological examination was performed for each and every group animals. The plant extract proved to have nephroprotective potentials may because of its known flavonoid contents and antioxidant properties.

Keywords: Bitter Orange, *Citrus aurantium*, gentamicin, nephrotoxicity, rabbits.

INTRODUCTION

Citrus aurantium L. (Rutaceae) also called Bitter orange, used as Traditional Chinese Medicine for more than 5,000 years (Castleman, 1995). The application of fruit extract as dietary supplement for losing body weight has recently been developed, with the active constituents as adrenergic amines and flavonoids (Kubo *et al*., 2005). Bitter orange used in herbal medicine as a stimulant (Sharpe *et al*., 2006), contains the tyramine metabolites N-methyltyramine, octopamine and synephrine (Gange *et al*., 2006). These substances are similar in action to that of epinephrine, act on the α_1 receptors responsible for hypertension (Hess and Sullivan, 2005). It had also been reported that *C. aurantium* has useful effects to control insect pests (Siskos *et al*., 2009), treatment of obesity and suppression of appetite (Monsef-Esfahani *et al*., 2004), as sedative and heart tonic (Calapai *et al*., 1999). Some of the studies showed their effectiveness on cardio vascular system, which includes; interruption in the blood pressure and excitability of cardiac muscles or adrenergic activity (Fang *et al*., 2003). The phytochemical study of fruit peel extract was reported to contain volatile oils, limonene, flavonoids, coumarins, triterpenes, vitamin C, carotene, and pectin. Flavonoids are being responsible for its anti-inflammatory, antibacterial, and antifungal properties (Jyotsna and Saonere, 2011). The plant extract was also reported with strong antimicrobial activities against a wide range of micro-organisms (kirbaşlar *et al*., 2009). Stimulation of antioxidant detoxification enzyme system by Limonene helps to control cancer (Satoh *et al*., 1996). Due to the usefulness of plant as discussed above and its antioxidant potential because of their strong flavonoids contents (Jyotsna and Saonere, 2011) and an local concept of peoples, about the protective role of decoction of fruit peel extract of *C. aurantium* in kidney problems like burning of urine, urinary hesitancy and kidney pain, the present work was attempted to investigate the nephroprotectant potentials of *C. aurantium* against gentamicin induced nephrotoxicity.

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MATERIALS AND METHODS

Plant extraction

C. aurantium fruit were purchased from Abbottabad, Pakistan in the year 2010 after identified by Professor Umar Farooq. Voucher specimen (1025) was deposited to the Department of Botany, Government College Abbottabad Pakistan. The peel was dried under shade and grinded to be powdered. Extracted with sufficient quantity of ethanol for about two weeks with continues stirring on alternate days and filtered under vacuum.

Phytochemical investigation

The preliminary phytochemical study was performed by using the procedure as previously described (Sofowora, 1993).

Experimental protocol

Twenty-four male rabbits, weighing between 1-1.5kg were purchased from local market Abbottabad Pakistan and were kept for acclimatization, fifteen days before the start of experiment. All the animals were maintained at same diet and divided into four groups. The handling and care of animals were strictly performed according to the

guidelines of the University of Malakand Chakdara, along with the international laws and policies (National Institutes of Health Guide for the Care and Use of Laboratory Animals, Publication no. 85-23, 1985) after the approval from University Research Committee. Gentamicin and extract was administered for the period of twenty one days according to the schedule as presented in table 1.

Table 1: Daily dosage regimen for a period of twenty one days

No.	Group	Daily dose received
1	C	Normosaline (0.9%) 2ml/kg/day.
2	G	Gentamicin 80mg/kg/day (toxic dose)
3	GC-au	Gentamicin 80mg/kg/day and *Citrus aurantium* 200mg/kg/day
4	C-au	*Citrus aurantium* 200mg/kg/day.

Both blood and urine samples were collected three times throughout experimental period for the estimation of BUN (Blood urea nitrogen), Creatinine, Uric acid, Sodium, Potassium, Calcium, urinary Protein and enzymes.

Reagent kits were used for estimation of BUN by Bertholot's indophenol assay, serum and urinary creatinine by Jaffe reaction and serum uric acid, with the help of chemistry analyzer (Smith, 1985). CPC (cresolphthalein complexone) method was used for the measurement serum calcium level while serum sodium and potassium were measured by using Flame photometric method (Blosser, 1985). Estimation of urinary protein (Johnson *et al*., 1999), Urinary ALP (Alkaline phosphatase) and LDH (Lactate dehydrogenase) was performed by using commercially available reagents following German Society of Clinical Chemistry (Deutsche, 1972). Microscopic examination of urine was also performed with the help of light microscope (Germany).

Histopathology

Three animals in each group were slaughtered on last day of study period for histological examination. Kidneys were isolated and fixed with formalin. Tissues were treated with alcohol and xylene and fixed in wax. The tissues were then cut down into a number of portions less than 3 μ, using Rotatory microtome (Micros, Germany). The slides were examined under light microscope after stained with hematoxylin and eosin dyes.

STATISTICAL ANALYSIS

Results were expressed as mean ± standard error of mean and compared by using one way analysis of variance followed by dunnett test by using 5th version of Graph-Pad prism. The difference was considered significant if the *P*-value was less than 0.05.

RESULTS

Preliminary phytochemical study

Preiminary phytochemical study of *C. aurantium* revealed the presence of excess amount of flavonoids and mild amount of carbohydrates, saponins, tannins, glycosides and terpenes. However, no significant presence of alkaloids was detected.

Measurement of body weight

All the animals found to decrease their body weight progressively throughout the experimental period. Control group animals observed to lost body weight (0.155± 0.91%) while gentamicin treated group lost (10.795± 1.09%). Group GC-au and C-au animals lost (1.28± 0.73%) and (1.24±3.65%) respectively as tabulated in table 2.

Blood urea nitrogen

Significant increase in blood urea nitrogen was observed in group G animals on 11th day of experimental period as 37.78±2.14 mg/dl vs control, 13.75±1.04 mg/dl, which further increased on the last day. The difference between Group GC-au and C-au were observed extremely significant when compared with group G, as given in table 2.

Serum creatinine level

Serum creatinine level elevated significantly on 11th (1.96 ± 0.14 mg/dl vs 0.71±0.10 mg/dl (control), $P<0.0001$) and 21st day of experiment (4.02±0.14 mg/dl vs 0.80±0.10 mg/dl, $P<0.0001$). The difference observed between Group G and all other groups were extremely significant as given in table 2.

Creatinine clearance

Creatinine clearance decreased as 2.08±0.25 ml/min vs control 5.08±0.82 ml/min ($P=0.0058$), which was significantly different from group GC-au and C-au as shown in table 2.

Serum uric acid

Serum uric acid increased significantly in toxic group as 2.34±0.12 mg/dl vs control 1.51±0.02 mg/dl significantly different from group GC-au and C-au as given in table 2.

Serum electrolytes

No significant increase or decrease in serum sodium was observed in any group throughout study period as tabulated in table 2. However extremely significant difference in serum potassium was noted between group G and control on both day 11th and 21st of study period. Group GC-au was found significantly different when compared with group G on 11th day of experiment (4.95± 0.26 mEq/l, 5.25±0.37 mEq/l vs group G 3.96±0.14

Table 2: Results according to day 0, 11th and 21st with various kidney functioning parameters

Parameter	Day	Group C	Group G	Group GC-au	Group C-au
% weight loss	21	0.155±0.91***	10.795±1.09	1.28±0.73***	1.24±3.65*
Serum BUN (mg/dl)	0	13.05±1.15	12.82±1.11	11.90±1.06	12.95±0.29
	11	13.75±1.04***	37.78±2.14	12.13±1.06***	13.79±0.19***
	21	14.14±1.12***	54.18±2.60	13.26±0.97***	15.60±0.46***
Serum Creatinine (mg/dl)	0	0.68±0.07	0.52±0.03	0.59±0.06	0.76±0.05
	11	0.71±0.10***	1.96±0.14	0.84±0.08***	0.83±0.03***
	21	0.80±0.10***	4.02±0.14	0.99±0.10***	0.89±0.03***
Creatinine clearance (ml/min)	0	5.65±0.63	5.28±0.46	7.05±0.90	4.50±0.57
	11	5.08±0.82**	2.08±0.25	4.39±0.48**	4.05±0.46**
	21	4.99±1.16**	0.76±0.09	3.37±0.42***	4.55±0.66***
Serum Uric acid (mg/dl)	0	1.23±0.07	1.11±0.08	1.21±0.06	0.98±0.05
	11	1.39±0.04*	1.56±0.05	1.18±0.07**	1.04±0.05***
	21	1.51±0.02***	2.34±0.12	1.53±0.23*	1.14±0.08***
Serum Sodium (mEq/l)	0	140.5±1.20	141.17±0.75	140.5±76	139.83±2.50
	11	139.6±0.56	140.5±0.56	140.16±0.74	139±2.33
	21	140.17±1.01	137.67±1.09	139.83±0.79	137.66±1.38
Serum Potassium (mEq/l)	0	5.30±0.19	5.21±0.21	5.13±0.30	5.80±0.49
	11	5.26±0.15***	3.96±0.14	4.95±0.26**	5.25±0.37**
	21	5.10±0.24***	3.43±0.17	4.70±0.20***	4.60±0.16*
Serum Calcium (md/dl)	0	10.12±0.16	10.28±0.29	9.86±0.15	9.96±0.20
	11	9.96±0.17**	8.48±0.34	9.70±0.10**	9.39±0.16*
	21	9.72±0.25***	7.68±0.21	9.48±0.27***	7.83±0.14
Urinary Protien (mg/dl)	0	1.98±0.25	1.56±0.20	1.94±0.27	2.10±0.30
	11	1.64±0.17*	2.51±0.30	1.81±0.24	2.0±0.29
	21	1.81±0.22***	3.86±0.32	2.31±0.21**	1.89±0.17***
Urinary Volume (ml)	0	203±12.13	180±9.92	220±13.92	190±19.76
	11	200±9.16	168±11.96	197.67±8.62	191.83±23.20
	21	217±19.77**	126±9.09	206±12.79***	227±29.90**

Results were expressed as Mean ± Standard error mean: *** extremely significant in comparison with group G, ** very significant and * significant.

mEq/l) and become extreme on day 21st as given in table 2. Serum calcium decreased significantly in group G when compared with control and group GC-au on last day of experiment as provided in table 2.

Total urinary protein excretion

Significant increase in urinary protein was observed in group G on day 11 (2.51±0.30 mg/dl vs control, 1.64± 0.17 mg/dl), which further increased on day 21st (3.86± 0.32 mg/dl vs controls, 1.81±0.22 mg/dl) significantly different from extracts treated groups.

Urinary lactate dehydrogenase and alkaline phospatase

On day 11, extremely significant difference in lactate dehydrogenase was observed in group C, GC-au and C-au when compared with gentamicin treated group which were not as significant on day 21 (fig. 1). Control, GC-au and only extract treated group exhibited no significant change in alkaline phosphatase excretion in comparison with group G throughout the study period (fig. 2).

Urinary volume and examination

Significant fall in group G (126±9.09 ml) was observed when comparison with group C and C-au (217±19.77 ml and 227±29.90 ml) respectively. Further, GC-au (206± 12.79 ml) was found significantly different from group G. Microscopic examination of urine revealed the presence of significant number of renal casts in gentamicin treated group, significantly different from group GC-au, C-au and C. Excess quantity of red blood cells, proteins and small quantity of leukocytes and bilirubin in gentamicin treated group were identified. However in group GC-au the presence of small quantity of RBC and WBC was diagnosed.

Histopathological Investigations

Glomerular atropy and hyper-cellularity were observed in group G animals when compared with control group animals. Ruptured tubules and flattening of cells with hydropic changes were also observed significantly in group G animals (fig. 4), different from control group

animals (fig. 3). Ruptured tubules was also diagnosed in group GC-au medulla up to little extant with hyaline filled Lumina (fig. 5). Cellular pattern was lost with necrosis, specially detected in proximal tubules in gentamicin treated group (fig. 4). However, necrosis was not detected in other groups as compared with group G. Further no significant changes observed in group C-au in comparison with control group (fig. 6).

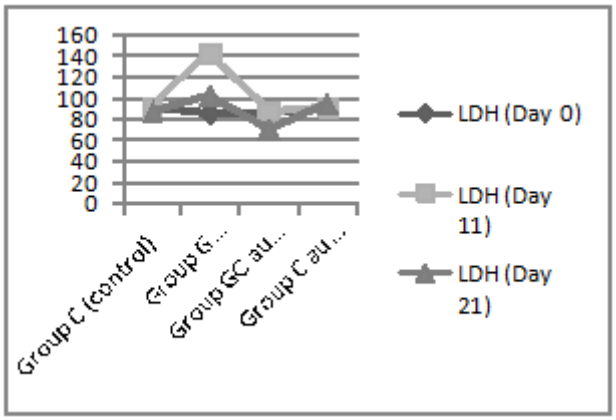

Fig. 1: Urinary Excretion of Lactate dehydrogenase (U/l) on day 0, 11th and 21st of study period

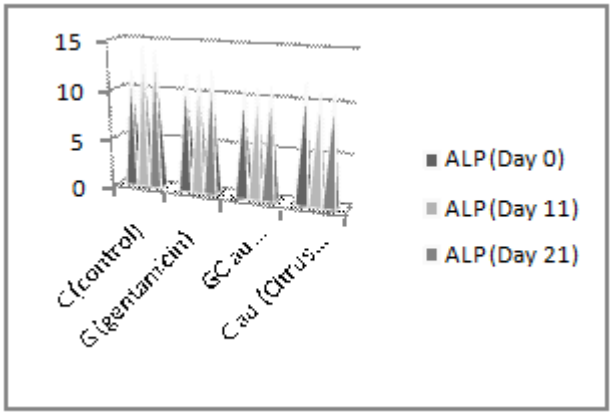

Fig. 2: Urinary excretion of Alkaline Phosphatase (U/l) on day 0, 11th and 21st of study period

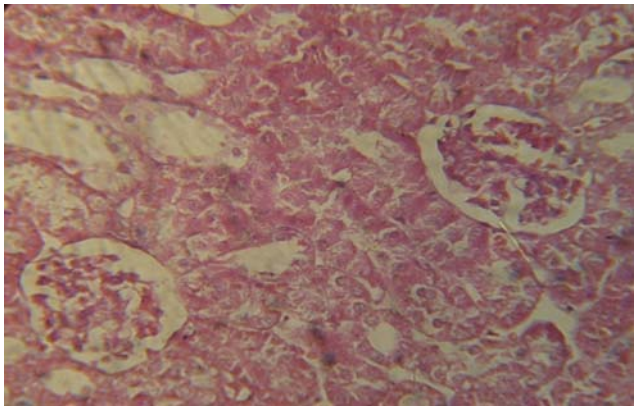

Fig. 3: Group C: Control group renal cortex presenting no common abnormality, presenting normal glomeruli and proximal tubules, no necrosis or ruptured tubules

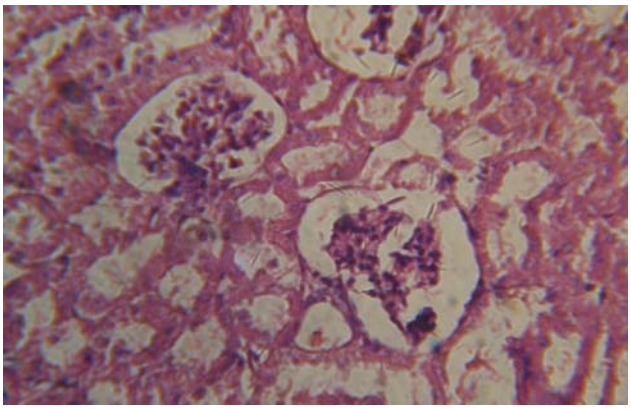

Fig. 4: Group G: Gentamicin treated group renal cortex presenting glomerular atropy and proximal tubular necrosis with a number of ruptured tubules

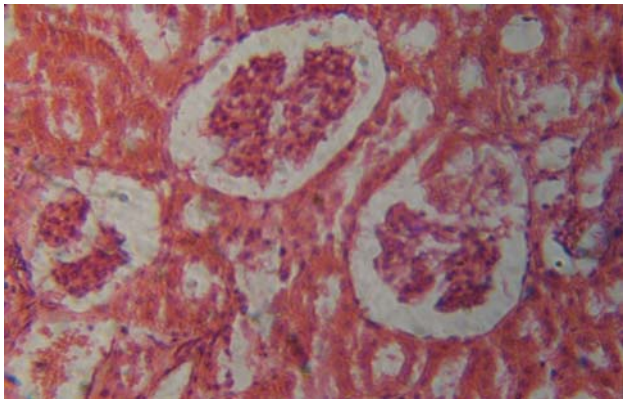

Fig. 5: Group GC-au: *C. aurantium* and gentamicin treated renal cortex presenting normal glumeruli with normal appearance of tubules

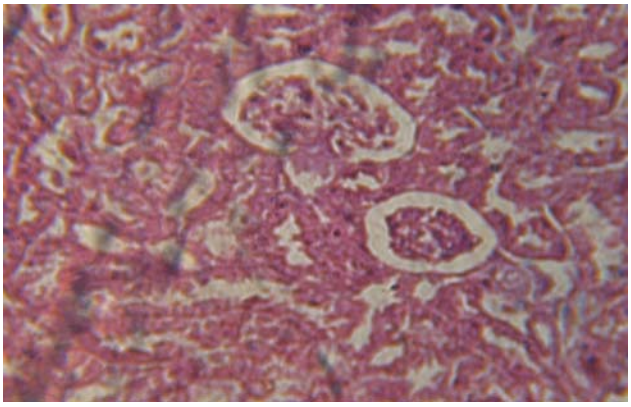

Fig. 6: Group C-au: *C. aurantium* treated renal cortex presenting normal glomeruli with normal appearance of tubules

DISCUSSION

To investigate nephrotoxicity associated with gentamicin and to observe the protective role of *C. aurantium* against this toxicity the present work was aimed to investigated. Humans and animals possess same cellular damage even

by taking normal doses of gentamicin (Kosek *et al*., 1974). However according to Bennett *et al*., (1991), these adverse effects occurs only, if the drug is taken 5-10 times increased dose. Therefore, in the current study we used daily dose of 80 mg/kg of gentamicin to produce significant nephrotoxicity. Blood urea nitrogen and serum creatinine increased with significant fall in glomerular filteration, measured by creatinine clearance was observed with gentamicin (Moghaddam *et al*., 2010). Similar results were observed in the present study. I.e. Significant increase in blood urea nitrogen, serum creatinine, serum uric acid and decreased creatinine clearance in gentamicin treated animals significantly different from group C, group GC au, and group C au was observed.

Elevation in the urinary excretion of sodium and decrease in the re-absorption of potassium was presented previously (Derakhshanfar *et al*., 2007). Gentamicin induced hypokalemia was reported by Cronin *et al*., (1980). Same results was observed in the current study for gentamicin treated group, however a little bit decrease in potassium was also diagnosed in group C au, but was still significantly different when compared with group G as reported previously (Asif *et al*., 2012). Serum calcium was decreased in the current findings in group G and group C au which were significantly changed when compared with control and GC au groups. These results were in agreement with Lambie *et al*., (1991), as a significant fall in serum calcium was observed with gentamicin treatment. But in the present findings, only extract treated animals were also found to have a decreased serum calcium levels. From this it can be concluded that the decreased calcium level may or may not be the effects of gentamicin, as according to Brinker *et al*., (1981), Serum calcium is not affected by gentamicin. Bennett *et al*., (1991) reported abnormal sodium level in gentamicin treated animals, however our findings no significant increase or decrease were observed in any group including gentamicin treated group. Significant loss in the body weight was observed in group G animals when compared with other groups, may be considered a common factor associated with kidney damage. It can be concluded from the current study that only extract treatment may also have weight reducing ability as in agreement with Colker *et al*., (1999), that in a double-blind, placebo-controlled study of *C. aurantium*, caffeine and St John's Wort decreased total body weight with strict diet and exercise.

Elevated urinary protein excretion and decrease in urinary volume in group G animals also showed abnormality. McCracken *et al*., (1986) studied urinary enzymes secretion for the identification of nephrotoxicity, same like in the present study an extremely significant increase in lactate dehydrogenase secretion were observed in group G, on day 11^{th} but decreased on day 21^{st}. However group GC au animals showed protective effects by inhibiting rise in Lactate dehydrogenase excretion. Further no change in alkaline phosphatase was observed in any group when compared with group G animals.

Luft *et al*. (1977) presented that there is no strong relationship between tubular damage and altered glomerular function. However, Solez (1983) described the relationship of tubular necrosis and rise in creatinine as; the raised creatinine level may because of the tubular obstruction by necrotic debris and the leaking of filtrate through these damaged tubules. Leaking of proteins may due to tubular cell degeneration caused by hyaline and granular casts in proximal and collecting tubules. Observation of urinary proteins may be because of granular casts or hyaline in the tubules, also observed in group GC au but not significantly. Further the blockade associated with the casts may responsible for renal damage (Solez, 1983). Histological examination showed normal renal cortex and medulla in group C, group GC au and group C au animals when compared with the toxic group animals.

CONCLUSIONS

Present work indicated that the combined administration of *C. aurantium* at a dose of 200 mg/kg/day for a period of twenty one days successfully protected renal damage associated with gentamicin, assessed by renal functional and histological examinations.

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