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Prophylactic and Protective Activity of Crude and Methanolic Extract of *Punica granatum* Peel against Gentamicin Induced Nephrotoxicity

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Authors' contributions

This work was carried out in collaboration between all authors. Author MA designed the study protocols. Author RW performed the analytical procedures and carried out all the experiments. Authors RA and AM co-supervised the experimental work and performed literature searches. Author SHK assisted in the extracts preparations, development of nephrotoxicity, total anti-oxidant analysis and manuscript correction. Author UA drafted the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

The aim of the current study was to investigate the nephroprotective effect of *Punica granatum* peel (pomegranate) against gentamicin induced nephrotoxicity. The crude pomegranate peel (600 mg/kg) and methanolic pomegranate extract (600 mg/kg) for 7 days showed significant ($p<0.05$) reduction in serum creatinine, urea, uric acid, serum electrolytes, alkaline phosphatase and total anti-oxidant activity as compared to gentamicin (2.5 mg/kg for 7 days) treated group. There was non-significant ($p>0.05$) increase in serum electrolytes, creatinine, urea, uric acid, alkaline phosphatase, total anti-oxidant activity in prophylactic group receiving 600 mg/kg of methanolic pomegranate extract for 5 days followed by the administration of gentamicin (2.5 mg/kg) for 7 days as compared to normal group. The result obtained demonstrates that pomegranate peel crude and

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methanolic extract are potent nephroprotective agents and prophylactic use prevents nephrotoxicity induced by gentamicin by decreasing oxidative stress in kidney.

Keywords: Punica granatum; pomegranate; gentamicin; nephrotoxicity.

1. INTRODUCTION

Worldwide aminoglycosides are still important in the treatment of life threatening infections and very commonly used antibiotics [1] including gentamicin having major side effects of nephrotoxicity and ototoxicity when employed for long term use in patients [2,3]. The main reason of nephrotoxicity is due to generation of reactive oxygen species (ROS) [4,5]. These species are highly reactive and cause cell death by different chain reactions including lipid peroxidation and formation of DNA adducts that are major cause of cancer promoting mutations and cell damage [6,7]. Gentamicin also accumulates in the kidney that leads to tubular necrosis [8,9]. There is significant variation in the activities of enzymes that leads to oxidative stress and impaired carbohydrate metabolism in rat intestine and kidney [10,11]. Nephrotoxicity induced by gentamicin is widely used to assess nephroprotective effect of medicinal plants *in vivo* [12-14].

Punica granatum (pomegranate) belonging to family Punicaceae and is cultivated mainly in the Mediterranean region. It is distributed worldwide and is used in baking, alcoholic beverages, juices, smoothies. It has potential therapeutic value in different diseases [15]. It's fruit is widely used for curing different types of diseases including red eyes, vomiting and headache [16]. Fruit of *Punica granatum* (aqueous extract) demonstrates nephroprotective effect [17]. It is also a promising alternative treatment from medicinal plant origin having anti-inflammatory, anti-infective, anti-oxidant [18,19], anti-diabetic effect [20], anti-malarial [21] and anti-diarrheal properties [22].

The main focus of the current research was to evaluate the nephroprotective effect of *Punica granatum* (pomegranate) in crude and it's methanolic extract against gentamicin induced nephrotoxicity in rabbits.

2. MATERIALS AND METHODS

2.1 Plant Material

*Punica granatum*was obtained from local market and peel was removed, cut into small pieces and dried under sunlight. These dried peels were then ground in pestle and mortar. Powdered peels obtained were then passed through sieve number 40 for content uniformity. Powder was preserved in air tight container to avoid moisture.

2.1.1 Preparation of methanolic extract of powdered peel

Powdered peel 400 g was soaked in 800 ml methanol for four days at room temperature with continuous stirring with magnetic stirrer. Macerate was then filtered and residue obtained on filter paper was dried. Dried residue was put in soxhlet apparatus to undergo extraction by hot percolation using 800 ml methanol. Extract was collected and evaporated under reduced pressure in rotary evaporator. The thick liquid obtained was placed in china dish and placed in oven at 37℃ till a sticky paste of reddish brown color was obtained.

2.1.2 Preparation of pomegranate peels suspension

Suspension of crude peel and its extract was prepared by using gum acacia in the ratio of 1:4. One part of gum acacia was used for four parts of peel powder/extract, water was added in quantity sufficient to make up the required volume.

2.2 Experimental Animals

Male rabbits weighing between 900-1200 gm were used as experimental animals. The animals were acclimatized with the animal house for seven days and were fed on green & fresh fodder with free access to water. All animals were treated according to the procedures approved by the Institutional ethical committee at the University College of Pharmacy, University of the Punjab, Lahore, Pakistan.

2.3 Experimental Design

Nephrotoxicity was induced by subcutaneous injection of gentamicin 2.5 mg/kg after every 8^{th} hour for seven days. The animals with significant difference in creatinine, uric acid, urea, alkaline

phosphatase (ALP), glucose, sodium, potassium, phosphate, calcium, magnesium and total antioxidant level were selected and included in experiment.

For study of protective effect, rabbits were divided into four groups of six animals each; group I Nor (normal rabbits); group II GemTr (gentamicin treated); group III GemCru (gentamicin treated with crude pomegranate peel 1.5 mg/kg); group IV GemMet (gentamicin treated with methanolic pomegranate peel extract 600 mg/kg).

For prophylactic study, rabbits were divided into three groups with six animals each; group I Nor (normal rabbits); group II MetTr (methanolic pomegranate peel extract for five days only) group III MetGem (methanolic pomegranate peel extract for five days then gentamicin injection.

2.4 Blood Biochemistry

Blood samples were taken from all groups of rabbits. Serum was isolated from blood of all animal groups after 24 hours of last dose from both protective and prophylactic group. The amount of serum creatinine, ALP (fortress diagnostic limited, U.K.), uric acid, urea, magnesium (crescent diagnostics, Saudi Arabia), glucose (pioneer diagnostics, Saudi Arabia), sodium, potassium, calcium (spectrum diagnostics, Germany), phosphorus (global's diagnostics U.K.) were estimated using kits according to the manufacturer's protocol and total anti-oxidant activity was calculated by estimating the inhibition of production of thiobarbituric reactive substances using uric acid as standard [23].

2.5 Statistical Analysis

Student t-test was applied to the data for statistical analysis. Data was expressed as ± SEM for different groups of protective and prophylactic groups. P-value less than 0.05 were statistically considered significant.

3. RESULTS

3.1 Effect of Gentamicin, Crude Pomegranate Peel and Methanolic Pomegranate Peel Extract on Serum Creatinine, Uric Acid and Urea

Creatinine, urea and uric acid are serum parameters were estimated to monitor kidney function test. Table 1 shows that there was significant increase ($p<0.05$) in serum creatinine (1.6±0.05 mg/dl), uric acid (10.2±1.0 mg/dl) and urea (73.98±3.4 mg/dl) in GenTr group as compared to normal group. In normal group, serum creatinine concentration was 0.87±0.08 mg/dl. With gentamicin injection it was significantly increased ($p<0.05$) when compared to normal, while GemCru (1±0.03 mg/dl) and GemMet (0.85±0.05 mg/dl) treatment groups showed significant reduction ($p<0.05$) in serum creatinine when compared to GenTr group (1.6±0.05 mg/dl).

In normal group, serum uric acid concentration was 7.86±0.01 mg/dl. With gentamicin injection it was significantly increased ($p<0.0025$) as compared to normal group, while GemCru (8.1±0.04 mg/dl) and GemMet (8.2±0.03 mg/dl) group showed significant reduction ($p<0.01$ and $p<0.05$ respectively) in serum uric acid as compared to GenTr group (10.2±1.0 mg/dl).

In normal group, serum urea concentration was 40±5 mg/dl. It was significantly increased ($p<0.005$) in GenTr group (73.98±3.4 mg/dl) as compared to normal, while GemCru (47.0±6.0 mg/dl) and GemMet (42.0±4.3 mg/dl) group showed significant reduction ($p<0.05$) in serum urea as compared to GenTr group (73.98±3.4 mg/dl).

Table 1. Effect of gentamicin, crude pomegranate peel and methanolic pomegranate peel extract on serum creatinine, uric acid and urea

Parameters	Nor	GenTr	GenCru	GenMet
Creatinine (mg/dl)	0.87±0.08	1.6±0.05*	1±0.03$^{\#}$	0.85±0.05$^{\#}$
Uric Acid (mg/dl)	7.86±0.01	10.2±1.0*	8.1±0.04$^{\#}$	8.2±0.03$^{\#}$
Urea (mg/dl)	40±5	73.98±3.4*	47.0±6.0$^{\#}$	42.0±4.3$^{\#}$

**p<0.05 GenTrvsNor, $^{\#}$p<0.05 GenCruvsGenTr, $^{\#}$p<0.05 GenMetvsGenTr*

3.2 Effect of Gentamicin, Crude Pomegranate Peel and Methanolic Pomegranate Peel Extract on Serum Electrolytes

Serum electrolytes such as sodium, potassium, phosphate, calcium, magnesium are important markers for kidney function. Table 2 showed that there is significant increase ($p<0.005$) in serum concentration of sodium in GenTr group (225±8 mEq/L) as compared to normal group which is 177±10 mEq/L. There is significant reduction ($p<0.005$) in serum of GenCru (170±10 mEq/L) and GenMet groups (173±10 mEq/L) when compared with GenTr group.

Table 2 showed that there is no significant increase ($p>0.3$) in serum potassium (6.1±0.2 mmol/L) and phosphate (5.4±0.1 mg/dl) in GenTr group as compared to normal group which is 6.2±0.1 mmol/L and 5.3±0.2 mg/dl respectively. While in case of calcium (16.7±0.3 mg/dl) and magnesium (2.71±0.07 mg/dl) there is significant increase ($p<0.005$) in GenTr group as compared to normal group which is 9.5±0.1 mg/dl and 1.65±0.02 mg/dl respectively but there is significant reduction ($p<0.005$) of calcium and magnesium in serum concentration in GenCru (11.1±0.3 mg/dl, 10.0±0.3 mg/dl respectively) and GenMet (2.0±0.04 mg/dl, 2.25±0.02 mg/dl respectively) groups when compared with GenTr group.

3.3 Effect of Gentamicin, Crude Pomegranate Peel and Methanolic Pomegranate Peel Extract on Serum Glucose Level

Table 3 showed that there is no significant effect of gentamicin treated group (83±4 mg/dl) on blood glucose level as compared to normal ($p>0.4$) group (83±4 mg/dl).

3.4 Effect of Gentamicin, Crude Pomegranate Peel and Methanolic Pomegranate Peel Extract on Serum Alkaline Phosphatase and Anti Oxidant Activity

There is significant increase ($p<0.005$) of ALP (230±10 U/L) and reduction in anti-oxidant activity (0.936±0.017 mmol/L) in GenTr group as compared to normal group 170±15 U/L and 1.827±0.099 mmol/L respectively. While, there is significant reduction of ALP concentration in GenCru (174±7 U/L) and GenMet (179±10 U/L) groups as compared with GemTr group as shown in Table 3 and also significant increase in anti-oxidant activity in GenCru (1.85±0.14 mmol/L) and GenMet (2.513±0.14 mmol/L) groups as compared with GenTr group.

3.5 Prophylactic Study

As shown in Table 4, administration of methanolic pomegranate peel extracts

Table 2. Effect of gentamicin, crude pomegranate peel and methanolic pomegranate peel extract on serum electrolytes

Parameters	Nor	GenTr	GenCru	GenMet
Sodium (mEq/L)	177±10	225±8*	170±10$^{\#}$	173±10$^{\#}$
Potassium(mmol/L)	6.2±0.1	6.1±0.2	6.1±0.2	5.9±0.3
Phosphate (mg/dl)	5.3±0.2	5.4±0.1	5.0±0.1$^{\#}$	5.4±0.09
Calcium (mg/dl)	9.5±0.1	16.7±0.3*	11.1±0.3$^{\#}$	10.0±0.3$^{\#}$
Magnesium (mg/dl)	1.65±0.02	2.71±0.07*	2.0±0.04$^{\#}$	2.25±0.02$^{\#}$

**p<0.05 GenTrvsNor, $^{\#}$p<0.05 GenCruvsGenTr, $^{\#}$p<0.05 GenMetvsGenTr*

Table 3. Effect of gentamicin, crude pomegranate peel and methanolic pomegranate peel extract on serum glucose, alkaline phosphatase (ALP) and total anti oxidant activity (Total AOA)

Parameters	Nor	GenTr	GenCru	GenMet
Glucose (mg/dl)	83±4	85±7.0	90±7.0	86±4.3
ALP (U/L)	170±15	230±10*	174±7$^{\#}$	179±10$^{\#}$
Total AOA (mmol/L)	1.827±0.099	0.936±0.017*	1.85±0.14$^{\#}$	2.513±0.14$^{\#}$

**p<0.05 GenTrvsNor, $^{\#}$p<0.05 GenCruvsGenTr, $^{\#}$p<0.05 GenMetvsGenTr*

600 mg/kg 12 hourly for 5 days followed by the administration of gentamicin 2.5 mg/kg 8 hourly through subcutaneous route for 7 days showed that there is no significant increase ($p>0.05$) of serum creatinine, uric acid, urea, sodium, calcium, magnesium and alkaline phosphatase as compared to normal group. There is significant ($p<0.05$) increase of total anti-oxidant activity in serum of MetTr group as compared to normal.

4. DISCUSSION

Gentamicin, aminoglycoside is considered effective and potential therapeutic alternative agent against microorganisms resistant to other antibiotics. It has well known nephrotoxic side effect. So, it has been used worldwide to study nephroprotective effect of different medicinal plants against gentamicin induced nephrotoxicity in experimental animals [24]. Gentamicin induced nephrotoxicity has been proposed through oxidative stress and inflammation due to central key role of reactive oxygen species that lead to reduction in glomerular filtration rate and tubular necrosis [4,25].

Significant increase in serum concentration of creatinine, urea and uric acid were evident suggesting as a marker in gentamicin induced nephrotoxicity [12,26]. Creatinine level is a more important serum marker in the first phase of renal disease but after parenchymal injury, the level of urea gradually increases in serum [27].

In current study, serum concentration of creatinine, urea and uric acid was markedly increased in GenTr group [12,24,28]. In present study, there was no increase of serum creatinine, urea and uric acid in GenCru and GenMet groups in comparison with GenTr group. Minimum parenchyma injury could be a reason for decreased concentration of serum urea in GemCru and GemMet groups [27]. Such results were previously demonstrated by using anti-oxidant agents [24,28,29] and also reported by using whole fruit aqueous extract of *Punica granatum* against gentamicin [17] but current study hypothesis was to investigate the effect of *Punica granatum* peel, crude and methanolic extract. In current study serum anti-oxidant activity was significantly increased ($p<0.05$) in GenCru (1.85±0.14 mmol/L) and GenMet (2.513±0.14 mmol/L) groups as compared to GemTr group (0.936±0.017 mmol/L). So, results of current study showed that crude and methanolic extract of pomegranate peel possess anti-oxidant activity that might have resulted reduction in the effect of gentamicin on kidneys. In prophylactic groups, methanolic extract administration for five days protects the kidney prophylactically from gentamicin induced nephrotoxicity. This effect could be attributed to the anti-oxidant nature of the pomegranate peel that might have inhibited the gentamicin uptake into the kidney tubules and prevented the induction of ROS by gentamicin in cellular structures of kidney. There was non-significant $p>0.05$ decrease in the total anti-oxidant status in MetTr group when compared with Normal group as presented in Table 4. Serum level of creatinine, uric acid, urea, sodium, calcium, magnesium and ALP showed no significant increase as compared to normal and only methanolic extract group.

Different studies reported that pomegranate peel is rich in ellagic acid, ellagitannins and gallic acids [30]. These polyphenols may be responsible for anti-oxidant effect of pomegranate peel [31]. Previous studies also reported that ethanolic extract of pomegranate had total phenolics, gallic acid equivalents and the nephroprotective effect of pomegranate peel was due to these compounds [32].

Table 4. Prophylactic study: Effect of gentamicin after the administration of methanolic pomegranate peel extract for five days on serum creatinine, uric acid, electrolytes, alkaline phosphatase (ALP) and total anti oxidant activity (Total AOA)

Parameters	Nor	MetTr	MetGen
Creatinine (mg/dl)	0.90±0.07	0.85±0.06 ^{ns}	1.0±0.03^{ns}
Uric Acid (mg/dl)	7.5±0.02	7.4±0.03 ^{ns}	7.45±0.02 ^{ns}
Urea (mg/dl)	39±2.5	41±3.0 ^{ns}	41.5±1.3 ^{ns}
Sodium (mEq/L)	180±3.5	178±5.0 ^{ns}	183±5.0 ^{ns}
Calcium (mg/dl)	9.0±0.02	8.9±0.03 ^{ns}	9.0±0.02 ^{ns}
Magnesium (mg/dl)	1.75±0.03	1.70±0.01 ^{ns}	1.72±0.1 ^{ns}
ALP (U/L)	185±10.2	190±11 ^{ns}	182±9 ^{ns}
Total AOA (mmol/L)	1.594±0.15	2.08±0.21*	1.925±0.12 ^{ns}

** p<0.05 MetTrvsNor, ^{ns} p>0.05 MetGenvs Nor, ^{ns} p>0.05 MetTrvs Nor*

In current study, crude pomegranate peel and methanolic pomegranate extract showed significant ($p<0.05$) nephroprotective effect against gentamicin induced nephrotoxicity. There is significant reduction ($p<0.05$) of serum sodium, calcium, magnesium in GenCru and GenMet groups as compared with GenTr group. GenCru group showed significant reduction ($p<0.01$) of phosphate as compared to GenTr while methanolic extract showed non-significant reduction in phosphate. There is also significant reduction of serum ALP in GenCru and GenMet groups as compared with GenTr group. The results of current study suggest that crude *Punica granatum* (pomegranate) and methanolic extract of *Punica granatum* protected gentamicin induced nephrotoxicity by increasing the anti-oxidant enzymes activity and improvement in renal function.

5. CONCLUSION

The crude and methanolic extract of *Punica granatum* peel possesses profound nephroprotective effect as demonstrated in our study. Co-administration of *Punica granatum* can reduce the potential side effect of gentamicin through restoration of anti-oxidant activity and improvement in kidney function. Further research is required to determine the mechanism involved in nephroprotective effect of *Punica granatum* peel.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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