INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY

Available online at www.ijrpc.com

Research Article

RP-HPLC METHOD OF SIMULTANEOUS NEPHROPROTECTIVE ROLE OF A. MARMELOS EXTRACT

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ABSTRACT

Present investigation was carried out to evaluate the Nephroprotective activity of an aqueous extract of Leaves of Aegle marmelos in Wistar rats. The aqueous extract of Aegle marmelos leaves (AEAM) was administered at three doses (250, 500, and 750 mg/kg, p.o.) to wistar rats in Gentamicin (GM)-induced nephrotoxicity model. The rats were pre-fed experimental diets for 8 days and then received GM (100 mg/kg body weight/day) treatment for 8 days while still on diet. Serum parameters, oxidative stress in rat kidney were analyzed. GM nephrotoxicity was recorded by increased serum creatinine and blood urea nitrogen level. GM increased MDA level whereas decreased catalase, reduced glutathione level, while AEAM significantly reduced the elevated MDA levels and increased GSH and catalase concentration. GM increased serum creatinine, urea and blood urea nitrogen level,while AEAM reduced serum creatinine, urea and blood urea nitrogen level in gentamicin toxicity indicating a nephroprotective effect. The aqueous extract of *A. marmelos* leaves possesses the Nephroprotective activity.However, further studies are necessary to isolate and characterize the phytoconstituents responsible for diuretic action and to explore the exact underlying mechanism of *A. marmelos.*

Keywords: Aegle Marmelos, Nephroprotective, Gentamicin, Nephrotoxicity.

INTRODUCTION

Nephrotoxicity can be defined as renal dysfunction that arises as a direct result if exposure to external agents such as drugs and environmental chemicals. Many therapeutic agents have been shown to induce clinically significant nephrotoxicity[1].

Aminoglycoside antibiotics have been widely used for gram-negative infections. However, their nephrotoxicity and their ototoxicity are major limitations in clinical use. Among several aminoglycosides, the grade of nephrotoxicity has been reported to be in the following order, neomycin > gentamicin > tobramycin[2]. GM nephrotoxicity, which occurs in about 15-30% of treated subjects, is manifested clinically as nonoliguric renal failure, with a slow rise in serum creatinine and hypoosmolar urinary output developing after several days of treatment[3]. Gentamicin is filtered through glomeruli into tubular urine, binds with anionic phospholipids, such as phosphatidylinositol or phospholipdylserine, in brush border membrane of proximal tubular cells reabsorbed actively via pinocytosis process into tubular cells, taken by lysosomes and thereafter produces phospholipidosis[2]. The drug enters cells by adsorptive/receptor mediated endocytosis after binding to acidic phospholipids and megalin and is found essentially in lysosomes. Animals treated with low, therapeutically

relevant doses of aminoglycosides show both lysosomal phospholipidosis and apoptosis in proximal tubular cells[4].

Bael (Aegle Marmelos (Linn), family Rutacae, is also known as Bale fruit tree, is a moderate sized, slender, aromatic tree, 6.0 -7.5 m in height, and 90 to 120 cm in girth, with a some what fluted bole of 3.0-4.5 meter growing wild throughout the deciduous forests of India, ascending to an altitude of 1200 meter in the western Himalayas and also occurring in Andaman island[5].

The different parts of Bael are used for various therapeutic purposes, such as for treatment of Asthma, Anaemia, Fractures, Healing of Wounds, Swollen Joints, High Blood Pressure, Jaundice, Diarrhoea Healthy Mind and Brain Typhoid Troubles during Pregnancy[6].Aegle marmelos has been used as a herbal medicine for the management of diabetes mellitus in Ayurvedic, Unani and Siddha systems of medicine in India[7], Bangladesh[8] and SriLanka[9]. This plant has been found to contain number of phytoconstituents like aegeline,agelinine, rutin, sterol, ß-sitosterol, ß-D-glucoside, marmesinine[10], lupeol, tannins, phlobatannins, flavonoids, umbelliferone, quercetin and volatile oils[11].It has been reported that leaves possess cardiotonic effect, antifungal, analgesic and antioxidant activities[12].

Therefore by taking base as an antioxidant, this experimental study was designed to investigate the possible protective effects of *Aegle marmelos* on nephrotoxicity induced by GEN in a rat model, and to clarify the association between body weight, kidney weight, malondialdehyde (MDA), catalase (CAT) activities, glutathione (GSH) content, Creatinine, urea, BUN levels in GM-induced nephrotoxicity.

MATERIALS AND METHODS

Plant Collection and authentication of Leaves

The dried Leaves of *Aegle marmelos* was procured from Pune region in the month of September-October (2008-09) and air-dried at room temperature (28 ± 2^{0}C) for a one week.

Preparation of crude extract

Leaves of *Aegle marmelos* were shade dried and coarsely powdered by using grinder mixer. The powdered material was macerated in sufficient quantity of distilled water with small quantity of chloroform to prevent fungal growth and kept for 7 days. During maceration it was shaken twice daily. On seventh day it was filtered & the filtrate was concentrated on water bath (50^{o}C) to remove the solvent and to get sticky brown coloured extract i.e. aqueous extract of *Aegle marmelos (AEAM)*. The extractive value of the extract was 18%.

Experimental design

The animal experiments were approved by the animal ethics committee of the institute. In this investigation, 36 healthy adult male Wistar rats weighting between 190 and 250 g were used. The animals were housed under standard laboratory conditions (12 h light and 12 h dark) in a room with controlled temperature (24±3 ◦C) during the experimental period. All experimental procedures were conducted in accordance with the guide to the care and use of laboratory animals. The rats were provided ad libitum with tap water and fed with standard commercial rat chow (Pranav agro industries, Sangali). Thirty-six rats were randomly assigned to five groups equally: (1) Normal control; injected intraperitoneal (i.p.) saline for 8 days, (2) GM treated group; injected intraperitoneal (i.p.) GM (100 mg/kg) for for 8 days, (3) GM + AEAM250; treated group; injected intraperitoneal (i.p.) GM (100 mg/kg) and AEAM (250 mg/kg) for for 8 days, 4) GM + AEAM500; treated group; injected intraperitoneal (i.p.) GM (100 mg/kg) and AEAM (500 mg/kg) for for 8 days, 5) GM + AEAM 750; treated group; injected intraperitoneal (i.p.) GM (100 mg/kg) and AEAM (750 mg/kg) for for 8 days. After the experimental period, blood samples were collected from animals of each treatment group by retro orbital puncture under light ether anesthesia. Serum was separated by centrifugation at 10000 x g for 10 min. Serum was analyzed for biochemical estimation. The rats were then sacrificed by cervical dislocation, the abdomen was opened and kidney was removed, decapsulated and was placed in KCL and stored for assayed of MDA, CAT activities, and GSH content.

Animals

Species: Swiss albino mice and albino rats were used in this study was obtained from the Yash farm and National Toxicological Center, Pune. Weight: Wistar rats - 150-250 gm.

Biochemical determination

The methods used for biochemical determinations, described briefly below, have been used and reported in our previous studies[13,14]. MDA levels in the kidney tissue were determined[15]. Kidney tissue (200 mg) was homogenized with ice-cold 1.15% KCl to form a 10% homogenate. Then, 0.5 ml of this homogenate was pipette into a 10ml centrifuge tube and 3.0 ml of 1% w/v H_3PO_4 and 1.0 ml of 0.6% v/v aqueous thiobarbituric acid solution were added. The tubes were heated for 45 min in a boiling water bath and the reaction mixture was then cooled in an ice-bath. This was followed by the addition of 4.0 ml of n-butanol. The contents were mixed for 40 s using a vortex mixer, centrifuged at 1200g for 10 min and the absorbance of the organic layer was measured at wavelengths of 535nm.

GSH was determined by a spectrophotometric method, based on the use of Ellman's reagent[16]. Tissue homogenates were mixed with 50% trichloroacetic acid in distilled water in glass tubes and centrifuged at 3000 rpm for 15 min. The supernatants were mixed with 0.4M Tris buffer, pH 8.9, and 0.01M 5,50-dithio-bis (2-nitrobenzoic acid) (DTNB) was added. After shaking the reaction mixture, its absorbance was measured at 412nm. The absorbance values were expressed as mmol/g tissue. CAT activity was determined according to the method of Aebi[17]. Briefly, 10 ml of kidney tissue supernatant was added to 2.99 ml of phosphate-buffered saline (PBS) and the absorbance was read at 240 nm using a UV spectrophotometer. Serum levels of Cr, urea and BUN were determined using the Autoanalyser (ChemMaster LabLife Instruments) according to manufacturers' instructions.

Statistical analysis

Data obtained for each set of anti-inflammatory model was expressed as means ± SEM and analysed by one-way ANOVA followed by Dunnett's test.

RESULTS

Effect of AEAM on serum creatinine, urea and blood urea nitrogen of Wistar rats in experimentally induced gentamicin nephrotoxicity.

In normal control, serum creatinine level was found 0.571± 0.013 whereas in gentamicin group was 1.417 ± 0.045 significantly ($p<0.001$) increased as compared to normal control. The animal treated with AEAM (250 mg/kg, 500 mg/kg and 750 mg/kg) showed significantly ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) decreased serum creatinine and value were 1.281 ± 0.048, 0.829 ± 0.025 and 0.642 ± 0.01 respectively as compared to gentamicin group.

In normal control, serum urea and blood urea nitrogen level were found 19.62 ± 0.18 and 9.16 ± 0.12 respectively. The serum urea and blood urea nitrogen in gentamicin group were 50.71 ± 0.63 and 23.69 ± 0.28 showed significantly ($p<0.001$) increased as compared to normal control. The animal treated with AEAM (500 mg/kg and 750 mg/kg) showed significantly ($p<0.01$ and $p<0.001$ respectively) decreased serum urea and blood urea nitrogen and value were 31.48± 0.99, 21.56 ± 0.65 and 14.70 ± 0.53, 10.07 ± 0.37 respectively, as compared to gentamicin group; whereas, AEAM 250 was not significant in this regards.

The gentamicin toxicity was reversed by treatment groups and an optimum creatinine, urea and blood urea nitrogen level was observed. The results thus indicated AEAM effective in reducing serum creatinine, urea and blood urea nitrogen level in gentamicin toxicity.

Table 1: Effect of AEAM on Serum Creatinine, Urea and Blood Urea Nitrogen in Gentamicin Induced Nephrotoxicity.

Treatment group	Serum creatinine(mg/dl) mean ±SEM	Serum urea (mg/dl) mean ±SEM	Serum BUN (mg/dl) mean ±SEM
Normal control	0.571 ± 0.013	19.62 ± 0.18	9.16 ± 0.12
GM	1.417 ± 0.045###	50.71 ± 0.63###	23.69 ± 0.28###
AEAM 250	1.281 ± 0.048*	44.12 ± 1.21	20.60 ± 0.71
AEAM 500	0.829 ± 0.025**	31.48 ± 0.99**	14.70 ± 0.53**

AEAM 750	0.642 ± 0.01***	21.56 ± 0.65***	10.07 ± 0.37***

Data are expressed as mean ± SEM., Data by one way ANOVA followed by Dunnett's test, n=6.
p<0.001 as compared to normal control; *p<0.05, **p<0.01 and ***p<0.001 as compared to GM group.

Effect of AEAM on lipid peroxidation (MDA), reduced glutathione (GSH), Catalase (CAT) Concentration in Isolated Kidney of Wistar Rats in Gentamicin Induced Nephrotoxicity.

In normal control, the concentration of MDA was 195.8 ± 42.23 whereas in gentamicin group the MDA concentration was significantly (p<0.01) increase to 499.4 ± 78.3 as compared to normal control. The animal treated with AEAM (500 mg/kg and 750 mg/kg) showed significantly (p<0.05 and p<0.01 respectively) decreased MDA concentration and value were 277 ± 56.25 and 204.7 ± 54.44 respectively, as compared to gentamicin group respectively; whereas AEAM 250 was not significant in this regards.

In normal control, GSH concentration was found 3.621 ± 0.371, whereas in gentamicin group the GSH concentration was 0.911 ± 0.260 showed significant (p<0.001) decreased as compared with normal control. The animal treated with AEAM (500 mg/kg and 750 mg/kg) showed significantly (p<0.05 and p<0.01 respectively) decreased GSH concentration and value were 2.209 ± 0.384 and 2.814 ± 0.502 respectively, as compared to acetaminophen group respectively, whereas AEAM 250 was not significant in this regards.

In normal control, the concentration catalase was 3.003 ± 0.469 whereas in gentamicin group the catalase concentration was 0.637± 0.189 showed significantly (p<0.001) decreased as compared with normal control. The animal treated with AEAM (500 mg/kg and 750 mg/kg) showed significantly (p<0.05 and p<0.01 respectively) decreased catalase concentration and value were 1.462 ± 0.184 and 2.371 ± 0.259 respectively, as compared to gentamicin group respectively; whereas AEAM 250 was not significant in this regards.

The result indicate that, gentamicin toxicity significantly increase the MDA concentration and decreased GSH and catalase concentration due to increase in oxidative stress, while on other hand, the AEAM significantly reduced the elevation of MDA levels and increased GSH and catalase concentration indicating a nephroprotective effect.

Table 2: Effect of AEAM on lipid peroxidation (MDA), Reduced Glutathione (GSH), Catalase (CAT) Concentration in Acetaminophen Induced Nephrotoxicity.

Treatment group	MDA (nmole/g tissue) mean ±SEM	GSH (µg/g tissue) mean ±SEM	CAT (µg/sec/g tissue) mean ±SEM
Normal control	195.8 ± 42.23	3.621 ± 0.371	3.003 ± 0.469
GM	499.4 ± 78.3##	0.911 ± 0.260###	0.637 ± 0.189###
AEAM 250	409.5 ± 63.42	1.138 ± 0.374	0.899 ± 0.280
AEAM 500	277 ± 56.25*	2.209 ± 0.384*	1.462 ± 0.184*
AEAM 750	204.7 ± 54.44**	2.814 ± 0.502**	2.371 ± 0.259**

Data are expressed as mean ± SEM., Data by one way ANOVA followed by Dunnett's test, n=6. ## p<0.01,
p<0.001 as compared to normal control; *p<0.05 and **p<0.01 as compared to GM group.

DISCUSSION

The kidney is a common target for toxic xenobiotics, due to its capacity to extract and concentrate toxic substances, and to its large blood flow share (about 20% of cardiac output)[18].

Development of nephrotoxicity can further increase load on the kidney leading to serious complications. This requires either stoppage of drug therapy or change over in the therapy. This raises a question whether any kind of nephroprotection is possible that can handle this problem. Screening of either substances from synthetic origin or herbal origin for nephroprotection can answer this question probably.

Several pharmacological uses of the *Aegle marmelos* have been documented but the leaves of the *Aegle marmelos* have not been subjected to the scientific study for its

nephroprotective activity. Hence, it was thought worthwhile to test the hydroalcohlic extract of fruits of *Aegle marmelos* for the nephroprotective activity against GM induced nephrotoxicity.

Gentamicin, an amino-glycoside antibiotic with a wide spectrum of activities against Gram-positive and Gram-negative bacterial infections but with high preference for the latter, is equally associated with nephrotoxicity as its side-effect[13]. Thus, gentamicin-induced nephrotoxicity are well established experimental models of drug-induced renal injury[19].

Administration of gentamicin (100 mg/kg i.p.) reported to cause nephrotoxicity[13,20,21]. In agreement in the study decrease in body weight, GSH and catalase concentration; increase in kidney weight, serum creatinine, urea, BUN and MDA as well as marked histopatological changes in kidney tissue of GM treated nephrotoxic rats was observed. Protective effect of AEAM was assessed by evaluating serum parameter, biochemical parameter and histopathological study of kidney as end point of renal damage.

GM reduces the glomerular filtration rate which is shown by an increase serum creatinine. The impairment in glomerular function was accompanied by an increase in blood urea[22]. The administration of GM showed significant increase in serum creatinine, serum urea and serum BUN as compared to normal control. Administration of AEAM 250 mg/kg, 500 mg/kg and 750 mg/kg has showed significant decrease in serum creatinine whereas AEAM 500 mg/kg and 750 mg/kg was showed significant decrease serum urea and serum BUN as compared to GM group.

In the complex pathogenesis of GM nephrotoxicity oxidative stress is probably the most common pathogenic[23]. The exact mechanism of GM which induces the renal damage is unknown (Maldonado et al., 2003). Recently, ROS were considered to be important mediators of GM-induced nephrotoxicity[24]. It has been found that the GM-treatment increases H_2O_2 production and it is known that H_2O_2 and O_2^- induce mesangial cells contraction, altering the filtration surface area and modifying the ultrafiltration coefficient factors that decrease the glomerular filtration rate (GFR). O_2^- this radical can react with nitric oxide (NO, a vasodilator) to form peroxynitrite, cytotoxic oxidant radical species. The inactivation of NO by O_2^- could also lead to a decrease in the GFR. It has been suggested that the oxidative stress induces tubular damage. It is known that the increase in ROS levels induces cytotoxicity due to a concerted action of oxygen and nitrogen-derived free radicals[25]. GM (100 mg/kg) has given alone significant increased in MDA level while CAT activities and GSH content were reduced in the kidney tissue. GM nephrotoxicity was associated with low activity of CAT and GSH content in the renal cortex. This decreased renal antioxidant enzymatic defense could aggravate the oxidative damage in these rats. The exaggerated production of ROS in GM-induced nephrotoxicity could induce inactivation of antioxidant enzymes[13].

In the current study GM (100 mg/kg) has significant increased in MDA level while decrease GSH content and CAT activities as compared to normal control. The administration of AEAM 500 mg/kg and 750 mg/kg showed significant decrease in MDA level whereas increase the GSH content and CAT activities as compared to GM group.

Thus agents having strong antioxidant and cellular anti-inflammatory properties may have ability to halt gentamicin-nephrotoxicity[26]. From this discussion it was strongly indicates that the aqueous extract of the *Aegle marmelos* protecting the kidney from GM-induced toxicity, through improvement in oxidant status and a possible antioxidant activity.

CONCLUSION

The present studies conclude that aqueous extract of *Aegle marmelos* leaves has nephroprotective activity, these effects of aqueous extract of *Aegle marmelos* leaves may be due to presence of phytochemicals like flavonoids, tannins which may act as an antioxidants individually or synergistically.

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