ANTIOXIDANT AND NEPHROPROTECTIVE ACTIVITY OF SPATHODEA CAMPANULATA BARK AGAINST GENTAMICIN INDUCED NEPHROTOXICITY

Shanmukha I*, Abubaker Siddiq, Gupt Kamlesh, Majam Khan and Ramachandra Setty S.

P.G.Dept. of Pharmacology, S.C.S.College of Pharmacy, Harapanahalli, Karnataka - 583131, India.

Summary

The present study investigated the protective effect of ethanolic extract of bark of Spathodea campanulata P. Beauv (EEBSC) on gentamicin induced nephrotoxicity in rats. EEBSC was administered to Wistar albino rats in two different doses (250 & 500mg/kg) orally for 11 days. Nephro toxicity was induced by intraperitonial administration of gentamicin at the dose of 80mg/kg for 8 days of treatment protocol. Gentamicin administration resulted in significant increase in the serum marker enzymes like blood urea nitrogen and serum creatinine. In addition these also exhibited significant increase in lipid peroxidation levels and there is marked depletion of reduced glutathione levels (GSH). Pretreatment of EEBSC orally was found to ameliorate the effects of gentamicin on lipid peroxide formation and showed a decrease in serum marker enzymes. It also prevented depletion of tissue GSH levels. The histopathological studies of the kidney revealed a protective role of EEBSC in gentamicin treated rats. The results exhibited that the pretreatment with ethanolic extract of Spathodea campanulata bark may be useful in preventing the damage induced by gentamicin in rat kidneys.

Key words: Antioxidant; GSH; Lipid peroxidation; Marker enzymes; Gentamicin.

Introduction

Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegenertion, Parkinson's diseases, mongolism, ageing process and perhaps dementias[1]. Experiment evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a high number of diseases. Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts. Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans[2].

Gentamicin is an important aminoglycoside anti-biotic commonly used in treating life-threatening gram-negative infections[3]. However its usefulness is limited by signs of nephrotoxicity, which may occur in 13-30% of treated patients[4]. Lipid peroxidation may occur in the course of gentamicin administration[5], giving rise to free radicals[6], which are highly toxic to tissue[7]. Oxidation and necrosis by apoptosis may occur. Several plant products are known to exhibit credible medicinal properties for the treatment of kidney ailments and need to be explored to identify their potential application in prevention and therapy of human ailments.

Spathodea campanulata P. Beauv of family Bignoniaceae commonly known as African tulip tree is planted in gardens and avenues, is reported to be useful in the treatment as diuretic, anti-inflammatory, for kidney diseases, antidote, enemas, herpes, stomach ache, antisecretolytic, anti parasitic, urethra inflammations, fungal skin diseases, diarrohea, anti-HIV, anti-malarial and hypo glycemic activity[8,9]. The major chemical constituents of the plant are steroids, cardiac glycosides, flavonoids, tannins and polyphenols. Ayurveda recommends Spathodea campanulata in the treatment of kidney disorders and the literature survey also confirms the use of the titled plant in kidney disorders. Hence the present study was undertaken to evaluate the in-vitro and invivo antioxidant and nephroprotective potential of ethanolic extract of Spathodea campanulata bark in gentamicin induced biochemical and histopathological changes.

Materials and methods

Plant material and extraction: Spathodea campanulata bark was collected from the Davanagere city around road side in the month of June 2009. The plant was identified and authenticated by Prof. K. Prabhu, Department of Pharmacognosy, S.C.S. College of Pharmacy, Harapanahalli. A herbarium specimen is preserved in the college museum. The bark was shade dried separately at room temperature and pulverized. The powder obtained was subjected to successive soxhlet extraction with the solvents with increasing order of polarity i.e. pet. Ether (60-80°), chloroform (59.5-61.5°), 70% ethanol (64.5-65.5°) and water. The shade-dried powder was extracted with 70% ethanol (hydro-alcoholic extract) after deffating with petroleum ether, which was used for biological investigations and *in-vitro* and *in-vivo* antioxidant studies, after subjecting it to preliminary qualitative phytochemical studies. The extracts were concentrated to a small volume using flash evaporator and further evaporated to dryness and stored in a vaccum dessicator until further use and the percentage yield of corresponding extracts were calculated.

Chemicals: Gentamicin is obtained from sorus labs, Pithampur, Paracetamol from S d fine chemicals, the biochemical kits from Erba Manheim, Germany and all other chemicals used were of analytical grade.

Animals: Wistar albino rats (weighing 150-250g) and albino mice (weighing 20-25g) of either sex were used in this study. They were procured from Sri Venkateshwara Enterprises, Bangalore. The animals were acclimatized for one week under laboratory conditions. They were housed in polypropylene cages and maintained at 27°C ± 2°C under 12 hrs dark / light cycle. They were fed with standard rat feed (Gold Mohur Lipton India Ltd.) and water *ad libitum* was provided. The husk in the cages was renewed thrice a week to ensure hygeinity and maximum comfort for animals. Ethical clearance for handling the animals was obtained from the Institutional animal ethical committee prior to the beginning of the project work, according to prescribed guidelines of committee for the purpose of control and supervision of

experiments on animals (CPCSEA), under the ministry of animal welfare division, Government of India, New Delhi.

Acute toxicity study: Acute toxicity study was carried out using albino mice (20-25g) for EEBSC in accordance with OECD guideline No. 420 given by CPCSEA[10] was adopted for toxicity studies. The extract was found to be devoid of mortality at 2000mg/kg. Hence, 2500 mg/kg was considered as LD_{50} cutoff value. Hence the 1/ 10^{th} (250 mg/kg, p.o.) and 1/ 5^{th} (500mg/kg, p.o.) of the dose selected for the screening of nephroprotective activity.

In vitro Antioxidant activity:

The following *in-vitro* models were carried out to evaluate antioxidant activity.

a) Reducing power.

b) Nitric oxide radical scavenging activity.

a) Reducing Power: This method is based on the principle of increase in the absorbance of the reaction mixture. Increase in the absorbance indicates increase in the antioxidant activity. In this method antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples. The reducing power of 70% ethanolic extract of bark of Spathodea campanulata P. Beauv was determined according to the method of Oyaizu (Oyaizu, 1986)[11, 12] **.**

Procedure: Different doses of 70% ethanolic extract of Spathodea campanulata P. Beauv were mixed in 1 ml of distilled water so as to get 25µg, 50µg, 75µg, 100µg, 125µg concentration. This was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes if precipitate occurs. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance (OD) was measured at 700nm.

Increased absorbance of the reaction mixture indicates increase in reducing power. The % reducing power was calculated by using following formula:

$$\% \text{ increase in absorbance} = \frac{\text{Test OD} - \text{Control OD}}{\text{Control OD}} \times 100$$

b) Nitric oxide radical scavenging activity [13]**:** Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals. This forms the basis of this experiment.

Procedure: Nitric oxide (NO) radical were generated from sodium nitroprusside solution at physiological pH. Sodium nitroprusside (1ml of 10mM) were mixed with 1ml of 70% ethanolic extract of bark of Spathodea campanulata P. Beauv of different concentration (25-125 µg/ml) in phosphate buffer (pH 7.4). The mixture was incubated at 25°C for 150 min. To 1 ml of the incubated solution, 1ml of Griess's reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm. Decreased absorbance of the reaction mixture indicates increased nitric oxide radical scavenging activity. % inhibition of OD was calculated by the formula.

$$\% \text{ inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Nephroprotective activity:

Evaluation of nephroprotective activity in Gentamicin induced nephrotoxicity[14]:

Procedure: Four groups of six rats in each were used in this model.

Group-I Animals (- ve Control) were administered saline (1ml/kg, p.o) for 8 days.

Group-II Animals (+ ve Control) were administered Gentamicin (80mg/kg, i.p.) for 8 days.

Group-III Animals were administered with 70% ethanolic extract 250mg/kg p.o., for 8 days.

Group-IV Animals were administered with 70% ethanolic extract

500 mg/kg p.o., for 8 days.

The animals of 3rd group and 4th group received 80 mg/kg of gentamicin i.p. nearly for eight days in addition to this they also received 250 mg/kg p.o. and 500 mg/kg of Spathodea campanulata bark extract orally, respectively. This was started three days prior to the gentamicin injections and continued with eight days gentamicin treatment. The body weights of all the animals were noted on every day.

At the end, the animals were sacrificed under mild ether anaesthesia and the kidney tissues and blood samples were collected. Thus collected kidney weighed: the samples of kidney tissue were analyzed for tissue GSH as per the method of Ellman et al and lipid peroxidation for measurement of thiobarbituric acid reactive substances (TBARS) and kidneys were stored in 10% formalin and proceeded for histopathology to evaluate the details of renal architecture in each group microscopically. The blood samples were used and measure serum creatinine and blood urea nitrogen (BUN).

Statistical analysis: Results were expressed as mean ± SEM, (n=6). Statistical analyses were performed with one way analysis of variance (ANOVA) followed by Tukey's Kramer comparison test by using Graph Pad Instat Software. P value less than 0.05 was considered to be statistically significant. *P<0.05, **<0.01 and ***<0.001, when compared with control and toxicant group as applicable.

Results

In-vitro Antioxidant activity

a) Reducing power activity:

It is observed that 70% EEBSC have demonstrated concentration dependent increase in the reducing property. Ascorbic acid (std.25µg) has 41.02 % reducing property. The test extract showed concentration dependent increase in reducing property. However, 50µg of 70 % EEBSC showed comparable reducing power property i.e. 55.12%. The results are summarized in table no.1.

b) Nitric oxide anion scavenging activity:

It is also observed that 70% EEBSC have demonstrated concentration dependent inhibition in the nitric oxide anion scavenging activity. Where as 25µg ascorbic acid (std.25µg) has 41.25 % nitric oxide radical scavenging activity. However, test extracts even at 120 µg showed lesser inhibition than standard in the antioxidant model. The results are summarized in table no.2.

Nephroprotective activity

Effect of 70% ethanolic extract of bark of Spathodea campanulata on Gentamicin induced nephrotoxicity:

Gentamicin treated rats showed a significant increase in serum marker enzymes like BUN, Serum creatinine and there is marked depletion of tissue GSH levels and increased lipid peroxidation levels when compared with control. It also showed a very high decrease of body weight and increased kidney weight over control group. EEBSC pretreated rats showed significantly (P<0.001) decreased levels of serum marker activities, dose dependent increase in tissue GSH, inhibition of lipid peroxidation levels and markedly reduce the decrease in bodyweight and reduced the elevated kidney weight as compared with gentamicin treated rats. The results are summarized in table no.3 and 4.

Histopathological Studies in Gentamicin induced nephrotoxicity:

Fig. A shows the light micrograph of control kidney showing normal architecture with normal glomeruli. The gentamicin induced rat kidney showed the glomerular congestion and severe interstitial congestions (Fig.B). Pretreatment with EEBSC 250 and 500mg/kg demonstrated marked improvement with maintained glomeruli and no interstitial congestion (Fig. C and D) as compared to gentamicin administered group.

Table 1: Reducing power activity of 70 % EEBSC:

Groups	Absorbance Mean ± SEM	% Increase
Control	0.078±0.000	--
Standard 25 µg	0.110±0.001***	41.02
Control + 70% Ethanolic Extract 25 µg	0.097±0.001***	24.35
Control + 70% Ethanolic Extract 50 µg	0.121±0.001***	55.12
Control + 70%.Ethanolic Extract 75 µg	0.144±0.000***	84.61
Control + 70% Ethanolic Extract 100 µg	0.148±0.001***	89.71
Control + 70% Ethanolic Extract 125 µg	0.151±0.000***	93.58

Values are the mean ± S.E.M., n=3, Significance *** P<0.001, compared to control.

Std: Ascorbic acid

Table 2: Nitric oxide scavenging activity of 70 % EEBSC:

Groups	Absorbance Mean ± SEM	%Increase
Control	0.143 ± 0.000	--
Control + standard 25 µg	0.084 ± 0.000***	41.25
Control + 70% Ethanolic Extract 25µg	0.136 ± 0.000***	04.89
Control + 70% Ethanolic Extract 50 µg	0.120 ± 0.002***	19.16
Control + 70%.Ethanolic Extract 75 µg	0.130 ± 0.000***	09.09
Control + 70% Ethanolic Extract 100 µg	0.120 ± 0.000***	19.16
Control + 70% Ethanolic Extract 125 µg	0.106 ± 0.001***	25.87

Values are the mean ± S.E.M., n=3,Significance *** P<0.001, compared to control.

Std: Ascorbic acid

Table 3: Effect of 70% EEBSC in Gentamicin induced renal damage in rats:

Gr. (n=6)	Treatment regimen	Kidney weight (g/100g)	Change in b.w. (%)	Blood urea (mg/dl)	Serum creatinine (mg/dl)
I	Vehicle treatment (Negative control)	0.664 ± 0.020	12.5 ± 1.118	45.09 ± 3.02	0.70 ± 0.05
II	Gentamicin 80 mg/kg i.p. for 8days (Positive control)	1.178 ± 0.058	8.33 ± 1.054	81.47 ± 6.22	1.60 ± 0.07
III	Gentamicin 80 mg/kg i.p. for last 8 days + 70% ethanolic extract 250 mg/kg p.o. for 11 days	0.740 ± 0.015***	4.16 ± 0.833*	43.83 ± 0.83***	0.90 ± 0.03***
IV	Gentamicin 80 mg/kg i.p. for last 8 days + 70% ethanolic extract 500 mg/kg p.o. for 11 days	0.718 ± 0.033***	2.50 ± 1.118**	40.58 ± 3.23***	0.63 ± 0.02***

Values are the Mean ± S.E.M. of six rats / treatment, Significance *P<0.05 and ***P<0.001 (vs. Control). b.w. – Body weight

Table 4: Effect of 70% EEBSC on tissue GSH and LPO levels in Gentamicin induced nephrotoxicity:

Treatment	GSH Absorbance Mean ± SEM	LPO Absorbance Mean ± SEM
Negative Control (1ml vehicle)	0.70 ± 0.01	0.167 ± 0.003
Gentamicin 80 mg/kg i.p. for 8days (Positive control)	0.35 ± 0.01	0.462 ± 0.029
Gentamicin 80 mg/kg i.p. for last 8 days + 70% ethanolic extract 250 mg/kg p.o. for 11days	0.41 ± 0.003***	0.039 ± 0.001***
Gentamicin 80 mg/kg i.p. for last 8 days + 70% ethanolic extract 500 mg/kg p.o. for 11days	0.49 ± 0.005***	0.025 ± 0.000***

Values are the mean ± S.E.M. of six rats/ treatment. Significance *P<0.05, ***P<0.001, compared to gentamicin treatment.

HISTOPATHOLOGY

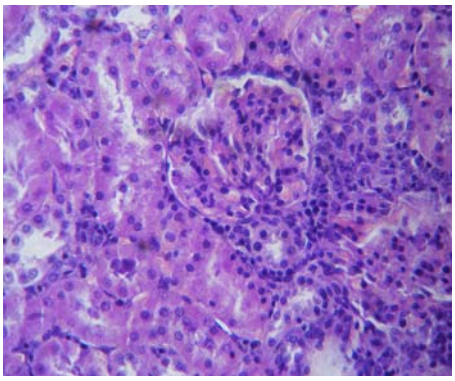

Fig. A (Control)

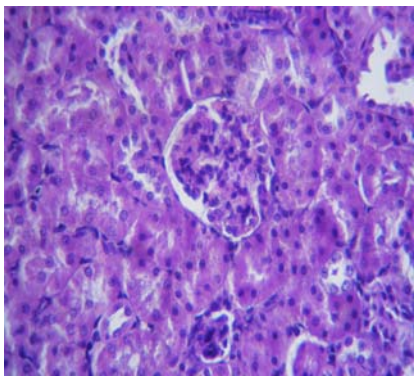

Fig. B (Gentamicin)

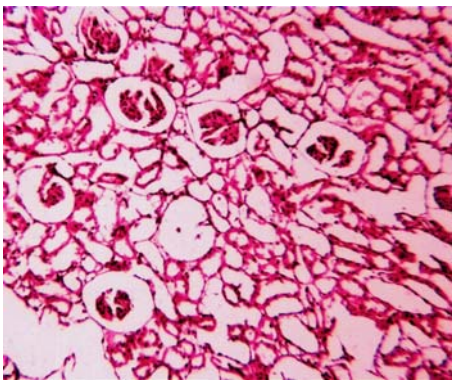

Fig. C (EEBSC250 mg/kg)

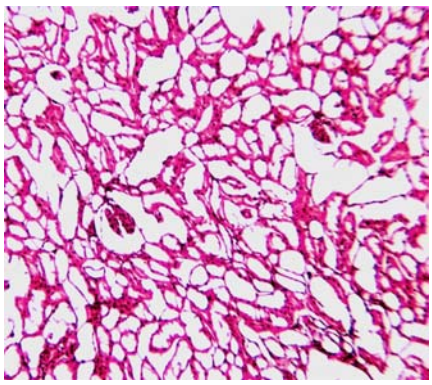

Fig. D (EEBSC500 mg/kg)

Discussion

Antioxidants are the chemical constituents, which are used for inhibiting the tissue damage by countering the free radicals; most of the antioxidants available in the markets are from natural origin eg- Vit-E, Vit-C, tocopherol, quercetin, β-carotene etc. In addition there are reports that polyphenolic compounds like flavonoids and tannins are useful as antioxidants and organ protectants. So, Ethanolic extract of bark is subjected to screen antioxidant activity using invitro models like reducing power and nitric oxide scavenging activities. EEBSC showed dose dependent reducing power similarly the extract also showed dose dependant NO radical scavenging activities. Since GSH is considered as inbuilt antioxidant substance which prevents lipid peroxidation, estimation of tissue GSH and extent of lipid peroxidation were considered as parameters of screening in-vivo antioxidant properties in gentamicin induced model. Kidneys are involved in the excretion of various xenobiotics pollutants, toxins and hence they are prone to liberate high quantities of free radicals. Therefore this organ is prone to be destroyed by such highly reactive free radicals.

In the present study the 70% ethanolic extract was subjected for screening the nephroprotective activity by using gentamicin induced nephrotoxicity in rats. Biochemical markers of kidney function like blood urea nitrogen, serum creatinine levels, body weight, kidney weight, tissue GSH and lipid peroxidation were considered for assessing the nephroprotective properties.

Gentamicin administration exhibited a marked decrease in body weight, tissue GSH level and increased kidney weight and LPO levels which is supported by a significant increase in serum markers like blood urea and serum creatinine. Co-administration of test extract normalized the raised kidney weight, blood urea, serum creatinine, tissue LPO level, and prevented the reduced tissue GSH level and prevented the fall in body weight. There are reports that gentamicin induced nephrotoxicity is due to generation of superoxide, hydroxyl radical and hydrogen peroxide free radicals[14]. In addition aminoglycoside antibiotics are protonated in body and bind to negatively charge phosphotidyl inositol or phosphotidylerin. This binding causes the inhibition of activities of lysosomal phospholipases resulting in lysosomal phospholipidosis. This excessive phospholipid overload causes cell necrosis by poorly understood mechanism. However in the present study, the test extract has demonstrated significant antioxidant property. Therefore the protective effect of the test extract may be attributed to the antioxidant activity of the plant. However our study does not rule out the possibility of prevention of renal lipidosis and cell death.

The nephro protective property of the extract is further confirmed by significant improvement of the kidney architecture by reversing the glomerular congestion, interstium with inflammatory cells, tubular necrosis, peritubular necrosis and presence of caspe suggesting massive total necrosis over gentamicin group. The data of the present study clearly showed EEBSC modulated most of the biochemical and Histopathological parameters were maintained to normal status in gentamicin treated rats, suggesting the beneficial action of EEBSC as a nephroprotective agent.

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