ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Nephroprotective Activity of *Psidium Guajava* Linn. Leaves Extract against Cisplatin Induced Nephrotoxicity In Rats.

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ABSTRACT

Cisplatin is an effective chemotherapeutic agent successfully used in the treatment of a wide range of tumors; however, nephrotoxicity has restricted its clinical use. Several studies have shown that reactive oxygen species are involved in cisplatin-induced nephrotoxicity. The aim of this study was to investigate nephroprotective activity of hydroalcoholic extract of *Psidium guajava* Linn. (HAPG) leaves in cisplatin induced nephrotoxicity in rats. Rats received a single injection of cisplatin (7.5 mg/kg, i.p.) on 5th day. HAPG was given to two groups (200 mg/kg and 400 mg/kg body weight p.o.) for 9 days. The HAPG treatment was able to ameliorate the reduced body weight, urinary creatinine, blood total protein and increased kidney weight, urine volume, urinary sodium, urinary potassium, urinary glucose, blood urea, blood creatinine levels due to cisplatin induced nephrotoxicity. HAPG significantly increased the tissue GSH levels and reduced lipid peroxide levels. Further it was confirmed by the histopathological observation that the degenerative changes caused by cisplatin were also restored by treatment with HAPG. These results suggested that HAPG possess nephroprotective activity against cisplatin induced kidney damage.

Key words: Nephrotoxicity; *Psidium guajava* Linn.; cisplatin.

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ISSN: 0975-8585

INTRODUCTION

Nephrotoxicity may be consequent to direct cytotoxic damage to kidney structures by toxicants, to immunologic processes, to indirect toxicity due to alterations in renal hemodynamics, or to the production of endogenous nephrotoxic substance. Toxic nephropathy is a disorder whose primary feature is impairment of the normal functions of the kidney. Because of the distinct anatomic and physiologic features, the kidney is uniquely susceptible to toxicity and is the target of many xenobiotic and environmental toxicants [1]. Medications have long been associated with the development of iatrogenic renal dysfunction and injury. The mechanism of drug induced nephrotoxicity can vary largely based on the pharmacological action, metabolism, and ultimate pathway of excretion of the drug administered [3]. Cisplatin is currently one of the most important chemotherapeutic drugs used in the treatment of a wide range of solid tumours of head, neck, ovarian and lung cancers. However, the clinical usefulness of this drug is limited due to nephrotoxicity induction. cisplatin gets accumulated in the tubular epithelial cells of proximal kidney tubule, causing nephrotoxicity, characterized by morphological destruction of intra cellular organelles, cellular necrosis, loss of microvilli, alterations in the number and size of lysosomes and mitochondrial vacuolization, followed by alterations including inhibition of protein synthesis, GSH depletion, lipid peroxidation and mitochondrial damage [3].

Psidium guajava Linn. is a fruit bearing tree commonly known as guava, which belongs to family Myrtaceae. The leaves and bark of guava tree have a long history of medicinal uses. In India, decoction of the leaves and bark of guava is used to cure diarrhea, dysentery, vomiting and sore throats and to regulate menstrual cycles. Guava leaves are free from fat and cholesterol. They are excellent source of fibre, potassium and vitamin A. The leaves of guava are rich in flavonoids, particularly quercetin. Guava has antioxidant property attributed to polyphenols found in its leaves [4].

MATERIALS AND METHODS

Plant material:

The leaves of *Pisidium guajava* Linn. used for the present study was collected from Bengaluru, Karnataka. The leaves were identified, confirmed and authenticated by Dr. K. Kempegowda, Professor and Head, Department of Horticulture, University of Agricultural Science, GKVK, Bengaluru.

Extraction:

The leaves were cleaned and shade dried. The dried material was then pulverized separately into coarse powder by a mechanical grinder. The powder was extracted directly with 70% v/v ethanol, which was used for biological investigations, after subjecting it to preliminary qualitative phytochemical studies [5]. The extract was concentrated under reduced pressure and stored in vacuum desiccators.

ISSN: 0975-8585

Determination of Acute Toxicity (LD_{50}):

The procedure was divided into two phases. Phase I (observation made on day one) and Phase II (observed the animals for next 14 days of drug administration). Two sets of healthy female rats (each set of 3 rats) were used for this experiment. First set of animals were divided into three groups, each of one in a group. Animals were fasted overnight with water *ad libitum.* Animals received a single dose of 2000 mg/kg, p.o. was selected for the test, as the test item was a source from herb. After administration of extract, food was withheld for 3-4 hrs [6].

Phytochemical screening:

The preliminary phytochemical analysis was carried out by using the standard procedures. Alkaloids, carbohydrates, flavonoids, glycosides, phytosterols/terpenes, proteins and saponins were qualitatively analysed [7].

Experimental animals:

Albino wistar rats weighing 150-250g was procured from Biogen, Bangalore. They were maintained in the animal house of Gautham College of Pharmacy. Animals were maintained under controlled condition of temperature at $27^{o} \pm 2^{o}$ C and 12-h light-dark cycles. They were housed in polypropylene cages and had a free access to standard pellets (Amruth) and water *ad libitum*. All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of Gautham College of Pharmacy, Bangalore (REF-IAEC/01/05/2011) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg No: 491/01/c/CPCSEA), Govt. of India.

Effect of HAPG on Cisplatin Induced Nephrotoxicity in Rats [8]:

The albino rats were divided in to 4 groups and each group contains 6 rats and treatment would be as follows

Group I: These group animals received only normal saline through i.p route throughout the course of the experiment served as control.

Group II: This group of animals were received a single dose of cisplatin (7.5 mg/kg, i.p) on 5^{th} day.

Group III: The animals of this group received 200 mg/kg, p.o. of HAPG for 4 days, on 5^{th} day single dose of cisplatin (7.5 mg/kg, i.p) was given, one hour after cisplatin administration, HAPG was administered to the group, and the HAPG treatment was continued for four days after 5^{th} day.

Group IV: The animals of this group received 400 mg/kg, p.o. of HAPG for 4 days, on 5^{th} day single dose of cisplatin (7.5, mg/kg i.p) was given, one hour after cisplatin administration, HAPG was administered to the group, and the HAPG treatment was continued for four days after 5^{th} day.

On the 9th day all group of animals were kept in metabolic cages. On the last day (10th) all animals were sacrificed under mild ether anesthesia and the kidneys, urine and blood samples were collected and assessed.

Physical Parameters:

Body Weight [9]:

The weight of the animals before starting and at the end of the treatment was measured and percentage change in body weight was calculated in cisplatin induced nephrotoxicity.

Kidney Weight [10]:

The weight of the kidneys of the animals at the end of the treatment was measured in cisplatin induced nephrotoxicity.

Urine Volume [10]:

The urine volume of the animals was measured in cisplatin induced nephrotoxicity.

Estimation of biochemical parameters:

The following parameters are estimated by using standard procedures of Excel, Beacon and Transasia diagnostics estimating kits: Urinary parameters: sodium, potassium, creatinine, glucose and Blood parameters: urea, creatinine, total protein.

Estimation of antioxidant activity:

Glutathione estimation [9]

Tissue samples were homogenized in ice cold Trichloroacetic acid (1 gm tissue plus 10 ml 10% TCA) in a tissue homogenizer. Glutathione measurements were performed using a modification of the Ellamn procedure (Aykae, et.al.) Briefly, after centrifugation at 3000 rpm for 10 minutes, 0.5 ml supernatant was added to 2 ml of 0.3 M disodium hydrogen phosphate solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml in 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. % increase in OD is directly proportional to the increase in the levels of Glutathione. Hence, % increase in OD is calculated.

Lipid peroxidation [9]

Stock solution of TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid. This solution may be mildly heated to assist in the dissolution of the thiobarbituric acid. Combine 1.0 ml of biological sample (0.1-2.0 mg of membrane protein or 0.1-0.2 μmol of lipid phosphate) with 2.0 ml of TCA-TBA-HCl and mix thoroughly. The solution is heated for 15 min in a boiling water bath. After cooling, the

flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the sample is determined at 535 nm against a blank that contains all the reagents minus the lipid. % decrease in OD is directly proportional to the decrease in the levels of lipid peroxidation. Hence, % decrease in OD is calculated.

Statistical analysis:

The values are expressed as Mean ± SEM. The data was analysed by using one way ANOVA followed by Dunnett's test using Graph pad prism software. Statistical significance was set at $P \leq 0.05$.

RESULTS

Effect of HAPG on Change in Body Weight, Urine Volume and Kidney Weight:

There was found to be high decrease of body weight in cisplatin treated group (II). However, there was dose dependent increase of body weight significantly in animals treated with HAPG 200 mg/kg, p.o ($p<0.001$) and 400 mg/kg, p.o ($p<0.001$) (III and IV) when compared with group (II). (Table no. 1)

There was found to be increase of urine volume and kidney weight in cisplatin treated group (II). However, there was dose dependent decrease of urine volume as well as kidney weight significantly in animals treated with HAPG 200 mg/kg, p.o ($p<0.001$) and 400 mg/kg, p.o ($p<0.001$) (III and IV) when compared with group (II). (Table no. 1).

Table No. 1: Effect of HAPG on Change in Body Weights, Urine Volume and Kidney Weight in Cisplatin Induced Nephrotoxic Rats.

Group	Dose	Change in Body Weight (g)	Urine Volume (ml)	Kidney Weight (g)
I	Vehicle	7.737 ±0.7622	5.767±0.1520	0.6767 ± 0.0291
II	Cisplatin 7.5 mg/kg, i.p.	-11.35 ± 0.6609	14.93 ± 0.9193	0.9500 ± 0.0201
III	Cisplatin 7.5 mg/kg i.p + 200 mg/kg, p.o HAPG	-6.272 ± 0.3046***	7.783 ± 0.0792***	0.7067 ± 0.0122***
IV	Cisplatin 7.5 mg/kg i.p + 400 mg/kg, p.o HAPG	-3.607 ± 0.3527***	6.767 ± 0.1256***	0.6217 ± 0.0195***

Values are Mean ± SEM (n=6) one way ANOVA followed by Dunnett's test. Where, ***P<0.001 and ** P<0.01. All values are compared with Toxicant control. HAPG- Hydro alcoholic extract of *Psidium guajava* Linn.

Effect of HAPG on Urinary Sodium, Potassium, Glucose and Creatinine:

There was an increase of sodium and potassium levels in cisplatin treated group (II) when compared to control (I). However 200 mg/kg, p.o (III) HAPG significantly ($p<0.01$) decreased the levels of sodium as well as potassium and in the case of 400 mg/kg, p.o (IV)

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HAPG decreased the levels of sodium and potassium significantly ($p<0.001$) in urine samples were observed when compared group (II). (Table no. 2). Glucose levels in cisplatin treated group (II) were highly increased when compared to control group (I). However 200 mg/kg, p.o HAPG (III) reduced the glucose levels significantly ($p<0.001$) and 400 mg/kg, p.o HAPG (IV) reduced the glucose levels significantly ($p<0.001$) in the urine samples when compared with group (II) (Table no. 2). Urinary creatinine levels in cisplatin treated group (II) were decreased when compared to control group (I). However 200 mg/kg, p.o HAPG (III) increased the levels of creatinine significantly ($p<0.05$) and in the case of 400 mg/kg, p.o HAPG (IV) increased the levels of creatinine significantly ($p<0.001$) in urine samples were observed when compared with group (II) (Table no. 2).

Effect of HAPG on Blood Urea, Blood Creatinine and Blood Total Protein:

Blood urea level increased in cisplatin treated group (II) when compared with control group (I). However 200 mg/kg, p.o HAPG (III) decreased urea levels significantly ($p<0.001$) and 400 mg/kg, p.o HAPG (IV) decreased urea levels significantly ($p<0.001$) in the blood samples when compared with group (II). (Table no. 3)

Blood creatinine level increased in cisplatin treated group (II) when compared with control group (I). However 200 mg/kg, p.o HAPG (III) slightly decrease the blood creatinine but not significant and 400 mg/kg, p.o HAPG (IV) decreased creatinine levels significantly ($p<0.01$) in the blood samples when compared with group (II). (Table no. 3)

Blood total protein level decreased in cisplatin treated group (II) when compared with control group (I). However 200 mg/kg, p.o HAPG (III) increased total protein levels significantly ($p<0.001$) and 400 mg/kg, p.o HAPG (IV) increased total protein levels significantly ($p<0.001$) in the blood samples when compared with group (II). (Table no. 3)

Table No. 2: Effect of HAPG on Urinary Sodium, Potassium, Glucose and Creatinine Levels in Cisplatin Induced Nephrotoxic Rats.

Group	Treatment	Urinary Sodium levels (mmol/l)	Urinary Potassium levels (mmol/l)	Urinary Glucose levels (mg/dl)	Urinary Creatinine levels (g/L)
I	Vehicle	105.8 ± 6.011	4.161 ± 0.2494	8.265 ± 0.5294	3.086 ± 0.3396
II	Cisplatin 7.5 mg/kg i.p.	135.7 ± 5.972	5.666 ± 0.4143	238.7 ± 22.10	0.6652 ± 0.2302
III	Cisplatin 7.5 mg/kg i.p + 200 mg/kg p.o HAPG	112.6 ± 2.095**	4.073 ± 0.2158**	120.7 ± 7.917***	1.699 ± 0.2650*
IV	Cisplatin 7.5 mg/kg i.p + 400 mg/kg p.o HAPG	104.0 ± 2.790***	3.345 ± 0.1802***	91.69 ± 2.745***	2.646 ± 0.1292***

Values are Mean ± SEM (n=6) one way ANOVA followed by Dunnett's test. Where, ***P<0.001, ** P<0.01, * P<0.05 and ns represents Not significant. All values are compared with Toxicant control. HAPG- Hydro alcoholic extract of *Psidium guajava* Linn.

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Table No. 3: Effect of HAPG on Blood Urea, Blood Creatinine and Blood Total Protein Levels in Cisplatin Induced Nephrotoxic Rats.

Groups	Treatment	Blood Urea (mg/dl)	Blood Creatinine (mg %)	Blood Total Protein (g/dl)
I	Vehicle	21.31 ± 2.194	1.894 ± 0.2768	7.482 ± 0.4286
II	Cisplatin 7.5 mg/kg i.p.	73.89 ± 5.696	4.666 ± 0.4320	2.930 ± 0.4427
III	Cisplatin 7.5 mg/kg i.p + 200 mg/kg p.o HAPG	42.99 ± 3.311***	3.652 ± 0.2934ns	6.350 ± 0.3085***
IV	Cisplatin 7.5 mg/kg i.p + 400 mg/kg p.o HAPG	23.11 ± 2.008***	2.589 ± 0.4042**	7.135 ± 0.2593***

Values are Mean ± SEM (n=6) one way ANOVA followed by Dunnett's test. Where, ***P<0.001, **P<0.01, *P<0.05 and ns represents Not significant. All values are compared with Toxicant control. HAPG- Hydro alcoholic extract of *Psidium guajava* Linn.

Effect of HAPG on Tissue Lipid Peroxidation (LP) and Glutathione (GSH):

There was dose dependent inhibition of *in-vivo* LP by both the doses of HAPG. 200 mg/kg p.o HAPG showed 50.916% inhibition, whereas 400 mg/kg, p.o HAPG showed 68.864% inhibition. There was a marked depletion of GSH level in cisplatin treated groups. HAPG showed a dose dependent increase in the level of GSH. However at 200 mg/kg, p.o HAPG showed 79.00% increased in GSH level and 400 mg/kg p.o HAPG showed 61.64% increased in GSH levels.

Table No. 4: Effect of HAPG on Tissue LP and GSH Levels in Cisplatin Induced Nephrotoxicity Model.

Group	Treatment	Absorbance (LP)	% Inhibition (LP)	Absorbance (GSH)	% Increase (GSH)
I	Vehicle	0.4590 ± 0.0005	-	0.7607 ± 0.0038	-
II	Cisplatin 7.5 mg/kg i.p.	0.8197 ± 0.0103	-	0.5377 ± 0.0034	-
III	Cisplatin 7.5 mg/kg i.p + 200 mg/kg p.o HAPG	0.4027 ± 0.0104***	50.916	0.6507 ± 0.0073***	79.00
IV	Cisplatin 7.5 mg/kg i.p + 400 mg/kg p.o HAPG	0.2553 ± 0.0031***	68.864	0.7437 ± 0.0029***	61.64

Values are Mean ± SEM (n=6) one way ANOVA followed by Dunnett's test. Where, ***P<0.001, **P<0.01, *P<0.05 and ns represents Not significant. All values are compared with Toxicant control. HAPG- Hydro alcoholic extract of *Psidium guajava* Linn.

ISSN: 0975-8585

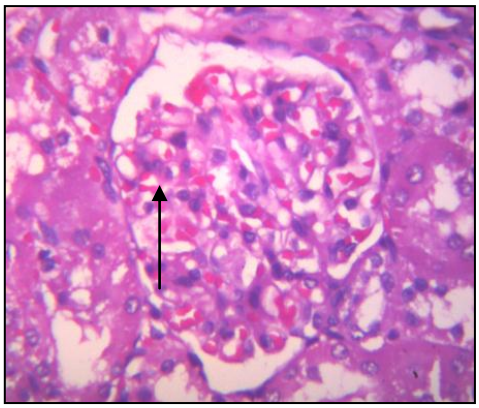
Histopathological Study of Kidneys in Cisplatin Induced Nephrotoxicity:

Group I: Negative control showed intact architecture of renal parenchyma. In glomerulus [Fig. A, Arrow] bowman's space and mesangial cells appeared intact. Intact renal tubules [Fig. B, Arrow] blood vessels and Interstitium were unremarkable.

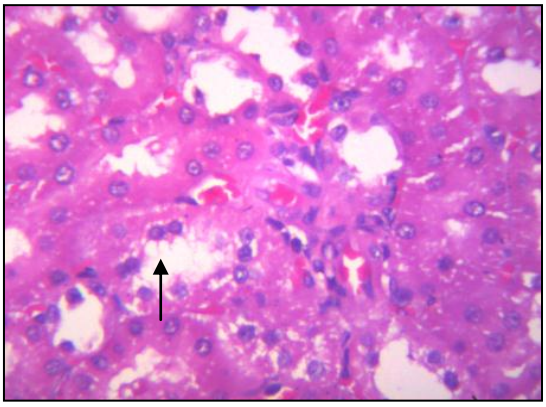
Group II: Positive control i.e. cisplatin treated showed focally distorted renal parenchyma architecture [mainly tubules]. In glomerulus [Fig. A, Arrow]: intact bowman's space, extravasation of erythrocytes seen mesangial cells appear increased. Most of the renal tubules showed degenerative changes [Fig. B, Arrow]. Blood vessels and Interstitium were unremarkable.

Group III: Treatment done with HAPG 200 mg/kg, p.o showed intact architecture of renal parenchyma. In glomerulus [Fig.A, Arrow]: Intact Bowman's space, Extravasation of erythrocytes was seen; Mesangial cells appear increased. Few renal tubules show degenerative changes [Fig.B, Arrow]. Blood vessels and Interstitium were unremarkable.

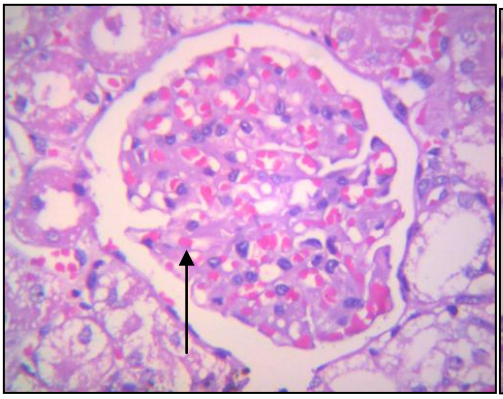
Group IV: Treatment done with HAPG 400 mg/kg, p.o showed intact architecture of renal parenchyma. In glomerulus [Fig.A, Arrow]: Hypercellular with increased extravasation of erythrocytes was observed. Renal tubules were unremarkable [Fig.B, Long - Arrow]. Blood Vessels and Interstitium remained unremarkable.



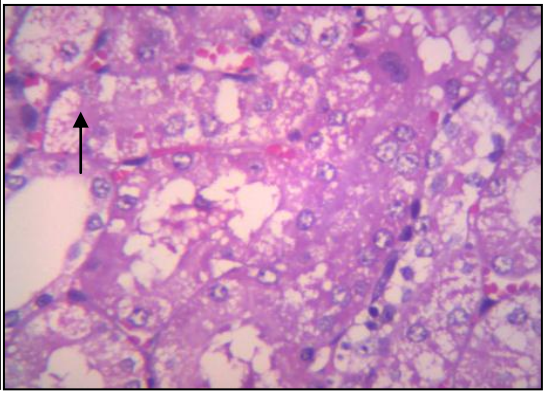
Group- I (Figure A)



Group- I (Figure B)



Group- II (Figure A)



Group- II (Figure B)

ISSN: 0975-8585

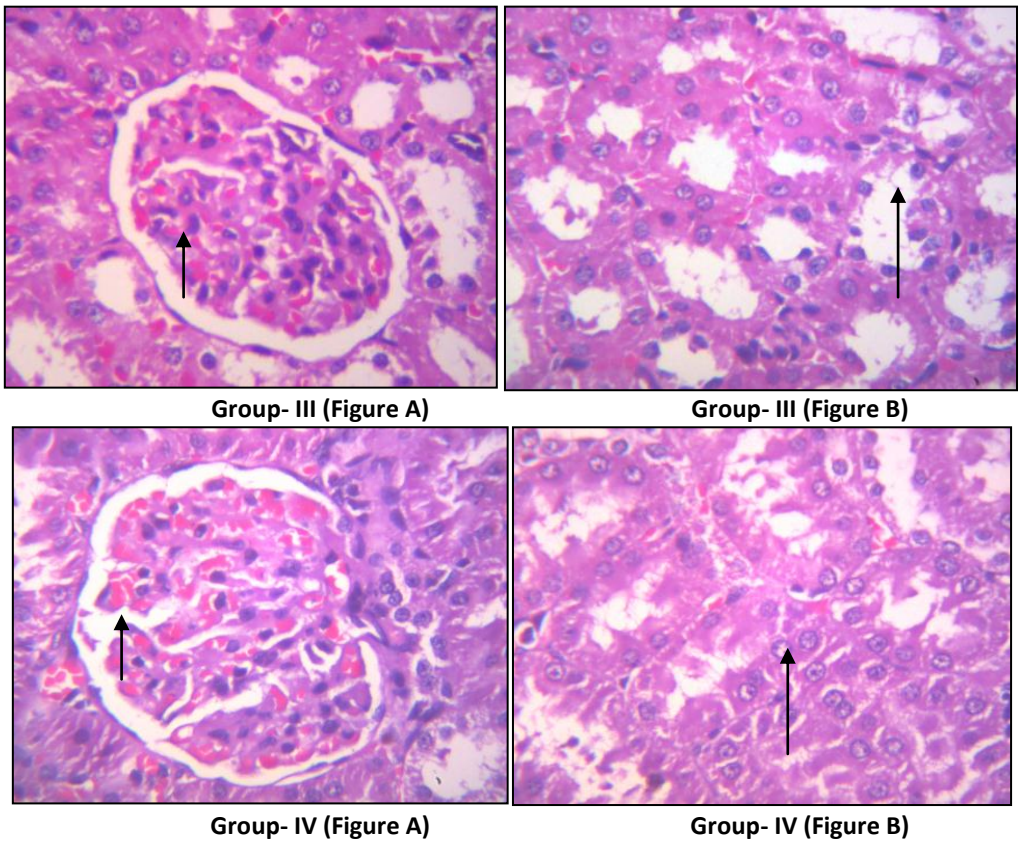


Figure No. 1: Histopathology of Kidney

DISCUSSION

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. Plants may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant. The intake of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing [11]. Hence in this study a plant *Psidium guajava* Linn has been selected for screening nephroprotective activity.

One report suggests that cisplatin increase the calcium independent nitric oxide synthase, alter the L-arginine metabolism resulting in the generation nitric oxide [12]. All these findings are indicating that cisplatin induced renal damage may be due to the oxidative stress.

The drug cisplatin (7.5 mg/kg i.p) reduced the body weight, urinary creatinine, blood total protein and increased kidney weight, urine volume, urinary sodium, urinary potassium, urinary glucose, blood urea, blood creatinine levels indicating that nephrotoxicity was

induced. It also depleted the levels of GSH and increased the levels of LP. Further confirmed by the histopathological observation that architecture of renal parenchyma was focally distorted, Extravasation of erythrocytes seen in glomerulus and most of the renal tubules show degenerative changes.

Upon treatment with 200 mg/kg and 400 mg/kg of HAPG p.o all the biochemical markers brought back to the near normal in a dose dependant manner. Even histopathological signs of nephrotoxicity were also reversed by HAPG. Since the test extract had shown significant and dose dependant antioxidant activity and prevented the depletion of GSH and decreased the lipid peroxidation. The nephroprotective activity of extract may be due to the antioxidant potential of it.

CONCLUSION

The HAPG has shown protection against cisplatin induced nephrotoxicity. It was observed that all the physical and biochemical parameters were brought back to the near normal levels with the HAPG treatment. The HAPG demonstrated significant dose dependant increase in depleted tissue GSH levels and reduction in lipid peroxidation caused by cisplatin induced nephrotoxicity. The histopathology reports concluded that the HAPG has improved the renal damage occurred due to cisplatin.

Hence, the HAPG possess the nephroprotective activity against cisplatin induced nephrotoxicity. Further studies will be necessary to establish the probable mechanism of action of the nephroprotective activity of the leaf extract *Psidium guajava* Linn.

ACKNOWLEDGEMENTS

I would like to sincerely thank Mrs.Kavitha Sarvesh, Chairperson and Mrs. Anitha Prasad, Management member of Gautham College of Pharmacy, for providing facilities and opportunity to accomplish this endeavour successfully.

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