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IN-VITRO ANTIOXIDANT AND PROTECTIVE EFFECT OF *BAUHINIA VARIEGATA* LINN ON GENTAMICIN-INDUCED NEPHROTOXICITY IN RATS

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ABSTRACT

The present study was aimed at evaluating the ethanolic and aqueous extracts of root of *Bauhinia variegata* Linn. for antioxidant and nephroprotective effect in gentamicin-induced nephrotoxicity in rats. The antioxidant activity of both ethanolic and aqueous extracts of root of *Bauhinia variegata* Linn. was carried out by *in-vitro* models such as scavenging of free radicals using 1,2-diphenyl1-2-picrylhydrazyl (DPPH), nitric oxide and superoxide. Nephrotoxicity was induced in Wistar rats by intraperitoneal administration of gentamicin 100 mg/kg/d for eight days. Effect of concurrent administration of ethanolic and aqueous extracts of root of *Bauhinia variegata* Linn. at dose of 200 and 400 mg/kg b.w. respectively was given by oral route. Serum creatinine, serum urea, urine creatinine and blood urea nitrogen (BUN) were determined on day 9. Histopathological study of kidney was also done. Both ethanolic and aqueous root extracts of *Bauhinia variegata* Linn produced significant free radical scavenging activity. Both the extracts produced significant nephroprotective activity in Gentamicin induced nephrotoxicity models as evident by decrease in serum creatinine, serum urea, urine creatinine and BUN levels in extract treated groups which was elevated by gentamicin, which was further confirmed by histopathological study. Gentamicin induced glomerular congestion, blood vessel congestion, and epithelial desquamation, accumulation of inflammatory cells and necrosis of the kidney cells were found to be reduced in the groups receiving the root extract of *Bauhinia variegata* Linn. along with gentamicin.

KEYWORDS: Antioxidant, Gentamicin, *Bauhinia variegata* Linn., Nephrotoxicity

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INTRODUCTION

Free radicals are highly reactive substances formed in the body as a result of metabolic processes. Many of these molecular species are oxygen (and some time nitrogen) centered, oxygen free radicals and its non radical products are associated with reactive oxygen species (ROS)[1]. The term ROS collectively denotes oxygen centered radicals (super oxide and hydroxyl radicals) as well as non-radical species derived from oxygen such as hydrogen peroxide, singlet oxygen and hypochlorus acid. The increased production of ROS seems to accompany most forms of tissue injury.[2] The involvement of ROS in aging and in many chronic diseases has been considered. The defense provided by antioxidant systems is crucial for the survival of organisms. Detoxification of ROS in the cell is provided by both enzymatic and non-enzymatic systems which constitute the antioxidant defense systems. These antioxidants play a role in delaying, intercepting, or preventing oxidative reactions catalysed by free radicals[3].

Aminoglycosides have long been one of the common causes of drug induced nephrotoxicity. Gentamicin induced nephrotoxicity is a model of acute renal failure caused by oxidative stress generated through the induction of superoxide[4]. It has been demonstrated that gentamicin-induced nephrotoxicity is characterized by direct tubular necrosis, which is localised mainly in the proximal tubules. It is complex phenomenon characterized by an increase in plasma creatinine and urea levels and severe proximal tubular necrosis,

followed by deterioration and renal failure[5]. The toxicity of gentamicin is believed to relate to generation of reactive oxygen species (ROS) in kidney. Because of the obvious mediation of ROS in gentamicin induced renal damage several antioxidant agents have been used to block gentamicin nephrotoxicity[6,7]

The plant *Bauhinia variegata* Linn *(Caesalpiniaceae)* is commonly known as Mountain Ebony is a medium-sized, deciduous tree, found throughout India. It has been used in dyspepsia, bronchitis, leprosy, ulcer, to prevent obesity, as an astringent, tonic and anthelmintic[8]. The stem contains β-sitosterol, lupeol, kaempferol-3-glucoside and 5,7-dihydroxy and 5,7-dimethoxy flavanone-4-O-α-L-rhamnopyranosyl-β-D-glucopyranosides. Flowers contain cyanidine-3-glucoside, malvidin-3-glucoside, malvidin-3-diglucoside, and peonidin 3-diglucoside, kaempferol-3-galactoside and kaempferol-3-rhamnoglucoside. Five flavonoids isolated from the different parts of *Bauhinia variegata* has been identified as quercetin, rutin, apigenin and apigenin 7-O-glucoside. Phytochemical analysis of the root bark of *Bauhinia variegata* Linn was reported to contain a new flavanone: (2S)–5,7-dimethoxy-3'-4'-methylene dioxyflavanone (1) and a new dihydrobenzoxepin 5,6-dihydro-1,7dihydroxy-3,4-dimethoxy-2-methyldibenz (b,f) oxepin[9.10].

Bauhinia variegata Linn. stem is reported to have antitumour[11], antimicrobial[12], anti-inflammatory[13], hepatoprotective[14], antihyperlipidemic[15] and immunomodulatory activities[16]. The present study was carried out to determine the effect of ethanolic and aqueous extracts of root of *Bauhinia variegata* Linn. for antioxidant and nephroprotective activity.

MATERIAL AND METHODS

Plant material

The root of *Bauhinia variegata* Linn was procured and authenticated by Shri A. V. Bhatt, survey officer, Regional Research Institute (Ay.), Bangalore, Karnataka. A voucher specimen of same has been deposited (voucher specimen no. RRCBI MCW 79/4)

Preparation of the root extract

The authenticated root was shade dried and powdered coarsely. Extraction was done according to standard procedures using analytical grade solvents. The powdered drug was defatted by extracting with pet-ether (60-80^0 C). Coarse powder of the root (1 Kg) was soxhlet extracted with 90% ethanol. The aqueous extract was prepared using same marc by the process of maceration. The extracts obtained were concentrated under reduced pressure to yield ethanolic (4.2%) and the aqueous extracts (2.4 %).

Chemicals

The chemical DPPH {(1, 2-diphenyl-2-picryhydrazyl), N-(1-Naphthyl) ethylendiamine dihydrochloride}, NADH, phenazine methosulphate, trichloro acetic acid and potassium ferricynide were purchased from Sigma chemical, St Louis, MO, USA. All other chemicals and reagents used were of analytical grade. UV-1700 Shimazu UV spectrophotometer was used for analytical studies.

Animals

The healthy Wistar albino rats of either sex weighing between 150-200 g were taken for the study. They were housed under controlled conditions of temperature (23±2^{o}c), humidity (55±5%) and 12h light and 12h dark cycles. The animals were fed with standard pellet diet and water *ad libitum*. Approval of the Institutional Animals Ethics Committee was taken (ref. no. IAEC/KLECP/ BNG/06/2009)

Acute toxicity studies

Acute toxicity studies for aqueous and ethanolic extracts of *Bauhinia variegata* Linn. were conducted as per OECD guidelines 423 using albino Wistar rats. Each animal was administered the aqueous solution of the extract by oral route. The animals were observed for any changes continuously for the first 2h and up to 24 h for mortality.

In-Vitro Antioxidant Models

DPPH radical scavenging activity

DPPH radical scavenging activity of the extracts was measured by the method of Shimada *et al*. Various concentrations of the extracts (10-50 µg/ml) in distilled water were added to solution of DPPH (1ml, 0.1mM in ethanol) and the mixture was shaken vigorously. After placing it in room temperature for 30 min, absorbance was measured at 517 nm[17].

Superoxide radical scavenging activity

Superoxide radical scavenging activity was determined by the method of Yen and Chen. To various concentrations of extract (100-500 µg/ml), phenazine methosulphate (1ml, 60 µM in phosphate buffer pH 7.4), NADH (1ml, 450 µM in phosphate buffer, pH 7.4) and NBT (1ml, 300 µM) were added and incubated at 25^0 C for 5 min. The absorbance was recorded at 560 nm against blank.

Butylated hydroxy anisole (BHA) and ascorbic acid were used as standards for the various *in-vitro* antioxidant studies. The percentage scavenging of various radicals were calculated using the formula

$$\% \text{ radical scavenged} = (A_0 - A_1)/ A_0 \text{ X}100$$

Where, A_0 is absorbance of the free radical alone and A_1 is absorbance of free radical in presence of

extract/standard. All the experiments were performed in triplicate[18].

Nitric oxide radical scavenging activity

It was determined by adding different concentrations of the extracts (100-500 µg/ml) to 0.6 ml sodium nitroprusside and phosphate buffered saline. The solution was incubated at 25^0 C for 120 min. To 1 ml of the incubated solution, 1ml of Greiss reagent (1% sulphanilamide and 0.1% naphthyl ethylene diamine dihydrochloride in 2% phosphoric acid) was added and absorbance was measured at 546 nm against blank solution[17].

Gentamicin induced nephrotoxicity in rats

Albino rats (150-200 gm) of either sex were used for the study. Animals were divided into six groups, each containing six animals. The study was carried out for nine days and treatment was given for eight days. Group I served as control group and received distilled water p.o. for eight days. Group II served as gentamicin group. The gentamicin treated group received 100 mg/kg/day gentamicin by the intraperitoneal (i.p.) route. Group III and IV received 200 and 400 mg/kg b.w. of aqueous extract of *Bauhinia variegata* Linn. root (BVRA) respectively. Group V and VI received 200 and 400 mg/kg b.w. of ethanolic extract of *Bauhinia variegata* Linn. root (BVRE)) respectively.

Animals of groups III to VI were administered 100 mg/kg b.w. of gentamicin i.p. along with extracts p.o. for 8 days. After dosing on the day 8, individual rats were placed in separate metabolic cages for 24h for urine collection to determine urine creatinine content. Blood samples were collected via retro-orbital puncture at the end of these 24h, the serum was rapidly separated and processed for determination of serum creatinine, serum urea, blood urea nitrogen (BUN), using of Span Diagnostic kits. Body weight of animal was also recorded. Rats were sacrificed and both kidneys were isolated from each rat. The kidneys were processed for histopathological examination[19].

RESULT AND DISCUSSION

There was no change in normal behavioral pattern of animals and no sign and symptoms of toxicity were observed during the first 2h and no mortality was observed till 24h. Extracts were safe up to a maximum dose of 2000 mg/ kg b.w. The biological evaluation was carried out at doses of 200 and 400 mg/kg b.w by oral route.

An *in-vitro antioxidant* study is based on the ability of the extracts to scavenge free radicals and by evaluating their reduction capability. The results of *in-vitro* antioxidant studies indicate that both ethanolic and aqueous extracts of root of *Bauhinia variegata* Linn possesses significant antioxidant potential. The IC 50 values for the various *in-vitro* antioxidant models are indicated in the **Table 1**.

DPPH radical scavenging activity

The various extracts produced significant DPPH radical scavenging activity from 10 µg/ml. The IC_{50} of the various extracts were compared to that of the standards-Ascorbic acid (AA) and Butylated hydroxyl anisole (BHA) were shown in Table 1. The DPPH radical is lipophilic, relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents, as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colour stoichometrically depending on the number of electrons taken up[20]. Hence, DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and DPPH radical which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. From results it is evident that extracts are acting as hydrogen donors and thus are able to scavenge DPPH free radical in a concentration dependent[21].

Super radical scavenging activity

Superoxide anion radical generation was inhibited by BHA (standard) and extracts from 100 µg/ml. The various extracts produced superoxide radical scavenging activity in a concentration dependent manner [Table1]. *In-vitro* superoxide radical scavenging activity was measured by NBT (Nitro blue tetrazolium) reduction[20]. Superoxide anion radicals are one of the most abundantly produced free radicals and are produced endogenously by flavoenzymes (eg. Xanthine Oxidase) which convert hypoxanthine to xanthine and subsequently to uric acid in ischemia reperfusion. The biologically generated superoxide anion breaks into molecular oxygen and H_2O_2 in the presence of protons. Superoxide and H_2O_2 serve as precursors of singlet oxygen and hydroxyl radicals and indirectly instigate lipid oxidation[22]. Superoxide anion derived from dissolved oxygen by coupling reaction reduces NBT to a blue coloured formazon that can be measured at 560nm, [21,22]. Decrease in absorbance at 560nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The antioxidant properties of flavonoids are found to be mainly via the scavenging of superoxide anions [21]. *Bauhinia variegata*

Linn contains flavonoids and in accordance with the above statement exhibited anti-oxidant activity.

Nitric oxide (NO) radical scavenging activity

Scavenging of nitric oxide by various extracts was found to be concentration dependent. [Table 1]. NO is a short lived free radical generated by endothelium, macrophages and neurons. It exerts influence on a number of functions including vasodilation, neurotransmission, synaptic plasticity and memory in the central nervous system and is an important chemical mediator. Overproduction of Nitric oxide can mediate toxic effects like DNA fragmentation, cell damage and neuronal cell death[23]. O_2 reacts with the excess NO to generate nitrite and peroxy nitrite anions which act as free radicals. Effect of various extracts on *in-vitro* NO radical scavenging activity was taken up using sodium nitroprusside (SNP) and phosphate buffered saline solution (PBS) to generate nitric oxide. The generated nitric oxide was measured by using Greiss reagent[24]. SNP in aqueous solution at physiological pH spontaneously generates NO which interacts with O_2 to produce a nitrite ion, which can be estimated using Greiss reagent. Nitric oxide generated from SNP at physiological pH reacts with oxygen to form nitrite ions. The compound that possesses NO scavenging activity inhibits nitrite formation by competing with O_2 to react with nitric oxide [23]. This leads to decrease in nitrite concentration in assay media. Extracts were found to scavenge NO radical at the various concentrations tested.

Nephroprotective Activity

Urine creatinine, serum creatinine, serum urea and blood urea nitrogen were found to be significantly *($P<0.001$)* increased in rats treated with only gentamicin, whereas treatment with the aqueous and ethanolic extracts of root of *Bauhinia variegata* Linn reversed the effect of gentamicin indicating nephroprotective activity. [**Table 2**]

There is a simultaneous significant decrease in the gentamicin-induced nephrotoxicity when the antioxidant defense system is effective. The increased production of ROS in gentamicin-induced nephrotoxicity may be a result of inactivation of antioxidant enzymes such as SOD and GSH-Px. A relationship between nephrotoxicity and oxidative stress has been confirmed by many investigations. The impairment in kidney functions is accompanied by increase in serum creatinine and urea level and kidney tissue MDA levels that indicates lipid peroxidation. It is one of the essential compounds for maintaining cell integrity participation in the cell metabolism[5,25]. The significant and progressive weight loss in gentamicin treated rats may possibly be due to the injury renal tubules and the subsequent loss of the tubular cells to reabsorb water, leading to dehydration and loss of body weight.

Phenolic compounds present in medicinal plants have been reported to possess powerful antioxidants activity. Root bark of *Bauhinia variegata* Linn is reported to contain polyphenolics, steroids, saponins and triterpenes. *Bauhinia variegata* has been reported to contain quercetin, rutin, apigenin and apigenin 7-O-glucoside. Flavonoids and quercetin in particular are potent antioxidants and are known to modulate the activities of various enzyme systems due to their interaction with various biomolecules[26]. They show antiatherogenic and anticarcinogenic activities by blocking LDL oxidation and inhibition of processes of bioactivation of carcinogens. Quercetin decreases the lipid peroxide formation, restoration of glutathione status and the activities of antioxidant enzymes during gentamicin-induced nephrotoxicity[15]. *Bauhinia variegata* might have exhibited nephroprotective activity by the virtue of its antioxidant activity.

Histopathological Examination

Control showed normal glomerular and tubular histology whereas gentamicin peritubular and blood vessel congestion and result presence of inflammatory cells in kidney section from the gentamicin-treated group. Mononuclear cell infiltrated mainly in the sub-capsular region and hyaline changes, vacuolization and necrosis in the proximal tubular epithelial cells was seen in gentamicin group. Gentamicin group also showed interstitial edema in tubular cells. Concurrent treatment with the extracts was found to reduce such changes in kidney histology induced by gentamicin [**Fig: 1**]. Treatment with extracts could prevent cell damage such as tubular vacuolization, glomerular congestion and interstitial edema. According the pathological result it can be stated that extracts of *Bauhinia variegata* Linn had protective effect against degenerative injury caused by gentamicin.

CONCLUSION

The present study reveals that ethanolic and aqueous extracts of *Bauhinia Variegata* is a good source of phytochemical with antioxidant properties. Extracts also reversed the nephrotoxicity induced by gentamicin which indicates that it can be used as adjuvant with Gentamicin. By this combination therapy, we can get the therapeutic benefit of the gentamicin without its prominent side effect; nephrotoxicity. Phytoconstituents flavonoids, tannins, steroids and saponins present in extracts may be responsible for antioxidant activity, through which nephroprotective activity might be produced.

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Table 1: $IC_{50\ values}$ ± SD (µg/ml) of AA, BHA, BVRA, BVRE for scavenging the free radicals: DPPH, Super oxide and Nitric oxide

Test/standard groups	IC_{50} values ± SD (µg/ml) for free radical scavenging activity		
	DPPH	Super oxide	Nitric oxide
Ascorbic acid	30.55 ± 1.12	-	-
BHA	29.11± 1.03	435.40± 7.78	368.00 ± 2.60
BVRA	37.73 ±1.37	502.10 ± 8.00	478.80 ± 3.40
BVRE	36.01 ± 1.25	445.30 ± 4.05	415.20 ± 2.88

n=3, Where BVRA, BVRE and BHA indicate of *Bauhinia variegata Linn.* root of aqueous extract and ethanolic extract and Butylated hydroxyl anisole respectively.

Table 2: Nephroprotective activity of ethanolic and aqueous extracts of *Bauhinia variegata* Linn in gentamicin induced nephrotoxicity in rats

Gp	Treatment	Serum creatinine (mg/dl)	Serum urea (mg/dl)	Urine creatinine (mg/dl)	BUN (mg/dl)	Body weight (% change)
I	Control	0.62±0.16	43.17±0.15	96.71±1.54	19.81±0.56	3.23±0.27
II	Gentamicin	3.11±0.67***[a]	174.4±1.45***[a]	281±2.06***[a]	81.44±1.92***[a]	-11.68±0.3***[a]
III	BVRA 200	1.42±0.52***[b]	102.4±1.23**[b]	199.2±0.86***[b]	47.78±1.58**[b]	-6.83±0.60*[b]
IV	BVRA 400	1.09±0.43***[b]	83.6±0.45***[b]	108.2±0.97***[b]	36.16±0.63***[b]	-6.38±0.67*[b]
V	BVRE 200	0.82±0.10***[b]	81.61±1.34***[b]	139.9±0.86***[b]	38.11±1.40***[b]	-6.57±1.07*[b]
VI	BVRE 400	0.66±0.11***[b]	57.43±0.98***[b]	101.6±1.22***[b]	26.8±0.79***[b]	-3.76±0.41***[b]

n=6, values are expressed as mean±S.D. BVRA and BVRE 200 and 400 indicates *Bauhinia variegata* Linn root aqueous and ethanolic extracts at 200 and 400 mg/kg b.w. respectively.
**p<0.05, **P<0.01, ***P<0.001,* a-indicates comparison with control, b-indicates comparison with gentamicin treated group.

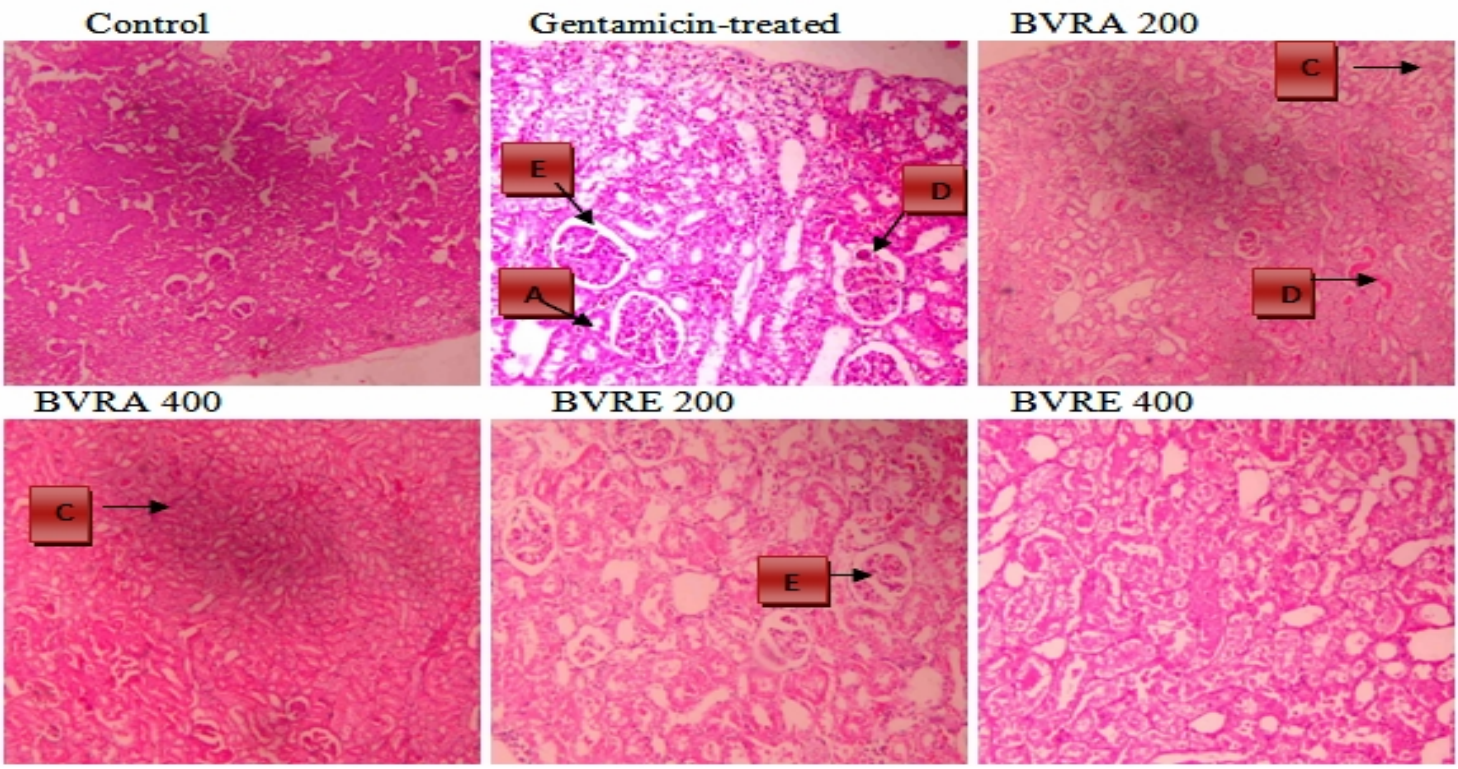

Fig 1: Histopathplogical view of renal section from different groups stained with hematoxylin and eosin
Gentamicin induced nephrotoxicity (Kidney section 200X)
A-Mononuclear infiltrate cells
B-Tubular casts
C-Interstitial edema
D-Blood vessel congestion
E-Glomerular congestion and peritubular congestion

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