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IN VITRO NEPHROPROTECTIVE AND ANTIOXIDANT ACTIVITY OF EXTRACTS OF *PITHECELLOBIUM DULCE* BARK

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ABSTRACT

Objective: The aim of this study was to investigate the In vitro antioxidant activity and nephroprotective activity of the extract of *Pithecellobium dulce* bark.

Methods: The *Pithecellobium dulce* bark was extracted using a solvent such as methanol and water. The antioxidant activity of these extracts was evaluated by using different assay such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, scavenging activity of superoxide anion and nitric oxide (NO) scavenging assay and in vitro nephroprotective screening of various concentrations of methanol and aqueous extract of bark were tested cytoprotective and epifluorescence staining assay was performed against normal kidney cell treated with gentamicin.

Results: The phytochemical study indicate the presence of phenols, tannin, flavonoids and steroid in the methanolic and aqueous extracts of the plant. Bark extracts clearly revealed the dose-dependent increase in nephroprotection. The presence of phytoconstituents in *Pithecellobium dulce* was inhibited the induced nephrotoxicity. Antioxidant activity results indicated that extracts were exhibited the ability to quench the DPPH radical, good antioxidant potential with free radical scavenging activity. The methanol and aqueous extracts at different concentration the prevent the toxicity effect to cell in place of inducer. According to IC_{50}, both extracts showed more than 100 so its non-toxic to cell as compared to

gentamicin itself. live cells and dead cells can be differentiated by epifluorescence staining. Both the extracts showed nontoxic on kidney cell. The maximum NO scavenging capacity was found in methanol extract with an IC_{50} value of which was more than water extract.

Conclusion: Phytochemical screening indicated the presences of steroids, saponins, flavonoids, tannins and phenolic compounds in methanol and aqueous extracts of *P. Dulce*. Our study also proved that this drug possesses considerable amount of flavonoid, phenolic compounds and tannin. In vitro antioxidant and cytotoxicity studies illustrated that *P. dulce* possessed significant free radical scavenging activity.

Keywords: *Pithecellobium dulce*, Phenols, Tannin, Flavonoids, Antioxidant, DPPH, Epifluorescence, Cytotoxicity

INTRODUCTION

Herbal plants are essential components of the natural world. After various survey and investigation many medicinal plants were identified as source of important medicine. Medicinal plants have been used since prehistoric period for the cure of various diseases. *Pithecellobium dulce* (Madras thorn) known as Vilayati babul, Vilayati imli, Jangle jalebi in Hindi, is known to have therapeutic effect against various ailment including diabetes. This plant is small to medium sized, evergreen, spiny and grows up to 18 m height. It is cultivated throughout the plains of India and Mexico through Central America to Colombia **[1]**.

The kidney is the important organ in human body which contributes to various physiological functions including detoxification, regulation of extracellular fluids, homeostasis and excretion of toxic metabolites. Variety of chemical substances can act as nephrotoxins. Nephrotoxins are substances that causes nephrotoxicity. As per definition, nephrotoxicity is a rapid deterioration in the renal function due to toxic effect of certain medications and chemicals **[2]**. In general, alternative medicines are usually used by the people who do not use or not benefitted by allopathic medicines. The chemical compounds of plants produce similar effects to the chemical compounds in conventional medicines. As ayurvedic medicines are cost effective, its role in the prevention and management of nephrotoxicity is quite obvious and the need of hour **[4]**. Several plants medicinal plants and their formulations are known to ameliorate the nephrotoxicity. The incidence of diabetes is increasing in general and in low- and middle-income countries in particular. Diabetes is a well-known risk factor for renal failure **[4-5]**. In the last few years more than 13 000 plants have been studied for the various diseases

and ailments all over the world. Various different type of activity of *P. dulce* bark has been investigated, however, search through the available literature have revealed a dearth of information of this medicinal plant as a nephroprotective. Therefore, the present study was conducted with an aim to investigate the role bark of *P. dulce* as a nephroprotective.

MATERIALS AND METHODS

Material collection and preparation

Collection

The Bark of the plant of *Pithecellobium dulce* was collected in the month of autumn season or spring or early summer from Jalgaon district of Maharashtra state. The plant was authenticated at Maharashtra Association for the Cultivation of Science, Agharkar Research Institute, Pune – 411004 (Certificate number AUTH 19-129). The plant material was thoroughly cleaned, powdered, and sieved for further analysis.

Chemicals

3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco`s Modified Eagle Media (DMEM) with low glucose -Cat No-11965-092 (Gibco, Invitrogen) Fetal bovine serum (FBS) - Cat No -10270106(Gibco, Invitrogen) Antibiotic – Antimycotic 100X solution (Thermofisher Scientific)-Cat No-15240062. 2,2-diphenyl-1-picrylhydrazyl (DPPH). Dimethyl Sulfoxide (DMSO), Hydrogen Peroxide (H_2O_2) and Propanol from Merck Ltd., Mumbai, India. All the chemicals and reagents used in our work are of analytic grade.

Cell lines and Culture medium

Cell line: HEK 293 (Human embryonic kidney) was procured from National Centre for Cell Sciences (NCCS), Pune, India.

Preparation of crude drug extract [6-8]

Bark of *Pithecellobium dulce* washed, dried, cut into pieces, followed by grinding. The powdered drug was extracted with solvents such as methanol and water. Furthermore, the sample was filtered through filter paper and then evaporated by the rotary evaporator.

Phytochemical screening [9-10]

The extract obtained was subjected to qualitative tests for identification of different phytoconstituents like alkaloids, tannins, glycosides, phenolic, flavonoids, proteins and steroids by using standard and simple qualitative methods.

In Vitro Antioxidant Activity

DPPH radical assay [11-12]

The ability of Compounds to scavenge DPPH radical was assessed using method with modification. Briefly1 ml of herbal extracts (200,400,600,800,1000 µg/ml) was mixed with 3.0 ml DPPH (0.5 mmol/l in methanol), the resultant absorbance was recorded at 517 nm after 30 min. incubation at 37^{O}C. All the analysis was done in

triplicates; ascorbic acid was taken as reference compound. A blank was also taken without adding the sample. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The percentage of scavenging activity was derived using the following formula,

Percentage of inhibition (%) = [(A control – A sample) / A control] x 100

Where $A_{control}$ - absorbance of DPPH

A sample - absorbance reaction mixture (DPPH with Sample)

Scavenging activity of superoxide anion [13]

To the reaction mixture containing 1 ml of alkaline DMSO, 0.3 ml of the drug samples at different concentrations (200, 400, 600, 800, 1000 µg/ ml) and standard ascorbic acid (1000 µg/ ml) was added in DMSO and water at various concentrations followed by 0.1 ml of NBT (0.1 mg) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm. absorbance pouring into a cuvette is measured at 546 nm. The decreasing absorbance indicates a high nitric oxide scavenging activity.

The amount of nitric oxide radical inhibition is calculated following this equation:

$$\% \text{ Inhibition of NO radical} = (A_0 - A_1) / A_0 \text{ X } 100$$

Where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place with Griess reagent

Nitric oxide (NO) scavenging activity [14]

One mL of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 1 ml of extracts in different concentration (200, 400, 600,800, 1000µg/ml). The mixture was incubated at 25^0C for 150 min. After incubation the reaction mixture mixed with 1.0 ml of pre-prepared Griess reagent [(1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture is then incubated at room temperature for 30 min and its

In Vitro Nephroprotective Studies

Epifluorescence staining [15]

Dual-staining procedure was used with epifluorescence microscopy which allows the detection of live cells and dead cells. In this method, normal kidney cells (vero cells) were suspended in 10 ml of phosphate buffered saline (PBS), and 200 µl of the suspension was incubated with gentamicin followed by the addition of 50 µL of the selected plant extracts in the concentration of 500 mg/ml. Another 200 µl of the suspension was incubated with gentamicin followed by the addition of vitamin E, which is used as a positive control. To the above suspensions 50 µl of

ethidiumbromide and 50 µl of acridine orange were added and was incubated for 1 hr. After the incubation the cells were viewed under epifluorescence microscope, in which the live cells emit green color and dead cells emit red color.

Cytoprotective assay [15]

HEK 293 (Human embryonic kidney) cells were collected. Tissue was mashed and washed with culture medium and made into single-cell suspension and Cells were seeded at a concentration (70µl) 10^4cells/well in 100 µl culture medium and 100 µl aqueous and methanolic extracts at different concentration (10,30,60,90,120 µg/ml) into micro plates along with the gentamicin added in control as well as test extracts well respectively (tissue culture grade, and 96 wells). The plates were incubated for further 48 hr at 37°C in a humidified incubator (Thermo scientific BB150) with 5% CO_2. Supernatant was removed from each well, and 10 µL of MTT (0.5 mg/mL) was added. MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g., isopropanol) and the released, solubilized formazan reagent is measuring the absorbance of each sample by a microplate reader (Benesphera E21) at a wavelength of 550 nm.

Statistical analysis

All in vitro assays data signify the mean ± standard deviation of triplicates and IC_{50} was calculated using GraphPad prism.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical Screening: Phytochemical screening results showed the presence of carbohydrates, flavonoids, proteins, phenol, steroid and tannin.

Antioxidant studies

DPPH scavenging activity

DPPH is the parameter of antioxidant activity. Radical scavenging activity of extract was observed from decrease in absorbance of DPPH with increase in concentration. IC_{50} value of ascorbic acid (standard) was 72.65 µg/ml. Methanolic and Aqueous extracts were found to be 85.86 µg/ml and 110.5 µg/ml respectively. These results indicated that extracts contained sufficient phytochemical constituents capable to donate 'H' for the conversion of free radical DPPH to non-free radical DPPH-H were seen in **Figure 1 & Table 1**.

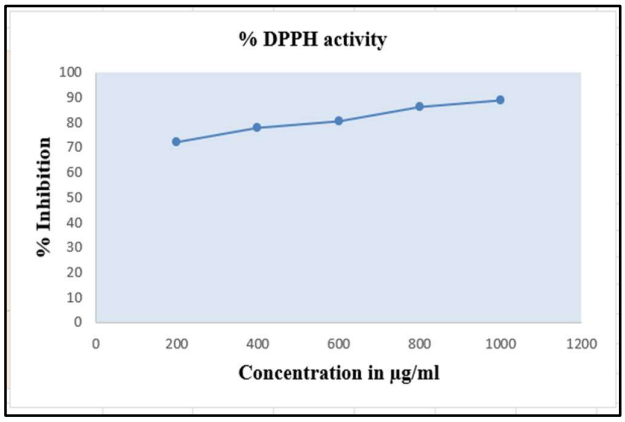

Figure 1: Percentage inhibition of DPPH radical scavenging activity of ascorbic acid

Table 1: DPPH radical scavenging activity of different extracts of *Pithecellobium dulce*

Treatment	Sample concentration (µg/ml) and its % inhibition					IC50(µg/ml)
	200	400	600	900	1000	
Ascorbic acid	72.23 ± 0.12	77.78 ± 0.18	80.54 ± 0.24	86.18 ±0.66	88.88 ±0.38	72.65
Methanol extract	69.40 ±0.76	72.20 ±0.18	78.21 ±0.78	82.55 ±0.33	86.33 ±0.10	85.86
Aqueous extract	64.10 ±0.07	69.63 ±0.22	72.41 ±0.41	75.65 ±0.46	79.56 ±0.33	110.5

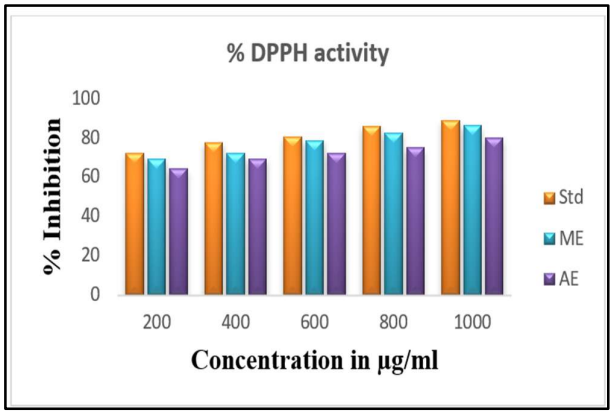

Figure 2: DPPH radical scavenging activity of different extracts of *Pithecellobium dulce*

Figure 2 shows the dose response of DPPH. radical scavenging activity of the extracts of *Pithecellobium dulce* compared with the reference standard ascorbic acid. The ascorbic acid has possessed significant free radical scavenging effect on DPPH. All the plant extracts shown a promising free radical scavenging activity in removing DPPH in a concentration dependent manner. The DPPH radical inhibition of *Pithecellobium dulce* extracts and standard reference ascorbic acid was in the following order: ascorbic acid > methanol > aqueous.

Superoxide ion scavenging activity

Superoxide radical scavenging activity of Methanol and Aqueous extracts were compared with the same dose of ascorbic

acid ranging from 200-1000 µg/ml. IC_{50} value of ascorbic acid, Methanol and Aqueous extracts were found to be 171.9µg/ml, 180.0 µg/ml and 200.3 µg/ml respectively. These results indicated that both extracts exhibited the ability to quench the DPPH radical, which indicated that extracts has good antioxidant potential with free radical scavenging activity. The super oxide radical scavenging activities of *P. dulce are* presented in **Figure 3 & Table 2**.

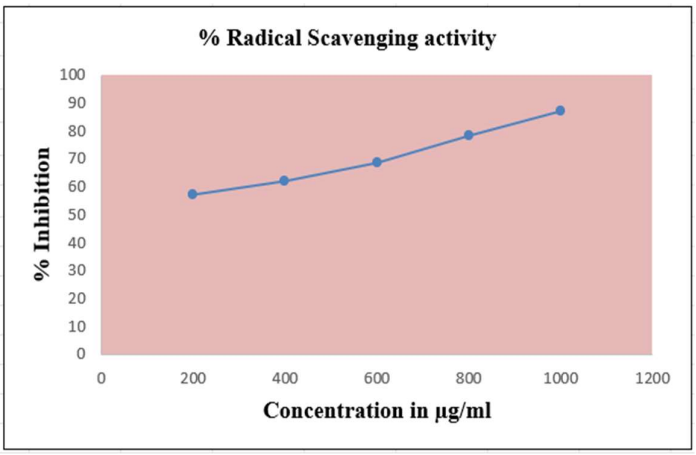

Figure 3: Percentage inhibition of Superoxide radical scavenging activity of ascorbic acid

Table 2: Superoxide radical scavenging activity of different extracts of *Pithecellobium dulce*

Treatment	Sample concentration (µg/ml) and its % inhibition					IC_{50}(µg/ml)
	200	400	600	800	1000	
Ascorbic acid	57.04 ± 0.06	61.84 ± 0.11	68.81 ± 0.08	78.25 ±0.05	87.15 ±0.04	171.9
Methanol extract	54.62 ±0.08	61.75 ±0.04	68.69 ±0.05	75.37 ±0.67	80.37 ±0.52	180.0
Aqueous extract	52.78 ±0.32	59.63 ±0.58	67.74 ±0.46	79.98 ±0.09	76.89 ±0.36	200.3

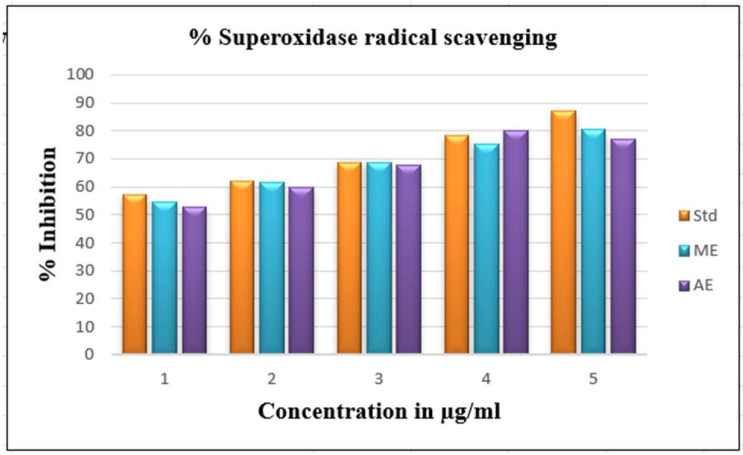

Figure 4: Superoxide radical scavenging activity of different extracts of *Pithecellobium dulce*

Superoxide is the major form of reactive oxygen species derived out of the electron transport chain process associated with the inner mitochondrial membrane. Formed superoxides, being highly reactive, reacts with another superoxide, resulting in the formation of peroxides. There was a decrease of absorbance at 560 nm with the plant extracts, which indicates the consumption of superoxide anion in the reaction mixture thus suggesting a promising scavenging activity. The scavenging activity of this radical by the treatment of Methanol and Aqueous is presented in **Figure 4**.

NO scavenging activity

The percentage inhibition was increased with increasing concentration of the extracts. IC_{50} value of Methanol and Aqueous extracts were found to be 254.1 mg/ml and 297.6 mg/ml respectively and that of ascorbic acid (standard) was 178.2 µg/ml. Standard ascorbic acid at a concentration of 200-1000 mg/ml inhibited production of nitric oxide radical by 57-95% whereas Methanolic and Aqueous extracts were inhibited nitric oxide radical generation by 46-86% and 43-84% thereby exhibited radical scavenging activity. Nitric oxide radical scavenging assay are depicted in **Figure 5 & Table 3**.

In biological systems, NO is generated by the catalytic action of nitric oxide synthase (NOS) on L-arginine; in inflammatory cells, nitric oxide is produced by the inducible isoform of NOS. In living biological systems, a high level of NO and its oxidized derivatives such as peroxynitrite are known to cause toxic. The NO scavenging capacity of some antioxidants present in the extracts were evaluated and is presented in the **Figure 6**. The maximum NO scavenging capacity was found in Methanol extract with an IC_{50} value of 254.1µg/ml which was more than Aqueous whose IC_{50} value was 297.6 µg/ml.

In vitro Nephroprotective activity of the extracts

Epifluorescence staining

Epifluorescence staining is carried out to evaluate the potential of extract on gentamicin treated kidney cells. Epifluorescence staining of healthy kidney cells indicates that the live cell stained with acrydine orange. Ethidium bromide is used for staining as stains the dead cells by entering into the nucleus and makes it appear in the fluorescence microscope, whereas acrydine orange being permeable to the cell membrane, enters and makes it appear green which denotes live cells. Hence live cells and dead cells can be differentiated by epifluorescence staining. Both the extracts showed nontoxic on HEK 293 (Human embryonic kidney). Results obtained are shown in **Figure 7 (a, b, c and d)**.

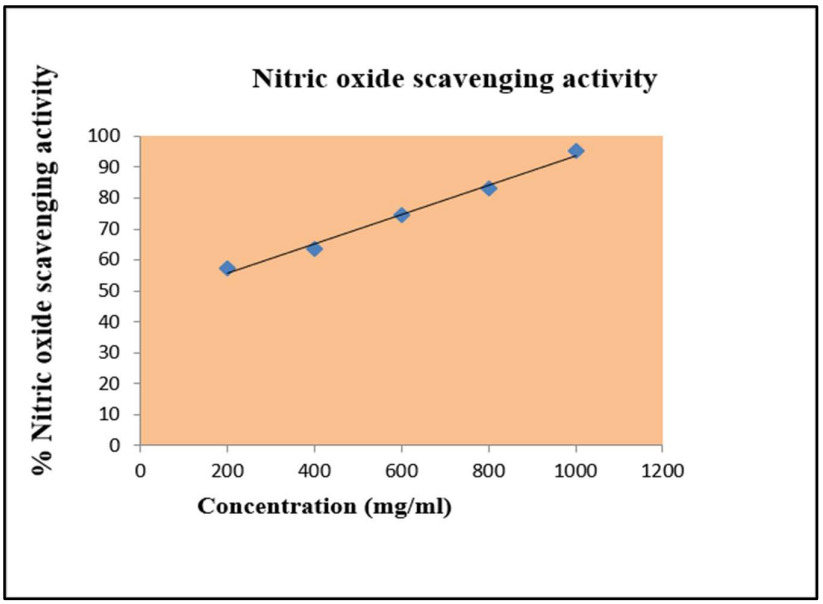

Figure 5: Percentage inhibition of Nitric oxide radical scavenging activity of ascorbic acid

Table 3: Nitric oxide radical scavenging activity of different extracts of *Pithecellobium dulce*

Treatment	Sample concentration (mg/ml) and its % inhibition					IC_{50}(mg/ml)
	200	400	600	900	1000	
Ascorbic acid	57.22 ± 0.54	63.37 ± 0.64	74.55 ± 0.56	83.21 ±0.50	95.12 ±0.31	178.2
Methanol extract	46.21 ±0.97	58.83 ±0.54	67.89 ±0.54	79.10 ±0.48	86.11 ±0.40	254.1
Aqueous extract	43.12 ±0.06	52.89 ±0.95	65.98 ±0.33	74.92 ±0.87	84.23 ±0.28	297.6

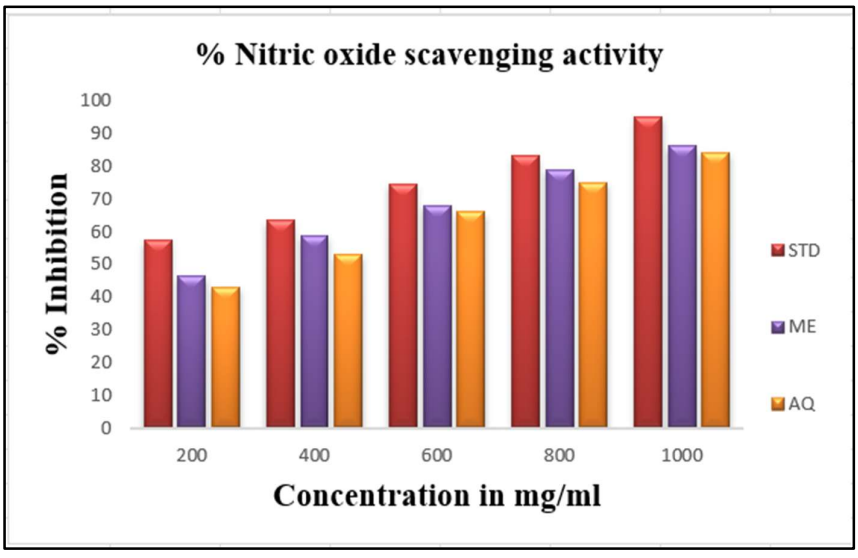

Figure 6: Nitric oxide scavenging activity of different extracts of *Pithecellobium dulce*

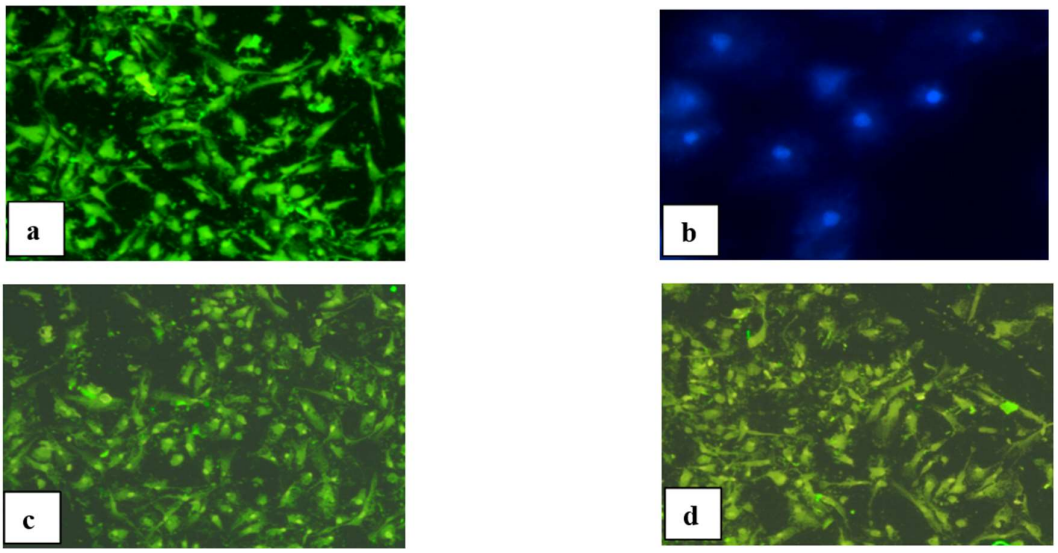

Figure 7: Epifluorescence staining of normal kidney cells treated with acrydine orange (a), kidney cell treated with gentamicin (b), kidney cell treated with Methanol (c) and Aqueous extracts (d)

Cytoprotective assay

MTT cell viability assay

Cytotoxicity activities of various concentrations of methanolic and aqueous extracts of *P. dulce* bark against human embryonic kidney 293 (HEK 293) cell lines were determined and evaluate the potential of extract on gentamicin treated kidney cells. Cells treated with control showed 100% cell viability. Methanol extract at dose of 10, 30, 60, 90, 120 µg/ml showed 79.38%, 84.16%, 86.45%, 87.40% and 89.50%, of cell growth and aqueous extract showed 69.65%, 77.48%, 79.38%, 87.59% & 91.79% respectively. Significant ($P<0.0001$) cell growth inhibition was observed only on cells which were treated with higher concentration of Methanol & Aqueous extracts were compared to control. Graph plotted with percentage of viable cells against the concentration showed a significant dose-dependent inhibition on cell growth are seen in **Table 4, Figure 8 and Figure 9**.

According to table the gentamicin itself indicates toxicity to the cell by inhibition of cell growth (62.01%) while the methanol and aqueous extracts at different concentration the prevent the toxicity effect to cell in place of inducer. According to IC_{50}, both extracts showed more than 100 so its non-toxic to cell as compared to gentamicin (33.32 µg/ml) itself.

Table 4: Effects of compound against HEK 293 (Human embryonic kidney) by MTT assay

Sr. no	Sample	Concentration (µg/ml)	% Viability	% Inhibition	IC50 (µg/ml)
1	Gentamicin only treated cell	10	52.29	47.70	33.32
		30	45.99	54.00	
		60	44.08	55.91	
		90	42.74	57.21	
		120	37.97	62.01	
2	Methanol extracts with gentamicin treated cell	10	79.38	20.62	<100
		30	84.16	15.84	
		60	86.45	13.55	
		90	87.40	12.60	
		120	89.50	10.40	
3	Aqueous extracts with gentamicin treated cell	10	69.65	30.35	<100
		30	77.48	22.52	
		60	79.38	20.62	
		90	87.59	12.41	
		120	91.79	08.21	

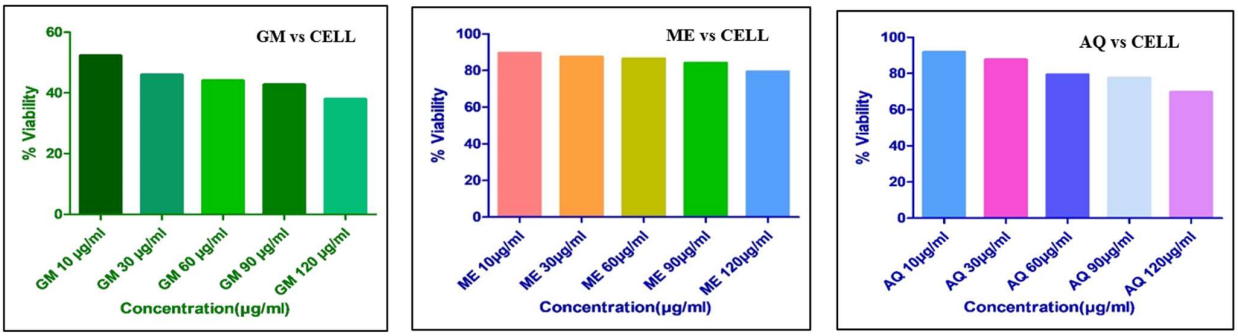

Figure 8: Cytotoxicity of GM (a) (IC_{50} = 33.32 µg/mL), methanol extract of *P. dulce* bark (b) (IC_{50} = <100) and aqueous extract of *P. dulce* bark (c) (IC_{50} = <100) on HEK293 cell lines

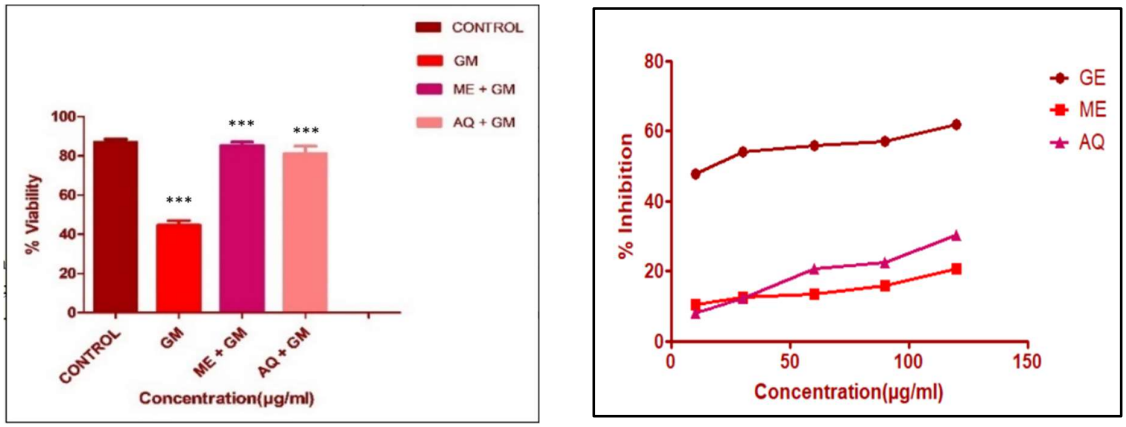

Figure 9: The nephroprotective activity of on the GM-induced HEK293 cell line (a). 1: normal control group; 2: disease control group; 3: methanolic extract group; 4: aqueous extract group. * indicates a significant difference with the disease control group (p < 0.0001) and disease control group is compared with normal control group (b)**

CONCLUSION

Antioxidant and nephroprotective potentials of methanol and aqueous extracts obtained from bark of *Pithecellobium dulce* were evaluated. The bark of *Pithecellobium dulce* was evaluated for their potential to DPPH, scavenge nitric oxide radicals and radical scavenging activity. In vitro nephroprotective effects differentiation between live and dead cells was done by epifluorescence staining.

In general, results indicated that the extracts possess potent bioactivities. In the present study, it is found that the methanol and aqueous extract of bark of *Pithecellobium dulce* contains a substantial amount of phenol, tannins and flavonoids, and due to the presence of these phytoconstituents remarkable antioxidant activity and nephroprotective activity has been observed. The methanolic extract of *Pithecellobium dulce* had higher antioxidant activity compared to aqueous extract. In vitro nephroprotective activity of methanolic and aqueous extract of bark of *Pithecellobium dulce* in gentamicin induced toxicity in Human embryonic kidney cells has been evaluated the pharmacological action of bark extract suitable for nephro protection and is proved to be safe and valuable. The positive results obtained for the crude extract could be due to the presence of phytochemical constituents. The methanol and aqueous extract of *Pithecellobium dulce* bark is not toxic to the human embryonic kidney (HEK)293 cell line.

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