

In vitro nephroprotective activity of selected herbal plants on vero cell line

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Abstract

Nephrotoxicity is common side effect of many drugs like anticancer, aminoglycoside antibiotic and radioactive compounds. In allopathy there is no effective treatment for the cure of kidney diseases. *Aerva javanica*, *Ocimum basilicum* and *Kalanchoe pinnata* are nephroprotective plant to treat various kidney diseases. This experiment was aimed to evaluate nephroprotective activity of above plants on Vero cell line. Optimization of dose of Cisplatin was done using MTT assay against Vero cell line upto 50% of cytotoxicity. Optimized concentration of Cisplatin was further used for the treatment of different extracts of *Aerva javanica*, *Ocimum basilicum* and *Kalanchoe pinnata* against Vero cell line individually. Full factorial design was performed to develop eight different combinations of extracts of selected plants. They were evaluated using MTT cell viability assay. The 50 µg/ml dose of Cisplatin was found to produce upto 50% of cell cytotoxicity. In the treatment model, % inhibition of *Aerva javanica* and *Kalanchoe pinnata* plant extracts at dose level 250 and 500 µg/ml were found more effective than *Ocimum basilicum* when compared with Cisplatin. In the treatment model, % inhibition of combination formula no. 3, 4 and 7 were found significant effective than other combinations with compared to Cisplatin. These experiments showed the plant *Aerva javanica* was most active than other two selected plants by using Cisplatin against Vero cell line. The combination of plants *Aerva javanica* and *Kalanchoe pinnata* were shows better effect at dose level 500 and 100 µg/ml compared to other combinations.

Keywords: *Aerva javanica*, *Ocimum basilicum*, *kalanchoe pinnata*, vero cell line, cisplatin

1. Introduction

The drug used as Pasanabheda means which breaks the kidney stone ^[1]. Roots and flowers of *Aerva javanica* are reported to possess medicinal properties against rheumatism and kidney troubles ^[2]. *Ocimum basilicum* used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunction ^[3]. *Kalanchoe pinnata* leaves are very good for removing kidney stone and multiple small gall bladder stone. ⁴ Cisplatin (*cis* - diamminedichloroplatinum or CDDP) is a potent anticancer drug ^[5]. The clinical use of Cisplatin is often complicated by nephrotoxicity ^[6]. Vero cells are a lineage of cells used in cell cultures. Vero cells are used in research for many purposes like Assessment of the effects of chemicals, toxins and other substances on mammalian cells at the molecular level ^[7]. Nephrotoxicity is importantly modulated as a result of biotransformation. Tubular dysfunction has also been demonstrated very early after Cisplatin administration ^[5, 6]. There is a continuous search for agent that provide nephroprotection against the renal impairment caused by Cisplatin for which allopathy offers no remedial measures. Also Selected nephroprotective plants were already screened on pre-clinical study. The mechanism of action for nephroprotective activity of above selected plants was not found till now. Hence, this study reveals the “*in vitro* nephroprotective activity of selected herbal plants on vero cell line.”

2. Materials and Methods

2.1 Collection of plants

The fresh roots of *Aerva javanica* were collected from Mahua costal region of Bhavanagar, Gujarat, India and fresh

leaves of *Ocimum basilicum* and *Kalanchoe pinnata* were collected from the botanical garden of K. B. Institute of Pharmaceutical Education and research Gandhinagar, Gujarat, India.

Raw material was subjected to washing with distilled water and then allowed for drying for 5 days under shade and powdered to 60# separately and stored in well close container.

2.2 Preparation of extracts

Preparation of hydroalcoholic extract of leaf of *Ocimum basilicum*

30 gm of leaf powder of *Ocimum basilicum* was weighed and 100 ml of hydro-alcoholic solvent (70:30) was added in to the conical flask. It was kept on water bath for reflux up to 1hr. Filter it and repeat this procedure twice with same solvent. Combine the filtrate and Evaporate to concentrate in porcelain dish. The obtained extract was hydroalcoholic extract of leaf of *Ocimum basilicum*.

Preparation of alcoholic extract of root of *Aerva Javanica*

30 gm of root powder of *Aerva Javanica* was weighed and 100 ml of methanol was added in conical flask. It was kept on water bath for reflux up to 1hr. Filter it and repeat this procedure twice with same solvent. Combine the filtrate and Evaporate to concentrate in porcelain dish. The obtained extract was alcoholic extract of root of *Aerva Javanica*.

Preparation of aqueous extract of leaf of *kalanchoe pinnata*

30 gm of leaf powder of *kalanchoe pinnata* was weighed

and 100 ml of distilled water was added in conical flask. It was kept on water bath for reflux up to 1hr. Filter it and repeat this procedure twice with same solvent. Combine the filtrate and evaporate the aqueous portion of leaf extract of *kalanchoe pinnata* by lyophilization process through lyophilizer.

2.3 Cytotoxicity Screening

All the above prepared extracts of different parts of selected plants were further screened for its cytotoxicity against Vero cell line

Introduction to Vero cell line

Details of cell line as mentioned below:

Cell line	Morphology	Origin	Species	supplier
Vero	Epithelial	Kidney	Monkey	NCCS, PUNE

2.3.1 Preparation of cell suspension

Vero cells were procured in 25 cm² T-flask. Increase or decrease the amount of dissociation medium needed proportionally for culture vessels of other size. T-25 flask is recommended for sub culturing these cells. Remove and discard culture medium which should be there in procured flask. Rinse the cell layer with 0.25% (W/V) trypsin- 0.53 mM EDTA solution remove all traces of serum that contains trypsin inhibitor. Add 2.0 to 3.0 mL of trypsin EDTA solution to flask and observe cells under an inverted microscope until cell layer is disappeared (usually within 5 to 15 mins). Trypan blue dye exclusion technique was done to cheque the percentage of cell viability. Add 6.0 to 8.0 ml of complete growth medium and aspirate cell by gently pipetting. Add aliquots of the cell suspension to new culture vessels. Incubate the culture at 37°C. (13)

Culture condition

Temperature: 37°C

Atmosphere: 95 % humidified air, ±5 % Carbon dioxide (CO₂)

Subculturing ratio: 1:3 to 1:6 is recommended

Medium renewal: 2 to 3 time per week

2.3.2 Trypan blue dye exclusion technique

Trypan Blue is a blue acid dye that has two azo chromophore groups. Trypan blue will not enter into the cell wall of plant cells grown in culture. Trypan Blue is an

essential dye, use in estimating the number of viable cells present in a population.

Make a cell suspension in a fixed volume of cells (e.g. 1ml). Although an aseptic technique is not essential in all stages of this procedure, it is good laboratory practice to maintain sterility throughout the procedure¹⁷. Take 50uL of cell suspension and mix it with an equal volume of trypan blue. Mix solution well using a pipette. Transfer to a hemocytometer and count the live cell as clear form and dead cell as blue cells. After staining with trypan blue solution counting should commence in less than 5minutes as after that time the cells will begin to take up the dye.

Using a pipette place some of the cell suspension: trypan blue mixture into the hemocytometer and overlay with a cover slip. The cell suspension will pass under the cover slip by capillary action unless there is an air bubble. Make sure the wells are not overfilled and that the cover slip is not moved once it is placed on the grid and the cell solution is added. Place the hemocytometer on the stage of an inverted microscope. Adjust focus and power until a single counting square fills the field. Calculate the number of cells per ml, and the total number of cells, using the following formula (18).

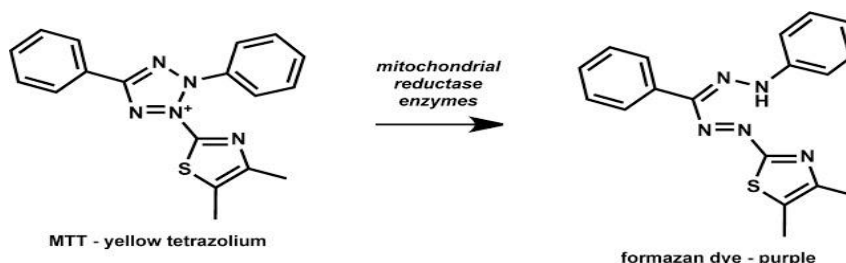
Calculate percent viability by using formula:

$$\% \text{ viability} = (\text{live cell count} / \text{total cell count}) * 100$$

2.3.3 Microculture tetrazolium (MTT) assay

Principle

The MTT assay is a colourimetric assay for assessing cell metabolic activity. NAD(p)H-dependent cellular oxidoreductase enzyme are capable of reducing the tertazolium dye MTT 3-(4,5dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble frozen, which has purple colour. Tetrazolium dye reduction is dependent on NAD(p)H-dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell. Therefore, reduction of MTT and other tetrazolium dyes depends on cellular metabolic activity due to NAD(p)H flux. Cells with a low metabolism such as thymocytes and splenocytes reduce very little MTT. In contrast, rapidly dividing cells exhibit high rates of MTT reduction. Viable cells with active metabolism convert MTT into a purple coloured formazan product with an absorbance maximum near 570 nm. When cells dye, they lose the ability to convert MTT into formazan, thus colour formation serves as useful and convenient marker of only the viable cells (15).



Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 3 lakh cells/ml using medium containing 10% foetal bovine serum. Cells were seeded in a flat bottomed 96 well plate and incubated for 24 hour at 37°C and in 5% CO₂. Vero cell line was treated with different plant extracts at various concentrations (1000µg/ml, 500µg/ml and 100µg/ml) for 48 hours. Cells

were then treated with MTT reagent (0.5 mg/ml as final concentration, i.e. 20µl/well of stock) for 4 h at 37°C. All the media and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; thiazolyl blue) reagent was removed from the wells and DMSO (200 µl) was added to each well to dissolve the formazan crystals.

The optical density (OD) was recorded at 570 nm in a Microplate (ELISA) reader.²¹

Percentage of cell viability was determined as (Avg. OD of treated cells/Avg. OD of control cells) × 100.

2.3.4 *In Vitro* assessment of Nephroprotective activity of different extracts of selected plants against damage induced by Cisplatin on Vero cell line through MTT assay

Optimization of Cisplatin concentration for nephrotoxicity against Vero cell line

Different concentrations of Cisplatin (100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/ml) were used to optimize the percentage (%) cytotoxicity against Vero cell line. Percentage (%) cytotoxicity above 50 % was considered for the optimized nephrotoxicity on Vero cells. The concentration of Cisplatin 50 µg/ml was showed 67.85 ± 1.7 cytotoxicity on Vero cells as mentioned in table. These results were also correlate with histological parameters. The results indicate that 50 µg/ml Cisplatin concentration taken as optimized concentration for the further study. (14)

Procedure for drug treatment

Cells were plated in the flat bottomed 96 well plate. Incubate cells at 37°C in 5% ± CO₂ for one day. Cisplatin 50µL concentrations dissolves in media and different concentrations of test drug dissolves in DMSO solvent and incubate at 37°C for 1 day. For concentration of test drug is (1000, 500, 250, 100µg/ml)

- Sample A: *Aerva javanica*
- Sample B: *Ocimum basilicum*
- Sample C: *Kalanecho pinnata*

For the combination of drug applied biostatistical formula full

factorial design L^V

L= 2 (+1, -1), V=3(A, B, C), 2³ = 8

So, 8 Combination of drug are taken.

A µg/ml	B µg/ml	C µg/ml
500	500	500
500	500	100
500	100	500
500	100	100
100	500	500
100	500	100
100	100	500
100	100	100

Full factorial design

A	B	C
+1	+1	+1
+1	+1	-1
+1	-1	+1
+1	-1	-1
-1	+1	+1
-1	+1	-1
-1	-1	+1
1	-1	-1

3. Results

Preparation of different extracts of selected Nephroprotective plants

Three different extracts of selected plants were prepared using organic solvents. The percentage yields of different extracts of selected plants were shown in Table 1.

Table 1: % yield of different extracts of selected plants

Drugs	alcoholic extract of <i>Aerva javanica</i>	Hydro-alcoholic extract of <i>Ocimum basilicum</i>	Aqueous extract of <i>Kalanchoe pinnata</i>
% yield	6.25% w/w	7.4 % w/w	16.2 % w/w
Color	Brown	Brownish black	Yellowish brown
Consistency	Semi-solid	Semi-solid	Solid

Optimization of Cisplatin concentration for nephrotoxicity against Vero cell line

Percentage (%) cytotoxicity above 50 % was considered for the optimized nephrotoxicity on Vero cells. The concentration of Cisplatin 25 µg/ml was showed 59 %

cytotoxicity on Vero cells as mentioned in Table 2. We report the cytotoxicity on the Vero cell line. These results indicate that 50 µg/ml Cisplatin concentration taken as optimized concentration for the further study.

Table 2: Optimization of Cisplatin concentration for nephrotoxicity against Vero cell line

Sr. no	Concentration	% Cytotoxicity
1	100 (µg/ml)	77.07 ± 1.21
2	50 (µg/ml)	67.85 ± 1.7
3	25 (µg/ml)	45.42 ± 4.5
4	12.5 (µg/ml)	37.38 ± 3.99
5	6.25 (µg/ml)	33.8 ± 2.14
6	3.12 (µg/ml)	29.23 ± 4.19
7	1.56 (µg/ml)	18.9 ± 4.79

In Vitro assessment of Nephroprotective activity of different extracts of selected plants against damage induced by Cisplatin on vero cell line through MTT assay

All the three different extracts of selected plants were

treated against vero cell line with the 50 µg cisplatin. Percentage inhibition of Vero cells were shown in Table 3. All the three different extracts of plants show very less inhibition at the concentration of 1000 µg/ml.

Table 3: Evaluation of drugs (*Aerva javanica*, *Ocimum basilicum* and *Kalanchoe pinnata*) treatment on vero cell line

Concentration in $\mu\text{g/ml}$	% inhibition		
	alcoholic extract of <i>Aerva javanica</i>	Hydro-alcoholic extract of <i>Ocimum basilicum</i>	Aqueous extract of <i>Kalanchoe pinnata</i>
1000	21.85 \pm 0.043	21.49 \pm 0.181	25.51 \pm 0.171
500	47.89 \pm 0.061	52.39 \pm 0.080	49.25 \pm 0.022
250	50.59 \pm 0.108	55.72 \pm 0.036	55.61 \pm 0.046
100	60.79 \pm 0.022	61.22 \pm 0.023	61.49 \pm 0.018

Alcoholic extract of *Aerva javanica* shows maximum nephroprotective activity then the Hydro-alcoholic extract of *Ocimum basilicum* and aqueous extract of *Kalanchoe pinnata*. Hydro-alcoholic extract of *Ocimum basilicum* and aqueous extract of *Kalanchoe pinnata* also shows similar Nephroprotective activity on vero cells. Higher dose may induce toxicity on vero cell. Because of this reason we select 500 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ concentration of different extract of selected plants.

In Vitro assessment of Nephroprotective activity of different combinations of different extracts of selected plants against damage induced by Cisplatin on vero cell line through MTT assay

Table 4: evaluation of combination of different extracts of selected plants against damage induced by Cisplatin on vero cell line through MTT assay

Combinations of drugs	% Inhibition
1	55.69 \pm 0.016
2	51.79 \pm 0.034
3	41.96 \pm 0.177
4	45.86 \pm 0.032
5	58.11 \pm 0.160
6	50.25 \pm 0.303
7	47.49 \pm 0.062
8	52.93 \pm 0.070

We report the combinations study of alcoholic extract of *Aerva javanica*, Hydro alcoholic extract of *Ocimum basilicum* and aqueous extract of *Kalanchoe pinnata* shows batter activity then the individual drugs. Combination no. 3, 4 and 7 shows maximum nephroprotective activity as compared to other combinations of different extracts of selected plants. Combination no.3 has 500 $\mu\text{g/ml}$ of alcoholic extract of *Aerva javanica* and aqueous extract of *Kalanchoe pinnata* and 100 $\mu\text{g/ml}$ of alcoholic extract of *Ocimum basilicum*. Combination no.4 has 500 $\mu\text{g/ml}$ of alcoholic extract of *Aerva javanica* and 100 $\mu\text{g/ml}$ of aqueous extract of *Kalanchoe pinnata* and Hydro alcoholic extract of *Ocimum basilicum*. Combination no.7 has 500 $\mu\text{g/ml}$ of aqueous extract of *Kalanchoe pinnata* and 100 $\mu\text{g/ml}$ of alcoholic extract of *Aerva javanica* and alcoholic extract of *Ocimum basilicum*.

4. Discussion

Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to a drug or toxin.1 A number of therapeutic agents can adversely affect the kidney resulting in acute renal failure, chronic interstitial nephritis and nephritic syndrome because there is an increasing number of potent therapeutic drugs like amino glycoside antibiotics, chemotherapeutic agents and NSAIDS.2 Exposure to chemical reagents like ethylene glycol, carbon tetrachloride, sodium oxalate and heavy

metals such as lead, mercury, cadmium and arsenic also induces nephrotoxicity. Prompt recognition of the disease and cessation of responsible drugs are usually the only necessary therapy.3 Nephroprotective agents are the substances, which possess protective activity against Nephrotoxicity. Medicinal plants have curative properties due to the presence of various complex chemical substances. Early literatures have prescribed various herbs for the cure of renal disorders. Co-administration of various medicinal plants possessing Nephroprotective activity along with different nephrotoxic agents, which may attenuate its toxicity. The term renal failure primarily denotes failure of the excretory function of kidney, leading to retention of nitrogenous waste products of metabolism in the blood.

The plant *Aerva javanica* belonging to the family Amaranthaceae is used as Pasanabheda means which breaks the kidney stone. Roots are reported to possess medicinal properties against rheumatism and kidney troubles. The nephroprotective activity of alcoholic extract and aqueous extract of root of *Aerva javanica* were separately reported by Suttee A. *et al.* [19] and Movaliya V. *et al.* [20] respectively. In addition, Isolation of ursolic acid from *Aerva javanica* plant was reported by Khan *et al.* [21]. Also, comparison of the alcoholic and aqueous extract of root of *Aerva javanica* for its nephroprotective activity with reference standard ursolic acid were reported [22]. The plant *Aerva javanica* was authenticated by macroscopic and microscopic studies as per reported work [23].

Cisplatin induced renal toxicity study

Relevant perhaps to the nephrotoxicity of Cisplatin are the observations that the kidney accumulates and retains platinum largely than other organs. The changes in renal function correlate well with the nephrotoxic effects of Cisplatin [24]. Change in creatinine clearance and serum creatinine levels taken as indications of an abnormal glomerular function [25]. Cisplatin caused a marked reduction in the glomerular filtration rate, indicating induction of acute renal failure [26]. Various mechanism proposed for the Cisplatin cytotoxicity, which include direct DNA damage [27], activation of caspase [28] and mitochondrial dysfunction [29]. The formation of reactive oxygen species (ROS) [30] effects on the endoplasmic reticulum [31] and activation of TNF- α -mediated apoptotic pathways are considered important for Cisplatin cytotoxicity. Cisplatin-induced nephrotoxicity is closely associated with an increase in lipid peroxidation in the kidney. There is a large body of evidence on the chemo protective activities of vitamin C, curcumin, selenium, bixin and other dietary components that scavenge free radicals induced by exposure to Cisplatin [32]. Cisplatin-induced nephrotoxicity is considered sensitive and significant [33] that showed increase in serum creatinine and urea level and reduced the protein and albumin level on 6th day of the study. The disease-control group showed definite sign of

nephrotoxicity, as evidenced by significant decrease in % change in body weight. The reduction in bodyweight may possibly due to the injured renal tubules and the subsequent loss of tubular cells to reabsorb water, leading to dehydration and loss of body weight^[34]. The alleviation of Cisplatin induced body weight reduction is a reflection of the general palliative effect of hexane fraction of alcoholic extract of root of *Aerva javanica* on the nephrotoxicity. The hexane fraction of alcoholic extract of root of *Aerva javanica* showed almost similar result as compared to ursolic acid as reference standard. Elevation of serum creatinine and serum urea considered as the most important manifestation of severe tubular necrosis of kidney^[35]. Cisplatin-induced nephrotoxicity showed decrease serum protein and serum albumin level. Based on results, hexane fraction of alcoholic extract of root of *A. javanica* showed significant recovery in serum protein level as compared to other fractions and disease-control group. Available evidence suggests that Cisplatin exerts its nephrotoxic effects by the generation of reactive free radicals^[36, 37]. Reasonable cellular-protective agents against Cisplatin toxicity may have at least some antioxidant properties to prevent GSH depletion and/or scavenge the intracellular ROS. Hence, antioxidants and free radical scavengers of natural and synthetic origin might provide nephroprotection in Cisplatin-induced renal injury^[38]. Treatment with hexane fraction of alcoholic extract of *Aerva javanica* root decrease the TBARS and increase the antioxidant enzymes levels of GSH and tissue protein as compared to the Cisplatin treated group, which indicate its nephroprotective activity. Hence, the significant effect is mainly due to the ability of the hexane fraction of alcoholic extract of root of *Aerva javanica* to restore renal antioxidant defence system as compared to other fractions. To conclude, the hexane fraction of alcoholic extract of root of *A. javanica* possesses marked nephroprotective activity as compared to other fractions and thus can have a promising role in the treatment of acute renal injury induced by Cisplatin. Further isolation of active components and its nephroprotective activity in chronic renal failure model need to be evaluate.

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