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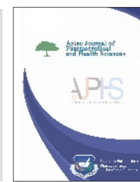

In vitro cytoprotective effect of *Ipomoea sepiaria* Roxb. extract on Gentamicin induced toxicity in HEK 293 cells

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ABSTRACT

Ipomoea sepiaria Roxb. also known as Purple heart glory is a slender vine belonging to family Convolvulaceae. The plant is found on sea coasts and on saline soil. In Sanskrit it is known as lakshmana, in Hindi as Bankalmi and in Malayalam as Tirutali. It is one among the ten sacred plants known collectively as "Dasapushpam" in Kerala. The present study is intended to check the effectiveness of the ethyl acetate extract of *Ipomoea sepiaria* for cytoprotective effect in Gentamicin induced toxicity in Human Embryonic Kidney Cells. The cytoprotective effect of the ethyl acetate extracts was studied after pretreatment of the HEK (Human Embryonic Kidney) 293 cells with gentamicin, anephrotoxic aminoglycoside antibiotic. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and MTT assay method. The cell lines incubated with 50 µg /mL and 100 µg /mL of ethyl acetate extract demonstrated 91% and 96.23% cell viability respectively indicating high degree of cytoprotection to gentamicin induced toxicity and apoptosis. The percentages of cell viability in different groups substantiate the results of cytotoxic evaluation by direct microscopic observation.

INTRODUCTION

Ipomoea sepiaria Roxb. also known as Purple heart glory is a slender vine belonging to family Convolvulaceae. The plant is found on sea coasts and on saline soil. In Sanskrit it is known as lakshmana, in Hindi as Bankalmi and in Malayalam as Tirutali. It is one among the ten sacred plants known collectively as "Dasapushpam" in Kerala. These plants are used for rejuvenating the body in the form of karkidakakanji in the monsoon season in kerala which is also prescribed in text books of ayurveda. This plant preparation is useful in preventing the aggravation of vata dosha which usually occurs in the monsoon. In traditional practice the juice of the leaf is instilled in the right nostril during second and third months of ante-natal period to the pregnant women for begetting male progeny known as "Pumsavana karma" mentioned in Ayurveda classics. The root powder in the dose of 1 teaspoon is administered with rice water for leucorrhoea in Nalgonda, Mehaboobnagar District of Andhra Pradesh. Juice of the plant is used as deobstruent, diuretic, hypotensive, uterine tonic, antidote to arsenic poisoning. Seeds are used as cardiac depressant, hypotensive, spasmolytic. Plant is also used in the treatment of sterility in women, urinary retention, constipation and gynaecological disorders. Ipomoea resin in the seeds contain non-ergoline type indole alkaloids as ipobscurine A & B, and alkaloids called ipobscurines C[1-3] The present study is intended to check the effectiveness of the *Ipomoea sepiaria* plant extract for cytoprotective effect in Gentamicin induced toxicity in Human Embryonic Kidney Cells.

MATERIALS AND METHODS

Plant material

The plant *Ipomoea sepiaria* Roxb. was collected during monsoon season from Thodupuzha part of Idukki District, Kerala. The botanical identity was confirmed by Dr. Tessy Joseph, Head of the Department, Botany, Nirmala College of Arts and Sciences, Muvattupuzha, Kerala. The plant was identified by using "Biodiversity documentation for Kerala Part VI : FLOWERING PLANTS (KFRI Handbook No: 17)". A voucher specimen (No. NCH/ 2012/ NCP /537) was deposited in the department for future reference. The plant material was dried in shade and was coarsely powdered in a grinding machine. It was then stored in polythene bags at room temperature.

Preparation of the extracts of *Ipomoea sepiaria*

Coarsely powdered whole plant material (1kg) of *Ipomoea sepiaria* was extracted with ethyl acetate using soxhlet apparatus. The extracts were concentrated in vacuum and

evaporated to dryness and calculated the yield. Dried extracts were kept in refrigerator.

***In vitro* cytoprotective effect of ethyl acetate extracts on gentamicin induced toxicity in HEK 293 cells.**

The cytoprotective effect of the ethyl acetate extracts was studied after pretreatment of the HEK (Human Embryonic Kidney) 293 cells with gentamicin, a nephrotoxic aminoglycoside antibiotic.

Cell lines: HEK 293 (Human Embryonic Kidney) cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecos Modified Eagles Medium (DMEM).The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% Foetal Bovine Serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution containing Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37º C in a humidified 5% CO_2 incubator.

Preparation of stock solution of ethyl acetate extract: 1mg of the ethyl acetate extract was completely dissolved in 1mL DMEM using a cyclomixer. The extract was filtered through 0.22 µm millipore syringe filter to ensure the sterility. 6.25, 12.5, 25, 50 and 100 µg/mL solutions were prepared by dilution of the stock solution with freshly prepared DMEM.

Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and were suspended in 10% growth medium. 100 µL cell suspension ($5x10^4$ cells/well) was seeded in tissue culture plate having 96 wells and incubated at 37ºC in a humidified 5% CO_2 incubator. After attaining sufficient growth 50 mM gentamicin was added to induce toxicity to each well and incubated for 1 h. 100 µL of different concentrations viz, 6.25, 12.5, 25, 50 and 100 µg/mL of ethyl acetate extracts were added in triplicates to the respective wells and incubated at 37ºC for 24 h in a humidified 5% CO_2 incubator.Positive control incubated with gentamicin alone was also maintained. A control was performed with untreated HEK 293 cells. The viability of cells were evaluated by

1. Direct observation of cells by Inverted phase contrast microscope .

2. MTT assay method.

Cytotoxicity evaluation by direct microscopic observation

Entire plate was observed after 24 h of incubation in an inverted phase contrast tissue culture microscope and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity assay by MTT Method

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS (Phosphate buffered saline pH 7.4) until completely dissolved and sterilized by filter sterilization. After 24 h of incubation period the sample content in wells were removed and 30 µL of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 h. After the incubation period, the supernatant was removed and 100µL of MTT solubilization solution (Dimethyl Sulfoxide) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm [4].

The percentage of cell viability was calculated using the formula below.

$$\% \text{ of viability} = \frac{\text{Mean Absorbance of sample X 100}}{\text{Mean Absorbance of control}}$$

The experiment was done in triplicate for each concentration of ethyl acetate extract and the absorbance readings were expressed as Mean Values ± SD.

RESULTS AND DISCUSSION

Extraction

The extract was found to be have light greenish colour with a semisolid paste like consistency**.** The percentage yield of the ethyl acetate extract was found to be 1.5 % w/w.

***In vitro* cytoprotective effect of ethyl acetate extracts on gentamicininduced toxicity in HEK 293 cells.**

Cytotoxicity evaluation by direct microscopic observation:

The results are given in figure 1-7.

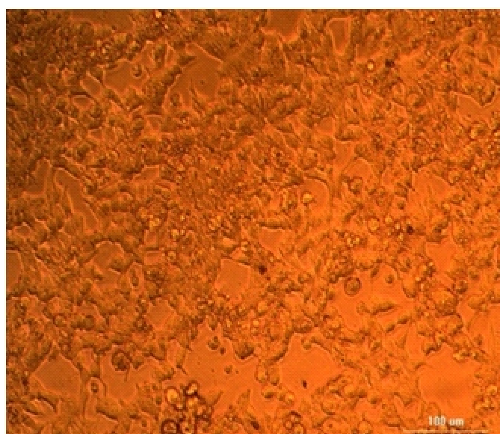

Fig 1 : Control

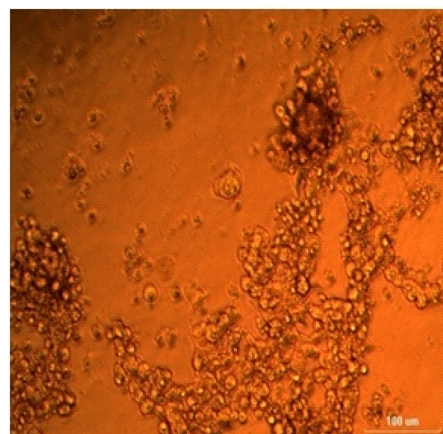

Fig 2 : Positive Control

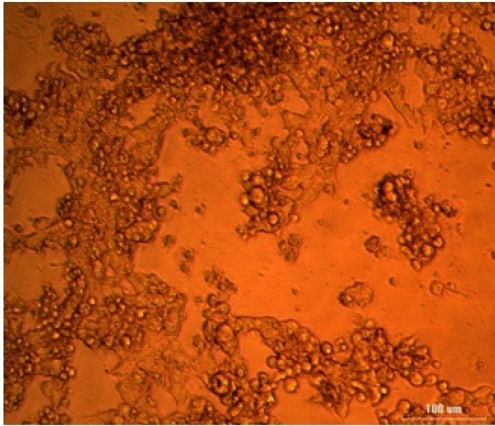

Fig 3 : 6.25µg/mL ofethyl acetate extract

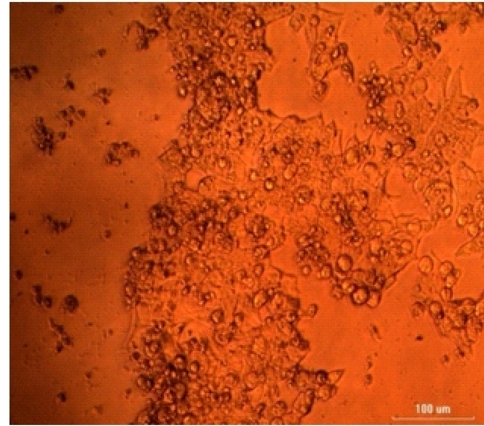

Fig 4 : 12.5 µg /mL of of ethyl acetate

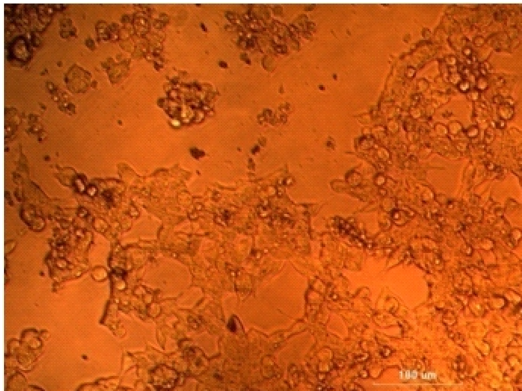

Fig 5 : 25µg/mL ofethyl acetate extract

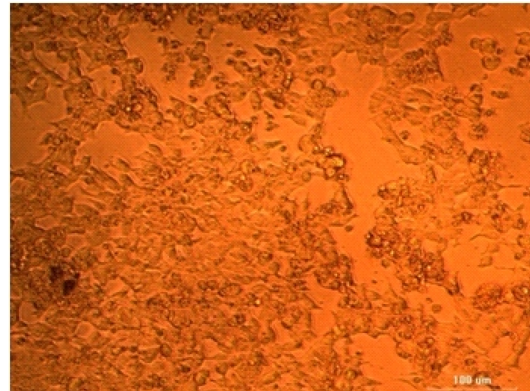

Fig 6 : 50µg/mL of ethyl acetate extract

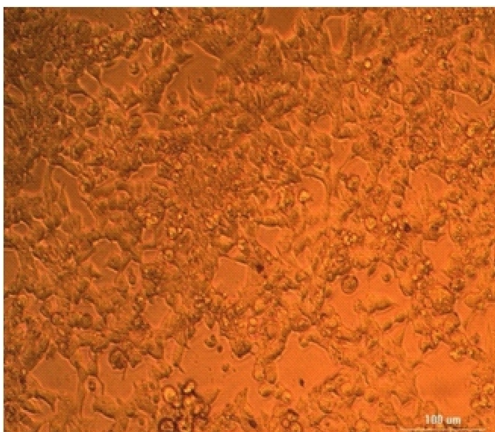

Fig 7 : 100 µg/mL of ethyl acetate extract

The HEK 293 cell lines incubated with gentamicin only (Figure 2) as well as those incubated with 6.25 µg/mL (Figure 3) and 12.5 µg/mL (Figure 4) and 25µg/mL (Figure 5) of ethyl acetate extract showed condensed nuclei, granulation and vacuolization in the cytoplasm, cell shrinkage**,** membrane blebbing**,** apoptotic bodies**,** bubbling and echinoid spikes. The cell lines incubated with with 50 µg/mL(Figure 6) and 100 µg/mL (Figure 7) of ethyl acetate extract offered high degree of cytoprotection to gentamicin toxicity as indicated by the normal morphology of the cells with absence of granulation and vacuolization in the cytoplasm.

Cytotoxicity evaluation by MTT Assay

The results of cytotoxicity evaluation by MTT assay are given in table 1 and figure 8,9.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening. The MTT substrate prepared in a physiologically balanced solution was added to cells in culture usually at a final concentration of 0.2 - 0.5 mg/mL and incubated for 1 to 4 h. The quantity of formazan (presumably directly proportional to the number of viable cells) was measured by recording changes in absorbance at 540 nm using a plate reading spectrophotometer. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum at 540 nm. When cells die, they lose the ability to convert MTT into formazan, and thus colour formation serves as a useful and convenient marker of viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT.

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Table 1 : Cytoprotective effects of ethyl acetate extracts on gentamicin induced toxicity in HEK 293 cells.

Sl. No.		Concentration (µg/mL)	Mean Absorbance	Percentage viability
1.	Control	-	0.6686±0.0037	100.00
2.	Gentamicin	-	0.3189±0.0356	47.70
3.	Ethyl acetate extract	6.25	0.4384±0.0052	65.57
		12.5	0.4479±0.0034	66.99
		25	0.4878±0.1458	72.96
		50	0.6084±0.0417	91.00
		100	0.4878±0.0095	96.23

Absorbance values are expressed as Mean ± SD, n=3

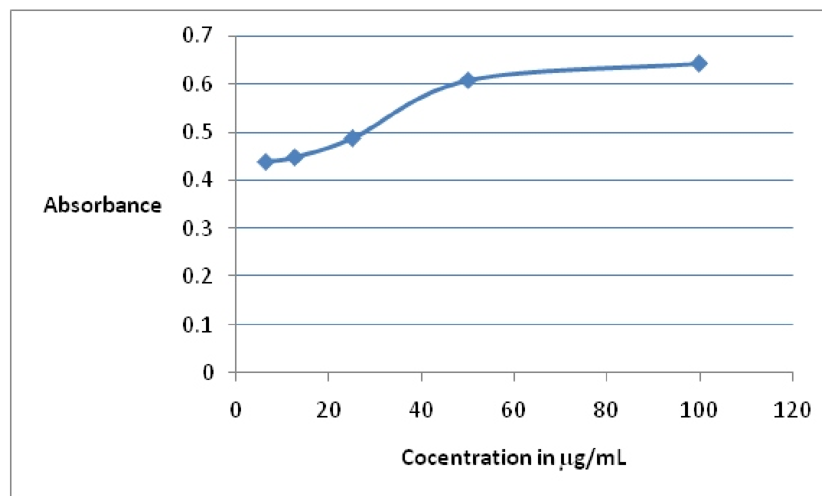

Figure 8 : Effect of concentration of ethyl acetate extracts on absorbance in MTT assay using HEK 293 cells.

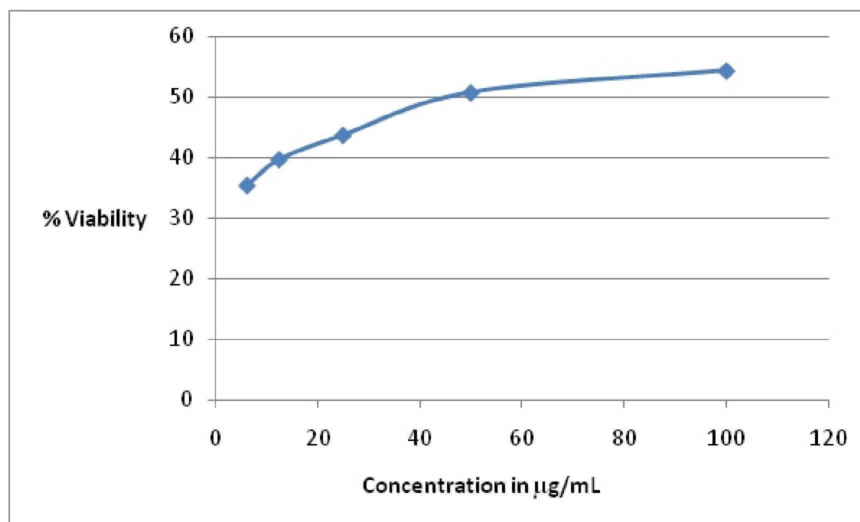

Figure 9: Cytoprotective effect of ethyl acetate extracts on gentamicin induced toxicity in HEK 293 cells.

reduction into formazan is not well understood but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT[5].

The formazan accumulates as an insoluble precipitate inside the cells as well as being deposited near the cell surface and in the culture medium. The formazan must be solubilized prior to recording absorbance readings. A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components. Various solubilization methods include using acidified isopropanol, DMSO, dimethylformamide, SDS and combinations of detergent and organic solvent [6-8] The amount of signal generated is dependent on several parameters including the concentration of MTT, the length of the incubation period, the number of viable cells and their metabolic activity [9].

Gentamicin, a nephrotoxic aminoglycoside causes cellular apoptosis during *invitro* cell differentiation. Reduced cell viability of human embryonic cells were observed at 25-200 µg/mL concentration of gentamicin. The cell viability decreased steadily with an increase in concentration from 25-200 µg/mL for both hepatic and neural differentiation of human embryonic(H9) cells in previous studies [10].

In the present study, a concentration dependent increase in absorbance was found in the range of concentration of ethyl acetate extracts from 6.25 µg/mL to 100 µg /mL. The increase in absorbance demonstrates a positive correlation of formazan formation with increase in concentration of ethyl acetate extracts as shown in Figure 8. The quantity of formazan was presumably directly proportional to the number of viable cells. In cell cultures of HEK 293 cells incubated with gentamicin (positive control), the cell viability was only 47.70 % which indicates marked toxicity. The cell lines incubated with 50 µg /mL and 100 µg /mL of ethyl acetate extract demonstrated 91% and 96.23% cell viability respectively indicating high degree of cytoprotection to gentamicin induced toxicity and apoptosis. The percentages of cell viability in different groups substantiate the results of cytotoxic evaluation by direct microscopic observation.

CONCLUSION

The cell viability of human embryonickidney cells was found to decrease with increasing concentration of Gentamicin as it affects the hepatic and neural differentiation of cells*in vitro* as indicated in previous studies. In the present *in vitro*study,the ethyl acetate extract of *Ipomoea sepiaria* Roxb. showed high degree of cytoprotection to gentamicin induced toxicity and apoptosis in Human embryonic kidney (HEK 293) cells. So further studies can be proposed in the direction of understanding the mechanism underlying the prevention of gentamicin induced toxicity and apoptosis in the human embryonic kidney cells by the compounds isolated from the extract.

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