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RESEARCH ARTICLE

THE NEUROPROTECTIVE EFFECT OF *MONOCHORIA VAGINALIS* PRESL. IN SHSY5Y CELL LINES

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ABSTRACT

Background: The ability to produce in-vitro cultures of neuronal cells has been the base for advancing the knowledge of the nervous system functioning. There is a rise in memory loss and associated disorders in the present era. The present day research focuses on developing cost effective medications for such conditions. There are many such medications found in classical Ayurvedic textbooks which if revalidated scientifically may get global acceptance and benefit the ailing society. *Ashtangahrdaya*, describes '*Chathushkuvalaya Rasayana*' and claims it to be a potent memory enhancer. In it *Kuvalaya*, botanically identified as *Monochoria vaginalis* Presl. is the only herbal ingredient. There is scarce published work regarding the neuroprotective potential of the plant. **Objective:** To evaluate the Neuroprotective effect of Ethanolic extract of *Monochoria vaginalis* Presl. in SHSY5Y cell lines followed by flow cytometry. **Method:** The ethanolic extract of the plant (rhizome, leaf, stem, flower) (KEE) was used as the study material in 5 concentrations (6.25µg/ml, 12.5µg/ml, 25µg/ml, 50µg/ml and 100µg/ml). Hydrogen peroxide was used to induce toxicity. **Result:** The concentrations 25µg/ml and 50µg/ml was found to have statistically significant neuroprotection ($p<0.01$). Flow cytometry was done with concentration providing maximum protection (50µg/ml). Treatment with KEE (ethanolic extract of *Kuvalaya*) produced decrease of apoptotic cells to below 50%, increasing the cell viability to 46% which confirmed the fact that KEE provided neuroprotection. **Conclusion:** The present study focuses on the importance of exploring such locally available plants and this study may serve as a base for further higher studies on the neuroprotective potential of the *Monochoria vaginalis* Presl.

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INTRODUCTION

The ability to produce in vitro cultures of neuronal cells has been fundamental to advancing our understanding of the functioning of the nervous system. The culture of neuronal cells is particularly challenging since mature neurons do not undergo cell division. The present day medical research is highly focusses on the development of Neuroprotective agents so as to develop cost effective solutions for the betterment of memory loss and other associated conditions. Classical Ayurvedic texts describe a set of measures to impart biological sustenance to body tissues known as '*Rasayana*' and those specific to brain tissue are called as '*Medhya Rasayana*'. They help in slowing down brain ageing and help in regeneration of neural tissues. Thus they can be effectively utilized for delaying the loss of memory and can also be used for the preventive aspect of the same (Singh, 2008).

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Ashtangahrdaya, a classical Ayurvedic text presents a preparation namely *Chathushkuvalaya Rasayana* (Vaghbhata, 1995) and emphasizes its applicability as a potent memory enhancer. In it, *Kuvalaya* is the only herbal ingredient and there is scarce published literature regarding the Neuroprotective effects of the plant. In Indian Medicinal Plants, (Anonymous, 2007) the plant with local name '*Karimkoovalam*' with botanical identity *Monochoria vaginalis* Presl. of Pontederiaceae has been identified as *Kuvalaya*. *Hortus Malbaricus* also accepts the botanical identity of '*Karimkuvalam*' as *Monochoria vaginalis* Presl.[(5)]. The present study aims to assess the in vitro Neuroprotective effect of the ethanolic extract of *Monochoria vaginalis* using SHSY5Y Cell lines followed by flow cytometry.

Study-setting: An approved analytical laboratory which conduct in-vitro studies.

METHODOLOGY

Study Material: Fresh stem, rhizome, leaf and flowers of the plant was collected from Oachira, Kerala, was shade dried and

powdered into fine powder. The dried powder of the sample was subjected to refluxing with ethanol and fractional distillation in Leibig's condensor to obtain the Ethanolic extract.

Procedure: SHSY-5Y (Neuroblastoma cells) cell line was purchased from National Centre For Cell Sciences Pune and was maintained in Dulbecco's modified eagles media (HIMEDIA) from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium(DMEM) (Sigma Aldrich, USA). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method. Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. 1mg of the sample was weighed and completely dissolved in 1mL DMEM using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. H_2O_2 (150µM) was added to induce toxicity.

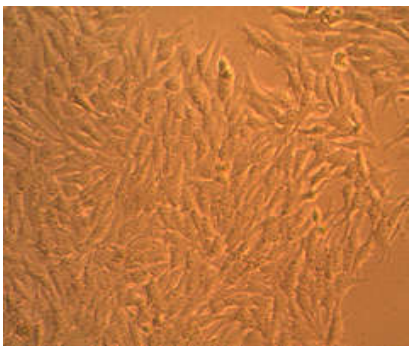
Neuroprotective Evaluation: After attaining sufficient growth, H_2O_2 (150µM) was added to induce toxicity and incubated for one hour, prepared extracts in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator.Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation 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.

Neuroprotective activity by MTT Method: Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml Phosphate Buffered Saline until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 3.0µl 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 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution DMSO 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. The percentage of growth inhibition was calculated using the formula:

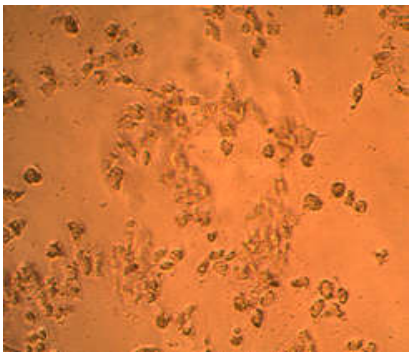
Flow cytometry: SHSY5Y (Neuroblastoma) cells were cultured as per standard procedures described earlier.
After attaining sufficient growth, H_2O_2 (150µM) was added to induce toxicity and incubated for one hour, and treated with selected concentration of sample (KEE- 50µg/mL) and incubated for 24 hours. The cells were trypsinized after incubation and 100 µL of cells in suspension was transferred to separate tubes. To the tubes added 100 µL of the Muse™ Annexin V & Dead Cell Reagent to each tube. The tubes were mixed thoroughly by pipetting up and down or vortexing at a medium speed for 3 to 5 seconds followed by incubation for 20 minutes at room temperature in the dark. The cells were analyzed in a flow cytometer and analyzed using Muse flow cytometry software. Cells were gated against untreated control cells and analyzed for apoptosis using Muse FCS 3.0 software.

OBSERVATIONS AND RESULTS

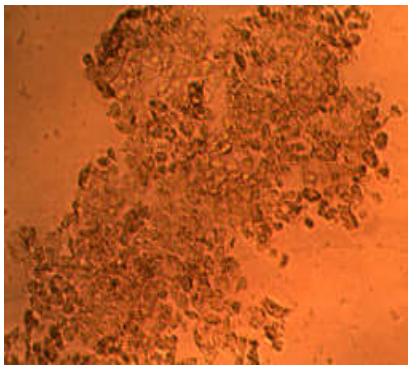
Results of MTT ASSAY



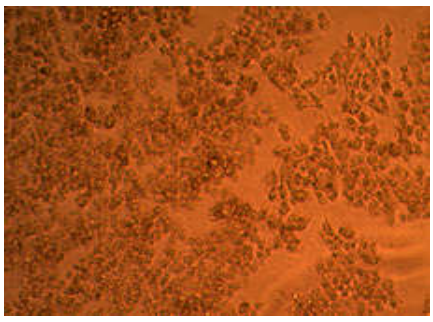
Control



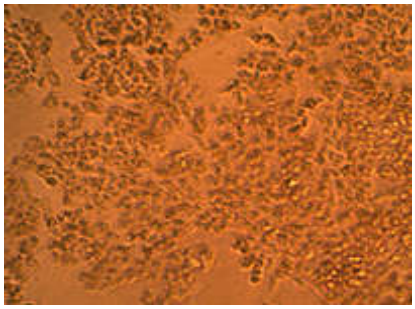
H_2O_2 Cells



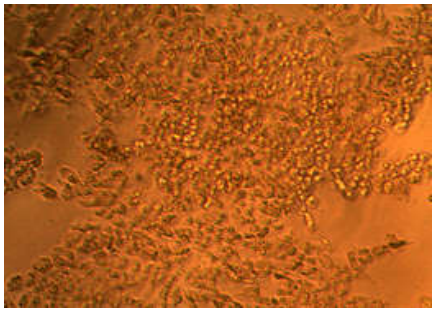
6.25µg/ml



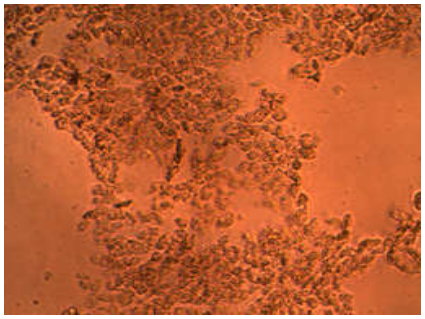
12.5µg/ml



25µg/ml



50µg/ml



100µg/ml

Fig 1. Results of MTT ASSAY

Table 1. Results of ANOVA

Sample	N	Mean	Std. Deviation	% of viability
Control	3	0.608	0.033	100
H2O2	3	0.145	0.006	23.8
6.25	3	0.163	0.021	26.9
12.5	3	0.18	0.02	29.5
25	3	0.267	0.032	43.9
50	3	0.276	0.03	45.4
100	3	0.172	0.02	28.2

Table 2. Table showing multiple comparison between different treatment groups

Multiple comparison	Mean Difference	Std. Error	P
Control VS H2O2	0.463	0.020	<0.001
Control VS 6.25	0.445	0.020	<0.001
Control VS 12.5	0.429	0.020	<0.001
Control VS 25	0.341	0.020	<0.001
Control VS 50	0.332	0.020	<0.001
Control VS 100	0.437	0.020	<0.001
H_2O_2 VS 6.25	-0.018	0.020	0.373
H_2O_2 VS 12.5	-0.035	0.020	0.106
H_2O_2 VS 25	-0.122	0.020	<0.001
H_2O_2 VS 50	-0.131	0.020	<0.001
H_2O_2 VS 100	-0.027	0.020	0.206
6.25 VS 12.5	-0.016	0.020	0.433
6.25 VS 25	-0.104	0.020	<0.001
6.25 VS 50	-0.113	0.020	<0.001
6.25 VS 100	-0.008	0.020	0.692
12.5 VS 25	-0.088	0.020	0.001
12.5 VS 50	-0.097	0.020	<0.001
12.5 VS 100	0.008	0.020	0.692
25 VS 50	-0.009	0.020	0.652
25 VS 100	0.096	0.020	<0.001
50 VS 100	0.105	0.020	<0.001

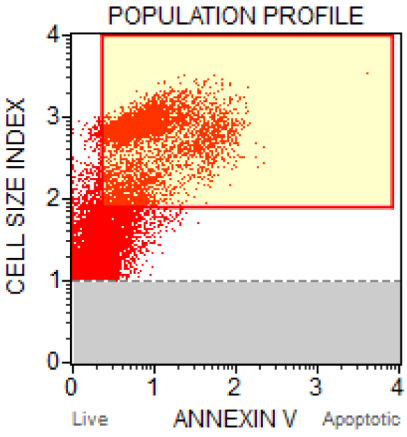


Fig. 2. Population profile of untreated control cells- SHSY5Y

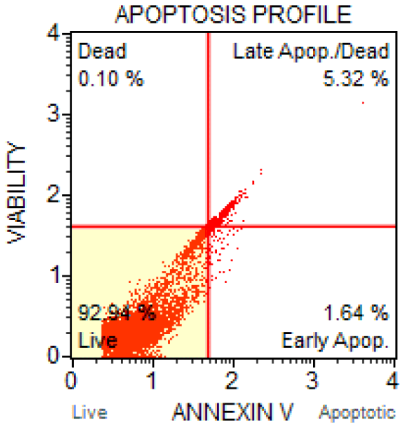


Fig. 3. Apoptosis profile of untreated control cells- SHSY5Y

	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.20E+06	92.94 %
Early Apoptotic (LR) :	2.12E+04	1.64 %
Late Apop./ Dead (UR) :	6.86E+04	5.32 %
Debris (UL) :	1.29E+03	0.10 %
Total Apoptotic :	8.98E+04	6.96 %

Fig 4. Cell distribution in untreated control cells- SHSY5Y

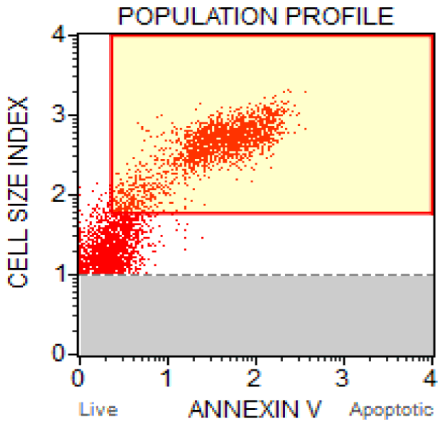


Fig 5. Population profile of cells treated with H_2O_2

Statistical Analysis: The results were statistically analyzed using one way ANOVA. It was observed that the concentrations of 25µg/ml and 50µg/ml had significant protection with a p value < 0.001.

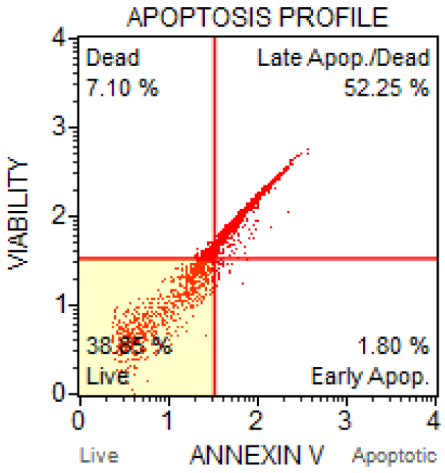


Fig. 6. Apoptosis profile of cells treated with H_2O_2

	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	9.48E+05	38.85 %
Early Apoptotic (LR) :	4.39E+04	1.80 %
Late Apop./ Dead (UR) :	1.28E+06	52.25 %
Debris (UL) :	1.73E+05	7.10 %
Total Apoptotic :	1.32E+06	54.05 %

Fig. 7. Cell distribution in cells treated with H_2O_2

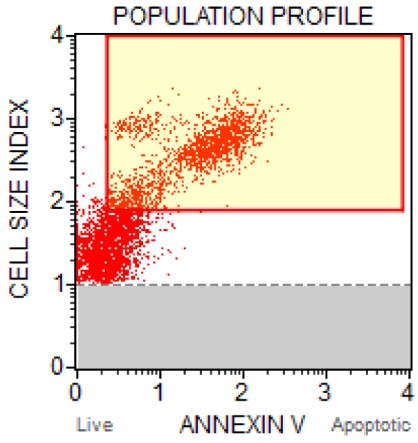


Fig. 8. Population profile of cells treated with Sample KEE

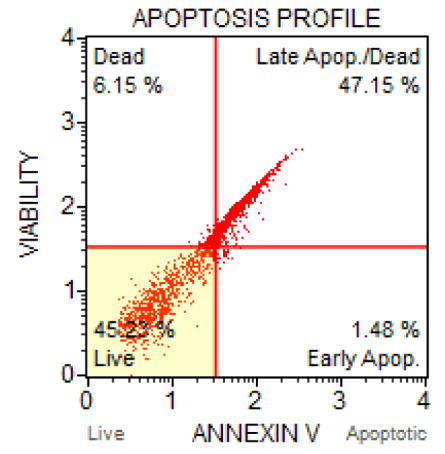


Fig 9. Apoptosis profile of cells treated with Sample KEE

	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.42E+06	45.23 %
Early Apoptotic (LR) :	4.66E+04	1.48 %
Late Apop./ Dead (UR) :	1.48E+06	47.15 %
Debris (UL) :	1.93E+05	6.15 %
Total Apoptotic :	1.53E+06	48.63 %

Fig 10. Cell distribution in cells treated with Sample KEE

To analyze and compare within groups multiple comparison was done using Post-Hoc Analysis. On Comparing between different groups by Post-Hoc analysis it was observed that 25µg/ml and 50µg/ml shows a significant protection with p <0.001 compared to other groups.

Results of Flow Cytometry: Annexin V– FITC analysis has shown significant increase in apoptotic cells (58%) of H_2O_2 treated cells when compared with untreated control cells (6%). Treatment with KEE (Ethanolic extract of *Kuvalaya*) has produced decrease of apoptotic cells to below 50%, increasing the cell viability to 46%.

DISCUSSION

There is an emerging need for developing cost effective medicines with less side effects for providing betterment of health of ailing society. Much research should be focused on locally available medicinal plants for their unexplored uses which can pave a long way in providing new medicines. This was such a study which explored the neuroprotective potential of a plant *Kuvalaya* locally identified as *Monochoria vaginalis* Presl. in Kerala which is very widely available in the marshy regions of the state. The study consisted of evaluating the Neuroprotective effect of Ethanolic extract of *Kuvalaya* (KEE) in SHSY5Y cell lines followed by its flow cytometry. The ability to produce in vitro cultures of neuronal cells has been fundamental to advancing our understanding of the functioning of the nervous system. SH-SY5Y, which has been cultured and used extensively in research on neuronal cells which was also used in the present study. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method. After conducting the MTT assay results were statistically analyzed using one way ANOVA. It was observed that the concentrations of 25µg/ml and 50µg/ml had significant protection with a p value < 0.001. On doing Flow Cytometry SHSY5Y (Neuroblastoma) cells were cultured as per standard procedures described earlier. The results revealed that the sample KEE showed a total apoptosis of 48.63% which was less than that shown by Hydrogen peroxide (54.04%) which proved its neuroprotective effect. Though the total apoptotic percentage was not comparable with the untreated cell sample (6.96%) the sample KEE still provided Neuroprotection.

Conclusion

The study thus proved that the Ethanolic extract of Kuvalaya does have neuroprotective effects as proved by the results of MTT Assay and Flow Cytometry. Further studies should be conducted for in depth analysis of the Neuroprotective potential and this study may serve as a base for further studies.

Conflict of interest: Nil

Funding: Nil

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