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IN VITRO NEPHROPROTECTIVE ACTIVITY OF MARINE ALGAE *CHLOROCOCCUM HUMICOLA*

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ABSTRACT

The impact of gravity of renal failure for the current study on human health is well known and as there is no specific pharmacotherapy for renal failure, undertaken to evaluate the effect of seaweed extract of *Chlorococcumhumicola*, belongs to chlorophyta and is a unicellular green algae. The algae were collected from Mandabam, Rameshwaram, Tamil Nadu, India. Marine algae have been the source of active compounds where, they are used for various applications. The medicinaluse in traditional medicine has been reported since time immemorial. The present study is on the marine algae *Chlorococcumhumicola*was investigated. The ethanol, ethyl acetate and hexane extracts of *Chlorococcumhumicola* were analyzed and subjected to phytochemical to know the secondary metabolites present in the ethanol extracts and showed the better activity so it was subjected for *in vitro* Nephroprotective activity.

KEYWORDS: *Chlorococcumhumicola*, Marine Algae, Nephroprotective Activity, MTT Assay

INTRODUCTION

Ninety percent of the world's living biomass is found in the oceans with marine species comprising approximately half of the total global biodiversity (Kim andWijesekara, 2010; Swing, 2003). This wide diversity of organisms is being recognized as a reservoir of potent molecules which are elicited by marine organisms to help them survive in the hostile environment (Swing, 2003; Alonso*et al*., 2005). Among marine organisms, marine algae have been identified as an under-exploited plant resources (Heo*et al*., 2009; Pangestuti*et al.*, 2010). The term marine algae, as used herein, generally refer to marine macro-algae or sometimes referred to as seaweeds.

In the early stage of drug development when selecting new drug candidates the renal failure is of global concern (Uehara *et al*., 2007). Important target of the toxicity of drugs, xenobiotics, oxidative stress and toxic materials is kidney (Uehara *et al.*, 2007). In addition to the mechanism, the reactive oxygen species (ROS) derived from chemical materials which are exposed to renal cells appear to mediate renal necrosis, of free radical toxicity are not well understood (Nabavi*et al*., 2012). Therefore, it is important to understand the role played by antioxidants e.g., phenolic compounds such as flavonoids, phenolic acids and tannins show protective effect against oxidative stress induced by reactive intermediates produced by various chemical materials during drug-mediated toxicity (Wu *et al.*, 2007).

Several million people get diagnosed with kidney disorders every year around the world. Acute kidney disorders

are most often due to synthetic drugs and are called Drug-induced nephrotoxicity (DIN). Nephrotoxicity is a serious consequence of H_2O_2 (Wink *et al.*, 1996) which is a diffusible reactive oxygen metabolite and a commonly used liquid to fight germs. Several parameters in blood enable the easy diagnosis of renal failures. Such failures require immediate medical attention or otherwise may often lead to death.

Vero cells are the cells which are derived from the kidney of an African green monkey and are one of the most commonly used mammalian cell lines in microbiology, molecular and cell biology research Sheets (Rebecca, 2000). The cell viability measurement and proliferation are the prominent of a cell population's for various *in vitro* assays response to external factors. The MTT Assay is reliable quantitative colorimetric assay that measures viability, proliferation and activation of cells of a most responsive, assay in living cells will be transformed from the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a purple formazan product that is insoluble in water(Mosmann and Tim., 1983). Precipitates of MTT in the cellular cytosol can be dissolved by cell lysis in viable cells, while the dead cells will not transform the MTT assay which is directly proportional to the number of viable cells and inversely proportional to the degree of Cytotoxicity. MTT can be reduced through the mediation by NADH or NADPH within the cells and out of mitochondria (Berridge*et al*., 1992; Fotakiset *al*., 2006).

Chlorococcum, is currently a distinct genus of chlorophyta, was first described as vegetative cells occurring as solitary in groups of indefinite form where the cells are ellipsoidal to spherical with smooth cell walls and variable size and the Extracellular products of *Chlorococcum* were isolated from culture filtrates . Upon the extracellular products of *Chlorococcum*isolation, five groups of substances which are steam-volatile acids, yellow water-soluble phenolic compounds, lipophilic substances, proteins and polysaccharides were found (Bhat and Madyastha, 2001). In the present study, the cell viability by MTT Assay screening of *Chlorococcumhumicola* was performed and also the determination ofNephroprotective activity was carried out.

MATERIALS AND METHODS

Collection of Plant Materials

The fresh sample of *Chlorococcumhumicola* were collected from the Rameshwaram sea shore, Tamil Nadu. Sample resources were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles in refrigerator.

Preparation of Extract

Crude sample extract was prepared by Soxhlet extraction method. About 20 gm of powdered material was uniformly packed into a thimble and extracted with 250 ml of solvents (methanol, ethyl acetate and hexane) extract separately. The process of extraction has to be continued for 24 hours or till the solvent in siphon tube of extractor become colourless. After that the extract was taken in a beaker and kept on hot plate and heated at 30-40ºC till all the solvent got evaporated. Dried extract was refrigerated at 4ºC till future use.

Chemicals

The chemicals namely, 3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Modified Eagle's Medium (MEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. Antibiotics from Hi-Media Laboratories Ltd, Mumbai. Dimethyl Sulfoxide (DMSO),

Hydrogen Peroxide (H_2O_2) and Propanol from E. Merck Ltd., Mumbai, India. All the chemicals and reagents used in our work are of analytic grade.

Cell Lines and Culture Medium

VERO (African Green Monkey Kidney) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of VERO cells were cultured in MEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in amoistened atmosphere of 5% CO_2 at 37°C until confluent. The cells were detached with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 micro-titre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, the weighed sample drugs were separately dissolved in distilled DMSO and volume was made up with MEM supplemented with 2% deactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out nephroprotective studies.

DETERMINATION OF CELL VIABILITY BY MTT ASSAY

Principle

The basic ability of the cells to survive a toxic insult has been the most Cytotoxicity assays. This assay is based on the hypothesis that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used (Pedraza-Chaverri*et al*., 2008).

Procedure

The monolayer cell culture was trypsinzed and the cell count was adjusted to 1.0 x 10^5 cells/ml using MEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was brushed off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and the observations were noted every 24 hrs interval.

After 72 hrs, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken well and incubated for 3 hrs at 37^o C in 5% CO_2 atmosphere. The supernatant was discarded and 100 µl of propanol was added to the plates and were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

DETERMINATION NEPHROPROTECTIVE ACTIVITY

The monolayer of the cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using MEM medium containing 10% FBS. To each well of the 96 well micro-titre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was brushed off, washed the monolayer once with medium and 50 µl of MEM with nontoxic concentration (1mM H_2O_2) of toxicant and 50 µl of different non-toxic test concentrations of test drugs were added. The plates were then incubated at 37^o C for 24 hrs in 5% CO_2 atmosphere. After 24 hrs, the cell supernatants were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hrs at 37^o C in 5% CO_2 atmosphere. The supernatant was detached and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage cell viability was determined, based on which the percentage protection offered by test and standard drugs was calculated over the H_2O_2control.

RESULTS AND DISCUSSIONS

In vitro confirmation of *Chlorococcumhumicola*toxicity in Vero cell lines was conducted. Trypan blue dye exclusion technique was used to determine the percentage of cell viability. The cytotoxicity activity was carried out by using MTT assay. The effect of methanolic extract of *Chlorococcumhumicola* (test) and H_2O_2 (control) on the growth of Vero cellline was examined by MTT assay. The susceptibility of cells to the extract exposure was characterized by CTC_{50}values. Results are tabulated in Tables.

Cytotoxicity Activity

The cytotoxicity study was carried out from the *Chlorococcumhumicola*methanol extract. Its extract was screened forits cytotoxicity against Vero cell lines at different concentrations to determine the CTC_{50} (50% growth inhibition) byMTT assay.

CYTOTOXIC EFFECT OF THE SAMPLE ON VERO CELL LINE

Inference

The test for Cytotoxicity was conducted for the MTT assay for Vero cell line (kidney epithelial cells extracted from an African green monkey) for the methanolicextract with different test concentrations of 62.5, 125, 250, 500 and 1000. For the L2 cell line highest activity is seen in 1000 µg/ml with 16.46 ± 1.30cytotoxicity with CTC_{50}value of >1000 and for the vero cell line highest activity is seen in 1000 µg/ml with 71.78 ± 2.72cytotoxicity with CTC_{50}value of 237.96.

Table 1: Cytotoxic Effect of the Sample on L6 Cell Line

S. NO	Conc (µg/ml)	% Cell viability	% CTC_{50}	CTC_{50}
1	62.5	99.82	0.18 ± 0.01	>1000
2	125	99.55	0.45 ± 0.02	
3	250	98.75	1.25 ± 0.02	
4	500	85.50	14.50 ± 1.58	
5	1000	83.54	16.46 ± 1.30	

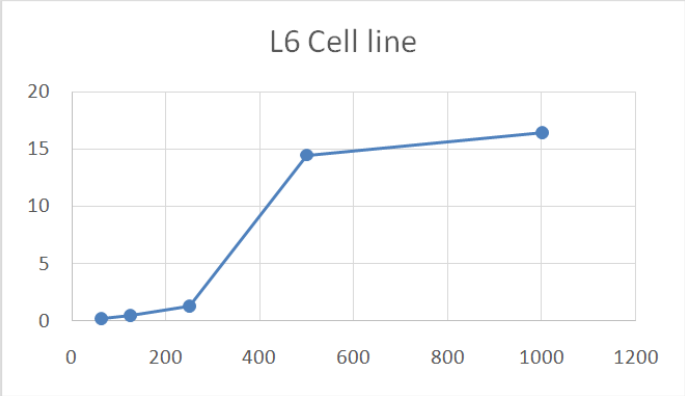

Figure 1: Cytotoxic Effect of the Sample on L6 Cell Line

Table 2: Cytotoxic Effect of the Sample on VERO Cell Line

S. NO	Conc (µg/ml)	% Cell viability	% CTC_{50}	CTC_{50}
1	62.5	77.68	22.32 ± 2.49	
2	125	58.24	41.76 ± 4.52	
3	250	48.87	51.13 ± 1.59	237.96
4	500	34.78	65.22 ± 1.04	
5	1000	28.22	71.78 ± 2.72	

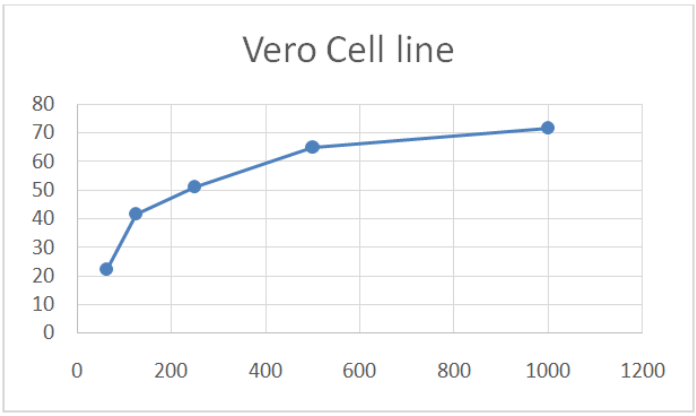

Figure 2: Cytotoxic Effect of the Sample on VERO Cell Line

CONCLUSIONS

In this paper, nephrotoxicity was induced by H_2O_2 in vero cell lines of African green monkey. *Chlorococcumhumicola*has been evaluated for its nephron-protectiveness. The presence of phyto-constituents in *Chlorococcumhumicola*has inhibited the induced nephrotoxicity. This has been revealed through the results, that shown moderate protection over toxicant control during test trials with different concentrations of test sample.

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