In vitro hepatoprotective activity of *Sargassum wightii* against CCl_4 induced hepatic damage

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Abstract: *In vitro* hepatoprotective activity of *Sargassum wightii* methanolic extract was checked using established cell culture (Hep G2) and primary rat hepatocytes. In Hep G2 cells, the extract concentrations varying from 31.25-500 µg/ml were studied using 1% CCl_4 as toxicant. All the extracts showed moderate protectivity against toxicity induced by CCl_4 in Hep G2 cells. All the biochemical constraints were determined and compared with that of the control. In this study, an innovator product (silymarin) was used along with the test extracts. *In vitro* hepatoprotective activity was evaluated by analyzing various parameters such as ALAT, ASAT, ALP, total protein, total bilirubin, TGL and albumin. The results stated that the hepatoprotective potential exhibited by the methanolic extract of *Sargassum wightii* (SWMH) is active and comparable with the standard.

Keywords: Hep G2, Hepatocytes, Carbon tetrachloride, *Sargassum wightii.*

INTRODUCTION

The liver is an intricate and imperative organ of human body. Its principal role is to direct the flow of the substances absorbed from the digestive tract before it gets distributed to the systematic circulatory system. The liver is also denoted as laboratory of human body as it carries out over 500 functions. It is coupled with all bodily processes since it filters all ingested food and fluids. In the body, detoxification of the supplied nutrients depends upon the liver (Robin, 2012).

There are several drugs that cause adverse reactions to the liver. Chronic liver ailment and cirrhosis collectively account for approximately 1% of annual mortality in United States (about 26,000 of 2,50,000 death/year) (Nourjah *et al.,* 2006). Liver disease is due to high consumption of alcohol is the most prevalent drug induced liver disease. Almost all the other human organ systems are affected by the liver function.

Further, a hepatoprotective activity has not yet been determined in modern medicines. One could notice a global trend for the revitalization of interest in the traditional Indian system of medicine. Screening of natural compounds has turned out to be a potential source of biodynamic compounds of therapeutic value (Deb *et al.,* 2014).

In the marine ecology, marine algae is one among the potent natural resource. They possess diverse bioactive principles used as medicine (Faulkner, 2000). Macroalgae act as source for more than 2400 marine natural products (Manilal *et al.,* 2009). Marine algae produce immense compounds functioning as chemical defense systems helping them to endure extreme competitive environments. Based on their biological activities reported, algae have been proposed as a promising resource of bioactive principles with pharmaceutical purposes (Blunden, 1993).

The study deals with evaluating the *in vitro* hepatoprotectivity of *Sargassum wightii* and further suggested for isolation, identification and *in vivo* studies for potential hepatoprotective agent.

MATERIALS AND METHODS

Cell lines and culture conditions

Liver cancer cell line (Hep G2) and normal fibroblast cells (Vero) procured from National Centre for Cell Sciences, Pune were used in this study. The cell lines were grown in suitable medium [MEM basal medium with 1mM Sodium pyruvate, 2mM glutamine, 2g/L sodium bicarbonate and 10% Fetal Bovine Serum (Sigma)]. 5% CO_2 incubator with moisture content was used to grow the cell lines.

Preparation of algal extract

The brown algae *Sargassum wightii* was collected from Marakkanam (Lat. 9.280 N and Lon.79.130 E), Tamil Nadu, India. The collected seaweeds were identified based on the Central Marine Fisheries research Institute (CFTRI) manual and authenticated by Dr. P. Anantharaman, Associate Professor, CAS in Marine Biology, Parangipettai, Tamil Nadu, India. Distilled water was used to clean the collected algae and was air dried. Maceration technique by using methanol for a period of 3 days (10g/100ml) was used for algal extraction. The macerated extract was clarified, filtered and refrigerated until use.

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Trypan blue exclusion test

Trypan blue exclusion test was used to determine the cell viability. At a density of 1×10^6 cells/well the Hep G2 cells were seeded and incubated at optimal condition (37°C, 5% CO_2 concentration). Cells were trypsinized after 72 hrs. 20 µl each of medium and trypan blue were mixed and viability was determined using Neubauer haemocytometer. The cells were confirmed to have 95-98% cell viability.

MTT assay

In 96-well tissue culture plate at a concentration of 1×10^6 cells/well, cell lines were added. Initially, stock solutions of extracts (1mg/ml) were prepared by using DMSO. Different concentrations (1000, 500, 250, 125, 62.5 and 31.25µg/ml) were made by diluting with cell culture medium. To the cultures, apt concentrations were added and incubated at 37°C for 72 hrs. Non-treated cells were used as control. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done using incubated cultured cells and concentration were determined by colorimetric assay. In order to determine cell viability in assays of cell proliferation and cytotoxicity, the tetrazolium is used. An insoluble purple formazan product is produced in metabolically active cells on reduction of MTT. The assay reading was taken using an ELISA reader at 520nm. Determination of absorbance and corresponding extract concentrations standardizes the cytotoxicity data. A dose dependent curve was plotted using data generated by the extract concentration where 50% of cell population was killed (IC_{50}/CTC_{50}) (Jeffrey *et al.*, 2004).

Inhibition (%) = 100- [(Mean test OD/control OD) × 100]

Preparation of freshly isolated rat hepatocytes

Firstly, the liver sample was subjected to a non-recirculating perfusion using calcium free buffer or a calcium chelator like EDTA, which causes irreversible separation of desmosomal cell contacts. It was then followed by perfusion of liver using collagenase inorder to dissolve the extra cellular matrix, calcium added back to ensure maximum enzyme activity. This is an optimal treatment where the liver is completely dissociated within 10-15 mins, that is, abundantly rapid to prevent the requirement for continuous oxygenation during perfusion. Thawing of HEPES buffer and collagenase solution was done by keeping in a water bath (38^oC-39^oC to achieve 37^oC in the liver). Flow rate of the pump was adjusted to 30ml/min. Thiopental sodium 45mg/kg b.w. was administered intra peritonealy to anaesthetize the rat (180-200gms). After the opening of the abdomen, a loosely tied ligature was placed around the portal vein and the ligature was tightened after the insertion of the cannula up to the liver followed by the injection of heparin (1000 IU) into the femoral vein. Inorder to avoid the excess pressure, sub hepatic vessels were hastily incised. HEPES buffer (600 ml calcium free) was perfused for 20 minutes at a flow rate of 30ml/min. At the same time, the liver swells slowly and a color change from dark red to greyish white was observed.

The perfusion of 300ml of collagenase solution for 20 minutes at a flow rate of 15ml/min led to the swelling of the lobes. After the disruption of the Glison capsule, the lobes were detached and washed with HEPES buffer. This was followed by, trypsinization and centrifugation of the cell suspension at 1000rpm to remove the collagenase, non-parenchymal cells and damaged cells. Ham's F12 medium was used for the collection of hepatocytes.

In vitro hepatocyte injury using carbon tetrachloride

The hepatocytes isolated were kept for incubation for 30 minutes at 37^oC for stabilization according to Surendran *et al.*, 2011. Ham's F12 coons modified medium was used to dilute the cells with the cell count of $5x10^5$ cells/ml. 100 µl of cell suspension was used to seed each well of the 96 well titre plate. The medium was replaced with fresh medium after 2 hours of pre-incubation. Then the hepatocytes were pretreated with solvent extracts for one hour followed by 1% carbon tetrachloride induced treatment (50µl of different extract and 50µl of carbon tetrachloride). 250µg/ml of the standard drug was used. Hepatocytes injury was brought about by incubating hepatocytes with carbon tetrachloride for 24 hours at 37^oC. After incubation, the toxicant and drug treated cell suspensions were pooled into eppendroff tubes and centrifugation (4000rpm) was done for 10-15min. The supernatant was then collected and the following enzyme levels were determined Alanine aminotransferase (ALAT), Aspartate aminotransferase (ASAT), Triglycerids (TGL), Alkaline phosphatase (ALP), Total Protein, Albumin and Total Bilirubin using diagnostic kits (Ecoline).

STATISTICAL ANALYSIS

The results were expressed as Mean ± SD. Using student's "t" test, data was statistically analyzed. P values set as lower than 0.01 were considered as statistically significant.

RESULTS

The present work was aimed to screen the *in vitro* hepatoprotective activity of SWMH extract in established hepatocellular carcinoma cell line Hep G2 and in freshly isolated rat hepatocytes, intoxicated with carbon tetrachloride. The experimental groups were compared with the control, toxicant and standard silymarin.

Table 1 depicts that SWMH extract was used at five different concentrations (500, 250, 125, 62.5, 31.25 µg/ml) to find out the cell toxic concentration by MTT assay. The tetrazolium salt is taken by the living cells and in turn produces a formazan product. The mitochondrial integrity is directly connected with the percentage

viability and the determination provides the CTC_{50} value of 425±0.04µg/ml. The dose value below the CTC_{50} was selected for the purpose of hepatoprotective activity.

Table 1: Determination of CTC_{50} by MTT assay in Hep G2 cells

Sample	Concentration (µg/ml)	CTC_{50} (µg/ml)
SWMH	500, 250, 125, 62.5, 31.25	425±0.04

Table 2 shows that survival of control cells was 100% and the cells intoxicated with CCl_4 were not able to survive the toxic insult and showed 15% viability. The cells on treating with the test extract SWMH at different concentrations showed an increase in viability in a dose dependent manner. This result had a significance of P< 0.01 on comparison with CCl_4 intoxicated cells. The extract concentrations were between 100-400µg/ml (value below CTC_{50}) and corresponding to the viability percentage ranging between 65-88%. The concentration of 400µg/ml was significant with 88% with a significance level of P<0.01 with standard silymarin.

Table 2: Hepatoprotective activity of SWMH extract on CCl_4 intoxicated Hep G2 cell

Treatment	Concentration (µg/ml)	% Viability
Control	-	100
Carbon tetrachloride	1%	15.20 ± 1.13^a
Carbon tetrachloride + Standard (Silymarin)	250	92.13 ± 4.23^b
Carbon tetrachloride + SWMH	400	88.25 ± 1.35^b
	300	78.35 ± 3.30^b
	200	72.03 ± 4.23^b
	100	65.42 ± 4.25^b

Averages of six independent determinations (n=6), values are mean ± SEM

a = P < 0.01 when compared to control

b = P < 0.01 when compared to CCl_4 intoxicated cells

In table 3 the effect of SWMH extract on freshly isolated rat hepatocytes intoxicated with CCl_4 is tabulated. The dosage of CCl_4 resulted in hepatic damage, which can be concluded, from the changes in liver enzymes. A comparison between control and toxicant infers a major increase in the parameters of ALAT, ASAT, ALP and total bilirubin (P<0.01) and a major decrease in albumin, total protein and TGL (P<0.01). Silymarin, which is a standard drug, has nearly restored all the levels as that of control. A restoration capacity of biochemical parameters were evident towards the control in the cells subjected to different extract concentrations (100-400µg/ml). In comparison with test and standard ASAT, ALAT, ALP and total bilirubin level decreased as the SWMH extract concentration was increased. At the concentration of 200µg/ml, all the parametric components tested had significance with standard and control. Further low concentration (100µg/ml) was not significant with control and standard groups.

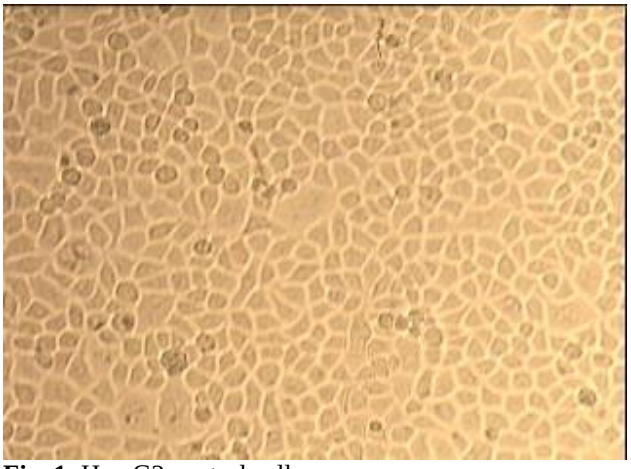

Fig. 1: Hep G2 control cells

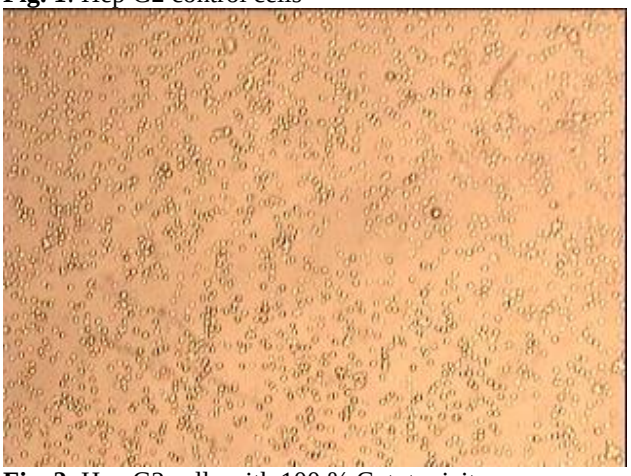

Fig. 2: Hep G2 cells with 100 % Cytotoxicity

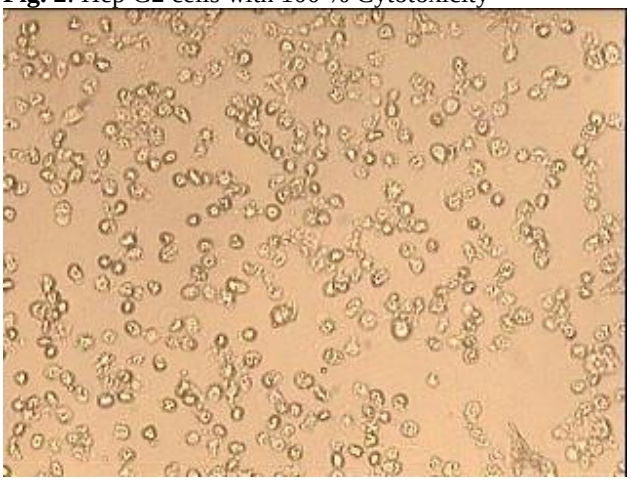

Fig. 3: Hep G2 cells with 50 % Cytotoxicity

DISCUSSION

Susceptibility to injury from various drugs and toxic substances is high in liver due to its distinctive

Table 3: Effect of SWMH extract on the biochemical parameters of CCl_4 intoxicated rat hepatocytes

Treatment	Concentration (µg/ml)	ASAT U/L	ALAT U/L	ALP U/L	TGL mg/ ml	Albumin g/L	Total protein g/dL	Total bilirubin mg /L
Cell Control	-	16±4.53	11±15.2	32±1.24	175±6.14	2.54±0.3	3.5±0.66	0.19±0.01
Carbon tetrachloride	1%	90±6.20[a]	77±0.46[a]	84±1.44[a]	65±4.22[a]	0.8±0.04[a]	0.6±0.01[a]	0.73±1.33[a]
Carbon tetrachloride +Standard (Silymarin)	250	19±1.62[b]	15±1.09[b]	37±1.66[b]	135±3.60[b]	1.1±0.43[b]	1.2±0.24[b]	0.12±0.03[b]
Carbon tetrachloride+ SWMH	400	35±4.16[b]	17±0.01[b]	40±0.25[b]	146±4.80[b]	1.6±0.06[b]	1.51±0.5[b]	0.13±1.04[b]
Carbon tetrachloride+ SWMH	300	33±1.02[b]	19±0.92[b]	42±1.52[b]	157±5.6[b]	1.8±0.05[b]	2.08±1.4[b]	0.15±3.6[b]
Carbon tetrachloride+ SWMH	200	30±5.1[b]	20±0.21[b]	43±1.30[b]	162±6.3[b]	1.92±0.42[b]	2.5±0.05[b]	0.22±0.24[b]
Carbon tetrachloride+ SWMH	100	38±1.06[b]	25±0.06[b]	47±1.78[b]	140±4.05[b]	0.9±0.14[b]	1.05±2.4[b]	0.31±1.60[b]

Averages of six independent determinations (n=6), values are mean ± SEM
a = $P < 0.01$ when compared to control
b = $P < 0.01$ when compared to CCl_4 intoxicated hepatocyte cells

metabolism. Numerous mechanisms are liable for causing hepatic injury and damage the hepatocytes. The toxic metabolites alter the plasma membrane, mitochondria, intra-cellular iron flux and enzyme activity. Liver disorders and diseases are considered as a major health problem and still have no cure in recent medicines. Both the developed and developing countries struggle to manage the impact of liver injury and continue to grow with other health complications such as type II diabetes, hypertension, heart diseases and obesity (Aggarwal *et al.,* 2004).

The cytoprotective potential of any extract or compound can be determined by *in vitro* and *in vivo* methods. Hep G2 is an apt *in vitro* model to study the polarized human hepatocytes and in turn displays a robust morphological and functional differentiation that resembles the *in vivo* model. Hepatic damage caused by CCl_4 causes instability of liver cells and leads to biochemical changes. CCl_4 is a prevalent industrial waste and acts as a cancer-causing agent and mutagen towards humans and animals. For hepatoprotective evaluation of liver, the best featured system of xenobiotic induced hepatotoxicity is liver damage by CCl_4 (Alqasoumi *et al.,* 2010).

The efficiency of hepatoprotective medicine depends on its viability of either decreasing the toxic effect or to maintain the normal hepatic physiology disturbed by the hepatotoxins. As liver executes various activities in the human body, damage of liver leads to vulnerable problems and thus the study of hepatoprotective potential are vital. Previous reports suggest that oxidative imbalance and injury generated by the hepatotoxin leads to membrane breakage and instability (Lima *et al.,* 2007).

The discovery and advancement of efficient therapeutic agents from natural sources could provide an evident novel drug. Marine algae are the natural resources with diverse bioactive compounds, which are used as a source of food, feed and medicine. The present work was done to learn the cytoprotective potential of methanolic extract of *Sargassum whigtii* against CCl_4 induced hepatotoxicity in established hepatocellular carcinoma cell Hep G2 and in freshly isolated rat hepatocytes by *in vitro* method.

The results of biochemical parameters exposed the alteration of enzyme levels in CCl_4 and thus stimulate liver damage. From the result, it is evident that extract of *Sargassum whigtii* improved the liver function by decreasing the serum ASAT up to 200µg/ml. Previous study showed similar results in ionized treated groups (Jiang *et al.,* 2004).

Total bilirubin, which is the byproduct of the breakdown of RBC, serves as a good indicator of liver function. In comparison to the standard, the level of total bilirubin at 400µg/ml treated group was moderately potent. If increased levels were seen it leads to jaundice and indicates bile and liver duct damage (Rajesh *et al.,* 2005).

The biochemical marker enzymes ALP, ALAT, TGL, albumin and protein show an increase level compared with control and toxicant. This result comparably agreed with earlier report of Anandan *et al.* (1999) in which the

quantity of diagnostic marker enzymes in the plasma is directly proportional to the count of necrotic cells in the liver tissue.

The marine algae taken in the study possessed a significant hepatoprotective potential by lowering the elevated level of liver marker enzymes. CCl_4 intoxication has increased the ASAT, ALP, ALAT and bilirubin that indicates permeability increase, membrane damage and leads to liver damage.

Significant elevated serum marker enzyme levels suggest oxidative destruction in the CCl_4 control, which shows adverse hepatic injury (Surendran, 2011). It is observable as an important reduction in protein and TGL as the concentration increases when compared with control. Concentration of extract above 425±0.04µg/ml was toxic to the cells and so concentration below this range was opted for *in vitro* studies, and the biochemical parameters determined the comparable result at 200µg/ml. The CCl_4 induces changes in the Hep G2 cells and isolated rat hepatocytes and were significantly ameliorated by the co-treatment of the toxin along with the crude extract and provided a reasonable protection against cells.

CONCLUSION

The results stated that the hepatoprotective activity shown by the methanolic extract of *Sargassum whigtii* was found to be comparable with innovator product. Earlier report suggests the methanol extract has phytochemicals like flavonoids and alkaloids that could lead to the potential activity in protecting the hepatocytes. Additional studies are essential to authenticate through *in vivo* experiments and characterization of active principles responsible for the activity.

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