# THE APOPTOGENIC POTENTIAL OF DIFFERENT FRACTIONS OF SRI LANKAN BROWN SEAWEED *Chnoospora minima* AGAINST HUMAN BREAST ADENOCARCINOMA (MCF-7) CELL LINE

***M.D.T.L. Gunathilaka[1], K.W. Samarakoon[3], P. Ranasinghe[2], L.D.C. Peiris[1*]***

*[1]Department of Zoology (Center for Biotechnology), Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka, [2]Industrial Technology Institute, Halbarawa Gardens, Sri Lanka, [3]Institute for Combinatorial Advanced Research and Education (KDU-CARE), Research and Development Office, General Sir John Kotelawala Defence University, Sri Lanka.*

## INTRODUCTION

Among the diversified source of marine environment, seaweeds play a major role in drug development due to the presence of bioactive secondary metabolites such as polyphenols, alkaloids, flavonoids and sterols. Bioactive compounds present in marine algae exhibited potent biological activities as an antioxidant, as well as being anti-cancer, anti-diabetic etc. Oxidative stress is tightly linked with the development of several degenerative diseases such as cancer and diabetes, cardiovascular disorders, and inflammation and aging etc (Abd-Elnaby et al., 2015). As marine algae are a rich source of bioactive secondary metabolites, it has the ability to combat most degenerative diseases. *Choonospora minima* is a brown alga that belongs to the family Scytosiphonaceae. Polyphenols purified from brown algae are considered to be a rich source of antioxidants (Shanura et al., 2017). Hence, in lieu of herbal drug development, natural antioxidant rich marine seaweeds can be utilised for the development of potential anti-cancer therapy. Therefore, the present study aimed to determine the *in-vitro* cytotoxic activity of different fractions of Sri Lankan marine brown algae *Choonospora minima* (Hering 1841).

## METHODOLOGY

### Collection of algae samples (*Choonospora minima*)

The permission to collect algae samples was obtained from Department of Wild Life Conservation (permit number- WL/3/280/17). The marine brown algae (*Choonospora minima*) was manually collected from the Kalpitiya area and the specimen of algae sample was deposited at the Department of Zoology, University of Sri Jayewardenepura. The collected samples were cleaned and washed with fresh water to remove salt, sand, attached epiphytes and organic matter. The samples were air dried and ground into a fine powder and stored at -20°C until further use.

### Preparation of *Choonospora minima* fractions

Homogenized *Choonospora minima* powder (10.0g) was extracted three times using 70% methanol, subjected to sonication at 25 °C for three 90 min periods. The polyphenols were separated by precipitating crude polysaccharides using 70% ethanol. The supernatant was separated as polyphenols and the portion of the supernatant was used to solvent-solvent partition with hexane, chloroform and ethyl acetate, respectively (Lakmal et al., 2014).

### Maintenance of the human breast adenocarcinoma (MCF-7) cell line

The human breast adenocarcinoma (MCF-7) cell line was obtained from the Department of Biochemistry, Faculty of Medical Sciences, University of Colombo, and the RD cell line was cultured in high glucose DMEM medium supplemented with 10% v/v FBS and 1% penicillin-streptomycin antibiotic solution. The cells were maintained in 5% $CO_2$ incubator at 37°C.

### *In-vitro* cytotoxic activity

*In-vitro* cytotoxic activities were assessed by 3-(4, 5-Dimethylthiazol-2-cyl)-2, 5-Diphenyltetrazolium Bromide (MTT) (Mosmann, 1983) and neutral red assays (Syed et al, 2013).

### *In-vitro* apoptotic activity

Apoptotic activity was examined by cellular morphology, DNA fragmentation and Caspase 3/7 assays (Tor et al, 2015 and RahbarSaadat et al, 2017). The cellular morphology was observed using phase-contrast inverted microscope and florescence staining (Hoechst stain) method. DNA fragmentation was performed to observe the typical DNA ladder pattern of apoptosis in the DNA extracted from the treated cells compared to the untreated cells. Caspase 3/7 assay was performed using Apo-one Homogeneous Caspase 3/7 kit.

## RESULTS AND DISCUSSION

### *In-vitro* cytotoxicity assays

The results on the cytotoxicity of the fractions of *C.minima* against MCF-7 cell line through the MTT assay are shown in Table 1. Based on the results obtained, the hexane and chloroform fractions of *C.minima* exhibited potential cytotoxic effect with $IC_{50}$ 90.58±4.34µg/ml and 97.73±2.92µg/ml, respectively, compared to the standard cycloheximide ($IC_{50}$: 28.76±0.55µg/ml). In addition, a significant cytotoxic activity was observed between hexane and chloroform fraction with ethyl acetate fraction and aqueous fraction against MCF-7 cell lines ($P>0.05$). Parveen and Nadumane (2020) have further confirmed the cytotoxicity effect of methanol extract of *C.minima* by MTT assay against MCF-7, HeLa and HepG2 cancer cell lines, and found that 100 µg/ml of methanol extract was more effective against MCF-7 cell line with 63% viability.

The neutral red assay was performed to confirm the cytotoxic activity of active hexane and chloroform fractions of *C. minima* against human MCF-7 cells. As in MTT results, the neutral red assay confirmed the dose-dependent decrease in cell viability. As presented in Table 1 and Figure 1, the hexane fraction treated MCF-7 cells ($IC_{50}$: 119.46±1.32 $\mu$g/ml) exhibited the highest cytotoxic activity compared to the standard cycloheximide ($IC_{50MCF-7}$: 27.84±0.33 $\mu$g/ml). Both MTT and neutral red assay measures cell viability in different approaches. Neutral red assay is based on the accumulation of neutral red dye in the lysosomes of viable cells, while MTT is based on the reduction of yellow tetrazolium MTT reagent by mitochondria of viable cells to purple formazan (Green et al., 1984).

Table 1: $IC_{50}$ (µg/ml) values obtained from MTT and neutral red assay for human MCF-7 cell line after being treated with different extract of *C.minima* for 24 hours

| *C.minima* fractions Cytotoxicity:$IC_{50}$ | MTT; % Cytotoxicity: $IC_{50}$ (µg/ml) | Neutral red; % Cytotoxicity: $IC_{50}$ (µg/ml) |
|---|---|---|
| Hexane fraction | 90.58±4.34$^{b}$ | 119.46±1.32$^{a}$ |
| Chloroform fraction | 97.73±2.92$^{b}$ | 135.89±1.16$^{b}$ |
| EA fraction | 140.39±1.48$^{c}$ | NA |
| Aqueous fraction | 162.14±4.44$^{d}$ | NA |
| Standard (Cycloheximide) | 28.76±0.55$^{e}$ | 27.84±0.33$^{c}$ |

Results are expressed as mean ± SD based on triplicates. Mean values in a column superscripted by different letters (a–e) are significantly different at $p < 0.05$.

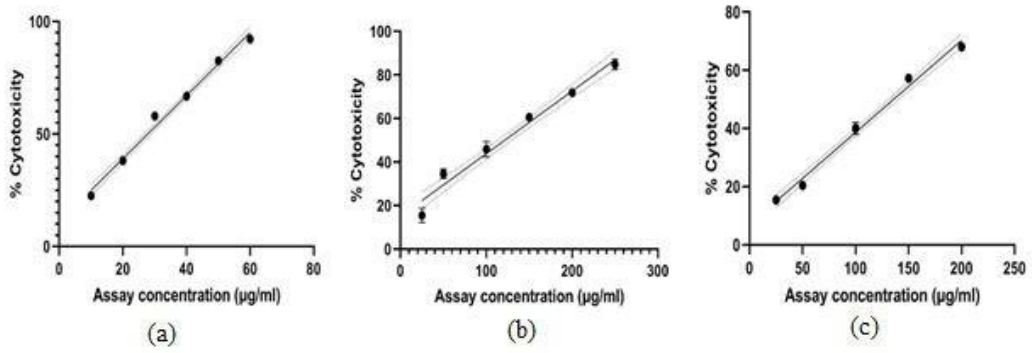


**Figure 1**: Cytotoxic assessment of human MCF-7 cells by neural red assay following the exposure of (a) cycloheximide (standard), (b) hexane fraction and (c) chloroform fraction of *C .minima*. Data is expressed as mean ± SD; n=3.

Further, apoptotic morphological features of different fractions treated MCF-7 cells were observed using crystal violet and Hoechst stain in addition to the phase-contrast inverted microscope. During the apoptosis, cells displayed typical morphological features such as cell membrane blebbing, formation of membrane bound vesicle, nuclear fragmentation and micro nuclei formation, cellular shrinkage, cellular aggregation and the formation of cell clumps, and chromatin condensation (Nur et al., 2013). Hence, to confirm the apoptosis, morphological alterations in treated MCF-7 cells were observed after 24 hours incubation compared to untreated cells (control). According to the observation, hexane and chloroform fractions treated cells revealed significant apoptotic morphological features compared to the standard cycloheximide while untreated cells maintained their original morphology. Figure 2 shows the apoptotic morphological features of MCF-7 cells treated with standard (cycloheximide), hexane and chloroform fractions of *C.minima* compared to untreated cells (control).

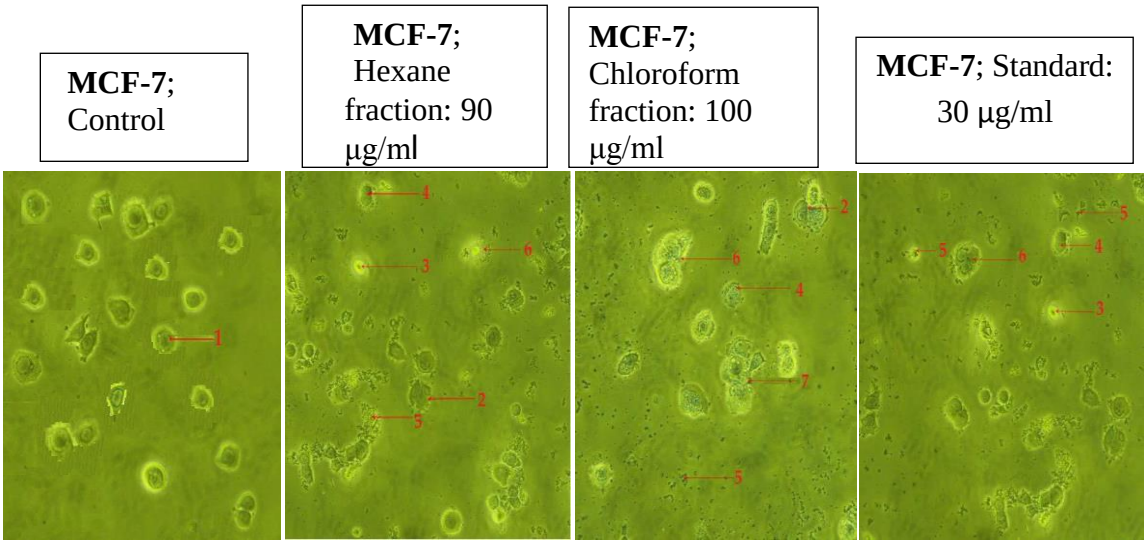


Figure 2: Apoptotic effects of enriched fraction of control, Standard Cycloheximide (30 µg/ml), *C.minima* hexane fraction (90µg/ml) and chloroform fraction (100 µg/ml) in human MCF-7. Phase contrast inverted microscopy. Arrows indicate 1.Cells with normal nuclei; 2. Cellular aggregation and formation of cell clumps; 3. Chromatin condensation; 4.Nuclear fragmentation

and bulging towards cell membrane; 5.Cell shrinkage and cellular death; 6.Cell membrane blebbing; and 7.Formation of membrane bound vesicle.

The caspase family of proteases is involved in the apoptosis pathways (Lamkanfi and Kanneganti, 2010). Therefore, the effect of hexane and chloroform fractions of *C. minima* on caspase 3/7 activities in MCF-7 cells was evaluated using the Ac-DEVD-AFC substrate, and the recognition site of caspases-3 and -7. According to the results, prominent activation of caspase 3/7 was observed in the hexane and chloroform fraction treated (24 h) MCF-7 cells after 3 h of caspase treatment compared to the standard Staurosporine (figure 3).

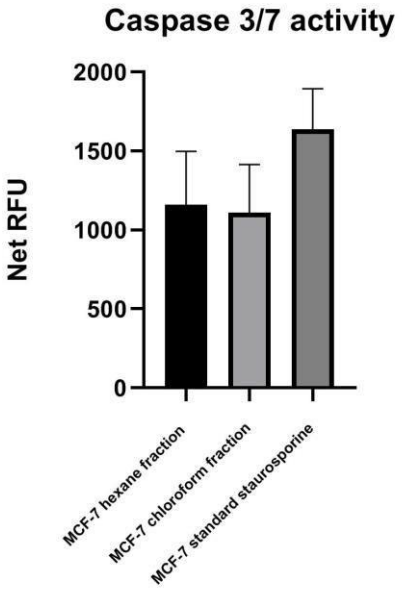


Figure 3:- Caspase 3/7 activities of MCF-7 after the treatment with various concentrations of hexane, chloroform fractions and standard staurosporine for 24 hours. Data is expressed as mean

± SD based on triplicates.

DNA fragmentation is a hallmark of apoptosis (Parveen and Nadumane, 2020). The MCF-7 cells treated with the hexane fraction did not show any typical ladder pattern. However, the positive control (cycloheximide-30µg/ml) showed a band similar to the typical DNA laddering in apoptosis compared to the untreated cells (Figure 4).

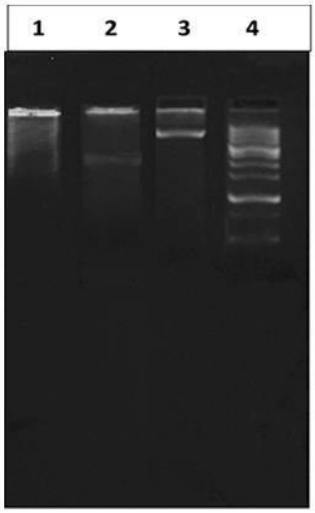


Figure 4: DNA fragmentation shown by agarose gel electrophoresis of MCF-7 cells treated with the *C .mi*nima hexane fraction (90 µg/ml) and standard cycloheximide (30 µg/ml). 1. hexane fraction treated MCF-7 cells; 2. Standard cycloheximide treated MCF-7 cells; 3. Control; and 4. DNA ladder; Concentrations were selected based on the $IC_{50}$ values of cytotoxicity.

## CONCLUSIONS/RECOMMENDATIONS

The results suggest that the apoptogenic potential of the hexane fraction of *C. minima* against human breast adenocarcinoma cells. Further studies should be carried out to isolate the active compounds in hexane fraction of *C.minima*.

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