

Abstract

Anti-proliferative and apoptotic activity of polyphenol-rich crude methanol extract of *Gracillaria edulis* against human Rhabdomyosarcoma (RD) and breast adenocarcinoma (MCF-7) cell lines

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Abstract

Seaweeds are an important source of bioactive metabolites in the development of drugs and nutraceuticals. This study aimed to analyze the anti-proliferative and apoptotic activity of methanol extract (ME) of *G.edulis* on human Rhabdomyosarcoma (RD) and breast adenocarcinoma (MCF-7) cell lines. De-polysaccharide ME of *G.edulis* was extracted to determine the anti-proliferative and apoptotic activity. The cytotoxic activity was evaluated by MTT and neutral red uptake assay, while apoptotic activity was characterized by cellular morphology, DNA fragmentation and Caspase 3/7 assay. The results of cytotoxicity assay showed that the decrease of the percentage of cell viability in a dose-dependent manner as signified by cell death. The apoptosis was induced by activating the Caspase 3/7 pathway and the RD cell line showed prominent activation of Caspase 3 compared to the MCF-7 cells have low activation. Further, the typical DNA ladder pattern was observed in RD cells, whereas MCF-7 cells did not show a typical ladder pattern. Thus, it can be concluded that the ME of *G.edulis* possesses anticancer activity via activating the Caspases pathway.

Keywords: *G.edulis*, Anti-cancer, Rhabdomyosarcoma, MCF-7, MTT, Neutral red, Caspase pathway.

1. Introduction

Recently, the burden of different types of cancer increased significantly around the globe. The World Health Organization projects that, by 2035, the world could see 24 million new cancer cases and 14.5 million cancer-related deaths a year [1]. According to the Cancer Registry of Sri Lanka, about 23,530 cancer cases were reported in 2018, accompanied by 14013 deaths [2]). Breast cancer, skin cancer, and ovarian cancer are the leading cancers affecting women globally [1]. Available cancer treatments cause several side effects with an increased risk of developing secondary cancers due to their long term potential toxic effects. Hence, it is vital to keep searching for an effective drug with

minimum side effects that may benefit patients suffering from different types of cancer (Fourquet *et al.*, 2008). Among the natural resources, seaweeds play a major role in drug development due to the presence of bioactive secondary metabolites that exhibit potent biological activities such as antioxidant, anti-cancer, anti-diabetic, etc. *Gracillaria edulis* (Gmelin) Silva is a red alga that belongs to the family Gracilariaceae. Polyphenols purified from red seaweeds are considered a rich source of antioxidants. In the search of new plant-derived drugs, natural antioxidant-rich marine seaweeds can be utilized to develop potential anti-cancer therapies. Therefore, the present study aimed to analyze the anti-proliferative and apoptotic activity of the methanol extract of *G.edulis* on human Rhabdomyosarcoma (RD) and human breast adenocarcinoma (MCF-7) cell lines.

2. Materials and Methods

2.1. Collection of red algae *Gracillaria edulis*

The permission to collect the algae sample was obtained from the Department of Wild Life Conservation (permit number- WL/3/280/17). The red algae *Gracillaria edulis* was manually collected from Kalpitiya lagoon area, Puttalam (8° 14' 0" North, 79° 46' 0" East), Sri Lanka, 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.

2.2. Preparation of the methanol extract

Homogenized *Gracillaria edulis* powder (10 g) 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 (1:20 v/v). The supernatant was separated as polyphenol-rich methanol extract [4].

2.3. Maintenance of cell lines

The human rhabdomyosarcoma (RD) and breast adenocarcinoma (MCF-7) cell lines were obtained from the Department of Biochemistry, Faculty of Medical Sciences, University of Colombo, and cells were cultured in high glucose DMEM medium supplemented with 10% v/v FBS and 1% penicillin-streptomycin antibiotic solution. The cells were maintained in a 5% CO₂ incubator at 37°C.

2.4. *In-vitro* anti-proliferative and apoptotic activity

In-vitro cytotoxic activities were determined by 3-(4, 5-Dimethylthiazol-2-yl)-5-Diphenyltetrazolium Bromide (MTT) and neutral red assays. Apoptotic activity was characterized by cellular morphology, DNA fragmentation, and Caspase 3/7 assay[5-6].

3. Results

3.1. *In-vitro* cytotoxic activity

The results of cytotoxic activity against the human RD and MCF-7 cells as determined by MTT and neutral red assays are shown in Table 1. The results exhibited a dose-dependent decrease in cell viability as signified by cell death. The extract of *G.edulis* exhibited potent cytotoxicity against RD (IC₅₀: 50.31 µg/ml) and MCF-7 (IC₅₀: 32.05 µg/ml) carcinoma cells when compared to the standard cycloheximide (IC_{50RD}: 36.26 µg/ml; IC_{50MCF-7}: 28.87 µg/ml) as confirmed by the MTT assay. The neutral red assay, further, confirmed the potential cytotoxic activity of the extract (IC_{50RD}: 45.95 µg/ml; IC_{50MCF-7}: 35.13 µg/ml) against RD and MCF-7, compared to the standard cycloheximide (IC_{50RD}: 31.80 µg/ml; IC_{50MCF-7}: 27.84 µg/ml).

Table 1: IC₅₀ (µg/ml) values of human RD and MCF-7 cell lines as determined by MTT and neutral red assays, after 24 h of treatment with the methanol extract of *G. edulis* and standard cycloheximide

Extracts	IC ₅₀ value (µg/ml)			
	MTT assay		Neutral red assay	
	RD cells	MCF-7 cells	RD cells	MCF-7 cells
<i>G.edulis</i> methanol extract	50.31	32.05	45.95	35.13
Standard Cycloheximide	36.26	28.87	31.80	27.84

3.2. Apoptotic activity

The apoptotic activity of RD and MCF-7 cells after 24 hours of the treatment with the methanol extract was characterized by cellular morphology, DNA fragmentation, and Caspase 3/7 assay. prominent activation of Caspase 3/7 was observed in the RD cells compared to the MCF-7 cells. Also, the highest Caspase 3/7 activity was observed after 4 hours in RD cells, whereas in MCF-7 high Caspase activity was observed after 3 hours of the treatment.

Similarly, the DNA ladder pattern was observed in RD cells treated with methanol extract and the standard cycloheximide whereas MCF-7 cells did not show any typical DNA ladder pattern (Figure 2).

Further, apoptotic morphological features of RD and MCF-7 cells were observed using fluorescence staining (Hoechst stain) method in addition to the phase-contrast inverted microscope. As shown in figure 3, treated cells with *G.edulis* methanol extract and standard cycloheximide displayed typical morphological features compared to the untreated cells (control).

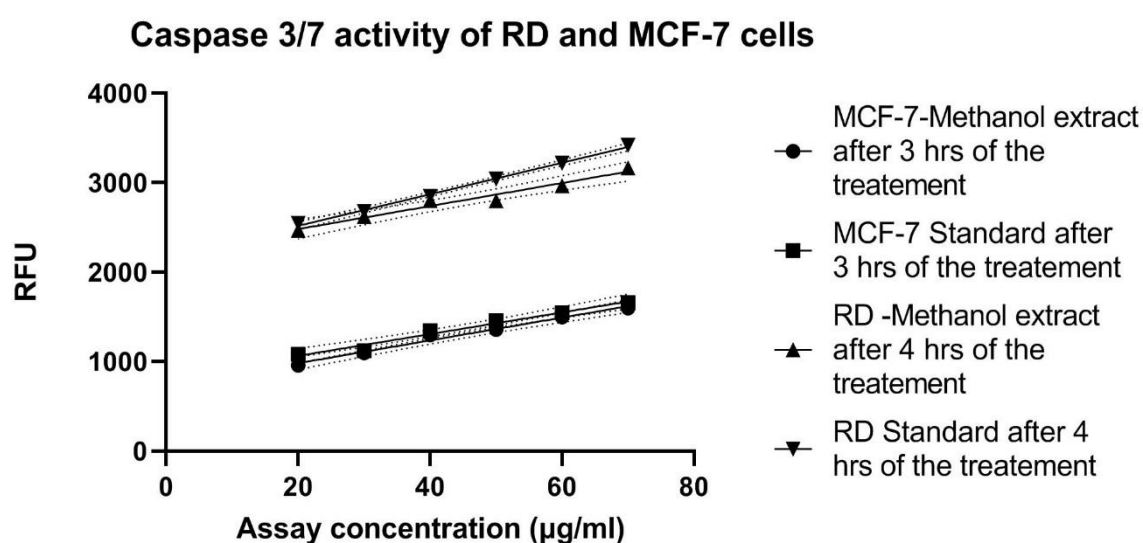


Figure 1: Caspase 3/7 activities of MCF-7 and RD cells obtained after 24 hours of treatment with various concentrations of the methanol extract of *G.edulis* and standard cycloheximide. Data are shown as mean ± SD of triplicates.

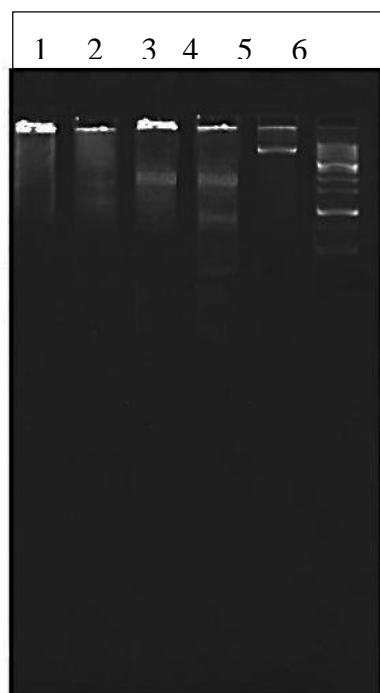


Figure 2: DNA fragmentation shown by agarose gel electrophoresis of MCF-7 and RD cells treated with *G.edulis* methanol extract (Concentrations; RD-50 $\mu\text{g/mL}$, MCF-7 30 $\mu\text{g/mL}$) and standard cycloheximide (30 $\mu\text{g/mL}$). 1-Methanol extract treated MCF-7 cells; 2-Standard cycloheximide treated MCF-7 cells; 3. Methanol extract treated RD cells; 4. Standard cycloheximide treated RD cells. Concentrations were selected based on the IC_{50} values of cytotoxicity.

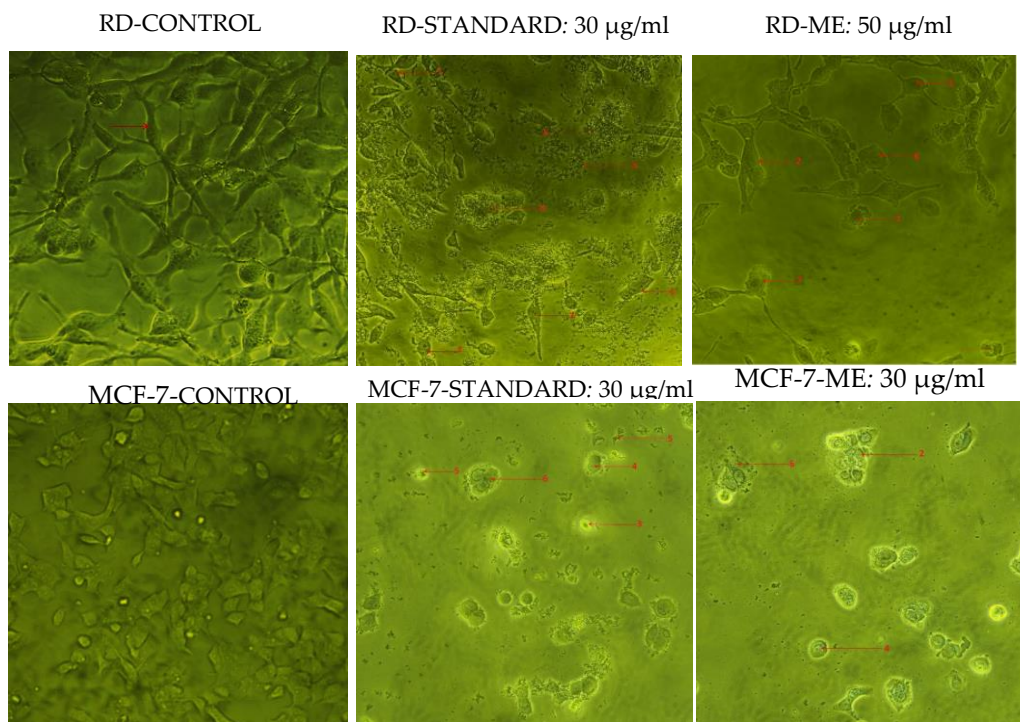


Figure 3: Phase contrast inverted microscopic images of the enriched fraction of *G.edulis* methanol extract and standard cycloheximide in human RD and MCF-7 cell lines. Apoptotic morphological features -1. Cells with normal nuclei; 2. Cellular aggregation and formation of cell clumps; 3. Chromatin condensation; 5. Cell shrinkage and cellular death; 6. Cell membrane blebbing

4. Discussion

The present study showed the potent cytotoxic and apoptotic effect of methanol extract of *G.edulis* against human RD and MCF-7 cell lines. The *G.edulis* methanol extract can reduce the cell count (visible) and it caused the apoptotic response.

According to the cytotoxic assay, higher cytotoxicity was observed in the RD cells treated with the methanol extract of *G.edulis* compared to the MCF-7 cells as determined by MTT and neutral red assays. When comparing the cytotoxicity results, both MTT and neutral red assays showed more or less similar results as both assays measure the cell viability in different approaches. The MTT assay is based on the reduction of yellow tetrazolium MTT reagent to purple formazan crystals by cellular oxidoreductase enzymes present in mitochondria of viable cells whereas the neutral red assay is based on the accumulation of neutral red dye which is a eurythodine dye in the lysosomes of viable cells [7].

Lakshmi *et al* (2016), found that the anti-proliferative activity of the methanol extract of *G. edulis* as 89.35% cell viability for 100 µg of methanol extract against MCF-7 cell line [8]. In the present study, 69.70% of cell viability was observed for 20 µg/ml of methanol extract. The observed high anti-inhibitory activity of the present study might be due to the variation in extraction techniques as the present study extracted only the polyphenolic portion using the sonication method whereas the previous study was against the whole crude algae using the Soxhlet method for extraction.

Apoptosis is the programmed cell death that is regulated by different families of proteins. Among them, caspase is one of the important protein families involved in the cell death pathway (apoptosis). Caspases are arranged in a proteolytic cascade and coordinate the stereotypical events that occur during apoptosis by transmitting the death signal. Among the different types of caspases, caspase 3 is important in the process of apoptosis as it is activated by both intrinsic (mitochondrial) and extrinsic (death ligand) pathways. The activation of caspase 3 leads to the cleavage of different structural proteins such as poly(ADP-ribose) polymerase, caspase-activated DNase (ICAD), and DNA fragmentation factor resulting in morphological alterations of cells such as cell membrane blebbing and cellular condensation. Further, DNA fragmentation apoptosis resulted in the cleavage of caspase-activated DNase (ICAD) [9].

In addition to caspase 3, caspase 7 is also an executioner protein of apoptosis, which is activated by cell death stimuli to induces apoptosis [10]. In the present study, a prominent elevation of Caspase 3/7 is observed in the RD cells after the 4 hours of the treatment when compared to the MCF-7 cells. The results of the present study showed that the extract induces very low activation of Caspase 3/7 after 24 hours of treatment. Further, Wang *et al* (2016), confirmed that Caspase 3 is deficient in MCF-7 due to the partial deletion of the *CASP-3* gene important for cell death, thus leaving the MCF-7 cells without typical apoptotic properties [11]. This was further confirmed by the DNA ladder pattern of the extract-treated MCF-7 cells. However, one band observed in the MCF-7 cells treated with the standard due to the activation of Caspase 7 causing cell membrane blebbing and DNA fragmentation [12].

During apoptosis, cells display typical morphological features such as cell membrane blebbing, the formation of the membrane-bounded vesicle, nuclear fragmentation, micronuclei formation, cellular shrinkage, cellular aggregation, the formation of cell clumps, and chromatin condensation. Most of these features were observed in both RD and MCF-7 cells treated with the methanol extract of *G.edulis* and standard cycloheximide compared to the untreated cells.

5. Conclusions

It can be concluded that the crude methanol extract of *G. edulis* exhibits significant anti-proliferative and apoptotic activity against RD and MCF-7 cell lines. The RD cell line showed prominent activation of Caspase 3 compared to the MCF-7 cells have low activation.

Author Contributions: Conceptualization, L.D.C.P., P.R. and K.W.S.; methodology, L.D.C.P., P.R., and K.W.S.; software, T.L.G.; formal analysis, T.L.G. and P.R.; investigation, T.L.G. and P.R.; data curation, T.L.G.; writing—original draft preparation, T.L.G.; writing—review and editing, L.D.C.P., and K.W.S.; supervision, L.D.C.P., P.R. and K.W.S.; project administration, L.D.C.P.; funding acquisition, L.D.C.P. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest

References

1. World Health Organization. *Cancer*, 2017. Available at: <http://www.who.int/mediacentre/factsheets/fs297/en/>
2. BC Cancer Agency. BC Cancer Registry Annual Report, 2018. Retrieved from <http://www.bccancer.bc.ca/about-site/Documents/BCCancerResearch-AnnualReport2018.pdf>
3. Fourquet, A., Kirova, Y., Bollet, M., Tournat, H., Dendale, R. and Campana, F. Meta-analyses of the effects of radiotherapy in breast cancer: the ultimate evidence, *Cancer* 2008, 12(6-7), pp.554-558.
4. Lakmal, H., Samarakoon, K., Lee, W., Lee, J., Abeytunga, D., Lee, H. and Jeon, Y. Anticancer and antioxidant effects of selected Sri Lankan marine algae. *Journal of the National Science Foundation of Sri Lanka*. 2014, 42(4).
5. Mosmann, T. 'Rapid Colorimetric Assay for Cellular Growth and Survival : Application to Proliferation and Cytotoxicity Assays', *Journal of Immunological Methods* 2008, Elsevier, 65, pp. 55–63.
6. Gnst-itc, G. *et al.* 'Induction of Apoptosis by Gluconasturtiin-Isothiocyanate (GNST-ITC) in Human Hepatocarcinoma HepG2 Cells and Human Breast Adenocarcinoma MCF-7 Cells', *Molecules* 2008, 25, 1240; doi:10.3390/molecules25051240.
7. Green, L. M., Reade, J. L. and Ware, C. F. 'Rapid colorimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines', *Journal of Immunological Methods* 1984, 70(2), pp. 257–268. doi: 10.1016/0022-1759(84)90190-X.
8. Sheeja, L. *et al.* 'Anticancer activity of phytol purified from *Gracilaria edulis* against human breast cancer cell line (MCF-7)', *INT J CURR SCI* 2016, 19(4): E 36-46.
9. Ponder, K., G. and Boise, L. H. 'The prodomain of caspase-3 regulates its own removal and caspase activation', *Cell Death Discovery*. Springer US 2019, pp. 1–10. doi: 10.1038/s41420-019-0142-1.
10. Lamkanfi and Kanneganti, Caspase-7: a protease involved in apoptosis and inflammation, *Int J Biochem Cell Biol* 2010, 42(1): 21–24. doi:10.1016/j.biocel.2009.09.013.
11. Wang, S. *et al.* 'Breast Cancer Cell-in-Cell Death Is Not Restricted by Caspase-3 Deficiency in MCF-7 Cells', *Journal of Cancer Breast* 2016, 19(3), pp. 231–241.
12. Mc, M. M. *et al.* 'Caspase-3 is not essential for DNA fragmentation in MCF-7 cells during apoptosis induced by the pyrrolo-1, 5-benzoxazepine, PBOX-6', *Febs letters*, Elsevier 2002, 515, pp. 66–70.

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