

Accumulation of Cells in Sub-G1 Phase and Apoptosis Induction by a Bioactive Fraction from the Seaweed *Gelidiella acerosa*

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ABSTRACT

Marine algae or seaweeds serve as excellent resources of bioactive secondary metabolites with wide range of therapeutic applications and multiple biological activities. Despite the growing worth of algae as a source of pharmacological compounds, the biotechnological or anticancer application of marine algae are still under-exploited. Hence the present study is aimed at exploring the anti proliferative activity of the marine macroalga, *Gelidiella acerosa* against HeLa, HepG2 and MCF-7 cancer cell lines and to identify the bioactive compound. The secondary metabolites of *G. acerosa* were extracted using methanol, the extract was purified by TLC. The bioactive fraction was selected through bioassay guided fractionation and the cytotoxic, apoptogenic potential of this fraction was analysed by different in vitro assays such as MTT assay, LDH assay, Trypan blue dye exclusion, DNA fragmentation, caspase activity and cell cycle analysis by flow cytometry. The characterization of the bioactive fraction was performed through GC MS analysis. The results of MTT and trypan blue assays indicated significant cytotoxicities to the GF7 treated HeLa, HepG2 and MCF-7 cancer cells and at the same time demonstrated non-toxicity to normal human lymphocytes at 50µg/mL concentration. The mechanism of action of this fraction on the cancer cells was observed as apoptosis induction as indicated by significantly elevated caspase activity, decreased cell counts, DNA fragmentation pattern and elevated LDH enzyme activities. Cell cycle analysis showed majority of cells accumulating in the sub-G1 phase that further confirmed apoptosis induction by the algal fraction. GC-MS analysis indicated the presence of hexadecanoic acid, previously documented for anticancer activity, that might be responsible for its bioactivity. It can be concluded that this algal bioactive fraction (GF7) has significant anti-cancer potential at low concentrations and shows promise for future in-vivo studies that might lead towards a safer anti cancer compound..

KEY WORDS: ANTI-CANCER, APOPTOSIS, CELL CYCLE, GELIDIELLA ACEROSA, SUB-G1.

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INTRODUCTION

Cancer is one of the major challenging diseases that leads to the death of several million people globally in an annual basis (Aggarwal et al., 2009). The fundamental characteristic of all types of cancers is uncontrolled cell division (Tay et al 2019). With most of the existing treatment modalities ineffective in completely curing the disease, there is a need to search for ideal anti-cancer agents from natural resources. It has been reported that uncontrolled symmetric cell division is the major factor that contributes to cancer. Hence it is quite natural for scientists to aim anticancer drugs that control cell cycle machineries and that induce apoptosis (Bai et al., 2017, Ashraf 2020).

Marine organisms with a wide range of bioactive secondary metabolites of pharmaceutical significance have the potential towards the development of anti cancer agents (Newman and Gordon, 2016). Marine algae comprise one of the rich sources of a wide range of secondary metabolites. The protective effect of edible seaweeds has been established against mammary, skin and intestinal carcinogenesis (Yuan and Walsh 2006). Marine algae have been used as food and also as traditional medicine in the eastern hemisphere (Kilinc et al., 2013). The red alga, *Gelidiella acerosa* is abundantly found in the coastal area of South India, especially in inter tidal region of Gulf of Mannar (Taskin et al., 2007; Bernecker et al., 2009). *G. acerosa* is mainly used for agar production and also has many phytochemical constituents having biological activities including anti cancer activities (Duraikannu et al., 2014; Begum et al., 2018).

Though anticancer activity was reported for *G. acerosa*, previous studies (both *in-vitro* and *in-vivo*) were carried out using crude extracts of this promising algal species. Hence we felt the necessity to purify and characterize the bioactive compounds from this red alga and aimed to study the mechanism of action of its bioactive components on cell cycle stages of cancer cell lines in the present study.

Figure 1: Macroscopic view of *Gelidiella acerosa*



MATERIAL AND METHODS

Preparation of algal extract and extraction of metabolites: Seaweed was collected from the Mandapam camp at Rameshwaram, Tamilnadu in India. The collected seaweed was identified as *Gelidiella acerosa* (Figure 1) and authenticated by Dr.Eswaran, Principle scientist at marine algal research station, Rameshwaram. *G. acerosa* was washed and dried for 7 days under the shade. The dried sample was powdered with the help of a mixer grinder and the metabolites were extracted in a soxhlet apparatus using methanol as the solvent. The extract was further concentrated in a rotary evaporator (IKA, Germany) at 40° C.

The methanol extract of *G.acerosa* was initially screened for cytotoxicity to cancer cell lines (HepG2, HeLa and MCF-7) by MTT cell viability assay. The methanol extract was further fractionated by TLC sheets using different solvent combinations. Through bioassay guided fractionation, the bioactive fraction was chosen for further cytotoxicity assays.

MTT Cell viability assay: MTT[3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide] assay is a colorimetric assay to determine the viability of the cells treated with the samples (Mosmann, 1983). The percentage viability of the cells was calculated as follows:

$$\text{Percentage viability (\%)} = \frac{\text{O.D 540 of treated}}{\text{O.D 540 of control}} \times 100.$$

Trypan Blue assay: Trypan blue dye exclusion test was carried out to determine the concentration of viable and dead cells in a cell suspension (Strober, 1997). Cells were treated with 50µg/ml of the sample for 48 hrs. The cell concentration was determined by counting the number of viable cells in the treated group with the help of a hemocytometer.

LDH cytotoxicity assay: The extent of damage in the treated cells was analysed by LDH cytotoxicity assay in the treated cells as per the protocol given in the kit manual (Weyermann et al., 2005).

Caspase -3,7 and 10 activities: The activity of caspase enzyme in the sample treated cancer cells was measured using CasPASE(tm) Apoptosis Colorimetric Assay kit (G Biosciences Ltd, USA), as per the instructions given in the manual of the kit manufacturer.

DNA fragmentation assay: DNA fragmentation is a semi quantitative method to analyse the fragmentation of DNA in the treated group in which the cells were harvested, DNA was extracted and loaded to 0.8% agarose gel for electrophoresis (Shidoji and Ogawa, 2004).

Flow cytometry analysis with Propidium Iodide staining: Flow cytometry analysis of the sample treated and untreated cancer cells was performed to analyse the distribution of DNA at different stages of the cell cycle. After 48 hrs of sample treatment, the cells were harvested

and the cell cycle was analysed as per the standard methodology (Pazarowski and Darzynkiewick, 2004).

GC-MS analysis: The GF7 fraction of *G. acerosa* was subjected to analysis through GC-MS method at the facility of Central Silk Technological Research Institute, Bangalore. The resulting mass spectral peaks of unknown compounds were analyzed and compared with the database of anti-cancer compounds so as to identify the bioactive component .

RESULTS AND DISCUSSION

Drug discovery from natural sources such as marine algae is an important area of recent research in cancer Biology. Many marine algae were found to produce structurally diverse secondary metabolites of therapeutic significance. In the current study, we mainly focussed to evaluate the anti cancer potential of the red alga *G. acerosa* towards in-vitro cancer cell lines. The methanol extract of *G. acerosa* was found to have anti proliferative activity to all the tested cancer cell lines at 24,48,72 and 96 h of treatment. The maximum inhibition of cell proliferation was observed at the higher concentration of 100µg/ml. The viability was 46.82% for HepG2, 40.38% for MCF-7 cells and 36.59% for HeLa cells after 96 h of treatment with 100µg/ml of the methanol extract of *G. acerosa* (Table 1).

Table 1. Effect of methanol extract of *G. acerosa* to various cancer cell lines at different concentrations.

Concentration (µg/ml)	Control	Viability (%) HeLa			
		24 h	48 h	72 h	96 h
1	100	100	83.31	71.65	49.97
10	100	100	82.61	62.77	68.51
50	100	100	76.63	53.40	37.72
100	100	98.1	66.20	51.82	36.59
	HepG2				
1	100	100	100	80.63	72.72
10	100	100	82.37	75.25	67.58
50	100	89.6	81.55	62.96	53.58
100	100	73.7	73.77	57.74	46.82
	MCF-7				
1	100	99.48	97.1	92.13	80.87
10	100	79.23	73.3	70.48	58.90
50	100	72.43	73.0	69.6	46.67
100	100	66.66	55.1	54.8	40.38

The methanol extract of *G. acerosa* was partially purified by thin layer chromatography using the solvent combination of Acetonitrile: chloroform: dichloromethane: Toulene (1:2:2:1). Seven different fractions were observed under UV and visible lights. When each of the fractions were tested by MTT assay, the 7th fraction (GF7) demonstrated maximum cytotoxicity

than the other fractions (results not shown). When the effect of GF7 was analysed on different cancer cell lines, an increase in the treatment concentration of GF7 (from 1 to 50 µg/ml) resulting in a decreased viability of cells was seen. The cell viability at 50µg/ml was 36.5 % for HeLa, 46.8% for HepG2 and 40.3% for MCF-7 cells after 96h treatment (Figure 2). The IC50 value of GF7 was calculated as 39 µg/ml for HeLa, 27µg/ml for HepG2 and 37µg/ml for MCF-7 cells for 96h of treatment.

Figure 2: Percentage cell viability of the cancer cells treated with GF7 at different treatment periods of 24, 48, 72 and 96 hrs * represents significance at p<0.05, ** represents significance at p<0.01.

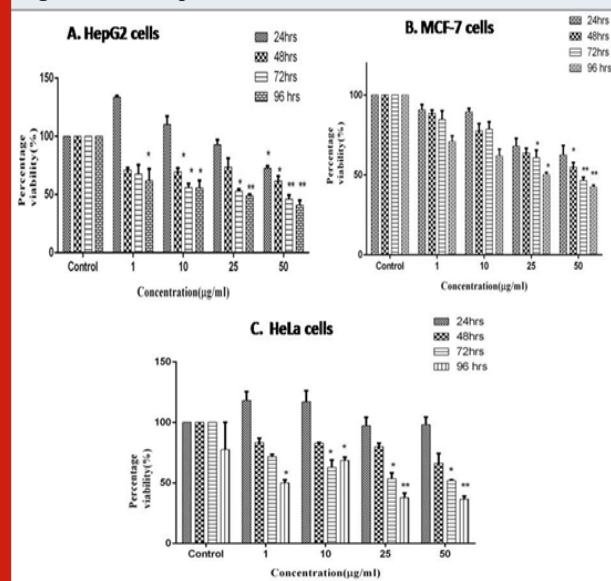


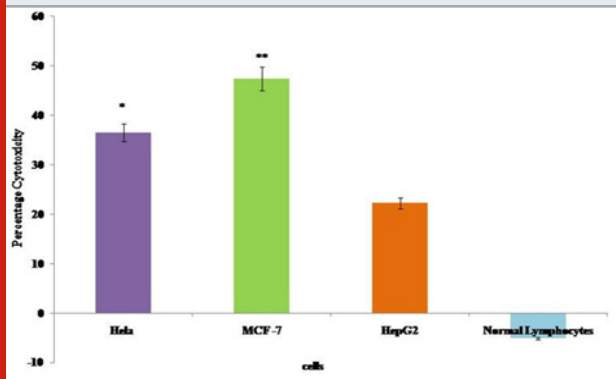
Table 2. Determination of cell count and cell viability by Trypan blue method.

Cell lines	Untreated Control		GF7 Treated	
	Viable Cell count (1x106 cells/ml)	Viability (%)	Viable Cell count (1x106 cells/ml)	Viability (%)
HeLa	8.56	99.76	3.29	38.79
MCF-7	9.93	96.59	3.62	35.01
HepG2	9.81	98.29	3.57	35.87
Normal Lymphocytes	1.51	98.69	0.97	97.53

In a previous report (Lakmal et al., 2014), where the anticancer activity of six different seaweeds were analysed, *G. acerosa* had moderate inhibition against HL-60 cells and had no cytotoxicity against mouse melanoma (B16F10) and human lung carcinoma (A549) cells. As compared to this report, the present study

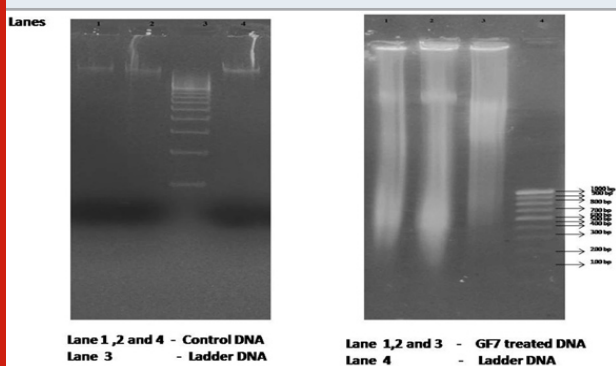
shows promising anti-cancer activity of *G. acerosa*. When the effect of GF7 on HepG2, HeLa and MCF-7 cell concentration was examined by trypan blue assay, it was clear that all the cell lines treated with the bioactive fraction GF7 had a decreased viable cell count as compared to the control cells (Table 2). There were 3.29×10^6 cells/ml in HeLa, 3.2×10^6 cells/ml in MCF-7 and 3.57×10^6 cells/ml in HepG2 cells after 48h of treatment with the viability ranging between 35.0-38.79%. No cytotoxicity was observed to the treated normal lymphocytes.

Figure 3: LDH cytotoxicity of the cancer cells treated with GF7.



The cytotoxic effect of the bioactive fraction GF7 to the cancer cells was assessed by LDH assay. Changes in the membrane integrity or membrane damage cause the release of LDH enzyme into extra cellular media. The cytotoxicity caused by GF7 fraction was 36.47% on HeLa, 47.38% on MCF-7 and 22.25% on HepG2 cells as compared to the control cells. No cytotoxic effect was observed to normal human lymphocytes by GF7 treatment (Figure 3).

Figure 4: DNA fragmentation analysis of cells treated with fraction GF7



The hallmark of apoptosis is the degradation of DNA into fragments by endogenous DNAses. The mechanism of cell death caused by GF7 is determined by DNA fragmentation analysis (Saraste and Pulkki, 2000; Elmore, 2007). Based on the results of this analysis, we could see shearing of DNA in the treated cancer cells as compared to the control cells, where the DNA was intact and a single band was visible (Figure 4).

Caspases are a family of aspartate-specific cysteine proteases and detection of activation of caspase activity is a valid method for assessing apoptosis (Chang and Yang, 2000). In our study, cells treated with GF7 had significantly higher caspase activity (Figure 5). The percentage increase in caspase activity of HepG2, HeLa and MCF-7 cells were 67.20%, 16.02% and 7.9% respectively. When cell cycle stages were analysed by flow cytometry, we observed that GF7 treated cancer cells had increased number of dead cells which accumulated in the sub G1 phase of the cell cycle (Figure 6). Sub G1 indicates apoptotic cells. The concentration of sub-G1 phase was 81.7% in HepG2, 68.4% in MCF-7 and 80.4% in HeLa cells.

Figure 5: Caspase activity in cancer cells treated with 50µg/ml of fraction GF7. * represent significant difference (p<0.05) between treated and untreated cells

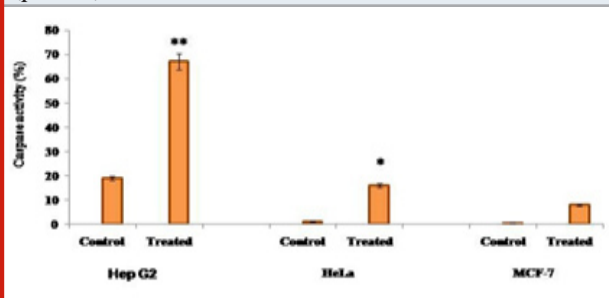
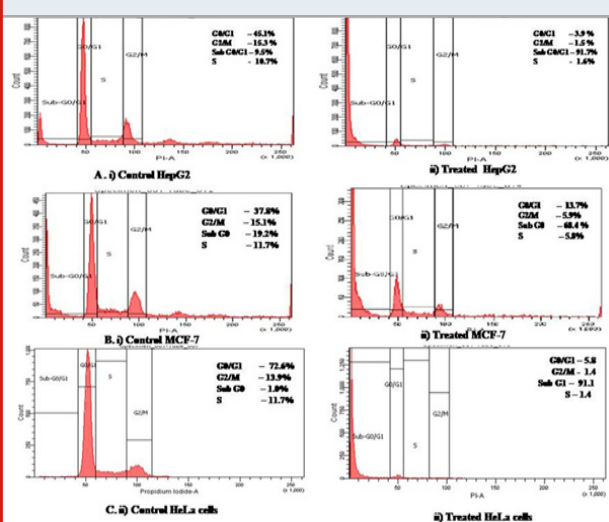


Figure 6: Flow cytometry analysis of cell cycle stages by propidium iodide staining in cancer cells untreated and treated with GF7.

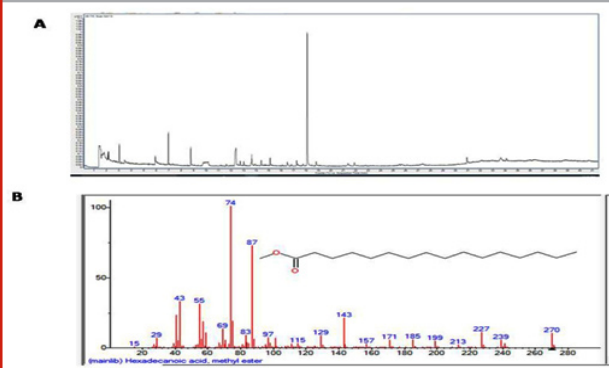


As the bioactive fraction GF7 from *G. acerosa* showed significant apoptogenic potential towards cancer cells, this fraction was subjected to GC-MS method for characterization. From the results, we found hexadecanoic acid in the major peak at RT 18.3 min (Figure 7).

Apoptosis induction is an important strategy to control and manage cancer. Cell cycle arrest at key points such as G1, S, G2 and G2/M drives the cells through apoptosis.

Many synthetic or natural anticancer compounds lead to cell cycle arrest and apoptosis as reported by many researchers (Alabsi et al., 2013; Wafa et al., 2020). In the current study, we could establish the anticancer potential of the methanol extract of the marine alga *G. acerosa*, to cervical, breast and liver cancer cell lines at lower concentrations of 25 and 50 µg/ml when compared to earlier reports (Murugan and Iyer, 2013; Lakmal et al., 2014; Duraikannu et al., 2014; Begum et al., 2018).

Figure 7: GC-MS analysis of fraction GF7. A. Gas Chromatogram and B. mass spectrum of fraction GF7 along with the Structure of Hexadecanoic acid



In a previous study, the methanol extract of *G. acerosa* has shown cytotoxicity to HL60 cancer cell line at higher concentrations of 100 and 200 µg/ml, where the IC₅₀ value was reported as 104.4 µg/ml (Lakmal et al., 2014). In another much recent study the IC₅₀ value of *G. acerosa* against lung cancer cells was reported as 1.5 mg/ml (Begum et al., 2018). As compared to these earlier study reports, the crude methanol extract of *G. acerosa* in our present study we got higher cytotoxicity to all the tested cancer cell lines with IC₅₀ values <50 µg/ml for 96h of treatment duration and after purification by TLC, the bioactive GF7 fraction had much greater cytotoxicity with IC₅₀ values with IC₅₀ values further decreasing, with a range of 27 µg/ml -39 µg/ml towards HeLa, MCF-7 & HepG2 cancer cells.

The bioactive compounds which are present in crude extracts at very low concentrations get concentrated during the purification process and that could be the reason for higher specificity and low IC₅₀ value of *G. Acerosa* purified fraction in the current study. There are reports from many other red algae having cytotoxicity to human cancer cell lines (Harada and Kamei, 1997; El, Baroty et al., 2007). In a previous study, *G. acerosa* was reported to reduce cancer cell growth and tumor weight in mice at 200mg/kg body weight (Duraikannu et al., 2014). In another study *G. acerosa* crude extract was reported as to have cytotoxicity to cancer cells and they also reported that the presence of polyphenols and flavonoids in the ethyl acetate extract of *G. acerosa* (GAE) being is the reason for inhibition of lung cancer cell proliferation at 1.5 mg/mL (Begum et al., 2018).

On the contrary, in our study we report the presence of Hexadecanoic acid in the bioactive fraction of *G. acerosa* as per the results of GC-MS analysis. Hexadecanoic acid is a well documented for anticancer potential and is reported from some other marine algae also (Parveen and Nadumane 2020). To the best of our knowledge, we are reporting its presence for the first time from *G. acerosa*. We also found that the basis for the anticancer mechanism of *G. acerosa* is cell cycle arrest, accumulation of cells in subG1 phase leading to the apoptosis of treated cancer cells. In our study apoptosis induction was clearly seen in DNA fragmentation, increased caspase activity, cell cycle stages, LDH cytotoxicity and highly decreased cancer cell counts due to *G. acerosa* treatment. Though there are previous studies reporting the in-vitro and in-vivo cytotoxicity of the crude extracts of *G. acerosa*, no attempts were made to purify the active component involved in the reported activity.

To the best of our knowledge, this study is the first attempt to analyse the mechanism of anti cancer action of TLC purified fraction of *G. acerosa* and characterizing the bioactive compound. Through the results of the present study, we report the presence of hexadecanoic acid in the bioactive fraction and assume it to be the reason for the observed anti cancer activity of *G. acerosa*. Fatty acids comprise one of the predominant phytochemical constituents in marine macroalgae and reported to have many biological activities (Hema et al., 2011). Palmitic acid (n-hexadecanoic acid) is documented to have anticancer potential with apoptosis inducing ability in human cancer cell lines (Yoo et al., 2007; Mericli et al., 2017; Parveen and Nadumane 2020). Our results too are validating these earlier reports.

CONCLUSION

Through the present work, it can be concluded that *G. acerosa* has cytotoxic activity against human cancer cell lines along with no toxicity to normal human lymphocytes. Presence of Hexadecanoic acid in the bioactive fraction GF7, might be the reason for the anti cancer activity of *G. acerosa*. Further studies need to be carried out for the development of an efficient drug against cancer from this promising Marine red alga.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

REFERENCES

- Aggarwal, B.B., Danda, D., Gupta, S. and Gehlot, P. (2009). Models for prevention and treatment of cancer: Problems vs. promises. *Biochemical pharmacology*, 78(9):1083–1094
- Ahn C.B., Jeon Y.J., Kang D.S., Shin T.S. & Jung B.M. (2004). Free radical scavenging activity of enzymatic extracts from a brown seaweed *Scytosiphon lomentaria* by electron spin resonance spectrometry. *Food Research International*, 37: 253 – 258.
- Alabsi AM, Ali R, Ali AM, Al-Dubai SA, Harun H, Abu Kasim NH, Alsalahi A. (2012). Apoptosis induction, cell cycle arrest and in vitro anticancer activity of gonothalamin in a cancer cell lines. *Asian Pac J Cancer Prev.*; 13(10):5131–5136.
- Aneiros A. & Garateix A. (2004). Bioactive peptides from marine sources: pharmacological properties and isolation procedures. *Journal of Chromatography B* 803: 41 – 53.
- Begum, F. M. S., Chitra, K., Joseph, B., Sundararajan, R., & Hemalatha, S. (2018). *Gelidiella acerosa* inhibits lung cancer proliferation. *BMC complementary and alternative medicine*, 18.
- Ashraf M A (2020). Phytochemicals as Potential Anticancer Drugs: Time to Ponder Nature's Bounty. *BioMed Research International*. 2020, Article ID 8602879, 7 pages.
- Bai, J. Li, Y. and Zhang G (2017). Cell cycle regulation and anticancer drug discovery, *Cancer Biology & Medicine*, 14: 348.
- Bernecker, A., In Whertmann, I.S. and Cortes, J. (2009). Marine benthic algae, *Marine Biodiversity of Costa Rica, Central America Springer*, 538: 109-118
- Blunt J.W., Copp B.R., Keyzers R.A., Munro M.H.G., Prinsep M.R. (2014). Marine natural products. *Nat Prod Rep*. 31(2):160.
- Boopathy, S.N. and Kathiresan, K (2010). Anticancer drugs from marine flora: an overview, *Journal of oncology*. 1-18.
- Chang, H.Y. and Yang, X. (2000). Proteases for cell suicide: functions and regulation of caspases, *Microbiology and Molecular Biology Reviews*. 64(4): 821-46.
- Coppejans E., Leliaert F., Dargent O., Guneseakra R. & Clerck O.D. (2009). Sri Lankan seaweeds-methodologies and field guide to the dominant species. *Abc Taxa*, 6: 265.
- Devi K.P., Suganthi N., Kesika P., Karutha Pandian S. (2008). Bioprotective properties of seaweeds: In vitro evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. *BMC Complementary Alternative Medicine*. 8:38.
- Dimmick, I. (2011). Multiple flow cytometry applications: use of DAPI. *Biomedical Scientist*, 55(8), 541.
- Duraikannu, K., Rani K.S., Anithajothi, R., Umagowsalya G, Ramakritinan CM. (2014). In-vivo anticancer activity of red algae (*Gelidiella acerosa* and *Acanthophora spicifera*). *International Journal of Pharmaceutical Sciences and Research*. 5(8): 3347–52.
- El-Baroty, G.S., Moussa, M.Y., Shallan, M.A., Ali, M.A. et al. (2007). Contribution to the aroma, biological activities, minerals, protein, pigments and lipid contents of the red alga: *Aspara gopsistaxiformis* (Delile) Trevisan. *Journal of applied science Research* 3(12): 1825–1834.
- Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic pathology*, 35(4), 495–516
- Harada, H. and Kamei, Y (1997). Selective cytotoxicity of marine algae extracts to several human leukemic cell lines, *Cytotechnology*. 25(1–3), 213
- Hema R., Kumaravel S and Alagusundaram K (2011). GC/MS Determination of Bioactive Components of *Murraya koenigii*. *Journal of American Science*. 7(1):80–83
- Jayaseelan E.C., Kothai S., Kavitha R., Tharmila S. & Thavaranjit A.C. (2012). Antibacterial activity of some selected algae present in the coastal lines of Jaffna Peninsula. *International Journal of Pharmaceutical and Biological Archives*. 3: 352 – 356.
- Kılınc, Berna., Semra C., Gamze T. et al. (2013). "Seaweeds for food and industrial applications," In *Food industry*. IntechOpen. 735–748
- Lakmal, H.H.C., Samarakoon, K.W., Lee, W.W. et al. (2014). Anticancer and antioxidant effects of selected Sri Lankan marine algae. *Journal of the National Science Foundation of Sri Lanka*, 42(4): 315–23.
- Mericali, F., Becer, E., Kabaday, H., Hanoglu, A. and Hanoglu, D.Y. et al., (2017). Fatty acid composition and anticancer activity in colon carcinoma cell lines of *Prunus dulcis* seed oil. *Pharmaceutical biology*. 55(1): 1239–1248.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*. 65(1-2): 55–63
- Murugan, K. and Iyer, V.V. (2013). Differential growth inhibition of cancer cell lines and antioxidant activity of extracts of red, brown, and green marine algae. *In Vitro Cellular & Developmental Biology-Animal*. 49(5): 324–34
- Newman, D.J. and Gordon M.C. (2016). Drugs and drug candidates from marine sources: An assessment of the current "state of play". *Planta medica*. 82(09/10): 775–789
- Ngo D.H., Wijesekara I., Vo T.S., Ta Q.V. & Kim S.K. (2011). Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. *Food Research International*. 44: 523 – 529
- Parveen S and Nadumane VK (2020). Anti-angiogenesis and apoptogenic potential of the brown marine alga,

- Chnoospora minima*. Future Journal of Pharmaceutical Sciences, 6:19.
- Pazarowski, P. and Darzynkiewicz, Z. (2004). Analysis of cell cycle by flow cytometry. In Checkpoint controls and cancer. 281: 301-11
- Samarakoon K.W., Nam O.K., Ko J.Y., Lee J.H., Kang M.C., Kim D., Lee B.J., Lee J.S. & Jeon Y.J. (2013). Purification and identification of novel angiotensin-I converting enzyme (ACE) inhibitory peptides from cultured marine microalgae (*Nannochloropsis oculata*) protein hydrolysate. Journal of Applied Phycology. 25: 1595 – 1606.
- Saraste, A. and Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. Cardiovascular research. 45(3): 528-37
- Shidoji, Y and Ogawa, H. (2004) Natural occurrence of cancer-preventive geranylgeranoic acid in medicinal herbs. Journal of lipid research. 45(6): 1092-1103
- Strober W. (1997) Trypan blue exclusion test of cell viability. Current protocols in immunology, 21 (1); A-3B
- Taskin, E., Ozturk, M., Taskin, E. and Kurt, O. (2007). Antibacterial activities of some marine algae from the Aegean Sea (Turkey). African journal of Biotechnology, 6(24): 2746-2751
- Tay K C., Tan LTH., Chan C K. et al. (2019). Formononetin: a review of its anticancer potentials and mechanisms. Frontiers in Pharmacology, 10.
- Wafa OMA, Daabees HMG, Badawi WA. (2020). 2-Anilinopyrimidine derivatives: Design, synthesis, in vitro anti-proliferative activity, EGFR and ARO inhibitory activity, cell cycle analysis and molecular docking study. Bioorganic Chemistry. 99:103798.
- Weyermann, J., Lochmann, D. and Zimmer, A. (2005). A practical note on the use of cytotoxicity assays. International journal of pharmaceuticals, 288(2): 369-376
- Yoo, Y.C., Shin, B.H., Hong, J.H., Lee, J., et al. (2007). Isolation of fatty acids with anticancer activity from *Protoetia brevitarsis* Larva. Archives of pharmacal research. 30(3): 361-365
- Yuan YV, Walsh NA (2006). Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds. Food and Chemical Toxicology. 44(7):1144-1150
- Yubin J, Guangmei Z. Pharmacological Action and Application of Available Antitumor Composition of Traditional Chinese Medicine. Heilongjiang, China: Heilongjiang Science and Technology Press; 1998