Isolation of Fatty Acid Constituents from *Ulva Fasciata* and its Anticancer Activity on HeLa Cells

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Abstract
Macroalgae are found as a great source of biologically active compounds important in pharmaceutical industry. In this study, 8 algal samples namely Ulvafasciata (Green algae), Padinagymnosphora, Sargassamwightii, and Turbinariaornate (brown algae), Gracilariacorticata, Halimedamacrolopa, Halymeniadilatata, and Gracilariacrassa (red algae) were collected and identified its fatty acid compositions. U. fasciatawhich showed high fatty acid constituents was further analysed for its anticancer activity against Hela cells by MTT assay and AO/EtBr staining. The results confirmed that U. fasciata could inhibit the Hela cells by cytotoxic activity and by inducing apoptosis and thus could be used as apontential source in treating cancers.

Keywords: Anti-Cancer Activity, Cytotoxicity, Fatty Acid Constituents, FAME, Macroalgae, Ulvafsciata

Introduction

Marine natural productsor MNPs have a wide range of biological actions, including anti-diabetic, antibacterial, anti-inflammatory, antiviral, and anticancer properties (Mayer et al., 2013; Petit and Biard, 2013). More than 22,000 compounds from the marine environment have been identified (Petit and Biard, 2013). Fungi, bacteria, sponges, coral, and micro- and macroalgae are among the organisms that produce MNP. Terpenes, alkaloids, peptides, toxins, and lipids are the most common bioactive compounds discovered in marine species. Furthermore, the isolation of novel compounds from marine sources frequently leads to the discovery of molecules with a wide range of medicinal and industrial applications (Blunt et al., 2015).

Algae are photosynthetic creatures that are divided into three phyla: Ocrophyta, Chlorophyta, and Rhodophyta. Algae are important sources of a variety of complex lipids with a variety of potential applications in the food, cosmetics, and pharmaceutical industries (Hossain et al 2005; Abd El Baky et al 2009; Abd El Baky and Baroty, 2013). Antibacterial, antiviral, anticancer, antiinflammatory, antiprolierative, and antioxidant properties have been found in the lipids of marine macroalgae (Gerasimenko et al 2010; Goecke et al 2010; El Baz et al 2013).

Polyunsaturated fatty acids (PUFA) and vitamins are abundant in algal lipids, in addition to bioactive substances such as phenolic and terpene chemicals. Algae from the sea are recognised to be a good source of nutrition. PUFAs help to prevent atherosclerosis and lower the risk of heart and blood vessel disease (Gerasimenko et al 2010). They have a high potential for adaptation to harsh environmental situations.

They have long been utilized as a food supplement as well as for medical purposes. Many crude extracts and isolated compounds from algae have been researched for their anticancer effects as some algae have been utilised in the treatment of tumours, and these investigations revealed that algae include some new compounds with therapeutic characteristics for human diseases.Unlike terrestrial plants, algae can synthesise a wide range of unique substances such phlorotannins (oligomers of phloroglucinol produced via the acetate–malonate pathway) and specialised polysaccharides (e.g. sulfated) (Li et al., 2011; Brownet al., 2014; Moussavou et al 2014).

Fatty acids (FA), particularly omega-3 polyunsaturated fatty acids (n-3 PUFA), are among the marine substances with remarkable action, providing numerous health benefits for chronic diseases such as cancer (Berquin et al., 2008), hyperlipidemia, and coronary heart disease (Jump et al., 2012).

Lipids and fatty acids have a wide range of structural complexity, which contributes to their medicinal efficacy. Small levels of saturated fatty acids have been shown to help reduce cardiovascular disease. Polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) found in marine algae are good to human health and may assist to prevent cardiovascular disease [Ruxton et al 2004].

Humans, on the other hand, are unable to synthesise PUFAs, which are prevalent in both macro and microalgae. Omega-3 and omega-6 fatty acids (e.g., EPA and AA) make up the majority of PUFAs in microalgae [Zheng et al 2013]. PUFAs influence blood coagulation and blood pressure, as well as brain and nervous system function. Furthermore, they lower the risk of a variety of chronic disorders, including diabetes and cancer. Eicosanoids, well-known inflammation mediators, are also produced by them, which modulate inflammatory responses [Wall et al 2010]. Dietary supplements containing omega-3 and omega-6 PUFA from macroalgae are already available [Fleurence et al 1994]. Omega-3 fatty acids (EPA and GLA) and omega-6 fatty acids are abundant in red and brown algae (e.g., AA and linoleic acid). Octadecatetraenoic acid, an omega-3 PUFA, is abundant in brown algae *Laminariaochroleuca* and *Undariapinnatifida* [Sanchez-Machado et al 2004]. Hexadecatetraenoic (omega-3), oleic (omega-9), and palmitic acids were discovered to be abundant in the green seaweed *Ulvapertusa* (SFA). Oleic, palmitic, and linolenic acids are found in the lipid fraction of the microalgae C. vulgaris. Short-chain fatty acids are also found in the green microalga Haematococcus sp. Long-chain PUFAs are frequently employed as food additives and nutritional supplements [Rodríguez-Meizoso et al 2010]. GLAs are a precursor of leukotrienes, prostaglandins, and thromboxans, which govern inflammatory, immunological, and cardiovascular problems. Spirulina sp. is a promising source of GLAs. Along with DHA, cyanobacteria and some green algae include bioactive fatty acids such as palmitic, oleic, and lauric acids.

Sterols are a type of lipid found in a wide range of macro and microalgae. Sterols and certain of its derivatives have biological properties, such as anti-inflammatory properties. In vascular endothelial cells, sterols from Spirulina cause the synthesis of plasminogen-activating factor. Brown algae include fucosterol, ergosterol, and chondrillasterol, while red algae have cholesterol [Lordan et al 2011, Bouzidi et al 2019].

Furthermore, earlier research on algae and plants has revealed that natural compounds derived from Brazilian red propolis and Antarctic macroalgae have antitumoral properties (Frozza et al., 2013; Gambato et al., 2014; Martins et al 2018). Cancer is one of the most serious diseases endangering humanity's survival today, and there is no known cause. Chemotherapy, radiation therapy, and/or surgical intervention are used to treat breast cancer, which is the most common cancer in women and the second most common cancer worldwide (Ilhan et al., 2017; Miller et al.,2016). Most anticancer medications are ineffective against specific stages of carcinogenesis, prompting researchers to look for new treatments for breast cancer (Zanoaga et al., 2018). Administration of red algae G acerosa and A spicifera extracts significantly alters the level in cancer-bearing animals (Duraikannuet al., 2014). The anticancer activity has been observed in many algal based compounds like polysaccharides, steroids, fatty acids, carotenoids, halogenated compounds, and peptides. In addition

to plants, many widely described chemotherapeutic agents from microorganism such as cyanobacteria and algae have been discovered by in vitro assays (ImenSaadaouiet al., 2020). The reduction in cell viability and induction of apoptosis of cancer cells were observed from the anticancer compounds extracted from macroalgae. *Dictyotadichotoma (D. dichotoma)* and four other brown algae species: *Bifurcariabifurcata, Cystoseiratamariscifolia, Desmarestialigulata*, and *Halidryssiliquosa* have exhibited a substantial cytotoxic activity to cancer cell lines (Amina El-Shaibanyet al., 2020).

PUFA has been demonstrated in epidemiological studies to reduce the incidence of a variety of disorders, including cancer (Gerber, 2012). In addition, D'Archivio et al., 2018; Zanoaga et al., 2018) have discovered that n-3 PUFA have an inhibitory impact in the development and progression of breast cancer. Though the exact mechanism by which n-3 PUFAs affect cancer cell proliferation is unknown, it is assumed that these acids may disrupt the cell cycle or cause cell death through necrosis or apoptosis (Corsetto et al., 2011). Furthermore, recent research has revealed that n-3 PUFA can be integrated into membranephospholipids and lipid rafts on breast cancer tumour cells, resulting in alterations in cell membrane fluidity and structure (Corsetto et al., 2012). Protein composition or activities of proteins that serve as ion channels, transporters, receptors, signal transducers, or enzymes may be affected by changes in membrane physiology (Fabian et al., 2015; Vander Sluis et al., 2017). Although the fatty acid makeup of several algal species has been investigated, a huge number of taxa remain unknown (El Gamal, 2010; Garcia-Vaquero and Hayes, 2016).

In the present research, we carried out the screening and identification of algal samples, estimated the total fatty acid content and their constituents. The algae with high fatty acid content was further proceeded to investigate its anticancer activity on HeLa cells.

Materials and Methods

Collection of Macroalgal Samples

The macroalgal samples were collected from

Extraction and Determination of Total Lipid Content

The soxhlet extraction method was implemented with 100 g biomass (small pieces 2–3 cm) on a soxhlet system for 6 h of extraction process with 250 ml of solvents. The lipid residue was dried in an oven at 60°C and weighed in order to obtain the lipid content (%) in dry biomass (Satpati et al., 2015).

Estimation of Free Fatty Acids

The amount of fatty acids present in the sample was estimated by applying the protocol developed by Sadhasivam and Manickam, 1996. 1G of oil or melted fat was dissolved in 50mL of the ethyl acetate solvent in a 250mL conical flask. A few drops of phenolphthalein were added to the mixture. Then, titration of the contents was carried out against 0.1N potassium hydroxide. The mixture was shaken well until a pink colour was developed that persisted for fifteen seconds. The amount of free fatty acids in the sample was then calculated by the formula

Acid value (mg KOH/g) = Titer value x Normality of KOH x 56.1/ Weight of the sample (g)

Fatty Acid Methyl Ester (FAME) Production by Transesterification

FAMEs were produced by the transesterification method. The lipid samples after extraction were taken into a 10 mL screw-cap glass tube (BOROSIL, Mumbai, India) in which the transesterification reagents methanolic hydrochloric acid (1 : 4 v/v) was added. The tube was kept in a glass beaker containing double distilled water and heated in an hot air oven at 70°C for 6-8h. The solution was allowed to cool and centrifuged at 10,000rpm for 10min to avoid particulate matters. The FAME extract was then transferred to GC-MS auto sample vials for analysis (Satpati et al., 2015).

Thin Layer Chromatography

The components of extracted lipids were separated by modified thin-layer chromatography (TLC) method. The merck ready made thin layer sheets were used (50mm X10mm). The lipid content was dissolved in chloroform or methanol and 50µL of lipid was loaded on TLC plate and was placed in the chamber using solvent system (chloroform:

acetone: methanol: aceticacid:water (50:20:10:10:5 v/v/v/v/v). The chamber was immediately closed with the cover and run for approximately 1 h, until the solvent front reached the upper line. The plate was removed and left to dry under the fume hood. After complete dryness, the lipids were visualized by exposing to iodine vapour under the fume hood.

Extraction of Lipids with Different Solvents

Lipid extraction was done using five different solvents (1) Hexane, (2) Chloroform: methanol (2:1), (3) Chloroform: hexane (1:1), (4) Chloroform: hexane (1:2), (5) Dichloromethane: methanol (2:1) (R. Byreddy et al., 2015).

Determination of Cytotoxicity Effects on HeLa Cell Lines

Minimal Essential Media Preparation

Weighed 9.5 grams of MEM dissolved in 950ml of pre-sterilized double distilled water and mixed well. Sodium hydrogen carbonate 2.2 grams was dissolved in 50ml of pre-sterilized double distilled water and mixed well. Both bottles were sterilized at 15 lbs, 121°C, for 15 min and allowed to cool at room temperature. Then, 0.3grams of L-glutamine was weighed and dissolved in 10ml of pre-sterilized double distilled water and poured. Antibiotics – streptomycin, penicillin G and amphotericin B, 1mg of each was weighed. Each antibiotic was dissolved separately in 1ml of pre-sterilized double distilled water. pH was checked and adjusted to 7.2-7.4 with diluted HCL. All the contents were syringe filtered and stored at 4°C.

10% Fetal Calf Serum (FBS)

10ml FBS was syringe filtered and added 90ml of MEM media. One aliquot of the prepared MEM was kept for 2 days at 37°C and checked for sterility, pH drop and floating particles.It was then checked for contamination and stored at +4 oC.

Cell Culture-HeLa Cells

The medium and TPVG were brought to room temperature. The tissue culture flask was monitored for growth, cell degeneration, pH and turbidity and selected the flask for splitting. The HeLa cells were washed with MEM medium twice and added 4ml (pre-warmed to 37°C) over the cells and allowed TPVG for the reaction to occur for 1-2 minutes. TPVG was discarded and added 5ml of 10% MEM. The cell clusters were broken by gently pipetting back and forth with pipette. It was then added with 20ml of growth medium to tissue culture flask and transferred the cells into 96 well plates.

Cell-Viability Assay

The viability of HELA cellswas determined by the MTT assay (Mosmann et al., 1983). Cells (1 × 10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO_2 incubator for 72 hours. Then, added various concentrations of the samples in 0.1% DMSO for 24 hrs at 5 % CO_2 incubator. The images were viewed under Inverted microscope 40X. After removal of the sample solution and 20µl/well MTT reagent was added. Viable cells were determined by the absorbance at 540nm. 50% inhibition of cell viability (IC50) value was determined graphically. The effect of the samples on the proliferation of HELA cells was expressed as the % cell viability, using the following formula:

Calculation

% cell viability = A540 of treated cells / A540 of control cells × 100%

AO/EtBr Staining

Morphological studies related to apoptosis by Acridine orange/Ethidium Bromide (AO/EtBr) staining was carried out by employing the method developed by Darzynkiewicz et al 1994.

Results and Discussion

The collected algal samples were identified as Green algae (*Ulvafasciata*), brown algae (*Padinagymnosphora*, *Sargassamwightii*, and *Turbinariaornata*), red algae (*Gracilariacorticata*, *Halimedamacrolopa*, *Halymeniadilatata*, and *Gracilariacrassa*).

The total fatty acid content found in the algae was shown in the Table 1 that ranged between 0.2% and 1.68%. The high amount of fatty acids was seen in Brown Algae: *Ulvafasciata* (1.68%) followed by which Green Algae: *Turbinaria ornate* (1.63%) was

the second algae sample with high fatty acid content. A very low amount of fatty acids was present in Red algae: *Halimedamacrolopa* (0.20%).

Table 1 Estimation of Fatty Acid Constituents from the Collected Macroalgal Samples

Sample name	Fatty acid Value (%)
Green Algae: *Ulvafasciata*	1.68
Brown Algae: *Padinagymnosphora*	1.09
Brown Algae: *Sargassamwightii*	1.27
Brown Algae: *Turbinariaornata*	1.63
Red Algae: *Gracilariacorticata*	1.28
Halimedamacrolopa	0.20 %
Halymeniadilatata	0.40 %
Gracilariacrassa	0.21 %

The fatty acid composition analysis showed the presence of 40 various fatty acid constituents from the 8 collected macroalgal samples. Our alga of interest (based on the highest fatty acid content) - *Ulvafascita* possessed 5 compounds namely Octadecane, Hexadecane, 1-Nonadecene, 1-Pentadecene, and 4-Trifluoroacetoxypentadecane. The results were shown in the Table 2.

Table 2 Fatty Acid Constituents of Green Algae:

Retention time	Name of the Compound	Molecular formula	M. Wt. $gmol^{-1}$	Area %
14.197	Octadecane	$C_{18}H_{38}$	254.494	0.26
14.374	Hexadecane	$C_{16}H_{34}$	226.448	0.29
16.574	1-Nonadecene	$C_{19}H_{38}$	266.5	0.20
16.763	1-Pentadecene	$C_{15}H_{30}$	210.3987	2.77
18.096	4-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	324.42205	0.23

The separation of fatty acids by thin layer chromatography using five different solvents revealed that Chloroform: methanol (2:1) showed maximum lipid extraction 4.21% and followed by hexane (2.91%). The results were shown in the Table 3. It is important to note that similar type of results was observed in the research carried out by Romalo et al. (2019).

Table 3 Effect of different Solvents in Separation of Fatty Acids in *U. fasciata*

Solvents	Fatty acid Value (%)
Hexane	2.91
Chloroform: methanol (2:1)	4.21
Chloroform: hexane (1:1)	0.82
Chloroform: hexane (1:2)	1.74
Dichloromethane: methanol (2:1)	1.1

The cytotoxicity and cell viability assay of HeLa cell lines by U. *fasciata* showed decrease in cells after treatment with various concentrations of the lipid extract. From the Table 4, we could analyse that the cell proliferation was strongly inhibited in a concentration dependent manner as the number of viable cells got decreased as the concentration of the algal extracts increased. Furthermore, DMSO showed reduced number of viable cells when compared to control. The anticancer activity of the alga on HeLa cell lines was shown in the Fig. 1. The cytotoxic activity by the lipid extracts of *U. fasciata* might be due to the cytotoxic action of the lipids.

The morphological analysis performed with AO/EtBr dual staining revealed that the majority of HeLa cells after treatment with *U. fasciata* macroalgal sample underwent apoptosis. The results were shown in the Fig. 2 which depicted the apoptosis induction by chromosome condensation.

Table 4 Anticancer Activity on HeLa Celllines by *U. fasciata*

Concentration µg/ml	Absorbance 540nm	% cell Viability
500	0.7	5.1
250	0.13	9.6
125	0.24	17.7
62.5	0.49	36.2
31.2	0.66	48.8

15.6	0.81	60
DMSO	1.32	97.7
Control Cells	1.35	100

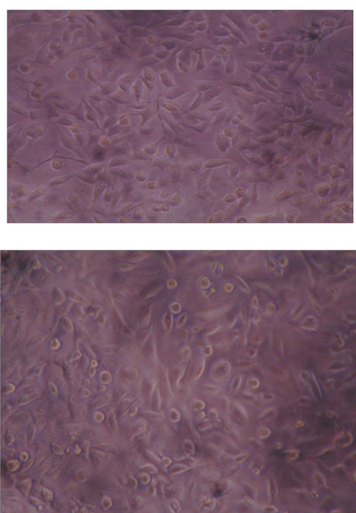

Fig 1 Anticancer Activity of *U. fasciata* on HeLa Cell Lines

Macroalgae such as marine green, brown and red algae can be regarded as natural bioactive chemical factories. Fucoidan, a sulfated polysaccharide isolated from Fucus spp., Sargassum spp., Turbinaria spp., and Padina spp., has been found to have anti-carcinogenic effect against colon and breast cancer cell lines (Isnansetyo et al., 2017). Various chemicals isolated from Laminariadigitata, Cymopoliabarbata, Lithothamnioncalcareum, and Undariapinnatifida have been shown to exhibit anticancer action against HT-29 colon cancer cells in a number of investigations (Yang et al., 2013; Badal et al., 2012). Furthermore, several chlorophyte-derived compounds have shown cytotoxic action against breast, skin, lung, and prostate cancer cell lines (Tavares-Carreón et al., 2020).The inflammatory markers nitric oxide, TNF-, and IL-6 were reduced by two enone fatty acids from Gracilariaverrucosa, (E)-10-Oxooctadec-8-enoic acid and (E)-9-Oxooctadec-10-enoic acid (Arif et al 2004). *Sargassummarginatum* inhibited the growth of human promelocytic leukaemia cells. In vitro, lipid extracts of Ginsens marc inhibited the proliferation of human hepatoma (HepG2) and breast (MCF7) cancer cells (Lee et al 2009).Araki et al (1990) discovered that the overall lipid content of macroalgal species varied depending on their genetic origin. In addition, the accumulation of lipids in macroalgal species was influenced by climate and geography (Manivannan et al 2008). El Baz et al (2014) also found higher amount of fatty acids in U. *fasciata* among the samples investigated.

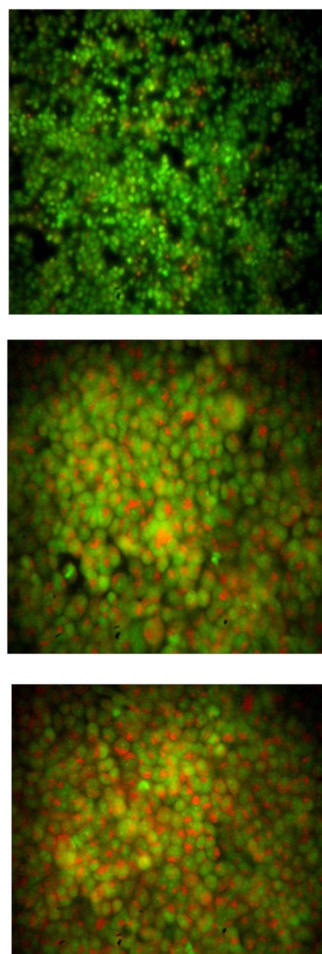

Fig 2 Acridine Orange/Ethidium Bromide (AO/EtBr) Dual Staining of HeLa Cells Treated with *U. fasciata*. A-control; B-62.5µg; C- 500 µg. Green Colour Indicates Live Cells; Orange Colour Indicates Apoptotic Bodies with Chromatin Condensation

It is known that apoptosis is a controlled cell death process that removes damaged cells. Apoptosis induction is a useful strategy in cancer treatment. Apoptotic cells exhibit a number of cellular and molecular biological characteristics, including cell

shrinkage, DNA fragmentation, and activation of the caspase cascade (the extrinsic pathway, which is one of the two main signaling pathways; the other one the extrinsic pathway, in which external stimuli operate on an intracellular target, with mitochondria playing a key part) [Germain et al 1999; Santos et al 2016]. The Bcl 2 family of proteins is a major regulator of mitochondrial outer membrane permeabilization and can inhibit the release of cytochrome c and other components that are required for caspase activation and apoptosis [Cui and Placzek, 2018]. Apoptosis necessitates the involvement of two types of caspases. The initiator caspases are the first type, whereas the executioner caspase 3 [Kim and Kim, 2018] is the second. The latter catalyzes the cleavage of PARP-1 which is overexpressed in various types of cancers. Upregulation of the p53 protein and the p53-regulated gene p21 is also linked to caspase 3 cell death [Ercolano et al 2019]. When p21 is activated, it upregulates p53 and causes the cell cycle to be blocked in the G1 and/or S phases [Liu et al 2017]. Resistance to cell-death stimuli is one of the most common features of malignancies.

According to studies, active chemicals bind to specific cancer-associated receptors or cancer cell-specific molecules, triggering pathways that cause cancer cell death [Harada et al 1997].

Amina El-Shaibany et al.,(2020) observed the reduction in cell viability and induction of apoptosis of cancer cells from the anticancer compounds extracted from macroalgae: *Dictyotadichotoma (D. dichotoma)* and four other brown algae species: *Bifurcariabifurcata*, *Cystoseiratamariscifolia*, *Desmarestialigulata*, and *Halidryssiliquosa* showing a substantial cytotoxic activity to cancer cell lines. Thus, macroalgae are found as reservoirs of various metabolic compounds involved in curing many diseases, including cancer.

Conclusion

In conclusion, lipids are found in macroalgaes which are a natural source of variety of biologically active compounds. Among the 8 algae samples, *Ulvafasciata* has been known to have higher fatty acid contents when compared to other algal samples under study. The alga was identified with 5 various fatty acid compounds that confirmed anticancer activity against HeLa cells by reducing the number of viable cells due to the cytotoxic action and also by apoptosis. Thus, the fatty acid constituents from the alga could be a potentcompound in pharmaceutical researches.

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