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EVALUATION OF CYTOTOXIC ACTIVITY OF *CHORDA FILUM* FROM THE LEBANESE COAST AGAINST MCF-7 CANCER CELL LINE

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ABSTRACT
The discovery of cancer drugs and the identification of agents that effectively destroy cancer cells or stop their growth without toxicity to normal cells, and reduce the opportunity for the development of drug resistance by cancer cells and reduce side effects would have an important impact on cancer therapy. Many studies have demonstrated the anti-cancer effect of natural materials from marine resources especially marine algae that elicit unique and unexpected biological activities. In this report, we investigated the cytotoxic activity of a crude extract from *Chorda filum* (*Chordaceae*), a marine brown algae collected from the Lebanese coast, against MCF-7 human breast cancer cell line using trypan blue exclusion test. This ethanol-acetone extract showed cytotoxic activity against MCF-7 cancer cell line with IC50 value of 15,373 µg/ mL after 48 hours of treatment. Further research designed to isolate and identify novel and efficient anticancer drug candidates from this seaweed extract need to be explored.

KEYWORDS: brown algae; *Chorda filum*; cytotoxic activity; MCF-7; trypan blue exclusion test.

INTRODUCTION
Cancer is globally the first-leading cause of death in economically developed countries and the second-leading cause of death in developing countries.[1] Cancer is a continually increasing threat for the global population, and considered to be raised with aging and adaptation to cancer-causing behaviors.[2] Breast cancer is one of the most common malignancies in women worldwide,[3] with half of the cases seen in economically developing countries.[4] Besides, 14% cancer deaths out of 23% of total breast cancer-cases in females were reported in 2008.[5] Current treatment of breast cancer involves chemotherapy, surgery, radiation therapy and hormonal therapy.[6] Of these, chemotherapy and radiotherapy are known to cause severe side effects. Other treatments like targeted therapies such as monoclonal antibodies and hormonal therapies are ineffective in several breast cancer types.[7] So, because of the particularity of more than one hundred cancer types and the high cytotoxicity of chemotherapy and other actual cancer treatments to normal cells and their undesirable side effects, there's a huge search for new bioactive molecules capable of substituting the ones that are being used.[8] In this context, natural products derived from plants, marine organisms and micro-organisms have interested many scientists.[9] In fact, the marine environment, the cradle of life, is an ecosystem made unique because of the amazing biodiversity it contains. The seas and oceans were and still are a place where the evolutionary processes and development flourish and diversify. The oceans are the largest volume offered to life on the "blue" planet and now between 235 000 and 250 000 species living in the oceans are described. So long, the marine environment has proven to be a rich source of biological and chemical biodiversity.[10] Indeed, from a relatively small number of marine species that have been studied till now, thousands of chemical compounds have been isolated and studied for their potential as useful products in various fields. Oceans then represent a largely untapped resource for the discovery of new products with potential as pharmaceuticals, cosmetics, agrochemicals, nutritional supplements, molecular probes, enzymes, and other chemicals used in different sectors.[11] Among these marine organisms, marine algae (also called seaweed) have been one of the natural sources of bioactive compound.[12] Marine algae serve as an outstanding potential natural resource for a wide range of

biologically active compounds that are reported to exhibit potential prospective in the medicinal, nutritional and cosmetic applications. Bioactive compounds obtained from marine seaweeds prove to be exceptionally beneficial with chemical diversity. Nowadays, various species of algae are important not only in food, but also in the production of extracts such as polysaccharides (agar, carrageenans, alginates ...), polyphenolic compounds, terpenes, alkaloids... These extracts are used in several applications in food, medical, pharmaceutical, cosmetic and industrial fields.[13-14] This study is particularly interested in the application of marine macroalgae in the medical field. Indeed, in the search for new therapeutic responses to mitigate the inefficiency or lack of certain treatments, the underwater world is still a great place of discovery and a considerable reservoir of molecules. Among all marine and freshwater organisms, it is the algae that are the main sources of biomolecules of interest. There have been significant recent interest in the use of macroalgae based on their valuable chemical components many of which have multiple biological activities with significant applications in food, in cosmetics, in the agricultural and horticultural sectors and in human health (medicine and pharmacy). The compounds of particular interest include pigments, lipids, fatty acids, proteins, polysaccharides and phenolic compounds which have all a considerable diversity between and within taxa.[15] The chemical composition of natural algal populations is influenced by changes in environmental parameters, including light, temperature, nutrients and salinity, as well as biotic interactions.[16] In fact, marine algae produce these bioactive substances to repair tissues, to protect against stress conditions, UV rays, herbivores, oxidative stress.[17] Thus, the algal compounds and their derivatives are a source of new therapies with original and specific biological properties that can be exploited for therapeutic purposes.[18] Numerous investigations have demonstrated the therapeutic potential of algae and their compounds for major pathologies in several medical fields as oncology, hematology, infectious diseases and restorative and regenerative medicine.[19] Seaweed is classified into three broad groups based on pigmentation-brown, red and green seaweed. They are ubiquitous in our daily lives, and their uses are many and varied. Besides food uses, they take an important place kin agriculture (fertilizers), in the pharmaceutical industry (coating of medications) and in the field of cosmetics (soap, cream, toothpaste), and products cleaning, etc.[20] Among the macroalgae, brown algae (*Pheophyceae*) appear to be major producers of metabolites and interesting molecules such as flavonoids, fucans, fucoïdanes, phlorotannins (specific types of brown algae tannins), carotenoids, and a wide variety of other compounds. These substances were found to have antioxidant, antibacterial, antiviral, antifungal, anticancer, anti-inflammatory and other significant therapeutic activities.[21-22] *Chorda filum* is an edible brown seaweed that was found abundantly at the Lebanese coast. It was recently introduced to the food market. Several studies have previously shown its potential as a source of biomolecules in diverse biological activities and several new researches investigating this potential would be interesting to develop. Furthermore, studies have shown that the alga is rich in potentially bioactive compounds, especially fucoidans some of which have been isolated from this algae.[23] Studies also show that *C. L. filum* has a significant antioxidant effect.[24] This study aims at assessing the cytotoxic activity of ethanol: acetone extract (50: 50, V/V) prepared from *Chorda filum* against the human breast cancer cell line MCF-7 using trypan blue exclusion method. Since no researcher in Lebanon and abroad examined the cytotoxic activity of the crude extracts or the bioactive compounds derived from *Chorda filum*, a brown seaweed species collected on the Lebanese coast against human breast cancer cell lines, we decided to select the MCF-7 cell line for this study.

MATERIALS AND METHODS

Chemicals

Chemicals and reagents used to study anti-proliferative activities were purchased from Sigma-Aldrich Co. (Beirut, Lebanon) while the other chemicals, solvents, and reagents were purchased from Alpha Co. (Beirut, Lebanon). The RPMI 1640 and the antibiotics (penicillin-streptomycin) were obtained from Lonza (Basel, Switzerland). The trypsin of the EDTA was the only chemical obtained from Gibco (New York, USA).

Plant materials

Algae were manually collected from El Barbara beach in Lebanon at the end of May 2015. The collected samples were transported to the laboratory where they were first washed thoroughly with fresh water to remove all the possible impurities (salts, sand, shells and epiphytes), then with hydrochloric acid 10% for a few seconds to remove the organic matter, and finally with distilled water. Then, the algae were weighted then frozen at -80ºC and lyophilized. After drying, the algae were ground to powder in a blender. Then the powder was placed in a sterile falcon protected from light.[25] Voucher specimen No 1150 was botanically authenticated by Dr Mona Tannoury, Biology Department, Faculty of Sciences II, Lebanese University and deposited in the Biology Department Herbarium, Faculty of Sciences II, Lebanese University.

Sample extraction

A solvent mixture of 50 % ethanol and 50 % acetone was added to a mass of 35 grams of powder of the algae *Chorda filum* (10 mL of solvent was added to every 1 gram of the alga's powder). The resulting mixture was then subjected to a continuous stirring using a magnetic stirrer for 24 hours (maceration). The material was filtered by using Whatman paper No.1. The extract was then obtained after vacuum filtration on a Büchner funnel and then evaporation of ethanol and acetone, in 50 ml-round-bottom flask, under reduced pressure at 40 ° C

using a rotary evaporator Buchi type. The extraction was performed three times.[26]

Cell lines and culture condition

MCF-7 is the name of the line of mammary tumor cells frequently used in research laboratories on breast cancer. MCF-7 cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Sigma Aldrich, St Louis, USA) and 1% penicillin-streptomycin (Lonza, Basel, Switzerland). The fetal bovine serum (FBS) is essential for cell growth. It has various attachment factors that allow cell proliferation, hormones, proteins, attachment factors such as fibronectin, which contribute to good adhesion essential for cell division and ensures a protective role. Antibiotic solution (penicillin-streptomycin) is used to reduce the risk of contamination in cell culture. The cells were preserved at 37^0C in a wet atmosphere of 95% air and 5% humidified CO_2. Cells were passaged in a ratio 1: 4 every 48 hours (80-85% confluence) by removing the medium then by washing with phosphate buffered saline (PBS, Sigma, St. Louis, USA) and then they were detached by the trypsin of the EDTA (Gibco, New York, USA). The cells were centrifuged at 900 rpm and the residue was suspended again in 4 mL of medium for seeding later in flasks (Corning, New York, USA) for the passage or in 24-well plates for experimentation.[27]

Cell viability assay

Cytotoxicity of the crude extract of brown algae *Chorda filum* was determined by the trypan blue exclusion assay to determine the number of living and dead cells in a population. Trypan blue is a dye incapable of crossing the membrane of living cells in contrast to dead cells. Then, dead cells take the blue color under a microscope while living cells remain clear. To test, 25000 MCF-7 cells were seeded in 24-well plates. When the cells reached 40-60% confluence, they were treated in duplicate with 10, 30, 60, 90 and 120 $\mu g.mL^{-1}$ of the extract, dissolved in DMSO, for 24 and 48 hours of incubation. At each time, the medium was removed and the cells were washed with PBS and detached by trypsin-EDTA. Then, they were centrifuged at 900 rpm for 5 minutes and the residue was suspended in 100 uL of fresh medium. The cell suspension was mixed for a dillution 1: 1 (v / v) with 0.4% trypan blue and 20 µl of the mixture was loaded on a Neubauer hemacytometer and examined under a microscope to count the viable cells (clear, not blue). Finally, the number of living cells was determined using the formula: Number of Cells per ml = average counted in the side tiles 1mm x dilution factor x 10^4.[28]

Statistical analysis

All experiments were carried out in duplicate. The data were expressed as means ± SD and the differences were evaluated by one-way analysis of variance (ANOVA) test completed by Dunnett's test. The differences were considered significant at $^{**}p < 0.01$. The 50% inhibitory concentration (IC_{50}) was calculated by nonlinear regression curve with the use of Prism Graphpad Prism version 4.0 for Windows[GraphPad Software, San Diego, CA, USA (Hwww.graphpad.com)H].

RESULTS AND DISCUSSION

The brown alga *Chorda filum (L.)* (Laminariales), called tsuru-mo in Japanese is harvested and consumed only in a few towns that lie on the coast of the Japan Sea. In Wajima, Ishikawa, Japan, the alga was dried under the sun and stored for one year before being sold in retail shops. The dried product of *C. filum* was washed and rubbed well with plenty of water five to ten times before cooking.[22] The crude extraction of dried brown seaweed *C. filum* prepared by solvent mixtures was used to calculate the yield of each extract expressed as a percentage for 35 g of dry and ground plant material. The highest yield is marked for the acetone: ethanol extract with a value of 20.10 % w/w. Since *C. filum* is edible seaweed, it's interesting to investigate its potential as a medicinal food. This contribution focuses on the anti-cancer effect of *C. filum*'s extract against MCF-7 human breast cancer cells. A mixture of ethanol and acetone was chosen as a solvent mixture to prepare an extract from the brown seaweed *Chorda filum*. We chose this solvent mixture because ethanol is known as an extraction solvent for polar compounds and the main molecules of interest, characteristics of brown algae, are phlorotannins, and acetone increases the total yield by inhibiting interactions between tannins and proteins during extraction or by cutting Hydrogen bonds between complex of tannin-protein. Therefore, the most commonly used solvents to extract bioactive compounds especially phlorotannins from brown algae are a mixture of ethanol and acetone.[29] Exclusion test using trypan blue was conducted to evaluate the cytotoxicity of the ethanol: acetone extract of the alga *Chorda filum*. 25,000 MCf-7 cells were seeded in multiwell plates and then treated in triplicate with 10, 30, 60, 90, 120 and 180 $\mu g.mL^{-1}$ of the algal extract for 24 and 48 hours. Consequently, the ethanol: acetone extract prepared from *C. filum L.* was able to exert anti-proliferative activities against MCF-7 human cancer cells with IC50 values 97.81 µg/mL and 29 µg/mL, respectively after 24 and 48 hours of treatment (Figs.1& 2). The counting of dead cells and living cells in hematocytometer was possible due to trypan blue staining that shows dead cells in blue while the living cells remain clear. The number of cells per ml was obtained by multiplying the average counted in the tiles of 1 mm side by the dilution factor and 10^4. The number of living cells and dead cells were recorded after 24 hours and 48h of treatment. After 24 or 48 hours of treatment, the number of living cells in the control (RPMI 1640) and in the vehicle (DMSO) was high. In the case of the tested concentrations ranging from 10 to 180 $\mu g.mL^{-1}$, the number of living cells decreased by increasing the extract's concentration, while the number of dead cells increased. At the highest concentration tested (180 $\mu g.mL^{-1}$), the percentage of living cells was the lowest (almost no living cells after 24 and 48 h treatment) while the percentage of dead cells was the

highest. This shows that the extract of brown algae *Chorda filum L.* has an effect against the cancer cells MCF-7 starting from a concentration of 30 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$ after 24 and 48 h of treatment respectively, and this anti-cancer effect was dose and time dependent. So, the trypan blue exclusion assay showed that with an increase in concentrations of algal extract and in the duration of treatment (24 and 48 hours), MCF-7 viability was significantly decreased according to the negative control free extract. This infers the existence of time and dose dependent properties of *Chorda filum* extract against MCF-7 tumor cell lines. The IC 50 (concentration inhibiting 50% of cell viability) were calculated for 24 and 48h of incubation of the cells according to the equation y = ax + b, where y = 50, x = IC50. Some literature highlight the potential implications of marine algae which exhibit antitumor activity.[30] Our preliminary results show the cytotoxic activity of the crude extract of the marine brown algae *Chorda filum* collected from the Lebanese coast against MCF-7 cancer cell line using trypan blue exclusion assay. This extract then has a significant anti-cancer potential against this cell line. This anti-proliferative effect of *Chorda filum*'s extract may be related to the richness of this brown seaweed in many bioactive compounds such as phlorotannins and sulfated polysaccharides (PSS). Phlorotannins have been reported to possess anticarcinogenic effects.[31] Phlorotannin extract derived from brown algae *Laminaria japonica* has exhibited a remarkable anti-proliferative activity in the human hepatocellular carcinoma cell line (BEL-7402) and on murine leukemic cell line (P388) in a dose dependent manner. The half-inhibitory concentration of the phlorotannins extract (IC50) on P388 and BEL-7402 cells was 120 g/ml and >200 g/ml, respectively. Microscopic observations have revealed that the morphologic features of tumor cells treated with PE and 5-fluorouracil (a commercial chemotherapy drug) are markedly different from the normal control group suggesting the anti-proliferative effect of PE.[32] Dioxinodehydroeckol isolated from *E. cava* has shown a considerable anti-proliferative effect on human breast cancer cells (MCF-7). Dioxinodehydroeckol inhibited the proliferation of MCF-7 cells with rates of approximately 25%, 40%, 53%, 56% and 64% at concentrations of 1, 5, 10, 50 and 100 M, respectively, compared to the control group. *In vitro* studies suggest that dioxinodehydroeckol's potential anti-proliferative activity might be associated with the induction of apoptosis through nuclear factor kappa-light-chain-enhance of activated B cells (NF-_B) family and NF-_B dependent pathway.[33] The enzymatic extract of *E. cava* together with its crude polysaccharide (CpoF) and crude polyphenolic fractions (CphF) have been reported to be a promising alternative to synthetic substances as natural compounds with high antiproliferative and antiradical activity. Especially the CphF, at an IC50 value 5.1 g/ml, has inhibited cell proliferation inmurine colon cancer cell line (CT-26). The anti-proliferative effect of CphF is believed to be associated with apoptotic cell demise in CT-26 confirmed by the nuclear staining experiment.[34] Other phlorotannins derivatives of this alga have cytotoxic potential against other human cancer cell lines (HeLa, HT1080, A549 and HT- 29).[35] The antiproliferative efficacy of these algal extracts are positively correlated with the total polyphenol contents.[36] Studies show also that sulfated polysaccharides (PSS) are the main antitumor components found in algae. The PSS from seaweed are polymers with hemiesters sulphate groups covalently linked to their monosaccharide residues. The degree of substitution can vary considerably, but he still has a negative influence on the polysaccharides in question. This property enables PSS to connect to more basic molecules, including proteins, thusdeveloping their activities.[37] The purification studies and algae PSS characterization focused on their pharmacological properties. The knowledge acquired till now have led to the conclusion that the algae from different taxa synthesize different polysaccharides. Brown algae (*Phaeophycae*) synthesize homo- and hetero-fucose, known as fucans and sulfated fucoids. Each type of alga synthesizes its sulfated polysaccharide with unique structural features which are reflected in the biological, pharmacological and biotechnological polysaccharide. Moreover, these structural characteristics can be modified by biotic and abiotic factors to which the algae is exposed, and the extraction and purification methods used to try to obtain PSS.[37] Fucoidan is a term used for a class of sulfated, fucose rich, polysaccharides found in the fibrillar cell walls and intercellular spaces of brown seaweeds (class of Phaeophyceae). These fucose-containing sulfated polysaccharides (FCSPs) principally consist of a backbone of (1→3)- and (1→4)-linked α-l-fucopyranose residues, that may be organized in stretches of (1→3)-α-fucan or of alternating α(1→3)- and α(1→4)-bonded l-fucopyranose resin.[38-39-40] In the past few years, various structures of algal fucanes such as FCSPs from brown seaweeds of the order of Laminariales have been solved, and many aspects of their biological activity have been elucidated.[38] FCSPs isolated from *C. filum* (Laminariales) have been shown to consist of a poly-α(1→3)-fucopyranose backbone with a high degree of branching mainly as α(1→2)-linked single α-l-fucopyranosyl,[40-41] the fucopyranosyl residues were found to be sulfated mainly at C-4 and sometimes at the C-2 position, whereas some of the α(1→3)-linked fucose residues were shown by NMR to be C-2 acetylated.[40] Usov et al. reported a similar structure for the FCSPs isolated from *Laminaria saccharina* (Laminariales) which are mainly built of (1→3)-linked α-l-fucopyranose with sulfation at C-4 and sometimes at the C-2 position or with possible α-l-fucopyranosyl at C-2 (1998). This FCSPs structure has also been found to be present in the body wall layer of a marine invertebrate, the sea cucumber *Ludwigothurea grisea* (Ribeiro, Vieira, Mourão, & Mulloy, 1994). Many reports have been published which indicate the antitumor and immune-response modulating activity of FCSPs in both *in vivo* and *in vitro* studies.[42-43-44] Several studies have reported

that polysaccharides (PS) have *in vitro* anti-proliferative activity against tumor cells, as well as inhibition of tumor growth in animal models. In addition, polysaccharides exhibit anti - metastatic activity by inhibition of tumor cell adhesion and migration of the molecules of the extracellular matrix, they act as metalloproteinase inhibitors, and are also anti-angiogenic and / or immunostimulants. They are also compounds of chemoprevention, because they exhibit scavenging activity of free radicals and antioxidants. As an example of anti-cancer effect of PSS, a study on adenocarcinomic human cells (MCF-7 and MDAMB231) ascertained that fucans of brown algae have anti-adhesive activities that depend mainly on the structure of the PS, its sulfates content and its molecular weight.[45] The authors showed that this activity is due to direct interaction between fucans and the matrix's molecules (fibronectine spcecifically), preventing adhesion molecules (integrins, proteoglycans…) from recognizing binding sites existing in the extracellular matrix.[46]

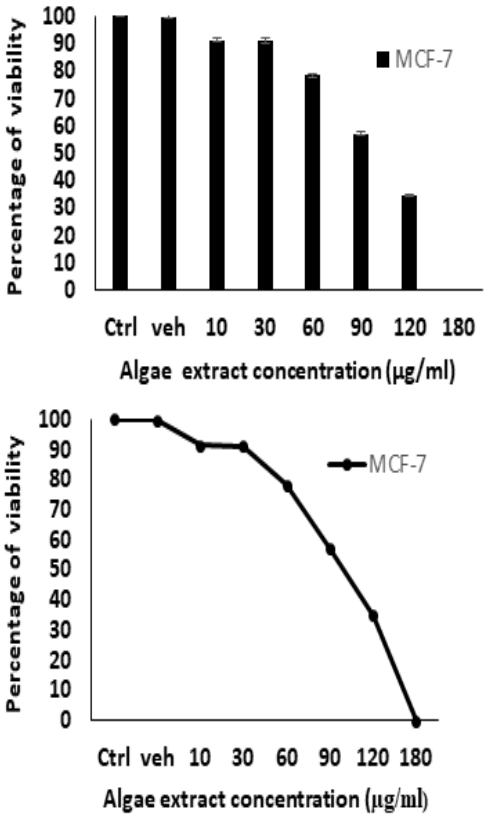

Figure 1. Treatment with ethanol/acetone algae extract after 24 h

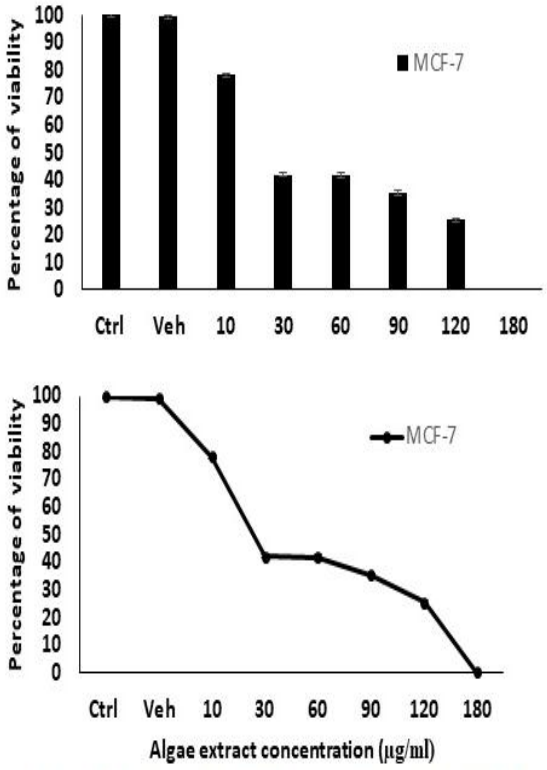

Figure 2. Treatment with ethanol/acetone algae extract after 48 hours

CONCLUSION

The overall findings of the present study conclude that brown marine algae *Chorda filum* collected from the Lebanese coast have promising anticancer activity. This result highlights how the marine algae collected from the Lebanese coast can be an important source to discover new effective antitumor compounds. In fact, this investigation proves the effectiveness of algae extracts in controlling tumor growth and brings front a fact that marine algae could serve beneficial for anti-cancer properties. Further research is needed to isolate and identify the bioactive compound of *Chorda filum* collected on the Lebanese coast, and also to elucidate the mechanism procedure by which the compound produces the cytotoxic effect - the compound's effect on the cell cycle and their ability to induce apoptosis.

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