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EVALUATION OF ANTI-PROLIFERATIVE EFFECT OF A SEAWEED CALLED "*PADINA PAVONICA*" (LINNAEUS THIVY N.D.) FROM THE LEBANESE COAST AGAINST JURKAT CANCER CELL LINE

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ABSTRACT
Most researches in the field of cancer nowadays, have the purpose of finding a drug with a selective cytotoxicity to kill the cancer cells with fewer side effects than those of the current therapies. Among all the marine organisms, brown seaweeds seem to have a potent anti-cancer effect against many cancer cell lines. In our paper, we assessed the cytotoxic activity of three crude extracts of brown algae named *Padina pavonica* (Linnaeus) Thivy, n.d. collected from the Lebanese coast, on Jurkat human cancer cell line by using the trypan blue exclusion assay. After 72h of treatment, all the extracts prepared from *P. pavonica* were cytotoxic on Jurkat cancer cells: the aqueous extract with IC_{50} values of 21.78 µg/mL, methanol: water extract with IC_{50} values of 31.1µg/mL, and the n-hexane extract with IC_{50} values of 40 µg/mL. More researches concerning the identity of the cytotoxic compounds in the crude extracts are crucial to identify new and efficient anti-cancer drugs.

KEYWORDS: brown algae; *Padina pavonica*; cytotoxic activity; Jurkat cell line; trypan blue exclusion assay.

INTRODUCTION
Cancer is a group of dreadful diseases characterized by a tumor formation where cells show multiple features like uncontrolled cell division, total independence toward growth factors, insensibility toward apoptotic, anti-proliferative signs, enhanced capacity to induce angiogenesis, and a remarkable ability of invasion and metastasis.[1] This disease is considered the second leading cause of death in the world. Every year an estimated 12.7 million new cases are registered with 7.6 million deaths.[2] Nowadays, chemotherapy remains the standard treatment used along with surgery and radiation therapy. However, due to their toxicity that targets all the cells in the body, these therapies cause a wide range of severe side effects that include bleeding, sensory abnormalities, cognitive impairment, infertility, and damage to hematopoietic tissue (anemia), hair-loss (alopecia).[3] The cancer researchers have done worldwide aims at finding a drug with selective cytotoxicity leading to fewer side effects.[4] More than 50% of all drugs clinically used in the world are either from natural products or from their derivatives.[5] Moreover, the approach of employing natural agents in preventing or suppressing invasive tumors progression has been considered to have an enormous potential.[6]

Covering more than 70% of the planet's surface, oceans and seas are the habitats of extremely large and diverse eco-systems. Many marine organisms have a sedentary life style, which means that they necessitate chemical means of defense. That is why they have evolved the ability to synthesize toxic compounds with a broad range of biological effects.[7] Among all the marine organisms, macro-algae, also called seaweeds, are largely productive and extremely diverse plants that generate a multitude of bioactive compounds.[8] About 6000 species of macro-algae have been identified so far and classified into three different groups: green (Chlorophytes), brown (Phaeophytes)-, and red (Rhodophytes) algae.[9] The highest amount of bioactive compounds has been reported to be found in brown seaweeds (Sea food and nutrition, 2008). Since they were used to treat malignant tumors in Asian countries a long time ago, brown seaweeds seem to have a clear and potent anticancer effect. Many pieces of evidence suggest that this effect is exerted through multiple mechanisms of action such as the inhibition of cell proliferation, the inhibition of invasion and metastasis, and/or the induction of apoptosis in cancer cells.[10] *Laminarin*, a polysaccharide extracted from the brown algal genus *Laminaria*, was able to inhibit the proliferation of colorectal cancer

cells.[11] Fucoidan, another polysaccharide, is a sulfated one found in many brown algal species. It has been demonstrated that the crude fucoidan extracted from the algae *Fucus vesiculosus* was able to reduce the number of highly metastatic breast cancer cells 4T1 by activating the caspase-3, and decreasing the expression of many proteins such as the B-Cell Lymphoma gene 2 (BCl-2), the Extracellular Regulated Kinase (ERK), and the Vascular Endothelial Growth Factor (VEGF).[12] The enzyme-digested fucoidan, derived from brown seaweed *Mozuku of Cladosiphon Novae-Caledoniae kylin,* exerts a cytotoxic effect on colorectal cancer cells by suppressing cell proliferation, inhibiting the metastatic process, and/or decreasing angiogenesis.[13] Furthermore, it has been proved that the treatment of human leukemic cancer cells U-937 with fucoidan extracted from a brown algal species called *Cladosiphon okamuranus* (which is commercially cultured around the Okinawa Island, Japan) induces apoptosis in these cells by activating the caspase-3 and 7.[14] Moreover, Namvar *et al.* (2013) have provided that the methanol extract from brown algae named *Sargassum muticum* had the ability to decrease the cellular proliferation of two breast cancer cell lines (MCF-7 and MDA-MB-231) by inducing apoptosis.[15] *Padina* is a genus of marine brown algae from the *Phaeophyceae*, class, *Dictyodiales* order and the *Dictyotaceae* family. Based on 'Algaebase', this genus includes 74 different species. One of these species is *Padina pavonica* (Linnaeus) Thivy, n.d. which is also known as Peacock's tail that grows on the Lebanese coast (*Padina pavonica* (Linnaeus) Thivy, n.d.) Just like many other brown algal species, *Padina pavonica* extracts show a cytotoxic activity. Taskin *et al.* (2010) have proven that the crude methanol extract of *P. pavonica* inhibits the proliferation of breast adenocarcinoma cells MCF7.[16] Moreover, in a study conducted by Stanojković *et al.* (2013), *P. pavonica* methanol extract showed a cytotoxic activity on the HeLa (cervix) and MDAMB-453 (breast) cancer cell line.[17] Ktari *et al.* (1999) have demonstrated that dichloromethane extract of *P. pavonica* has a potent cytotoxic activity (IC_{50}=10µg/mL) against KB cells (Obtained through Hela cells contamination). The responsible compound of this effect is a sterol, and precisely an oxysterol called hydroperoxy-24 vinyl-24 cholesterol.[18] Finally, in order to explore the cytotoxic effect of *P. pavonica* extracts on other cancer cell lines, we are studying whether this type of seaweed has an effect against Jurkat cancer cells (acute lymphoblastic leukemia) or not. That is why we have collected *Padina pavonica* from the Lebanese coast and prepared three different extracts using three different solvents: water, methanol 50%, and n-hexane 100% before applying them on cancer cells.

MATERIALS AND METHODS

Algae material

In the beginning of Aprił 2016, algae were manually harvested from Sarafand, south Lebanon, and transported to the laboratory. The samples were first washed with fresh water to remove impurities such as salt, sands, and epiphytes and then algae material were taken to the freezer at -80˚C before lyophilization for drying. After an optimal drying, the algae material was transformed into powder by using a blender.

Chemicals

The fetal bovine serum (FBS) was obtained from CELBIO (Milano, Italy). The chemicals used to study the anti-proliferative activity were purchased from sigma Aldrich Co. While all the other chemicals, solvents and reagents were purchased from Sigma Aldrich Co (Beirut, Lebanon).

Extraction

Every time a mass (15 grams) of powdered dry material of *Padina pavonica* was macerated either with a volume of 150 ml of pure water, 150 ml of a mixture methanol/water (v:v), or 150 ml of n-hexane. By using a magnetic stirrer, and after 24 hours of stirring the three mixtures in the shadow, the extracted liquid obtained was filtrated under vacuum, by using a Buchner. When the solvent used was pure water, the filtrate was later transferred into the freezer at -80˚ C before lyophilization. In order to get rid of the methanol, the filtrate of methanol/water mixture was evaporated by using a rotary evaporator, and then the residual liquid extract was frozen at -80℃ before the optimal drying by lyophilization. With respect to the n-hexane extract, the filtrate was concentrated in the rotary evaporator at 45˚C to finally obtain a viscous material that was subsequently transferred under an inert atmosphere for an optimal drying.[19]

Cell line culture and maintenance

Jurkat cells (T lymphocyte cells) are non-adherent lymphoblast (cells in suspension). All these cells were cultured in a complete RPMI medium (RPMI 1640 (Gibco/Invitrogen)) supplemented with 10% fetal bovine serum (Hyclone), 10 mM Hepes 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated under 5 % CO2 and at 37˚C. In order to store these cells in liquid nitrogen, we had a complete growth medium supplemented with 5% (v/v) DMSO in 1 mL aliquots of approximately 5 x 10^6 cells. RPMI 1640 medium for suspension cells with fetal bovine serum (FBS).[20-21]

Cell viability assay

Cells were cultured in 24-well plates at a density of 1 x 10^5 cells per well. Two wells were prepared for each concentration. We have compared treated cells with the three different *P.pavonica* extracts (water; methanol 50%; n-hexane) at different concentrations with untreated controls. The plates were treated for either one, two, or three days. The cytotoxicity was determined by using a trypan blue exclusion assay. A volume of 10 microliters of cell suspension taken from the well was mixed with an identical volume of trypan blue. In the case of dead cells, the damaged membrane allows the entrance of the dye and consequently the staining of the

cell, whereas the membrane of living cells prevents the dye from entering the cell which stays uncolored. After 24, 48 and 72 h the trypan blue exclusion test was executed, and the percentage of cell viability was calculated based on the following formula:

$$\%\ of\ cell\ viability\ = \frac{number\ of\ viable\ cells\ in\ the\ well}{total\ number\ of\ cells\ (control)} \times 100$$

Statistical analysis

All the data are presented as mean ± S.DSD and the differences were evaluated by one-way analysis of variance (ANOVA) test completed by Dunnett's test. Using Graphpad Prism 5.02 software, the statistical analysis and generated curves were performed. All the data obtained from the experiments were subjected to grouped analysis by a two-way analysis of variance (ANOVA) followed by Bonferroni post-tests keeping $p < 0.05$ in all cases.

RESULTS AND DISCUSSION

As we mentioned before, the extraction process has been done by using three different solvents: pure water, methanol/water (50/50; v:v), and n-hexane. Water is a polar solvent dissolving hydrophilic molecules. N-hexane is a non-polar solvent which means that it dissolves hydrophobic compounds, while methanol/water (50/50; v:v) is a polar mixture, whose polarity is clearly lower than water itself. The crude extraction of dried brown seaweed *Padina pavonica* prepared by the three solvents was used to calculate the yield of each extract expressed as a percentage for 15.g of dry and ground plant material. The yields' values marked of water/methanol, and water extracts are respectively 6.50 % and 5 %, w/w while a very low yield of 0.2%. w/w is recorded for the n-hexane extract. To evaluate the three different extracts activity on the Jurkat cells, we have seeded the Jurkat cells at an initial concentration of 100000 cells/ml and then cultured for one, two, and three days in the presence or absence of the algal extracts. By using the trypan blue exclusion assay to evaluate the cell viability, we have noticed that for every extract applied, the number of viable cancer cells has significantly decreased with the concentration of the extracts as well as the duration 24, 48 and 72 hours of the treatment according to the negative control (extract-free).). After 72 hours of treatment, the IC_{50} of the *Padina pavonica* water extract was 21.78 $\pm$ 0. 28 µg/mL, 31.1 $\pm$ 0.56 µg/mL for the methanol/water extract, and 40 $\pm$ 2.12µg/mL for the n-hexane extract (**Figures 1,2&3**). Thus, the lowest IC_{50} is obtained with the water extract in comparison with the IC_{50} of others extracts with methanol and hexane. These results shed the lights on the importance of the solvent choice in the extraction in the cytotoxic activity of the extract. This highlights the time- and dose- dependant activity of these extracts against Jurkat cancer cells. Hence, the water, methanol 50%, and the n-hexane extracts of *P. pavonica* exert anti-proliferative activity on Jurkat cell line. In addition, Tannoury *et al*, (2017) revealed that the two crude extracts prepared from the marine red algae *Laurencia papillosa* collected from the Lebanese coast are cytotoxic agaisnt the Jurkat human cancer cell line in a time and dose dependent manner, via the trypan blue exclusion assay. The IC50 values are 121.642 µg/ mL and 57.77 µg/ mL for the ethanol: water extract and the ethanol: chloroform extract, respectively after 72 hours of treatment.[22-23] Moreover, Mary *et al.* (2012) reported that the treatment with a concentration range of 100 to 300 µg/mL of ethanol extract of *Sargassum sp.* reduces the cell viability of both Hep-2 and MCF-7 cell lines in a dose dependent manner with an IC 50 value of 200µg/mL and 250µg/mL, respectively.[24] On the other hand, the methanolic extract prepared from *Sargassum muticum* inhibits the growth of both cell lines MCF-7 and MDA-MB-231 in a dose and time-dependent manner, with IC50 values of 22 µg/mL and 55 µg/mL, respectively, after a 24 hours of treatment.[25] For example, Namvar *et al*. (2014) have verified that the red algae *Gracilaria corticata* methanol extract exert an anti-proliferative activity against MCF-7 cancer cell line dose-dependently.[26] Furthermore, Calves *et al.* (2016) have demonstrated that the extracts of *Fucus spiralis*, *Asparagopsis armata*, *Plocamium cartilagineum* and *Sphaerococcus coronopifolius* show a time-dependent antitumor activity on hepatocellular carcinoma HepG-2.[27] Our work is compatible with results of Tannoury *et al.* (2016) that emphasizes the influence of the choice of solvent extraction on the cytotoxic activity of seaweed extracts against Jurkat cancer cell line. In this study, we assessed the cytotoxic activity of two crude extracts from *Sargassum vulgare* (*Sargassaceae*), a marine brown algae collected from the Lebanese coast, against Jurkat human cancer cell line using trypan blue exclusion test in a time- and dose-dependent manner. Both extracts, water: ethanol extract and chloroform: ethanol extract, showed cytotoxic activity against Jurkat cancer cell line with IC50 values of 136.907 µg/ mL and 49.056 µg/ mL, respectively after 72 hours of treatment. Many other researchers have evaluated the cytotoxic effect of the genus *Padina* on many cancer cell lines. A significant growth inhibition effect of the methanol and ethanol extracts of *Padina gymsospora* was seen against A549 (adenocarcinomic human alveolar basal epithelial cells), MG-63 (*Homo sapiens* osteosarcoma), and PC-3 cells (*Homo sapiens* prostate; derived from metastatic site: bone). In addition to this, signs of apoptosis (formation of apoptotic bodies or blebbing) were also observed in a study conducted by Murugan *et al* (2013).[28] Similarly, the chloroform fraction of the dichloromethane extract of *P. gymnospora* has shown a great anti-proliferative effect against K562 (human immortalised myelogenous leukemia cell line; IC_{50} =11.0 µg/mL) and HEp-2 (HeLa contaminant) (IC_{50} =8.2 µg.mL^{-1}) cells.[29] It has also been provided by Paul *et al*. (2014) that the fucoidan, extracted from *Padina distromatica Hauck and* collected in Hare Island (India), inhibit potently the proliferation of A-549 and moderately the proliferation of HL-60 (human promyelocytic leukemia cells) and A-431 (human epidermoid carcinoma) cancer cells.[30] Also, and by using the sulforhodamine B (SRB) colorimetric

assay, Oliveira *et al.* (2015) have proved that three different extracts of *P. boergesenii* (Hexane, butanol, and chloroform) present inhibition proliferation values of MCF-7 cells around 60%, which is similar to those induced by the positive control used in their study (colchicine, 67.0 ± 4.0%).[31]

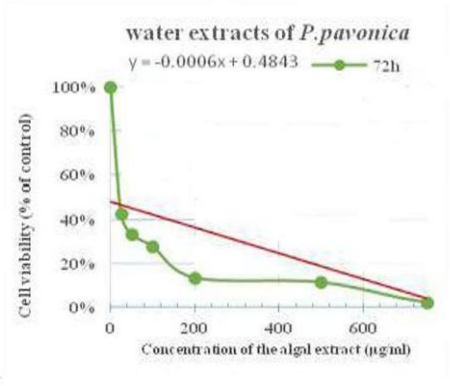

Figure 1 : treatment with water extract after 72 hours

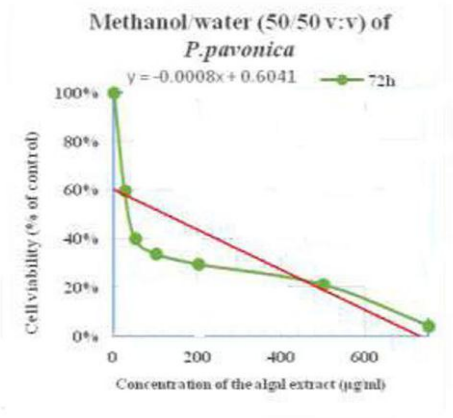

Figure 2: treatment with methanol/water extract (V:V) after 72 hours

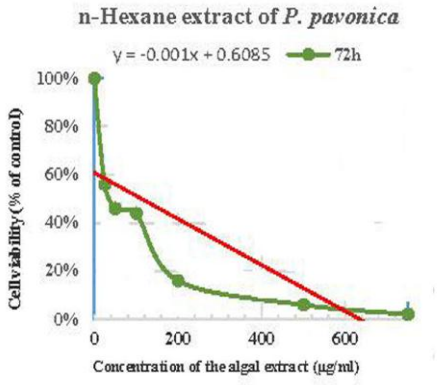

Figure 3: treatment with n-hexane extract after 72 hours

CONCLUSION

Our paper shows that the brown marine algae *Padina pavonica* collected on the Lebanese coast have a promising anti-cancer activity. Among the three extracts prepared (water, methanol: water, and n-hexane) the aqueous extract gave the best result with the IC_{50} values of 21.78 µg/mL. Hence, this paper highlights the importance of the extraction solvent choice in the anti-tumor activity. Furthermore, to be considered as an anti-cancer drug source, the crude extracts should be fractionalized, the compounds in charge for this activity have to be identified, as well as the mechanism of action of each bioactive compound in the cancer cell should be clarified.

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Conflict of Interests: There are no conflicts of interest.

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