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Lipidic compounds from the muscle of white shrimp (*Litopenaeus vannamei*): chemical structure and effect on the proliferation and morphology of human cancer cell lines

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Abstract

Cancer represents the second leading cause of death worldwide, therefore, the search for chemoprotective agents is on the rise. The muscle of white shrimp (*Litopenaeus vannamei*) is one of the species analyzed as it has been reported as a source of compounds with antiproliferative activity. The aim of this study was to evaluate the effect of shrimp muscle-isolated compounds on cell proliferation and morphology of human cancer cell lines. The muscle underwent a process of extraction and fractionation of compounds; their effect on cell viability assays (MTT) on lung adenocarcinoma (A549), prostate carcinoma (22 Rv-1), invasive breast adenocarcinoma (MDA MB 231), colon carcinoma (HTC 116), cervix adenocarcinoma (HeLa) and non-cancerous retinal cells (ARPE-19) was measured. Morphological changes were observed using fluorescence microscopy and chemical structure data was obtained using nuclear magnetic resonance. Fraction named C5 showed the highest antiproliferative activity on HCT-116 and MDA-MB-231, without significantly affecting the control cells. Subfractions C5-3 and C5-4 presented significant antiproliferative potential in MDA-MB-231; this cell line showed morphological changes that could be related to apoptosis, and spectroscopic analysis revealed the presence of b-carotene and eicosapentaenoic acid in C5, nevertheless, further studies are needed to determine the effect of each compound.

Keywords: bioactive compounds; chemoprevention; cancer.

Practical Application: It provides the basis for further pharmacological studies in marine bioactive compounds.

1 Introduction

Currently, non-communicable diseases are responsible for most human deaths worldwide, among these illnesses, cancer is the second cause (World Health Organization, 2020). The term cancer refers to a condition that is primarily characterized by the disordered and rapid growth of malignant cells, which grow beyond their usual limits (Nosrati et al., 2017). Among the diverse types of cancer, lung cancer is the most frequently diagnosed with the highest mortality rate (Denisenko et al., 2018), which accounts for 18.4% of total deaths, followed by breast cancer, which mainly affects women with 6.6% mortality and the third is colorectal cancer (Bray et al., 2018). According to the World Health Organization (2020), 9.6 million people died from this disease in 2018 and it has been estimated that cases will continue to increase up to 28.4 million new cases in 2040 (International Agency for Research on Cancer, 2020). Despite the efforts to optimize treatments against this problem and increase the survival rates of patients, there has been many limitations to achieve it.

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most aquaculture-produced species of crustaceans, due to its high nutritional value and market acceptability, accounting for 52.9% of total aquaculture production (Food and Agriculture Organization of the United Nations, 2020); besides, there is evidence that its muscle is a natural source of compounds with chemopreventive properties such as antioxidant (Prameela et al., 2017), antimutagenic (López-Saiz et al., 2016) and antiproliferative activities (López-Saiz et al., 2014). Polyunsaturated fatty acids (PUFA's) have been reported among these compounds with antiproliferative activity, such as eicosapentaenoic acid: that could prevent cancer cell proliferation by modifying the expression of genes involved in the cell cycle (López-Saiz et al., 2014). Also, carotenoids such as astaxanthin which is the most abundant pigment (75-95%), has been evidenced to have antiproliferative activity on the MCF-7 breast cancerous cell line (Sowmya et al., 2017). However, the chemical structure of the compounds extracted from *L. vannamei*, which are responsible for the antiproliferative potential as well as for the mechanisms of action by which these

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compounds act, are still unknown. Therefore, the objective of this study was to evaluate the effect of lipid fractions isolated from the *L. vannamei* muscle on the proliferation and morphological changes of human cancer cell lines, and to elucidate the chemical components present in those fractions.

2 Materials and methods

2.1 Shrimp specimens

Litopenaeus vannamei specimens were obtained from a local market and transported in ice to the Microbiology and Mycotoxin Laboratory of the University of Sonora, where the shrimps were hand eviscerated and stored at -20 °C until further use.

2.2 Extraction and isolation of compounds with antiproliferative potential

In order to extract the bioactive compounds from the shrimp muscle, the methodology described by López-Saiz et al. (2014) was followed. Shrimp muscle tissue was homogenized with chloroform (1:5 w/v ratio), constantly stirred for an hour and filtered under negative pressure using Whatman Paper No. 1. The filtrate was concentrated, using a rotary evaporator (Yamato, RE300) at 30 °C, recovered with a mixture of methanol:hexane (150:150 mL), and partitioned overnight.-Filtrates were concentrated using the rotary evaporator and recovered with a known amount of chloroform and the obtained extract was stored at -20 °C until further use. Once the methanolic phase was obtained, compounds were isolated using Ruíz-Almada (2020) was carried out by using open column chromatography. A glass column was (5 cm i.d., 120 cm length) packed with silica (SIGMA, 70-230 mesh) as stationary phase. Different mixtures of 500 mL of hexane:acetone (100:0, 95:5, 9:1, 85:15, 80:20, 75:25, 70:30 and 65:35) were used as mobile phase to fractionate methanolic phase.

Thin layer chromatography was used to monitor the eluates obtained from chromatographic column, using silica gel as a stationary phase and hexane-acetone (80:20) as mobile phase; compounds were revealed using high and low frequency UV light. The delay factor was calculated employing Equation 1.

$$Rf = \frac{Cd}{Mpd} \quad (1)$$

where: Rf – retention factor, Cd – distance from the baseline to the point of the compound (cm) and Mpd – distance from the baseline to the solvent front (cm).

Samples with similar Rf signals were considered as the same fraction; these were dried under nitrogen stream and brought to a final concentration of 20 mg/mL using dimethyl sulfoxide (DMSO).

2.3 Cell lines

Five cancerous cell lines were used: Lung adenocarcinoma (A549), prostate carcinoma (22Rv-1), cervix adenocarcinoma (HeLa), invasive breast adenocarcinoma (MDA-MB-231), colon carcinoma (HTC-116) and non-cancerous retinal cells (ARPE-19) were used as control. All of them were kindly donated by the Molecular Biology Laboratory at the Department of Scientific and Technological Research of the University of Sonora.

Cell lines A549, MDA-MB-231, HCT-116, HeLa, and ARPE-19 were optimally preserved in Dulbecco's Modified Eagle Medium (DMEM) and 22Rv-1 in Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich, St. Louis, MO, USA), supplemented with 5% heat-inactivated fetal bovine serum (Gibco ™, USA) and incubated under the following conditions: 37 °C, 5% CO_2 and 80% humidity.

2.4 Cell viability assay

The standard MTT test (Mosmann, 1983) was conducted to determine whether compounds affect cell viability. Briefly, 10,000 cells per well were seeded, exposed to different treatment concentrations (200, 100, 50, and 25 µg/mL), and incubated during 48 h. Subsequently, 10 µL of MTT salt (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazole bromide) were added to the cell culture and incubated for 4 h more; then, 100 µL of dimethyl sulfoxide (DMSO) were added to solubilize the formazan crystals. Optical density was read using a microplate reader (BioTek Instruments, Inc 800 TS) at 570, using 650 nm as reference. DMSO and cisplatin (15663-27-1 Cis-diaminodichloroplatin II, Sigma-Aldrich) were used as negative controls, respectively. Three independent experiments in triplicate were carried out for each treatment.

2.5 Fluorescence cell staining and microscopy assay

To observe the effect of the treatment on cell morphology and structure, the method described by Van Vuuren et al. (2019) was followed. The cell line was seeded in a 96-well microplate and incubated for 24 h, then treated with fraction at the half maximal inhibitory concentration (IC_{50}). After 24 h, cells were fixed with 3.7% formaldehyde in PBS (Phosphate-buffered saline) for 15 min and cell membranes were permeated using 0.2% Triton X-100 in PBS during 15 min. Cells were stained with 50 µg/mL phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin) (Sigma-Aldrich, MFCD00278840) solution to visualize F-actin and with 1.5 µg/mL of 4′, 6-Diamidino-2-phenyindole, dilactate (DAPI) (Sigma-Aldrich, D9564) solution to stain the genetic material (DNA). Cell structures were observed under an inverted epifluorescence microscope (Leica DMi8).

2.6 Nuclear magnetic resonance (^{13}C and ^{1}H- NMR)

The chemical-structural characterization of the compounds responsible for the antiproliferative potential was carried out on a Bruker Avance III 400 spectrometer, ^{1}H (400 MHz) and ^{13}C (100 MHz). The sub-fractions C5-3 and C5-4 were dissolved in $CDCl_3$ (Sigma-Aldrich, Saint Louis, MI, USA) at 20 mg/mL and tetramethylsilane (TMS) was used as an internal standard; the samples were placed in 5-mm diameter ultra-precision NMR tubes. Chemical shifts were recorded in ppm.

2.7 Statistical design and analysis

Cell viability trials were analyzed implementing a split-plot experimental design and a Tukey-HSD test was performed as a mean separation procedure between the different concentrations evaluated for each treatment. These analyses were carried out with using the JMP 13.0 statistical program. To obtain de IC_{50} values for fractions with the higher antiproliferative activity, a probit analysis was run using the XLSTAT statistical program.

3 Results and discussion

3.1 Extraction and isolation of bioactive compounds

To obtain the fraction that has been previously reported with the highest antiproliferative activity in murine cancer cells (López-Saiz et al., 2014), two partitions were obtained from white shrimp muscle chloroform extract (0.30% yield): hexane and methanolic partitions (0.24% yield); the latter was subjected to open column chromatography, to isolate the compounds responsible for the highest activity.

Column eluates were monitored using high and low frequency UV light. Eluates were separated in seven fractions, according to the rf signals revealed; these fractions were named as C1, C2, C3, C4, C5, C6, and C7, being C1 the fraction with the lowest polarity and gradually increasing to C7.

Each fraction could contain different compounds; lipid fraction has been reported to be composed of phospholipids, neutral lipids such as cholesterol, triglycerides, diglycerides and monoglycerides, as well as of free fatty acids such as polyunsaturated fatty acids. Another lipidic component are carotenoids, which have been extensively studied since there is evidence that they possess chemopreventive properties (Saini et al., 2020). Due to the variety of compounds that could be found in the fractions obtained at this stage, it is necessary to identify and purify the molecule(s) responsible for the greatest antiproliferative potential.

3.2 Cell viability assay

Fractions

Cell viability assay was performed to test the seven fractions obtained by open column chromatography, at different concentrations (200, 100, 50, 25 µg/mL) (Table 1). When 22Rv1 cancerous cell line was treated with C3 and C5 fractions, at a concentration of 200 g/mL, cell viability significantly ($p \leq 0.05$) decreased compared to that observed in cell exposed to other fractions. These results might suggest an antiproliferative potential for these fractions on this cancerous cell line; results that are consistent with those reported by García-Romo et al. (2022), in which a lipid fraction extracted from the *L. vannamei* muscle, composed of eicosapentaenoic acid, dioctyl phthalate and an indolocarbazole derivative, was reported to have antiproliferative activity on the same cancerous cell line.

In the lung adenocarcinoma cell line (A549), a lower percentage of viability was observed when treated with C5 fraction at a concentration of 200 µg/mL, which could suggest antiproliferative activity. These results are important since globally, lung cancer has the higher mortality rate, and specifically adenocarcinoma is the most frequently diagnosed among the types of lung cancer (Zappa & Mousa, 2016). Colorectal cancer is the third most diagnosed and represents 10% of all cancer cases (Razali et al., 2016; International Agency for Research on Cancer, 2020). In this study, when HCT-116 was exposed to the C5 fraction at the highest concentration, showed significantly ($p \leq 0.05$) low percentages of viability, even lower than in other studies with the same cell line (Rafiq et al., 2020; Eor et al., 2021).

Regarding the antiproliferative potential of fractions on breast adenocarcinoma (MDA-MB-231) cancerous cell line, the lowest percentages of viability were also observed when cells were treated with fraction C5; these results are considered relevant since breast cancer remain the second leading cause of death in women worldwide (Siegel et al., 2016).

On the other hand, no significant antiproliferative effect was observed on cervical adenocarcinoma cell lines when exposed to the fractions. Cervical cancer is the fourth most common type of cancer in women; the standard treatment implemented against this are cisplatinum and 5-fluororacil (Farooqui et al., 2018); however, the use of agents that have as target molecule HER2 (human epidermal growth factor receptor-2), has been considered as a more specific alternative treatment since their activity on cervical adenocarcinoma has been demonstrated to be effective (Nakamura et al., 2019; Ueda et al., 2017); therefore, the fact that the fractions evaluated in this study on this specific cancerous cell line, did not show any antiproliferative effect, might be to the incapacity of the compounds present in these fractions of acting on HER2; this is consistent with the testing fractions having antiproliferative activity on MDA-MD-231, a cell line that does not respond to treatments that have influence on HER2.

Cell viability of non-cancerous ARPE-19 cell line was significantly ($p \leq 0.05$) unaffected by any of the testing fractions (Table 1); this may suggest certain bioselectivity of the fractions over cancerous cells. Cell selectivity is one of the desirable characteristics in drugs used in chemotherapy. However, to assure bioselectivity of any compound, antiproliferative studies using both, cancerous- and noncancerous-cell lines from the same type of tissue, must be carried out.

Sub-fractions

From the seven fractions evaluated over the different cancerous cell lines, fraction C5 (0.055% yield) showed the highest antiproliferative activity on HCT-116 and MDA-MB-231 cell lines, therefore, C5 was further fractionated using open column chromatography into six sub-fractions (named as C5-1, C5-2, C5-3, C5-4, C5-5, and C5-6) (Figure 1) and their effect on cell viability was studied. No significant ($p \leq 0.05$) differences in cell viability of HCT-116 was observed when treated with all the six subfractions, showing viability values very close to 100% (Table 2); that is, the antiproliferative activity previously observed when C5 was used, was not detected in neither of the sub-fractions. The apparently loss of activity might be due either the fact that a lower concentration was used for the subfractions or to a possible synergistic effect that was lost when the compounds contained in C5 were separated after column

Table 1. Cell viability percentage exposed to seven methanol-soluble fractions at different concentrations.

Fraction	Concentration (µg/mL)	Cell line (%)					
		HeLa	22-Rv1	A549	HCT-116	MDA-MB-231	ARPE-19
C1	25	89.2 ± 21.0	79.8 ± 7.1^{ab}	104.9 ± 5.3^{a}	114.4 ± 14.9^{ab}	97.3 ± 10.9^{ab}	100.1 ± 0.3^{ab}
	50	108.5 ± 13.3	72.9 ± 18.2^{abc}	80.9 ± 5.7^{cd}	110.0 ± 11.6^{ab}	101.2 ± 13.1^{ab}	104.0 ± 3.5^{ab}
	100	118.5 ± 15.9	79.4 ± 11.5^{abc}	77.6 ± 5.4^{cd}	92.7 ± 3.9^{ab}	104.7 ± 13.5^{a}	91.1 ± 8.9^{b}
	200	103.7 ± 1.8	65.1 ± 7.1^{abc}	78.4 ± 4.8^{cd}	97.0 ± 4.02^{ab}	102.8 ± 15.4^{a}	100.2 ± 4.5^{ab}
C2	25	97.1 ± 20.9	69.8 ± 0.6^{abc}	84.4 ± 3.5^{abcd}	108.7 ± 0.6^{ab}	98.1 ± 8.2^{ab}	105.8 ± 9.1^{ab}
	50	128.5 ± 38.2	79.7 ± 5.8^{ab}	83.6 ± 4.9^{bcd}	119.4 ± 13.8^{ab}	100.8 ± 10.1^{ab}	101.2 ± 16.2^{ab}
	100	118.8 ± 14.9	74.7 ± 7.3^{abc}	83.8 ± 4.5^{abcd}	106.8 ± 27.8^{ab}	96.9 ± 17.7^{ab}	83.2 ± 6.4^{b}
	200	108.5 ± 4.6	67.7 ± 4.1^{abc}	82.5 ± 3.1^{bcd}	103.3 ± 4.9^{ab}	91.6 1 ± 3.5^{ab}	102.7 ± 7.2^{ab}
C3	25	88.6 ± 21.9	68.04 ± 13.0^{abc}	103.5 ± 8.3^{ab}	83.1 ± 7.5^{ab}	104.1 ± 9.04^{a}	101.7 ± 8.8^{ab}
	50	105.1 ± 12.8	59.9 ± 7.3^{abc}	89.2 ± 7.3^{abcd}	79.9 ± 10.7^{ab}	118.9 ± 9.4^{a}	108.4 ± 14.2^{ab}
	100	113.1 ± 5.9	53.8 ± 1.0^{abc}	86.1 ± 7.7^{abcd}	85.3 ± 7.8^{ab}	117.4 ± 18.1^{a}	82.1 ± 7.7^{b}
	200	124.6 ± 4.6	41.1 ± 3.3^{c}	89.2 ± 5.4^{abcd}	82.8 ± 8.6^{ab}	112.6 ± 20.1^{a}	109.7 ± 11.5^{ab}
C4	25	89.5 ± 11.9	65.8 ± 1.6^{abc}	86.9 ± 2.6^{abcd}	90.2 ± 4.8^{ab}	97.1 ± 4.2^{ab}	99.5 ± 6.2^{ab}
	50	114.2 ± 25.4	73.8 ± 0.1^{abc}	90.9 ± 2.6^{abcd}	104.3 ± 15.3^{ab}	105.1 ± 0.3^{a}	117.0 ± 10.6^{ab}
	100	99.4 ± 8.6	75.2 ± 0.5^{abc}	92.6 ± 1.5^{abcd}	95.9 ± 5.7^{ab}	99.0 ± 16.0^{ab}	100.4 ± 3.1^{ab}
	200	110.6 ± 14.0	75.4 ± 0.4^{abc}	96.8 ± 1.0^{abc}	107.3 ± 6.2^{ab}	105.8 ± 9.4^{a}	139.4 ± 9.3^{a}
C5	25	83.5 ± 19.9	77.4 ± 3.0^{abc}	98.0 ± 6.3^{abc}	100.9 ± 7.7^{ab}	98.8 ± 15.6^{ab}	113.0 ± 5.1^{ab}
	50	94.2 ± 19.4	80.3 ± 4.0^{ab}	90.3 ± 9.6^{abcd}	106.55 ± 4.5^{ab}	95.0 ± 11.3^{ab}	111.9 ± 8.6^{ab}
	100	121.4 ± 6.9	74.3 ± 0.6^{abc}	84.9 ± 4.6^{abcd}	96.5 ± 8.9^{ab}	76.0 ± 27.0^{ab}	92.6 ± 6.8^{ab}
	200	110.9 ± 10.4	46.7 ± 11.4^{bc}	41.5 ± 6.5^{e}	39.9 ± 11.1^{b}	37.3 ± 1.1^{ab}	80.1 ± 19.5^{b}
C6	25	87.7 ± 8.6	69.3 ± 0.3^{abc}	83.8 ± 1.1^{abcd}	101.7 ± 3.9^{ab}	98.0 ± 3.2^{ab}	105.5 ± 0.5^{ab}
	50	107.9 ± 13.8	79.3 ± 0.01^{abc}	85.3 ± 2.7^{abcd}	107.4 ± 6.8^{ab}	98.3 ± 7.1^{ab}	102.4 ± 13.4^{ab}
	100	100.1 ± 13.9	75.4 ± 1.6^{abc}	88.9 ± 4.9^{abcd}	95.2 ± 2.2^{ab}	90.6 ± 13.9^{ab}	99.0 ± 4.5^{ab}
	200	76.2 ± 10.4	69.6 ± 1.9^{abc}	83.9 ± 5^{abcd}	96.8 ± 5.6^{ab}	82.3 ± 12.3^{ab}	93.3 ± 11.7^{ab}
C7	25	95.5 ± 2.7	75.7 ± 8.2^{abc}	87.9 ± 3.4^{abcd}	94.5 ± 19.3^{ab}	101.5 ± 9.6^{ab}	118.0 ± 15.6^{ab}
	50	118.7 ± 8.9	90.3 ± 0.1^{a}	80.2 ± 8.5^{cd}	83.5 ± 15.2^{ab}	101.2 ± 8.0^{ab}	100.6 ± 7.3^{ab}
	100	107.6 ± 16.6	82.4 ± 2.4^{ab}	77.3 ± 8.1^{cd}	130.9 ± 38.6^{a}	97.2 ± 7.9^{ab}	105.0 ± 2.7^{ab}
	200	99.1 ± 12.0	71.9 ± 12.2^{abc}	74.6 ± 2.2^{d}	101.8 ± 22.5^{ab}	82.89 ± 2.4^{ab}	108.0 ± 7.5^{ab}

The values represent mean from triplicate determinations ± standard error. Means followed by superscript are significantly different (P ≤ 0.05). Control cell cultures were incubated with DMSO and represent 100% viability.

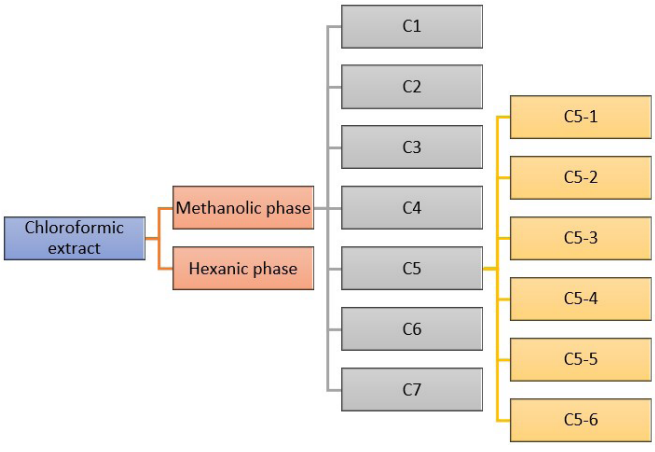

Figure 1. Scheme of extraction and isolation of fractions with antiproliferative potential of shrimp.

chromatography. An effect is recognized as synergistic when two or more chemical compounds or physiologic processes show a greater effect when they are acting together than individually (Tavadyan & Minasyan, 2019).

On the other hand, it was observed that the sub-fractions C5-3 and C5-4 at a concentration of 100 µg/mL, significantly ($p \leq 0.05$) decreased cell viability of MDA-MB-231 cell line, when compared to that observed with other sub-fractions. These values are even lower than those observed in cells treated with cisplatin; a drug that was used as a positive control, and lower than that observed for astaxanthin; a carotenoid present in crustaceans which antioxidant and antiproliferative activity have been reported (Sowmya et al., 2017; Nirmal et al., 2020). These results suggest an antiproliferative potential of the lipid sub-fractions of *L. vannamei* on MDA-MB-231 cell line. In previous studies, the antiproliferative activity of lipid compounds (such as hexadecanoic acid and 2-hexadecanol), extracted from the marine organism *Virgularia gustaviana*, on the same cancerous cell line, has been reported (Sharifi et al., 2020). Eicosapentaenoic acid (EPA), a fatty acid commonly found in marine organisms, has also been reported to cause arrest in MDA-MB-231 cell line (Barascu et al., 2006). Breast cancer is one of the most diagnosed, accounting for 11.6% of combined cases and 6.6% mortality (World Health Organization, 2020), representing the second cause of woman death (Siegel et al., 2016; Liu et al., 2022). In addition, this type of cancer is characterized by its capability to

Table 2. Viability percentage of colon carcinoma (HCT-116), breast adenocarcinoma (MDA-MB-231), and retinal pigment epithelia cell (ARPE-19) exposed to six sub-fractions at different concentrations.

Sub-fraction	Concentration (μg/mL)	Cell lines (%)		
		HCT-116	MDA-MB-231	ARPE-19
C5-1	12.5	122.2 ± 7.3	82.6 ± 8.7^{bcde}	112.7 ± 5.4^{ab}
	25	116.9 ± 3.8	132.1 ± 9.8^{ab}	112.0 ± 17.7^{ab}
	50	118.4 ± 9.9	126.4 ± 9.2^{abc}	91.5 ± 9.9^{abc}
	100	97.9 ± 10.1	145.7 ± 13.8^{a}	82.0 ± 2.6^{abcd}
C5-2	12.5	81.2 ± 4.5	76.8 ± 6.2^{cde}	108.0 ± 9.8^{ab}
	25	91.3 ± 7.5	102.4 ± 8.1^{abcde}	131.0 ± 13.7^{a}
	50	101.7 ± 10.3	106.9 ± 11.8^{abcde}	101.6 ± 14.3^{abc}
	100	103.3 ± 8.45	95.1 ± 17.0^{bcde}	98.5 ± 7.6^{abc}
C5-3	12.5	97.5 ± 5.4	79.3 ± 10.1^{cde}	105.0 ± 14.5^{ab}
	25	82.8 ± 7.0	158.0 ± 10.0^{a}	124.2 ± 10.2^{a}
	50	94.3 ± 7.6	117.6 ± 12.4^{abcd}	101.1 ± 4.0^{abc}
	100	86.6 ± 8.3	14.1 ± 4.4^{g}	31.7 ± 10.5^{d}
C5-4	12.5	83.1 ± 7.0	134.9 ± 14.0^{ab}	118.3 ± 5.4^{ab}
	25	96.0 ± 6.1	92.1 ± 4.8^{bcde}	112.6 ± 16.9^{ab}
	50	96.4 ± 8.23	68.5 ± 3.7^{defg}	88.2 ± 12.7^{abcd}
	100	94.5 ± 7.4	18.4 ± 4.9^{fg}	44.3 ± 18.2^{cd}
C5-5	12.5	81.5 ± 7.3	102.9 ± 13.9^{abcde}	118.1 ± 9.5^{ab}
	25	111.7 ± 6.9	115.2 ± 9.7^{abcd}	100.6 ± 6.5^{abc}
	50	94.4 ± 14.0	109.7 ± 7.3^{abcde}	92.2 ± 12.4^{abc}
	100	87.7 ± 6.6	71.1 ± 11.8^{cdef}	61.6 ± 10.1^{bcd}
C5-6	12.5	89.6 ± 8.3	105.5 ± 9.7^{abcde}	77.3 ± 4.4^{abcd}
	25	101.0 ± 6.8	122.2 ± 13.1^{abc}	102.3 ± 7.7^{abc}
	50	96.6 ± 9.9	69.6 ± 7.2^{defg}	96.5 ± 12.0^{abc}
	100	87.7 ± 8.3	92.8 ± 10.8^{bcde}	91.1 ± 7.8^{abcd}
Cis-platin	100	11.3 ± 2.78	23.1 ± 3.5	37.5 ± 5.8
Astaxanthin	100	-	113.4 ± 14.7	-

Viability percentage in response to six sub-fractions at different concentrations (12.5-100 mg/mL). The values represent mean of triplicates determinations ± standard error. Means indicated with different superscripts are significantly different ($P \leq 0.05$). Control cell cultures were incubated with DMSO and represent 100% viability.

invade other organs and by being triple negative, this means that it lacks the expression of estrogen (ER) and progesterone (PR) receptors, as well as HER2 (human epidermal growth factor receptor), that is, the treatments that have HER2 and hormones as target molecules have been reported as ineffective against this cell line (Komi et al., 2021), therefore, it could be said that the fractions evaluated in the present study do not act on HER2.

Similarly, in the ARPE-19 cell line, significantly ($p \leq 0.05$) lower cell viability values were achieved by C5-3 and C5-4 sub-fractions at their highest concentration, compared to that obtained when cisplatin was used; however, cell viability values were closer to 100% when sub-fractions concentration was 50 mg/mL (Table 2); based on that, the study of these sub-fractions at concentrations between 50 and 100 g/mL (as well as to calculate the bioselectivity index), is suggested.

3.3 Effect of C5-3 and C5-4 on the structure of MDA-MB-231

In order to detect changes in MDA-MB-231 cell structure, cells were stained with phalloidin and DAPI, which allowed the observation of some morphological changes in the cells structures after being treated with IC_{50} of sub-fractions C5-3 and C5-4 (Figure 2). These changes include chromatin condensation and cellular volume reduction (pyknosis); usually, one is accompanied by the other, these aspects are consistent with some others that occur during apoptosis (Battistelli & Falcieri, 2020). Protrusion of the plasmatic membrane, development of blebs, as well as microtubule peaks, were also possible to observe (Figure 2); these characteristics typically correspond to the later stage of apoptosis (Battistelli & Falcieri, 2020). It is pertinent to mention that one of the attributes sought in compounds with anticancer potential is that they cause cell death by apoptosis (Sipahli et al., 2022). However, further studies are considered necessary to determine the mechanism of death cell by which sub-fractions C5-3 and C5-4 might operate.

3.4 Proton nuclear magnetic resonance (^{13}C and ^{1}H- NMR)

Elucidation of the molecules contained in sub-fractions C5-3 and C5-4 was performed by means of proton nuclear magnetic resonance (^{1}H-NMR), the spectra of both sub-fractions are shown in Figure 3A-3B as well as an amplification of the chemical displacement of 2.5 to 8 parts per million (ppm). In both fractions, in high field are observed the protons of attached to bis-allylic carbons (2.7-20.8 ppm), the protons

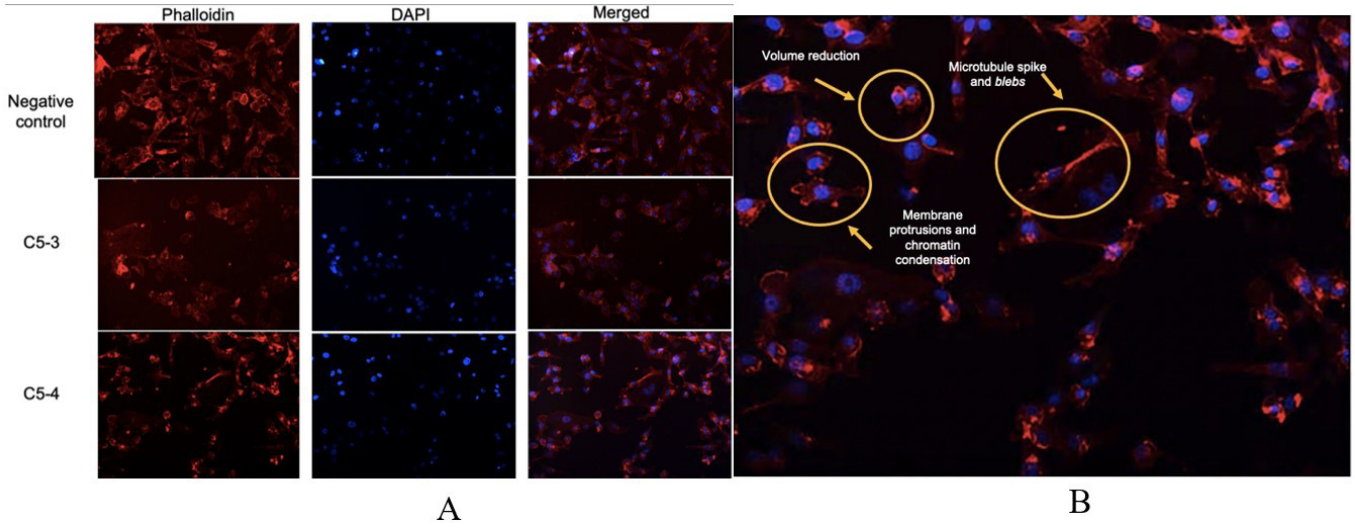

Figure 2. Morphological changes in MDA-MB-231 after 24 h of treatment. (A) rNegative control (without treatment); C5-3 (IC_{50} = 95.2 µg/mL); C5-4 (IC_{50} = 72.9 µg/mL); (B) Cells treated with sub-fraction C5-4. Actin cytoskeleton and DNA were stained with phalloidin and DAPI, respectively. Cells were observed at 20x.

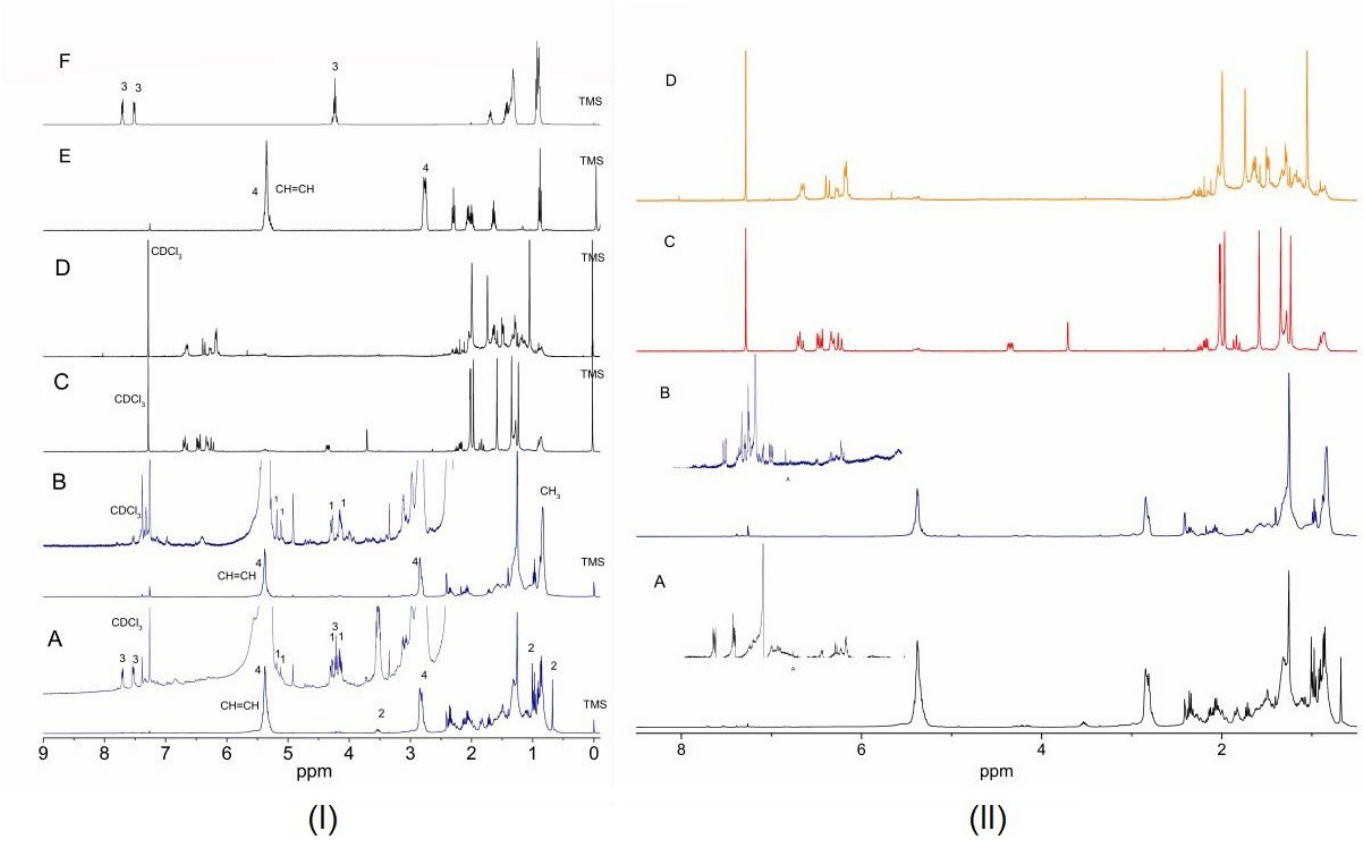

Figure 3. ^{1}H NMR Spectra: (I) A - sub-fraction C5-3; B - sub-fraction C5-4; C - Astaxanthin standard; D - β-Carotene standard; E - Eicosapeaenoic acid standard and F - Dioctyl phthalate standard. (II) ^{1}H NMR Spectra. A - sub-fraction C5-3; B - sub-fraction C5-4; C - Astaxanthin standard; D - β-Carotene standard.

attached to the allylic carbons (2.0-2.1 ppm) and the protons attached to methylene carbons (1.8-0.9 ppm) and terminal methyl groups (0.9-0.6 ppm) are observed (Knothe & Kenar, 2004). In both fractions, two compounds were identified by their chemical displacements. The first and most abundant presents chemical shift at 5.39, 2.83, 2.37, 2.15, 2.08, 1.74 and 0.87 ppm, corresponding to EPA, the ^{1}H NMR spectra of the pure EPA compound is shown in Figure 3E and into spectra 3A

and 3B the corresponding signals are marked with the number 4. The other compound identified in the two samples is glycerol, for its chemical shift at 4.14, 4.29, 5.19 and 5.12 ppm, probably this signal corresponds to the presence of triglycerides present in the sample, in the spectra 3A and 3B the corresponding signals are marked with the number 1; by the intensity of the signals, indicates that this compound is in low concentration when compared to the EPA. In the case of subfraction C5-3, two additional compounds were identified, the signals at 0.67 ppm and 3.54 ppm coincide with the chemical shift of the cholesterol, in the spectra 3A corresponding signals marked with the number 2. The signals at 4.23, 7.54 and 7.71 ppm coincide with the chemical displacements of phthalate (Figure 3F), in the spectra 3A corresponding signals marked with the number 3. To determine which compound was responsible for the color of the solution, the region of 6 to 9 ppm was enlarged, and the spectra obtained are shown in Figure 3. Although they are in very low concentration, the signals observed in the region of 6-7 ppm indicate the presence of β-carotene. In previous studies of our research group, signals corresponding to double bonds (-CH=CH-) between 5.3 - 5.4 ppm corresponding to the signals identified by López-Saiz et al. (2014) Figure 3 shows a low field extension (6 to 9 ppm) of C5-3 and C5-4 where the signals corresponded to those detected for b-carotene standard, this molecule is known primarily for its antioxidant activity and for being a precursor of vitamin A, however, there have been studies where its antiproliferative potential has been proved in some cancer cell lines (Zhang et al., 2016b; Shankaranarayanan et al., 2018). Furthermore, in addition to the spectra of sub-fractions C5-3 and C5-4 (A and B), shows eicosapentaenoic acid (EPA) and dioctyl phthalate (C and D respectively) spectra. In the spectra of both sub-fractions the signals corresponding to the EPA signals are shown, therefore, the presence of this fatty acid in the treatments can be confirmed; there is currently evidence that this fatty acid has potential antiproliferative on lines of murine cancer (López-Saiz et al., 2014) and on MDA-MB-231 (Li et al., 2019; Brown et al., 2020). On the other hand, in C5-3 an extension was made where signals corresponding to dioctyl phthalate are observed, phthalates are plasticizers and can be found in various marine organisms such as octopus (Cruz-Ramírez et al., 2015), turtles (Savoca et al., 2018), different fish species (Panio et al., 2020), amongst others. The presence of phthalates is related to anthropogenic pollution (Makkaew et al., 2022), nevertheless, there are studies where antiproliferative activity for phthalate has been disproved (López-Saiz et al., 2014). A link between the capacity of phthalates of binding and therefore activating estrogen receptors associated to signaling cascades for progression, and breast cancer, has been suggested by several *in vitro* studies (Zuccarello et al., 2018). In a study conducted by Fu et al. (2017) a relationship between the presence of di(2-ethylhexyl) phthalate metabolites and an increased incidence of breast cancer was observed. Another study found that di(2-ethylhexyl) phthalate significantly enhanced the invasion ability of MDA-MB-231 cells (Zhang et al., 2016a). Therefore, in this specific cell line, phthalates may be associated to a loss of antiproliferative potential of subfractions C5-3; since the presence of phthalates in this subfraction did not increase, or at least maintained, the antiproliferative activity, a lack of antiproliferative potential of this phthalate might be suggested.

4 Conclusion

Compounds extracted from white shrimp muscle possess antiproliferative potential over human cancerous cell lines, specifically, sub-fractions C5-3 and C5-4 that have lipid compounds such as β-carotene, polyunsaturated fatty acids such as EPA, cholesterol, and phthalate. It can also be concluded that lipid subfraction C5-4 showed significant antiproliferative capacity in breast adenocarcinoma (MDA-MB-231). In addition, the same cell line treated with C5-4 presents morphological changes associated with cell death by apoptosis, so it is suggested to perform relevant tests to determine the mechanism of cell death.

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