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# ISOLATION AND IDENTIFICATION OF A NEW ANTIPROLIFERATIVE INDOLOCARBAZOLE ALKALOID DERIVATIVE EXTRACTED FROM FARMED SHRIMP (*Litopenaeus vannamei*) MUSCLE

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**ABSTRACT**

Farmed shrimp *Litopenaeus vannamei*, one of the most consumed seafood in the planet, is a source of antiproliferative extracts still to be fully characterized. This study the isolation and identification of these antiproliferative compounds. From a chloroform-soluble extract from shrimp muscle, hexane- and methanol-soluble fractions were obtained and tested for antiproliferative activity (MTT), a bioassay that guided the fractionation and isolation of bioactive fractions using open column chromatography. MeOH-soluble fraction resulted bioactive and was subjected to further fractionation from which one subfraction outstand for being highly active against prostate cancer cell line. Antiproliferative effects were evaluated using colorimetric assays and cell morphology observations. Further chromatographic procedures resulted in sub-fractions from which one was effective in causing DNA damage and F-actin polymerization, which suggests cellular collapse and apoptosis. According to the structural chemical characterization carried out, dioctyl phthalate, eicosapentaenoic acid, and an indolocarbazole alkaloid type of compound were identified. This last compound, which resulted majorly responsible for the bioactivity, was not found reported in the available databases. Pure EPA control was used to compare it with the subfraction, observing greater activity in the subfraction than when EPA was used, suggesting that another compound different from EPA is providing the highest activity; but, more investigation is needed for a full chemical and structural characterization.

**Keywords:** *Litopenaeus vannamei*, bioactive compounds, marine source

## INTRODUCTION

Academic and pharmaceutical institutions have developed several chemotherapeutic compounds, but their efficacy and adverse effects are limitations for their use (**Buzdar *et al.*, 2005; DeVita & Chu, 2008; Martin *et al.*, 2014**). Another problem in cancer chemotherapy is drug resistance (**Austreid, Lonning, & Eikesdal, 2014**). Therefore, the search for more effective and safer bioactive molecules gains importance (**Livstone, 2019**).

The search for novel natural bioactive products is not an easy task; there are many challenges throughout the process of obtaining new effective and efficient drugs. Despite that, various organizations have decided to reduce mortality and morbidity rates through investigation on biologically active compounds from nature (**De Kok, Van Breda, & Manson, 2008; Thomson, LeWinn, Newton, Alberts, & Martinez, 2003**), and the search for these new molecules in the marine environment is each time more frequently considered. Nowadays, several studies have reported the existence of potential anticancer compounds (**Lordan, Ross, & Stanton, 2011; Stankevicins, Aiub, Maria, Lobo-Hajdu, & Felzenszwalb, 2008**), which include bacterial, fungal, but also higher animals including commonly consumed seafood; among them are shrimp species.

Shrimp, one of the top consumed seafood in the world, has been reported to contain potential anticancer compounds in its lipid fraction (**López-Saiz *et al.*, 2016; López-Saiz, Suárez-Jiménez, Plascencia-Jatomea, & Burgos-Hernández, 2013; Wilson-Sanchez *et al.*, 2010**). Examples include carotenoids, which have been associated to apoptosis induction and oxidative stress promotion in cancer cells lines (**Sila *et al.*, 2013; Sowmya, Arathi, Vijay, Baskaran, & Lakshminarayana, 2017**). Also, hemocyanin has reported as a cancerous cell-growth inhibitor and apoptosis inductor (**Zheng *et al.*, 2016**), and to exert antitumorigenicity in *in vivo* studies (**S. Liu *et al.*, 2017**).

Previously, a triglyceride esterified containing polyunsaturated fatty acids was reported to have the capability of inhibiting proliferation of a murine lymphoma cell line (**López-Saiz *et al.*, 2014**); however, other biologically active fractions obtained during this study were also reported. Polyunsaturated fatty acids are well known for providing health benefits from seafood consumption, since they are present in significant amounts; however, other minoritarian compounds with great bioactivity have been detected and identified as interesting molecules that might be proposed for their chemical synthesis and further research as potential chemotherapeutic agents. Recently, a highly chemoprotective indolocarbazole alkaloid type of compound, present in low concentrations in wild shrimp (*Litopenaeus stylirostris*), was reported (**García-Romo et al., 2020**). Based on the above, he present study aimed the characterization of these antiproliferative fractions previously reported in farmed shrimp *Litopenaeus vannamei* and to study the possible presence of the indolocarbazole alkaloid type of compound and the ways by which these molecules might act.

## MATERIALS AND METHODS

### Testing species

Farmed shrimp (*Litopenaeus vannamei*), acquired at Hermosillo, Sonora, México from a local market, was taken in ice to the University of Sonora seafood laboratory. Edible portions of shrimp (muscle) were separated and immediately processed for extraction according to reported procedure **López-Saiz *et al.*, (2014)**.

### Crude extraction and solvent partition

Shrimp muscle (100 g) extraction and solvent partitioning procedures were conducted according to reported procedure **López-Saiz *et al.*, (2014)**, from which hexane- and methanol-soluble fractions were obtained and dried under $N_2$ stream for further biological activity testing. All the studies performed in the present

research work were carried out using shrimp cultured from the same culturing season.

**Column chromatography**

Methanol-soluble fraction was reconstituted again in methanol and was subjected to fractionation using open column chromatography to obtain same fractions reported by **López-Saiz *et al*., (2014)**. All the fractions were tested to confirm their biological activity.

**Semi-preparative thin layer chromatography (TLC) and partial purification**

The fraction with the highest biological activity were further fractionated using silica gel-preparative TLC, using hexane-ethyl acetate (95:5) as a mobile phase (**W. Liu *et al*., 2019**). All the sub-fractions were evaluated for biological activity. For further purification of the most active compounds, undesired substances were eliminated by solvent partitioning.

**Cell lines**

Five cancerous- [A-549 (lung carcinoma), HCT116 (colon carcinoma), HeLa (epithelioid cervix carcinoma), MDA-MB-231 (breast adenocarcinoma), and 22Rv1 (prostate carcinoma)] and 1 non-cancerous [ARPE-19 (retinal pigmented epithelium)] human epithelial cell lines were used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 Medium (Sigma Aldrich, St. Louis, MO, USA), amended with 10 and 15 % heat-inactivated fetal bovine serum (FBS) (Corning, NY, USA), respectively, and incubated in an atmosphere of 5 % $CO_2$ at 37 °C.

**3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay**

Antiproliferative activity was evaluated using the MTT assay (Roche, cell proliferation kit I, Roche, Cat. No. 11-465-007-001), according to manufacturer's instructions. Briefly, 1 x $10^4$ and 2 x $10^4$ cells/well were suspended in 100 µL DMEM or RPMI at 10 – 15 % FBS, according to ATCC specifications (**Boyd & Paull, 1995**); they were seeded on flat-bottom 96-well plates before being incubated (24 h). Then, cell were incubated in 100 µL of medium amended with each of the samples (dissolved in DMSO) at different concentrations, diluted in supplemented medium at 10 – 15 % of FBS, and incubated for another 48 h. Control cultures consisted of cells in medium only (final DMSO concentrations: 0.06 % – 0.5 % v/v). After 4 h, 10 µL of MTT (5 mg/mL) were added to every well, and a 100 µL sodium dodecyl sulfate (SDS) aliquot was added to dissolve formazan crystals before the absorbance of each well was read (overnight) in an ELISA plate reader equipment (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Test and reference wavelengths were 570 and 650 nm, respectively. Cisplatin (CIS) (15663-27-1, Sigma-Aldrich) and docetaxel (DOC) (01885, Sigma-Aldrich) were used as positive antiproliferation controls. Dioctyl phthalate (DOP) (D201154, Sigma-Aldrich) and eicosapentaenoic acid (EPA) (E-2011, Sigma-Aldrich) were used as confirmatory standards for selected bioactive fractions. To evaluate the growth behavior of the most sensitive cell line to a specific fraction, a cellular growth kinetic was made from 0 to 72 h. Three independent assays were made, and all testing concentrations were assayed in triplicate (**K. Liu, Xiao, Wang, Chen, & Hu, 2017**).

**Phalloidin–tetramethylrhodamine B isothiocyanate and 4′, 6-Diamidino-2-phenyindole, dilactate fluorescence cell staining**

To observe the effect of shrimp fractions on internal morphological/structural aspects of cell lines, staining procedures were performed according to **Van Vuuren, Botes, Jurgens, Joubert, & Van Den Bout, 2019**, with modifications. Testing cultures were incubated in 96-well microplates for 24 h and treated with sub-fractions at their $GI_{50}$. With the aim of seeking the incubation time at which morphological changes cell can be observed, 3.7 % formaldehyde in PBS was used to fix cells and 0.2 % Triton X-100 in PBS was added for 15 min to permeabilize them, after 4, 12, 24, and 48 h. Then, phalloidin–tetramethylrhodamine B isothiocyanate (phalloidin) (Sigma-Aldrich, MFCD00278840) and 4′, 6-Diamidino-2-phenyindole, dilactate (DAPI) (Sigma-Aldrich, D9564) were used to stain cells and visualize F-actin and DNA, respectively. Microplates were placed onto a microscope (inverted epifluorescence, Leica DMi8) to carried out the observations.

**Ultraviolet–visible (UV-Vis) and fluorescence spectroscopies**

Bioactive subfraction was subjected to UV-Vis and fluorescence studies following the procedure described by **García-Romo *et al*. (2020)**.

**Proton-nuclear magnetic resonance ($^1$H- NMR)**

Sample preparation and measurements were carried out as described by **García-Romo *et al*. (2020)**. TMS proton signal was used an internal standard to record chemical shifts (ppm).

**Electrospray ionization tandem mass spectrometry (ESI/MS)**

Mass spectrometry analyses on bioactive fraction was performed according to **García-Romo *et al*. (2020)**.

**Statistical analysis**

An analysis of variance (ANOVA) along with the Tukey multiple comparisons test, with a confidence interval of 95 % was used to analyze data (SPSS). A probit analysis was carried out to obtain $GI_{50}$ values for bioactive fractions, using the Number Cruncher Statistical Software (NCSS), version 2001, NCSS Statistical Software, USA. Data is presented as the mean value ± S.D. Data computation was made following procedures reported by the National Cancer Institute (NCI) / National Institutes of Health (NIH). Developmental Therapeutics Program (DTP), Human Tumor Cell Line Screen Process (http://dtp.nci.nih.gov/branches/btb/ivclsp.html) and as described by **Monks *et al*., 1991**.

**RESULTS AND DISCUSSION**

**Extraction and fractions obtained**

Chloroform-soluble extract (yield of 5.58 g/kg of shrimp muscle) was obtained from which two partitions were achieved: a hexane- and a methanol-soluble partitions. Methanol-soluble partition resulted bioactive and it was fractionated using open column chromatography, from which were obtained 15 packages of fractions, denominating them fraction M (methanol partition) 1 - 15, whose yield (in mg per g of methanol partition) of each fractions are the following: 1, 7.9; 2, 23.5; 3, 24.1; 4, 15.4; 5, 8.4; 6, 11.9; 7, 12.9; 8, 10.9; 9, 188.7; 10, 401.5; 11, 43.6; 12, 67.0; 13, 76.7; 14, 32.7; 15, 23.6. To localize the most antiproliferative compounds among the 15 fractions, they were tested against different human cancer cell line; then a sub-division (highest level of purity) of the most bioactive fraction (M.11), calls sub-fraction M.11.A, M.11.B, M.11.C, and M.11.D, where the % obtained was 30, 10, 60 and 10, respectively; once the present compounds were identified, a partial purification of bioactive sub-fraction, was carried out.

**Bioactivity-guided isolation of antiproliferative compounds**

The potential that sample must prevent the proliferation of cancerous cells is known as antiproliferative activity; detection of this bioactivity in all the fractions obtained was performed using the MTT assay and the selection of the most bioactive fractions was based on their half-maximal growth inhibition ($GI_{50}$) values, the lower this value the higher the bioactivity. As shown in **Table 1**, only methanol partition could reduce ($GI_{50}$ values of 177 ± 8 µg/mL) the proliferation of 22Rv1 cell line, without affecting a non-cancerous cell line (ARPE-19). Methanol partition was then fractionated resulting in 15 fractions, form which M.11, M.12, and M.15 showed antiproliferative activity on human cell line 22Rv1 (prostate cancer), with $GI_{50}$ values of 70.6 ± 5.8, 71.0 ± 4.5, and 70.3 ± 5.6 µg/mL, respectively (**Table 2**). However, as shown also in **Table 2**, fraction M.11 was significantly ($P \leq 0.05$) more active against HeLa, MDA-MB-231, and A549 cell lines than the other 2 fractions, therefore it was selected for further fractionation.

**Table 1** Half-maximal growth inhibition ($GI_{50}$) for crude extract and partitions obtained from farmed shrimp (*Litopenaeus vannamei*) muscle on human epithelial cell lines after 48 h.

| | $GI_{50}$ (µg/mL) | |
|---|---|---|
| | *Cancerous cell* | *Non-cancerous cell* |
| | 22Rv1 | ARPE-19 |
| Chloroform crude extract | > 200 b | > 200 a |
| Hexane partition | > 200 b | > 200 a |
| Methanol partition | 177 ± 8 a | > 200 a |

Values represent means ± S.D. from 3 determinations. Distinct letters in a column are significantly different ($P \leq 0.05$). Control cultures were performed in DMSO (0.5 %) representing 100 % proliferation. These values were statistically estimated from a dose response curve using concentrations of 200 µg/mL downwards.

**Table 2** Half-maximal growth inhibition ($GI_{50}$) for fractions obtained from column chromatography fractionation of the methanol-partition, on human cancer epithelial cell lines after 48 h.

| | $GI_{50}$ (µg/mL) | | | | |
|---|---|---|---|---|---|
| | *Adenocarcinomas* | | *Carcinomas* | | |
| | HeLa | MDA-MB-231 | A549 | 22Rv1 | HCT116 |
| M.1 | > 200 e | > 200 e | 172 ± 16 d | 135 ± 17 cd | > 200 e |
| M.2 | > 200 e | > 200 e | 86 ± 10 b | 195 ± 11 f | > 200 e |
| M.3 | > 200 e | > 200 e | > 200 e | > 200 f | > 200 e |
| M.4 | > 200 e | > 200 e | > 200 e | > 200 f | > 200 e |
| M.5 | > 200 e | > 200 e | > 200 e | 142 ± 7 d | > 200 e |
| M.6 | > 200 e | 148 ± 10 cd | > 200 e | 117 ± 7 c | > 200 e |
| M.7 | > 200 e | > 200 e | > 200 e | > 200 f | > 200 e |
| M.8 | > 200 e | > 200 e | > 200 e | > 200 f | > 200 e |
| M.9 | > 200 e | > 200 e | 167 ± 11 d | 173 ± 11 e | 189 ± 9 e |
| M.10 | 161 ± 9 d | > 200 e | 131 ± 8 c | 84.0 ± 6.2 b | 130 ± 19 d |
| M.11 | 144 ± 13 c | 101 ± 10 b | 83.5 ± 12.2 b | 70.6 ± 5.8 b | 92.7 ± 16 c |
| M.12 | 148 ± 8 c | 132 ± 6 c | 174 ± 13 d | 71.0 ± 4.5 b | 112 ± 16 cd |
| M.13 | > 200 e | > 200 e | > 200 e | > 200 f | > 200 e |
| M.14 | > 200 e | > 200 e | > 200 e | > 200 f | > 200 e |
| M.15 | > 200 e | 138 ± 8 cd | > 200 e | 70.3 ± 5.6 b | 107 ± 15 cd |
| CIS | 47.1 ± 3.8 b | 152 ± 5 d | 33.6 ± 2.6 a | 21.6 ± 2.4 a | 61.2 ± 5.1 b |
| DOC | 10.9 ± 3.6 a | 21.0 ± 3.5 a | 18.5 ± 2.6 a | 24.1 ± 6.6 a | 12.8 ± 2.4 a |

Values represent means ± S.D. from 3 determinations. Distinct letters in a column are significantly different ($P \leq 0.05$). Control cultures were performed in DMSO (0.5 %) representing 100 % proliferation. These values were statistically estimated from a dose response curve using concentrations of 200 µg/mL downwards. Cisplatin (CIS) and docetaxel (DOC) were used as a positive control for cell growth inhibition. Adenocarcinomas and carcinomas refer to the cell disease.

**Table 3** Half-maximal growth inhibition ($GI_{50}$) for sub-fractions obtained from thin layer chromatography fractionation of fraction M.11, on human epithelial cell lines at 48 h.

| | $GI_{50}$ (µg/mL) | | Bioselective index |
|---|---|---|---|
| | *Cancerous cell* | *Non-cancerous cell* | |
| | 22Rv1 | ARPE-19 | |
| M.11.A | 134 ± 11 c | > 200 c | > 1.48 |
| M.11.B | 46.6 ± 2.2 b | > 200 c | > 4.29 |
| - M.11.B.1 | 26.6 ± 8.0 a | 180 ± 3 b | 6.77 |
| M.11.C | > 200 d | > 200 c | --- |
| M.11.D | > 200 d | > 200 c | --- |
| DOP | > 200 d | > 200 c | --- |
| EPA | 58.3 ± 10 b | > 200 c | > 3.42 |
| CIS | 21.6 ± 2.4 a | 184 ± 15 b | 8.52 |
| DOC | 24.1 ± 6.6 a | 155 ± 4 a | 6.44 |

Values represent means ± S.D. from 3 determinations. Distinct letters in a column are significantly different ($P \leq 0.05$). Control cultures were performed in DMSO (0.5 %) representing 100 % proliferation. Dioctyl phthalate (DOP) and eicosapentaenoic acid (EPA) were used as standard controls. Cisplatin (CIS) and docetaxel (DOC) were used as a positive antiproliferative control. (---) means "was not determined".

To reach a higher level of isolation of the bioactive compounds, fraction M.11 was carried out by TLC in 4 groups of compounds with different polarities, which were coded sub-fractions M.11.A, M.11.B, M.11.C, and M.11.D. As shown in **Table 3,** sub-fraction M.11.B, was the most bioactive against the 22RV1 cell line, with a $GI_{50}$ = 46.6 ± 2.2 µg/ml; shows that the fraction procedure increased the antiproliferative activity, since the $GI_{50}$ value decreased from 70.6 ± 5.8 (in M.11) to 46.6 ± 2.2 µg/ml (in M.11.B), making it more effective against prostate cancerous cell line.

**Characterization of sub-fraction M.11.B**

For chemical/structural characterization of fraction M.11.B, $^1$H-NMR, UV-Vis, ESI/MS, and fluorescence were used. UV-Vis analysis showed that compounds present in M.11.B have maximum absorption at wavelength of 205, 223, and 274 nm, these transitions are attributed to $\pi \rightarrow \pi*$, which are related to the aromatic structures (**Fig. 3 A**). In addition, two bands with low absorbance at 313 and 350 nm and associated to $n \rightarrow \pi*$ transition were also observed. This kind of transitions are related to aromatics rings that have attached atoms with available electrons (e.g., O and N). The fluorescence study (**Fig. 3 B**) showed an emission band centered at λ = 311 nm when the sample was excited at λ = 250 or 274 nm, suggesting the presence of a single compound responsible for this emission. These results confirm the presence of aromatics rings present in the extract.

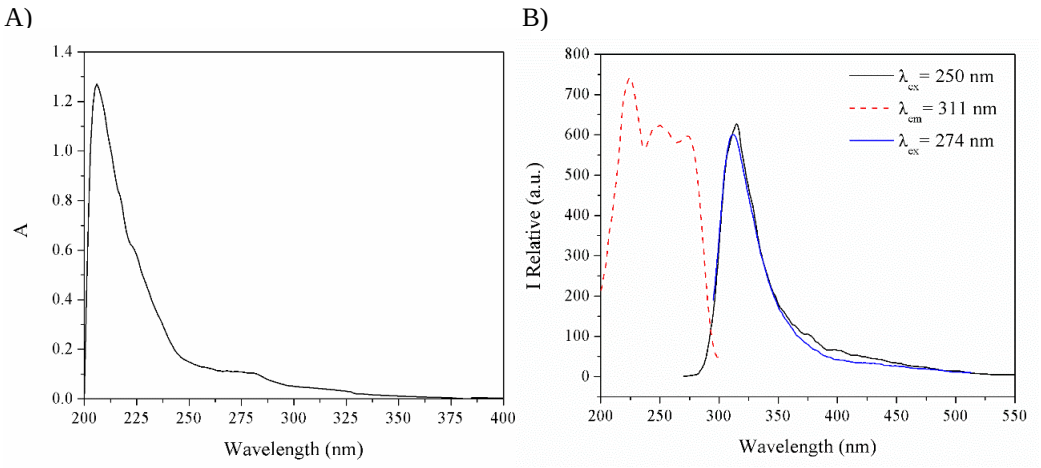


**Figure 3** UV-Vis (A) and fluorescence (B) spectra of sub-fraction M.11.B diluted in methanol.

Results from $^1$H-NMR showed that M.11.B consists of more than one compound (**Table 4**). A characteristic pattern of 1, 4 di-substituted aromatic ring [δ = 7.71 ppm (*dd*, J= 5.7, 3.3 Hz)], protons of aromatic rings [7.53 ppm (*dd*, J= 5.7, 3.3 Hz)], signals obtained at δ of 4.22 ppm (associated to O-$CH_2$), at δ high field of 1.25 ppm ($CH_2$) and signals at δ 0.88 ppm ($CH_3$ groups) suggest dioctyl phthalate (DOP), which has been reported by others (**Cruz-Ramírez *et al*., 2015; López-Saiz *et al*., 2014; García-Romo *et al*., 2018**).

On the other hand, signals (ppm) on 5.33 (*m*), 2.83 (*m*), 2.35 (*t*, J= 7.5 Hz, 3H), 2.03 (*m*) suggested the presence of eicosapentaenoic acid (EPA), and they are in accordance with those reported in the literature (**Tyl, Brecker, & Wagner, 2008**). At this stage, results suggested the presence of DOP and EPA in M.11.B; however, other signals were also detected.

Likewise, signals that appeared at δ = 9.77 (s) ppm; associated to hydroxyl groups, δ = 7.54 (*d*), 7.36 (*d*) and 7.13 (*dd*) ppm; related to protons of substituted aromatic rings, signals at δ = 3.8 – 3.4 (*m*) ppm; belong to aliphatic protons, and that at δ = 1.15 (*d*) ppm; associated to methyl protons, were also observed. The signals in $^1$H-NMR spectrum of the proposed molecule presenting the activity show signals in the aromatic region as in the aliphatic region. The complex coupling pattern of the signals that appear in the aromatic region indicate that there is a trisubstituted type aromatic ring of type 1, 2, 4. The signals in the region 3-4 ppm indicate that the protons are attached electronegative elements such as oxygen or nitrogen.

**Table 4** $^1$H-NMR spectra of sub-fraction M.11.B in $CDCl_3$ at 400 Hz.

| **Dioctyl phthalate (DOP)** | | **Eicosapentaenoic acid (EPA)** | | **Indolocarbazole alkaloids derivative (IAD)** | |
|---|---|---|---|---|---|
| ***No.*** | ***δH*** | ***No.*** | ***δH*** | ***No.*** | ***δH*** |
| 2 | 7.71 (dd, J= 5.7, 3.3 Hz, 2H) | 5, 6, 8, 9, 11, | | 18 | 9.77 (s, 2H) |
| 1 | 7.53 (dd, J= 5.7, 3.3 Hz, 2H) | 12, 14, 15, 17, | 5.35 (m, 10H) | 9 | 7.54 (d, J=8.7 Hz, 2H) |
| 5 | 4.22 (m, 4H) | 18 | | 6 | 7.36 (d, J=2.1 Hz, 2H) |
| 6, 7, 8, | 1.25 (s, 18H) | 4, 7, 10, 13, | 2.83 (m, 12H) | 8 | 7.13 (dd, J=8.8, 2.6 Hz, 2H) |
| 9, 11 | | 16, 19 | | 12, 13, 14 | 3.8 – 3.4 (m, 5H) |
| 10, 12 | 0.88 (m, 12H) | 2 | 2.35 (t, J= 7.5 Hz, 3H) | 15 | 1.15 (d, J= 6.28 Hz, 6H) |
| | | 4 | 2.03 (m, 2H) | | |

In addition, results from ESI-MS, in negative mode (**Fig. 4**), indicated a molecular weight of 540.06 m/z, which is not consisting of neither with DOP or EPA, which indicates that the signal observed possibly corresponds to an indolocarbazole alkaloid derivative (IAD). Considering the signals observed in the NMR spectrum and the observed molecular weight found, it suggests symmetry in the molecule. The shape of the signals in the aromatic region coincides with those in Arcyriaflavin C (**Kotha, Saifuddin, & Aswar, 2016; Nakatani *et al.*, 2003; Steglich, Steffan, Kopanski, & Eckhardt, 1980**). Based on the information obtained by the different spectroscopic data the molecules suggested as components of M.11.B (**Fig. 5**). **Table 4** shows the chemical shifts as well as the integrals of the signals observed in the NMR spectrum.

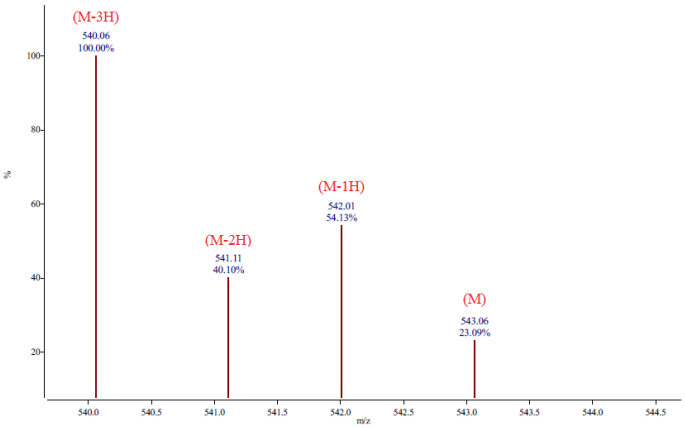


**Figure 4** Mode negative ESI /MS of sub-fraction M.11.B, pointing the mass / charge of IAD and their isotopes (M-H).

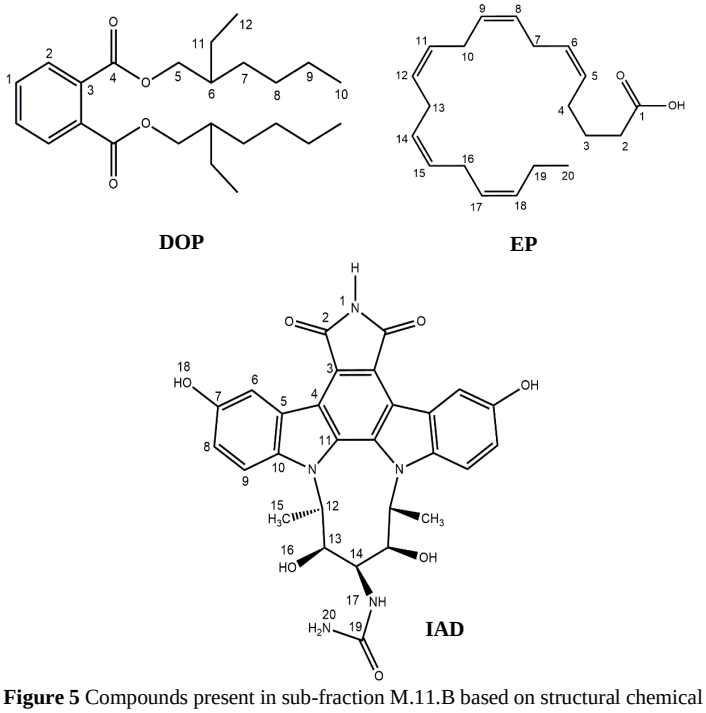


**Figure 5** Compounds present in sub-fraction M.11.B based on structural chemical characterization.

The molecule proposed as representative of antiproliferative activity is 1-((5S,7r,9R)-2,6,8,12-tetrahydroxy-5,9-dimethyl-14,16-dioxo-6,7,8,9,15,16-hexahydro-5H,14H-4b,9a,15 triazadibenzo [b, h] cyclonona [jkl] cyclopenta [e] -as-indacen-7-yl) urea (IUPAC nomenclature), with chemical formula $C_{28}H_{25}N_5O_7$ and with mass of 543 Da, this molecule has the characteristics of a new indolocarbazole alkaloid derivative (IAD). The molecule contains atoms of nitrogen related to alkaloid compounds, presenting heterocyclic aromatic organic rings characteristic of the indoles, these are commonly present in natural ecosystems and can be produced by a wide variety of bacteria; being structures derived from the indoles, the indocarbazoles.

On the other hand, chemical structures derived from them and biologically active are the arcyriaflavin, whose molecule is structural base (substituted or not) of the compound isolated in the sample of shrimp; arcyriaflavin is a compound that is structurally related to staurosporine. The staurosporine, is a chemical structures similar to our molecule present in M.11.B, example of these with anticancer activity are: 7-oxo-3, 8, 9-trihidroxiestaurosporine (more similarity with our molecule) and 7-oxo -8, 9-dihydroxy-4'-N demethystastaporine which are present in the ascidia marine *Cystodytes solitus* and both showed cytotoxicity to cell lines HT-29, A549, and MDA-MB-231 (**Reyes *et al.*, 2008**), as well as isolated in higher organisms (**Jimenez *et al.*, 2012**). Therefore, indolocarbazole are a group of structures currently being studied because of their anticancer potential, as well as to the extensive and possible number of derivatives from them; since then, a many molecules have been found throughout the planet, as well as their important uses and applications (**Sánchez, Méndez, & Salas, 2006; Wang, Zhang, Li, Ding, & Ma, 2018**); and as we know there is an extensive list of indole alkaloids type of molecules that have been isolated from different marine organisms whit bioactivity potential (**Netz & Opatz, 2015**).

**Antiproliferative activity of sub-fraction M.11.B.1 and bioselectivity**

Chemical / structural characterization carried out on fraction M.11.B allowed the identification of 2 of at least three compounds present in this fraction; therefore, they were commercially obtaining and tested for bioactivity confirmation (**Table 3**). Resulting for DOP ($GI_{50}$ greater than 200 µg/ml) against 22Rv1 cell line suggested that DOP does not contribute to M.11.B antiproliferative activity. On the other hand, a $GI_{50}$ = 58.3 ± 10 µg/ml value obtained for EPA indicated contribution of this compound to M.11.B bioactivity. Other studies have reported similar $GI_{50}$ values for EPA (**Chiu & Wan, 1999; Lai, Ross, Fearon, Anderson, & Carter, 1996; Li, Hou, Yeh, & Yeh, 2017; Turan, Kaya, & Erdem, 2011**). However, by being this $GI_{50}$ value higher than that obtained for M.11.B, the presence of a third more active compound is suggested. As an additional test, M.11.B was partially purified by removing DOP as much as possible with a wash with ethyl ether (10:1 DMSO v/v), and the resulted purified fraction (M.11.B.1) was tested again for bioactivity.

Sub-fraction M.11.B.1 showed the highest antiproliferative activity (**Table 3**), showing a $GI_{50}$ = 26.6 ± 8 µg/mL; according to American National Cancer Institute (NCI) criteria, the $GI_{50}$ limit value of a crude extract to be considered worthy for further purification is < 30 µg/mL (Upper limit) (**Abdel-Hameed, Bazaid, Shohayeb, El-Sayed & El-Wakil, 2012**).

Bioselectivity index (BI) is the term is used to refer to an agent that exhibits a difference growth inhibitory effect between normal and cancerous cell lines. An agent is labeled as "bioselective" if it shows a ratio of at least 3 times its value between its $GI_{50}$ of normal and cancerous cells (**Ciavatta *et al.*, 2017**). In this case, the sub-fraction M.11.B.1 was bioselective against 22Rv1 cell line (**Table 3**); the BI was 6.77, and it has been established that the higher the BI, a greater chance has the agent of being effective on cancerous cells without harming non-cancerous cells.

Growth kinetics allows us to determine the number of cells affected by M.11.B.1; once the dose-response relationship is obtained, the number of affected (dead/ inhibited) cells by a certain concentration (at a determined time of incubation under stimulus) can be determined. This was obtained for 22Rv1 cell line in 10 % FBS (**Fig. 1**). The effective concentration (EC) is the proportion of a substance in a

medium that causes a certain effect in each model (cells); the number of prostate cancer cells obtained after 48 h of incubation were 32500, meaning that 22500 cells proliferated in 48 h since 10000 cells were seeded. Therefore, the EC to affect 50 % ($EC_{50}$) of cell growth (11250 cells) is 26.6 ± 8 µg/mL, considering this as antiproliferation.

To obtain inhibition values equal or lower than 10000 cells on *in vitro* proliferation systems, we would be talking about cytostatic and cytotoxic terms, like total growth inhibition (TGI), which refers to 0 % of proliferations cells (22500) and lethal concentration medium ($LC_{50}$) that refers to -50 % of viable cells (<10000, which refers to the number of cells seeded), respectively (**Boyd & Paull, 1995**), whose required concentrations to these values are greater than 200 µg/mL.

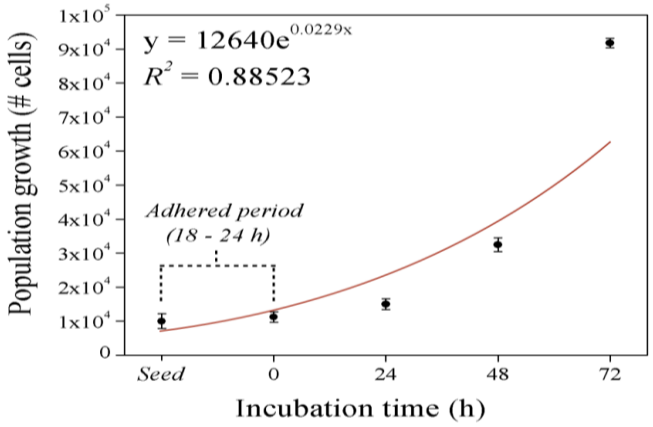


**Figure 1** Growth kinetics of 22Rv1 cell line (human prostate epithelial carcinoma). Values represent means ± S.D. from 3 determinations. Cells were plated in medium RPMI-1640 with 10 % FBS. These means optimal growth, when the cells were incubated in the presence of DMSO (0.5 %), representing 100 % proliferation on assays.

According to the ATCC, the 22Rv1 (ATCC® CRL-2505™) cell line, is a human epithelial prostate and tumorigenic carcinoma (possessing an androgen receptor), which is stimulated by epidermal growth factor (EGF); however, transforming growth factor beta-1 (TGF beta- 1) does not inhibit it. Moreover, the compounds could be conducting an antiproliferative activity by means of this receptor, this if the extrinsic pathway were considered; however, another way of action would be the intrinsic or mitochondrial pathway where it is related to oxidative stress (**Kroemer *et al.*, 2009**). To make it clear, according to the $GI_{50}$ obtained for the rest of the fractions by the Probit test, they are not worthy to be further investigated for bioactivity since their $GI_{50}$ do not meet the NCI criteria for the development of drugs (**Boyd & Paull, 1995; Chakravarti & Klopman, 2008**).

**Cell morphology changes induced by sub-fraction M.11.B.1**

The purpose of this part of the current study was to observe the effects that M.11.B.1 has on cell structure and how these possibly might be related to cell death. Cell morphology changes induced by M.11.B.1 were discussed based on observations made after staining cells F-actin and nuclear material, using fluorescence microscopy; results are shown the in **Fig. 2**. Cells treated with 26.6 ± 8 µg/mL M.11.B.1 showed, after 4 h of exposure, cellular structures morphologically similar to those of control, which were cultured only in the presence of DMSO (<0.5 %). DNA staining with DAPI allowed the observation of cellular damages such as chromatin condensation and nuclear shrinkage (also called pyknosis); moreover, in most cells, DNA diffusion (karyolysis) was observed and, in other cells, karyorrhexis was observed as well, being both phenomena associated to DNA fragmentation. These morphological issues are characteristic of cells that undergoing dead pathways.

Different from control cells, in those treated with M.11.B.1 a decrease in the cell volume (shrinkage) was observed. Large plasma membrane protrusion (blebs) in 22Rv1 cells resulted from F-actin polymerization, which is associated to cell collapse (**Coleman *et al.*, 2001; Taylor, Cullen, & Martin, 2008; Wickman, Julian, & Olson, 2012**). Likewise, microtubule spike and beaded apoptopodia, and apoptotic bodies, which have been associated to cell fragmentation (**Poon *et al.*, 2014; Rubartelli, Poggi, & Zocchi, 1997; Witasp *et al.*, 2007**), were also observed. Results suggest that only a certain percentage of cells were in apoptotic processes, since plasma membrane rupture (which is associated to cellular and nuclear lysis as well as to necrosis pathways) could not be distinguished in cells. However, more studies are recommended to determine the proportions of cells that undergo either pathway.

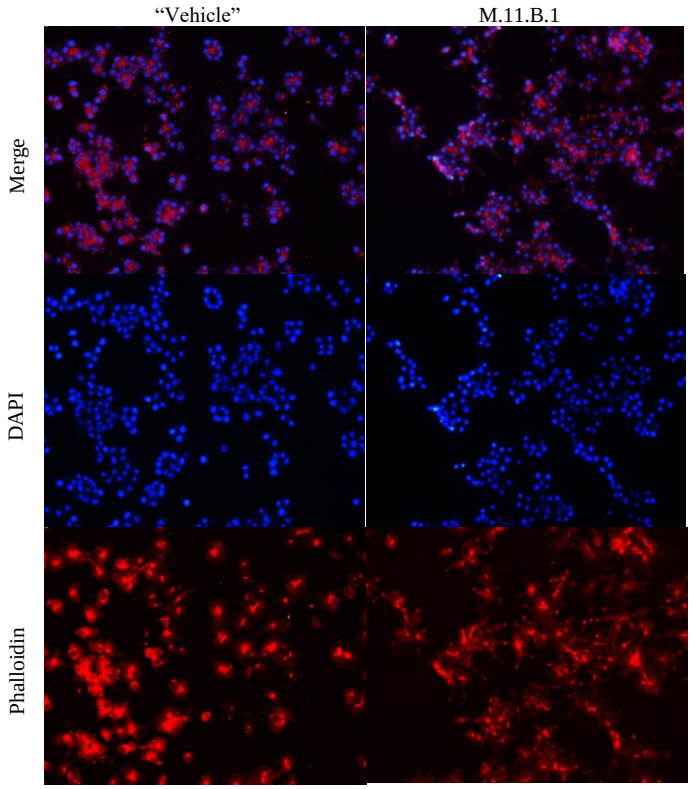


**Figure 2** Effect of sub-fraction M.11.B.1 on 22Rv1 cell line (human prostate epithelial carcinoma) morphology changes after 4 h of incubation. Cell line 22Rv1 treated with solvent (DMSO to 0.5 %) only was considered the negative control ("Vehicle"). Fraction M.11.B.1 at $GI_{50}$ (26.6 ± 8 µg/mL) was used as the treatment. DNA (blue) and actin cytoskeleton (red) were observed by DAPI and phalloidin–tetramethylrhodamine B isothiocyanate staining, respectively. The cells were observed at 20x.

These results are evidence that the compound present in M.11.B.1 responsible for decreasing cancerous cell population, and possibly for inducing apoptosis on prostate carcinoma *in vitro*, is another one in addition to carotenoids, a triglyceride esterified containing PUFAs or even free PUFAs (compounds already reported in several previous studies). A general diagram of the bioassay guided isolation and the identification of the IAD extracted from *L. vannamei* with *in vitro* anticancer potential is shown in **Fig. 6**. In addition, recently, antihemolysis, antioxidant and retinoprotective potential activities have been reported for these same compounds (**García-Romo, *et al.*, 2020**) which suggests that, in addition to reduce proliferation of cancerous cells, they may also act in a protective way at the same time.

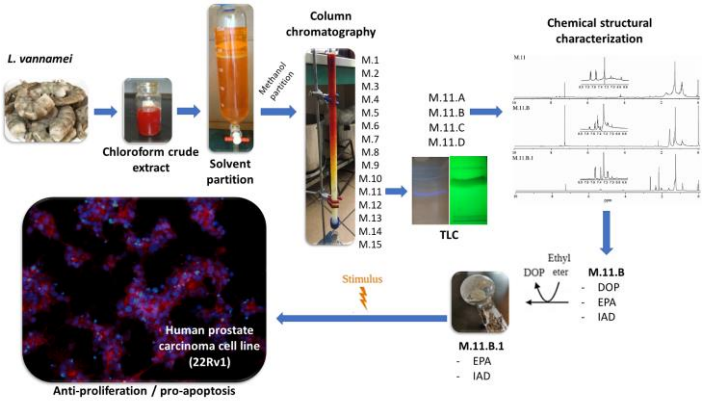


**Figure 6** General diagram of the bio-assay guided isolation from farmed shrimp (*Litopenaeus vannamei*) muscle.

**CONCLUSIONS**

The present study demonstrated that the antiproliferative effect of M.11.B.1 on a prostate cancerous cell line is, in addition to EPA, majorly caused by another compound yet to be fully characterized (IAD). Also, this compound possibly has the capability of inducing cellular collapse associated to apoptosis. These findings open the possibility of chemically synthesize IAD and test it in its pure form in order to biochemically understand its possible beneficial effect on health and be proposed as potential chemotherapeutic agents. However, further investigation is

needed to confirm the apoptotic activity of components of M.11.B.1; specifically, early and late apoptosis by annexin-V / propidium iodide, activation of intrinsic and extrinsic caspases, and even alteration of oxidative stress by ROS (2 ', 7'-Dichlorodihydrofluorescein, a fluorescent probe).

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