academicJournals

Vol. 15(8), pp. 214-220, 24 February, 2016
DOI: 10.5897/AJB2014.13783
Article Number: 3709FA357324
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antiproliferative activity of protein extracts from the black clam (*Chione fluctifraga*) on human cervical and breast cancer cell lines

Hiesu García-Morales[1], Luis Enrique Gutiérrez-Millán[1], Miguel A.Valdez[2], Armando Burgos-Hernández[3], Teresa Gollas-Galván[4] and María G. Burboa[1]*

[1]Posgrado en Biociencias, Departamento de Investigaciones Científicas y Tecnológicas (DICTUS), Universidad de Sonora, Hermosillo, Sonora, 83000, México.
[2]Departamento de Física, Universidad de Sonora, Hermosillo, Sonora, 83000, México.
[3]Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora, Hermosillo, Sonora, 83000, México.
[4]CTAOA, Centro de Investigaciones en Alimentación y Desarrollo A.C, Carretera a la Victoria Km 0.6, Hermosillo, Sonora, 83304, México.

Received 6 March, 2014; Accepted 13 October, 2015

The wide diversity of the marine environment has been an important resource for the discovery of new bioactive agents from marine organisms. The aim of this study was to obtain protein extracts from the clam *Chione fluctifraga* and determine its antiproliferative activity against cervical and breast cancer cells. The extracts were obtained by ammonium sulfate fractionation, gel filtration and ion exchange chromatography. Antiproliferative activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT). The results showed that 3F3 had antiproliferative effect against HeLa and MDA-MB-231 cell lines with IC_{50} values of 138.035 and 157.19 µg/ml, respectively, and 2F2 showed IC_{50} values of 67.46 µg/ml on HeLa cells. These results suggest that protein extracts from *C. fluctifraga* might be potential anticancer agents.

Key words: Protein extracts, clam, antiproliferative activity, breast cancer, cervical cancer.

INTRODUCTION

Cancer is a worldwide health issue; it is not just one disease but a variety of different diseases, which have in common uncontrolled cell proliferation. There are more than 100 different types of cancer, most of them take the name of the organ or cell in which they begin to grow (Hawkins, 1992; Martinez et al., 2003). Breast and cervical cancer are the main cancer related cause of death in women around the world. The high prevalence of these types of cancer is due to a lack of strategies that allow detection at early cancer stages (Palacio-Mejía et al., 2009; Gök et al., 2011). Chemotherapy is the most commonly used treatment against cancer, however some

*Corresponding author. E-mail: mburboa@correom.uson.mx. Tel: (662) 2592169. Fax: (662) 2592197.

patients develop resistance against the drugs and this has become a major problem in the past years. Resistance to treatment can be caused by a large variety of factors, which include individual variations between patients and genetic differences among tumor cells (Gottesman, 2002; Jordan and Wilson, 2004; Bhutia and Maiti, 2008). The discovery of new compounds from natural sources has been very important in pharmaceutical science research. The past decade has seen an increase in the number of compounds obtained from the screening of diverse marine invertebrates, such as soft corals, tunicates and mollusks, that are advancing to preclinical trials (Liang et al., 2008; Simmons et al., 2005; Amador, 2003; Bhatnagar and Kim, 2010). Examples of anticancer agents extracted from marine organisms include bryostatin-1, a macrocyclic lactone from *Bugula neritine*, with antitumor activity; ziconotide, a peptide from cone snail, is an N-type calcium-channel blocker in chronic pain treatment; ectenaisdin 743 is a tetrahydroisoquinolone alkaloid, isolated from *Ectinascidia turbinate*, with cytotoxic activity. It has been previously reported that the peptide dolastatin, obtained from the marine mollusk *Dollabella auricularia*, possesses an impressive growth inhibition of cancer cells. Another example is Kahalalide F, a cyclic depsipeptide obtained from *Elysia rufescens*; this peptide shows antitumor activity against colorectal, lung and prostate cancer cells, being lysosomes the cellular target (Haefner, 2003; Amador, 2003; Aneiros and Garateix, 2004; Faircloth and Cuevas, 2006; Bhatnagar and Kim, 2010).

Many peptides with different biological activities such as antioxidant (Wenyan et al., 2012), immune-regulatory (Lixin et al., 2005), antihyperglycemia and antihyperlipemia activity (Tsai et al., 2008) have been isolated from the clam *Meretrix meretrix*; polypeptide Mer2, MGP_{0501} and MGP_{0405} inhibits cell proliferation of several cancer cell lines; Mere-15 shows antitumor activity *in vitro* and *in vivo;* the protein (MML) shows growth inhibition in several cancer cell lines such as breast, colon and hepatic (Leng et al., 2005; Ning et al., 2009; Wang et al., 2012). Two peptides, 18 and 16 kDa, with cytotoxic activity *in vitro* against HeLa cells have been purified from *Arca subcrenata* (Song et al., 2008). The Gulf of California has a great diversity of clams, such as the black clam (*Chione fluctifraga*), that represent a popular seafood and a very important income source for the community (Martinez-Cordova and Martinez-Porchas, 2006). The aim of the present study was to evaluate the capability of *C. fluctifraga's* protein extract to inhibit the cellular growth on HeLa and MDA-MB-231 cultured cells.

MATERIALS AND METHODS

Materials

The clams (*C. fluctifraga*) were obtained from the experimental unit of the University of Sonora (Kino Bay, Sonora). All the reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of the crude extract

The clams were cut into small pieces and homogenized with 0.01 M phosphate buffer (0.1 M NaCl, pH 7.2) in a 1:3 ratio (w/v). The homogenate was centrifuged at 3000 *x g*. The supernatant was fractionated by ammonium sulfate precipitation up to 60% saturation. After 12 h of stirring the precipitate was collected by centrifugation (3000 *x g*, 10 min) and the pellet was suspended in phosphate buffer and collected as the crude extract.

Preparation of protein extract

The crude extract was passed through a Sephadex G-25 column (1.5 × 50 cm) pre-equilibrated with 0.01 M phosphate buffer (0.1 M NaCl, pH 7.2) at a flow rate of 1 ml/min and the absorbance was monitored at 280 nm. The fraction collected was dialyzed against 0.025 M Tris-HCl and then loaded onto a HiPrep 16/10 Q XL column (BioRad) pre-equilibrated with 0.025 M Tris-HCl pH 8.2. The column was eluted with a linear gradient 0 to 1 M NaCl prepared in Tris-HCl buffer at a flow rate of 1 ml/min, each fraction was collected at a volume of 5 ml and detection was performed at 280 nm. Fractions were lyophilized then dialyzed and antiproliferative activities were determined.

Measurement of protein content

The protein concentration was measured by the BCA method using the BCA protein assay kit (Pierce, USA) and bovine serum albumin as standard.

Tricine-SDS-PAGE

The extraction process was monitored by electrophoresis tricine-SDS-PAGE (Schägger, 2006) using a Bio-Rad Mini-Protean II electrophoresis unit (Bio-Rad, CA, USA) with an acrylamide concentration of 16% for the running gel. The protein bands were detected by silver stain.

Cell culture

The breast cancer cell (MDA-MB-231), cervical cancer cell (HeLa) and a human retinal pigment epithelial cell line (ARPE-19) were obtained from American Type Culture Collection (Rockrville, MD). HeLa and ARPE-19 cell were grown in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, BRL, Grand Island, NY), 1% penicillin-streptomycin and 1% gentamicin; MDA-MB-231 was grown in DMEM supplemented with 15% fetal bovine serum, 2 mM glutamine, 1% penicillin-streptomycin, 1% gentamicin and 1% non-essential amino acids (SIGMA-Aldrich, St. Louis, MO, USA) and were maintained at 37°C under humidified atmosphere with 5% CO_2.

Antiproliferative assay

MDA-MB-231, HeLa and ARPE-19 cells were cultured in a 96 wells microplate (1×10^4 cell/ml) for 24 hours; then 50 µl of the extracts were added in serial concentrations, PBS or Cisplatin as controls, and incubated for 48 h. At the end of the incubation period, 10 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

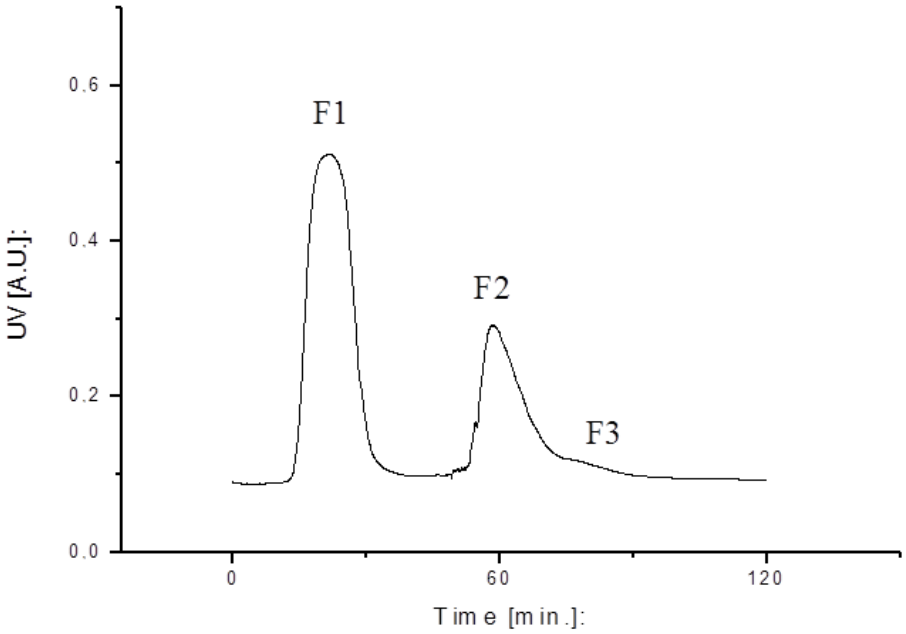

Figure 1. Fractionation of crude extract by gel filtration chromatography on a Sephadex G-25 column. Column specification: 1.5 x 50 cm; the elution was performed with 0.1M phosphate buffer at a flow rate of 1 ml/min. The elution was monitored at 280 nm.

solution (SIGMA-Aldrich, St. Louis, MO, USA) were added to each well and the microplate was incubated for 4 h to allow the formation of formazan by the viable cell. The formazan crystals were dissolved in 150 µl of solubilization solution. The absorbance was read on an ELISA reader (Beckman Coulter AD 340) at 570 nm. The percentage of growth inhibition was calculated as [(values from experimental conditions) – (values from control conditions)] × 100 / (Values from untreated control).

Statistical analysis

The experimental data were expressed as the mean ± standard deviation. Statistical analysis was carried out using the GraphPad PRISM 6 program (GraphPad Software, Inc., San Diego, CA), statistical significance ($p < 0.05$) was determined using a Student´s t-test.

RESULTS AND DISCUSSION

Marine organisms represent a large source of new compounds with biological activities. Direct extraction is one way to obtain bioactive compounds from marine organisms; this approach is widely used for isolated and purified biologically active peptides (Chen et al., 2013). Figure 1 shows the crude extract's chromatogram. Three fractions were obtained after passing the crude extract through the Sephadex column. The separation is given based on the size, the high molecular weight proteins were obtained in the first fraction and afterwards the low molecular weight ones. The fractions that resulted (F1, F2, and F3) from gel filtration chromatography were separated by ion-exchange chromatography through elution with a NaCl gradient on a HiPrep 16/10 QXL column, ten fractions were obtained as follows: four fractions from F1, three fractions from F2 and three fractions from F3 (Figure 2). Antiproliferative activity of all fractions was tested using the MTT assay, a well-established *in vitro* model for citotoxicity against cancer cell lines that has been used as a conventional technique for the screening of new compounds with antiproliferative activity (Song et al., 2008). F1 showed a antiproliferative effect of 87% of over MDA-MB-231 cell and 43.17% over HeLa cell. F3 shows inhibition of the cellular growth of 64.36 and 70% on HeLa and MDA-MB-231 cell, respectively (Figure 3). When a high concentration of the extract is required to inhibit the cellular growth on 50% of the population, it is considered a low cytotoxicity (Hsu et al., 2010). The concentrations used ranged from 0.054 to 1.97 mg/ml, inhibition on cancer cell line has been reported with 1 mg/ml of extracts (Picot et al., 2006; Liang et al., 2008). The fractions did not inhibit cellular proliferation beyond 60% on the human retinal pigment epithelial cells (ARPE-19). Ten fractions were obtained by ion-exchange chromatography, on the antiproliferative evaluation of these samples (Table 1), the fraction 3F3 showed to inhibit the proliferation of HeLa and MDA-MB-231 cell with IC_{50} values of 138.035 and 157.19 µg/ml respectively and 2F2 fraction showed IC_{50} values of 67.46 µg/ml over HeLa cells. Wang et al. (2012) have

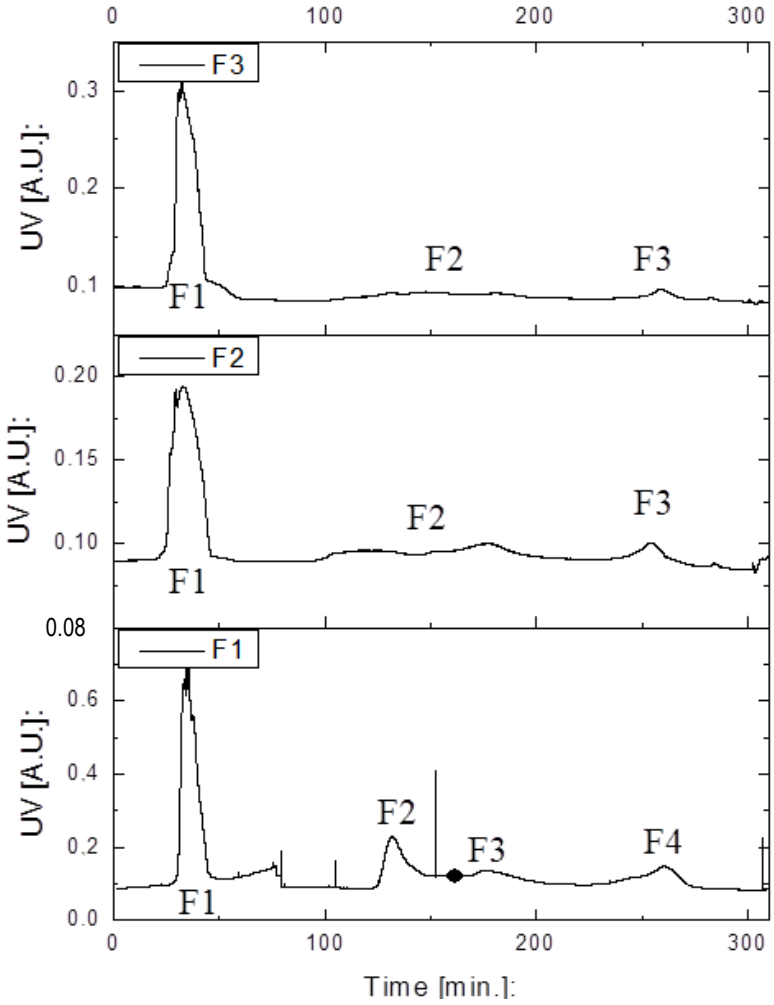

Figure 2. Fractionation by anion exchange chromatography on a HiPrep 16/10 QXL column. The elution was performed with a linear gradient of NaCl (0-1 M) in 25 mM Tris-HCl buffer at a flow rate of 1ml/min. The elution was monitored at 280 nm. F1: Fraction 1 from gel filtration chromatography; F2: Fraction 2 from gel filtration chromatography and F3: Fraction 1 from gel filtration chromatography.

reported IC_{50} values of 84.9 and 215.3 µg/ml over A549 and CCRF-CEM cells, for *Syngnathus acus's* protein purified by ion-exchange cromatography; from *A. subcrenata* two fractions have shown cytotoxic activity against HL-60 (IC_{50}: 123.2 µg/ml) and HeLa cells (IC_{50}: 38.2 µg/ml) (Song et al., 2008).

Several biologically active peptides from marine organisms have been identified and have shown different activities including antioxidant, antimicrobial, antiproliferative and antihypertensive actions (Kim et al., 2013). Some of these compounds are in the process of entering clinicals trials. Peptides from marine sources (Jaspamide, aplidine, didemnin, MML, Mere 15 and dolastatin) induce cell death with different mechanisms, including apoptosis, disruption of microtubular function; interaction with tubulin, alteration of microtubule assembly, or angiogenesis inhibition (Bai et al., 1990; Zheng et al., 2011). Also some of these compunds can bind the vinca alkaloids or the colchine-binding domain inhibiting the polimerization of microtubules (Simmons et al., 2005; Amador, 2003). Several bands with molecular weights between 45 and 18 kDa were observed in the electrophoresis gel (Figure 4). Several compounds derived from marine organisms that have been evaluated on pre-clinical and clinical trials as potential anticancer drug are low molecular weight compounds (Ning et al., 2009).

Cyplisin, a 56 kDa protein isolated from *Aplysia punctate,* has shown citotoxicity against several cancer cell lines; MML, a 40 kDa protein from *M. meretrix,* has inhibitory effects on the proliferation of human hepatoma,

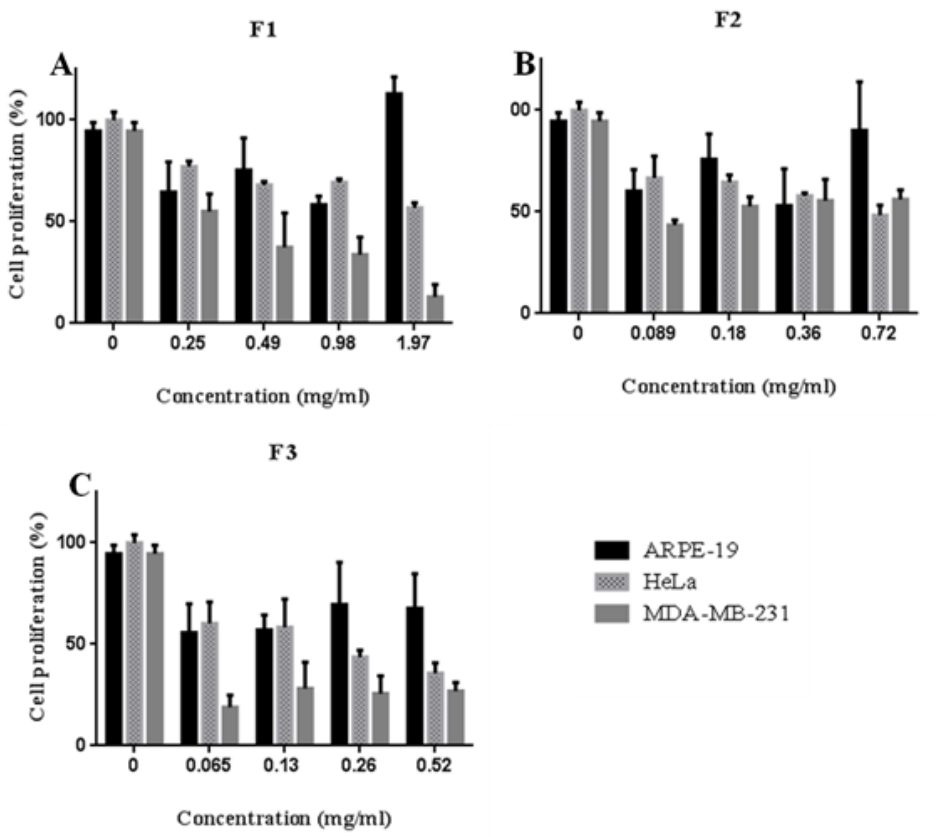

Figure 3. The inhibitory effect of fractions obtained by gel filtration chromatography. The cancer cell lines were treated with the indicated concentrattion of protein and cell proliferation was determinated by MTT assay. A: Fraction 1; B: Fraction 2; C: Fraction 3.

Table 1. Antiproliferative activity of fractions obtained by anion exchange chromatography against different cell lines (IC_{50} µg/ml ± SD).

Fraction	IC_{50} (µg/ml)		
	ARPE-19	HeLa	MDA-MB-231
1 F1	345.76 ± 0.0092	ND	105.89 ± 0.036*
1 F2	174.42± 0.0074	ND	101.83 ± 0.0068*
1 F3	281.29 ± 0.082	ND	150.35 ± 0.0005
1 F4	497.84 ± 0.167	ND	187.02 ± 0.0066*
2F1	260.18 ± 0.022	227.18 ± 0.13	ND
2F2	146.22 ± 0.030	67.46 ± 0.0026*	ND
2F3	235.94 ± 0.053	143.02 ± 0.0024*	ND
3F1	1339.91 ± 1.42	119.35 ± 0.013	1667.70 ± 1.075
3F2	1201.5 ± 0.21	155.09 ± 0.032*	186.67 ± 0.057*
3F3	240.54 ± 0.022	138.035 ± 0.0017*	157.19 ± 0.052

ND: No determinate; 1F1: Fraction 1 from F1; 1F2: Fraction 2 from F1; 1F3: Fraction 3 from F1; 1F4: Fraction 4 from F1; 2F1: Fraction 1 from F2; 2F2: Fraction 2 from F2; 2F3: Fraction 3 from F2; 3F1: Fraction 1 from F3; 2F3: Fraction 2 from F3; 3F3: Fraction 3 from F3. (*) Significant difference between the normal cell and cancer cell line at $p<0.05$ as analyzed by Student´s t-test.

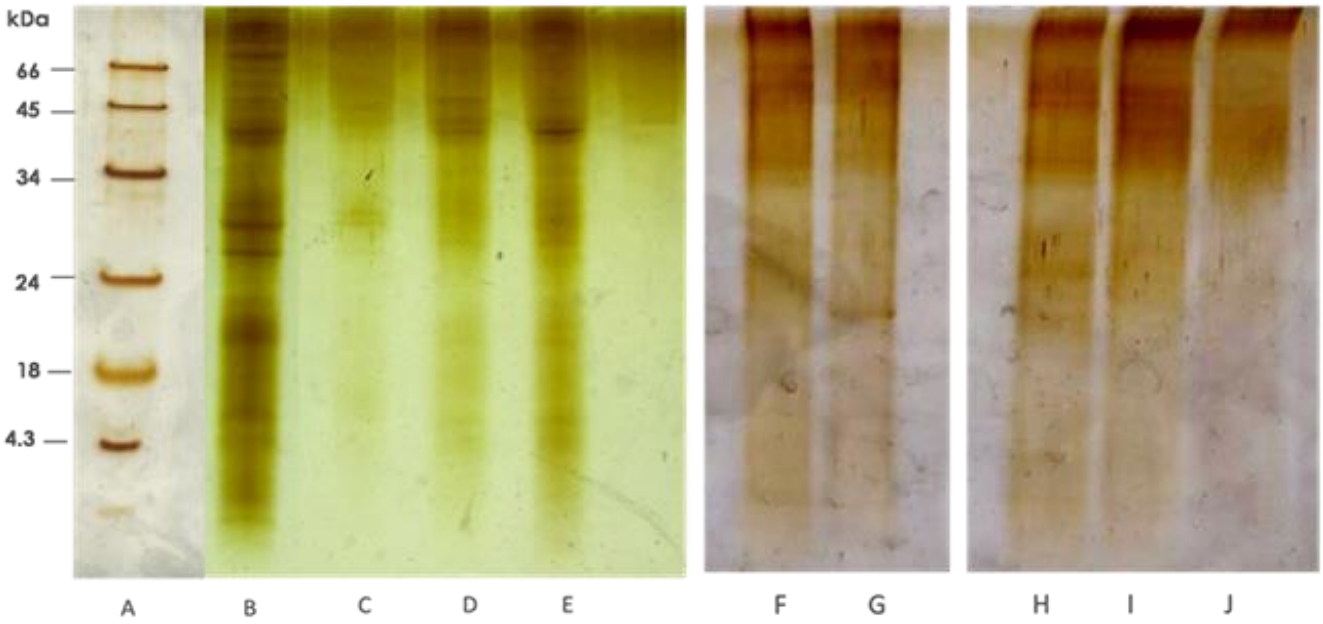

Figure 4. Tricine-SDS-PAGE of fractions obtained by anion exchange chromatography; protein bands were detected by silver stain. Lane A: Low range molecular weight marker; B: Fraction 1 from F1; C: Fraction 2 from F1; D: Fraction 3 from F1; E: Fraction 4 from F1; F: Fraction 1 from F2; G: Fraction 2 from F2; H: Fraction 1 from F3; I: Fraction 2 from F3; J: Fraction 3 from F3.

apparently increasing the permeability of the cell membrane and inhibiting tubulin polymerization. Also, peptides with antiproliferative activity, such as MGP_{0405} and MGP_{0501} (9.6 and 15.8 kDa, respectively), have been reported (Hoarau et al., 2002; Lixin et al., 2005; Nakano et al., 2006; Takahashi et al., 2008; Zheng et al., 2011; Wang et al., 2012). Takahashi et al. reported a protein with antimicrobial activity of 58 kDa from clam (*Ruditapes philippinarum*) and Hoarau found fractions of 20 to 28 kDa with detoxify activity. It has been reported that bioactive peptides or small proteins are highly influenced by their molecular weight and structure, which are affected by the isolation process; different approaches for isolation can lead to different bioactive agents (Guo-fang et al., 2011; Wang et al., 2012).

In the present study, the *in vitro* antiproliferative activity of protein extracts from *C. fluctifraga* was evaluated for the first time, the importance of these results is evidenced by previous reports of many anticancer chemical entities found by the screening of natural sources, such as marine organisms. Our results suggest that protein extracts from *C. fluctifraga* could induce cellular growth inhibition against HeLa and MDA-MB-231 cells, however further studies are needed.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Amador ML (2003). Progress in the development and acquisition of anticancer agents from marine sources. Ann. Oncol. 14:1607–1615.

Aneiros A, Garateix A (2004). Bioactive peptides from marine sources: pharmacological properties and isolation procedures. J. Chromatogr. B. Anal. Technol. Biomed. Life Sci. 803:41–53.

Bai RL, Pettit GR, Hamel E (1990). Binding of dolastatin 10 to tubulin at a distinct site for peptide antimitotic agents near the exchangeable nucleotide and vinca alkaloid sites. J. Biol. Chem. 265:17141–17149.

Bhatnagar I, Kim S-K. (2010). Marine antitumor drugs: status, shortfalls and strategies. Mar. Drugs 8:2702–2720.

Bhutia SK, Maiti TK (2008). Targeting tumors with peptides from natural sources. Trends Biotechnol. 26:210–217.

Chen L, Song L, Li T, Zhu J, Xu J, Zheng Q, Yu R (2013). A new antiproliferative and antioxidant peptide isolated from Arca subcrenata. Mar. Drugs 11:1800–1814.

Faircloth G, Cuevas C(2006). Kahalalide F and ES285: potent anticancer agents from marine molluscs. Prog. Mol. Subcell. Biol. 43: 363–379.

Gök M, Rozendaal L, Berkhof J, Visser O, Meijer CJLM, van Kemenade FJ (2011). Cytology history preceding cervical cancer diagnosis: a regional analysis of 286 cases. Br. J. Cancer 104:685–692.

Gottesman MM (2002). Mechanism of cancer drug resistance. Annu. Rev. Med. 53:615–627.

Guo-fang D, Fang-fang H, Zui-su Y, Di YU, Yong-fang Y (2011). Anticancer Activity of an Oligopeptide Isolated from Hydrolysates of Sepia Ink. Chin. J. Nat. Med. 9:151–155.

Haefner B (2003). Drugs from the deep: marine natural products as drug candidates. Drug Discov. Today 8:536–544.

Hawkins MJ (1992). National Cancer Institute. JNCI J. Natl. Cancer Inst. 84:1699–1702.

Hoarau P, Garello G, Gnassia-Barelli M, Romeo M, Girard J-P (2002). Purification and partial characterization of seven glutathione S-transferase isoforms from the clam Ruditapes decussatus. Eur. J. Biochem. 269:4359–4366.

Hsu H-F, Wu Y-C, Chang C-C, Houng J-Y (2010). Apoptotic effects of bioactive fraction isolated from *Glossogyne tenuifolia* on A549 human lung cancer cells. J. Taiwan Inst. Chem. Eng. 1–7.

Jordan MA, Wilson L (2004). Microtubules as a target for anticancer drugs. Nature 4:253–265.

Kim E-K, Kim Y-S, Hwang J-W, Lee JS, Moon S-H, Jeon B-T, Park P-J (2013). Purification and characterization of a novel anticancer peptide derived from *Ruditapes philippinarum*. Process Biochem. 48:1086–1090.

Leng B, Liu X-D, Chen Q-X (2005). Inhibitory effects of anticancer peptide from Mercenaria on the BGC-823 cells and several enzymes. FEBS Lett. 579:1187–1190.

Liang C-H, Wang G-H, Liaw C-C, Lee M-F, Wang S-H, Cheng D-L, Chou T-H (2008). Extracts from *Cladiella australis*, *Clavularia viridis* and *Klyxum simplex* (soft corals) are capable of inhibiting the growth of human oral squamous cell carcinoma cells. Mar. Drugs 6:595–606.

Lixin Z, Xiao F, Lijun H (2005). Antitumor and immune regulation activities of the extracts of some Chinese marine invertebrates. Chin. J. Oceanol. Limnol. 23:110–117.

Martinez JD, Parker MT, Fultz KE, Ignatenko AN, Gerner EW (2003). Molecular biology of cancer. In Burger's Medicinal Chemistry and Drug Discovery, D. J. Abraham, ed. (John Wiley & Sons, Inc.), pp. 1–50.

Martinez-Cordova LR, Martinez-Porchas M (2006). Polyculture of Pacific white shrimp, *Litopenaeus vannamei*, giant oyster, *Crassostrea gigas* and black clam, *Chione fluctifraga* in ponds in Sonora, Mexico. Aquaculture 258:321–326.

Nakano T, Matsushima-Hibiya Y, Yamamoto M, Enomoto S, Matsumoto Y, Totsuka Y, Watanabe M, Sugimura T, Wakabayashi K (2006). Purification and molecular cloning of a DNA ADP-ribosylating protein, CARP-1, from the edible clam *Meretrix lamarckii*. Proc. Natl. Acad. Sci. U. S. A. 103:13652–13657.

Ning X, Zhao J, Zhang Y, Cao S, Liu M, Ling P, Lin X (2009). A novel anti-tumor protein extracted from *Meretrix meretrix* Linnaeus induces cell death by increasing cell permeability and inhibiting tubulin polymerization. Int. J. Oncol. 35:805–812.

Palacio-Mejía LS, Lazcano-Ponce E, Allen-Leigh B, Hernández-Avila M (2009). Diferencias regionales en la mortalidad por cáncer de mama y cérvix en México entre 1979 y 2006. Salud Publica Mex. 51:208–219.

Picot L, Bordenave S, Didelot S, Fruitierarnaudin I, Sannier F, Thorkelsson G, Berge J, Guerard F, Chabeaud A, Piot J (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. Process Biochem. 41:1217–1222.

Schägger H (2006). Tricine-SDS-PAGE. Nat. Protoc. 1:16–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17406207 [Accessed July 15, 2011].

Simmons TL, Andrianasolo E, Mcphail K, Flatt P, Gerwick WH (2005). Minireview Marine natural products as anticancer drugs. Mol. Cancer Ther. 4:333–342.

Song L, Ren S, Yu R, Yan C, Li T, Zhao Y (2008). Purification, characterization and in vitro anti-tumor activity of proteins from Arca subcrenata Lischke. Mar. Drugs 6:418–430.

Takahashi KG, Kuroda T, Muroga K (2008). Purification and antibacterial characterization of a novel isoform of the Manila clam lectin (MCL-4) from the plasma of the Manila clam, *Ruditapes philippinarum*. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 150: 45–52.

Tsai J-S, Chen J-L, Pan BS (2008). ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). Process Biochem. 43:743–747.

Wang C, Liu M, Cheng L, Wei J, Wu N, Zheng L, Lin X (2012). A novel polypeptide from *Meretrix meretrix* Linnaeus inhibits the growth of human lung adenocarcinoma. Exp. Biol. Med. 237:442–450.

Wenyan X, Chen C, Xiaoshuang L, Bo W, Ying S, Maocang Y, Xiaoying Z (2012). Meretrix Meretrix: Active Components and Their Bioactivities. Life Sci. J. 9:756–762.

Zheng L-H, Wang Y-J, Sheng J, Wang F, Zheng Y, Lin X-K, Sun M (2011). Antitumor peptides from marine organisms. Mar. Drugs 9: 1840–1859.