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Antiproliferative Effect of Extracts and Fractions from the Calcareous Sponge *Leucetta aff. floridana* from the Colombian Caribbean

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***SUMMARY*. Three tumor cell lines of lung (A549), colon (HT29) and breast (MDA-MB-231) were used to evaluate the antiproliferative effect of ethanol and dichloromethane extracts and seven fractions obtained by flash column chromatography from the most bioactive extract of the sponge *Leucetta aff. floridana.* Ethanol extract showed antiproliferative activity on three cell lines, whereas dichloromethane extract exhibited low activity. Four ethanol fractions showed antiproliferative activity, which was higher on A549 (IC_{50} for all four fractions: < 1.0 μg/mL), followed by HT29 (IC_{50}: 2.5 μg/mL; 2.2 μg/mL; 13.2 μg/mL and 15.8 μg/mL) and finally, MDA-MB-231 (IC_{50}: 2.8 μg/mL, 8.3 μg/mL; 13.3 μg/mL and 20.5 μg/mL). GC-MS analysis of the highest activity fraction permitted to identify sixteen fatty acids among saturated, branched-saturated, monounsaturated and polyunsaturated of which hexadecanoic and hexadecenoic acids were the most abundant fatty acids.**

INTRODUCTION

The *Leucetta aff. floridana* calcareous sponge (class: Calcareous, order: Clathrinida, family: Leucettidae), commonly lives in superficial waters in tropical zones [1] and constitutes one of the four species of the *Leucetta* genus (Haeckel, 1982), altogether with the species *L. cf chagosensis, L. aff. microrhaphis* and *L. villosa.* Likewise other calcareous sponges, they present extracellular spicules formed of diactine, triactine or tetractine and a spicular skeleton with calcium and Mg [2]. Spicules and larval development differentiate Calcareous class sponges from Demospongiae and Hexactinellida classes, while the soft flesh organization and the type of cells differentiate the Hexactinellida class from the other two kinds of sponges [3-4]. Besides, the Calcareous class sponges do not present long chain fatty acids (> C24) with carbon-carbon double bond system cis-5, cis-9, characteristic of the demospongic acids [5-7], present on the Demospongiae and Hexactinellida classes [5, 8-9].

The few chemical studies made on the *Leucetta* genus species show the presence of antimicrobial [10-12] and antitumor [13-14] compounds. Among the antimicrobial alkaloid anticryptococcal and nitric oxide synthase inhibitors in the *L. cf chagosensis* sponge [10], guanidine type alkaloids, spiroleucettadine with activity against the *Enterococcus durans* in the *Leucetta sp.* sponge, (CMI< 6.25 μg/mL) [12], sphingolipids with antimicrobial activity in the *L. aff. microrhaphis* sponge [11], and methanolic, chloroformic and hexanic extracts with biological activity against microorganisms *E. coli, S. aureus* and *C. albicans* in the *L. aff. floridana* sponge have been found [15]. Among the antitumor compounds naamidine A, an imidazol type alkaloid with antitumor activity isolated from the *L. chagosensis* sponge [13], and the sphingolipid leucettamol-A with antitumor activity in the *L. aff. microrhaphis* sponge [14] have been identified, and the presence of these kind of compounds are unknown in the *L. villosa* and *L. aff.*

KEY WORDS: Antiproliferative activity, Fatty acids, *Leucetta aff. floridana*.

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floridana species, which have not been chemically studied for this purpose.

Aiming to contribute with the search of antitumor compounds of potential interest in the cancer treatment and to generate data on the calcareous sponge chemistry, the effect of the extracts and fractions obtained from the ethanolic extract of the *L. aff. floridana* sponge on the growth of the colon (HT29), lung (A549) and breast (MDA-MB-231) tumor cell lines was evaluated and the chemical composition of the most active fraction was analyzed.

MATERIALS AND METHODS

Solvents employed for extraction and fractions were analysis grade (Merck®), and HPLC-grade solvents were utilized without further purification in HPLC separations. HPLC purifications were carried out on a Waters equipment (pump 600 E, autoinjector 417, and photodiode array detector 996) coupled with an evaporative light-scattering detector (ELSD) SEDEX 55. The following chromatographic conditions were used: a Phenomenex® column C18 (250mm x 10mm, 5µm), using as mobile phase acetonitrile/water (60:40) under isocratic conditions for 5 minutes and later gradient mode up to 30 min at 100 % of acetonitrile. A flow rate, 3 mL/min; 75 µL volume injection and detector wavelength, 210 nm were used. NMR experiments were performed on a Bruker ARX 500 spectrometer. Chemical shifts are recorded in ppm with $CDCl_3$ (δ 7.26 for ^1H) as internal standard.

The analyses by gas chromatography - mass spectrometry (GC-MS) were performed in an Agilent® 6890N gas chromatographer coupled to an Agilent® 5973N mass spectrometer. For the (GC-MS) analysis of the methyl esters of the fatty acids a 19091S, HP5MS Agilent® (0.25mm x 30m x 0.25 µm) column at a maximum 350 °C temperature was used. The schedule of the oven was as follows: starting temperature 150 °C at a 10 °C gradient up to a final temperature of 300 °C. Splitless mode at 200 °C temperature, 13.2 psi pressure and 14.1 mL/min total flow for the injection. The auxiliary detector temperature was 300 °C. The scan mode in the mass detector was used at a mass interval of 30-600 uma. The injection volume was 5.0 µL and the analysis time was 35 min. For the GC-MS analysis of the pyrrolidine derivatives from the fatty acids a 19091J-433, HP-5 Agilent® (0.25 mm x 30 m x 0.25 µm) column was used at 350 °C top temperature. The schedule of the column was as follows: starting temperature 150 °C (1 min) – 200 °C (5 °C/min- 3 min) – 250 °C (3 °C/min - 4 min) – 300 °C (5 °C/min - 5.33 min). Splitless mode was used for the injection. The injector's temperature was 250 °C, 13.1 psi pressure and 14.1 mL/min total flow. Auxiliary detector's temperature was 300 ºC. Scan mode in the mass detector at 30-600 uma mass interval was used. The injection volume was 3.0 µl and the analysis time was 90 min.

Sampling and identification

Samples were collected by scuba divers from reef in habitats at depths of 15 to 21 m in the Golfo de Urabá (Colombia) in 2002 and were kept frozen until its use. Strange materials and/or organisms were removed from samples with a knife. Samples were frozen (-10 °C) as soon as possible and transferred to the laboratory where they were cut in small pieces and dried (40 °C). Identification was carried out by Dr. Sven Zea (Colombia), sponge taxonomy expert, and two reference samples are in the Laboratorio de Productos Naturales Marinos de la Universidad de Antioquia (Medellín, Colombia).

Extraction and fractionation

Dried samples were extracted with two different solvents: CH_2Cl_2 (2 x 50 mL) and CH_3OH (2 x 50 mL). Each extraction was developed with mechanical shaking in amber glass flasks, at room temperature. Each extract was filtered and concentrated under vacuum on a rotary evaporator (Heidolph) at low temperature (40 °C). The ethanol extract was subjected to a C18 reversed-phase flash column, eluting with 500 mL of the following eluotropic series: H_2O, H_2O/CH_3OH (1:1), H_2O/CH_3OH (1:3), CH_3OH, CH_3OH/CH_2Cl_2 (3:1), CH_3OH/CH_2Cl_2 (1:1), and CH_2Cl_2. Each fraction was concentrated under vacuum on a rotary evaporator at low temperature (40 °C) and after evaluated on tumor cell derived cell lines. Fractions were named in the same order each solvent elution was made, that is: F1, F2, F3, F4, F5, F6, F7, and the F4 fraction was separated by reversed-phase HPLC.

Biological assay

The cell growth and viability were determined by a colorimeter assay using sulforhodamine B, SRB [16]. The colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB 16. The *in vitro* activity of the extracts was evaluated against cultured human cancer cells of A-549 lung carcinoma, HT-29 colon adenocarcinoma and MDA-

MB-231 breast carcinoma at three concentrations 1 µg/mL, 5 µg/mL and 25 µg/mL. According to National Cancer Institute guidelines, extracts and fractions with IC_{50} values < 20 µg/mL were considered active [17].

Preparation of methyl esters and pyrrolidine derivatives of the fatty acids

The HPLC obtained fractions of fatty acids from the *L. aff. floridana* sponge were added diazomethane aiming to get methyl esters from the fatty acids. 100 µg of methyl esters were added 1000 µL of pyrrolidine and 100 µL glacial acetic acid for getting pyrrolidine derivatives. The mix was boiled at 100 °C in an open flow for 90 min. Reaction was monitored using thin layer chromatography comparing with a sample of methyl esters using hexane/ethyl acetate (2:1) as mobile phase and silica gel F254 as stationary phase. Both derivative samples were analyzed by GC-MS.

RESULTS

Bioassay of extracts

The ethanol extract in the 5 µg/mL concentration did not meaningfully affected the evaluated cell line growth, while at 25 µg/mL concentration, the colon tumor line (IC_{50} > 25 µg/mL) dropped to 55 %, the breast tumor line (IC_{50} 17.3 µg/mL) dropped to 15 % and completely inhibited the lung derived line growth (IC_{50} 15.0 µg/mL) (Fig. 1). In contrast, the dichloromethane extract at 5 µg/mL and 25 µg/mL concentrations, reduced only 15% and 10%, respectively, the breast cell line growth and did not affect the lung and colon cell lines, showing IC_{50} > 25 µg/mL for all the evaluated cell lines.

Bioassay of fractions

Out of the seven evaluated fractions, F4 and F3 fractions were the most active due to a 5 µg/mL concentration showed total inhibition of the A549 lung, HT29 colon tumor lines growth and a 74 % and 41 % inhibition in MDA-MB-231 breast cancer cell line, respectively. At a 25 µg/mL concentration, F5 and F2 fractions showed total inhibition of the three cell line growth, but at 5 µg/mL, they inhibited 84 % and 68 %, respectively, of the A549 tumor line and showed minor or equal to 20 % inhibition percentages in the other cell lines. The four fractions showed a greater lung A549 tumor line inhibitory activity (IC_{50} of all fractions: < 1.0 µg/mL), followed by the HT29 colon tumor line (IC_{50} F3: 2.2 µg/mL; F4: 2.5 µg/mL; F5: 13.2 µg/mL and F2: 15.8 µg/mL) and finally the MDA-MB-231 breast tumor line (IC_{50} F4: 2.8 µg/mL, IC_{50} F3: 8.3 µg/mL; F5: 13.3 µg/mL; F2: 20.5 µg/mL). F1, F6 and F7 fractions showed low biological activity due to the inhibition percentage lower to 20 % (Fig. 2).

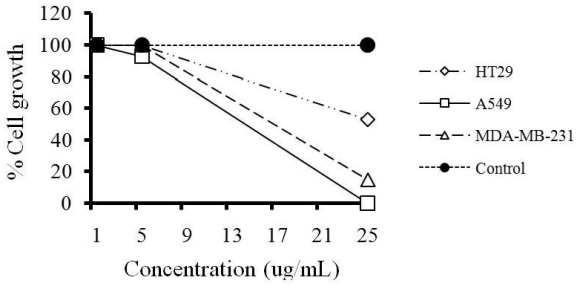

Figure 1. Effect of the ethanol extract from the *L. aff. floridana* sponge on the growth of HT29, A549 and MDA-MB-231 tumor cell lines.

Fractionation of F4 fraction by HPLC

The HPLC fractioning of the most active fraction (F4) resulted on the collection of eight fractions named L1 to L8 (Fig. 3).

L. aff. floridana sponge HPLC obtained fractions were analyzed by RMN-^{1}H. Fatty acid characteristic signals were observed on the obtained

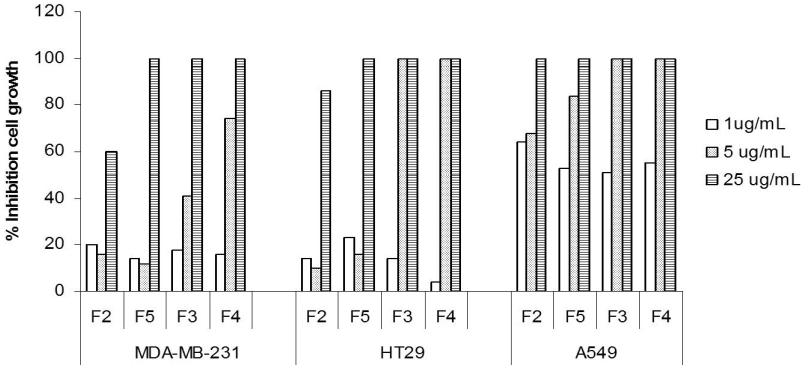

Figure 2. Effect of ethanolic fractions concentration from the *L. aff. floridana* sponge on the MDA-MB-231, HT29 and A549 tumor cell lines growth.

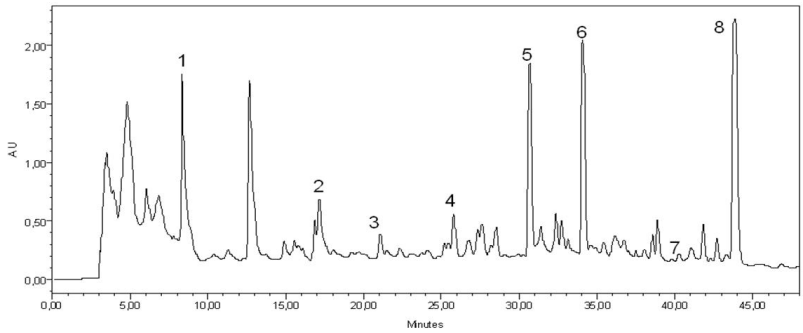

Figure 3. UV chromatogram of the F4 fraction from the *L. aff. floridana* sponge measured at 210 nm.

spectrum such as: a triplet δ 0.8-0.9 ppm signal corresponding to methyl group, a δ 1.2–1.4 ppm characteristic signal of methylene groups, the displaced multiplet signals between δ 1.5 ppm and δ 3.0 ppm correspond to methyl groups close to carboxyl group, being evident a triplet signal in δ 2.3 ppm corresponding to the two protons of the methyl group directed linked to the carbonyl group. Olefinic protons characteristic signals were also observed due to the presence of a multiplet removed to δ 5.3 ppm [18]. Carbohydrates protons characteristic signals between δ 3.5 ppm and δ 5 ppm were observed in some fractions, which permitted to establish the presence of possible glycolipids [19].

Fatty acids structure identification was possible by GC-MS analysis of the methyl ester and pyrrolidine derivatives. The mass spectra of methyl ester derivatives allowed determining whereas the compound was a saturated fatty acid by observing the presence of characteristic fragments such as *m/z* 74 base peak, which comes from a McLafferty type rearrangement, the ion at *m/z* [M-43]$^+$ which represents the lost of C3 units (2 to 4 carbons) and the ion at *m/z* [M-31]$^+$ which represents the lost of a methoxyl ion [20]. The mass spectra of methyl esters of unsaturated fatty acids showed the presence of an ion at *m/z* [M-32]$^+$ which represents the lost of methanol, the ion at *m/z* 74 which is less abundant than in saturated fatty acids and the ion at *m/z* 55 which is usually the base peak of the $[C_nH_{2n-1}]^+$ series [21]. The pyrrolidine derivatives permitted the location of the double bonds and branches [22]. A double bond is present between the *n* and *n*+1 carbons in the molecule if a 12 uma interval instead of 14 uma among the most intense peaks of the fragments containing *n* and *n*-1 carbon atoms is observed [18]. The identification of the compounds was based on the comparison of the published mass spectra and retention times [23].

For the case of the sulfur-containing fatty acid identified in the L3 fraction, methyl ester mass spectrum (Fig. 4) shows the *m/z* 324 molecular ion, corresponding to the molecular formula $C_{19}H_{32}O_2S$. The ion at *m/z* 293 [M-31]+ corresponding to the methoxyl ion lost is observed 24. The ion at *m/z* 253 [M-71]+ is characteristic and corresponds to the initial fragment which is formed by the cleavage beta of the ring, in other words, the bond between the C-13/C-14 carbons and the ion at *m/z* 181 [M-143]$^+$, is the base peak which corresponds to the terminal fragment formed by the cleavage *beta* of the other side of the ring, that is to say, the bond between the C-6/C-7 carbons, both breakings give origin to the ion at *m/z* 111, which corresponds to the ring with the two lateral carbons. The ion at *m/z* 227 corresponds to the cleavage of the bond between the C-11/C-12 carbons, follow by the loss of 32 uma which is equivalent to a sulfur atom, gives origin to the ion at *m/z* 195 [23].

A series of fragments of the general formula [MeO-CO-(CH_2)n]$^+$ *m/z* 181 [M-143]$^+$, 153 [M-171]$^+$, 138 [M-186]$^+$, 124 [M-200]$^+$, 100 [M-224]$^+$ which correspond to the lost of 14 uma (–CH_2) and the $[C_nH_{2n+1}]$+ alkylic and $[C_nH_{2n-1}]$+ alkenylic series with the ions at *m/z* 57 and 55,

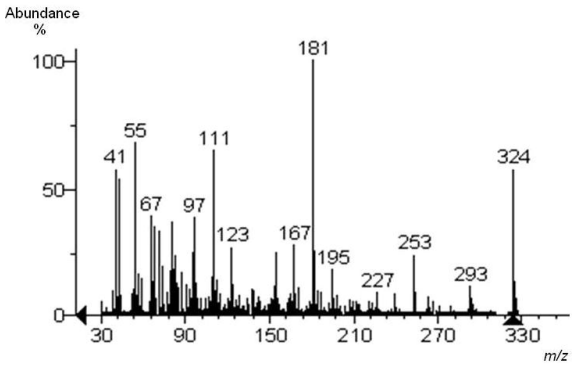

Figure 4. Mass spectrum of 9, 12-epithio-9, 11-octadecanoic acid methyl ester from the *L. aff. floridana* sponge.

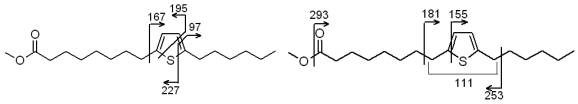

Figure 5. Mass spectrum observed fragments of 9,12-epithio-9,11-octadecanoic acid methyl ester from the *L. aff. floridana* sponge.

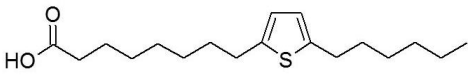

Figure 6. The structure of 9,12-epithio-9,11-octadecanoic acid.

m/z 71 and 69, *m/z* 85 and 83, *m/z* 99 and 97 are observed in the mass spectrum of the methyl ester [18]. Figure 5 shows a fragmentation schema for the analyzed compound methyl ester mass spectrum. This analysis permitted to establish that this compound is 9,12-epithio-9,11-octadecanoic acid (Fig. 6).

Sixteen fatty acids distributed in the different fractions obtained by HPLC were identified from the fraction of free fatty acids of the *L. aff. floridana* sponge. Results are shown in Table 1.

Results show the *L. aff. floridana* sponge has saturated, branched-saturated and unsaturated fatty acids with C_{15}-C_{20} carbon numbers. The most abundant fatty acids are the 9-hexadecenoic acid with 14.28 % and the hexadecanoic acid (the most abundant) with 82.66 %. The other fatty acids showed abundance below 1.00 % in the F4 fraction. The fatty acid types found in the F4 fraction are branched-saturated (33.3%), followed by monounsaturated (26.7 %), and finally polyunsaturated and saturated, which are found in the same abundance (20.0 %).

DISCUSSION

The most relevant results of this work are related to: (a) the presence of compounds with antiproliferative activity in the ethanol extract and several of its fractions in the *L. aff. floridana* calcareous sponge and (b) the identification of nine fatty acids, which have not been reported in previous studies made on *Leucetta* genus sponges.

The results of the biological activity obtained in this work indicate the used tumor lines show a differential sensibility to the compounds present in the evaluated fractions. For instance, the greatest growth inhibitory activity was observed in the lung tumor cells, which was affected by four out of the seven evaluated fractions (IC_{50} of all fractions: < 1.0 µg/mL), the colon tumor line followed (IC_{50} F4: 2.5 µg/mL; F3: 2.2 µg/mL) and finally the breast one (IC_{50} F4: 2.8 µg/mL, IC_{50} F3: 8.3 µg/mL), in which only two fractions were responsible of the greatest antiproliferative activity.

The differential sensibility of the cell lines has been evidenced in several previous works

N°	Fraction	Compound	Abundance %	methyl ester M^+
1	L1	Nonadecenoic acid (C19:1)	0.11	310
2	L2	14-Methylheptadecanoic acid (14-Me-C18:0)	0.04	298
3	L3	9,12-Epithio-9,11-octadecanoic acid (C18:2)	0.38	324
4	L4	Hexadecadienoic acid (C16:2)	0.07	266
5	L5	9,12,15-Octadecatrienoic acid ($\Delta^{9,12,15}$-C18:3, *n*-3)	0.2	292
6	L6	9-Hexadecenoic acid (Δ^9 C16:1, *n*-7)	14.28	268
7	L7	13-Methylpentadecanoic acid (13-Me-C16:0)	0.21	270
8		Octadecenoic acid (C18:1)	0.92	296
9		2-Methyltetradecanoic acid (2-Me-C15:0)	0.10	256
10	L8	2,6,10-Trimethyldodecanoic acid (2,6,10-Trime-C15:0)	0.21	256
11		8-Hexadecenoic acid (C16:1, *n*-8)	0.09	268
13		n-Hexadecanoic acid (C16:0)	82.66	270
14		n-Heptadecanoic acid (C17:0)	0.25	284
15		Heptadecenoic acid (C17:1)	0.41	282
16		3,7,11,15-tetramethylhexadecanoic acid (3,7,11,15-tetrame-C20:0)*	0.09	326

Table 1. Fatty acids identified in the *L. aff. floridana* sponge. Me: methyl; Dime: dimethyl; Trime: trimethyl; Tetrame: tetramethyl, * Phytanic acid.

[24,25], which led to propose the use of a reduced high sensible human tumor cell line panel for the selection of the products with potential anticancer activity and then assess in a broad 60 line panel the promissory products in the first level screening [25]. Comparing the IC_{50} doses obtained in this work with a 20 µg/mL reference limit value, suggested for the extracts by the US National Cancer Institute [17], F4 fraction exhibited antiproliferative activity in the three evaluated cell lines, which merited continuing their chemical analysis.

In the chemically analyzed antiproliferative fraction, three compound groups which individually or in group could be responsible of the inhibition in the growth of the analyzed tumor lines were found: the monounsaturated fatty acids (C_{14}-C_{22}) possibly with double bond in cis position, among which the most abundant is the 9-hexadecenoic acid, the hexadecanoic acid (palmitic acid) and the 9,12,15-octadecatrienoic acid.

The monounsaturated fatty acids (C_{14}-C_{22}) were found abundantly in the analyzed fraction, which matches with the results obtained in other species of the *Leucetta* genus [9]. Even though the presence of the double bond in cis position was not determined in this work, the abundant presence of monounsaturated fatty acids in the antiproliferative fraction suggests this kind of compounds could be present in such fraction. These compounds are recognized as DNA topoisomerase I inhibitors, an essential enzyme in the DNA rupture and repairing processes which is currently action target of new anticancer drugs [26].

The other abundant compound of the analyzed fraction is the hexadecanoic acid (palmitic acid), which has demonstrated to have selective cytotoxic activity against human leukemia cells but not on HDF normal cells at a 12.5 to 50 µM/mL concentration, it induces apoptosis in the MOLT-4 human leukemia cell line at 50 µg/mL concentration, and presents *in vivo* antitumor activity in assays performed with mouse. Topoisomerase I is considered a molecular target for this compound without affecting the topoisomerase II [27].

Another interesting antiproliferative fraction component is the 9,12,15-octadecatrienoic acid (*n*-3 acid), which is found in traces in the L5 fraction (0.20 %). It has been indicated that *n*-3 fatty acids have important pharmacological activity, especially in cardiovascular and inflammatory illnesses. These fatty acids are powerful inhibitors of the lipoxygenases, enzymes participating in the inflammation process and found participating in illnesses such as cancer, arterioscleroses and Alzheimer [28].

Besides the previously mentioned antiproliferative compounds, nine non published fatty acids in other species of the *Leucetta* genus analyzed fraction were identified: nonadecenoic, 14-methylpentadecanoic, 9,12-epithio-9,11-octadecanoic, hexadecadienoic, 9,12,15-octadecatrienoic, 2-methyltetradecanoic, 2,6,10-trimethyldodecanoic, heptadecenoic and 3,7,11,15-tetramethylhexadecanoic fatty acids [9]. These results reflect the chemical diversity among this genus species and could be useful for understanding their evolutionary and adaptational relations.

Finally, it is left to explore the analyzed fraction effect in other cell lines; elucidate the double bond geometry, in particular the 9-hexadecenoic acid, the most abundant of the monounsaturated fatty acids of this fraction; and study in depth the chemical differences among the *Leucetta* species and their possible evolutionary and adaptational role. The results of this work contribute to the chemical knowledge of the specie and permit estimate its usefulness in the search of pharmacological interest substances.

CONCLUSIONS

Sixteen fatty acids among saturated, branched-saturated, and unsaturated were identified out of the F4 fraction with activity on the cell lines derived of the lung carcinoma cells A-549, colon carcinoma HT29 and breast cancer MDA-MB-231. This is the first report on biological activity of extracts and fractions of the *L. aff. floridana* sponge on cell lines derived from lung carcinoma cells A-549, colon carcinoma HT29 and breast cancer MDA-MB-231 and on the chemical composition of a bioactive fraction of this sponge. These results show the growth inhibitory effect shown by the extracts and fractions on the tumor cell lines depends on the dose.

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