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Article

Characterization of bioactive molecules isolated from sea cucumber *Athyonidium chilensis*

Caracterización de moléculas bioactivas aisladas del pepino de mar *Athyonidium chilensis*

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Resumen.- En la última década se ha incrementado el interés por la búsqueda de moléculas con potencial biomédico y amigable para el ambiente. Desde esta perspectiva, la búsqueda de principios bioactivos de origen marino ha permitido valorar la diversidad biológica presente en los ecosistemas acuáticos. En el Phylum Equinodermata, se destaca la familia Holothuridae, por su capacidad de sintetizar moléculas como saponinas y otros metabolitos secundarios de alto interés farmacológico debido a su capacidad hemolítica, antitumoral, anti-inflamatoria, antimicrobiana, citostática y antineoplásica. El interés del presente trabajo se centró en la caracterización de un extracto purificado obtenido desde *Athyonidium chilensis* (Holothuria) mediante técnicas cromatográficas y de espectrometría de masas, con la posterior evaluación de su potencial bioactivo sobre modelos *in vitro*. Como resultado de estos análisis, se identificaron 2 saponinas. La primera con un peso molecular de 1522 Da y altamente conjugada con monosacáridos. La segunda con un peso molecular de 764 Da fue identificada como holoturinósido D. Respecto a los resultados de la actividad biológica del extracto purificado, éste mostró actividad antibacteriana, antifúngica y citotóxica frente a una línea celular de neuroblastoma. Los resultados de este estudio se convierten en la primera caracterización de moléculas con actividad biológica desde *Athyonidium chilensis*.

Palabras clave: Holothuria, holoturinósido D, saponina

Abstract.- In the last decade, the interest for searching molecules with biomedical potential as well as innocuous for the environment has been increased. Under this perspective, the search for bioactive principles of marine origin has allowed to value the biological diversity present in aquatic systems. In phylum Echinodermata, the family Holothuridae is distinguished by its capacity of synthesizing molecules such as saponins and other secondary metabolites of high pharmacological interest because of their interesting haemolytic, antitumor, anti-inflammatory, antimicrobial, cytostatic and antineoplastic capacity. The aim of the present study was focused on the characterization of a purified extract obtained from *Athyonidium chilensis* (Holothuria) by chromatographic and mass spectrometric techniques and the later assessment of its bioactive potential on *in vitro* models. As a result of these analyses 2 saponins were identified. The first with a molecular weight of 1522 Da and highly conjugated with monosaccharides. The second with a molecular weight of 764 Da was identified as holothurinoside D. The biological activity of the purified extract showed antibacterial, antifungal and cytotoxic activity on a neuroblastoma cell line. Outcomes of this study correspond to the first characterization of molecules with biological activity from *Athyonidium chilensis.*

Key words: Holothuria, holothurinoside D, saponins

Introduction

Advances in technology and chemical sciences of natural products have allowed to researchers get focused on sea floor biological diversity, allowing characterizing species and identifying the chemical composition of new molecules with biopharmacological potential (Munro *et al*. 1999), in order to increase both scientific and commercial value of such species (Ruggieri 1976, Scheuer 1990, Faulkner 2000, Kelly 2005, Bhakuni & Rawat 2005).

There are several examples of bioactive products, naturally produced by marine organisms as defense against a highly competitive environment (Bhakuni & Rawat 2005). Among others, the identification of molecules with anticarcinogenic capacity obtained from the Bryozoa *Bugula neritina* (Pettit *et al.* 1996, 2002) can be mentioned, as well as molecules with antitumor and antiviral properties, from the tunicate *Trididemnum solidum* (Rinehart *et al.* 1981, Mayer & Gustafson 2008). Among the molecules with greater pharmacological value found in holothurids, the presence of saponins has been identified; an important bioactive substance originally described only in plants (Li *et al*. 2006), but later isolated from marine organisms such starfishes and sponges (Regalado *et al*. 2010, Levina *et al*. 2010). Their production has been associated to defensive molecules (Kalinin *et al*. 1996, Van Dyck *et al*. 2009).

Holothurids are able to synthesize toxins and diverse metabolites from lanosterol (Makarieva *et al*. 1993). This kind of molecules have pharmacological activity comparable to other species belonging to the same phylum, with antimicrobial and antifungal properties (Neira *et al*. 1985, Haug *et al.* 2002, Ismail *et al*. 2008) and interesting antifouling capacity (Selvin & Lipton 2004), as well as powerful molecules with anticarcinogenic properties (Bryan *et al*. 1996, Chludil *et al.* 2002, Mayer & Gustafson 2004). Class Holoturoidea belonging to the phylum Echinodermata is represented by 6 orders and 74 species, from which *Athyonidium chilensis* is the most abundant and widely distributed, from Ancón in Southern Peru and the Southern end of Chile (Larrain 1995). Exploitation of this resource is based exclusively for export, for which 109 tons yr^{-1} are extracted (SERNAPESCA 2010), destined to Asian countries where it is consumed as a high cost and high nutritional value gastronomic product, having a high source of lipids, polyunsaturated fatty acids and sterols (Drazen *et al*. 2008). However, there is a lack of studies on potential bioactive compounds found in *A. chilensis*. For this reason the main objective of this study was focused on the identification of potential bioactive compounds from tissues of *A. chilensis* by means of a number of analytical techniques and the *in vitro* assessment of their pharmacological potential.

Materials and methods

Biological material collection

Specimens of *Athyonidium chilensis* (Semper, 1868) were collected from the intertidal zone of the Cocholgüe beach (39º36''S; 72º59''W; Fig. 1) during April, 2009. Specimens were maintained in sea water at room temperature and then were transported with constant aeration (100 L h^{-1}) to the Cell Culture and Marine Genomics Laboratory at the Universidad de Concepción until processing.

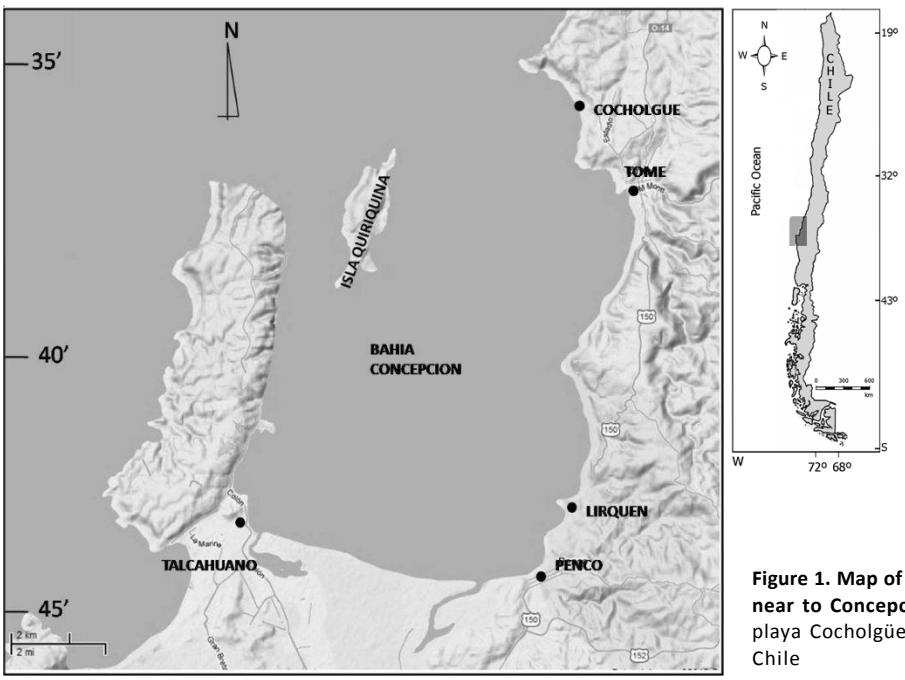

Figure 1. Map of the study area of Cocholgüe beach located near to Concepción, Chile / Mapa del área de estudio de playa Cocholgüe, ubicada en la Provincia de Concepción, Chile

Preparation of pure extracts of *A. chilensis*, preparative chromatography and analysis by LC-MS-MS

Specimens were eviscerated, to avoid contamination, and minced to pieces of 1 cm^2 or less. From 5 kg of pooled sample, approximately 1.4 kg were frozen in glass containers of 500 ml capacity. Samples were then lyophilized in a Lyph Lock 12 Freeze System equipment (Labconco). Once lyophilization was over, dried tissue was transferred to a heater at 40ºC in order to assure elimination of water. Dehydrated samples were weighted again and the water percentage was calculated. Later, samples containing 10-13 g were placed in Whatman filter paper cartridges (11 µm pore size). Extraction was carried out following Soxhlet method (Bialy *et al*. 2004) at 60ºC, and two kinds of solvent were used. First extraction was performed using dichlormethane (CH_2Cl_2) in order to extract fatty or low polarity compounds (lipophilic) during 3 d through cycles of 8 h per day. This process was followed by an extraction in methanol (CH_3OH) in order to get the hydrophilic compounds. Extraction in methanol was carried out following the same method indicated above.

Samples were dissolved in 200 ml of distilled water and loaded per 12 h in an amberlite XAD-7 column (Sigma-Aldrich) and later washed with distilled water to remove salt from the extract and finally eluted using methanol.

Purification of extracted compounds was carried out by means of column chromatography, using a 3.5 cm diameter glass column filled with silica gel 60 F_{254} (Merck). Samples were impregnated with 3 g silica gel in a porcelain mortar until dryness. Then, samples were carefully mounted in the column, in order to obtain a development of the sample and an optimum separation of the mobile phase constituted by a gradient of chloroform ($CHCl_3$) and methanol (CH_3OH). Fractions obtained were monitored through fine layer chromatography, using silica gel 60 F_{254} chromatographic plates, with a mobile phase constituted by 80% chloroform ($CHCl_3$), 19% methanol (CH_3OH) and 1% water (H_2O). At the same time, in some cases reverse phase chromatographic phases (RP-18) were used.

Chromatographic identification and determination of molecular weight

LC-MS-MS analysis was carried out in a MDS SCIEX liquid chromatographer (Applied Biosystems), equipped with an Agilent 1200 series quaternary pump, Agilent 1200 vacuum degasser, Agilent Hip-Als, automatic sampler with temperature control, Agilent 1200 column thermostatizer, and 3200 Q trap LC-MS-MS System detector. Fractions were injected in the equipment loop, in a volume of 15 µl per sample. As mobile phase a CH_3CN:H_2O gradient (HPLC quality) in C_{18} column (Sunfire Waters Company) was used with a 2.5 µm particle size and 21 cm length. In order to improve chromatographic peaks, 1% formic acid was added in the mobile phase. Determination of molecular weight of the fraction 18 was carried out by means of an Applied Biosystems Q trap 3200 mass spectrometer. This was injected with different solvents in order to obtain a comparison of the molecular weights in the different injections. The first one was added to a CH_3OH:H_2O (50:50%) mixture; the second one, in CH_3OH:H_2O: formic acetate mixture and the third one was performed in a mixture of CH_3OH:H_2O and 1% formic acid. A direct infusion to the spectrometer was performed at 20 µl min^{-1} of the methanolic fraction in order to obtain first order spectra of the fraction (elution having analytes if interest) previously evaporated under nitrogen current at 40ºC. The extract was dissolved in a 1:1 methanol: water mixture, with addition of 5 nM ammonium acetate and filtered through a 0.22 µm PTFE membrane. Ionization was carried out by means of the electrospray mode (ESI) both positive and negative, with parameters of the ion source of 70, -70 for DP, 10, -10 for EP and 20, -20 for CEP. Capillary voltage was 400 and -4500 Volt in positive and negative mode, respectively. A scanning between 700 and 1700 uma was performed for both ionization modes at 50ºC; with a GS1 and GS2 curtain gas of 40 and 50 arbitrary units respectively. Q1 Quadrapole resolution was of 0.4 uma. In the case of the chromatographic analysis 10 µL of the extract were injected in a TSK-gel column of 2.1 x 150 mm, with 3.5 µm particle size. Used flow was 0.25 µl min^{-1} and oven temperature of 45ºC. Elution was performed in gradient. With 100% aqueous phase in the beginning, and later linearly increased the organic phase B at 95% at 25 min and kept for other 5 min in order to remove impurities of the column and return to the initial condition at 35 min for a new injection.

Bioprospecting of biological activity

Hemolytic test

To determine the presence of saponins in the primary extracts of dichloromethane and methanol, the hemolytic test was used following a method described by Gestetner *et al*. (1968). Saturated discs with the extracts were placed on blood agar plates for 24 h. Hemolytic halos formed around the discs were proportional to the hemolytic

potential or saponin concentration. Also, this test was performed with the fractions purified from the methanolic extract.

Antibacterial test

The evaluation of the antibacterial activity was performed against Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633), and against Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Salmonella* sp. and *Pseudomonas* sp., obtained from the bacterial collection of the Environmental Microbiology Laboratory at the Faculty of Biological Sciences of the Universidad de Concepción. Bacteria were incubated at a concentration of 10^6 UFC ml^{-1} for 24 h at 37ºC in Trypticase soy agar at 37ºC on 10 cm Petri dishes. Extract of *A. chilensis* was used at 10 mg ml^{-1}, and a mix of penicillin and streptomycin (10000 IU and 10 mg ml^{-1}, respectively) was used as a reference antibiotic. The diameter of the halo of bacteria growth inhibition, or zone of inhibition, was measured after 24 h of incubation with the extract.

Antifungal test

In order to assess antifungal properties *Aspergillus* sp., *Botrytis* sp., *Rhizopus* sp. and *C. albicans* coming from the fungal culture collection of the Natural Products Chemistry Laboratory (Universidad de Concepción) were used. Spores of *Aspergillus* sp., *Botrytis* sp. and *Rhizopus* sp. were placed in a H_2O:Tween 20 (10:1) solution and quantified using a hemocytometer. Standard concentration used was $1x10^6$ spores per 10 cm Petri dish in HA agar. Purified extract was place in wells of 100 µl at a concentration of 10 mg ml^{-1} and incubated at 20ºC. Finally, the zone of inhibition generated around the extract samples was measured at 72 h. *C. albicans* was tested with the same conditions used for bacteria (incubation in Trypticase soy agar for 24 h). As positive control 10 mg ml^{-1} Ketoconazole was used.

Cytotoxicity test on mammal cells

Neuro 2a (ATCC CCL-131) mouse neuroblastoma cell line was allowed to grow in RPMI 1640 (Invitrogen) culture media, supplemented with 10% fetal bovine serum (FBS, Invitrogen), 0.25 µg ml^{-1} amphoteromicin B (Invitrogen), 100 IU ml^{-1} 100 µg ml^{-1} penicillin - streptomycin (Invitrogen), 1 mM sodium piruvate (Invitrogen) and 2 mM glutamine (Invitrogen). Cells were maintained at 37ºC in an incubator (Thermo Scientifics), with injection of 5% CO_2. In order to determine the cytotoxicity of *A. chilensis* extracts on the tumor cell line of mouse neuroblastoma N2a, the inhibition of cell growth was evaluated at 24 h post-exposure, by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) colorimetric assay, according to the method described by Manger *et al*. (1993) for determination of cell viability. A density of $15x10^4$ cells ml^{-1} in 200 µL of culture medium supplemented with 5% FBS was grown in 96 wells-plates (Nunc) at 37ºC and 5% CO_2 for 24 h. Later, 30 µl of different concentrations of *A. chilensis* extract (from 3.9 µg ml^{-1} to 1500 µg ml^{-1}) were added. Also, control without extract was tested. Absorbance was quantified at 570 nm, in a Biotek EL-800 plate reader. The cell viability of N2a cells was expressed as a percentage of the control without extract. Every value was indicated as the average ± SD (n = 8) with 2 experimental replicates.

Results

The first methanolic extract of *A. chilensis* showed hemolytic activity (data not shown). Therefore, this extract was under further purification. This purification was carried out by means of column chromatography, using chloroform, methanol and water. This method allowed the separation of the extract in 19 different fractions, from which only 3 exhibit hemolytic activity, but fraction 18 showed the greatest bioactivity on a specific test for saponins (blood agar test). Its yield reached only 0.03% related to the dry weight of the sample.

The characterization of the polar compounds from the methanolic fraction of the extraction of *A. chilensis* by means of HPLC showed the presence of 3 compounds, with retention times (RT) of 1.20, 3.25 and 6.06 min, and molecular weights of 508.5, 1015.8 and 1544 Da, respectively (Fig. 2).

By means of LC-MS-MS, molecules contained in fraction 18 were characterized. The first order mass spectrum in positive mode (Fig. 3A) presents 5 clusters with species of the form $[M+H]^+$, with masses of 507 (M1), 765 (M2), 1015 (M3), 1272 (M4) and 1523 (M5) Da. Species forming adducts with sodium are also observed, generating mass differences of 23 Da for every one of them.

In negative electrospray mode (Fig. 3B) the presence of 5 clusters can also be observed with masses of 566 (M1), 823 (M2), 1073.8 (M3), 1330 (M4) and 1581 (M5) Da. Every one of these species corresponds to a number of adducts with acetate ion (A) present in the ionization

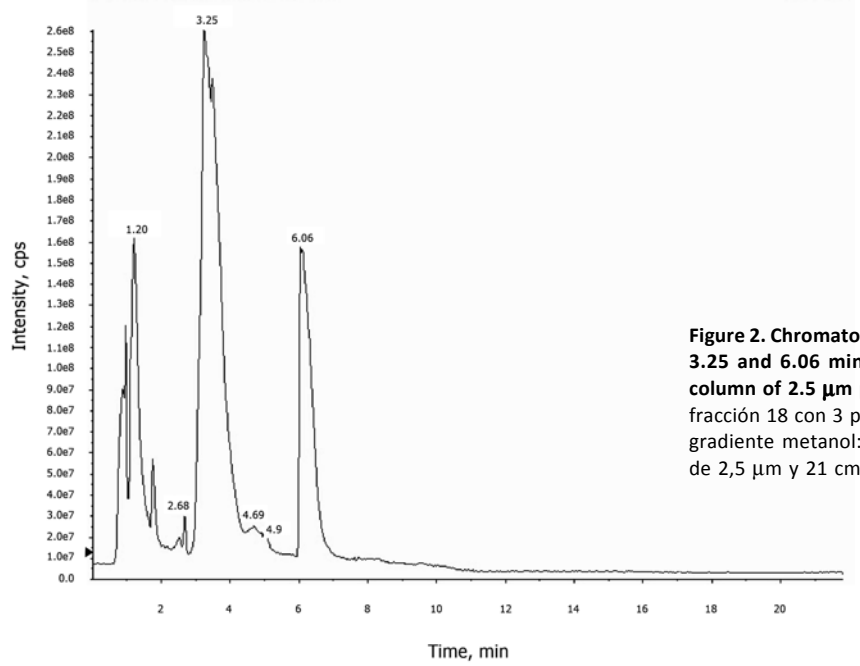

Figure 2. Chromatogram of fraction 18 with 3 peaks with RT of 1.20, 3.25 and 6.06 min. Gradient scanning methanol: H2O with C-8 column of 2.5 µm particle size and 21 cm length / Cromatograma fracción 18 con 3 picos con TR de 1,20, 3,25 y 6,06 min. Corrida en gradiente metanol: H2O con columna C-8 de tamaño de partícula de 2,5 µm y 21 cm de largo

Table 1. **Species and molecular masses asigned for fraction 18. Ionization was carried out by means of the electrospray mode (ESI) both positive and negative** / Especies y masas moleculares asignadas para la fracción 18. La ionización fue llevada a cabo en modo electrospray (ESI) positivo y negativo

Compound	ESI +	m/z (amu)	ESI -	m/z (amu)	Molecular weight (amua)
M1	$[M1+H]^+$	507	$[M1+A]^-$	566	506
	$[M1+Na]^+$	530			
	$[M1+K]^+$	546			
M2	$[M2+H]^+$	765	$[M2+A]^-$	823	764
	$[M2+Na]^+$	787			Holothurinoside D
	$[M2+K]^+$	803			
M3	$[M3+H]^+$	1015	$[M3-A]^-$	1073,8	1014
	$[M3+Na]^+$	1037			
	$[M3+K]^+$	1053			
M4	$[M4+H]^+$	1272	$[M4+A]^-$	1330,8	1271
	$[M4+Na]^+$	1294			
	$[M4+K]^+$	NF			
M5	$[M4+H]^+$	1523	$[M5+A]^-$	1581	1522
	$[M5+Na]^+$	1545			
	$[M5+K]^+$	1561			

ESI: Electrospray Ionization. m/z: mass to charge ratio. amu: atomic mass unit. A: acetate. NF: not found

vehicle. Masses distribution is correlated with the spectrum in a positive way, where molecular weight is possible to be assigned (Table 1).

The results from the evaluation of microbiological activity for the chromatographically purified fraction 18 from *A. chilensis* against 5 clinically important microorganisms showed antimicrobial activity against Gram-positive bacteria such as *S. aureus* and *B. subtilis*, with a zone of inhibition of 22 ± 2 and 19 ± 1 mm, respectively. On the other side, there was no evidence of activity against the Gram-negative bacteria analyzed (Fig. 4).

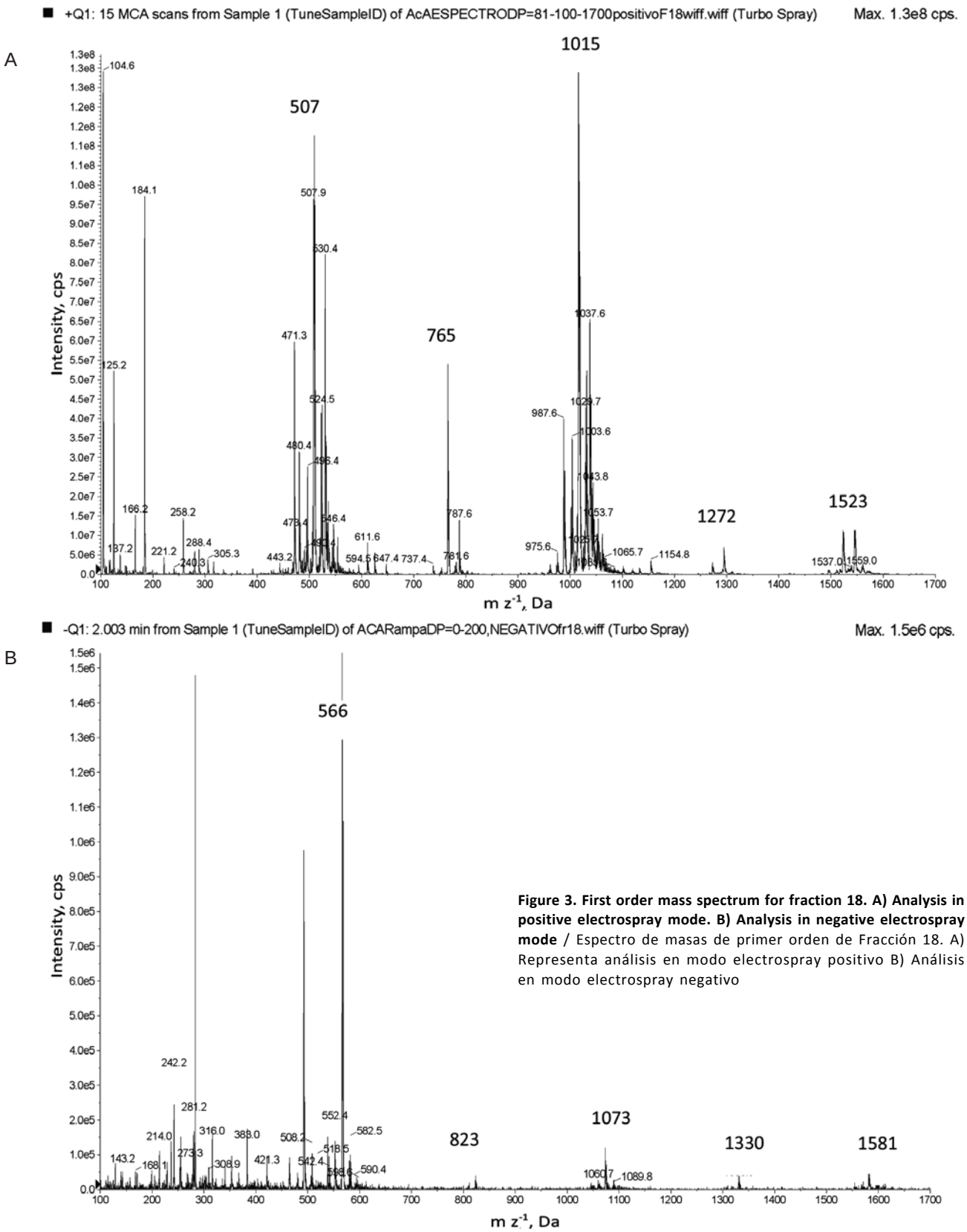

Figure 3. First order mass spectrum for fraction 18. A) Analysis in positive electrospray mode. B) Analysis in negative electrospray mode / Espectro de masas de primer orden de Fracción 18. A) Representa análisis en modo electrospray positivo B) Análisis en modo electrospray negativo

Antifungal activity of the extract was evaluated on 4 microorganisms medically and commercially important (Zahavi *et al*. 2000, Pasqualotto & Denning 2008). Fraction 18 showed activity against *Aspergillus* sp., *Botrytis* sp. and *C. albicans*, with a zone of inhibition of 24.5 ± 4, 13.5 ± 3 and 22 ± 1 mm, respectively. There was no activity against *Rhizopus* sp. (Fig. 5).

Finally, the extract from *A. chilensis* characterized by the presence of saponins (fraction 18) showed citotoxic activity on the tumor cell line N2A. A dose-depending reduction in the cell viability was detected with an IC_{50} of 77.34 ± 1.6 µg ml^{-1} (Fig. 6).

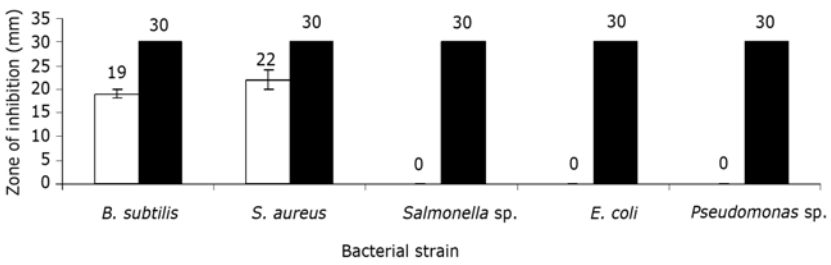

Figure 4. Representation of the antibacterial activity between fraction 18 (10 mg ml^{-1}, white bars) and reference antibiotic (Penicillin-Streptomycin 10 mg ml^{-1}, black bars) / Representación de la actividad antibacteriana entre la fracción 18 (10 mg ml^{-1}, barras blancas) y el antibiótico de referencia (Penicilina-Estreptomicina 10 mg ml^{-1}, barras negras)

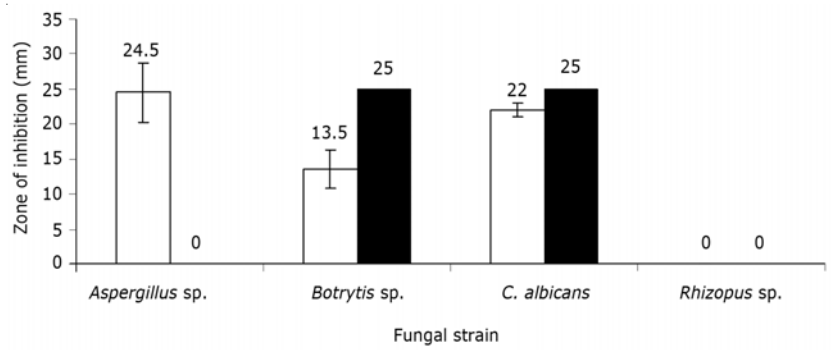

Figure 5. Representation of the antifungal activity between fraction 18 (10 mg ml^{-1}, white bars) and referenced antifungal (10 mg ml^{-1}, black bars) / Representación de la actividad antifúngica, entre la fracción 18 (10 mg ml^{-1}, barras blancas) y el antifúngico de referencia (10 mg ml^{-1}, barras negras)

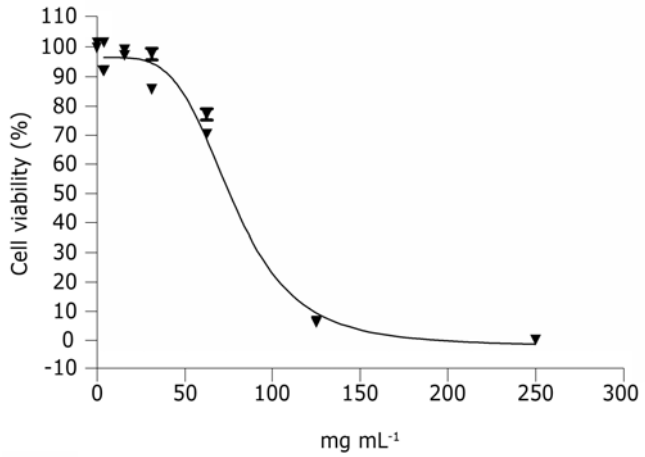

Figure 6. Dose-response curve of the fraction 18 on the cell viability of mouse neuroblastoma N2a cells / Curva de dosis-respuesta de la fracción 18 sobre la viabilidad celular en las células N2a de neuroblastoma de ratón

Discussion

A considerable number of reports have demonstrated the presence of bioactive metabolites in holothurids, with an important diversity of glycosylated triterpenes, called saponins (Kobayashi *et al.* 1991, Stonik *et al.* 1999, Chludil *et al.* 2003, Kalinin *et al.* 2005, Antonov *et al.* 2011). These saponins are described for sea cucumber species collected in tropical zones of the Pacific and Indian Oceans (Stonik *et al.* 1999), Mediterranean Sea (Silchenko *et al.* 2005), North Atlantic (Silchenko *et al.* 2007), North Pacific (Silchenko *et al.* 2008) and some sea cucumber from the Antarctic Sea (Maier *et al.* 2001, Antonov *et al.* 2008, 2009). However, there is a lack of reports about secondary metabolites and saponins of sea cucumber from South American coasts.

The election of solvents and the methodological extraction strategy of active metabolites from marine organisms are fundamental steps for the following evaluation of biological activity, because it allows concentrate, separate and clean the potential bioactive molecules from the biological matrix of the marine organisms. Specifically, in marine organisms it has been suggested the use of alcoholic extractions based on ethanol or methanol. However, this kind of extraction is useful for the preparation of total extracts, which are more complex extracts, with low yields of the total sample weight, and makes difficult to identify the bioactive principles as unique compounds (Silchenko *et al.* 2008, Avilov *et al.* 2008). Therefore, the strategy used in this work included differentiated extraction with solvents of increasing polarity. This methodology can be divided in 2 steps: a first non-polar extraction with dichloromethane, in which similar compounds as lipids and non-polar metabolites are extracted; and a second hydrophilic extraction using methanol as solvent in order to obtain more polar compounds. This approximation allowed the enrichment of the extracts into highly hydroxylated metabolites, which would correspond to the phenolic compounds described by Mamelona *et al.* (2007), who described greater yields in the presence of aqueous solvents, especially from muscle and gonads in sea cucumbers from the Atlantic Sea.

Identification of saponins

Currently, the utilization of LC-MS-MS is emphasized by using triple quadrupole mass spectrometry with turbulonspray ionization interface in order to obtain molecular ions and the molecule fragmentation (Van Dyck *et al.* 2009). Thus, being know molecular weight and atoms distribution, a chemical characterization of them can be built.

The major problem in characterizing glycosides is the capacity of forming adducts with other molecules. This induces the integration of molecules of the analyte with other molecules from the mobile phase, originating clusters with certain deviation, which are presented as [M+H]. Isotopic distribution of the molecules can be observed around every cluster, as well as the formation of adducts with H^+ or Na^+. This kind of adduct has been reported by several authors for saponin analyses (Van Dyck *et al.* 2009). In the spectrum of the first order masses, on positive mode, the presence of 5 clusters can be observed. In every one of them, molecular species that correspond to adducts with protons and cations coming from the ionization additive used can be noticed. The most abundant species are those with protons and sodium. Such profile is analogous to that one obtained from *Holothuria forskali* (Van Dyck *et al.* 2009). The first species, denominated M1, of 507 Da, could correspond to 2 different structures and both correspond to a chain of monosaccharides and the aglucon, conjugated with a proton. When it is associated to a sodium atom, its mass increases to 530 Da. Similar behaviours can be observed for the 4 remaining species, being those species of masses 765, 1015, 1272 y 1523 Da, of the form $[M+H]^+$ for M2, M3, M4 and M5 (Table 1). Also, molecular species composed by sodium with mass differences of 23 Da for every one of them can be observed (Table 1).

In negative electrospray mode, 5 ion clusters, with lower number of adducts and intensity can be observed. This behaviour is characteristic in this kind of ionization. In this spectrum, the presence of 5 clusters can be observed: 566 (M1), 823 (M2), 1073.8 (M3), 1330 (M4) and 1581 (M5) Da. Every one of them corresponds to adducts with ion acetate (A) present in the ionization vehicle. Such mass distribution is correlated to the spectrum in positive mode, being possible to assign the molecular weight to the fragments or molecules after the ionization present in fraction 18. Thus, a molecular weight of 764 Da was assigned to the M2 molecule. Then, species of the form $[M2+H]^+$ and $[M2+Na]^+$ of 765 and 787 Da are confirmed in positive mode with differences of 1 and 23 Da, respectively. In negative mode, the species $[M2+A]^-$ of 823 Da with a difference of 60 Da is observed. Molecular weights of the other saponins were equally assigned. From the species identified in Table 1, the one with a mass of 787 Da corresponds to the saponin

holothurinoside D (Fig. 7), isolated from *Holothuria forskali* by Rodriguez *et al*. (1991). The remaining chemical species described would correspond to new saponins isolated from *Athyonidium chilensis*. However, the high complexity of the saponins mixtures from these extracts makes difficult their structural elution and the evaluation of their potential biological role.

The evaluation of the biological activity of purified extracts isolated from *A. chilensis* showed a remarkable activity against Gram positive bacteria such as *S. aureus* and *B. subtilis*, but not against Gram negative bacteria tested in this study. These results are in agreement with observations described by Haug *et al*. (2002), who determined that most of the extracts prepared from different organs of the holothurid *Cucumaria frondosa* showed antibacterial activity against Gram positive and no activity against Gram negative such as *E. coli* and *Vibrio* sp., also suggesting that the antimicrobial activity would not be exclusively attributed to the presence of enzymes such as lisozime or other antimicrobial peptides (Haug *et al*. 2002), but it would be related to the presence of secondary metabolites like saponin in the extracts from *A. chilensis.*

According to the evaluation of the antifungal activity, though they suggest the presence of antagonistic molecules on proliferation of fungi such as *Aspergillus* sp. and *Botrytis* sp. did not show activity against *Rhizopus sp*. This would be explained exclusively on the basis of the specific inhibition mechanisms, which were not considered in this study. However, these results are congruent with what has been reported for holothurid species *C. frondosa* and *C. japonica* (Attaway & Zaborsky 1993).

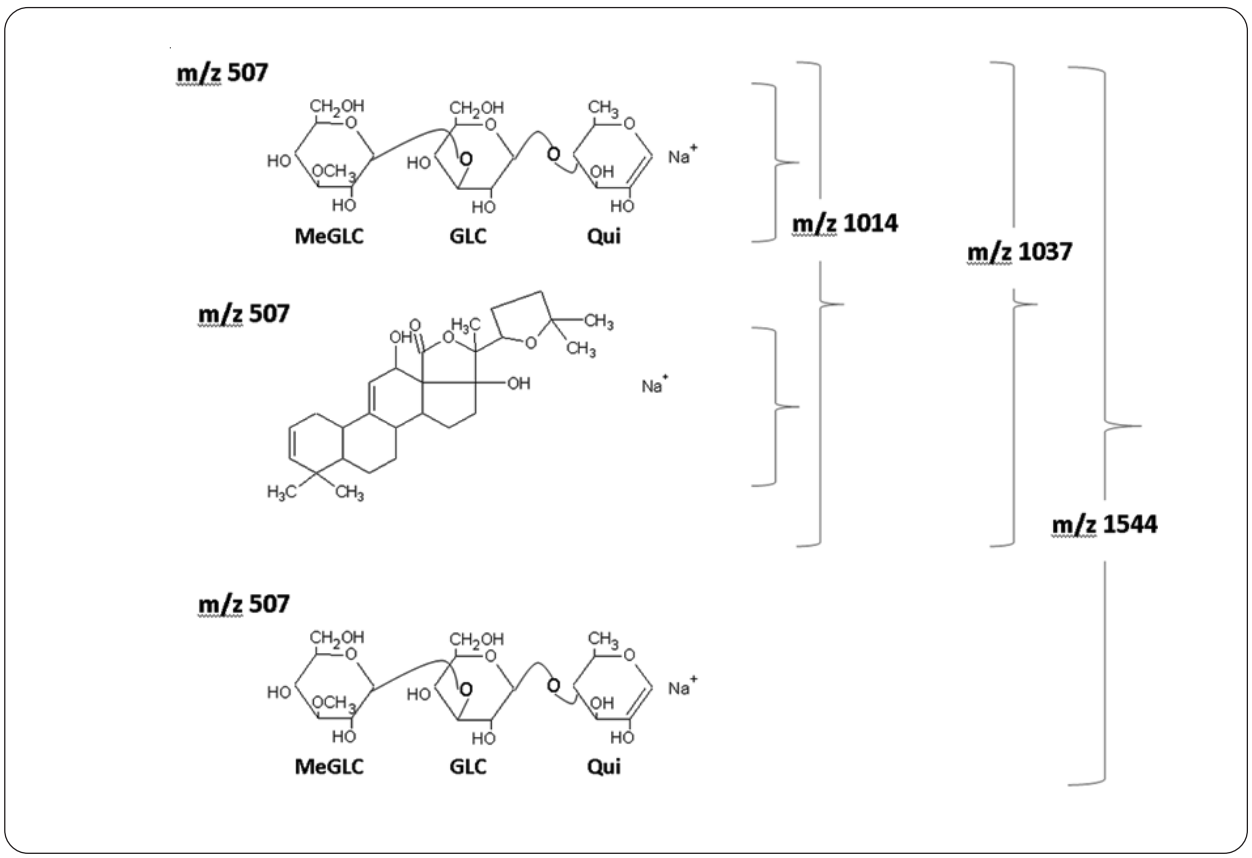

Figure 7. Presumptive molecular structure of a derivative holothurinoside D isolated from *Athyonidium chilensis* / Presumible estructura molecular de un derivado de holoturinósido D aislado desde la especie *Athyonidium chilensis*

There are reports about the antiproliferative potential of sphingolipids and branched chain fatty acids obtained from extracts of sea cucumbers on cell lines of peripheral tumours such as prostate cancer cells (Yang *et al.* 2003) and colon cancer cells (Sugawara *et al*. 2006). However, there are no records of its evaluation on cell lines from neuroblastoma. Our results showed that the extract purified of saponins is capable to reduce both proliferation and cell viability of the N2a tumour cell line after 24 h, with an IC_{50} of 77.3 ± 1.6 µg ml^{-1} (Fig. 6). These results of cytotoxicity on N2a cells are correlated with the antiproliferative properties described for total or enriched extracts isolated from other species of sea cucumbers in cell lines of human tumours of peripheral origin such as A549, MCF-7, 1A9, CAKI-1, U-87-MG, PC-3, KB, KB-VIN, SK-MEL-2, HCT-8 (Zou *et al.* 2006, Sugawara *et al.* 2006). Antiproliferative activity has been attributed to the purification or enrichment of certain molecules like saponins and triterpenic glycosides for which different mechanisms are suggested. Among these mechanisms, selective inhibition by arrest of the cell cycle (Mujoo *et al*. 2001), specific cytotoxic activity influenced by the glycosylated portion of the saponin structure (Kuroda *et al.* 2001), and non-specific cytotoxicity caused by detergent action (Mimaki *et al*. 2001) can be mentioned. Additionally, there are previous information on the basis that sphingolipids isolated from sea cucumber are able to induce apoptosis on colon cancer cells (Sugawara *et al*. 2006). On the other side, other molecules such as leptosines are able to produce inhibition of the *in vitro* proliferation on tumour cell lines by means of the specific inhibition of the protein kinase and topoisomerase II (Jha & Zi-rong 2004).

In conclusion, this study demonstrated that the fraction 18 obtained from *A. chilensis* contains 2 saponins, which are identified through LC-MS-MS. The first saponin has a molecular weight of 1522 Da, whis is fragmented in diverse small molecules, such as monosaccharides or the aglucon (Fig. 7). The other saponin that could be identified was of 764 Da and would correspond to the saponin named holothurinoside D, previously identified in *Holothuria forskali* by Rodriguez *et al*. (1991). The other chemical species described in this study would correspond to new saponins identified from *A. chilensis*. Additionally, saponin purified extracts showed an interesting hemolytic, antibacterial and antifungal activity. However, the efficiency of the last 2 activities is lower respect to what was observed for other members of the family Holothuridae. On the other side, in relation to the evaluation of the *in vitro* antiproliferative properties of the extracts obtained from *A. chilensis*, it can be concluded that it is presented as an interesting focus of study as potential cytotoxic biomedicines of marine origin for the treatment of cancer. An example of this is Frondoside A, isolated from sea cucumber which demonstrated an important antimetastatic activity (Ma *et al*. 2012).

Altogether, our results have become into the first characterization of molecules with biological activity from *Anthyonidium chilensis* which, in the long term, would give an added value to this resource and it can be propose it as a potential source of biomedicines. Even though its poor yield does complex its commercial exploitation, it is a good source of new molecules which can be produced at a bigger scale.

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