Biological activity of extract from *Styela plicata* and *Ascidia mentula* (Ascidiacea)

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Abstract

The biological activity of extract obtained from the ascidians *Styela plicata* and *Ascidia mentula*, living in the coastal lake Faro (Messina, Italy) was ascertained with specific regard to cytotoxic effect, tested on cultured human embryonic kidney (HEK 293 Phoenix) cells after 12, 24 or 48 h, and to antimicrobial effect (10 mg/mL), assayed on both Gram positive and negative strains. Nitrosative stress, possibly induced by the extract on HEK 293 cells and assessed by NO_2^-/NO_3^- production measurement, was also verified. With regard to cytotoxic activity, *A. mentula* extract was more effective than *S. plicata* one, as shown by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, with toxic action due to nitrosative stress. *S. plicata* extract had a more significant antimicrobial activity than *A. mentula*. The present findings provide a first evidence of the biological power exhibited by both *S. plicata* and *A. mentula* extracts, thus increasing the knowledge about the biological activity of marine compounds and providing novel information to possibly correlate the different toxicity pattern displayed by both specimens and their distribution in the lake Faro (Messina).

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Introduction

The biological and chemical diversity of the marine environment constitute a great source of new bioactive products, the knowledge of which has greatly increased the interest of researchers over the past twenty years.[1,2] Several marine organisms produce a variety of chemical compounds for predation strategies and for defence against potential predators and competitors. Such compounds are critically important for soft bodied organisms such as marine algae, sponges, ascidians (sea squirts) to deter predators and compete for space.

Amongst marine organisms, ascidians (*Phylum* Chordata, Class Ascidiacea) are distributed on hard bottoms from the intertidal to the subtidal area, abyssal depths, including coral reef and mangroves. Approximately 3000 living species of Ascidians have been identified so far[3] and, due to their distribution, they are considered good indicators for water quality monitoring.[4] In addition, they represent a potential source of marine natural products,[1] whose characterization has risen the interest of marine biologists, chemists and toxicologists.

Marine toxicological studies have shed light on numerous compounds extracted from several marine animals[5,6] focusing on their structure and biological activity. In this latter regard, different biological assays were set up to verify the effect of such compounds on cell targets, thus opening the way to consider their possible use as novel tools for pathophysiological and/or therapeutical investigations.[7] Amongst biological assays, the hemolytic one, together with the cytotoxicity assay on cultured cells, are the most suitable.[8]

With regard to Ascidians, more than 800 different biologically active compounds exhibiting a range of biological activities, such as cytotoxic, antibiotic or immunosuppressive activities, inhibition of topoisomerases and cyclin kinases, have been described.[1,2,9] Amongst ascidian specimens, *Trididemnum solidum*, *Lissoclinum patella* and *Styela clava* have been studied (as reviewed by Aneiros and Garateix),[10] with Didemnin A and B, Lissoclinamides and Styelin D as major compounds displaying antitumoral, antiviral and antimicrobial activity, respectively. Recently,

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an acetylcholinesterase inhibitor has been isolated from the sub-Arctic colonial ascidian *Synoicum pulmonaria*.[11]

Based on these premises, the aim of the present study was to provide a first characterization of the biological activity of extract from two ascidian specimens, *Styela plicata* and *Ascidia mentula*, never investigated so far, in an attempt to add more information about the Ascidian fauna inhabiting the ecosystem surrounding the Strait of Messina (Italy),[12] one of the most complex Mediterranean area in terms of biodiversity and species distribution.

For this purpose, the extract, obtained by alcoholic extraction method from both species, has been assayed for cytotoxic activity on cultured embryonic kidney (HEK 293 Phoenix) cells and for a possible antimicrobial activity on Gram positive and negative strains. Moreover, oxidative nitrite formation, known to be produced in response to inflammatory or mitogenic stimuli,[13,14] has been also evaluated on HEK 293 cells after treatment with the extracts.

Materials and Methods

Sample collection

The specimens chosen for the present study, *Styela plicata* (Lesueur, 1823) and *Ascidia mentula* (Müller, 1776), were collected from the coastal Lake Faro (Messina Italy) at 2 to 10 m depth, during January to April 2014. The animals were then transferred in laboratory and stored at -20°C until extraction procedures.

Extraction procedures

Extracts from either *S. plicata* or *A. mentula* were obtained by MeOH/$CHCl_3$ Soxhlet extraction, sonication method.[15] The extract was then re-suspended in MeOH to precipitate salt, filtered and afterwards diluted with water in a 7:3 ratio (MeOH:H_2O). A successive partition of the aqueous phase was performed with CH_2Cl_2 (2:1, v/v, 3-fold) and then with Na_2SO_4. The solvent was removed under reduced pressure and the obtained extract underwent protein determination (mg/mL) according to Bradford[16] and then addressed to cytotoxic and antimicrobial activity assessment.

Cytotoxic activity assay

Cell culture

Human renal HEK 293 Phoenix cells, kindly provided by Paracelsus Medizinische Privatuniversität (Salzburg, Austria), were cultured in minimum essential eagle medium [(MEM); Sigma Aldrich, St. Louis, MO, USA] supplemented with 10% fetal bovine serum [(FBS); Cambrex Bio Science, East Rutherford, NJ, USA], 2 mM L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 1 mM pyruvic acid (sodium salt). Cells were maintained at 37°C, 5% CO_2, 95% air and 100% humidity. Subcultures were routinely established by seeding the cells into 100 mm diameter Petri dishes after trypsin/ethylene diamine tetraacetic acid (EDTA) treatment.

To evaluate the cytotoxic activity of extract from either *S. plicata* or, alternatively, *A. mentula*, cells were seeded in 96-microwell culture plates at a density of $3x10^4$ cells/well. Cells were allowed to grow for 24 h (37°C, 5% CO_2, 95% air and 100% humidity) and incubated for various time intervals (12, 24 and 48 h) in a medium containing extract at different concentrations (1 to 10 mg/mL). Cell viability was then estimated and compared to untreated cells.

Cell viability assay

To test the cytotoxicity of extract, cell viability was estimated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT; Sigma Adlrich) assay, which measures the levels of mitochondrial dehydrogenase activity, using MTT reduction assay. The assay is based on the redox ability of living mitochondria to convert dissolved MTT into insoluble formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. Extract from both ascidian species was dissolved in dimethyl sulfoxide (DMSO, 1% v/v final concentration). DMSO alone was tested on control cells to exclude any possible damage. After treatment with extract, the medium was removed and cells incubated with MTT solution (0.5 mg/mL) at 37°C for 1 h in the dark. The incubation was stopped by removing MTT solution and adding DMSO (1% v/v final concentration) to solubilize the formazan. Optical density (OD) was spectrophotometrically measured at 540 nm by a microplate reader (SLT-Lab Instruments, Salzburg, Austria).

Measurement of nitrite/nitrate production

Nitrite (NO_2^-)/nitrate (NO_3^-) production is an indicator of NO synthesis as the final product of NO reacting with molecules present in biological fluids.[13] To measure NO_2^-/NO_3^- levels, medium from both treated and untreated cells was incubated with nitrate reductase (0.1 $U{\cdot}mL^{-1}$), nicotinamide adenine dinucleotide phosphate (1 mM) and flavin adenine dinucleotide (50 mM) at 37°C for 15 min, followed by further incubation with lactate dehydrogenase (100 $U{\cdot}mL^{-1}$) and sodium pyruvate (10 mM) for 5 min. NO_2^- concentration was measured by Griess reaction, by adding 100 µL of Griess reagent [0.1% (*w/v*) naphthylethylenediamide dihydrochloride in H_2O and 1% (*w/v*) sulphanilamide in 5% (*v/v*) H_2PO_4; vol. 1:1 to the 100 µL sample]. The OD at 550 nm (OD_{550}), measured using a microplate reader (SLT-Lab Instruments), was compared with OD_{550} of standard solutions containing sodium nitrate in saline solution to calculate NO_3^- concentrations.[14]

Antimicrobial activity assay

A first screening of the possible antimicrobial activity of ascidian extracts was performed by the standard disk diffusion method, the most used test for antimicrobial susceptibility assessment, as described by Matuschek and co-authors.[17] The test was performed on the following microbial strains: *Burkholderia mallei* (B7), *Klebsiella pneumonia* (B19), *Staphylococcus aureus* (B23), *Pseudomonas sp* (B831), *K. pneumonia* (B938), *Escherichia coli* (U743) and *E. coli* (U744). A volume of 25 µL of extract (10 mg/mL) from both species was tested *per* disk and disks were placed on plates within 15 min of inoculum. Samples were then incubated overnight at 37°C with 5% CO_2 and the diameter (mm) of inhibition area after treatment with both extracts was measured in triplicate and compared with inhibition area after ampicillin application, assumed as control.

Experimental data and statistics

GraphPad Prism software (version 5.00 for Windows; GraphPad software, San Diego, CA, USA) was used and data expressed as arithmetic means±standard error of the mean (SEM) for statistical analysis. Significant differences between means were tested by paired Student's *t* test or one-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test for multiple comparisons. Statistically significant differences were assumed at $P<0.05$ (*$P<0.05$, **$P<0.01$, ***$P<0.001$). *N* represents the number of independent experiments performed on different experimental days.

Results

Cytotoxic activity assay

DMSO (1% v/v final concentration) did not elicit any cell damage when applied alone, cell viability being comparable to that observed in control cells (Figure 1).

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Styela plicata

The exposure of HEK 293 Phoenix cells to different extract concentrations (1, 2, 5 and 10 mg/mL) for 12, 24 or 48 h provoked a significant (P<0.001) reduction in cell viability, if compared to control cells, as depicted in Figure 1A. There was no statistical difference between the different times of exposure to extract (12-24-48 h) at each dose employed, so that no time dependence of extract effect was observed. With regard to 12 h exposure (Figure 1A), no statistical difference was seen between cell viability measured at each dose. With regard to 24 h exposure to extract, cell viability after 10 mg/mL treatment was significantly (P<0.001) lower with respect to both 1 and 2 mg/mL treatment (Figure 1A). Cell viability after 1 mg/mL treatment was significantly (P<0.001) higher than that measured after treatment with the other doses (Figure 1A). When cells were exposed for 48 h to the extract, no statistical difference was seen between cell viability measured at each dose (Figure 1A). Hence, a dose-dependent effect of extract was shown only in case of 24 h exposure.

Ascidia mentula

The exposure of HEK 293 Phoenix cells to different concentrations of extract (1, 2, 5 and 10 mg/mL) for 12, 24 and 48 h caused a significant (P<0.001) reduction in cell viability with respect to control cells (Figure 1B), as shown by MTT assay. There was no time dependence for extract toxicity, as cell viability was not reduced by increasing time of exposure (Figure 1B). With regard to 12 h treatment, cell viability measured at 1 mg/mL was significantly (P<0.01) higher than that measured at the other doses (Figure 1B), while there was no statistically significant difference between cell viability measured at 2-5 and 10 mg/mL.

With regard to both 24 and 48 h incubation, there was no statistically significant difference between cell viability measured at each dose (Figure 1B). Hence, 1 mg/mL extract was the minimum dose capable of significant cell viability reduction.

There was no statistically significant difference between cell viability detected after 12 h treatment with *A. mentula* extract at each dose (Figure 1B) and the one measured after *S. plicata* extract treatment (Figure 1A). Moreover, cell viability measured after either 24 or 48 h exposure to *A. mentula* extract (Figure 1B) was significantly (P<0.01 and P<0.001) lower than that observed after *S. plicata* extract treatment, at all doses (Figure 1A), thus showing a more toxic effect of *A. mentula* extract (Figure 1B).

Measurement of intracellular nitrite/nitrate production

DMSO (1% v/v final concentration) did not induce NO_2^-/NO_3^- production when applied alone (Figure 2).

Styela plicata

The exposure of HEK 293 Phoenix cells to different *S. plicata* extract concentrations (1, 2, 5 and 10 mg/mL) for 12, 24 or 48 h caused a significant (P<0.001) increase in NO_2^-/NO_3^- production, when compared to control cells (Figure 2A). Moreover, NO_2^-/NO_3^- measured after 10 mg/mL was significantly (P<0.001) higher than that measured at the other doses, at any time of exposure (Figure 2A), thus showing a dose-dependent effect. NO_2^-/NO_3^- production measured after 48 h was significantly (P<0.001) higher than those measured at both 12 and 24 h (Figure 2A), thus exhibiting a time-dependent effect.

Ascidia mentula

The exposure of HEK 293 Phoenix cells to different concentrations of extract (1, 2, 5 and 10 mg/mL) for 12, 24 or 48 h caused a significant (P<0.001) increase in NO_2^-/NO_3^- production, with respect to control conditions (Figure 2B). Nitrite production was significantly (P<0.001) higher after 10 mg/mL extract treatment than after applying the other doses, at any observation time (Figure 2B), showing a dose-dependent effect. Nitrite production measured after 10 mg/mL treatment was significantly (P<0.001) higher after 48 h incubation than both 12 and 24 h (Figure 2B), exhibiting a time-dependent effect of extract, not seen when lower doses were considered. Nitrate production after treatment with *A. mentula* extract was significantly higher than that produced after *S. plicata* extract application at both 5 and 10 mg/mL after 12 h incubation (P<0.001 and P<0.05 respectively; Figure 2B), while significantly (P<0.001) higher at all doses after both 24 and 48 h (Figure 2B).

Antimicrobial activity assay

The antimicrobial activity of ascidian extracts assessed on 7 microbial strains is reported in Table 1. As far as *S. plicata* extract is concerned, the antimicrobial activity detected on *Pseudomonas* sp (B831)

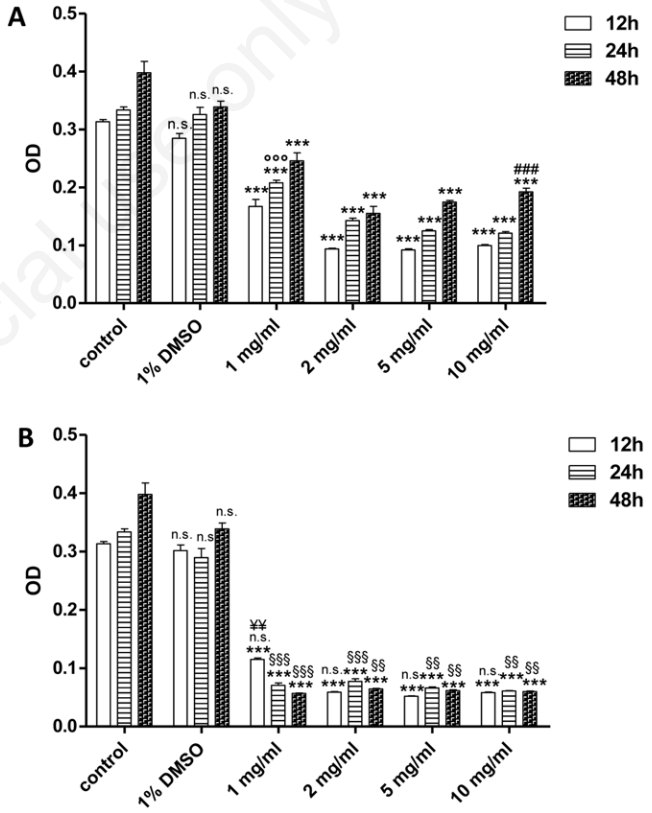

Figure 1. Effect of *Styela plicata* (A) and *Ascidia mentula* (B) crude extract on cultured human embryonic kidney cells viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were treated with different concentrations of extract (1, 2, 5 and 10 mg/mL) for the indicated times: 12, 24 and 48 h. A) ***P<0.001, significantly different *vs* control cells; ns, not significant *vs* control; ###P<0.001, significantly different *vs* both 1 and 2 mg/mL; °°°P<0.001, significantly different *vs* 2, 5 and 10 mg/mL. B) ***P<0.001, significantly different *vs* control cells; ns (DMSO), not significant *vs* control; ¥¥P<0.01 significantly different *vs* 2, 5 and 10 mg/mL; §§§P<0.001 significantly different *vs S. plicata;* §§P<0.01 significantly different *vs S. plicata;* ns (all doses in B) not significant *vs S. plicata.* DMSO, dimethyl sulfoxide; OD, optical density.

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and U743 was significantly (P<0.001 and P<0.05, respectively) higher with respect to what observed in control conditions.

The antimicrobial activity of *S. plicata* extract on *MRSA Staphylococcus aureus* (B23) and on (B938) strain was not significantly different (ns) with respect to the control, while the antimicrobial activity on U744 and B7 strain was significantly (P<0.05) lower than that one observed in control conditions.

With regard to *A. mentula* extract, the antimicrobial activity detected on U743 was significantly (P<0.001) higher than what observed in control conditions. The antimicrobial activity on B938, B831 and B23 was not significantly different (ns) with respect to control conditions, while the activity on B7 and U744 was significantly (P<0.05 and P<0.001, respectively) lower than that observed in control conditions. No antimicrobial activity was detected on B19 strain after treatment with extract from both species.

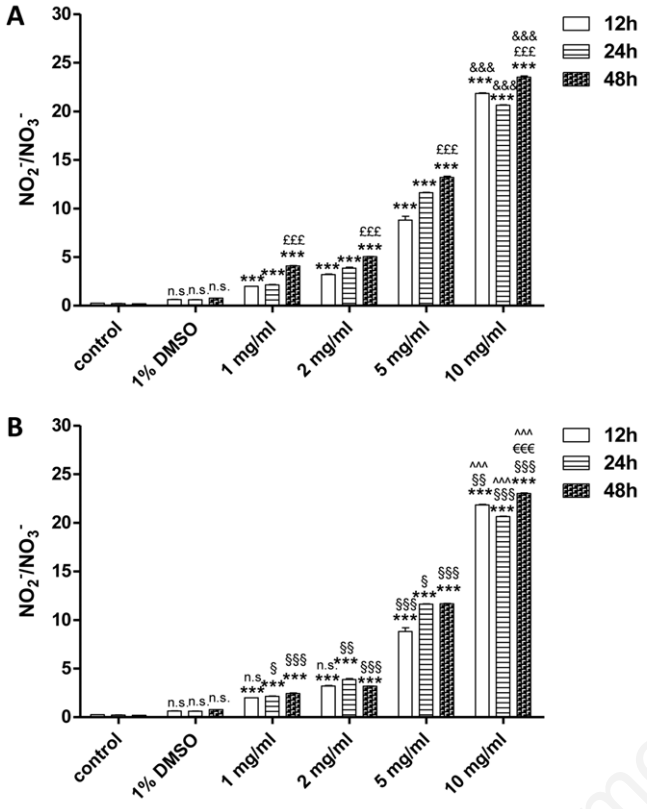

Figure 2. Nitrite/nitrate levels measured in cultured human embryonic kidney cells after exposure to *Styela plicata* (A) and *Ascidia mentula* (B) crude extract. Cells were treated with different concentrations of extract (1, 2, 5 and 10 mg/mL) for the indicated times: 12, 24 and 48 h. A) ***P<0.001, significantly different *vs* control cells; ns, not significant *vs* control; &&&P<0.001, significantly different *vs* 1, 2 and 5 mg/mL; £££P<0.001, significantly different *vs* 12 and 24 h. B) ***P<0.001, significantly different *vs* control cells; ns (DMSO), not significant *vs* control; ^^^P<0.001, significantly different *vs* 1, 2 and 5 mg/mL; €€€P<0.001, significantly different *vs* 12 and 24 h; ns (1 and 2 mg/mL), not significantly different *vs S. plicata*; §§§P<0.001, significantly different *vs S. plicata*; §§P<0.01, significantly different *vs S. plicata*; §P<0.05, significantly different *vs S. plicata*. DMSO, dimethyl sulfoxide.

Discussion

The first goal of the present investigation was to add more information to the field of marine toxins by studying the biological power of extract obtained from *S. plicata* and *A. mentula*, Ascidians inhabiting the coastal lake Faro, close to the Strait of Messina (Italy).

As a general feature, the assessment of biological activity of marine compounds has long attracted the interest of many researchers,[2] in an attempt to characterize substances with biological activity possibly providing beneficial effects.[7] To prove this latter feature, a variety of assays has been validated so far, with the cytotoxic assay, performed on erythrocytes[18,19] or on cultured cells[20-22] chosen as the most suitable for *in vitro* studies. For the present investigation, human epithelial kidney cultured cells (HEK 293 Phoenix) were used as a model to verify the cytotoxic effect of both *S. plicata* and *A. mentula* extracts.

Our findings show that the extracts from both ascidian species exhibit a cytotoxic action on HEK 293 cells in a dose-dependent manner. *A. mentula* extract seems to be more cytotoxic since its effect, after 24 and 48 h of incubation, is higher than that of *S. plicata*. Such discrepancy could be due to the different habitats where the two species live, in the wide and complex ecosystem of the Strait of Messina and surrounding lakes. *S. plicata* is an introduced species which is dominant in harbour areas and in zones with highly modified substrates, a low rate of water renewal and excess silting and suspended matter.[23] It mainly colonized the mussel farm structures in the inner part of the Lake Faro, with high benthic production and related space completion. By contrast, the native *A. mentula*, colonizing both natural rocks in the outer zone of the bay and vertical walls of ports, is generally absent from internal harbour areas with low water movement.[12] In the Lake Faro, it settled near the canals, where sea-water exchanges dilute the local organic load, lowering benthic production and space competition. This consideration may putatively explain the different toxicological pattern exhibited by the extracts of both specimens, though the exact biological potency of each metabolite has not been studied so far.

The evidence that the toxicological profile of a marine species can be related to its habitat has been already provided.[24] In this regard, it has

Table 1. Antimicrobial activity of extract from *Styela plicata* and *Ascidia mentula*.

Strain name	B7	B19	B23	B831	B938	U743	U744
Ampicillin (control)	23	32	11	11	10	2	20
Styela plicata	12^{**}	-	10^{ns}	16^{***}	9^{ns}	8^{**}	10^{**}
Ascidia mentula	$12^{\#\#}$	-	11^{ns}	10^{ns}	11^{ns}	$10^{\#\#\#}$	$13^{\#\#\#}$

Inhibition zone (mm) after application of 25 µL extract (10 mg/mL) per disk. B7, *Burkholderia mallei*: Gram negative, rod shape; B19, *Klebsiella pneumonia*: Gram negative, rod shape; B23, *MRSA Staphylococcus aureus*: Gram positive, cocci shape; B831, *Pseudomonas sp.*: Gram negative, rod shape; B938, *Klebsiella pneunoniae*: Gram negative, rod shape; U743, *Escherichia coli*: Gram negative, rod shape; U744, *Escherichia coli*: Gram negative, rod shape. **P<0.05; ***P<0.001 *vs* control; ##P<0.05; ###P<0.001 *vs* control; ns, not significant.

been previously shown that crude venom from the scyphozoan *Pelagia noctiluca* (mesoplankton) induces a more powerful hemolytic effect than the anthozoan *Aiptasia mutabilis* (sessile), while, on the other hand, crude venom from the latter species elicits a more significant cytotoxic effect on cultured cells than *P. noctiluca*.[25,26] Therefore, it can be hypothesized that the biological power of crude venom from some marine organisms may correlate with different strategies performed to guarantee survival in their habitat. On this basis, a similar consideration can be proposed here to explain the difference in the toxic power displayed by *S. plicata* and *A. mentula*, collected in the coastal lake Faro, communicating with sea water of the Strait of Messina and submitted to many significant changes in chemico-physical parameters due to both climatic and anthropic factors.

Although the action mechanism of extract from both ascidians was not the focus of the present study, a first attempt to verify whether cell viability reduction under extract treatment was due to nitrosative stress has been performed. NO• is produced by NO• synthases and its major metabolic fate is oxidation to NO_2^- and eventually to NO_3^-. NO• plays a pivotal role in vascular homeostasis and neurotransmission, whereas unregulated NO• production induces nitrosative stress leading to damage of macromolecules and potentially to disease.[13] In this regard, our data clearly show that the extract from both ascidian specimens elicits a significant nitrosative stress on HEK 293 cells, with a dose-dependent effect and species-related differences, as *A. mentula* extract induced a more significant nitrosative effect than *S. plicata*, at each observation time. Nonetheless, since a significant cytotoxic effect of both extracts was clearly detected at low concentrations (1 mg/mL) without a significant production of nitrates, it is reasonable to suggest that cell damage is independent from nitrosative stress at that dose.

As a general feature, it has been reported that, natural toxins may damage cell target through two main mechanisms of action: i) by forming pores into the membrane phospholipid bilayer, with consequent ionic unbalance and, hence, osmotic lysis of the cell; ii) through the induction of oxidative stress.[27] Further investigations on both *S. plicata* and *A. mentula* extract will better focus on mechanism of action.

In addition to the cytotoxic effect on kidney-cultured cells, the antimicrobial activity was also assayed to add more information on the toxicological profile of *S. plicata* and *A. mentula*. The antimicrobial effect of marine drugs was already demonstrated[28,29] and, along with the cytotoxic effect elicited on cultured cells, represents a good basis to suggest a possible future application of marine compounds in the field of drug development, in line with what suggested by other authors.[8,30]

Our findings reveal a significant antimicrobial activity elicited by crude extract of both species on different microbial strains, here used as a model, suggesting a promising anti-microbial power against human microbial pathogens. Similarly to what observed after cytotoxic assay on HEK 293 cells, also in this case a different antimicrobial effect was elicited, depending on the species. In particular, *S. plicata* extract induced a significant inhibition zone against the Gram negative *Pseudomonas* sp (B831) and Gram negative *Escherichia coli* (U743), while *A. mentula* extract provoked a significant inhibition zone only against *Escherichia coli* (U743). Such observations allow to suggest that the ascidian extract, namely from *S. plicata*, can be reasonably considered in the development of antimicrobial agents, consistently with previous studies.[24,28,29] These latter studies have already identified a modified octapeptide, plicatamide and analogues from the hemolymph of the ascidian *S. plicata* collected from San Diego Bay (San Diego, CA, USA), displaying a significant anti-microbial activity by causing K^+ efflux in *Staphylococcus aureus,* along with a significant hemolytic effect on human erythrocytes, by forming cation-selective channels.[24] According to Lee,[28] two phenylalanine-rich antimicrobial peptides Styelin A and Styelin B were reported from the hemocytes of solitary tunicate *Styela clava* collected in California coast. Both styelins are active against Gram positive and Gram negative bacterial pathogens of humans (MIC 0.5 µm), even in the presence of 100 mM NaCl. Then, also Styelin C, D and E, detected in *S. clava*, have been demonstrated to elicit antimicrobial activity against Gram positive and Gram negative bacteria.[31] Styelins also killed marine bacteria, *Psychrobacter immobilis* and *Planococcus citreus*, in media containing 0.4 mM NaCl. This feature is consistent with the anti-microbial activity of *S. plicata* extract revealed by the present data, namely against the Gram negative bacteria strain B 831 of *Pseudomonas sp*.

Conclusions

Collectively, our results provide additional information on the ascidian fauna inhabiting the area of the Strait of Messina (Italy) and demonstrate the biological power of extract from two different ascidian specimens (*S. plicata* and *A. mentula*). The extract from *A. mentula* seems to be more cytotoxic than *S. plicata* but with a less effective antimicrobial action, putatively depending on the different habitat where each specimen lives. Further studies, including fractions separation, are recommended to better focus on the toxicological properties of both ascidians extracts, which can be hopefully included in drug development investigations. Moreover, the assessment of ascidians toxicity may be possibly considered as a tool to monitor species distribution and ecological interactions in the coastal lake Faro.

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