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Methodology article

A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products

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Abstract

Background: The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. It has also been suggested for screening pharmacological activities in plant extracts. However, we think that it is necessary to evaluate the suitability of the brine shrimp methods before they are used as a general bio-assay to test natural marine products for pharmacological activity.

Material and Methods: The bioactivity of the isopropanolic (2-PrOH) extracts of 14 species of marine invertebrates and 6 species of macroalgae was evaluated with the shrimp lethality assay (lethality assay), as well as with another assay based on the inhibition of hatching of the cyst (hatchability assay). The extracts were also assayed for cytotoxicity against two human cell lines, lung carcinoma A-549 and colon carcinoma HT-29, in order to assess the sensitivity of the shrimp assays to detect cytotoxic activity.

Results: Two sponges (*Hyatella* sp, *Dysidea* sp.), two gorgonians (*Pacifigorgia adamsii*, *Muricea* sp.), one tunicate (*Polyclinum laxum*), and three echinoderms (*Holothuria impatiens*, *Pseudoconus californica* and *Pharia pyramidata*) showed a strong cytostatic (growth inhibition) and cytotoxic effect. The hatchability assay showed a strong activity in 4 of the species active against the two human cell lines tested (*Hyatella* sp, *Dysidea* sp., *Pacifigorgia adamsii* and *Muricea* sp.), and the lethality assay also showed a high lethality in 4 of them (*Pacifigorgia adamsii*, *Muricea* sp., *Polyclinum laxum*, and *Pharia pyramidata*). Each bioassay detected activity in 50% of the species that were considered active against the two human cell lines tested. However, the simultaneous use of both bioassays increased the percentage to 75%.

Conclusions: Our results seem consistent with the correlation previously established between cytotoxicity and brine shrimp lethality in plant extracts. We suggest using both bioassays simultaneously to test natural marine products for pharmacological activity.

Background

The shrimp lethality assay was proposed by Michael et al. [1], and later developed by Vanhaecke et al. [2], and Sleet and Brendel [3]. It is based on the ability to kill laborato-

ry-cultured *Artemia* nauplii brine shrimp. The assay is considered a useful tool for preliminary assessment of toxicity [4], and it has been used for the detection of fungal toxins [5], plant extract toxicity [6], heavy metals [7], cyanobacteria toxins [8], pesticides [9], and cytotoxicity testing of dental materials [10].

On the other hand, although most researchers have made use of the hatched nauplii, other assays based on the inhibition of hatching of the cyst (encased embryos that are metabolically inactive) have also been used [11].

We think that it is necessary to evaluate the suitability of both methods before the brine shrimp method is used as a general bio-assay technique to test natural marine products for biological and pharmacological activity.

Our aim was to assess the bioactivity of organic extracts from 20 species of marine organisms (mainly invertebrates) with two *Artemia* brine shrimp assays, and then compare the results with those obtained for cytotoxic activity against two human cell lines: lung carcinoma A-549 and colon carcinoma HT-29.

For this purpose, we developed a method based on the percentage of hatching of the cyst which was incubated in a medium with different concentrations of organic extracts. Toxicity was measured by comparing the percentage of hatched nauplii to a control.

Our study is part of a program for screening a variety of biological activities in marine fauna in order to find new substances with potential pharmaceutical applications.

Results and discussion

Cytotoxicity assay against human lung carcinoma (A-549) and human colon carcinoma (HT-29)

Only the extracts which showed GI values higher than 60% at the three concentrations tested were considered active. The species that presented the highest cytotoxicity at the highest concentration tested were the sponges *Hyatella* sp. (161% Gl for A-549, and 129% for HT-29) and *Dysidea* sp. (106 and 88% GI), the cnidarian *Pacifigorgia adamsii* (127 and 86% GI), the tunicate *Polyclinum laxum* (87 and 102% GI), the equinoderm *Pseudoconus californica* (122 and 139% GI), and the macroalgae *Colpomenia tuberculata* (91 and 91% GI) (Table 1). All of them had values greater than 60% GI at the three concentrations tested. The GI data obtained in these species allow us to predict their potential not only because of the cystostatic effect, but in terms of potential for tumor reduction (values higher than 100%).

Brine shrimp bioassays

Only a few species were bioactive against the brine shrimp bioassays at 10 and 100 μg de extract per ml, and a low relationship between the brine shrimp bioassays and the cytotoxicity assays was found. However, most of the invertebrates presented toxicity in some of the bioassays at 1000 μg/ml in a way that was consistent with the citotoxicity results. The macroalgae was the group where least activity was detected. A strong hatch inhibition (hatchability assay) was present in the extracts of the sponges *Hyatella* sp. (51%), *Mycale parishii* (64%) and *Dysidea* sp. (50%), and in the extracts of the gorgonians *Lophogorgia* sp. (81%), *Pacifigorgia adamsii* (76%) and *Muricea* sp. (89%). After 12 h of exposure, the percentage of active species increased very slightly. After 24 h of exposure there were no significant changes in the activity of the extracts, although a few species such as *Muricea* sp. and *Lophogorgia* were more active at 48 h than at 24 h.

On the other hand, a high lethality was found in the extracts of the gorgonians *Pacifigorgia adamsii* (68%), *Muricea* sp. (83%), the tunicate *Polyclinum laxum* (96%) and the echinoderms *Toxopneustes roseus* (96%), *Isostichopus fuscus* (96%) and *Pharia pyramidata* (93%) (Table 1). In this case, activity increased significantly with up to 48 h of exposure.

With respect to the effect of the time of exposure, in the hatchability test the highest percentage of toxicity was detected at 12 h or 24 h of exposure, and significant changes in toxicity were not detected in subsequent times of exposure. The very low hatching rate detected after the 12 h treatment was probably due to an alteration in the development of *Artemia* embryos. It has been shown that *Artemia* is highly vulnerable to toxins at the early developmental stages [12,13]. In contrast, in the brine shrimp lethality test, maximum sensibility was reached after 48 h of exposure (the oldest age class tested by us) [14]. At this stage in their life cycle the nauplii have reached their second and third instar and exhibit their greatest sensitivity to test compounds [15].

The hatchability test detected toxicity in a number of species similar to the lethality test, but seemed less sensitive to detect toxicity of macroalgae extracts. In general, the groups with the highest percentage of toxic species, and with the most toxic extracts, were the invertebrates. Some species such as the echinoderms, the sponges *Mycale parishii, Dysidea*, sp, and the gorgonians *Muricea* sp. etc, significantly lowered hatching in the hatchability test, interfering with normal development of the nauplii. The echinoderms *Toxopneustes roseus*, *Isostichopus fuscus*, etc. presented a high lethality (almost 100%).

Table 1: Toxicity of the isopropanolic extracts against lung (A-549), colon carcinoma (HT-29), and brine shrimp assays

	A-549	HT-29	HI-12 h	HI-24 h	HI-48 h	M-12 h	M-24 h	M-48 h
Sponges								
Chondrosia tenochca	16	22	19	15	11	0	0	77
Hyatella sp.	161	129	28	51	46	13	15	40
Mycale parishii	4	25	44	62	64	5	10	10
Dysidea sp.	106	88	35	50	50	0	3	59
Gorgonians								
Lophogorgia sp.	33	26	51	72	81	0	0	0
Pacifigorgia adamsii	127	86	56	72	76	8	68	68
Muricea sp.	93	131	42	64	89	40	83	83
Tunicates								
Polyclinum laxum	87	102	3.4			67	96.5	95.5
Echinoderms								
Toxopneustes roseus	14	14	8.4	24.4	29.6	17	96.5	95.5
Isostichopus fuscus	31	13	8.4	24.4	29.8	32	91.5	95.5
Holoturia impatiens	181	84				4.3	23.7	60
Pseudocus californica	122	139	17.9	27.8	30	1.5	18	58
Phataria unifascialis	29	10	5.6	8.8	0.2	1.8	8.7	20
Pharia pyramidata	194	88	17.9	27.8	30	1.5	0.5	93
Macroalgae								
Colpomenia tuberculata	91	91	4.4	12.8	19.1	0	8.3	8.3
Enteromorpha intestinalis	2	47	8	20.3	20.6	1.8	5	38.1
Gelidiopsis tenuis	81	91	5.6	8.5	18.6	0	3.33	0.8
Ralfsia hesperia	11	7	8	5.1	0.9	1.8	2.5	13.1
Ceramium sp.	4	5	7.4	18	24.1	0	0.8	0.8
Codium dichotomum	110	-9	2.6	0.5	2.8	0	0	0

The percentage of growth inhibition at 50 μg/ml is shown below A-549 and HT-29. The percentage of hatch inhibition, and percentage of mortality at 1000 μg/ml at 12, 24 and 48 h of exposure are shown below HI (brine shrimp hatchability test)and M (brine shrimp lethality test).

The high incidence of toxicity in sponges and echinoderms seems to be an effective defense mechanism against many predation fishes, which increases closer to the tropics (almost 100% of all species tested) [16].

For the past 30 years, the *Artemia* nauplii have been used to detect general toxicity [17], in teratology screens [3,13,18,19] and in ecotoxicology [12,17]. From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumoral compounds in terrestrial plant extracts [4,20,21].

These two bioassays show that standard tests employing organisms such as brine shrimp are useful in identifying metabolites with a high potential for activity against marine organisms. Our results show that the shrimp hatchability test together with the lethality test could be an easy bioassay to screen marine natural products.

Methods

Collection and preparation of extracts

4 species of sponges, 3 gorgonians, 6 echinoderms, 1 tunicate, and 6 macroalgae (Table 1) were collected by snorkeling and scuba diving from low tide to a depth of 20 m along the Mexican Pacific coast. The specimens of each species were freeze-dried and ground together. The lyophilized material (2–5 g) was extracted three times with 2-PrOH (1/20) w/v. The extracts were evaporated under reduced pressure and dissolved in acetone/methanol (1:1) to prepare the stock solution.

Preparation of the bioassays

The tests were conducted in multiwell plates in filtered (0.45 μm pore diameter) and sterilized seawater (final volume 5 ml). Each of the extracts for each species was tested at 1000, 100 and 10 μg of extract per ml. The concentrations were obtained by transferring the corresponding volume from the stock solution to different wells for evaporation [21]. After evaporation, 5 ml of seawater were added to each well with gentle shaking to ensure that the compounds diffused adequately in the aqueous solution. Four replicates were used for each treatment and control. The control was performed by adding the solvent used to dissolve the extracts in the assays, and it was allowed to evaporate. Before the assays, the average time of appearance of the first free nauplii and the subsequent develop-

mental stages was calculated. The first cysts hatched approximately after 12 h of incubation (average time); the maximum percentage of instar II (55.33%) appeared 12 h later (24 h after the start of incubation). All the tests were performed in a temperature-controlled room at 28°C, under a continuous light regime. The extracts were subjected to the following tests:

Brine shrimp hatchability test

The brine shrimp hatchability test is based on Migliore et al. [11]. They calculated the hatch, harvesting the free nauplii from 1 g of cysts on a Millipore 45 μm filter, weighed and placed in a desiccator at 60 C for 24 h to obtain the dry weight. In our case, the percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control and the whole procedure was standardized (see below).

Following the procedure in Amat [22], 0.5 g of dried cysts were separated from their shells using the commercial brine shrimp hatchers solution. After that, the cysts were hatched in seawater (1 g cyst per liter) at 28°C, under conditions of continuous illumination and strong aeration. After 2 h aliquots measuring 250 μl were placed in each well where the extracts had previously been deposited, and they were incubated at the same conditions of temperature and illumination under gentle shaking. After 12, 24 and 48 h of exposure the free nauplii were counted under a stereoscopic microscope. The percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control. Later the percentage of hatch inhibition (%HI) was calculated as: % HI = % hatchability in the control - % hatchability in each treatment.

Brine shrimp lethality test

Dried cysts were performed as indicated above, and incubated (1 g cyst per liter) in a hatcher at 28–30°C with strong aeration, under a continuous light regime. Approximately 12 h after hatching the phototropic nauplii were collected with a pipette from the lighted side and concentrated in a small vial. Ten brine shrimp were transferred to each well using adequate pipettes. Each test consisted of exposing groups of 10 Artemia aged 12 h to various concentrations of the toxic compound. The toxicity was determined after 12 h (mainly nauplii in instar I/II), 24 h (nauplii in instar II/III) and 48 h (mainly nauplii in instar III/IV) of exposure.

The numbers of survivors were counted and percentage of deaths were calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation.

The larvae did not receive food. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not to starvation; we compared the dead larvae in each treatment to the dead larvae in the control. In any case, hatched brine shrimp nauplii can survive for up to 48 h without food [15] because they still feed on their yolk-sac [10]. However, in cases where control deaths were detected, the percentage of mortality (% M) was calculated as: % M = percentage of survival in the control - percentage of survival in the treatment.

Bioassays for cytotoxicity

The cytotoxicity of the extracts was assessed employing two human cell lines: lung carcinoma (A-549), and colon carcinoma (HT-29). A colorimetric type of assay using a sulforhodamine B (SRB) reaction was adapted for a quantitative measurement of cell growth and viability, following the technique described by Skehan et al. [23]. Cells were seeded in 96-well microtiter plates, at 5×10^3 cells per well in aliquots of 190 μl, and they were allowed to attach to the plate surface by growing in a drug free medium for 18 hours. Afterward, samples were added in aliquots of 10 μl (dissolved in DMSO/H_2O 1:9). After 48 hours of exposure, the cytotoxicity was measured by the SRB methodology: cells were fixed by adding 50 μl of cold 50% (w/v) trichloroacetic acid (TCA), and incubating for 60 minutes at 4°C. Plates were washed with deionized water and dried. One hundred μl of SRB solution (0.4% w/v in 1% acetic acid) was added to each microtiter well, and incubated for 10 minutes at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried and bound stain was dissolved with Tris buffer. Optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 490 nm. Data analysis was generated automatically by LIMS implementation at PharmaMar and some parameters for cellular responses were calculated. The activity of the isopropanolic extracts was given in % GI (growth inhibition) for each concentration tested (50, 17 and 5 μg per ml). Values could be as follows:

- negative GI = no growth inhibition (NOT ACTIVE)

< 50% GI = week growth inhibition (NOT ACTIVE)

50 to 100% GI = moderate to high growth inhibition (ACTIVE, growth inhibition effect)

100% GI = Total growth inhibition (ACTIVE, cytostatic effect)

>100% GI = Cell killing (ACTIVE, cytotoxic effect).

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