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Effects of the green alga, *Bornetella oligospora* (Solms-Laubach), metabolite fractions on the cleavage and free-swimming hatching stages of sea urchin embryos

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The ethanolic extract of the green alga *Bornetella oligospora* was sequentially fractionated with hexane, chloroform, ethyl acetate, and water, and concentrated *in vacuo*. Different concentrations of the fractions, ranging from 1 to 150 ppm, were prepared in 80% DMSO, and tested on sea urchin embryos at cleavage and free-swimming hatching. The chloroform concentrate at 150 ppm was the most potent in altering sea urchin embryonic development among fractions. The rate of cleavage in embryos exposed to this fraction was significantly inhibited. Moreover, anomalous morphological features were observed among treated embryos with less apoptosis during cleavage and no cell arrest during blastulation and the free-swimming larval stage. The motility of treated larvae was also slowed. All embryos exposed to 80% DMSO control remained unaffected. Preliminary phytochemical analysis detected the presence of terpenoids in the hexane, chloroform, and aqueous fractions. The ethyl acetate fraction tested positive for saponins. The chloroform fraction clearly altered embryonic development, which could be indicative of its potential chemotherapeutic activity since sea urchin embryos are used as a pre-screen model for determining potential anticancer property.

Keywords: bioactivity, embryotoxicity, marine natural products, secondary metabolites

Introduction

Marine organisms account for more than 80% of total world biodiversity [1]. Studies on tunicates, sponges, nudibranchs, soft corals, cnidarians, macroalgae, and others have led to the isolation of around 10,000 metabolites [1] belonging to specific classes like isoprenoids, eicosanoids, phenolic compounds, polyketides, alkaloids, and acetogenins [2]. Many of these compounds exhibit novel pharmacological activities against selected viruses, Gram-positive and-negative bacteria, fungi, and cancer cell lines [3].

Marine macroalgae, in particular, have been the source of natural products with a broad range of bioactivities. Specific metabolites are synthesized by different macroalgal divisions:

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rhodophytes produce the halogenated compounds isoprenoids and acetogenins; phaeophytes synthesize phlorotannins; and chlorophytes generate terpenoids. The different stress factors present in the marine benthic communities are thought to have led to the structural modifications of these compounds enhancing further their diversity [2, 4]. In spite of this, macroalgae have received less bioassay attention as compared to other marine forms like sponges and coelenterates [1].

The green alga, *Bornetella oligospora* (Solms-Laubach), is one of the many algae not explored for biological activity, as marked by the absence of published work and pharmacological data for this species. This study was undertaken to sequentially fractionate *B. oligospora* secondary metabolites and determine the potential chemotherapeutic property of its fractions using sea urchin embryos at cleavage and free-swimming hatching as a pre-screen model.

Experimental

Marine sample collection and processing. *Bornetella oligospora* (Solms-Laubach) was collected by hand from the shallow waters of Piapi Beach, Dumaguete City, Philippines and brought to the Silliman University Institute of Environmental and Marine Sciences (IEMS) for identification (Fig 1). The algal sample was then cleaned with filtered seawater (FSW) to get rid of sediments and epiphytes, and dried in containers using silica gel for 2–3 weeks.

Figure 1. The green macroalga *B. oligospora* (Solms-Laubach)

Fractionation of secondary metabolites. Two kilograms of dried sample was extracted in 2.5 L of 95% ethanol. The ethanolic extract was concentrated to syrupy consistency *in vacuo* at 35°C. The components of the ethanolic extract were partitioned sequentially according to increasing polarity of solvent. Using a separatory funnel, a 1:1 ratio of hexane and water was added to the ethanolic concentrate. The nonpolar fraction was withdrawn, concentrated *in vacuo*, and set aside. The remaining aqueous layer was successively partitioned with chloroform and ethyl acetate in 1:1 proportion. Each of these fractions was concentrated *in vacuo*. Further removal of the solvent was achieved with the aid of a vacuum dessicator.

Preparation of sea urchin gametes. Sea urchins, *Tripneustes gratilla* (Linnaeus, 1758), were collected from the coast of Canday-ong, Dumaguete City, Philippines. Spawning was induced in the lab by intracoelomic injection of 0.5 M potassium chloride (KCl) [5]. Gametes were collected from each urchin in separate labelled beakers. Ova were washed three times and strained to remove the jelly coat. Egg density was obtained by counting the ova in 1 mL of egg suspension. The sperm suspension was diluted 1:50 with FSW. The gametes were set aside and maintained at room temperature.

Experimental treatment. A 1000 ppm stock solution was prepared by dissolving 250 mg of each fraction with 250 mL of 80% dimethyl sulfoxide (DMSO). The screening concentration for all test fractions was 150 ppm, prepared by taking a 4.5 mL aliquot of stock solution and diluting it with 30 mL FSW. Following screening, the most bioactive fraction was tested at various concentrations,

wherein aliquots of 0.03, 0.30, 0.75, 1.50, 3.0, and 4.50 mL of stock metabolite solution were diluted to 30 mL FSW to make 1, 10, 25, 50, 100, and 150 ppm solutions, respectively. The negative control was FSW, while the positive control was 80% DMSO.

Fertilized eggs were prepared by adding 2 mL of diluted sperm to 200 mL of egg suspension. Immediately after fertilization, 5×10^4 embryos per milliliter solution were stocked on each replicate. Each treatment was then replicated five times, each receiving 30 mL test solution.

Microscopic observation. Embryos were observed at 100 and 400× magnifications. Untreated embryos at different developmental stages (early cleavage, blastula, and free-swimming hatching) were cultured in beakers with a motor driven paddle (60 rpm) to ensure good aeration. Healthy embryos at early cleavage were exposed to the test fractions and monitored every 30 min until one of the test replicates reached the 16-cell stage.

Cell counts at early cleavage were determined after the embryos were fixed with 1% formaldehyde. Embryos at blastula were observed until more than 50% of the population hatched in one of the test replicates. During the onset of free-swimming, several batches of the larvae were incubated with the fractions and observed after 1 h exposure.

Anomalous morphological development during cleavage and free-swimming stages were recorded following the specified defect categories (Table 1).

For the morphological examination, the embryos and larvae were not chemically fixed to avoid visualization of artefacts. A total of 50 embryos and larvae per replicate were examined. A dye exclusion test using erythosin B was carried out to determine the number of dead embryos and larvae at each test. Apoptotic anomalies were indicated by embryonic blebbing.

Statistical treatment. To determine significant differences in the rate of cell division during cleavage, one-way analysis of variance (ANOVA) was used. To conform to the normality and homogeneity of variances assumptions of ANOVA, the data set was transformed to percentages. To determine where the significant difference lies in terms of the proportion of cells in the embryos

Table 1. Defect categories for evaluating anomalous development from early cleavage to hatching.

Developmental Stage	Anomalies
A. Cleavage	
1. Early cleavage (from 1-cell to 16-cell stage)	I – Dead II – Abnormal cleavage III – Normal
2. Late cleavage/blastula (until >50% hatch)	I – Dead II – Anomalous blastula III – Normal blastula IV – Anomalous and free-swimming V – Normal and free-swimming
B. Free-swimming (After 1 h incubation)	I – Dead II – Living but immobile III – Living, mobile, but lacks forward movement IV – Living, mobile, but with slow forward movement V – Living, mobile, and with fast forward movement

exposed to the fractions and controls, Tukey HSD (Honest Significant Difference) Test was undertaken. For all the inferential tests, the level of significance (α) was set at 0.05 and was analyzed using STATISTICA® (Student Edition) software.

The specified defect category data gathered from embryos treated at early cleavage, blastula, and free-swimming hatching were presented in percentages.

Screening for phytochemicals. Phytochemicals in all fractions were categorized following standard protocols [6, 7].

Results

Effects of* B. oligospora *metabolite fractions at 150 ppm on embryogenesis. During early cleavage, the rate of cell division was followed. After 60 min incubation, 95–99% of the 250 embryos in the treatment and control progressed to 2-cell stage except for the chloroform fraction where 71% remained at the 1-cell stage and only 29% reached the 2-cell stage (Fig. 2A). The difference between the chloroform fraction and the other treatments during the first division was statistically significant ($p = 2.16\times10^{-39}$).

After 90 min exposure, development in the chloroform fraction continued to lag. Sixteen percent remained at the 1-cell stage and 84% of embryos reached the 2-cell stage. In the other treatments, none remained in the 1-cell stage, 69–76% were at 2-cell, 20–26% at 4-cell, and 2–5% reached the 8-cell stage (Fig. 2B). No inhibition was evident in the controls. The difference elicited by the chloroform fraction at 8-cell stage was found to be statistically significant ($p = 0.004$).

After 120 min incubation, development of embryos treated with the chloroform fraction continued to lag and the ethyl acetate fraction showed delayed development from the 4- to 8-cell stage. In the chloroform fraction, 31% remained at the 2-cell stage and 69% of embryos progressed to 4-cell stage. In all other treatments, excluding ethyl acetate fraction, 20–22% were in the 2-cell stage, 48–50% at 4-cell, 28% at 8-cell, and 2–3% at 16-cell. In the ethyl acetate fraction, 19% were at the 2-cell stage, 69% at 4-cell, 8% at 8-cell, and none reached 16-cell (Fig. 2C). Consequently, the difference in the number of embryos that progressed to 16-cell stage among the controls, aqueous fraction, and hexane fraction revealed to be insignificant ($p = 0.723$).

Aside from monitoring the rate of cell division, the presence of cleavage anomalies (Fig. 4A-E) was also determined. After 120 min exposure, all embryos appeared normal in the controls (Fig. 3A-E), aqueous and hexane

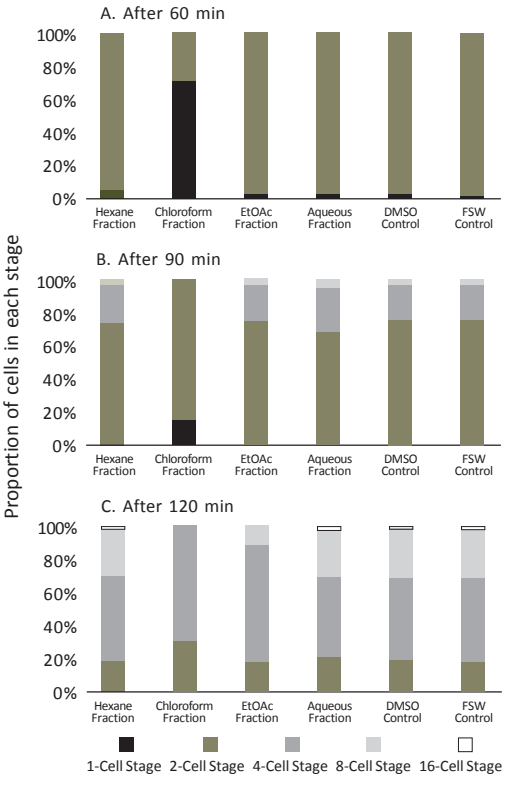

Figure 2. The rate of cell division after exposure of sea urchin embryos to different fractions of *B. oligospora* at 150 ppm

fractions. However, of the embryos exposed to the ethyl acetate fraction, 16.4% showed abnormal cleavage. Of those exposed to the chloroform fraction, 23.2% exhibited abnormal cleavage and 9.6% were dead as revealed by the dye exclusion test. Of the dead embryos, only a few exhibited blebbing (Fig. 4D).

Embryos at the blastula stage (8 h after fertilization) were also treated with the metabolites. After 4 h incubation, more than 50% of the embryos in the controls (Fig. 3F), hexane and aqueous fractions hatched and none, either hatched or unhatched, showed any anomalous development. In the ethyl acetate fraction, 45.6% hatched and 5.6% exhibited anomalous development. Of the remaining embryos at blastula, 36% were normal and 12.8% showed defects. All of the embryos treated with the chloroform fraction remained at the blastula stage and of these, 35.6% were normal and 64.4% showed deformities (Fig. 4E).

On the other hand, free-swimming larvae (12 h after fertilization) were also exposed to the metabolites. After 1 h incubation, all larvae showed normal, active swimming behavior (Fig. 3G) except those exposed to the chloroform fraction where embryos moved much slower (Fig. 4F).

Effects of varying concentrations (1–150 ppm) of chloroform fraction on embryogenesis. Being the most bioactive fraction, the chloroform concentrate was further tested to determine its effect to the development of sea urchin embryos and larvae.

After 60 min incubation (Fig. 5A), 98–99% of control embryos reached the 2-cell stage. Of the embryos exposed to 150 ppm of chloroform fraction, 25% were at 2-cell and 75% remained at 1-cell. Of the embryos exposed to 25, 50, and 100 ppm, only 33–47% reached the 2-cell stage. The rest of the embryos did not divide. More than 50% of the embryos in 1 and 10 ppm test concentrations reached 2-cell (Fig. 5A). The differences in all concentrations were statistically significant compared to the controls ($p = 0.00$).

After 90 min incubation (Fig. 5B), the embryos in the controls underwent second, third, and fourth divisions. At 150 ppm of treatment, 82% of embryos progressed to 2-cell stage and 18% remained at 1-cell. In all other test concentrations excluding 1 ppm, 3–15% reached the 4-cell stage while the rest of the embryos continued to lag at 1-cell and 2-cell. As in the controls, the embryos treated with the 1 ppm test concentration divided as far as 8-cell stage. The difference at 8-cell stage

Figure 3. Normal embryonic development of *T. gratilla*: 1-cell stage (A), 2-cell stage (B), 4-cell stage (C), 8-cell stage (D), 16-cell stage (E), blastula (F), and free-swimming hatching (G).

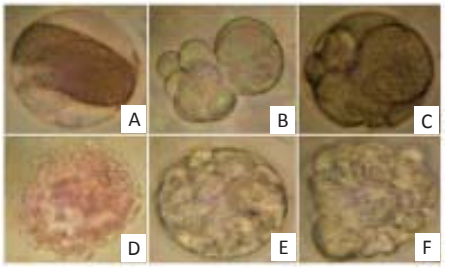

Figure 4. Anomalous development of *T. gratilla* embryos treated with 150 ppm *B. oligospora* chloroform fraction: Deformed morphology (A-C) and apoptotic blebbing (D) at early cleavage; and malformation with no or less defined coelom at blastula (E) and at free-swimming hatching (F).

between the controls and the chloroform fraction at 1ppm was found to be statistically significant ($p = 0.002$).

After 120 min exposure (Fig. 5C), development in the controls continued to progress as far as the 16-cell stage. Of the embryos exposed to 150 ppm, 68% reached the 4-cell stage and 32% were at 2-cell. More than 70% of the embryos in 50 and 100 ppm test concentrations reached the 4-cell stage while others remained at 2-cell. All embryos exposed to 10 and 25 ppm divided up to 8-cell stage. Chloroform fraction at 1 ppm caused 1% of embryos to divide to 16-cell stage. However, the difference at 16-cell stage between the controls and the chloroform fraction at 1 ppm showed to be statistically significant ($p = 0.02$).

As in the first experiment, embryos exposed to chloroform fraction consistently showed anomalies at cleavage, blastula, and free-swimming hatching. The effect was concentration-dependent. Cell death, with few blebbing, occurred from 50 to 150 ppm and abnormal cleavage from 10 to 150 ppm. Both anomalies were highest at 150 ppm. No morphological defects were evident at 1 ppm. More than 60% of the embryos exposed to the rest of the concentrations of the fraction appeared normal. All cleaving embryos remained unaffected in the controls.

Furthermore, embryos at the blastula stage (8 h after fertilization) were treated with the metabolites. After 4 h incubation, less than 50% of the embryos in the 1ppm to 50 ppm test concentrations reached free-swimming. Most were at blastula showing malformations. As the concentration was increased to 150 ppm by 50 ppm interval, the development of the embryos was completely retarded at the blastula stage where more than 50% exhibited defective morphology. As in the first experiment, no anomalies or malformations were observed in the embryos exposed to the controls.

Lastly, the free-swimming larvae (12 h after fertilization) were also treated with the chloroform fraction. After 1 h incubation, the active swimming pattern of the larvae in the 1–25 ppm test concentrations remained unaltered as in the controls. Meanwhile, at 50–150 ppm, most of the larvae exhibited slow motility, while a few showed abnormal morphology. The effect of the metabolites in the swimming behavior of the larvae appeared to be dose-dependent, being greatest at 150 ppm.

Phytochemical screening revealed the presence of terpenoids in the hexane, chloroform, and aqueous fractions. On the other hand, the ethyl acetate fraction tested positive for saponins.

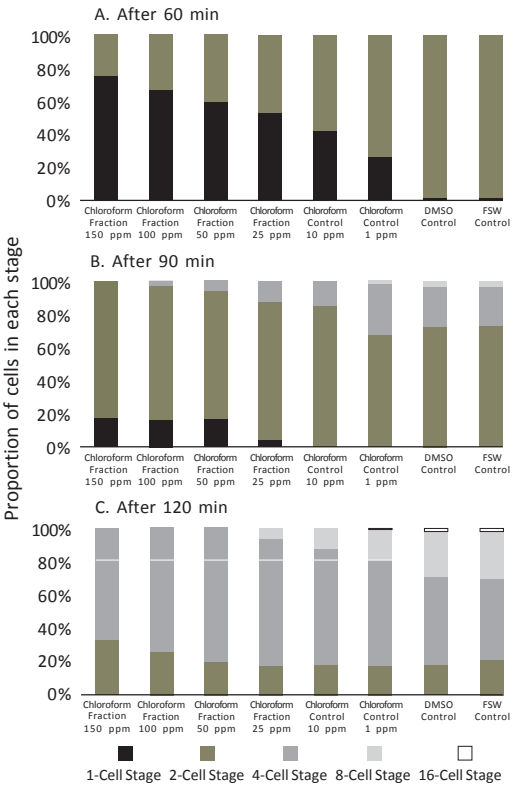

Figure 5. The rate of cell division after exposure of sea urchin embryos to chloroform fraction at different concentrations (1–150 ppm)

Discussion

The adverse effects induced by the chloroform fraction were concentration-dependent. During early cleavage, the fraction inhibited the rate of cell division and triggered anomalous morphology with less apoptosis indicated by blebbing of the cells. Furthermore, the development of embryos at blastula was completely halted by the 100 and 150 ppm concentrations without causing apoptosis. Most of the blastulae did not reach the free-swimming larval stage upon exposure to the other concentrations. Blastulae exposed to the test solutions below 100 ppm showed malformations. All treated larvae showed slow swimming behavior in contrast to their normal, active motility with no apoptotic anomalies.

Based from the results of the study, it is evident that the metabolites present in the chloroform fraction altered normal cytological structures and processes that eventually led to the observed malformations on sea urchin embryos. Clearly, the metabolites impaired normal ciliary beating of hatched embryos resulting to slower locomotion.

Previous study [8] revealed that the cilia in free-swimming larvae are stabilized by the components of the cytoskeleton, particularly the microtubules. Hence, it could be possible that slow ciliary beating was caused by the activity of the metabolites that affected the stability of these structures since the success of the sea urchin early embryogenesis requires proper functioning of the cytoskeleton, most importantly the microtubules.

At cleavage, microtubule-containing spindle fibers regulate mitosis by controlling the movement and segregation of chromosomes [9]. Consequently, with metabolites attacking the microtubules in the spindle fibers, mitotic inhibition and eventually cell death may occur in the embryos.

Likewise, during blastulation, ectodermal cells assemble microtubule-containing cilia. This event is coupled by the increasing rate of synthesis of tubulin subunits which form the microtubules. The cilia then become functional at free-swimming and start to beat rhythmically [8]. Active metabolites might have altered these microtubule-mediated functions causing defects during blastulation and during the free-swimming larval stage. The chemotherapeutic drugs vinblastine, colchicine, nocodazole, and cytochalasin were found to elicit anticancer property by targeting the stability of the cytoskeleton on sea urchin embryos [10].

Analysis for possible phytochemicals revealed the presence of terpenoids in the chloroform, hexane, and aqueous fractions. This suggests that the bioactive chemical found in the chloroform fraction must be terpenoids. Terpenoids are secondary metabolites made up of isoprene units [11]. They have been reported to exhibit anticancer, antimalarial, anti-ulcer, and antimicrobial properties [12].

Many marine organisms are known to synthesize terpenoids. The soft coral genus *Sinularia* was reported to produce africanene, a tricyclic sesquiterpene compound. Clinically, this was demonstrated to possess anti-inflammatory activity against carrageenan-induced rat edema and cytotoxic properties against Ehrlich ascites carcinoma and Dalton's lymphoma ascites tumor cell lines [13].

The red alga *Portieria hornemanii* synthesizes the pentahalogenated monoterpene halomon that was demonstrated *in vitro* to have high cytotoxicity profile against brain, renal, and colon tumor cell lines and was even reconsidered by the U.S. National Cancer Institute for preclinical drug development [14].

The same secondary metabolites were also found in the aqueous and hexane fractions of

B. oligospora but showed no developmental targeting effects on sea urchin embryos. This indicates that, although the compounds in these fractions belong to one class, their individual chemical structure varies rendering different biological properties for each terpenoid compound in the fractions. Furthermore, the performed test might have been unsuitable for the metabolites extracted by hexane and water and should therefore be screened for more bioassays that may help unravel any bioactivity of these compounds.

Saponins, on the other hand, were detected in the ethyl acetate fraction. Saponins are steroid or triterpenoid glycosides that are able to form stable, soap-like foams in aqueous solutions. Recent research showed that some saponins have anticarcinogenic, antioxidant, hypocholesterolaemic, and antiviral properties [15]. The ethyl acetate fraction inhibited cell division slightly but the result was not compelling enough for it to be tested intensively on sea urchin embryos. However, the metabolites extracted by ethyl acetate are still worth investigating using other bioassays because, like terpenoids, saponins in the fraction could be a good source of natural products with a wide range of pharmacological activities.

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