



Selective cytotoxicity of bioluminescent bacteria isolated from fresh ink of Philippine squid on human colon cancer and normal cell lines

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ABSTRACT

Aims: The current work determined the cytotoxicity of the symbiotic bioluminescent bacteria isolated from fresh ink of the squid, *Photololigo duvaucelii* for human colon cancer cell lines HT-29 and HCT116 and normal human dermal fibroblasts.

Methodology and results: The crude sterile culture supernatants of the bacterial isolates grown in marine broth containing 2.8% of NaCl were tested for their cytotoxic activities for HT-29 and HCT-116 human colon cancer cells and the normal human dermal fibroblasts HDFn using Presto Blue™ Resazurin Assay. Zeocin served as the positive control. The cytotoxicity index profiles of all culture supernatants and negative control (marine broth with 2.8% NaCl) for HDFn suggest non-toxicity to the cells, whereas most culture supernatants were observed to be cytotoxic to the two colon cancer cell lines. The culture supernatants of the isolates were found to be more cytotoxic for the HT-29 colon cancer cells than to the HCT-116 colon cancer cells. At the same time, the IC₅₀ values showed that 85% (17/20) and 40% (8/20) of the culture supernatants tested were significantly lower, hence more potent than zeocin for HT-29 and HCT-116, respectively ($p < 0.05$). The rest were equally potent ($p > 0.05$). The 16S rRNA gene sequence analysis of the bioluminescent isolates studied revealed that they have a 97-99% similarity identity with *Photobacterium leiognathi*.

Conclusion, significance and impact of study: This may be the first report on the cytotoxic activities on cancer cells of *P. leiognathi* from the Philippine squid and suggests the potential use of the secondary metabolites of these bioluminescent bacteria as anti-cancer agents.

Keywords: Bioluminescent bacteria, cytotoxicity, secondary metabolites, squid ink, cancer

INTRODUCTION

Colon cancer is reported to be among the top causes of cancer-related deaths in many countries. Statistics from the American Cancer Society (2016) showed that in the United States alone, colon cancer ranks 3rd in both men and women. Among Asian countries, it is considered the fourth most common cancer in men, with the highest incidence of age-standardized incidence rates for cancer in the proximal and distal colon and rectum (Park *et al.*, 2016).

The incidence and mortality due to cancer are likely to increase worldwide due to lifestyle, nutrition, pollution, and environmental changes such as global warming among other factors. Moreover, reports show that the currently available drugs for cancer treatments are not specific for the cancer cells, resulting in toxicity to normal cells at the same time. According to the World Health

Organization (WHO), 80% of the population from the developing countries have been dependent on medicinal plants in treating many diseases including cancer (Boopathy and Kathiresan, 2010).

Currently, the diverse marine organisms and their associated microorganisms, such as bacteria and fungi, are being targeted as potential sources of naturally derived compounds. These organisms have been found to contain medically important polyphenols, sulphated polysaccharides and other chemicals, which are reported to have antioxidant, antitumor and immuno-stimulatory activities. Despite this, the marine environment is still unexplored for anticancer substances (Boopathy and Kathiresan, 2010).

Among the marine organisms is the bioluminescent squid belonging to the genus *Photololigo*. Unlike the usual bioluminescent organisms, its bioluminescence is symbiotically associated with bioluminescent bacteria that

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are resident flora of the squid's photophores (light organ). Generally, bioluminescent organism can produce its own cold light through chemical reaction with the help of an enzyme and proteins in the presence of oxygen (Haddock *et al.*, 2010). In the sea, bioluminescence serves many functions such as a source of light in the deep waters, as the predominant form of communication among species for attracting prey or mates, and as protection from predators in the form of counter-illumination (Altun *et al.*, 2008; Haddock *et al.*, 2010).

Cephalopods, which include squids, are well-known among the bioluminescent marine molluscs. Herring in 1977 was able to identify at least 70 luminous genera of squids (Haddock *et al.*, 2010) wherein bacterial symbionts produce luminescence for families Sepiolidae and Loliginidae (Ruby and McFall-Ngai, 1992). Other squids have exhibited intrinsic bioluminescence using luciferin-luciferase interaction, creating a variety of luminescence displays. Some use their ventral photophores for counter-illumination while others, specifically *Taningia danae*, has its light organs at its arm tips for intraspecific communication as well as to stun its potential prey (Haddock *et al.*, 2010). Bacterial colonization in squid has been investigated in detail, particularly for *Euprymna scolopes*, which harbors *Allivibrio fisheri*, another bioluminescent bacterium, as its symbiont. Apparently, the squid acquired its symbiont bioluminescent bacteria shortly after hatching (Dunlap *et al.*, 2008).

At present, the production of important secondary metabolites by the associated microorganisms in marine invertebrates has been recognized due to their significant biological activities including antibacterial and anticancer (Berrue *et al.*, 2011; Mansson *et al.*, 2011).

In the Philippines, recent studies report the presence of bioluminescent bacterial symbiont in the local marine fishes, squids and octopus (Naguit *et al.*, 2014; Arante *et al.*, 2015). Furthermore, the discovery of the anticancer compounds from Philippine marine organisms focused primarily on the ascidians and sponges with their associated microorganisms (Concepcion *et al.*, 2014).

In support to these findings, the present study isolated and identified bioluminescent bacteria from the squid *Photololigo duvaucelii* d'Orbigny, 1835 caught locally in the Philippine seas, and determined the cytotoxicity of their secondary metabolites on human colon cancer cell lines HT-29 and HCT116.

MATERIALS AND METHODS

Isolation of bioluminescent bacteria from the squid ink

Fresh squid belonging to genus *Photololigo* (Figure 1) were obtained from a wet market in Pritil, Tondo, Manila, Philippines, and immediately transported in an ice chest to the laboratory for processing. The squids were washed with sterile distilled water to remove excess ink. Ink was inoculated unto marine agar plates (MAP), which was composed of 28.13 g NaCl, 3.5 g MgSO₄·7H₂O, 2.55 g MgCl₂, 1.2 g CaCl₂, 0.77 g KCl, 0.11 g NaHCO₃, 13 g

nutrient broth and 15 g agar per liter of distilled water (Atlas, 2004). The inoculated plates were incubated at room temperature (25-27 °C) for 18-24 h. Twenty well-isolated glowing colonies from the five squids sampled were included in the study. Figure 2 shows the blue-green light emitted by the bioluminescent bacteria isolated from squid ink, digitally photographed in the dark room using a 16 Megapixel Samsung S6 SM-G9287C set in professional mode.

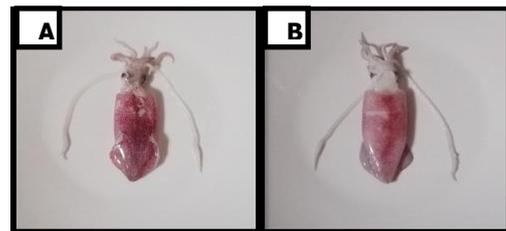


Figure 1: Dorsal (A) and ventral (B) view of the local squid specimen identified as *Photololigo duvaucelii* d'Orbigny, 1835 from which the bioluminescent bacteria were isolated.

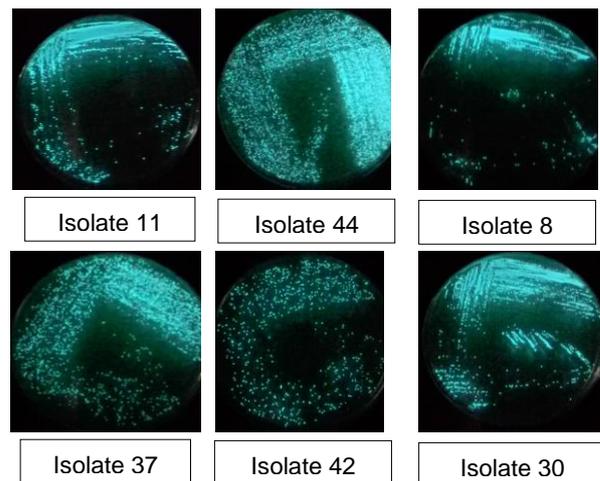


Figure 2: Colonies of bioluminescent bacterial isolates from the squid ink grown on marine agar plates (with 2.8% NaCl) at 25-27 °C for 18-24 h, identified as *Photobacterium leiognathi* using 16S rRNA gene sequences and digitally photographed by the continuous light they produced in the dark room.

The identification of the squid specimen was authenticated by the National Museum, Philippines as *P. duvaucelii* d'Orbigny, 1835 (Cephalopoda, Loliginidae) with Control #: CONCHO 2016-52. The squid was placed in 70% ethanol prior to identification.

Extraction of genomic DNA of the bioluminescent bacteria

The total genomic DNA from the bioluminescent bacterial cells was extracted using an InstaGene Matrix (Bio-Rad)

DNA Extraction Kit following the manufacturer's protocol. Briefly, an isolated bacterial colony was suspended in 1 mL of autoclaved water and centrifuged at 13,000 rpm for 2 min. The pellet was suspended in 100 μ L of InstaGene Matrix, incubated at 56 °C for 30 min, vortexed at high speed for 10 sec, then placed in a 100 °C boiling water bath for 8 min. The mixture was centrifuged at 13,000 rpm for 3 min and the supernatant served as the source of genomic DNA.

Amplification of the 16S rRNA gene and molecular identification of bioluminescent bacterial isolates

The PCR mixture was comprised of 1 \times PCR buffer, 2.5 mM MgCl₂, 10 μ M dNTP, 10 μ M forward primers 16S, 10 μ M reverse primers 16S, 5 u/L *Taq* polymerase and sterile distilled water with 1 μ L template DNA. The sequences of the universal 16S rDNA primers were: 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Lin *et al.*, 2005).

Amplification of the gene was carried out under the following conditions: Initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Final elongation was at 72 °C for 1 min. The approximately 1.4 kb amplicons were viewed after agarose gel electrophoresis (Figure 3) and sent to 1st Base, Malaysia for sequencing. Chromas Lite version 2.1.1 software was used for the chromatogram editing of the resulting sequences. The results were submitted to the National Center for Biotechnology Information (NCBI) for Basic Local Alignment Tool (BLAST) analysis for comparison of the resulting gene sequences to the known sequences in the GeneBank nucleotide database. The species level was identified as the nearest phylogenetic neighbor with sequences 97-99% sequence similarity (Lin *et al.*, 2005).

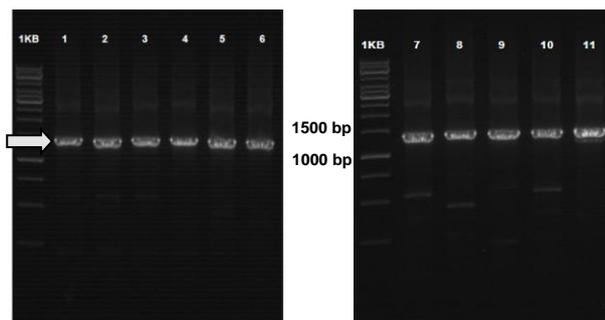


Figure 3: Agarose gel electrophoresis results showing 16S rRNA gene amplicons of approximately 1400 bp from bioluminescent bacterial isolates from squid ink (indicated by the red arrow). 1 kb DNA ladder (bp: 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000; Lanes 1 to 11: Isolates 8, 12, 11, 16, 18, 22, 24, 39, 37, 27 and 3).

Phylogenetic analysis of bioluminescent bacterial isolates

The phylogenetic tree of the 11 bioluminescent bacterial isolates was established using the Molecular Evolutionary Genetics Analysis version 7 software (MEGA7) (Kumar *et al.*, 2015). Following the previous study, the 16S rRNA gene sequences of known species of bioluminescent bacteria were downloaded from NCBI database with the following accession numbers: AB243250.1, AF003548.1, AF493804.1, AJ746357.1, AY455872.1, FJ240416.1, and X74685.1 (Naguít *et al.*, 2014). All of these were aligned with the 16S rRNA gene sequences of the isolates of this study using ClustalX2. The evolutionary history was inferred using the Neighbor-joining method (Saitou and Nei, 1987), while the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). Bootstrap values were conducted at 1000 replicates displaying only branches with > 50% bootstrap support values. The tree was rooted with *Photorhabdus* sp. (AY278667.1), a terrestrial bioluminescent bacterium (Naguít *et al.*, 2014).

Figure 4 shows that the bioluminescent bacterial isolates from the squid host clustered with representative members of Vibrionaceae family including *Photobacterium* and *Vibrio*. Moreover, the results showed that the isolates have closer evolutionary relationship with *P. leiognathi* (Isolates 3, 11, 8, 18 and 39) and *Photobacterium mandapamensis* (Isolates 12, 24, 27, 16, 22 and 37). *Photobacterium mandapamensis* is closely related phenotypically to *P. leiognathi*, but they can be distinguished phylogenetically by their luminescence genes (*luxCDAB(F)E*): *luxF* is absent in *P. leiognathi*, while it is present in *P. mandapamensis* (Kaeding *et al.*, 2007).

Preparation of sterile culture supernatant of bioluminescent bacteria from squid for cell viability assay

The bioluminescent bacterial isolates were grown in 5 mL marine broth (MB) for 24 h at room temperature of 25-27 °C. The turbidity of the resulting bacterial cultures was adjusted to equal that of 0.5 MacFarland corresponding to 1.5 \times 10⁸ cells/mL. The bacterial cultures were centrifuged at 13,000 rpm for 3 min, and the supernatant was filter-sterilized using a 0.2 μ m syringe filter. The pHs of each supernatant and control MB were determined to ensure that this will not be a factor for cytotoxicity to the test cell lines.

Culture of cell lines

The human colon cancer cell lines HT-29 and HCT-116 were kindly provided by the Molecular Science Unit Laboratory (MSUL) of the Center for Natural Science and Environmental Research (CENSER) of De La Salle University. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA) and 1 \times anti-mycotic

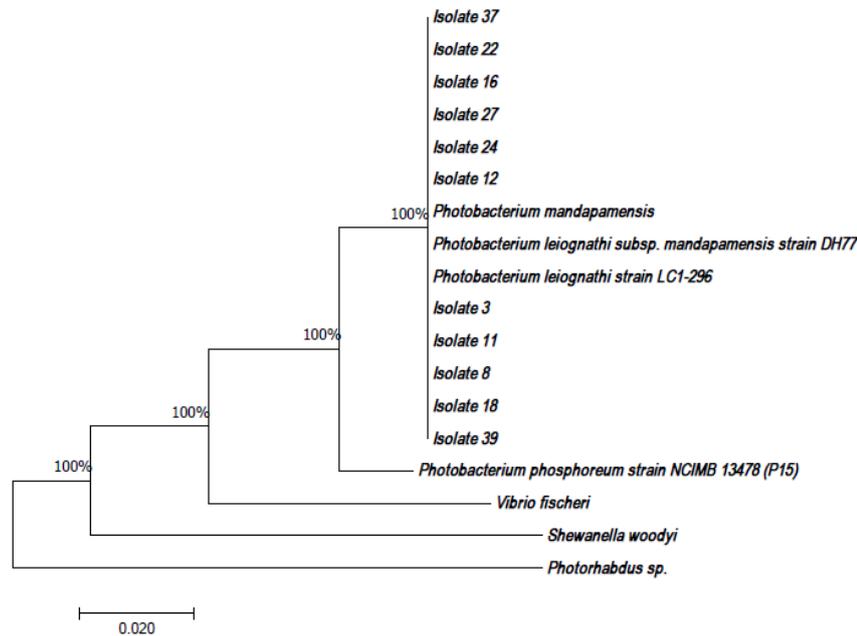


Figure 4: Phylogenetic tree of the 11 bioluminescent bacterial isolates isolated from fresh ink of Philippine squid samples (*Photololigo duvaucelii* d' Orbigny, 1835) based on ~1,400 bp 16S rRNA gene sequence and representative sequences from Genbank. Neighbor-Joining was employed using MEGA 7. Bootstrap values are based on 1000 replicates displaying only bootstrap values support of > 50. Following Naguit *et al.* (2014), the tree was rooted using *Photorhabdus* sp. (AY278667.1). The bar represents substitution per site.

antibiotic. The culture flasks were incubated at 37 °C with 5% CO₂ and 95% humidity.

Cell viability assay

Cell viability assay was conducted following the method of Shyu *et al.* (2014). All cell lines were cultured to 90% confluence, harvested and separately seeded in 100 µL volume per well in a 96-well culture plate. Each 100 µL inoculum had a density of 2.4×10^5 viable cells/mL as determined using the Trypan Blue Exclusion method. The plates were incubated for 6 days under the aforementioned conditions for cell lines. After attachment of cells to the bottom of wells as monolayers, 100 µL of twofold serial dilutions of the prepared filter-sterilized culture supernatants and the positive control zeocin were added into each well. DMEM broth with 10% FBS and MB served as the negative controls. Each treatment was done in three replicates and the plates were incubated for 72 h (Vijayarathna and Sasidharan, 2012) under the same conditions.

After incubation, 10 µL of Presto Blue (Invitrogen, USA) were added to each well, and the plates were further incubated for 4 h under the same conditions. A microplate reader (Biotek ELx800, Bio Tek Instruments, USA) was used for colorimetric detection of the reduction of resazurin to resorufin at 570 nm. The resulting cytotoxicity index values (%) were plotted against the concentrations of the sterile culture supernatants to obtain

the corresponding linear equations for the calculation of the concentration that was cytotoxic to 50% of the cells or IC₅₀.

Statistical analysis

The presence of significant differences between treatments was determined using the students' paired t-test. A $p < 0.05$ was designated as the reference value to indicate statistical difference between treatment results.

RESULTS AND DISCUSSION

Bioluminescent bacterial colonies emitting a continuous cold, blue-green light were isolated on marine agar plates from squid ink. The 16S rRNA gene sequences of the 11 isolates (Isolates 3, 8, 11, 12, 16, 18, 22, 24, 27, 37, 39) assayed showed that these are *P. leiognathi* isolates, with e values of 0.0 and 97-99% similarities in their identities when submitted for BLAST analysis (Table 1). The results are similar to those of Naguit *et al.* (2014) who reported the occurrence of *P. leiognathi* in the squid and octopus specimens procured from a local fish market in the Philippines. This occurrence is part of the evolutionary strategy employed by at least 35 species of squid distributed worldwide (Ruby and McFall-Ngai, 1992). This is not surprising because the Philippine seas, being a tropical coastal water, could favour the survival of *Photobacterium* species, specifically *P. leiognathi* where it

is recorded to thrive more commonly in coastal and warmer waters, and usually in bioluminescent symbiosis with certain shallow-dwelling fish and squid (Urbanczyk *et al.*, 2011).

Photobacterium leiognathi is a Gram-negative marine bacterium under Vibrionaceae (Proteobacteria, Gammaproteobacteria) family. As previously reported, it is a mesophile thriving in warmer waters and symbiotically linked with bioluminescent marine fish and squid. Fukusawa and Dunlap (1986) and Nirmale *et al.* (2002) both recognized that only one type of luminescent bacterium, namely *P. leiognathi*, inhabits the light organ of *L. duvauceli* (Indian squid) and *Doryteuthis kensaki*. In addition, *P. leiognathi* isolated from Indian squid is distinguished to be associated with its ink sac.

Environmental congruence, notably temperature, takes part in the dispersal as well as in the continued existence of the genus *Photobacterium* in the ocean. Among the three closely related species, it was the mesophilic *P. leiognathi* or the *P. mandapamensis*, as light-organ symbionts of bacterially luminous fish such as ponyfish (leiognathids) that was documented to flourish widely in warmer coastal waters, while *Photobacterium kishitanii* was found to be subsisted in the deeper and colder waters. The ponyfish (*Secutor megalolepis*: Leiognathidae) caught from Iloilo, Philippines was identified as a host of *P. leiognathi* (Urbanczyk *et al.*, 2011). The Philippines, being tropical coastal water may have favoured the bioluminescent symbiosis of *P. leiognathi* with its squid host *P. duvaucelii* (d'Orbigny, 1835) identified in this study.

Table 1: Identities of bioluminescent bacteria isolated from squid ink based on 16S rRNA gene sequences.

| Isolate # | Identity (Similarity of 100%) | E value | Accession | The most related species |
|-----------|-------------------------------|---------|------------|------------------------------------|
| 8 | 97% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 12 | 98% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 11 | 98% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 16 | 98% | 0.0 | FJ240423.1 | <i>P. leiognathi</i> strain DH162 |
| 18 | 99% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 22 | 99% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 24 | 99% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 39 | 99% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 37 | 99% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 27 | 98% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |
| 3 | 99% | 0.0 | HM008703.1 | <i>P. leiognathi</i> strain NB0902 |

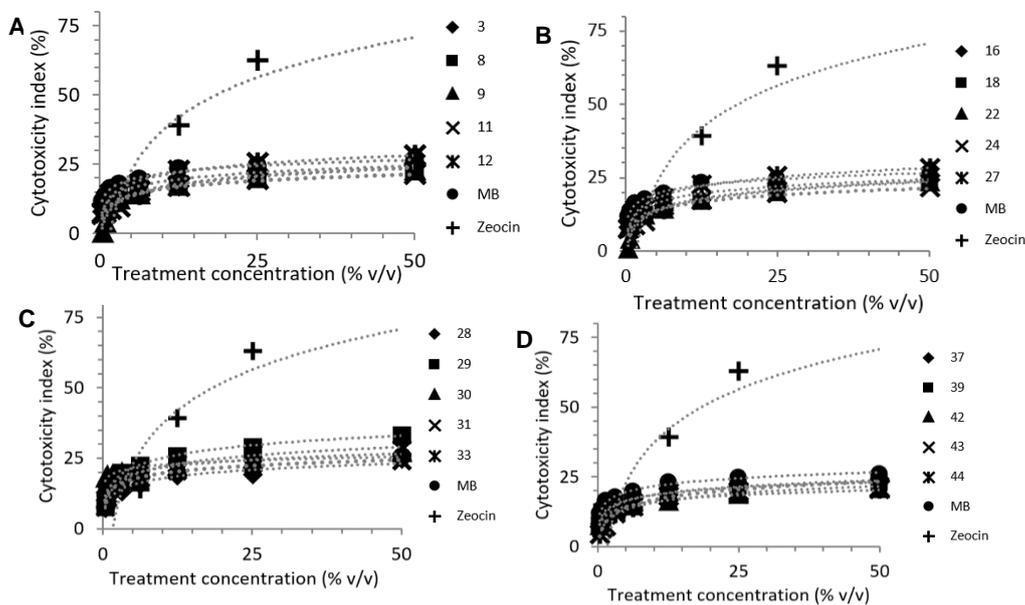


Figure 5: Cytotoxicity index (CI%) plots for HDFn cells after 72-hour exposure to the sterile culture supernatants of the 20 bioluminescent bacterial isolates grown in marine broth medium (2.8% NaCl). All supernatants, together with the marine broth (MB) as negative control were nontoxic to HDFn as shown by the absence of IC₅₀, in contrast to the positive control zeocin.

Cytotoxicity assay

The sterile culture supernatants of 20 different bioluminescent bacteria from ink of squid were tested for their cytotoxicity to colon cancer cell lines HT-29 and HCT-116 and to the normal human fibroblast HDFn. Figure 5 shows that all of the supernatants were not toxic to the normal human fibroblasts (HDFn), as the highest concentrations tested had cytotoxicity indices of less than the IC₅₀, in contrast to the positive control zeocin (Chicca *et al.*, 2008).

On the other hand, all of the 20 bacterial isolates had cytotoxic activities for both the colon cancer cell lines. All

were cytotoxic to HT-29 and HCT-116, as suggested by their IC₅₀ values. However, between the two cell lines, the culture supernatants of the isolates were found to be more cytotoxic for the HT-29 colon cancer cells than to the HCT-116 colon cancer cells. At the same time, the IC₅₀ values showed that 85% (17/20) and 40% (8/20) of the culture supernatants tested were significantly lower, hence more potent, than zeocin for HT-29 and HCT-116, respectively ($p < 0.05$) (Figure 6 to Figure 7; Table 2). Table 2 shows the computed IC₅₀ mean values of the isolates for the colon cancer cell lines HT29 and HCT-116.

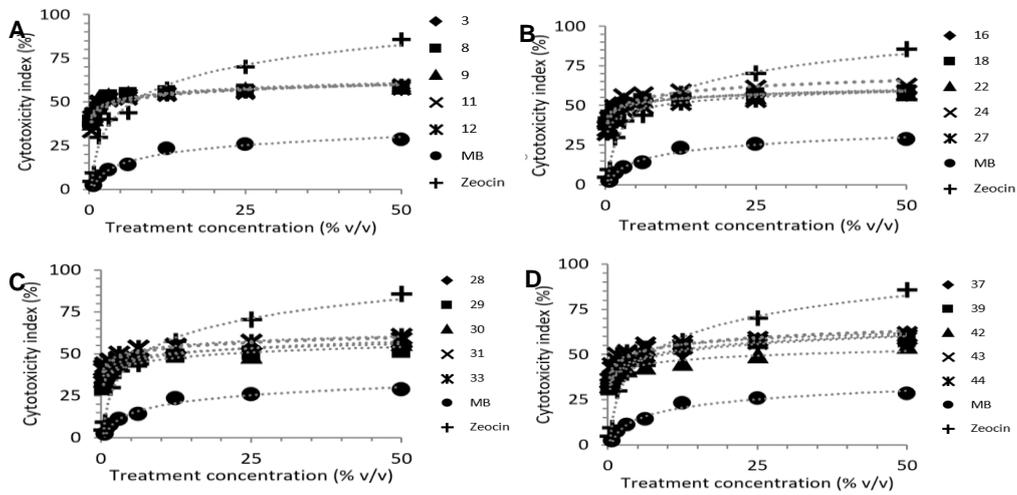


Figure 6A-D: Cytotoxicity index (CI%) plots for human colon cancer cells HCT-116 after 72-hour exposure to the culture supernatants of 20 luminous bacterial isolates grown in marine broth. (+) control: Zeocin; (-) control: marine broth medium with 2.8% NaCl (MB).

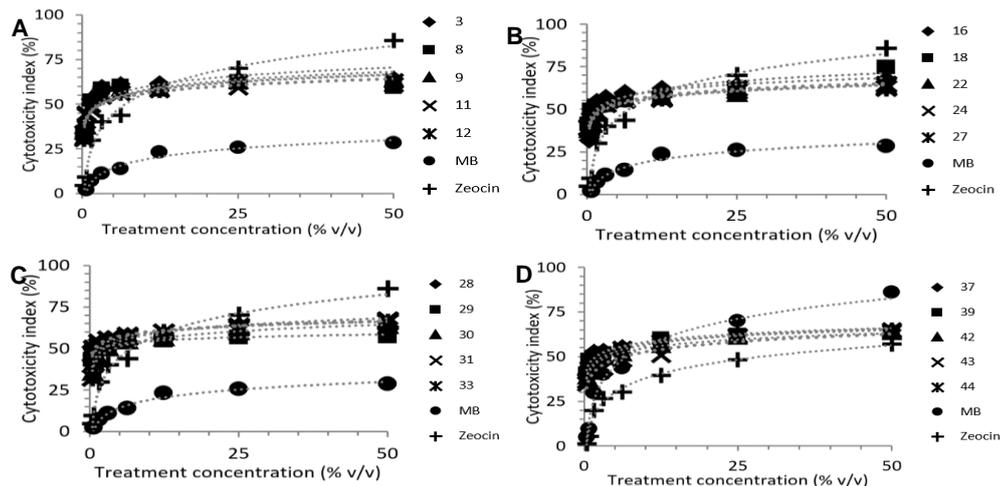


Figure 7A-D: Cytotoxicity index (CI%) plots for HT-29 cells after 72-hour exposure to the culture supernatants of 20 luminous bacterial isolates grown in marine broth. (+) control: Zeocin; (-) control: marine broth medium with 2.8% NaCl (MB).

Table 2: The mean IC₅₀ values of the sterile culture supernatants of 20 bioluminescent bacteria from squid ink and zeocin for colon cancer cell lines HCT-116 and HT-29.

| Isolate # | HCT-116 IC ₅₀ (% v/v) | HT-29 IC ₅₀ (% v/v) |
|-----------|----------------------------------|--------------------------------|
| 3 | 11.64 ± 3.46* | 2.77 ± 0.82* |
| 8 | 10.32 ± 0.47* | 4.99 ± 5.06* |
| 9 | 9.11 ± 1.76* | 6.36 ± 2.96* |
| 11 | 14.50 ± 2.57 | 7.49 ± 0.50† |
| 12 | 8.34 ± 6.07 | 6.86 ± 1.57† |
| 16 | 6.36 ± 6.65* | 2.15 ± 1.81† |
| 18 | 12.53 ± 4.50 | 2.03 ± 0.55† |
| 22 | 9.22 ± 0.89* | 8.54 ± 4.00† |
| 24 | 6.12 ± 7.75 | 5.94 ± 0.85† |
| 27 | 21.64 ± 0.96* | 7.02 ± 0.74† |
| 28 | 26.04 ± 2.93* | 5.23 ± 0.39† |
| 29 | 34.00 ± 2.30* | 4.52 ± 0.07† |
| 30 | 24.24 ± 6.56 | 16.67 ± 0.12† |
| 31 | 13.99 ± 1.36* | 13.82 ± 0.15 |
| 33 | 10.36 ± 3.28* | 6.11 ± 0.15† |
| 37 | 18.74 ± 3.31 | 1.25 ± 0.43† |
| 39 | 22.14 ± 0.36* | 2.46 ± 0.75 |
| 42 | 32.59 ± 6.04* | 12.76 ± 4.49* |
| 43 | 11.90 ± 3.30* | 16.67 ± 0.14 |
| 44 | 10.01 ± 7.29 | 8.07 ± 0.36† |
| Zeocin | 17.65 ± 0.27 | |

*Significant difference between the treatment and zeocin ($p < 0.05$).

It is noteworthy that the supernatants were more cytotoxic to the colon cancer cell line HT-29 than to HCT-116, with IC₅₀ values that ranged from 0.14% v/v to 16.67% v/v (Table 2). The IC₅₀ value of zeocin for HT-29 was 17.65% v/v. Statistical analysis showed that the sterile crude culture supernatants of 17 isolates (Isolates 3, 8, 9, 11, 12, 16, 18, 22, 24, 27, 28, 29, 30, 33, 37, 42 and 44) had significantly lower IC₅₀ values than zeocin ($p < 0.05$), indicating that these are more potent than the anticancer agent. The IC₅₀ values of the remaining 3 isolates were likewise lower than that of zeocin, but were not significantly different ($p > 0.05$), indicating equal potencies.

The IC₅₀ values of the culture supernatants for HCT-116 colon cancer cells ranged from 6.12% v/v to 34.59% v/v (Table 2). Eight (8) isolates (Isolates 3, 8, 9, 16, 22, 31, 33 and 43) had IC₅₀ values significantly lower than that of zeocin's 17.65% v/v ($p < 0.05$) indicating higher potency. On the other hand, isolates 27, 28, 29, 39 and 42 had significantly higher IC₅₀ values than zeocin ($p < 0.05$), showing lower activity than zeocin. The IC₅₀ of the remaining seven (7) isolates rest were not significantly different from that of zeocin ($p > 0.05$).

The results of this study significantly showed that in terms of calculated IC₅₀, the supernatants were more cytotoxic to HT-29 than to the HCT-116 cell lines. The difference between the responses of the two colorectal cancer cell lines might be attributed to their distinct characteristics. HCT-116 is known for its aggressiveness and is likewise non-differentiating, whereas HT-29 has an intermediate capacity to differentiate into enterocytes and mucin-expressing lineages (Yeung *et al.*, 2010). Sagar *et al.* (2013) showed that the specific activities of different

extracts for particular cell lines may be due to the presence of different polarity compounds and cancer type.

Overall, it was observed that bioluminescent marine bacterial supernatants exhibited strong cytotoxic effects against the human colon cancer cell lines. Similar findings were also observed in previous studies on the strong cytotoxic effects of marine bacterial metabolites (Lin *et al.*, 2005; Zheng *et al.*, 2005). This could be due to the more novel and unique structures of the secondary metabolites produced by marine microorganisms resulting to stronger bioactivities (Zheng *et al.*, 2005). Moreover, Lin *et al.* (2005) showed the associated marine bacteria isolated from seaweeds and invertebrates had higher percentage of cytotoxicity than bacteria extracted from seawater and sediment samples. Several authors have supported that the higher rate/percentage of associated bacteria with cytotoxic activities may be connected to the interaction between the host and its associated bacteria and vice versa, such as: 1) the control mechanism of the host organism to its associated bacteria is through the production of antibiotics (through hydroxyl radicals toxic to bacteria); or 2) the associated bacteria may eliminate or control its host organism by cell growth inhibition or direct attack (Lin *et al.*, 2005).

It is well-known that squid and its ink have antioxidant properties, and this could be attributed to the presence of bioactive molecules in the ink itself (Sasaki *et al.*, 1997), or from its symbiont bioluminescent bacteria such as *Photobacterium* species. The latter can produce bacteriocupreins, a specific kind of superoxide dismutase (SOD), and luciferin, a kind of protein important to the bioluminescent effect/reaction. These compounds both

have anticancer activities and cellular protection (Dunlap and Steinman, 1986; Boopathy and Kathiresan, 2010).

The present work is probably the first documentation on the cytotoxicity of the secondary metabolites of bioluminescent bacteria isolated from the Philippine squid. As of today, it is widely recognized that the mutualistic association of microorganisms with their marine invertebrate hosts have a distinctive role in the production of secondary metabolites. Similarly, this distinction could also provide a new substitute to the establishment of potential drugs isolated from marine invertebrates (Berrue *et al.*, 2011). In vibrios, the production of secondary metabolites has been linked to antagonism, intra-species communication and pathogenicity which produce compounds with a broad range of interesting biological activities such as anti-cancer and antibacterial among others (Mansson *et al.*, 2011).

CONCLUSION

The results of the study showed that the sterile crude culture supernatants of bioluminescent bacteria grown in marine broth with 2.8% NaCl isolated from the ink of the Philippine squid *P. duvaucelii* (d'Orbigny, 1835) were not cytotoxic to the normal human fibroblast (HDFn), but were significantly cytotoxic for both the cancer cell lines tested. These were more cytotoxic to HT-29 colon cancer cell lines than to HCT-116 based on their respective IC₅₀ values. At the same time, the IC₅₀ values showed that 85% (17/20) and 40% (8/20) of the culture supernatants tested were significantly lower, hence more potent, than zeocin against HT-29 and HCT-116, respectively ($p < 0.05$).

The results warrant more studies on these bioluminescent bacterial isolates from the Philippine squid such as the isolation and identification of novel active secondary metabolites with anticancer activities which may be more potent, economical and non-toxic for human use.

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