Black sea cucumber (*Holothuria atra* Jaeger, 1833) protects *Caenorhabditis elegans* against *Pseudomonas aeruginosa* infection

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- **Wan-Ting Lee: Conducted killing assays, anti-virulence and gene expression studies, draft of manuscript**
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Abstract:

Ethnopharmacological relevance: Sea cucumbers are known to have beneficial effects towards many ailments. Among traditional documented use of edible sea cucumbers are as anti-infective tonic food. Metabolites with immunomodulatory properties have also been reported from temperate sea cucumber species. The black sea cucumber (*Holothuria atra*) is a widely distributed tropical marine sea cucumber species traditionally consumed by Asian populations for its medicinal values.

Aim of the study: A strategy to circumvent the problem of multidrug resistant *Pseudomonas aeruginosa* is discovery of novel anti-infectives targeting bacterial virulence or host immunity. This study aims to decipher the anti-infective properties of *H. atra*.

Materials and methods: Here, we utilized the *Caenorhabditis elegans-P. aeruginosa* infection model to assess the anti-infective properties of *H. atra*. We conducted the *C. elegans-P. aeruginosa* slow killing assay to isolate a fraction with capable of conferring survival to pathogen-infected worms, followed by a series of tests to assess the anti-virulence and immune-related properties of the sea cucumber bioactive fraction.

Results: The *H. atra* methanol extract improved survival of infected worms without affecting pathogen viability. Further extraction and assays sequestered a fraction with significant anti-infective activities and was able to attenuate the secretion of virulence factors and biofilm formation. Treatment of *P. aeruginosa*-infected transgenic *lys-7*::GFP worms with this fraction also restored the repressed expression of host *lys-7*, a defense enzyme. QTOF-LCMS analysis of this fraction revealed the presence of aspidospermatidine, an indole alkaloid and inosine.

Conclusion: Collectively, results shows that *H. atra* confers survival advantage against *P. aeruginosa* infection through inhibition of pathogen virulence and the restitution of host *lys-7* expression.

Keywords: *Pseudomonas aeruginosa; Caenorhabditis elegans; Holothuria atra*; anti-virulence; immunomodulator

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen involved in urinary tract and bloodstream infection, nosocomial pneumonia and contamination of surgical sites (Driscoll et al., 2007; Taylor et al., 2014). Increasing number of antibiotic resistance *P. aeruginosa* strains are being reported globally (Yayan et al., 2015). Attempts to overcome bacteria resistance through modification of existing drugs or drug discovery are often ineffectual (Parisien et al., 2008). Collectively, these issues underscore the need for alternative therapies with different modes of targeting mechanism involving host immune response or

pathogen virulence. In comparison to direct killing of pathogens, these paths are projected to invoke milder evolutionary pressure responsible for resistance development (Allen et al., 2014).

The nematode *Caenorhabditis elegans* is a proven model for deciphering host–pathogen interactions due to its readiness for infection by numerous human pathogens (Moy et al., 2006). As a model organism, it is also supported by an array of tools useful for deciphering immunity related pathways (Aballay and Ausubel, 2002). The *C. elegans* host-pathogen interaction is a proven powerful platform for discovery of anti-infectives, and is frequently utilized to screen for novel anti-infectives against a myriad of pathogens, (Conery et al., 2014; Kim et al., 2017; Maria et al., 2017; Utari and Quax, 2013; Zhu et al., 2015). An advantage in having both host and pathogen in a screening assay is early detection of undesirable toxic properties in hit candidates (Squiban and Kurz, 2011).

The diverse marine environment impose the selection of an array of metabolites produced by an immeasurable source of organisms (Molinski et al., 2009). Secondary metabolites with anti-infective properties against *P. aeruginosa* have been identified from numerous marine organisms including marine bacteria and seaweeds (Fatin et al., 2017; Kandasamy et al., 2012; Kulshreshtha et al., 2016; Liu et al., 2013; Naik et al., 2013; Papa et al., 2015). . To date, the only published report of an anti-infective from marine invertebrates using the *C. elegan*s platform is from a tropical mollusk (Kong et al., 2014).

The consumption of sea cucumber, a marine echinoderm as medicine can be traced back to medical records documented during the Ming Dynasty (Chen 2003). Sea cucumbers prepared for medicinal purposes are known as `gamat' in Malaysia, with different forms of gamat being utilized for various types of ailments (Fredalina *et al.* 1999). From the estimated 1000 varieties of sea cucumbers worldwide, around 20 species have known ethnopharmacological values (Bordbar et al., 2011). Asians typically regard sea cucumbers as effective remedy of asthma, hypertension, joint pain, sprains intestinal and urinary problems (Chen, 2004; Dakrory et al., 2015). Correspondingly, studies on sea cucumber metabolites have reported multiple activities including anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, antihyperglycemic, anti-ageing and wound healing (Aminin et al., 2015; Bordbar et al., 2011; Janakiram et al., 2015; Jattujan et al., 2018; Mourão et al., 1998; Song, M. et al., 2013). These bioactivities are linked to a wide range of metabolites, such as triterpene glycosides, peptides, collagen, mucopolysaccharides, carotenoids, polyunsaturated fatty acids and phospholipids

(Aminin et al., 2015; Bahrami and Franco, 2016; Ibrahim, 2012; Saito et al., 2002; Shi et al., 2016).

Holothuria atra Jaeger 1833 (Holothuridae), colloquially known as black sea cucumber or lollyfish, is one of the most commonly found sea cucumber species in the Indo-Pacific region, dwelling in seagrass beds and rocky reefs (Conand, 2004). In Fiji, this species is traditionally cooked or marinated before consumption (Vuki and Viala, 1990). Among the many medicinal properties linked with sea cucumbers, sea cucumbers are also traditionally consumed for its immune boosting properties (Chen, 2004). In tandem, metabolites with *in vitro* or *in vivo* immunomodulatory activities have been isolated from several sea cucumber species (Aminin et al., 2016; Aminin et al., 2008; Aminin et al., 2006; Song, Y. et al., 2013). Therefore, sea cucumber is an opportune organism for discovery of novel and potent anti-infective entities. Using the *C. elegans* host-pathogen platform, we investigated the anti-infective properties of *H. atra*. Overall, we demonstrate the capacity of an extract from this sea cucumber in rescuing worms from a lethal *P. aeruginosa* infection. The capacity of *H. atra* metabolites in impeding the production of well-characterized *P. aeruginosa* virulence factors and boosting an immune-related gene were also studied.

2. Materials and Methods

2.1. Bacterial and Nematode Strains

P. aeruginosa strain PA14 (Rahme et al., 1995), which is rifampicin-resistant, was grown in King's B broth supplemented with rifampicin (100 µg/mL) while the streptomycin-resistant *E. coli* strain OP50 (Brenner, 1974) was cultured in Luria Bertani (LB) broth with streptomycin (100 µg/mL). Cultures were grown overnight with aeration at 37°C. The wild type *C. elegans* Bristol N2 (N2) and transgenic *lys-7*::GFP worm strain SAL105 were obtained from the *Caenorhabditis* Genetics Center (CGC), USA, with growth and handling as described elsewhere (Alper et al., 2007; Brenner, 1974).

2.2. Preparation of Holothuria atra extract

Holothuria atra was collected from Pulau Songsong Kedah situated at the northern region of Peninsular Malaysia (N5°48'33.72" E100°17'32.63") and authenticated by the Centre for Marine and Coastal Studies, Universiti Sains Malaysia. Samples were stored at -20°C until further processing. Frozen samples were cleaned with distilled water, sliced into

smaller pieces and freeze-dried for about 5-7 days. Dried samples were extracted with either methanol or acetone, followed by 15 minutes of sonication at room temperature and lastly, filtration with Whatman filter paper No. 1. Extraction procedure was repeated thrice. Filtrates were then concentrated using a rotary evaporator at 39°C. All dried extracts were kept at -20°C before dilution in DMSO at 20mg/mL, followed by storage at 4°C. Depending on assays, the extract stock solution is further diluted to achieve the desired concentrations.

2.3. C. elegans Survival Assay

The slow killing assay was performed according to the protocol described elsewhere (Dharmalingam et al., 2012). PA14 cultured overnight on King's B broth was evenly spread on Pseudomonas Infection Agar (PIA) assay plates supplemented with natural products, followed by incubation at 37^{o}C for 24 hours to allow growth of PA14. Triplicate plates containing 50-60 young adult worms each were used. At least three independent repeats were performed.

Percentage survival of the infected worm population was determined after 48 hours of incubation at 25°C. DMSO (0.5%) and curcumin (100 µg/mL), a known anti-infective agent, was used as negative and positive control, respectively. (Rudrappa and Bais, 2008).

2.4. Antimicrobial Test

The minimum inhibitory concentration (MIC) broth microdilution antimicrobial test was carried out to determine bactericidal activities of natural product. Stock solution of extracts or fractions were further diluted in DMSO to achieve concentration ranging from 25 µg/mL to 1000 µg/mL. Overnight culture of *P. aeruginosa* PA14 was adjusted to 0.5 McFarland standard turbidity and diluted to 1:20. A total of 10 µL of inoculum was added into the wells, followed by incubation at 37°C for 24 hours. Streptomycin (100 µg/mL) and DMSO (0.5%) were also tested as positive and negative controls, respectively.

2.5. Bacterial Growth Assay

The assay was conducted with similar preparations as the MIC broth microdilution test. Upon inoculum addition, the well plate was incubated at 37°C with aeration and bacterial cell density was measured at OD_{600} every 2 hours.

2.6. Liquid-liquid Partitioning of Methanolic H. atra Extract

The methanol extract was subjected to liquid-liquid chromatography, to separate compounds according to polarity. The extract was suspended in distilled water and subsequently partitioned with butanol. The partition was collected and dried with a rotatory evaporator, followed by storage at -20°C. Stock solution for the partition was prepared in DMSO and stored at 4°C.

2.7. Fractionation of H. atra Butanol Partition

The butanol partition was column chromatographed over silica gel 60 (70-230 mesh) and eluted stepwise from 100:0:1 to 0:100:1 with the chloroform:methanol:water solvent system at ratio of 80:20:1. The process was repeated until sufficient fractions were obtained for further analysis.

2.8. Virulence Factors and Biofilm Production Assays

Protease and elastase activity assays were performed as described elsewhere, with minor modifications (Prithiviraj et al., 2005). Briefly, *P. aeruginosa* PA14 was grown in the presence of bioactive fraction at 200 µg/mL or DMSO (0.5%) for 24 hours at 37°C. The supernatants were collected and filtered with a 0.22 µm nylon filter. Reaction mixture consisting of 0.8% azocasein in 500 µL of 50mM K_2HPO_4 (pH7) and 100 µL of the purified supernatant were incubated at 25°C for 3 hours. A total of 0.5 mL of 1.5M HCl was added into the reaction, followed by placement on ice for 30 minutes and centrifugation. After the addition of 0.5 mL 1M NaOH, the absorbance was measured at 400nm (SpectraMax M5).

As for elastase activity, 100 µL of the purified supernatant described above was added into tubes pre-prepared with 1 mL of 10mM Na_2HPO_4 and 20 mg of elastin-Congo red. The mixtures were then incubated at 37°C for 4 hours with agitation. After the incubation, the mixtures were centrifuged at 14 000 rpm for 10 minutes and absorbance determined at 495nm.

The pyocyanin quantification assay was conducted as described (Essar et al., 1990). *P. aeruginosa* PA14 was grown in the presence of bioactive fraction for up to 24 hours at 37°C. Cultures were collected at 6, 12 and 24 hours after treatments and centrifuged. A total of 3 mL chloroform was then added into the resulting supernatant, vortexed briefly and centrifuged for 10 minutes at 10 000 rpm. The bottom blue layer was isolated, and the extraction processes were repeated using 1.5 mL of 1M HCl. The absorbance at 520 nm of

192 the pink layer was measured. Pyocyanin concentration was determined by multiplying the
193 absorbance value with 17.072.

194 A microtiter biofilm formation assay was used to determine the effect of *H. atra* fraction
195 on *P. aeruginosa* PA14 biofilm formation (O' Toole, 2011). *P. aeruginosa* PA14 was grown
196 overnight followed by dilution to 1:100. A total of 100 µL of the inoculum was added into 96
197 wells plate preseeded with *H. atra* fraction or 0.5% DMSO. The plate was then incubated for
198 24 hours at 37°C. Liquid and cells were discarded, followed by rinsing of plate with distilled
199 water. For visualisation, 125 µL of 0.1% crystal violet solution was added into each well,
200 followed by incubation for 15 minutes at room temperature. Excessive stain was washed off
201 with water and wells left air-dried for 2 hours. Stained biofilm was treated with 125 µL of
202 30% acetic acid followed by 10-15 min incubation. A total of 125 µL of solubilized crystal
203 violet was transferred into a new microtiter for measurement at 550 nm .

204 *2.9. Visualization of Transgenic lys-7::GFP C. elegans*

205 Transgenic *C. elegans lys-7*::GFP strain SAL105 were exposed *P. aeruginosa* PA14 in
206 the presence or absence of *H. atra* fractions as described in the slow killing assay. The
207 fluorescence intensities of young adult worms at 12 hpi for all the experimental conditions
208 were visualized using Leica Microsystem M205 FA microscope and digitally captured with
209 Leica DFC310 FX camera (60x magnification)

210 *2.10. Real-time PCR based quantification of lys-7 expression in C. elegans*

211 Primers were designed accordingly for the amplification of *C. elegans lys-7* and
212 pan-actin as housekeeping gene. Total RNA was treated with RNase-free DNase (Promega,
213 USA) for removal of genomic DNA. PCR optimization and amplification was performed
214 under condition described previously (Dharmalingam et al., 2012). Melt curve analysis was
215 performed to ensure the specificity of the PCR products. Quantitative expression of *lys-7*
216 was calculated using CFX Manager software (BioRad, USA) by normalizing its level to
217 housekeeping gene. Three independent replicates were performed for each analysis.

218 *2.11. Chemical Profiling of Fractions*

219 Compounds identification of both the *H. atra* active fractions were performed using the
220 Agilent 6520 accurate-mass quadrupole time-of-flight liquid chromatography mass
221 spectroscopy (QTOF-LCMS). Zorbax SB-C18 column (1.5µm particle size, 0.5x150mm)
222 was used and separation was achieved with a 22.5 minutes gradient of 3-90% acetonitrile,

followed by 3% acetonitrile for 10 minutes at a flow rate of 20 µL/min. Solvents contained 0.1% formic acid. The compound prediction was achieved by comparing the obtained individual MS/MS fingerprinting spectrum with METLIN, MassBank and Chemspider databases.

2.12. Statistical Analysis

Data were analyzed using Student's *t*-test whereby in all comparisons, $p<0.05$, $p<0.01$ and $p<0.001$ were accepted as statistically significant.

3. Results

3.1. H. Atra Promotes Survival of P. aeruginosa PA14 Infected C. elegans Without Displaying Any Direct Bactericidal Effect

The slow-killing assay showed methanol extract of *H. atra* causing a significantly higher percentage of worm survival (60.21± 6.7 %) at 48 hours after treatment, which is comparable to value obtained with curcumin (60.68 ± 8.6%), a known anti-infective against PA14 infection (Figure 1). A lower percentage of survival (56 $\pm$ 7.6%) was obtained with acetone extract of *H. atra*.

Next, we determined the bactericidal effect of the methanol extract on *P. aeruginosa* PA14. Extract concentrations ranging from 25 µg/mL to 1000 µg/mL did not reduce *P. aeruginosa* PA14 growth, as compared to streptomycin at 100 µg/mL (Supplementary 1). This extract was also non-detrimental towards the growth kinetic of *P. aeruginosa* PA14, as no significant difference was observed between *H. atra* and DMSO exposed bacteria population (Figure 2).

The lethality of *P. aeruginosa* PA14 infection in the slow killing assay involves the colonization of *C. elegans* intestinal tract (Tan et al., 1999). Therefore, we ascertain if the improved survival of worms treated with extract is an outcome of reduced worm feeding activities. We observed the worm pharyngeal pumping rate, a reliable indicator of bacterial feeding in this species (Kong et al., 2014). Results show no significant difference in feeding activities between OP50 fed worms and untreated PA14 fed worms, 0.5% DMSO treated PA14 fed worms or *H. atra* methanol treated PA14 fed worms, respectively

(Figure 3). Therefore, worms exposed *H. atra* methanol extract still display normal feeding activities, leading to colonization of gut by PA14.

Column chromatography fractionation of the *H. atra* butanol partition resulted in 9 fractions (F1-F9). Based on the slow-killing assay, fractions F2 (55.37 ± 8.4%), F3 (64.05 ± 8.8%), F4 (52.75 ± 6.3%) and F9 (52.00 ± 8.0%) resulted in significantly higher worm survival as compared to 0.5% DMSO treatment (Figure 4a). Fraction F3 also did not appear to perturb *P. aeruginosa* PA14 growth (Figure 4b).

Dose dependent assay for fraction F3 showed that 200 µg/mL was the minimal concentration resulting in significantly highest percentage of worm survival, comparable to value obtained with curcumin (Figure 5). At higher concentrations, a decreasing trend of survival rates was observed.

3.2. H. atra fraction Inhibits the Production of P. aeruginosa PA14 Elastase, Protease, Pyocyanin and Formation of Biofilm

In comparison with 0.5% DMSO, treatment of *P. aeruginosa* PA14 with F3 significantly suppressed the production of elastase and protease at 24 hr after treatment (Figure 6a). Formation of biofilm in microtiter plate was also attenuated with F3. As for pyocyanin, significant reduction in pyocyanin production was obtained at 6 and 12 hr after incubation, respectively, but not 24 hr. (Figure 6b).

3.3. H. atra Induces the Expression of C. elegans Immune Effector lys-7 in PA14 Infected Worms

At 12 hr after infection, GFP intensity of *P. aeruginosa* PA14 infected worms was lower than uninfected worms, indicating diminished *lys-7* expression (Figure 7a,b). Treatment with *H. atra* restored *lys-7* expression to a level comparable with uninfected worms (Figure 7c). Correspondingly, qPCR analysis of *lys-7* in worms also showed higher expression levels in worms exposed to F3 at 12 or 24 hours after treatment (Figure 7d). *H. atra* fraction *also* resulted in higher *lys-7* expression as compared to curcumin.

2.4. Tentative identification of major compound in H. atra F3 and F4 Fractions

QTOF-LCMS separation detected two major peaks in F3 at Retention Times (R_T) 17.764 min and 18.395. The compounds were identified using an HR MS (accurate mass in

negative mode) and HR MS/MS spectra (fragmentation) that matched with METLIN, MassBank and Chemspider databases. From the accurate mass analysis (Table 1), the molecular formula was calculated as $C_{18}H_{22}N_2$ and $C_{10}H_{12}N_4O_5$, respectively. The compounds were putatively identified as aspidospermatidine and inosine.

4. Discussion

An ongoing strategy to overcome the problem of drug-resistant pathogens is discovery of anti-infective activity, which either disrupt virulence-related pathways (Clatworthy et al., 2007) or boost host immunity (Hamill et al., 2008). Marine natural products with anti-virulence (Naik et al., 2013; Papa et al., 2015) or immunomodulatory properties (Kandasamy et al., 2012) have been reported. Among edible marine organisms, sea cucumber is regarded as a potent source of novel entities useful for both nutraceutical and pharmaceutical industries. There is an increasing reports of bioactivities from *H. atra*, a widely distributed tropical sea cucumber species. This includes antibacterial, antifungal, anti-proliferative, anti-apoptotic and antioxidant properties (Dakrory et al., 2015; Dhinakaran and Lipton, 2014; Dhinakaran and Lipton, 2015; Esmat et al., 2013; Nishikawa et al., 2015; Ridzwan et al., 1995; Saad et al., 2016).

Our results identified a sea cucumber *H. atra* methanol extract with capacity to confer survival in *P. aeruginosa* PA14-infected *C. elegans*. This extract does not possess direct bactericidal activity nor impedes growth of *P. aeruginosa* PA14, which rules out bacteria mortality as reason for improved worm survival. It was previously reported that a methanol extract of *H. atra* did not exert any bactericidal effect on several pathogenic bacteria species, including *P. aeruginosa* (Lawrence et al., 2010). Taken together with other investigations, *H. atra* bioactivities is therefore dependent on extraction solvent and target pathogen species (Abraham et al., 2002; Ibrahim, 2012). Further separation of the *H. atra* methanol extract yielded a fraction with similar bioactive properties. The range of worm survival obtained with this fraction is comparable to levels using a similar slow-killing assay where infected worms were treated with a terrestrial plant seed extract (Dharmalingam et al., 2012) or curcumin (Rudrappa and Bais, 2008). These levels however, are lower than a fraction isolated from a tropical marine actinomycete (Fatin et al., 2017).

In order to further understand the mechanisms responsible for the survival of infected worms, we assessed the effect of *H. atra* towards several *P. aeruginosa* PA14 virulence

313 factors. Although the complete spectrum of the pathogenesis of *C. elegans-P. aeruginosa*
314 slow killing assay is not fully understood, the involvement of multiple virulence factors such
315 as pyocanin, pyoverdine, various proteases, elastase, phospholipase and exotoxin is
316 canonical (Kipnis et al., 2006; Rahme et al., 1995; Tan et al., 1999). Our results indicate *H.*
317 *atra* lowered proteolytic and elastolytic activities, diminished the production of pyocyanin
318 and the formation of biofilm, collectively signifying attenuation of *P. aeruginosa* PA14
319 virulence. In addition, the collective mitigation of these virulence factors suggests a possible
320 interference of PA14 quorum sensing (QS) by *H. atra metabolites*. QS have been
321 corroborated as one of the crucial factor responsible for host mortality in the *C. elegans* slow
322 killing assay (Papaioannou et al., 2009). Elsewhere, a marine seaweed extract was reported to
323 repress *P. aeruginosa* QS genes, leading to diminished production of virulence factors and
324 survival of infected worms (Kulshreshtha et al., 2016). Future work should ascertain the
325 capacities of *H. atra* metabolites in interfering with pivotal elements of the *P. aeruginosa*
326 QS.

327 The bioactive fraction was also able to restore the expression of *lys-7,* which encodes an
328 immune-specific lysozyme which facilitates the clearance of bacterial infection through
329 disruption of bacterial cell wall peptidoglycans (Evans et al., 2008; Mallo et al., 2002). Two
330 plausible reasons could explain the restitution of *lys-7*. A known characteristic of *P.*
331 *aeruginosa* infection in *C. elegans* is the suppression of host *lys-7* through the subversion of
332 the DAF-2/DAF-16 insulin-like signaling pathway, leading to the downstream
333 transcriptional suppression of several antimicrobial factors, including *lys-7* (Evans et al.,
334 2008; Garsin et al., 2003; Murphy et al., 2003). Since the *H. atra* F3 fraction could mutually
335 reduce the production of virulence factors and restore expression of *lys-7* in infected worms,
336 it is possible that the latter observation is a consequence of halting the *P. aeruginosa*
337 virulence activities upstream of the DAF2-DAF16 pathway. This would mimic the effects
338 exerted by a marine brown seaweed extract in overcoming the pathogenicity of PA14
339 towards *C. elegans* (Kandasamy et al., 2012). Alternately, *H. atra* could also contain
340 compounds capable of directly inducing *lys-7* activities, as reported elsewhere (Fatin et al.,
341 2017; Li et al., 2014).

342 Compounds with immunomodulatory activities have been isolated from edible sea
343 cucumber species such as *Cucumaria japonica*, *C. frondosa* and *Stichopus japonicus*
344 (Aminin et al., 2016; Aminin et al., 2008; Aminin et al., 2006; Song, Y. et al., 2013).
345 Among these anti-infective molecules, polysaccharides are the most studied group (Kale et

al., 2013; Song, Y. et al., 2013; Wang et al., 2017). In addition, some triterpene glycosides, which are prominent secondary metabolites in sea cucumbers, also possessed immune-boosting effects (Bahrami and Franco, 2016). Two examples, cucumariosides and frondoside, were also reported to promote cellular immunity through stimulation of phagocystosis and lysosomal activities (Aminin et al., 2016; Aminin et al., 2001; Aminin et al., 2008). In our case, the main constituents detected in the bioactive *H. atra* fraction are aspidospermatidine and inosine. Aspidospermatidine is an indole alkaloid, a large and growing group of potentially useful marine-based metabolites (Netz and Opatz, 2015). Elsewhere, alkaloids have been identified in *H. atra* extract (Dhinakaran and Lipton, 2014). Indole and its derivatives have been demonstrated to repress the *P. aeruginosa* QS system, leading to diminished production of protease, pyocyanin and biofilm (Kim and Park, 2013; Lee et al., 2009; Lee et al., 2012). Inosine, a deaminated product of adenosine, was initially speculated as not having useful biological effects (Hasko et al., 2004). However, various studies have highlighted immunomodulatory and neuroprotective properties in inosine (Benowitz et al., 2002; Chen et al., 2002; Jin et al., 1997).

5.0 Conclusion

In conclusion, we propose that *H. atra* has anti-infective properties against *P. aeruginosa*. A bioactive fraction of this sea cucumber species inhibits the production of pathogen virulence factors. Concomitantly, *H. atra* also restored the expression level of host *lys-7*. High levels of an indole alkaloid and inosine were detected in this bioactive fraction.

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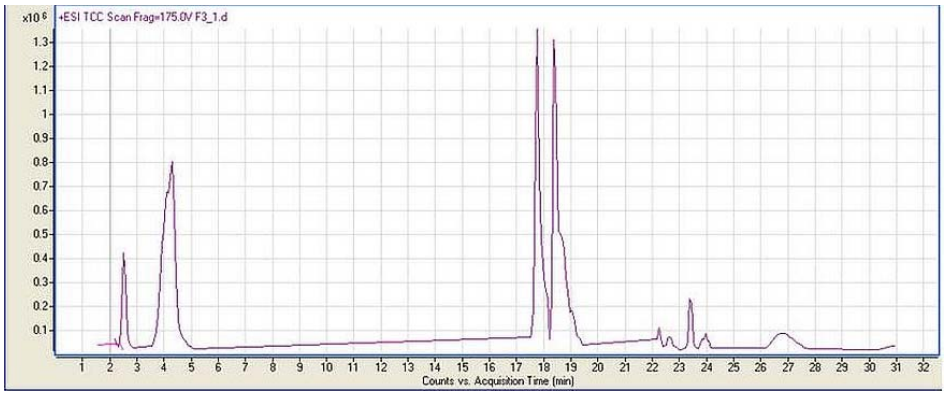
570

571

Table 1. Aspidospermatidine and inosine, the main compounds identified in F3 fraction of *Holothuria atra*

ESI-MS ***R*T (min)**	Detected ion **(*m/z*) [M-H]-**	**Fragments**	**Molecular Formula**	**Identification**
17.764	**266.1789**	**266, 144, 136, 130**	**$C_{18}H_{22}N_2$**	**Aspidospermatidine**
18.395	**268.194**	**268, 215, 185, 136, 133, 109, 93, 82, 54**	**$C_{10}H_{12}N_4O_5$**	**Inosine**



Supplementary 1

The antimicrobial effect of *Holothuria atra* on *P. aeruginosa* PA14 as determined by Minimum Inhibitory Concentration (MIC) test*.

	Concentration (µg/mL)				
Samples	25	50	100	500	1000
Holothuria atra-methanol	-	-	-	-	-
Holothuria atra-fraction F3	-	-	-	-	-
Streptomycin**			+		

* + denotes inhibition; − denotes no inhibition of PA14 growth

** 100 ug/ml streptomycin was used as control

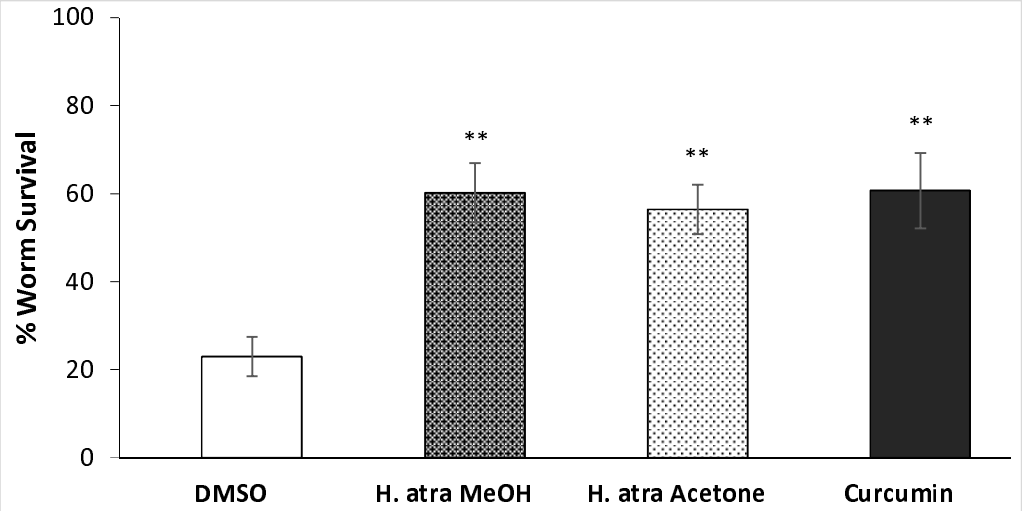


Figure 1. Improved survival of *P. aeruginosa* PA14-infected *C. elegans* at 48 hours after treatment with *Holothuria atra* extracts (200 µg/mL). The methanol extract showed the highest percentage of survival. Data were analyzed using Student's *t*-test where ** denotes significance at $p<0.01$ when compared to 0.5% DMSO treatment. Curcumin was used as positive control.

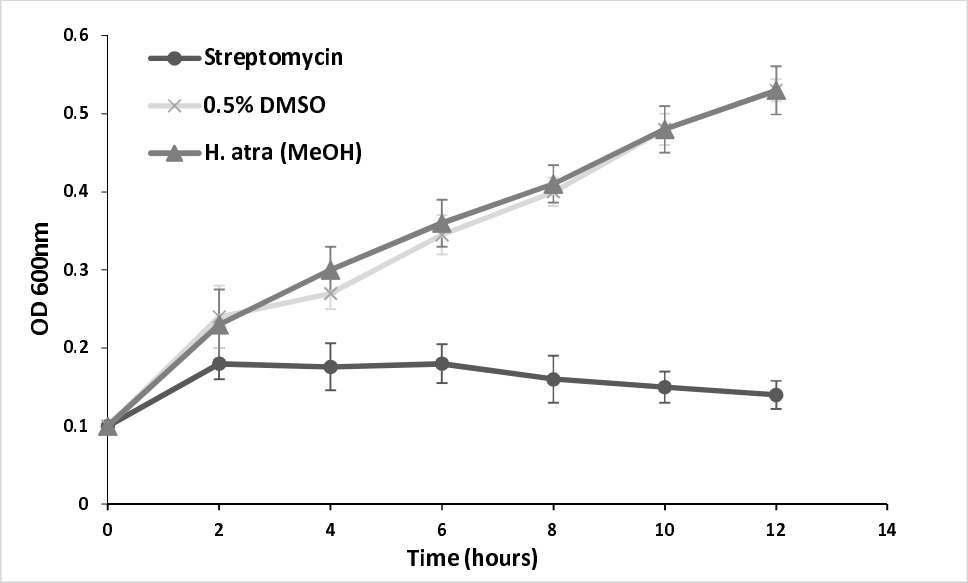


Figure 2. Growth rate of *P. aeruginosa* PA14 in the presence of 200 µg/mL *Holothuria atra* methanolic extract was comparable to 0.5% DMSO treatment while streptomycin (100 µg/mL) reduced bacterial proliferation.

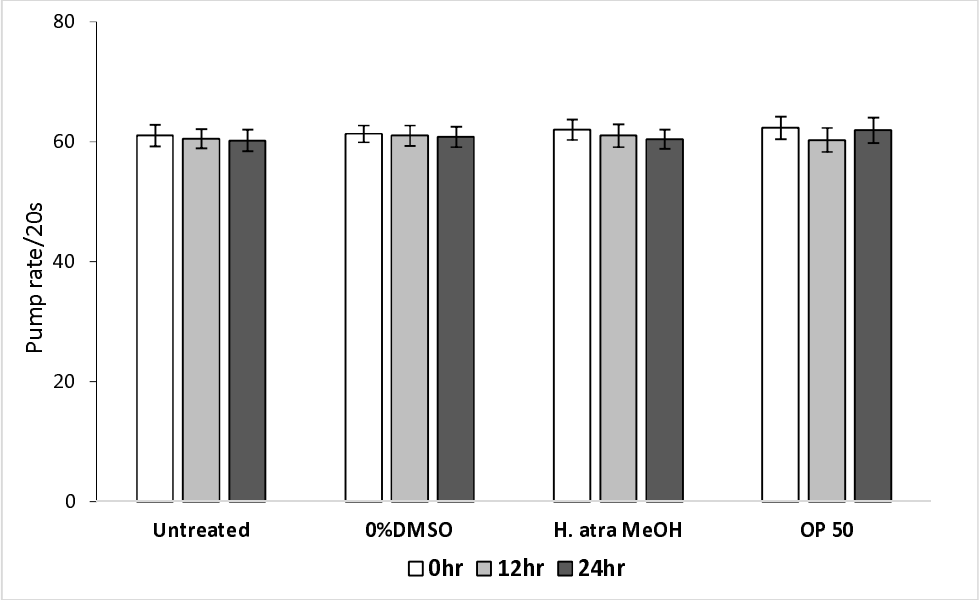
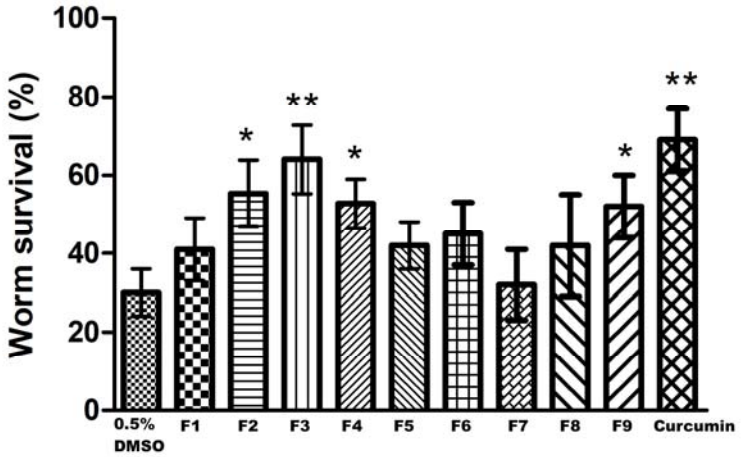


Figure 3. Pharyngeal pumping rate of *C. elegans* fed OP-50, fed PA14 (untreated), fed PA14 (0.5% DMSO treated) and fed PA14 (*Holuthuria atra* methanol extract at 200 µg/mL) at 0, 12 or 24hr after treatment.

A



B

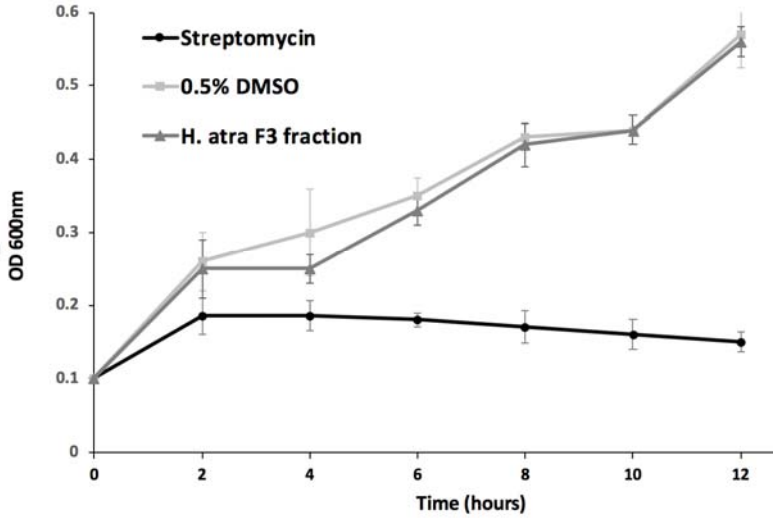


Figure 4. a) Survival of *P. aeruginosa* PA14-infected *C. elegans* at 48 hours after treatment with respective *Holothuria atra* fractions. The F3 fraction showed the highest percentage of survival. Data were analyzed using Student's *t*-test where * and ** denotes significance at $p<0.05$ and $p<0.01$ respectively when compared to 0.5% DMSO treatment. Curcumin, used as positive control, also prolonged worm survival significantly. Survival of *P. aeruginosa* PA14-infected *C. elegans* at 48 hours after treatment with respective *Holothuria atra* fractions (a). The F3 fraction showed the highest percentage of survival. Data were analyzed using Student's *t*-test where * and ** denotes significance at $p<0.05$ and $p<0.01$ respectively when compared to 0.5% DMSO treatment. Curcumin, as positive control, also prolonged worm survival significantly. b) Growth rate of *P. aeruginosa* PA14 in the presence of 200 µg/mL *Holothuria atra* fraction F3 was comparable to 0.5% DMSO treatment (b). Streptomycin (100 µg/mL) reduced bacterial proliferation.

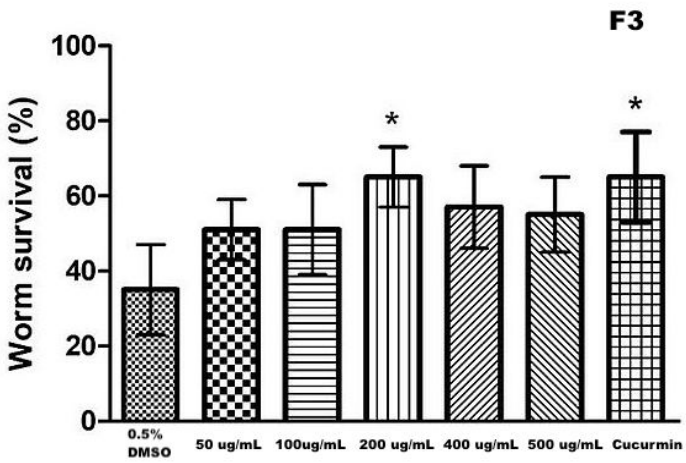
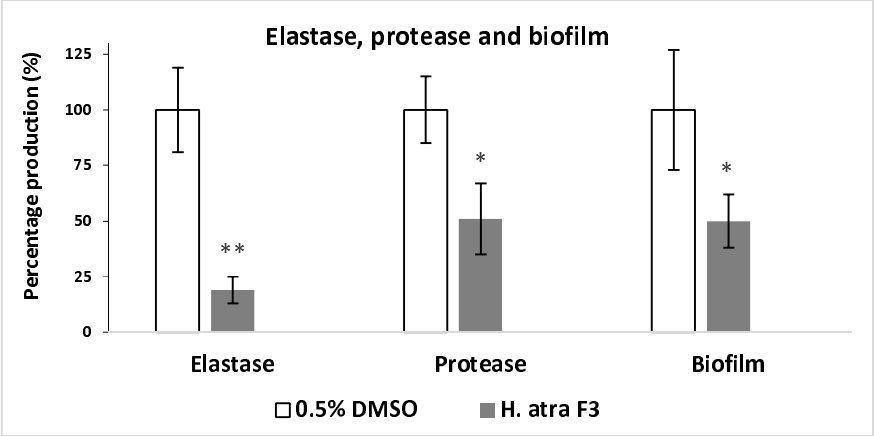


Figure 5. Dose dependent response of *P. aeruginosa* PA14-infected *C. elegans* at 48 hours after treatment with *Holothuria atra* fraction F3. Data were analyzed using Student's t-test where * at $p<0.05$ when compared to 0.5% DMSO treatment. Curcumin, as positive control, also prolonged worm survival significantly.



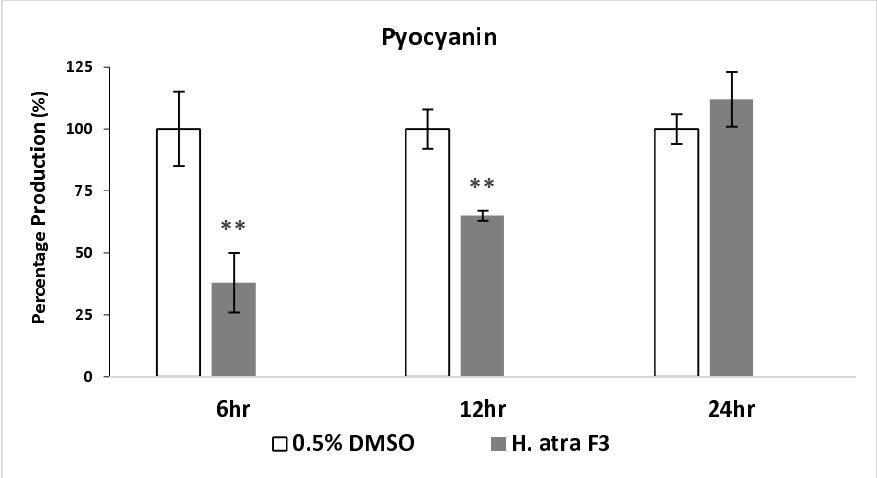


Figure 6 Effect of *Holothuria atra* F3 fraction on production of *P. aeruginosa* PA14 virulence factors (**a**) Reduced production elastase, protease and biofilm at 24 hr after exposure to F3 (**b**) Reduced pyocyanin production at 6 and 12 hr after exposure. Data were analyzed using Student's *t*-test where * and ** denotes significance at $p<0.05$ and $p<0.01$ respectively when compared to 0.5% DMSO treatment.

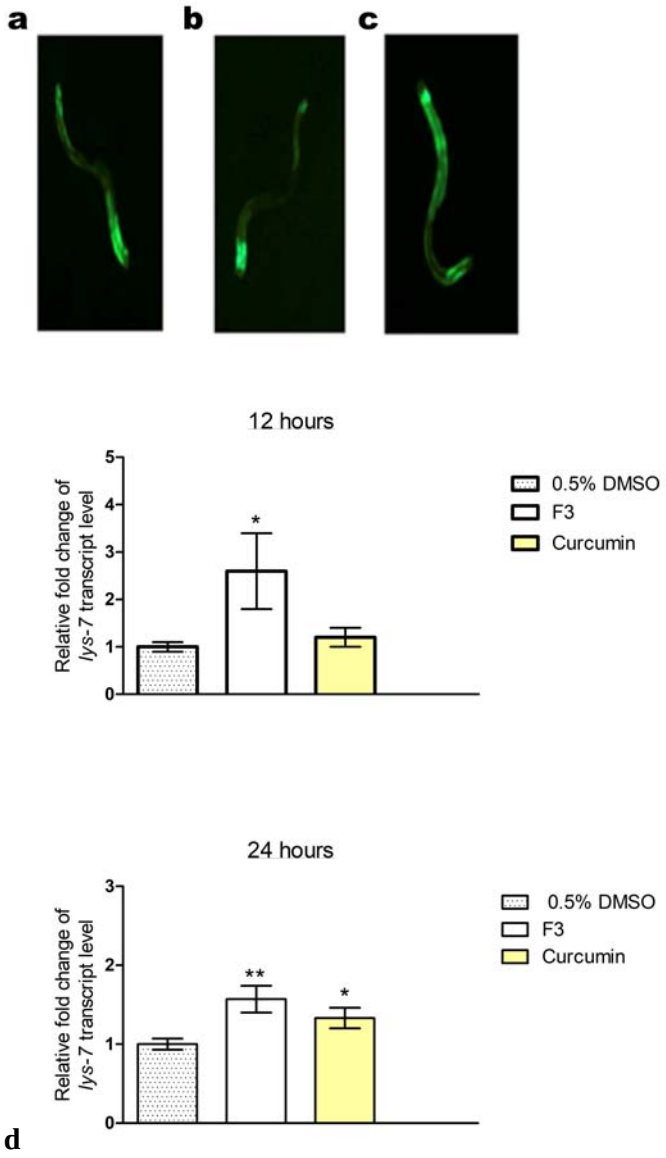


Figure 7. *Holothuria atra* restore *lys-7* expression in *P. aeruginosa* PA14 infected *C. elegans*. Representative fluorescence micrographs (60x magnification) of transgenic *lys-7*::GFP worms (**a**) fed with *E. coli* OP50 (**b**) infected with *P. aeruginosa* PA14 and treatment with 0.5% DMSO (**c**) infected with *P. aeruginosa* PA14 and treatment with F3. Images were captured at 12 hr after treatment. (**d**) qPCR of *C. elegans lys-7* in PA14 infected worms treated with 0.5% DMSO, *H. atra* F3 fraction or curcumin at 12 or 24 hrs. Data were analyzed using Student's *t*-test where * and ** denotes significance at $p<0.05$ and $p<0.01$ respectively when normalized to 0.5% DMSO treatment.