# SCREENING OF ANTI INFECTIVES FROM MALAYSIAN MARINE ORGANISMS USING *CAENORHABDITIS ELEGANS* AS MODEL ORGANISM

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**By**

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## LISTS OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | One way analysis of variance |
| $CaCl_2$ | Calcium chloride |
| CDC | Centers for disease control |
| cDNA | Complementary deoxyribonucleic acid |
| CEMACS | Centre for marine and coastal studies |
| ChAT | Choline acetyltransferase |
| $CHCl_3$ | Chloroform |
| DCM | Dichloromethane |
| DFI | Differential fluorescence induction |
| $dH_2O$ | Distilled water |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DR | Diet restriction |
| EtOH | Ethanol |
| FIM | Fluorescence intensity manager |
| FTIR | Fourier transform infrared |
| GFP | Green fluorescence protein |
| HCl | Hydrochloric acid |
| HMEs | Horizontally mobile elements |
| HIV | Human immunodeficiency virus |
| ICU | Intensive care unit |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| IVET | *In-vivo* expression technology |
| $KPO_4$ | Potassium phosphate |
| $K_2HPO_4$ | Dipotassium phosphate |
| $KH_2PO_4$ | Monopotassium phosphate |
| LB | Luria bertani |
| LCMS | Liquid chromatography mass spectroscopy |
| LRTI | Lower respiratory tract infection |
| MDR-TB | Multidrug-resistant tuberculosis |
| MeOH | Methanol |
| MIC | Minimum inhibitory concentration |
| $MgCl_2$ | Magnesium chloride |
| $MgSO_4$ | Magnesium sulphate |
| MH | Mueller-Hinton |
| MS | Mass spectroscopy |

| | |
|---|---|
| NaOH | Sodium hydroxide |
| $Na_2HPO_4$ | Disodium phosphate |
| *n*-BuOH | *n*-Butanol |
| NCE | New chemical entities |
| NGM | Nematode growth medium |
| nm | Nanometer |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PIA | Pseudomonas infection agar |
| PK/PD | Pharmacokinetic/pharmacodynamic |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| QS | Quorum sensing |
| QTOF-LCMS | Quadrupole time-of-flight liquid chromatography mass spectroscopy |
| RNA | Ribonucleic acid |
| RP-HPLC | Reverse phase high performance liquid chromatography |
| rpm | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| TLC | Thin layer chromatography |
| UV/Vis | Ultraviolet-visible |

**SARINGAN AKTIVITI ANTI-INFEKTIF DARI ORGANISMA MARIN DI MALAYSIA DENGAN MENGAPLIKASIKAN *Caenorhabditis elegans* SEBAGAI ORGANISMA MODEL**

## ABSTRAK

Kejadian rintangan antibiotik telah menjadi fenomena sering muncul yang belum ditemukan penawarnya. Penyelesaian alternatif untuk menghapuskan patogen boleh dilakukan melalui peningkatan sistem imun tuan rumah dan pengurangan virulensi patogen tanpa membunuh patogen dalam tuan rumah secara langung. Produk semula jadi sentiasa menjadi sumber utama untuk penemuan anti-infektif. Organisma marin merupakan sumber yang mempunyai entiti kima baru dengan aktiviti biologi. Dalam kajian ini, penyaringan anti-infektif 20 invertebrata marin dijalankan dengan menggunakan *C. elegans* sebagai perumah dan *P. aeruginosa* sebagai patogen. Hasil kajian menunjukkan estrak metanol *Holothuria atra*, lollyfish, dapat melangsungkan hidup *C. elegans* daripada jangkitan *P. aeruginosa* tanpa menjejaskan pertumbuhan bakteria dan pemakanan biasa perumah. Kebolehupayaan *C. elegans* untuk meningkatkan tahap gen *lys-7* juga diperhatikan apabila diserangi jangkitan *P. aerugniosa*. *H. atra* telah dipilih untuk menjalankan pengasingan berpandukan bio-asai and hasilnya, fraksi F3 dan F4 dapat melanjutkan jangka hayat *C. elegans* yang dijangkiti PA14. Kesan kedua-dua fraksi telah dicadangkan untuk bertindak terhadap faktor virulensi PA14 dan/atau meningkatkan sistem imun *C. elegans* menerusi laluan transduksi isyarat DAF2/DAF16. Analisis kimia F3 dan F4 menunjukkan bahawa kehadiran sebatian aspidospermatidine, EPA, alkaloid dan sebatian lain berpotensi untuk menjana akitiviti anti-infektif. Kesimpulannya, F3 dan F4 dari *H. atra* mengandungi sebatian anti-infektif yang dapat menyelamatkan *C. elegans*

terhadap jangkitan *P. aeruginosa* dengan menggunakan kesan gabungan dalam peningkatan sistem imun perumah dan juga pengurangan virulensi patogen.

## SCREENING OF ANTI INFECTIVES FROM MALAYSIAN MARINE ORGANISMS USING *Caenorhabditis elegans* AS MODEL ORGANISM


### ABSTRACT

Antibiotic resistance has been a reoccurring phenomenon that has yet to find an effective cure. There are alternatives in eliminating pathogens through increasing hosts' immune system and reducing pathogen virulence without directly killing the pathogen in hosts. Natural products have always been the main sources of anti-infective drugs. Marine organisms have been found to possess new chemical entities with potent biological activity. In this study, anti-infective activity of 20 marine invertebrates was screened using live-animal infection model, *Caenorhabditis elegans-Pseudomonas aeruginosa* host-pathogen screening assay. The results showed a methanolic crude extract of *Holothuria atra*, to double the survival of *P. aeruginosa* infected *C. elegans* without affecting both the bacteria growth and viability, as well as the normal feeding ability of the nematodes. An increase of *lys-7* expression was observed in the treated nematodes, indicating the secretion of innate immune response gene upon pathogen attack. Virulence factors of *P. aeruginosa* in *C. elegans* infection are also greatly reduced upon treatment Bioassay-guided fractionation was carried out on lollyfish, resulting in two semi-purified fractions (F3 and F4) which produced positive results in all the assays. The effects of the active fractions have suggested to be acting on both the virulence factors of *Pseudomonas aeruginosa* and DAF2/DAF16 signalling pathway. Chemical analysis on F3 and F4 indicated the co-existing constituents of aspidospermatidine, EPA and other alkaloids responsible for the activity. All in all, both F3 and F4 contained possible anti-

infective molecules in improving the sustainability of *C. elegans* against *P. aeruginosa* infection by a combination effects on boosting up immunity of the host and attenuation of pathogen's virulence factors.

# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

Antibiotic resistance has become so common that it affected both the prevention and treatment of infections caused by various pathogens (WHO, 2014). The phenomenon exists all over the world, indicating the desperate need to develop new anti-infective drugs in pharmaceutical industry (Tadesse *et al.*, 2008). The frequent usage of traditional broad-spectrum antibiotics causes the emergence of not only resistant bacteria (Hoiby *et al.*, 2001), but viruses (Mendes *et al.*, 2011) and fungi (Chandra *et al.*, 2001) as well. As direct killing of the pathogens do not serve much purpose nowadays due to the presence of antibiotic resistance, the main objective would be to find an alternative to combat the pathogen by reducing its virulence or by increasing hosts' immune system, being termed as anti-infective in short (Smith *et al.*, 2010).

The novelty in mode of actions for the anti-infective drugs is vital, with targeted mechanism of action in pathogen killing. The development of novel anti-infective drugs has been very much focused in pharmaceutical industry, especially incorporating natural produtcs. As the compounds isolated natural products give the best overall protective property to human, no doubt natural product have been and will continue to be the major player for drug development as there are higher hit rates on discovering active new chemical entities (NCE) from natural resources which are targeted on secondary metabolites in both terrestrial and marine organisms (Sarker *et al.*, 2005). Plants and terrestrial animals have gone through investigations for anti-bacterial, anti-fungal, anti-malarial compounds identification (Cowan, 1999),

whereas marine environment has been a huge untapped reservoir with possible rich source of anti-infective compounds in a variety of marine natural products which is pharmacologically important (Bhadury *et al.*, 2006).

The marine environment is so harsh yet diverse that the production of biologically active compounds by these marine organisms are found to have low scaffold similarities compared to those extracted from terrestrial organisms, and at the same time possess potent biological activity with targeted mechanism of actions. As such, scientists and researchers are keen to move towards the ocean for novel drug discovery as the new chemical entities obtained are novel with huge range of therapeutic properties (Newman and Cragg, 2007). With the latest technology and resources available, making use of marine natural products as the source of searching for potential anti-infective drugs has been a very promising journey, with 262 marine compounds currently in preclinical pharmaceutical pipeline (Mayer *et al.*, 2013).

The effectiveness of anti-infective agents is best determined with the host-pathogen assay, where the impacts to both the host and pathogens can be detected simultaneously (Moy *et al.*, 2006). With the increasing consciousness of animal welfare in scientific research, it is essential to search for model organisms to study the interactions. The nematode worm *Caenorhabditis elegans* is found to be the most suitable model organism for this particular purpose (Walhout *et al.*, 1998). Despite being the simplest organism, the nematode is well-differentiated with muscle cells, hypodermis, a nervous system, gonads, intestine, glands and an excretory system in its 1mm long transparent body (Artal-Sanz *et al.*, 2006). Besides, it has also been fully sequenced with 60-80% of genes orthologue to humans. Research have showed that the biochemical pathways of *C. elegans* and humans are high conserved;

allowing researchers to probe further in on biological drug effects as well as identify its molecular targets with this model organism (Culetto and Sattele, 2000).

This study discusses the screening of anti-infectives found in marine invertebrates using a type of live-animal infection model, *Caenorhabditis elegans*-*Pseudomonas aeruginosa* host-pathogen screening assay. The Gram-negative bacterium, *Pseudomonas aeruginosa*, is the common cause of nosocomial contamination in medical care facilities, causing hospital-acquired infections in mostly cystic fibrosis patients and immune-compromised patients (DiazGranados *et al.*, 2009). The ability of *P. aeruginosa* to infect *C. elegans* has given an opportunity to venture into the possibility of pinpointing the effect of potential anti-infective compounds extracted to the host (Tan *et al.*, 1999).

## 1.2 Aim of the study

The increasing amount of incidents in multi-resistant pathogens have brought a huge negative impact on general public health and it is important to tackle the problem with an effective, fast and safe alternative to combat the pathogens by reducing its virulence or to counterstrike it by increasing hosts' immune system. The untapped marine environment is definitely a good place to initiate the investigation on searching for novel active metabolites with anti-infective property. The aim of this present study was to identify the possible anti-infective compounds from marine natural products using *Caenorhabditis elegans* as a model system against *Pseudomonas aeruginosa* infection.

This study is carried out to accomplish following objectives:

i. To screen crude extracts of marine invertebrates from Northern Peninsular Malaysia costal waters for anti-infective activity.
ii. To predict bioactive compounds with anti-infective properties from the selected marine organisms.
iii. To determine the mode of action of the marine fractions on *P. aeruginosa* infected *C.elegans*.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Antibiotic drug usage and resistance

The use of antibiotics in humans has been made common since the introduction of penicillin in 1914 by Alexander Fleming. Since then, antibiotic is a life-saving medicine for those who are suffering from mild to chronic bacterial infections. Bacteria are capable to survive after exposure to antibiotics with multiple ways of adaptations, for instance, changing cell wall permeability, modify or change the original target cell, and produce lysozymes (Levy, 1998). Even though antibiotics are able to successfully retard bacterial reproduction, DNA transmission out of the dead bacteria remains possible through plasmids. This enables most of the resistance and virulence genes stored in horizontally mobile elements (HMEs) to be transferred out through conjugation, which allow gene transfer from one resistant bacterium to other bacteria, or what is even worse, possibly to eukaryotes (Heinemann, 1999). This secondary effect of antibiotics should be taken into serious consideration when it comes to the search of alternatives for combating resistance.

The presence of resistance genes in one bacterium will not only be retained, but they will spread within a short period of time, leading to uncontrollable antibiotic-resistant bacteria population (WHO, 2013). Antibiotic-resistant bacteria started off as hospital-acquired infections, whereby only patients who are immunocompromised and stay in hospital for long terms would be exposed. For now, the evolution has moved to the global community due to the misuse and overuse of broad spectrum antibiotics by most of the medical practitioners, leading to

accelerated emergence of plasmid-borne antibiotic resistance bacteria (Svara and Rankin, 2011).

Multidrug-resistant tuberculosis (MDR-TB) has been a major issue as the infection rates have been increasing distinctively with respect to its growing resistant strains towards isonizaid and rifampicin, especially with the increased amount of HIV and immune-compromised patients in the hospital facilities (Jacobs, 1994). According to World Health Organization (2012), an estimated total of 8.7 million people are infected with tuberculosis in 2011. A shocking result of 18.2% and 13.6% respectively of drug resistance rate for the age group of 0-14 years old and 15-24 years old proved the importancy to overcome resistance (Jacobs, 1996). Another recent incident to be taken note is the multi-antibiotics resistant human pathogen *Pseudomonas aeruginosa*, which was found to be associated with fatal nosocomial infections in ICUs. Ozer *et al.* (2012) have studied intensively on patients who suffered from lower respiratory tract infection (LRTI) in intensive care unit (ICU) of Mustafa Kemal University Hospital and they have found out that 86% of them were determined to carry at least one resistant gene, with *NfxB* gene being the most common resistant gene found which causes resistant to Norfloxacin and Ciprofloxacin (Ozen *et al.*, 2012). The examples listed are merely one among millions of them. With such, conventional antibiotics are said to be nearing the end of effectiveness.

Understanding the impact of the antibiotic-resistant bacteria occurrence to the world, it is essential to come up with new generation drugs to solve the problem. (Spratt, 1996). The creation of stronger antibiotics has also proven to be an unsuccessful alternative as it only causes increased rapid emergence of stronger antibiotic-resistant bacteria. This crisis of modern medicine has to be controlled, or

even reduced significantly with a novel cure for bacterial infections, especially those with virulence and resistance genes. Various methods have been implemented to combat the problem, such as antibiotic heterogeneity (Sandiumenge *et al*, 2006.), but none of them found to be a permanent solution. There is an urgent need to come out with a novel cure against multidrug-resistant bacteria, in an unconventional modes of action, opening up for possibility to give desirable anti-infective properties without causing antibiotic resistance.

### 2.2 *Pseudomonas aeruginosa* as pathogen

According to Centers for Disease Control (CDC), approximately 1.7 million patients suffer from hospital-acquired infections annually, which are the fourth leading cause of death in the United States (Klevens *et al.*, 2007). There are various types of bacteria present in the hospital environment which are pathogenic enough to cause hospital-acquired infections especially in immunocompromised patients. *P. aeruginosa*, the rod-shaped gram negative bacterium, is the fifth most frequently isolated nosocomial pathogen, in which there is a 71% increase in resistance rates within 6 years timeframe (NNIS system, 2004). The blue-green colored bacteria is one of the most prevalent opportunistic pathogen, which responsible for 14-16% of nosocomial pneumonia, 7-11% of urinary tract infections, 8% of surgical site infections and 2-6% of bloodstream infections (Emori *et al.*, 1993; NNIS system, 1998).

Typically, *P. aeruginosa* is found in moist environment, for instance, in soil, water, fruits, vegetables, flowers and all sorts of solutions. In hospital environment, the well adapted pathogen may be spread through healthcare personnel, equipments, devices and solutions in hospitals. Patients with prolonged usage of breathing

machines, catheters and patients with wounds from surgery and from burns are potentially at high risk for *Pseudomonas* infections (DiazGranados *et al.*, 2009; CDC, 2013). Along the way, the pathogen is capable to accommodate environmental variation via transformation, transduction and conjugation for genetic information exchange to acquire resistance genes on mobile genetic elements. With the single and supercoiled chromosome in *P.* aeruginosa, the bacterium carries chromosome-mobilizing plasmids encoded for β-lactamase production (Fick, 1993). It breaks down polycyclic aromatic hydrocarbons, hence it became resistance towards β-lactam and quinolone antibiotics such as tobramycin, making it one of the toughest pathogen to counterattack (Lister *et al.*, 2009).

Many researchers and scientists have been working on to halt the propagation of this opportunistic pathogen with various ways, since antibiotic does not seem to work well with multi-resistant *P. aeruginosa*. The pathogenesis of *P. aeruginosa* works via cell association as well as secretion of virulence factors. It is capable to make use of flagella to deliver effector proteins into the host cells and lipopolysaccharides that are capable to suppress immune system of the host and establish persistent infections (Lister *et al.*, 2009). As for virulence factors, the pathogen secretes elastase and protease, which both lead to the degradation of host proteins such as elastin, collagen and transferrins. Besides, *P. aeruginosa* possesses type IV pilli, exotoxin A, alkaline protease, phospholipase C and type III secretion system, whereby all of them also play major roles in bacterial pathogenicity (Salyers and Whitt, 1998; Wareham *et al.*, 2005).

In this study, clinical isolate of *P. aeruginosa* from a human burn patient, strain PA14 has been selected due to its capability to infect a large variety of hosts, with *Caenorhabditis elegans* being one of them. Pathogenic interaction between *P.*

*aeruginosa* strain PA14 and genetically tractable host *C. elegans* will serve as a model system to understand the mechanism behind mammalian pathogenesis, which involves host defence mechanism and bacterial virulence factors (Tan *et al.*, 1999). Interestingly, the virulence factors required for PA14 to infect *C. elegans* is evolutionary conserved, in which similar set of virulence factors is capable of infecting plants, insects and vertebrates as well (Rahme *et al.*, 1995). With the hope of fighting this superbug, it is essential to form a strong defense against *P. aeruginosa* to halt the continue resistance towards broad spectrum antibiotics by studying its pathogenesis in molecular level, as well as find out effective approaches to counterattack it.

**2.3 The discovery of anti-infective compounds**

With the continuous increasing microbial resistance, it is crucial to control and minimize the spread, especially for those multi-resistant pathogens. Researchers and scientists have been focusing on dose, duration, pharmacokinetic/ pharmacodynamic (PK/PD) principles to provide new insight in designing the appropriate dosage to maximize the clinical cure of antimicrobial drugs, at the same time reduce toxicity and also resistance (Mouton *et al.*, 2011; Alvan *et al.*, 2011). Sadly, this alternative only provides temporary resistance control and more research has to be done in understanding the mechanism of emergence, into the spread of resistant as well as into drug exposure-resistance relationships (Theuretzbacher and Mouton, 2011). Numerous researches on drug discovery have decided to approach this crisis with antibiotics with multiple mode of actions in one, or counterattack the pathogens another way round instead of using the traditional approach of drug discovery on developing antibiotics, such as cell wall biosynthesis disruption, protein synthesis inhibition, a halt in DNA replication and reducing virulence factors of the

pathogen (Monaghan and Barrett, 2006). There are new findings reported consistently on combating this problem with various approaches.

Instead of eliminating the invading pathogens directly like what usual antimicrobial drugs do, the active ingredients in anti-infective agents attack the pathogens through different mode of actions: in which some prevent pathogen from adhering to host tissues (Zopf and Roth, 1996), some work by reducing pathogen's virulence factor (Marra, 2004) and some tap on modulating the host's immune system to counteract with the pathogen. As it is impossible to put a halt in the usage of antibiotics, the creation of novel anti-infective agents plays an important role in preventing us from falling back to pre-antibiotic era. There are no easy ways to combat resistance, multiple counter measures and actions are necessary on all different aspects in this long term war, especially in terms of knowledge/information exchange, coordination and cooperation throughout the whole science community.

An interesting approach proposed by Zopf and Roth (1996) will be the use of soluble oligosaccharides as anti-infective agents, by developing anti-adhesive therapeutic agents in the form of small (~1kDa) non-immunogenic human oligosaccharide component to prevent microbial adhesin from binding to the specific carbohydrate receptor on the host (Wick *et al.,* 1991). This strategy is less likely to cause resistance as it works mechanically, whereby non-adherent microorganisms are easily cleared through normal functioning human mucosal surfaces. Marra (2004) proposed another novel molecular approach in targeting virulence genes of the specific attacking pathogen. Signature-tagged mutagenesis, *in-vivo* expression technology (IVET), differential fluorescence induction (DFI) technology and other genomics and micro-array technology are applied to screen for essential virulence genes to cause an infection and monitor both their *in vivo* expression level and

pattern whereby the products are involved in the biosynthesis and nutrient source of the pathogen of interest (Saenz and Dehio, 2005; Angelichio and Camilli, 2002; Mahan *et al.*, 1995).

Scientists have also been searching for compounds which possess antimicrobial or immunomodulatory properties without a similar structural motif and mode of actions with existing drugs to combat bacteria resistance from both terrestrial and marine natural products. The results are promising as compared to synthetic libraries, whereby there is an increase in ten-fold of hit rate. Purple coneflower, *Echinacea angustifolia* is known to be plant immunostimulants, where cichoric acid, polysaccharides and alkylamides are thought to contribute to stimulate the phagocytic activity of neutrophils and macrophages *in vitro* and *in vivo* (Parnham and Verbanac, 2011). Methanolic extract of marine sponge *Spongosorites halichondriodes*, which contains glycospingolipids are thought to possess immunomodulating activity whereby it reduces the total white blood cell count and prevents myelosuppression in cyclophosphamide drug treated rats (Kumar *et al*., 2012). Various new chemical entities are mostly found in active secondary metabolites of natural products available in both terrestrial and marine environment, often with a very complicated scaffold and excellent biological potency.

### 2.4 Host-pathogen interaction

Every living organism, let it be plants, invertebrates or vertebrates, all of them utilizes innate immunity to repel infectious agents. It is the first line of defense against infection, whereby the organism undergoes three main responses: pathogen recognition, immune signaling and protective effecter responses; in which it will

only then mobilizes adaptive immune responses, provided if the organism has one (Gravato-Nobre and Hodgkin, 2005). Studying the mode of action on pathogen invasion through both host and pathogen perspectives is of best interest for the immunologists and researchers to understand the underlying mechanism of innate immunity that is most likely conserved among all living organisms (Aballay and Ausubel, 2002).

Besides highly conserved innate immunity across phylogeny, Taylor *et al* (2001) also show that out of 1415 species of pathogen that is able to infect human, 61% of them has the capability to be transmitted between humans and animals. In other words, it indicates the ability of using vertebrates to facilitate the understanding of innate host defence mechanisms. There are several ways on studying host-pathogen interactions, which often complements with animal testing to observe the severity of the infections at whole organism level. The ethical concerns on the animal welfare in science research have shifted the study to search for model organisms of similar ancient evolutionary origins and conserved signaling mechanisms of humans' innate immune system.

The extensive studies have revealed interesting findings, whereby there are several genetically tractable model organisms available to conduct host-pathogen interactions study, namely *Arabidopsis thaliana* (Schlaich, 2011), *Drosophila melanogaster* (Limer *et al.*, 2011), *Danio rerio* (Trede *et al.*, 2004) and *Caenorhabditis elegans* (Tan *et al.*, 1999). All of them contain 60-80% common mechanisms of innate immunity compared to mammals, which includes similar universal defense genes and biochemical pathways on controlling gene expression upon the increase of infection. Hence, it has been widely applied on investigating host-pathogen interactions to give a new level of insights of the pathways in both the

host and pathogen, subsequently lead to the development of possible cure to certain diseases.

Upon understanding how host-pathogen interactions work, a simple non-vertebrate host, *Caenorhabditis elegans*, is used to address the pathogenicity question that involves host-pathogen interaction of *C. elegans* upon infection of human ubiquitous opportunistic pathogen, *P. aeruginosa*. The high degree of conservation between the innate immune system of *C. elegans* and human has made it possible for the nematode worm to be coupled with human ubiquitous pathogen *P. aeruginosa*, which has the capability to cause an infection in the nematode worm, to identify the universal virulence factors and to study the conserved mechanism of innate immunity (Tan *et al.*, 1999; Ewbank, 2002).

### 2.4.1 *Caenorhabditis elegans* as host

*Caenorhabditis elegans* is a free-living soil nematode which grows up to 1mm in length and it feeds on various bacteria present in its natural habitat. The population of *C. elegans* is predominantly hermaphrodite, with only 0.1% of them being male in wild-type populations (refer Figure 2.1). A self-fertilized hermaphroditic nematode worm produces up to 300 progeny, while it can produce up to 1000 progeny when a hermaphrodite is male-fertilized. Every adult *C. elegans* contains 959 somatic cells and they are similar in position and identity between individuals (Strange, 2010). A healthy adult nematode worm has an average life span of 3-4 weeks in 25$^{o}$C upon optimal laboratory conditions, where it only needs 12 hours to undergo a full cycle of embryogenesis with another 28 hours for postembryonic development from L1-L4, as per shown in Figure 2.2 (Altun and Hall,

2006). When there is insufficient food supply, *C. elegans* produces dauer hormone, whereby it enters dauer larvae stage which has structural, behavioral and metabolic adaptations to resist stress and starvation for up to 4 months (Golden and Riddle, 1982; Hu, 2007). Dauer larvae will resume back to normal development stage when food becomes available.

The unique features of *C. elegans* has attracted attention from Sydney Brenner and he adopted it as a laboratory model. The founder of *C. elegans*, Brenner initiates the study of the nematode worm for developmental and behavioural study in the early sixties, whereby the work won him and his colleagues, John Sulston and H. Robert Horvitz the Nobel Prize in Physiology or Medicine in 2002 (Nobelprize.org, n.d.). The model organism he found is capable of being used as a genetic analysis tool to elucidate different types of molecular mechanisms, with great experimental advantages. *C. elegans* can be easily cultured on nematode growth medium (NGM), seed with *Escherichia coli* OP50 as food source (Brenner, 1974). The development of the nematode worm can easily be manipulated by the incubation temperature, which is advantageous for experimental purposes. As the main infection site for *C. elegans* is the intestine, infection can easily be triggered by changing the food source to the pathogen of choice. The body transparency of *C. elegans* enables physical observation of the stimuli effect on the nematode directly under the microscope without the need of sacrificing it, such as the monitoring of animal survival, locomotion, pharyngeal pumping rate and so on. The body transparency of the worms allow GFP reporters to be present in the living animals for *in vivo* monitoring studies at the cellular and intracellular level (Ewbank, 2002).

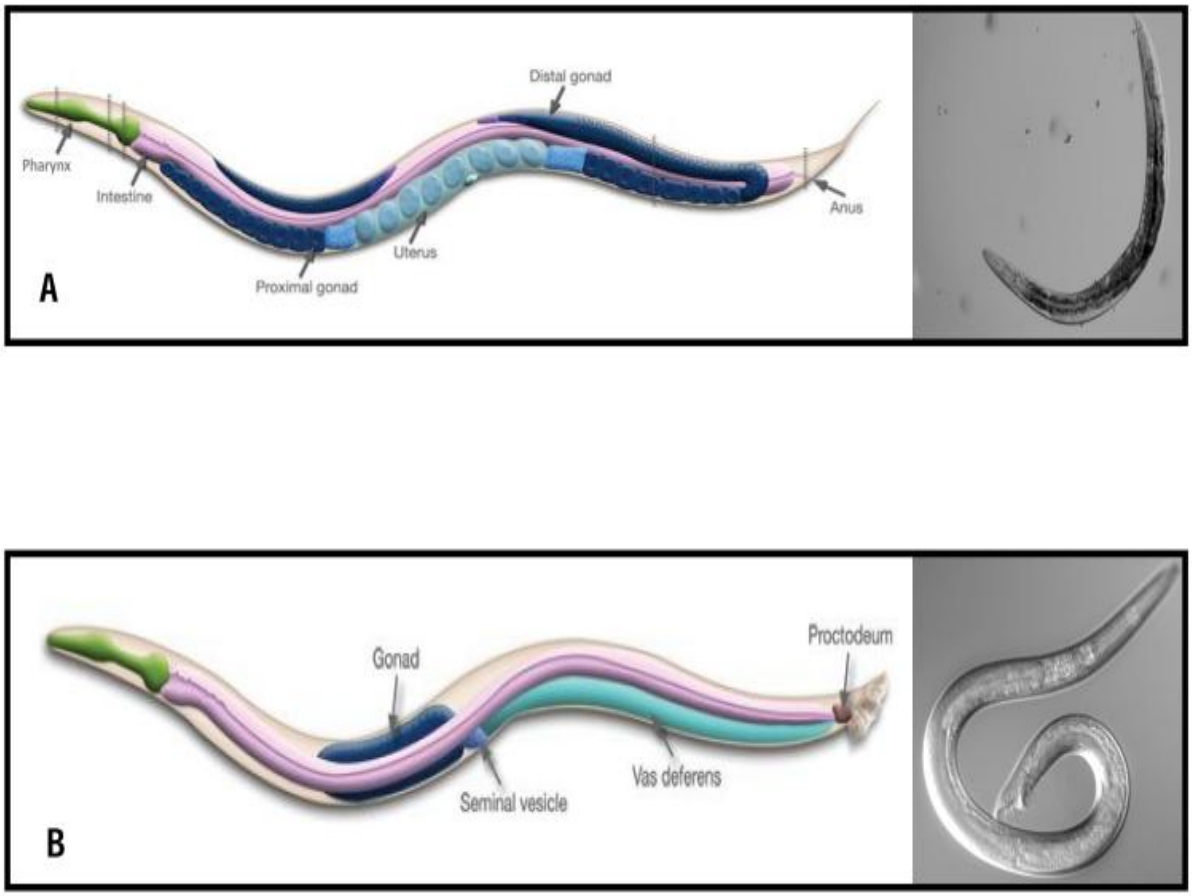


Figure 2.1. The hermaphrodite and male *C. elegans*. Schematic (left) and microscopic (right) images of both hermaphrodite (A) and male (B) *C. elegans*. The distinctive features for hermaphrodite worms would be the presence of uterus, eggs and vulva while the male worms possess fan shaped tail.

(Altun and Hall, 2006)

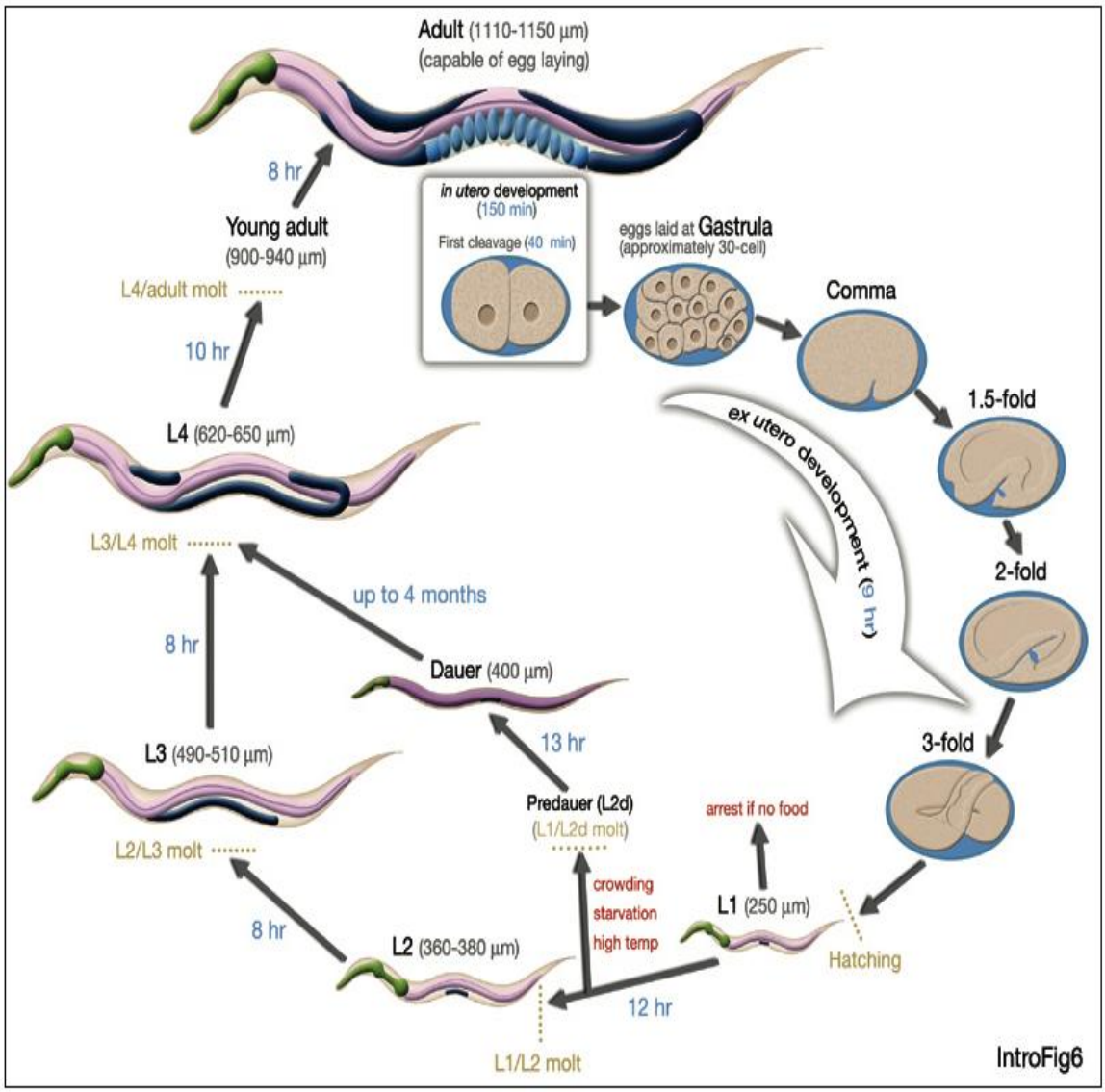


Figure 2.2. The life cycle of *C. elegans.* The nematode worm goes through embryogenesis, which then proceeds to larval stage development (L1-L4) to be a fertile adult. The larval development can be bypassed to form dauer larvae when food resource is limited, which they will then continue with the life cycle when conditions are favorable.

(Altun and Hall, 2006)

The *C. elegans* Genome Sequencing Consortium has completed the genome sequencing project, which contains 100Mb in total and encodes for more than 19099 proteins (*C. elegans* Sequencing Consortium, 1998). Being the first multicellular organism to have its genome fully sequenced, *C. elegans* has been extensively researched to extrapolate the relationship between this model organism and human. The combination of good genetic maps, low cost and maintenance, easily visualization and efficient system for transgenic animals' generation enables *C. elegans* to be selected as the model organism for pharmacological studies which then contributes large to a bigger picture.

Tan (2002) have showed the ability of various human pathogens to infect multiple hosts, from invertebrates, plants to vertebrates. The huge similarities of bacterial virulence factors required for an occurrence of *P.* infection in mammals and for *C. elegans* killing has demonstrated the key features of conserved innate immune response. There are three different killing mechanisms of pathogens on nematode: toxin-mediated, intestinal infections and biofilm formation. When the food source of *C. elegans* is being replaced by candidate pathogens under particular culture conditions, the worms produce fast-acting nematicidal toxins and other diffusible factors. The toxin-mediated effect is termed as 'fast killing', where worms are killed within 4-24 hours. The examples of pathogen that can exert such killing mechanisms are *Burkholderia multivorans* (O'Quinn *et al.*, 2001) and *Bacillus thuringiensis* (Marroquin *et al.*, 2000). Some pathogens, for instance *P. aeruginosa* strain PA14 (Tan *et al.*, 1999), *Salmonella enterica* (Aballay *et al.*, 2000), *Enterococcus faecalis*

(Garsin *et al.*, 2001), can establish intestinal infections by colonizing the intestine of the worms and kill the animal over several days, termed as slow-killing.

Another type of killing mechanism possess by bacteria *Yersinia pestis* and *Yersinia pseudotuberculosis* would be biofilm formation (Joshua *et al.*, 2003). It is a widespread attribute to increase survival ability under stress (Costerton *et al.*, 1995). The pathogen adheres to the surface region of the mouth and vulva to form biofilm, whereby the biofilm prevents the nematode from feeding. In the context of biofilms, *P. aeruginosa* remains as the most studied microbes (Costerton, 2001). The organism forms multicellular aggregates within the lungs of cystic fibrosis patients and surfaces of catheters (Deretic, 2000). Hence, it is crucial to determine the genes involved in the production of biofilm matrix to develop new therapeutic strategies (Friedman and Kolter, 2004).

Upon understanding these killing mechanisms of pathogen to *C. elegans*, it expands the possibility of using the nematode worm to identify the underlying innate immune response which is evolutionarily conserved, as well as to determine the candidate universal virulence factors of these pathogens. The test system involving *C. elegans* is relatively simple, rapid and inexpensive as compared with the use of other vertebrate hosts (Kim, 2008). The pathogenicity of *P. aeruginosa* is of particular interest to be studied due to the society concerns towards occurrence of hospital-acquired infections, whereby a solution in tackling both the host and pathogen all at once would be desirable.

### 2.4.2 *C. elegans* as viable screening system for drug discovery

The added advantages of having highly conserved biochemical pathways and gene functions between *C. elegans* and human have gained confidence that this tiny

organism could be a viable screening assays for drug identification. As traditional *in vitro* and cell-based screening assays in drug discovery have possibilities of yielding toxic hit compounds or compounds with same mode of action with current known antibiotics, a unique approach of drug screenings through an environment that imitates infected human host is recommended by Moy and coworkers (2006). This is achieved by assaying the nematode, *C. elegans* infected with *Enterococcus faecalis*. From small-scale manual screening assay of 6,000 compounds to high-throughput screening of 37, 200 compounds, a total of 44 of them were found to promote the nematode survival upon *E. faecalis* infection. It has also been observed that the extracts acted differently on the pathogenic infection: some cure the bacterial infection *in vivo* without affecting the growth of pathogen, while some possess antimicrobial properties (Moy *et al.*, 2006; Moy *et al.*, 2009). Hence, it can be said that the use of live-animal infection model has added advantage in addressing novel compound identification of potential medical interest, and even the mechanism of action could be made known.

The contribution of live-animal infection model in drug screenings has enabled further advancement of the current paradigm in several key approaches. It directly assesses the drug efficacy and eliminates compounds that are toxic to host in the early stage of analysis, which would only possible when host-pathogen relationship presents. Unlike *in vitro* and cell-based assays, the drug target could also be known when it corrects a phenotype in mutant strains of *C. elegans*, giving information on where the molecule act upon, as well as the target of the molecule in a specific biochemical pathway (Segalat, 2009). In this study, the use of live-animal infection screening assay of *C. elegans* to search for potential anti-infective molecules in marine natural product against *P. aeruginosa* infection serves as a

powerful yet unique assay. It holds the potential of simultaneously identify the effect of marine natural product on both the pathogen virulence-related genes and host immune-related antimicrobial molecules, which then the findings reach an implication on the conserved bacterial pathogenesis from *C. elegans* to human (Mellies and Lawrence-Pine, 2010).

**2.5 Potential of natural product in drug development**

From a 30-year review of new drugs from 1981-2010, there are 71% of the 1350 new approved drugs are from natural products, derived or molecularly inspired by natural products (Newman and Cragg, 2012). With this in mind, no doubt natural product, let it be from terrestrial or marine origins, will be the major player for drug development as there are higher hits on discovering new chemical entities from natural resources as compared to synthetic scaffold formulation, which are targeted on secondary metabolites in particular. Secondary metabolites are usually available in a particular group of living organisms, species or strain under specific environmental positions (Sarker *et al.*, 2005). Interestingly, the exact biological functions of these small molecules with molecular weight less than 2000 atomic mass units are unknown, and are not strictly essential for the survival or the organisms. This raises many biological questions to further invest their potential benefits to mankind with endless possibilities (Lou, 2011). The scaffolds derived from natural resources are in such complex form that it is impossible to synthesize chemically without living organisms. This indicates the possibilities of utilizing the natural resources, as well as making a promising trend for upcoming plans on drug development (Claeson and Bohlin, 2012).

Nature has wide range of biodiversity for various supplies, such as food, medicine, skincare regimes, agricultural chemicals, cleaning products; you name it. They offer a very huge untapped reservoir of chemical compounds and metabolites which may contribute to beneficial usages back in ancient times (Singh and Barrett, 2006). "Ebers Papyrus" is known to be the oldest record of ancient Egyptian medicine, which documented over 700 drugs back in 1500BC, and most of the drugs are originated from plant origin, for instance, the oils of *Cedrus* species, the resin of *Commiphora myrrha* and the juice of poppy seed *Papaver somniferum* (Newman *et al*, 2000). The record is further proven as there are several records from China and Mesopotamia which records over 1000 substances of plant origins, including *Aloe vera* and *Boswellia caeteri* (Zhong and Wan, 1999; Cragg *et al.*, 2009).

Since plants have been known to aid in treating various ailments, the search for potential new drugs begins with plants. E. Merck has successfully commercialized morphine in 1826, in which its pure form is isolated from opium poppy *Papaver somniferum* (Newman *et al*., 2000). For early discoveries, it can be seen that almost all of the new chemical entities found are from terrestrial origin. There are compounds with different pharmacological activities isolated from plants being reported consistently throughout all these years, including anti-bacterial (Kim *et al*, 2002; Medeiros *et al*, 2011), anti-cancer (Bardona *et al*., 2002; Shoeb *et al.*, 2006), anti-inflammatory (Chen *et al.*, 2001; Ueda *et al*., 2002), anti-HIV (Qian-Cutrone, 1996; Wang and Ng, 2001; Sung *et al.*, 2005) and those with other therapeutic effects. Natural products of terrestrial origins, especially plants, have been constantly found to be the effective.

Marine natural products only begin to catch the attention of the scientists in the mid-seventies and the development increases rapidly during 1980s when

Faulkner (1986) began a series of annual reviews about marine natural products. As marine environment comprises of half the global diversity, the contribution of natural products in searching for novel and active compounds has no boundary. Due to the completely different living conditions for marine organisms, unique structures are discovered continuously with the current technology. The continuous success in searching for novel compounds in marine natural products in the past 25 years have proven that refocusing the efforts on natural products is definitely the correct alternative (Hu *et al.*, 2011).

**2.6 Marine natural products**

Marine environment is considered unexplored, with excellent potential of obtaining novel bioactive compounds from 225,000 described species of marine resources (World Register of Marine Species, 2014). Marine plants, bacteria and animals are living in very harsh and diverse natural habitats; some may live at shallow water with high temperature and sunlight penetration, while some live deep under the ocean with extremely high pressure, low temperature and without sunlight penetration (De Vries and Hall, 2004). The differences in marine ecosystem is so diverse that the production of biologically active compounds are found to have no scaffold similarities compared to those extracted from terrestrial organisms, and at the same time possess potent biological activity. As such, scientists and researchers are keen to move towards the ocean for novel drug discovery as the molecules obtained are molecularly unique with huge range of therapeutic properties (Newman and Cragg, 2007).

The first reported discovery of bioactivity from marine resources was nucleosides spongothymidine and its derivative spongouridine from Caribbean

sponge *Crypthitheca crypta* in 1951 by Bergmann and Feeny. Two drugs are developed with the compounds found fifteen years later, which were anticancer agent Ara-C, for non-Hodgkin's lymphoma and acute myelocytic leukemia, and antiviral compound Ara-A for herpes treatment (Bergmann and Fenny, 1951). Continuous investigation has been performed to gain access to the marine natural products, especially on those soft-bodied and/or slow moving marine organisms without a physical defense system. These creatures rely largely on producing toxic secondary metabolites as potential predators' deterrence. The active compounds obtained from marine natural products are said to exhibit excellent therapeutic effect with unique yet targeted mode of actions. The results have been promising, with Ziconotide, the toxin from tropical snail, being the first marine peptidic drugs to be approved for chronic pain treatment which works as calcium channel blocker, interrupting pain signaling at the spinal cord level (Miljanich, 2004).

There are a large variety of marine natural resources having the ability to produce secondary metabolites for different medicinal purposes. For instance, marine microbes have been heavily researched due to its unique ability to be semi-synthesized to a sufficient amount of active pharmaceutical ingredients (API) to construct drugs for large commercial production (Piel, 2009; Gulder and Moore, 2009). The structural similarity of compounds derived from marine invertebrates and in bacteria provide circumstantial evidences that they may be of bacterial origin. Table 2.1 shows the compounds isolated from marine invertebrates and their microbial symbionts that are currently undergoing clinical trials (Waters *et al.*, 2010). Marizomib, a naturally-occurring salinosporamide isolated from marine actinomycete *Salinospora tropica* is working its way towards clinical trials. It has unique scaffold as well as unique β-lactone-γ-lactam proteasome inhibitor, which is

found to have novel mode of action for anticancer treatment (Potts *et al*., 2011). It thus can be known that marine microbes own a huge role in the drug development pipeline, and at the same time play a major role in biosynthesis of drug like molecules for drug construction.

Marine plants such as seaweed and algae have also been reported to contain unique chemical structures with active bio-activity for human health improvement, often being used as functional food ingredients. For instance, the production of karlotoxins by dinoflagellate *K. veneficum* is capable of imitating high density lipoprotein (HDL) in human body to increase the transport rate of cholesterol back to liver for excretion, which will significantly reduce cholesterol level (Pandey and Sassetti, 2008). Another successful study done by Lin and coworkers (2010) have isolated four new bromophycolides (R,S,T and U), with all of them exhibit cytotoxicity against 12 selected human breast, colon, prostate, ovarian, lung and leukemia cancerous cell lines of while Bromophycolide S also show submicromolar activity with $IC_{50}$ value of 0.9μM against human malarial parasite *Plasmodium falciparum*.

Marine invertebrates are also one of the major contributor in producing potent secondary metabolites, in which its pharmacological activity includes anticancer, antiviral, antibacterial, antifouling and anti-inflammatory activities as shown in Table 2.1 (Hu *et al*., 2011). There are five main taxonomic group for invertebrates, which includes Porifera, Cnidaria, Mollusca, Arthropoda, Echinodermata and other minor phyla. As most of the marine invertebrates are soft-bodied without physical defense system, usually they will initiate chemical defense mechanisms such as synthesis of defensins or toxin compounds which have the capability to instill fear to predators, maintain a safe distance from competitors and immobilize their prey. Hence, they