Antifouling Activity of Asian Clam (*Corbicula fluminea*) Periostracum Extract

By

Nur Shazlin Binti Mat Said

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Student

Name: Nur Shazlin Binti Mat Said

Date:

I certify that the report of this final year project entitled "Antifouling Activity of Asian Clam (*Corbicula fluminea*) Periostracum Extract" by Nur Shazlin Binti Mat Said, matric number F14A0256 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

_____

Supervisor

Name: Dr. Suniza Anis Binti Mohamad Sukri

Date:

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ABSTRACT

A research was conducted to determine the antifouling activity of periostracum extract from *Corbicula fluminea* against *Staphylococcus aureus* and *Escherichia coli*. Dried periostracum was extracted with different concentrations of dichloromethane (DCM), ethyl acetate (EA) and methanol (MeOH). Due to the polarity properties of the solvents, high antifouling activity was observed on DCM extract whilst moderate and low antifouling activity was observed on EA and MeOH extract respectively. Disc diffusion method was used to test antibacterial activity against *S. aureus* and *E. coli*. Three solvents which are DCM, EA and MeOH with different concentration were used as solvents in testing the antibacterial activity of bacteria. Antibacterial activity of the crude extract was obtained by measuring the inhibition zone. The results using this test revealed that, maximum inhibition zone of 11.50 ± 0.76 mm and 10.50 ± 0.27 mm were produced by DCM extract at with concentration of 200 ppm against *S. aureus* and *E. coli* respectively. Minimum inhibition zone of 9.17 mm ± 0.17 mm was produce by DCM extract with concentration of 70 ppm against *S. aureus.* As for *E. coli,* a minimum zone of 9.33 ± 0.16 mm was produced by EA extract with concentration of 150 ppm. From the study, it can be concluded that extract using DCM is the best for antifouling activity as it showed the highest antifouling activity for both tests.

Keywords: Antifouling, periostracum, *Corbicula fluminea*, antibacterial

Aktiviti Anti-kotoran oleh Ekstrak Periostrakum Etok (*Corbicula fluminea*)

ABSTRAK

Satu kajian telah dijalankan untuk mengesan aktiviti anti-kotoran pada periostrakum *Corbicula fluminea* terhadap dua bakteria, iaitu *Staphylococcus aureus* dan *Escherichia coli*. Periostracum kering diekstrak dengan diklorometana (DCM), etil asetat (EA) dan metanol (MeOH) dengan kepekatan berbeza. Oleh kerana sifat-sifat polar pelarut, aktiviti antifouling yang tinggi telah dilihat pada ekstrak DCM manakala aktiviti antifouling sederhana dan rendah telah diperhatikan pada ekstrak EA dan MeOH. Kaedah penyebaran cakera digunakan untuk menguji aktiviti antibakteria terhadap *S. aureus* dan *E. coli*. Tiga pelarut iaitu DCM, EA dan MeOH dengan kepekatan yang berbeza digunakan sebagai pelarut dalam menguji aktiviti antibakteria bakteria. Aktiviti anti bakteria diperoleh dengan mengukur zon perencatan. Keputusan menggunakan kaedah penyebaran cakera mendedahkan bahawa, zon perencatan maksimum 11.50 ± 0.76 mm dan 10.50 ± 0.27 mm dihasilkan oleh ekstrak DCM pada kepekatan 200 ppm terhadap *S. aureus* dan *E. coli*. Zon perencatan rendah sebanyak 9.17 ± 0.17 mm dihasilkan oleh ekstrak DCM pada kepekatan 70 ppm terhadap *S. Aureus.* Manakala bagi *E. coli,* zon perencatan terendah sebanyak 9.33 ± 0.16 mm dihasilkan oleh ekstrak EA pada kepekatan 150 ppm. Sebagai kesimpulan, ekstrak DCM merupakan ekstrak terbaik bagi aktiviti anti fouling.

Kata kunci: Anti-kotoran, periostrakum, *Corbicula fluminea*, extraks, anti bakteria

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LIST OF ABBREVIATIONS AND SYMBOLS

TBT	Tributylin
DCM	Dichloromethane
EA	Ethyl acetate
MeOH	Methanol
Ppm	Part per million
mL	Milliliter
Rpm	Revolutions per minute
CFU	Colony forming unit
Mm	Micrometer
Nm	Nanometer
°C	Degree Celsius
µL	Microliter
%	Percentage

CHAPTER 1

INTRODUCTION

1.0 Research Background

The attachment of unwanted organisms of both microorganisms and macro-organisms on the surface of both marine organisms and man-made structure immersed in the sea are known as biofouling (Nikolaou, Neofitou, Skordas, Castritsi-Catharios, & Tziantziou, 2014; Grandison, Scardino, & Ovenden, 2011). The very first step of marine biofouling was caused by the formation of biofilm or slime in which derived from the adhesion of microorganisms such as bacteria, diatoms and microalgae to submerged surfaces (Kim et al., 2009; Mitbavkar & Anil, 2008). According to Kim et al. (2009), those marine biofilm such as bacteria and diatom are difficult to be controlled by using pretreatment methods. This is due to some microorganisms managed to survive after pretreatment. The form of biofouling can be characterised by the level of thickness, structure, composition, strength of bioadhesive and the weight of fouling organisms (Gregory & Bhusan, 2012).

Antifouling is the technique of discarding or restricting the accumulation of biofouling from forming. There are several types of antifouling agents that widely being used. This include copper, zinc and herbicide which contain toxic coatings such as

tributylin (Grandison et al., 2011; Yebra, Kiil, & Dam-Johansen, 2004). According to Nikolau et al. (2014) copper and zinc were known as effective agents against the biofouling but their uses as antifoulant agents were displeasing for marine environment, health and marketing prospects. These metals are listed under EU Dangerous Substances Directive, as harmful substances against aquatic organisms because of their toxicity.

Corbicula fluminea is one of the species under kingdom Animalia. It derived from phylum Mollusca under the class of Bivalvia. It is a species of Order Veneroida, genus Corbicula and family Corbiculidae. It is also noted as Asian clam. *Corbicula flumidnea* is a filter feeder in which can be distinguish by their shell morphology and colour (United State Fish and Wildlife Service, 2011; Sousa et al., 2007). This Asian clam is differentiated by the yellow-green to light yellowish-brown external shell and the inside of their shells are white to light blue or purple in colour (Thorp & Covish, 2010). The outline of the shell is either triangular or round. The beak is placed at the centre of dorsal slide and inflated (Kramer-Wilt, 2008).

The Asian clam can be abundantly found at or slightly below the surface of the sediment, in freshwater ecosystems. It also can be found on brackish and estuarine habitat. Most species of mollusc have a thin, pliable, proteinaceous covering on the outer shell, known as periostracum. According to Grandison, Scardino and Ovenden (2011), mollusc's mussel is able to resist fouling when the shell coating is in good condition. It also stated that the shell coating may contain some of chemical defense against unwanted fouling organism.

1.1 Problem Statement

Antifouling is referred as the potential of both natural and synthetic objects to eliminate or inhibit biofouling. Biofouling is the attachment of microorganisms and macro-organisms on the surface of submerged objects. According to Cao, Wang, Chen and Chen (2011), in order to eliminate fouling organisms, a broad-spectrum of biocides are used. Mussel adhesive protein is one of the example of antifouling agent that can be found in the world. Most molluscan shells have specific chemical defense mechanisms which will protect them from predators and epibiotics (Sui et al., 2017; Paul, Puglisi, & Ritson-Williams, 2006). Potential chemical defense of molluscs is rarely been studied. A study by Bers, D'Souza, Klinjstra, Willemsen and Wahl (2006), mussels are able to resist fouling when in good physiological condition. It was stated that the blue mussel *Mytilus edulis*'s shells are free from biofouling as long as the shell is covered by periostracum. The result showed that mussels with periostracum has high resistance to fouling organisms.

1.2 Objectives

1. To determine the antifouling activity of *Corbicula fluminea* through periostracum extract.
2. To determine the antibacterial properties of *Corbicula fluminea*.
3. To investigate the effective solvent for antifouling and antibacterial agents.

1.3 Hypothesis

H_0: There is no antifouling and antibacterial activity of *Corbicula fluminea*

H_1: There is antifouling and antibacterial activity of *Corbicula fluminea*

If $P > 0.05$, H_0 is rejected.

If $P < 0.05$, H_0 is accepted.

1.4 Scope of Study

This research focus on whether or not there is an antifouling activity of shell coating of *Corbicula fluminea* against marine fouling organisms and bacteria that can be found in seawater. The research was performed on periostracum layer of *C. fluminea*. Periostracum can be extracted from the shell of Asian clam. It can be obtained by serial extraction with three solvent. The extract sample was tested by antifouling assay. Gram staining method was used to analyse the end result (Grandison et al., 2011).

1.5 Significance of Study

The antifouling test and the disc diffusion method was helpful to determine antifouling activity of periostracum peels of *C. fluminea*. The preliminary method in the determination of the antifouling activity will be functional for further research to investigate whether the crude extract of methanol, ethyl acetate or dichloromethane has the highest antifouling activity. Besides, in the future, the antifouling agent found in the Asian clam can be combined with current antifouling agent to lessen the side effect of the synthetic

antifouling coating such as copper and zinc in the environment as well as health and economic aspect. The important of this study is to reduce the negative effect of biofouling in environment as well as to reduce the use of toxic material as antifouling agents. The purpose of choosing Asian clam as antifouling agent because of its abundancy in Kelantan as well as there was limited research about its potency as antifouling agent.

Biofouling is commonly related with marine fields where notable aquatic growth appears on a ship hull and structure immersed in the sea. The negative impact of biofouling include an increase in maintenance and operation costs especially in marine industry, corrosion as well as fuel requirements due to the increment of ship hull drag. There have been a slow progress in searching for an effective antifouling agents. The example of antifoulant available are mostly contain harmful metal such as zinc, copper, and tributyltin. Because of high level of toxic, a tributyltin-free self-polishing paints based on copper were developed. Nonetheless this tributyltin-free paints were ineffective against certain widespread copper tolerance algal species.

Few research have been developed in order to search for toxic-free anti-foulant and this leads to the development of periostracum of mollusc as antifouling agents. A study on the function of periostracum of blue mussel, *Mytilus edilus* showed that mollusks are able to resist fouling organism as long as the perioustracum is in a good condition. This prove that periostracum can be a pontential sources of chemical and physical antifouling defense. Hence, the aim of the study is to determine the potential of periostracum extract of Asian clam as a source of antifouling substance.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofouling

Biofouling is defined as the accumulation of organisms on submerged surfaces. Both natural and synthetic object immersed in the marine environment are typically colonised by microorganisms. The initial caused of biofouling is the formation of biofilm, including microorganisms and macro-organisms such as bacteria, barnacles and others (Holm, 2012). Biofilm or slime can be defined as the colonisation of microfouling and macrofouling organisms over the fouled surface either in the form of film or layer. The example of microfoulers are bacteria, diatom, protozoa and other microorganism, whilst macrofoulers divided to 'hard fouling' and 'soft fouling'. Hard fouling include barnacles and mussels, whilst soft fouling include algae and invertebrates, such as soft corals, sponges, and anemonoes (Flemming & Wingender, 2010; Kim et al., 2009; Callow & Callow, 2002).

Bacteria and diatom play a crucial role in manipulating the biofilm development, in which they provided information for the settlement of macrofouling. Generally, bacteria play majored role of triggering attachment and early colonisation on the submerged surface, altering it to favour the development of more complex community. The first formation of biofilm associated with bacteria, but sometimes diatom also can become the

first coloniser (Khandeparker, D'Costa, Anil, & Sawant, 2014). Fouling can rapidly developed within days to weeks depending on their communities. The driven factors caused the fouling to vary with each other include the introduction of new recruits, growth period, or times of dormancy and regression (Fitridge, Dempster, Guenther & Rocky de Nys, 2012).

Environment, deposition of inorganic matter and organisms affected the form and level of fouling. The morphology of biofouling can be categorised by the thickness, density, structure, composition, strength of bioadhesive and weight of fouling organisms (Khandeparker et al., 2014; Gregory & Bhushan, 2012). The microbial biofilm composed of a complex and heterogenous microbial population in the form of gelatinous matrix or 'slime'. The structure of these slimes varies on microscopic level, from filamentous growth to gelatinous films, which are barely visible to the naked eye.

2.1.1 Factor affecting biofouling formation

The control of fouling are commonly based on physiology and dispersal of fouling organisms. Factors that influenced the attachment of fouling onto a surface when exposed in the sea or water are geographical and seasonal distribution, movement of water relative to the surface, texture of the surface, gravity effect, light and illumination, electricity usage, wetting of surface, exfoliation, and slime (Madin & Chong, 2015).

Generally, the geographical location defines whether certain structure were colonised with intense fouling attachment. The number of fouling organisms present in the water differ in each regions and in certain area at different times of year. Madin and Chong (2015) stated that fouling organisms in tropical waters is more diverse compared in temperate water region despite a short period of immersion. Furthermore, the composition and domination of the species onto the surface of floating fish cages were site specific. The intensity of fouling organisms are difficult to predict based on general aspect of region since local variations are importance. The movement of water can influenced the attachment of fouling. According to study performed by Delauney, Compere, and Lehaitre (2010), active ships tend to be less fouled compared to those stationary ships.

Another factor that affect biofouling is the texture surface of the structure. Both micro- and macro-fouling organisms are incapable to colonise onto smooth surfaces. Most manufacturers tried to produce smooth surface paints in order to minimise the frictional resistance. Biofouling also depends on the effect of gravity. Several studies have proven that fouling organisms will react to the force of gravity. Intense fouled was observed on the under-side of a horizontal surface compared to surface exposed at any other angels.

Fouling organisms tend to accumulate on darker surface since they are sensitive to light. This can be seen in the distribution of fouling organisms that produced by gravity. The underside of structures is darker than upper-side. The quantity of fouling accumulated on the surface of the structure vary depends upon its shape, material, and the service used.

2.1.2 Impact of Biofouling

Biofouling not narrowed purely on natural system, it also can occur in man-made systems involving the flowing water such boats, barges, buoys, ropes, nets, cages, pipelines, heat exchanger, cooling towers, filter beds, and other packing operated in waste-water treatments (Fitridge et al., 2012). From the listed above, it showed that biofouling in aquaculture is more specific into structure, material, and site and stock. Sometimes, marine biofouling also can occurred on shellfish stock such as oysters, mussels, scallops and prawns. These shellfish stocks are consumed by human, thus it also can affected human health. In order to tackle this problem, the shellfish stocks needed to undergo antifouling treatment before they can be consumed (Simone Dürr & Jeremy, 2009). Non-flowing systems are also prone to biofouling for instance, in water-sealed gas holder and calorimeter.

The impact of biofouling includes physical damage, mechanical interference, disease outbreak on aquatic organisms, environmental impact, restriction of water exchange, cages and structure deformation, high consumption of fuel, and increase economic costs for maintenance. As for example, biofouling effect the ship hulls to increase in frictional resistance, thus it will cause the rise in power and the consumption of fuel as it required more power and fuel to increase the speed (Fitridge et al., 2012; Briand, 2009; Yebra et al., 2004). Besides, it also increased labour costs because there is high requirement for cleaning. Biofouling also can has a detrimental effect on environment (Simone Dürr & Jeremy, 2009; Callow & Callow, 2002).

Marine industry uses some technologies and husbandry technique to minimise the consequence of biofouling. Biofouling control is a specific and complex problem depends to each industry. The development of technologies is required to reduce the consequence on non-target organisms and the culture environment (Fitridge et al., 2012). Besides, common control also existed to control and reduce biofouling. A low-drag surface and low-adhesion surfaces are used in fluid flow systems. The function of low-drag is to enhance microorganisms' removal, while low-adhesion surface inhibit colonization of microorganisms through reduced adhesive strength (Gregory & Bhushan, 2012).

2.2 Antifouling

Antifouling is the process of discarding or restricting the accumulation of biofouling from forming. Previous stated by Magin, Cooper and Brennan (2010), antifouling was associated with biocidal compound, until recently, researcher discovered more environmentally antifouling which is less or non-toxic. The force required to discard organisms accumulate to a surface is termed as fouling-release. There were several methods to treat and remove biofouling which include both chemical and physical methods, and a combination of both chemical and physical methods (Gregory & Bhushan, 2012). Ogunola and Onada (2016) and Railkin (2004) said that physical defense against biofouling is creating a mechanical boundary to block the colonisation of both micro- and macro-fouling. The example of physical methods include low–drag, low adhesion, wettability, micro-texture, grooming, and sloughing (Gregory & Bhushan, 2012). Railkin (2004) also stated that chemical defense against biofouling is based on the action of

chemical factors on the foulers. The example of chemical techniques comprise variety of secretions, antifouling coatings, copper sulphate treatment and others.

2.2.1 Antifouling coatings

Antifouling coatings had been used as a medium for protection against fouler for a really long time. Antifouling paint has been used widely in marine industry especially on surface of marine transport, and oil and gas industries. Antifouling paints have undergone dramatic transformation since 1952. It involved insoluble and soluble matrix paints, the aid of biocides, including heavy metals like cooper and zinc, self-polishing copolymer paints and other compound (Yebra et al., 2004). Most of the chemicals and heavy metals used in antifouling paint are dangerous in the environment and has a harmful impact on the survival and growth of shellfish (Fitridge et al., 2012). The used of tributyltin has been banned by the International Maritime Organisation (IMO) due to its toxicity which affect the environment negatively (Kang et al., 2016).

Even though these synthetic coatings can protect the immersed surface from the fouler, it also has a detrimental effect on environment and it can attacked non-targeted species if they do not degraded rapidly (Callow & Callow, 2002). For example, the use of lead sheathing caused decomposition of iron constituents of ships. Besides, the use of tributyltin, TBT in antifouling coating triggered a loss in production of oyster (Yebra, 2004). Because of that, most countries boycotted the use of TBT-based paints for small vessels. From the table below, it can be summerised that most antifouling coatings available in market used toxic heavy metals such as zinc, copper, and tin.

Table 2.1: Antifouling coatings (Gregory & Bhusan, 2012)

Type	Mechanism
Metal coating	Toxic copper or silver-coated surfaces
Self-polishing copolymer	The used of copper, zinc, tin or biocides in self-polishing paint released during vessel movement
Hydrogels biological	The used of hydrophilic surface can confuses settlers deterrents such as bacteria, algae and invertebrates
Enzymes	Decreases bioadhesion and lyses bacteria
Hormones	Stimulates premature metamorphosis of larva
Short fibres	Fibres or spikes can confuses attached biofouling
Conductive paints	Disinfection of electrochemical process toxic flexible metal surface
Plastic film	Attached biofouling will be removed with disposable film
Photoactive film	Self-cleaning film with the application of UV or visible radiation
Cayenne pepper	Deterrent pepper with the grease of silicone

2.2.2 Antifouling Test

Most antifouling compounds that have been screened by the submerged take longer time to remove the biofouling and the composition of the compounds have a high level of toxicity. A number of countries have come into agreement to ban the use of antifouling coatings based on organotin compounds such as tributyltin. This leads to an

increase in demand for environmental friendly antifouling compounds. Several methods have been developed using natural products for instance, physical mechanisms of defense of invertebrates such as topography, mucus production, and partly by producing biochemical that provide them with a chemical defense against a precise group of fouling organisms. A development of bioassay method was introduced in order to know the implication of natural product on inhibition of biofouling organisms (Briand, 2009).

Blue mussel, *Mytilus edulis* has been used as a new bioassay technique for screening antifouling substances. Antifouling substances that have been used were cuprous sulfate and triphenyltin acetate. The result showed that repellent activity of the mussel could not be estimated clearly due to high toxicity. The advantages of this technique is it is time-saving to screen for a compound that have repellent activity and with less or no toxicity (Kang et al., 2016).

Another antifouling bioassay that have been developed from external shell covering is periostracum of marine mussels. A study was conducted by Grandison et al. (2011) is to determine antifouling activity of blue mussels' periostracum extract. The extracts were produced using three different solvents, which were dichloromethane (DCM), ethyl acetate (EA), and methanol (MeOH). These extract were tested against three common fouling organisms. The result showed that extracts produced from DCM and EA have high antifouling activity compared to extract from MeOH. DCM and EA displayed non-polar characteristics therefore it easier for the compound to extract both solvent. From the study by Grandison et al. (2011), it reported that there was no antifouling activity using 50 ppm methanol, ethyl acetate and dichloromethane. As for 100 ppm solvent, only ethyl

acetate and dichloromethane showed antifouling activity against biofouling. A high antifouling activity was recorded on 200 ppm and 250 ppm of crude extract of dichloromethane.

Moreover, a study on algal metabolites by Lachnit, Wahl and Harder (2010) have been developed in short-term field assay. The secondary metabolites of algae were immobilized in hydrogels form. After seven days, they found that the hydrogels had a limiting activity against both micro- and macrofouling. Another similar technique also been used to investigate the effectiveness of algal metabolite against biofouling. In a study conducted by Persson et al. (2011), sesquiterpene elatol and dolabellane were isolated from red algae and brown algae and immobolised for weeks. The result showed, the hydrogels have a significant antifouling activity. Thus, they concluded that the secondary metabolites of algae were suitable to become bioassay.

2.3 Periostracum

Periostracum is a chitinous layer which covered the shell of molluscs. This layer served as a protection from acid dissolution, boring organisms and predator, a medium for the deposition of calcium carbonate crystals and to rise the recruitment and fertilisation success. Periostracum is the first part of shell that formed. Periostracum is secreted from the mantle edge, but the formation mode is vary from species to species. The periostracum is divided into three compartments, forming, free and outer periostracum. The first part which is forming periostracum is located at the base of periostracal groove. The free periostracum is located from the edge of mantle lobe to the edge of the valve. (Salas,

Marina, Checa & Rueda, 2012). The thickness depends on the amount of mechanical abrasion (Shumway & Parsons, 2006). Bers et al. (2006) stated that molluscs are able to resist fouling when in good physical condition. Generally, periostracum is a pontential chemical defense mechanisms of many molluscs.

2.4 *Corbicula fluminea*

Corbicula fluminea or Asia clam is distributed mainly in Asia, Africa and Australia. It is then been spread worldwide as a result from human activity (McMahon, 2002). The taxonomic of *C. fluminea* is it is a species under domain Eukaryota, kingdom Metazoa and phylum Mollusca. *Corbicula fluminae* is species under class bivalvia, subclass Heterodonta and order Veneroida. Corbicula fluminea is under family Corbiculidae and genus Corbicula. The most distributed *Corbicula* species are *C. fluminea* and *C. fluminalis*. These two are often mistaken due to their almost identical morphology. *Corbicula fluminea* is a freshwater habitat, whilst *C. fluminalis* is found at estuarine ecosystem (Centre of Agriculture and Biosciences International, 2017; Hiebert, 2015; Aldridge, Madhyastha, & Van Damme, 2012).

Figure 1.0: *Corbicula fluminea*

Corbicula fluminea can be categorised based on shell morphology and colour. The shape of Asian clam is oval-triangular with a dorsal beak at the top of the shell. *Corbicula fluminea* has a tan exterior and a white in the exterior part of the shell or sometimes with purple marking. In general, bivalve shell consists of outer periostracum, middle prismatic layer or ostracum, and inner lamellar layer or hypostracum. *Corbicula fluminea* has a well-developed and visible periostracum and the inner layers. However, its ostracum layer is reduced. A thick periostracum is present on surface of the shell. The colour of periostracum range from olive, yellowish or black-brown and composed of protein conchiolin. This shell covering will become darker as the clam ages. The inner side of periostracum is a chalky white prismatic layer which composed of calcium carbonate crystals. The nacre of *C. fluminea* is white-bluish in colour (Hiebert, 2015; Thorp & Roger, 2015).

As stated by Sousa and Guilhermino (2008), species of genus *Corbicula* embrace different reproductive modes, which related to its huge ecological spectrum. *Corbicula fluminea* also described as hermaphrodites species which is capable of self-fertilisation. Asian clam can lay up to 70,000 eggs per year. Because of their high productivity, Asian clam compete with other species for food and space. They can also cause major biofouling in power plants, water treatments systems and pipes (http://www.seagrant.wisc.edu, 2013). Another characteristics of Asian clam are they has become a main component of benthic communities, can be found in both clean and polluted environment, can easily be maintained in the laboratory and they have an excellent filtration capacity.

2.5 Bacteria as biofouling agents

Interactions between microfouling components influence the biofilm community and the flowing events subsequently playing an important role in the biofouling process (Khandeparker, Costa, Anil & Sawant, 2014). Bacteria play a crucial role in manipulating the development of the biofilm, and are closely associated with diatoms (Holm, 2012). Bacteria provide signals for the settlement and metamorphosis of various macrofouling organisms which are algae, ascidians, barnacles, bryozoans, hydroids, oysters and polychaetes (Khandeparker et al., 2014).

Both gram positive and gram negative bacteria were used as biofouling agents in antifouling test. The example of bacteria used range from *S. aureus, S. epidermidis, S. marcescens, K. pneumoniae, S. typhi,* and *E. coli*. Gram negative bacteria are more susceptible to antifouling agents compared to gram positive bacteria. In a study conducted by Palanicharmy and Subramanian (2017), antifouling agents showed more activity against gram positive bacteria.

2.5.1 Characteristic of *Staphylococcus aureus*

Staphylococcus aureus is a gram positive bacteria that can be found in respiratory tract, nose and on the skin. It is also known as golden staph. *Staphylococcus aureus* is a round shaped bacterium under phylum Firmicutes (Liu et al., 2015). Golden staph is a

facultative anaerobe which can produce energy either in presence or absence of oxygen. *Staphylococcus aureus* is non-motile bacterium and it does not procedure spores.

The morphology of *S. aureus* is in grape-like cluster. The gram staining colours of *S. aureus* is purple. Typically, gram positive bacteria have massive cell walls composed of peptidoglycan. The reproduction mode of *S. aureus* is asexual through binary fission (Hale, 2013). One of the characteristic of *S. aureus* is it can produce the catalase enzyme. *Staphylococcus* bacteria is often mistook as enterococci bacteria and *streptococcus* bacteria. In order to distinguish these three, catalase activity tests were used (Kateete et al., 2010). *Staphylococcus aureus* is found in the formation of biofilm on the surface of medical device implanted in the body or on human tissue (Schlecht et al., 2015).

2.5.2 Characteristic of *Escherichia coli*

Escherichia coli is a rod-shaped gram negative bacteria which abundantly found in the lower intestine of warm-blooded organisms. It is a facultative anaerobe bacterium under phylum Proteobacteria. Most *E. coli* strain is harmless however it can cause severe food poisoning in human. *Escherichia coli* strain can be either motile or non-motile and it does not form spores. It is motile due to peritrichous flagella. Capsules and fimbriae are present in some strains (Todar, 2012).

Escherichia coli arranged singly or in pairs and can grow over a wide range of temperature from 15 – 45°C. The optimum temperature for the bacteria to grow is 37°C

(Aryal, 2016). The gram staining colour of *E. coli* is pink as its cell wall composed of thin peptidoglycan layer and an outer membrane. *Escherichia coli* takes up the colour of counterstain safranin and stains pink (Gould, 2014).

2.6 Gram Stain

Gram stain is the simplest technique to identify bacteria. It is a method of staining cells either in pink or purple depending on their cell wall properties. This technique was discovered by Christian Gram. Crystal violet, iodine, alcohol and safranin are used in Gram stain method. Both iodine and crystal violet will form large complexes which attached to the cell and turn the cell into purple. The function of alcohol in this method is decolourised the bacteria by washing the outer lipid layer away from the cell (Spear, 2017).

During this process, gram positive bacteria will lose some of its massive chunky peptidoglycan cell wall. The remaining cell wall is enough to retain the purple colour whilst, the gram negative bacteria with small peptidoglycan layer appeared colourless. When the safranin were introduced, gram negative bacteria will change from colourless to pink. However, the safranin stained is not strong enough to disturb the purple stained of gram positive cell. Gram negative bacteria are unaffected to antibiotics, nevertheless it is more prone to detergent (Gould, 2014).

2.7 Disc Diffusion Method

There are several methods for antibacterial susceptibility testing of bacteria. One of the method is disc diffusion method. This test allows categorisation of most bacterial isolates either as susceptible, intermediate, or resistant to a variety of antibacterial agents. To carry out the test, prepared filter paper discs impregnated with a specified single concentration of an antibacterial agent are applied to the surface of an agar medium that has been inoculated with test organism. The extract in the disc diffuses through the agar. The result of the test is determine by the diameter of inhibition zone. If there is no growth occurs in the areas where the concentration of extract is inhibitory, inhibition zone around disc is forming (Patel, Tenover, Turnidge, & Jorgensen, 2011).

CHAPTER 3

METHODOLOGY

3.1 Collection of *Corbicula fluminea*

The sample were collected from Pergau, Kelantan. The sample were stored in freezer at -20°C prior to sampling in the laboratory of University Malaysia Kelantan Jeli Campus area (6°09′52″N 102°16′57″E). The shell of the sample were cleaned for solvent extraction.

3.2 Removal of periostracum

The soft body of Asian clams were removed from the shell (Bers et al., 2006). The samples were submersed in a 1:2 vinegar seawater mixture to loosen the periostracum and retained in the solution for 24 hours. The periostracum was peeled from the shell using forceps and kept in seawater. The extracts were dried and ground to a fine powder using an electric grinder (Grandison, 2011).

3.3 Solvent Extraction

The extractions were performed on the ground periostracum. Three solvents which were dichloromethane (DCM), ethyl acetate (EA) and methanol (MeOH) were used. The

solvents were utilised to increase polarity. The solvents and the ground periostracum samples were extracted in soxhlet apparatus (Ibtissam et al., 2009). The extraction using Methanol were performed twice. Extracts were filtered and concentrated using rotary evaporator. Samples were transferred to the glass petri dish after the petri dish have been weighed. The samples were dried under the fume hood. Samples were weighed for the second time to obtain the weight of the sample. To know the concentrations, the samples were reconstituted using appropriate bioassay solvent (Grandison et al., 2011).

3.4 Inoculum preparation

Single colony of *Staphylococcus aureus* and *Escherichia coli* were streaked from agar plate that can be obtained using a sterile loop. The colony were transferred into a falcon tube containing 40ml of nutrient broth (NB) and incubated while shaking at 150 rpm at 37°C overnight. The organism suspension were adjusted to the density of 0.5 McFarland standards. It is equivalent to 1 x 10^7 colony-forming units (CFU/ml). The falcon tube were centrifuged, in which the supernatant were discarded and the pellet were diluted in the sterile solution. To blank the spectrophotometer, the distilled water was used at 600 nm wavelength. The bacterial absorbance were measured and the density was adjusted by adding sterile saline water. The cuvette was wiped to make sure that there is no obstruction from light. The suspension were used within 10 minutes (Gurunathan, Han, Kwon & Kim, 2014).

3.5 Antifouling test

The test was carried out to identify bacterial growth and attachment on surfaced immersed in seawater. Approximately 25 ml of nutrient broth was added into 50 ml coning

tube. The glass slides were soaked in 70% ethanol and placed in each coning tube. The tubes were then been autoclaved. Next, the cultured bacteria which were *S. aureus* and *E. coli* were inoculated 100µl in each tube. The tubes were incubated for 37°C overnight. The extracts were pipette 90µl into the tube and were rolled overnight. Approximately 1% of crystal violet was used to stain the slide for one minute. Then, the slide was rinsed with distilled water. The slide was covered with iodine for one minute. The slide was rinsed again with distilled water. Acetone was used to clean the slide. Then, the slide was covered with safranin for 45 seconds and rinsed with tap water. Clear slide shows that there is antifouling activity. Bacteria will be grown on the glass slides and incubated without any extract is the negative control (Palanicharmy & Subramanian, 2016).

3.6 Disc Diffusion Method

Nutrient agar were prepared and autoclaved. Sufficient amount of agar were poured into petri dishes under the laminar flow. The plates were inverted after the agar has hardened. A punctured Whattman filter paper were sterilised to obtain a 7 mm filter paper disc. The crude extract sample were serially diluted to obtain four different concentration, 70 ppm, 100 ppm, 150 ppm and 200 ppm. From each dilution, 30µl was impregnated on the sterile filter paper disc. It then was allowed to dry before placing on the prepared nutrient agar plates. The lid of the petri dish was partially moved for filter paper disc application. The forceps were sterilised by soaking in ethanol and ignited them each time a new disc will be applied. The plate was sealed with a parafilm after all discs have been prepared to minimise exposure to air. The plates were incubated for 24 hours and the zones of inhibition will be observed. A plastic ruler were used to measure the diameter of the inhibition zones (Redfern, Kinninmonth, & Verran, 2014).

3.7 Data Analysis

The result of antifouling test were analysed by observing the stained appearance on the slides. Whilst, the data of disc diffusion test was analysed by using one way ANOVA, Analysis of Variance with post-hoc using SSPS software version 21 to determine the significant value.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Crude extract preparation

Solvents extraction were performed by using soxhlet apparatus and rotary evaporator. The solvents were extracted for eight hours in soxhlet apparatus. Approximately, 30 gram of ground periostracum were used for solvent extraction. Dichloromethane (DCM), ethyl acetate (EA) and methanol (MeOH) were used as extractor as these three are different in polarity. MeOH has the highest polarity compared to DCM and EA. While DCM is characterised as moderate polar solvent and is termed as best solvent for wide range of reactions. Moreover, all three solvents being used is known as non-participatory solvent which served to dissolve the reactants. The function of polar solvent is to dissolve polar reactant, whilst non-polar solvent is used to dissolve non-polar reactant (James, 2012). The solvents were wrapped with aluminum foil and stored in the chiller at 4°C after extraction to avoid adsorption of the extract into the air.

4.2 Antifouling test

A total of three crude from one species of mollusc *Corbicula fluminea* were test against two bacteria for their antifouling activities. Two bacteria that been used are *Staphylococcus aureus* and *Escherichia coli*. The antifouling activity was observed on the glass slide. Table 4.1 showed the result of gram stain by observation of clearness on glass slides. High antifouling activity was recorded on glass slides with DCM extract for *S. aureus* whilst moderate antifouling activity occurred on glass slide with EA extract for gram positive bacteria. Low antifouling activity was observed on glass slide with MeOH extract for *S. aureus* whilst no antifouling activity was detected on the glass slide incubated with *E. coli*.

Table 4.1: Antifouling activity of periostracum extract

Bacteria	Crude			
	Control	Dichloromethane	Ethyl Acetate	Methanol
S. aureus	-	+++	++	+
	-	++	+	+
	-	++	+	-
E. coli	-	++	+	-
	-	++	+	-
	-	+	+	-

Key:

(-) no antifouling activity

(+) low antifouling activity

(++) moderate antifouling activity

(+++) high antifouling activity

Table 4.2 showed the appearance of post gram stain of the glass slide against *S. aureus* and *E. coli*. For *S. aureus*, glass slide with DCM extract showed high antifouling activity as the glass slide shows low staining of gram positive bacteria. The glass slide of EA extract was moderately stained in purple, whilst low staining of *S. aureus* was detected on the glass slide with MeOH. As for *E. coli*, moderate stained was observed on glass slide with DCM extract. Glass slide with EA extract showed low staining whilst, glass slide with MeOH extract showed no staining.

Table 4.2: The appearance of slide of various periostracum extract after gram stain

Bacterial strain	Crude		
	Dichloromethane	Ethyl Acetate	Methanol
Staphylococcus aurues			
Escherichia coli			

From the Table 4.1 and 4.2, it can be observed that the periostracum of *C. fluminea* can resist biofouling. The result of this test support previous published research by Grandison et al. (2011) concerning biocidal activity of periostracum extracts. Several research been conducted regarding the antifouling properties of the periostracum against common fouling organisms. Bers et al. (2009) stated that mussels have a multilevel antifouling defense on their shells. The defense include cumulative filtration and chemical repellents in the periostracum. The finding of the research indicated that the periostracum of the mussel does inhibit bacterial settlement on the surface immersed in the water. However, the inhibition of bacterial colonisation were considered low as majority of the extract exhibited low antifouling activity.

Some of the errors that occurred during the study are the electric grinder was not completely dried and crushed when grinded the clams. The sample cannot be efficiently extracted because the solvent cannot easily penetrated the moist sample as the sample would accumulated together and this will reduce the surface area of the sample. According to Refern, Kinninmonth and Verran (2014), greater surface area allows the solvent to dissolve or extract the majority of the samples. Besides, during storing the extract in the chiller, the media bottle was not secured with parafilm. Some of the extract may escape into the air. Another factor that should be reconsidered when performing antifouling test are the surface condition of the immersed structure. According to Delauny et al. (2010), microfouling and macrofouling organisms are incapable to colonise onto smooth surfaces. For further research, it is advice to use coarse surface of the structure instead of using glass surface.

4.3 Disc Diffusion Method

Table 4.3 showed the antibacterial activities of different concentration of periostracum extracts against test bacteria. For dichloromethane extract, a maximum inhibition zone of 11.5000 ± 0.7638 mm was produced by 200 ppm extract concentration against *Staphylococcus aureus* and 10.5000 ± 0.2887 mm against *Escherichia coli*. At concentration of 70 ppm, a minimum inhibition zone of 9.1667 ± 0.1667 mm was produced against *S. aureus* and 9.0000 ± 0.0000 mm against *E. coli.* Moderate inhibition zone of 9.5000 ± 0.2887 mm were produced by extract concentration at 100 ppm and 150 ppm against *E. coli.* As for *S. aureus,* at concentration of 100 ppm and 150 ppm, inhibition zone of 9.3333 ± 0.3333 mm and 9.8333 ± 0.4410 mm was produce respectively.

For ethyl acetate extract, a maximum inhibition zone of 10.000 ± 0.2887 mm was produce by concentration of 200 ppm against *S. aureus*. Moderate inhibition zone of 9.5000 ± 0.2887 mm was produced by 150 ppm against *S. aureus* and 200 ppm against *E. coli*. Whilst, a minimum inhibition zone of 9.3333 ± 0.1667 mm was produced by 150 ppm concentration against *E. coli*. No inhibition zone was produced by 70 ppm and 100 ppm concentration. As for methanol extract, only extract at concentration of 200 ppm produced inhibition zone of 9.6667 ± 0.2887 mm against *S. aureus.*

From Table 4.3, it can be seen that there was significant difference ($P < 0.05$) between the concentrations of dichloromethane against both bacteria. For *S. aureus*, concentration of 200 ppm was significantly difference from 70 ppm and 100 ppm. However, there was no significant difference ($P > 0.05$) between the concentration of 70 ppm, 100

ppm and 150 ppm. As for *E. coli*, there was significant difference between 200 ppm and 70 ppm and 100 ppm and 200 ppm.

Table 4.3: Zone of inhibition of different concentration of Dichloromethane extract against test bacteria

Concentration of solvent (ppm)		Bacterial strain showing inhibition zone (mm)	
		Staphylococcus aureus	*Escherichia coli*
Dichloromethane	70	9.1667 ± 0.1667[a]	9.0000 ± 0.0000[a]
	100	9.3333 ± 0.3333[a]	9.5000 ± 0.2887[a]
	150	9.8333 ± 0.4410[ab]	9.5000 ± 0.2887[a]
	200	11.5000 ± 0.7638[b]	10.5000 ± 0.2887[b]
Ethyl Acetate	70	-	-
	100	-	-
	150	9.5000 ± 0.2887[b]	9.3333 ± 0.1667[b]
	200	10.000 ± 0.2887[b]	9.5000 ± 0.2887[b]
Methanol	70	-	-
	100	-	-
	150	-	-
	200	9.6667 ± 0.2887[b]	-

Mean ± S.E. (Standard Error)

This test has evaluated the antibacterial activity of periostracum extracts of *C. fluminea*. Dichloromethane was proved to be good solvent in extracting the inhibitory compound from periostracum. The result obtained can support the finding of previous

studies. According to a study conducted by by Grandison et al. (2011), on antifouling activity of periostracum extract of blue mussel showed that DCM and EA extract have high antifouling activity than MeOH extract. Besides, a study performed by Bakht, Shaheen and Shafi (2014) showed that ethyl acetate showed inhibitory activity against all tested microbial species. The same result were obtained from a study conducted by Martins et al. (2013). High antibacterial activity of test samples was observed on DCM and EA extracts compared to MeOH extracts. Moreover, it also showed that the crude extract was more effective inhibiting the growth of gram positive bacteria than gram negative bacteria.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From this research, it can be concluded that periostracum extract of *Corbicula fluminea* display some antifouling effects, thus the H_1 was accepted. The result of antifouling test showed that slides with crude extracts inhibit the colonisation of bacteria on the structure immersed in the water as clear slides can be seen on the slides. The best solvent for extraction is dichloromethane (DCM). Furthermore, the study also revealed that the *C. fluminea* also possessed some antibacterial effects against gram positive and gram negative bacteria. DCM extract produced a highest inhibition zone of 11.5000 ± 0.7638 mm against *S. aureus*. The highest inhibition zone of 10.5000 ± 0.2887 mm was produced by DCM extract against *E. coli*. From the result obtained, majority extract concentration of 200 ppm produced the highest inhibition zone against gram positive and gram negative bacteria.

5.2 Recommendation

For further research, it is recommended to improve the methods of removing the without degrading the periostracum layer. Besides, for solvent extraction, it is recommended to use sonicator probe instead of soxhlet apparatus. Sonication treatment can improved the antibacterial activity of various methanol extract compared to extraction using soxhlet apparatus. Moreover, it is recommended to analyses the accuracy of antifouling activity by using Nuclear Magnetic Resonance (NMR), Gas Chromatography Mass Spectrometry (GCMS), Liquid Chromatography Mass Spectrometry (LCMS) or Fourier Transform Mass Spectrometry (FTMS).

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APPENDIX A

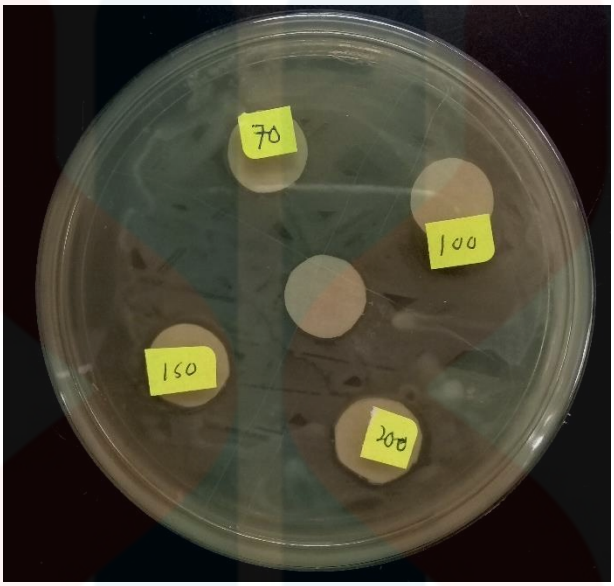

Figure A.1: Inhibition zone of *S. aureus* using different concentration of DCM extract

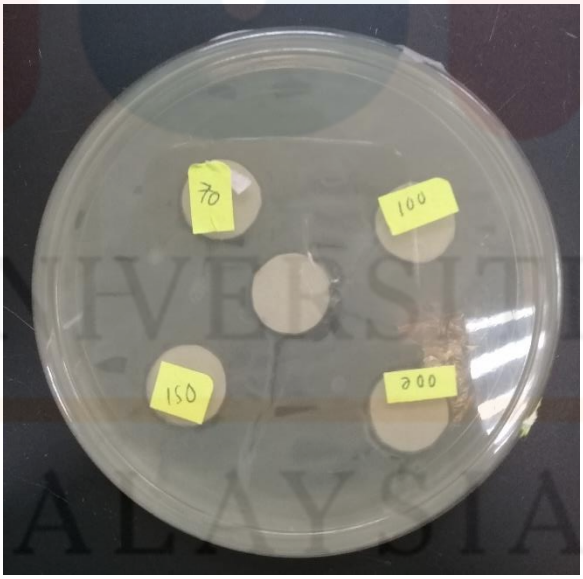

Figure A.2: Inhibition zone of *Escherichia coli* using different concentration of DCM extract

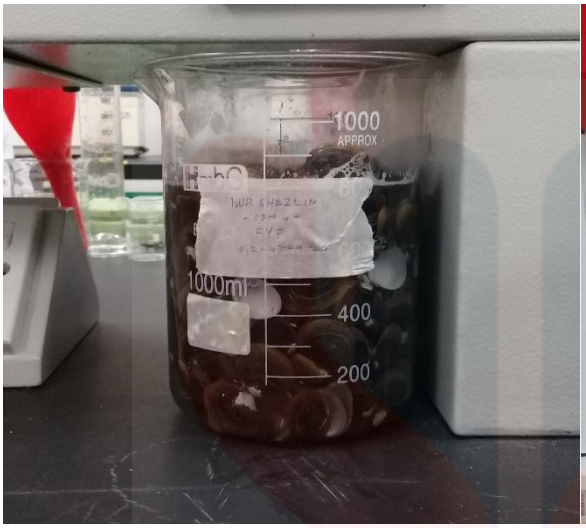

Figure A.3.: Immersion of *C. fluminea* in vinegar seawater solution

Figure A.4: Solvent extraction using soxhlet apparatus

Figure A.5: Filteration and concentration of extracts using rotary evaporator

Table A.1: One-way ANOVA of dichloromethane extract against test bacteria

		Sum of Squares	Df	Mean Square	F	Sig
S. aureus	Between Groups	8.750	3	2.917	4.118	.049
	Within Groups	5.667	8	.708		
	Total	14.417	11			
E. coli	Between Groups	3.563	3	1.188	6.333	.017
	Within Groups	1.500	8	.188		
	Total	5.063	11			

Table A.2: One-way ANOVA of ethyl acetate against test bacteria

		Sum of Squares	Df	Mean Square	F	Sig
S. aureus	Between Groups	285.563	3	95.188	761.500	.000
	Within Groups	1.000	8	.125		
	Total	285.563	11			
E. coli	Between Groups	266.063	3	88.688	1064.25	.000
	Within Groups	.667	8	.083		
	Total	266.729	11			

Table A.3: One-way ANOVA of methanol extract against test bacteria

		Sum of Squares	Df	Mean Square	F	Sig
S. aureus	Between Groups	285.563	3	95.188	761.500	.000
	Within Groups	1.000	8	.125		
	Total	285.563	11			

Table A.4: Post Hoc Analysis using Duncan Multiple Test for inhibition zone of different concentration of dichloromethane solvent against *S. aureus*

Concentration (ppm)	N	Subset for alpha = 0.05	
		a	b
70	3	9.1667	
100	3	9.3333	
150	3	9.8333	9.8333
200	3		11.3333
Significant		0.3790	0.0610

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.5: Post Hoc Analysis using Duncan Multiple Test for inhibition zone of different concentration of dichloromethane solvent against *E. coli*

Concentration (ppm)	N	Subset for alpha = 0.05	
		a	b
70	3	9.0000	
100	3	9.5000	
150	3	9.5000	9.5000
200	3		10.5000
Significant		0.2120	0.0850

Means for groups in homogeneous subsets are displayed.

b. Uses Harmonic Mean Sample Size = 3.000.

Table A.6: Post Hoc Analysis using Duncan Multiple Test for inhibition zone of different concentration of ethyl acetate solvent against *S. aureus*

Concentration (ppm)	N	Subset for alpha = 0.05	
		a	b
70	3	0.0000	
100	3	0.5000	
150	3		9.5000
200	3		9.8333
Significant		1.000	0.195

Means for groups in homogeneous subsets are displayed.

c. Uses Harmonic Mean Sample Size = 3.000.

Table A.7: Post Hoc Analysis using Duncan Multiple Test for inhibition zone of different concentration of ethyl acetate solvent against *E. coli*

Concentration (ppm)	N	Subset for alpha = 0.05	
		a	b
70	3	0.0000	
100	3	0.5000	
150	3		9.5000
200	3		9.8333
Significant		1.000	0.1950

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.8: Post Hoc Analysis using Duncan Multiple Test for inhibition zone of different concentration of methanol solvent against *S. aureus*

Concentration (ppm)	N	Subset for alpha = 0.05	
		a	b
70	3	0.0000	
100	3	0.0000	
150	3	0.0000	
200	3		9.5000
Significant		1.0000	1.0000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.