IN VITRO HAEMATOLOGICAL STUDIES OF *STICHOPODIDAE SP.* AND *HOLOTHURIIDAE SP.*

By

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CERTIFICATE OF APPROVAL

This is to certify that the dissertation entitled,

" *IN VITRO* HAEMATOLOGICAL STUDIES OF *STICHOPODIDAE SP.* AND *HOLOTHURIIDAE SP.*"

is the bonafide record of research work done by Siti Norhaiza Binti Hadzir during the period from July 2004 to March 2005 under our supervision.

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Thrombosis is a pathologic condition that occurs when the body forms arterial or venous blood clots that are excessively large and obstruct blood flow. Aspirin and ticlopidine has been widely used as prophylactic inhibitors for platelet aggregation and anticoagulants such as coumadin (warfarin) and heparin are used as a conventional treatment for thrombosis prevention, it is however, difficult to control them due to many factors that can contribute to coagulation. Further, prolonged usage of aspirin and ticlopidine have been implicated with peptic ulceration and gastrointestinal disturbances; and the use of warfarin and heparin have been shown to cause haemorrhage, necrosis of skin, gastro intestinal disturbances, thrombocytopenia and allergy reactions. Therefore, the search for more effective agents that can secure full antithrombotic benefits while minimizing the antihaemostatic outcome should be pursued. This could help to prevent the often lethal consequences of the conditions mentioned above. A part from hastening wound healing, opsonizations of leucocytes to the wound site were observed with 10% gamat solution in an earlier unpublished observation. (SSJ Mohsin-personal communication). This observation prompted the idea of looking at the effects of sea cucumber extracts (*gamat* in Malay) on haematological parameters.

In this study, the extracts of sea cucumbers (Holothuriidae sp and Stichopodidae sp.), will be investigated as a potential antithrombotic agents

An initial study will be undertaken to look for changes in mean platelet volume (MPV) using automated Cell-Dyn 4000 haematology analyzer. Six different healthy voluntary donors were chosen randomly and used as sample. One (1) ml of blood was placed into seven tubes containing 0.01ml of PBS (control) and extracts of two species of sea cucumber at different concentrations were used. In this study the concentration used were at 0.1mg/ml, 1.0mg/ml, and 10mg/ml *Stichopodidae sp.*; 0.1mg/ml, 1.0mg/ml and 10mg/ml *Holothuriidae sp.* The effective dose on blood was determined for duration of 2 hours with 30 minutes interval from the time of the extract were added to the blood specimen. RM ANOVA revealed that there were no significant different of mean platelet volume between the seven groups tested. However, for within group analysis (time effect) of the species, only *Holothuriidae sp.* showed significant different.

The results from these experiments will establish the direction of subsequent studies in vivo using laboratory animals. The proposed *in vivo* studies were to assess the effect of gamat extracts on platelet functions at various concentrations of *Holothuriidae* and *Stichopodidae* species. Test group animals which have been initially induced to have hypercoagulable states will be used to compare with the non-treated and vehical treated groups. Blood viscosity, coagulation parameters, haematological parameters and platelet morphology and platelet function tests will be done on the blood samples of treated and control groups of animals. The potential anti-thrombotic properties of different species of gamat can only be elucidated following the extensive *in vivo* studies as mentioned above.

ABSTRAK (versi Bahasa Melayu)

Trombosis adalah satu keadaan patologi yang berlaku apabila tubuh membentuk bekuan darah yang besar dan boleh menyebabkan penyumbatan di dalam arteri atau vena. Aspirin dan tiklopidin telah digunakan dengan meluas sebagai perencat secara profilaksis untuk mencegah aggregasi platelet manakala antikoagulan seperti kaomadin (warfarin) dan heparin digunakan sebagai rawatan konvensional untuk mencegah trombosis. Walau bagaimanapun, adalah sukar untuk mengawal trombosis kerana terdapat pelbagai faktor yang menyumbang kepada koagulasi. Tambahan pula, penggunaan aspirin dan tiklopidin untuk jangka masa panjang boleh menyebabkan ulser perut dan gangguan gastrousus. Penggunaan warfarin dan heparin telah terbukti menyebabkan pendarahan, kematian sel kulit, gangguan gastrousus, pengurangan dalam bilangan platelet, dan tindakbalas alergi. Maka, kajian ke arah mencari bahan yang lebih efektif dan mampu memberi manfaat sebagai antitrombus serta meminimumkan kesan antihemostatik, perlu dilakukan. Ini dapat membantu menghindar kesan-kesan negatif seperti yang dinyatakan di atas. Selain mempercepatkan penyembuhan luka, penghijrahan sel darah putih ke kawasan luka juga dipercepatkan. Ini terbukti dari pemerhatian dalam kajian yang sebelum ini yang tidak diterbitkan (SSJ Mohsin – komunikasi personal) yang menggunakan 10% larutan gamat. Pemerhatian ini mencetuskan idea untuk melihat kesan ekstrak gamat ke atas parameter hematology.

Dalam kajian ini, ekstrak timun laut (*Holothuriidae sp* and *Stichopodidae sp.*), digunakan dalam penyelidikan untuk mencari agen yang berpotensi sebagai

antitrombosis. Kajian awal dilakukan dengan melihat secara khusus kepada perubahan pada isipadu purata platelet (MPV) dengan menggunakan penganalisis hematologi automatik (Abbot Cel-Dyn 4000. Enam spesimen darah penderma sukarela yang sihat dipilih secara rawak dan digunakan sebagai sampel dalam kajian ini. Satu (1) ml darah dimasukkan ke dalam tujuh tabung uji yang mengandungi 0.01ml PBS sebagai kawalan dan ekstrak timun laut dari kedua-dua spesis pada kepekatan yang berbeza iaitu 0.1mg/ml, 1.0mg/ml dan, 10mg/ml *Stichopodidae sp.*; 0.1mg/ml , 1.0mg/ml dan 10mg/ml *Holothuriidae sp.* Dos yang efektif ke atas darah ditentukan untuk selang masa pada setiap 30 minit hingga

2 jam bermula dari masa ekstrak dimasukkan ke dalam spesimen darah.

RM ANOVA mendapati tidak terdapat perubahan yang signifikan pada isipadu purata platelet (MPV) bagi tujuh kumpulan rawatan pada kepekatan yang berbeza. Namun, bagi analisa kesan masa ke atas ekstrak, *Holothuriidae sp.* menunjukkan perubahan peningkatan yang signifikan.

Keputusan daripada eksperimen ini dapat memberi panduan ke arah kajian secara *in vivo* menggunakan haiwan makmal. Kajian *in vivo* akan dilakukan ke atas tikus yang telah dirawat dengan ekstrak dari kedua-dua spesis pada kepekatan yang berbeza. Kumpulan tikus yang diuji diaruhkan ke tahap hiperkoaguagulabel dan dibandingkan dengan tikus yang tidak dirawat. Kelikatan darah, parameter koagulasi, parameter hematologi, morfologi dan kajian fungsi platelet akan dilakukan ke atas sampel darah kumpulan ujian dan kawalan. Hanya melalui kajian lanjutan ini, kesesuaian beberapa spesies gamat sebagai bahan antitrombus gamat dapat diketahui.

1. INTRODUCTION

Sea cucumber or *holothuroids* are marine invertebrates belonging to phylum Echinoderms; class Holothuridae (Bakus, 1973; Rowe, 1995). It is estimated that the class consist of 900 species (Moore & Clark, 1962). Phylum Echinoderm consist of 5 classes, that are Holothuridae (sea cucumber), Crinoidea (sea lilies), Echinodea (sea urchins), Ophiuroidae (brittle stars) and Asteroidae (starfish) (Hyman, 1955). There are 6 orders that are Dendrochirotida, Dactylochirotida, Aspidochirotida, Elasipodida, Apodida and finally Malpodida (Bakus, 1973; George and George, 1979). For this research, I will be studying the phylum and class mentioned above and under the order of Aspidiochirotida and both the family of *Holothuridae* and *Stichopodidae* . The taxonomy detail is shown in Figure 1.

They are found in nearly every marine environment, but are most diverse on tropical shallow-water coral reefs (Reseck, 1979; Barth and Broshears, 1982). They can be found from very superficial waters to great depths. Some live buried in the sand and only expose their tentacles above the sand to attract food. Others live exposed in the water on rocks or sand (Hickman, Cleveland, P.Jr., 1998). Aspidochirotide holothurians are sediment feeders which pass large amounts of sediment through their gut system in order to assimilate a fraction of the low content of organic, mainly living diatoms, bacteria and detritus (Yingst, 1976; Moriarty, 1982 and Uthicke, 1998). In coral reef ecosystems, these animals are important recyclers of inorganic nutrients and are thus a part of the close cyling of material (Moriarty *et. al.*, 1985 and Uthicke, 1998).

Holothuroid or sea cucumber can be found in variety of sizes, shape and colors depending on their species (Ridzwan & Che Bashah, 1985). However, most *holothuroids* are black, orange or grey depended on the pigment in dermal layer or existed as nodes in dermal and epidermal layers (Hyman, 1955). Sea cucumbers resemble the shaped of a big sausage. They have a distinct upper (dorsal) and lower (ventral) surface with tube feet only being found on the upper or surface (Hickman, Cleveland, P.Jr., 1998). The ventral part of sea cucumber are more horizontal compared to dorsal part and have structure called 'podium' to help in locomotion (Barnes, 1974). The bodies of sea cucumbers are elongated, leathery and muscular; spines are contained within the skin. (EnchantedLearning.com, 2004). Their hard calcareous skeleton, reduced to microscopic spicules or ossicles, is buried under the skin (Nicholas & Cooke, 1979). Surrounding the mouth are 8 to 30 tentacles (modified tube feet) (Enchanted learning.com, 2004). These tentacles are used to mop up food particles. Some have tentacles that they put up into water column to filter the water overhead. These tentacles can also be used to trap passing plankton or, depending on the species, used to sweep up the sandy mud in which they live. The mud is swallowed, the food particles must be removed, and the sand is passed out through the body (Encyclopedia.com, 2004).

Five double rows of tube feet (with tiny suction cups) run along the body. They are used for crawling along the sea bed or anchoring to a rock. Sea cucumbers move in a slug-like manner, using these series of little tube feet (Enchanted learning.com, 2004). Not all sea cucumbers live above the sand. There are some sea cucumbers which spend their lives burrowing through the sediments. As they burrow,

the sediments are ingested and sorted, the edible particles are absorbed by the body. The rest of the sediment (grit and rubble) are expelled out the back end of the sea cucumber. Celomic fluids are produced by ciliated structure at the peritoneum part of sea cucumber. These fluids are function in general substance circulation in the body (Ruppert & Barnes, 1994).

A sea cucumber breathes by pumping sea water in and out of an internal organ called a respiratory tree. Respiratory trees attached to the intestine near the arms. Sea cucumbers also have hemal system that function in absorption and transportation of nutrients (Ruppert & Barnes, 1994). In some species, branches called tubules of Cuvier, attached to or near the bases of the respiratory trees, are ejected when the organism is attacked; they swell and become sticky, entangling the pursuer. In some sea cucumbers the ability for local, softening of the connective tissue enables them to forcibly eject parts of their internal organs or body in response to attack. Others literally melt when attacked, an occurrence many people have experienced when picking up *Stichopus sp.* If the animal is returned to the water immediately this disintegration is reversed (Lambeth, 1999). The anatomy of external and internal part of sea cucumber is shown in Figure 4 and 5.

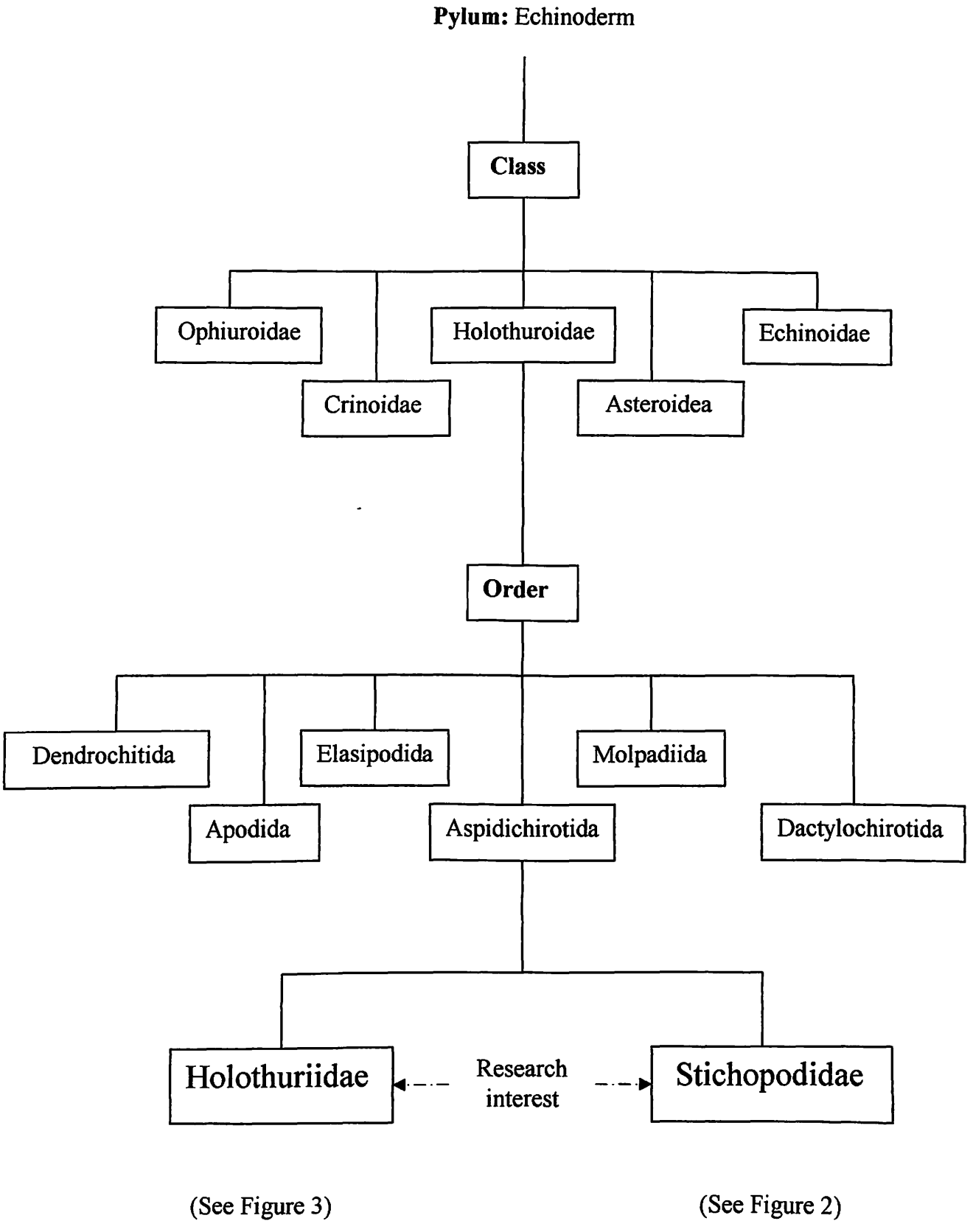


Figure 1: Taxonomy of Sea cucumber

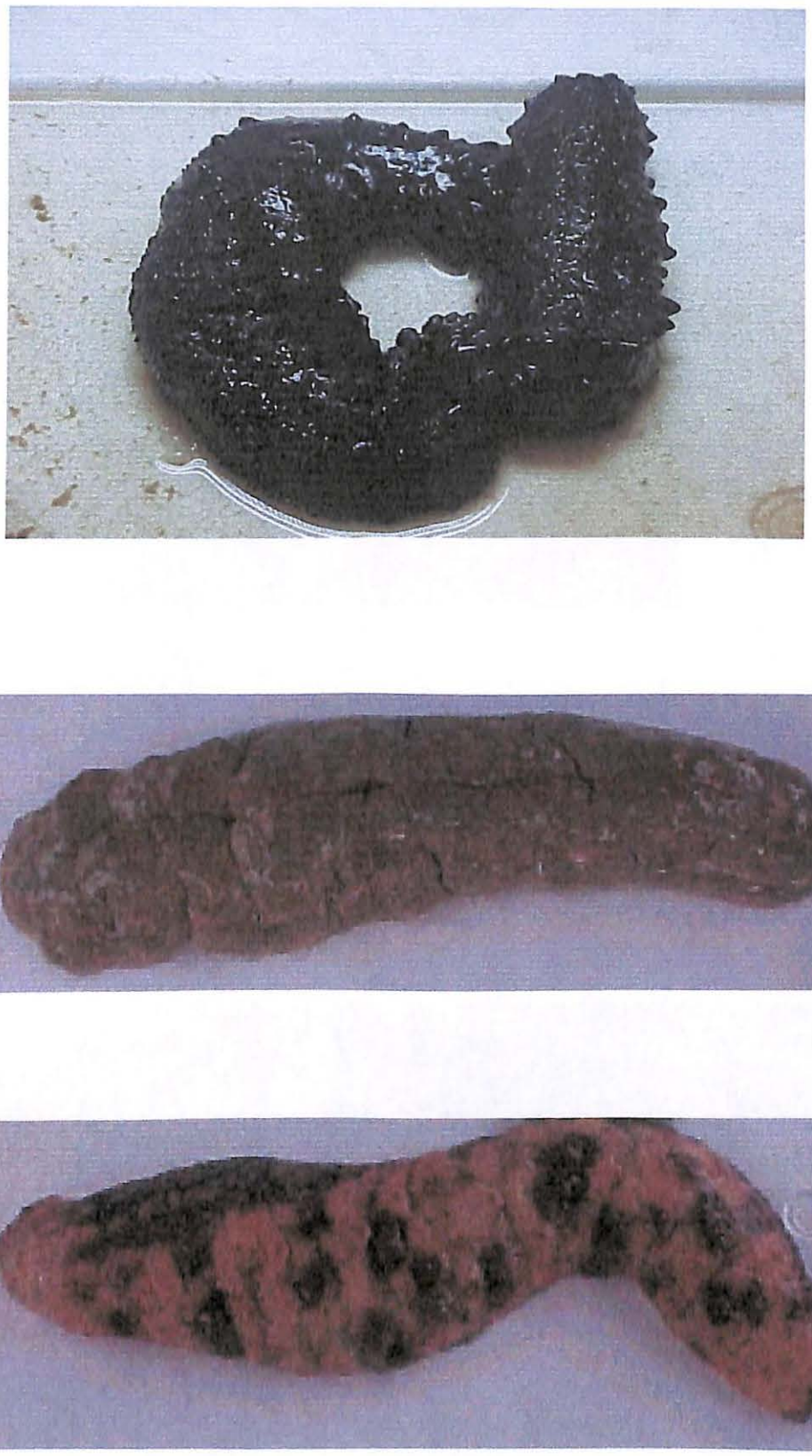


Figure 2: Sea cucumber (Family Stichopodidae)

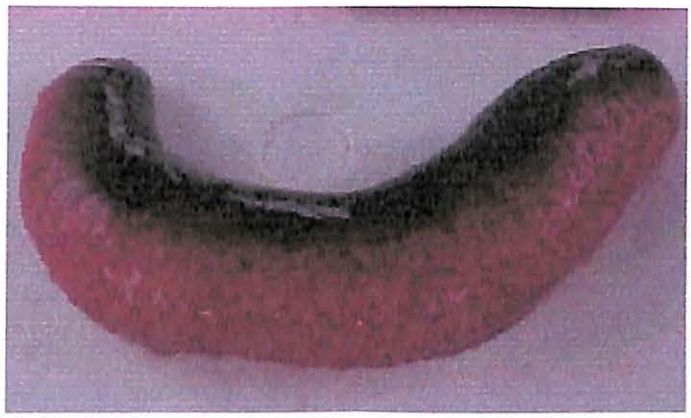


Figure 3: Sea cucumber (Family Holothuriidae)

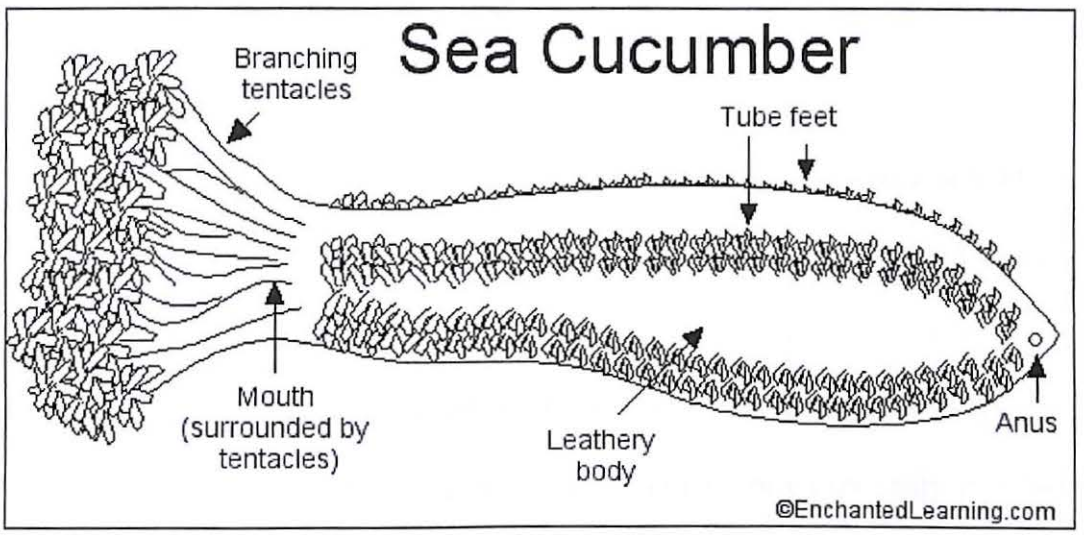
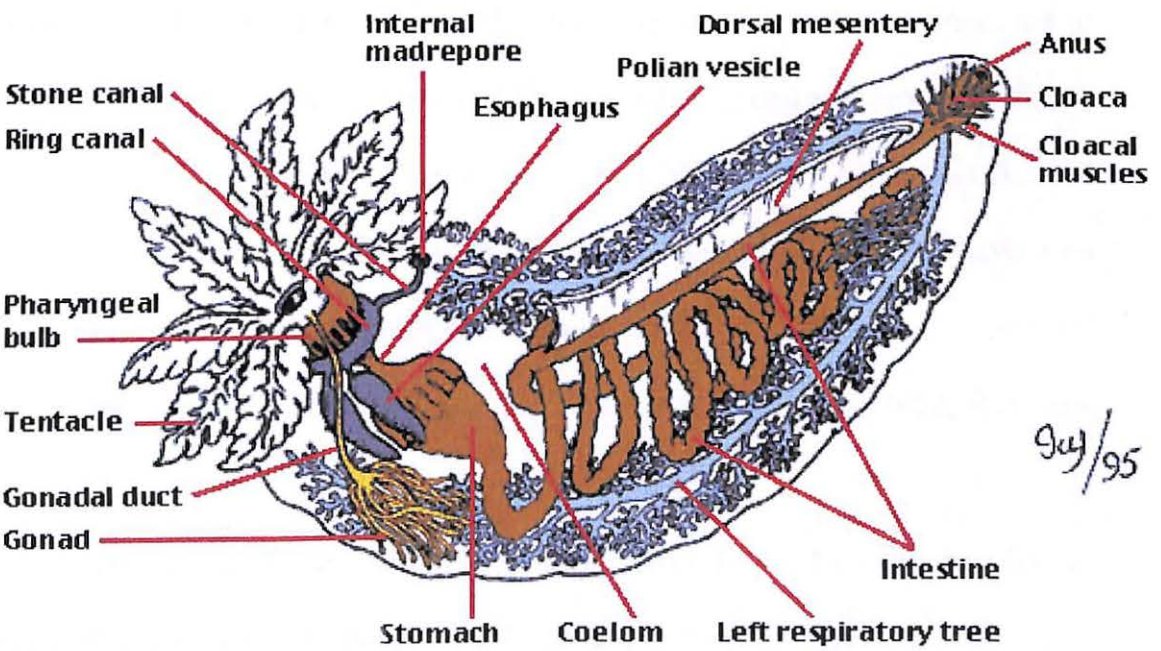


Figure 4: External anatomy of sea cucumber

Adapted from: EnchantedLearning.com.

Date of assessed: November 3, 2004.



Adapted from: Encyclopedia.com

Date of assessed: November 3, 2004.

Figure 5: Internal part of sea cucumber

2. REVIEW OF LITERATURE

2.1 GENERAL

Sea cucumber known as *gamat* in the Malay language or *hai-som* in hokkien or *becher-de-mer*, is a remedy derived from a marine animal closely related to starfish and sea urchins. It is also called *brunok*, *bat* or *balat* in various South East Asian languages. The extracts from sea cucumber have been used as therapeutic remedy by the Malays and are well-known among local ethnic community especially in South East Asia. It is claimed to reduce pain in ailments of inflammatory origin or stiffness in arthritis sufferers (Mohsin, S.S.J *et. al.*, 2003).

Researches on *holothuroids* from the ecological and physiological aspects have been reported (Bernhard, 1974; Clarck, 1976; Sloan and Bodugen, 1980; Robert and Bryce, 1982). There was no complete statement reported upon the important of *holothuroid* as a food sources. However, Hyman (1955) stated that sea cucumber in Indo Pacific region contained protein, mineral and water, whereas fat and carbohydrate are not found. In Malaysia, there was no scientific research have been done on this matter , even though *holothuroid* have become food source by local ethnic groups in Sabah and immigrants from the Philipines (Anuar,1984; Ridzwan, 1993).

As through the review, researches done mainly focused on the morphology (Nicholas & Cooke 1979; Hyman, 1955; Ridzwan, 1993), distribution (Hyman 1955; Bakus, 1973), food source (Ridzwan & Che Bashah, 1985), composition content (Ridzwan 1987a) and its use (Tarlochan, 1980; Chan & Liew, 1986). There were also other researches done on the sea cucumber looking into their self defenses

mechanism. It has been reported in the previous studies that *Holothuroidae* and *Asteroidae* are able to produce a toxin called 'saponin' when threatened (Yasumoto *et. al,* 1967). It has also been reported that sea cucumber does have holotoxin (Shimada, 1969). In recent study, the reproductive cycle of the sea cucumber was studied (Guzman *et. al.*, 2003; Ramofafia *et. al*, 2003). Researches on antifungus and antitumor effect of sea cucumber have also been conducted (Shimada, 1969). Recently, antibacterial study was done by local research group (Saruddin, 1997; Zaki, 2000).

2.2 SEA CUCUMBER AND WOUND HEALING

Wound healing is a complex mechanism in human body towards injury. It involved many cells such as epithelial cell to produce cell growth factor, WBCs to release cytokine in order to attract more cells for further reaction, platelet to contribute their factors and granule content and others. Besides, to hasten wound healing, it required a favorable environment that free from microorganisms (anti microbial properties), enough nutrient and oxygen supply, and well vasculated with blood vessels.

The Chinese eat sea cucumber to cure disease such as high blood pressure, asthma, internal bleeding, body weakness, impotence and wound healing after giving birth or having an operation (Tarlochan, 1980; Chan & Liew, 1986). The ability of the extracts of the Stichopus species to hasten wound healing was first reported by Hassan Yaacob *et. al.*, (1994). This was proved by the study done by Ridzwan 1990 *et. al.*, which showed *Holothuria sp.*, could heal wounds even faster than controlled

petroleum jelly. In this study, they used the extract of *Holothuria atra, Bohadchia marmorata, Stichopus badionotus* and *Actinopyga sp.* The duration taken by these extracts to heal the wound were 168, 264, 302, 257 and 192 hours respectively compared to controlled petroleum jelly that took 408 hours. Since then, many researches have been carried out to find the potential bioactive substance that increased the rate of wound healing. In the researches done this far, there were no documented experiment done on the effects of the sea cucumber extracts on normal haematological parameters. Ridzwan *et. al.*, 1987b, used crude extracts from 5 edible species of *holothuroid* (*Holothuria scraba, H.edulis, Stichopus bodionotus, S. variegates* and *Bohadschia marmota*) from the Sabah coastal area to test their effect on red cell hemolysis. Results showed that the extracts from all the species hemolysed 100% red blood cells tested. However, their result still need to be verified because they have not stated the dose that caused hemolysis, the type of buffer and pH that was used as all these shortcomings may also contribute to the negative results.

Noor Ibrahim and Lim (2000) studied the effects of methanol extracts of *Stichopus variegates* and *Holothuria atra* on cutaneous wound healing in guinea pigs. Based on histological studies using both light and scanning electron microscopes, both the extracts were found to enhance the rate of healing as well as the quality of the resultant scar.

In more recent study, Mohsin *et. al.*, (2003) stated that *Stichopus variegates* extracts seems to have a dynamic potential in enhancing keloid healing by virtue of its excellent permeability and adherence in the tissues micro-vessels. The extracts seem to display a fatal attraction towards the morphology of epithelial and connective

tissue cells, that is fibroblast and endothelial cells, but does nothing to the collagen presentation and orientation.

A part from hastening wound healing, opsonization of leokocytes to the wound site were observed with 10% *gamat* solution in an earlier unpublished observation (Mohsin SSJ. -personal communication). This observation prompted the idea of looking at the effects of *gamat* extracts on haematological parameters.

2.3 OVERVIEW OF HEMOSTASIS

A thrombus is a mass formed from blood constituents within a vessel during life. Blood clotting is a physiological protective mechanism but thrombosis is a pathological process with serious consequences (Macfarlane P.S. *et. al.*, 2000).

Human body has intricate system designed to keep the blood in a fluid state under physiologic conditions. This system also is primed to stem blood loss when the integrity of the vascular system is interrupted. The normal vascular endothelium maintains blood fluidity by inhibiting blood coagulation and platelet aggregation while promoting fibrinolysis. The endothelium also provides a protective barrier that separates the blood cells and plasma factors from the highly reactive and thrombogenic elements of the matrix in the deeper layers of the vessel wall. The thrombogenic elements of the matrix include adhesive proteins, such as collagen and von Willbrand factor (vWF) (both of which promote platelet adhesion), and tissue factor (TF) (a membrane protein located in fibroblasts and macrophages) that triggers blood coagulation. When a vessel is severed, it constricts to divert blood from the site of injury. However, the extravasated blood comes into contact with the exposed

subendothelial matrix, which stimulates the formation of the hemostatic plug by promoting activation of platelets and blood coagulation (Colman *et. al.*, 1994).

2.4 PLATELET ACTIVATION

Platelets play a fundamental role in hemostasis. When a blood injury occurs, platelets exhibit a sequence of events. These events include adhesion of platelets to the injury site, spreading of adherent platelets over the exposed subendothelial surface, secretion of platelet granule constituents, platelet aggregation and platelet coagulation activity (Colman RW *et. al.*, 1994; Thomson AR *et. al.*, 1984).

Many agonist, such as thrombin, adenosine diphophate (ADP), collagen, arachidonic acid, and epinephrine, have the ability to induce platelet aggregation and secretion (Colman RW *et. al.*, 1994). Platelet granule contents include ADP, serotonin, fibrinogen, lysosomal enzymes, β-thromboglobulin and heparin neutralizing factor (platelet factor 4) (Hoffbrand, 1993). Arachidonic acid is converted by the enzyme cyclooxegenase (COX) into prostaglandin endoperoxides. Ultimately, it is converted into the potent platelet agonist thromboxane A_2, as well as into stable prostaglandins such as PGD_2, that inhibit platelet activation. Thromboxane A_2 is a potent mediator of platelet aggregation and secretion (Colman *et. al.*, 1994).

2.5 ASPIRIN

Aspirin is one of non-steroidal anti-inflammatory drug (NSAID). It inhibits the synthesis of prostaglandin. There is evidence that prostaglandins are important mediators of inflammation and perhaps of pain such as headache and pyrexia. One of the principal actions of aspirin is hypoprothrombinemia also as antiplatelet aggregation activity (Laurence D.R., 1993).

The enzymatic activity of COX is blocked by aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). The effectiveness of aspirin as anti-thrombotic agent appears to be dependent on its ability to block the formation of thromboxane A_2 irreversibly by blocking the COX activity of the PGG/H synthase system (Moran N, *et. al.*, 1994; Marcus AJ *et. al.*, 1994).

However, the use of aspirin as anti-thrombotic agent is limited because of several adverse effects include gastric bleeding, allergy (asthma, angioneurotic edema, urticaria, rashes, rhinorhoea) aspirin may also aggravate chronic urticaria. If a particle of aspirin is placed on human buccal mucosa, within 30 minutes the mucosa becomes white, opaque and wrinkled and a slough that readily peels away is formed (Laurence D.R., 1973).

Previous research had shown that sea cucumbers are two times better than aspirin anti-inflammatory and analgesic effects (Ridzwan Me 'GAMAT' & My Doc, 2004). The mechanism of sea cucumber to reduce pain and exhibit anti-inflammatory effect is proposed to be similar to aspirin. Our research using this natural product would be in view of identifying alternative agents that are more effective in securing full anti-thrombotic benefits while minimizing the antihaemostatic outcome.

3. AIMS OF THE STUDY:

1. To find potential antithrombotic agent in *Holothuriidae sp.* and *Stichopodidae sp.*
2. To test a number of hypothesis arising out
    i) Change in mean platelet volume (MPV) could be used as indicator of platelet activation
    ii) The blood treated with the extract would give significant changes after certain time incubation (30, 60, 90,120 minutes incubations).
    iii) There are dose- dependent changes on mean platelet volume after treated with different dose of extract.

3.1 OBJECTIVES OF THE STUDY:

1. To review published material relevant to the effect of *holothuroids* (sea cucumbers) on wound healing.
2. To take samples on voluntary basis and carry out haematological studies of (*Stichopodiidae sp & Holothuriidae sp.*) *in vitro.*
3. To analyze the data and use it to test the hypothesis described in the aims.
4. To make other recommendation of methodology in order to reveal potential antithrombotic agent in *Holothuriidae sp.*and *Stichopodidae sp.*

4.0 MATERIAL AND METHODS

4.1 EQUIPMENT

Below is a list of the equipment that was used in this research project. All the equipment used is located in at the Lab Facility Unit (UKM- Unit Kemudahan Makmal).

1. Weighing scale (Sartorius BP 221 S) [Max 220g , d = 0.1mg]
2. Refrigerator (National NR-B53FE)
3. Freeze drier (ilShin Lab. Co., Ltd.)
4. Hematology analyzer (Abbot Cell Dyn-4000)
5. Sonicator (Sonicor SC – 221)
6. Ph Meter (HANNA Instrument pH 211 Microprocessor pH meter)
7. Centrifuge (Hettich Zentrifugen Universal 32R)

4.2 MATERIAL

4.2.1 Sea cucumber

Sea cucumber was harvested freshly from Perhentian Island, Redang Island and Lang Tengah Island. Five species were identified from 2 families, which were the *Holothuriidae* and *Stichopididae* (Figure 2 and 3). From each of this species, water extraction was conducted from its body tissue. Non-organic extraction was not done because the bioactive substances in sea cucumber are believed to be water soluble. Generally it is easier to use water extract and the result is more promising. (Zury, 2004).

4.2.1.1 Processing of sea cucumber

Fresh sea cucumbers were separated according to their species and were then washed first with tap water and rinsed with distilled water. The body tissue and body fluids were separated. The body tissues were dried in hot air oven.

After drying in hot air oven, the result was a piece of tight and packed body tissue. A grinder was used to blend the body tissue into powdered form. All these procedures have been done by previous student, Zury Azreen bin Azizul Rahman.

4.2.1.2 Extraction Process

A vast majority of analyses related problems confronting the researcher are the determination of low concentrations of one or several substances in a complex biologic media consisting of multitude of both inorganic and organic methods, many of which are present in large concentration. Most procedures require preparatory separations prior to analysis.

In the event of forming a solution between water and the biologic medium, sonnicator was of used to break the cell of sea cucumber powder. This step was important to form very small particle and any substances in the cell were revealed. The break down process was done by mean of ultrasonic with very high frequency. Ultrasonic extraction is the use of sound waves beyond the range of human

audibility to perform scrubbing of material in a solution. The transmission of these waves into the fluid causes the formation of millions of microscopic bubbles which collapse and release an intense amount of energy to literally "blast" the extract from the external walls of the sea cucumber. An ultrasonic system consists of a "generator" which is an electronic device capable of generating electric energy at an ultrasonic frequency and a "transducerized tank" which holds liquid and parts. Together they create a "scrubbing action" in the liquid which results in thorough break of the sea cucumber wall. The "SC" systems are one piece consoles housing both the generator and transducerized tank in a rugged cabinet (Anonymus B, Sonicor Instruction Manual, 2000).

About 15 gram of sea cucumber powder was mixed with 150ml distilled water. This mixture was placed in a beaker and closed with aluminium foil. The tank was filled to the desired level with distilled water. A minimum of two inches (heated systems must have three inches) of liquid should always be in the tank to prevent damage. The beaker with the mixture was operated in the tank for 15 minutes. After 15 minutes, the mixtures were spun in centrifuge at 5000rpm for 10 minutes. After centrifugation, the supernatant was taken as water-based extract solution. The sediment was disposed. These steps were repeated until 100gram of sea cucumber powder was used. The extracts were

labeled and transferred into media bottle before placing them into a refrigerator for freezing and preserving.

4.2.1.3 Principle of separation technique

The mixtures were separated by means of centrifugation is the technique in which the sample in a suspension was placed in a centrifuge tube and spun at a high angular velocity (high numbers of revolutions per minute, rpm). Particles experiencing a greater centrifugal force have faster sedimentation rates are preferentially pulled toward the bottom of the centrifuge tube. For particles of equal density the separation is based on mass, with heavier particles having greater sedimentation rates. When the particles are of equal mass, those with high density have the greatest sedimentation rate.

4.2.1.4 Freeze Dry

Following centrifugation the samples were placed in an appropriate container for freeze drying. In this process, water was sucked out by using pressure from cold air, which results in a dry sample. The operating temperature and pressure was 55°F and 200atm respectively. This process took 3 days to dry completely. When running the samples, extreme care had to be observed to ensure that the bubbles were not formed from the sample as such spilling and contamination with other samples would not occur.

Powder extract was end the result of this process. The powder was then stored in refrigerator before being used in dose preparation.

4.2.2 Blood Specimen

The specimen for this research was blood. 6 different healthy voluntary donors were chosen randomly. From the 6 donors, 7ml of blood was obtained by intravenous venesection. The blood specimen was placed in EDTA tube and used immediately as any delay may permits morphological and biological changes of the blood constituents.

4.2.3 Anticoagulant

The potassium salt of EDTA is the most commonly used anticoagulant in hematology. Blood coagulation mechanism is inhibited by the chelation or binding of calcium ions with EDTA salts. The optimal anticoagulant concentration used was 1.5mg/ml of blood. This quantity has no adverse effects on routine cell counts and preserves cellular morphology when blood films are made within 2 hours of collection (Stiene *et. al.*, 1998)

4.2.4 Buffer

4.2.4.1 Preparation of Phosphate buffered saline (pH 7.3)[Green, *et. al.*, 1986]

Solution A: 0.1M KH_2PO_4 (BDH laboratory supplies)

- 13.6 of KH_2PO_4 was dissolved in approximately 600ml of normal saline and made up to 1 liter with normal saline.

Solution B: 0.1 MNa_2HPO_4 (BDH laboratory supplies).

- 14.2 of Na_2HPO_4 was dissolved in approximately 600ml of normal saline and made up to1 liter with normal saline.

* The pH was adjusted to 7.3 by pH meter to suit with microenvironment of the blood in the body.

I. In the preparation of 1 liter of PBS (pH7.3) 900ml of physiological saline (0.90%w/v) were mixed with 23.6ml of Solution A(0.1M KH_2PO_4) and 76.4ml of Solution B(0.1 MNa_2HPO_4).

II. The pH of the solution was checked, and adjusted if necessary to required pH, by adding 0.1 M HCl or 0.1M NaOH.

4.2.5 Preparing stock solution of the extract

The stock solution was prepared at the concentration of1mg/ml by adding 10mg of sea cucumber extracts (for each species) and dissolved in 10 ml PBS (pH 7.3). The stock was placed in the appropriately labeled universal bottle

4.2.5.1 Preparation of sea cucumber's water extract at different concentration:

For the pilot study, three different concentrations of water extract were prepared from the stock solution. The concentrations were at 0.1mg/ml, 1.0mg/ml, and 10mg/ml respectively.

0.1mg/ml of *Holothuriidae*

This concentration was prepared based on the formula:

$M_1V_1 = M_2V_2$

M_1 = concentration of stock solution

= 1mg/ml

V_1 = volume that needed to be taken from the stock solution

= ?

M_2 = concentration that we want to prepare

= 0.1mg/ml

V_2 = volume of solution that we want to prepare

= 10ml

$M_1V_1 = M_2V_2$

(1mg/ml) (V_1) = (0.1mg/ml) (10)

= 1 ml

1ml was taken from stock solution and placed in 10 ml volumetric flask and made up to 10ml with PBS.

1mg/ml of *Holothuriidae*

This concentration was obtained directly from the stock solution (1mg/ml).

10mg/ml of *Holothuriidae*

100mg (0.1 gram) of extract was dissolved with 10 ml of PBS.

The same steps were used in the preparation extract of another species. For each concentration that have been prepared, (0.1mg/ml, 1mg/ml and 10mg/ml), 0.01 ml was taken and tested on 1ml of blood. The effects of different concentration of extracts were observed for duration of 2 hours.

4.2.6 Doses used in the study

There were 3 different concentrations used in this study:

i) 0.1mg/ml

ii) 1mg/ml

iii) 10mg/ml

From three concentrations above, the doses that have been chosen for testing on blood were:

$$\frac{0.01\text{ml} \times 0.1\text{mg/ml}}{1.01} = 0.001\text{mg/ml}$$

$$\frac{0.01\text{ml} \times 1\text{mg/ml}}{1.01} = 0.01\text{mg/ml}$$

$$\frac{0.01\text{ml} \times 10\text{mg/ml}}{1.01} = 0.1\text{mg/ml}$$

0.01ml of the extract was added to 1ml of blood, giving the total volume of 1.01ml of blood and the extract.

Out of the 3 doses above, the effective dose on blood were determined for a duration of 2 hours from the time of the extract were added to the blood specimen.

Zury (2003) have conducted a previous study to determine the presence of toxicity of the *Holothuriidae sp.* and *Stichopodidae sp.* on the central nervous system (CNS) .The entire test that were conducted, with the dose set at 1mg of powdered extract per kg body weight of mice (1mg/ml). The dose chosen were based on the assumption that 1mg of the extract will give a reasonable and tolerable effect for 1kg of body weight.

1mg ⟶ 1 kg

Average weight of men = 50kg

50mg ⟶ 50kg

In normal human body, they were approximately 5000ml of blood.

So, 50mg ⟶ 5000ml blood

1ml blood ⟶ $\frac{50}{5000}$

= 0.01mg

Based on the results of the previous study by Zury, the concentration used in this study were at 0.1, 0.01 and 0.001mg/ml of blood respectively.