TOTAL SULFATED GLYCOSAMINOGLYCAN (GAG) FROM MALAYSIAN SEA CUCUMBERS *Stichopus hermanni* AND *Stichopus vastus* AND ITS EFFECTS ON WOUND HEALING IN RATS

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2011

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by

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Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

(Biomedicine)

JUNE 2011

DECLARATION

I declare that the contents presented in this thesis are my own work which was done at School of Health Sciences, Universiti Sains Malaysia unless stated otherwise. The thesis has not been previously submitted for any other degree.

Siti Fathiah Binti Masre

ACKNOWLEDGEMENT

Alhamdulillah, praise be to Allah S.W.T, the Most Gracious and ever Merciful. I would sincerely like to express my deepest appreciation to all my supervisors; Assoc. Prof. Dr. Farid Che Ghazali, Assoc. Prof. Dr. George W. Yip and Assoc. Prof. K.N.S Sirajudeen for their supervision, guidance, continuous moral support and encouragement during the course of my master's degree project. My sincere gratitude to Skim Latihan Akademi Bumiputera (SLAB) Universiti Kebangsaan Malaysia (UKM), for awarding me the scholarship and to Universiti Sains Malaysia (USM) for the short term grant (304/ppsk/6139058) from theSchool of Health Sciences and the postgraduate incentive grant (1001/ppsk/6122001) from the Institute of Postgraduate Studies (IPS).

I would also like to express my appreciation to Miss Koo Chuay Yeng and all the students in the Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore (NUS) for their kindness in giving help and guidance during my master project. A special thanks to all the officers and technicians in the Electron Microscopy Unit from University of Malaya (UM), Universiti Kebangsaan Malaysia (UKM) and Universiti Sains Malaysia (USM) for their kind assistance and expertise in the electron microscopy work.

My appreciation to Assoc. Prof. Dr. Norsa'adah Bachok and Dr. Muhamad Saiful Bahri Yusoff from the Department of Biostatistics, USM for their assistance in determining the right statistical analysis for my master's project. I would like to express my sincere thanks to Dr. Norziah Ghani and Dr. Rosilawati Kamaruddin from the Laboratory of Animal Research Unit (LARU), USM and to Mohd. Amri Maslan and Mohd. Shahrizal for their guidance and assistance in this master's project. My warmest thanks to Encik Razak Bin Hamzah and all the staffs at MAFPREC for the sea cucumber sources. Not forgetting thanks to my Dean, deputy Deans, lecturers and all staffs of School of Health Sciences for their continuous support during my master program.

My gratitude to my beloved family, in particular to my loving father and mother, Encik Masre Bin Jaafar and Puan Zainon Binti Suratman for their moral support, patience and constant prayers that have helped me to apprehend the difficulties faced throughout my master's program.

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LIST OF DIAGRAMS

LIST OF ABBREVIATIONS

%	Percentage
cm	Centimeter
CS	Chondroitin sulfate
DC	Discoid crystal
ECM	Extracellular matrix
ED	Endothelial cell
EFTEM	Energy Filter Transmission Electron Microscope
FAO	Food and Agricultural Organization
g	Gram
G	Granules
GAG	Glycosaminoglycan
HA	Hyaluronan
H&E	Hematoxylin and Eosin
HS	Heparan sulfate
IqR	Interquartile range
K	Keratinization
kg	Kilogram
m	Meter
M	Molar
mg	Milligram
µg	Microgram
ml	Milliliter
µl	Microliter
mm	Millimeter
NaOH	Natrium hidroxide
nm	Nanometer
PBS	Phosphate buffer saline
PMNL	Polymorphonuclear leucocyte
RBC	Red blood cell
RER	Reticuloendothelial ribosomes
S. hermanni	*Stichopus hermanni*
S. vastus	*Stichopus vastus*
SEM	Scanning electron microscope
SPSS	Statistical Package for Social Sciences
TEM	Transmission electron microscope
VS	Vesicles

VC	Vacuoles
VPSEM	Variable Pressure Scanning Electron Microscope
WD	Working distance

ABSTRAK

GLIKOSAMINOGLIKAN (GAG) SULFAT DARIPADA SPESIES *Stichopus hermanni* DAN *Stichopus vastus* TIMUN LAUT MALAYSIA DAN KESAN TERHADAP PENYEMBUHAN LUKA KE ATAS TIKUS

Timun laut telah lama dimanfaatkan sebagai sumber bahan perubatan semulajadi disebabkan kandungan glikosaminoglikan (GAG) sulfat. Tujuan kajian ini adalah untuk menyiasat kesan jumlah GAG sulfat daripada dinding tubuh integumen, organ viseral dalaman dan cecair selom pada spesies *Stichopus hermanni* dan *Stichopus vastus* timun laut Malaysia terhadap penyembuhan luka pada tikus dengan menggunakan penilaian makroskopik dan mikroskopik.

Bagi kedua-dua spesies, dinding tubuh integumen menunjukkan jumlah GAG sulfat, O- dan N- yang tertinggi, diikuti organ viseral dalaman dan cecair selom. Bahagian anatomikal kedua-dua spesies menunjukkan peratusan (%) GAG sulfat-O lebih tinggi berbanding GAG sulfat-N. Pada kajian penyembuhan luka menggunakan 47 ekor tikus betina *Sprague-dawley* sebagai model luka eksisi ketebalan penuh, 20 µl dari kepekatan 1 µg/ml jumlah GAG sulfat dari setiap bahagian anatomikal kedua-dua spesies telah diaplikasikan ke luka (diameter 6 mm) dari $hari_0$ hingga $hari_{12}$. Manakala salina penimbal fosfat (PBS) telah diaplikasikan sebagai kumpulan kawalan. Kesan penyembuhan luka dianalisis melalui penilaian makroskopik dan migrasi epithelial, respon inflamatori, proliferasi fibroblas, pembentukan pembuluh darah baru dan

organsisai pembentukan kolagen dengan mikroskop cahaya (LM), mikroskop elektron transmisi (TEM) dan mikroskop elektron pengimbas (SEM).

Penilaian makroskopik menunjukkan signifikan (p<0.0167) berlaku antara kumpulan dirawat dengan GAG sulfat dari cecair selom *S. vastus* [$hari_1$ (8.33, IqR 9.38); $hari_6$ (33.33, IqR 6.25); dan $hari_{12}$ (75.00, IqR 2.08)] berbanding kumpulan kawalan [$hari_1$ (0.00, IqR 0.00); $hari_6$ (8.33, IqR 9.38); dan $hari_{12}$ (54.17, IqR 18.75)]. Kemajuan migrasi epithelial kumpulan GAG sulfat daripada dinding tubuh integumen dan cecair selom *S. vastus* adalah lebih baik secara signifikan (p<0.0167) berbanding kumpulan kawalan. Evaluasi LM dan SEM menunjukkan luka semua kumpulan rawatan telah bercantum semula pada $hari_{12}$. Penilaian LM dan TEM menunjukkan GAG sulfat dapat mempertingkatkan migrasi fibroblas ke kawasan luka dan signifikan berlaku (p<0.0167) antara GAG sulfat kumpulan rawatan dengan cecair selom *S. vastus* berbanding kumpulan kawalan. Untuk pembentukan pembuluh darah baru, penilaian LM dan TEM menunjukkan keputusan signifikan (p<0.05) pada kumpulan rawatan GAG sulfat dari ketiga-tiga bahagian *S. vastus*. Evaluasi LM, TEM dan SEM mencadangkan bahawa GAG sulfat dari ketiga-tiga bahagian *S. vastus* amat merangsang organisasi fiber kolagen secara signifikan (p<0.05) pada pemerhatian $hari_{12}$.

Hal ini menunjukkan bahawa GAG sulfat khususnya dari cecair selom *S. vastus* dilihat dapat mempercepatkan pemulihan luka dan ini dapat dilihat dari kesan positif pada peratusan (%) kecepatan kontraksi luka, peningkatan migrasi epithelial, proliferasi fibroblas, proses angiogenesis dan penyusunan kolagen.

ABSTRACT

TOTAL SULFATED GLYCOSAMINOGLYCAN (GAG) OF MALAYSIAN SEA CUCUMBERS *Stichopus hermanni* AND *Stichopus vastus* AND ITS EFFECTS ON WOUND HEALING IN RATS

Sea cucumbers have long been exploited as a source of medicinal compounds due to the presence of sulfated glycosaminoglycans (GAGs). The aim of this study was to investigate the occurrence of total sulfated GAG from the integument body wall, the visceral internal organs and the coelomic fluid of Malaysian sea cucumbers *Stichopus hermanni* and *Stichopus vastus* and evaluate the effect of total sulfated GAG on wound healing in rats using macroscopic and microscopic evaluations.

In both species, the integument body wall was the highest source of total, O- and N-sulfated GAGs followed by the visceral internal organs and the coelomic fluid. There was more O-sulfated GAGs compared to N-sulfated GAGs for percentage (%) division in both species. In the full-thickness excisional wound model using 47 female *Sprague-dawley* rats, 20 µl of 1 µg/ml concentration of total sulfated GAG from each anatomical part of each sea cucumber species were applied to the wound area (6 mm diameter) from Day_0 to Day_{12}, while phosphate buffered saline (PBS) was applied to control group. The progress of healing was assessed through macroscopic examination and analysis of epithelization, inflammatory cells, fibroblasts proliferation, new vessels formation and collagen fibers organisation using light microscope (LM), transmission electron microscope (TEM) and scanning electron microscope (SEM).

Macroscopic examination revealed significantly (p<0.0167) wound contraction percentage (%) on each observation occurred in sulfated GAGs treated group from *S. vastus* coelomic fluid [day_1 (8.33, IqR 9.38), day_6 (33.33, IqR 6.25) and day_{12} (75.00, IqR 2.08)] as compared to control group [day_1 (0.00, IqR 0.00), day_6 (8.33, IqR 9.38) and day_{12} (54.17, IqR 18.75)]. The epithelization progress of *S. vastus* integument body wall and coelomic fluid sulfated GAGs treated groups was significantly (p<0.0167) greater compared to control group. LM and SEM evaluations showed that all treatment groups have fully bridged the excised wound on the 12^{th} day of observations. LM and TEM evaluations showed enhanced fibroblasts proliferation with significant (p<0.0167) finding occurred in sulfated GAGs treated group from *S. vastus* coelomic fluid compared to control group. For new vessels formation, LM and TEM showed a significant (p<0.05) increase in the sulfated GAGs treated group from *S. vastus* anatomical parts compared to control group. LM, TEM and SEM evaluations showed that sulfated GAGs from *S. vastus* anatomical parts stimulate dense organisation of collagen fibers on the 12^{th} day of observation, significantly (p<0.05) compared to control group.

This study strongly indicate that sulfated GAGs in particularly from *S. vastus* coelomic fluid, seems to hasten the wound healing event through positive effect on acceleration of wound contraction percentage (%), enhance epithelization migration, fibroblast proliferation, angiogenesis process and collagen organization.

CHAPTER 1

INTRODUCTION

1.1 Title

Total sulfated glycosaminoglycan (GAG) from Malaysian sea cucumbers *Stichopus hermanni* and *Stichopus vastus* and its effects on wound healing in rats.

1.2 Background Overview

Sea cucumbers are marine invertebrates from the phylum Echinodermata (Kamarul *et al.* 2010). These blind cylindrical invertebrates that live throughout the worlds' ocean intertidal beds are known as gamat in Malay (Fredalina *et al.* 1999). There are more than 2500 species available of varying morphology and colours throughout the world (Ibrahim 2003; Baine & Forbes 1998). Out of Malaysia's approximately 47 sea cucumber species, 7 are believed to possess therapeutic properties. Within the coastal areas of Malaysia, sea cucumbers can be located in Semporna Island, Sabah; Pulau Pangkor in Perak, Pulau Tioman, coastal areas of Terengganu and Pulau Langkawi, Kedah. Among the most populous species are *Stichopus hermanni, Stichopus badionotus, Stichopus chloronotus, Holothuria atra, Holothuria edulis* and *Holothuria scabra* (Ridzwan *et al.* 1995). According to Ridzwan & Che Bashah (1985), among the 23 species of sea cucumbers found in coastal areas of Sabah, a few of them are toxic, but most are seafood delicacies. These have been eaten since ancient times and are among the most commonly consumed of echinoderms (Hirimuthugoda *et al.* 2006). Even in South-East Asia, sea cucumbers are taken as an important food supplement as they have high nutritional values (Ridzwan & Che Bashaah 1985; Ridzwan 1993).

In Malaysia, sea cucumbers and their products have long been purported as a source of traditional medicines due to their various important nutritional and medicinal values (Langkawi Magazine 2006). Furthermore, these invertebrates can also help cure certain ailments and diseases (Shimada 1969; Sit 1998). Practitioners of traditional remedies often consume the fluid portion of sea cucumber to remain fit and healthy, and this is also practiced by fishermen while out of sea for long periods of time. They believe that, if consumed regularly, sea cucumbers can reduce hypertension and asthma, help in the healing of internal wounds, and prevent or cure cancer. In addition, the coelomic fluid of certain sea cucumbers has been reported to contain high bioactive substances that suggest orchestrating an important role in wound healing (Pechenik 1996).

Ibrahim (2003) reported that sea cucumbers are a rich source of collagen and GAGs, also known as mucopolysaccharides. Biochemically, these GAGs can be presented as sulfated and non-sulfated groups. Literatures indicate that the sulfated GAGs can improve skin appearance and wound healing, as well as being important for healthy joints. Studies have shown that sulfated GAGs such as chondroitin sulfate and heparan sulfate can assist in the positive wound healing processes and are involved in the formation of connective tissue components (Zou *et al.* 2004; Annika *et al.* 2007; Stringer & Gallagher 1997).

Wound healing is a complex pathophysiological event that consists of a series of highly complex interdependent and overlapping stages (Inkinen 2003). The wound healing process has four phases: the hemostasis or coagulation phase, the inflammatory phase, the proliferative phase, and the remodeling phase (Laurence 2006). The effectiveness of this wound healing phases is commonly depending on a complex interplay of inflammatory mediators released, nitric oxide, and cellular elements (Granick & Chehade 2007). Wound healing disorders present a serious clinical problem and are likely to increase since they are associated with diseases such as diabetes, hypertension, and obesity. Moreover, increasing life expectancies will cause more people to face such disorders and further aggravate this medical problem (Frank & Kampfer 2005). Nowadays, there are various modern and traditional medicinal products in the market that purport to have wound healing properties. There has also been an explosion in research and findings in recent years to develop the products and approaches to wound healing. The development of wound care products, such as bio-active wound dressings, bioengineered skin substitutes, and exogenous growth factors, was only possible through an increased understanding of the roles of cellular factors in regulating normal healing (Schultz 2007).

Various approaches for using natural products as new remedies have been explored for both acute and chronic wounds over the past decade. Although some people view these ideas as somewhat primitive or ignorant, many of the remedies are the result of thousands of years of empiric observation. These remedies have their roots in the ancient civilizations of the East as well as those of the Native American and Native South American cultures (Davis & Perez 2009).

Several natural products from marine invertebrates have been reported to promote the process of wound healing (Pujol *et al.* 2007). Although many natural products have been claimed to have healing effects, most of these claims are not backed by scientific data. Therefore, this study will act as a stepping stone for new exploratory scientific data, testing beneficial pharmaceutically active compounds from Malaysian sea cucumbers for wound healing treatment. Sea cucumbers have been widely revealed as traditional remedies for healing various internal and external wounds. As part of the efforts to elucidate its pharmaceutical activities and hence medicinal potential, this study will focus on the wound healing properties of sulfated GAGs extracted from the integument body wall, visceral internal organs and coelomic fluid of two species from the Stichopodidae family (*S. hermanni* and *S. vastus*) using experimentally created wound on rats.

1.3 Justification of The Study

The essence of the research here is the freshly harvested Malaysian sea cucumbers, *S. hermanni* (Semper 1868) and *S. vastus* (Sluiter 1887), from which to identify the possible presence of sulfated GAGs, extracted from the three anatomical parts: integument body wall, visceral internal organs and coelomic fluid and the roles of sulfated GAGs in the wound healing process on a rat dorsal attributed as a wound model which was evaluated via macroscopic and microscopic investigations. This Stichopodidae family of sea cucumbers has been chosen as they dominate in terms of diversity in the hot shallow-water tropics to the warm temperate regions in coastal areas of Malaysia. *S. vastus* species is an indigenous commensal invertebrate of the coastal areas of Terengganu while *S. hermanni* can be found throughout the west coastal areas of Peninsular Malaysia.

In term of behavior, *S. hermanni* is a diurnal invertebrate while *S. vastus* is a nocturnal (Zulfigar *et al.* 2000). These behaviors plus a characteristic feature of echinodermata, in which absence of visceral internal organs during certain period of time may postulate potential possibility when an actual whole individual sea cucumber can be fully viable or feasible as a therapeutic compound biomass. Therefore these features might be suggestive as one of the contributor factors of GAGs level in the sea cucumber itself. Plus, both *S. hermanni* and *S. vastus* are well known as commercially important sea cucumber species, globally (Toral-Granda 2007). In addition, this is the first well-controlled scientific study conducted to explore the therapeutic role of sulfated GAGs from the three different anatomical parts of *S. hermanni* and *S. vastus* species in wound healing as no scientific study had been done previously.

For the elucidation of total sulfated GAG roles in the wound healing process, macroscopic analysis by calculating the percentage (%) of wound contraction using standard formula and microscopic analysis (light microscope, transmission electron microscope and scanning electron microscope) were studied by using rats as a wound model. There are relatively few combinations of transmission electron microscope (TEM) and scanning electron microscope (SEM) study reports on the wound healing observations. Furthermore, most investigations using TEM and SEM were performed on individual aspects of wound healing. Therefore, this study has put an effort to combine the three elemental microscopic approaches which consists of light microscopy, TEM and SEM to study the wound healing process as a whole.

This research may have relevant impact to wider areas of clinical concern such as exploration of new therapeutic agents as traditional medicines from sulfated GAGs of the hot temperate *Stichopus* spp. to assist in the wound healing process. In addition, the government supports efforts to apply traditional medicines to the public as Malaysia has rich sources of tropical biodiversity. The ultimate reasons for the use of traditional medicines are due to the faith and belief that traditional medicines contain natural substances which do not contain any harmful chemicals as they are naturally-derived and are without side effects especially when compared to pharmaceutical drugs (Abas 2001).

1.4 RESEARCH OBJECTIVES

1.4.1 Main Objective:

To assess the effects of total sulfated GAG extracted from integument body wall, visceral internal organs and coelomic fluid of Malaysian sea cucumbers, *Stichopus hermanni* and *Stichopus vastus* on wound healing in rats.

1.4.2 Specific Objectives:

1. To extract total GAG from the three anatomical parts (integument body wall, visceral internal organs and coelomic fluid) of *S. hermanni* and *S. vastus*.
2. To determine the level of total sulfated GAG together with total O- and N-sulfated GAG level in the three anatomical parts of *S. hermanni* and *S. vastus.*
3. To compare the rates of wound healing in sulfated GAGs treated groups using the three anatomical parts of *S. hermanni* and *S. vastus* and a control group through macroscopical and microscopical analysis using experimental rats.

1.5 Research Hypothesis

There is a significant positive effect on wound treated with sulfated GAGs from the three anatomical parts of *S. hermanni* and *S. vastus*, compared to the control group.

CHAPTER 2

LITERATURE REVIEW

2.1 Sea Cucumber

The sea cucumber in Malaysia is better known as “Gamat” (Fredalina *et al.* 1999) by the Malays and “Hoisam” by the Chinese (Teoh 2004). They are also called trepang or bêche-de-mer, (Miriam 1999), while in Sabah it is called “bat” or “balat” and in Sarawak it is named “brunok” (Chan & Liew 1986). It is an extent class of echinoderms, Holothuroidea (Gilliland 1993). Generally, animals in the Holothuroidea class are characterized by their soft body tissue, bilaterally symmetrical, and lie on one side with an elongated body axis between the mouth and the anus (Sim 2005; Moore 2006). They also have three cell layers and a coelom (Moore 2006). Sea cucumbers are a soft-bodied, invertebrate relative of the starfish and sea urchin (Charles Darwin 2006). They are tube-shaped animals somewhat like worms and come in a variety of colors (George 2008) and sizes reaching up to 5m of length and over 5kg of weight (Kerr & Kim 2001). Sea cucumbers have a mouth at one end and an anus at the other end, and these creatures do not have a real head (LeBlanc 2005). The integument body wall of sea cucumbers is muscular and has embedded spicules, but these invertebrates are unique by having no bones. The spicules are in many different shapes that correspond to each sea cucumber species (Pechenik 2000). The integument body wall of sea cucumber consists of a thick dermal region including loose and dense connective tissues, circular and longitudinal muscles and coelomic epithelium (Miguel-Ruiz & García-Arrarás 2007).

Meanwhile, the visceral internal parts of sea cucumber consist of many subdivisions of the sea cucumber coelom, which are the water vascular system and the body coelom. The body coelom of sea cucumber contains of the visceral internal organs and it is the chief body cavity in the internal parts of sea cucumber. For the coelomic fluid of sea cucumber, it is produced by the water vascular system and filled up the coelom space (Hyman 1955; Lawrence 1987; Smiley 1994; Fox 2001). Sea cucumbers have a single, branched gonad. Respiratory trees with highly branched tubes attached to the intestine, which facilitate in taking the oxygenated sea water, are also a characteristic of sea cucumbers (Figure 2.1) (Moore 2006; Byrne 2001).

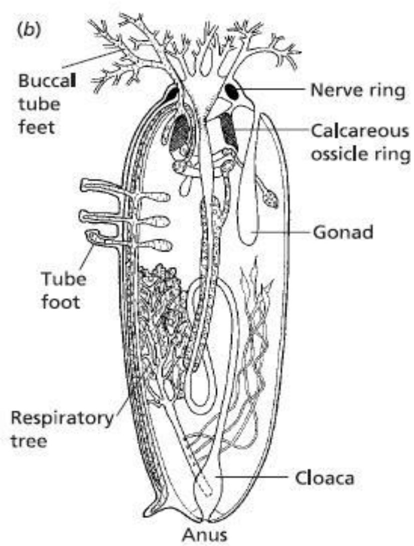


Figure 2.1: Vertical section of a holothuroid (Moore 2006)

Most sea cucumbers are deposit-feeders that ingest sediment with organic matter (Choe 1963; Uthicke 1999; Michio *et al.* 2003). They belong to the family Aspidochirotida which includes the Holothuriidae and Stichopodidae. Other sea cucumbers are suspension feeders and these belong to the family Dendrochirotida, which include the genus *Cucumaria* (Kelly 2005). Their anterior tube feet will form long buccal tentacles that are held out around the mouth like a net or gather food from the sand (Pechenik 2000; Moore 2006). Sea cucumber locomotion is slow, via two ways either by worm-like wriggling of the muscular body wall or by tube feet in ambulacral grooves on the underside of its body (Moore 2006). These invertebrates have a life span of five to ten years and most of the time they reproduce at two to six years (Ibrahim 2003; Kelly 2005). Sea cucumbers can reproduce through sexual reproduction as well as asexual reproduction. Commonly, they reproduce sexually but the process is not too intimate, where the eggs and sperm are ejected in the water and fertilized. This is followed by the formation of larvae which float in the ocean until they settle in an appropriate place (Charles Darwin 2006). Then juvenile sea cucumbers are formed from the larvae and will develop into adult sea cucumbers (Pechenik 2000; Charles Darwin Foundation 2001; Charles Darwin 2006). In asexual reproduction, sea cucumbers propagate through the process of transverse fission that occurr under natural conditions, and then regenerate either the anterior or posterior end (Mackey & Hentschel 2002). These invertebrates can be found at all depth of the sea from the intertidal zone to the deepest oceanic trenches, and are distributed over all latitudes from the poles to the tropics (Hawa *et al.* 2004; Kerr & Kim 2001).

Generally, there are two main important features of sea cucumber. First, undoubtedly were their medicinal values. Researchers isolate their active compound to produce antimicrobial activities, to act as anti-inflammatory agents and to serve as anticoagulants. Second, sea cucumber could generate multimillion-dollar as they form a gourmet food item in the orient (Kerr 2000). Sea cucumber could increase the development of food industry throughout the world. *Holothuria*, *Actinopyga* and *Bohadschia* are among genuses that are frequently exploited for food (Choo 2008). Chinese philosophy has long considered that food and medicine are one entity. It is, therefore, very popular for Chinese to regard food as a medicine for prevention and treatment of disease. Hence, the popular Chinese name for sea cucumber is "haishen", which means "ginseng of the sea" (Chen 2004).

2.1.1 Taxonomy of Sea Cucumber

Taxonomy of sea cucumber (Hawa *et al.* 2004):

Phylum: Echinodermata

Class: Holothuroidea

Order: Aspidochirota

Family: Stichopodidae

Genus: *Stichopus*

Species: *Stichopus* spp.

Inevitably, the taxonomy for several groups of sea cucumber remains unclear and even certain species have been redefined in the past decade (Toral-Granda *et al.* 2008). To date, this particular matter also not excluded from our Malaysian sea cucumbers which are still unclear (Kamarul *et al.* 2009). Echinodermata are among the most familiar marine invertebrates and they fall into defined class of Holothuroidea, sea cucumbers (Zito *et al.* 2005). There are three subclasses under Holothuroidea; Apodacea, Aspidochirotacea and Dendrochirotacea (Myers *et al.* 2008) and six orders under Holothuroidea class; i) Aspidochirotida, ii) Apodida, iii) Dactylochirotida, iv) Dendrochirotida, v) Elasipodida, and vi) Molpadiida (Smithsonian National Museum of National History 2009). Furthermore, Holothuriidae and Stichopodidae are the two well-known family of sea cucumber in Malaysia from the Aspidochirotida order that are commonly well explored (Choo, 2008; Myers *et al.* 2008). Then, under Stichopodidae family, there are two genuses which are *Stichopus* and *Neostichopus* (Smithsonian National Museum of National History 2009). While under Holothuriidae family, the two genuses are *Holothuria* and *Bohadschia* (Hyman 1955).

The important keys for identification of sea cucumber until species phase could depend on spicules and ossicles shape found in the dermis layer around the sea cucumber's integument body wall and also depend on visceral internal organs like gonad system and respiratory tree (Cannon & Silver 1986; Chan & Liew 1986). Local Malaysia sea cucumber, *Stichopus* spp. is comprised only 7 species from overall 47 sea cucumber species in Malaysia and this amount comprised only a small portion from total of 2500 species in the world. Of the 47 species, 7 species have been taxonomically described as gamat. This includes S*tichopus hermanni, Stichopis horrens, Stichopus variegates* and *Stichopus vastus* (Baine & Forbes 1998). One unique characteristic finding about sea cucumbers *Stichopus spp.* is that, in certain circumstances, they will eviscerate where the anterior or posterior end of the sea cucumber ruptures and parts of the gut and associated organs are expelled (Byrne 1985).

However, identification of taxonomy for the species in universally way, is a scientific discipline that is still controversial and often seen as a burden rather than a facility by a large number of biologists (Samyn 2000). These can be approved by the example of *S. variegatus* which hold its name from 1868 until 1995 when researchers changed its name to *S. horrens* in 1995 when they found that it is synonym to *S. variegatus* (Rowe & Gates 1995; Samyn 2000). Furthermore, *S. variegatus* also misunderstood as similar with *S. hermanni*. However, with detail observations, the behavioral differences have been detected where it is proved that sea cucumber *S. hermanni* is a diurnal species while the other species is a nocturnal type (Zulfigar *et al.* 2000). Therefore, the erroneous in identifying taxonomy of sea cucumber should be avoided as it could bring incorrect conclusions in fundamental and applied level in the future (Samyn 2000).

2.1.2 Sea cucumber *Stichopus hermanni*

Figure 2.2 Sea cucumber *Stichopus hermanni*

Phylum: Echinodermata

Class: Holothuroidea

Order: Aspidochirota

Family: Stichopodidae

Genus: *Stichopus*

Species: *Stichopus hermanni*

Sea cucumber *S. hermanni* (Figure 2.2) which is commonly known as Curryfish or “gamat putih” in local is a tropical sea cucumber species. This species prefer to live in sea grass beds, rubble and sandy-muddy bottoms. For its characteristics, the body length is commonly reaching 35cm, while for the weight; its average is 1.0kg (Conand 1998; Guille *et al.* 1986). The key identification of this species is yellow-orange with numerous conical warts in eight rows and the anus is situated midway between the dorsal and ventral parts called anus terminal (Secretariat of the Pacific Community 2008). *S. hermanni* are among Stichopus spp. that are commonly exploited in pacific island for subsistence use and some market sale (Dalzell *et al.* 1996). This species is most commonly collected and processed for export (Schoppe 2000).

There are several documented research to explore the ability of sea cucumbers *S. hermanni* to regenerate into complete individual from two halves. Then the results showed that, adult *S. hermanni* (with a median wet weight of 3,650 g) were able to regenerate in around 100 days for only the posterior part into a whole animal, with zero per cent survival of the anterior parts and 80 per cent survival of the posterior parts. Meanwhile, for medium (medium weight 1,300 g) and small (medium weight 600 g) *S. hermanni,* they were able to regenerate from 40 to 80 days for both anterior and posterior parts (with 100 per cent survival) into whole animals (Lambeth 2000). For natural spawning, Desurmont (2003) has successfully observed that *S. hermanni* was erected on top of a small rocky pinnacle and it was slowly swaying while releasing dribbles of gametes. From the observations by Zaidnuddin & Forbes (2000) at Pulau Payar, it seems to indicate that *S. hermanni* can be seen in the deeper area of intertidal sea level.

2.1.3 Sea cucumber *Stichopus vastus*

Figure 2.3 Sea cucumber *Stichopus vastus*

Phylum: Echinodermata

Class: Holothuroidea

Order: Aspidochirota

Family: Stichopodidae

Genus: *Stichopus*

Species: *Stichopus vastus*

Sea cucumber *S. vastus* (Figure 2.3) which is a tropical sea cucumber is also known by the name of brown curryfish or “ngimes” in Palau (Secretariat of the Pacific Community 2008; Secretariat of the Pacific Community Fisheries Newsletter 2008) and “gamat sawa” in local. *S. vastus* prefers to live in shallow coastal muddy-sand areas with depth range between 0.5 to 2 m (Secretariat of the Pacific Community 2008). For its characteristics, the integument body wall can grow up to 31 cm (Massin 1999). The key identification of this species is green and yellow harlequin pattern and firm body with quadrangular section (Secretariat of the Pacific Community 2008). *S. vastus* has been widely exploited around the Pacific Island and it’s commonly reach medium to high value (Friedman 2008). Besides, this species also shows commercially important to other countries like Australia and India (Toral-Granda 2007). It is found that, sea cucumber *S. vastus* tend to forcibly eject their integument body walls as responses when attacked. This is due to the soft connective tissue factors of the sea cucumber (Lambeth 2000). The lumen of the dorsal vessel of *S. vastus* is identified by numerous hollow blind processes which it bears, hanging freely into the body–cavity (Habheb & Shipley 1959).

2.1.4 Nutritional and Medicinal Values of Sea Cucumber

Historically for over many centuries, sea cucumbers have been an exotic food delicacy and utilized in folk medicines for the Asians. This indirectly creates potential contribution to economies and livelihoods of coastal communities, being the most economically important fishery and non-finfish export in many countries (Toral-Granda *et al.* 2008). Sea cucumber can be consumed in many ways, but the most significant product is the dried integument body wall known as beche-de-mer (Baine 2004). People in Western Pacific regularly consumed these sea cucumbers especially the organ parts as a source of protein for traditional diets (Toral-Granda *et al.* 2008). Moreover, the intestine parts of sea cucumbers are believed to be good for pregnant women and new mothers (Lambeth 2000). Since sixteenth century, the Chinese had treat sea cucumber as medicinal substances (Miriam 1999). According to legend, the healing properties of the sea cucumber became evident when fishermen who hurt themselves applied the coelomic fluid from sea cucumbers on their wounds and discovered that the wounds healed faster (Langkawi Magazine 2006).

Primarily, sea cucumber has been collected for food, but extensive research on sea cucumber has explored it as a source of medicinal components (Dharmananda 2010). Sea cucumbers have good therapeutic value and potential to be commercialized in the field of modern treatment and cosmetics. These creatures are well known of their high protein contents and absence of cholesterol (Food and Agricultural Organization 1991). The amino acids profile, especially the essential amino acids and the presence of necessary trace elements makes sea cucumber a healthy food item (Wang 1997). In addition, sea cucumbers do contain rich nutritional contents of glycosaminoglycans, chondroitins, protein, lysine, arginine, tryptophan, vitamins A & C, riboflavin, niacin, calcium, iron, magnesium, zinc, sodium, and carbohydrates (Food and Agricultural Organization 1991; Chen 2004). The integument body wall of sea cucumber consists of rich insoluble collagen, which have been used for treating anaemia and acted as a nutrient supplement of haematogenesis (Liu *et al.* 1984). Furthermore, the extracts from digestive tract, gonad, muscles, and respiratory apparatus of sea cucumber, *Cucumaria frondosa* showed a good potency of an antioxidant activity (Mamelona *et al.* 2007).

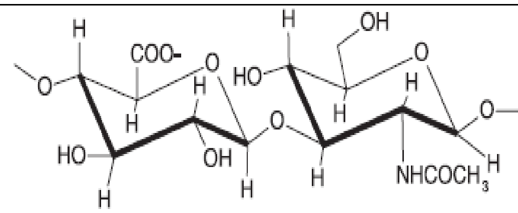
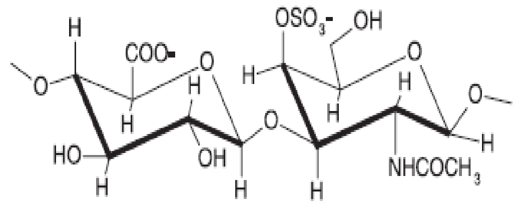
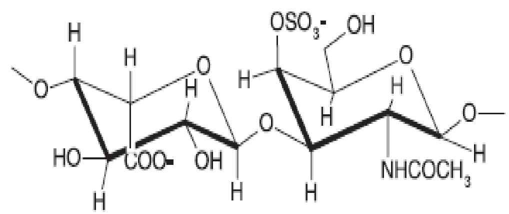
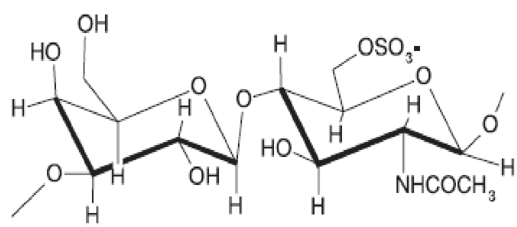
Sea cucumber has been nominated as poly-anion-rich food due to the presence of GAGs (Dharmananda 2010; Liu *et al*. 2002b) that has a physiologically active function, for example, (a) inhibition of some cancers including lung cancer and galactophore cancer (Ma *et al.* 1982); (b) enforcing immune function (Li *et al.* 1985; Chen *et al*. 1987; Sun *et al*. 1991); (c) anti-aggregation of platelet (Li *et al*. 1985); and (d) other functions of pharmaceutical value. GAGs which are sometimes known as mucopolysaccharides (Neha & Ricardo 2008) are large complex carbohydrate molecules that interact with a wide range of proteins involved in physiological and pathological processes (Jackson *et al.* 1991; Casu & Lindahl 2001). GAGs have a special effect on growth, recovery from illness, anti-inflammation, bone formation and prevention of tissue ageing and arteriosclerosis. At the same time, GAGs have been shown to possess an extensive anti-tumour potential (Food and Agricultural Organization 1991). The sulfated GAGs compound has been patented to have antiviral properties. For instance, sea cucumber chondroitin sulfate has displayed to act as HIV therapy and other sulfated GAGs compound from sea weed have been patented as inhibitors of herpes viruses (Dharmananda 2010).

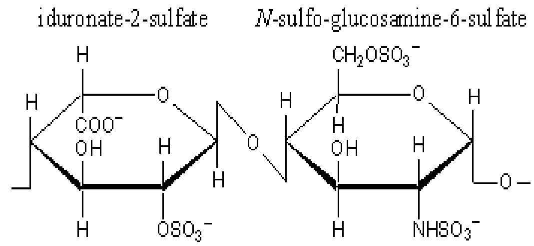
Literatures have indicated that the sea cucumber extracts have antibacterial compound (Afiyatullov *et al.* 2002; Dybas & Fankboner 1986; Aminin *et al.* 2001; Sedov *et al.* 1984). Farouk *et al.* (2007) showed certain Malaysian sea cucumber species (*Holothuria atra*, *Cucumaria fundosa*) recorded to have moderate antibacterial activity against *Klebsiella pneumoniae*, *Serratia marscens*, *Pseudomonas aeruginosa* and *Enterococcus feacalis*. Sea cucumbers have been proven to have curative effects to many diseases. In a manual training report, Food and Agricultural Organization (1991) stated that the integument body wall of *S. japonicus* is known to be a cure for kidney diseases, constipation, lung tuberculosis, anaemia, diabetes and many other diseases. Meanwhile, the invertebrate's viscera was purported to be a cure for epilepsy and its intestine for various curative roles on stomach and duodenal ulcers (Food and Agricultural Organization 1991). The medical important of sea cucumber is convincing because of the increasing market demand, and consequently this stimulates the development of both farming and fishing of sea cucumber (Chen 2004)

2.2 Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) are long, unbranched polysaccharides composed of repeating disaccharide units consisting of alternating uronic acids (D-glucoronic acid or L-iduronic acid) and amino sugars (D-galactosamine or D-glucosamine) (Table 2.1), typically sulfated disaccharides and are capable of interacting with diverse molecules (Esko 1999; Neha & Ricardo 2008). These polymers are negatively charged due to the presence of sulfate groups in their structure and/or carboxyl groups from the uronic acids, which contribute to the highly polyanionic nature of the GAGs (Sampaio & Nader 2006). There are two types of GAGs, sulfated GAGs and non-sulfated GAGs (Neha & Ricardo 2008). Sulfated GAGs include chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and heparin (Kimata *et al.* 2007; Vijayagopal *et al.* 1980). Hyaluronan is the only GAGs without sulfate groups (Neha & Ricardo 2008). Physiologically, most GAGs are covalently attached to core proteins to form proteoglycans. These GAGs endow proteoglycans with unique biochemical as well as biological properties (George *et al.* 2006).

Table 2.1 Repeating disaccharide units of various glycosaminoglycans (GAGs).

Glycosaminoglycans	Dissacharide units
Hyaluronan (HA)	 D-GlcA- β(1→ 4)-D-GlcNAc-α(1→ 4)
Chondroitin sulfate (CS)	 D-GlcA-β(1→ 3)-D-GalNAc4S-β(1→ 4)
Dermatan sulfate (DS)	 L-IdoA-α(1→ 3)-D-GalNAc4S-β(1→ 4)
Keratan sulfate (KS)	 D-Gal-β(1→ 4)-D-GalNAc6S-β(1→ 3)

Heparan sulfate (HS) or Heparin	 α-*N*-sulpho-6-sulphoglucosaminyl-(l→4)- iduronate 2-sulphate

Source: Neha & Ricardo 2008; Turnbull & Gallagher 1990; Diwan 2008; Bishop *et al.* 2007; Lyon & Gallagher 1998; Bernfield, *et al.* 1999; Penc *et al.* 1999, Gartner *et al.* 2006

GAGs are widely distributed in animal tissues (Harada *et al.* 1969; Linhardt & Toida 2003). These molecules are present on all animal cell surfaces, in the extracellular matrix (ECM), and in the intracellular compartment (Sampaio & Nader 2006). Some of the GAGs are known to bind and regulate a number of distinct proteins, including chemokines, cytokines, growth factors, morphogens, enzymes and adhesion molecules (Jackson *et al.* 1991; Conrad 1998) that are important in cell growth and cell communication (Linhardt & Toida 1997). Nevertheless, GAGs are also found in plant tissues (Hooper *et al.* 1996). Some algae species like *fucoidans* and *carrageenans* do have GAGs in their tissues and the GAGs serve as a protective role to the plants (Witvrouw & DeClerq 1997; Toshihiko *et al.* 2003).

GAGs have shown various chemical and biological functions that are benefits to human and even living organisms (Toshihiko *et al.* 2003). Lehninger *et al.* (2004) stated that anti-coagulation was the first described function for sulfated GAGs since first discovered in 1917. GAGs play a major role in cell signalling and development, angiogenesis (Iozzo & San Antonio 2001), axonal growth (Holt & Dickson 2005), tumour progression (Liu *et al.* 2002a; Timar *et al.* 2002), metastasis (Liu *et al.* 2002a; Sanderson 2001) and anti-coagulation (Casu *et al.* 2004; Fareed *et al.* 2000), affect hemostasis and platelet aggregation (Vijayagopal *et al.* 1980). Both GAGs and proteoglycans (PGs) are believed to play a very important role in cell proliferation because they act as co-receptors for growth factors of the fibroblast growth factor (FGF) family (Neha & Ricardo 2008).

2.2.1 Sulfated GAGs

2.2.1(a) Chondroitin sulfate

Chondroitin sulfate (CS) chains are linear polymers comprising disaccharide composition of N-acetyl-D-galactosamine (GalNAc) (β 1, 4) glucuronic acid (GlcA) which may be sulfated at C-4 or C-6 of GalNAc (Nandini & Sugahara 2006; Turnbull *et al.*1995). The structure of CS chains is varies widely. These variations include length, arrangement of disaccharide units, charge density, sulfation pattern, and configuration (Sugahara & Yamada 2000). CS is the most abundant GAG within the body and widely distributed in humans, (Lauder *et al.* 2001) other mammals (Lauder *et al.* 2000) and invertebrates, (Mourão *et al.* 1996; Yamada *et al*. 2007). CS can be found in cartilage, tendon, ligament and aorta (Neha & Ricardo 2008). In invertebrates, the occurrence of CS type E was originally described in squid cartilage (Suzuki *et al.* 1968). Several researches have proved the percentage of CS type E is about 65% in squid cartilage (Yoshida *et al.* 1989; Kawai *et al.* 1966), 12.1% in bovine kidney and 7% in shark fin

(Yoshida *et al.* 1989). CS has also been detected in squid cornea (Karamanos *et al.* 1991) and the integument body wall of sea cucumber *Ludwigothurea grisea* and *S. japonicus* (Kariya *et al.* 1990; Vieira & Mourão 1988). In addition, sea cucumber muscles contain high concentrations of fucosylated CS which surrounds the muscle fibers (Landeira-Fernandez *et al.* 2000).

CS does not have a unique structure, it is a molecular type with a very wide range of structures (Lauder *et al.* 2000) and the impact of this diversity upon function is significant (Fried *et al.* 2000). CS is most frequently found as a proteoglycan in which CS is covalently bound to the core protein by way of 10 to 100 xylolose-modified Ser residues (Gilbert *et al.* 2004). CS proteoglycans (aggrecan, versican, and decorin) have various biologic functions, including collagen fibril assembly (Danielson *et al.* 1997), intracellular signaling, cell recognition, connection of ECM constituents to cell surface glycoproteins (Ayad *et al.* 1994) and cell division and development of the central nervous system (Sugahara *et al.* 2003; Sugahara & Mikami 2007; Nandini & Sugahara 2006). In the recent years, CS have become a focus of attention by virtue of its tangible roles in wound healing, as neurite outgrowth promoters, as well as axonal regeneration, cell adhesion, cell division and in regulatory roles of growth factors (Nandini & Sugahara 2006). In concurrent with the research by Kirker and friends (2002) showed that, the use of an experimental, biocompatible, nonimunogenic, pliable CS hydrogel seems to have benefits in the healing of full thickness cutaneous wounds observed in a mouse model and this was highlighted as a superior treatment than the HA hydrogel.

2.2.1(b) Heparan sulfate

Heparan sulfate (HS) is a linear polysaccharide and is common with other GAGs that has a repeating disaccharide unit structure composed of N-substituted (Nacetyl or N-sulpho) glucosamine (GlcNAc; $GlcNSO_3$) and hexuronic acid (HexA) (Lyon & Gallagher 1998). HS can be distinguished by the presence of N-sulfate groups and α-linked hexosamines (Turnbull *et al.* 1995). HS proteoglycans (HSPGs) are the major component of ECM in mammals (Bishop *et al*. 2007) and are able to bind to and regulate the activity of many growth and signalling factors (Guo *et al.* 2007). HSPG is a normal constituent of basement membranes that presumably plays an important role in the organization of basement membranes components (John *et al.* 1980). HS which is originally called heparitin sulfate is structurally similar to heparin. HS contains a higher level of acetylated glucosamine (Lindahl & Kjellen 1991) and is less sulfated than heparin (Conrad 1998). Heparin is synthesized by and stored exclusively in granules of mast cells (Nader & Dietrich 1989), whereas HS is expressed on cell surfaces of all species (Gomes & Dietrich 1982; Toledo & Dietrich 1977) and in the ECM as part of a proteoglycan (Varki 1999). However, both HS and heparin differ from other sulfated GAGs by having N-sulfated hexosamines within their molecular structure (Esko & Lindahl 2001).

Experimentally, HS chains have been shown to interact with a wider and rather varieties of proteins (e.g., growth factors, chemokines, ECM proteins, enzymes, and enzyme inhibitors) of which, some of these interactions are mediated via specific intra-chain sequences (Gallagher 1995; Salmivirta *et al.* 1996). Such specificities are assumed to be indicative of significant biological relevance. Studies utilising organisms' model and human diseases have also demonstrated HS importance in development and sustaining normal physiology (Bishop *et al.* 2007). Research by Guo *et al.* (2007) has recognized HS as a regulator of cell adhesion and migration. HS was reported to be involved in regulating cancer cell adhesion, migration and focal adhesion complex formation (Guo *et al.* 2007). However, HS from different tissues may have potentially significant differences structure, thus presumably different in biological activities. Hence, failure to obtain any interesting biological activities with HS from a single source may not be direct reflection of the actual potential activities of HS from other multiple sources (Conrad 1998).

2.2.1(c) Dermatan Sulfate

Dermatan sulfate (DS) is composed of linear polysaccharides with alternating disaccharide units containing a hexuronic acid that is either D-glucuronic (GlcA) acid or L-iduronic acid (IdoA) and hexosamine, N-acetyl galactosamine (GalNAc) joined by B 1, 4 or 1, 3 linkages (Trowbridge & Gallo 2002; Osborne *et al.* 2007). DS is also known as chondroitin sulfate B (CS-B) by its presence of GalNAc, but DS can be distinguished from CS by the presence of iduronic acid (IdoA). It is shown that many mammalian tissues do expressed DS and in addition, DS is the predominant glycan present in skin (Trowbridge & Gallo 2002). While in marine invertebrate, DS is the predominant GAG in the integument (Pelli *et al.* 2007). DS could be isolated from various connective tissues like articular cartilage, blood vessel walls, cornea, sclera, skin, tendon, follicular fluid and yolk sac tumor (Poole 1986).