WOUND HEALING EFFECT OF *Acanthaster Planci* SULFATED GLYCOSAMINOGLYCANS EXTRACTED FROM DIABETIC INDUCED RATS

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF DIAGRAMS

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celcius
A. Planci	*Acanthaster Planci*
cm	Centimeter
CS	Chondroitin sulfate
COI	Cytochrome oxidase I
COT's	Crown-of-Thorns
$CuSO_4$	Copper sulphate
D	Dermis
DC	Discoid crystal
DNA	Deoxyribonucleic acid
DS	Dermatan sulfate
E	Epidermis
EC	Endothelial cell
ECM	Extracellular matrix
ED	Endothelial cell
EG	Eosinophilic granule
FEGSEM	Field Emission Gun Scanning Electron Microscope
FucCS	Fucosylated chondroitin sulfate
g	Gram
G	Granules

GAGs	Glycosaminoglycans
HA	Hyaluronan
H&E	Hematoxylin and Eosin
HMDS	Hexamethyldisilazane
HS	Heparan sulfate
IC_{50}	Low half maximal inhibitory concentration
IL	Interleukin
IqR	Interquartile range
ITIS	Integrated Taxonomic Information System
K	Keratin
kg	Kilogram
KS	Keratan sulfate
L	Lysosome
LIRRF	Lizard Island Reef Research Foundation
m	Meter
M	Molar
mg	Milligram
µg	Microgram
ml	Milliliter
µl	Microliter
mm	Millimeter
NaOH	Natrium hidroxide
NCI	National Cancer Institute

nm	Nanometer
PBS	Phosphate buffer saline
PMNL	Polymorphonuclear leucocyte
RBC	Red blood cell
RER	Reticuloendothelial ribosomes
SEM	Scanning electron microscope
SPSS	Statistical Package for Social Sciences
STZ	Streptozotocin
TEM	Transmission electron microscope
TGF	Tumor Growth Factor
US	United States of America
VEGF	Vascular endothelial growth factor
VS	Vesicles
VC	Vacuoles
WD	Working distance
WHO	World health organization

KESAN PENYEMBUHAN LUKA EKSTRAK GLIKOSAMINOGLIKAN BERSULFAT *Acanthaster Planci* KE ATAS TIKUS DIABETIS

ABSTRAK

Tapak sulaiman adalah echidonermata yang mendiami ekosistem perairan marin tropika dan subtropika Indo-Pacifik. Organisma marin ini adalah pemangsa utama kepada kelesterian ekosistem terumbu karang. Tapak sulaiman: *A.Planci* menyebabkan kemusnahan kehidupan ekosistem terumbu karang secara endemik. Tesis ini mengupas tentang satu kaedah untuk tatacara penggunaan biomas tapak sulaiman sebagai strategi kawalan dan eksploitasi biomas. Pada millinea kini permasalahan mengawalselia aktiviti pemangsa Tapak sulaiman merupakan suatu dilema. Kandungan glikosaminoglikan (GAGs) yang tinggi dijumpai pada Tapak sulaiman. Matlamat utama kajian adalah untuk memanfaatkan glikosaminoglikan (GAGs) yang diekstrak daripada cecair selom badan dan dinding integumen tapak sulaiman pada proses penyembuhan luka diabetik. Penilaian pengamatan makroskopik iaitu saiz luka dan respon peradangan (inflamasi) dan mikroskopik dengan menggunakan mikroskop cahaya dan pengimbaan FEGSEM digunapakai untuk membahas tindakbalas dan penemuan penting analisis histologi berkaitan migrasi sel epitelial, penyusunan kembali serat kolagen, proliferasi sel fibroblas dan pembentukan salur pembuluh darah baharu. Tiga dos kepekatan 1 µg/ml, 3 µg/ml dan 5 µg/ml disapu secara topikal pada luka eksisi ketebalan penuh pada bahagian dosal tubuh tikus streptozotocin (STZ) *Sprague Dawley*. Aplikasi 10 mg dari kepekatan 1 µg/ml, 3 µg/ml dan 5 µg/ml GAGs pada luka berdiameter 6mm disepadukan dengan polimer transdermal berasaskan bahan carbopol dari hari 0 hingga hari ke 12 kajian. Peratusan pengecutan luka yang signifikan ($p<0.0167$) berlaku dalam kumpulan GAGs

daripada cairan selom badan Tapak sulaiman dengan dos kepekatan 5 µg/ml. Keputusan menunjukkan bahawa GAGs dengan dos 5 µg/ml mempercepatkan proses patofisiologi luka, apabila migrasi sel epitelial di amati adalah lebih baik dan signifikan ($p < 0.0167$) berbanding kumpulan kawalan. Walaubagaimanpun tiada perbezaan tercatat antara skor median migrasi sel epitelial untuk kumpulan rawatan apabila dihubungkait dengan kedua-dua ekstrak. Penilaian data LM dan FEGSEM menunjukkan semua luka kumpulan rawatan tercantum erat pada hari ke-12. Wujud pengurangan kehadiran sel mikrofaj dan sel PMNL (sel leukosit polimorphonuklear) dan sel mast dalam semua kumpulan rawatan dan kawalan. Tidak wujud perbezaan statistik pada hari ke-12. Peningkatan proliferasi sel fibroblas ($p < 0.0167$) diamati pada kumpulan rawatan (dengan dos cairan selom kepekatan 5 µg/ml). Pembentukan salur pembuluh darah baru secara signifikan ($p < 0.05$) pada kumpulan dengan cecair selom badan tercatat. Kumpulan dengan ekstrak daripada selom badan di amati dapat meransang penyusunan kembali serat kolagen khususnya pada hari ke-12 ($p < 0.05$). Kajian menunjukkan dengan jelas bahawa GAGs daripada biomas tapak sulaiman dengan tahap dos kepekatan yang berbeza memberi ciri manfestasi penyembuhan luka yang berbeza. Walaubagaimanapun ekstrak daripada cairan selom (dengan dos kepekatan 5 µg/ml) mempamerkan kesan penyembuhan luka yang lebih optimum khususnya pada peradangan kronik (pada model luka tikus diabetik induksi STZ). Penemuan ini memberi harapan baru kepada permasalahan pengurusan luka diabetik.

WOUND HEALING EFFECT OF *Acanthaster Planci* SULFATED GLYCOSAMINOGLYCANS EXTRACTED FROM DIABETIC INDUCED RATS

ABSTRACT

The Crown-of-Thorns starfish is an echidonermata that inhabits tropical and subtropical marine ecosystem within the Indo-Pacific. The starfish: *A. Planci* causes endemic devegetation of coral reef ecosystem. This study extrapolate a industrial able effort to exploite the starfish biomass as strategy management. In this millinea, the strategic management of the starfish: *A. Planci* is a dilemma. High content of GAGs can be extracted from COT's starfish: *A. Planci* biomass. The main aim of this study was to harness the prowess of wound healing capabilities of COT's starfish: *A. Planci* sulfated GAGs extracted from its body's coelomic fluid and body's integument wall in management of chronic (diabetic) wound. Macroscopic observation (wound contraction and inflammatory response) and microscopic assessment (LM and FEGSEM) was adapted to discuss the important features and histological analysis findings related to epithelial cell migration, collagen fibers reorganization, PMNL and fibroblasts cell proliferation and the formation of new blood vessels (angiogenesis). Three concentrations dose of 1 μg/ml, 3 μg/ml and 5 μg/ml were topically applied on the full thickness excision wound at the streptozotocin (STZ) induced diabetic Sprague Dawley male rats. Applications of 10 mg from 1 μg/ml, 3 μg/ml and 5 μg/ml doses of GAGs concentrations applied within a 6mm diameter wound were integrated with carbopol-based transdermal polymer from day 0 to day 12. The wound contraction percentage was significantly ($p < 0.0167$), in sulfated GAGs from COT's starfish: *A. Planci* body's

coelomic fluid of 5 μg/ml concentration study as compared to control group. The results revealed that GAGs with 5 μg/ml doses accelerated patho-physiology dynamics of epithelial cell migration far better and significantly ($p < 0.0167$) than as observed in the control group. However there were no recorded differences between the median score for epithelial cell migration from the treatment group when both doses of extracts was analysed. Assessment from the LM and FEGSEM data revealed complete epithelial bridging of the wound area to all treatment groups on day 12. There is a reduction in the presence of macrophage and PMNL cells (polymorphonuclear leukocytes cells) and mast cells in all treatment and control groups. There was no statistical difference on day 12. The increase in fibroblast cell proliferation ($p < 0.0167$) was observed in the treatment group (with coelomic fluid concentration of 5 μg/ml). The formation of new blood vessels was significantly ($p < 0.05$) in the group treated with body's coelomic fluid. Groups with extracts from coelomic fluid was observed able to stimulate reorganization of collagen fibers, especially on day 12 ($p < 0.05$). This study seems to reveal that GAGs from starfish biomass with different concentration levels manifest different wound healing dynamic features. However, the extracts from coelomic fluid (5 μg/ml concentrations) showed the optimum effect of wound healing, especially in chronic inflammation (on STZ induced diabetic rats models). This discovery gives new hope to the management problem of diabetic wound healing.

CHAPTER 1

INTRODUCTION

1.1 Overview of the research

Within the tropics, Malaysia is one of the biggest continental shelf areas. *Vis a vis*, in comparison with other geographical areas, this region ecosystem is rich in marvellous, magnificent biodiversity, thus considered a gifted habitat and prime with wild population of marine phylum and species diversity (flora and fauna) (Mazlan, *et al*., 2004). Comley, *et al*., (2003) revealing that Malaysia has been blessed with exotic coral reefs. Coral reef organisms display a remarkable specialization, intensive predator prey evolutionary life pathways, and competitive interactions within and among species. In Malaysia, this natural hazard of coral bleaching become a threat to the coral reefs ecosystem and its associated endosymbiotics. In tandem to this, some corallivores species like *Acanthaster Planci* and *Drupella spp.* become one of the cause for coral mortality and divegetation (Mazlan, *et al*., 2004). Crown-of-Thorns (COT's): *Acanthaster Planci*, marine invertebrates can cause the endemic of the coral reefs and will further aggravate the damage that can lead to total coral reef deforestation. These invertebrates occur in large aggregations and high densities (Harborne, *et al*., 2000).

Marine creatures signify a valuable source for new bioactive compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new active substances in the development of bioactive products field. The Crown-of-Thorns, *A. Planci* is well known for its poisonous spines and its body surface (Shiomi, *et al*., 2004; Karasudani, *et al.,* 1996). Various research relating to *A. Planci* includes distribution characteristic (Marsh and Tsuda, 1973;

Yokochi, *et al*., 1991), toxicity and constituentss to its venom (Shiomi, *et al.*, 1989, 1990; Ota, *et al.*, 2006), reproductive system (Nishida and Lucas, 1988; Nina *et al*., 2009), nurturing behavior (Hanscomb, *et al*., 1976; Teruya, *et al*., 2001) and control strategies (Kencington and Kelleher, 1992).

In order to control the outbreak of *A. Planci* and lessen its destruction to coral reefs, some traditional measures have been adopted. But, these monitoring actions (manual captures and cutting up the COT's) are laborious, (injection of chemical regents) are expensive and also some other measures are harmfully to other organisms (Brian, 1995). Therefore, these control measures seem difficult and impossible to widely adapted and practiced in order to control COT's outbreak. As a consequence, a new alternative source is needed to be outsourced for pharmacologically active biocompounds within *A. Planci* biomass and thus can be maximize its added value medical tangibles.

The best known sulfated glycans are the glycosaminoglycans (GAGs) in which composed of a structurally complex and widely diverse class of carbohydrates (Ariana and Vitor, 2017). Glycosaminoglycans, is a major extracellular matrix biocompounds in various pharmaco-physiological events (Shuhei Yamada, *et al*., 2011) in animal (vertebrates and invertebrates) tissues. GAGs are capable in interacting with various functional proteins of numerous pathophysiological systems such as wound repair (Kosir, *et al*., 2000; Ghatak, *et al*., 2015), coagulation (Bourin and Lindahl, 1993), cancer growth and metastasis (Afratis, *et al*., 2012; Liu *et al*., 2002), inflammation (Pomin, 2015), neovascularization (Pardue, *et al*., 2008), tissue development (Esko, *et al*., 2009), regeneration (Salbach, *et al*., 2012) and repair (Peplow, 2005), cellular

growth (Yamada, *et al*., 2008), differentiation (Smith, *et al*., 2011) and migration (Tanino, *et al*., 2010). Their high anionic character and enormous structural heterogeneity throughout their chains becomes one of the factor to that situation. As a consequence, GAGs (mainly those isolated from mammalian sources) are highly in demand and widely explored in medicine as therapeutics and neutraceutical (Ariana & Vitor, 2017).

Until now, approximately 7000 marine natural products, 25 percent of which are from algae, 33 percent from sponges, 18 percent from coelenterates (sea whips, sea fans and soft corals), and 24 percent from representatives of other invertebrates phyla such as ascidians (also called tunicates), opisthobranch molluscs (nudibranchs, sea hares), echinoderms (starfish and sea cucumber) and bryozoans (moss animals) have been isolated by the researchers (Dhivya, *et al*., 2012). About 14,000 pharmacologically bioactive compounds have been extracted from a wide diversity of marine flora and fauna (Adrian, 2007). Some recent studies suggested that glycosaminoglycans which also known as mucopolysaccharides can easily be isolated from an echinoderm. These echinoderms are rich in glycosaminoglycans that can give an effective effect in improving skin appearance, healing wounds and are also important for the healthy functioning of joints (Shirahata, 1990). Glycosaminoglycans play a critical role in the formation of connective tissue components and have been of intrigued for many years, particularly among patient who associated with problems of wound healing (Bentley, 1967).

Wound healing series of critical event by which an impaired tissue is replace, regenerate as closely as possible to its normal state whereas the shrinkage process of

wound area is called the wound contraction. The process of wound healing is mainly depending on the extent of the injury and current state of health tissue and the type of tissue itself. The granulation tissue of the wound injury is principally made out of fibroblast, collagen, edema, and small new blood vessels (Shivananda Nayak, *et al.*, 2006). The wound healing process is an immune-mediated physiologic mechanism that can be categorized by four distinct but overlapping phases as follows: hemostasis, inflammation, proliferation and remodeling (collagen deposition) (Hon Kwon, *et al.*, 2009). In order to accelerate wound healing process, numerous growth factors are secreted during the process.

Diabetic wound healing is a very serious phenomenon both in experimental and clinical diabetes (Lioupis, 2005). This impaired wound healing in diabetes mellitus is a major clinical problem. High blood glucose inhibits proliferation of cells and also decreases collagen production (Hehenberger, *et al.*, 1998). Impaired wound healing also can occur because of the decreased in chemotaxis and phagocytosis (Marhoffer, *et al*., 1992), a reduction in the levels of growth factors, and the inhibition of fibroblast proliferation (Hehenberger, *et al.*, 1998). A serious clinical problem such as diabetes, hypertension and obesity are closely associated with wound healing disorders. Moreover, within this new era, more systemic disorder will occur because of high life expectancies and this will further aggravate medical quandaries especially in diabetis wound healing scope (Frank and Kampfer, 2005).

Yet, despite these obstacles, there are increasing concern for optimism in the treatment of chronic wound focusing in the diabetic wound. A further improvement in the standard care, a technological breakthrough on biological agents and the enhanced in

understanding and correction of pathogenic factors will give a new hope to the problem of impaired healing. Various new approaches for new treatments have become readily available for patients with both acute and chronic wounds over the past decade (Bello and Phillips, 2000). Farid Che Ghazali (2011) have scientifically shown that GAGs from *S. Vastus* and *S. Horrens* accelerate normal wound healing. Therefore, a new milestone is very needed to further harness the prowess of wound healing capabilities of COT's total sulfated GAGs in diabetic wound so as to cross check it's potential to actual clinical scenario to be adapted in diabetic wound management.

Carbomer is suitable for aqueous formulations for topical dosage form. Various types of commercial topical products today was formulated by using the polymer of this type and they have a variety of uses. Carbopol polymers are safe and effective use of the topical gels, creams, lotions and the 'balm'. The use of this polymer has been supported by extensive toxicological studies. In addition, carbopol polymer also shows the effect of low irritation and is not sensitive to continuous use. In addition, carbopol polymer had no effect on the biological activity of drugs used. Carbopol polymer also serves as the medium for the delivery of a very good medicine. Carbopol molecular weight polymers are very high that this polymer cannot penetrate the skin and thus does not interfere with drug activity. In addition, carbopol polymer is thickening agent, emulsification and very good for topical formulations without the use of surfactants. Carbopol polymers can be used to hang permanently active ingredient in the formulation of transdermal topical gel and cream (Gumma, 1971; Strauss, *et al*., 1978; Briede, 1979; Machida, *et al*., 1980; Al-Thursday, *et al*., 1986; Sanghavi, *et al*., 1989; Thoma & Klimek, 1991; Yusef, 1993; Ruiz, *et al*.1994). Thus, the above paragraph gives a new perspective that carbopol

polymer transdermal carrier GAGs will transformed and enhanced GAGs application in diabetic wound management.

1.2 Justification of the study

Entering this millennium industrial revolution era, problems arising from diabetic disease is still a dilemma. This non-communicable disease has become cost management issues. Most patients have a prolonged detrimental effect to their bodies as well as to their lives. The wound takes a long time to heal and easily infected and can lead to amputation. Natural bioactive compound is known to be able in reduce diabetic wound effect and complications. GAGs from other species of echinoderms such as the sea cucumber *Holothuria scabra* and *Stichopus japonicus* (Pacheco, *et al*., 2000), *Stichopus hermanni* and *Stichopus vastus* (Siti Fathiah, *et al*., 2011), sea cucumber Ludwigothurea *grisea* and also from two other species of sea urchins *Lytechinus variegatus* and *Arbacia lixula* (Pereira, *et al*., 1999), sand dollar *Mellita quinquisperforata* and crustacean *Ucides cordatus* (Medeiros, *et al*., 2000) have been biotechnologically extracted. Glycosaminoglycans extracted from a waste marine predator, Crown-of-Thorns (COT's) is hypothesized to be able to accelerate the wound healing process. Little is known about the therapeutic value of sulfated GAGs from COT's invertebrates. Nur Afiqah, *et al.,* (2014) and Siti Fathiah, *et al.,* (2011) has revealed the possible extraction, presence and chemical character of GAGs from body parts of COT's and sea cucumbers. The pharmacologically active biocompounds harvested from these marine sources which have never been ever before scientifically illustrated was found to be compatible in managing diabetic wound dilemma. Thus, this study hopes to extrapolate its prowess, and as such purify and harness purported folk-medicine nature products (from marine-

based-biomass) claims. Glycosaminoglycans (GAGs) that extracted from body region of local COT's will be investigated on streptozotocin (STZ)-induced diabetic rats so as it's macroscopic and microscopic features in diabetic wound healing will be asssed. The resulting outcome will better illustrate how linearity and sensitivity of marine GAGs from COT's biomass orchestrate mechanistic role in diabetic wounds scenario that applied topically on the wound using a carbopol polymer transdermal carrier. The outcome of this research will provide an alternative solution for outsourcing of GAGs and chronic wound healing pharmacologically active and empowered possibility of a GAGs of (non-adulterated) homogenous biocompounds especially for diabetic wound managements.

1.3 RESEARCH OBJECTIVES

1.3.1 Main objective:

To investigate the effect of sulfated GAGs extracted from Crown-of-Thorns biomass on the macroscopic and microscopic wound features in Streptozotocin induced diabetic rat model.

1.3.2 Specific objectives:

1) To investigate the effect of sulfated glycosaminoglycans (GAGs) extract from the invertebrate's coelomic fluid and integument wall on the body weight and blood glucose of streptozotocin induced diabetic rat models.

2) To determine and extrapolate the effect of sulfated glycosaminoglycans (GAGs) extract of macroscopic wound features via wound contraction percentage analysis.
3) To determine and extrapolate the macroscopic (wound contraction percentage) and microscopic features (by using Light Microscope and Field Emission Gun Scanning Electron Microscope) of wound healing on topical application of (1 µg/ml, 3 µg/ml and 5 µg/ml) of GAGs from COT's in diabetic rat wound model.

1.4 Research hypothesis

It is hypothesized that Crown-of-Thorns sulfated glycosaminoglycans have positive wound healing effect on diabetic induced wound on experimental rat model.

CHAPTER 2
LITERATURE REVIEW

2.1 Bioactive compound from marine organism

The sea covered 70% of the world surface. The marine environments have been taxonomied, and 34 among 36 known living phyla have been classified, of which more than 30000 unusual known type of marine flora and fauna was noted (Jimeno, *et al*., 2004 & Anake & Pichan, 2004). There are numerous bioresource of new bioactive substance to be outsourced in marine environment. Their biodiversity and associated biological resource is needed in the development and optimization of novel sustainable commercially exploitable bioactive marine based product (Carte, 1996; Anake & Pichan, 2004).

For a past decade, marine organisms have been known to own a medicinal curative power. But until now, their valuable commercial potential such as neutraceutical properties, bioactive compounds and green technology remained mystery. The marine world exemplifies a largely unexploited reservoir of bioactive components that can be applied in various aspects of food processing, storage and fortification. Thus, the marine environment is special reservoir of bioactive natural products, many of which unveil structural/chemical features not scientifically proof located within terrestrial natural products. These marine-derived functional components like certain polysaccharides (sulfated or total), polyphenols, polyunsaturated fatty acids and carotenoids is asssumed to have a global role in refining human health and nutrition (Newman & Cragg, 2012).

Rhetorically, search for new drug candidate from nature (also called "biorespecting") are focusing on the plants and terrestrial microorganisms (especially those from soil samples biomass). Nowadays, the search for new drug-like chemical shifted to less investigated organism from unique habitat such as the sea rather than focusing on the repeated reisolation of already known compounds (Ebada, *et al.*, 2011). To date, seven drugs derived from marine natural products are already listed either in the EU or in the US and many more are under clinical or pre-clinical investigation.

Neutraceutical notion has been derived by coining the terms of "nutrition" and "pharmaceutical". In this perspective, food based approaches was chosen in supplying the active substances with pharmaceutical properties to humans in order to treat and prevent certain disease conditions. There is various role of the active substance from diverse group of marine organisms in neutraceutical industry because that source recognized as natural and safe for human consumption. Polysaccharides, polyunsaturated fatty acid, polyphenols, bioactive peptide and carotenoids which are known to have anticancer, anti-obese, anti-inflammatory, antioxidant, antimicrobial, prebiotic and probiotic activity are some of the marine derived active ingredients that enabling them to be applied as neutraceutical.

Marine organisms from different species origin yield varieties of sulfated glycosaminoglycans with different characteristic of sugar composition and sulfation patterns. The distinct molecular structures and biophysical properties of marine GAGs reflect the evolutionary adaptation and diversification of sulfated polysaccharides to complex and changing habitats. Marine GAGs are derived from organism that have

significant regenerative capacity. They may have interesting effects on the activities of many growth factors, morphogens that are activated by heparin sulfate co-receptors.

2.2 Crown-of-Thorns (COT's) starfish, *Acanthaster Planci*

The Crown-of-Thorns a starfish (Asteroidae) at the phylum echinodermata. *A. Planci* is a major predator to corals reefness habitat (Pratchett, *et al*., 2009) because it can cause a large-scale coral reefs destruction during its outbreak (Kenneth, 1994; Sweatman, 2008). Crown-of-Thorns starfish, *Acanthaster Planci* are an indigenous intertidal marine organism that inhabitant coral reefs ecosystem throughout the Indo-Pacific and Red Sea ocean. These starfish are the most influential corallivores in the Pacific that feed exclusively on corals polyps (facultative corallivores) (Birkeland, 1989). The Crown-of-Thorns starfish is an Echinodermata sea star that cover its dorsal body and arms by its venomous spines (CRC Reef Research Centre, 2003). The Crown-of-Thorns starfish, a ravenous predator to the coral reefness is an unusually large starfish which may grow to more than one metre in diameter that propagates sexually and asexually (Lassig, 1995; Harriot, *et al*., 2003). Their body is flattened, and there are between 7 and 23 radiating arms from a large central disk body (Madl, 1998). These invertebrates move slowly around its habitat by using their small tubular feet extensions positioned below of its arm (Madl, 2002)

COT's starfish is a bilateral radial symmetry marine invertebrate (Kosarek, 2000). A Crown-of-Thorns starfish propagate when it is of 2-3 years old and this reproductive ability continue for five to seven years. These COT's feeds mainly on the tubular structured coral species; *Acropora spp.* specifically and its coral polyps. The take up of corals match their body size for meal per day. These starfish reproduce by several

females and males releasing their eggs and sperm into the water column above the reef at the same time. This propagates behavior known as broadcast spawning. By this method, the eggs will successfully fertilize and safe from the predators on the reef surface (Oceana Protecting the world ocean). COT's starfish can propagate very rapidly because each COT's female is able to produce a quite huge number of fertilized eggs for about 60 million eggs in a singleseason (Sikorski, 2006). Because of that, it is not surprising if more COT's starfish are being found at every corner of coral reef system.

Two main anatomical build up characterized of the COT's. This is the body and the expanding arms with its appendages (Figure 2.1). The internal tissue of COT's body enclosing several other organs within its body region. Those organs are cardiac stomach and pyloric caeca that accountable in the absorption, digestion and nutrients storage (Pechenik, 2005). On the aboral surface of the arm region, there is tubular feet which is accountable in the movement of COT's (Goldschmid & Madl, 2002), and within each arm region, there are internal tissue indented with several neighboring organs, such as its gonads, ampulla and digestive glands (Grzimek, 1972). On the aboral surface of COT's there are a cylindrical shape of spine with sharp tips whereas on the oral surface, the spines are flat and bent in order to cover the mouth arifice of the starfish (Motokawa, 1986). COT's protect themselves by pricking and stinging with its spines, thus inflicting great pain that can last for hours. The stinging pain will cause a persistent bleeding to the human due to the haemolytic effect of these saponins, as well as nausea and tissue swelling that may persist for a week or more (Birkeland & Lucas, 1990). That's why the starfish have been characterized of having saponins known as asterosaponins in their tissues (Birkeland & Lucas, 1990). The Crown-of-Thorns starfish is believed to have a

very sharp spines, about 5 cm in length, and liable to breaking off in wounds. Injury can occur because of the spine and associated toxic compounds (called asterosaponins, a group of chemicals related to steroids) which are deposited in the tissues on penetration. Skin permeation by the spines is painful. The spines, which are brittle, can lead to swelling and secondary infections and may also break off and become embedded in the tissue and need to remove surgically. These COT's have a very unique way of eating the coral. They positioned themselves over a piece of coral and then release their stomach out of their ventrally placed mouth. The stomach will spread above the fresh hard coral and then the digestive juices will discharge which ultimately dissolve the soft fleshy layer of the coral (Kosarek, 2000). Finally, the underlying coral tissue will be digested by COT's and leaves a white coral skeleton. The digestive juices are very toxic and contained a triglycosides chemical called saponin that can harm other marine organisms.

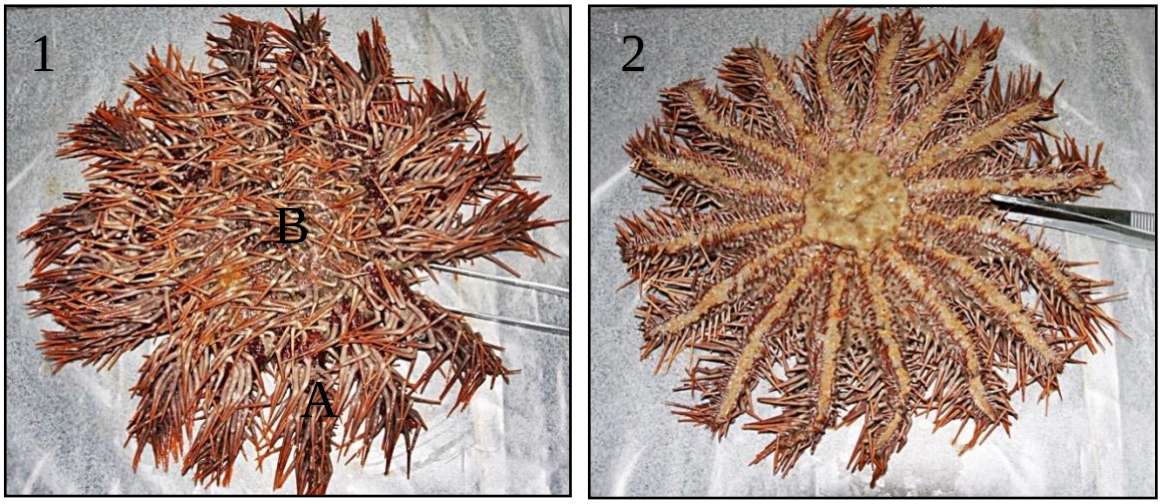


Figure 2.1 A digital photograph of the aboral surface (1) and oral surface (2) of adult Crown-of-Thorns starfish (*Acanthaster planci*). *A = Arm region; B = Body region (Adapted from Nur Afiqah, *et al*., 2012).

2.2.1 Population outbreak of COT's and control strategies

Worldwide, coral reefs are facing the danger of Astroidea COT's outbreaks. Coral reefs are well known with their tangible sources for high quality protein, ornamental products as well as medicinal exploited elements (Bentley, 1998). Coral reefs and its associated eco habitats contained over 4,000 species of fishes and other benthic invertebrates and contribute greatly to fisheries industry. These coral reefs not only produce a raw biomass material from within the coast region but also acts as the wave breaker and habitat protection from storm damage and erosion (Katherine, *et al.*, 2000). The widespread destruction of these coral reefs will give a negative effect in tourism sector, as well as contribute to imbalance in world fisheries population ecosystem. This will lead to economy loss and financial insecurity (Brodie, *et al.*, 2005; Walbran, *et al.*, 1989).

In the last decades, one of the major causes of coral mortality is the mass outbreak of thousands of COT's. This COT's demolish most of the corals on reef and have significantly contributed to coral reef declining (De'ath, *et al.*, 2012). Thus, major controlling issue of these COT's is necessary (Veron, 2008). Fluctuation is one of the major contributor to the incidence of outbreaks occurrence (Brodie, *et al.*, 2005) of COT's because it can harvest high number of egg in the lifespan of single female. Apart from that, human use of the coastal zone (Potts, 1981) and the season of high rainfall or extended dry seasons (Birkeland, 1982) causing water with low salinity, high sediment and loads of nutrient being flushed out into the sea water. These phenomena will increase larvae survival level and lead to larger adult starfish populations. High nutrient

level will increase the population of microscopic algae in the water, thus providing food for the starfish larvae development (Randall, 1972).

The local government and researches take this population outbreaks of COT's as a major issue. The new and advanced strategies in controlling this outbreak is needed. One of the common method that practice worldwide is by physically removes the starfish and buried it to shore (Harriot, *et al.,* 2003; Lassig, 1995). Although this mode of COT's removal seems not being the best approach, but this standard procedure has been applied worldwide. Aside from that, this approach requires the involvement of hundreds of divers to expel manually these echinoderms from the coral reefs to prevent them from the damage (Fraser, *et al*., 2003).

The other conventional approach is by cutting the COT's into a number of pieces (Lassig, 1995). However, this method seems not effectively success in controlling the COT's outbreak because COT's body can generate and survived from the detached part (Lassig, 1995). Instead of reducing the number of COT's, this method may increase the number of COT's population. A high number of experience divers also required in doing this process because it could easily damage the corals reef.

The most effective method is by using the poison to kill these starfish. Copper sulphate ($CuSO_4$) solution was injected to the starfish in order to kill them. This method was relatively inexpensive and safe. However, this method can give an adverse effect to the environment (heavy metal pollution) that could be hazardous to the organisms of coral reefs where the poison injections exercise take place (Lassig, 1995). There are other alternatives poison that used in this method such as concentrated ammonium solution, hydrochloric acid and formalin, but these chemicals are harmful to human and

also tend to damage the poison injection gun itself (Lassig, 1995). As a conclusion, this crown-of-thorns starfish activities need to stop and a proper management must be considered as this predators will cause a lot of adverse effects to the valuable coral reef system. A new alternative that is cost effective and also biological beneficial needed in controlling COT's outbreaks as these echinoderms comprise of biological active substance which possess pharmaceutical and neutraceutical values.

2.3 Glycosaminoglycans (GAGs)

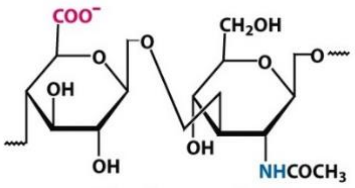
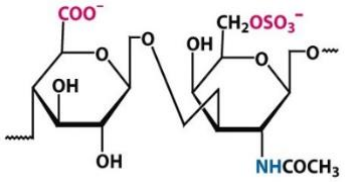
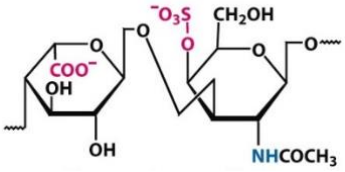
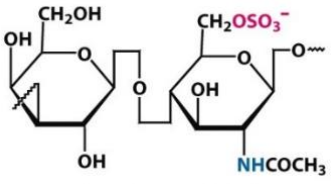
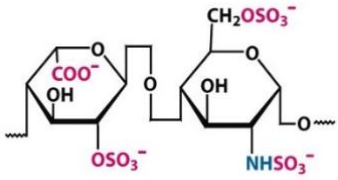
2.3.1 General overview of Glycosaminoglycans (GAGs)

Glycosaminoglycans 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) (Esko, 1999; Neha & Ricardo, 2008). Glycosaminoglycans (GAGs) typically sulfated disaccharides and display a variety of important biological roles. The presence of sulfate group in their structure and carboxyl group from uronic acids causing these polymers in negatively charged, in which can contribute to the greatly polyanionic nature of the GAGs (Lucia & Helena, 2006). Sulfated GAGs and non-sulfated GAGs are the two types of the glycosaminoglycans (Neha & Ricardo, 2008). Glycosaminoglycans can be categorized into four main groups: heparin/heparan sulfate, chondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronan. The differences in its repeating disaccharide units will determined their groups (Fuming Zhang, *et al*., 2010). The structures of these sulfated GAGs and of hyaluronan (the only GAGs without sulfate groups) (Neha & Ricardo, 2008) are all described on (Table 2.1). Physiologically, most GAGs are covalently attached to core proteins to form proteoglycans (George, *et al*., 2006).

GAGs can be positioned in the extracellular matrix (ECM), on all animal cell surface or in the intracellular compartment (Karim Senni, *et al*., 2011). Glycosaminoglycan plays an important role in diverse physiological event in the extracellular matrix of animal tissues. (Shuhei, *et al*., 2011). Glycosaminoglycans denoting preserved functions in all animal kingdoms and there are a lot of research pertaining the presence of GAGs in various vertebrates as well as invertebrates (Shuhei, *et al.,* 2011). Some of the GAGs are known to bind and regulate a number of distinct proteins such as chemokines, cytokines, growth factors, morphogens, enzymes and adhesion molecules (Jackson, *et al*., 1991; Conrad, 1998) that are important in cell growth, cell signaling and cell matrix interactions (Linhardt & Toida, 1997). There is a great potential of these bioactive molecules in medical, pharmaceutical and biotechnological application like tissue regeneration, wound dressings, biomaterials, 3D culture scaffolds and even drugs (Karim Senni, 2011).

Tissues repair abilities and inflammatory response regulated by these polysaccharides biological activities (Lindahl, *et al*., 1997; Ernst & Magnani, 2009; Gandhi & Mancera, 2008; Mulloy & Lindhart, 2001) and play a significant role in various number of diseases. The structure analysis of GAGs improves the understanding and development of structure-activity interactions as well as their biological functions (Fuming Zhan, *et al*., 2010). Glycosaminoglycans has been used in various sectors like cosmetic, food and medical areas (Ronca, *et al.,* 1998 & Kogan, *et al*., 2007).

Table 2.1: Repeating disaccharide units of various glycosaminoglycans (GAGs) (Adapted from Berg, *et al*., 2012).

Glycosaminoglycans	Dissacharide units
Hyaluronan (HA) β (l→3) and β (l→4) linkages	
Chondroitin sulfate (CS) β (l→3) and β (l→4) linkages	
Dermatan sulfate (DS) α (l→3) and β (l→4) linkages	
Keratan sulfate (KS) β (l→4) linkages	
Heparan sulfate (HS) or Heparin α (l→4) linkages	

2.3.2 Sulfated Glycosaminoglycans

A linear polymer comprising of 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 is known as Chondroitin sulfate (CS) chains (Nandini & Sugahara, 2006; Turnbull, *et al.,* 1995). Chondroitin sulfate is an anionic linear polysaccharide, which is synthesized as part of proteoglycan (PG) molecules in vertebrates and invertebrates and a prominent GAGs that can be found in articular cartilage (Karla, *et al*., 2011). The structure and concentration of CS are subjected to the function of tissues and organism itself. CS from inland and aquatic sources comprises of diverse chain lengths and oversulfated disaccharides (Gamjanagoonchom, *et al*., 2007 & Lauder, 2009). Suzuki, *et al.,* (1968) mentioning that the occurance of CS type E in invertebrates was originally found in squid cartilage. 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). The integument body wall of sea cucumber *Ludwigothurea grisea* and *S. japonicas* and the squid cornea (Karamanos, *et al.,* 1991) also contain CS (Kariya, *et al.,* 1990; Vieira & Mourão, 1988). In addition, high concentrations of fucosylated CS also detected in sea cucumber muscles in which it surrounds the muscle fibers (Landeira-Fernandez, *et al.,* 2000).

There are diversity ranges of CS molecular structures with its significant functions (Lauder, *et al.,* 2000) and it does not have a unique structure (Fried, *et al.,* 2000). There are diverse biologic functions of CS proteoglycans (aggrecan, versican, and decorin) such as collagen fibril association (Danielson, *et al.,* 1997), cell division and development of the central nervous system (Sugahara, *et al.,* 2003; Sugahara &

Mikami, 2007; Nandini & Sugahara, 2006), intracellular signaling, cell recognition and the connection of ECM constituents to cell surface glycoproteins (Ayad, *et al.*, 1994). Up to date, because of its noticeable roles as neurite outgrowth promoters, as well as axonal regeneration, cell adhesion, cell division and in regulatory roles of growth factors in wound healing, CS have rising attention among researchers (Nandini & Sugahara, 2006). In concurrent with the research by Kirker, *et al.,* (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. HA and CS which are rich in mammalian tissues are high in activity and diverse physiological functions are the most valuable types of GAGs (Vazquez, *et al*., 2013).

Heparan sulfate (HS) is a linear polysaccharide. It has a repeating disaccharide unit structure composed of N-substituted (Nacetyl or N-sulpho) glucosamine (GlcNAc; $GlcNSO_3$) and hexuronic acid (HexA) and this structure is common with other GAGs (Lyon & Gallagher, 1998). HS can be differentiated by the presence of N-sulfate groups and α-linked hexosamines (Turnbull, *et al.,* 1995). In mammalian, HS proteoglycans (HSPGs) are the major component of ECM (Bishop, *et al*., 2007) and are able to bind 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 is less sulfated than heparin (Conrad, 1998) and contains a higher level of acetylated glucosamine (Lindahl & Kjellen, 1991). HS is expressed on cell surfaces of all species

(Gomes & Dietrich, 1982; Toledo & Dietrich, 1977) whereas heparin is synthesized by and stored in the ECM as part of a proteoglycan (Varki, 1999) and in granules of mast cells (Nader & Dietrich, 1989). Despite that, their molecular structure that composed of N-sulfated hexosamines making both HA and heparin differ from other sulfated GAGs (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). 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 can be differentiated from CS by the presence of iduronic acid (IdoA) but by the presence of GalNAc in its structure, DS is known as chondroitin sulfate B (CS-B). Numerous mammalian tissues do expressed DS. Apart from that, DS is the predominant glycan existing in skin (Trowbridge & Gallo, 2002). In marine invertebrate, DS is the predominant GAGs in the integument (Pelli, *et al.,* 2007) and can be isolated from various connective tissues like articular cartilage, blood vessel walls, cornea, sclera, skin, tendon, follicular fluid and yolk sac tumor (Poole, 1986).

DS which are the major elements of skin have an essential role in coagulation, cell growth and immune defense (Penc, *et al.,* 1998; Trowbridge & Gallo, 2002). DS could affect signaling molecules in response to cellular damage, like wounding,

infection and tumorigenesis (Rostand & Esko, 1997; Schmidtchen, *et al.*, 2001) and could serve as stabilizers, cofactors and/or coreceptors for growth factors, cytokines and chemokines. Researches have actively investigated the role of DS in wound repair activity. Yet, several studies have come out with good implication results of DS in wound repair. Penc, *et al.*, (1998) proved that, DS derived from wounds activates endothelial leukocyte adhesion through stimulation of inter-cellular adhesion molecule 1 (ICAM-1), plus DS showed a good potent promoter of the activity of FGF-2, which is a growth factor that involved in several aspects of the repair response.

Keratan sulfate (KS) is a heteropolysaccharide compound which composed of N-acetyllactosamine repeating units (Hirano, *et al.*, 1961; Mathews & Cifonelli, 1965). KS is short GAG chains consist mainly of mono- and di-sulfated disaccharides of basic structure GlcNAc β 1, 3 Gal (Turnbull, *et al.*, 1995). KS can be found specifically distributed in the ECM of the cartilage, cornea and brain (Funderburgh, 2000). 2 classes of KS are available in which they are classified as KS I and KS II. Initially, the designations of these KS classes were based on differences between KS from cornea and cartilage. For KS biosynthesis, it is broaden through the action of glycosyltransferases which alternately add Gal and GlcNAc to the growing polymer (Funderburgh, 2000).

KS do involve in numerous biological functions. Keratocytes in the corneal stroma is secreted by high abundance of KS in cornea (Funderburgh, *et al.*, 1996). KS is important for the structure and physiology plus the maintenance of tissue hydration in the cornea (Funderburgh, *et al.*, 1991; Funderburgh, 2000). KS also has been suggested to implicate in motility of corneal endothelial cells which is the single layer epithelium that lines the corneal posterior surface. This is approved by the reduction or absent of

KS on migrating cells after wounding occurred at the apical surface (Davies, *et al.*, 1999).

2.3.3 Glycosaminoglycans from marine species

It is now well documented about the presence of sulfated glycosaminoglycans in some phyla of invertebrates (Rahemtula, *et al*., 1974). The world capture of marine organisms including aquaculture (mainly fish, mollusks and crustaceans) amounts to 132 million tons (Food and Agriculture Organization, 2004). Among them, more than 35% of the total weight is handled as by-product and waste that include animal fractions (skeletons, heads, viscera) generated in seafood production or species, sizes or qualities without commercial value (discards and by-catch) (Vazquez, 2013). Mammalian sulfated polysaccharides or glycoconjugates are the most studied polysaccharides for their biological properties because it's comprise by glycosaminoglycans (GAGs) that composed of negatively charged osidic chains, most of them covalently linked to proteins. The emerging of a new modern research has started by the discovery of the biological importance of the mammalian glycoconjugates that focusing on the carbohydrate based recognition phenomena, glycobiology (Lindahl, 1997; Shriver, *et al.*, 2004).

There is tremendous biodiversity in marine environment. Thus, original polysaccharides have been discovered presenting a great chemical diversity that is largely species specific (Karim Senni, *et al.*, 2011). Marine polysaccharides present an enormous variety of structures; they are still under- exploited and they should therefore be considered as an extraordinary source of chemical diversity for drug discovery (Laurienzo, 2011). The high content of GAGs from the wasted COT's biomass can thus

be a better tangible source of collagenous threads as compared with other out sourced Echinodermata mass. Literatures have been documented that body's coelomic fluid contained the highest amount of total sulfated GAGs, followed by body's integument, arm's internal tissue, arm's integument, body's internal tissue and the lowest amount was extracted from arm's coelomic fluid.

GAGs has never been reported in plants. Reports also revealed structural diversity in vertebrate GAGs. Table 2.2 shows a comparison for why GAGs harvesting from inland or marine invertebrates sources is better considered (Proksch, *et al.,* 2002).

Table 2.2: Comparison for why GAGs harvesting from inland or marine invertebrates sources is better considered.

Vertebrates / Inland	**Marine Invertebrates**
1. Heterogenous structure	1. Homogenous structure
2. Diverse sulfation pattern	2. Sulfated total-, N-, and O-sulfated Glycosaminoglycans
3. Mutational defects in most genes – biosynthetically derived enzyme which causes severe consequences.	3. Stable expansion of sulfated structures: Pharmacologically active compounds are associated to a hetero undescribed compounds (reaction of waste to benefit opportunities)
	4. No alteration in structure (morphologically undefended)

According to (Nur Afiqah, *et al*., 2012), it is a better yield of sulfated GAGs has been successfully extracted from crown-of-thorns *A. Planci*, and the quantitative studies conducted suggest that a sustainable production technology is achievable. The high content of GAGs from the wasted COT's biomass can thus be a better tangible source of