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THE EFFECT OF TOTAL SULFATED GLYCOSAMINOGLYCANS FROM CROWN-OF-THORNS *Acanthaster planci* ON WOUND HEALING

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UNIVERSITI SAINS MALAYSIA
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THE EFFECT OF TOTAL SULFATED GLYCOSAMINOGLYCANS FROM CROWN-OF-THORNS *Acanthaster planci* ON WOUND HEALING

by

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Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

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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.

Nur Afiqah Binti Bahrom

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The most beneficent and ever merciful.

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KESAN JUMLAH GLIKOSAMINOGLIKAN SULFAT DARIPADA TAPAK SULAIMAN MAHKOTA BERDURI *Acanthaster planci* TERHADAP PENYEMBUHAN LUKA

ABSTRAK

Pemusnahan terumbu karang oleh mahluk perosak tapak sulaiman mahkota berduri (COTS) *Acanthaster planci* boleh menyebabkan kesan yang berbahaya terhadap kuantiti dan kualiti terumbu karang. Usaha perlu dilakukan untuk menjana kekayaan daripada COTS, bahan yang tidak mempunyai nilai komersial, seperti kajian untuk mengenal pasti potensi terapeutik daripada COTS tersebut. Tujuan kajian ini adalah untuk menyiasat kesan jumlah GAG sulfat daripada integumen, tisu dalaman dan cecair selom daripada dua bahagian utama COTS, iaitu komponen badan dan lengan, terhadap penyembuhan luka pada tikus dengan menggunakan penilaian makroskopik dan mikroskopik.

Integumen dan cecair selom daripada bahagian badan adalah sumber GAG sulfat jumlah dan - N yang tertinggi. Kesemua bahagian anatomikal daripada kedua-dua bahagian badan COTS menunjukkan peratusan (%) GAG sulfat - N lebih tinggi berbanding GAG sulfat - O. Penemuan ini mencadangkan bahawa di dalam komponen badan COTS terutamanya dari integumen dan bahagian cecair selom, terdapat heparin dan / atau heparin sulfat, satu-satunya spesies GAGs yang mempunyai kumpulan sulfat - N di dalam rantaian disakaridanya. 20 µl daripada kepekatan 1 µg/ml jumlah GAG sulfat dari setiap bahagian anatomikal kedua-dua bahagian COTS telah diaplikasikan ke

luka (diameter 6 mm) dengan PBS diaplikasikan sebagai kumpulan kawalan dari hari 0 hingga hari ke-12. Kesan penyembuhan luka dianalisis melalui penilaian makroskopik dan migrasi epitelial, 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).

Pemeriksaan makroskopik menunjukkan terdapat signifikan ($p<0.0167$) peratusan (%) pengecutan luka pada setiap hari pemerhatian (Hari 1, Hari ke-6 dan Hari ke-12) dan kemajuan dalam migrasi sel epitelial berlaku dalam semua kumpulan yang dirawat GAG sulfat daripada bahagian badan COTS berbanding dengan kumpulan kawalan. Penilaian LM dan SEM menunjukkan bahawa luka telah bercantum sepenuhnya pada semua kumpulan yang dirawat GAG sulfat pada hari ke-12 pemerhatian. Penilaian LM dan TEM menunjukkan GAG sulfat dapat mempertingkatkan migrasi sel fibroblas ke kawasan luka secara signifikan ($p<0.0167$) dalam kesemua kumpulan yang dirawat dengan GAG sulfat daripada bahagian badan dan anatomikal integumen daripada bahagian lengan berbanding kumpulan kawalan. Untuk pembentukan pembuluh darah baru, penilaian LM dan TEM menunjukkan keputusan signifikan ($p<0.0167$) pada integumen daripada kedua-dua bahagian badan (2.00, IqR 0.17) dan lengan (2.02, IqR 0.11) COTS berbanding dengan kumpulan kawalan . Evaluasi LM, TEM dan SEM mencadangkan bahawa semua kumpulan yang dirawat dengan GAG sulfat merangsang susunan organisasi serat kolagen secara signifikan ($p<0.0167$) pada hari ke-12 pemerhatian berbanding dengan kumpulan kawalan.

Hal ini menunjukkan bahawa GAG sulfat khususnya dari integumen dan cecair selom dari bahagian badan COTS dilihat dapat mempercepatkan pemulihan luka dan ini dapat dilihat dari kesan positif pada peratusan (%) kecepatan kontraksi luka, peningkatan migrasi epitelial, proliferasi sel fibroblas, proses angiogenesis dan penyusunan serat kolagen.

THE EFFECT OF TOTAL SULFATED GLYCOSAMINOGLYCAN FROM CROWN-OF-THORNS *Acanthaster planci* ON WOUND HEALING

ABSTRACT

Coral reef deforestation by corallivore crown-of-thorns (COTS) *Acanthaster planci* could result into a hazardous effect towards the quantity and quality of the majestic coral reefs. Effort should be made to generate wealth from COTS, a material that does not have any commercial value, such as studies to identify the therapeutic potentials from the COT. This study investigate the occurrence of total sulfated GAGs from the integument, internal tissue and coelomic fluid of COTS starfish and evaluate the effect of total sulfated GAGs on wound healing in rats using macroscopic and microscopic evaluations.

The integument and coelomic fluid from body region were the highest source of total and N-sulfated GAGs. There was more N-sulfated GAGs compared to O-sulfated GAGs for percentage (%) division in both regions. This finding suggested heparin and/or heparin sulphate, the only species of GAGs that have N-sulfated group in its disaccharide chain, could be found in body region of COTS, particularly integument and coelomic fluid parts. 20 µl of 1 µg/ml concentration of total sulfated GAG from each anatomical part of each COTS region were applied to the full-thickness excisional wound model from Day 0 to Day 12, with PBS application as 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 significant (p<0.0167) wound contraction percentage (%) on each observation (Day 1, Day 6 and Day 12) and epithelization progress occurred in all sulfated GAGs treated group from COTS' body region as compared to control group. LM and SEM evaluations showed that all treatment groups have fully bridged the excised wound on the 12th day of observations. LM and TEM evaluations showed enhanced fibroblasts proliferation with significant (p<0.0167) finding occurred in all sulfated GAGs treated group from COTS' body region and integument of arm region compared to control group. For new vessels formation, LM and TEM analysis showed a significant (p<0.0167) increase in the sulfated GAGs treated group from integument of body region (2.00, IqR 0.17) and integument of arm region (2.02, IqR 0.11) as compared to control group. LM, TEM and SEM evaluations showed that sulfated GAGs from all anatomical parts of both COTS' regions stimulate dense organisation of collagen fibers on the 12th day of observation, significantly (p<0.0167) compared to control group.

This study strongly indicate that sulfated GAGs in particularly from its integument and coelomic fluid of COTS' body region, 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.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celcius
A. planci	*Acanthaster planci*
cm	Centimeter
CS	Chondroitin sulfate
COI	Cytochrome oxidase I
COTS	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
FucCS	Fucosylated chondroitin sulfate
g	Gram
G	Granules
GAG	Glycosaminoglycan
HA	Hyaluronan
H&E	Hematoxylin and Eosin
HMDS	Hexamethyldisilazane
HS	Heparan sulfate
IC_{50}	Low half maximal inhibitory concentration
IqR	Interquartile range
ITIS	Integrated Taxonomic Information System
IUCN	International Union for Conservation of Nature
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
s-GAG	Sulfated glycosaminoglycans
SPSS	Statistical Package for Social Sciences
TCBS	Thiosulfate-citrate-bile-sucrose
TEM	Transmission electron microscope
US	United States of America
VEGF	Vascular endothelial growth factor
VS	Vesicles
VC	Vacuoles
WD	Working distance
WHO	World health organization

CHAPTER 1

INTRODUCTION

1.1 Title

The Effect of Total sulfated glycosaminoglycans from Crown-of-thorns *Acanthaster planci* on Wound Healing.

1.2 Background overview

Malaysia is geographically situated in one of the most biodiverse region, hence blessed with exotic coral reefs build up along with its tropical marine life world resources (Comley, *et al*., 2003). However, these build up of coral reefs are now facing an ever increasing detrimental threats related also to global warming that could result into a hazardous effect towards the quantity and quality of its majestic coral reefs. Such an effect can also be caused by crown-of-thorns (COTS) as a predator. *Acanthaster planci* which is a species of star fish that feed on polyps of coral reef is identified as one of the threats, along with other factors such as nutrient runoff from human activities, presence of sediment substrate from forest clearing and coral bleaching due to ever increasing water temperatures (Fenner, 2010). It is feared that these crown-of-thorns (COTS) *Acanthaster planci* will further aggravate the damage when these invertebrates occur in large aggregations numbers and at high densities prevalence which ultimately will leads to total coral reef deforestation scenario (Harborne, *et al*., 2000).

The main concern regarding COTS is that at present, there is no gold standard or scientific ‘SOP’ method available or even nationwide survey done nationally to really identify its actual endemic level. Up-to-date, the only recorded prompt management towards these COTS endemic was to collect and buried these invertebrates in on shore sand bed. This is actually an adaptation of a rhetoric effort practice worldwide. This effort can be assumed as a non-problem solving and inappropriate as it lacks of out-of-the-box-thinking. Effort should be made to generate wealth from this wasted mass, such as studies to identify the therapeutic potentials from the COTS. Until now, the biomass compound of COTS are just being wasted away without applying present knowledge that Echinoderms have unique features such as regeneration properties. Sea cucumbers biomass is at small weight and these invertebrates are not a predator. However, COTS biomass is a wasted entity, hence should be researched for any possible present of health or therapeutic role indicators. We believe that being Echinoderms, COTS should contain a wide range of therapeutic biocompound. Hopefully via these research objectives, we hope that we will be able to extract sulfated glycosaminoglycan (GAG) from the biomass of local COTS, which will be then tested its potential especially in wound healing dynamics and activities.

Wound healing is a complex pathophysiological event that consists of a series of highly complex interdependent and overlapping stages (Inkinen, 2003). Disorders of wound healing present a serious clinical problem and are likely to increase since they are associated with diseases such as diabetes, hypertension, and obesity (Inkinen, 2003). Additionally, increasing life expectancies will cause more people to face such disorders and further aggravate this medical problem (Frank & Kampfer, 2005). Thus, chronic

wounds represent a significant burden to patients and health care professionals. In the US health care system, they affect 5.7 million patients and cost an estimated 20 billion dollars annually while the incidence and prevalence of chronic wounds has risen to epidemic proportion (Branski, *et al*., 2005).

Although many physicians routinely treat acute wounds in their day-to-day practice, the variety and complexity of non-healing chronic wounds presents a particular challenge (Brem, *et al*., 2006). As with any chronic disease process, a wound, regardless of its cause, requires intervention by multiple health care disciplines to address the many conditions and co-morbidities that impact wound healing. Advanced wound care products have been criticized for their expense and perceived overuse, given the relative lack of clinical evidence to support such use. In pertinent to this, this research objectives hopes to come up with an exploitation and commercialization activities globally to a much cheaper solution on producing another wound healing care product using natural product considered waste sources but with tangible scientifically proven results.

The process of wound healing occurs in four phases: (i) hemostasis (or coagulation), which prevents blood loss, (ii) inflammation and debridement of wound, (iii) repair, including cellular proliferation and (iv) tissue remodelling and collagen deposition (Sarma, *et al*., 1990). Thus, any agent which accelerates the above process is a promoter of wound healing. Therefore in this research, instead of just wasting away the COTS, this study aims to explore any possibilities of this creature biomass that could be at beneficial to mankind. GAGs extracted from other sources have been linked to a wide range of applications in the pharmaceutical, cosmetic and food industries (Nakano,

2001). This biocompounds plays a key role in the bioavailability of heparin-binding growth factors by providing storage and protection sites, thus improving wound healing (Giancotti & Ruoslahti, 1999). Hence, GAG derived from biomass of COTS is still a scientific lacuna that should be considered for discovery research as alternative source from marine sources. As part of the effort to elucidate its medicinal or therapeutic potential, this study aimed to focus on the wound healing properties of sulfated GAGs extracted from the integument, internal tissue and coelomic fluid of COTS' body mass and radiating arm regions. The extracted GAGs will then be experimentally tested on dorsal wound of experimental rats.

1.3 Justification of the study

The innovation effort of this research undertaking is that, up-to-date there is no such similar research documented or reported has ever been conducted pertaining to extraction of sulfated glycosaminoglycan (GAGs) from these echinoderms or crown-of-thorns (COTS) *Acanthaster planci* (Linnaeus,1758) starfish wasted biomass. A review of literatures seem to reflect that there are previous researches that have extracted GAG from other species of echinoderms such as the sea cucumber *Holothuria scabra* and *Stichopus japonicus* (Pacheco, *et al*., 2000), *Stichopus hermanni* and *Stichopus vastus* (Masre, *et al*., 2011), sand dollar *Mellita quinquisperforata* and crustacean *Ucides cordatus* (Medeiros, *et al*., 2000), sea cucumber *Ludwigothurea grisea* and also from two other species of sea urchins *Lytechinus variegatus* and *Arbacia lixula* (Pereira, *et al*., 1999). However, very little is known about the isolation or presence of therapeutic sulfated GAG in COTS biomass. If successful, this research may have relevant product

impact and application to a wider areas of clinical concern as the exploration of new therapeutic biocompound agents from COTS biomass involve more weight of biomass as compared to other marine sources.

Glycosaminoglycan (GAG) is well associated to wound healing process. Hence, instead of letting these coral's predators becoming a waste, we hope to generate a potential wealth therapeutic compound from these invertebrates by harvesting GAGs from their biomass for future commercialization in biotechnological and nutraceutical activities. For acknowledgement, the outbreak of COTS starfish affects high-costing management of the vegetation within the marine coral area and severely damages the fishery environment (Bentley, 1998). Since sulfated GAGs are possible to be obtained from COTS, a material that does not have any commercial value, thus it can become a source of bioactive compounds with pharmaceutical potential. Proper scientific management in the elimination of these COTS as wasted wealth-generating products via trials utilizing its body biomass can be an economic success story and management treatise can then providing more choices and sources for wound healing repairs management. The uniqueness of this research is that the COTS starfish, from which to identify the possible presence of sulfated GAGs, extracted from the three anatomical parts: integument, internal tissue and coelomic fluid from two COTS' regions, body and arm. Later, the effect of sulfated GAGs in the wound healing process on a rat dorsal attributed as a wound model was evaluated via macroscopic and microscopic investigations.

1.4 RESEARCH OBJECTIVES

1.4.1 Main objective:

To isolate, identify and to extract glycosaminoglycans (GAGs) from crown-of-thorns (COTS) *Acanthaster planci* starfish biomass harvested in Malaysia marine coast and assess its potential and effect on wound healing in ethically consented animal model.

1.4.2 Specific objectives:

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

1.5 Research hypothesis

Sulfated glycosaminoglycans (GAGs) can be isolated and identified in Malaysia's crown-of-thorns (COTS) *Acanthaster planci* starfish and it can have positive effect on wound healinng.

CHAPTER 2

LITERATURE REVIEW

2.1 Crown-of-thorns (COTS) *Acanthaster planci*

2.1.1 General overview of Crown-of-thorns (COTS)

Crown-of-thorns starfish (COTS) *Acanthaster planci* (Linnaeus, 1758) (Figure 2.1) is a starfish that belongs to the phylum Echinodermata, thus it is a radial marine invertebrate (Kosarek, 2000). COTS *A. planci* is the second largest starfish categorized under the phylum Echinodermata that propagates sexually and asexually (Harriott, *et al*., 2003). The average growth recorded is between 25 to 35 cm in diameter (Sikorski, 2006). Due to its large mass size, these corallivores can feed on coral tissue equal to its own diameter at any one time interval. The starfish is well known for its nature signature as a coral predator that contributes to the widespread devastation of coral reefs vegetation throughout Indian and Pacific Oceans for decades **(**Madl, 2002**)**. These invertebrates are not able to swim, but move slowly around its habitat with the help of tiny tubular feet extensions located underneath each of its many arms (Madl, 2002). Adults COTS have 16 to 21 arms (Harriott, *et al*., 2003) and are multi-colored in presentation with thousands of 4 to 5 cm spinous protuberances (Kosarek, 2000).

COTS are divided into two main regions, the body and the arms appendages (Figure 2.1). Body region consist of mouth at its oral surface with anus and madreporite

are located on the aboral surface (Kosarek, 2000). Madreporite is an opening where water is filtered through it and fills in the water-vascular system of a starfish (Kosarek, 2000). Within the body region, there are internal tissue enclosing several other organs, such as cardiac stomach and pyloric caeca which responsible for absorption, digestion and storage of nutrients (Pechenik, 2005). Tubular feet which responsible in locomotion of the COTS are located on the oral surface of the arm region (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). The spines on the aboral surface of COTS are in cylinder shape with sharp tips whereas the spines on the oral surface are flat and they are bent to cover the mouth of the starfish (Motokawa, 1986). COTS protect themselves with these spines and are capable of pricking and stinging, thus inflicting great pain that can last for hours. Per se, starfish have been characterized of having saponins known as asterosaponins in their tissues (Birkeland & Lucas, 1990). In humans, the stinging pain will caused a persistent bleeding 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). The spines, which are brittle, may also break off and become embedded in the tissue where if this do happen, then they must be removed surgically. The COTS feed mainly on the tubular coral species, *Acropora* specifically and coral polyps (Harriott, *et al*., 2003). These COTS 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 and spread its stomach over the fresh hard coral. It then secretes digestive juices which ultimately dissolve the soft fleshy layer of the coral (Kosarek, 2000). Finally, it digests the underlying coral tissue and leaves a white coral skeleton (Figure 2.2). The digestive

juices are very toxic and contained a triglycosides chemical called saponin that harms other marine organisms.

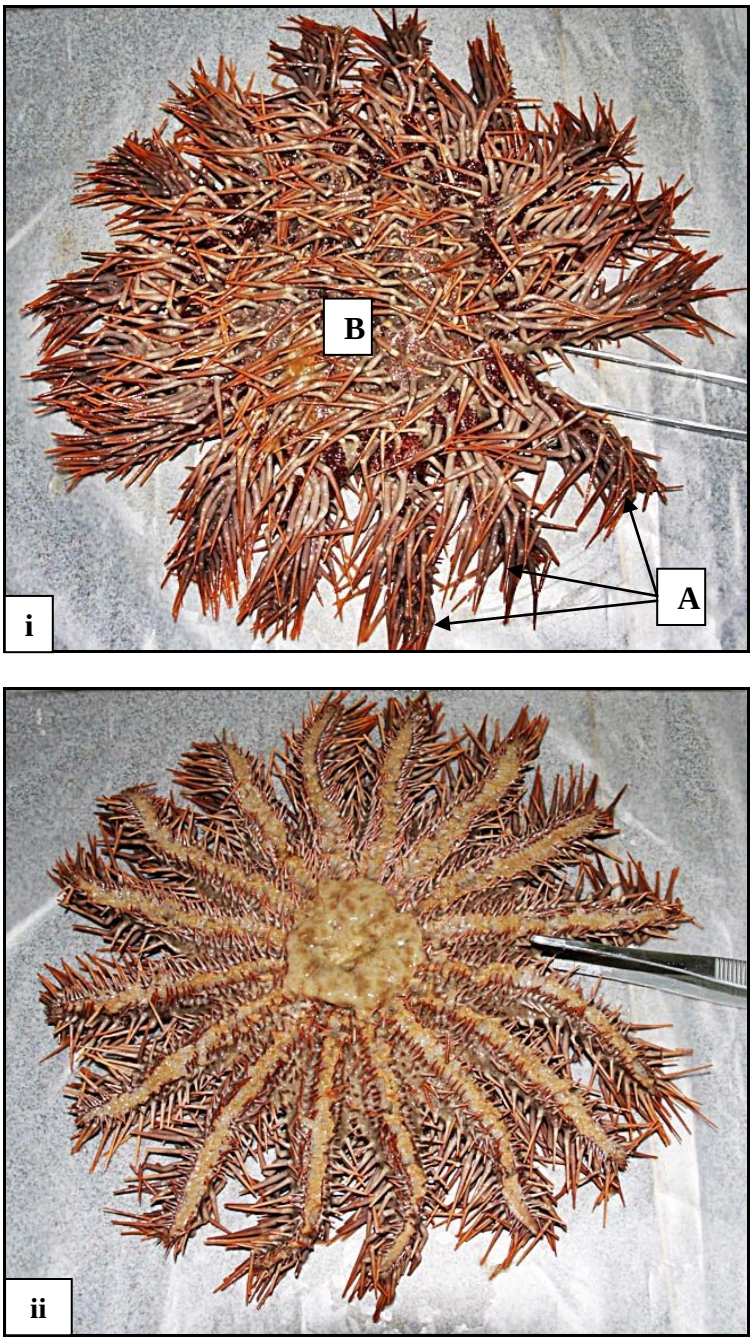


Figure 2.1 A digital photograph of the aboral surface (i) and oral surface (ii) of adult crown-of-thorns starfish (*Acanthaster planci*). *A = Arm region; B = Body region

COTS propagate very rapidly because each female are able to produce 60 million eggs in a single season, a huge number of fertilized eggs (Sikorski, 2006). For that main reason alone, it is not surprising more COTS are being found at every corner where there is coral reef. If the presence and activities of these crown-of-thorns starfish is not stopped, these invertebrates will cause many adverse problems for the coral reefs and surrounding areas which will cause a nation more to manage the eco-system dilemma (Birkeland & Lucas, 1990).

2.1.2 Taxonomy of Crown-of-thorns (COTS)

Table 2.1 A tabulated taxonomic hierarchy of crown-of-thorns starfish.

Taxonomic ranks	Taxonomic hierarchy
Kingdom	Animalia
Phylum	Echinodermata
Subphylum	Eleutherozoa
Superclass	Asterozoa
Class	Asteroidea
Order	Spinulosida
Suborder	Leptognathina
Family	Acanthasteridae
Genus	Acanthaster
Species	*Acanthaster planci*

(Source: ITIS, 2004)

Crown-of-thorns starfish (COTS) *Acanthaster planci* (Linnaeus, 1758) can be found throughout Indo-Pacific region which ranging from the tropical sea water from Indian Ocean to the western and central of Pacific Ocean (Ault, *et al.*, 2011). Table 2.1 revealed the taxonomical hierarchy of crown-of-thorns starfish. Up until the year 2008, COTS has been regarded as a single species throughout its distribution, and therefore the same ecological and behavioural traits are assumed worldwide (Haszprunar & Spies, 2014). However, a molecular research by Vogler, *et al*. (2008) revealed that these Echinoderms are in fact a complex species, due to the molecular and biogeographic investigation of "barcoding fragment" in the mitochondrial COI-gene that revealed there are four different kinds of clades of *A. Planci*. These clades show distinct geographical distribution patterns across the Indo-Pacific, with one species restricted only to the Red Sea, one each occurring in the northern and southern Indian Ocean, and the fourth showing a pan-Pacific distribution (Vogler, *et al*., 2008). This geography speciation differences was probably due to sea-level changes (Pillans, *et al*., 1998), isolating these populations between the world major oceans (Voris, 2000), thus restricted circulation patterns which could reduced larval interchange between populations (Pollock, 1993) and creating ecological differences among lineages (Reid, *et al*., 2006).

2.1.3 Population outbreak of COTS and control strategies dilemma

Coral reefs and its associated habitats are important biodiversity aquatic entities to millions of people around the world as tangible sources for high quality protein, medicinal exploited elements, and ornamental products (Bentley, 1998). They contained over 4,000 species of fish as well as other edible invertebrates and aid lavishly towards

the fisheries industry. They also provide raw materials for dwellings along the coast, and as such protect the fragile shorelines from storm damage and erosion (Katherine, *et al*., 2000). The widespread destroy of coral reefs will not only contribute to the imbalance of ecosystem at the affected areas, but also gives a negative effect on tourism industry, leading to substantial economic loss (Brodie, *et al.*, 2005; Walbran, *et al.*, 1989). Thus, many economies are thus dependent on these reefs and their products.

However, episodic "mass outbreaks" during which tens of thousands of COTS devour most if not all corals on a reef, are one of the major causes of coral mortality in many countries and have significantly contributed to coral reef decline in the last decades (De´ath , *et al*. 2012), thus making it a major management issue (Veron, 2008). The outbreaks resulting in extensive of up to 90% mortality among reef-building and habitat-forming corals (Pratchett, *et al*. 2009).

Figure 2.2 A photograph indicating COTS starfish devouring on the surface of a coral reef. Dead coral reef can be identified by the white area presentation of coral skeleton (Source: LIRRF, 2014)

Fluctuations in crown-of-thorns starfish population is one of the major contributor to the occurance of outbreaks phenomenon (Brodie, *et al.*, 2005). COTS starfish produce large numbers of potentially eggs in the lifetime of a single female. Fluctuations in the environment can change the survival rate of the larvae. Once there is a small increase in the starfish population, the success of subsequent spawning aggregations would ensure that even more larvae could survive in successive generations (Lucas, 1982). Furthermore, periods of high rainfall after drought or extended dry periods (Birkeland, 1982) and human use of the coastal zone (Potts, 1981) cause water with low salinity, high sediment and high nutrient loads to be washed into the sea waters. High nutrient levels can cause an increase in microscopic algae in the water, providing food for the developing crown-of-thorns starfish larvae (Randall, 1972), thus increase the number of larvae that survive and lead to larger adult starfish populations..

Other possible reason for the outbreak is the removal of natural predators of the COTS starfish, which are giant triton snail (*Charonia tritonis*), humphead maori wrasse (*Cheilinus undulatus*), starry pufferfish (*Arothron hispidus*) and titan triggerfish (*Balistoides viridescens*) (Brodie, *et al.*, 2005). The giant triton snail was heavily collected and commercially exploited prior to its protection in 1969 (Harriott, *et al*., 2003), thus the livelihood numbers of these giant snails still remain low. The triton shell can eat only about one COTS starfish per week so its capacity to prevent starfish outbreaks seems limited (Walbran, *et al.* 1989). While the rest of the COTS' predators were heavily fished, thus it seems that the outbreaks of COTS would not be physically stop possible in near the future.

2.1.4 Controlling COTS population outbreaks

The widespread devastations of coral reefs by the population outbreak of COTS have received increasing attention from researchers and local government to control the outbreak. Several techniques have been employed to control the outbreak of COTS. Regardless of techniques or method applied, efforts should be made to generate wealth from this wasted biomass, such as studies to identify the therapeutic potentials available from these COTS.

One of the common method of controling the population outbreak of COTS and have been practising wordwide is to physically removes the starfish and buried it ashore (Harriott, *et al*., 2003; Lassig, 1995). A sharpen stick or hooked steel rod are found to be effective to pull the COTS from the coral reefs (Anthony, *et al*., 1996; Lassig, 1995). This mode of COTS removal although may not be the best of practice for the time being, seems to be the standard procedure applied worldwide. The invasion of these venomous spiny creatures was so apparent that it requires immediate actions by Marine Park Malaysia Department annually at popular sites of islands in Malaysia (Ibrahim, 2010). These involve hundreds of divers to expel manually these ehinoderms from continually devouring the precious coral reefs (Fraser, *et al*., 2003). However, this approach requires experienced divers to pull the starfish from coral reefs. This is to prevent the damage of coral reef itself when COTS are being removed.

The other conventional approach to control the number of the starfish was to cut COTS into a number of pieces (Lassig, 1995). It was found that the survival rate for

COTS which have been cut into pieces was very low. However, this method was not effective to control the population of COTS. This is because, COTS were able to bodily regenerate and survive from the detached part (Lassig, 1995). Therefore, this method may readily increase the number of COTS instead of reducing it. In addition, this method also requires experienced diver to do it. This is because it will easily damage the corals which are firmly surrounded or attached by COTS during these cutting process.

Lassig (1995) reported that the most efficient technique to control the outbreak is by killing the COTS through induced poison injection. Copper sulphate ($CuSO_4$) solution injection was found to be an efficient approach for every killing of the COTS as it was relatively inexpensive and safe method. However, the $CuSO_4$ injection method is associated with the potential for local environmental heavy metal pollution. The heavy metal could be hazardous to the organisms of coral reefs where the poison injections exercise had take place (Lassig, 1995). Although small amount of copper sulphate is used to control the COTS outbreak, there are other alternative poison that also been used for this regards such as concentrated ammonium solution, hydrochloric acid and formalin, but these chemicals are also harmful to human and they also tend to damage the poison injection gun itself. In recent years, sodium bisulphate or dry acid, which is a common swimming pool chemical additive was used to kill the COTS (IUCN, 2008; Koonjul, *et al.*, 2004). Sodium bisulphate offers a more readily available, inexpensive and harmless approach to control the population outbreak of COTS as compare to the hazardous poison of chemicals as stated above (Lassig, 1995). In addition, after this said sodium bisulphate injection, the whole body mass of COTS will undergoes shrinkage and eventually die within a 48 hours time period (Koonjul *et al.*, 2004). As the poison is

injected "*in situ*" to COTS, it is harmless to the neighboring corals and benthic organisms at its surrounding area (Koonjul, *et al.*, 2004).

Latest study also showed that bile salts and thiosulfate-citrate-bile-sucrose agar (TCBS) could be the next potential COTS outbreak control method (Posada, *et al*., 2014). Both injections induced marked epithelial desquamation, epithelial cell destruction and reduction of thickness and disorganization of COTS connective tissue. These injections into COTS induces a rapid condemning disease that is transmitted to incontact COTS. Additionally, there is no introduction of new pathogens into the environment and it is a rapid and simple procedure with immense economic advantages (Posada, *et al*., 2014). The susceptibility of COTS to disease could provide an option for controlling population outbreaks.

2.1.5 Bioactive compounds from COTS

Over the past decades, marine organisms have been considered as new source of biological active substance and compound which possess nutraceutical and pharmaceutical values. The venom in spines of COTS has been widely studied over the past decades by scientists in order to explore the potential application of the venom. The venom COTS was shown to have various biological activities. Glandular cells that are located in the epithelium that covers each spine help to release toxic chemicals into the skin when it stings (Grzimek, 1972). The effect is temporary paralyzation at the sight of sting accompanied by nausea. The crude toxin extracted from these venom glands exhibits many diverse biological activities; edema-formation presentation (Shiroma, *et*

al., 1998), histamine-releasing activities from mast cells (Shiomi, *et al*., 1989), cardio-vascular actions (Yara, *et al*., 1992) and anticoagulant activity (Karasudani, *et al*., 1996).

Two lethal factors have been isolated from the venom of COTS' spines, which are plancitoxins I as the major toxin and plancitoxin II as the minor one (Shiomi, *et al*., 2004). Plancitoxins I are quite unique not only in having potent hepatoxicity (Shiomi, *et al*., 1990), but also in structural resemblance to mammalian deoxyribonucleases II (Shiomi, *et al*., 2004), which are implicated in deoxyribonucleic acid (DNA) degradation during apoptosis (Counis & Torriglia, 2000). Plancitoxin I from COTS' venom also showed to have induced a cytotoxic effect in A375.S2 cells, which is the human malignant melanoma cell and act via the apoptotic procedure (Lee, *et al*., 2014). This suggested that protein toxin from COTS' venom could be commercially exploited for skin cancer treatment. Other than that, two types of phospholipase A_2 enzymes were extracted from venom of COTS' spines, known as AP-PLA_2-I and AP-PLA_2-II (Shiomi, *et al*., 1998). Phospholipase A_2, has hemorrhagic and increasing capillary permeability activities that involved in the local inflammation action line.

A research by Mutee, *et al*. (2012) revealed extract from the whole body mass of COTS can induce significant potent cytotoxic effect on MCF-7 cells, the human breast cancer cell line. The starfish crude extract exhibited significant potent cytotoxic activity on MCF-7 cells with a low half maximal inhibitory concentration (IC_{50}) value of 15.6 μg/ml, $p < 0.01$. The National Cancer Institute (NCI) has reported that crude extract which exhibits cytotoxicity activity with IC_{50} value less than 20 μg/ml is considered as an active compound against cancer cells (Chen, *et al*., 1988). The research also

demonstrated that the COTS extract induces apoptosis in MCF-7 cells. Interestingly, the apoptotic effect of the COTS extract was induced within 2 hours of treatment while apoptotic effect by the positive control drug Tamoxifen was seen only after 4 hours of treatment (Mutee, *et al*., 2012). This showed how potent the COTS extract was against breast cancer cell line

Furthermore, two antiviral compounds, AP-I and AP-II was successfully purified from COTS by Shimizu, 1971. The extracted biological active compounds were found to inhibit the multiplication of influenza virus in chicken embryos. The study also showed that similar antiviral compounds present in two other species of starfish, i.e. *Asterias forbesi* and *Asterina pectinifera* (Shimizu, 1971). In addition to that, COTS integument and internal tissue were believed to contained collagen when the said tissues were examined microscopically (Bahrom, *et al*., 2011; 2012). Collagen were successfully extracted from the body wall COTS (Tan, *et al*., 2013; O'Neil, 1989; Matsumura, 1973). However, the studies on the physico-chemical properties and application of collagen from COTS are still limited.

2.2 Glycosaminoglycans (GAGs)

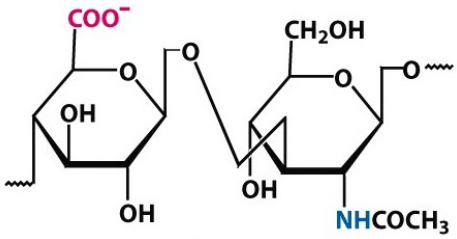
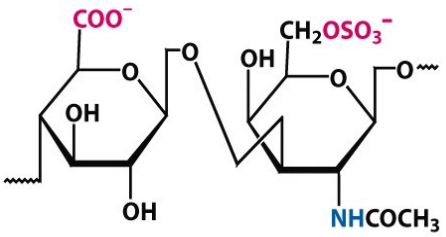
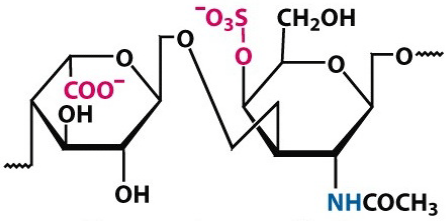
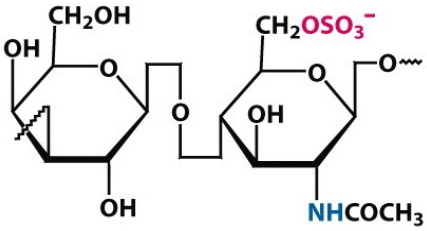
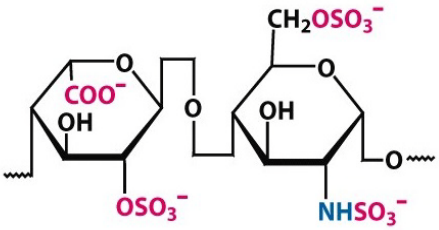
2.2.1 General overview of Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) or mucopolysaccharides are anionic polysaccharides, typically sulfated disaccharides and are capable of interacting with diverse molecules (Neha & Ricardo, 2008; Esko, 1999). These GAGs chains are

composed of alternating units of amino sugar (N-acetylglucoseamine, GlcNAc or N-acetylgalactosamine, GalNAc) and uronic acid (D-glucuronic acid, GlcA or L-iduronic acid, IdoA) (Yamada, *et al*., 2011). There are two types of GAGs, sulfated GAGs and non-sulfated GAGs (Neha & Ricardo, 2008). Sulfated GAGs include chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS) and heparin (Kimata, *et al.* 2007; Vijayagopal, *et al.,* 1980). Hyaluronan (HA) is the only GAG without sulfate groups (Neha & Ricardo, 2008). With the exception of hyaluronan, which exists as a free polymer, all GAGs are covalently attached to core proteins to form proteoglycans in tissues (Kjelle´n & Lindahl, 1991). Proteoglycans are proteins consist of a "core protein" with one or more covalently attached glycosaminoglycans (GAGs) chain (Meisenberg & Simmons, 2006).

GAGs are widely distributed in animal tissues (Harada, *et al.,* 1969; Linhardt & Toida, 2003). These natural substances 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 (Toshihiko, *et al.,* 2003).

Table 2.2 A tabulated repeating disaccharide units of various glycosaminoglycans (GAGs).

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	

(Source: Berg, *et al*., 2012)

2.2.2 Sulfated Glycosaminoglcans (GAGs)

2.2.2 (a) Chondroitin sulfate

Chondroitin sulfate (CS) consists of alternating dissacharide units of N-acetylgalactosamine, GalNAc with sulfate on either C-4 (forming chondroitin 4-sulfate) or C-6 (forming chondroitin 6-sulfate) and D-glucuronic acid, GlcA joined by β (l→3) and β (l→4) linkages (Champe, *et al*., 2005). 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 (Mourao, *et al.,* 1996). CS is produced by cells called chondrocytes, found primarily in cartilage and connective tissue (Yamada, *et al.* 2007). As human beings age, the CS that is produced by the chondrocytes decreases contributing to wrinkled skin, arthritis and other ailments. Supplemental chondroitin sulfates have been part of nutritional intake studies. These studies have shown that the consumption of CS resulted in measurable boosts in their levels within the affected tissues (Kitagawa, *et al*., 1997). In animal, CS has been detected in squid cornea (Karamanos, *et al.,* 1991) and the integument of sea cucumber *Ludwigothurea grisea* and *S. japonicus* (Kariya, *et al.,* 1990; Vieira & Mourao, 1988). In addition, sea cucumber muscles contain high concentrations of fucosylated CS which surrounds the muscle fibers (Landeira-Fernandez, *et al.,* 2000).

CS is well known for 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). A 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. CS proteoglycans have various biologic functions, including collagen fibril assembly (Danielson, *et al.*, 1997), intracellular signaling, cell recognition, cell division and development of the central nervous system (Sugahara & Mikami, 2007).

2.2.2 (b) Heparan sulfate

Heparan sulfate (HS) consists of alternating dissacharide units of D-glucuronic acid, GlcA or Iduronic acid, IdoA and N-acetylglucosamine, GlcA joined by α (l→4) linkages (Lyon & Gallagher, 1998). Heparin have the same disaccharide structure as HS, except HS contains a higher level of acetylated glucosamine (Lindahl & Kjellen, 1991) and is less sulfated than heparin (Conrad, 1998). Both HS and heparin differ from other sulfated GAGs by having N-sulfated hexosamines within their molecular structure (Esko & Lindahl, 2001). Heparin is synthesized by and stored exclusively in granules of mast cells (Medeiros, *et al*., 2000), whereas HS is expressed on cell surfaces of all species (Gomes & Dietrich, 1982) and in the ECM as part of a proteoglycan (Varki, 1999).

HS play a role as a regulator of cell adhesion and migration and also involved in regulating cancer cell adhesion, migration and focal adhesion complex formation (Guo, *et al.*, 2007). HS proteoglycans are the major component of ECM in mammals (Bishop,

et al., 2007) and plays an important role in binding and regulating the activity of many growth and signalling factors (Guo, *et al.,* 2007) and involved in the organization of basement membranes components (John, *et al.,* 1980), angiogenesis and possess a growth factor-dependent activity (Carey, 1997). Heparin, in the presence of heparin-binding growth factors such as FGF-1 and FGF-2, modulates endothelial cell proliferation (Gillis, *et al*., 1995) and migration (Ha¨mmerle, *et al*., 1991) and involved in enhancing the effect of FGF-1 and an inhibitory effect on FGF-2 mitogenic activity.

2.2.2 (c) Dermatan sulfate

Dermatan sulfate (DS) consists of alternating dissacharide units of L-iduronic acid, IdoA and N-acetylgalactosamine, GalNAc joined by α (l→3) and β (l→4) linkages (Osborne, *et al.,* 2007; Trowbridge & Gallo, 2002). There are two species of DS, first is the primary high molecular weight GalNAc-IdoA and second is the low molecular weight GalNAc-GlcA (Matsunaga & Shinkai, 1986). This sequence variability leads to substantial microheterogeneity in the DS polymer and may play a role in its biological activities (Linhardt, *et al*., 1991). DS can be distinguished from CS by the presence of iduronic acid. DS is the predominant glycan present in mammalian skin tissue (Trowbridge & Gallo, 2002) and in marine invertebrates, it can be found in articular cartilage, blood vessel walls, cornea, sclera, skin, tendon and follicular fluid (Poole, 1986). DS which is the major constituent of skin have a vital role in coagulation (Penc, *et al.,* 1998), cell growth, immune defense and as coreceptors for growth factors, cytokines and chemokines (Trowbridge & Gallo, 2002). It also could affect signaling

molecules in response to cellular damage, such as wounding, infection and tumorigenesis (Schmidtchen, *et al.,* 2001). DS proteoglycans will bind to the collagen fibrils in ECM and participate in intercellular signaling (Trowbridge & Gallo, 2002) as well as structural integrity of connective tissue matrices (Schmidt, *et al.,* 1987).

2.2.2 (d) Keratan sulfate

Keratan sulfate (KS) consists of alternating dissacharide units of N-acetylglucosamine, GlcNAc and galactose, Gal joined by β (l→4) linkages (Mathews & Cifonelli, 1965). KS is a short GAG and the only GAGs without uronic acid in its chain (Turnbull, *et al.,* 1995). KS can be found specifically distributed in the ECM of the cartilage, cornea and brain (Funderburgh, 2000). KS which showed high abundance in cornea was secreted by keratocytes in the corneal stroma (Funderburgh, *et al.,* 1996). In the cornea, KS is important for the structure and physiology plus the maintenance of tissue hydration (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).

2.2.3 Sulfated GAGs from invertebrates

GAGs are found not only in vertebrates but also in many invertebrates, implying a conserved presence and function within the animal kingdom (Yamada, *et al*., 2011).