

**COMPARISON OF FUCOSYLATED CHONDROITIN  
SULFATE STRUCTURE FROM THREE  
HOLOTHURIAN SPECIES IN  
SABAH, MALAYSIA**

PERPUSTAKAAN  
UNIVERSITI MALAYSIA SABAH



**MYRON PANG JYAN YU**

UNIVERSITI MALAYSIA SABAH

**BORNEO MARINE RESEARCH INSTITUTE  
UNIVERSITI MALAYSIA SABAH**

**2016**

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**MYRON PANG JYAN YU**

**THESIS SUBMITTED IN FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE**



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**BORNEO MARINE RESEARCH INSTITUTE  
UNIVERSITI MALAYSIA SABAH**

**2016**

# UNIVERSITI MALAYSIA SABAH

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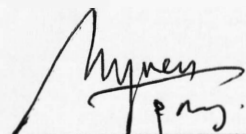
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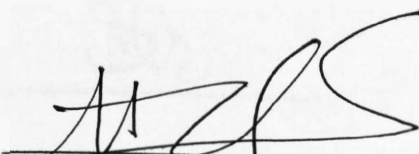
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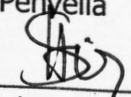
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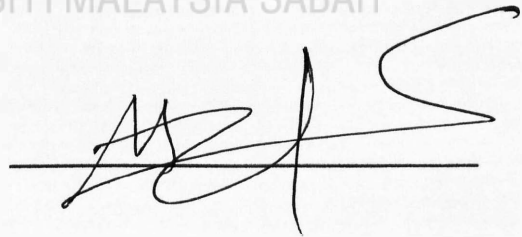
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## ABSTRACT

Fucosylated chondroitin sulfate (FuCS) is a unique compound from commercially important holothurians that possess pharmaceutical bioproduct through its many physicochemical activities but was not explored in non-targeted species of *Stichopus horrens*, *Holothuria atra* and *Holothuria arenicola*. This study has examined the levels and structure of FuCS in these three holothurian species of Sabah using combination of spectrometry, spectroscopy and chemometrics techniques. Through extraction and purification, the FuCS levels for *H. arenicola* was  $66.24 \pm 0.307$   $\mu\text{g/ml}$ , *H. atra* was  $73.16 \pm 0.293$   $\mu\text{g/ml}$  and *S. horrens* was  $104.47 \pm 0.338$   $\mu\text{g/ml}$ . High performance liquid chromatography analysis confirmed each of the extracts had approximately equimolar of D-glucuronic acid (GlcUA) and D-N-acetylgalactosamine (GalNAc), resembling chondroitin sulfate analogs. *Stichopus horrens* had significant higher fucose ratio at 0.78 per mol GalNAc followed by *H. atra* at 0.45 per mol GalNAc and *H. arenicola* at 0.44 per mol GalNAc. Liquid chromatography quadrupole time-of-flight mass spectrometry (LCQTOF/MS) analysis had deduced the mode of mass breakage of the FuCS components and determined the extracts purity of  $>96.1\%$ . Gas chromatography mass spectrometry (GCMS) analysis suggested sulfation was on the fourth and sixth carbon of acetylgalactosamine. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analysis showed additional evidence of sulfate and sulfation positions on the compound. The second derivatives IR spectra of each holothurian species had distinct absorptions difference in the region  $860\text{-}800\text{ cm}^{-1}$ , indicating the sulfation position from each species was different. Principal component analysis (PCA) models had facilitated the IR discrimination and found the greatest influential variables were from carbonyl region, seconded by sulfation region and to a lesser extend of ring vibration region. Both PCA models and hierarchical clustering analysis (HCA) clustered the samples precisely to three clusters. The HCA analysis showed low degree of heterogeneity among the sample branches while samples from *S. horrens* forming an isolated branch at distance height of 285.234 using classical PCA scores and 0.057 using robust PCA scores. In conclusion, these three previously unexplored holothurian species had been proven to possess analogous FuCS chemical structure from commercially important species counterparts.

## ABSTRAK

### **PERBANDINGAN STRUKTUR KON드로ITIN SULFAT FUCOSYLATED DARIPADA TIGA SPECIES TIMUN LAUT DI SABAH, MALAYSIA**

Kondroitin sulfat fucosylated (FuCS) merupakan sebatian unik daripada timun laut bermutu tinggi malah mempunyai nilai bioprospek farmaseutikal melalui pelbagai aktiviti fizikokimia tetapi belum dikaji dalam spesies kurang mutu seperti *Stichopus horrens*, *Holothuria atra* and *Holothuria arenicola*. Kajian ini meneliti tahap dan struktur FuCS dalam ketiga-tiga spesies timun laut daripada Sabah menggunakan kombinasi teknik spektrometri, spektroskopi dan chemometrik. FuCS telah diekstrak daripada *Holothuria arenicola*, *Holothuria atra* dan *Stichopus horrens* dengan memperoleh kepekatan  $66.24 \pm 0.307$ ,  $73.16 \pm 0.293$  dan  $104.47 \pm 0.338$   $\mu\text{g/ml}$ , masing-masing. Analisis kromatografi cecair berprestasi tinggi menunjukkan kesemua ekstrak timun laut mempunyai asid D-glucuronic dan D-N-acetylgalactosamine dengan nisbah yang equimolar, menyerupai struktur kondroitin sulfate. *Stichopus horrens* mempunyai nisbah fucose yang lebih tinggi pada nisbah 0.78/mol GalNAc diikuti *H. atra* pada nisbah 0.45/mol GalNAc dan *H. arenicola* pada nisbah 0.44/mol GalNAc. Analisis kromatografi cecair spektrometri jisim (LCQTOF/MS) berjaya menyimpulkan mod pecahan jisim komponen FuCS dan menghitung ketulenan ekstrak adalah lebih daripada 96.1%. Analisis kromatografi gas spektrometri jisim (GCMS) mencadangkan bukti ikatan ester sulfat pada karbon keempat dan keenam acetylgalactosamine. Analisis pelemahan jumlah pantulan - jelmaan Fourier spektroskopi inframerah (ATR-FTIR) menunjukkan corak sulfat dan kewujudan sulfat pada sebatian. Terbitan kedua IR spektrum setiap species mempunyai perbezaan penyerapan inframerah sekitar  $860\text{-}800\text{cm}^{-1}$ , menunjukkan corak sulfat setiap species timun laut adalah berbeza. Model analisis principal component (PCA) telah memudahkan pembezaan IR dan mendapati pembolehubah paling berpengaruh merangkumi rantau karbonil, sulfat dan getaran cincin. Kedua-dua model PCA dan analisis pengelompokan hierarki (HCA) mengumpulkan sampel tepat kepada tiga kumpulan. Analisis HCA menunjukkan tahap rendah kepelbagaian di kalangan cawangan sampel manakala sampel dari *S. horrens* membentuk cawangan tersendiri pada jarak paksi-y 285.234 menggunakan skor PCA klasik dan 0.057 menggunakan skor PCA teguh. Kesimpulannya, ketiga-tiga spesies timun laut ini telah terbukti mempunyai ciri-ciri FuCS yang sama dengan spesies timun laut yang bernilai komersial tinggi.



## **ABSTRAK**

### **PERBANDINGAN STRUKTUR KON드로ITIN SULFAT FUCOSYLATED DARIPADA TIGA SPECIES TIMUN LAUT DI SABAH, MALAYSIA**

Kondroitin sulfat fucosylated (FuCS) merupakan sebatian unik daripada timun laut bermutu tinggi malah mempunyai nilai bioprospek farmaseutikal melalui pelbagai aktiviti fizikokimia tetapi belum dikaji dalam spesies kurang mutu seperti *Stichopus horrens*, *Holothuria atra* and *Holothuria arenicola*. Kajian ini meneliti tahap dan struktur FuCS dalam ketiga-tiga spesies timun laut daripada Sabah menggunakan kombinasi teknik spektrometri, spektroskopi dan chemometrik. FuCS telah diekstrak daripada *Holothuria arenicola*, *Holothuria atra* dan *Stichopus horrens* dengan memperoleh kepekatan  $66.24 \pm 0.307$ ,  $73.16 \pm 0.293$  dan  $104.47 \pm 0.338$   $\mu\text{g/ml}$ , masing-masing. Analisis kromatografi cecair berprestasi tinggi menunjukkan kesemua ekstrak timun laut mempunyai asid D-glucuronic dan D-N-acetylgalactosamine dengan nisbah yang equimolar, menyerupai struktur kondroitin sulfat. *Stichopus horrens* mempunyai nisbah fucose yang lebih tinggi pada nisbah 0.78/mol GalNAc diikuti *H. atra* pada nisbah 0.45/mol GalNAc dan *H. arenicola* pada nisbah 0.44/mol GalNAc. Analisis kromatografi cecair spektrometri jisim (LCQTOF/MS) berjaya menyimpulkan mod pecahan jisim komponen FuCS dan menghitung ketulenan ekstrak adalah lebih daripada 96.1%. Analisis kromatografi gas spektrometri jisim (GCMS) mencadangkan bukti ikatan ester sulfat pada karbon keempat dan keenam acetylgalactosamine. Analisis pelemahan jumlah pantulan - jelmaan Fourier spektroskopi inframerah (ATR-FTIR) menunjukkan corak sulfat dan kewujudan sulfat pada sebatian. Terbitan kedua IR spektrum setiap species mempunyai perbezaan penyerapan inframerah sekitar  $860\text{-}800\text{cm}^{-1}$ , menunjukkan corak sulfat setiap species timun laut adalah berbeza. Model analisis principal component (PCA) telah memudahkan pembezaan IR dan mendapati pembolehubah paling berpengaruh merangkumi rantau karbonil, sulfat dan getaran cincin. Kedua-dua model PCA dan analisis pengelompokan hierarki (HCA) mengumpulkan sampel tepat kepada tiga kumpulan. Analisis HCA menunjukkan tahap rendah kepelbagaian di kalangan cawangan sampel manakala sampel dari *S. horrens* membentuk cawangan tersendiri pada jarak paksi-y 285.234 menggunakan skor PCA klasik dan 0.057 menggunakan skor PCA teguh. Kesimpulannya, ketiga-tiga spesies timun laut ini telah terbukti mempunyai ciri-ciri FuCS yang sama dengan spesies timun laut yang bernilai komersial tinggi.

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## LIST OF SYMBOLS & ABBREVIATIONS

<b>%</b>	-	Percentage
<b>(S/N)</b>	-	Signal to noise ratio
<b>(v/v)</b>	-	Volume by Volume
<b>°C</b>	-	degree Celsius
<b>®</b>	-	Registered trademark
<b>µm</b>	-	micrometer
<b><sup>60</sup>Co</b>	-	Cobalt 60
<b>Å</b>	-	Angstrom
<b>ACN</b>	-	Acetonitrile
<b>ANOVA</b>	-	Analysis of Variance
<b>aPTT</b>	-	activated partial thromboplastin time
<b>AT III</b>	-	AntiThrombin type 3
<b>ATR-FTIR</b>	-	Attenuated total reflectance-Fourier transform infrared
<b>AUFS</b>	-	Absorbance Unit Full Scale
<b>B.C.</b>	-	Before Christ
<b>BSTFA</b>	-	N,O-Bis-trifluoroacetamide
<b>Ca<sup>2+</sup></b>	-	Calcium ion
<b>CD4</b>	-	Clusters of Differentiation 4
<b>CITES</b>	-	Convention on International Trade in Endangered species
<b>cm<sup>-1</sup></b>	-	Unit for wavenumber; # of waves per centimeter
<b>CNS</b>	-	Central Nervous System
<b>CPC</b>	-	Cetylpyridinium chloride
<b>cPCA</b>	-	Classical Principal Component Analysis
<b>CS D</b>	-	Chondroitin sulfate type D
<b>CS E</b>	-	Chondroitin sulfate type E or Chondroitin 4,6-di-sulfate
<b>CS</b>	-	Chondroitin Sulfate
<b>CXCR4/ CCR5-</b>	-	Chemokine Receptor Type 4 or 5
<b>DHG</b>	-	Depolymerized holothurian glycosaminoglycan
<b>DMMB</b>	-	1,9-dimethylmethylene Blue
<b>DS</b>	-	Dermatan sulfate or Chondroitin sulfate type B
<b>EC<sub>50</sub></b>	-	Effective drug concentration at 50% maximal

<b>ECM</b>	-	Extra cellular matrix
<b>EDTA</b>	-	Ethylenediaminetetraacetic acid
<b>FPLC</b>	-	Fast Protein Liquid Chromatography
<b>Fuc</b>	-	Fucose
<b>Fuc2S4S</b>	-	Fucose, sulfated at carbon-2 and 4
<b>Fuc3S</b>	-	Fucose, sulfated at carbon-3
<b>FuCS</b>	-	Fucosylated chondroitin Sulfate
<b>g</b>	-	Relative centrifugal force to earth's gravity
<b>GAGs</b>	-	Glycosaminoglycans
<b>Gal</b>	-	Galactose
<b>GalNAc</b>	-	N-acetylgalactosamine
<b>GCMS</b>	-	Gas Chromatography Mass Spectrometry
<b>GlcA</b>	-	Glucuronic acid
<b>GLUT4</b>	-	Glucose Transporter 4
<b>GPI</b>	-	Glycosylphosphatidylinositol
<b>GuHCl</b>	-	Guanidine Hydrochloride
<b>H<sub>2</sub>SO<sub>4</sub></b>	-	Sulfuric acid
<b>HA</b>	-	Hyaluronic acid
<b>HC II</b>	-	Heparin Cofactor type 2
<b>HCA</b>	-	Hierarchical Clustering Analysis
<b>HCl</b>	-	Hydrochloric acid
<b>HDL</b>	-	High density lipoprotein
<b>Hep</b>	-	Heparin
<b>HG</b>	-	Holothurian Glycosaminoglycan
<b>HIV</b>	-	Human Immunodeficiency Virus
<b>HS</b>	-	Heparan sulfate
<b>HUVEC</b>	-	Human umbilical vein endothelial cell
<b>IdoaA</b>	-	Iduronic acid
<b>IU/mg</b>	-	International Unit per milligram
<b>kDa</b>	-	kiloDalton or atomic mass unit
<b>KS</b>	-	Keratan sulfate
<b>L-/D-</b>	-	conformation L or D
<b>LDL</b>	-	Low density lipoprotein



<b>LMWH</b>	-	Low Molecular Weight Heparin
<b>M</b>	-	Mole
<b>m/z</b>	-	mass per charge
<b>Man</b>	-	Mannose
<b>MeOH</b>	-	Methanol
<b>mg/g</b>	-	milligram per gram
<b>mg/kg</b>	-	milligram per kilogram
<b>mg/ml</b>	-	milligram per milliliter
<b>MS</b>	-	Mass Spectrometry
<b>MW</b>	-	Molecular Weight
<b>NaCl</b>	-	Sodium Chloride
<b>NIST</b>	-	National Institute of Standards and Technology
<b>nm</b>	-	nanometer
<b>NMR</b>	-	Nuclear magnetic resonance
<b>PAD</b>	-	Pulsed Amperometry Detector
<b>PF4</b>	-	Platelet Factor 4
<b>PI3K</b>	-	Phosphoinositide 3-kinase
<b>PKB</b>	-	protein kinase B
<b>pmol</b>	-	Pico Mole
<b>PMP</b>	-	1-Phenyl-3-methyl-5-pyrazolone
<b>ppm</b>	-	Parts per Million
<b>QTOF</b>	-	Quadruple Time Of Flight
<b>rPCA</b>	-	Robust Principal Component Analysis
<b>RP-HPLC</b>	-	Reverse Phase-High Performance Liquid Chromatography
<b>rpm</b>	-	Revolution per Minute
<b>RSG</b>	-	Rosiglitazone drug
<b>Ser</b>	-	Serine, amino acid
<b>T-20</b>	-	Enfuvirtide drug
<b>TFA</b>	-	Trifluoroacetic acid
<b>TMS</b>	-	Trimethyl-silyl
<b>™</b>	-	Trademark
<b>UFH</b>	-	Unfractionated Heparin
<b>ug/ml</b>	-	microgram per milliliter

- UV** - Ultra Violet
- Xyl** - Xylose
- µL** - microliter



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# CHAPTER 1

## INTRODUCTION

### 1.1 Overview

Among the twelve “mega-biodiversity” countries, Malaysia is accounted for its great biological diversity and species endemism. It is also stated in the Malaysia National Biodiversity Policy (1998) that East Malaysia is blessed with greater diversity and endemism of holothurian species compared to Peninsular Malaysia (MOSTI, 1998). Albeit sea cucumber is considered as small scale fisheries to compared with other fisheries sectors, such as marine finfish and shrimp, the sea cucumber fisheries in Sabah remain significant in terms of commodity contribution to sea cucumber fisheries in Malaysia (Choo, 2004). Sea cucumber harvesting is considered as artisanal fisheries in Malaysia, but plays an important part as a food source and traditional remedies such as for cuts and burns, impotence and rheumatism (Bordbar et al., 2011; Choo, 2004). Out of the estimated 80 species in Malaysia, only a handful are edible (Choo, 2008) such as *Holothuria scabra*, *Thelenota ananas*, *Thelenota anax* and *Stichopus hermanni*, while *Stichopus horrens* and *Stichopus hermanni* are choice species used for making traditional medicine (Choo, 2004). The body wall or internal organs are consumed depending on traditional medicinal beliefs or serves as a food source (Hartati and Yanti, 2006).

Sea cucumber possesses wide array of bioactive components such as saponins, proteoglycans, sulfated polysaccharides, sterols, phenolics, cerebrosides, lectins and fatty acids (Bordbar et al., 2011). These bioactives are gaining recognition in modern biomedical research to explain the properties exhibited in traditional medicine practices such as wound healing, rheumatism, body nourishment and asthma (Chen, 2003). The compound fucosylated chondroitin sulfate (FuCS) is a characteristic sulfated polysaccharide belonging to sea cucumber

species (Yamada et al., 2011). This compound is unique in various bioactivities having a research record for the last three decades from many research groups covering the structural characterization to physicochemical activities (Pomin, 2014a; Myron et al., 2014a). However, structural characterization of FuCS were mainly addressed in a few commercially important species such as *Thelenota ananas* (Wu et al, 2012), *Ludwigothuria grisea* (Mourão, 1996), *Apostichopus japonicus* (Kariya et al, 1997) and *Holothuria forskali* (Panagos et al., 2014).

## **1.2 A unique bioactive compound: fucosylated chondroitin sulfate (FuCS) in sea cucumber**

Fucosylated chondroitin sulfate is an anionic mucopolysaccharide found in the body wall of holothurian (Pomin et al, 2014a). The origin of the compound is thought to be an integral part of holothurian stiffening-liquidation mechanism by being the complex glycosaminoglycan that interacts with core proteins to form proteoglycans found in the cellular environment (Mourão, 1991; Trotter et al., 1998; Koob et al., 1999). FuCS has demonstrated various biological activities such as antithrombotic and anticoagulant (Mourão et al., 1996; Kariya et al., 1997; Wu et al., 2012), angiogenesis and anti-angiogenesis (Tapon-Bretonnière et al., 2000), antitumor (Borsig et al., 2007; Zhang et al., 2009; Lu et al., 2010; Zhao et al., 2013a), wound healing (Masre et al., 2010; Patar et al., 2012a; Patar et al., 2012b), antihyperlipidemic (Tovar and Mourão, 1996; Liu et al., 2002; Liu et al., 2012), antidiabetic (Hu et al., 2013; Hu et al., 2014a) and anti-human immunodeficiency virus-HIV (Hoshino and Heiwamachi, 1990; Huang et al., 2013; Lian et al., 2013). Among the biomedical effects, anticoagulation-related physicochemical activity has been well documented in two species of holothurian namely: *Ludwigothurea grisea* (Mourão et al., 1996), and *Stichopus japonicus* (Kariya et al., 1997). Moreover, the sulfation patterns on the fucose side chain are responsible for the biomedical properties (Pomin, 2014a). Zhao et al. (2012) mentioned the elucidation of whole molecular sequence of FuCS remains a challenge and FuCS structures related to its physicochemical activity was only partially known.

Studies in Malaysia have covered some promising results from the sulfated polysaccharide of three locally available species namely *Holothuria scabra*, *Stichopus hermanii* and *Holothuria leucospilota*. Extracts from *S. hermanii* have

shown potent bioactivity on wound healing (Masre et al., 2010; Patar et al., 2012b) and spinal astrocytes cell line growth (Patar et al., 2012a); An unknown sulfated polysaccharides compound from *H. leucospilota* exhibited antitumor metastasis (Zhang et al., 2009); and extracts from *H. scabra* showed hypolipidemic activity (Liu et al., 2002). The structural characteristics of their extract were not identified in aforementioned studies. Nevertheless, the authors suggested the active compound could be FuCS or FuCS analogs (Masre et al., 2010; Patar et al., 2012a; Liu et al., 2002; Zhang et al., 2009). The general aim of this study is to explore the structural characteristics and levels of FuCS in non-targeted holothurian species found in Sabah using separation techniques and infrared spectroscopy. Understanding the structural characteristics of FuCS will provide fundamental information for future pharmaceutical applications in regards to drug synthesis.

### **1.3 Research objectives**

This study were focused on two main objectives to achieve the aim of providing fundamental structural characterization of FuCS from non-targeted holothurian species:-

- I. To compare the FuCS fine structure extracted from three non-targeted local holothurian species.
- II. To Determine the FuCS levels in three non-targeted local holothurian species.

The specific objectives of this study were:

- a. To optimize FuCS extraction method.
- b. To determine FuCS monosaccharide composition using 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization through reverse phase high performance liquid chromatography (RP-HPLC).
- c. To elucidate the structure of FuCS using mass spectrometry techniques.
- d. To elucidate the structure of FuCS using ATR-FTIR technique.
- e. To compare amongst the FuCS IR spectra of three holothurian species using chemometric analysis.

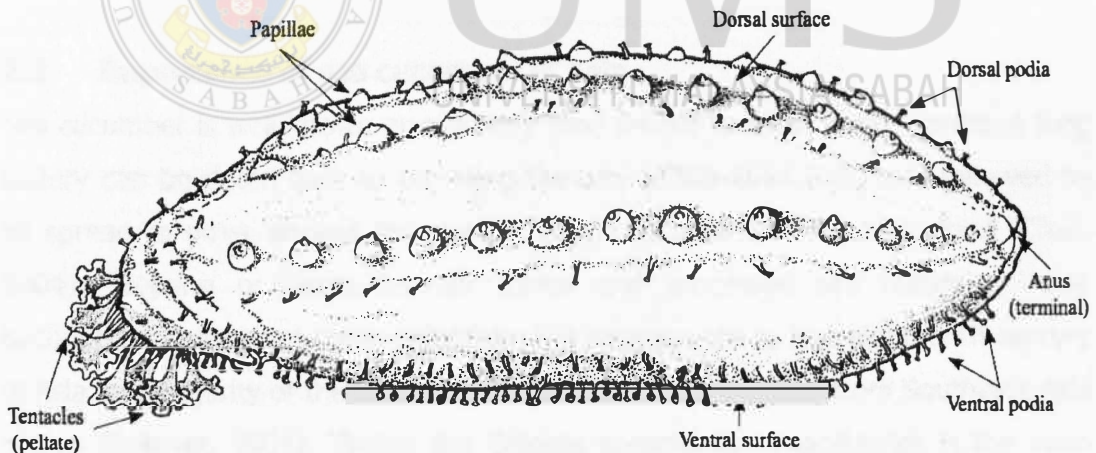


## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Sea cucumber status in Sabah

Sea cucumber or its interchangeable common name, holothurian belongs to the taxonomy class Holothuroidea. The class is further subdivided into three subclass namely Aspidochirotea, Apodacea and Dendrochirotea. The usual external morphology of the body shape is elongated longitudinally with different height of papillae. The stout, thick and firm body has a variable degree of tegument roughness dorsally and usually darker than the ventral (Figure 2.1). Microscopic spicules or "ossicles" are enclosed within the connective tissues in the dermis and used as identification key of species.



**Figure 2.1: External morphology of holothurians (dorsal view).**

Source: Purcell et al., 2012

A total of 62 species belonging to three Orders and five families were reported by Choo (2008) in Malaysian waters. Kamarudin et al., (2010) reviewed the species diversity and estimated there were more than 80 species in Malaysia. The Southeastern coast of Sabah around Semporna which located in the Wallace's