Discovery of novel saponins as potential future drugs from sea cucumber viscera

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For my father, my mother and my family;

Elham and Artin

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ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µL	Microliter
AAM	Antibiotic assay medium no. 1
Agl	Aglycone
C	Carbon
CH_2Cl_2	Dichloromethane
CHCA	Alpha-cyano-4-hydroxycinnamic acid
$CHCl_3$	Chloroform
CID	Collision- Induced Dissociation
CO_2	Carbon dioxide
CPC	Centrifugal Partition Chromatography
Da	Dalton
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
ESI MS/MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
g	Gram
Glc	Glucose
H_2O	Water
HPCPC	High Performance Centrifugal Partition Chromatography
HPDA	Half strength Potato Dextrose Agar
HPLC	High Performance/Pressure Liquid Chromatography
Iso-BuOH	Iso- Butanol
L	liter
L/h	Liter per hour
LC-MS	Liquid Chromatography- Mass Spectrometry
m	meter
m/z	Mass to charge ratio
MALDI MS/MS	Matrix-Assisted Laser Desorption/Ionization mass spectrometry
MeGlc	3-*O*-methylglucose
MeOH	Methanol
mg	Milligram
mL	Millilitre

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaHSO_4$	Sodium monohydrogen sulphate
NaI	Sodium iodide
NMR	Nuclear Magnetic Resonance
PDA	Potato Dextrose Agar
Qui	Quinovose
sulXyl	Sulphated xylose
t	ton
TLC	Thin Layer Chromatography
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UV	Ultraviolet
V	Volt
v/v	Volume per volume
Xyl	Xylose

ABSTRACT

Sea cucumbers are prolific producers of a wide range of bioactive compounds, which are potential sources of agrichemical, nutraceutical, pharmaceutical and cosmeceutical products.

Sea cucumbers expel their internal organs as a defence mechanism called evisceration. We hypothesize that the reason for their ingenious form of defence is because their internal organs contain high levels of compounds that repel predators. To our knowledge, no study has investigated the contribution of saponins from the viscera of any sea cucumber species. Therefore, this project is aimed at the characterisation of the triterpene glycosides, saponins, from the viscera (and body wall) of selected Australian sea cucumber species using high-throughput technologies such as HPCPC and mass spectrometry. The longer term aim is to develop the novel compounds for pharmaceutical or nutraceutical or cosmeceutical application. We will describe the saponin distributions of *Holothuria lessoni* and *Stichopus hermanni* in detailed as representatives of two different families to reveal how their saponin profiles are different.

The saponins were extracted from the viscera or body wall and enriched by a standard liquid-liquid partition process followed by adsorption column chromatography and partition of the eluate into isobutanol. The isobutanol saponin-enriched mixture was further purified by high performance centrifugal partition chromatography (HPCPC) to a high level of purity and recovery. The resultant purified polar samples were analysed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)/MS and electrospray ionization mass spectrometry (ESI-MS)/MS to identify saponin congeners and characterise their molecular structures.

Our results revealed over 100 saponin congeners in the viscera and body wall of *H. lessoni* with a high range of structural diversity, including 45 new sulphated, non-sulphated and acetylated triterpene glycosides.

This study also identified the presence of more than 85 saponin congeners in the viscera of *S. hermanni* of which around half are new compounds. The majority of major identified triterpene

glycosides from the viscera of *S. hermanni* were acetylated, but non-sulphated compounds, contacting six monosaccharaide units, where the abundant saponin congeners from the viscera of *H. lessoni* were mainly sulphated compounds. All of these highlighted the chemical diversity of triterpene glycosides from sea cucumber species. Moreover, the identified saponin congeners have shown strong antifungal property in addition to antioxidant and antiviral activity.

The conventional procedures to differentiate between isomeric saponins, including chemical derivatization and stereoscopic analysis, are tedious and time-consuming. Tandem mass spectrometry was conducted to obtain more structural information about the saccharide moiety and elucidate their structural features. Collision-Induced Dissociation (CID) preferentially cleaves glycosides at glycosidic linkages, which makes the assignment of the sugar residues and elucidation of the structure relatively straight forward.

This study revealed the presence of the highest number of saponin congeners reported from any sea cucumber species in the viscera of examined species, *H. lessoni* and S. hermanni. These congeners contain a diverse range of molecular weights and structures. The mass of reported saponins for these species ranged from 759 Da to 1600 Da. So far we have identified more than 15 aglycone structures in these species.

This research discovered around 100 new compounds from the viscera and body wall of different sea cucumber species with a high range of structural diversity, including sulphated, non-sulphated, and acetylated congeners. In conclusion, our findings showed that the viscera were found to be an excellent repository of numerous unique and novel saponins which have a broad range of potential applications in the health industry as nutraceutical, pharmaceutical, and cosmeceutical products.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed.....

Date.....

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CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Nature is an ancient pharmacy (Montaser & Luesch 2011) with a unique source of pharmaceutical compounds. Oceans, counting for more than 70% of the earth's area (Blunt, J *et al.* 2012; Gomes *et al.* 2014; Montaser & Luesch 2011), contain numerous organisms which are a rich source of diverse therapeutic compounds. Marine organisms exert higher prevalence of bioactive compounds compared to terrestrial organisms (Montaser & Luesch 2011), since biodiversity seems to be much greater in the marine world than on land (Sugumaran & Robinson 2010). The marine environment is exceptionally complex, containing numerous organisms which produce an extremely diverse range of biochemicals attracting the attention of scientists and manufacturers worldwide hoping to discover new substitutes for biologically active materials. In the past five decades, over 24662 new compounds sourced from the marine environment with interesting biological activities have been reported many of which yield a large variety of highly complex chemical structures (Blunt, JW *et al.* 2015). These compounds possess valuable pharmaceutical, nutraceutical and other health beneficial compounds (Ngo *et al.* 2012).

1.2 Benefit of marine organisms; Potential source of new leads

Natural products have played a crucial role in discovery and development of new therapeutic agents. To date over hundreds of molecules have been identified from numerous marine organisms including algae, sponge, coelenterates (sea whips, sea fans and soft corals), echinoderms (sea cucumbers, starfish, etc.), ascidians (also called tunicates), microorganisms, opisthobranch molluscs and bryozoans (Blunt, JW *et al.* 2013; Mayer, A *et al.* 2013). Researchers from 36 countries contributed more than 279 marine compounds to the preclinical pharmaceutical pipeline targeting a small number of diseases (Mayer, A *et al.* 2013). Currently there are six

commercial marine origin compounds approved by U.S. Food and Drug Administration (F&DA) in addition to 11 drug leads which are in Phase I, II and III clinical trials (Mayer, A *et al.* 2013). These medicines have either come directly from marine organisms or have been synthesised as analogies of natural compounds (Blunt, JW *et al.* 2014). These compounds are utilised to treat a range of diseases such as cancer, relieve pain and kill virus and fungi (Montaser & Luesch 2011).

1.3 Taxonomy and classification and general characteristics of sea cucumbers

Sea cucumbers belong to the Animal kingdom, the Echinodermata phylum, and the Holothuroidea class (from the Greek *holothurion*, "sea polyps").

Echinoderms are distinguished by their radial symmetry body plan and are well-known for their ability to regenerate. They are the largest phylum of exclusively marine animals, including around 7,000 known species (Blunt, J *et al.* 2012) which are recognised by the numerous morphological variations (diversity) of its members (Chludil *et al.* 2003). Echinoderms are classified to five main classes (groups) including Holothuroidea (sea cucumbers), Crinoidea (crinoids and sea lilies), Echinoidea (sea urchins, sea biscuits and sand dollars), Asteriodea (starfish), and Ophiurioids (snake stars, brittle stars and basket stars) (Brusca *et al.* 1990; Hashimoto & Yasumoto 1960; Matranga 2005).

The name holothuroid was coined around 23 centuries ago by the Greek philosopher, Aristotle ("holos: whole" and "thurios: rushing") and defined them as "kind of motionless marine organisms" (Pitt & Duy 2004; Samyn 2003) while the scientific name "*Cucumis marimus*" which means "sea cucumber" was created by Pliny (Bordbar *et al.* 2011; Conand 1990b; Croneis & Cormack 1932; Ridzwan 2007). Holothurians are sedentary marine invertebrates, commonly known as sea cucumbers, trepang, bêche-de-mer, or gamat, which vary in size from an inch in length to up to three feet long.

Holothuroidea are divided into three subclasses; Dendrochirotidae, Aspidochirotacea and Apodacea. To date, Holothurians consist of over 1500 species categorised into six main orders namely, Aspidochirotida, Dendrochirotida, Apodida, Dactylochirotide, Molpadiida and Elasipodida,

which include twenty-five families and about 200 genera (Conand 2005a, 2005b; Pawson, D & Fell 1965). These orders are distinguished in according to their anatomical features.

Sea cucumbers are classified into order, family, genus, and species on the basis of their internal and external morphological characteristics (Olivera-Castillo *et al.* 2014), such as general body shape, structure, arrangement of ambulacral feet, tentacle shape, calcareous ring shape, and spicule shape and combination (Pawson, DL *et al.* 2010). Classification at the species level is based on the combination and shape of calcareous deposits or spicules (Olivera-Castillo *et al.* 2014).

Chemical fingerprinting of saponins in holothurians can give insight on the correct taxonomic position of a species. These congeners have the potential to be used as a chemotaxonomic marker for the whole family instead of the usual holothurins (Bondoc *et al.* 2013).

1.4 Habitat, diversity and distribution of sea cucumbers

Holothurians are widespread throughout all oceans and seas around the world at all latitudes, but are most diverse in tropical shallow-waters, from the shore down to abyssal plains (Kim, SK *et al.* 2012; Purcell *et al.* 2012). Sea cucumbers are found at depths ranging from 0.50 to 61.0 m, however their normal inhabit is above a depth of 33 m. They are known as slow-moving invertebrates, most species are nocturnal and benthic. Sea cucumbers are ecologically important as they play a crucial role as bioturbators, recyclers of lagoons and processing of the detritus and organic matter from the sea bed (Lampe 2013). The current knowledge of Holothurian diversity is virtually unknown. Biodiversity may provide chemical diversity (chemodiversity) which increases the chance of exploring novel therapeutic compounds.

Among the commercial coastal holothurians, the Aspidochirotida are found predominantly in the tropics, while the Dendrochirotida, which are generally of little commercial interest, are more common in temperate regions (Conand 1990a; Purcell *et al.* 2012; Stutterd & Williams 2003). However, Lampe (2013) stated the physical factors, such as water temperature, turbidity of the water, salinity degrees and depth at which the species inhabit, as well as nutrient composition, may

have a direct influence on sea cucumbers distribution and prevalence.

1.4.1 Australian sea cucumbers

Over 1,500 species of holothurians are known world-wide. The Australian fauna, when catalogued in 1995, comprised of fifteen families, 69 genera and 211 species, although additional cryptic species of small holothurians continue to be identified from temperate Australian environments (Rowe & Gates 1995). Since then more than 20 species have been recognised from Australian waters, mainly southern Australia (O'loughlin *et al.* 2011, 2012; O'loughlin *et al.* 2014; Shackleton *et al.* 1998). Currently, over 60 species of the family Holothuriidae have been reported in the Australian fauna (Rowe & Gates 1995).

1.4.2 Abundance of commercial and non-commercial species

The most consumable and valuable echinoderms are sea cucumbers (Holothurians) thanks to their food and medicinal application. Over 1500 species of holothurians have been taxonomically described but only a small portion of these are commercially important. Sea cucumbers are fished and traded in over 70 countries around the world. Over seventy species are currently harvested commercially worldwide (Purcell *et al.* 2010; Purcell *et al.* 2012), with most of them including tropical and sub-tropical species. The species that are commercially exploited as food belong to the families Holothuriidae and Stichopodidae, comprising the genus *Bohadschia*, *Holothuria*, *Actinopyga*, *Isostichopus*, *Stichopus*, *Astichopus*, *Parastichopus, Thelenota, Isostichopus* and *Australostichopus* (Purcell *et al.* 2010; Purcell *et al.* 2012; Toral-Granda *et al.* 2008). In addition, three genera of the Dendrochirotids; *Cucumaria, Athyonidium and Pseudocolochirus* cordate to the family Cucumariidae are also commercially important (Conand 2004b; Purcell *et al.* 2012).

In the Western Central Pacific region; Australia and Melanesian countries are the largest exporters of bêche-de-mer in the region (Toral-Granda *et al.* 2008). Currently, 35 known sea cucumber species in the families Holothuriidae and Stichopodidae are harvested for the production of bêche-demer in this region (Purcell *et al.* 2012; Toral-Granda *et al.* 2008). They belong to the order Aspidochirotida. Commercial spices are well-known by having generally a thick body wall.

The large numbers of commercially harvested species belong to the order Aspidochirotida,

exclusively to the families Holothuriidae and Stichopodidae, which are mostly tropical. In addition, fisheries also harvest a number of species belonging to the order Dendrochirotida, family Cucumariidae which are traded to keep in the aquarium as an ornament. In 2010, an estimation of global harvest of sea cucumbers was in the order of 100,000 t per annum (Bechtel *et al.* 2013).

Species and processing conditions of sea cucumbers are two main factors which affect the quality, and therefore price of the products. In general, the Philippines, Indonesia, and China produce lower-quality product, whereas higher-quality product originates from Japan, Australia, South Africa and the Pacific Coast of South America (Olivera-Castillo *et al.* 2014).

The most profitable species in Australia are *A. ecbinites, A. miliaris, A. mauritiana, H. atra, H. wbitmaei, H. scabra, H. lessoni, H. fuscogilva, H. fuscopunctata, S. chloronotus, S. berrmanni* and *T. ananas* (Toral-Granda et al. 2008)**.**

1.5 The biology of sea cucumber

Sea cucumbers have a simple structure; the mouth, at the anterior end, where the tentacles are attached and the anus at the posterior end. They have a leathery skin and gelatinous body wall, and internal organs called viscera (gut) composing of a pharynx, an esophagus, a stomach, each of which are short structures, and a very long intestine ended in a cloaca (Figure 1.1). Some species possess Cuvierian tubules, found in several species of Aspidochirotida, which are generally considered as defensive structures, which are connected to the base of the respiratory trees and can be ejected to evade predators (Purcell *et al.* 2012). The body wall comprises connective tissue, the endoskeleton ossicles or spicules, which are key diagnostic tools for taxonomic identification, and a layer of circular muscles. Their reproductive system, in contrast to other echinoderms, comprises of a single gonad or genital gland (Figure 1.1). Tentacles are also used as a key characteristic for taxonomic classification.

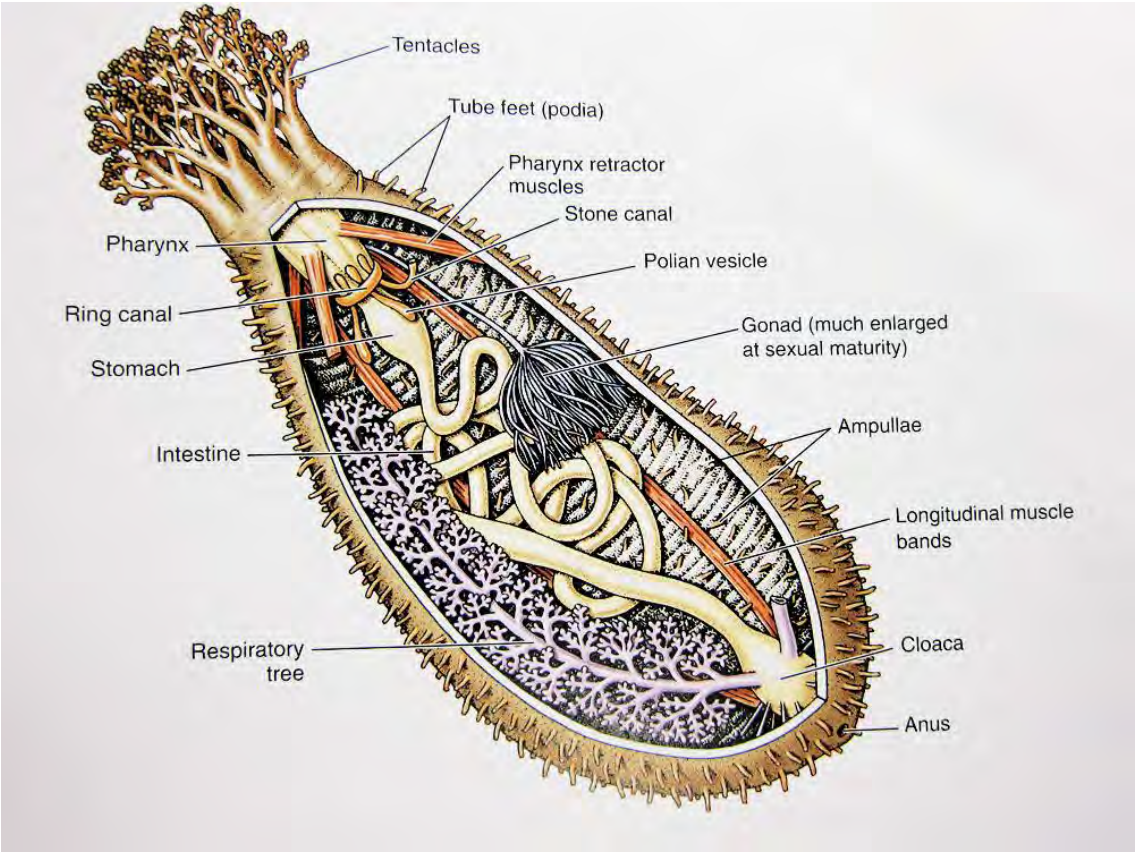

Figure 1.1. Diagram of anatomy of a generalised holothuroid.
Illustration adapted from wikimedia commons.
(http://www.gbri.org.au/SpeciesList/Actinopygaechinites|DaltonBaker?PageContentID=3829)

1.6 Immune system (defence) in Sea cucumbers

Sea cucumbers entirely depend on an innate immune system, including anti-microbial peptides (AMPs), lectins, lysozyme, saponins etc., which is the first line of inducible host defence against bacteria, fungal, viral pathogens (Fusetani 2010). In addition, the endoskeleton calcareous ossicles or "spicules" in the outer body wall of holothurians also function as a structural defence (Toral-Granda *et al.* 2008).

1.6.1 Cuvierian Tubules

Cuvierian tubules are found in several species of Aspidochirotida. They are considered as the chemical and physical defence mechanisms of sea cucumbers which can be expelled (collagenous fibres that are extremely sticky) to evade predators (Elyakov *et al.* 1973; Kobayashi *et al.* 1991; Matsuno & Ishida 1969a). Besides, sea cucumbers are able to eviscerate parts of their internal organs (Toral-Granda *et al.* 2008). Some species can expel their cuvierian tubules. For example the cuvierian tubules of *Bohadschia* species are evicted easily if aggravated while those of

Actinopyga species are ejected rarely, unless are expose to intense aggravation (Levin 1989). *Actinopyga mauritiana*, *A. echinities* and *A. miliaris* are not able to eject their cuvierian tubules (Lawrence, JM 2001; VandenSpiegel & Jangoux 1993). Some species such as *H. lessoni*, *H. atra* and *Stichopus hermanni* do not have this organ. Some holothurians e.g. *H. atra* excrete a strong toxin (mainly saponins), generally known as "holothurin" which may react with the fish branchiae (Bakus 1968, 1973).

1.7 Sea cucumbers as functional foods or tonics

Sea cucumbers are economically important. They are considered as a gourmet food ingredient in the Asian cuisine. They are widely consumed as a healthy food. Holothurians also contain compounds with pharmaceutical properties.

Sea cucumbers, commonly called bêche de-mer, or gamat, or hai-shen, have long been used for food and folk medicine in the communities of Asia and Middle East. Most of the harvestable species of sea cucumbers, which are mainly targeted as beche-de-mer, belong to two families (Holothuriidae, Stichopodidae) and ten genera of the Aspidochirotids including *Bohadschia*, *Holothuria*, *Actinopyga*, *Isostichopus*, *Stichopus*, *Astichopus*, *Parastichopus, Thelenota, Isostichopus* and *Australostichopus,* and one family (Cucumariidae) and two genera of the Dendrochirotids: *Cucumaria and Athyonidium* (Bordbar *et al.* 2011; Purcell *et al.* 2010; Purcell *et al.* 2012).

Sea cucumbers have been used as a food item and tonic food for over 1000 years ago in China. In East Asia, particularly China and Japan, sea cucumbers are a highly appreciated and prized food item.

1.8 Sea cucumbers as a source of bioactive compounds

Sea cucumber is a prolific source of bioactive secondary metabolites with the potential to cure or prevent several diseases. The bioactive compounds from sea cucumbers are well-known.

In the last three decades, the functional properties of molecules from the body wall of holothuroids have been studied extensively, since this part is the most frequently utilised (Kelly 2005; Olivera-

Castillo *et al.* 2014). The sea cucumber body wall is known to possess lectins (Mojica & Merca 2005a, 2005b), saponins (Chludil *et al.* 2003; Honey-Escandón *et al.* 2015; Kalinin, VI *et al.* 2008; Van Dyck *et al.* 2009), glucosaminoglucans (GAGs) (Hossain *et al.* 2011; Kariya *et al.* 1997; Liu, HH *et al.* 2002; Pacheco *et al.* 2000), cerebrosides (Careaga & Maier 2014; Ikeda *et al.* 2009; Sugawara *et al.* 2006), sulphated polysaccharides (Hu, Shiwei *et al.* 2014c; Wang, Y *et al.* 2012; Yu *et al.* 2014a), bioactive peptides (Zheng *et al.* 2012), phenols and flavonoids (Althunibat *et al.* 2009; Mamelona *et al.* 2007; Zhong *et al.* 2007), glycoproteins, mucopolysaccharides (Lu *et al.* 2010), polyunsaturated fatty acids (PUFAs) (Wen *et al.* 2010), gangliosides (Yamada *et al.* 2001), chondroitin sulphates, fucosylated chondroitin sulphate (FuCS), fucoidan (Zhang, Y *et al.* 2010), fucan (Mourão, PA & Pereira 1999), sterols (glycosides and sulphates), carotenoids (Sugawara *et al.* 2006), Ω-6 and Ω-3 fatty acids, branched-chain fatty acid, peptides, collagen, gelatin, enzymes, glycoprotein, glycosphingolipids and opsonins (Findlay & Daljeet 1984; Gowda *et al.* 2008; Himeshima *et al.* 1994; Mojica & Merca 2005a). This high chemical diversity is a potential source of nutraceutical, pharmaceutical and cosmetic agents. Many of which have been of interest in pharmaceutical development.

1.8.1 Fucoidan

In recent years, studies on oligosaccharides have increased significantly. Fucans are a heterogeneous group of polysaccharides and contain fucoidans, xylofucoglycuronans, and glycouronogalactofucans (Barrow & Shahidi 2007). Fucoidans possess therapeutic properties and potential drug delivery applications (Huang & Liu 2012).

Sea cucumber fucoidan (SC-FUC), which mainly comprises of fucose and sulphate ester groups (Mulloy *et al.* 1994; Yu *et al.* 2013), is one of the important classes of bioactive compounds in sea cucumbers. The structures of a few native and derived SC-FUCs have been elucidated and their anticoagulant activities and osteoclastogenesis inhibition have been reported (Mulloy *et al.* 1994; Yu *et al.* 2013). It has also been reported that the SC-FUC protects against ethanol-induced gastric ulcer (Wang, Y *et al.* 2012). It is noteworthy that the bioactivity function of fucoidan relies highly on its structure.

Fucoidan from sea cucumber generally contains a simple and regular structure when compared with algal fucoidan. In contrast to fucoidan from algae, sea cucumber fucoidans possess a liner structure without any branches, and are composed of fucose, some of which are sulphated. For example, Yu *et al.* (2013) reported that the body wall of *Acaudina molpadioides* possesses 3.8% SC-FUC (dried weight), composed of fucose with 26.3 ± 2.7% sulphate. Yu *et. al*. (2013) suggested that the sulphate pattern of fucoidan might be distinct in different species of sea cucumbers.

Zhang, Y *et al.* (2010) isolated sulphated polysaccharide, known as Haishen (HS) from the body wall of the sea cucumber *Stichopus japonicas* which promoted viability and proliferation of neural stem/progenitor cells (NSPCs), possibly through an interaction with FGF-2 signalling pathways. HS is a highly sulphated fucoidan with a molecular weight of 4.23×10^5 Da and can serve as an adjuvant for promoting the proliferation of NSPCs and can be utilised in the treatment of neurodegenerative disorders. The bioactivity of identified fucoidans is listed in Table 1.1.

Table 1.1. Bioactivity of identified fucoidans from holothurians

Sea cucumber species	Activity	References
Acaudina malpadioides	Anti-inflammatory	(Wang, Y *et al.* 2012)
Acaudina malpadioides	Not reported	(Yu *et al.* 2013)
Acaudina malpadioides	Not reported	(Chang *et al.* 2010)
Acaudina malpadioides	Not reported	(Yu *et al.* 2014a)
Acaudina malpadioides	Anti-hyperglycaemia	(Hu, Shiwei *et al.* 2014c)
Acaudina malpadioides	Antioxidase activities, gastric matrix hydrolysis suppression, and anti-inflammation	(Wang, Y *et al.* 2012)
Apostichopus japonica	Not reported	(Chang *et al.* 2010)
Bohadschia marmorata	Not reported	(Chang *et al.* 2010)
Holothuria atra	Not reported	(Chang *et al.* 2010)
Isostichopus badionotus	Anticoagulant and antithrombotic	(Chen, S *et al.* 2012)
Ludwigothurea grisea	Not reported	(MourÃO, PAS & Bastos 1987)
Ludwigothurea grisea	Anticoagulant	(Mulloy *et al.* 1994)
Stichopus japonicus	Inhibit osteoclastogenesis	(Kariya *et al.* 2004)
Thelenota ananas	Not reported	(Yu *et al.* 2014b)

The antioxidant, antimetastatic, antivenom, antibacterial, antiviral, anti-inflammatory and anticoagulant activities of fucoidans have been reported (Hayes 2012). Fucoidan is a sulphated polysaccharide with a breadth of therapeutic properties.

1.8.2 Glucosaminoglycones (GAGs)

The presence of glucosaminoglycan (GAG) and fucan in the body wall and the viscera of sea cucumber is another characteristic of this animal. These two mucopolysaccharides also called "poly-anion elements" are idiographic components of sea cucumber, (Fan, H *et al.* 1983), found in higher levels in the sea cucumber body. Hence, sea cucumbers have been recognised as "poly-anion-rich food" which possess various physiologically and biological properties, such as (a) inhibit some cancerous cells including galactophore cancer and lung cancer (Ma, K *et al.* 1982; Su *et al.* 2011); (b) activate insulin signalling (Hu, Shiwei. *et al.* 2014; Hu, Shiwei *et al.* 2014a); (c) stimulate the immune system (Li et al., 1985; Chen et al., 1987; Sun et al., 1991); and (d) prevent the aggregation of platelets (Li et al., 1985). GAGs are categorised into non-sulphated and sulphated GAGs.

It has been reported that GAGs enhance the human immune system, have anticancer and anti-tumour effects, suppress inflammation and relieve pain, accelerate wound healing, reduce blood sugar and blood viscosity, preventing blood clot, regulate blood lipid profile, reducing triglyceride and cholesterol, anti-aging and possess antiviral and anti-radioactive effects (Kiew, Peck Loo & Don 2012; Mindell, Earl 2002). In recent years, holothurians have been utilised in the manufacture of arthritis medicines (Stutterd & Williams 2003).

1.8.2.1 Chondroitin sulphate

Chondroitin sulphate is a sulphated polysaccharide (glycosaminoglycan); primarily found in cartilage tissue. It is consumed as a constituent in food supplement or health food for the cure of osteoarthritis. Sea cucumber, having a cartilagenous body, serves as a rich source of mucopolysaccharides, mainly chondroitin sulphate, which is well known for its ability to reduce arthritis pain, especially that of osteoarthritis (Dharmananda 2003).

The major alteration between chondroitin sulphates of various sea cucumber species is the

sulphation profile of their fucose residues. Sea cucumbers have been utilised as a source of chondroitin sulphate, also known "sea chondroitin", which is well-documented in its effect in decreasing arthritic pain (Chen, J 2004). For instance, Schaafsma (2007) stated that the anti-inflammatory properties of chondroitin sulphate is mainly due to the sulphate part of the molecule. Recently, there has been a marked increase in the number of marketable products from sea cucumber in the market place, such as ArthriSea®, SeaCuMAX®, being employed to treat osteoarthritis, rheumatoid arthritis and ankylosing spondylitis (Chen, J 2004). There are no publications as to whether marine chondroitin sulphate is more effective than mammalian chondroitin sulphate.

Masre *et al.* (2011) has studied N-, O-sulphated and total sulphated GAG content of three different anatomical areas (integument, internal organs and coelomic fluid) of *S. hermanni* and *S. vastus* and found the highest quality in the body wall followed by the viscera.

1.8.2.2 Fucosylated chondroitin sulphate

Another sulphated polysaccharide found in sea cucumbers is fucosylated chondroitin sulphate (FuCS) comprising of β-D-glucuronic acid and N-acetyl-β-D-galactosamine moieties (Wu, M *et al.* 2012). The sulphated fucose branches are crucial for the anticoagulant function of FuCS, and this powerful effect is probably associated with the presence of 2,4-di-*O*-sulphated fucose residues which is unique to sea cucumbers. This finding was in agreement with Chen, S *et al.* (2011) and Fonseca *et al.* (2009) who stated the sulphation pattern of the fucose branch of the chondroitin sulphate, and the present of 2,4-di-*O*-disulphation are the main factors accounting for the anticoagulant activity. However, Luo *et al.* (2013) stated both monosaccharide composition and sulphate components attributed to the activities (Figure 1.2).

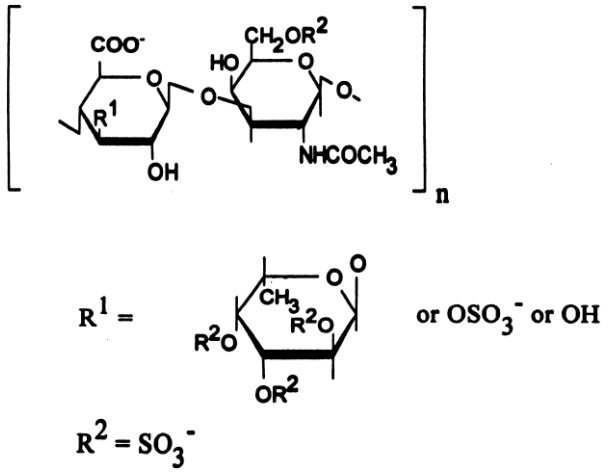

Figure 1.2. Structure of the FucCS from sea cucumber,
similar backbone structure with mammalian chondroitin sulphate (Mourão, PA *et al.* 2001).

FuCS isolated from *Cucumaria frondosa* has anti-hyperglycaemic properties (Hu, S *et al.* 2013a). This group also stated that FuCS reduced blood glucose, TNF-α levels, insulin, and enhanced adiponectin levels by increasing Bcl-2 and Bcl-xL mRNA expressions and down-regulation of cytochrome c in cytoplasm, t-Bid, Bax, caspase 9, and cleaved-caspase 3 proteins, and up-regulation of Bcl-2 and Bcl-xL proteins, which suggests the inhibition of mitochondrial apoptosis pathway (Hu, Shiwei *et al.* 2014b). Table 1.2. lists the medical properties of GAGs from sea cucumbers.

In addition, it has been stated that FuCS isolated from body wall of sea cucumber exhibit remarkable anti-angiogenic activity, comparable with that of the positive control, hydrocortisone/heparin, and even stronger than shark cartilage chondroitin-6-sulphate (Collin, P. D. 1999).

Table 1.2. Glucoseaminoglycones in sea cucumber species and their medicinal properteis.

species	Compounds	Activity	References
Acaudina molpadioides	FuCS	Anti-adipogenic (by activation of Wnt/β-catenin), Anti-hyperglycemia	(Xu, H et al. 2015) (Hu, S et al. 2013b)
Apostichopus japonicas	FuCS, Sulphated fucan	Anticoagulant	(Luo et al. 2013)
Cucumaria frondosa	FuCS	Anti-hyperglycemia, Improves Insulin Sensitivity	(Hu, Shiwei et al. 2014b) (Hu, Shiwei. et al. 2014)
Cucumaria frondosa	Fraction termed B1000	anti-invasive and antiangiogenic	(Collin, P. D. 1999)

Holothuria edulis	FuCS, Sulphated fucan	Anticoagulant	(Luo et al. 2013)
Holothuria nobilis	FuCS, Sulphated fucan	Anticoagulant	(Luo et al. 2013)
Holothuria vagabunda	FuCS	Anticoagulant	(Chen, S et al. 2011)
Isostichopus badionotus	FuCS, Sulphated fucan	Anticoagulant Antithrombotic	(Chen, S et al. 2012)
Ludwigothurea grisea	FuCS	Anticoagulant, Antithrombotic	(Mourao et al. 1996b; Wu, M et al. 2015)
Ludwigothurea grisea	Fucan sulphate, glucosamine, chondroitin	Anticoagulant, Antithrombotic, Anti-tumour, Regulate angiogenesis	(Mourao et al. 1996a) (Borsig *et al.* 2007; Tapon-Bretaudiere *et al.* 2002)
Pearsonothuria graeffei	FuCS	Anticoagulant	(Chen, S et al. 2011)
Stichopus tremulus	FuCS	Anticoagulant	(Chen, S et al. 2011)
Stichopus japonicus	GAGs, Fucan sulphate, glucosamine, chondroitin	Anticoagulant, Antithrombotic, Osteoarthritis, Anti-proliferation	(Bordbar et al. 2011; Suzuki et al. 1991) (Hu, RJ et al. 1997)
Thelenota ananas	FuCS	Anticoagulant	(Wu, M et al. 2012)

The promising anticoagulant activity and possible lack of bleeding side effect make these polysaccharides from sea cucumbers promising compounds for antithrombotic therapy (Mourão, PA *et al.* 1996).

1.8.3 AMPs

Antimicrobial peptides are characterised as small cationic and amphipathic, having both hydrophilic and hydrophobic domains, cationic (a net positive charge) with low molecular weight (majority 12 to 50 amino acids (aa)) which have been shown to have a wide range of antimicrobial activity such as bactericidal, veridical and antifungal (Fusetani 2010; Li, C *et al.* 2010; Mookherjee & Hancock 2007). The cationic properties are mostly due to the presence of arginine residues. Sea cucumbers also produce a wide spectrum of AMPs of which many have been determined. For instance, small antimicrobial peptides (≤ 6 kDa) have been described for the sea cucumber *Cucumaria frondosa*, which were reported to be active at low pH (5.0 - 6.5) toward bacterial strains including *Pseudomonas aruginosa* and *Staphylococcus aureus* (Beauregard *et al.* 2001). It has been documented that these compounds function principally by producing pores in the microbial

membranes (Brogden 2005; Jenssen *et al.* 2006; Yeaman & Yount 2003).

1.8.4 Collagen

The protein content of dried sea cucumber has been reported to be higher than 50 % in most edible species. Collagen is one of the main classes of extracellular matrix (ECM) proteins, comprising of three polypeptide α-chains, forming triple helix structure. Collagen has been reported in various species of sea cucumbers, as marine sources are sought after due to the fears related to the high risk of bovine derived spongiform encephalopathy. The body wall of sea cucumber mainly comprises of collagen, which accounts for roughly 70% of the total protein (Saito *et al.* 2002).

It is reported that collagen promotes wound healing, maintains health of joint and bone, prevents osteoporosis, rejuvenate skin and enhance beauty as an anti-aging (Abou Neel *et al.* 2013; Dharmananda 2003), and a treatment for arthritis. Collagen use in the biomedical industry as principally used in cartilage reconstruction (Chattopadhyay & Raines 2014; Parenteau-Bareil *et al.* 2010; Rose & Chrisope 2004).

Partial hydrolysis of collagen can produce gelatine. Sea cucumber gelatine is a putative bioactive material. Gelatine possess antioxidant activity and shows promise as an important constituent in functional foods, cosmetics and pharmaceuticals or nutraceuticals (Wang, J *et al.* 2010).

1.9 Saponins

Nigrelli, R (1952) and (Yamanouchi 1955) were the pioneers to investigate the presence of glycosides in the marine environment in particular in sea cucumbers. To the best of our knowledge, no extensive study has been performed to entirely cover the medicinal, pharmaceutical, nutraceutical and cosmeceutical applications of sea cucumber saponins. Although several reviews published the biological activities and roles of saponins (Anisimov 1987; Anisimov & Chirva 1980; Caulier *et al.* 2011; Chludil *et al.* 2003; Kalinin, V *et al.* 1996; Kalinin, VI *et al.* 2008; Kalyani *et al.* 1988) , none have covered all the recent studies in this field. The present study outlines a comprehensive overview of the structural characteristic of triterpenoid glycosides and their biological properties in addition to their potential applications. Although some clinical applications

of sea cucumber saponins were partially reviewed by Bordbar *et al.* (2011), and two groups briefly reviewed the diversity of saponins in the family Holothuriidae (Caulier *et al.* 2011; Honey-Escandón *et al.* 2015). Here an extensive review was conducted (covering the last 70 years) on diversity, isolation and structural elucidation of sea cucumber saponins in addition to the medicinal functions of these complex molecules.

Saponins are naturally highly polar compounds with low volatility. These amphipathic compounds generally possess a triterpene or steroid backbone. Triterpene glycosides or triterpene saponins are the most abundant category of secondary metabolites in terrestrial plants (Kim, SK & Himaya 2012). The ecological and agronomic functions of plant saponins are vital to crop plants, which relate to pest and pathogen resistance and to food quality (Osbourn *et al.* 2011).

Indeed, the name 'saponin' originated from sapo (the Latin word soap) since they possess surfactant properties and creates stable, soap-like foams once shaken in aqueous solution. Generally saponins are naturally occurring bioactive compounds and characterised by their surface-active properties, solubilise in water forming a foam-solution because of their tension-activity (Chaieb 2010; Hostettmann & Marston 1995). Saponins are constituents of many plant drugs and folk medicines, especially from the Orient. They have been used as emulsification and foaming agents (Güçlü-Üstünda & Mazza 2007; Hostettmann & Marston 1995; Kjellin & Johansson 2010). They are also consumed as a preservative, flavour modifiers and cholesterol- lowering agents.

1.9.1 Terrestrial vs. marine saponins

It is noteworthy to state that ancient Chinese medical books highlight that unspecified elements in sea cucumber can activate the human immune system, thereby boosting resistance to diseases and relieve stress and mental exhaustion. Sea cucumbers have been used in Asian traditional medicine since ancient time as tonics and delicacies, and frequently reported as a complement for the treatment of certain diseases, and now is gaining popularity as a dietary supplement in western countries (Bordbar *et al.* 2011; Kiew, Peck Loo & Don 2012). Even though sea cucumbers contain different types of natural compounds, saponins are the most important and abundant secondary

metabolites (Caulier *et al.* 2011; Dong *et al.* 2008; Han *et al.* 2010c; Naidu 2000; Zhang, S-L *et al.* 2006; Zhang, S-L *et al.* 2004; Zhang, S-Y *et al.* 2006b). More than 20,000 triterpenoids have been reported from nature, which belong to different chemical groups (Hill & Connolly 2013; Liby *et al.* 2007). Saponins are generally perceived as highly active natural products and the sea cucumber saponins have been well characterized for their biological activities.

In sea cucumbers, the sugar residue has only one branch (Kalinin, VI *et al.* 2005), whereas plant saponins may contain one, two or three saccharide chains, with a few having an acyl group bound to the sugar moiety (Xu, R *et al.* 2012). Besides, terrestrial saponins are sulphated in both aglycone and sugar residues and contain some monosaccharides such as pentoses (arabinose and apiose) and rhamnose (methylpentoses or 6-deoxy-hexoses) (Güçlü-Üstünda & Mazza 2007), which have not been reported in marine glycosides. In contrast, 6-deoxyhexose monosaccharides such as fucose (6-deoxygalactose), quinovose (6-deoxyglucose), are often found in marine saponins. Further the numbers of sugar moieties in the plant saponins are varied from two to eleven moieties, but generally they contain three to five moieties.

1.9.2 Marine saponins

Triterpenoid saponins are typical metabolites of higher plant origin, however, a limited number of marine species including holothuroids (Bahrami *et al.* 2014b; Kalinin, VI *et al.* 2008; Kitagawa *et al.* 1989a; Kitagawa *et al.* 1981a; Van Dyck *et al.* 2010b), asteroids (Demeyer *et al.* 2014), sponges (Campagnuolo *et al.* 2001; Chludil *et al.* 2002; Thompson *et al.* 1985), and bacteria have also produced saponins (Chapagain & Wiesman 2008). Saponins are also reported in the defensive secretions of certain insects (Plasman et al. 2000). Holothuroidea and Asteriodea are the most studied echinoderms. However, the structures of isolated saponins are different among these classes. Sea cucumber saponins are usually triterpene glycosides (derived from lanostane) while those from starfish (astero-saponins) are steroid glycosides (Minale *et al.* 1982). The presences of saponins in this species is a unique characteristic among the animal kingdom differentiating them from other echinoderms and from each other (Blunt, J *et al.* 2012). The main characteristic feature of the holothurians is the presence of particular holostane type triterpene glycosides.

The first animal saponin called holothurin was isolated from the sea cucumber *Holothuria vagabunda* by Yamanouchi (1955) and the sea cucumber saponins have been generally named "holothurins." However, Holotoxins A and B were the first saponins which were entirely characterised from sea cucumber *Stichopus japonicus* (*Holothuria Leucospilota)* by Kitagawa (Kitagawa *et al.* 1976; Kitagawa *et al.* 1978b), even though Yamanouchi described a haemolytic toxin from the same species in 1943 (Fusetani & Kem 2009; Hostettmann & Marston 1995; Yamanouchi 1943). Since then Holothurins A and B have been found in several species of sea cucumbers.

1.9.3 Function and biological roles of saponins in sea cucumbers

Triterpene glycosides have been recognised as a defence mechanism, as they are deleterious for most of organisms (Bakus 1968, 1973). Therefore, their role in nature is likely to be in defence against pathogens, pests and predators. In contrast, a recent study has shown that these repellent chemicals are also kairomones that attract symbionts and are used as chemical "signals" (Caulier *et al.* 2013). Moreover, it has been reported that triterpene saponins act as allelochemicals because some of these molecules possess phytotoxic properties. Chemical communication and sensory ecology are important areas in marine chemical ecology which have developed significantly in the last decade.

The content of triterpene glycosides have been shown to vary in several internal organs of sea cucumbers (gonads, ovaries) by season and age, which suggests a contributory role of these toxins in the reproductive processes. For instance, the content of glycosides was constant in the body wall of *H. leucospilota* in changing seasons, while it was highest in the gonads prior to the beginning of spawning (Matsuno & Ishida 1969b). However, in sea cucumber, it was suggested that saponins may play two more regulatory roles during reproduction, synchronising processes of oocyte: (1) to prevent oocyte maturation and (2) to act as a mediator of gametogenesis (Kalinin, VI *et al.* 2008; Mercier *et al.* 2009). The main role of saponins has not been known entirely in plants although some defence mechanisms have been reported to be associated with saponins.

1.9.4 Chemical structure of saponins

Saponins are the most important characteristic and abundant secondary metabolite in these species (Bahrami *et al.* 2014b). Saponins are polar complex compounds, heterosides, composed of a saccharide moiety (hydrophilic part, water-soluble), connected glycosidically to a hydrophobic aglycone (sapogenin), which is a triterpene or steroid backbone (lipo-soluble) (Chapagain & Wiesman 2008; Kerr & Chen 1995; Williams & Gong 2004).

Saponins are generally divided into three main groups in accordance with their aglycone (genin) structure: triterpenoidic, steroidal and steroid alkaloid glycosides (Hostettmann & Marston 1995). Triterpenoid saponins have aglycones that consist of 30 carbons, whereas steroidal saponins possess aglycones with 27 carbons, which are rare in nature (Hostettmann & Marston 1995). Sea cucumber saponins are usually triterpene glycosides (derived from lanostane) rather than nonholostane (Bahrami *et al.* 2014b; Dang *et al.* 2007; Kerr & Chen 1995), which is comprised of a lanostane-3β-ol type aglycone containing a γ-18(20)-lactone in the D-ring of tetracyclic triterpene (3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone) (Kim, SK & Himaya 2012) and can contain shortened side chains and a carbohydrate moiety consisting of up to six monosaccharide units covalently connected to C-3 of the aglycone (Chludil *et al.* 2002; Han *et al.* 2010c; Zhang, S-L *et al.* 2006). The basic structure of the holostane type aglycone, which is a characteristic aglycone moiety for sea cucumber saponin is shown in Figure 1.3.

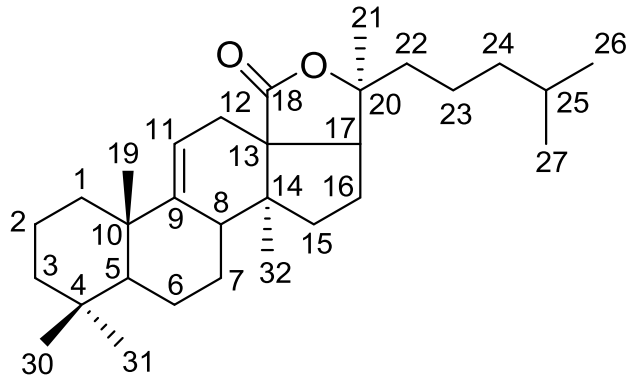

Figure 1.3. Structure of the holostane group, which is the characteristic aglycone moiety in sea cucumber glycosides

The sugar moieties mainly consist of D-xylose (Xyl), D-quinovose (Qui), 3-*O*-methyl-D-glucose

(MeGlc), 3-*O*-methyl-D-xylose (MeXyl) and D-glucose (Glc), and sometimes 3-*O*-methyl-Dquinovose, 3-*O*-methyl-D-glucuronic acid and 6-*O*-acetyl-D-glucose (Avilov, S. A. *et al.* 2008; Iniguez-Martinez *et al.* 2005; Kalinin, VI *et al.* 2005; Stonik, VA *et al.* 1999). In the oligosaccharide chain, the first monosaccharide unit is always a xylose, whereas MeGlc and/or MeXyl are always the terminal sugars. One of the most noteworthy characteristics of many of the saponins from marine organisms is the sulphation of aglycone or sugar moieties (Hostettmann & Marston 1995), and in sea cucumbers the sulphation of one or more of Xyl, Glc, MeGlc and Qui residues have been reported (Bahrami *et al.* 2014b; Stonik, VA *et al.* 1999; Zhang, S-L *et al.* 2008). Most of them are mono-sulphated glycosides with few occurrences of di- and tri-sulphated glycosides (Chludil *et al.* 2003; Kalinin, VI *et al.* 2005). A literature review has revealed that saponins with di- and tri-sulphated substitutes are mainly reported in the order *Dendrochirotida*. However, in asterosaponin the aglycone is usually sulphated.

Over 700 triterpene glycosides have been reported in wide range of sea cucumbers species collected from many areas including tropical Pacific, Indian, and Atlantic oceans and the Mediterranean Sea (Bahrami & Franco 2015). These glycosides are classified into four main structural categories based on their aglycone moieties: three holostane types containing a (1) 3β-hydroxyholost-9(11)-ene aglycone skeleton, (2) a 3β-hydroxyholost-7-ene skeleton and (3) an aglycone moiety different to other two holostane type aglycones, and a nonholostane aglycone. The majority of saponins belong to the holostane type group. The molecular mass and formula of identified saponin congeners from sea cucumber species over the past six decades, together with their taxonomic information are summarized in Table 1.3. This table indicates the immense diversity of saponin congeners including sulphated, non-sulphated, methylated and acetylated triterpene glycosides

Table 1.3. Distribution of triterpene glycosides in the sea cucumber species belonging to the class Holothuroidea

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Achlionice violaecuspidata (Rhipidothuria racowitzai)	Achlioniceoside A_1 Achlioniceoside A_2 Achlioniceoside A_3	1445.5 $[M_{2Na} –Na]^-$ 1445.5 $[M_{2Na} –Na]^-$ 1443.5 $[M_{2Na} –Na]^-$	$C_{60}H_{94}Na_2O_{34}S_2$ $C_{60}H_{94}Na_2O_{34}S_2$ $C_{60}H_{92}Na_2O_{34}S_2$	(Antonov *et al.* 2009)
Actinocucumis typica	Typicoside A_1 Typicoside A_2 Typicoside B_1 Typicoside C_1 Typicoside C_2	1221.5 $[M_{Na} + Na]^+$ 1223.5 $[M_{Na} + Na]^+$ 1253.5 $[M_{Na} + Na]^+$ 1313.4 $[M_{2Na} + Na]^+$ 1355.4 $[M_{2Na} + Na]^+$	$C_{55}H_{83}NaO_{25}S$ $C_{55}H_{85}NaO_{25}S$ $C_{56}H_{87}NaO_{26}S$ $C_{54}H_{84}Na_2O_{28}S_2$ $C_{56}H_{86}Na_2O_{29}S_2$	(Silchenko *et al.* 2013b)
Actinopyga agassizi	24-dehydroechinoside A Holothurin A (Nobiliside I)	 1243.5 $[M + Na]^+$	 $C_{54}H_{85}NaO_{27}S$	(Kitagawa *et al.* 1982)
Actinopyga agassizi	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1975)
Actinopyga echinites	Echinoside A (Holothurin A_2) Echinoside B (Holothurin B_1)	1229.5 $[M + Na]^+$	$C_{54}H_{87}NaO_{26}S$ $C_{41}H_{65}NaO_{16}S$	(Kitagawa *et al.* 1980; Kitagawa *et al.* 1985)
Actinopyga echinites	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Actinopyga echinites	Holothurin B_3 Holothurin B_1 Holothurins B/B_4 Holothurin B_2 Fuscocinerosides B/C Holothurin A_2 Holothurin A (Nobiliside I)	889 $[M + Na]^+$ 891 $[M + Na]^+$ 905 $[M + Na]^+$ 907 $[M + Na]^+$ 1227 $[M + Na]^+$ 1229 $[M + Na]^+$ 1243 $[M + Na]^+$	 $C_{41}H_{65}O_{11}$ $C_{54}H_{87}NaO_{26}S$ $C_{54}H_{85}NaO_{27}S$	(Van Dyck *et al.* 2010b)
Actinopyga flammea	Holothurin A Holothurin B Echinoside A (Holothurin A_2) 24-dehydroechinoside A 22-hydroxyechinoside A (Holothurin A_1) 24(s)-hydroxy-25-dehydro-echinoside A 22-hydroxy-24-dehydroechinoside A 22-acetoxy-echinoside A 25-hydroxy-dehydroechinoside A	1243.5 $[M + Na]^+$ 905 $[M + Na]^+$ 1229 $[M + Na]^+$ 1227 $[M + Na]^+$ 1245 5 $[M + Na]^+$ 1243 $[M + Na]^+$ 1243 $[M + Na]^+$ 1287 $[M + Na]^+$ 1243 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$ $C_{54}H_{87}NaO_{26}S$ $C_{54}H_{85}NaO_{26}S$ $C_{54}H_{87}NaO_{27}S$ $C_{54}H_{87}NaO_{27}S$ $C_{54}H_{85}NaO_{27}S$ $C_{56}H_{89}NaO_{28}S$ $C_{54}H_{85}NaO_{27}S$	(Bhatnagar *et al.* 1985)
Actinopyga lecanora	lecanoroside A lecanoroside B Holothurin A Holothurin A_1 Holothurin B	1007.3 $[M + Na]^+$ 1230.5 $[M + Na]^+$ 1243 $[M + Na]^+$ 1245 5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{41}H_{62}Na_2O_{20}S_2$ $C_{53}H_{84}Na_2O_{27}S$ $C_{54}H_{85}NaO_{27}S$ $C_{54}H_{87}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Zhang, S-L *et al.* 2008)
Actinopyga lecanora	Holothurin A Holothurin B	1243 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Actinopyga mauritana	Echinoside B	N/A	N/A	(Radhika *et al.* 2002)
Actinopyga mauritiana	24-dehydroechinoside B Echinoside A	N/A	N/A	(Kitagawa *et al.* 1985;

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
	Echinoside B 24-dehydroechinoside A			Kitagawa *et al.* 1982; Kobayashi *et al.* 1991)
Actinopyga mauritiana	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Actinopyga miliaris	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Actinopyga sp.	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Apostichopus japonicus	3-O-[β-D-quinovopranosyl-(1→2)-4-O-sodium sulfate-β-D-xylopranosyl]-holosta-9(11)-ene-3β,12α,17α-trio	N/A	$C_{41}H_{65}NaO_{16}S$	(Li, C *et al.* 2013)
Apostichopus japonicus	26-nor-25-oxo-Holotoxin A_1 Holotoxin D Holotoxin E Holotoxin F Holotoxin G Holotoxin A_1 Holotoxin B Cladoloside B	1417.6 $[M + Na]^+$ 1431.6 $[M + Na]^+$ 1401.6 $[M + Na]^+$ 1227.6 $[M + Na]^+$ 1213.6 $[M + Na]^+$ Known Known Known	$C_{65}H_{102}NaO_{32}$ $C_{66}H_{104}NaO_{32}$ $C_{65}H_{102}NaO_{31}$?? $C_{59}H_{96}NaO_{25}$ $C_{58}H_{94}NaO_{25}$	(Wang, Z *et al.* 2012b)
Apostichopus japonicus	Holotoxin D_1 25,26-Dihydroxy-holotoxin A_1 Stichloroside C_1 Bivittoside D	1417.6 $[M + Na]^+$ 1449.6 $[M + Na]^+$ Known 1449.7 $[M + Na]^+$	$C_{65}H_{102}NaO_{32}$ $C_{66}H_{106}NaO_{33}$ $C_{67}H_{110}O_{32}$	(Wang, Z *et al.* 2012a)
Astichopus multifidus	Astichoposide C	N/A	N/A	(Stonik, VA *et al.* 1982b)
Astichopus multifidus	Stichoposide B	N/A	N/A	(Elyakov *et al.* 1975)
Athyonidium chilensis	Holothurinoside D	787 $[M + Na]^+$	N/A	(Sottorff *et al.* 2013)
Australostichopus mollis	Mollisoside A Mollisoside B_1 Mollisoside B_2	1209.5 $[M_{Na} + Na]^+$ 1195.5 $[M_{Na} + Na]^+$ 1195.5 $[M_{Na} + Na]^+$	$C_{54}H_{83}NaO_{25}S$ $C_{53}H_{81}NaO_{25}S$ $C_{53}H_{81}NaO_{25}S$	(Moraes, G. *et al.* 2005)
Australostichopus mollis	Neothyonidioside	1153 $[M_{Na}\text{-}Na]^-$	N/A	(Moraes, Greta *et al.* 2004)
Bohadschia aff.	Bivittoside D Bivittoside C	1449.7 $[M + Na]^+$	$C_{67}H_{110}O_{32}$	(Radhika *et al.* 2002)
Bohadschia argus	Arguside A	1167.6 $[M + Na]^+$	$C_{56}H_{88}O_{24}$	(Liu, BS *et al.* 2007)
Bohadschia argus	Holothurin C	N/A	N/A	(Elyakov *et al.* 1973)
Bohadschia argus	Arguside D Arguside E	1199.5 $[M + Na]^+$ 1141.5 $[M + Na]^+$	$C_{56}H_{88}O_{26}$ $C_{54}H_{86}O_{24}$	(Liu, BS *et al.* 2008a)
Bohadschia argus	Aglycone	472 M^+ 456	N/A	(Stonik, VA *et al.* 1982d)
Bohadschia argus	Arguside B Arguside C	1465.7 $[M + Na]^+$ 1465.7 $[M + Na]^+$	$C_{67}H_{110}O_{33}$ $C_{67}H_{110}O_{33}$	(Liu, BS *et al.* 2008b)
Bohadschia argus	Bivittoside C Bivittoside D	1449.7 $[M + Na]^+$	$C_{67}H_{110}O_{32}$	(Kitagawa *et al.* 1989a; Kobayashi *et al.* 1991)
Bohadschia bivittata	Bivittoside A	773 $[M + Na]^+$	$C_{41}H_{66}O_{12}$	(Kitagawa *et*

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
	Bivittoside B	111 $[M + Na]^+$	$C_{54}H_{88}O_{22}$	*al.* 1989a)
	Bivittoside C	1433 $[M + Na]^+$	$C_{67}H_{110}O_{31}$	
	Bivittoside D	1449 $[M + Na]^+$	$C_{67}H_{110}O_{32}$	
	Seychellogenin	454 M^+	$C_{30}H_{46}O_3$	
	Isobivittogenin	472 M^+	$C_{30}H_{48}O_4$	
	Seychellogenin acetate	496 M^+	$C_{32}H_{48}O_4$	
Bohadschia cousteaui	Cousteside A	1463.7 $[M + Na]^+$	$C_{67}H_{108}O_{33}$	(Elbandy *et al.* 2014)
	Cousteside B	1445.7 $[M + Na]^+$	$C_{67}H_{106}O_{32}$	
	Cousteside C	1303.6 $[M + Na]^+$	$C_{60}H_{96}O_{29}$	
	Cousteside D	1345.6 $[M + Na]^+$	$C_{62}H_{98}O_{30}$	
	Cousteside E	1287.6 $[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Cousteside F	1287.6 $[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Cousteside G	1269.6 $[M + Na]^+$	$C_{60}H_{94}O_{27}$	
	Cousteside H	1271.6 $[M + Na]^+$	$C_{60}H_{96}O_{27}$	
	Cousteside I	1289.6 $[M + Na]^+$	$C_{60}H_{98}O_{28}$	
	Cousteside J	1273.6 $[M + Na]^+$	$C_{60}H_{98}O_{27}$	
Bohadschia graeffei	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Kitagawa *et al.* 1985; Kobayashi *et al.* 1991)
	Echinoside A			
Bohadschia graeffei	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1973)
	Holothurin B	905.2 $[M + Na]^+$	$C_{41}H_{63}NaO_{17}S$	
Bohadschia marmorata	17-hydroxy fuscocineroside B,	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Yuan, W *et al.* 2008)
	25-hydroxy fuscocineroside B	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	
	Fuscocineroside B	1227.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{26}S$	
Bohadschia marmorata	Marmoratoside A,	1447.7 $[M + Na]^+$	$C_{67}H_{108}O_{32}$	(Yuan *et al.* 2009b)
	17α-hydroxyimpatienside A,	1463.7 $[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Marmoratoside B,	1463.7 $[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	25-acetoxy bivittoside D,	1507.7 $[M + Na]^+$	$C_{69}H_{112}O_{34}$	
	Impatienside A			
	Bivittoside D	1449.7 $[M + Na]^+$	$C_{67}H_{110}O_{32}$	
Bohadschia marmorata	Holothurin C	N/A	N/A	(Elyakov *et al.* 1973)
Bohadschia sp.	Holothurin C	N/A	N/A	(Elyakov *et al.* 1973)
Bohadschia subrubra	Holothurinoside J_1	1157 $[M + Na]^+$		(Van Dyck *et al.* 2010b)
	Holothurinoside F	1433 $[M + Na]^+$		
	Bivittoside C	1433 $[M + Na]^+$		
	Impatienside A (Marmoratoside A)	1447 $[M + Na]^+$		
	Bivittoside D	1449 $[M + Na]^+$	$C_{67}H_{110}O_{32}$	
	Holothurinoside H	1463 $[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Holothurinoside H_1	1463 $[M + Na]^+$		
	Arguside C	1465 $[M + Na]^+$		
	Holothurinoside I	1479 $[M + Na]^+$		
	Holothurinoside I_1	1479 $[M + Na]^+$		
	Holothurinoside K_1	1495 $[M + Na]^+$		
Bohadschia vitiensis	Bivittoside D	1449.7 $[M + Na]^+$	$C_{67}H_{110}O_{32}$	(Lakshmi *et al.* 2012)
Bohadschia vitiensis	Bivittoside B	1111 $[M + Na]^+$		(Caulier *et al.* 2013)
	Holothurinoside A	1303 $[M + Na]^+$		
	Holothurinoside F	1433 $[M + Na]^+$		

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
	Holothurinoside G Holothurinoside H Arguside C	1449 $[M + Na]^+$ 1463 $[M + Na]^+$ 1465 $[M + Na]^+$		
Cladolabes schmeltzii	Cladoloside A_1 Cladoloside A_2 Cladoloside A_3 Cladoloside A_4 Cladoloside A_5 Cladoloside A_6	1181.6 $[M + Na]^+$ 1179.6 $[M + Na]^+$ 1121.6 $[M + Na]^+$ 1077.5 $[M + Na]^+$ 1093.5 $[M + Na]^+$ 1209.6 $[M + Na]^+$	$C_{57}H_{90}O_{24}$ $C_{57}H_{88}O_{24}$ $C_{55}H_{86}O_{22}$ $C_{53}H_{82}O_{21}$ $C_{53}H_{82}O_{22}$ $C_{58}H_{90}O_{25}$	(Silchenko *et al.* 2013c)
Cladolabes schmeltzii	Cladoloside B_1 Cladoloside B_2 Cladoloside C Cladoloside C_1 Cladoloside C_2 Cladoloside D Holotoxin A_1	1343.6 [M + Na]+ 1341.6 [M + Na]+ 1517.7 [M +Na]+ 1519.7 [M + Na]+ 1417.7 [M + Na]+ 1473.6 $[M + Na]^+$ -	$C_{63}H_{100}O_{29}$ $C_{63}H_{98}O_{29}$ $C_{70}H_{110}O_{34}$ $C_{70}H_{112}O_{34}$ $C_{66}H_{106}O_{31}$ $C_{68}H_{106}O_{33}$ -	(Silchenko *et al.* 2013c)
Cladoloside sp.	Cladoloside A Cladoloside B	N/A	N/A	(Avilov, S. A. & Stonik 1988)
Cucumaria conicospermium	Cucumarioside A_2-5 Cucumarioside A_3-2 Cucumarioside A_3-3 Isokoreoside A Koreoside A	1401 $[M_{Na} + Na]^+$ 1129 $[M + Na]^+$	$C_{61}H_{95}NaO_{31}S$ $C_{53}H_{86}NaO_{24}$	(Avilov, S.A. *et al.* 2003)
Cucumaria echinata	Cucumechinoside A Cucumechinoside B Cucumechinoside C Cucumechinoside D Cucumechinoside E Cucumechinoside F	1281.4 $[M - H]^-$ 1251.4 $[M - H]^-$ 1267.4 $[M - H]^-$ 1383.4 $[M - H]^-$ 1347.4 $[M - H]^-$ 1369.4 $[M - H]^-$	$C_{54}H_{82}Na_2O_{29}S_2$ $C_{53}H_{80}Na_2O_{28}S_2$ $C_{54}H_{84}Na_2O_{28}S_2$ $C_{54}H_{81}Na_3O_{32}S_3$ $C_{53}H_{80}KO_{31}S_3$ $C_{54}H_{83}Na_3O_{31}S_3$	(Miyamoto, T. *et al.* 1990)
Cucumaria echinata	Cucumechinol A Cucumechinol B Cucumechinol C	484.3 M^+ 470.4 M^+ 484 M^+	$C_{30}H_{44}O_5$ $C_{30}H_{46}O_4$	(Miyamoto, Tomofumi *et al.* 1990)
Cucumaria fraudatrix	Genin	554 M^+ 556 M^+ 556 M^+	$C_{34}H_{50}O_6$ $C_{34}H_{52}O_6$ $C_{34}H_{50}O_6$	(Afiyatullov, Sh Sh *et al.* 1983)
Cucumaria frondosa	Frondoside A	1357 $[M + Na]^+$		(Girard *et al.* 1990)
Cucumaria frondosa	Frondoside F Frondoside E_1 (Cucumarioside A_2-2) Frondoside E_2	N/A	N/A	(Yayli 2001)
Cucumaria frondosa	Frondoside A	1357 $[M + Na]^+$	N/A	(Kalinin, VI *et al.* 2008)
Cucumaria frondosa	Frondoside A Frondoside A_1	1357 $[M + Na]^+$	N/A	(Avilov, S. A. *et al.* 1993)
Cucumaria frondosa	Frondoside B_1 Frondoside B_2	N/A	N/A	(Burnell & Apsimon 1983)
Cucumaria frondosa	Frondoside B Frondoside	N/A	$C_{59}H_{92}M_2O_{31}S_2$ $C_{58}H_{92}M_6O_{63}S_6$ M=Na or K	(Findlay *et al.* 1992)
Cucumaria frondosa	Frondogenin	N/A	N/A	(Findlay & Daljeet 1984)
Cucumaria frondosa	Frondoside A_2-1 Frondoside A_2-2 Frondoside A_7-3 Frondoside A_2-6	1341.5 $[M_{Na} + Na]^+$ 1341.5 $[M_{Na} + Na]^+$ 1343.5 $[M_{Na} + Na]^+$ 1341.5 $[M_{Na} + Na]^+$	$C_{59}H_{91}NaO_{29}S$ $C_{59}H_{91}NaO_{29}S$ $C_{59}H_{93}NaO_{29}S$ $C_{59}H_{91}NaO_{29}S$	(Silchenko *et al.* 2005a)
Cucumaria frondosa	Frondoside D	1373 $[M + Na]^+$	$C_{60}H_{95}NaO_{30}S$	(Yayli & Findlay 1999)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Cucumaria frondosa	Frondoside $A_{2-}4$ Frondoside $A_{2-}7$ Frondoside $A_{2-}8$	1327.5 $[M_{Na} + Na]^+$ 1373.6 $[M_{Na} + Na]^+$ 1373.6 $[M_{Na} + Na]^+$	$C_{59}H_{93}NaO_{28}S$ $C_{61}H_{99}NaO_{29}S$ $C_{61}H_{99}NaO_{29}S$	(Silchenko *et al.* 2005b)
Cucumaria frondosa	Frondoside $A_{2-}1$ Frondoside $A_{2-}2$ Frondoside $A_{2-}3$ Frondoside $A_{2-}6$	1341 $[M_{Na} + Na]^+$ 1341 $[M_{Na} + Na]^+$ 1341 $[M_{Na} + Na]^+$ 1341 $[M_{Na} + Na]^+$	$C_{59}H_{91}NaO_{29}S$ $C_{59}H_{91}NaO_{29}S$ $C_{59}H_{91}NaO_{29}S$ $C_{59}H_{91}NaO_{29}S$	(Silchenko *et al.* 2005a)
Cucumaria frondosa	Frondoside C Desulfated frondoside C	 1271 $[M + Na]^+$	 $C_{61}H_{100}O_{26}$	(Avilov, S. A. *et al.* 1998)
Cucumaria japonica	Cucumarioside A_4-2	N/A	N/A	(Aminin, D. L. *et al.* 2001)
Cucumaria japonica	Cucumarioside A_2-2	N/A	N/A	(Avilov, S. A. *et al.* 1984)
Cucumaria japonica	Cucumarioside A_3 Cucumarioside A_6-2	1438 $[M_{k.Na} + 2H]^+$ 1454 $[M_{k.k} + 2H]^+$	$C_{59}H_{90}KNa_2O_{32}S_2$ $C_{59}H_{90}K_2O_{32}S_2$	(Drozdova *et al.* 1997)
Cucumaria japonica	Cucumarioside A_1-2 Cucumarioside A_0-1 Cucumarioside A_0-2 Cucumarioside A_0-3	 1355 $[M_{Na} + Na]^+$ 1311 $[M_{Na} + Na]^+$	N/A	(Drozdova, O *et al.* 1993a)
Cucumaria japonica	Cucumarioside A_1-2 Cucumarioside A_2-3 Cucumarioside A_2-4 Cucumarioside A_4-2	N/A	N/A	(Avilov, S. A. *et al.* 1990b)
Cucumaria japonica	Cucumarioside A_7-1 Cucumarioside A_7-2 Cucumarioside A_7-3	1499 $[M_{3Na} - Na]^-$	N/A	(Drozdova, OA *et al.* 1993)
Cucumaria japonica	Cucumarioside A_2-4 Cucumarioside A_2-3 Cucumarioside A_7-2 Cucumarioside A_2-2 Cucumarioside A_7-1 Cucumarioside A_4-2	N/A	N/A	(Drozdova, O *et al.* 1993b)
Cucumaria japonica	Cucumarioside A_2 -2	N/A	N/A	(Menchinskaya *et al.* 2013)
Cucumaria japonica	Genin	468 454	$C_{30}H_{44}O_4$ $C_{30}H_{46}O_3$	(Sharypov *et al.* 1985)
Cucumaria koraiensis	Koreoside A	1429	$C_{53}H_{83}O_{33}S_3M_3$ (M represents Na, K, or H)	(Avilov, S. A. *et al.* 1997)
Cucumaria koraiensis	Koreoside A	1429	$C_{53}H_{83}O_{33}S_3M_3$ (M represents Na, K, or H)	(Avilov, S. A. *et al.* 1997)
Cucumaria lefevrei	Lefevreioside A_1 Lefevreioside A_2 Lefevreioside B Lefevreioside C	1123 $[M + Na]^+$ 1225 $[M_{Na} + Na]^+$ 1223 $[M_{Na} + Na]^+$ 1223 $[M_{Na} + Na]^+$	N/A	(Rodriguez & Riguera 1989)
Cucumaria miniata	Cucumarioside A_7-3	N/A	N/A	(Drozdova *et al.* 1997)
Cucumaria okhotensis	Okhotoside B_1 Okhotoside B_2 Okhotoside B_3 Frondoside A	1269.5 $[M_{Na} + Na]^+$ 1371.5 $[M_{2Na} + Na]^+$ 1371.5 $[M_{2Na} + Na]^+$ 1357 $[M + Na]^+$	$C_{56}H_{87}NaO_{27}S$ $C_{56}H_{86}Na_2O_{30}S_2$ $C_{56}H_{86}Na_2O_{30}S_2$	(Silchenko *et al.* 2008)
Cucumaria okhotensis	Frondoside A_1 Okhotoside B_1 Okhotoside A_1-1 Frondoside A Okhotoside A_2-1	N/A 1269.5 $[M_{Na} + Na]^+$ 1239 $[M_{Na} + Na]^+$ 1357 $[M + Na]^+$ 1401.5 $[M_{Na} + Na]^+$	N/A $C_{56}H_{87}NaO_{27}S$ $C_{55}H_{85}NaO_{26}S$ N/A $C_{61}H_{95}NaO_{31}S$	(Aminin, D. L. *et al.* 2010b)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
	Cucumarioside A_2-5			
Cucumaria okhotensis	Okhotoside A_1-1 Okhotoside A_2-1 Okhotoside A_0-1	1239 $[M_{Na} + Na]^+$ 1401.5 $[M_{Na} + Na]^+$ N/A	$C_{55}H_{85}NaO_{26}S$ $C_{61}H_{95}NaO_{31}S$ N/A	(Silchenko *et al.* 2007)
Duasmodactyla kurilensis	Kuriloside A Kuriloside C	N/A	N/A	(Avilov, S. A. *et al.* 1991)
Eupentacta fraudatrix	Cucumarioside I_1 Cucumarioside I_3 Cucumarioside I_4	1457.5 $[M_{2Na} + Na]^+$ 1473.5 $[M_{2Na} + Na]^+$ 1329.4 $[M_{2Na} + Na]^+$	$C_{60}H_{92}Na_2O_{32}S_2$ $C_{60}H_{92}Na_2O_{33}S_2$ $C_{53}H_{80}Na_2O_{30}S_2$	(Silchenko *et al.* 2013a)
Eupentacta fraudatrix	Cucumarioside H_5 Cucumarioside H_6 Cucumarioside H_7 Cucumarioside H_8 Cucumarioside H	1307.5 $[M_{Na}– Na]^-$ 1309.5 $[M_{Na}– Na]^-$ 1311.6 $[M_{Na}– Na]^-$ 1327.5 $[M_{Na}+ Na]^+$ 1281.5 $[M_{Na}– Na]^-$	$C_{60}H_{91}NaO_{29}S$ $C_{60}H_{93}NaO_{29}S$ $C_{60}H_{95}NaO_{29}S$ $C_{58}H_{89}NaO_{29}S$	(Silchenko *et al.* 2011a)
Eupentacta fraudatrix	Cucumarioside A_2 Cucumarioside A_7 Cucumarioside A_9 Cucumarioside A_{10} Cucumarioside A_{11} Cucumarioside A_{13} Cucumarioside A_{14}	1179.5 $[M + Na]^+$ 1137.5 $[M + Na]^+$ 1141.6 $[M + Na]^+$ - - 1135.5 $[M + Na]^+$ 1155.5 $[M + Na]^+$	$C_{57}H_{88}O_{24}$ $C_{55}H_{86}O_{23}$ $C_{55}H_{90}O_{23}$ $C_{55}H_{84}O_{23}$ $C_{55}H_{88}O_{24}$	(Silchenko *et al.* 2012c)
Eupentacta fraudatrix	Cucumarioside A_1 Cucumarioside A_3 Cucumarioside A_4 Cucumarioside A_5 Cucumarioside A_6 Cucumarioside A_{12} Cucumarioside A_{15}	- 1193.6 $[M + Na]^+$ 1165.6 $[M + Na]^+$ - 1119.5 $[M + Na]^+$ 1123.5 $[M + Na]^+$ 1123.6 $[M + Na]^+$	- $C_{59}H_{94}O_{23}$ $C_{57}H_{90}O_{23}$ - $C_{55}H_{84}O_{22}$ $C_{54}H_{84}O_{23}$ $C_{55}H_{88}O_{22}$	(Silchenko *et al.* 2012a)
Eupentacta fraudatrix	Cucumarioside G_1 Cucumarioside G_2	1223 $[M_{Na} + Na]^+$ 1095 $[M_{Na} + Na]^+$	N/A	(Kalinin, VI *et al.* 1996)
Eupentacta fraudatrix	Cucumarioside G_4 Des- Cucumarioside G_4	1239 $[M_{Na} + Na]^+$	N/A	(Kalinin, V *et al.* 1992)
Eupentacta fraudatrix	Cucumarioside G_2	1095 $[M_{Na} + Na]^+$	N/A	(Avilov, S. A. *et al.* 1994)
Eupentacta fraudatrix	Cucumarioside G_3	N/A	N/A	(Kalinin, VI *et al.* 1992a)
Eupentacta fraudatrix	Cucumarioside I_2 Cucumarioside H Cucumarioside A_5 Cucumarioside A_6 Cucumarioside B_2 Cucumarioside B_1	1409.5 $[M_{2Na} –Na]^-$ 943.5 $[M + Na]^+$ 943.5 $[M + Na]^+$	$C_{60}H_{90}Na_2O_{32}S_2$ $C_{48}H_{72}O_{17}$ $C_{48}H_{72}O_{17}$	(Silchenko *et al.* 2012b)
Eupentacta fraudatrix	Cucumarioside A_8	1125.6 $[M_{Na} + Na]^+$	$C_{55}H_{90}O_{22}$	(Silchenko *et al.* 2012e)
Eupentacta fraudatrix	cucumarioside H_2 cucumarioside H_3 cucumarioside H_4	1325.5 $[M_{Na} - Na]^-$ 1181.5 $[M_{Na} - Na]^-$ 1353.6 $[M_{Na} - Na]^-$	$C_{60}H_{93}NaO_{30}S$ $C_{53}H_{81}NaO_{27}S$ $C_{62}H_{97}NaO_{30}S$	(Silchenko *et al.* 2012d)
Eupentacta fraudatrix	Cucumarioside F_1 Cucumarioside F_2	1279 $[M_{2Na} – Na]^-$ 1323.5 $[M_{2Na} + Na]^+$	$C_{55}H_{84}Na_2O_{28}S_2$ $C_{55}H_{82}Na_2O_{27}S_2$	(Popov, RS *et al.* 2014)
Eupentacta fraudatrix	Cucumarioside G_1 Cucumarioside C_1 Cucumarioside C_2	1223 $[M_{Na} + Na]^+$	N/A	(Afiyatullov, S. S. *et al.* 1987; Afiyatullov, S. S. *et al.* 1985)
Eupentacta pseudoquinquinquisemita	Cucumarioside C_2 Cucumarioside H	N/A	N/A	(Kalinin, VI *et al.* 1988)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Hemoiedema spectabilis	Hemoiedemoside A Hemoiedemoside B	1311 $[M + Na]^+$ 1413 $[M + Na]^+$	$C_{54}H_{82}Na_2O_{28}S_2$ $C_{54}H_{81}Na_3O_{31}S_3$	(Chludil *et al.* 2002)
Holothuria leucospilota	Leucospilotaside B Holothurin B_2 Holothurin B Holothurin A Leucospilotaside A Leucospilotaside C Echinoside B	907.4 $[M + Na]^+$ 907 $[M + Na]^+$ 905 2 $[M + Na]^+$ 1243.4 $[M + Na]^+$ 921.4 $[M + Na]^+$ 759.2 $[M + Na]^+$ 914. 2 $[M + Na]^+$	$C_{41}H_{65}NaO_{17}S$ $C_{41}H_{63}NaO_{17}S$ $C_{41}H_{63}NaO_{18}S$ $C_{35}H_{53}NaO_{13}S$ 	(Han *et al.* 2009c; Han *et al.* 2010c)
Holothuria leucospilota	Des-holothurin A (Nobiliside 2A) Holothurinoside E_1 Bivittoside D Holothurin B_3 Holothurins B/B_4	1141 $[M + Na]^+$ 1187 $[M + Na]^+$ 1449 $[M + Na]^+$ 889 $[M + Na]^+$ 905 $[M + Na]^+$	$C_{54}H_{86}O_{24}$ $C_{67}H_{110}O_{32}$ 	(Van Dyck *et al.* 2010b)
Holothuria (Microthele) axiloga	Arguside F Impatienside B Pervicoside D Holothurin B	1329.6 $[M + Na]^+$ 1271.6 $[M + Na]^+$ 1331.6 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{62}H_{98}O_{29}$ $C_{60}H_{96}O_{27}$ $C_{62}H_{100}O_{29}$ $C_{41}H_{63}NaO_{17}S$	(Yuan *et al.* 2009a)
Holothuria (Microthele) axiloga	Axilogoside A Holothurin B	859.4 $[M - H]^-$ 905.2 $[M + Na]^+$	$C_{41}H_{64}O_{17}S$ $C_{41}H_{63}NaO_{17}S$	(Yuan, WH *et al.* 2008)
Holothuria arenicola	Holothurin A Holothurin B	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1975)
Holothuria arenicola	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1973)
Holothuria atra	Holothurin A Echinoside A Holothurin B Echinoside B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$ 	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$ 	(Kobayashi *et al.* 1991)
Holothuria atra	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Stonik, VA *et al.* 1979)
Holothuria atra	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria atra	Holothurin B_3 Holothurin B_1 Holothurins B/B_4 Holothurin B_2	889 $[M + Na]^+$ 891 $[M + Na]^+$ 905 $[M + Na]^+$ 907 $[M + Na]^+$	- $C_{41}H_{65}O_{11}$ 	(Van Dyck *et al.* 2010b)
Holothuria axiologa	Holothurin A Echinoside A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Kobayashi *et al.* 1991)
Holothuria cinerascents	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1973)
Holothuria coluber	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria cubana	Holothurin A Holothurin B	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1975)
Holothuria difficilis	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1973)
Holothuria edulis	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Kobayashi *et al.* 1991)
Holothuria edulis	Holothurin A_2	1229.5 $[M + Na]^+$	$C_{54}H_{87}NaO_{26}S$	(Kalinin, V & Stonik 1982)
Holothuria edulis	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria floridana	Holothurin A_1 Holothurin A_2	1245 5 $[M + Na]^+$ 1229 5 $[M + Na]^+$	$C_{54}H_{87}NaO_{27}S$ $C_{54}H_{87}NaO_{26}S$	(Oleinikova *et al.* 1982a)
Holothuria floridana	Holothurin B_1	891 $[M + Na]^+$	$C_{41}H_{65}O_{11}$	(Elyakov *et al.* 1982)
Holothuria forskali	Holothurinoside A	1303 $[M + Na]^+$	$C_{60}H_{96}O_{29}$	(Rodriguez *et*

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*		Molecular formula	References
	Holothurinoside B	1345	$[M + Na]^+$	$C_{62}H_{98}O_{30}$	*al.* 1991)
	Holothurinoside C	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	
	Holothurinoside D	787	$[M + Na]^+$	$C_{41}H_{64}O_{13}$	
	Des-holothurin A (Nobiliside 2A)	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Compound 6	1127	$[M + Na]^+$		
Holothuria forskali	Holothurinoside C	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	(Van Dyck *et al.* 2010a)
	Holothurinoside C_1	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	
	Des-holothurin A (Nobiliside 2A)	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Des-holothurin A_1	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Holothurinoside E	1287	$[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Holothurinoside E_1	1287	$[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Holothurinoside A	1303	$[M + Na]^+$	$C_{60}H_{96}O_{29}$	
	Holothurinoside A_1	1303	$[M + Na]^+$	$C_{60}H_{96}O_{29}$	
	Holothurinoside F	1433	$[M + Na]^+$	$C_{66}H_{106}O_{32}$	
	Holothurinoside F_1	1433	$[M + Na]^+$	$C_{66}H_{106}O_{32}$	
	Holothurinoside G	1449	$[M + Na]^+$	$C_{66}H_{106}O_{33}$	
	Holothurinoside G_1	1449	$[M + Na]^+$	$C_{66}H_{106}O_{33}$	
	Holothurinoside H	1463	$[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Holothurinoside H_1	1463	$[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Holothurinoside I	1479	$[M + Na]^+$	$C_{67}H_{108}O_{34}$	
	Holothurinoside I_1	1479	$[M + Na]^+$	$C_{67}H_{108}O_{34}$	
Holothuria forskali	Holothurinoside C	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	(Caulier *et al.* 2013)
	Des-holothurin A (Nobiliside 2A)	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Holothurinoside F	1433	$[M + Na]^+$	$C_{66}H_{106}O_{32}$	
	Holothurinoside M	1301	$[M + Na]^+$		
	Holothurinoside G	1449	$[M + Na]^+$	$C_{66}H_{106}O_{33}$	
	Holothurinoside N	1317	$[M + Na]^+$		
Holothuria forskali	Holothurinoside C	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	(Van Dyck *et al.* 2009)
	Holothurinoside C_1	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	
	Desholothurin A (Nobiliside 2A)	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Desholothurin A_1	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Holothurinoside E	1287	$[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Holothurinoside E_1	1287	$[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Holothurinoside A	1303	$[M + Na]^+$	$C_{60}H_{96}O_{29}$	
	Holothurinoside A_1	1303	$[M + Na]^+$	$C_{60}H_{96}O_{29}$	
	Holothurinoside F	1433	$[M + Na]^+$	$C_{66}H_{106}O_{32}$	
	Holothurinoside F_1	1433	$[M + Na]^+$	$C_{66}H_{106}O_{32}$	
	Holothurinoside G	1449	$[M + Na]^+$	$C_{66}H_{106}O_{33}$	
	Holothurinoside G_1	1449	$[M + Na]^+$	$C_{66}H_{106}O_{33}$	
	Holothurinoside H	1463	$[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Holothurinoside H_1	1463	$[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Holothurinoside I	1479	$[M + Na]^+$	$C_{67}H_{108}O_{34}$	
	Holothurinoside I_1	1479	$[M + Na]^+$	$C_{67}H_{108}O_{34}$	
Holothuria forskali	Holothurinoside C	1125	$[M + Na]^+$	$C_{54}H_{86}O_{23}$	(Van Dyck *et al.* 2011)
	Desholothurin A (Nobiliside 2A)	1141	$[M + Na]^+$	$C_{54}H_{86}O_{24}$	
	Holothurinoside E	1287	$[M + Na]^+$	$C_{60}H_{96}O_{28}$	
	Holothurinoside A	1303	$[M + Na]^+$	$C_{60}H_{96}O_{29}$	
	Holothurinoside F	1433	$[M + Na]^+$	$C_{66}H_{106}O_{32}$	
	Holothurinoside G	1449	$[M + Na]^+$	$C_{66}H_{106}O_{33}$	
	Holothurinoside H	1463	$[M + Na]^+$	$C_{67}H_{108}O_{33}$	
	Holothurinoside I	1479	$[M + Na]^+$	$C_{67}H_{108}O_{34}$	
	Holothurinoside M	1301	$[M + Na]^+$		
	Holothurinoside L	1317	$[M + Na]^+$		

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Holothuria fuscocinerea	Fuscocineroside A Fuscocineroside B Fuscocineroside C Pervicoside C Holothurin A	1285.5 $[M + Na]^+$ 1227.5 $[M + Na]^+$ 1227.5 $[M + Na]^+$ 1243.5 $[M + Na]^+$	$C_{56}H_{87}NaO_{28}S$ $C_{54}H_{85}NaO_{26}S$ $C_{54}H_{85}NaO_{26}S$ $C_{54}H_{85}NaO_{27}S$	(Zhang, S-Y *et al.* 2006a)
Holothuria fuscocinerea	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria gracilis	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria grisea	17-dehydroxyholothurinoside A Griseaside A	1287.6 $[M + Na]^+$ 1289.6 $[M + Na]^+$	$C_{60}H_{96}O_{28}$ $C_{60}H_{98}O_{28}$	(Sun, GQ *et al.* 2008)
Holothuria grisea	Holothurin B Holothurin A	905.2 $[M + Na]^+$ 1243.5 $[M + Na]^+$	$C_{41}H_{63}NaO_{17}S$ $C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1975)
Holothuria grisea	Holothurin A_1	1245 5 $[M + Na]^+$	$C_{54}H_{87}NaO_{27}S$	(Oleinikova *et al.* 1982a)
Holothuria hilla	Hillaside A Hillaside B Holothuria B	623.3 $[M + Na]^+$ 717.4 $[M + Na]^+$	$C_{35}H_{52}O_8$ $C_{37}H_{58}O_{12}$	(Wu, J *et al.* 2007)
Holothuria hilla	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria hilla	Hillaside C	893.3 $[M + Na]^+$	$C_{40}H_{63}NaO_{17}S$	(Wu, J *et al.* 2006c)
Holothuria hilla	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Kuznetsova *et al.* 1982)
Holothuria impatiens	Impatienside A Bivittoside D	1447.7 $[M + Na]^+$ 1449.7 $[M + Na]^+$	$C_{67}H_{108}O_{32}$ $C_{67}H_{110}O_{32}$	(Sun, P *et al.* 2007)
Holothuria impatiens	Holothurins A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1973)
Holothuria lessoni	Des-holothurin A (Nobiliside 2A) Holothurin A_2 Scabraside B Holothurinoside H	1141 $[M + Na]^+$ 1229 $[M + Na]^+$ 1243 $[M + Na]^+$ 1463 $[M + Na]^+$	$C_{54}H_{86}O_{24}$ $C_{54}H_{87}NaO_{26}S$ $C_{67}H_{108}O_{33}$	(Caulier *et al.* 2013)
Holothuria leucospilota	Leucospilotaside C	759.2 $[M + Na]^+$	$C_{35}H_{53}NaO_{13}S$	(Han *et al.* 2008)
Holothuria leucospilota	Leucospilotaside A Echinoside B	921 $[M + Na]^+$ 914 $[M + Na]^+$	$C_{41}H_{63}NaO_{18}S$	(Han *et al.* 2009c)
Holothuria leucospilota	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Kitagawa *et al.* 1979)
Holothuria leucospilota	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria leucospilota	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Kitagawa *et al.* 1981c; Kitagawa *et al.* 1978a)
Holothuria leucospilota	Leucospilotaside C 3-O-(4 0-O-sodiumsulfate- b-D-xylopyranosyl)-holosta-22,25-epoxy-9-ene-3 b,12 a,17 a-triol	759.2 $[M + Na]^+$	$C_{35}H_{53}NaO_{13}S$	(Han *et al.* 2008)
Holothuria mixecana	Holothurin B Holothurin A	905.2 $[M + Na]^+$ 1243.5 $[M + Na]^+$	$C_{41}H_{63}NaO_{17}S$ $C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1975)
Holothuria nobilis	Nobiliside A Nobiliside B Nobiliside C	621.4 $[M + Na]^+$ 905.4 $[M + Na]^+$ 715.4 $[M + Na]^+$	$C_{35}H_{50}O_8$ $C_{41}H_{63}NaO_{17}S$ $C_{37}H_{56}O_{12}$	(Wu, J *et al.* 2006b)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Holothuria nobilis	Echinoside A	known	known	(Li, M *et al.* 2010)
Holothuria nobilis	Holothurin A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1973)
Holothuria nobilis	Holothurin B	905.2 $[M + Na]^+$	$C_{41}H_{63}NaO_{17}S$	(Radhika *et al.* 2002)
Holothuria pervicax	Pervicoside A Pervicoside B Pervicoside C	N/A	N/A	(Kitagawa *et al.* 1989b)
Holothuria pervicax	DS-pervicoside A DS-pervicoside B DS-pervicoside C Pervicoside A Pervicoside B Pervicoside C	1169 $[M + Na]^+$ 1109 $[M + Na]^+$ 1111 $[M + Na]^+$ 1271 $[M + Na]^+$ 1211 $[M + Na]^+$ 1213 $[M + Na]^+$	$C_{56}H_{90}O_{24}$ $C_{54}H_{86}O_{22}$ $C_{54}H_{88}O_{22}$	(Kitagawa *et al.* 1989b)
Holothuria pervicax	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria Polii	Holothurin B_2 Holothurin B_3 Holothurin B_4 Holothurin A Holothurin B	907.4 $[M + Na]^+$ 889.3 $[M + Na]^+$ 905.4 $[M + Na]^+$ 1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{41}H_{65}NaO_{17}S$ $C_{41}H_{63}NaO_{16}S$ $C_{41}H_{63}NaO_{17}S$ $C_{54}H_{85}NaO_{27}S$ $C_{41}H_{62}NaO_{17}S$	(Silchenko *et al.* 2005c)
Holothuria pulla	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria Scabra	Scabraside A Scabraside B	1227.5 $[M + Na]^+$ 1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{26}S$ $C_{54}H_{85}NaO_{27}S$	(Han *et al.* 2009a)
Holothuria Scabra	Holothurinogenin B Holothurin A Holothurin A_2 Holothurin B	762.7 $[M + Na]^+$ 1243.5 $[M + Na]^+$ 1229 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{41}H_{62}O_{13}$ $C_{54}H_{85}NaO_{27}S$ $C_{54}H_{87}NaO_{26}S$ $C_{41}H_{62}NaO_{17}S$	(Thanh *et al.* 2006)
Holothuria Scabra	Holothurin B	905.2 $[M + Na]^+$	$C_{41}H_{62}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria scabra	Scabraside D Fuscocineroside C 24-dehydroechinoside A (Scabraside A)	1245.5 $[M + Na]^+$ 1227.5 $[M + Na]^+$ 1227.5 $[M + Na]^+$	$C_{54}H_{87}NaO_{27}S$ $C_{54}H_{85}NaO_{26}S$ $C_{54}H_{85}NaO_{26}S$	(Han *et al.* 2012)
Holothuria scabra	Desholothurin A (Nobiliside 2A) Bivittoside D Holothurinoside H	1141.5 $[M + Na]^+$ 1449.7 $[M + Na]^+$ 1463.7 $[M + Na]^+$	$C_{54}H_{86}O_{24}$ $C_{67}H_{110}O_{32}$ $C_{67}H_{108}O_{33}$	(Bondoc *et al.* 2013)
Holothuria scabra	Holothurin A_3 Holothurin A_4	1259.5 $[M + Na]^+$ 1245.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{28}S$ $C_{54}H_{87}NaO_{27}S$	(Dang *et al.* 2007)
Holothuria scabra	Holothurin A Echinoside A 24-dehydroechinoside A	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Kitagawa *et al.* 1985; Kitagawa *et al.* 1982; Kobayashi *et al.* 1991)
Holothuria scabra	Scabraside A Echinoside A Holothurin A_1	1227.5 $[M + Na]^+$ 1229 5 $[M + Na]^+$ 1245 5 $[M + Na]^+$	$C_{54}H_{85}NaO_{26}S$ $C_{54}H_{87}NaO_{26}S$ $C_{54}H_{87}NaO_{27}S$	(Han *et al.* 2009b)
Holothuria scabra	Holothurinoside C Scabraside A Holothurin A_2 Scabraside B	1125 $[M + Na]^+$ 1227.5 $[M + Na]^+$ 1229 $[M + Na]^+$ 1243 $[M + Na]^+$	$C_{54}H_{87}NaO_{26}S$	(Caulier *et al.* 2013)
Holothuria sp.	Holothurin A Holothurin B	1243.5 $[M + Na]^+$ 905.2 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$ $C_{41}H_{63}NaO_{17}S$	(Elyakov *et al.* 1973)
Holothuria subrubra	Bivitoside C	1434 $[M + Na]^+$		(Caulier *et al.*

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
	Holothurinoside G Holothurinoside H	1449 $[M + Na]^+$ 1463 $[M + Na]^+$	 $C_{67}H_{108}O_{33}$	2013)
Holothuria surinamensis	Holothurin A Holothurin B	1243.5 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Elyakov *et al.* 1975)
Holothuria vagabunde	Holothurin B	905.2 $[M + Na]^+$	$C_{41}H_{63}NaO_{17}S$	(Kitagawa *et al.* 1978a)
Kolga hyalina	Kolgaoside A Kolgaoside B Holothurinoside B	1303.6 $[M + Na]^+$ 1303.6 $[M + Na]^+$	$C_{60}H_{96}O_{29}$ $C_{60}H_{96}O_{29}$	(Silchenko *et al.* 2014b)
Mensamaria intercedens	Intercedenside D Intercedenside E Intercedenside F Intercedenside G Intercedenside H Intercedenside I	1253.5 $[M + Na]^+$ 1223 $[M + Na]^+$ 1255.5 $[M + Na]^+$ 1207.5 $[M + Na]^+$ 1237 $[M + Na]^+$ 1239 $[M + Na]^+$	$C_{55}H_{83}NaO_{27}S$ $C_{54}H_{81}NaO_{26}S$ $C_{55}H_{85}NaO_{27}S$ $C_{54}H_{81}NaO_{25}S$ $C_{55}H_{83}NaO_{26}S$ $C_{55}H_{85}NaO_{26}S$	(Zou *et al.* 2005)
Mensamaria intercedens	Intercedenside A Intercedenside B Intercedenside C	1221 $[M + Na]^+$ 1325.3 $[M + Na]^+$ 1253.5 $[M + Na]^+$	$C_{55}H_{83}NaO_{25}S$ $C_{55}H_{84}Na_2O_{28}S_2$ $C_{55}H_{83}NaO_{27}S$	(Zou *et al.* 2003)
Neothyone gibbosa	Neothyoside A Neothyoside B	1271 $[M + Na]^+$ 911 [M + Na]+	$C_{56}H_{89}NaO_{27}S$ $C_{43}H_{67}NaO_{17}S$	(Encarnación D *et al.* 1996; Encarnacion *et al.* 1989)
Neothyonidium magnum	Neothyonidioside C	N/A	N/A	(Avilov, S. A. *et al.* 1990a)
Parathyona sp	Parathyoside R Parathyoside T	N/A	N/A	(Smetanina *et al.* 1983)
Pearsonothuria graeffei	Holothurin A Echinoside A	1243 $[M + Na]^+$ 1229 $[M + Na]^+$	$C_{54}H_{85}NaO_{27}S$	(Dong *et al.* 2008)
Pearsonothuria graeffei	Holothurinoside C Des-holothurin A (Nobiliside 2a) Holothurins B/B_4 Fuscocinerosides B/C Holothurin A_2 Holothurin A	1125 $[M + Na]^+$ 1141 $[M + Na]^+$ 905 $[M + Na]^+$ 1227 $[M + Na]^+$ 1229 $[M + Na]^+$ 1243 $[M + Na]^+$	 $C_{54}H_{86}O_{24}$ $C_{54}H_{87}NaO_{26}S$ $C_{54}H_{85}NaO_{27}S$	(Van Dyck *et al.* 2010b)
Pearsonothuria graeffei	Holothurin A_1 24-dehydroechinoside A	1245 5 $[M + Na]^+$	$C_{54}H_{87}NaO_{27}S$ $C_{54}H_{85}NaO_{26}S$	(Zhao *et al.* 2010)
Pearsonothuria graeffei	Des-echinoside A	N/A	$C_{54}H_{88}O_{23}$	(Zhao *et al.* 2011)
Pearsonothuria graeffei	Echinoside A	N/A	$C_{54}H_{87}NaO_{26}S$	(Zhao *et al.* 2012)
Pentacta australis	DS-peanustroside A DS-peanustroside B DS-peanustroside C DS-peanustroside D	1191.6 $[M - H]^-$ 1189.6 $[M - H]^-$ 1215.6 $[M - H]^-$ 1217.6 $[M - H]^-$	$C_{59}H_{99}O_{24}$ $C_{59}H_{97}O_{24}$ $C_{59}H_{92}O_{26}$ $C_{59}H_{94}O_{26}$	(Miyamoto *et al.* 1992)
Pentacta quadrangularis	PentaClaside I PentaClaside II PentaClaside III Philinopside A Philinopside B	1047 $[M + Na]^+$ 1047 $[M + Na]^+$ 915 $[M + Na]^+$ 1223.5 $[M + Na]^+$ 1325.2 $[M + Na]^+$	$C_{48}H_{73}NaO_{20}S$ $C_{48}H_{73}NaO_{20}S$ $C_{43}H_{65}NaO_{16}S$ $C_{55}H_{85}NaO_{25}S$ $C_{55}H_{85}Na_2O_{28}S_2$	(Han *et al.* 2010a)
Pentacta quadrangularis	Philinopside A Philinopside B	1223.5 $[M + Na]^+$ 1325.2 $[M + Na]^+$	$C_{55}H_{85}NaO_{25}S$ $C_{55}H_{85}Na_2O_{28}S_2$	(Yi, Y-H *et al.* 2006)
Pentacta quadrangularis	Philinopside E	1179 $[M + Na]^+$	N/A	(Tian *et al.* 2005)
Pentacta quadrangularis	Philinopside E Philinopside F	1179 $[M + Na]^+$ 1239 $[M + Na]^+$	N/A	(Zhang, S-L *et al.* 2006)
Pentacta quadrangularis	PentaClaside B PentaClaside C	1325.6 $[M + Na]^+$ 1325.6 $[M + Na]^+$	$C_{55}H_{84}Na_2O_{28}S_2$ $C_{55}H_{84}Na_2O_{28}S_2$	(Han *et al.* 2010b)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Pentacta quadrangularis	Philinopside A	known	known	(Tong *et al.* 2005)
Pentacta quadrangulasis	Philinopgenin A Philinopgenin B Philinopgenin C	535 $[M + Na]^+$ 493 $[M + Na]^+$ 535 $[M + Na]^+$	$C_{32}H_{48}O_5$ $C_{30}H_{46}O_4$ $C_{32}H_{48}O_5$	(Zhang, S-L *et al.* 2004)
Pentamera calcigera I	Calcigeroside B Calcigeroside C_1 Calcigeroside C_2 Cucumarioside G_2	1241 $[M_{Na} + Na]^+$ 1257 $[M_{Na} + Na]^+$ 1343 $[M_{Na} + Na]^+$	$C_{54}H_{83}NaO_{27}S$ $C_{54}H_{83}NaO_{28}S$ -	(Avilov, S. A. *et al.* 2000b)
Pentamera calcigera II	Calcigeroside D_1 Calcigeroside D_2 Calcigeroside E	1359 $[M_{Na,Na} + Na]^+$ 1445 $[M_{Na,Na} + Na]^+$ 1527 $[M_{Na,K} + H]^+$	$C_{54}H_{82}Na_2O_{31}S_2$ $C_{59}H_{92}Na_2O_{32}S_2$ $C_{62}H_{96}Na_2O_{35}S_2$	(Avilov, S. A. *et al.* 2000a)
Pseudocnus dubiosus leoninus	Pseudocnoside A	1267.4 $[M – Na]^-$	$C_{54}H_{84}Na_2O_{28}S_2$	(Careaga *et al.* 2014)
Pseudocolochirus violaceus	Violaceusoside C Violaceusoside D Violaceusoside E Violaceusoside G	1133.5 $[M_{Na}-Na]^-$ 1309.5 $[M_{2Na}–Na]^-$ 1235.4 $[M_{2Na}– Na]^-$ 1381.4 $[M_{3Na}– Na]^-$	$C_{53}H_{81}NaO_{24}S$ $C_{56}H_{86}Na_2O_{29}S_2$ $C_{53}H_{80}Na_2O_{27}S_2$ $C_{55}H_{83}Na_3O_{31}S_3$	(Silchenko *et al.* 2014a)
Pseudocolochirus violaceus	Intercedenside B	1457 $[M + Na]^+$	$C_{60}H_{92}Na_2O_{32}S_2$	(Zhang, S-Y *et al.* 2007)
Pseudocolochirus violaceus	Violaceuside A Violaceuside B	1223.5 $[M + Na]^+$ 1239.5 $[M + Na]^+$	$C_{55}H_{85}NaO_{25}S$ $C_{55}H_{85}NaO_{26}S$	(Zhang, S-Y *et al.* 2006b)
Pseudocolochirus violaceus	Violaceuside I Violaceuside II Violaceuside III	1195.5 $[M + Na]^+$ 1281 $[M + Na]^+$ 1281 $[M + Na]^+$	$C_{53}H_{81}NaO_{25}S$ $C_{53}H_{80}Na_2O_{27}S_2$ $C_{53}H_{80}Na_2O_{27}S_2$	(Zhang, SY *et al.* 2006)
Psolus eximius	Eximisoside A	1153 $[M + Na]^+$	$C_{55}H_{86}O_{24}$	(Kalinin, VI *et al.* 1997)
Psolus fabricii	Psolusoside A	N/A	N/A	(Kalinin, VI *et al.* 1985)
Psolus fabricii	Psolusoside B	N/A	N/A	(Kalinin, VI *et al.* 1989)
Psolus fabricii	Psoluthurin A	N/A	N/A	(Garneau *et al.* 1983)
Psolus patagonicus	Patagonicoside A Ds- Patagonicoside A Patagonicoside B	1285 $[M_{Na} –Na]^-$	$C_{54}H_{86}Na_2O_{29}S_2$	(Murray, AP *et al.* 2001)
Psolus patagonicus	Patagonicoside B Patagonicoside C	1151.5 $[M –Na]^-$ 1285.4 $[M –Na]^-$	$C_{53}H_{83}NaO_{25}S$ $C_{54}H_{86}Na_2O_{29}S_2$	(Careaga *et al.* 2011)
Staurocucumis liouvillei	Liouvilloside A Liouvilloside B	1457 $[M + Na]^+$ 1459 $[M + Na]^+$	$C_{56}H_{85}Na_3O_{32}S_3$ $C_{56}H_{87}Na_3O_{32}S_3$	(Maier *et al.* 2001)
Staurocucumis liouvillei	Liouvilloside A_4 Liouvilloside A_5	1295.5 $[M_{2Na} + Na]^+$ 1355.5 $[M_{2Na} + Na]^+$	$C_{54}H_{82}Na_2O_{27}S_2$ $C_{56}H_{86}Na_2O_{29}S_2$	(Antonov *et al.* 2011)
Staurocucumis liouvillei	Liouvilloside A_1 Liouvilloside A_2 Liouvilloside A_3 Liouvilloside B_1 Liouvilloside B_2	1265.4 $[M_{2Na}- Na]^-$ 1249.4 $[M_{2Na}- Na]^-$ 1293.5 $[M_{2Na}- Na]^-$ 1367.4 $[M_{3Na}- Na]^-$ 1411.4 $[M_{3Na}- Na]^-$	$C_{54}H_{82}Na_2O_{28}S_2$ $C_{54}H_{82}Na_2O_{27}S_2$ $C_{56}H_{86}Na_2O_{28}S_2$ $C_{54}H_{81}Na_3O_{31}S_3$ $C_{56}H_{85}Na_3O_{32}S_3$	(Antonov *et al.* 2008)
Staurocucumis liouvillei	Liouvilloside A_1 Liouvilloside A_2 Liouvilloside A_3 Liouvilloside B_1 Liouvilloside B_2	1265.4 $[M_{2Na} –Na]^-$ 1249.4 $[M_{2Na} –Na]^-$ 1293.5 $[M_{2Na} –Na]^-$ 1367.4 $[M_{3Na} –Na]^-$ 1411.4 $[M_{3Na} –Na]^-$	$C_{54}H_{82}Na_2O_{28}S_2$ $C_{54}H_{82}Na_2O_{27}S_2$ $C_{56}H_{86}Na_2O_{28}S_2$ $C_{54}H_{81}Na_3O_{31}S_3$ $C_{56}H_{85}Na_3O_{32}S_3$	(Antonov *et al.* 2008)
Staurocucumis turqueti	Turquetoside A	1229.5 $[M_{2Na}-2Na+H]^-$	$C_{54}H_{84}Na_2O_{27}S_2$	(Silchenko *et al.* 2013d)
Stichopus japonicus	Holotoxin A Holotoxin B	N/A	$C_{67}H_{108}O_{32}$ $C_{66}H_{104}O_{32}$	(Kitagawa *et al.* 1978b)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Stichopus chloronotus	Stichloroside A Stichloroside B	genin	N/A	(Sharypov *et al.* 1981)
Stichopus chloronotus	Stichloroside A_1 Stichloroside A_2 Stichloroside B_1 Stichloroside B_2 Stichloroside C_1 Stichloroside C_2 Stichlorogenol dehydrostichlorogenol	1454 1452 1454 1452 1438 1436 472 470	$C_{68}H_{110}O_{33}$ $C_{68}H_{108}O_{33}$ $C_{68}H_{110}O_{33}$ $C_{68}H_{108}O_{33}$ $C_{68}H_{110}O_{32}$ $C_{68}H_{108}O_{32}$ $C_{30}H_{48}O_4$ $C_{30}H_{46}O_4$	(Kitagawa *et al.* 1981b)
Stichopus chloronotus	Stichloroside A_1 Stichloroside A_2 Stichloroside B_1 Stichloroside B_2 Stichloroside C_1 Stichloroside C_2	N/A	N/A	(Kitagawa *et al.* 1981a; Kitagawa *et al.* 1981b)
Stichopus chloronotus	Stichoposide C Stichoposide D	N/A	N/A	(Kuznetsova *et al.* 1982)
Stichopus chloronotus	Stichoposide A Stichoposide B	N/A	N/A	(Sharypov *et al.* 1981)
Stichopus chloronotus	Stichoposide A Stichoposide C Stichoposide D Stichoposide E	N/A	N/A	(Mal'tsev *et al.* 1985)
Stichopus chloronotus	Stichoposide C	N/A	N/A	(Stonik, VA *et al.* 1982b)
Stichopus chloronotus	Stichoposide D	N/A	N/A	(Stonik, VA *et al.* 1982c)
Stichopus chloronotus	Stichoposide E	1454 $[M]^+$	$C_{68}H_{110}O_{33}$	(Maltsev *et al.* 1983)
Stichopus chloronotus	Stichloroside F Stichoposide D Stichloroside A_2 Stichoposide E Neothyonidioside Holothurin B	935.5 $[M + Na]^+$ Known Known Known Known 905.2 $[M + Na]^+$	$C_{47}H_{76}O_{17}$ $C_{41}H_{63}NaO_{17}S$	(Thao *et al.* 2014)
Stichopus hermanni	Stichloroside A_1 Stichloroside A_2 Stichloroside B_1 Stichloroside B_2 Stichloroside C_1 Stichloroside C_2	N/A	known	(Kobayashi *et al.* 1991)
Stichopus japonicus	Stichoposide A Stichoposide A_1	N/A	N/A	(Anisimov *et al.* 1972)
Stichopus japonicus	Stichoposide A Stichoposide C	N/A	N/A	(Elyakov *et al.* 1973)
Stichopus japonicus	Holotoxin A	N/A	known	(Mal'tsev *et al.* 1985)
Stichopus japonicus	Holotoxin A_1 Holotoxin B_1	N/A	known	(Maltsev *et al.* 1984)
Stichopus parvimensis	Parvimoside A Parvimoside B	1410 M^+,1433 $[M + Na]^+$ 1380 M^+ ,1403 $[M + Na]^+$	$C_{66}H_{106}O_{32}$ $C_{65}H_{104}O_{31}$	(Iniguez-Martinez *et al.* 2005)
Stichopus variegatus	Variegatuside C Variegatuside D Variegatuside E Variegatuside F	1095.5 $[M + Na]^+$ 1259.6 $[M + Na]^+$ 1435.7 $[M + Na]^+$ 1435.7 $[M + Na]^+$	$C_{53}H_{83}O_{22}^-$ $C_{59}H_{96}O_{27}$ $C_{66}H_{107}O_{32}^-$ $C_{66}H_{107}O_{32}^-$	(Wang, X-H *et al.* 2014)

Sea cucumbers species	Name of compounds	Molecular Mass *m/z*	Molecular formula	References
Stichopus variegatus	Variegatuside A Variegatuside B	1097 $[M + Na]^+$ 1097 $[M + Na]^+$	$C_{53}H_{86}O_{22}$ $C_{53}H_{86}O_{22}$	(Wang, XH *et al.* 2006)
Stichopus variegatus	Astichoposide C	N/A	N/A	(Stonik, VA *et al.* 1982b)
Synallactes nozawai	Synallactoside A_1 Synallactoside A_2 Synallactoside B_1 Synallactoside B_2 Synallactoside C	1121.5 $[M + Na]^+$ 1399.6 $[M + Na]^+$ 1429.6 $[M + Na]^+$ 1253.6 $[M + Na]^+$ 1283.6 $[M + Na]^+$	$C_{55}H_{86}O_{22}$ $C_{66}H_{104}O_{30}$ $C_{67}H_{106}O_{31}$ $C_{60}H_{94}O_{26}$ $C_{61}H_{96}O_{27}$	(Silchenko *et al.* 2002)
Synapta maculata	Synaptoside A Synaptoside A_1	1409.5 $[M + Na]^+$ 1423.5 $[M + Na]^+$	$C_{60}H_{92}NaO_{31}S$ $C_{60}H_{90}NaO_{32}S$	(Avilov, S. A. *et al.* 2008)
Synapta maculata	Synaptoside S-2 Synaptoside S-3	N/A	N/A	(Kuznetsova *et al.* 1985)
Thelenota ananas	Thelenotoside A Thelenotoside B	1123 $[M + Na]^+$ 1139 $[M + Na]^+$	$C_{55}H_{88}O_{22}$ $C_{55}H_{88}O_{23}$	(Mal'tsev *et al.* 1985; Stonik, VA *et al.* 1982a)
Thelonota ananas	Stichloroside A_1 Stichloroside A_2 Stichloroside B_1 Stichloroside B_2 Stichloroside C_1 Stichloroside C_2	N/A	known	(Kobayashi *et al.* 1991)
Thelonota ananas	Stichloroside C	N/A	N/A	(Yun *et al.* 2012)
Thelonota ananas	Compound 1 Compound 2	965 $[M + Na]^+$ N/A	$C_{47}H_{74}O_{19}$ N/A	(Hegde *et al.* 2002)
Thelonota anax	Stichloroside A_1 Stichloroside B_1 Stichloroside C_1	N/A	known	(Kobayashi *et al.* 1991)
Thyone aurea	Thyonoside A Thyonoside B	1325.4 $[M + Na]^+$ 1193.5 $[M + Na]^+$	$C_{55}H_{84}Na_2O_{28}S_2$ $C_{54}H_{83}NaO_{24}S$	(Bonnard & Rinehart 2004)

N/A= information was not available.

1.9.5 Nonholostane type glycosides

Nonholostane type glycosides are rare saponins found in sea cucumber species (Chludil *et al.* 2003; Kim, SK & Himaya 2012). Triterpene saponins with non-holostane aglycone structures lack the lactone group and bear a short lateral side chain compared to holostane type. Only twenty four non-holostane type congeners hitherto have been reported from sea cucumber species, which mainly belong to the order Dendrochirotida. The identified nonholostane type saponins, habitats and taxonomic information are listed in Table 1.4.

Table 1.4. Nonholostane (without lactone) type triterpene glycosides isolated from sea cucumbers

Species	Compounds	Habitat	Reference
Apostichopus japonicus	Holotoxins F/G	Dalian coast, Bohai Sea of China	(Wang, Z *et al.* 2012b)
Cucumaria conicospermium	Cucumarioside A_3-2 Cucumarioside A_3-3 Isokoreoside A Koreoside A	Sigsbi trawl, Sea of Japan	(Avilov, S.A. *et al.* 2003)
Cucumaria frondosa	Frondoside A_2-7 Frondoside A_2-8	Penobscot Bay, Gulf of Maine, USA	(Silchenko *et al.* 2005b)
Cucumaria frondosa	Frondoside C, DS-frondoside C	Dalnezelenetskaya Bay, Kolsky Peninsula, Barents Sea	(Avilov, S. A. *et al.* 1998)
Cucumaria koraiensis	Koreoside A	Kurile Island, Sea of Okhotsk	(Avilov, S. A. *et al.* 1997)
Duasmodactyla kurilensis	Kurilosides A/C	Kurile Islands, Sea of Okhotsk	(Avilov, S A. *et al.* 1991)
Eupentacta fraudatrix	Cucumarioside G_2	Gulf of Posiet, Sea of Japan	(Avilov, S. A. *et al.* 1994)
Eupentacta fraudatrix	Cucumariosides $A_8/A_9/A_{10}$	Peter the Great Gulf, Sea of Japan	(Silchenko *et al.* 2012c; Silchenko *et al.* 2012e)
Eupentacta fraudatrix	Cucumarioside H_3	Peter the Great Gulf, Sea of Japan	(Silchenko *et al.* 2011b)
Pentamera calcigera	Calcigerosides B/C_1 Cucumarioside G_2	Peter the Great Gulf, Sea of Japan	(Avilov, S. A. *et al.* 2000b)
Pentamera calcigera	Calcigeroside D_1, DS-calcigeroside D_1	Peter the Great Gulf, Sea of Japan	(Avilov, S. A. *et al.* 2000a)
Pentarta australis	DS-penaustrosides A/B	Ariake Sea in Saga Prefecture	(Miyamoto *et al.* 1992)
Psolus fabricii	Psolusoside B	Kurile Island, Sea of Okhotsk	(Kalinin, VI *et al.* 1989)

1.9.6 Extraction, isolation and structural elucidation of saponins

Due to the nature of saponins, they are mainly extracted with alcohol, water or a mixture of two. While water, alcohols (methanol, ethanol) and aqueous alcohols are the most common extraction solvents for saponins, solubility of some saponins in ether, chloroform, benzene, ethyl acetate, or glacial acetic acid has also been documented (Güçlü-Üstünda & Mazza 2007; Hostettmann & Marston 1995). Purification of the crude saponin extract usually requires a sequential approach. A common method for the preliminary purification of saponins after the extraction step involves the partitioning of saponins between aqueous extracts and a water immiscible solvent such as n-

butanol (Kitagawa 1986).

Briefly, for the purification of saponins, following evaporation of alcohol, the extract might be partitioned between a relatively non-polar solvent such as n- hexane, petroleum ether, ethyl acetate, carbon tetrachloride or chloroform and water to eliminate low polarity and apolar components. The aqueous extract is then subjected to the microporous resin such as Amberlite® XAD-4 (Van Dyck *et al.* 2009) or Amberlite® XAD-2 (Hostettmann & Marston 1995; Roccatagliata *et al.* 1994), or Diaion® MCI Gel HP20, or Kogel® BG4600, or Diaion® HP-20 macroporous resin, or Polychrom-1 or DA-101, or DA-201 or/and silica gels to purify saponins (Jia & Qian 2011). This is then followed by partition between water saturated iso-butanol or *n*-butanol and water to obtain saponins in the iso-butanol fraction. It is noteworthy that some acetylated saponins are lipophilic owing to the acetylation of a sugar moiety, and thereby dissolve in the chloroform or ethyl acetate or other non-polar solvents.

An alternative approach, which has been established as the primary alternative to organic solvents for the processing of natural materials, such as saponins, with advantages such as ease of solvent removal, solvent free products, and an oxygen free environment is supercritical fluids (SCFs) technology such as supercritical CO_2 technique ($SCCO_2$) (Hamburger *et al.* 2004). However, the application of $SCCO_2$ technology to the processing of polar solutes such as polyphenolic and glycosidic components has been restricted by the low solvent power of $SCCO_2$ for these solutes, which can be improved by the addition of co-solvents.

1.9.7 Spectroscopic analysis of triterpenoids

Several different spectroscopic analysis methods namely ultraviolet spectrum (UV), infrared spectrum (IR), and NMR have been applied for the structure elucidation of saponins. However, most triterpenoids do not have strong UV absorption owing to the absence of conjugated functional groups. Some triterpenoids with α,β- unsaturated carbonyl functional groups absorb UV and exhibit signal peaks. Saturated aglycones of steroidal saponins show no absorption between 200 and 400nm. However, introduction of a single double bond, carbonyl groups, α,β- unsaturated ketones, or conjugated double bonds may result in UV absorption at 205 to 225nm and 285nm

(Xu, R *et al.* 2012).

MS is an accurate, rapid, effective, and convenient analytical method for the identification of a broad range of saponins in sea cucumber. It is also high sensitive, reliable and a fast processing technique for the structure elucidation of carbohydrates. This technique has been used to analyse saponins in sea cucumber in this study, therefore, it will be discussed in detail.

Applying tandem mass spectrometry (MS/MS, MS^2) or (MS/MS/MS) using collision- induced dissociation (CID), on parent ions cleave the glycoside bonds and generate a signature mass chromatogram due the loss of sugar unit. The mass differences between the fragment ion peaks which indicate the present of five-carbon sugars (-132), six-deoxy-carbon sugars (-146), hexoses (-162), uronic acids or methyl hexoses (-176) and sequence of sugars in the saccharides moiety can be determined.

1.9.8 Biosynthesis of saponins in Holothurians

In *Holothuria*, there appear to be two main biosynthetic pathways for saponins, one via lanosterol and the other via parkeol. It has been postulated that lanosterol glycosides may serve as a putative biosynthetic precursor to the holostane triterpene glycosides (Makarieva *et al.* 1993). Saponins have an isoprenoid structure (especially triterpene- or steroid- derived) and are synthesised from mevalonate via farnesyl diphosphate and squalene in plants (Osbourn *et al.* 2011).

Saponin generation is initiated by the oxidation of squalene to 2,3-oxidosqualene in which is catalysed by squalene epoxidase (Osbourn *et al.* 2011). It has been reported, by measuring radioactivity from tritium-labelled squalene and labelled parkeol, which squalene is a precursor for biosynthesis of steroid and triterpenoid metabolites in the sea cucumbers (Kerr & Chen 1995; Makarieva *et al.* 1993). It is established that parkeol is generated from the cyclisation of squalene followed by further modification and converted into triterpenoide glycosides.

Saponins are glycoside compounds. It has been noted that glycosylation patterns in natural products are usually a crucial modification for bioactivity. This modification is catalysed by glycosyltransferases that are important enzymes in generation of saponins.

Sea cucumber saponins which are primarily of the lanostane type tetracyclic triterpenoids are characterised by the *trans* junction of rings A/B, B/C, and C/D, β-oriented methyl at C-10 and C-13, α-oriented methyl at C-14, β-oriented side chain at C-17, and the R configuration for C-20. In contrast, the oleanane type saponins are the most common triterpenoid saponins in plants, and most contain hydroxyl group at C-3, $\Delta^{12,13}$ and a carboxylic group at C-28 (Xu, R *et al.* 2012).

1.10 Biological properties, application of saponins and future prospects

Triterpene glycosides, an important class of naturally occurring products, from sea cucumbers have attracted the attention of researchers including chemists, biochemists, pharmacologists and biologists-taxonomists, for more than sixty years (Kalinin, VI *et al.* 2008), and increasingly in the last decade because of their properties and commercial applications as natural products, natural detergents, and their cardiac, immunomodulator, cytotoxic, antibacterial anti-viral, anti-cancer, antifungal, haemolytic, cytostatic and as well as other health promotion such as cosmetic and age caring (Aminin, D *et al.* 2015; Chludil *et al.* 2002; Dong *et al.* 2008; Maier *et al.* 2001; Osbourn *et al.* 2011).

Traditional knowledge of the medicinal value of sea cucumbers has a long history. The therapeutic use of sea cucumber for healing has traditions dating back at least 5000 years in China where they were used for joint pain, tendonitis and sprains (Zaki 2005). Among the locals in China, Malaysia and the Philippines, cuvierian tubules are generally applied as crude plaster in treatment of minor wounds, and sea cucumber extracts from the body wall are used to treat tumours, fungal infection, high blood pressure, arthritis and muscular disorders (Chen, J 2004; Choo 2004; Conand 2004a).

Since then sea cucumber products have been marketed for their pharmaceutical and medicinal benefit as effective natural products. As a result, numerous commercial products from sea cucumbers have been commercialised such as the arthritis medicines ArthriSea®, ArthriSea® Plus, Sea-Q and SeaCuMax, and SeaFlex, Green-Bones, Sea Soap, NutriSea® Biscuits, Sea Jerky, which are used to treat joint problems in canines; Gold-G Bio sea cucumber for enhancing the immune system (obtained from golden sea cucumber); and Sea Cucumber Plus Syrup for

relieving cough (Len Fa Med. Supplies) are among the most routine products on the shelves and in the market (Al Marzouqi *et al.* 2011; Alfonso *et al.* 2007; Asha *et al.* 2007; Coastside Bio Resources 2012; Janakiram *et al.* 2010; Mindell, E. 1998).

However, a range of biological and pharmaceutical activities have been ascribed to saponins such as antifungal (Chludil *et al.* 2002; Francis *et al.* 2002; Han *et al.* 2010c) anti-viral (Francis *et al.* 2002), anti-tumour (Dang *et al.* 2007; Roginsky *et al.* 2004; Zhang, SY *et al.* 2006), anti-inflammatory (Collin 1998; Collin, P.D. 1999; Collin 2004; Herencia *et al.* 1998; Whitehouse & Fairlie 1994), ichthyotoxic (Nigrelli, RF 1952; Nigrelli & Jakowska 1960; Stonik, VA *et al.* 1999; Yamanouchi 1955), anti-herbivore (Osbourn *et al.* 2011) cytotoxic (Dang *et al.* 2007; Han *et al.* 2010c; Stonik, VA *et al.* 1999) cytostatic (Han *et al.* 2010c; Zhang, SY *et al.* 2006) haemolytic (Han *et al.* 2010c; Kalinin, V *et al.* 1996; Stonik, VA *et al.* 1999) immunomdulatory (Francis *et al.* 2002; Han *et al.* 2010c; Stonik, VA *et al.* 1999) larvicidal, antibacterial (Sedov *et al.* 1990), antimolluscide, antioxidants (Althunibat *et al.* 2009; Francis *et al.* 2002; Zhong *et al.* 2007), hypoglycaemic (Rao & Gurfinkel 2000), antihepatotoxic (Rao & Gurfinkel 2000) and antineoplastic functions. Many of these properties have originated from their tensioactive characteristics.

1.10.1 Pharmaceutical and Medicinal Properties

Sea cucumbers are consumed as a medicinal food in East Asia. Sea cucumber is famously known as *haishen* in Chinese, which roughly means ginseng of the sea (Bruckner *et al.* 2003; Chen, J 2003). It is part of traditional Chinese medicine because of its pharmaceutical and aphrodisiac properties. Apart from its reputation as an aphrodisiac (Aydın *et al.* 2011; Choo 2004; Purcell *et al.* 2010), sea cucumber is widely used as a traditional remedy for weakness, constipation, asthma, hypertension, rheumatism, sinus, cuts, and burns. Sea cucumber is also known to accelerate internal healing, especially after clinical surgery, injury, or caesarean surgery (Anderson 1990; Chen, J 2003; Fredalina *et al.* 1999; Jilin & Peck 1995; San Miguel-Ruiz & Garcia-Arraras 2007; Weici 1987; Wen *et al.* 2010; Yaacob *et al.* 1997; Zhong *et al.* 2007; Zohdi *et al.* 2011). Fredalina *et al.* (1999) stated that the present of high omega fatty acid content, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in *S. chloronotus* contributes to its ability to trigger

tissue repair, proposing potential and active involvement of sea cucumbers in tissue regeneration.

1.10.2 Anti-microbial activity

It has been reported that saponins possess antibacterial properties. For instance, Jamali *et al.* (2009) evaluated the antibacterial activity of polar and apolar extracts from the body wall of sea cucumbers collected from the Persian Gulf against three strains of *E. coli*, namely Top 10 F0, TG1 and K12 and noticed significant antibacterial property.

Moreover, the antibacterial and antifungal activities of alcoholic extracts of holothurian species including *Actinopyga echinites, A. miliaris*. *Holothuria atra* and *H. scabra* were assessed towards a number of gram-positive and gram-negative bacteria*,* and fish-borne mould using saponin and ampicillin as positive controls (Abraham *et al.* 2002). Their results demonstrated that the extracts of *A. miliaris, H. atra* and *H. scabra* inhibited strongly the growth of above microorganisms. However, the holothurian extracts did not exhibit growth inhibitory effects on *Bacillus sp*. In addition, Park *et al.* reported the antibacterial effects of water-soluble fractions from red sea cucumber *S. japonicus* against *S. aureus* and *S. epidermidis* (Park, SY *et al.* 2011).

In contrast some researchers stated that the crude extracts from sea cucumber show no significant antibacterial activity towards the examined species (Lawrence, AJ *et al.* 2010; Mokhlesi *et al.* 2012; Ridzwan *et al.* 1995).

Ridzwan *et al.* (1995) stated that the sea cucumber extracts inhibit bacterial growth in a dose-dependent manner. They also compared the growth inhibitory effect of extracts from the outer layer and inner part body wall of *H. atra*, and found that the inner layer extract has higher bacterial growth inhibitory property.

Throughout the efforts to discover novel biological active compounds from marine organisms, the antifungal activities of sea cucumber saponins have been broadly studied (Fredalina *et al.* 2004; Lawrence, AJ *et al.* 2010; Yuan, WH *et al.* 2008), and is the second most studied biological activities of these metabolites (Kim, SK & Himaya 2012). Several triterpene glycosides have been noted to possess antifungal activities. The antifungal property of studied sea cucumber saponin

congeners is summarised in Table 1.5.

Table 1.5. Anti-fungal property of triterpene glycosides from holothurians

Species	Triterpene glycosides	Fungi	References
Actinocucumis typica	*Typicosides A_1, A_2, B_1, C_1 and C_2*	*Aspergillus niger, Fusarium oxysporum and Candida albicans*	*(Silchenko et al. 2013b)*
Actinopyga echinites, Bohadschia bivittata,	*Holotoxin A_1, Holotoxin B_1, Stichloroside C_2, Bivittosides A, B, C and D, Echinosides A and B*	*A. niger, A. oryzae, Penicillium citricum, Mucor spinescens, Cladosporium herbarum, Rhodotorula rubra, Trichophyton mentagrophytes, T. rubrum, C. albicans, C. utilis*	*(Kitagawa 1988; Kitagawa et al. 1989a; Kitagawa et al. 1985)*
Actinopyga lecanora	*Methanol extract*	*C. albicans, Candida neoformans, Sporothrix schenckii, T. mentagrophytes and A. fumigatus*	*(Kumar et al. 2007)*
Apostichopus japonicas	*Holotoxin A_1*	*C. albicans, A. niger, Penicillum niger, Tomila uiilis, Hormodendro pedrosoi, T. mentagraphytes, S. carlsbergensis,*	*(Mal'tsev et al. 1985)*
Australostichopus mollis	*Neothyonidioside and Mollisoside A*	*Saccharomyces cerevisiae*	*(Yibmantasiri et al. 2012)*
B. marmorata	*Marmoratoside A, 17α-hydroxy impatienside A, Marmoratoside B, 25-acetoxy bivittoside D, Impatienside A and Bivittoside D*	*C. albicans, Cryptococcus neoformans, Aspergillus fumigates, T. rubrum, C. tropicalls and C. krusei.*	*(Yuan et al. 2009b)*
B. marmorata	*17-hydroxy fuscocineroside B, 25-hydroxy fuscocineroside B, Fuscocineroside B*	*Candida albicans, C. neoformans, A. fumigates, T rubrum, C. tropicalls and C. krusei.*	*(Yuan, W et al. 2008)*
Cucumaria echinata	*Cucumechinosides A, B, C, D, E and F*	*C. albicans, Trichomonas foetus, T. mentagrophytes, A. niger, Mucor hiemalis, and Penicillum chrysogenum*	*(Miyamoto, T. et al. 1990)*
Cucumaria japonica	*Cucumariosides I and II,*	*C. albicans and C. tropicalis*	*(Batrakov et al. 1980)*
Eupentacta fraudatrix	*Cucumariosides I_1, I_3 and I_4*	*C. albicans, A. niger, and F. oxysporum*	*(Silchenko et al. 2013a)*
E. fraudatrix	*Cucumariosides A_1, A_3, A_4, A_5, A_6, A_{12} and A_{15},*	*C. albicans, A. niger, Septoria glycines, Cercospora sojina and F. oxysporum*	*(Silchenko et al. 2012a)*
E. fraudatrix	*Cucumariosides A_2, A_7, A_8, A_9, A_{10}, A_{11},*	*C. albicans, A. niger, S. glycines, C. sojina and F.*	*(Silchenko et al. 2012c)*

Species	Triterpene glycosides	Fungi	References
	A_{13} and A_{14},	oxysporum	
H. Fuscocinerea	n-BuOH extract	P. oryzae	(Zhang, S-Y et al. 2006a)
H. fuscogilva, Stichopus hermanni, Actinopyga mauritiana, A. crassa, Bohadschia vitiensis, B. tenuissima, Pearonothuria graeffei, B. cousteaui, H. atra, H. leucospilota, and H. nobilis.	Ethanol extract	C. albicans	(Lawrence, AJ et al. 2010)
H. scabra	Scabraside A, Echinoside A and Holothurin A_1	C. albicans, C. pseudotropicalis, C. neoformans, A. fumigates, T. rubrum, Fonsecaea compacta and Microsporum gypseum	(Han 2009)
Hemoiedema spectabilis	Hemoiedemosides A and B	Cladosporium cucumerinum	(Chludil et al. 2002)
H. (Microthele) axiloga	Axilogoside A and Holothurin B	C. albicans, C. neoformans and A. fumigates	(Yuan, WH et al. 2008)
H. (Microthele) axiloga	Arguside F, Impatienside B, and Pervicoside D	C. albicans, C. neoformans, A. fumigates, T. rubrum, C. tropicalls and C. krusei	(Yuan et al. 2009a)
Psolus patagonicus	Patagonicoside A Ds-patagonicoside A	Cladosporium fulvum, Fusarium oxysporum and Monilia sp.	(Muniain et al. 2008)
P. patagonicus	Patagonicoside A	Cladosporium cucumerinum	(Murray, AP et al. 2001)
P. patagonicus	Patagonicosides A, B and C	Cladosporium cladosporoides	(Careaga et al. 2011)
S. chloronotus	Methanol extract	C. albicans, C. tropicalis, T. glabrata, Crytococcus neoformans and M. canis	(Kiew, Peck Loo & Don 2012)
Stichopus chloronotus	Stichoposides A, C, D, E	C. albicans, A. niger, Penicillum niger, Tomila uiilis, Hormodendro pedrosoi, T. mentagraphytes and S. carlsbergensis,	(Mal'tsev et al. 1985)
S. chloronotus	Ethanol extracts	Microsporum canis and T. mentagrophytes	(Fredalina et al. 2004)
Thelenota ananas	Thelenotosides A and B	C. albicans, A. niger, Penicillum niger, Tomila uiilis, Hormodendro pedrosoi, T. mentagraphytes and S. carlsbergensis,	(Mal'tsev et al. 1985)

It has been established that there is a strong correlation between the structure and activity of saponins. The numbers and position of sulphate groups, hydroxy groups and double bonds can influence the activity. For instance, Chludil *et al.* (2002) reported hemoiedemosides A and B, isolated from sea cucumber *Hemoiedema spectabilis* have significant antifungal activity with the phytopathogenic fungus *Cladosporium cucumerinum*. These two saponins possess similar structures and vary only in level of sulphation in their sugar moieties. They reported hemoiedemoside A exhibits stronger activity compared to hemoiedemoside B, and concluded that having a third sulphate group at C-6 of hemoiedemosides B reduce the activity.

It was reported that holotoxin A_1 clearly inhibits the RNA synthesis in *C. albicans* and *Saccharomyces carlsbergensis.* Furthermore, Stichoposide A from *Stichopus japonicus* inhibited biosynthesis of squalene, lanosterol and ergosterol in *S. carlsbergensis* (Anisimov *et al.* 1978). Also a crude of holothurin arrested mitosis and inhibited DNA synthesis, which is resulted from the loss of precursors throughout plasma membranes.

In conclusion the antifungal activity of saponins depends on the structure of triterpenoid aglycone, and the present and the number of sulphate groups in the oligosaccharide moiety. Having a sulphate group in the carbohydrate moity plays an important role in this activity, and the position of this groups in the molecule make different impacts (negative or positive) on antifungal activity (Kalinin, VI *et al.* 2008).

1.10.3 Antiprotozoal activity

The antiprotozoal activity of several glycosides from sea cucumbers such as holothurin and holotoxins have been studied by few scientists (Kitagawa *et al.* 1976; Nigrelli & Jakowska 1960). It has been documented that holotoxins from *A. japonicas* exhibit activity against *Trichomonas vaginalis,* and holothurin show activity towards *Amoeba proteus.* Besides, it is reported that sea cucumbers possess antileishmani activity. Singh *et al.* (2008) investigated antileishmania activity of methanol extract and n-butanol fraction of sea cucumber *Actinopyga lecanora* against *Leishmania donovani in vitro* and *in vivo*. They noted both fractions have potent *Leishmania* inhibitory activity. According to their finding, between the two glycosides isolated from n-butanol fraction, holothurin B

exhibited remarkable *in vitro* and moderate *in vivo* leishmanicidal activity, while holothurin A revealed only moderate action (Singh *et al.* 2008).

1.10.4 Anti-viral activity

A number of investigations revealed the antiviral activities of saponins. Several derived sea cucumbers and plant saponins have been reported to exhibit inhibitory activity towards a variety of DNA and RNA pathogenic viruses including human immunodeficiency virus (HIV) and herpes simplex virus type- I (HSV-1). For instance, Lee *et al.* (2012) reported that plant saponins inhibit replication of hepatitis C virus (HCV) with suppressing of cytokine signalling 2 (SOCS2) protein level which lead to inhibition of HCV replication. Therefore saponins inhibit HCV replication by SOCS2 signalling pathway.

Maier *et al.* (2001) reported the cytotoxicity of sea cucumber saponins toward HSV-1 (Table 1.6). The inhibitory mechanism of saponins in HSV was explained to be either throughout a direct virocidal effect or interference with early stage of the viral replicative cycle (Simões *et al.* 1999).

Table 1.6. Antiviral activity of saponins form sea cucumbers

Species	Saponin	Virus	Mode of action	References
Holothuria forskali	Holothurinosides A, C and D	HSV-1	Inhibit cytopathic effect	(Rodriguez *et al.* 1991)
Staurocucumis liouvillei	Liouvillosides A and B	HSV-1	Virucidal effect or by interference with an early step of the viral replicative cycle	(Maier *et al.* 2001)
Telenata ananas	Bivittoside D	HIV-1	Inhibitory activity in chemokine receptor subtype 5 (CRC 5)	(Hegde *et al.* 2002)

Further, Kalinin, VI *et al.* (2008) stated that a mechanism of antiviral activity of sea cucumber triterpene glycosides could be associated with antiviral protection at the stage of virus-cell interaction.

All of these studies indicate the ability of triterpene glycoside to function as antiviral agents against several viruses, indicating the wide anti-viral properties of saponins.

1.10.5 Relationship between chemical structure and functions

Many biological activities of holothurian saponins occur through their membranolytic function. Despite several studies that have investigated the membranotropic properties of sea cucumber glycosides during the past three decades further investigation needs to be done. It has been reported that the interaction of glycosides with the Δ^5-sterols of membranes, preferably with cholesterol (ergosterol in fungi), are the major factor, but not the only factor for the determination of the vast biological activities of sea cucumber glycosides (Anisimov 1987; Augustin *et al.* 2011; Kalinin, VI *et al.* 2008). It is recognised that the holothuroid triterpene glycosides possess strong membanolytic function towards biological and model membranes owing Δ^5 – sterols due to formation of single- ion channels and larger pores (channels) which is the basis of haemolytic, antifungal and cytotoxic features of these substances, which addresses the wide spectrum of their biological activities. It has been documented that the existence of an 18(20)-lactone, and 9(11) double bond in lanostane of the aglycone moiety, and having a linear tetrasaccharide residue in the sugar chain are very crucial for the membranotropicity of these compounds (Kalinin, VI *et al.* 2008; Kalinin, VI *et al.* 1996; Miyamoto, T. *et al.* 1990). However, the presence of a 16-ketone group in glycosides with a 7(8)-double bond in their aglycone structure diminished their activity in comparison with those lack of this group (Kalinin, VI *et al.* 1996). Furthermore, it has been stated that the presence of a sulphate group at C-4 of the first xylose residue also elevates the membranolytic activity, whereas the presence of a sulphate group at the C-6 position of the terminus glucose and 3-*O*-methylglucose units sharply reduce activity. The absence or very low concentration of Δ^5 – sterols in sea cucumber membranes suggested the role of this molecule in membranolytic activity (Kalinin, VI *et al.* 2008; Miyamoto, T. *et al.* 1990). The membranolytic activity of saponins probably indicates role of these substances in the defence mechanism against predators and correlates with their cytotoxicity towards tumour cells (Drozdova *et al.* 1997; Kalinin, VI *et al.* 1996). Sea cucumber glycosides showed membranotropic activity on permeability at lower temperatures compared to plants glycosides (Shcheglov *et al.* 1979a; Shcheglov *et al.* 1979b). However, cell membranes of sea cucumber are resistance to both their own glycosides and the terrestrial higher plant ones. This resistance is associated with low amount of free sterols in their

membranes and the existence of Δ^7 –sterols and sulphated and glycosylated sterols instead of Δ^5 –sterols (Kalinovskaya *et al.* 1983; Stonik, VA & Elyakov 1988).

Having a quinovose at the second position of monosaccharide unit (not Glc or Xyl) helps glycoside penetration into the cellular membrane of the target. Thus two structural elements, lanostane nucleus and quinovose residue, are determined the penetration of saponins into the membranes target. The stereochemistry of lanostane nucleus plays a major role in the creation of complex between glycoside and membrane sterols, besides, the presence of an 18(20) -lactone is also critical (Kalinin, VI *et al.* 1992b; Kitagawa 1988). The sugar moiety is also crucial for creation and maintenance of the channels and pores, determines their sizes and forms. Furthermore, the linear tetrasaccharide fragments have great effect.

Triterpene glycosides influence the physico-chemical properties of membranes such as stability, permeability and microviscosity of lipid bilayers and lipid- protein interaction and conformation of membrane proteins (Gorshkova *et al.* 1989; Popov, AM *et al.* 1982), for instance, reduction or inhibition the activity of some membrane enzymes, in particular ATPases (Stonik, VA *et al.* 1999). Such sterol/saponin interactions result in an efflux of some ions, substances of the nucleotide pool and peptides, disruption of ion homeostasis and osmolarity followed by lysis and cell death (Aminin, D. L. *et al.* 2010a; Anisimov 1987; Kalinin, VI *et al.* 2008; Popov, AM 2003). Triterpene glycosides influence with most of membrane normal functions such as ions transports, membrane permeability.

In short, the formation of complex between glycoside and membranes sterols followed by the creation of single ion channels and more large pores are fundamental for haemolytic, antitumor and cytotoxic properties of sea cucumber glycosides.

1.10.6 Haemolytic activity

It has been pointed out that Holothurin from sea cucumbers are lytic to mammalian and fish erythrocytes in high concentrations. Studies on the haemolytic activities of saponins from different sea cucumbers were revealed that the haemolytic activity of the holostane-type glycosides (having

a lactone) are much higher than, almost 100-fold stronger, non-holostane type (Kalinin, VI *et al.* 1996).

The haemolytic property of several saponins including cucumarioside G_1, cucumarioside G_2, cucumarioside C_2 and cucumarioside H obtained from *Eupentacta fraudatrix*, and frondoside A obtained from *Cucumaria frondosa* , and cucumarioside A_4-2 obtained from *Cucumaria japonica* all belonging to the order of Dendrochirotida was assessed by Kalinin and co-workers (Kalinin, VI *et al.* 1992b). They concluded that the haemolytic activity of these compounds relied on both aglycone and sugar moiety structures, and their results uncovered that the existence of a linear tetrasaccharide fragment and an 18(16)-lactone in the aglycone moiety of molecules are essential for haemolytic function. It has been reported that glycosides extracted from sea cucumbers inhibit both Na^+ and K^+ ATPase of rat brine (Gorshkov *et al.* 1982).

1.10.7 Cytotoxicity of saponins

The ability of saponins to bond with sterols and cause membrane permeabilisation is well known. However, these secondary metabolites can also influence the normal functions of cells that are mediated through specific interactions with metabolic processes, cellular receptors and structural proteins (Osbourn *et al.* 2011; Simons *et al.* 2006; Siu *et al.* 2008). For instance, the triterpene glycoside avicin D and ginsenoside Rh2 have been reported to activate apoptosis by means of triggering Fas-mediated cell death through influence with membrane lipids (Osbourn *et al.* 2011).

The cytotoxicity of cucumarioside A_2-2, monosulphated pentaoside, and cucumarioside A_7-1, trisulphated pentaoside, along with their analogues aglycones, from the edible sea cucumber *Cucumaria japonica* were studied (Agafonova *et al.* 2003). It has been pointed out that cucumariosides indicate high cytotoxic properties in a sea urchin embryo test. They proposed that cucumariosides, particularly A_2-2, might act as a Ca^{2+} agonists because of their membranolytic activities.

It has been reported that the presence of the 25-hydroxy group in aglycone moiety significantly decrease the cytotoxicity of saponins (Silchenko *et al.* 2011b). The cytotoxicity effects, the name of

cell lined and features of examined sea cucumber glycosides is summarised in Table 1.7.

Table 1.7. Sea cucumber triterpene glycosides as cytotoxic agents

Species	Cell lines	Compounds	References
Actinopyga lecanora	LH-60 and BEL-7402	Lecanorosides A/B, and Holothurins A/A_1	(Zhang, S-L *et al.* 2008)
Actinopyga lecanora	HL-60 and BEL-7402	Lecanorosides A/B, Holothurins A/A_1/B	(Zhang, S-L *et al.* 2008)
Bohadschia argus	A-549, HCT- 116, HepG2 and MCF-7	Argusides B/C/D/E	(Liu, BS *et al.* 2008a; Liu, BS *et al.* 2008b)
Cucumaria japonica	BALB/C	Cucumariosides A_2-2/A_7-1,	(Agafonova *et al.* 2003)
H. hilla	A-549, MCF-7, IA9, CAKI-1, PC-3, KB, KB-VIN and HCT-8	Hillasides A/B	(Wu, J *et al.* 2007)
H. scabra	KB and Hep-G2	Holothurins A_3/A_4	(Dang *et al.* 2007)
Holothuria leucospilota	HL-60, MOLT-4, A-549 and BEL-7402	Leucospilotaside B	(Han *et al.* 2010c)
Pentacta quadrangularis	A-549, MCF-7, IA9, CAKI-1, PC-3,KB, KB-VIN and HCT-8	Hillaside C	(Wu, J *et al.* 2006c)
Pentacta quadrangularis	CAK1, HOS, KB-VIN, KB SK-MEL-2,U87-MG,HCT-8, IA9, A549 and PC3	Philinopsides A/B	(Yi, YH *et al.* 2006)
Staurocucumis liouvillei	Vero cells	Liouvillosides A/B	(Maier *et al.* 2001)

1.10.8 Anti-tumour activity

Despite remarkable advanced technology in medical research, cancer remains the second most common cause of death following heart disease, and based on the World Health Organization it will kill more than 10 million people in 2020 (Karagozlu & Kim 2015). Therefore it is vital to discover new powerful and effective anticancer reagents with high biodegradable and biocompatible properties. In this section we want to highlight the possible mechanism and mode of action of studied saponins.

Several studies not only have been shown that the glycosides are major compounds in traditional oriental medicines but also highlighted the cytotoxic activities of saponins against various type of human cancer cell lines (Li, X *et al.* 2008; Liu, BS *et al.* 2008a; Liu, BS *et al.* 2008b; Liu, BS *et al.* 2007; Miyamoto, T. *et al.* 1990; Roginsky *et al.* 2010; Sun, GQ *et al.* 2008; Sun, P *et al.* 2007;

Zhang, S-Y *et al.* 2006a) by inducing apoptosis in these cells via mitochondrial integrity pathway (the intrinsic) (Yuen-Nei Cheung *et al.* 2005).

The anti-cancer activities of holothurinosides A, B, C and D and desholothurin A from *Holothuria forskalii* were assessed against P388, A 549, HeLa and B-16 cell lines *in vitro* (Rodriguez *et al.* 1991). This group reported that holothurinosides A and C, having a linear carbohydrate chains, were the strongest compounds among all examined substances against these given four lines.

Apart from above studies, the cytotoxicity of triterpene glycosides fuscocinerosides A, B, and C, along with pervicoside C and holothurin A, isolated from *H. Fuscocinerea* was examined against two human tumour cell lines namely HL-60 and BEL-7402 using HCP as positive reference (Zhang, S-Y *et al.* 2006a). They reported that all compounds possess cytotoxic activities against both cell lines, while fuscocineroside C was most effective against HL-60 with average IC_{50} value of 0.88 ± 0.32 μmol/L. However, fuscocineroside C, pervicoside C and holothurin A were remarkable cytotoxicity toward the BEL-7402 cell line. Even though the structures of fuscocineroside C and holothurin A are almost the same, only difference in C-17 substitutes (OH in holothurin A), this little change led to the large difference in cytotoxicity against HL-60 cells.

Liu, BS *et al.* (2008a) reported that argusides D and E exhibit lower cytotoxicity activity toward the four different cell lines compared to argusides B and C. This finding clearly indicated that the length and type of carbohydrate moieties of such glycosides play a core role in terms of cytotoxicity against tumour cell lines (Liu, BS *et al.* 2008a; Liu, BS *et al.* 2008b).

Roginsky *et al*. (2004) isolated a polar extract called Frondanol-A5P; a mixture of (%18) saponins, (%61) protein and (%21) sulphated polysaccharides, form *C. frondosa* and examined its effects on growth inhibitory and apoptosis in two human pancreatic cancer cell lines; S2013 and AsPC-1 (Roginsky *et al.* 2010; Roginsky *et al.* 2004). They pointed out this fraction inhibit proliferation and induce G2/M phase cell cycle arrest in both cell lines by the reduction in expression of cyclin A, cyclin B, and cdc25c; a phosphatise which is a vital mediator in the progression to mitosis through the G_2 phase of the cell cycle. They indicated the cell cycle and apoptosis in human pancreatic

cancer cells were affected by Frondanol-A5P via reduce expression of cyclin A, cyclin B, and cdc25c and enhancement of the expression of p21 (Roginsky *et al.* 2010). Moreover, Frondanol-A5P showed inhibition of proliferation of both human pancreatic cell lines, however, it was more effective in the AsPC-1 in comparison with other one. They stated that this fraction also triggered apoptosis in both pancreatic cancer cell lines by a remarkable increase in annexin V binding and increase activity of caspase 3. However, the anti-tumour properties of some saponins are resulted from their immunomodulatory function (Xu, R *et al.* 2012).

The cytotoxic activity of phylinopsides A and B isolated from *Pentacta quadrangularis* were examined against ten human cell lines (Table 1.8) *in vitro* (Kalinin, VI *et al.* 2008; Yi, YH *et al.* 2006). They showed that these compounds exhibit a marked activity with ED_{50} values less than 3.5 μg/ml. Phylinopside B, contains additional sulphate group at Xyl and the terminal double bond in the aglycone, showed more activity. In addition, phylinopside A remarkably inhibited the proliferation, migration and tube formation of human microvascular endothelial cells (HMECs) with average IC_{50} values of 1.4 ± 0.17, 0.89 ± 0.23 and 0.98 ± 0.19 respectively.

Interestingly, cucumarioside H_2, having 25-hydroxy group in the aglycone side chain, was not active against lymphocytes and tumour cells and had very low haemolytic activity (Silchenko *et al.* 2011b). Therefore, the presence of 25-hydroxy group in aglycone moiety significantly decreased the activities. Cucumarioside H_6 having an 24(25) double bond in the aglycone side chain was less active in comparison with others, where cucumarioside H showed the more cytotoxic activity with ED_{50} = 4.3 (Silchenko *et al.* 2011a).

Table 1.8. summarises the studies have been done on anti-cancer properties of saponin congeners in sea cucumber species.

Table 1.8. Anticancer property of some saponins from sea cucumbers species.

Species	Compounds	Cell lines	References
Apostichopus japonicus	Water-soluble holothurian glycosides	HeLa cells, A-549, SGC-7901, Bel-7402 and S180	(Fan, TJ *et al.* 2009)
Bohadschia argus	Arguside A	HL-60, HCT-116, MKN-45 and BEL-7402	(Liu, BS *et al.* 2007)
Bohadschia argus	Argusides B/C/D/E	A-549, HCT- 116, HepG2 and MCF-7	(Liu, BS *et al.* 2008a; Liu, BS *et al.* 2008b)
C. echinata	Cucumechinosides A, B, C, D, E and F	L 1210 and KB cells	(Miyamoto, T. *et al.* 1990)
C. frondosa	Frondoside A	P-388, Schabel, A-549, HT-29 and Mel-28	(Avilov, S. A. *et al.* 1998)
C. japonica	Cucumariosides A_3 and A_6-2	P-388, Schabel, A-549, HT-29 and Mel-28,	(Drozdova *et al.* 1997)
Cucumaria frondosa	Frondanol-A5	S2013 and AsPC-1	(Roginsky *et al.* 2010; Roginsky *et al.* 2004)
Cucumaria okhotensis	Okhotosides B_1, B_2 and B_3, Frondoside A, Cucumarioside A_2-5 and Koreoside A	HeLa cells, THP-1	(Silchenko *et al.* 2008)
Eupentacta fraudatrix	Cucumariosides H, H_2, H_3 H_4, H_5, H_6 and H_7	Spleen lymphocytes	(Silchenko *et al.* 2011a, 2012d)
H. Fuscocinerea	Fuscocinerosides A/B/C, Pervicoside C and Holothurin A	HL-60 and BEL-7402	(Zhang, S-Y *et al.* 2006a)
Holothuria forskalii	Holothurinosides A, B, C and D and Desholothurin A	P388, A549, HeLa and B-16	(Rodriguez *et al.* 1991)
Holothuria fuscocinerea	Fuscocinerosides A, B, C and Pervicoside C, and Holothurin A	HL-60 and BEL-7402	(Zhang, S-Y *et al.* 2006a)
Holothuria fuscocinerea	Pervicoside C	HCT-116 and A549	(Sun, P *et al.* 2007)
Holothuria grisea	17-Dehydroxyholothurinoside A and Griseaside A	A549, HL-60, BEL-7402, and Molt-4	(Sun, GQ *et al.* 2008)
Holothuria hilla	Hillaside C	A-549, MCF-7, IA9, CAKI-1, PC-3, KB, KB-VIN, and HCT-8	(Wu, J *et al.* 2006c)
Holothuria impatiens	Impatienside A and Bivittoside D	HCT-116, HT-29, A549, HepG2, DU145, MCF-7 and KB	(Sun, P *et al.* 2007)
Holothuria nobilis	Nobilisides A, B and C	A-549, MCF-7, IA9, CAKI-1, PC-3, KB, KB-VIN, and HCT-8	(Wu, J *et al.* 2006b)
Mensamria intercedens	Intercedensides A, B, C D, E, F, G, H and I	A549, MCF-7, IA9, CAKI-1, U-87-MG, PC-3, KB, KB-VIN, SK-MEL-2 and HCT-8	(Zou *et al.* 2003; Zou *et al.* 2005)
Pentacta australis	DS-penaustrosides A and B,	L 1210 and KB cells	(Miyamoto *et al.* 1992)

Species	Compounds	Cell lines	References
	DS-penaustrosides C and D		
Pentacta quadrangularis	Philinopside E	P388, HL-60, A549, SPC A4, MKN28, SGC7901, BEL-7402, HO8901, W138 and A431	(Zhang, S-L *et al.* 2006)
Pentacta quadrangularis	Philinopsides A and B	CAKI, HOS, KB-VIN, KB, SM-MEL-2, U87-MG, HCT-8, IA9, A549, and PC3	(Yi, YH *et al.* 2006)
Pentacta quadrangularis	Phylinopsides A and B	A549, HOS, IA9, CAKI-1, U-87-MG, PC-3, KB, KB-VIN, SK-MEL-2 and HCT-8	(Kalinin, VI *et al.* 2008; Yi, YH *et al.* 2006)
Pentamera calcigera	Calcigerosides B, C_1 and C_2	P-388, Schabel, A-549, HT-29 and Mel-28	(Avilov, S. A. *et al.* 2000b)
Pseudocolochirus violaceus	Intercedenside B	MKN-45 and HCT-116	(Zhang, S-Y *et al.* 2007)
Synapta maculata	Synaptosides A and A_1	Hela cells	(Avilov, S. A. *et al.* 2008)

Collectively, all these sea cucumber saponins are very potent cytotoxic agents against a broad range of cancer types. However, further studies on the anticancer properties of these metabolites are desired to be done to confirm their activities and use them in the cancer therapy.

1.10.9 Anti-angiogenic activity

Angiogenesis is a hub process in menstruation, wound healing and restoration of blood flow to tissues after injuries, however, uncontrolled angiogenesis may result to incurable disease. Angiogenesis is fundamental part in tumour growth, progression, invasion and metastasis, understanding the mechanism and inhibiting of this process provides a potential strategy for cancer treatment (Folkman 1995; Matter 2001; Tian *et al.* 2005). Thus, inhibition of angiogenesis is another key target in the fight against cancer. Researchers highlighted that sea cucumber extracts not only could prevent the formation of new blood vessels, but also suppressed the RTK biding of growth factors (Chi 2005; Tian *et al.* 2005).

For instance, Tian *et al.* (2005) reported a sulphated saponin termed philinopside E (PE) from sea cucumber *pentacta quadrangularis*, and investigated its anti-angiogenesis and anti-tumour

activities. A few examples of anti-angiogenesis saponins from sea cucumber species are listed in Table 1.9. PE was reported to exert the anti-tumour activity by reducing the proliferation of tumour cells and increasing apoptosis of both endothelial cells and tumour cells (Tian *et al.* 2005). They also stated that PE, which is member of drugs called "smart bombs" shows the antiangiogenic activity via inhibition on KDR phosphorylation and downstream signalling (Tian *et al.* 2007).

In addition, the effects of frondoside A, isolated from *Cucumaria frondosa* (Girard *et al.* 1990) and cucumariosides A_2-2 and A_4-2, from *Cucumaria japonica* (Aminin, D. L. *et al.* 2001) were evaluated for cell death-inducing capability in leukaemia cells including HL-60, NB4, THP-1 and K562, (Jin *et al.* 2009), with more focus on structure–activity relationships. The main structural difference between frondoside A and cucumarioside A_2-2 is in the functional group at C-16 of the aglycone (acetoxy or keto group) and the third carbohydrate unit in the carbohydrate moiety while they all are monosulphated pentaoside. They presented that both frondoside A and cucumarioside A_2-2 strongly induce apoptosis of leukemic cells however frondoside A-induced apoptosis was more potent and rapid than cucumarioside A_2-2-induced apoptosis. They stated that cucumarioside A_2-2 induced apoptosis was caspase-dependent in contrast with frondoside A-induced apoptosis (Jin *et al.* 2009). In according to their findings, frondoside A provokes apoptosis in HL-60 cells and THP-1 cells in a dose-dependent manner. They proposed that holothurians may induce apoptosis of leukemic cells caspase dependently or -independently, depending on the holothurian structure (Jin *et al.* 2009). In addition, It has been also reported that some saponins induced apoptosis by upregulating the Apaf-1 (apoptotic protease- activating factor 1), caspase-3, and acetylcholinesterase (Cho 2011).

Table 1.9. Saponins examined for anti-angiogenesis

Spices	Compounds	Cell lines	Mode of actions	References
C. frondosa	Frondoside A	F344 rats and HCT116	Growth inhibitory via decreasing Cdc25c, anti-proliferative and induce apoptosis related to H2AX phosphorylation and activate caspase-2	(Janakiram *et al.* 2010)
C. frondosa	Frondoside A	Balb/cByJ mouse and 66.1 mammary tumour cell	Antimetastatic property, antagonises the prostaglandin E receptors EP2 and EP4	(Ma, X *et al.* 2011)
C. japonica and Thelenota anax	Cucumariosides A_2-2 and A_4-2, Stichoposides C, and D	HL-60, THP-1 and NB-4 K562	Stimulate apoptosis	(Fedorov *et al.* 2007)
Cucumaria frondosa *Cucumaria japonica*	Frondoside A and Cucumariosides A_2-2 and A_4-2	HL-60, NB4, THP-1 and K562	Induce apoptosis	(Jin *et al.* 2009)
Cucumaria frondosa	frondoside A	AsPC-1	Inhibit proliferation of AsPC-1, induce apoptosis (caspases 3/7 and 9)	(Li, X et al. 2008)
Cucumaria frondosa	Frondoside A	MDA-MB- 231	Inhibit cell migration and invasion, Apoptotic through the activation of p53 (via the intrinsic pathway)	(Al Marzouqi et al. 2011)
H. leucospilota, H. scabra, S. chloronotus	Aqueous and organic extracts	A549 and C33A	Antiproliferative and antioxidant	(Althunibat *et al.* 2009)
Holothuria nobilis	Echinoside A	SGC-7901, BEL-7402 and SMMC-7721, Hela and HO-8910, A549, HL-60, K562, Hep-G2, KB, MDA-MB-468, MDBMB-231, SK-BR-3, HT-29, MKN-28, MKN-45, HCT-116 and LoVo	Target Top2α by interfering with the binding of Top2 to DNA and by damaging the Top2-regulated DNA	(Li, M *et al.* 2010)
P. graeffei	Echinoside A and Ds-echinoside A	HepG2	Antimetastatic property through the specific inhibition of NF-κB-dependent MMP-9 and VEGF expressions, induce apoptosis via caspase-3 activation	(Zhao *et al.* 2012)

Pearsonothuria graeffei	Holothurin A_1 and 24-dehydroechinoside A	HepG2	Suppress the expression of the matrix metallo-proteinase-9 (MMP-9) and upregulate the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), antimetastatic activities by inhibiting MMP-9 and VEGF expression and enhancing TIMP-1 expression,	(Zhao *et al.* 2010)
Pentacta quadrangulari	Philinopside A	Mouse sarcoma 180	Inhibit the proliferation, migration, and tube formation of HMECs, induce apoptosis	(Tong et al. 2005)
Pentacta quadrangularis	Philinopside E (PE)	Mouse sarcoma 180	Inhibit proliferation of HMECs and HUVECs, induce endothelial cell apoptosis, suppress the phosphorylation of protein kinases including VEGFR2, Akt, ERK, FAK and paxillin	(Tian et al. 2007)
Psolus patagonicus	Patagonicoside A DS-Patagonicoside A	Hep3B, MDA-MB231 and A549	Antiproliferative	(Careaga *et al.* 2009)

1.10.10 Immunomodulatory activity

The immune system is core part for health. Medicines stimulating the immune system can be used for treatment of the incurable diseases like cancer, AIDS, and other infectious, and also decrease anti-aging effects. Recently the immunomodulatory activity of saponins has attracted much attention (Hostettmann & Marston 1995). Moreover, saponins are used as adjuvant with antigens which improve the integration of these macromolecules through the mucosal membrane and promote absorption, which boost the effectiveness of injected and oral vaccines (Xu, R *et al.* 2012). For instance, it has been reported cucumariosides isolated from *C. japonica* exhibit adjuvant properties increasing the number of produced antibodies in corpuscle whooping-cough vaccine and boosting the protective property of vaccine (Sedov *et al.* 1984), or remarkably increased the lysosomal activity of macrophage in mouse even with very low concentration (around 0.02 μg/ mouse)(Aminin, D. L. *et al.* 2001). The most effective immunostimulants are monosulphated

glycosides but di- and trisulphated saponins are immunosuppressors (Kalinin, VI *et al.* 2008). In contrast, it has been reported some of those saponins show immune suppressive functions which might have resulted from their anti-inflammatory properties.

Besides, a complex of monosulphated triterpene glycosides (obtained from *C. japonica* mainly cucumarioside A_2-2) and cholesterol in an approximate molar ratio of 1:2 termed Cumaside was reported to have immunomodulatory properties (Aminin, D. L. *et al.* 2006). Furthermore, frondoside A, isolated from *C. frondosa* also exhibited immunomodulatory activities in sub-toxin dose in mouse (Aminin, D. L. *et al.* 2008). It was ascribed this secondary metabolite enthused lysosomal activity of macrophages *in vivo* at the dose of 0.2 µg/ mouse.

Unicorn pacific, a company in Vanuatu, produces TBL-12, an immune-therapy treatment that is obtained from sea cucumbers and other marine organisms (Kinch *et al.* 2008). Additionally, it was reported that dietary saponins from sea cucumber *Pearsonothuria graeffei* alleviate orotic acid-induced fatty liver in rats via PPARα and SREBP-1c signalling (Hu, XQ *et al.* 2010). Three ganglioside molecular species, HLG-1, HLG-2, and HLG-3 isolated from the lipid fraction of the chloroform/methanol extract of the sea cucumber *Holothuria leucospilota* showed neuritogenic activity against the rat pheochromocytoma cell line, PC-12 cell (Yamada *et al.* 2001). Some saponins are able to stimulate both the Th1 immune response and the production of cytotoxic T lymphocytes against exogenous antigens (QA-21 as an example). Some nanoparticle containing plant saponins stimulate IgG response in mice.

1.10.11 Anti-diabetic activity

In addition, some saponins inhibit the absorption of glucose (hypoglycaemia) and ethanol. In Malaysia, *S. hermanii* and *S. horrens* are favourable sea cucumbers species being consumed in traditional medicines to decrease blood glucose level in patients suffering from diabetes mellitus in which patients use the extract or fluid from sea cucumbers in order to control their glucose level (Ridzwan 2007).

1.10.12 Anti-arthritis, anti-inflammatory, anti-edema activity

There is no doubt a large population of the world suffer from arthritis and joint problems. A wide range of arthritis (more than 110 types of arthritis) from wear of the cartilage to the auto-immune disease known as rheumatoid arthritis has been recognised so far. Sea cucumbers have a traditional reputation for decreasing arthritis pain and artharalgia in East Asia (Toral-Granda 2006). Besides, sea cucumbers are considered as a good source of arthritic pain reliever, and have been utilized in China for treatment of tendonitis and arthritis over thousands of years (Balch 2006; Zhong *et al.* 2007). Thus, studies confirm that sea cucumber extract have potent therapeutic substances for the treatment of inflammation. In addition, modern studies strongly believe that sea cucumbers possess components that can control the balance of prostaglandins involved in the regulation of the inflammatory process.

1.10.13 Cardiovascular property and hypolipidemic effect

Cardiovascular diseases are one of the most common diseases with high prevalence in human with high risk of mortality. Despite declines in mortality rates, cardiovascular disease remains one of the leading causes of death in Australia. Many studies revealed the important role of saponins in preventing cardiovascular diseases including lowering cholesterol, anti-hypobaric hypoxia, anti-arryhthmia, positive inotropic effect, and capillary protection activity.

For example, some saponins, by binding to sterols make them into insoluble forms, were reported to lower body cholesterol levels. Studies highlighted that a diet containing black sea cucumber remarkably lowers serum and hepatic cholesterol content in Sprague-Dawley rats fed with 0.2% cholesterol diet (Tanaka *et al.* 2003). Saponins also combine with bile salts in the gut and form micelle, hence reducing the absorption of these components, and presumably also of cholesterol.

1.10.14 Functional food and nutraceuticals

It was announced that the majority of world's population (65 – 80 %) is applying traditional medicines as the principal form of healthcare (Akerele 1992). Without doubt, marine derivative compounds will contribute high proportion to the nutraceutical, cosmeceutical and pharmaceutical products in near future. In terms of nutraceutical values, sea cucumbers are considered to be of

high nutritional value reflected their amino acid and fatty acid profiles since they are rich in proteins, trace elements, and are low in fat content. Traditionally, sea cucumbers were consumed for their tonic value rather than their seafood tastes.

Sea cucumbers have been utilised as a folk remedy and a medicine for prevention and treatment of disease since the Ming Dynasty (1368 – 1644 BC) in China (Chen, J 2004). The clinical function of sea cucumbers was reviewed by Chen, J (2004). According to the principles and theory of traditional Chinese medicine, it is believed that the sea cucumber feeds the blood, vital essence (*jing*), kidney (*qi*) (treats syndromes of the kidney system, comprising reproductive organs) and decreases dryness (especially of the intestines) (Chen, J 2004). Commonly use to cure impotence, weakness, debility of the aged, constipation due to intestinal dryness, and frequent urination in addition to treat anaemia, prevent cancer, increase immune system function, decrease arthritic pain. Therefore, in China, sea cucumber is considered as a tonic rather than a seafood item. Hence, in Chinese the sea cucumber is called “haishen”, which means, “ginseng of the sea” (Chen, J 2004).

Several surveys have been conducted to assess amino acid and fatty acid compositions in different species of sea cucumbers to estimate their nutritional value to consumers (Drazen *et al.* 2008; Wen *et al.* 2010; Zhong *et al.* 2007). These studies concluded that sea cucumbers contain high protein and necessary trace elements with low fat levels. For example, Wen *et al.* (2010) evaluated the chemical and nutritional composition of eight common commercially processed sea cucumber species namely *S. herrmanni, T. ananas, T. anax, H. fuscogilva, H. fuscopunctata, A. mauritiana, A. caerulea* and *B. argus,* and found the protein contents to be high within the range of 40.7 to 63.3%. Additionally, the given sea cucumbers contain very low levels of fat (0.3–1.9%) except *T. anax* and *A. caerulea* while the ash content is notably high (15.4–39.6%). However, the fully dried sea cucumber material may contain protein content as high as 83% and is marketed as nutraceutical in tabulated or capsulated forms (Chen, J 2003). Interestingly sea cucumbers have an ideal composition of valuable aa. For instance the ratio of lysine to arginine in Sea cucumbers is remarkably lower than other fishery products (Wen *et al.* 2010), by knowing that the concentration

of cholesterol in serum and aorta is changed by the composition of the proteins and amino acids, which is led to exert hypocholesterolemic effects (Sugano *et al.* 1984), therefore ,sea cucumber has been consumed as an ideal tonic food for those who deal with hyperlipidemia (Wen *et al.* 2010). Furthermore, presenting free fatty acids such as C20:5, eicosapentaenoic acid (EPA) and C22:6, DHA from *S. chloronotus* and *Cucumaria frondosa* (Fredalina *et al.* 1999; Zhong *et al.* 2007) highlighted the value of sea cucumber in cardiovascular diseases. Both of these omega acids showed hemodynamic and antiatherogenic properties, which may abolish blood coagulation in the blood vessels and reduce serum triglycerides or serum cholesterol levels.

Besides Holothurins are rich source of trace elements and minerals *i.e.* Chromium (Cr), manganese (Mn), zinc (Zn), iron (Fe), nickel (Ni), cobalt (Co) and copper (Cu). It is notable that there is a different between the heavy metals from organic source with those from inorganic ones. Inorganic source of heavy metals are toxic Cr, Ni, Cadmium (Cd) and lead (Pb). Sea cucumbers are also rich in vitamins such as A, B1 (thiamine), B2 (riboflavin), B3 (niacin) and C, which are essential for healthiness (Balch 2006; Chen, J 2003). Sea cucumber also contains other components such as triterpene glycosides, gangliosides, branched-chain fatty acids, lectins and opsonins (Kalinin, VI *et al.* 2008; Kelly 2005).

Besides the features mentioned above, vanadium was found in a high concentration (12 ppm) in the intestine of *A. japonicus*, which has been applied to treat gastrointestinal ulcers (Yaohai 1993).

Sea cucumbers have been a staple in Japan, China and other parts of East Asia since ancient times, are going to consider as a popular dietary supplement in western countries (Kiew, P. L. & Don 2011; Kim, CG & Kwak 2015). Species such as *Stichopus horrens* is used as medicinal drugs. The viscera organs, including fermented intestine (konowata) and dried gonad (kuchiko) of commercial species are also marketed in Korea, Japan and China (Stutterd & Williams 2003).

In Malaysia, sea cucumber co-products have been utilised to produce a range of hygienic products such as roselle gamat, gamat oral jelly, gamat topical gel, body lotion, gamat cream, gamat hair shampoo, gamat whitening cream and toothpaste, as well as gamat "tongkat ali" (a plant

aphrodisiac) (Purcell *et al.* 2010; Toral-Granda *et al.* 2008).

SeaCare^{R} (a registered human food supplement), made of dried extracts of selected species of holothurians: 95% w/w sea cucumbers and sea plants (Sargassum pallidum) from northern Australia, showed anti-inflammatory activity against adjuvant-induced polyarthritis rats (Whitehouse & Fairlie 1994). Therefore, sea cucumbers are economically important as they have been utilised as traditional medicine and traditional healthy food, or eaten as delicacies in Asian cuisine for thousands of years (Dong *et al.* 2008; Thao *et al.* 2014).

1.10.15 Cosmeceutical activity

In general, all classes of organic compounds use in skin care; however, some of those such as saponins, polyphenols, flavonoids or other phytosterols, fatty acids, and waxes exhibit the highest variety of relevant biological properties in dermatology and skin care (Burlando *et al.* 2010).

The sapogenin part of saponins and saponins are generally used in cosmetics for instance in the Japanese Cosmetic Ingredient Codex. Owing to their anti-dermatophytic activity they are very promising as raw materials for ingredients of cosmetics and dermatologic products (Burlando *et al.* 2010; Kjellin & Johansson 2010). Their antifungal and antibacterial properties are also important in cosmetic applications, in addition to their emollient effects. Certain saponins possessing antimicrobial activity have been consumed in cosmetics.

1.10.16 Agricultural and insecticides

Saponins are interesting potential pesticides because of their unique strictures. Indeed these secondary metabolites are famous for their toxicity to harmful insects (anti-feeding, growth regulation, mortality and so on), resulting from interaction of saponins with cholesterol, leading to disturb the synthesis of ecdysteroids (Chaieb 2010). These bioactive metabolites also inhibit the activity of some proteases in certain insects.

The insecticides activity of saponins may occur within three different mechanisms including interfering with the feeding behaviour, effecting growth regulation and entomotoxicity. It has been noticed that saponins inhibit the food uptake by mite, caterpillar's species, larvae and many pest

insects. Several authors also reported that saponins (purified or crude extract) are able to control the regulation of cellular growth in many insect species (Adel *et al.* 2000). Moreover, binding saponins with cholesterol create insoluble complex, which is not up-taken by the digestive system of many animal species. However, due to there is a limitation in using saponins in phytoprotection now, for example instability of the structure of saponins in the environment because of degradation of sugar moiety, which can lead to loss of biological activity.

1.11 Taxonomic application using saponin profiles

Saponins might be a good tool for taxonomy determination of sea cucumbers. Some studies have shown the prospective relationship between the chemical structures of triterpenoid glycosides and geographical and ecological position of corresponding species (Elyakov *et al.* 1973; Stonik, VA *et al.* 1999). The relationship between the structure of saponins and the taxonomy of sea cucumbers were reviewed by several authors (Stonik, VA *et al.* 1999) and show meaningful correlations. Caulier *et al.* (2011) have also stated most species of sea cucumbers apparently have an individual congener mixture of saponins, which is a worthwhile chemotaxonomic feature allow the assignment of a holothuriid species to a certain taxa in accordance with its chemical fingerprint.

We have studied the saponin profiles of 16 different sea cucumber species, and found unique profiles for each species (data not shown). Therefore, it is possible to classify sea cucumber species based on their matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) saponin profiles since it has been applied for efficient and rapid classification and identification of human pathogenic bacteria (Freiwald & Sauer 2009; Hsieh *et al.* 2008; Murray, PR 2010; Sauer & Kliem 2010; Seng *et al.* 2009).

1.12 Pros and cons in drug development from sea cucumbers

The immense diversity of sea cucumbers paved the way to natural product scientists to mine for novel bioactive compounds. Among them, sea cucumber triterpene glycosides are the most studied ones. Furthermore, there are few sea cucumber product including ArthriSea, ArthriSea Plus, Sea-Q, SeaCuMax, MobiliTea, NutriSea and Gold-G Bio sea cucumber on the shelf for

human consumption (Al Marzouqi *et al.* 2011; Coastside Bio Resources 2012; Janakiram *et al.* 2010). Although there are many lead compounds that show promise to be developed as drugs for cancer therapy, the cytotoxicity itself would be a limitation for this purpose, because most of the compounds might be cytotoxic against normal cells in addition to the cancerous cells (Kim, SK & Himaya 2012). Therefore, extensive cytotoxic studies should be carried to obtain a lead compound before introducing them to the drug development phase.

1.13 Future perspectives

More than half of all therapeutic drugs are derived from terrestrial natural product scaffolds (Montaser & Luesch 2011). The unexplored marine environment which is harbours an enormous biodiversity could be the next source of medicinal compounds with a novel modes of action.

In technological terms innovations in nanoscale nuclear magnetic resonance (NMR) for structure elucidation, coupled with advances in MS and genetic engineering of biosynthetic pathways, are all vital to the achievement of marine natural products as medicinal leads.

1.14 Aims and objectives and research plan

We hypothesise that the reason for evisceration as a form of defence is because these internal organs (viscera) contain high levels of bioactive compounds that deter predators. It is therefore hypothesised that viscera are rich in bioactive compounds such as saponins. Furthermore, the results of this project may identify the potential economic benefits of transforming viscera of the sea cucumber into high value co-products important to human health and industry

The overall aims of this study were to discover the bioactive compounds (saponins) from the viscera of selected Australian sea cucumber species using high-throughput technologies such as HPCPC, mass spectrometry and metabolomics, and investigate their poetical activities. The major mission of this research was to isolate, purify, identify and characterize the structure of novel saponins from sea cucumbers and evaluate their antimicrobial activities, and develop the novel technology for seafood processing co-products.

CHAPTER 2

DISCOVERY OF NOVEL SAPONINS FROM THE VISCERA OF THE SEA CUCUMBER *HOLOTHURIA LESSONI*

This chapter is a paper published in the Journal "Marine Drugs". The paper is cited as "Bahrami, Y., Zhang W., Franco C.M.M. (2014). Discovery of Novel Saponins from the Viscera of the Sea Cucumber *Holothuria lessoni*. Marine Drugs, 2014, 12, 2633-2667; doi:10.3390/md12052633"

This chapter addresses the isolation, purification and structure elucidation of saponins in the viscera of the Australian sea cucumber *Holothuria lessoni* (previously known as *H. scabra* var. *versicolour,* Jaeger 1833), (Figure 5.1) in the class *Holothuroidea.* The paper covers an introduction, a methods section; chromatographic purification, and describes the MS analyses in detail.

I conducted the experiments with the guidance of my supervisors Prof. Chris Franco and Prof. Wei Zhang, who assisted in setting up the HCPCP. I purified and analysed the samples at the Flinders Advanced Analytical laboratory and elucidated the chemical structure of the compounds, and then I wrote the draft manuscript. CF confirmed the analyses and proofread the manuscript. I drafted the responses to the reviewers' comments which were revised by CF.

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Article

Discovery of Novel Saponins from the Viscera of the Sea Cucumber *Holothuria lessoni*

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Abstract: Sea cucumbers, sometimes referred to as marine ginseng, produce numerous compounds with diverse functions and are potential sources of active ingredients for agricultural, nutraceutical, pharmaceutical and cosmeceutical products. We examined the viscera of an Australian sea cucumber *Holothuria lessoni* Massin *et al.* 2009, for novel bioactive compounds, with an emphasis on the triterpene glycosides, saponins. The viscera were extracted with 70% ethanol, and this extract was purified by a liquid-liquid partition process and column chromatography, followed by isobutanol extraction. The isobutanol saponin-enriched mixture was further purified by high performance centrifugal partition chromatography (HPCPC) with high purity and recovery. The

resultant purified polar samples were analyzed using ***matrix-assisted laser desorption/ionization mass spectrometry*** (MALDI-MS)/MS and electrospray ionization mass spectrometry (ESI-MS)/MS to identify saponins and characterize their molecular structures. As a result, at least 39 new saponins were identified in the viscera of *H. lessoni* with a high structural diversity, and another 36 reported triterpene glycosides, containing different aglycones and sugar moieties. Viscera samples have provided a higher diversity and yield of compounds than observed from the body wall. The high structural diversity and novelty of saponins from *H. lessoni* with potential functional activities presents a great opportunity to exploit their applications for industrial, agricultural and pharmaceutical use.

Keywords: sea cucumber viscera; saponins; *Holothuria lessoni*; bioactive compounds; MALDI; mass spectrometry; ESI; HPCPC; triterpene glycosides; structure elucidation; marine invertebrate; *Echinodermata*; holothurian

2.1 Introduction

Holothurians are sedentary marine invertebrates, commonly known as sea cucumbers, trepang, bêche-de-mer, or gamat [1,2], belonging to the class Holothuroidea of the *Echinodermata* phylum. Sea cucumbers produce numerous compounds with diverse functions and are potential sources of agricultural or agrochemical, nutraceutical, pharmaceutical and cosmeceutical products [3–5]. It is for this reason they are called "marine ginseng" in Mandarin.

Even though sea cucumbers contain different types of natural compounds, saponins are their most important and abundant secondary metabolites [6–12]. Saponins are reported as the major bioactive compound in many effective traditional Chinese and Indian herbal medicines.

Sea cucumber saponins are known to have a wide range of medicinal properties due to their cardiovascular, immunomodulator, cytotoxic, anti-asthma, anti-eczema, anti-inflammatory, anti-arthritis, anti-oxidant, anti-diabetics, anti-bacterial, anti-viral, anti-cancer, anti-angiogenesis, anti-fungal, hemolytic, cytostatic, cholesterol-lowering, hypoglycemia and anti-dementia activities [4,7,13–24].

Saponins are amphipathic compounds that generally possess a triterpene or steroid backbone or aglycone. Triterpenoid saponins have aglycones that consist of 30 carbons, whereas steroidal saponins possess aglycones with 27 carbons, which are rare in nature [4].

Triterpene saponins belong to one of the most numerous and diverse groups of natural occurring products, which are produced in relatively high abundance. They are reported primarily as typical metabolites of terrestrial plants [25]. A few marine species belonging to the phylum *Echinodermata* [26] namely holothuroids (sea cucumbers) [7,10,13,27–33] and asteroids, and sponges from the phylum *Porifera* [13,34,35] produce saponins.

The majority of sea cucumber saponins, generally known as Holothurins, are usually triterpene glycosides, belonging to the holostane type group rather than nonholostane [36,37], which is

comprised of a lanostane-3β-ol type aglycone containing a γ-18 (20)-lactone in the D-ring of tetracyclic triterpene (3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone) [25] sometimes containing shortened side chains, and a carbohydrate moiety consisting of up to six monosaccharide units covalently connected to C-3 of the aglycone [7,8,13,37–42].

The sugar moiety of the sea cucumber saponins consists mainly of D-xylose, D-quinovose, 3-*O*-methyl-D-glucose, 3-*O*-methyl-D-xylose and D-glucose and sometimes 3-*O*-methyl-D-quinovose, 3-*O*-methyl-D-glucuronic acid and 6-*O*-acetyl-D-glucose [40,41,43–48]. In the oligosaccharide chain, the first monosaccharide unit is always a xylose, whereas either 3-*O*-methylglucose or 3-*O*-methylxylose is always the terminal sugar.

Although some identical saponins have been given different names by independent research groups [6] as they could be isomeric compounds, our comprehensive literature review showed that more than 250 triterpene glycosides have been reported from various species of sea cucumbers [7,13,18,25,29,41,44,49,50]. They are classified into four main structural categories based on their aglycone moieties; three holostane type glycoside group saponins containing a (1) 3β-hydroxyholost-9 (11)-ene aglycone skeleton; (2) saponins with a 3β-hydroxyholost-7-ene skeleton and (3) saponins with an aglycone moiety different to the other two holostane type aglycones (other holostane type aglycones); and (4) a nonholostane aglycone [25,38,46,51,52].

One of the most noteworthy characteristics of many of the saponins from marine organisms is the sulfation of aglycones or sugar moieties [4]. In sea cucumber saponins, sulfation of the oligosaccharide chain in the Xyl, Glc and MeGlc residues has been reported [38,40,46,53,54]. Most of them are mono-sulfated glycosides with few occurrences of di- and tri-sulfated glycosides. Saponin diversity can be further enhanced by the position of double bonds and lateral groups in the aglycone.

Triterpene glycosides have been considered a defense mechanism, as they are deleterious for most organisms [6–10,12,55–57]. In contrast, a recent study has shown that these repellent chemicals are also kairomones that attract the symbionts and are used as chemical "signals" [58]. However, in the sea cucumber, it has been suggested that saponins may also have two regulatory roles during reproduction: (1) to prevent oocyte maturation and (2) to act as a mediator of gametogenesis [18,59].

The wide range of biological properties and various physiological functions of sea cucumber extracts with high chemical structural diversity and the abundance of their metabolites have spurred researchers to study the ability of sea cucumbers to be used as an effective alternative source for potential future drugs. However, the large number of very similar saponin glycosides structures has led to difficulties in purification, and the complete structure elucidation of these molecules (especially isomers), has made it difficult to conduct tests to determine structure-activity relationships, which can lead to the development of new compounds with commercial applications [16]. Therefore, in order to overcome this problem, we employed High Performance Centrifugal Partition Chromatography (HPCPC) to successfully purify saponins in this study. HPCPC is more efficient in purifying large amounts of a given sample and also lower solvent consumption with high yields compared to other conventional chromatography methods.

This project aims to identify and characterize the novel bioactive compounds from the viscera (all internal organs other than the body wall) of an Australian sea cucumber *Holothuria lessoni* Massin *et al.* 2009 (golden sandfish) with an emphasis on saponins. *H. lessoni* was selected because it is a newly-identified Holothurian species, which is abundant in Australian waters. While only a few studies have compared the saponin contents of the body wall with that of the cuvierian tubules in other species [50,60–62], to our knowledge, no study has investigated the contribution of saponins of the body wall or the viscera of *Holothuria lessoni*. Sea cucumbers expel their internal organs as a defense mechanism called evisceration, a reaction that includes release of the respiratory tree, intestine, cuvierian tubules and gonads through the anal opening [50,58,61,63–68]. We hypothesize that the reason for this ingenious form of defense is because these organs contain high levels of compounds that repel predators [60,61,69,70]. Furthermore, the results of this project may identify the potential economic benefits of transforming viscera of the sea cucumber into high value co-products important to human health and industry.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF/MS) and electrospray ionization mass spectrometry (ESI-MS) techniques allow the "soft" ionization of large biomolecules, which has been a big challenge until recently [71]. Therefore, MALDI and ESI-MS, and MS/MS were performed to detect saponins and to elucidate their structures.

2.2 Results and Discussion

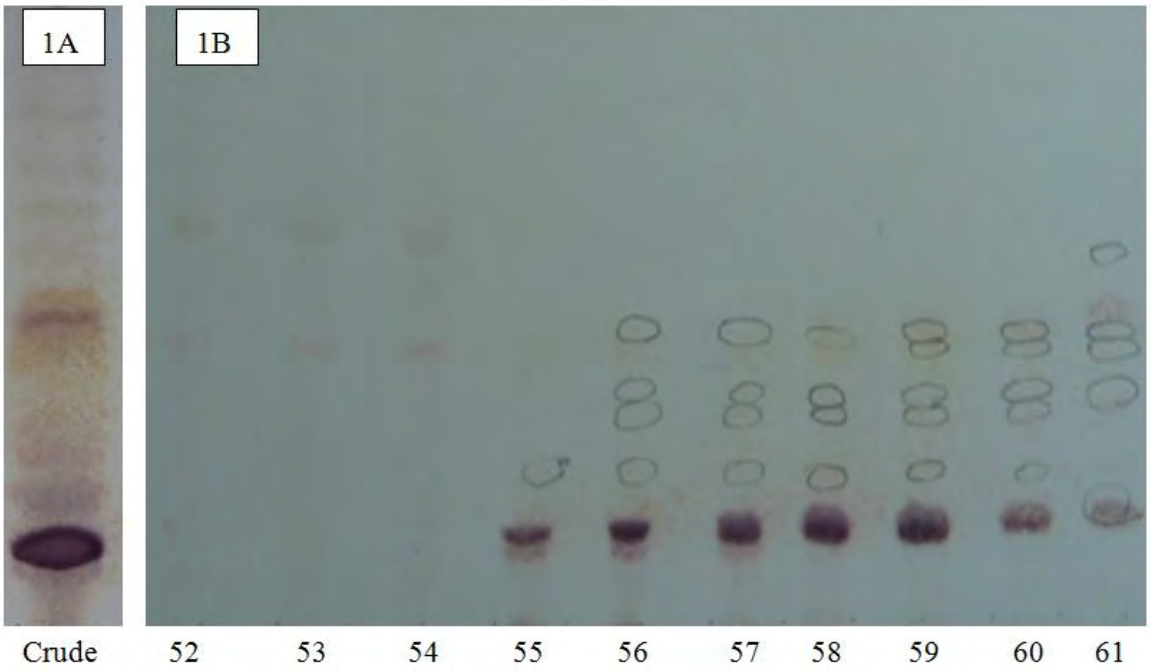
An effective method for the purification of saponins has been developed, and several saponins were isolated and purified from the viscera of *H. lessoni*. The enriched saponin mixtures of the viscera extract were successfully purified further by HPCPC, which is very efficient in purifying compounds with low polarity as well as in processing large amounts of sample. This method yielded saponins with higher than a 98% recovery of sample with high purities [72]. Purifying saponins from mixtures of saponins also helps to overcome the problem associated with identifying multiple saponins with liquid chromatography-tandem mass spectrometry (LC-MS) and ESI-MS. Mass spectrometry has been applied for the structure elucidation of saponins in both negative and positive ion modes [73–79]. In this study, identification of the saponin compounds was attempted by soft ionization MS techniques including MALDI and ESI in the positive mode. Previous studies have reported that the fragment ions of alkali metal adducts of saponins provide valuable structural information about the feature of the aglycone and the sequence and linkage site of the sugar residues [80]. Therefore, the MS analyses were conducted by introducing sodium ions to the samples. However, saponin spectra can also be detected without adding a sodium salt. Because of the high affinity of alkali cations for triterpene glycosides, all saponins detected in the positive ion mode spectra were predominantly singly charged sodium adducts of the molecules $[M + Na]^+$ [19,81]. The main fragmentation of saponins generated by cleavage of the glycosidic bond yielded oligosaccharide and monosaccharide fragments [19]. Other visible peaks and fragments were generated by the loss of other neutral moieties such as CO_2, H_2O or CO_2 coupled with H_2O.

The saponins obtained from the viscera of this tropical holothurian were profiled using MALDI-MS and ESI-MS. MALDI is referred to as a "soft" ionization technique, because the spectrum

shows mostly intact, singly charged ions for the analyte molecules. However, in some cases, MALDI causes minimal fragmentation of analytes [71].

The chromatographic purification of isobutanol-soluble saponin-enriched fractions of *H. lessoni* viscera was monitored on pre-coated thin-layer chromatography (TLC) plates (Figure 1A) showing the presence of several bands. As a typical example, the TLC profile of HPCPC Fractions 52–61 of the isobutanol-saponin enriched fraction from the viscera of the *H. lessoni* sea cucumber is shown in Figure 1B. The centrifugal partition chromatography (CPC) technique not only allowed for the purification of saponins, but in some cases it could separate isomeric saponins e.g., separation of the isomers detected in the ion peak at *m/z* 1303.6, which will be discussed later.

Figure 1. The thin-layer chromatography (TLC) pattern of a saponin mixture (**A**) and the high performance centrifugal partition chromatography (HCPCP) fractions (**B**) from the purified extracts of the viscera of the *Holothuria lessoni* sea cucumber using the lower phase of $CHCl_3$-MeOH-H_2O (7:13:8) system. The numbers under each lane indicate the fraction number of fractions in the fraction collector. Here, only the fractions 52 to 61 of one analysis (of 110 fractions) are shown as a representative.



Mass spectrometry has been used extensively for the characterization of saponins and their structural confirmation. One of the powerful methods, which are widely used for the analysis of high molecular weight, non-volatile molecules is MALDI [82]. The appropriate HPCPC fractions were consequently pooled based on their TLC profiles and concentrated to dryness and analyzed by MALDI MS and MS/MS, and ESI MS/MS. In the positive ion mode, all detected ions were sodium-coordinated species such as $[M + Na]^+$ corresponding to sulfated and non-sulfated saponins [64]. The prominence of the parent ions $[M + Na]^+$ in MS spectra also enables the analysis of saponins in mixtures or fractions. The MALDI results indicate that the saponin fractions are quite pure, which is consistent with the TLC data. As a representative example, the full-scan MALDI mass spectrum of the saponin extract obtained from HPCPC Fraction 55 of the *H. lessoni* viscera is shown in Figure 2.

This spectrum displays the major intense peak detected at *m/z* 1243.4, which corresponds to Holothurin A, with an elemental composition of $C_{54}H_{85}NaO_{27}S$ $[M + Na]^+$. Other visible peaks seem to correspond to the sugar moieties and aglycone ions generated by the losses of sugars and/or losses of water and/or carbon dioxide from cationized saponins upon MALDI ionization. These analyses show that this fraction contains one main saponin. Therefore, even though the HPCPC fractionation separated the saponin mixture, some saponin congeners, due to the similarity in their TLC migration, were detected in some of the pooled fractions. It was found that the total separation

of the saponins was difficult within a single HPCPC run. However, this technique allowed the separation of a number of saponins, including some isomers (Figure 3).

Figure 2. The full-scan ***matrix-assisted laser desorption/ionization mass spectrometry*** (MALDI) mass spectrum of HPCPC Fraction 55 in the (+) ion mode.

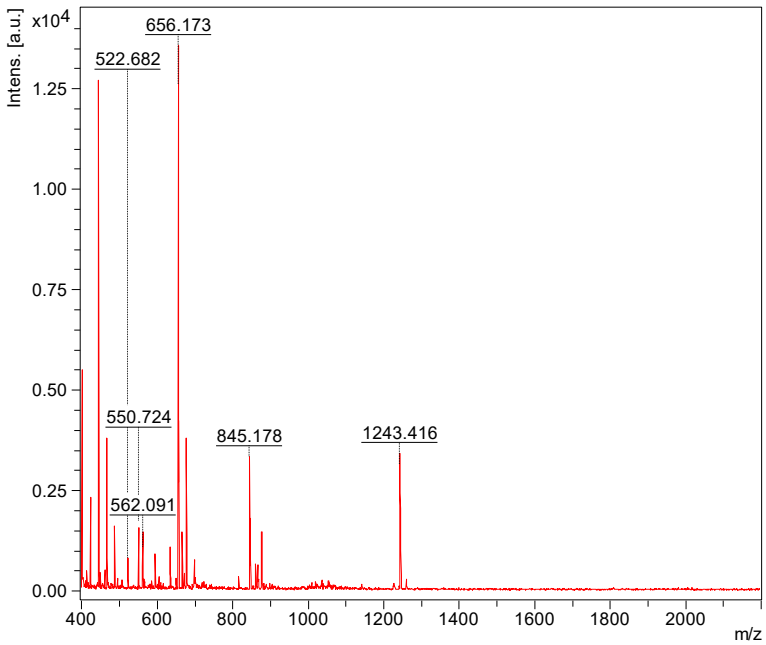
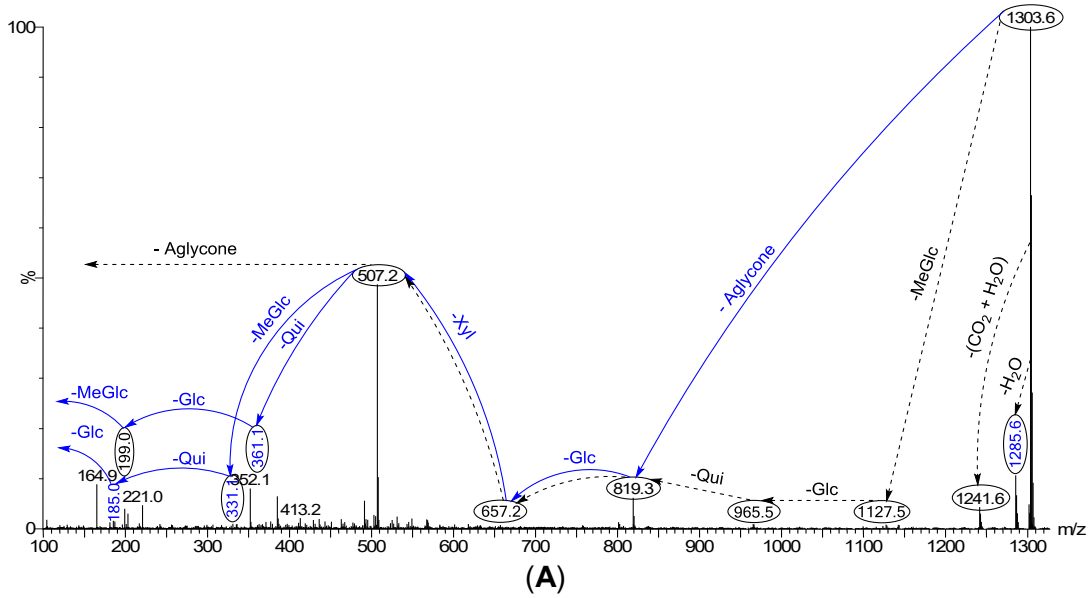


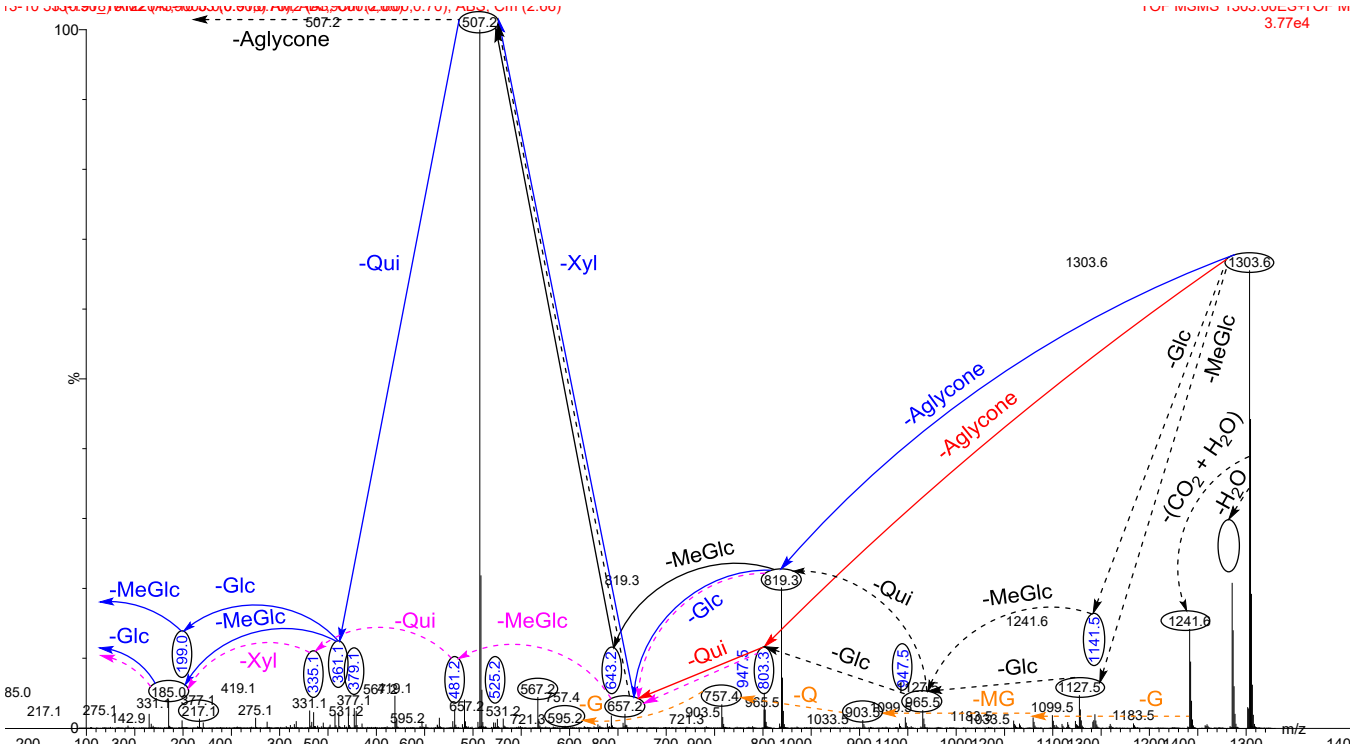
Figure 3. Schematic fragmentation patterns of the ion detected at *m/z* 1303.6; (**A**) Fraction 15; (**B**) Fraction 14 and (**C**) Fraction 12. Full and dotted arrows show the two main feasible fragmentation pathways. The predominant peak (**A** and **B**) at *m/z* 507 corresponds to the key sugar residue and aglycone moiety. The major abundant peak (**C**) at *m/z* 523 corresponds to both the key sugar residue and aglycone moiety. Abbreviations;

G = Glc, MG = MeGlc, Q = Qui, X = Xyl.

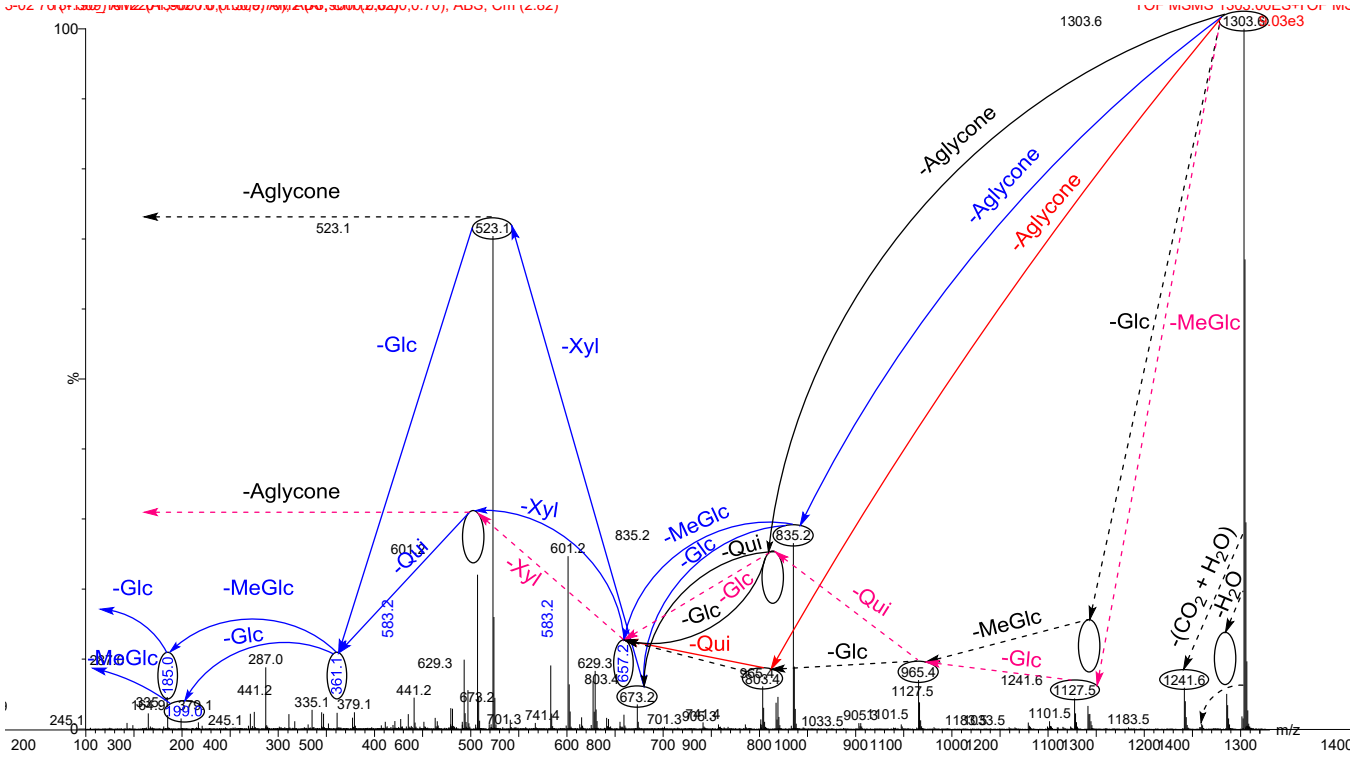


(**A**)

Figure 3. *Cont.*



(**B**)



(**C**)

The full-scan MALDI mass spectrum of the isobutanol-enriched saponin extract obtained from the viscera of the *H. lessoni* is shown in Figure 4. A diverse range of saponins with various intensities was identified. This spectrum displays 13 intense peaks that could each correspond to at least one saponin congener. The most abundant ions observed under positive ion conditions were detected at *m/z* 1335, 1303, 1289, 1287, 1259, 1245, 1243, 1229, 1227, 1149, 1141, 1123 and 845. Further analysis revealed that some of these MS peaks represented more than one compound. For instance the peaks at *m/z* 1303 and 1287 were shown to contain at least six and five different congeners, respectively (Figures 3 and 5–7).

Figure 4. The full-scan MALDI mass spectrum of the isobutanol-enriched saponin extract from the viscera of the *H. lessoni*. A mass range of 600 to 1500 Da is shown here. It is noted that this spectrum is unique for this species.

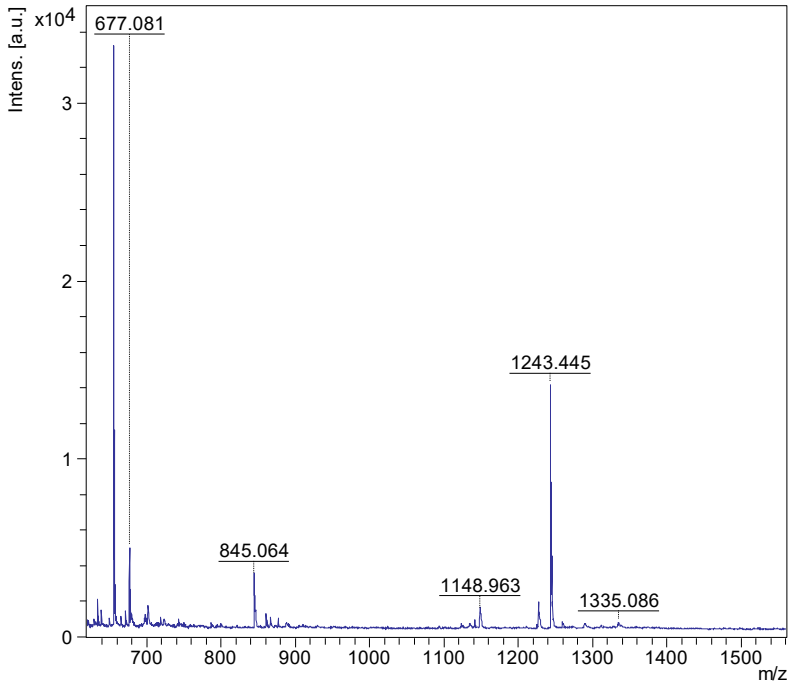


Figure 5. The schematic diagram of the proposed isomeric structures of ion at *m/z* 1303.6.

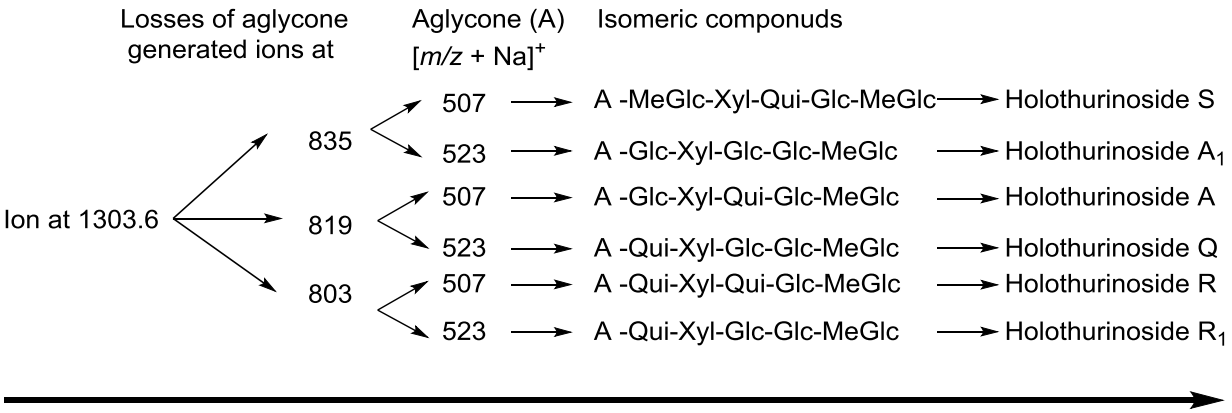


Figure 6. (+) ion mode ESI-MS/MS spectrum of saponins detected at *m/z* 1287.6. This spectrum shows the presence of two different aglycones, which led to the isomeric saponins. Full and dotted arrows illustrate the two main possible fragmentation pathways.

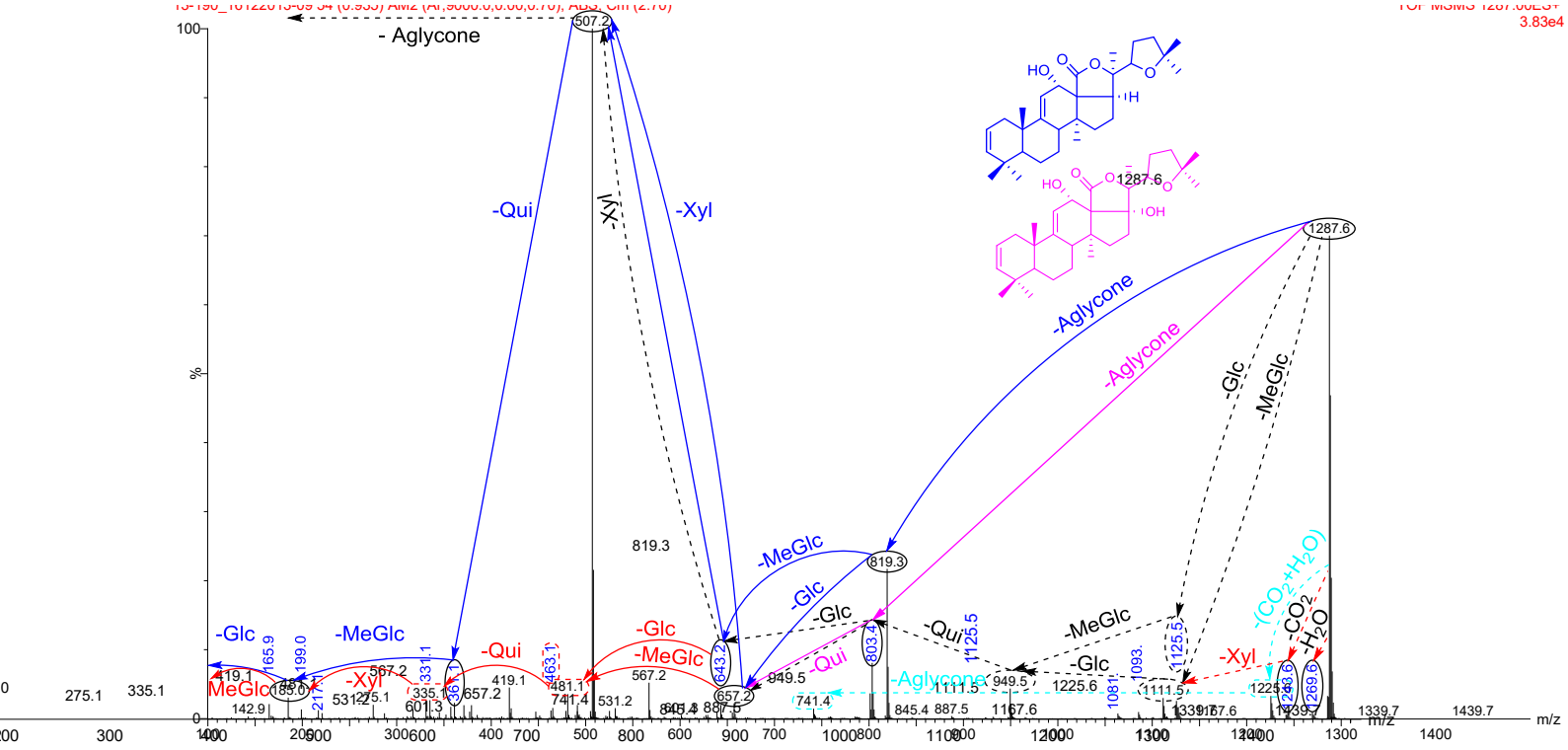
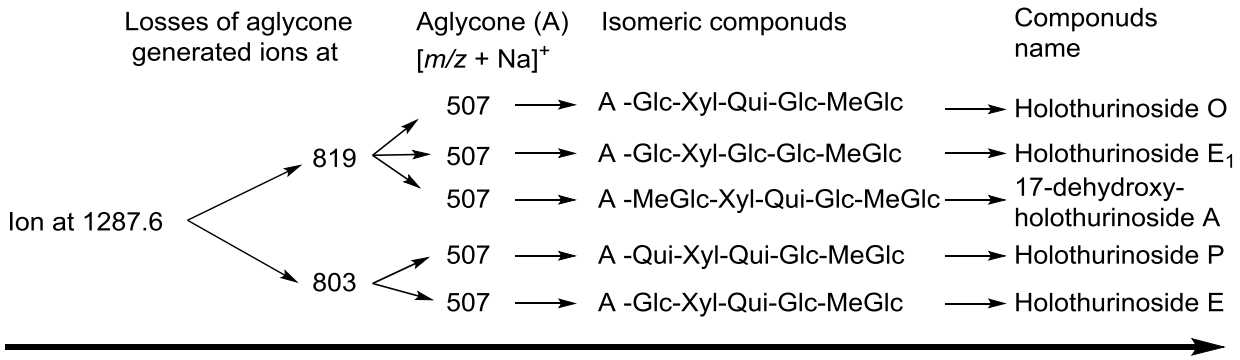


Figure 7. A schematic diagram of the proposed isomeric structures of ion at *m/z* 1287.6.



The accurate mass measurements acquired by MALDI-MS detected the saponin peaks, and molecular formulae and elemental compositions were assigned by ESI-MS/MS as summarized in Table 1. Our results revealed that at least 75 saponins were detected in *H. lessoni*, including 39 new sulfated, non-sulfated and acetylated triterpene glycosides, containing a wide range of aglycone and sugar moieties.

Table 1. Summary of saponins identified from the viscera of *H. lessoni* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) and electrospray-ionization mass spectrometry (ESI-MS). This table illustrates the 39 novel identified compounds (N) along with the 36 known compounds (P). This table also shows some identical saponins, which have been given different names by different researchers in which they might be isomeric congeners.

$[M + Na]^+$ *m/z*	MW	Formula	Compound's Name	Novel (N)/ Published (P)	References
889.4	866	$C_{41}H_{63}NaO_{16}S$	Holothurin B_3	P	[83]
		$C_{42}H_{67}NaO_{15}S$	Unidentified	N	–
905.4	882	$C_{41}H_{63}NaO_{17}S$	Holothurin B_4	P	[83]
			Holothurin B	P	[61,84–86]
			Nobiliside B	P	[87]
907.4	884	$C_{41}H_{65}NaO_{17}S$	Holothurin B_2	P	[83]
			Leucospilotaside B	P	[8]
911.6	888	$C_{45}H_{92}O_{16}$	Unidentified	N	–
917.4	994	$C_{44}H_{71}NaO_{15}S$	Unidentified	N	–
921.4	898	$C_{41}H_{63}NaO_{18}S$	Leucospilotaside A	P	[84]
1034.1	1011	a*	Unidentified	N	–
1065.5	1042	$C_{48}H_{82}O_{24}$	Unidentified	N	–
1071.5	1048	$C_{47}H_{93}NaO_{21}S$	Unidentified	N	–
1078.5	1055	a *	Unidentified	N	–
1083.3	1060	$C_{58}H_{64}O_{25}$	Unidentified	N	–
1087.6	1064	$C_{47}H_{93}NaO_{22}S$	Unidentified	N	–
1123.5	1100	$C_{54}H_{84}O_{23}$	Unidentified	N	–
1125.5	1102	$C_{54}H_{86}O_{23}$	Holothurinoside C Holothurinoside C_1	P	[62,69,88,89]
1127.6	1104	$C_{54}H_{88}O_{23}$	Unidentified	N	–

		$C_{53}H_{84}O_{24}$	Unidentified	N	−
1141.6	1118	$C_{54}H_{86}O_{24}$	Desholothurin A (Nobiliside 2a), Desholothurin A_1 (Arguside E)	P	[5,62,69,88–90]
1149.2	1126	a *	Unidentified	N	−
1157.5	1134	$C_{54}H_{109}O_{25}$	Holothurinoside J_1	P	[50]
		$C_{49}H_{91}NaO_{25}S$	Unidentified	N	−
1193.5	1170	$C_{55}H_{87}NaO_{23}S$	Unidentified	N	−
1199.4	1176	$C_{54}H_{64}O_{29}$	Unidentified	N	−
1221.5 **	1198	$C_{56}H_{78}O_{28}$	Unidentified	N	−
1225.5	1202	$C_{54}H_{83}NaO_{26}S$	Unidentified	N	−
1227.5	1204	$C_{54}H_{85}NaO_{26}S$	Fuscocineroside B/C, Scabraside A or 24-Dehydroechinoside A	P	[29,56,89,91,92]
1229.5	1206	$C_{54}H_{87}NaO_{26}S$	Holothurin A_2, Echinoside A	P	[7,61,91,93–95]
1243.5	1220	$C_{54}H_{85}NaO_{27}S$	Holothurin A Scabraside B 17-Hydroxy fuscocineroside B 25-Hydroxy fuscocinerosiden B	P	[29,58,61,95–97]
1245.5	1222	$C_{54}H_{87}NaO_{27}S$	Holothurin A_1 Holothurin A_4 Scabraside D	P	[91] [36] [92]
1259.5	1236	$C_{54}H_{85}NaO_{28}S$	Holothurin A_3	P	[36]
			Unidentified	N	−
1265.5	1242	$C_{56}H_{83}NaO_{27}S$	Unidentified	N	−
1271.6	1248	$C_{60}H_{96}O_{27}$	Impatienside B	P	[5,98]
1287.6	1264	$C_{60}H_{96}O_{28}$	Holothurinoside E, Holothurinoside E_1	P	[62,69]
			Unidentified	N	−

			Unidentified	N	–
			17-Dehydroxyholothurinoside A	P	[5,99]
1289.6	1266	$C_{60}H_{98}O_{28}$	Griseaside A	P	[99]
1301.6	1278	$C_{61}H_{98}O_{28}$	Holothurinoside M	P	[65]
		$C_{60}H_{94}O_{29}$	Unidentified	N	–
1303.6	1280	$C_{60}H_{96}O_{29}$	Holothurinoside A Holothurinoside A_1	P	[5,62,69,88]
			Unidentified	N	–
			Unidentified	N	–
			Unidentified	N	–
			Unidentified	N	–
1305.6	1282	a *	Unidentified	N	–
1317.6	1294	$C_{61}H_{98}O_{29}$	Unidentified	N	–
1335.3	1312	a *	Unidentified	N	–
1356.4	1333	a *	Unidentified	N	–
1409.4	1386	$C_{61}H_{78}O_{36}$	Unidentified	N	–
1411.7	1388	$C_{62}H_{116}O_{33}$	Unidentified	N	–
1419.7	1396	$C_{66}H_{108}O_{31}$	Unidentified	N	–
1435.7	1412	$C_{66}H_{108}O_{32}$	Unidentified	N	–
1465.7	1442	$C_{67}H_{110}O_{33}$	Arguside B Arguside C	P	[5,32]
1475.6	1452	$C_{65}H_{96}O_{36}$	Unidentified	N	–
1477.7 **	1454	$C_{61}H_{114}O_{38}$	Unidentified	N	–
1481.7	1458	$C_{66}H_{106}O_{35}$	Unidentified	N	–
1493.7	1470	$C_{65}H_{114}O_{36}$	Unidentified	N	–

1495.7	1472	$C_{61}H_{116}O_{39}$	Holothurinoside K_1	P	[50]
		$C_{72}H_{112}O_{31}$	Unidentified	N	–
1591.7	1568	$C_{66}H_{120}O_{41}$	Unidentified	N	–

a * The composition was not measured through the ESI analysis; ** acetylated compounds.

A number of studies have reported the presence of multiple saponins. Elbandy *et al.* [5] described the structures of 21 non-sulfated saponins from the body wall of *Bohadschia cousteaui*. These authors reported 10 new compounds together with 11 known triterpene glycosides including Holothurinoside I, Holothurinoside H, Holothurinoside A, Desholothurin A, 17-dehydroxyholothurinoside A, Arguside C, Arguside F, Impatienside B, Impatienside A, Marmoratoside A and Bivittoside. Bondoc *et al.* [64] investigated saponin congeners in three species from Holothuriidae (*H. scabra* Jaeger 1833, *H. fuscocinerea* Jaeger 1833, and *H. impatiens* Forskal 1775). This group reported 20 saponin ion peaks, with an even number of sulfated and non-sulfated types, in *H. scabra,* which contained the highest saponin diversity among the examined species, followed by *H. fuscocinerea* and *H. impatiens* with 17 and 16 saponin peaks, respectively. These authors also described a total of 32 compounds in *H. scabra* and *H. impatiens* and 33 compounds in *H. fuscocinerea*. The saponin content of five tropical sea cucumbers including *H. atra*, *H. leucospilota*, *P. graeffei*, *A. echinites* and *B. subrubra* was also studied by Van Dyck *et al.* [50]. These authors reported the presence of four, six, eight, ten and nineteen saponin congeners in these species, respectively. In addition, this group [69] also detected a higher number of saponins (26) in the cuvierian tubules of *H. forskali* compared to the body wall (12 saponins). These results further support the evidence, suggested by the present study, of greater saponin congeners in viscera.

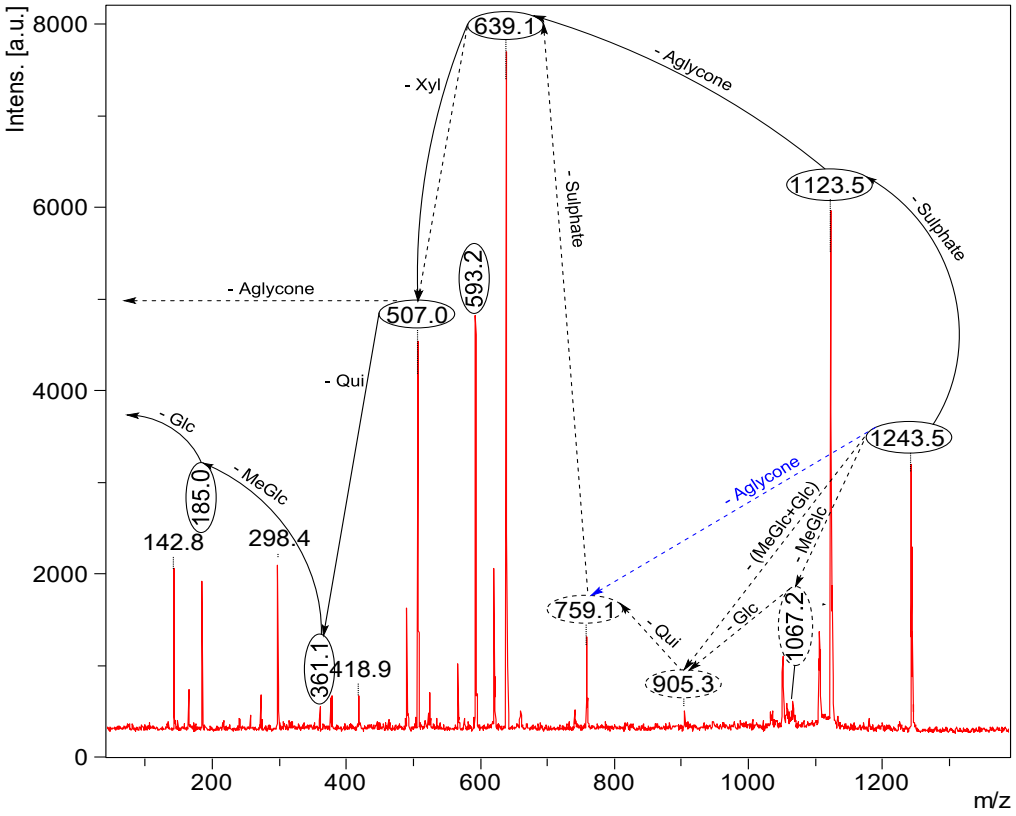
2.2.1 MALDI-MS/MS Data of Compound Holothurin A in the Positive Ion Mode

The conventional procedures to differentiate between isomeric saponins, including chemical derivatization and stereoscopic analysis, are tedious and time-consuming [100]. Tandem mass spectrometry was conducted to obtain more structural information about the saccharide moiety and elucidate their structural features. In order to ascertain that ions (signals) detected in the full-scan MALDI MS spectrum indeed correspond to saponin ions, tandem mass spectrometry analyses were performed for each ion, and saponin ion peaks were further analyzed using MS/MS fingerprints generated with the aid of collision-induced dissociation (CID) from their respective glycan structures. CID can provide a wealth of structural information about the nature of the carbohydrate components, as it preferentially cleaves glycosides at glycosidic linkages, allowing a straightforward interpretation of data. Almost all of observed daughter ions originated from the cleavage of glycosidic bonds (Figure 8). Therefore, the reconstruction of their fingerprints (fragmentation patterns) created by the glycosidic bond cleavages was utilized to deduce the structure of sugar moieties. This technique was also able to distinguish the structural differences between the isomers following HPCPC separation. However, in some cases, the MS/MS spectra obtained from the CID could be essentially identical for isomeric precursor ions. As a typical

example, the MALDI-MS/MS mass spectrum for the ion detected at *m/z* 1243.5 is shown in Figure 8. The fragmentation pattern of the sodiated compound at *m/z* 1243.5 $[M + Na]^+$ in successive MS experiments is discussed in detail below for stepwise elucidation of the molecular structure of these compounds.

Collisional induced-dissociation activates two feasible fragmentation pathways of cationized parent ions shown in full and dotted arrows. First, the loss of the sugar unit; the successive losses of 3-*O*-methylglucose (-MeGlc), glucose (-Glc), quinovose (-Qui), sulfate and xylose (-Xyl) units generate ion products detected at *m/z* 1067, 905, 759, 639 and 507, respectively. As this figure illustrates, the consecutive losses of the (MeGlc + Glc) simultaneously generated the ion at *m/z* 905.3, and Qui (−146 Da) resulted in the peak at *m/z* 759.1 which corresponds to [Aglycone + sulXyl-H + 2Na]$^+$.

Figure 8. Positive tandem MALDI spectrum analysis of the precursor ion (saponin) detected at *m/z* 1243.5. The figure shows the collision-induced fragmentation of parent ions at *m/z* 1243.5. The consecutive losses of sulfate group, aglycone, xylose (Xyl), quinovose (Qui) and 3-*O*-methylglucose (MeGlc) residues affords product ions detected at *m/z* 1123, 639, 507, 361and 185, respectively.



Secondly the decomposition of the precursor ions can also be triggered by the loss of the aglycone residue, creating peaks at *m/z* 759 (Figure 8) corresponding to the sugar moieties of 1243.5. The losses of the $NaHSO_4$ (ion generated at *m/z* 1123.5), aglycone residue (ion generated at *m/z* 639.1), and xylose (ion generated at *m/z* 507.0), respectively, were produced by glycone and aglycone fingerprint peaks from the precursor ion. Therefore, the consecutive losses of the sodium monohydrogen sulfate ($NaHSO_4$) from 1243.5 and aglycone unit produced signals observed at *m/z*

1123 and 639 (Figure 8); the latter peak corresponding to the total desulfated sugar moiety. Furthermore, the consecutive losses of Xyl, Qui and MeGlc presenting signals observed at *m/z* 507, 361 and 185, respectively, additionally proved that the decomposing ions were definitely generated from sodiated Holothurin A (*m/z* 1243.5). The implementations of these molecular techniques on all ions detected in the MALDI spectra allow us to identify the molecular structures of the saponins. All spectra were analyzed and fragmented, and some of them shared common fragmentation patterns. Key fragments from the tandem MS spectra of the positive ion mode of MALDI and ESI were reconstructed according to the example illustrated in order to propose the saponin structures. On the bases of these fragment signatures, 39 new saponins can be postulated. Some of these compounds, which share the common *m/z* 507 or/and *m/z* 523 key signals as a signature of the sodiated MeGlc-Glc-Qui and the sodiated MeGlc-Glc-Glc oligosaccharide residues, respectively, were easily identified. The identified saponins possess different aglycone structural elements.

The loss of 18 Da from the sodiated molecular ion, suggested the elimination of a neutral molecule (H_2O) from the sugar group [19]. The simultaneous loss of two sugar units indicated characteristics of a branched sugar chain. Other visible peaks correspond to saponin product ions produced by the losses of water and/or carbon dioxide from sodiated saponins upon MALDI ionization. Hereby the sugar sequence of saponins can be determined by applying CID. The MALDI MS/MS data for this *m/z* value were in complete agreement with those reported in a previous study [50,64]. The predominant fragment signal at *m/z* 593.2 results from $\alpha\ ^{1,5}A_4$ cross-ring cleavage of the sulXyl residue, which was consistent with previous findings for the MS/MS analyses of sea cucumber saponins [64]. However, this peak was only detected as an intense signal in the sulfated saponins such as Holothurin A, whereas it was not observed in the non-sulfated saponins such as Holothurinoside A. Therefore, this cross-ring cleavage seems to occur only with the sulfated Xyl. Analysis by MALDI resulted in an information rich tandem mass spectrum containing glycosidic bond and cross-ring cleavages that provided more structural information than previous studies on the same precursor ion. The sugar moiety of saponins developed from non-sulfated hexaosides to sulfated tetraosides [64]. The assignment of the sulfate group was determined by the mass difference between the parent ion at *m/z* 1243 and daughter ion *m/z* 1123 peaks based on knowing the molecular weight of the sulfate unit (120 Da). Complete glycosidic bond cleavage was observed, which enabled us to determine the locations of the sulfate (*m/z* 1123), the entire sugar moieties (*m/z* 639), and each component of sugar residue.

The losses of the aglycone and sugar residues are largely observed from glycosidic bond cleavages. Even though one cross-ring cleavage is assigned, the generation of glycosidic bond cleavages in combination with accurate mass is sufficient to assign the position of the sulfate group along the tetrasaccharide sequence for Holothurin A. The ion detected at *m/z* 1105 (Figure 8) is the water-loss ion derived from the ion at *m/z* 1123, whereas the ion observed at *m/z* 1061 corresponds to the neutral loss of CO_2 (44 Da).

As described by Song *et al.* [100], the cross-ring cleavages that occurred in the CID spectra of saccharides with α 1–2 linkage, such as the sugar residue for Holothurin A, are X and A types, whereas the glycoside bond cleavages are C and B types. The major peak at *m/z* 593.2 was attributed to cross-ring cleavage of the sugar unit.

This MS/MS spectrum allows us to reconstruct the collision-induced fragmentation pattern of the parent ion (Figure 4) and consequently to confirm that ions monitored at *m/z* 1243.5 correspond to the Holothurin A elucidated by Van Dyck *et al.* [50], Kitagawa *et al.* [96] and Rodriguez *et al.* [88].

The occurrence of a sulfate group ($NaHSO_4$) in saponin compounds, such as in the case of Holothurin A, was assigned by a loss of 120 Da during the MS/MS. By the combination of accurate mass and MS/MS information, saponins were categorized into seven distinct carbohydrate structural types: (A) MeGlc-Glc-Qui-Xyl-Aglycone; (B) MeGlc-Glc-Glc-Xyl-Aglycone; (C) (MeGlc-Glc)-Qui-sulXyl-Aglycone; (D) MeGlc-Glc-Qui-(Qui-Glc)-Xyl-Aglycone; (E) MeGlc-Glc-Qui-(MeGlc-Glc)-Xyl-Aglycone; (F) MeGlc-Glc-Glc- (MeGlc-Glc)-Xyl-Aglycone; and (G) MeGlc-Glc-Glc-(Qui-Glc)-Xyl-Aglycone. Non-sulfated saponins had one to six monosaccharide units and six distinct structural types. All sulfated saponins ranging from *m/z* 889 to 1259 had a structure (C), in which Xyl was sulfated. However, in some cases, the sulfation of Xyl, MeGlc and Glc was reported [13]. The MS analyses also indicated that this sea cucumber species produced a mixture of common and unique saponin types. Unique saponin types were also identified when the mass spectra of this species were compared with others. Saponin peaks with the ion signatures at *m/z* values of 1477, 1335, 1221, 1149 and 1123 were unique in *H. lessoni*. In the tandem MS, in general, the most abundant ions were attributed to the losses of aglycones and/or both key diagnostic sugar moieties (507 and 523). For 1243.5, the most abundant ions observed under positive ion conditions were at *m/z* 1123, 639 and 507, corresponding to the losses of sulfate, aglycone and Xyl moieties. The major ion at *m/z* 621.2 corresponded to the loss of water from ion at *m/z* 639. Some saponins were commonly found among species (e.g., Holothurins A and B), whereas others were unique to each species (e.g., 1221 in *H. lessoni*), as Bondoc *et al.* [64] and Caulier *et al.* [6] have also indicated. The saponin profile (peaks) of sea cucumbers indicated the different relative intensities of saponins in the viscera. The peaks observed (Figure 4) at *m/z* 1149.0, 1227.5, 1229.5, 1243.5, and 1259.5 in the positive ion mode corresponded to an unidentified saponin, Scabraside A or Fuscocinerosides B/C (isomers), Holothurin A_2 (Echinoside A), Holothurin A, and Holothurin A_3, respectively [36,56,61,89,93,94]. Most of these sulfated saponins were also reported by Kitagawa *et al.* [89] and Bondoc *et al.* [64]. The ion peaks of the non-sulfated saponins at *m/z* 1125, 1141, 1287, 1289, 1301 and 1303 corresponded to Holothurinosides C/C_1 (isomers), Desholothurin A (synonymous with Nobiliside 2A) or Desholothurin A_1, Holothurinosides E/E_1, Griseaside A, Holothurinosides M and A, respectively [69]. *H. scabra*, *H. impatiens* and *H. fuscocinerea* were also reported to contain Holothurin A, Scabraside B and Holothurinoside C [64]. This group also detected 24-dehydroechinoside A and Scabraside A in *H. scabra*. The presence of Holothurinosides C/C_1 (isomers), Holothurinosides A/A_1 (isomers), Desholothurin A (synonymous with Nobiliside 2A), Desholothurin A_1 and Holothurinosides E/E_1 were also described in *H. forskali* by several groups [65,69,88]. We were not able to identify all the saponin congeners detected in the semi-pure extract in the HPCPC-fractionated samples. Bondoc *et al.* [64] experienced a similar issue in that they observed some peaks in MALDI MS, which were not seen in the isomeric separation done in LC-ESI MS. For instance, we could not find ions at *m/z* 1149 and 1335 in the spectra of HPCPC fractions by ESI-MS. The MALDI mass spectra of the semi-pure and HPCPC fractionated samples of the *H. lessoni* revealed 75 ions (29 sulfated and 46 non-sulfated) in which a total of 13 isomers was found (Table

1), of which 36 congeners had previously been identified in other holothurians. It is the first time that the presence of these identified saponins has been reported in *H. lessoni,* apart from the saponins reported by Caulier *et al.* [58] that were found in the seawater surrounding *H. lessoni*. They reported saponins with *m/z* values of 1141, 1229, 1243 and 1463 namely Desholothurin A, Holothurin A_2, Scabraside B (synonymous with Holothurin A) and Holothurinoside H, respectively [58]. However, we could not detect the ion at *m/z* 1463 in our sample.

Most of the sulfated saponins that had previously been reported were detected in this species, including Holothurin B_3 (*m/z* 889), Holothurin B/B_4 (*m/z* 905), Holothurin B_2 (*m/z* 907), Fuscocinerosides B or C, which are functional group isomers (*m/z* 1227), Holothurin A_2 (*m/z* 1229), Holothurin A (*m/z* 1243), Holothurin A_1/A_4 (*m/z* 1245), and Holothurin A_3 (*m/z* 1259). The common sulfated congeners among this species and other sea cucumbers are Holothurin B (*m/z* 905) and Holothurin A (*m/z* 1243). Among these saponins, Holothurin A is the reported to be the major congener with the highest relative abundance in this species.

To illustrate the identification of a novel compound at *m/z* 1149.0, the parent ion at *m/z* 1149.0 was subjected to MS/MS fragmentation. The MALDI fingerprints revealed that the compound contained a novel aglycone at *m/z* 493 and a tetrasaccharide moiety with *m/z* value of 656 Da including -Xyl, -Qui, -Glc and -MeGlc in the ratio of 1:1:1:1. This saponin possessed the common *m/z* 507 key signal as a fingerprint of MeGlc-Glc-Qui + Na^+. We propose to name Holothurinoside T.

The isomers within one sample showed different MS^n spectra [101] allowing their structures to be elucidated based on the ion fingerprints. Here we indicate that the occurrence of many product ions in the spectrum of viscera extract is due to the presence of a mixture of saponins and isomeric saponins (Figures 3 and 5–7). This observation is consistent with the findings proposed by Van Dyck and associates [69] for the Cuvierian tubules of *H. forskali*. Mass spectrometry alone, however, is not powerful enough to obtain more structural information about the isomeric congeners. Nonetheless, it provides a quick and straightforward characterization of the element components and saponin distributions by the presence of ions at *m/z* 507 and 523 in the tandem spectra of the viscera extracts.

2.2.2 Key Fragments and Structure Elucidation of Novel Saponins

The common key fragments facilitated the structure elucidation of novel saponins. Tandem mass spectrometry analyses of saponins led to identification of several diagnostic key fragments corresponding to certain common structural element of saponins as summarized in Table 2.

Table 2. Key diagnostic ions in the MS/MS of the holothurians saponins.

Diagnostic ions in CID Spectra of $[M + Na]^+$			
	***m/z* Signals (Da)**		
	507	**523**	**639**
Chemical signatures	MeGlc-Glc-Qui + Na	MeGlc-Glc-Glc + Na	MeGlc-Glc-Qui-Xyl + Na

The structures of saponins were deduced by the identification and implementation of the key fragment ions generated by tandem mass spectrometry. The presence of these oligosaccharide residues (*m/z* 507 and/or 523) facilitated the determination of the saponin structure. However, some compounds with a *m/z* value of less than 1100 Da including 921, 907, 905 and 889 did not yield the peak *m/z* 523, which reflected the lack of this oligosaccharide unit in their structures. Unlike other compounds, the MS/MS spectrum of the ion at *m/z* 1477.7 illustrated the unique fingerprint profile, which contained ions at *m/z* 511 and 493 instead of an ion at *m/z* 507. The structure of compound was further confirmed by MS/MS analyses.

The MALDI analysis revealed that the ion with *m/z* 1243.5 was the prominent peak in the spectrum, which corresponded to Holothurin A, which was found in several species of sea cucumbers [6,29,50,58,61,64,89,95–97]. The MALDI data were confirmed by ESI-MS.

Table 1 summarizes data of all analyses performed on the saponin-enriched sample and HPCPC fractionated samples using MALDI and ESI on compounds from the viscera of *H. lessoni*. The identified saponin mixture contains a diverse range of molecular weights and structures. The chemical structures of the identified compounds are illustrated in Figure 9. The isobutanol and HPCPC fractionated samples indicated 29 sulfated and 46 non-sulfated saponin ions. The number of MS ion peaks was lower than the number of isomers identified by MS/MS following HPCPC separation (Figure 3).

Figure 9. The structure of identified saponins in the viscera of *H. lessoni*.

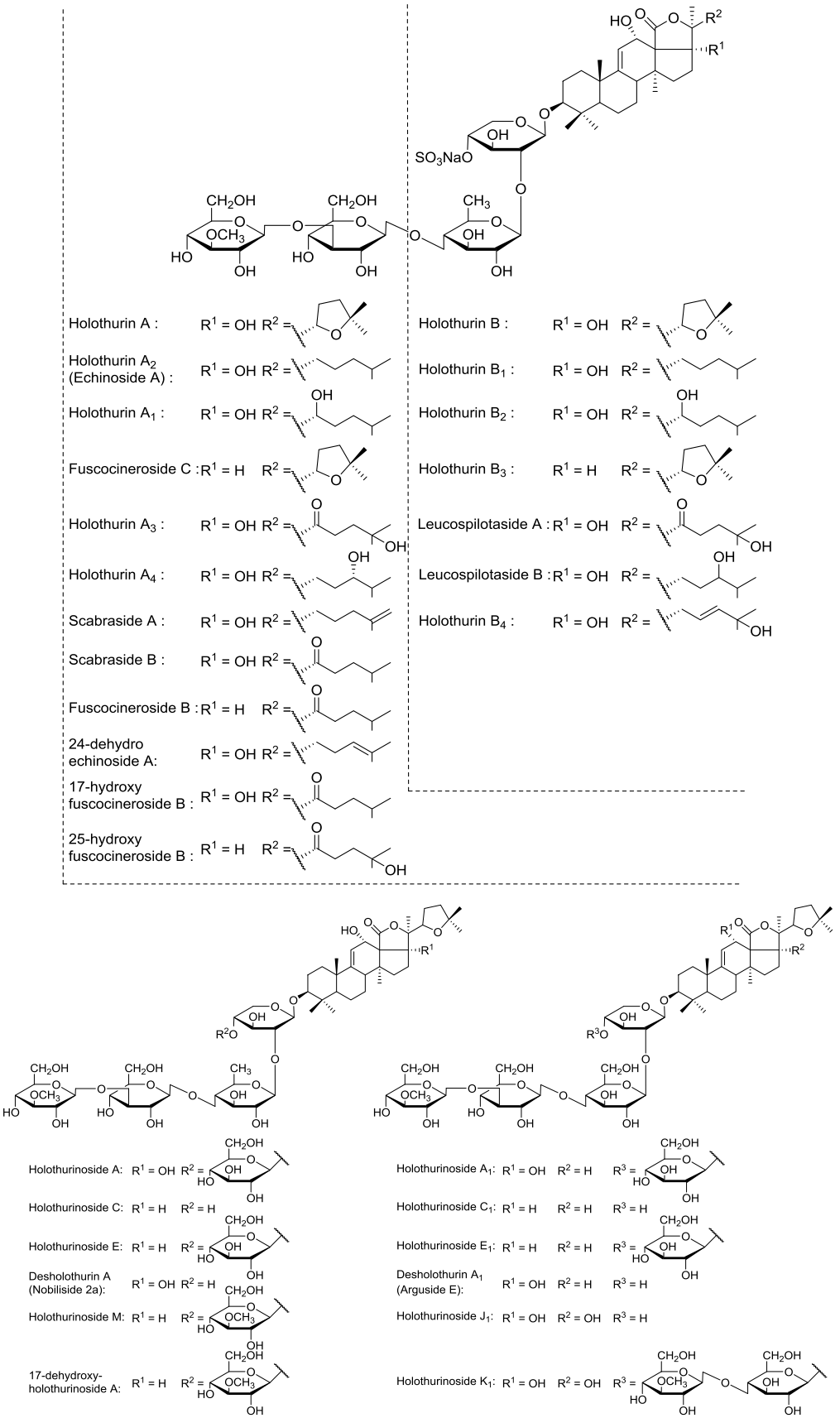
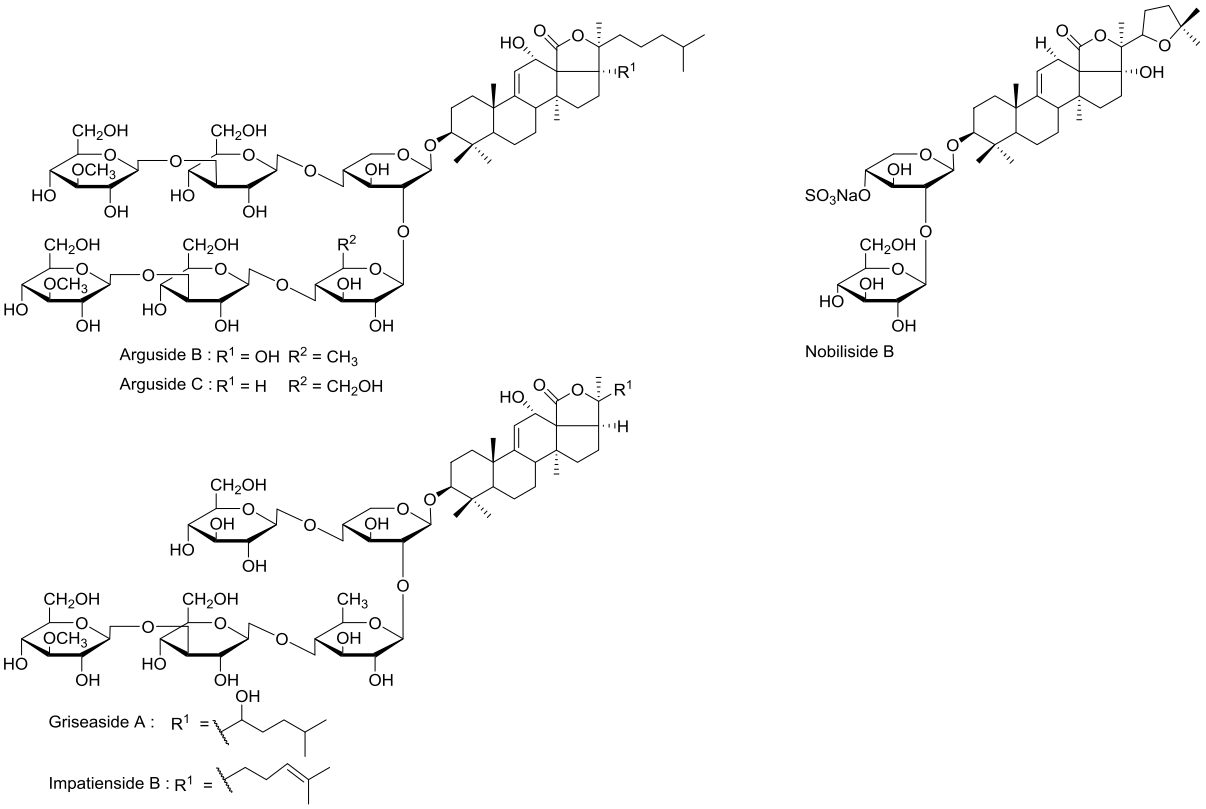


Figure 9. *Cont.*



2.2.3 Analyses of Saponins by ESI-MS

The positive ion mode ESI-MS analyses were also conducted on the samples. ESI mass spectra of the saponins are dominated by $[M + Na]^+$. There were some instances where peaks observed in the MALDI-MS spectra were not monitored from the isomer separation done in the ESI-MS, such as the peak detected at *m/z* 1149 in the MALDI spectra. Other researchers had experienced the same

issue [50,64].

ESI-MS^n is a very effective and powerful technique to differentiate isomeric saponins [100]. Tandem MS analyses on $[M + Na]^+$ ions provided abundant structural information about saponins. The positive ion mode ESI-MS/MS analyses were also performed on all compound ions detected in the ESI-MS spectrum of HPCPC fractions. This technique also confirmed the existence of saponins reported in the literature and allowed the discovery of new saponin congeners in the species examined. The molecular masses of the identified compounds are summarized in Table 1. The ESI-MS spectrum of the saponin extract from the viscera of *H. lessoni* is shown in the Figure 10.

Several major peaks were detected. The peaks at *m/z* 1123 and 1243 correspond to a novel compound and Holothurin A with the elemental compositions of $C_{54}H_{84}O_{23}$ and $C_{54}H_{85}NaO_{27}S$, respectively. The ESI-MS analyses were also carried out on all HPCPC fractions. As a typical example, Figure 11 shows the ESI-MS spectrum of Fraction 14.

Figure 10. (+) ESI-MS spectrum of saponins extract from the viscera of *H. lessoni*.

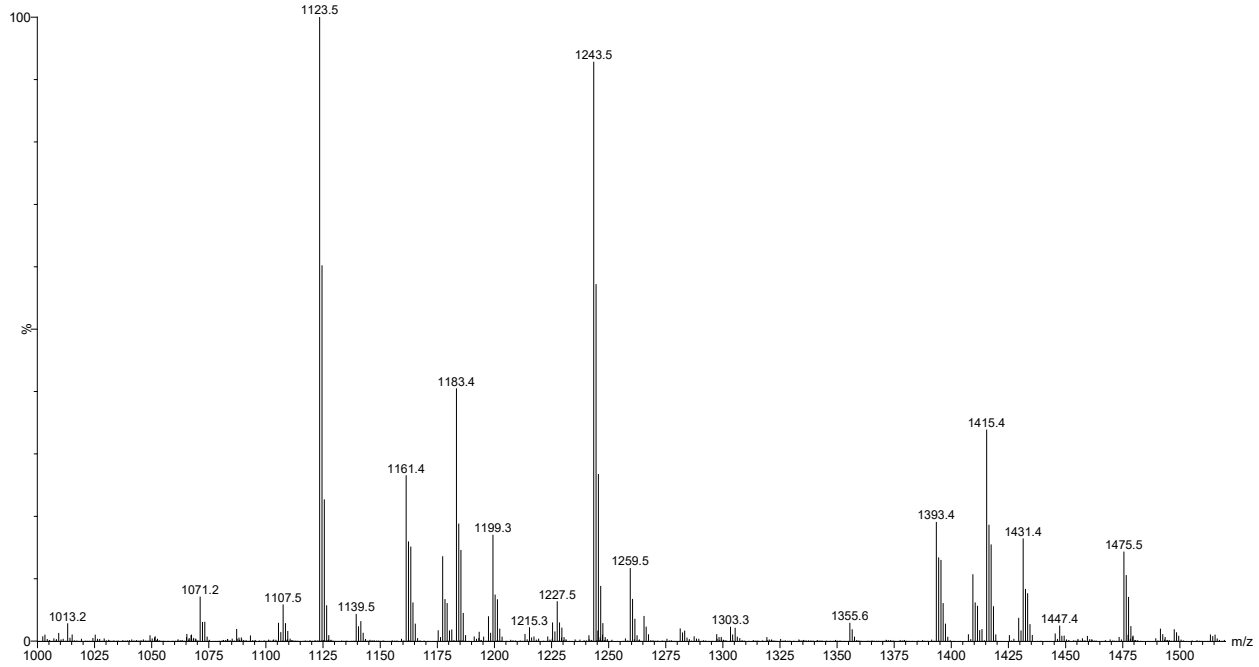
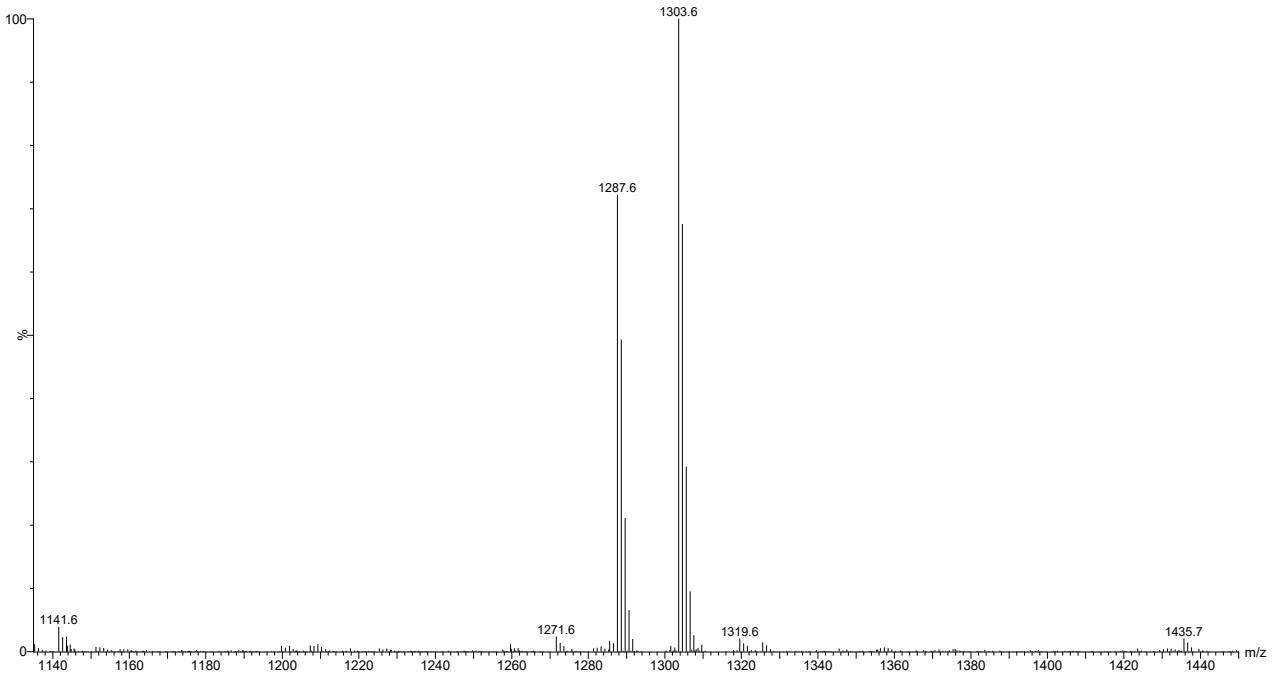


Figure 11. (+) ion mode ESI-MS spectrum of saponins extract from Fraction 14.



As can be seen in Figure 11, there are two major peaks at *m/z* 1287.6 and 1303.6, which correspond to Holothurinosides E/E_1 and Holothurinosides A/A_1, respectively. These two peaks, as the MS/MS analyses show which will be discussed later, were found to correspond to at least five and six isomers, respectively (Figures 3, 6 and Supplementary Figure S2). A comparison of the molecular weights of both saponins revealed some mass differences between them, such as a 16 Da (O) mass differences between Holothurinosides E/E_1 and Holothurinosides A/A_1, reflecting the

small structural alterations and the intrinsic connections between them. Their MS/MS analyses indicated, as will be discussed later, the presence of some identical aglycones in both ions.

2.2.4 Molecular Mass of Saponins by ESI

ESI/MS provide considerable structural information with very high sensitivity for saponins [60,63]. Peaks corresponding to the sodium adduct of the complete sugar side chains were often quite intense in the product ion spectra of the sodiated saponin precursor. Tandem mass spectra of saponins reflected the different fingerprints with different relative intensities.

2.2.5 Structure Elucidation of the Saponins by ESI-MS/MS

Seventy-five different triterpene saponins purified from sea cucumber were investigated by MALDI and electrospray ionization tandem mass spectrometry (ESI-MS/MS) in the positive ion modes. All spectra were analyzed and fragmented, and some of them shared common fragmentation patterns. Key fragments from the positive ion mode MS/MS spectra of MALDI and ESI were reconstructed with an example illustrated that proposes the saponin structures. Peak intensities of fragment ions in MS/MS spectra were also correlated with structural features and fragmentation preferences of the investigated saponins. In general, the formation of fragments occurred predominantly by cleavages of glycosidic bonds in the positive mode (Figure 12), which was applied to identify the structure of saponins. Interpretation of fragment ions of MS/MS spectra provided the key information for the structural elucidation of saponins as exemplified in Figure 12.

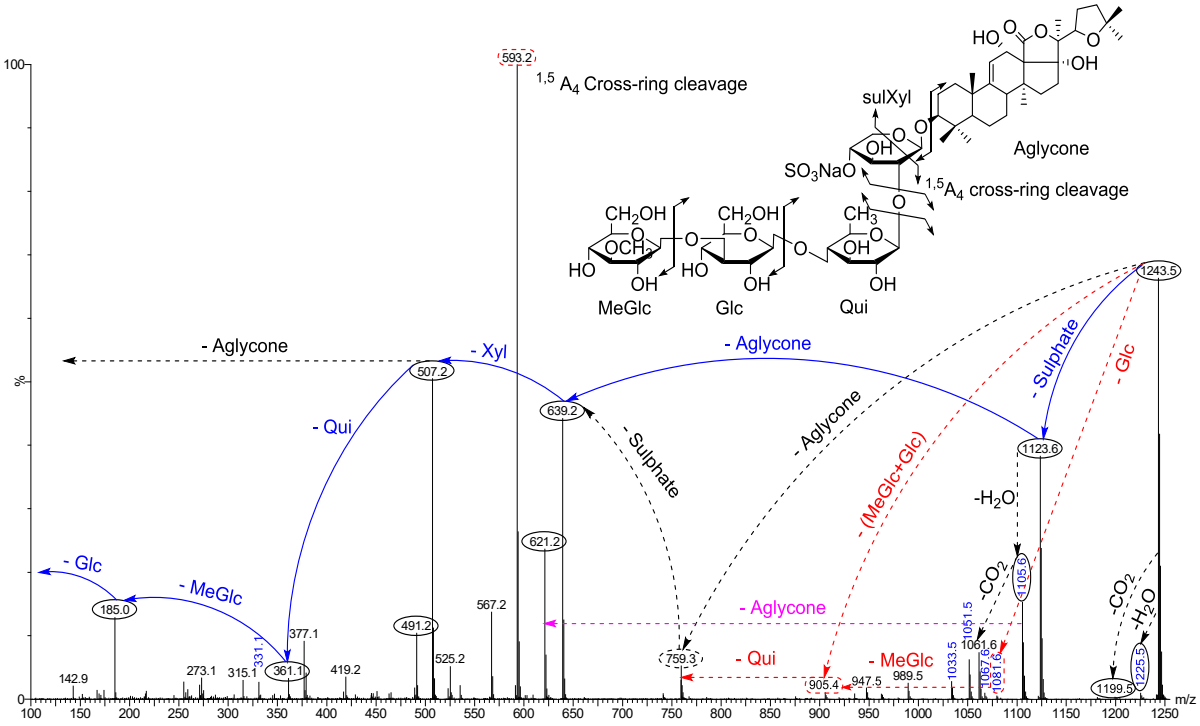
Fragmentation of the ion at *m/z* 1243.5 (sulfated saponin) under collisionally activated dissociation (CAD) conditions is shown in Figure 12. Full and dotted arrows show the two main fragmentation pathways in this saponin. The peak at *m/z* 507 corresponds to both the aglycone and the key diagnostic fragment of sugar moiety.

The most abundant peaks were detected at *m/z* 1123 $[M + Na - 120 \text{ (sulfate)}]^+$, 639 $[M + Na - 120 - 484 \text{ (aglycone)}]^+$ and 507 $[M + Na - 120 - 484 - 132]^+$. In addition, the peaks observed at *m/z* 1225.5 and 1199.5 were generated by the losses of H_2O and CO_2 from their respective parent ion.

The most intensive peak was observed at *m/z* 593 stemming from a cross-ring cleavage. The observed fragments are consistent with the structure of the Holothurin A proposed by Van Dyck *et al.* [50]. This ESI-MS/MS analysis confirmed the MALDI data on the ion at *m/z* 1243.5. The full analysis can be seen in Supplementary Figure S1.

ESI-MS was applied to distinguish the isomeric saponins by Song *et al.* [100]. Isomers of saponins were also identified using tandem mass spectrometry combined with electrospray ionization (ESI-MS/MS) following HPCPC separation. MS/MS spectra of these ions gave detailed structural information and enabled differentiation of the isomeric saponins. The results are exemplified in the following figures. The analyses applied on the ion at *m/z* 1303.6 (non-sulfated saponins), which was obtained from Fractions 15, 14 and 12, are shown in Figure 3A–C. The main fragmentation patterns observed for this isomeric compound are shown with full and dotted arrows.

Figure 12. (+) Ion mode ESI-MS/MS spectrum of saponin detected at 1243.5 (Holothurin A). Full and dotted arrows show the two main feasible fragmentation pathways. The structure of saponin was elucidated on the base of tandem mass spectrometry.



The structures of six isomeric saponins were ascribed to the ions detected at *m/z* 1303.6 (Figure 3A–C). These isomers have at least three different aglycone structures with *m/z* 468, 484 and 500 and contain five different monosaccharaide residues. These figures illustrate different isomers of ions detected at *m/z* 1303. For instance, Figure 3A (Fraction 15) shows the stepwise structure elucidation of Holothurinoside A. The consecutive losses of MeGlc, Glc, Qui, Glc, and Xyl units generate signals detected at *m/z* 1127.5, 965.5, 819.3, 657.2 and 507.2, respectively, which correspond to Holothurinoside A [5,62,69,88]. As can be seen in Figure 3A, this saponin fraction is quite pure.

In one of these isomers (Figure 3B), the consecutive losses of aglycone, Glc, Xyl, Qui and Glc units provided signals detected at *m/z* 819, 657, 507, 361 and 199, respectively, further confirming that the fragment ions unambiguously originate from sodium-cationized Holothurinoside A. In addition (*m/z* 1303.6), the precursor ion sequentially lost MeGlc (*m/z* 1127.5), Glc (*m/z* 965.5), Glc (*m/z* 803.4), Qui (*m/z* 657.2) and Xyl (*m/z* 507.2) (Figure 3B) thereby indicating the structure of another isomer of this molecule. The characteristic peak observed at *m/z* 507.2 generated by tandem MS was identified either as a sodiated MeGlc-Glc-Qui residue or sodiated aglycone residue. The ion at *m/z* 803 resulted from the loss of aglycone from the parent ion at *m/z* 1303.6, which is the fragment ion corresponding to the complete saccharide chain, which subsequently (Figure 3B) produces the ions at *m/z* 657 and ion at *m/z* 507 by the losses of Qui and Xyl residues. Moreover, the ions (*m/z* 507) further fragmented to form ions of the same *m/z* value at *m/z* 361 and *m/z* 199 or 185. The observation of ions at *m/z* 507 and 657 further supports the above conclusion. The ions detected at *m/z* 1285.5 and 1241.6 correspond to the losses of H_2O and H_2O + CO_2, respectively.

These two fragments correspond to the sequential losses of water and carbon dioxide. It is notable that the configurations of all the sugars in all previously known sea cucumber triterpene glycosides are D-configurations.

A similar analysis was carried out (Figure 3C) on the ion at *m/z* 1303.6 of Fraction 12. As can be seen in the figure, the spectrum has a different fragmentation pattern compared to the spectra in Figure 3A,B even though they have the same *m/z* value. In one of the isomers, the consecutive losses of aglycone, Glc, Xyl, Glc and MeGlc units generated signals detected at *m/z* 835.2, 673.2, 523.1, 361.1 and 185, respectively, further confirming the structure of one of the isomeric compounds
(Figures 3C and 5). The full analysis can be seen in the Supplementary Figure S2 (Fraction 12).

Further, the cleavage of the C_2 ion at *m/z* 673, 643, 629, 601, 583, 541 and 523 (Figure 3C) produced the ion at *m/z* 613, 583, 569, 541, 523, 481 and 463, respectively, through the loss of $C_2H_4O_2$ (60 Da) which indicates an α 1–4-linked glycosidic bond in the α-chain, which is in agreement with a previous study [100]. This observation is consistent with the fragmentation rules for ions of 1–4-linked disaccharides.

The MS/MS spectra show the presence of three different aglycone structures, namely ions detected at *m/z* 835.2, 819.2 and 803.4 by the losses of aglycone moieties. This analysis reveal the presence of at least six different isomers with different aglycones and sugar moieties, as the MS/MS spectra generate both key diagnostic fragments at *m/z* 507 and 523. These isomers are composed of five monosaccharaides including MeGlc-Glc-Qui (Glc)-Xyl-MeGlc (Glc or Qui). The proposed structures are shown in Figure 5 and correspond to Holothurinoside A_1 and Holothurinoside A, and four novel saponins [5,69,88]. We propose to call these molecules Holothurinosides S, Q, R and R_1, respectively.

It should be noted that both major fragment ions (507 and 523) can correspond to partial glycoside compositions or aglycone moieties, further supporting the presence of isomeric saponins. The predominant fragment ion at *m/z* 507 results from the sodium adduct ion of the [MeGlc-Glc-Qui + Na] side chain or the aglycone. Similarly, the abundant fragment ion at *m/z* 523 arises from the sodium adduct ion of the $[MeGlc\text{-}Glc\text{-}Glc + Na]^+$ side chain or the aglycone. Since the masses of sodiated aglycones are identical with their relative partial sugar residues, namely $[MeGlc\text{-}Glc\text{-}Qui + Na]^+$ and $[MeGlc\text{-}Glc\text{-}Glc + Na]^+$, the ions at *m/z* 507.2 and 523.2, respectively, correspond to both sugar residues and their aglycones. When the decomposition of the parent ion (*m/z* 1303.6) is triggered by the losses of sugar residues, as an exemplified by the black and pink dotted arrows in Figure 3A–C, the ions at *m/z* 507.2 and 523.2 correspond to the aglycone moieties. Alternatively, the fragmentation of the parent ion can proceed by the losses of all five sugar residues, which generates ions at *m/z* 507.2 and 523.2, which correspond to the aglycone moieties. Similar conclusions were drawn by Van Dyck *et al.* (2009) [69] for triterpene glycosides. Losses of H_2O and CO_2 or their combination result from cleavage at the glycosidic linkages as noted by Waller and Yamasaki [3].

Different fractions of the HPCPC separation were compared to show the presence of one aglycone (Figure 3A), the presence of two different aglycones (Figure 3B) and the presence of three different aglycones (Figure 3C) indicating that the HPCPC allowed the separation of the isomers.

On comparison of the MS/MS spectra of 1303.6 and 1243.5 (Figures 3 and 12), it is notable that the *m/z* 523 fragment (aglycone loss) of the $[M + Na]^+$ ions was only observed with 1303.6, which corresponds to the presence of a new aglycone unit at *m/z* 500 (sodiated 523). Individual patterns were detected from sulfated and non-sulfated saponins as indicated in Holothurin A and Holothurinoside A as representative examples. This sequential decomposition confirms the proposed Holothurin A and Holothurinoside A structures.

Another typical chemical structure elucidation of isomeric saponins by tandem MS is exemplified in Figure 6. This spectrum shows the ion signature of the sample under tandem MS from the ion detected at *m/z* 1287.6. Tandem MS analyses revealed the presence of two different aglycones with *m/z* values of 484 and 468, confirming the presence of chemical isomeric structures. The same fragmentation behaviors have been observed from the positive ESI-MS/MS spectra of saponins with *m/z* 1303. The structures of aglycones are identical with those reported for the ion at *m/z* 1303. The possible fragmentation pathways were shown using full and dotted arrows. The losses of aglycone moieties (Figure 6) generated ions at *m/z* 819.3 and 803.4, which correspond to the complete sugar components. The successive losses of aglycone, Glc or MeGlc, Xyl, Qui and MeGlc yielded to ion fragments at *m/z* 819, 657 or 643, 507, 361 and 185, respectively.

The decomposition of the parent ion can also be triggered by the loss of a sugar moiety, namely MeGlc, Glc, Qui, Qui or Glc and Xyl, followed by the aglycone, which generates daughter ions at *m/z* 1111.5, 949.5, 803.4, 657.2 or 643.2 and 507.2. It is clear that the ion at *m/z* 507 is the most abundant fragment ion and is the signature of the sodiated aglycone and/or the key sugar component. The losses of water (−18 Da) and/or carbon dioxide (−44 Da) are observed from the spectrum, and some of the peaks are also designated to those molecules.

This analysis revealed the presence of at least five different isomers with different aglycones and sugar moieties. These isomers contain some identical aglycone structures with those identified in the ion at 1303 (Figure 5). These isomers are also pentaglycosidic saponins. The proposed structures are shown in Figure 7, which correspond to Holothurinoside E_1, Holothurinoside E, 17-dehydroxy-holothurinoside A and two novel saponins (the first and fourth compounds). We propose to name these molecules Holothurinosides O and P, respectively.

The data indicate that the terminal sugar is preferentially lost first in glycosidic bond cleavages. Since Holothurinosides A and E contain the same terminal sugar units in their sugar residue, they yield the ions with the same *m/z* value (*m/z* 507).

2.3 Experimental Section

2.3.1 Sea Cucumber Sample

Twenty sea cucumber samples of *Holothuria lessoni* Massin *et al.* 2009, commonly known as Golden sandfish were collected off Lizard Island (latitude 14°41′29.46″ S; longitude 145°26′23.33″ E), Queensland, Australia in September 2010. The viscera (all internal organs) were separated from the body wall and kept separately in zip-lock plastic bags which were snap-frozen, then transferred to the laboratory and kept at −20 °C until use.

2.3.2 Extraction Protocol

The debris and sand particles were separated from the viscera (all internal organs) manually and the visceral mass was freeze-dried (VirTis, BenchTop K, New York, NY, USA). The dried specimens were then pulverized to a fine powder using liquid nitrogen and a mortar and pestle.

All aqueous solutions were prepared with ultrapure water generated by a Milli-Q systems (18.2 MΩ, Millipore, Bedford, MA, USA). All organic solvents were purchased from Merck (Darmstadt, Germany) except when the supplier was mentioned, and were either of HPLC grade or the highest degree of purity.

2.3.3 Extraction of Saponins

The extraction and purification procedures were adapted from Campagnuolo *et al.* [34], Van Dyck *et al.* [69], Garneau *et al.* [102] and Grassia *et al.* [103]. The pulverized viscera sample (40 g) was extracted four times with 70% ethanol (EtOH) (400 mL) followed by filtration through Whatman filter paper (No.1, Millipore, Bedford, MA, USA) at room temperature. The extract was concentrated under reduced pressure at 30 °C using a rotary evaporator (Büchi AG, Flawil, Switzerland) to remove the ethanol, and the residual sample was freeze-dried to remove water (VirTis, BenchTop K, New York, NY, USA). The dried residue was successively extracted using a modified Kupchan partition procedure [104]: The dried extract (15 g) was dissolved in 90% aqueous methanol (MeOH) (any remaining solid residue was removed by filtration), and partitioned against 400 mL of *n*-hexane (v/v) twice. The water content of the hydromethanolic phase was then adjusted to 20% (v/v) and then to 40% (v/v) and the solutions partitioned against CH_2Cl_2 (450 mL) and $CHCl_3$ (350 mL), respectively. In the next step, the hydromethanolic phase was concentrated to dryness using a rotary evaporator and freeze-drier. The dry powder was solubilized in 10 mL of MilliQ water (the aqueous extract) in order to undergo chromatographic purification.

2.3.4 Purification of the Extract

A solution of the aqueous extract was then subjected to a prewashed Amberlite® XAD-4 column (250 g XAD-4 resin 20–60 mesh; Sigma-Aldrich, MO, USA; 4 × 30 cm column) chromatography. After washing the column extensively with water (1 L), the saponins were eluted sequentially with MeOH (450 mL) and acetone (350 mL) and water (250 mL). The eluates (methanolic, acetone and water fractions) were then concentrated, dried, and redissolved in 5 mL of MilliQ water. Finally, the aqueous extract was partitioned with 5 mL isobutanol (v/v). The isobutanolic saponin-enriched fraction was either stored for subsequent mass spectrometry analyses or concentrated to dryness and the components of the extract were further examined by HPCPC and RP-HPLC. The profile of fractions was also monitored by Thin Layer Chromatography (TLC) using the lower phase of $CHCl_3$/MeOH/H_2O (7:13:8 v/v/v) solvent system.

2.3.5 Thin Layer Chromatography (TLC)

Samples were dissolved in 90% or 50% aqueous MeOH and 10 microliters were loaded onto silica gel 60 F_{254} aluminum sheets (Merck #1.05554.0001) and developed with the lower phase of

$CHCl_3$/MeOH/H_2O (7:13:8) biphasic solvent system. The profile of separated compounds on the TLC plate was visualized by UV light and by spraying with a 15% sulfuric acid in EtOH solution and heating for 15 min at 110 °C until maroon-dark purple spots developed.

2.3.6 High Performance Centrifugal Partition Chromatography (HPCPC or CPC)

The solvent system containing $CHCl_3$/MeOH/H_2O–0.1% HCO_2H (7:13:8) was mixed vigorously using a separating funnel and allowed to reach hydrostatic equilibration. Following the separation of the two-immiscible phase solvent systems, both phases were degassed using a sonicator-degasser (Soniclean Pty Ltd., Adelaide, SA, Australia). Then the rotor column of HPCPC™, CPC240 (Ever Seiko Corporation, Tokyo, Japan) was filled with the liquid stationary phase at a flow rate of 5 mL/min by Dual Pump model 214 (Tokyo, Japan).

The CPC was loaded with the aqueous upper phase of the solvent system in the descending mode at a flow rate of 5 mL/min with a revolution speed of 300 rpm. The lower mobile phase was pumped in the descending mode at a flow rate of 1.2 mL/min with a rotation speed of 900 rpm within 2 h. One hundred and twenty milligrams of isobutanol-enriched saponin mixture was dissolved in 10 mL of the upper phase and lower phase in a ratio of 1:1 and injected to the machine from the head-end direction (descending mode) following hydrostatic equilibration of the two phases indicated by a clear mobile phase eluting at the tail outlet. This indicated that elution of the stationary phase had stopped and the back pressure was constant. The chromatogram was developed at 254 nm for 3.0 h at 1.2 mL/min and 900 rpm using the Variable Wavelength UV-VIS Detector S-3702 (Soma optics Ltd., Tokyo, Japan) and chart recorder (Ross Recorders, Model 202, Topac Inc., Cohasset, MA, USA). The fractions were collected in 3 mL/tubes using a Fraction collector. The elution of the sample with the lower organic phase proceeded to remove the compounds with low polarity from the sample, within 200 mL of which several peaks were eluted. At this point (Fraction 54), the elution mode was switched to ascending mode and the aqueous upper phase was pumped at the same flow rate for 3.0 h. Recovery of saponins was achieved by changing the elution mode to the aqueous phase which allowed the elution of the remaining compounds with high polarity in the stationary phase. A few minor peaks were also monitored. Fractions were analyzed by TLC using the lower phase of $CHCl_3$/MeOH/H_2O (7:13:8) as the developing system. The monitoring of the fractions is necessary, as most of the saponins were not detected by UV due to the lack of a chromophore structure. Fractions were concentrated with nitrogen gas.

2.3.7 Mass Spectrometry

The isobutanol saponin-enriched fractions and the resultant HPCPC purified polar samples were further analyzed by MALDI and ESI MS to elucidate and characterize the molecular structures of compounds.

2.3.8 MALDI-MS

MALDI analysis was performed on a Bruker Autoflex III Smartbeam (Bruker Daltonik, Bremen, Germany). All MALDI MS equipment, software and consumables were from Bruker Daltonics

(Bremen, Germany). The laser (355 nm) had a repetition rate of 200 Hz and operated in the positive reflectron ion mode for MS data over the mass range of 400 to 2200 Da under the control of the FlexControl and FlexAnalysis software (V 3.3 build 108, Bruker Daltonik, Bremen, Germany). External calibration was performed using PEG. MS spectra were processed in FlexAnalysis (version 3.3, Bruker Daltonik, Bremen, Germany). MALDI MS/MS spectra were obtained using the LIFT mode of the Bruker Autoflex III with the aid of CID. The isolated ions were submitted to collision against argon in the collision cell to collisionally activate and fragment, and afford intense product ion signals. For MALDI, a laser energy was used that provided both good signal levels and mass resolution, the laser energy for MS/MS analysis was generally 25% higher than for MS analysis.

The samples were placed onto a MALDI stainless steel MPT AnchorChip TM 600/384 target plate. Alpha-cyano-4-hydroxycinnamic acid (CHCA) in acetone/ iso-propanol in ratio of 2:1 (15 mg/mL) was used as a matrix to produce gas-phase ions. The matrix solution (1 μL) was spotted onto the MALDI target plate and air-dried. Subsequently 1μL of sample was added to the matrix crystals and air-dried. Finally, 1 μL of NaI (Sigma-Aldrich #383112, St Louis, MO, USA) solution (2 mg/mL in acetonitrile) was applied onto the sample spots. The samples were mixed on the probe surface and dried prior to analysis.

2.3.9 ESI-MS

The ESI mass spectra were obtained with a Waters Synapt HDMS (Waters, Manchester, UK). Mass spectra were obtained in the positive ion mode with a capillary voltage of 3.0 kV and a sampling cone voltage of 100 V.

The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 80 °C; desolvation temperature, 350 °C; desolvation gas flow rate, 500 L/h. Data acquisition was carried out using Waters MassLynx (V4.1, Waters Corporation, Milford, CT, USA). Positive ion mass spectra were acquired in the V resolution mode over a mass range of 100–2000 *m/z* using continuum mode acquisition. Mass calibration was performed by infusing sodium iodide solution (2 μg/μL, 1:1 (v/v) water/isopropanol). For accurate mass analysis a lock mass signal from the sodium attached molecular ion of Raffinose (*m/z* 527.1588) was used.

MS/MS spectra were obtained by mass selection of the ion of interest using the quadrupole, fragmentation in the trap cell where argon was used as collision gas. Typical collision energy (Trap) was 50.0 V. Samples were infused at a flow rate of 5 μL/min, if dilution of the sample was required then acetonitrile was used [100]. Chemical structures were determined from fragmentation schemes calculated on tandem mass spectra and from the literature.

2.4 Conclusions

The extract of the viscera of sea cucumber *H. lessoni* was processed by applying HPCPC to purify the saponin mixture and to isolate saponin congeners and isomeric saponins. The tandem MS approach enabled us to determine the structure of a range of saponins. The purity of HPCPC fractions allowed mass spectrometry analyses to reveal the structure of isomeric compounds

containing different aglycones and/or sugar residues. Several novel saponins, along with known compounds, were identified from the viscera of sea cucumber.

This study is the first on saponins from the viscera of sea cucumbers. Our results to date highlight that there are a larger number of novel saponins in the viscera compared to the body wall (data not shown) indicating the viscera as a major source of these compounds. This paper is the first not only to report the presence of several novel saponins in the viscera of *H. lessoni* but also to indicate the highest number of saponin congeners detected in the viscera of any sea cucumber species. The mass of reported saponins for this species ranged from 460 Da to 1600 Da. So far we have identified more than ten aglycone structures in this species. Evidence from MALDI-MS suggested that the most intensive saponin ion was *m/z* 1243.5, a major component which seemed to correspond to Holothurin A. However, in the tandem MS, the most abundant ions are generally attributed to the loss of aglycones and/or both key diagnostic sugar moieties (507 and 523). Our results also showed that the incidence of the cross-ring cleavages was higher in the sulfated compounds compared to non-sulfated glycosides. It can be concluded that the presence of a sulfate group in the sugar moiety of saponins made them more vulnerable to cross-ring cleavages.

At the moment, MS is one of the most sensitive techniques of molecular analysis to determine saponin structures. This methodology of molecular structure identification using fragmentation patterns acquired from MS/MS measurements helps to propose and identify the structure of saponins. It was found that under CID some of the identified saponins had the same ion fingerprints for their aglycone units, yielding the same *m/z* daughter ions. Some of these saponins were easily characterized based on MS/MS measurement since their CID spectra contained the key diagnostic signals at *m/z* 507 and 523, corresponding to the oligosaccharide chains [MeGlc-Glc-Qui + Na^+] and [MeGlc-Glc-Glc + Na^+], respectively. The simultaneous loss of two sugar units indicated characteristics of a branched sugar chain. This methodology also permitted the structural elucidation of isomers.

Sea cucumbers have developed a chemical defense against potential predators based upon saponins. Our finding indicates that the viscera are rich in saponins, in both diversity and quantity, and that these saponins are apparently more localized in the viscera than in the body wall.

The chromatography techniques used in this study were able to for the first time, separate high purity saponins from sea cucumber, highlight the diversity of saponin congeners, and stress the unique profile of saponins for this species. MALDI and ESI-MS proved to be sensitive, ultra-high-throughput methodologies to identify these secondary metabolites in a complex mixture. Therefore, mass spectrometry has become the preferred techniques for analysis of saponins, as both ESI-MS and the MALDI-MS spectra provide remarkable structural information. However, the MALDI data is simpler to interpret compared to ESI-MS data due to the singly charged ions. This ancient creature with a long evolutionary history is a unique source of high-value novel compounds.

This manuscript describes the structure elucidation of seven novel compounds; Holothurinoside O, Holothurinoside P, Holothurinoside Q, Holothurinoside R, Holothurinoside R_1, Holothurinoside S and Holothurinoside T in addition to six known compounds, including Holothurin A, Holothurinoside A, Holothurinoside A_1, Holothurinoside E, Holothurinoside E_1 and 17-dehydroxy-holothurinoside A.

In conclusion, our findings show that the viscera of *H. lessoni* contain numerous unique and novel saponins with a high range of structural diversity, including both sulfated and non-sulfated congeners, and with different aglycone and sugar moieties. Furthermore, the tremendous range of structural biodiversity of this class of natural metabolites, which enables them to present in a remarkable functional diversity, is potentially an important source for the discovery of high-value compounds for biotechnological applications.

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2.6 Author Contributions

Y.B., C.F. and W.Z. designed the experiments. Y.B. carried out the experiments with guidance of C.F. and W.Z., who assisted in setting up the HCPCP analysis. Y.B., C.F. and W.Z. worked together on chemical structure elucidation, and all three authors contributed in writing the manuscript.

2.7 Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 3

STRUCTURE ELUCIDATION OF NOVEL SAPONINS IN THE VISCERA OF THE SEA CUCUMBER *HOLOTHURIA LESSONI*

This chapter is my second paper published in the journal “Marine Drugs”.

The paper is cited as “Bahrami, Y.; Zhang, W.; Chataway, T.; Franco, C.M.M. (2014), Structural elucidation of novel saponins in the sea cucumber *Holothuria lessoni*. Mar. Drugs 12, 4439–4473.” doi:10.3390/md12084439

This chapter reports the isolation, purification and structure elucidation of 5 novel saponins in the viscera of sea cucumber *Holothuria lessoni*, including Holothurins D/E and Holothurinosides X/Y/Z, along with seven reported triterpene glycosides, and includes an introduction, material and methods and experimental sections; HPCPC purification, and MS analyses, and described the HPCPC purification and MS analysis in detail.

I performed the experiments with the guidance of my supervisors Prof. Chris Franco and Prof. Wei Zhang, who assisted in setting up the HCPCP. I purified and analysed the samples and deduced the chemical structure of the compounds, and then I wrote the draft manuscript. CF confirmed the analyses, proofread and edited the manuscript, and TC also proofread the manuscript and offered his comments. I drafted the responses to the reviewers’ comments which were revised by CF.

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Article

Structure Elucidation of Novel Saponins in the Sea Cucumber *Holothuria lessoni*

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Abstract: Sea cucumbers are prolific producers of a wide range of bioactive compounds. This study aimed to purify and characterize one class of compound, the saponins, from the viscera of the Australian sea cucumber *Holothuria lessoni*. The saponins were obtained by ethanolic extraction of the viscera and enriched by a liquid-liquid partition process and adsorption column chromatography. A high performance centrifugal partition chromatography (HPCPC) was applied to the saponin-enriched mixture to obtain saponins with high purity. The resultant purified saponins were

profiled using MALDI-MS/MS and ESI-MS/MS which revealed the structure of isomeric saponins to contain multiple aglycones and/or sugar residues. We have elucidated the structure of five novel saponins, Holothurins D/E and Holothurinosides X/Y/Z, along with seven reported triterpene glycosides, including sulfated and non-sulfated saponins containing a range of aglycones and sugar moieties, from the viscera of *H. lessoni*. The abundance of novel compounds from this species holds promise for biotechnological applications.

Keywords: sea cucumber viscera; saponins; bioactive compounds; MALDI; ESI; mass spectrometry; HPCPC; triterpene glycosides; structure elucidation; marine invertebrate; Echinodermata; holothurian

3.1 Introduction

Holothurians vary in size, shape and color, and belong to the class Holothuroidea of the *Echinodermata* phylum [1]. Sea cucumbers are known to produce a range of compounds which are present in agricultural or agrochemical, nutraceutical, pharmaceutical and cosmeceutical products [2–5].

Sea cucumbers are used in traditional Asian medicine to treat diseases such as rheumatoid arthritis, joint-pain, cardiovascular, tendonitis, gastric, osteoarthritis, ankylosing spondylitis, arthralgia, tumors, fungal infection, impotence, frequent urination and kidney deficiency, high blood pressure, arthritis and muscular disorders [6–10] and are also used as a general tonic [9,11,12]. Of the many chemical classes present in sea cucumbers, saponins are the most important and abundant secondary metabolites [13–19]. They are generally perceived as highly active natural products and the sea cucumber saponins have been well characterized for their biological activities. They possess a wide range of therapeutic applications due to their cardiovascular, immunomodulator, cytotoxic, anti-asthma, anti-eczema, anti-inflammatory, anti-arthritis, anti-oxidant, anti-diabetics, anti-bacterial, anti-viral, anti-cancer, anti-angiogenesis, anti-fungal, hemolytic, cytostatic, cholesterol-lowering, hypoglycemia and anti-dementia activities [3,12,14,20–33].

Saponins are produced by a limited number of marine species which belong to the phylum *Echinodermata* [34], namely holothuroids (sea cucumbers) [14,17,20,35–41], asteroids, and sponges from the phylum *Porifera* [20,42,43]. They are amphipathic compounds that generally possess a triterpene or steroid backbone or aglycone which in sea cucumbers is of the holostane type [44,45]. Although sea cucumber saponins usually share common features, their aglycones, also called sapogenins or genins, are significantly different from those reported in the plant kingdom [3]. They comprise a lanostane-3β-ol type aglycone containing a γ-18 (20)-lactone in the D-ring of tetracyclic triterpene (3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone) [46] and can contain shortened side chains and a carbohydrate moiety consisting of up to six monosaccharide units covalently connected to C-3 of the aglycone [14,15,20,45,47–51].

The sugar moiety of the sea cucumber saponins consists principally of D-xylose, D-quinovose, 3-*O*-methyl-D-glucose, 3-*O*-methyl-D-xylose and D-glucose and less frequently 3-*O*-methyl-D-

quinovose, 3-*O*-methyl-D-glucuronic acid and 6-*O*-acetyl-D-glucose [47,49,52–57]. In the oligosaccharide chain, the first monosaccharide unit is always a xylose, whereas 3-*O*-methylglucose or 3-*O*-methylxylose are always the terminal sugars. A plant saponin may contain one, two or three saccharide chains, often with an acyl group bound to the sugar moiety [58], whereas, in sea cucumbers, the sugar residue has only one branch [49].

Over 500 triterpene glycosides have been reported from various species of sea cucumbers [11,12,14,20,25,36,46,47,55,59] and are classified into four main structural categories based on their aglycone moieties; three holostane type glycoside group saponins containing a (1) 3β-hydroxyholost-9 (11)-ene aglycone skeleton; (2) saponins with a 3β-hydroxyholost-7-ene skeleton; and (3) saponins with an aglycone moiety different to the other two holostane type aglycones (other holostane type aglycones); and (4) a nonholostane aglycone [46,48,54,60,61].

Many of the saponins from marine organisms have sulfated aglycones or sugar moieties [3]. Sulfation of the oligosaccharide chain in the Xyl, Glc and MeGlc residues have been reported in sea cucumber saponins [48,49,54,62,63]. Most of them are mono-sulfated glycosides with few occurrences of di- and tri-sulfated glycosides. Saponin diversity can be further enhanced by the position of double bonds and lateral groups in the aglycone.

The most commonly accepted biological role for these secondary metabolites in nature is that they are a powerful defense mechanism for sea cucumbers as they are deleterious for most organisms [13–18,64–66] and are responsible for the organism's environmental defense mechanisms in general. Sea cucumbers expel their internal organs as a defense mechanism called evisceration, a reaction that includes release of the respiratory tree, intestine, cuvierian tubules and gonads through the anal opening [59,67–74]. The deterrent effect of saponins seems, therefore, to act as an aposematic signal, warning potential predators of the unpalatability of the holothuroid tissues [70]. In contrast, a recent study has shown that these repellent chemicals are also kairomones attracting the symbionts and are used as chemical "communicates" [67]. However, in the sea cucumber, it was suggested that saponins may also have two regulatory roles during reproduction: (1) to prevent oocyte maturation and (2) to act as a mediator of gametogenesis [25,75].

In this paper we describe the structural characterization of novel bioactive triterpene glycosides from the viscera (which comprises all internal organs other than the body wall) of an Australian sea cucumber *Holothuria lessoni* (golden sandfish) [76]. *H. lessoni* is a newly-identified Holothurian species, which is abundant in Australian waters. We hypothesize that the reason for their ingenious form of defense is because their internal organs contain high levels of compounds that repel predators [72,77–79]. The results of this project will assist in transforming viscera of the sea cucumber into high value co-products, important to human health and industry.

We have used matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS), and MS/MS to elucidate the structure of five novel isomeric saponins. Knowledge of the chemical structure of compounds is very important for determining the specific correlation between the structure and their molecular and biological mechanism(s) of actions [22,25,28,46].

3.2 Results and Discussion

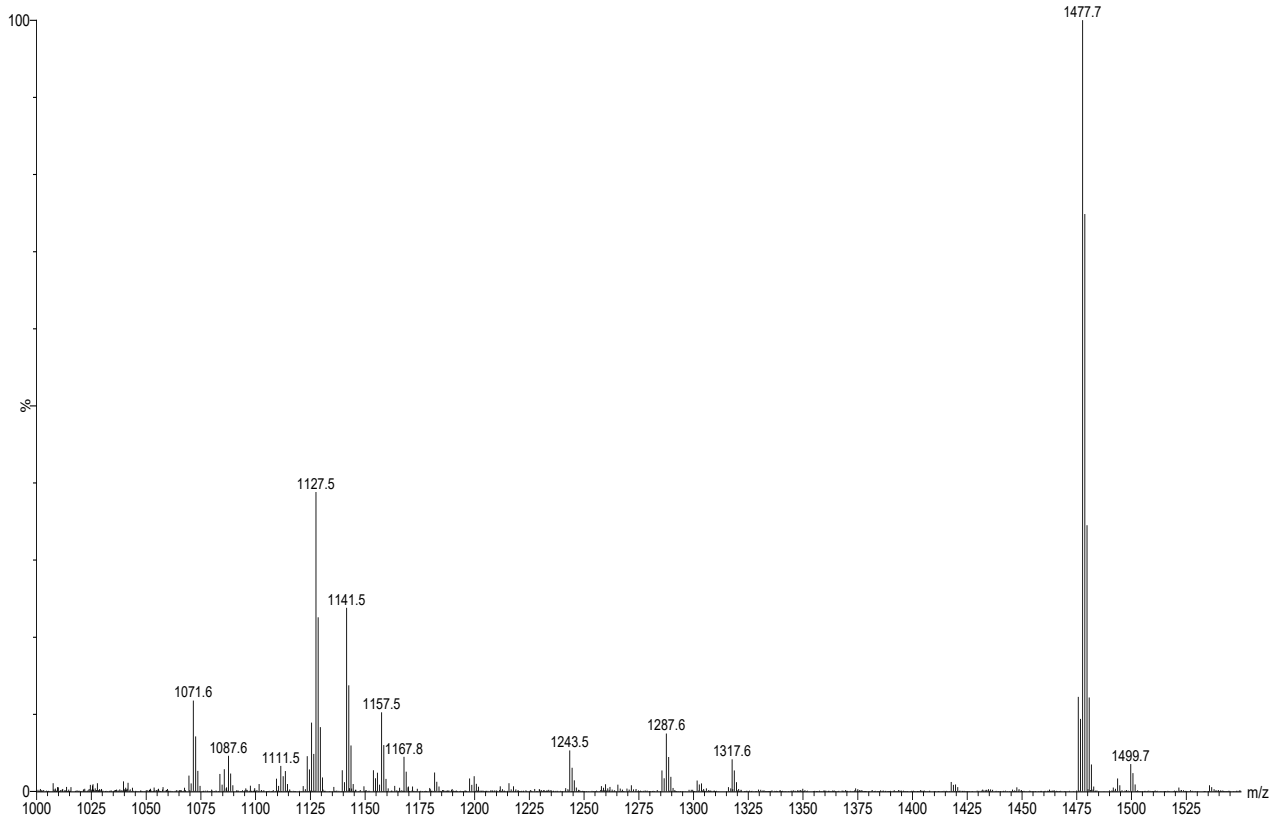
Several saponins were isolated and purified from the viscera of *H. lessoni* sea cucumber species using HPCPC. The extraction and purification procedures and the mass spectrometry analysis was described in detail previously [12]. Mass spectrometry has been applied for the structure elucidation of saponins in both negative and positive ion modes [80–86]. In this study, identification of the sugar component of saponin compounds was performed by soft ionization MS techniques including MALDI and ESI in positive ion mode. The low CID energy MS/MS techniques used here do not fragment the aglycone. In addition, LC was not used to separate the compounds before introduction into the mass spectrometer. Instead high performance centrifugal partition chromatography (HPCPC) was conducted which we believe is more efficient for the separation of saponins. Identification of the aglycone component of the saponins was performed by comparison with published data. In these papers, the structure elucidation of the aglycones was confirmed predominantly by NMR which is capable of determining detailed structural analysis. Therefore, while we are confident of the assignment of the aglycones, confirmation of these assignments should be made by NMR. We have previously highlighted the presence of isomers in the saponin mixture [12]. The MS analysis was conducted by introducing sodium ions to the samples. Because of the high affinity of alkali cations for triterpene glycosides, all saponins detected in the positive ion mode spectra were predominantly singly charged sodium adducts of the molecules $[M + Na]^+$. The main fragmentation of saponins generated by cleavage of the glycosidic bond yielded oligosaccharide and monosaccharide fragments [24]. Other visible peaks and fragments were generated by the loss of other neutral moieties such as CO_2, H_2O or CO_2 coupled with H_2O.

The appropriate HPCPC fractions were pooled based on their thin-layer chromatography (TLC) profiles (Supplementary Figure S1), concentrated to dryness, and analyzed by MALD-MS/MS and ESI-MS/MS. The ESI and MALDI spectra reflect the saponin profile of each HPCPC fraction.

3.3 Structure Elucidation of Saponins by ESI-MS

ESI-MS^n is a very effective and powerful technique to differentiate isomeric saponins as they exhibit different MS^n fingerprints spectra [77,87,88]. ESI-MS/MS analysis was conducted on all saponin ions detected in the ESI-MS spectrum of the HPCPC fractions in positive ion mode. ESI mass spectra of the saponins are dominated by $[M + Na]^+$. The ESI-MS spectrum of the saponin extract from Fraction 18 of the viscera of *H. lessoni* is shown in Figure 1.

Figure 1. (+) Electrospray ionization- mass spectrometry (ESI-MS) spectrum of saponins purified by HPCPC from Fraction # 18 of the extract from the viscera of *H. lessoni*.



Fifteen major peaks were detected which correspond to several novel and known triterpene compounds. It is notable that the observed ions generate from cationization of the neutral molecules in MALDI or ESI. The ions at *m/z* 1071.6 (Unidentified), 1087.6 (Unidentified), 1125.5 (Holothurinosides C/C_1), 1141.5 (Desholothurin A_1 and Desholothurin A (synonymous with Nobiliside 2A), 1157.5 (Holothurinoside J_1), 1227.4 (Fuscocinerosides B/C or Scabraside A or 24-dehydroechinoside A and a novel saponin), 1243.5 (Holothurin A), 1287.6 (Holothurinosides E/E_1/O/P), 1301.6 (Holothurinosides M), 1303.6 (Holothurinosides A/A_1/Q/R/R_1/S), 1305.4 (Unidentified), 1317.6 (Holothurinoside N), 1417.7 (Unidentified), 1477.7 (Unidentified), 1479.7 (Holothurinoside I), 1493.7 (Unidentified) and 1495.7 (Holothurinoside K_1) were detected. The spectrum displays one dominant peak at *m/z* 1477.7 which corresponds to an unidentified (novel) saponin(s), with an elemental composition of $C_{61}H_{114}O_{38}$ which requires further analysis and will be published later. The names, elemental compositions and producing organisms of saponins detected in this fraction are summarized in Table 1. Further analysis revealed that some of these peaks represented more than one compound. The ions at *m/z* 1227.4, 1229.5, 1243.5, and 1259.5 are sulfated compounds, whereas the ion peaks at *m/z* 1125.5, 1141.5, 1287.6, 1301.6 and 1303.6 correspond to the non-sulfated saponins.

Thus Fraction 18 contains several saponin congeners indicating that complete separation of the saponins was not possible within a single HPCPC run due to the high similarity of their structures. However, this technique allowed the separation of a number of saponins, including some isomers.

Our method to elucidate or propose the molecular structure of saponins was based on the MS/MS spectra as described in detail in Bahrami *et al*. [12]. Firstly, fragmentation patterns were built, and the generated fingerprint signals were reconstructed according to the measurement of the mass transitions between the successive collision-activated fragmentation signals. In other words, based on MS/MS spectra the molecular structures of the saponins were obtained by the identification of the mass transitions between the successive collision-induced fragmentation peaks.

The ESI and MALDI mass spectrum of the isobutanol-enriched saponin extract obtained from the viscera of the *H. lessoni* shows a diverse range of saponins [12]. Our results revealed that at least 75 saponins (29 sulfated and 46 non-sulfated) were detected in *H. lessoni*, including 39 new sulfated, non-sulfated and acetylated triterpene glycosides, containing a wide range of aglycone and sugar moieties of which 36 congeners were previously identified in other holothurians.

In this manuscript we describe the structure elucidation of ions at *m/z* 1127.6, 1227.4, 1243.5 and 1259.5 (from HPCPC Fractions 17, 18, 20 and 22). Among these saponins, Holothurin A is the reported major congener with the highest relative abundance in this species.

Table 1 summarizes the data of all analysis performed on the HPCPC fraction 18 using MALDI-MS and ESI-MS on compounds. This fractionated sample contained 14 novel saponins along with 27 reported triterpene glycosides, including 14 sulfated and 27 non-sulfated saponin ions.

Table 1. Summary of saponins identified from Fraction 18 of the viscera of *H. lessoni* by MALDI-MS/MS and ESI-MS/MS. This table includes the 14 novel identified compounds (N) along with the 27 published compounds (P).

$[M + Na]^+$ *m/z*	MW	Formula	Compound's Name	Novel (N)/ Published(P)	Sea Cucumber Species A. = *Actinopyga*; B. = *Bohadschia*; P. = *Pearsonothuria*; H. = *Holothuria*	References
1071.6	1048	$C_{47}H_{93}NaO_{21}S$	Unidentified	N	*H. lessoni*	[12]
1083.3	1060	$C_{58}H_{64}O_{25}$	Unidentified	N	*H. lessoni*	[12]
1087.6	1064	$C_{47}H_{93}NaO_{22}S$	Unidentified	N	*H. lessoni*	[12]
1123.5	1100	$C_{54}H_{84}O_{23}$	Unidentified	N	*H. lessoni*	[12]
1125.5	1102	$C_{54}H_{86}O_{23}$	Holothurinoside C Holothurinoside C_1	P P	*H. lessoni*, *H. forskali*, *A. agassizi*, *H. scabra*, *H. fuscocinerea* and *H. impatiens*	[12,67,69,77,89,90]
1127.6	1104	$C_{54}H_{88}O_{23}$	Unidentified	N	*H. lessoni*	-
		$C_{53}H_{84}O_{24}$	Unidentified	N	*H. lessoni*	-
			Unidentified	N	*H. lessoni*	-
1141.5	1118	$C_{54}H_{86}O_{24}$	Desholothurin A (Nobiliside 2a), Desholothurin A_1	P P	*H. lessoni*, *H. forskali*, *H. nobilis*, *A. agassizi*, *B. argus*, *B. cousteaui*, *H. leucospilota*, *P. graeffei*	[4,12,13,77,89–93]

$[M + Na]^+$ *m/z*	**MW**	**Formula**	**Compound's Name**	**Novel (N)/ Published(P)**	**Sea Cucumber Species** **A. = *Actinopyga*; B. = *Bohadschia*; P. = *Pearsonothuria*; H. = *Holothuria***	**References**
			(Arguside E)	P		
1149.2	1126	a *	Holothurinoside T	P	*H. lessoni*	[12]
1157.5	1134	$C_{54}H_{109}O_{25}$	Holothurinoside J_1	P	*H. lessoni*, *B. subrubra*	[12,59]
		$C_{49}H_{91}NaO_{25}S$	Unidentified	N	*H. lessoni*	-
1193.5	1170	$C_{55}H_{87}NaO_{23}S$	Unidentified	N	*H. lessoni*	-
1227.4	1204	$C_{54}H_{85}NaO_{26}S$	Fuscocinerosides B/C, Scabraside A or 24–dehydroechinoside A, Unidentified	P P P N	*B. subrubra*, *H. lessoni*, *H. scabra*, *H. leucospilota*, *H. fuscocinerea*, *A. agassizi*, and *H. impatiens*, *P. graeffei*, *A. echinites*	[12,13,36,64,67,69,90,94–96]
1243.5	1220	$C_{54}H_{85}NaO_{27}S$	Holothurin A Scabraside B 17-Hydroxy fuscocineroside B 25-Hydroxy fuscocinerosiden B	P P P P	*H. lessoni*, *H. scabra*, *H. atra*, *H. leucospilota*, *H. arenicola*, *H. cinerascens*, *H. coluber*, *H. cubana, H. difficilis*, *H. gracilis*, *H. pervicax*, *H. lubrica*, *H. polii*, *H. pulla*, *H. squamifera*, *H. surinamensis*, *H. tubulosa*, *P. graeffei*, *A. agassizi*, *A. echinites*, *A. lecanora*, *A. mauritana*, *H. grisea*, *H. hilla*, *H. Mexicana*, *H. moebi*, *H. nobilis*, *H. monacaria*, *H. forskali*,	[12,36,44,67,72,79,93,96–103]

$[M + Na]^+$ *m/z*	**MW**	**Formula**	**Compound's Name**	**Novel (N)/ Published(P)**	**Sea Cucumber Species** **A. = *Actinopyga*; B. = *Bohadschia*; P. = *Pearsonothuria*; H. = *Holothuria*****	**References**
					H. edulis, *H. axiloga*, *H. fuscocinerea* and *H. impatiens*	
1259.5	1236	$C_{54}H_{85}NaO_{28}S$	Holothurin A_3	P	*H. lessoni*, *H. scabra*, *H. fuscocinerea* and *H. impatiens*	[12,44,69]
			Unidentified	N	*H. lessoni*	-
1287.6	1264	$C_{60}H_{96}O_{28}$	Holothurinoside $E_,$	P	*H. lessoni*, *H. forskali*	[12,77]
			Holothurinoside E_1	P	*H. lessoni*, *H. forskali*	[12,77]
			Holothurinoside O	P	*H. lessoni*	[12]
			Holothurinoside P	P	*H. lessoni*	[12]
			17-dehydroxyholothurinoside A	P	*H. lessoni*, *H. grisea*, *B. cousteaui*	[4,12,104]
1301.6	1278	$C_{61}H_{98}O_{28}$ $C_{60}H_{94}O_{29}$	Holothurinoside M Unidentified	P N	*H. lessoni*, *H. forskali*, *H. scabra*, *H. fuscocinerea* and *H. impatiens H. lessoni*	[12,67,69,70] -
1303.6	1280	$C_{60}H_{96}O_{29}$	Holothurinoside A	P	*H. lessoni*, *H. forskali*, *B. vitiensis*, *B. cousteaui*	[4,12,67,77,89]

$[M + Na]^+$ *m/z*	**MW**	**Formula**	**Compound's Name**	**Novel (N)/ Published(P)**	**Sea Cucumber Species** **A. = *Actinopyga*; B. = *Bohadschia*; P. = *Pearsonothuria*; H. = *Holothuria***	**References**
			Holothurinoside A_1	P	*H. lessoni*, *H. forskali*, *B. vitiensis*, *B. cousteaui*	[4,12,67,77,89]
			Holothurinoside Q	P	*H. lessoni*	[12]
			Holothurinoside S	P	*H. lessoni*	[12]
			Holothurinoside R	P	*H. lessoni*	[12]
			Holothurinoside R_1	P	*H. lessoni*	[12]
1317.6	1294	$C_{61}H_{98}O_{29}$	Holothurinoside N	P	*H. lessoni*, *H. forskali*	[12,67]
1475.6	1452	$C_{65}H_{96}O_{36}$	Unidentified	N	*H. lessoni*	-
1477.7	1454	$C_{61}H_{114}O_{38}$	Unidentified	N	*H. lessoni*	-
1479.7	1456	$C_{67}H_{108}O_{34}$	Holothurinoside I	P	*H. lessoni*, *H. forskali*	[12,92]
1495.7	1472	$C_{61}H_{116}O_{39}$	Holothurinoside K_1	P	*B. subrubra*, *H. lessoni*	[12,59]
		$C_{72}H_{112}O_{31}$	Unidentified	N	*H. lessoni*	-

[a] *: The composition was not measured through ESI analysis.

3.3.1 Determination of the Saponin Structures by ESI-MS/MS

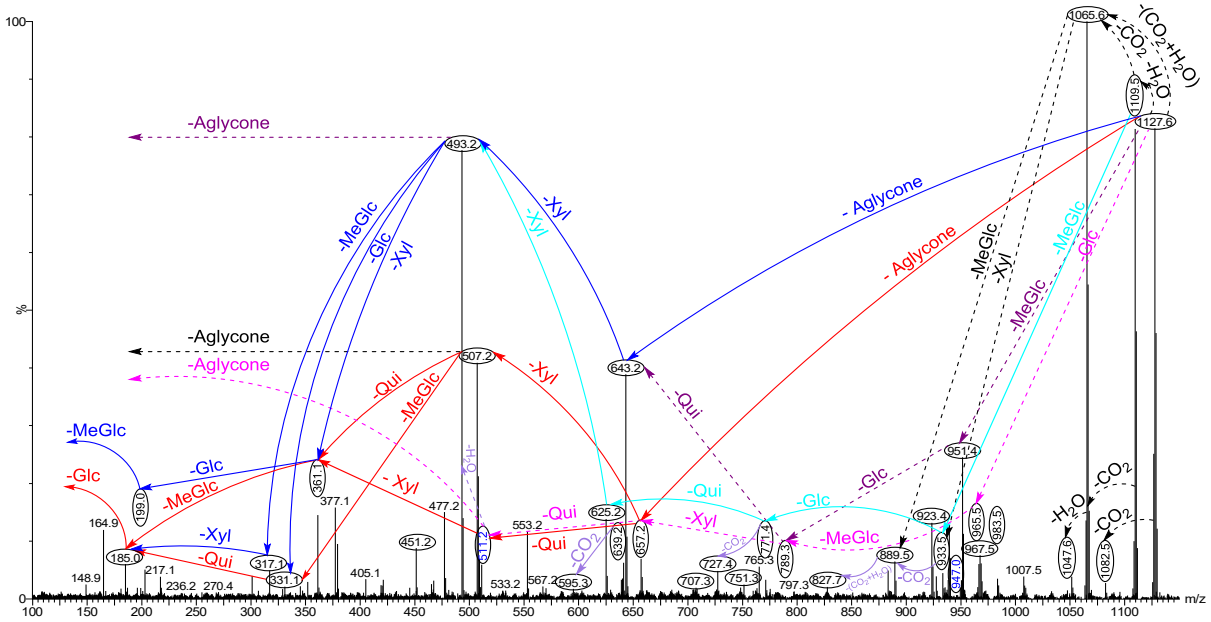
In order to differentiate between isomeric saponins following chromatographic separation, tandem mass spectrometry analysis was performed. Saponin ion peaks were analyzed by ESI MS/MS and confirmed using MALDI MS/MS. ESI-MS/MS was carried out using Collisional Induced-Dissociation (CID), creating ion fragments from the precursor ions. In general, the formation of fragments occurred predominantly by the cleavage of glycosidic bonds in the positive ion mode (Figure 2), which was applied to identify the structure of saponins. Interpretation and assignment of fragment ions of MS/MS spectra provided the key information for the structural elucidation of saponins. CID can provide valuable structural information about the nature of the carbohydrate residues, as it preferentially cleaves glycosides at glycosidic linkages, which makes assignment of the sugar residues and elucidation of the structure relatively straight forward.

ESI-MS was used to distinguish the isomeric saponins as described by Song *et al*. [87]. Following HPCPC separation, tandem mass spectrometry coupled with electrospray ionization (ESI-MS/MS) allowed the identification of isomers. MS/MS spectra of these ions provided detailed structural information and enabled differentiation of the isomeric saponins following HPCPC separation. The stepwise structure elucidation analysis applied to the ion at *m/z* 1127.6 (non-sulfated saponins), obtained from Fractions 17 and 18 is shown in Figure 2A,B. The peak at *m/z* 1127 was shown to contain at least three different saponin congeners.

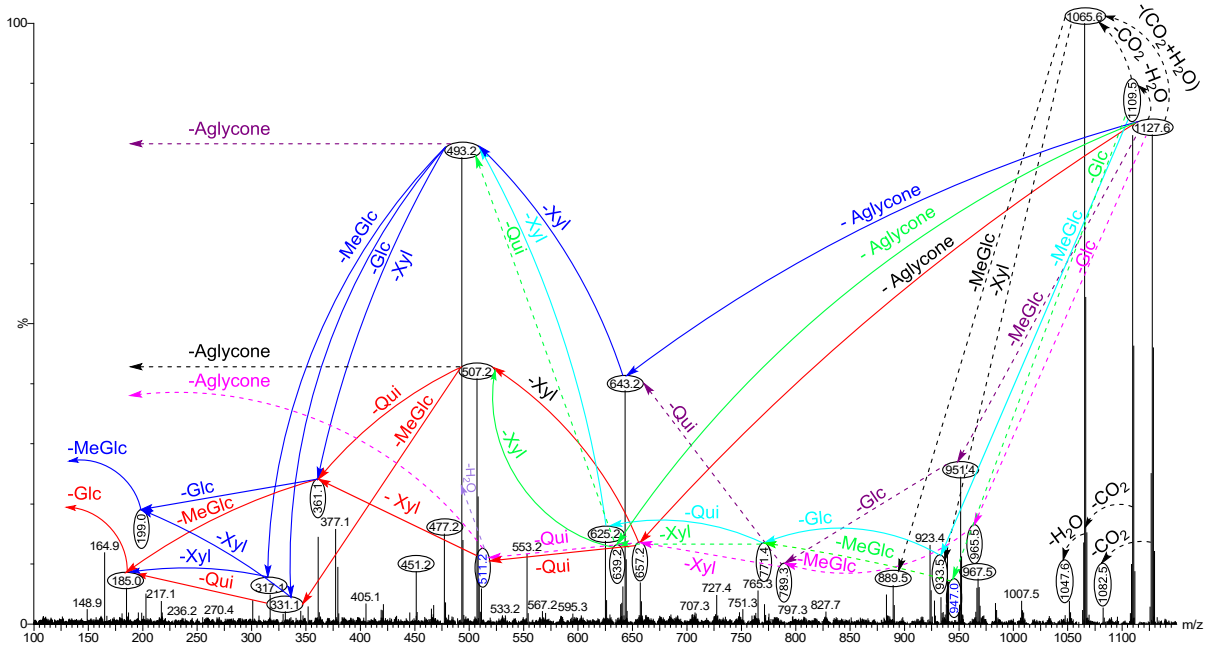
CID initiates two feasible fragmentation pathways of cationized parent ions shown in full with dotted arrows. First, the successive losses of the sugar moieties 3-*O*-methylglucose (MeGlc), glucose (Glc), quinovose (Qui) and xylose (Xyl) followed by the aglycone (Agl) unit generate ion products detected at *m/z* 951.4, 789.3, 643.2, and 493.2, respectively (Figure 2A,B), which proposed the structure of Holothurinoside Y (Figure 3a). In another isomer, the sequential losses of MeGlc, Glc, Xyl, and Xyl followed by the aglycone residue correspond to ions observed at *m/z* 951.4, 789.3, 657.2 and 507.2, respectively, which postulates the structure of Holothurinoside X. In this case, the ions at *m/z* 493.2 and 507.2 correspond to the sodiated aglycone moieties.

Further, the consecutive losses of MeGlc, Glc, Qui, and Xyl from the ion at *m/z* 1109.5 generated the fragment ions shown in Figure 3b, confirming the structure of Holothurinoside Y. In addition to 1109.6, the ion at 1065.6 can be fragmented and produced ion products exhibited in Figure 3c. The sugar moiety was found to be identical to those of Intercedenside D and Eximisoside A isolated from sea cucumbers *Mensamaria intercedens* [105] and *Psolus eximius* [37], respectively. Both groups also stated the ions at *m/z* 625.2 and 493.1 correspond to $[MeGlc + Xyl + Glc + Xyl + Na]^+$ and $[MeGlc + Xyl + Glc + Na]^+$, respectively, which agrees with our results.

Figure 2. Positive tandem ESI spectrum analysis of saponins detected at *m/z* 1127.6, Fraction 17; (**A**) and Fraction 18; (**B**) Full and dotted arrows show the two main feasible fragmentation pathways. The figures indicate the collision-induced fragmentation of parent ions at *m/z* 1127.6. The consecutive losses of the aglycone (Agl), xylose (Xyl), quinovose (Qui) and 3-*O*-methylglucose (MeGlc) residues affords product ions detected at *m/z* 657.2, 507.2, 361.1 and 185.0, respectively, which indicate the structure of a novel saponin. The predominant peak (**A** and **B**) at *m/z* 493.2 corresponds to either the diagnostic sugar residue or the aglycone moiety. The major abundant peak (**A** and **B**) at *m/z* 507 also corresponds to either the key sugar residue or the aglycone moiety.

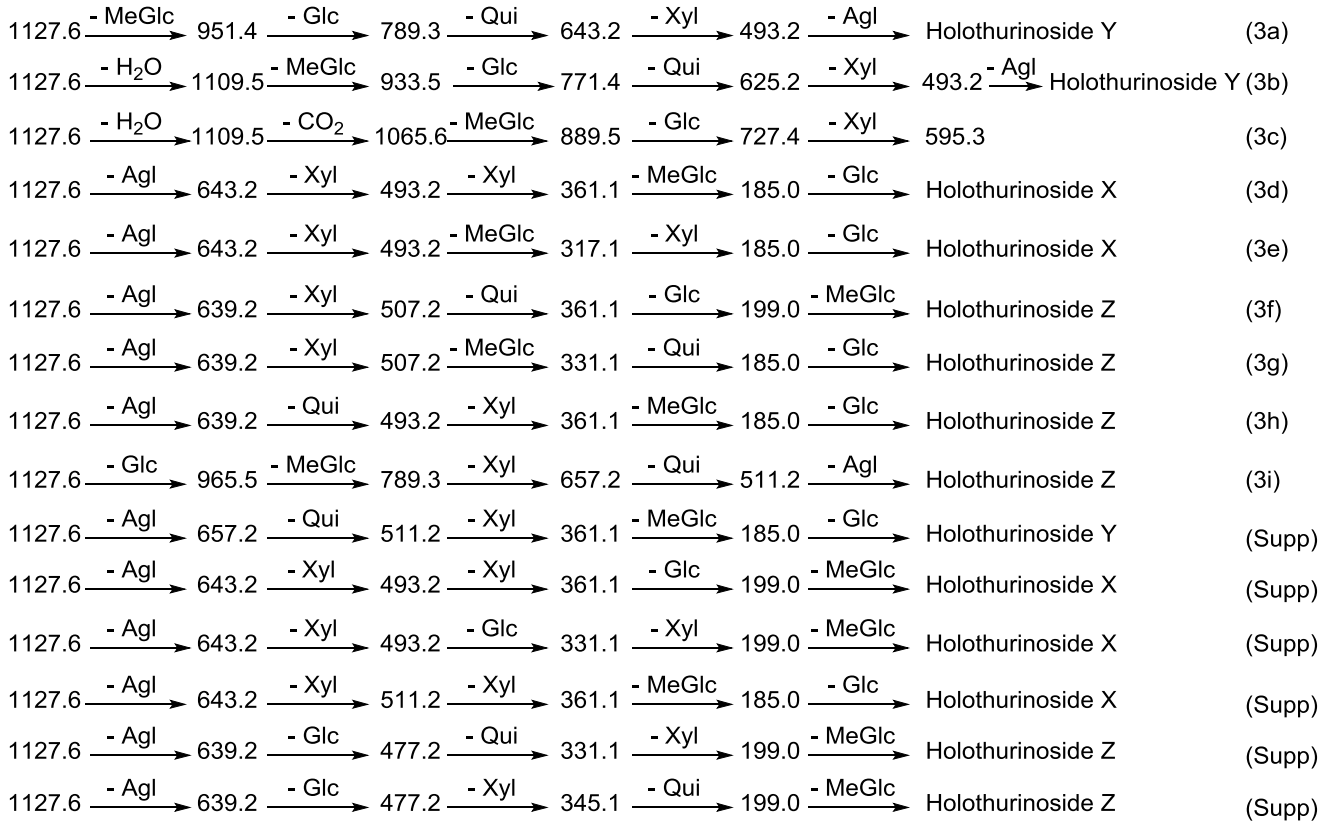


(**A**)



(**B**)

Figure 3. Schematic fragmentation patterns of the ion detected at *m/z* 1127.6.



Secondly the decomposition of the precursor ions can also be triggered by the losses of the aglycone residues, creating peaks at *m/z* 657.2, 643.2 and 639.2 (Figure 2A,B) corresponding to the complete sugar moieties of the ion at *m/z* 1127.6, and indicates the presence of three types of aglycones. The consecutive losses of the aglycone (generated ion at *m/z* 657.2), Xyl (generated ion at *m/z* 507.2), Qui (generated ion at *m/z* 361.1), and MeGlc (generated ion at *m/z* 185.0), respectively, were produced by glycone and aglycone fingerprint peaks from the precursor ion, confirming the structure of Holothurinoside Y. In the second isomer, the consecutive losses of the aglycone, Xyl, Xyl and MeGlc followed by Glc presenting masses described in Figure 3d, additionally indicate that the decomposing ions were generated from the sodiated ion at *m/z* 1127.6. In this case, the ions at *m/z* 493.2 and 507.2 correspond to the key diagnostic sugar resides. Moreover, the ion at *m/z* 493.2 generated ions at *m/z* 317.1 and 185.0 by the losses of MeGlc and Xyl, respectively (Figure 3e), further confirm that the fragment ions unambiguously originate from a novel sodium-cationized saponin which we name as Holothurinoside X.

The ions at *m/z* 657.2 and 643.2 resulted from the loss of aglycones from the parent ion at *m/z* 1127.6, and are the fragment ions corresponding to the complete saccharide chains, which subsequently (Figure 2A) produce the ions at *m/z* 511.2 or 507.2 and ion at *m/z* 493.2 due to the losses of Qui or Xyl and Xyl residues. The ions (*m/z* 511) further fragmented to form ions of the same *m/z* value at *m/z* 361 and *m/z* 199 or 185. The observation of ions at *m/z* 507.2 and 511.2 further supports the above conclusion. The ion at *m/z* 511.2 can also be ascribed to the mass of the sodiated aglycone.

Similar analysis was carried out on another isomer (Figure 2B). As can be seen in the figure, the spectrum has (green arrows) a different fragmentation pattern from that seen in other isomeric compounds even though they have the same *m/z* value which indicates the presence of another isomer. For this isomer, the consecutive losses of the aglycone, Xyl, Qui and Glc or MeGlc units generated signals shown in Figure 3f, further confirmed the structure of one of the isomeric compounds (Figure 2B). Alternatively, the ion at 507.2 led to the ions at *m/z* 331.1 and 185.0 by the sequential loss of MeGlc and Qui (Figure 3g). On the other hand, the consecutive losses of the aglycone, Qui, Xyl and Glc or MeGlc units generated ion fingerprints illustrated in Figure 3h, which postulates the structure of another isomer from this compound (Holothurinoside Z). The decomposition of the parent ion can also be triggered by the loss of sugar moiety, namely Glc, MeGlc, Xyl, and Qui followed by the aglycone which generated the product ions indicated in Figure 3i. It should be noted that the major characteristic peaks at 493, 507 and 511 correspond to either the sodiated partial glycoside compositions or the sodiated aglycone moieties [12,59,77,106], which further confirmed the presence of isomeric saponins. The full analysis can be seen in Supplementary Figure S2.

The implementation of these molecular techniques on all ions detected in the MALDI/ESI spectra allows us to identify the molecular structures of the saponins. Key fragments from the tandem MS spectra of the positive ion mode of MALDI and ESI were reconstructed according to the example illustrated in order to propose the saponin structures. On the basis of these fragment signatures, the structures of three novel isomeric saponins have been elucidated. Some of these compounds share the diagnostic *m/z* 493 and/or 507, and *m/z* 639 and/or 657 key signals as a signature of the sodiated oligosaccharide residues (Table 2).

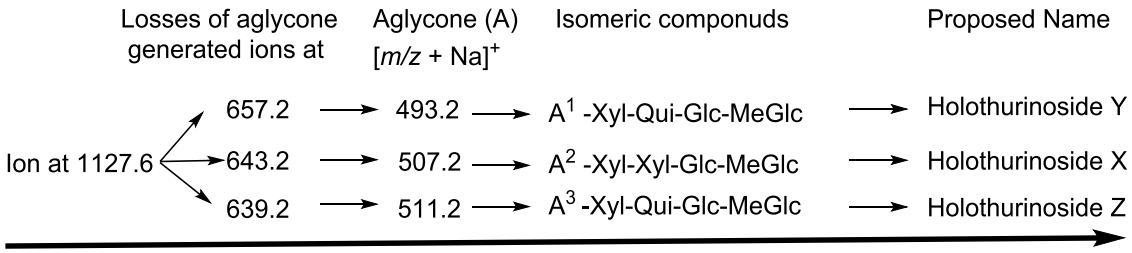
Table 2. Key diagnostic ions in the MS/MS of the holothurians saponins.

	Diagnostic Ions in CID Spectra of $[M + Na]^+$			
	m/z signals (Da)			
	493	507	523	657
Chemical signatures	MeGlc-Glc-Xyl + Na	MeGlc-Glc-Qui + Na	MeGlc-Glc-Glc +Na	MeGlc-Glc-Qui-Xyl + Na

The structures of three isomeric saponins were ascribed to the ions detected at *m/z* 1127.6 (Figures 2A,B and 3 and Supplementary Figure S2). The MS/MS spectra show the presence of three different aglycone structures, namely ions detected at *m/z* 657.2, 643.2 and 639.2 due to the losses of aglycone moieties. This analysis revealed the presence of at least three different isomers with different aglycone moieties *m/z* values of 470, 484 and 488 and differ in the lateral side as the MS/MS spectra generated the sodiated diagnostic fragments at *m/z* 493, 507 and 511, respectively. The structures of the aglycones were proposed based on the literature. The predominant fragment ion at *m/z* 493 corresponds to the sodium adduct sugar residue [MeGlc-Glc-Xyl + Na] side chain or the sodiated aglycone moiety. Similar conclusions were drawn by Zhang *et al.* [18] and Silchenko *et al*. [107] for triterpene glycosides.

Based on the literature [12,59,69,77] and as the above analysis indicates, ions detected at *m/z* 657, and 639 correspond to the sodiated Xyl-Qui-Glc-MeGlc. The ion at 643 yields ions at 511 and 493 by the loss of Xyl. These ions are recognized as the key diagnostic fragments in triterpenoid saponins. These isomers were composed of four monosaccharides including MeGlc-Glc-(Xyl or Qui)-Xyl. The proposed structures are shown in Figure 4, which correspond to three novel saponins for which we propose the names Holothurinosides Y, X and Z.

Figure 4. The schematic diagram of the proposed isomeric structures of ion at *m/z* 1127.6.



The loss of aglycone with *m/z* value of 470 Da from the ions at *m/z* 1109.5, 1095.5, 1065.6, 951.4, 947.0, 889.5 and 618.1 generated ions that were detected at *m/z* 639.2, 625.2, 595.3, 481.2, 477.2, 419.1 and 148.9, respectively. Moreover, the loss of another aglycone (*m/z* value of 484 Da) from the ions at *m/z* 1109.5, 1051.6, 951.4, 935.5 and 889.5 generated ions of *m/z* 625.2, 567.2, 467.2, 451.2 and 405.1 respectively. In addition the loss of the third aglycone (*m/z* value of 488 Da) from the ions at *m/z* 1007.5, 947.0, 939.4, 933.5 and 691.4 generated ions that were observed at *m/z* 519.3, 459.2, 451.2, 445.3 and 203.0, respectively.

Tandem MS analysis revealed the presence of three different aglycones with *m/z* values of 470, 484 and 488 confirming the presence of the isomeric structures. The carbohydrate moieties of the isomeric saponins were found to be composed of four sugar units, which were identical to the sugar component of Holothurinoside C, Desholothurin A, Intercedenside D and Eximisoside A [12,37,105].

As can be seen in Figure 2 the abundance of signals for the ions at *m/z* 493, 507 and 511 is 8:4:1, respectively. Our findings demonstrated that the ions at *m/z* 493 and 507 are formed from the decomposition of two isomers separately. In contrast, the ion at *m/z* 511 has resulted from the disintegration of one isomer which can explain this discrepancy in intensity. The losses of H_2O and CO_2 or their combination results from cleavage at the glycosidic linkages as noted by Waller and Yamasaki [2]. The ions detected at *m/z* 1109.5, 1082.5 and 1065.6 resulted from the sequential losses of H_2O, CO_2 and the combination of H_2O and CO_2 from the parent ion. The first two fragments correspond to the sequential losses of water and carbon dioxide.

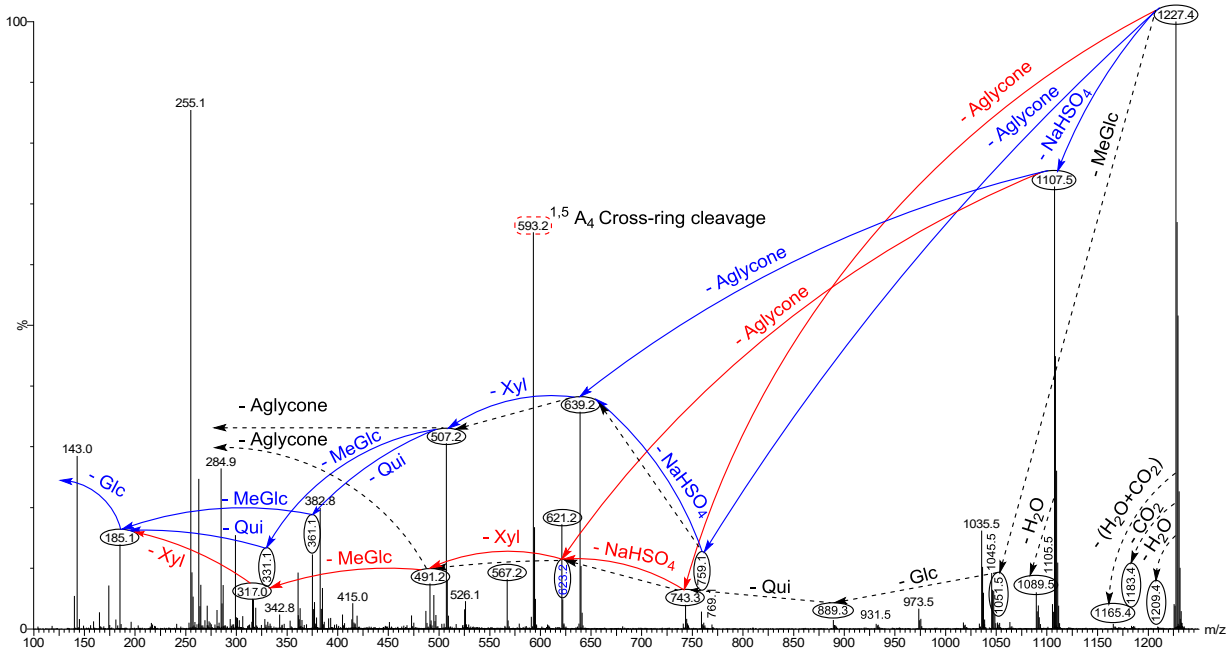
These two peaks at *m/z* 1127.6 and 1141.5 (Figures 1–3 and Table 1), were found to correspond to at least three and two isomers, respectively.

Kitagawa and associates [90] suggested the structure of the ion at *m/z* 1125.5, later named as Holothuriosides C/C_1, based on their NMR and field desorption mass spectrum (FD-MS) data. Therefore, the structure of one of the isomers in the ion at *m/z* 1127.6 is produced due to reduced structure in the lateral chain. This is similar to what we see in the formation of Holothurinoside Y.

Rodriguez and coworkers [89], however, demonstrated a different structure for the aglycone part of the ion observed at *m/z* 1125 compared to that reported by Kitagawa and co-workers.

The other example of an isomeric compound is the ion at *m/z* 1227.4. The structural elucidation of this ion using tandem MS is demonstrated in Figure 5. As can be seen in the MS/MS spectrum, the two ion peaks at *m/z* 743.3 and 759.1 correspond to the losses of different aglycone moieties with *m/z* values of 484 and 468, respectively, demonstrating the presence of isomeric compounds. Further, this MS/MS analysis revealed the presence of a sulfate group in the structure of the isomers. Similar to ions at *m/z* 1243.5 and 1259.5, after collisional activation, parent ions are subjected to three dissociation pathways shown using full and dotted arrows in Figure 5. First, the consecutive losses of the aglycone, sodium monohydrogen sulfate ($NaHSO_4$), Xyl, Qui and MeGlc residues followed by Glc afford product ions that were described in Figures 5 and 6a. Alternatively, the sequential losses of MeGlc and Qui from the key diagnostic peak (*m/z* 507.2) generated ions as shown in Figure 6b. Therefore, in this case, the ions at *m/z* 507.2 correspond to the sodiated key diagnostic sugar residue.

Figure 5. (+) ion mode ESI-MS/MS spectrum of sulfated saponins detected at 1227.4. Full and dotted arrows show the three main feasible fragmentation pathways. The consecutive losses of $NaHSO_4$, Agl, Xyl, MeGlc and Qui residues affords product ions detected at *m/z* 1107.5, 639.0, 507.0, 331.1 and 185.0, respectively.



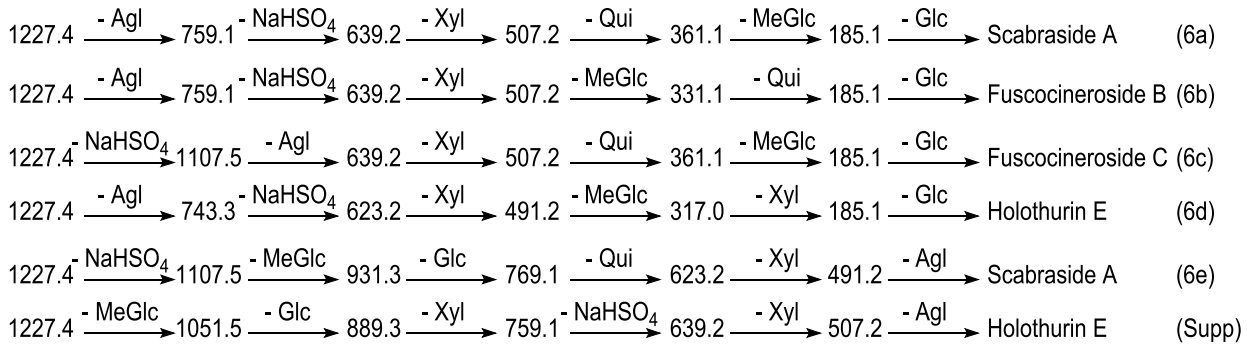
Secondly, the decomposition of the parent ion can also be triggered by the loss of sugar moiety, namely MeGlc, Glc, Qui, $NaHSO_4$ and Xyl followed by the aglycone residue which generated daughter ions at *m/z* 1051.5, 889.3, 743.3, 623.2 and 491.2, respectively. In this case, the ions at *m/z* 491.2 correspond to the sodiated aglycone moiety (*m/z* value of 468).

Finally, the fragmentation of the parent ions can also be initiated with the loss of the sulfate group. The consecutive losses of $NaHSO_4$ and the Agl units followed by the sequential losses of the sugar moiety, namely Xyl, Qui and MeGlc produced masses illustrated in Figures 5 and 6c; ions at

639.2, corresponds to the total desulfated sugar moiety. This saponin possesses the common *m/z* 507.2 key signal as a fingerprint of MeGlc-Glc-Qui + Na^+.

In another isomer, the consecutive losses of the aglycone, $NaHSO_4$, Xyl and MeGlc residues generate product ions of *m/z* 743.3, 623.2, 491.2 and 317.0, respectively, and postulate the structure of a new isomer, which we propose to name Holothurin E (Figure 6d). In this case the ion at *m/z* 491.2 corresponds to the sodiated sugar residues. Further, the consecutive loss of $NaHSO_4$ followed by the aglycone (*m/z* 484) and Xyl residue generated ions at *m/z* 1107.5, 623.2, and 491.2, respectively, confirming the structure of this isomer. Alternatively, the losses of $NaHSO_4$ followed by the sugar moiety, namely MeGlc, Glc, Qui and Xyl followed by the aglycone unit generated daughter ions indicated in Figure 6e, confirming the structure of Scabraside A.

Figure 6. Schematic fragmentation patterns of the ion detected at *m/z* 1227.4.



The loss of the aglycone (with *m/z* value of 468) from the ions at *m/z* 1107.5, 1089.5 and 1035.5 generated ions detected at *m/z* 639.2, 621.2 and 567.2, respectively. The loss of the aglycone (with *m/z* value of 484 Da) from the ions at *m/z* 1107.5, 1105.5 and 1051.5 generated ions observed at *m/z* 623.2, 621.2 and 567.2, respectively. The complete analysis can be seen in Supplementary Figure S3.

The losses of water and/or carbon dioxide were observed from the spectrum. For instance, the ions at *m/z* 1089.5 and 1045.5 were generated by the sequential losses of H_2O and CO_2, from the desulfated parent ions (*m/z* 1107.5), or the sequential losses of CO_2 and H_2O molecules from the parent ions generated ions at *m/z* 1183.5 and 1165.5, respectively.

The identification of compounds was confirmed by MS-MS analysis and was based on the published literature as shown in the last column of the Table 1. For the saponin detected at *m/z* 1227.4, the MS/MS spectrum exhibited the key diagnostic peaks at *m/z* 491, 507 and 639, which confirmed the presence of 24-dehydroechinoside A (synonymous with Scabraside A) and Fuscocinerosides B and C along with a novel isomeric saponin, Holothurin E. This finding was in agreement with those reported by Bondoc *et al.* [69]. They described this peak as corresponding to 24-dehydroechinoside A (synonymous with Scabraside A) or Fuscocinerosides B and C, or other isomers, differing only in the lateral side chain of their aglycone units. Cucumarioside H_3 possesses the same molecular weight (*m/z* 1227) with those isomeric compounds [108], however, the structure of this compound was not detected in this species.

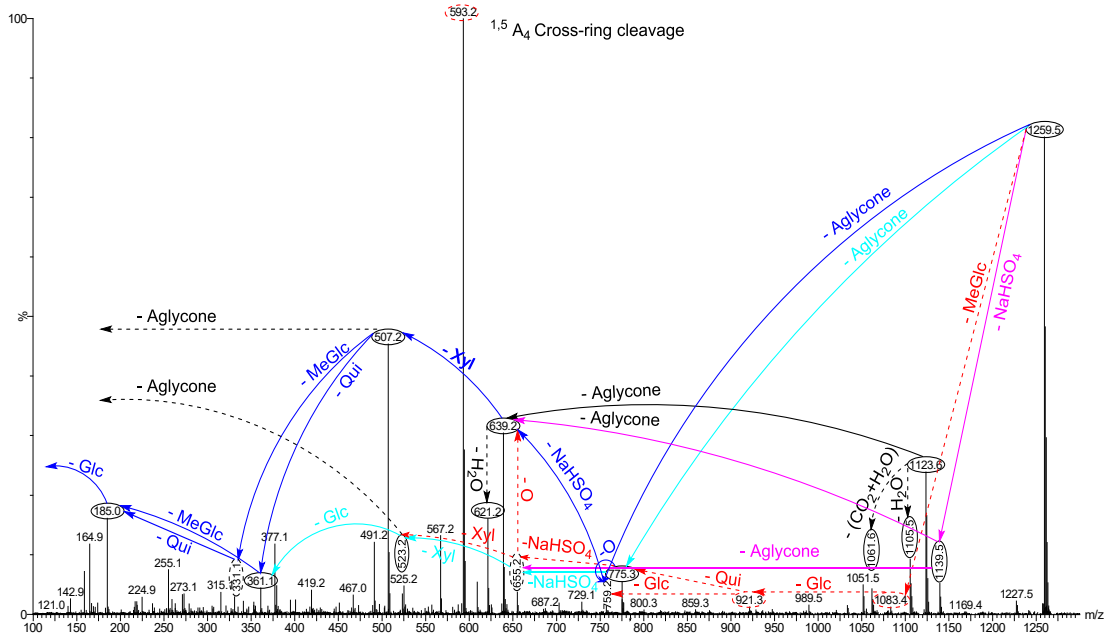
As Van Dyck *et al*. [59] also stated, the differences between isomeric saponins are not always measurable by applying MS methodology which only relies on low-kinetic energy CID. The

saponin detected at *m/z* 1227.4 could correspond to either Fuscocineroside B or C, the two molecules differ only at the level of the lateral chain of their aglycones.

The MS/MS analysis of ions at 1227.4 revealed a very similar fingerprint profile with those reported for Holothurin A and Holothurin A_3, which show the intrinsic relationship between these saponin congeners.

Another typical chemical structure elucidation of isomeric saponins by tandem MS is exemplified in Figure 7. This spectrum shows the ion signature of the ion detected at *m/z* 1259.5 from Fraction 22 under tandem MS. Tandem MS analysis revealed the presence of two different aglycones with *m/z* values of 484 and 500, confirming the presence of chemical isomeric structures. Tandem MS analysis also distinguished the presence of a sulfate group in the structure of isomers. After collisional activation, *m/z* 1259.5 cations are subjected to three dissociation pathways shown using full and dotted arrows. First, as described in Figure 7, consecutive losses of aglycone, $NaHSO_4$, Xyl, Qui and MeGlc residues generate the product ions demonstrated in Figures 8a and S4 Supplementary Data. Alternatively, the sequential losses of MeGlc and Qui from the key diagnostic peak (*m/z* 507) generated ions shown in Figure 8b. Therefore, in this case the ion at *m/z* 507.2 corresponds to the key diagnostic sugar residue.

Figure 7. (+) ion mode ESI-MS/MS spectrum of saponins detected at *m/z* 1259.5 from Fraction 22. This spectrum shows the presence of two different aglycones indicating the presence of isomeric saponins. The consecutive losses of $NaHSO_4$, Agl, Xyl, Qui and MeGlc followed by Glc residue affords product ions detected at *m/z* 1139.5, 639.2, 507.2, 361.1 and 185.0, respectively, which indicate the structure of Holothurin A_3. Full and dotted arrows illustrate the three main possible fragmentation pathways.



The decomposition of the parent ion can also be triggered by the loss of sugar moiety, namely MeGlc, Glc, Qui, $NaHSO_4$ and Xyl followed by the aglycone which generated daughter ions illustrated in Figure 8c, which confirms the structure of Holothurin A_3. It is clear that the ion at *m/z* 523.2 is a signature of the sodiated aglycone. The sequential losses of MeGlc, Glc, Glc, $NaHSO_4$ and Xyl followed by the aglycone afford ions as shown in Figure 8d for which we propose the name Holothurin D. In this case, the ion at *m/z* 507.2 is the second most abundant fragment ion of the signature of the sodiated aglycone.

Figure 8. Schematic fragmentation patterns of the ion detected at *m/z* 1259.5.

1259.5 $\xrightarrow{-\text{Agl}}$ 759.2 $\xrightarrow{-NaHSO_4}$ 639.2 $\xrightarrow{-\text{Xyl}}$ 507.2 $\xrightarrow{-\text{Qui}}$ 361.1 $\xrightarrow{-\text{MeGlc}}$ 185.0 $\xrightarrow{-\text{Glc}}$ Holothurin A_3 (8a)

1259.5 $\xrightarrow{-\text{Agl}}$ 759.2 $\xrightarrow{-NaHSO_4}$ 639.2 $\xrightarrow{-\text{Xyl}}$ 507.2 $\xrightarrow{-\text{MeGlc}}$ 331.1 $\xrightarrow{-\text{Qui}}$ 185.0 $\xrightarrow{-\text{Glc}}$ Holothurin A_3 (8b)

1259.5 $\xrightarrow{-\text{MeGlc}}$ 1083.4 $\xrightarrow{-\text{Glc}}$ 921.3 $\xrightarrow{-\text{Qui}}$ 775.3 $\xrightarrow{-NaHSO_4}$ 655.2 $\xrightarrow{-\text{Xyl}}$ 523.2 $\xrightarrow{-\text{Agl}}$ Holothurin A_3 (8c)

1259.5 $\xrightarrow{-\text{MeGlc}}$ 1083.4 $\xrightarrow{-\text{Glc}}$ 921.3 $\xrightarrow{-\text{Glc}}$ 759.2 $\xrightarrow{-NaHSO_4}$ 639.2 $\xrightarrow{-\text{Xyl}}$ 507.2 $\xrightarrow{-\text{Agl}}$ Holothurin D (8d)

1259.5 $\xrightarrow{-NaHSO_4}$ 1139.5 $\xrightarrow{-\text{Agl}}$ 655.2 $\xrightarrow{-\text{Xyl}}$ 523.2 $\xrightarrow{-\text{Glc}}$ 361.1 $\xrightarrow{-\text{MeGlc}}$ 185.0 $\xrightarrow{-\text{Glc}}$ Holothurin D (8e)

1259.5 $\xrightarrow{-\text{Agl}}$ 775.3 $\xrightarrow{-NaHSO_4}$ 655.2 $\xrightarrow{-\text{Xyl}}$ 523.2 $\xrightarrow{-\text{Glc}}$ 361.1 $\xrightarrow{-\text{MeGlc}}$ 185.0 $\xrightarrow{-\text{Glc}}$ Holothurin D (8f)

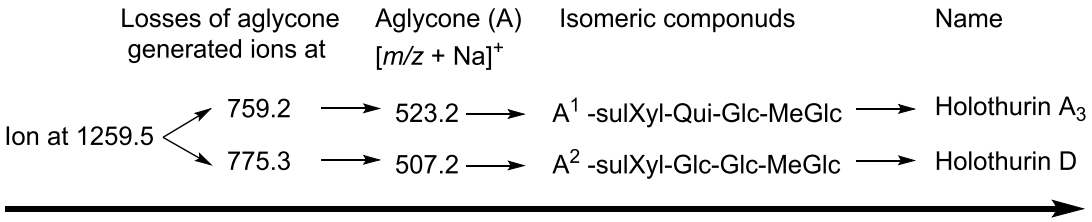
Alternatively, the fragmentation of the parent ions can also be initiated by the loss of the sulfate group. The consecutive losses of $NaHSO_4$ and the aglycone unit followed by the sequential losses of the sugar moiety, namely Xyl, Glc and MeGlc produced the masses exhibited in Figures 7 and 8e. The 655.2 ion corresponds to the total desulfated sugar moiety.

The reconstruction of fragment ions generated by tandem MS also identifies the existence of a novel isomeric saponin. The fragmentation of the precursor ion can also be initiated by the loss of aglycone moiety (Figure 7) which generated the mass at *m/z* 775.3 corresponding to the entire sulfated-sugar components. The continuous losses of the aglycone, $NaHSO_4$, Xyl, Glc and MeGlc followed by Glc yielded the ion fragments shown in Figure 8f. In this case the ion at *m/z* 523.2 corresponded to the sodiated sugar residue and further confirmed the presence of a novel saponin (Holothurin D).

The loss of aglycone (with *m/z* value of 500) from the ions at *m/z* 1139.5, 1083.4, 967.2, 963.2, 947.4, 890.4, 877.4 and 847.3 generated ions of *m/z* 639.2, 583.1, 467.0, 463.0, 447.2, 390.9, 377.1, and 347.1, respectively. The loss of aglycone (with *m/z* value of 484 Da) from the ions at *m/z* 1139.5, 1123.6, 1105.5, 1051.5, 1033.6, 757.2, 729.1 and 639.2 generated ions observed at *m/z* 655.2, 639.2, 621.2, 567.2, 549.3, 273.1, 245.0 and 155.0, respectively. The loss of the aglycone from the ion at *m/z* 1123.6 yields the ion at *m/z* 639.2. Therefore, the ions at *m/z* 639.2 and subsequently 507.2 can be generated by the losses of both aglycones and the key diagnostic sugar unit which can explain the higher intensity of these peaks compared to the ions at 655.2 and 523.2 in the new isomeric saponin, which is produced by the loss of one type of aglycone.

This analysis revealed the presence of at least two different isomers with diverse aglycone and sugar moieties. These isomers are also tetraglycosidic saponins. The proposed structures are shown in Figure 9, which corresponds to Holothurin A_3 and a novel saponin; Holothurin D.

Figure 9. A schematic diagram of the proposed isomeric structures of ion at *m/z* 1259.5.



The structures of aglycones are similar to those reported for Holothurin A, Holothurin A_3. The different fragmentation behaviors have been observed from the positive ESI-MS/MS spectra of saponins with *m/z* 1127.6. However, the tandem MS data of the carbohydrate moiety, in one isomer, was identical to those of the sugar component of Holothurin A, indicating the tetrasaccharide chain of Holothurin A_3 was composed of 4-*O*-sulfated Xyl, Qui, Glc and MeGlc residues with the ratio of 1:1:1:1. Further the spectrum was similar to the spectrum of Holothurin A, the signals were coincident with those of Holothurin A.

The molecular analysis revealed a series of ions consistent with the presence of Holothurin A_3 with an elemental composition of $C_{54}H_{85}NaO_{28}S$ in addition to one novel saponin. The observed fragments are consistent with the structure of the Holothurin A_3 proposed by Dang and coworkers [44]. Our data are in agreement with ESI-MS data obtained by Dang *et al*, identified as the 3β, 12α,

17α, 25-tetrahydroxyholost-9(11)-ene-22-one 3-*O*-[(3-*O*-methyl)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-β-D-quinovopyranosyl-(1→2)-(4-*O*-sulfo)-β-D-xylopyranoside] sodium salt.

The data indicate that the terminal sugar is the first moiety to be lost under CID. Since these isomers (A_3 and D) contain the same terminal sugar units in their sugar residue, they yielded the ions with the same *m/z* values (*m/z* 361 and 185).

3.3.2 Key Diagnostic Fragments in the Sea Cucumber Saponins

The common key fragments facilitated the structure elucidation of novel and reported saponins. Tandem mass spectrometry analysis of saponins revealed the presence of several diagnostic key fragments corresponding to characteristic structural element of saponins as summarized in Table 2. Here we report a new diagnostic key fragment at *m/z* 493 corresponding to either the aglycone moiety or the sugar residue.

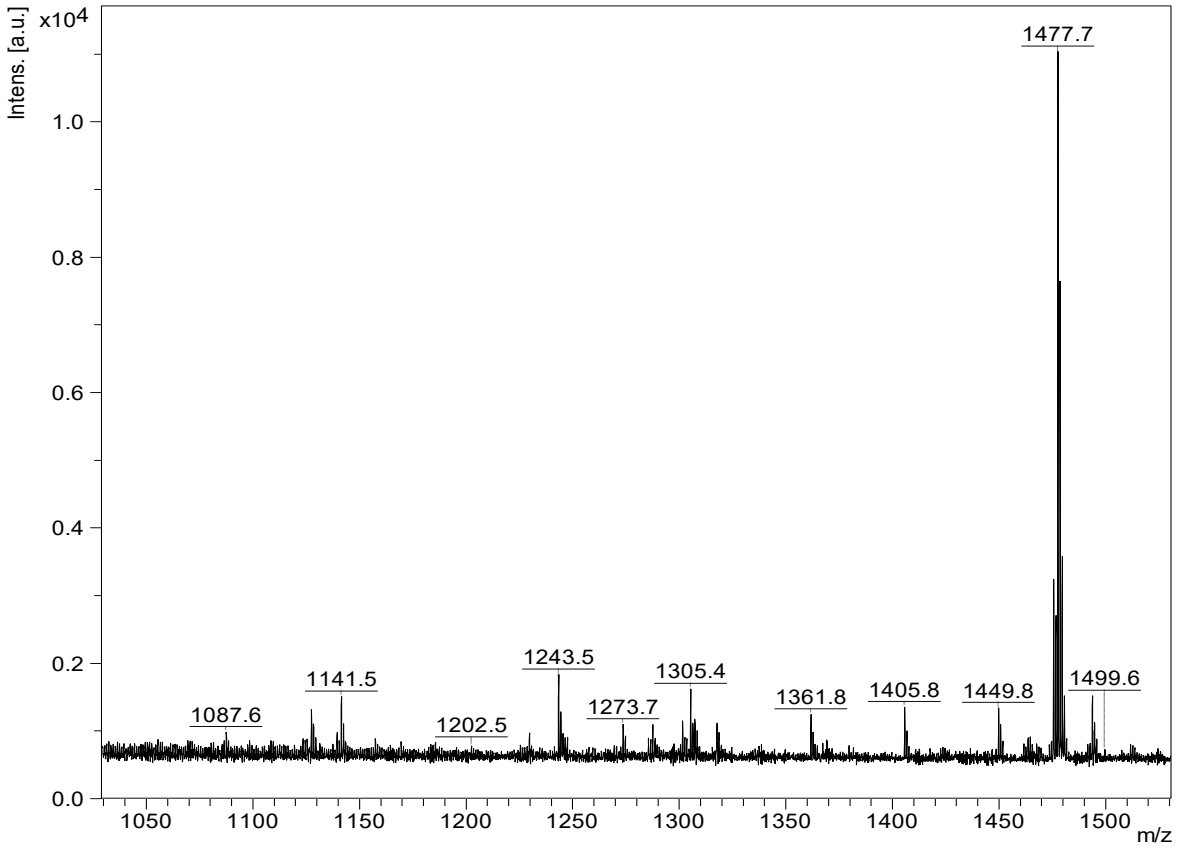
The structures of saponins were elucidated by the identification and implementation of the key fragment ions produced by tandem mass spectrometry. The presence of the oligosaccharide residue (*m/z* 493 and/or 507 and/or 511 and/or 523 and/or 657) simplified the determination of the saponin structure.

The ESI analysis revealed that the ion *m/z* 1259.5 is an isomeric compound that corresponded to a new saponin (Holothurin D) and Holothurin A_3, which was found in several species of sea cucumbers.

MALDI-MS analysis was also performed on the isolated saponins. The prominence of the parent ions $[M + Na]^+$ in MS spectra also enabled the analysis of saponins in mixtures or fractions. As a representative example, the MALDI mass spectrum (between 1000 and 1500 Da) of the saponin extract obtained from the HPCPC Fraction 18 is shown in Figure 10.

The peaks at *m/z* 1125.5 (Holothurinosides C/C_1), 1141.5 (Desholothurins A/A_1), 1157.5 (Holothurinoside J_1), 1227.4 (Fuscocinerosides B/C or Scabraside A and a novel isomer), 1243.5 (Holothurin A), 1287.6 (Holothurinosides E/E_1/O/P), 1301.6 (Holothurinoside M), 1303.6 (Holothurinosides A/A_1/Q/R/R_1/S), 1317.6 (Holothurinoside N) and 1495.7 (Holothurinoside K_1) represent known compounds whereas the following peaks represent novel saponins 1087.6, 1111.5, 1123.5, 1127.6, 1305.4, 1361.8, 1405.8, 1417.7, 1449.8 1475.7, 1477.7 and 1493.7 is shown in Table 1.

Figure 10. The MALDI mass spectrum of HPCPC Fraction 18 from the viscera of the *H. lessoni* in the (+) ion mode. A mass range of 1000 to 1500 Da is shown here.



3.3.3 MALDI-MS/MS Analysis of Saponins in Positive Ion Mode

Saponin ion peaks were further analyzed by MALDI MS/MS and reconfirmed the ESI results. The MALDI MS/MS figures can be found in Supplementary Figures S4 and S5. The techniques used are able to distinguish the structural differences among the isomers following HPCPC separation. This analysis also confirmed the presence of saponins reported in the literature and allowed the discovery of new saponin congeners. As a typical example, the MALDI-MS/MS mass spectrum for the ion detected at *m/z* 1259.5 (sulfated triterpene glycoside) is shown in Supplementary Figure S4. The fragmentation pattern of the sodiated compound at *m/z* 1259.5 from fraction 20 in successive MS experiments is discussed in detail below for stepwise elucidation of the molecular structure of these compounds.

CID activates three feasible fragmentation pathways of cationized parent ions shown in full and dotted arrows. First, the continuous losses of Agl, $NaHSO_4$, Xyl, Qui and MeGlc residues yielded to ion fragments described in Figure 8a. In this case the ion at *m/z* 507.2 corresponds to the sugar reside $[MeGlc\text{-}Glc\text{-}Qui + Na]^+$.

Secondly, the decomposition of the parent ion can also be triggered by the sequential loss of a sugar moiety, namely MeGlc, Glc, Qui, $NaHSO_4$ and Xyl followed by the aglycone which generated the daughter ions demonstrated in Figure 8c. This sequence of fragmentation corresponds

to the known structure of Holothurin A_3. For the second isomer, the consecutive losses of MeGlc, Glc, Glc, $NaHSO_4$ and Xyl followed by the aglycone produced ions corresponding to the structure of Holothurin D (Figure 8d).

The fragmentation of the parent ions can also be initiated by the loss of the sulfate group. Then consecutive losses of $NaHSO_4$ and the aglycone unit is followed by the sequential losses of the sugar moiety produced signals observed at *m/z* 1139.5 and 639.2 (Supplementary Figure S4); the latter peak corresponding to the entire desulfated sugar moiety; 639.2 [M + Na − 120 − 500 (aglycone)]$^+$ and 507 [M + Na − 120 − 500 − 132 (Xly)]$^+$. In addition, the sequential losses of the $NaHSO_4$ (*m/z* 1139.2), Agl (*m/z* 655.2), Xyl (*m/z* 523.2), Glc (*m/z* 361.1) and MeGlc (*m/z* 185.0) support the isomer being Holothurin D (Figure 8e).

Holothurin A_3 was originally isolated from the methanol extract of the sea cucumber *H. scabra* by Dang *et al.* [44]. This group indicated Holothurins A_3 as a sulfated tetrasaccharide triterpene glycoside, contacting sulXyl, Qui, Glc and MeGlc with the ratio of 1:1:1:1. Here we describe the structure of a novel isomer, Holothurin D, with the same *m/z* value but different aglycone and sugar residues. The structure of this sulfated tetrasaccharide triterpene comprised of sulXyl, Glc and MeGlc with the ratio of 1:2:1, respectively.

Peaks formed by the losses of water (18 Da) and/or carbon dioxide (44 Da) have been annotated in Supplementary Figure S4. For instance, the peaks observed at *m/z* 1241.5 and 1223.5 were generated by the sequential losses of two H_2O molecules from the parent ion and the major ion at *m/z* 621.2 corresponds to the loss of water from *m/z* 639.2. This peak could also be generated by the loss of aglycone from the ion at *m/z* 1105.5.

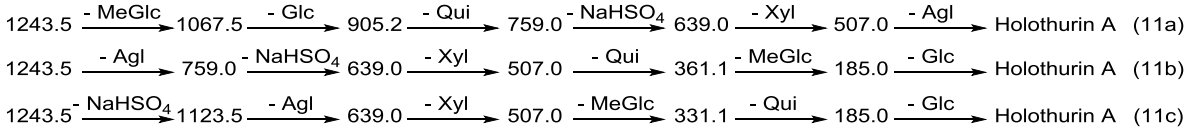
Another typical chemical structure elucidation of sulfated saponins by tandem MS is exemplified by the ion detected at *m/z* 1243.5 in Supplementary Figure S5. CID induces three feasible fragmentation pathways of the cationized sulfated parent ions like ion at *m/z* 1259.5 shown in full and dotted arrows. First, the loss of the sugar unit; the successive losses of MeGlc, Glc, Qui, sulfate and Xyl followed by the aglycone unit generate the ions described in Figure 11a. The fragmentation masses correspond to those which would be generated by Holothurin A. The ion at *m/z* 507.0 corresponds to the aglycone moiety.

Secondly, the decomposition of the precursor ions can also be triggered by the loss of the aglycone residue, creating peaks at *m/z* 759.0 (Figure 11b) corresponding to the sulfated sugar moiety. The consecutive losses of the Agl, NaHSO4, Xyl, Qui and MeGlc units generate the masses shown in Figure 11b. The third viable pathway is elicited by the loss of sulfate group. The consecutive losses of NaHSO4 and the Agl unit producing signals observed at *m/z* 1123.5 and 639.0, the latter peak corresponding to the complete desulfated sugar moiety exhibited in Figure 11c. All these possible fragmentation routes are consistent with ions corresponding to the fragmentation of sodiated Holothurin A (*m/z* 1243.5).

The loss of 18 Da from the sodiated molecular ion, suggests the elimination of a neutral molecule (H2O) from the sugar group [24]. A similar predominant peak at *m/z* 593.2 was noted and the assignment as previously given was confirmed [12].

This MS/MS spectrum allows us to reconstruct the collision-induced fragmentation pattern of the parent ion (Figure 10) and consequently to confirm that ions monitored at *m/z* 1243.5 correspond to the Holothurin A [12,59,89,96].

Figure 11. Schematic fragmentation patterns of the ion detected at *m/z* 1243.5, Holothurin A.



Mass spectrometry provides a relatively swift and straightforward characterization of the elemental composition, saponin structure and distribution by the presence of the key ions at *m/z* 493, 507, 523 and 639 in the tandem spectra of the viscera extracts as described in detail in our previous publication [12].

Based on the MS/MS data and mass accuracy, saponins were categorized into seven distinct carbohydrate structural types [12]. In general, non-sulfated saponins are more conjugated with glycosides compared to sulfated saponins. Non-sulfated saponins had one to six monosaccharide units and six distinct structural types [12]. All sulfated saponins ranging from *m/z* 889 to 1259 had the structure [(MeGlc-Glc)-Qui-sulXyl-Aglycone] in which Xyl was sulfated. However, in some cases the sulfation of MeGlc and Glc has also been reported [20]. The chemical structures of the identified compounds are illustrated in Figure 12.

Figure 12. The structure of identified saponins in the viscera of *H. lessoni*. Holothurins D/E and Holothurinosides X/Y/Z are the novel compounds described in this paper.

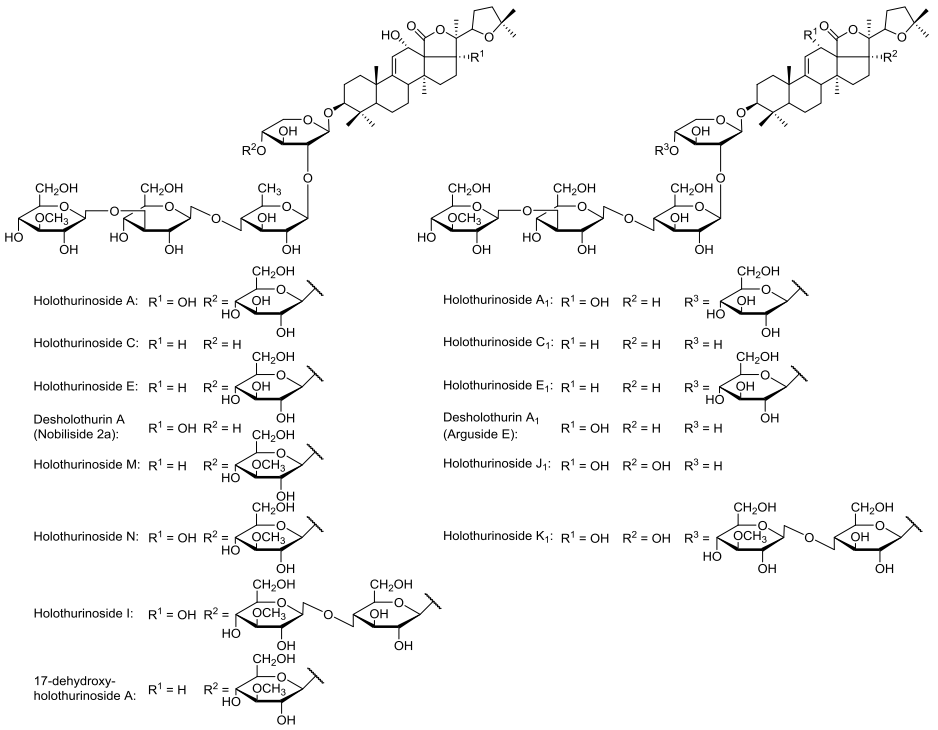
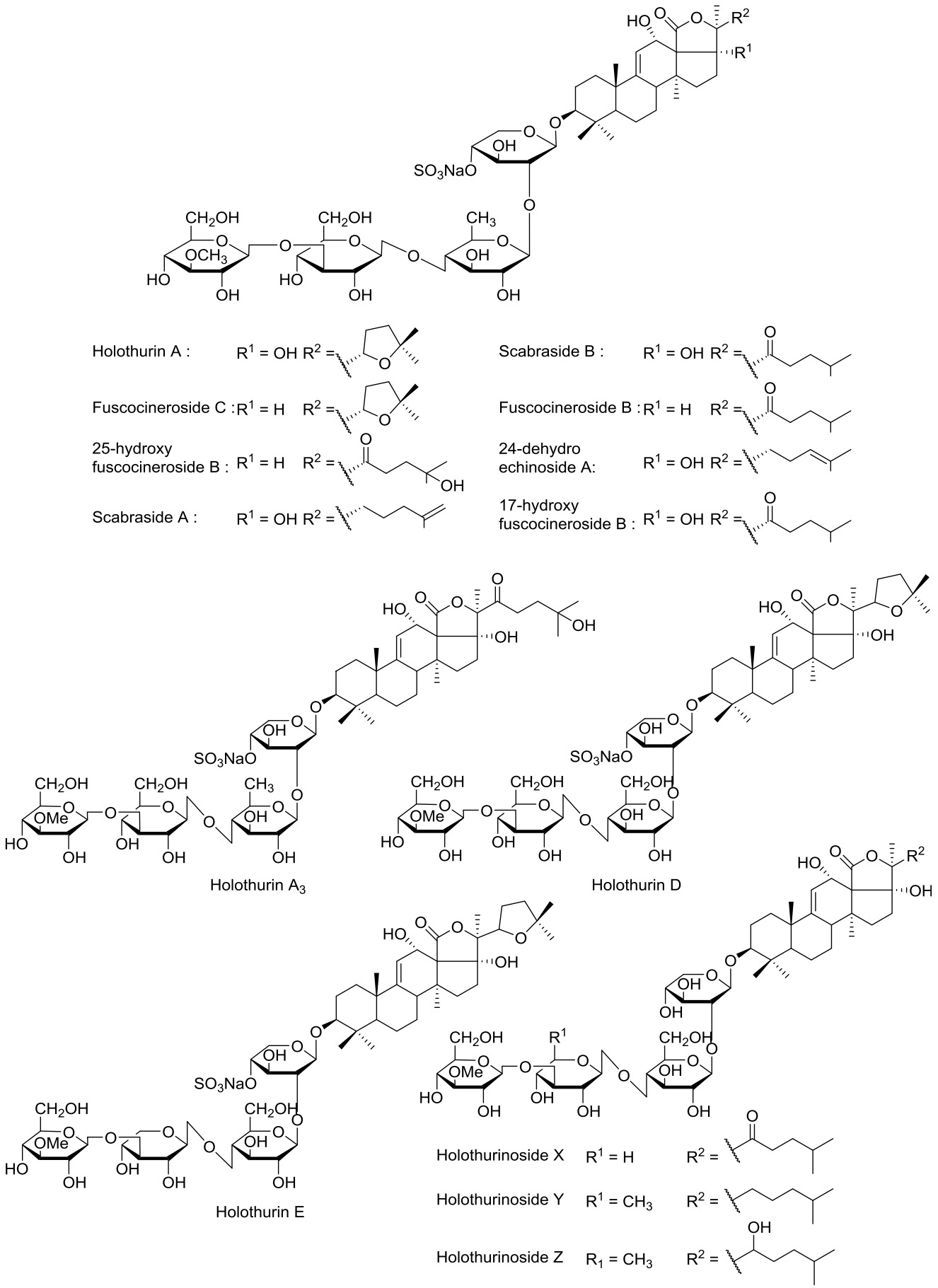


Figure 12. *Cont.*



When comparing the MS/MS spectra of 1127.6 and 1259.5 (Figures 2 and 7), it is notable that the *m/z* 523 fragment (the aglycone loss) of the $[M + Na]^+$ ions observed with 1259.5 only, which corresponds to the presence of a new aglycone unit at *m/z* 500 (sodiated 523). Individual patterns were detected from sulfated and non-sulfated saponins as indicated in Holothurin A_3 and Holothurinoside Y as representative examples. The main difference between both MS/MS spectra was the presence of a sulfate group in Holothurin A_3.

The presence of a sulfate group is also confirmed for the ion at *m/z* 1227.4. The loss of 120 Da from the parent ion is the signature of a sulfate group in the saponin. On the other hand, both MS/MS spectra (compared to 1259.5) share the common *m/z* 507 key signal as a signature of the sodiated MeGlc-Glc-Qui oligosaccharide chain.

By comparison of the molecular weights and structures of both saponins (Holothurin A_3, Holothurin A), they revealed mass differences between each other, such as 16 Da (*O*) mass differences between the aglycone of this saponin and Holothurin A, reflecting the small structural alterations and the intrinsic connections between them. Their MS/MS analysis indicated the presence of identical sugar moieties in both ions.

In some cases this methodology was not sufficiently precise to identify the molecular structure with certainty and several possibilities were offered by the literature. This paper is the first to describe the structure of these saponin congeners in this species and the first to show the MS/MS spectra of the ions at *m/z* 1127.6, 1227.4 and 1259.5 and assigned their fragmentation pattern. This sequential decomposition confirms the proposed structures Fuscocinerosides B/C, Scabraside A, Holothurins A/A_3/D/E and Holothurinosides X/Y/Z.

The predominant fragment signal at *m/z* 593.2 results from α $^{1,5}A_4$ cross-ring cleavage of the sulXyl residue which was consistent with previous findings for the MS/MS of sea cucumber saponins [12]. However, this peak was only detected as an intense signal in the sulfated saponins such as Scabraside A and Holothurin A_3 whereas it was not observed in the non-sulfated saponins such as 1127.6. Therefore, the occurrence of this cross-ring cleavage appears to occur only with the sulfated Xyl (sulXyl). Moreover, this data confirms that cross-ring cleavages are more frequent in sulfated saponins. This phenomenon may occur as a result of the loss of sulfate group creating double bound in the Xyl residue. The intensity of the ion at *m/z* 593.2 resulted from the cross-ring cleavage is higher in the ESI-MS/MS spectra compared to those in the MALDI MS/MS spectra which might be consequence of the ESI CID. As highlighted there are three feasible MS/MS fragmentation pathways for the sulfated compounds, while there are only two possible fragmentation pathways for non-sulfated saponins.

Peaks corresponding to the natriation of complete sugar side chains were often quite intense in the product ion spectra of the natriated saponin precursor. Tandem mass spectra of these saponins reflected the multiple fingerprints with a range of relative intensities.

3.4 Experimental Section

3.4.1 Sea Cucumber Sample

Twenty sea cucumber samples of *Holothuria lessoni* Massin *et al.*, 2009, commonly known as Golden sandfish were collected off near Lizard Island (latitude; 14°41′29.46″ S, longitude; 145°26′23.33″ E), Queensland, Australia in September 2010 as described in [12]. The viscera (all internal organs) were separated from the body wall and kept separately in zip-lock plastic bags which were snap-frozen, then transferred to the laboratory and kept at −20 °C until use.

3.4.2 Extraction Protocol

The debris and sand particles were separated from the viscera manually and the visceral mass was freeze dried (VirTis, BenchTop K, New York, NY, USA). The dried specimens were then pulverized to a fine powder using liquid nitrogen and a mortar and pestle.

All aqueous solutions were prepared with ultrapure water generated by a Milli-Q water purification system (18.2 MΩ, Millipore, Bedford, MA, USA). All organic solvents were purchased from Merck (Darmstadt, Germany) except when the supplier was mentioned, and were either of HPLC grade or the highest degree of purity.

3.4.3 Extraction of Saponins

The saponins were extracted as described previously [12]. The pulverized viscera sample (40 g) was extracted four times with 70% ethanol (EtOH) (400 mL) followed by filtration through Whatman filter paper (No.1, Whatman Ltd., Maidstone, England, UK) at room temperature. The extract was concentrated under reduced pressure at 30 °C using a rotary evaporator (Büchi AG, Flawil, Switzerland) to remove the ethanol, and the residual sample was freeze dried. The dried residue was successively extracted using a modified Kupchan partition procedure [109]: The dried extract (15 g) was dissolved in 90% aqueous methanol (MeOH), and partitioned against 400 mL of n-hexane (v/v) twice. The water content of the hydromethanolic phase was then adjusted to 20% (v/v) and then to 40% (v/v) and the solutions partitioned against CH_2Cl_2 (450 mL) and $CHCl_3$ (350 mL), respectively. The hydromethanolic phase was concentrated to dryness using a rotary evaporator and freeze drier. The dried powder was solubilized in 10 mL of MilliQ water (the aqueous extract) in readiness for undergo chromatographic purification.

3.4.4 Purification of the Extract

The aqueous extract was placed on a prewashed Amberlite® XAD-4 column (250 g XAD-4 resin 20–60 mesh; Sigma-Aldrich, MO, USA; 4 × 30 cm column) chromatography. After washing the column extensively with water (1 L), the saponins were eluted sequentially with MeOH (450 mL), acetone (350 mL) and water (250 mL). The eluates (methanolic, acetone and water fractions) were concentrated, dried, and redissolved in 5 mL of MilliQ water. Finally, the aqueous extract was partitioned with 5 mL isobutanol (v/v). The isobutanolic saponin-enriched fraction was either stored for subsequent mass spectrometry analysis or concentrated to dryness and the components of the extract were further examined by HPCPC and RP-HPLC. The profile of fractions was also

monitored by Thin Layer Chromatography (TLC) using the lower phase of $CHCl_3$:MeOH:H_2O (7:13:8 v/v/v) solvent system.

3.4.5 Thin Layer Chromatography (TLC)

Samples were dissolved in 90% or 50% aqueous MeOH and 10 microliters were loaded onto silica gel 60 F_{254} aluminum sheets (Merck # 1.05554.0001, Darmstadt, Germany) and developed with the lower phase of $CHCl_3$–MeOH–H_2O (7:13:8) biphasic solvent system. The profile of separated compounds on the TLC plate was visualized by UV light and by spraying with a 15% sulfuric acid in EtOH solution and heating for 15 min at 110 °C until maroon-dark purple spots developed.

3.4.6 High Performance Centrifugal Partition Chromatography (HPCPC or CPC)

The solvent system containing $CHCl_3$:MeOH:H_2O- 0.1% HCO_2H (7:13:8) was mixed vigorously using a separating funnel and allowed to reach hydrostatic equilibration. Following the separation of the two- immiscible phase solvent systems, both phases were degassed using a sonicator-degasser (Soniclean Pty Ltd. Adelaide, SA Australia). Then the rotor column of HPCPC™, CPC240 (Ever Seiko Corporation, Tokyo, Japan) was filled with the liquid stationary phase at a flow rate of 5 mL/min^{-1} by Dual Pump model 214 (Tokyo, Japan).

The CPC was loaded with the aqueous upper phase of the solvent system in the descending mode at a flow rate of 5 mL/min^{-1} with a revolution speed of 300 rpm. The lower mobile phase was pumped in the descending mode at a flow rate of 1.2 mL/min^{-1} with a rotation speed of 900 rpm within 2 h. One hundred and twenty milligrams of isobutanol- enriched saponins mixture was dissolved in 10 mL of the upper phase and lower phase in a ratio of 1:1 and injected to the machine from the head-end direction (descending mode) following hydrostatic equilibration of the two phases indicated by a clear mobile phase eluting at the tail outlet. This indicated that elution of the stationary phase had stopped and the back pressure was constant. The chromatogram was developed at 254 nm for 3.0 h at 1.2 mL/min^{-1} and 900 rpm using the Variable Wavelength UV-VIS Detector S-3702 (Soma optics, Ltd. Tokyo, Japan) and chart recorder (Ross Recorders, Model 202, Topac Inc. Cohasset, MA, USA). The fractions were collected in 3 mL/tubes using a Fraction collector. The elution of the sample with the lower organic phase proceeded to remove the compounds with low polarity from the sample within 200 mL of which several peaks were eluted. At this point (Fraction 54), the elution mode was switched to ascending mode and the aqueous upper phase was pumped at the same flow rate for 3.0 h. Recovery of saponins was achieved by changing the elution mode to the aqueous phase which allowed the elution of the remaining compounds with high polarity in the stationary phase. A few minor peaks were also monitored. Fractions were analyzed by TLC using the lower phase of $CHCl_3$:MeOH:H_2O (7:13:8) as the developing system. The monitoring of the fractions is necessary as most of the saponins were not detected by UV due to the lack of a chromophore structure. Fractions were concentrated with nitrogen gas.

3.4.7 Mass Spectrometry

The resultant HPCPC purified polar samples were further analyzed by MALDI and ESI MS to elucidate and characterize the molecular structures of compounds.

3.4.8 MALDI MS

MALDI analysis was performed on a Bruker Autoflex III Smartbeam (Bruker Daltonik, Bremen, Germany). The laser (355 nm) had a repetition rate of 200 Hz and operated in the positive reflectron ion mode for MS data over the mass range of 400 to 2200 Da under the control of the Flexcontrol V 3.3 build 108, Bruker Daltonik, Bremen, Germany). External calibration was performed using PEG. MS spectra were processed in FlexAnalysis (version 3.3, Bruker Daltonik, Bremen, Germany). MALDI MS/MS spectra were obtained using the LIFT mode of the Bruker Autoflex III with the aid of CID (Bruker Daltonik, Bremen, Germany). The isolated ions were submitted to collision against argon in the collision cell to collisionally activate and fragment, and afford intense product ion signals. For MALDI, a laser energy was used that provided both good signal levels and mass resolution, the laser energy for MS/MS analysis was generally 25% higher than for MS analysis.

The samples were placed onto a MALDI stainless steel MPT Anchorchip TM 600/384 target plate (Bruker Daltonik, Bremen, Germany). Alpha-cyano-4-hydroxycinnamic acid (CHCA) in acetone/iso-propanol in ratio of 2:1 (15 mg/mL) was used as a matrix to produce gas-phase ions. The matrix solution (1 μL) was spotted onto the MALDI target plate and air-dried. Subsequently 1 μL of sample was added to the matrix crystals and air dried. Finally, 1 μL of NaI (Sigma-Aldrich # 383112, St Louis, MI, USA) solution (2 mg/mL in acetonitrile) was applied onto the sample spots. The samples were mixed on the probe surface and dried prior to analysis.

3.4.9 ESI MS

The ESI mass spectra were obtained with a Waters Synapt HDMS (Waters, Manchester, UK). Mass spectra were obtained in the positive ion mode with a capillary voltage of 3.0 kV and a sampling cone voltage of 100 V.

The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 80 °C; desolvation temperature, 350 °C; desolvation gas flow rate, 500 L/h. Data acquisition was carried out using Waters MassLynx (V4.1, Waters Corporation, Milford, USA). Positive ion mass spectra were acquired in the V resolution mode over a mass range of 100–2000 *m/z* using continuum mode acquisition. Mass calibration was performed by infusing sodium iodide solution (2 μg/μL, 1:1 (v/v) water:isopropanol). For accurate mass analysis a lock mass signal from the sodium attached molecular ion of Raffinose (*m/z* 527.1588) was used.

MS/MS spectra were obtained by mass selection of the ion of interest using the quadrupole, fragmentation in the trap cell where argon was used as collision gas. Typical collision energy (Trap) was 50.0 V. Samples were infused at a flow rate of 5 μL/min, if dilution of the sample was required then acetonitrile was used [87]. Chemical structures were determined from fragmentation schemes calculated on tandem mass spectra and from the literature.

3.5 Conclusions

Marine invertebrates synthesize a plethora of small fascinating molecules with interesting chemical structures and potent biological properties. Holothurians are one class of marine invertebrate animals which are an important source of human food and traditional medicine, especially in some parts of Asia. In the past three decades, the scientific literature from several countries revealed that triterpene glycosides from sea cucumbers have a wide spectrum of biological effects.

The extract of the viscera of sea cucumber *H. lessoni* has been processed by applying HPCPC to purify the saponin mixture and to isolate saponin congeners and isomeric saponins. Other research groups have applied nuclear magnetic resonance (NMR) spectroscopy to obtain extensive structural information for saponins, but high-quantities of high-purity samples are usually required. In particular when the NMR signals are overlapping, the assignment of data is labor-intensive and very time consuming. Moreover, the measurement of the absolute configuration of the sugar moieties of a saponin cannot be completely solved by NMR methods alone [110]. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI- MS) and electrospray ionization mass spectrometry (ESI-MS) techniques have become the preferred techniques for analysis of saponins. Mass spectrometry provides a highly sensitive platform for the analysis of saponin structures by generating product ions by the cleavage of the glycosidic bond.

The tandem MS approach enabled us to determine the structure of a range of saponins. The purity of HPCPC fractions allowed mass spectrometry analysis to reveal the structure of isomeric compounds containing different aglycones and/or sugar residues. Several novel saponins, along with known compounds were identified from the viscera of sea cucumber. We performed tandem mass spectrometry analysis on both sulfated and non-sulfated compounds, and compared their fragmentation profiles.

Our results highlight that there are a large number of novel saponins in the viscera indicating the viscera as a major source of these compounds. This paper is the first not only to deduce the structure of several novel isomeric saponins, including ions at *m/z* 1127 (Holothurinosides X/Y/Z), 1227 (Holothurin E, Scabraside A) and 1259 (Holothurins D/A_3) in the viscera of *H. lessoni* but also to demonstrate the MS/MS profiles of the number of known triterpene glycoside congeners such as Fuscocinerosides B/C or Scabraside A and Holthurin A_3. Peak intensities of fragment ions in MS/MS spectra were also correlated with structural features and fragmentation preferences of the investigated saponins; therefore, we were able to estimate the proportion of each isomer in the isomeric compounds. Evidence from tandem mass spectrometry suggested that the most abundant ions are generally attributed to the losses of aglycones and/or the key diagnostic sugar moieties (493, 507, 523, 639 and 643). Our results also reconfirmed the incidence of cross-ring cleavages to be higher in the sulfated compounds compared to non-sulfated glycosides. It is likely that the loss of sulfate group in the sugar moiety of saponins made them more susceptible for cross-ring cleavages.

For now, MS is one of the most sensitive and straightforward techniques of molecular analysis to determine saponin structure. This methodology of molecular structure identification using fragmentation patterns acquired from MS/MS measurements helps to propose and identify the structure of the saponins. It was found that under CID some of the identified saponins have the

same ion fingerprints for their sugar units, yielding the same *m/z* product ions. Some of these saponins were easily characterized since their MS/MS spectra shared common fragmentation patterns for the key diagnostic signals at *m/z* 493 and/or 507 and 523, in addition to the vital peaks at *m/z* 639 and 657, corresponding to the oligosaccharide chain [MeGlc-Glc-Qui-Xyl + Na^+]. We used mass spectrometry to evaluate the isomeric heterogeneity of their precursor ions as well as the structurally informative product ions.

Our finding indicates that the viscera are rich in saponins, in both diversity and quantity, and therefore their localization in the viscera is apparently related to the use of these organs in the defense against potential predators.

The chromatography techniques that were used in this study were able to separate saponins and isomeric saponins from sea cucumber to a purity which permits the structure elucidation of isomeric saponin congeners.

These novel saponins (Holothurinosides X/Y/Z and Holothurins E/D/) have great potential to be used for functional food ingredients (tonic foods), dietary supplements, food additives, food preservatives (because of emulsifying and foaming properties) and development of high value products for various industrial applications. For instance, they can be used as nutritional supplements or functional foods for human and animals. Therefore, this marine invertebrate is a valuable source for functional food.

This manuscript described the structure elucidation of seven reported compounds, Holothurin A or Scabraside B, 17-dehydroxy-holothurinoside A, Fuscocinerosides B/C or Scabraside A, 24-dehydroechinoside A and Holothurin A_3 before obtaining the structure of five novel compounds, Holothurins D/E and Holothurinosides X/Y/Z.

This study confirms the viscera of *H. lessoni* as a source of saponins with a wide spectrum of structural diversity, including both novel sulfated and non-sulfated congeners. Our findings demonstrate that the study of new sea cucumber species has provided a large number of compounds that may have application as nutraceuticals, pharmaceuticals, agrochemicals, cosmeceuticals or as research reagents.

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3.7 Author Contributions

Y.B. and C.F. designed the experiments. Y.B. carried out the experiments with guidance of C.F., T.C. and WZ, who assisted in setting up the HCPCP analysis.

Y.B. worked on chemical structure elucidation and all four authors contributed in writing the manuscript.

3.8 Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 4

STRUCTURE ELUCIDATION OF NEW ACETYLATED SAPONINS, LESSONIOSIDES A, B, C, D, AND E, AND NON-ACETYLATED SAPONINS, LESSONIOSIDES F AND G, FROM THE VISCERA OF THE SEA CUCUMBER *HOLOTHURIA LESSONI*

This chapter is my third paper published in the journal "Marine Drugs". This paper is cited as "Bahrami Y., Franco C.M.M. (2015) Structure Elucidation of New Acetylated Saponins, Lessoniosides A, B, C, D, and E, and Non-Acetylated Saponins, Lessoniosides F and G, from the Viscera of the Sea Cucumber *Holothuria lessoni*. Marine Drugs 13 (1):597-617." doi:10.3390/md13010597

This chapter outlines the isolation, purification and structure elucidation of 7 novel acetylated saponins, Lessoniosides A, B, C, D, and E, and non-acetylated saponins in the viscera of the sea cucumber *Holothuria lessoni*. It contains a brief introduction, material and methods and experimental sections; and described the HPCPC purification and MS analyses in detail.

I carried out these experiments with the guidance of my supervisor Prof. Chris Franco. I purified and analysed the samples and elucidated the chemical structure of the compounds, and I wrote the draft manuscript. CF confirmed the analyses and did a thorough revision of the manuscript. I drafted the responses to the reviewers' com ments which were revised by CF.

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Article

Structure Elucidation of New Acetylated Saponins, Lessoniosides A, B, C, D, and E, and Non-Acetylated Saponins, Lessoniosides F and G, from the Viscera of the Sea Cucumber *Holothuria lessoni*

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Abstract: Sea cucumbers produce numerous compounds with a wide range of chemical structural diversity. Among these, saponins are the most diverse and include sulfated, non-sulfated, acetylated and methylated congeners with different aglycone and sugar moieties. In this study, MALDI and ESI tandem mass spectrometry, in the positive ion mode, were used to elucidate the structure of new saponins extracted from the viscera of *H. lessoni*. Fragmentation of the aglycone provided structural information on the presence of the acetyl group. The presence of the *O*-acetyl group was confirmed by

observing the mass transition of 60 u corresponding to the loss of a molecule of acetic acid. Ion fingerprints from the glycosidic cleavage provided information on the mass of the aglycone (core), and the sequence and type of monosaccharides that constitute the sugar moiety. The tandem mass spectra of the saponin precursor ions $[M + Na]^+$ provided a wealth of detailed structural information on the glycosidic bond cleavages. As a result, and in conjunction with existing literature, we characterized the structure of five new acetylated saponins, Lessoniosides A–E, along with two non-acetylated saponins Lessoniosides F and G at *m/z* 1477.7, which are promising candidates for future drug development. The presented strategy allows a rapid, reliable and complete analysis of native saponins.

Keywords: sea cucumber; viscera; saponins; mass spectrometry; MALDI; ESI; HPCPC; triterpene glycosides; structure elucidation; bioactive compounds; marine invertebrate; Echinodermata; holothurian

4.1 Introduction

Sea cucumbers belonging to the class *Holothuroidea* of the *Echinodermata* phylum are marine invertebrates that produce a range of compounds that have the potential to be used in agriculture, and as pharmaceuticals, nutraceuticals and cosmeceuticals [1,2].

Saponins are the most important characteristic and abundant secondary metabolites in this species [3]. Sea cucumber saponins exert a wide range of medicinal and pharmacological properties. Saponins are also the main bioactive compounds in many plant drugs and folk medicines, especially in the Orient.

Although sea cucumber saponins share common saponin features, their aglycones, also called sapogenins or genins, are significantly different from those reported in the plant kingdom [1]. These amphipathic compounds generally possess a triterpene or steroid backbone or aglycone (hydrophilic, lipid-soluble) connected glycosidically to a saccharide moiety (hydrophilic, water-soluble) [3–5]. Saponins are also produced by other marine organisms including asteroids [6], which also belongs to the phylum *Echinodermata,* and sponges of the phylum *Porifera* [7]. Sea cucumbers saponins are usually triterpene glycosides (derived from lanostane) [3] while those from starfish are steroid glycosides [6]. The sugar moieties mainly consist of D-xylose (Xyl), D-quinovose (Qui), 3-*O*-methyl-D-glucose (MeGlc), 3-*O*-methyl-D-xylose (MeXyl) and D-glucose (Glc), and sometimes 3-*O*-methyl-D-quinovose, 3-*O*-methyl-D-glucuronic acid and 6-*O*-acetyl-D-glucose. In the oligosaccharide chain, the first monosaccharide unit is always a xylose, whereas 3-*O*-methylglucose and/or 3-*O*-methylxylose are always the terminal sugars. The presence of two quinovose residues in a carbohydrate moiety is unique for sea cucumber and starfish glycosides.

There are more than 700 triterpene glycosides in various species of sea cucumbers [3,5,8–17], which are classified into four main structural categories based on their aglycone moieties: three holostane types containing a (1) 3β-hydroxyholost-9(11)-ene aglycone skeleton; (2) a 3β-

hydroxyholost-7-ene skeleton and (3) an aglycone moiety different to other two holostane type aglycones, and a nonholostane aglycone [5,12,17,18]. The majority of saponins belong to the holostane type group [3,12,13,19]. Most sea cucumber saponins comprise of a lanostane-3β-ol type aglycone with a γ-18 (20)-lactone in the D-ring of tetracyclic triterpene (3β,20*S*-dihydroxy-5α-lanostano-18,20-lactone) [5], sometimes containing shortened side chains; the glycone contains up to six monosaccharide units covalently connected to C-3 of the aglycone.

In sea cucumbers, the sugar residue has only one branch [13], whereas plant saponins may contain one, two or three saccharide chains, with a few having an acyl group bound to the sugar moiety [20]. One of the most noteworthy characteristics of many of the saponins from marine organisms is the sulfation of aglycone or sugar moieties [1], and in sea cucumbers the sulfation of one or more of Xyl, Glc, MeGlc and Qui residues have been reported [3,11]. Most of them are mono-sulfated glycosides with few occurrences of di- and tri-sulfated glycosides [13,17]. Another structural feature that has been found only in this series of aglycones is the presence of an acetoxyl group at C-16 and/ or in the lateral side of the aglycone (C-22 or C-23 and/or C-25). The other structural feature is the presence of a 12α-hydroxy group in the aglycone unit of saponins in the genus *Holothuria*; however some contain two hydroxy groups at positions 12α and 17α of the holostanol skeleton. Several triterpene glycosides (such as Holothurinoside X, Fuscocinerosides B and Scabraside B) isolated from the sea cucumber *Holothuria lessoni* contain a carbonyl group in the lateral chain [3,12]. The majority of saponins from Aspidochirotida sea cucumbers contain the 9(11)-double bond in their aglycone moiety, but most of the glycosides isolated from *Holothuria* are $\Delta^{9,11}$-glycosides.

Recently, a series of unusual non-holostane triterpene glycoside have been reported from sea cucumbers, belonging to order *Dendrochirotida*, which have a shortened side chain, and no lactone function. So far, only nine non-holostane acetylated saponins including Kurilosides A and C, Psolusoside B, Frondoside C, Cucumariosides A_2-7, A_2-8, A_8, A_9 and Koreoside A have been reported from the class *Holothuroidea*.

Similarities in structure of saponin glycosides leads to difficulties in purification, and this vitiates the complete structure elucidation of these molecules (especially isomers). High Performance Centrifugal Partition Chromatography (HPCPC) is effective in separating polar compounds and was employed successfully in obtaining purified saponins in this study.

The viscera of an Australian sea cucumber *Holothuria lessoni* (golden sandfish) [21] was selected as a source of saponins because we hypothesized that the internal organs contain high levels of compounds as the viscera are expelled from the sea cucumber in order to repel other sea animals. In relation to internal organs, the saponin content of the cuvierian tubules of *Holothuria* were found to be higher than the body wall on a weight basis [22,23]. We have recently reported [3,12] new saponins within the viscera of *H. lessoni*, and in this paper we present five new acetylated saponins and two related new non-acetylated isomers identified from the viscera using mass spectrometry.

Nuclear magnetic resonance (NMR) spectroscopy can provide extensive structural information for saponins, however larger quantities of high-purity samples are generally needed. This is complicated with fractions if the NMR signals overlap, making their assignments more difficult.

Moreover, the measurement of the absolute configuration of the sugar moieties of a saponin cannot be completely solved by NMR methods alone [24]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF/MS) and electrospray ionization mass spectrometry (ESI-MS) techniques have become the preferred techniques for analyses of saponins. Mass spectrometry provides a highly sensitive platform for the analyses of saponin structures by generating product ions by the cleavage of the glycosidic bond.

Several studies reported that individual species have specific saponin congeners. However some congeners are common among different species. Even if the diversity is great, saponins from closely related species still retain the same molecular motif [11,25] and this property of saponins can be utilized for their taxonomic classification. Because of their internal and external roles the molecular structure of these compounds was most likely to be conserved within the species.

Mass spectrometry has a long history in the structure elucidation of saponins in both negative and positive ion modes. It has been extensively used to determine the molecular weight and the structure of the native aglycones as well as the glycosidic linkages in the oligosaccharide chain without degradation of the glycosides. Knowledge of the chemical structure of compounds is very important to determine the specific correlation between the structure and their molecular and biological mechanism(s) of actions. We expect that the results of this project will transform the value of the viscera of sea cucumbers into sources of high value products, important to human health and industry.

4.2 Results and Discussion

We reported the isolation and purification of several saponins from the viscera of sea cucumber species, *H. lessoni*, using ethanolic extraction, followed by solvent partition, then HPCPC. The extraction and purification procedures and the mass spectrometry analyses were described in detail in our previous publications [3,12].

The appropriate HPCPC fractions were pooled, based on their similar Rf values when run on thin-layer chromatography (TLC), and concentrated to dryness. Sodium ions were introduced to the samples before conducting the MS analysis, ensuring all saponins observed in the positive ion mode were predominantly singly charged sodium adducts $[M + Na]^+$; triterpene glycosides have a high affinity to alkali cations. The prominence of $[M + Na]^+$ also facilitated the analysis of saponins in mixtures or fractions. The saponin profile of each HPCPC fraction was then revealed by MALDI MS and ESI-MS [3,12]. MS^2 analyses identified key diagnostic ions produced by cleavage of the glycosidic bond including oligosaccharide and monosaccharide fragments [3,12,26]. Other visible peaks and fragments detected corresponded to the loss of other neutral moieties such as CO_2, H_2O or CO_2 coupled with H_2O.

4.2.1 Structure Determination of Saponins by ESI-MS

ESI-MS^n is a very effective and powerful technique to distinguish isomeric saponins as they generate different MS^n fragmentation profiles [27,28]. All saponin ions perceived in the ESI-MS spectrum of the HPCPC fractions were also analyzed by ESI-MS^2 in the positive ion mode. Previous MS^2 studies on HPCPC fractions 12, 14, 15 [3], 17, 18, 20 and 22 [12] obtained from the butanolic

extract of viscera of sea cucumber *H. lessoni* yielded a number of new saponins. This analysis of fraction 18 gave complex spectra representing several saponin classes, also confirmed the presence of saponins reported in the literature and identified new saponin congeners (Supplementary Figure S1, and Figure 1 of [12]). Fifteen major peaks were detected which corresponded to several known triterpene compounds (as summarized in Table 1 of [12]), including Holothurinosides C/C_1, Desholothurin A_1 and Desholothurin A (synonymous with Nobiliside 2a), Holothurinoside J_1, Fuscocinerosides B/C or Scabraside A or 24-dehydroechinoside A and Holothurin E, Holothurin A, Holothurinosides E/E_1/O/P, Holothurinoside M, Holothurinosides A/A_1/R/R_1/S/Q, Holothurinoside N, Holothurinoside I and Holothurinoside K_1 in addition to several new saponins [3,12]. The spectrum displays one dominant peak at *m/z* 1477.7, which corresponds to unidentified (new) saponins, with elemental compositions of $C_{68}H_{110}O_{33}$, $C_{66}H_{102}O_{35}$ and $C_{66}H_{118}O_{34}$.

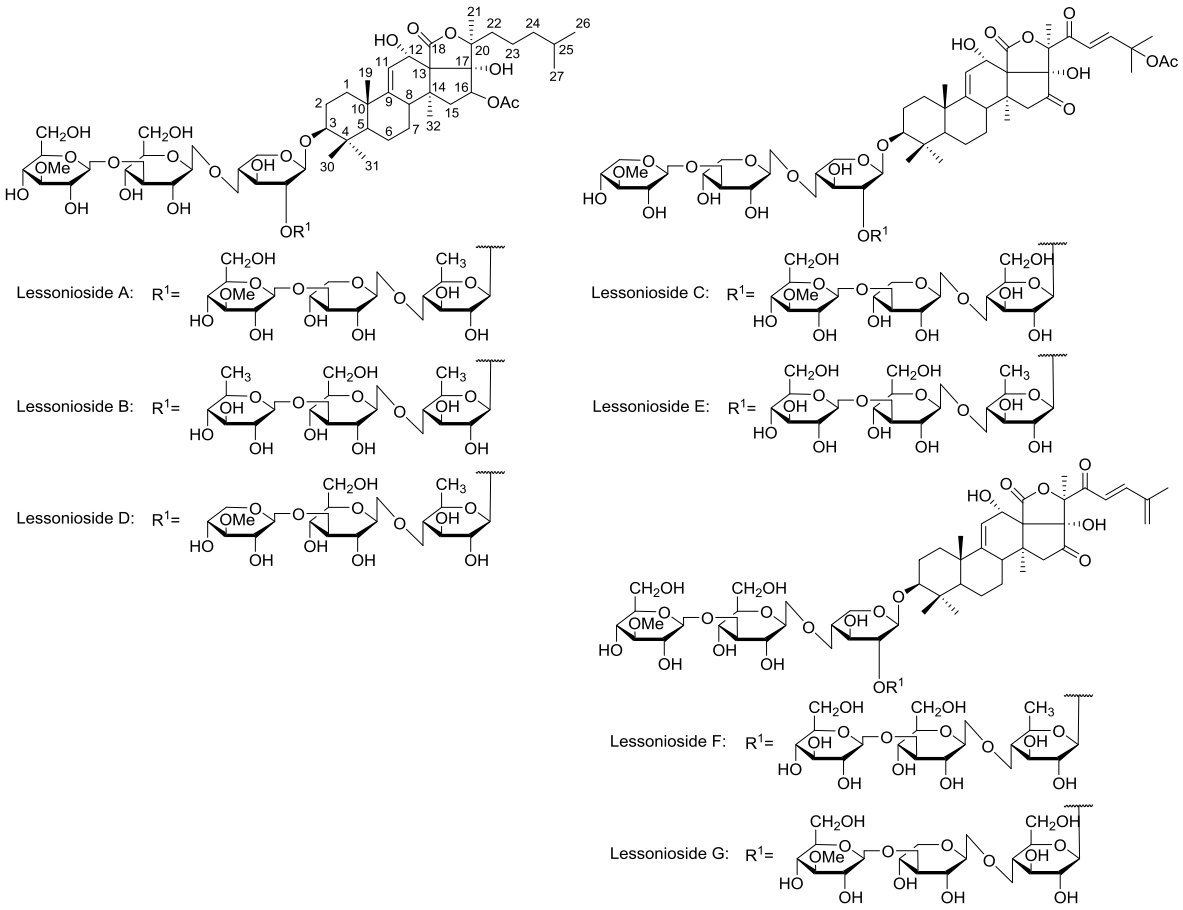

Figure 1. The structures of the new acetylated saponins in the viscera of *H. lessoni*, Lessoniosides (**A**–**E**) along with the non-acetylated Lessoniosides (**F**–**G**) compounds are described in this figure.

This analysis revealed that HPCPC Fraction 18 contains several saponin congeners showing that the absolute purification of the saponins was not possible within a single HPCPC run with these closely related compounds.

4.2.2 Structure Identification of Saponins by MALDI-MS

Similar to the ESI-MS, the MALDI MS of the isobutanol-enriched saponin extract obtained from the viscera of the *H. lessoni* revealed the presence of at least 75 saponin congeners, including 39 new sulfated, non-sulfated and acetylated triterpene glycosides, and 36 congeners which were previously reported in other holothurians [3].

To elucidate the chemical structure of saponins based on the MS^2 spectra, as described previously [3,12], precursor ions were selected, fragmented and fragmentation profiles built. The molecular structures of the saponins were determined by the identification of the mass transitions between the successive collision-induced fragmentation peaks on the basis of the accurate mass of the individual sugar components.

Based on the literature, MeGlc and MeXyl are always terminal sugars and Xyl is always the first sugar, which is bound to C-3 of the aglycone. Further, the exact mass of each sugar, such as MeGlc = 176 Da, Glc = 162 Da, Xyl = 132 Da, Qui = 146 Da, and the determination of the mass transitions between the peaks on the basis of the accurate mass of the individual sugar moieties, and mass and sequence of the key diagnostic peaks helped us build the sequence of these sugar moieties. Using this strategy the structure of seven new triterpene glycosides from *H. lessoni* with an *m/z* value of 1477.7 from HPCPC fraction 18 were characterized.

The chemical structures of the new acetylated saponins from the viscera of *H. lessoni* are illustrated in Figure 1. Lessoniosides A, B, C, D and E are the only published examples of glycosides from *H. lessoni* containing the side chain of the acetoxy group in their aglycone moieties. We now provide an account of the structure elucidation of these saponins using this approach.

4.2.3 MALDI-MS^2 Analysis of Saponins

Saponin ion peaks were further analyzed using MS^2 fingerprints generated with the collision-induced dissociation (CID) from their respective glycan structures. The techniques used are also able to distinguish the structural differences among the isomers following HPCPC separation. As a typical example, the MALDI-MS^2 fingerprints for the ion detected at *m/z* 1477.7 (triterpene glycoside) are shown in Figure 2. The schematic fragmentation of Lessonioside A as a representative is shown in Supplementary Figure S2. The fragmentation pattern of the sodiated compound at *m/z* 1477.7 in consecutive MS experiments is discussed in detail below for stepwise elucidation of the molecular structure of these compounds.

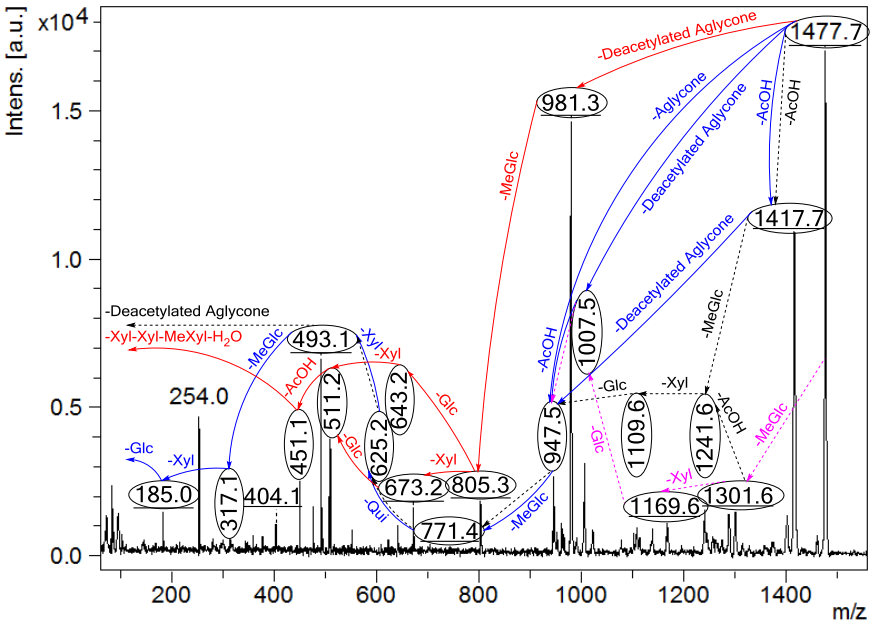

Figure 2. Positive tandem MALDI (matrix-assisted laser desorption/ionization) spectrum analyses of the precursor ion (saponin) detected at *m/z* 1477.7. The MS^2 fragmentation profile of the ion at *m/z* 1477.7. Figure shows the collision-induced fragmentation of parent ions at *m/z* 1477.7. The full and dotted arrows show the possible fragmentation pathways of this ion using CID (collision-induced dissociation). The blue arrows show the fragmentation of the isomeric congeners Lessonioside A where the red arrows indicate the decomposition patterns of Lessonioside C. These analyses revealed that this ion corresponds to isomeric compounds.

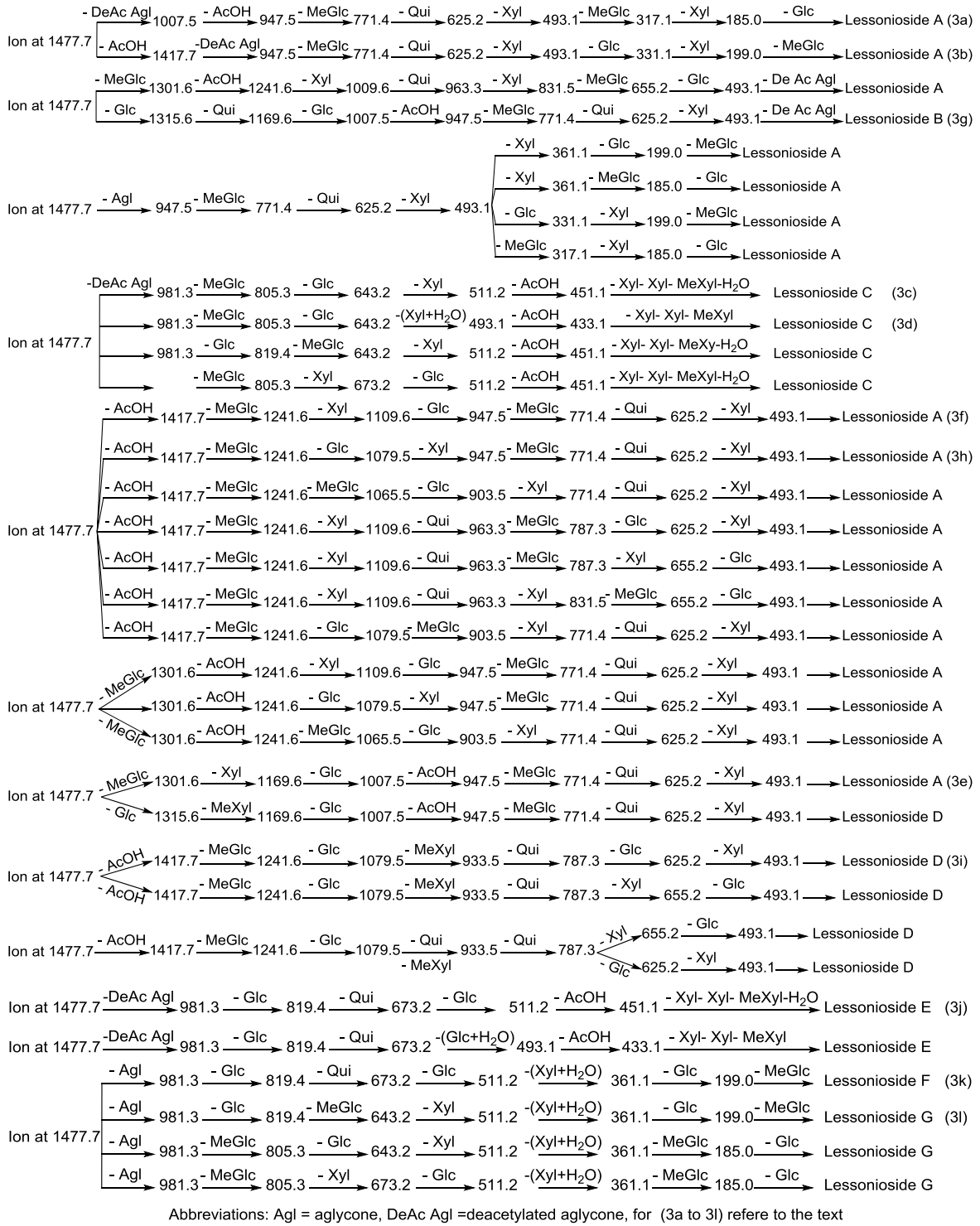

Figure 3. The schematic diagram of the proposed isomeric structures of the ion at *m/z* 1477.7. This figure indicates the comprehensive feasible fragmentation pathways of the isomeric acetylated, Lessoniosides (**A**–**E**), and non-acetylated, Lessoniosides (**F**–**G**), triterpene glycosides generated from the ion at *m/z* 1477.7.

CID activates three feasible independent fragmentation pathways of cationized parent ions shown in full and dotted arrows. First, as described in Figure 2, the consecutive losses of the deacetylated aglycone, acetic acid (AcOH), 3-*O*-methyl-D-glucose (MeGlc), D-quinovose (Qui), D-

xylose (Xyl), MeGlc and Xyl residues (blue arrows) followed by D-glucose (Glc) yielded ion fragments at *m/z* 1007.5, 947.5, 771.4, 625.2, 493.1, 317.1 and 185.0 (Figure 3a), respectively, in one of the new isomers for which we propose the name Lessonioside A. The loss of aglycone (Agl) generated the ion at *m/z* 947, corresponding to the complete sugar moiety. The ion at *m/z* 493.1 corresponds to the diagnostic sugar reside $[MeGlc\text{-}Glc\text{-}Xyl + Na]^+$. Further, the sequential losses of Glc and Xyl units from this key diagnostic peak (*m/z* 493.1) generated ions at *m/z* 331.1 and 199.0 (Figure 3b).

With another isomer, the consecutive losses of the deacetylated aglycone, MeGlc, Glc, Xyl and AcOH followed by the hydrated three sugar units (red arrows) produced ions at *m/z* 981.3, 805.3, 643.2, 511.2 and 451.1, respectively, (Figure 3c) revealed the structure of a second new saponin, which we named Lessonioside C. Further, the consecutive losses of the deacetylated aglycone, MeGlc, Glc, Xyl (at a terminal position) and an acetyl group from the parent ion generated the fragment ions at *m/z* 981.3, 805.3, 643.2, 493.1 and 433.1, (Figure 3d) respectively, confirming the structure of Lessonioside C.

Secondly, the decomposition of the parent ion can also be triggered by the sequential loss of sugar moiety namely MeGlc, Xyl, Glc, AcOH, MeGlc, Qui and Xyl followed by the deacetylated aglycone residue which generated daughter ions at *m/z* 1301.6, 1169.6, 1007.5, 947.5, 771.4, 625.2, and 493.1, respectively (Figure 3e). This sequence of fragmentation confirms the structure of new saponin, Lessonioside A. In this case, the ions at *m/z* 493.1 correspond to the sodiated deacetylated aglycone moiety (*m/z* value of 470).

The third viable pathway is elicited by the initial loss of an acetoxy group. In the case of Lessonioside A this initial loss (−60) is followed by the sequential loss of the sugars (including the diagnostic MeGlc-Glc-Xyl) to yield the key diagnostic DeAc Agl ion (*m/z* 493.1) (Figure 3f). In addition the sequential losses of the MeGlc (317.1) and Xyl (*m/z* 185.0) followed by Glc further confirmed the structure of the new isomer, Lessonioside A.

As was observed with the MALDI-MS^2 this saponin possesses the common *m/z* 493.1 key signal diagnostic of both the sugar moiety $[MeGlc\text{-}Glc\text{-}Xyl + Na]^+$ and the DeAc Agl moiety $[C_{32}H_{50}O_6 - AcOH + Na]^+$. This is consistent with previous findings for the MS^2 of sea cucumber saponins [12]. The ion 493.1 is also observed in Lessonioside C, however, this is formed as a result of a loss of DeAc Agl and of the sugar moiety MeGlc-Glc-Xyl (511) and H_2O (493). The ion at 643 yields ions at 511 and 493 by the loss of Xyl and Xyl + H_2O, respectively. These ions are recognized as the key diagnostic fragments in triterpenoid saponins.

These MS^2 analyses using both MALDI and ESI modes allowed the establishment of connectivities of the sugar residues and thus permit the assignment of the peaks. For example, the MALDI-MS^2 of the parent ion showed fragments at *m/z* 1417.7 $[M + Na - AcOH]^+$ which suggested the presence of an acetyl moiety and the innate sugar component at *m/z* 947.5 $[M + Na - Agl]^+$, an observation that was confirmed by ESI- MS^2 in the positive ion mode. The MALDI mass spectrum showed evidence of the presence of the acetoxy group in the aglycone.

4.2.4 Key Diagnostic Sugar Residues in the Sea Cucumber Saponins

Characterization of common key fragments expedited the structure elucidation of new and reported saponins. Tandem mass spectrometry analysis of saponins showed the presence of several diagnostic key fragments corresponding to certain common structural element of saponins as summarized in Table 1. Here we report a new diagnostic key fragment at *m/z* 643 corresponding to the sodiated hydrated sugar residue MeGlc-Glc-Xyl-Xyl.

Table 1. Key diagnostic ions in the MS^2 of the holothurians saponins. It has been adapted from [12] and modified.

Diagnostic Ions in CID Spectra of Saponins $[M + Na]^+$				
	***m/z* Signals (Da)**			
	493	507	643 or 625	639 or 657
Chemical signatures	MeGlc-Glc-Xyl + Na	MeGlc-Glc-Qui + Na	MeGlc-Glc-Xyl-Xyl + Na = 625 MeGlc-Glc-Xyl-Xyl + H_2O + Na = 643	MeGlc-Glc-Qui-Xyl + Na = 639 MeGlc-Glc-Qui-Xyl + H_2O + Na = 657

The structures of sugar components of saponins were established by the identification of these diagnostic ions produced by tandem mass spectrometry. Observing these oligosaccharide moieties (*m/z* 493 and/or 507 and/or 511 (493 + H_2O) and/or 523 and/or 643 and/or 657) simplified the characterization of the saponin structure.

4.2.5 Elucidation of the Saponin Structures by ESI-MS^2

ESI-MS^2 was carried out using CID, creating ion fragments from the precursor ions (Figure 4), and was applied to differentiate the structure of isomeric saponins as described by Song *et al.* [27]. The schematic fragmentation of Lessonioside A, as a representative, and the stepwise structure elucidation of the ion at *m/z* 1477.7 is shown in Figure 4 corroborates results from the MALDI-MS^2.

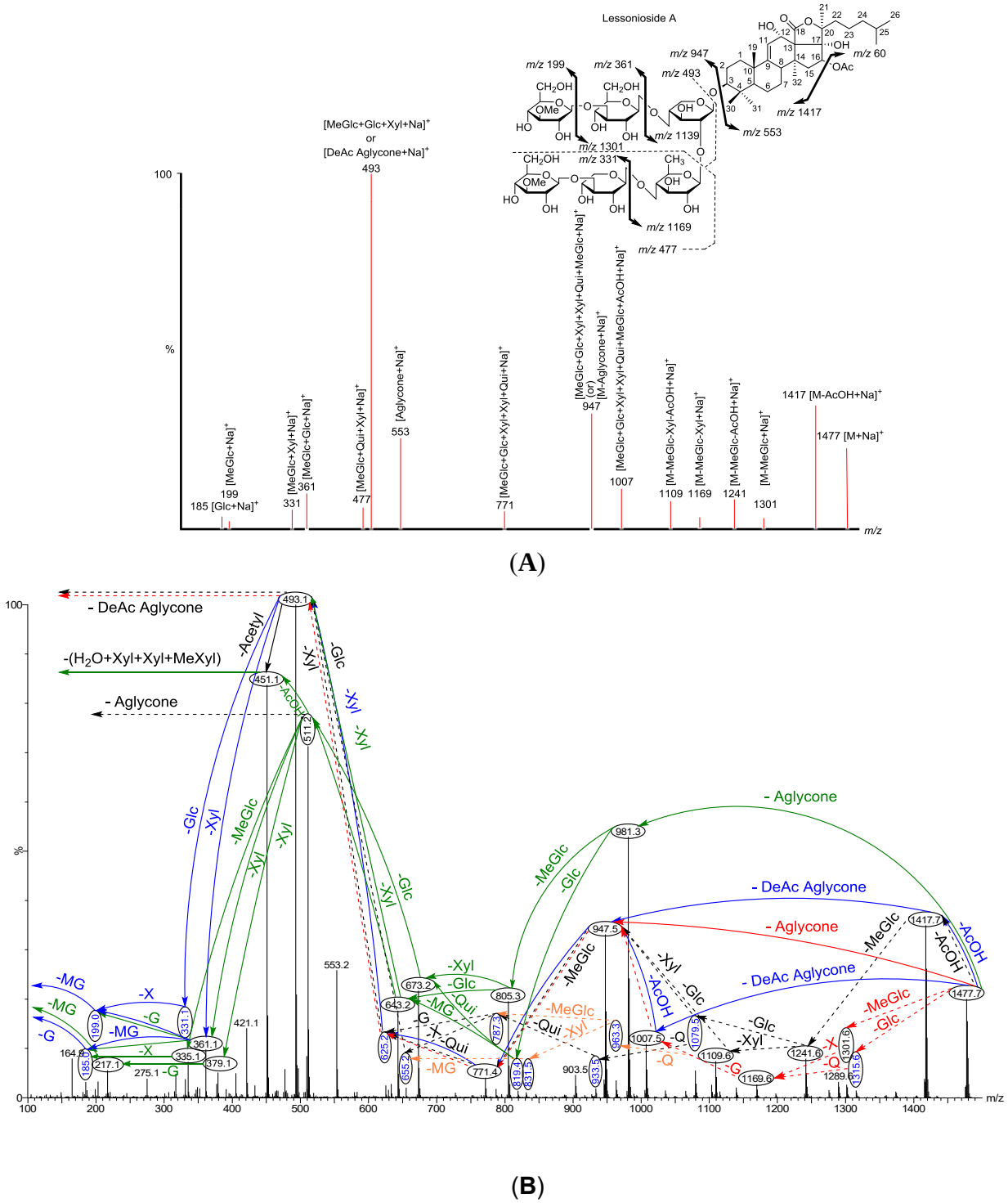

Figure 4. Positive ion mode ESI-MS^2 spectrum of acetylated saponins detected at *m/z* 1477.7 from Fraction 18. The schematic fragmentation of Lessonioside A as a representative (**A**), and the complete ESI-MS^2 fragmentation profile of the ion at *m/z* 1477.7 (**B**). Spectrum (**B**) shows the presence of two different aglycones in the isomeric saponins. Full and dotted arrows illustrate the three main feasible fragmentation pathways. The blue arrows show the decomposition of the isomeric congeners Lessoniosides A, B and D where the green arrows indicate the fragmentation patterns of

Lessoniosides C, E, F and G. The ion at *m/z* 451.1 corresponds to the hydrated three sugar units [Xyl-Xyl-MeXyl + H_2O + Na].

4.2.6 ESI- MS^2 Analyses of Ion at *m/z* 1477.7

Tandem MS analyses revealed the presence of two different peaks with *m/z* value of 947.5 and 981.3, corresponding to the losses of different aglycone moieties with *m/z* values of 530 and 496, respectively, confirming the presence of chemical structural isomers. Further this MS^2 analysis also distinguished the presence of an acetoxy group in both isomer types.

Similar to sulfated compounds, after collisional activation, the parent ions are subjected to three independent dissociation pathways shown using full and dotted arrows (Figure 4). First, the consecutive losses of the deacetylated aglycone, acetoxy group, MeGlc, Qui, Xyl, Xyl and MeGlc residues (blue arrows) followed by Glc afford product ions as shown in Figure 4 confirmed the structure of Lessonioside A. Therefore, in this case, the ions at *m/z* 493.1 correspond to the sodiated key diagnostic sugar residue; $[MeGlc\text{-}Glc\text{-}Xyl + Na]^+$.

Secondly, the decomposition of the parent ion could also be triggered by the loss of sugar moieties followed by the deacetylated aglycone residue which generated daughter ions as shown in Figure 3e confirming once more the structure of Lessonioside A. It is clear that the ions at *m/z* 493.1 correspond to the sodiated deacetylated aglycone moiety (*m/z* value of 470). Alternatively, ions corresponding to the sequential losses of Glc, Qui, Glc, AcOH, MeGlc, Qui, and Xyl (red dotted arrows) were detected in Figure 3g indicating the presence of another isomer, Lessonioside B, which possesses two Qui units. The presence of two Qui in the carbohydrate chain of sea cucumber glycoside is a very rare characteristic.

Finally, the fragmentation of the parent ions can also be initiated with the loss of the acetoxy group. The consecutive losses of the acetic acid (AcOH) and the deacetylated aglycone unit followed by the sequential losses of the sugar moiety (Figure 3b) further confirmed the structure of Lessonioside A. Alternatively, the decomposition of the deacetylated saponins can be accomplished by the sequential losses of monosaccharides in the sugar chain, namely ion detected at 1417.7 $[M - AcOH + Na]^+$ (black dotted arrows, Figure 3h). In this case, the ions at 493.1 corresponds to the DeAc Agl moiety $[M - \text{sugar residue} - AcOH + Na]^+$. Alternatively, the sequential losses of AcOH and sugars from the parent ions (Figure 3i), afforded daughter ions that assisted in postulating the structure of another new isomer, Lessonioside D. The above evidence suggested that Lessonioside A possesses the same aglycone as Lessoniosides B and D, but differs in the hexasaccharide chain. The complete analyses can be seen in Supplementary Figure S3.

The MALDI-MS^2 and ESI-MS^2 analyses for all possible isomers were carried out in a similar manner as described above for Lessoniosides A, B, C and D. A comprehensive list of possible fragmentation patterns based on the MS^2 ions generated from the ion at *m/z* 1477.7 is shown in Figure 3.

The sugar moiety of Lessonioside A was found to be identical to those of Cladolosides C_1 and C_2 isolated from the sea cucumber *Cladolabes schmeltzii* [16], confirming the constituents of the hexasaccharide chain (Figures 1 and 3). The sugar component also had some similarity to those of Violaceuside B isolated from the sea cucumber *Pseudocolochirus violaceus* [29]. This group also

stated the ions at *m/z* 625.2 and 493.1 corresponded to [MeGlc + Xyl + Glc + Xyl + Na]$^+$ and [MeGlc + Xyl + Glc + Na]$^+$, respectively, which confirmed our results. Yayli and associates [30], however, stated the ions at *m/z* 493 and 325, corresponding to [MeGlc-*O*-Xyl-*O*-Qui(*O*)-*O*]$^+$ and [MeGlc-*O*-Xyl]$^+$, respectively, which are under question. The structure of the aglycone moiety was also very similar to that of Holothurinoside Y [12], the difference being the addition of an acetoxy group at C-16. The assignments of the MS^2 signals associated with the aglycone moiety Lessoniosides A, B and D showed a close similarity to those reported for 16β-acetoxy-holosta-9-ene-3β,12α,17α-triol, the aglycone of Nobiliside C [*m/z* 715], *m/z* 656 [M − OAc + Na]$^+$, isolated from the sea cucumber *Holothuria nobilis* [31].

These saponin congeners identified from Fraction 18 are more conjugated with glycosides compared with the new saponins previously reported in this species [3,12]. Lessoniosides C, D and E possess the same terminal saccharide moiety (MeXyl), which is a rare structural feature among naturally occurring sea cucumber glycoside and has been infrequently reported.

4.2.7 Isomers that Generate the Deacetylated Aglycone at *m/z* 981.3

For other isomers, the loss of the deacetylated aglycone yields a large fragment at *m/z* 981.3. The alternative fragmentation patterns of the sugar residues for Lessoniosides C and E are described in Figure 3.

An interesting peculiarity of Lessonioside C and E is the presence of keto groups at both C-16 and C-22 positions, and 25β-*O*-Ac in the aglycone. This is the third example of the aglycone of sea cucumber glycosides bearing a ketone group at C-22 as Zhang *et al.* [32] and Liu *et al.* [33] stated the presence of a ketone group in Fuscocineroside A and Arguside D, respectively. The structure is characterized by the presence of an oligosaccharide chain composed of six units. The holostane-type aglycone features an endocyclic double bond at position C-9(11) and C-23 and a β-acetoxy group at C-25.

4.2.8 Non-Acetylated Isomeric Congeners

Based on the chemical evidence the structure of Lessoniosides F/G were defined as 16,22-diketo-holosta-9(11)-23(24)-25(27)-triene-3β,12α,17α-triol, which is shown in Figure 1. They are of the lanosterol type featuring the characteristic ring-D-fused γ-lactone (C=O) function and a Δ^9 double bond. The aglycone of Lessonioside F/G differs from E by the presence of a double bond at Δ^{25} and a loss of the AcO group at C-25 to form an exo double bond, Δ^{25}.

On fragmentation these isomers generate ions at 981.3 corresponding to the loss of aglycone. Subsequent losses of the sugar moieties are described in detail in Figure 3 (k and l) and Supplementary Figure S4 for Lessoniosides F and G. These non-acetylated Lessoniosides possess similar aglycones but different sugar moieties. It is notable that all three feasible independent fragmentation pathways might occur simultaneously which generated several different fragmentation sequences.

The molecular weights of the deacetylated aglycone moieties in some of these new saponins (Lessoniosides A, B and D) coincided with those reported for Philinopside B, from the sea cucumber *Pentacta quadrangularis* using ESI-MS by Zhang *et al.* [34], however, the structures are

very different, because they are from a different family of sea cucumber, with ring closure of the C-20 side chain.

The cleavage of the *O*-acetyl group of the aglycone residue results in the loss of AcOH (60 Da). However, Song and co-workers [27] noted the neutral losses of CH_2O (30 Da) and $C_2H_4O_2$ (60 Da) in cross-ring reactions of the sugar residues. The cross-ring cleavage of the sugar also leads to the loss of $C_2H_4O_2$ (60 Da).

The losses of H_2O and CO_2 or their combination results from cleavage at the glycosidic linkages as noted by Waller and Yamasaki [2]. The losses of CO_2 (44 Da), H_2O (18 Da), AcOH (60 Da), Acetyl group (42 Da) and CH_2O (30 Da) were detected from the spectra which affords different product ions and several peaks were assigned to those molecules. For instance, the ion at *m/z* 1373.7 was generated by the loss of CO_2 from the deacetylated parent ions (*m/z* 1417.7), or the sequential losses of H_2O and acetyl molecule from the ions at *m/z* 511.2 generated ions at *m/z* 493.1 and 451.1, respectively. Further the ion at *m/z* 493.1 can be stemmed from the ion at *m/z* 553.2 (sodiated aglycone) by the loss of the acetoxy group. Additionally, the loss of CH_2O from the ion at *m/z* 451.1 yields the ion at *m/z* 421.1. However, Kelecom and coworkers [35], and Kitagawa and associates [36], stated the latter ion as an aglycone fragment. The complete analyses can be seen in the Supplementary Figure S3.

The MS^2 analyses of ions at *m/z* 1477.7 revealed a similar fingerprint profile with those reported for Holothurinosides X, Y and Z, in particular in the area of 100 to 600 Da where the signals were coincident with those of ion at *m/z* 1127.6, which show the intrinsic relationship between these saponin congeners [12].

Lessoniosides A, B, C, D and E are the only examples of glycosides from *H. lessoni* containing an acetoxy side chain in their aglycone moieties. Most of the glycosides isolated from sea cucumber *Holothuria* are $\Delta^{9,11}$- glycosides. In general, 3β-hydroxyholost-9(ll)-ene based aglycones were characterized in Holothurins isolated from animals of the order *Aspidochirota* [17].

4.2.9 The Structure of Aglycones

This analysis revealed the presence of at least seven different isomers with diverse aglycone and sugar components for the ion at *m/z* 1477.7. The glycosides differ in their aglycone structures or sugar moieties. The mass of the cationized aglycone and the deacetylated aglycone in Lessonioside C and E are 556 Da $[M - \text{sugar residue} + Na]^+$ and 496 $[M - AcOH - \text{sugar residue} + Na]^+$, respectively, which are consistent with the mass of the aglycone reported by Elyakov and co-workers [37]. Their MS analyses showed *m/z* 556 $[M]^+$, 541 $[M - CH_3]^+$ and 496 $[M - CH_3COO]^+$ for an aglycone moiety. Further, Rothberg and associates noted an aglycone with the same molecular weight having an acetoxy group at the C-23 from *Stichopus chloronotus* [38]. Analysis of the MS data for these isomers and comparison with those published for related saponin aglycones [3,12,31,39] shows that the aglycone part of Lessoniosides are a holostane skeleton featuring hydroxy groups at C-12 and C17. With other isomers, the mass of the cationized aglycone and the deacetylated aglycone was found to be 530 Da and 493 Da, respectively. Other prominent high mass ions *m/z* 1417 $[M - AcOH + Na]^+$ and 1241 $[M - AcOH - MeGlc + Na]^+$ or 1241 $[1301 - AcOH + Na]^+$ provide additional support for the acetate and lactone functions.

The aglycone structure of Lessoniosides C/E appears to be similar to that of Fuscocineroside A reported from the sea cucumber *Holothuria fuscocinerea* [32]. The lateral C-20 side chain of Lessoniosides C and E was found to be similar to Fuscocineroside A [32], and Arguside D [33]. However, they differed from Fuscocineroside A and Arguside D by the addition of a keto at C-16, a C-23 double bond and a 17-OH.

Three of these compounds, Lessoniosides A, B and D have identical holostane aglycones containing an 18(20)-lactone with a 9(11)-double bond and acetoxy group at C-16 and differ from each other in their sugar component. On the other hand, they differ from Lessoniosides C/E in the presence of an acetoxy group at the C-16 (*vs.* keto group in C/E) and absence of a C-22 keto group, a C-23 double bond and a C-25 acetoxy at the lateral chain.

These glycosides have holotoxinogenin, a genin containing 9(11)-double bond and 16-oxidized group in the aglycone. They possess two hydroxy groups at 12α and 17α positions that are characteristic for Aspidochirotid sea cucumber (the family *Holothuriidae*). This aglycone is common for glycosides from many different sea cucumbers [11].

Therefore, elucidation of the aglycone component of the saponins was performed by comparison with published data. Because the new identified compounds clearly have aglycone structures similar to that of other previously characterized compounds we can be confident that it is possible to elucidate the structure of these compounds based on MS analysis alone. However, NMR analysis will be required to confirm the structure of the aglycones and also to ascertain the stereochemistry and linkages of the sugar moieties. Whereas, in the case of a novel compound without any similar previously characterized components, detailed chemical analysis including the application of NMR would be required.

4.2.10 Acetylated Saponins

So far more than 700 triterpene glycosidic saponins have been reported from sea cucumber species of which more than 130 are acetylated saponin. The majority of these are of the holostane type. The known acetylated triterpene glycosides (saponins), isolated from sea cucumbers of the class *Holothuroidea*, possess an acetyl group (acetoxy) in their aglycone residues. In the *Holothuriidae* family, the acetoxy group is either located at C-16 of the aglycone core moiety such as in Arguside F [40] and Nobiliside C [31], or at the C-22, C23 or C-25 of the lateral chain, ie at C-25 of the Pervicosides A and D [40,41]. However, Cucumarioside A_1-2 is the only example of a triterpene glycoside containing an acetate group at C-6 (6-OAc) of the terminal glucose unit (sugar residue) 6-*O*-acetylglucose [42].

The majority of the acetylated compounds, such as Fuscocineroside A from the sea cucumber *H. fuscocinerea,* contain a sulfate group in their structures [32]. However, the presence of a sulfate group was not observed in these new acetylated saponins from *H. lessoni*.

Acetylated saponins are mainly reported in the family *Cucucmarridae*. However, the presence of acetylated saponins for the genus *Holothuria* is only reported for *H. lessoni* (this work), *H. forskalii*, *H. nobilis*, *H. hilla*, *H. fuscocinerea*, *H. (Microthele) axiloga* and *H. pervicax* [17,31,32,40,41,43,44]. The majority of reported acetylated saponins possess only one acetoxy group in their structure, whereas saponins containing two *O*-acetic groups in their aglycone moieties have also been reported [16].

The presence of 12α and 17α–hydroxy, which are characteristic for glycosides from holothurians belonging to the family *Holothuriidae* (order *Aspidochirotida*), in glycosides of Dendrochirotids confirms parallel and relatively independent character of evolution of glycosides.

Observations from numerous studies confirm that the biological activity of saponins is influenced both by the aglycone and the sugar moiety. In other words there is a close relationship between the chemical structure of saponins and their biological activities. It has been reported that the presence of acetyl groups usually increases cytotoxic potency [45]. Therefore Lessoniosides seem to be potential candidates for anti-cancer drugs development.

4.3 Experimental Section

4.3.1 Sea Cucumber Sample

Twenty sea cucumber samples of *H. lessoni* [21], commonly known as Golden sandfish were collected off Lizard Island (latitude; 14°41′29.46″ S, longitude; 145°26′23.33″ E), Queensland, Australia on September 2010 [3,12]. The viscera (all internal organs) were separated from the body wall and kept separately in zip-lock plastic bags which were snap-frozen, then transferred to the laboratory and kept at −20 °C until use.

4.3.2 Extraction of Saponins

The saponins were extracted as described previously [3,12]. Briefly, the visceral masses were removed, freeze dried (VirTis, BenchTop K, New York, NY, USA) and pulverized to a fine powder using liquid nitrogen and a mortar and pestle. The pulverized viscera sample was extracted four

times with 70% ethanol (EtOH) (400 mL) and filtered using Whatman filter paper (No. 1, Whatman Ltd., Maidstone, England, UK) at room temperature. The extract was concentrated under reduced pressure at 30 °C using a rotary evaporator (Büchi AG, Flawil, Switzerland) to remove the ethanol, and the residual sample was freeze-dried. The dried extract was dissolved in 400 mL of 90% aqueous methanol (MeOH), and partitioned against 400 mL of *n*-hexane twice. The water content of the hydromethanolic phase was then adjusted to 20% (v/v) and then to 40% (v/v) and the solutions partitioned against CH_2Cl_2 and $CHCl_3$, respectively. The hydromethanolic phase was concentrated and then freeze-dried. The dried powder was solubilized in 10 mL of MilliQ water (18.2 MΩ, Millipore, Bedford, MA, USA) in readiness for chromatographic purification.

4.3.3 Purification of the Extract

The aqueous extract was applied to an Amberlite® XAD-4 column (250 g XAD-4 resin 20–60 mesh; Sigma-Aldrich, MO, USA; 4 × 30 cm column) [3,12], washed extensively with water (1 L) and the saponins eluted sequentially with MeOH (450 mL), acetone (350 mL) and water (250 mL). The MeOH, acetone and water eluates were concentrated, dried, and redissolved in 5 mL of MilliQ water. Finally, the aqueous extract was partitioned with 5 mL isobutanol (v/v). The isobutanolic saponin-enriched fraction was either stored for subsequent mass spectrometry analyses or concentrated to dryness and the components of the extract were further purified by HPCPC. The profile of fractions was also monitored by TLC.

4.3.4 Thin Layer Chromatography (TLC)

Samples were dissolved in 90% or 50% aqueous MeOH and 10 µL were loaded onto silica gel 60 F_{254} aluminum sheets (Merck # 1.05554.0001, Darmstadt, Germany) and developed with the lower phase of $CHCl_3$:MeOH:H_2O (7:13:8) biphasic solvent system [3]. The profile of separated compounds on the TLC plate was visualized by UV light and by spraying with a 15% sulfuric acid in EtOH solution and heating for 15 min at 110 °C until maroon-dark purple spots developed.

4.3.5 High Performance Centrifugal Partition Chromatography (HPCPC or CPC)

The solvent system containing $CHCl_3$:MeOH:H_2O–0.1% HCO_2H (7:13:8) was mixed vigorously in a separating funnel and allowed to reach hydrostatic equilibration [3,12]. Following the separation of the two-immiscible phase solvent systems, both phases were degassed using a sonicator-degasser (Soniclean Pty Ltd. Adelaide, SA Australia). Then the rotor column of HPCPC™, CPC240 (Ever Seiko Corporation, Tokyo, Japan) was filled with the stationary phase (the aqueous upper phase) in the descending mode at a flow rate of 5 mL min^{-1} by Dual Pump model 214 (Tokyo, Japan), with a revolution speed of 300 rpm. The lower mobile phase was pumped in the descending mode at a flow rate of 1.2 mL min^{-1} with a rotation speed of 900 rpm within 2 h. One hundred and twenty milligrams of isobutanol-enriched saponins mixture was injected into the machine in the descending mode. The chromatogram was developed for 3 hours at 1.2 mL min^{-1} and 900 rpm using the Variable Wavelength UV-VIS Detector S-3702 (Soma optics, Ltd. Tokyo, Japan) and chart recorder (Ross Recorders, Model 202, Topac Inc. Cohasset, MA, USA). The fractions were collected in 3 mL tubes using a Fraction collector. At Fraction 54, the elution mode

was switched to ascending mode and the aqueous upper phase was pumped at the same flow rate for 3 h to recover saponins. Fractions were monitored by TLC as described above. Monitoring of the fractions was necessary, as most of the saponins could not be detected by UV due to the lack of a chromophore structure. Fractions were concentrated with nitrogen gas.

4.3.6 Mass Spectrometry

The resultant HPCPC purified polar extracts were further analyzed by MALDI- and ESI-MS to elucidate and characterize the molecular structures of compounds.

4.3.7 MALDI MS

MALDI analysis was carried out using a Bruker Autoflex III Smartbeam (Bruker Daltonik, Bremen, Germany). All MALDI MS equipment, software and consumables were from Bruker Daltonics. The laser (355 nm) had a repetition rate of 200 Hz and operated in the positive reflectron ion mode for MS data over the mass range of 400 to 2200 Da under the control of the Flexcontrol and FlexAnalysis software (V 3.3 build 108). External calibration was performed using the sodium-attached ions from a Polyethylene Glycol of average molecular weight 1000. MS spectra were processed in FlexAnalysis (version 3.3, Bruker Daltonik, Bremen, Germany). MALDI MS^2 spectra were obtained using the LIFT mode of the Bruker Autoflex III with the aid of CID. The isolated ions were subjected to collision against argon in the collision cell to be fragmented, affording intense product ion signals. For MALDI a laser was used to provide both good signal levels and mass resolution with the laser energy for MS^2 analysis being generally 25% higher than for MS analysis.

The samples were spotted onto a MALDI stainless steel MPT Anchorchip TM 600/384 target plate. Alpha-cyano-4-hydroxycinnamic acid (CHCA) in acetone/iso-propanol in ratio of 2:1 (15 mg mL^{-1}) was used as a matrix to produce gas-phase ions. The matrix solution (1 μL) was placed onto the MALDI target plate and air-dried. Subsequently 1 μL of sample was added to the matrix crystals and air-dried [3,12]. Finally, 1 μL of NaI (Sigma-Aldrich # 383112, St Louis, MI, USA) solution (2 mg/mL in acetonitrile) was applied onto the sample spots. The samples were mixed on the probe surface and dried prior to analysis.

4.3.8 ESI MS

The ESI mass spectra were attained with a Waters Synapt HDMS (Waters, Manchester, UK). Mass spectra were acquired in the positive ion mode with a capillary voltage of 3.0 kV and a sampling cone voltage of 100 V.

The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 80 °C; desolvation temperature, 350 °C; desolvation gas flow rate, 500 L h^{-1} [3,12]. Data acquisition was performed using a Waters MassLynx (V4.1, Waters Corporation, Milford, CT, USA). Positive ion mass spectra were acquired in the V resolution mode over a mass range of 100–2000 *m/z* using continuum mode acquisition. Mass calibration was performed by infusing sodium iodide solution (2 μg/μL, 1:1 (v/v) water:isopropanol). For accurate mass analysis a lock mass signal from the

sodium attached molecular ion of Raffinose (*m/z* 527.1588) was used through the LockSpray source of the Synapt instrument.

MS^2 spectra were obtained by mass selection of the ion of interest using the quadrupole, fragmentation in the trap cell where argon was used as collision gas. Typical collision energy (Trap) was 50.0 V. Samples were infused at a flow rate of 5 μL/min, if dilution of the sample was required then acetonitrile was used [27]. Chemical structures were determined from fragmentation schemes calculated on tandem mass spectra and from the literature.

4.4 Conclusions

In recent years, there has been a great improvement in the number of MS applications. The tandem MS approach coupled with HPCPC separation revealed the structure of isomeric compounds containing different aglycones and/or sugar residues. Therefore, a creative and sensitive method has been developed for the structure elucidation of triterpene glycosides in sea cucumber and related products using HPCPC and MS. The result showed that this method is a rapid, accurate and reliable technique for the structure determination of triterpene glycosides in sea cucumber extracts.

This study proved the occurrence of both glycoside and cross-ring cleavages in the sugar moieties of sea cucumber saponins. The sequence of monosaccharide units and the presence of an acetoxy group, clearly reflected by the loss of 60 Da from the parent ions, were noted in five of the seven
new saponins.

Tandem mass spectrometry data suggested that the most prominent ions generally stemmed from the losses of aglycones and/or the key diagnostic sugar moieties (*m/z* 493, 507, 511, 639, 643 and 625).

Our results also illustrate that some saponins are unique to the species, whilst others are common between multiple species. The MS analysis revealed that individual species possesses a unique saponin pattern in which some congeners are very specific to one species. This feature can be used for the taxonomic classification of sea cucumber species.

Characterization of some of these saponins were easier since their MS^2 spectra possessed the key diagnostic signal at *m/z* 493, corresponding to the oligosaccharide chain [MeGlc-Glc-Xyl + Na^+] in addition to the vital peak at *m/z* 643, corresponding to the oligosaccharide moiety [MeGlc-Glc-Xyl-Xyl-H_2O + Na^+]. Lessoniosides C, D and E contain 3-*O*-methylxylose as a terminal monosaccharide unit, which is a rare structural feature in sea cucumber triterpene glycosides.

The ion at *m/z* 1477.7 was identified as the major component of the glycoside fraction 18, containing holostane aglycones with 9(11)-double bond and 18(20)-lactone, characteristic for most of the known sea cucumber glycosides. The structures of these glycosides are quite different from those reported in this species. These substances contain aglycones with an oxidized position at C-16, (acetate group or keto group). Lessoniosides have the aglycone unit like that in Holothurinoside Y with an acetoxyl instead of hydrogen at position 16, but another aglycone moiety with the saturated side chain and with an acetoxyl (a 16β-acetate group) instead of a ketone at position 16.

Our results to date highlight the abundance of new saponins in the viscera indicating the viscera as a major source of these compounds with diverse structures. This paper is the first not only to deduce the structure of several new acetylated isomeric saponins, (Lessoniosides A–G) but also to present the structural diversity of triterpene glycoside congeners in the viscera of *H. lessoni*.

These new saponins (Lessoniosides A–G) have the potential to be consumed with applications as dietary supplements, food preservatives (because of their emulsifying and foaming properties), food additives and development of high value products for various industrial applications and as anti-cancer agents.

Our findings demonstrate that the marine world, in particular sea cucumbers, have much to offer human society in the way of nutraceuticals, pharmaceuticals, agrochemicals, cosmeceuticals, and research biochemicals.

4.5 Acknowledgments

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4.6 Author Contributions

Y.B. and C.F. designed the experiments. Y.B. carried out the experiments with guidance from C.F. Y.B. worked on chemical structure elucidation and both authors contributed in writing the manuscript.

4.7 Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 5

SAPONIN DISTRIBUTION IN THE BODY WALL OF THE SEA CUCUMBER *HOLOTHURIA LESSONI*

Previously, we described the saponin profiles of the viscera (which includes all internal organs) of this commercial species. In this chapter the focus will be on the isolation, purification and structure elucidation of these secondary metabolites in the body wall of *H. lessoni* as a representative of family Holothuriidae. To the best of our knowledge, no research has been published on saponin profile from the body wall of *H. lessoni* yet. The aims of this study, therefore, were (1) to purify and identify novel bioactive saponins, (2) to determine saponin distribution in the body wall, (3) to compare saponin profiles between the body wall and viscera and finally (4) to evaluate the potential bioactivity of isolated saponins.

Figure 5.1 *H. lessoni* picture from New Caledonia reefs (Photographed by Dr. Steven Purcell).

5.1 Introduction

Sea cucumbers vary in size, shape, colour and flavours. They are obviously different in pharmacological, nutraceutical and medicinal activities due to the remarkable differences in the type and quantity of saponins, the abundance of metabolites, which result from various marine environments and food intake. This might also result from the localisation of saponins.

Holothuria lessoni, commonly known as golden sandfish, belongs to the family Holothuriidae, order Aspidochirotida. The coloration of this new-identified holothurian is highly variable from dark greyish black, to beige with black blotches and spots, or beige without black spots (Purcell *et al.* 2012). This species is among one of the highset demand species for luxury seafood in Asia (Purcell 2014). Purcell (2014) also stated that *H. lessoni* and *H. scabra* are the most valuable tropical holothurians in dried seafood markets in China. The processed (dried) *H. lessoni* is marketed in Hong Kong in retail markets with prices ranging from USD 242 to 787 per kg (Purcell *et al.* 2012).

In addition this chapter addresses the purification and structure elucidation of several holostane glycosides including many new saponins along with multiple known compounds from the body wall of this species using the same methods as described previously unless otherwise stated. In this study, the integration of the counter-current chromatography and mass spectrometry techniques utilised to deduce the structure of saponins.

5.2 Material and Methods

The material and methods were the same as previous chapters, except for a small modification in the ESI-MS analysis as the samples were analysed in both negative and positive ion modes.

5.2.1 Extraction protocol

The saponins were extracted as described previously (Bahrami & Franco 2015; Bahrami *et al.* 2014a), but replacing the viscera with the body wall. Mass spectrometry analysis combined with the existing literature led to discovery of many known and new glycosides.

5.2.2 ESI MS

The ESI mass spectra were attained with a Waters Synapt HDMS (Waters, Manchester, UK). Mass spectra were acquired in both the positive and negative ion modes with a capillary voltage of 3.0 kV and a sampling cone voltage of 60 V.

The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, 80

°C; desolvation temperature, 350 °C; desolvation gas flow rate, 500 $L.h^{-1}$ (Bahrami *et al.* 2014a; Bahrami *et al.* 2014b). Data acquisition was performed using a Waters MassLynx (V4.1, Waters Corporation, Milford, CT, USA). Positive ion mass spectra were acquired in the V resolution mode over a mass range of 600 – 1600 *m/z* using continuum mode acquisition. Mass calibration was performed by infusing sodium iodide solution (2 µg/µL, 1:1 (v/v) water:isopropanol). Accurate mass analysis was performed in the positive ion mode, a lock mass signal from the sodium attached molecular ion of Raffinose (1 ng/µL in 50% aqueous acetonitrile, *m/z* 527.1588) was used through the LockSpray source of the Synapt instrument.

MS^2 spectra were obtained by mass selection of the ion of interest using the quadrupole, fragmentation in the trap cell where argon was used as collision gas. Typical collision energy (Trap) was 50.0 V. Samples were infused at a flow rate of 5 µL/min, if dilution of the sample was required then acetonitrile was used (Song et al. 2004). Chemical structures were determined from fragmentation schemes calculated on tandem mass spectra and from the literature.

5.2.3 Bioactivity test

Microorganisms were grown on HPDA or TSB medium (Appendix I).

1.1.1.1 Antifungal activity assay (plug type diffusion assay)

The antifungal activities of the isobutanol-saponin enriched and HPCPC fractions (pure saponins) were tested against three strains including *Fusarium pseudograminearum, Pythium irregulare* and *Rhizoctonia solani* using a modified disc diffusion agar assay. The test fungi were grown on HPDA medium for 7 days, and a plug of the radial growth of each fungus was cut (0.5 x 0.5 cm cubes). The cubes were then placed onto the centre of a new HPDA plate and incubated at 27°C for 24 hours, or until the fungal growth surrounding the cube to 1.5 cm diameter. At this stage, 40 µL of the samples were spotted onto standard paper discs and air-dried. The six discs were then placed onto the fungal growth plates about 1.5 cm from the edge and pressed into the agar using sterile tweezers. The plates were then re-incubated at 27°C and checked for inhibition zones every 24 hours for four days. The negative controls were methanol and plates of each fungus culture with tested samples, while Benomyl ® (50 µg/mL) was used as a positive control.

1.1.1.2 Antibacterial activity assay

Antibacterial activity of saponin extracts were examined against resistant pathogen *Staphylococcus aureus* using a typical agar diffusion assay. Antibiotic assay medium (AAM) was used for the antibacterial activity assay. The test culture was grown in tryptone soy broth (TSB) and incubated at 37°C for 18 – 22 hrs. The growth of the culture was evaluated by measuring the optical density (OD) using a Shimadzu UV-160A spectrophotometer at 600 nm (OD600 nm), and OD was adjusted to 0.2. The AAM was seeded with the culture (1% v/v) and dispensed into 9 cm petri dish plates at 25 mL/plate, and cut using a cork borer to make 10 wells (6 mm). Each well was then filled with 40 µL of samples and the plates incubated at 37°C for 18 – 24 hours. Vancomycin (0.25 µg/mL) was used as a positive control.

5.3 HPCPC purification

We previously reported the isolation and purification of several saponins from the viscera of a sea cucumber species, *H. lessoni,* using standard chromatography and HPCPC. The saponin constituents of the body wall of *H. lessoni* were also investigated using the same protocol (Bahrami & Franco 2015; Bahrami *et al.* 2014a; Bahrami *et al.* 2014b).

HPCPC was performed in the ascending mode. One hundred and forty milligrams of butanolic saponin extract was loaded into the machine. One hundred and thirty fractions were collected and monitored by TLC as described previously. The TLC profile of the isobutanol saponin enriched fraction showed the presence of several bands Figure 5.2. (lane 1), whereas the TLC pattern of HPCPC fractions exhibited the existence of one band in the majority of fractions. As a typical example, the TLC profile of HPCPC Fractions 89–102 is shown in Figure 5.2.

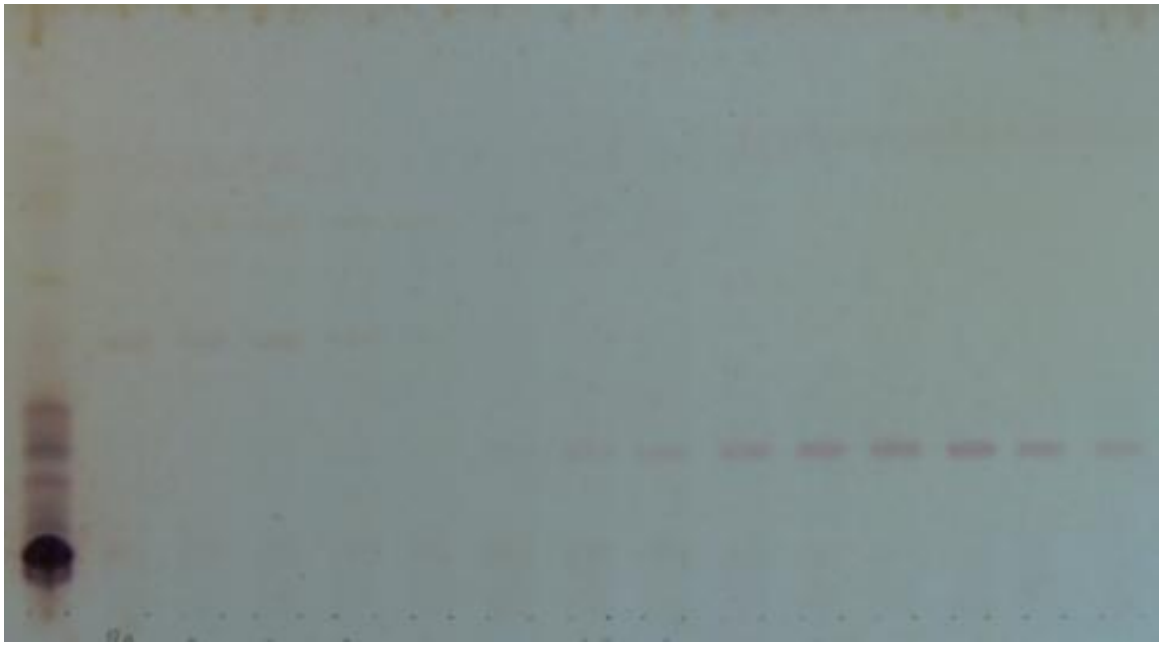

Figure 5.2. The thin-layer chromatography (TLC) pattern of the HCPCP fractions from the purified extracts of the body wall of the *H. lessoni* sea cucumber using the lower phase of $CHCl_3$–MeOH–H_2O (7:13:8) system. The numbers under each lane indicate the fraction number in the fraction collector. The Fractions 89 to 102 of one analysis (of 130 fractions) are shown. Lane 1 is iso-butanol saponin enriched extract.

5.4 Mass spectrometry analysis of saponins

This section will outline chemical structure analysis of saponins using MALD-MS/(MS) and ESI-MS/(MS) in the positive and/or negative ion mode(s).

5.4.1 MALDI-MS and ESI-MS analyses of saponins from the body wall of *H. lessoni*

Saponin HPCPC fractions from the body wall of *H. lessoni* were analysed by MALDI-MS and ESI-MS, and MS/MS as described in detail previously (Bahrami *et al.* 2014b). The MALDI-MS and MS/MS performed in the positive ion mode, while ESI-MS and MS/MS were conducted in both positive and negative ion modes. All detected ions in the positive ion mode, were sodium-coordinated species such as $[M - H + 2Na]^+$ and $[M + Na]^+$ corresponding to sulphated and non-sulphated saponins, respectively.

For instance, the positive ion mode MALDI-MS of Fraction 110 is shown in Figure 5.3. This spectrum illustrates the presence of one major peak at 1141 corresponding to Desholothurin A.

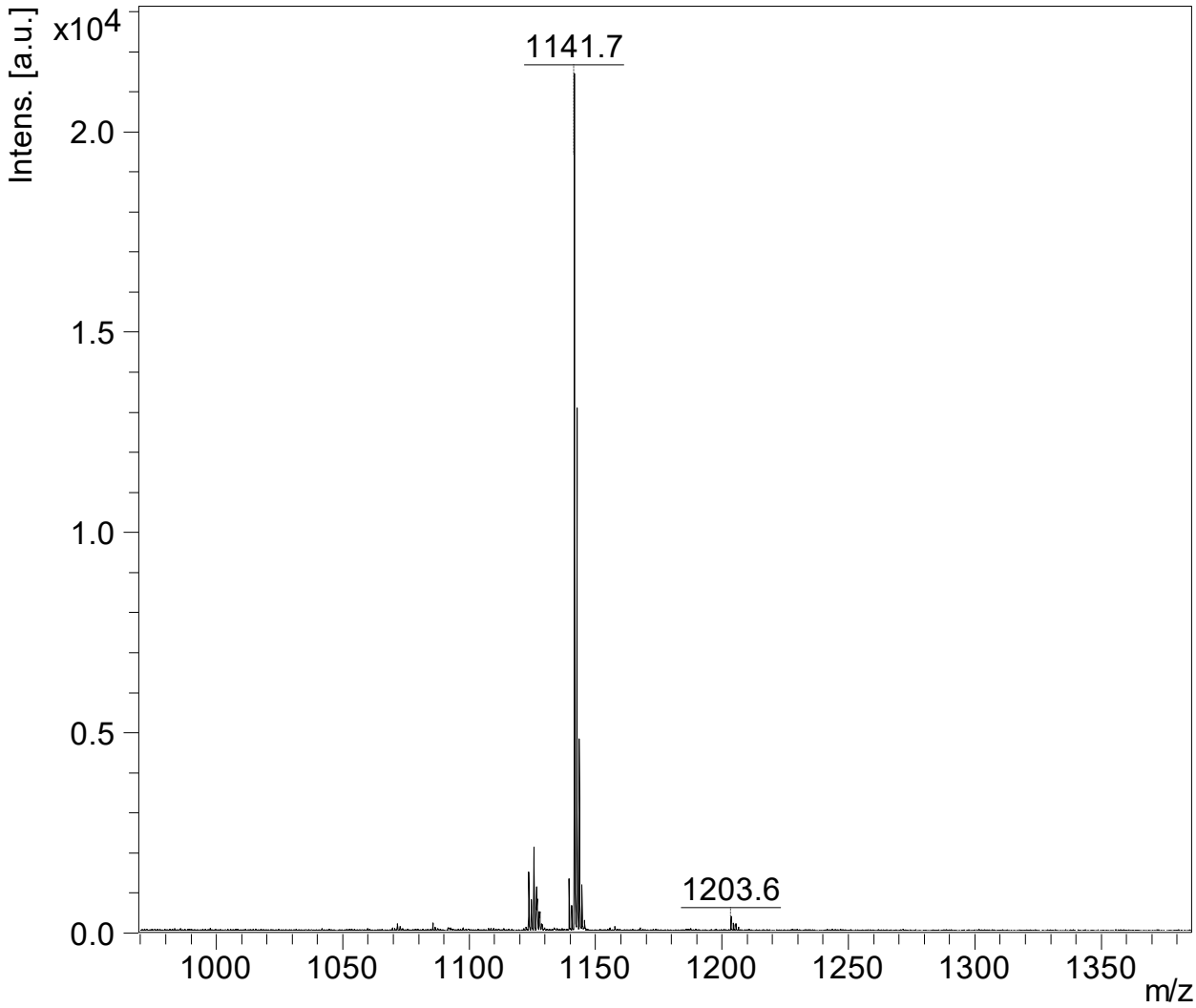

Figure 5.3. MALDI-MS fingerprint of Fraction 110. The major peak at *m/z* 1141 corresponded to Desholothurin A.

Both positive and negative ion modes ESI-MS also performed on the fractions. As an example, the positive ion mode ESI-MS spectrum of Fraction 51 is shown in Figure 5.3. This spectrum indicated the presence of the major ion at *m/z* 1477.7 corresponding to Lessonioside A. Therefore, the MALDI-MS data was corroborated by ESI-MS analysis.

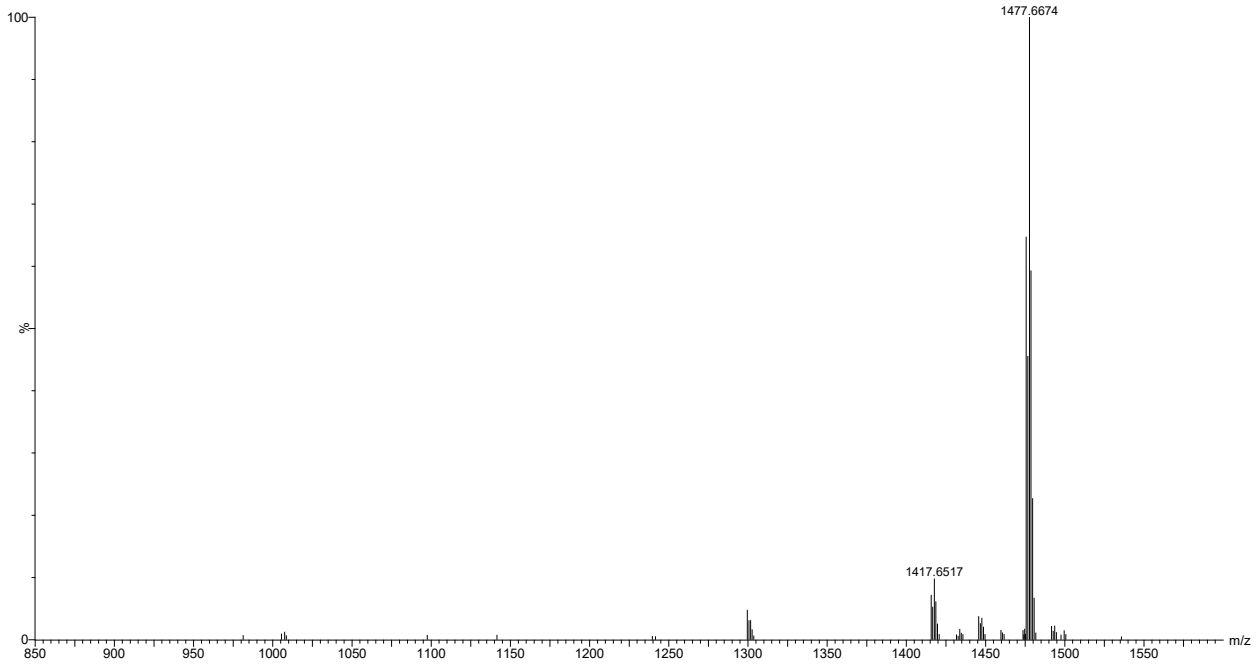

Figure 5.4. ESI-MS spectrum of Fraction 51. The major peaks corresponded to Lessonioside A.

More than 89 saponin congeners were found in the body wall of sea cucumber *H. lessoni*, which are summarised in Table 5.1. Around 80 saponins were common between the body wall and the viscera with various intensities. Nine saponin congeners were found solely in the body wall compared to viscera.

Twenty three major saponin peaks detected at *m/z* 905.4, 1069.5, 1071.5, 1087.5, 1107.5, 1109.5, 1123.5, 1125.5, 1141.6, 1199.5, 1211.5, 1227.5, 1229.5, 1243.5, 1287.6, 1289.6, 1303.6, 1305.6, 1361.7, 1461.7, 1463.7, 1475.7 and 1477.7 in the body wall of *H. lessoni*. These intense peaks could each correspond to at least one triterpene saponin congener.

The most abundant saponin peaks were detected at *m/z* 1141, 1227, 1229 and 1243 which corresponded to Desholothurin A (Nobiliside 2a) (*m/z* 1141) (Kitagawa *et al.* 1982; Rodriguez *et al.* 1991), Fuscocinerosides B or C (*m/z* 1227) which were functional group isomers (Han *et al.* 2012; Han *et al.* 2009a; Han *et al.* 2009b; Kitagawa *et al.* 1982; Zhang, S-Y *et al.* 2006a), Holothurin A_2 (*m/z* 1229) (Han *et al.* 2009b; Kalinin, V & Stonik 1982; Kitagawa *et al.* 1985) , Holothurin A (*m/z* 1243) (Bhatnagar *et al.* 1985; Elyakov *et al.* 1973; Han *et al.* 2009a; Kitagawa *et al.* 1982; Kitagawa *et al.* 1979; Kobayashi *et al.* 1991; Stonik, VA *et al.* 1979; Yuan, W *et al.* 2008), respectively. As can be seen in Table 5.1., these abundant saponin congeners were sulphated

triterpene glycosides except for the ion monitored at *m/z* 1141. Likewise in the viscera, the ion at *m/z* 1243 was the predominant peak, corresponding to Holothurin A, which was followed by the ions at *m/z* 1227, 1229, 1141 and 1305, respectively. In all sulphated saponins ranging from *m/z* 900 to 1400, xylose was sulphated. However in the viscera the ions at *m/z* 1243, 1141, 1305, 1259 and 1227 were the top five intense saponins.

The distribution of saponin in the cuvierian tubules and body wall of *H. forskali,* in the same family as *H. lessoni*, was investigated using both conventional MALDI and MALDI- mass spectrometric imaging (MALDI-MSI) analyses (Van Dyck *et al.* 2011; Van Dyck *et al.* 2010a; Van Dyck *et al.* 2009). Eight major intense peaks reported at *m/z* 1125, 1141, 1287, 1303, 1433, 1449, 1463 and 1479. All of these glycosides defined as non-sulphated saponins, while the major abundant saponins in the *H. lessoni* were sulphated congeners (except the ions at *m/z* 1141.5).

Chemical analysis by MALDI- and ESI –MS/MS of the HPCPC fractions identified several novel along with multiple known saponins. The molecular structures of some of the identified compounds are illustrated in Figure. 5.5. The isobutanol and HPCPC fractionated samples indicated 26 sulphated and 63 non-sulphated saponin ions.

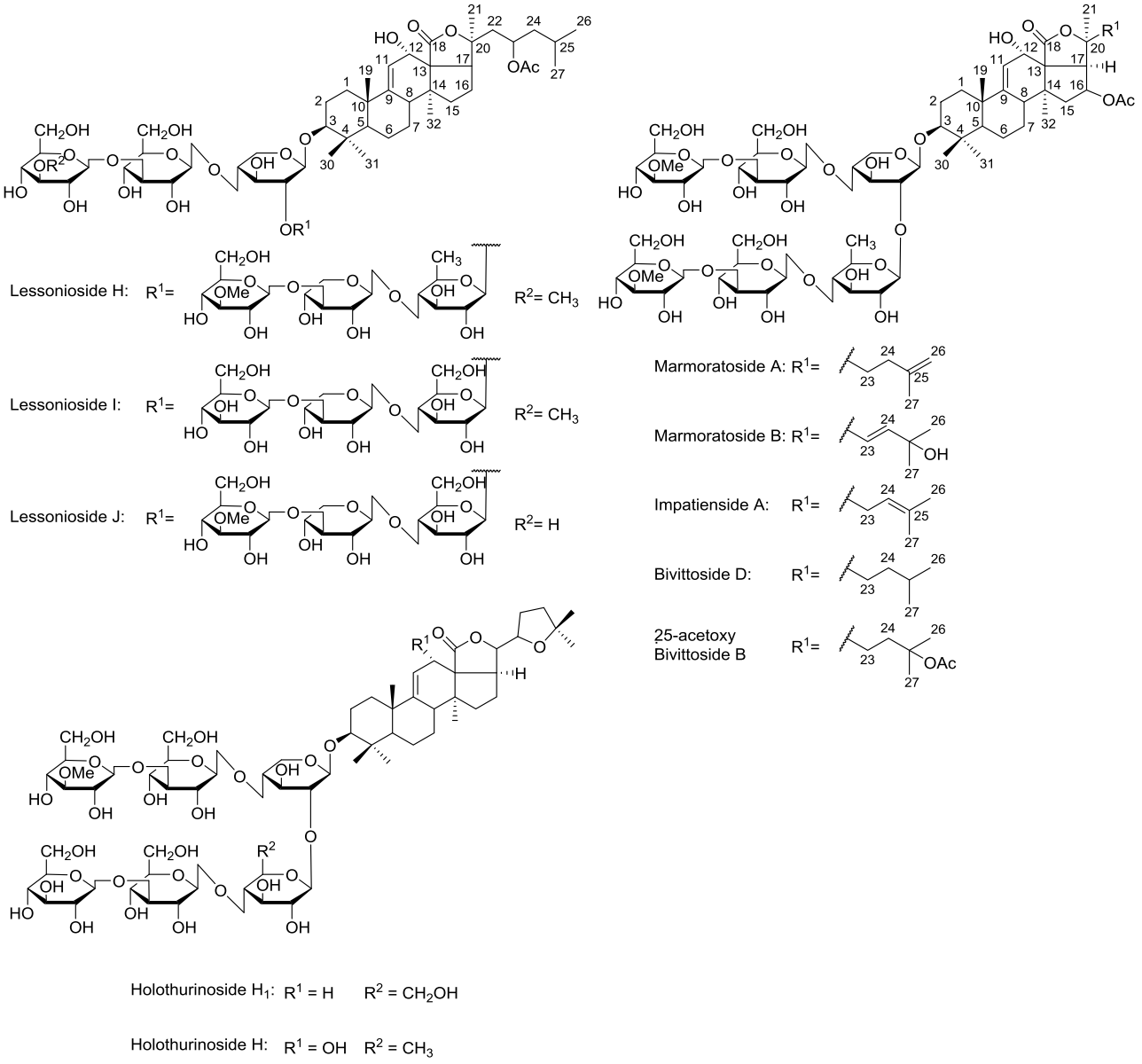

Figure 5.5. Structure of identified saponins from the body wall of *H. lessoni*

5.4.2 Saponin profiles by negative-ion ESI-MS

The result of positive ion mode was validated by the negative ion mode under condition similar to those used for the positive ion mode. The analysis of saponins by the negative ion mode facilitated calculation of the molecular formula of compounds as showed the presence of the number of Na in the molecules. For instance, the ions detected in both positive and negative ion modes ESI-MS of HPCPC Fraction 110 is displayed in Figure 5.6., which demonstrated ions detected in both positive $[M + Na]^+$ (the top two spectra) and negative $[M - H]^-$ (bottom one) ion modes between 1050 to 1275 Da. Three main peaks at *m/z* 1125, 1141 and 1163 in ESI-MS^+ generated peaks at *m/z*

1101, 1117 and 1139 in the negative ion mode ESI-MS, respectively, indicating the presence of only one Na atom in their chemical formulae. As can be noted from the spectra, the mass discrepancy between the positive and negative ion modes for an individual ion was 24 u or Da, representing the loss of a sodium molecule and that there was no sulphur present. However, in the case of a sulphated saponin the mass discrepancy between these two modes of ionisation was 46 u, showing the presence of a sulphur group.

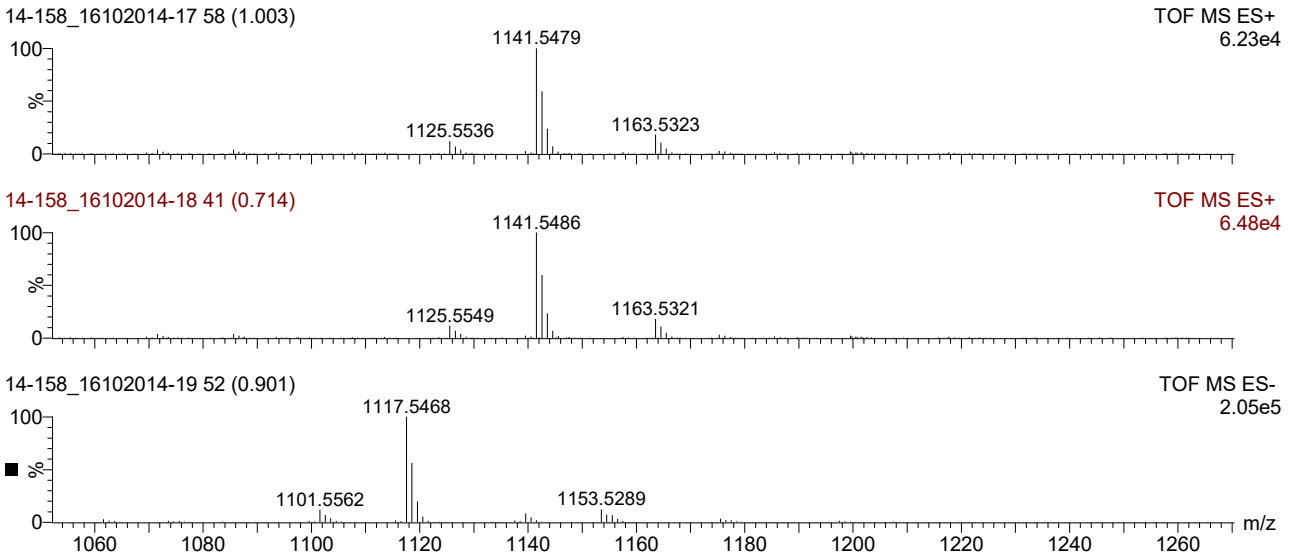

Figure 5.6. Saponin profile of Fraction 110 by ESI-MS in both positive (the top two spectra) and negative (bottom one) ion modes

5.4.3 Structure elucidation of saponins by tandem mass spectrometry analysis

The appropriate HPCPC fractions were pooled, on the basis of their similar Rf values on TLC, and concentrated to dryness. The saponin profile of each HPCPC fraction was then revealed by MALDI-MS and ESI-MS, and -MS^2. MALDI-MS/MS and ESI-MS/MS were carried out to provide more structural information about the components of individual saponin and differentiate isomeric saponins following HPCPC purification. MS^2 afforded crucial information about the structure of saponins such as the glycoside linkage, sequence and type of sugar components and the position of sulphate group. MS^2 analyses identified key diagnostic ions generated by cleavage of the glycosidic bond including oligosaccharide and monosaccharide fragments (Bahrami *et al.* 2014a; Bahrami *et al.* 2014b; Liu, J *et al.* 2007). However, in some case, the definitive structure elucidation of saponins is required NMR analysis. It is notable that the low kinetic energy CID used here had no fragment in the core of the aglycone, whereas cleaved the side chain of the aglycone in some

cases, which was consistence with observation by Demeyer *et al.* (2014). In this section tandem mass spectrometry analysis of a few saponin ions will be discussed as representative.

5.4.4 Structural determination of saponins by MALDI MS/MS

To validate the structure of saponin tandem mass spectrometry was conducted on detected ions. As a typical example MALDI-MS/MS profile of the ion at *m/z* 1141 from Fraction 55 is shown in Figure 5.7. Chemical analysis of this ion revealed the structure of Desholothurin A_1. This conclusion was established by fragment ion peaks at *m/z* 763, 523, 361, and 185 in the positive ion mode MALDI-MS^2, corresponding to the consecutive losses of aglycone, Xyl, Glc, MeGlc and Glc residues, respectively

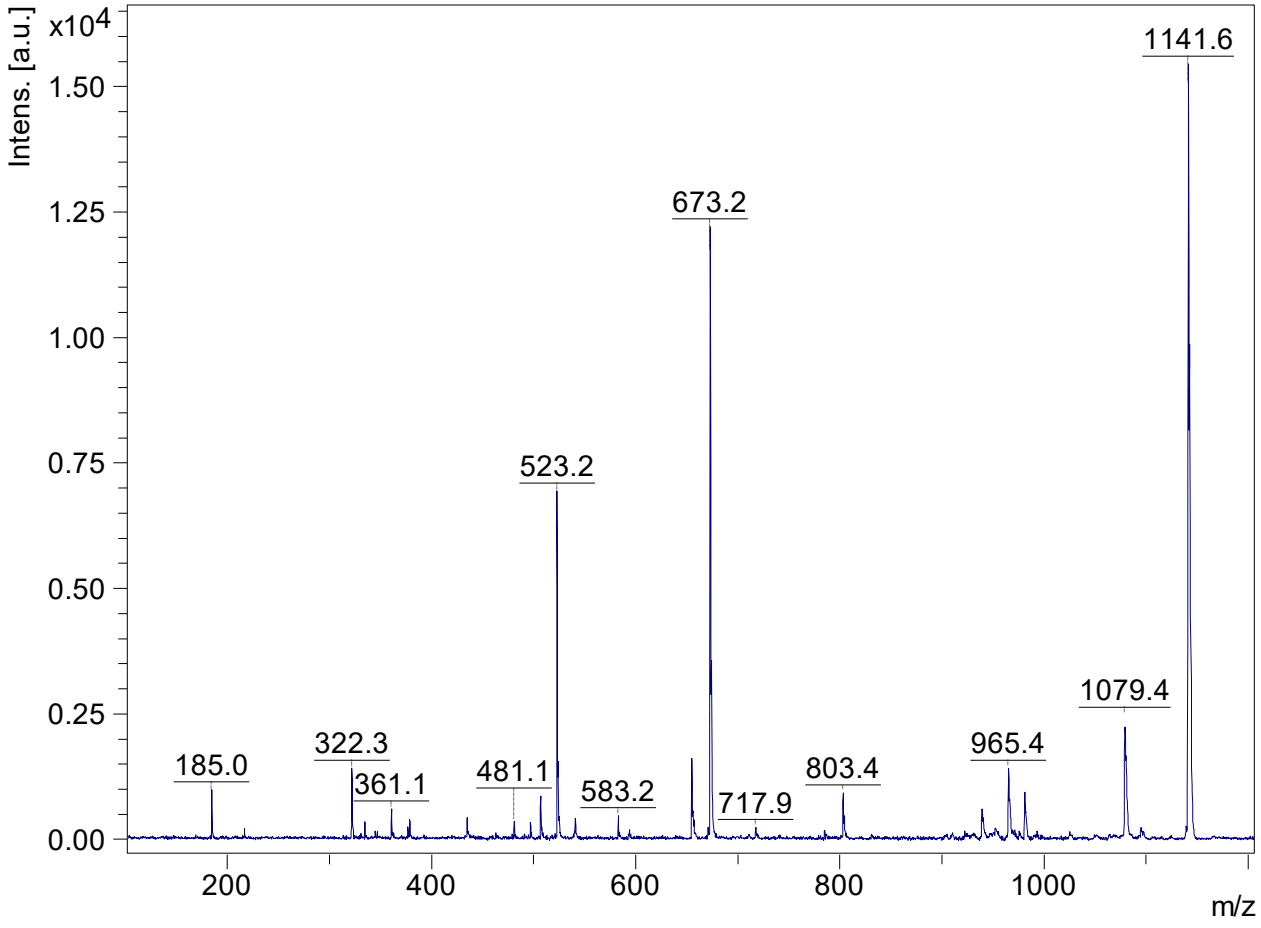

Figure 5.7. MALDI-MS/MS profile of the ion at *m/z* 1141 corresponding to Desholothurin A_1.

5.4.5 Chemical analysis of saponins by ESI-MS/MS

The effective ability of HPCPC in purifying saponins was described in the previous chapters. The capability of this apparatus in separating isomeric saponins such as ions detected at *m/z* 1141 is

exemplified in Figure 5.8.

The positive ion mode ESI-MS^2 spectra of the ion detected at *m/z* 1141 from the Fractions 55 (the top spectrum) and 110 (the bottom spectrum) are shown in Figure 5.8 as representative. These ions corresponded to Desholothurin A_1 (Arguside E) and Desholothurin A (Nobiliside 2a), respectively, which were different in both aglycone and sugar moieties from each other. The presence of *m/z* 507 and/or 523 ions as the key decomposition products was observed in the MS^2 spectra of these compounds.

As can be seen these isomeric compounds showed different MS/MS spectra. Major peak at *m/z* 523 (the top spectrum, Figure 5.8) corresponded to the sodiated key diagnostic peak [MeGlc-Glc-Glc+Na]$^+$, and the peak at *m/z* 673 generated by the loss of the Agl moiety, corresponding to the entire sodiated hydrated sugar residue [MeGlc-Glc-Glc-Xyl+Na]$^+$. Therefore this compound had an aglycone with an *m/z* value of 468. Our analysis inferred tetraosides structure for this ion. This analysis revealed the structure of tetrasaccharide triterpene glycoside corresponded to Desholothurin A_1.

While the prominent peaks at *m/z* 507 and 657 (the bottom spectrum, Figure 5.8.) corresponded to the sodiated key diagnostic peak [MeGlc-Glc-Qui+Na]$^+$, and the entire sodiated hydrated sugar residues [MeGlc-Glc-Qui-Xyl+Na]$^+$, respectively. The latter ion indicated that this compound had an aglycone with an *m/z* value of 484. These findings revealed the structure of this compound, corresponding to Desholothurin A. Therefore the analysis of data showed that HPCPC could separate the isomeric congeners in some cases. The integration of the counter-current chromatography and mass spectrometry techniques was an efficient and reliable approach for purification and structure elucidation of saponins.

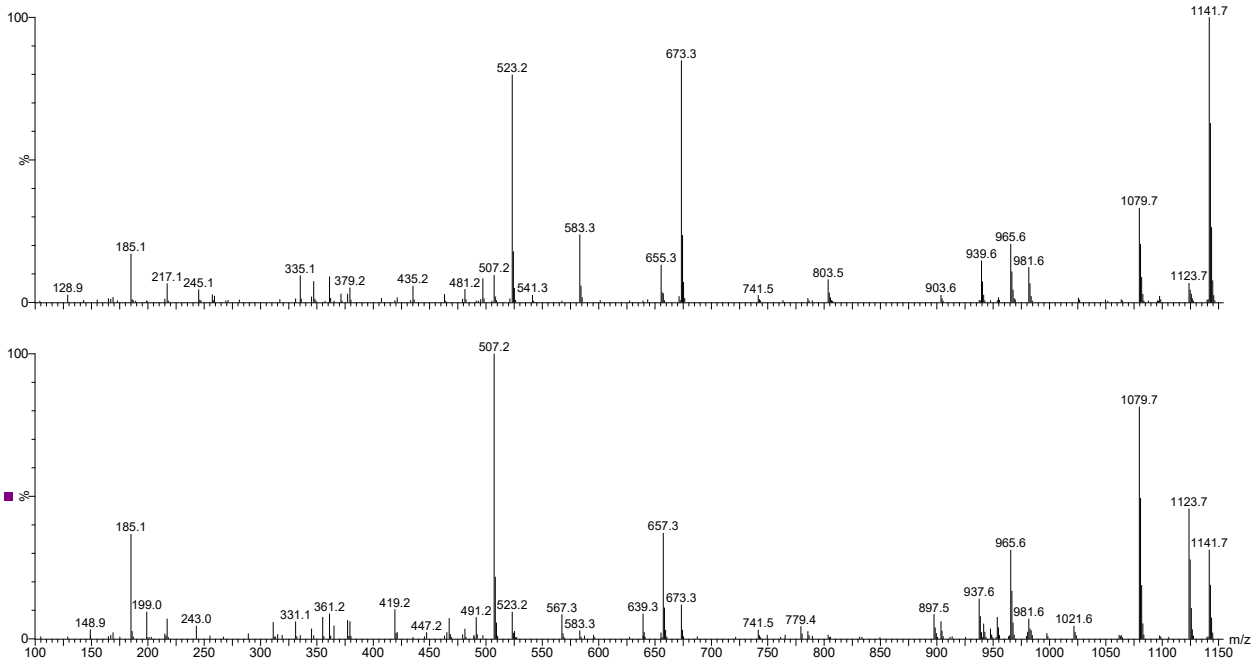

Figure 5.8. (+) ESI-MS/MS spectra of the ions at *m/z* 1141 in fractions 55 (top) and 110 (bottom). Figure indicated the presence of isomeric compounds. The key diagnostic peak at *m/z* 523 corresponding to $[MeGlc\text{-}Glc\text{-}Glc+Na]^+$ revealed the structure of Desholothurin A_1 (Figure A), while presenting the key diagnostic peak at *m/z* 507 corresponding to $[MeGlc\text{-}Glc\text{-}Qui+Na]^+$ indicted the structure of Desholothurin A.

As an example, the positive ion mode ESI-MS/MS for the ion detected at *m/z* 1461 $[M + Na]^+$ from Fraction 95 is shown in Figure 5.9. This ion displayed an *m/z* value of 1437 $[M - H]^-$ in the negative ion mode ESI-MS indicating had no sulphur group in the molecular structure.

Similar analysis applied on saponin ions to elucidate the chemical structures of saponins as described previously. As can be seen in Figure 5.9, this triterpene glycoside contained the ion at *m/z* 493.2 corresponding to the key diagnostic sugar residue $[MeGlc\text{-}Glc\text{-}Xyl+Na]^+$. The consecutive losses of the deacetylated aglycone and acetic acid (AcOH) followed by sugar residues yielded ion fragments at *m/z* 1007.5 and 947.5, respectively (for more details please refer to Chapter 4). The latter ion corresponded to the sodiated sugar moiety generating by the loss of the Agl. This sequence of fragmentation confirmed the presence of an acetoxy group.

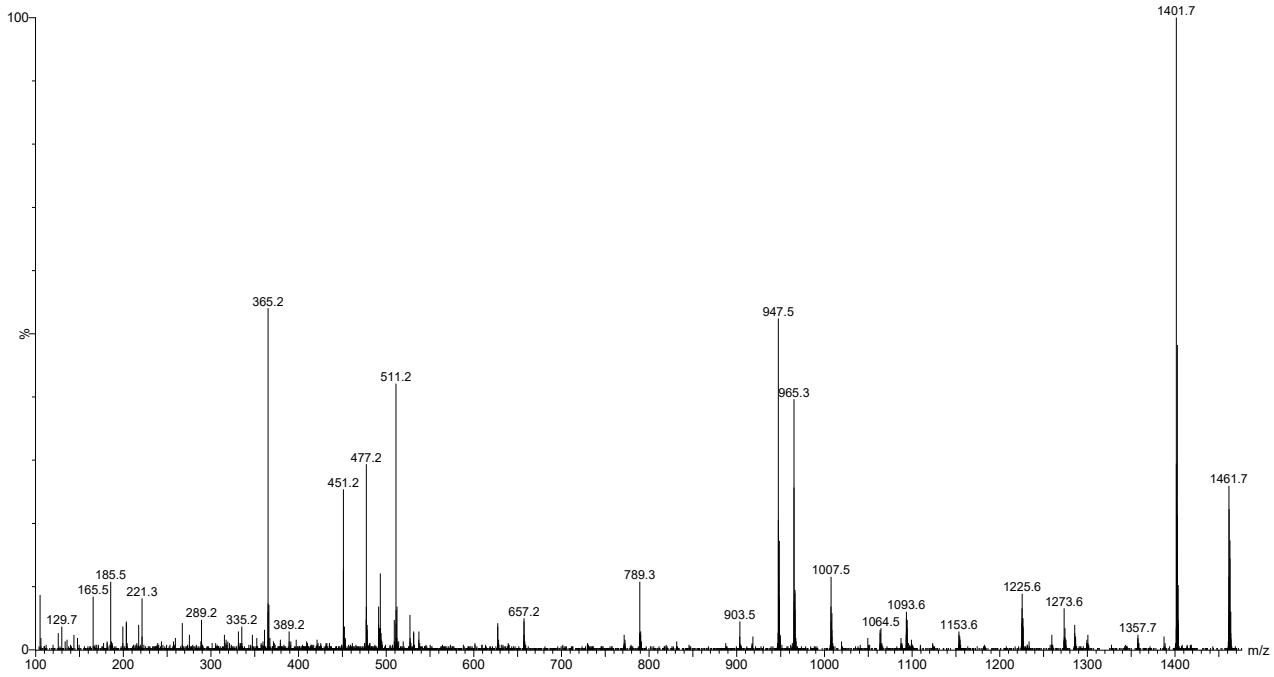

Figure 5.9. ESI MS/MS spectrum of ion at 1461.7 in the positive ion mode.

The MS^2 analyses of ions at *m/z* 1461.7 revealed a similar fingerprint profile with those reported for Lessoniosides, which were isolated and characterised from the viscera of this species, in particular with Lessonioside A where the signals were coincident (Bahrami & Franco 2015). In addition, the sugar moiety of this novel isomeric compound was found to be identical to those of Lessonioside A, confirming the constituents of the hexasaccharide chain. This novel triterpene glycoside had a holostane aglycone containing an 18(20)-lactone with a 9(11)-double bond and acetoxy group at C-23. We named these isomeric compounds Lessoniosides H, I and J.

Further, it differed from Holothurinoside H (Marmoratoside B) in the sugar moieties. Holothurinoside H generates a peak at *m/z* 507 corresponding to MeGlc-Glc-Qui under positive ion mode mass spectrometry (Van Dyck *et al.* 2009). However, no peak was detected at *m/z* 507 corresponding to the key diagnostic ion $[MeGlc\text{-}Glc\text{-}Qui+Na]^+$ from the ion at *m/z* 1461.

Moreover, Sun, P *et al.* (2007) reported a lanostane-type triterpene glycoside, Impatienside A with molecular weight $[M+Na]^+$ 1447 ($C_{67}H_{108}O_{32}$) which had peak at *m/z* 1423 $[M-H]^-$ in the negative ESI-MS, isolated from the sea cucumber *Holothuria impatiens*, which contained a double bond at C24 position (ions 507 and 493), along with a structurally related known compound, Bivittoside D $[M+Na]^+$ 1449 ($C_{67}H_{110}O_{32}$) and by negative ESI-MS *m/z* 1425 $[M-H]^-$, similar to Impatienside A

without double bond. However, Yuan *et al.* (2009b) described a structure with a double bond at the position of C25 instead C24 for this compound. This compound was detected in both viscera and body wall of *H. lessoni*. However it was found to be more intense in the body wall than viscera.

Yuan *et al.* (2009b) isolated several saponins including Marmoratoside A $[M+Na]^+$ 1447 ($C_{67}H_{108}O_{32}$), 17α-hydroxy impatienside A $[M+Na]^+$ 1463 ($C_{67}H_{108}O_{33}$), Marmoratoside B $[M+Na]^+$ 1463 ($C_{67}H_{108}O_{33}$), 25-acetoxy bivittoside D $[M+Na]^+$ 1507 ($C_{69}H_{112}O_{34}$), together with known glycosides Impatienside A and Bivittoside D from the sea cucumber *B. marmorata.* These compounds were also identified in *H. lessoni.*

Our analysis revealed the presence of ions peak at *m/z* 1435 $[M+Na]^+$ in the positive ion mode MS which showed a signal at *m/z* 1411 $[M-H]^-$ in the negative-ion mode ESI-MS. Tandem mass spectrometry revealed the isomeric structure of the ion at *m/z* 1435. The assignment of fragments revealed that this ion was an isomeric compound. This saponin was also common between body wall and viscera. Wang, X-H *et al.* (2014) reported Variegatuside D with a chemical formula $C_{59}H_{96}O_{27}$ at *m/z* 1259 $[M+Na]^+$ which might produce by loss of MeGlc from the ion at *m/z* 1435.

Another novel isomeric saponin ion detected at *m/z* 1221.5 which was common between viscera and body wall. This novel saponin contained four sugar residues. Silchenko *et al.* (2013b) also reported an acetylated-sulphated tetraosides triterpene glycoside, Typicosides A_1, isolated from the sea cucumber *Actinocucumis typica* (Family Cucumariidae, Order Dendrochirotida) with the identical *m/z* value (1221.5). However, the MS^2 spectrum of the ion at *m/z* 1221.5 had a different fragmentation pattern from that seen in Typicosides A_1 even though they had the same *m/z* value which indicated the presence of a new saponin congener. In addition to Typicosides A_1, Intercedenside A ($C_{55}H_{83}NaO_{25}S$), a sulfo-acetylated saponin isolated from *Mensamaria intercedens* sea cucumber had the same molecular weight, found to be identical with one of isomers in structure (Zou *et al.* 2003).

5.4.6 Negative ion mode ESI-MS/MS

Negative ion mode MS/MS analyses were also performed on compounds under experimental

conditions similar to those used for positive ion mode. It is clear that different fragmentation patterns produced in the negative ion mode MS/MS compared to those in the positive mode.

As a typical example, the ESI- MS^2 fingerprints of the ion at *m/z* 1117 $[M-H]^-$ in the negative ion mode from fraction 110 is shown in Figure 5.10. This ion was observed at *m/z* 1141.5 $[M+Na]^+$ in positive mode, which was corresponded to Desholothurin A (Nobiliside 2a). This peak detected at *m/z* 1117 in the negative ion mode ESI- MS with molecular formula $C_{54}H_{85}O_{24}$ $[M-H]^-$, indicating the presence of one Na atom [sodium adduct in the positive mode] which means no sulphur group exist in this compound.

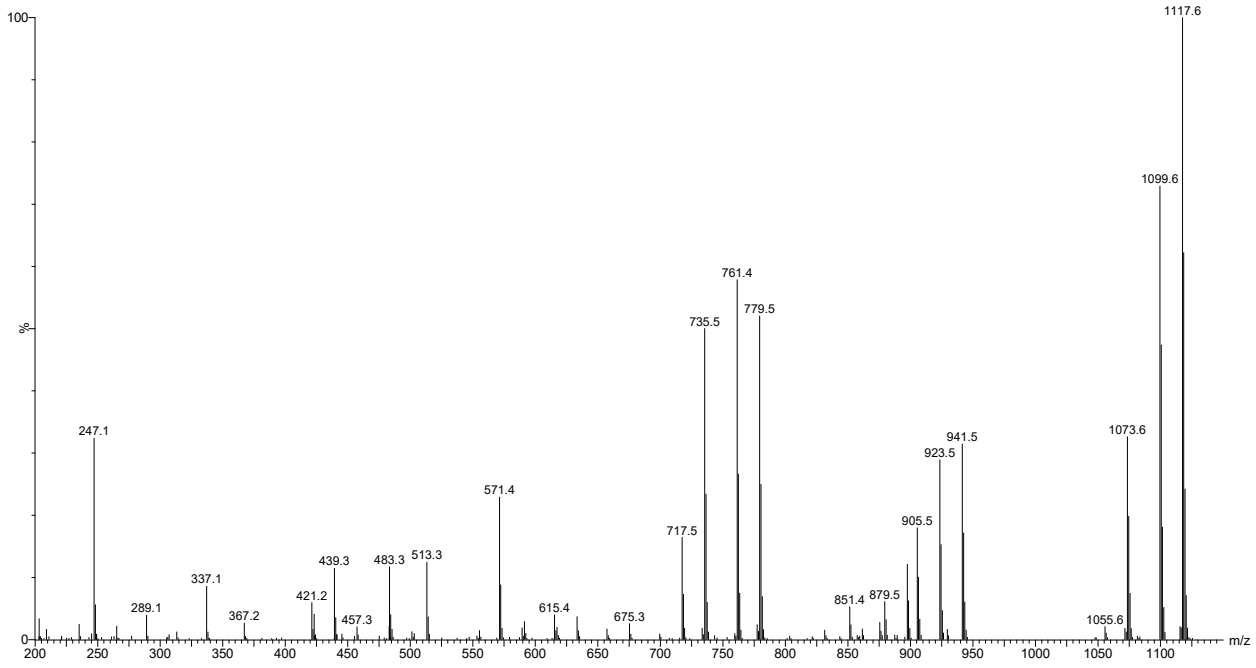

Figure 5.10. ESI-MS/MS spectrum of Desholothurin A in the negative ion mode

The mass discrepancy among these peaks and associated peaks in the positive ion mode were 24 u. For instances, the ions at *m/z* 337 and 483 corresponded to the ions at *m/z* 361 and 507 in the positive mode ESI-MS/MS, respectively. This analysis determined that the sugar compartment of this saponin comprised four sugar residues. This analysis further validated our results.

Table 5.1. Summary of saponins identified from the body wall of *H. lessoni* by MALDI- and ESI-MS^2.
This table illustrates the 35 novel identified compounds (N) along with the 54 known compounds (P). This table also shows some identical saponins, which have been given different names by different researchers in which they might be isomeric congeners.

$[M + Na]^+$ *m/z*	**MW**	**Formula**	**Compound Name**	**Body wall**	**Viscera**	**Novel (N)/ Published (P)**	**References**
889.4	866	$C_{41}H_{63}NaO_{16}S$	Holothurin B_3	Yes	Yes	P	(Silchenko *et al.* 2005c)
		$C_{42}H_{67}NaO_{15}S$	Unidentified	Yes	Yes	N	–
905.4	882	$C_{41}H_{63}NaO_{17}S$	Holothurin B_4	Yes	Yes	P	(Bahrami *et al.* 2014b; Silchenko *et al.* 2005c)
			Holothurin B	Yes	Yes	P	(Kitagawa *et al.* 1978a; Kobayashi *et al.* 1991)
			Nobiliside B	Yes	Yes	P	(Wu, J *et al.* 2006b)
907.4	884	$C_{41}H_{65}NaO_{17}S$	Holothurin B_2	No	Yes	P	(Silchenko *et al.* 2005c)
			Leucospilotaside B	No	Yes	P	(Han *et al.* 2010c)
911.6	888	$C_{45}H_{92}O_{16}$	Unidentified	Yes	Yes	N	–
917.4	994	$C_{44}H_{71}NaO_{15}S$	Unidentified	No	Yes	N	–
921.4	898	$C_{41}H_{63}NaO_{18}S$	Leucospilotaside A	No	Yes	P	(Han *et al.* 2007)
1034.1	1011	a*	Unidentified	Yes	Yes	N	–
1065.5	1042	$C_{48}H_{82}O_{24}$	Unidentified	No	Yes	N	–
1069.5	1046	$C_{52}H_{86}O_{21}$	Unidentified	Yes	No	N	-
1071.5	1048	$C_{47}H_{93}NaO_{21}S$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
1079.5	1056	$C_{53}H_{84}O_{21}$	Unidentified	Yes	Yes	N	-
1083.3	1060	$C_{58}H_{64}O_{25}$	Unidentified	No	Yes	N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
1085.5	1062	$C_{53}H_{90}O_{21}$	Unidentified	No	Yes	N	-
1087.5	1064	$C_{52}H_{88}O_{22}$ $C_{47}H_{93}NaO_{22}S$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)

$[M + Na]^+$ *m/z*	MW	Formula	Compound Name	Body wall	Viscera	Novel (N)/ Published (P)	References
1101.6	1078	$C_{52}H_{86}O_{23}$	Unidentified	Yes	Yes	N	-
1103.5	1080	$C_{52}H_{88}O_{23}$	Unidentified	Yes	No	N	-
1107.7	1084	$C_{54}H_{84}O_{22}$	Unidentified	Yes	Yes	N	-
1109.5	1086	$C_{54}H_{86}O_{22}$	DS-pervicoside B	Yes	Yes	P	(Kitagawa *et al.* 1989b)
1111.5	1088	$C_{54}H_{88}O_{22}$	Bivitoside B	Yes	Yes	P	(Caulier *et al.* 2013; Kitagawa *et al.* 1989a)
1121.5	1098	$C_{54}H_{82}O_{23}$	Unidentified	No	Yes	N	-
1123.5	1100	$C_{54}H_{84}O_{23}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
1125.5	1102	$C_{54}H_{86}O_{23}$	Holothurinosides C/C_1	Yes	Yes	P	(Kitagawa *et al.* 1982; Rodriguez *et al.* 1991)
1127.6	1104	$C_{53}H_{84}O_{24}$ $C_{54}H_{88}O_{23}$	Holothurinosides X/Y/Z	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
1139.5	1116	$C_{54}H_{84}O_{24}$	Unidentified	No	Yes	N	-
1141.5	1118	$C_{54}H_{86}O_{24}$	Desholothurin A (Nobiliside 2a), Desholothurin A_1 (Arguside E)	Yes	Yes	P	(Bahrami *et al.* 2014b; Elbandy *et al.* 2014; Kitagawa *et al.* 1982; Liu, BS *et al.* 2008a; Rodriguez *et al.* 1991; Van Dyck *et al.* 2009; Wu, J *et al.* 2006a)
1149.2	1126	a *	Holothurinoside T	No	Yes	P	–
1157.5	1134	$C_{54}H_{86}O_{25}$	Holothurinoside J_1 Unidentified	Yes	Yes	P N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b; Van Dyck *et al.* 2010b)
1163.5	1140	$C_{54}H_{92}O_{25}$	Unidentified	Yes	Yes	N	-
1167.8	1144	$C_{56}H_{88}O_{24}$	Arguside A	No	Yes	P	(Liu, BS *et al.* 2007)
1173.5	1150	$C_{57}H_{82}O_{24}$	Unidentified	Yes	Yes	N	-
1179.5	1156	$C_{57}H_{88}O_{24}$ $C_{54}H_{85}NaO_{23}S$	Unidentified	Yes	Yes	N	-
1181.4	1158	$C_{57}H_{90}O_{24}$	Unidentified	No	Yes	N	-

$[M + Na]^+$ *m/z*	MW	Formula	Compound Name	Body wall	Viscera	Novel (N)/ Published (P)	References
1189.5	1166	$C_{59}H_{97}O_{24}$	Unidentified	Yes	No	N	-
1193.5	1170	$C_{54}H_{83}NaO_{24}S$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
1197.5	1174	$C_{54}H_{87}NaO_{24}S$	Unidentified	Yes	Yes	N	-
1199.5	1176	$C_{54}H_{64}O_{29}$ $C_{56}H_{88}O_{26}$	Unidentified Arguside D	Yes	Yes	N P	(Bahrami *et al.* 2014b; Liu, BS *et al.* 2008a)
1205.5	1182	$C_{57}H_{82}O_{26}$ $C_{55}H_{83}NaO_{24}S$	Unidentified	Yes	Yes	N	-
1207.5	1184	$C_{55}H_{83}NaO_{24}S$	Unidentified	Yes	Yes	N	-
1211.5	1188	$C_{54}H_{85}NaO_{25}S$	Unidentified	Yes	Yes	N	-
1221.5	1198	$C_{56}H_{78}O_{28}$ $C_{55}H_{83}NaO_{25}S$	Unidentified Intercedenside A	Yes	Yes	N P	(Bahrami *et al.* 2014b; Zou *et al.* 2003)
1223.5	1200	$C_{55}H_{85}NaO_{25}S$	Unidentified	No	Yes	N	-
1225.5	1202	$C_{54}H_{83}NaO_{26}S$	Unidentified	No	Yes	N	–
1227.5	1204	$C_{54}H_{85}NaO_{26}S$	Fuscocinerosides B/C, Scabraside A or 24–dehydroechinoside A, Unidentified	Yes	Yes	P	(Bahrami *et al.* 2014a; Caulier *et al.* 2011; Han *et al.* 2012; Han *et al.* 2009a; Han *et al.* 2009b; Kitagawa *et al.* 1982; Kitagawa *et al.* 1979; Zhang, S-Y *et al.* 2006a)
1229.5	1206	$C_{54}H_{87}NaO_{26}S$	Holothurin A_2, Echinoside A Pervicoside B	Yes	Yes	P	(Caulier *et al.* 2013; Dong *et al.* 2008; Han *et al.* 2009b; Kalinin, V & Stonik 1982; Kitagawa *et al.* 1985; Kobayashi *et al.* 1991; Thanh *et al.* 2006)
1237.5	1214	$C_{56}H_{78}O_{29}$ $C_{55}H_{83}NaO_{26}S$	Unidentified	Yes	Yes	N	-
1243.5	1220	$C_{54}H_{85}NaO_{27}S$	Holothurin A Scabraside B 17-Hydroxy	Yes	Yes	P	(Chanley *et al.* 1959; Elyakov *et al.* 1975; Elyakov *et al.* 1973; Han *et al.* 2009a; Kitagawa *et al.* 1979; Kobayashi *et al.* 1991; Matsuno & Iba 1966;

$[M + Na]^+$ *m/z*	MW	Formula	Compound Name	Body wall	Viscera	Novel (N)/ Published (P)	References
			fuscocineroside B, 25-Hydroxy fuscocinerosiden B				Silchenko *et al.* 2005c; Thanh *et al.* 2006; Wu, J *et al.* 2006a; Yasumoto *et al.* 1967; Yuan, W *et al.* 2008)
1245.5	1222	$C_{54}H_{87}NaO_{27}S$	Holothurin A_1 Holothurin A_4 Scabraside D	No	Yes	P	(Dang *et al.* 2007; Han *et al.* 2012; Han *et al.* 2009b)
1259.5	1236	$C_{54}H_{85}NaO_{28}S$	Holothurin A_3 Holothurin D	Yes	Yes	P P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b; Dang *et al.* 2007)
1261.5	1238	$C_{54}H_{87}NaO_{28}S$	Unidentified	No	Yes	N	–
1265.5	1242	$C_{56}H_{83}NaO_{27}S$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1269.5	1246	$C_{60}H_{94}O_{27}$	Cousteside G	No	Yes	P	(Elbandy *et al.* 2014)
1271.6	1248	$C_{60}H_{96}O_{27}$	Impatienside B Cousteside H	Yes	Yes	P	(Elbandy *et al.* 2014; Yuan *et al.* 2009a)
1273.6	1250	$C_{60}H_{98}O_{27}$	Cousteside J	Yes	Yes	P	(Bahrami *et al.* 2014b; Elbandy *et al.* 2014)
1281.4	1258	$C_{54}H_{87}NaO_{29}S$	Unidentified	No	Yes	N	-
1283.4	1260	$C_{54}H_{89}NaO_{29}S$ $C_{61}H_{96}O_{27}$	Unidentified	No	Yes	N	-
1285.6	1262	$C_{56}H_{87}NaO_{28}S$	Fuscocineroside A	Yes	Yes	P	(Zhang, S-Y *et al.* 2006a)
1287.6	1264	$C_{60}H_{96}O_{28}$	Holothurinoside E,	Yes	Yes	P	(Van Dyck *et al.* 2010a; Van Dyck *et al.* 2009)
			Holothurinoside E_1	Yes	Yes	P	(Van Dyck *et al.* 2010a; Van Dyck *et al.* 2009)
			Holothurinoside O	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
			Holothurinoside P	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
			17-dehydroxy holothurinoside A	Yes	Yes	P	(Elbandy *et al.* 2014; Sun, GQ *et al.* 2008)

$[M + Na]^+$ *m/z*	MW	Formula	Compound Name	Body wall	Viscera	Novel (N)/ Published (P)	References
			Cousteside E	Yes	Yes	P	(Elbandy *et al.* 2014)
			Cousteside F	Yes	Yes	P	(Elbandy *et al.* 2014)
		$C_{56}H_{89}NaO_{28}S$	22-acetoxy-echinoside A	Yes	Yes	P	(Bhatnagar *et al.* 1985)
1289.6	1266	$C_{60}H_{98}O_{28}$	Griseaside A	Yes	Yes	P	(Sun, GQ *et al.* 2008)
			Cousteside I	Yes	Yes	P	(Elbandy *et al.* 2014)
1301.6	1278	$C_{61}H_{98}O_{28}$	Holothurinoside M	Yes	Yes	P	(Bahrami *et al.* 2014a; Van Dyck *et al.* 2011)
		$C_{60}H_{94}O_{29}$	Unidentified			N	
1303.6	1280	$C_{60}H_{96}O_{29}$	Holothurinoside A	Yes	Yes	P	(Rodriguez *et al.* 1991; Van Dyck *et al.* 2009)
			Holothurinoside A_1	Yes	Yes	P	(Rodriguez *et al.* 1991; Van Dyck *et al.* 2009)
			Holothurinoside Q	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
			Holothurinoside S	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
			Holothurinoside R	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
			Holothurinoside R_1	Yes	Yes	P	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b)
			Cousteside C	Yes	Yes	P	(Elbandy *et al.* 2014)
1305.6	1282	$C_{60}H_{98}O_{29}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1307.6	1284	$C_{60}H_{100}O_{29}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1317.6	1294	$C_{61}H_{98}O_{29}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014a; Bahrami *et al.* 2014b; Caulier *et al.* 2013)
			Holothurinoside L			P	
1319.5	1296	$C_{60}H_{96}O_{30}$	Unidentified	Yes	Yes	N	-
1329.7	1306	$C_{62}H_{98}O_{29}$	Arguside F	No	Yes	P	(Yuan *et al.* 2009a)
1335.3	1312	$C_{60}H_{96}O_{31}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1349.8	1326	$C_{61}H_{98}O_{31}$	Unidentified	No	Yes	N	-

$[M + Na]^+$ *m/z*	MW	Formula	Compound Name	Body wall	Viscera	Novel (N)/ Published (P)	References
1356.4	1333	a *	Unidentified	No	Yes	N	–
1361.7	1338	$C_{63}H_{102}O_{30}$	Unidentified	Yes	Yes	N	-
1377.3	1354	$C_{63}H_{102}O_{31}$	Unidentified	No	Yes	N	-
1409.4	1386	$C_{61}H_{78}O_{36}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1411.7	1388	$C_{62}H_{116}O_{33}$	Unidentified	No	Yes	N	–
1415.7	1392	$C_{66}H_{104}O_{31}$	Unidentified	No	Yes	N	-
1417.7	1394	$C_{66}H_{106}O_{31}$	Unidentified	Yes	Yes	N	-
1419.7	1396	$C_{66}H_{108}O_{31}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1431.4	1408	$C_{66}H_{104}O_{32}$	Unidentified	No	Yes	N	-
1435.7	1412	$C_{66}H_{108}O_{32}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1447.7	1424	$C_{67}H_{108}O_{32}$	Unidentified Impatienside A Marmoratoside A	Yes	Yes	N P	- (Yuan *et al.* 2009b)
1449.7	1426	$C_{67}H_{110}O_{32}$	Bivittoside D	No	Yes	P	(Kitagawa *et al.* 1989a)
1453.6	1430	$C_{66}H_{94}O_{34}$	Unidentified	Yes	Yes	N	-
1459.7	1436	$C_{68}H_{108}O_{32}$	Unidentified	Yes	No	N	-
1461.7	1438	$C_{68}H_{110}O_{32}$	Unidentified	Yes	No	N	-
1463.7	1440	$C_{67}H_{108}O_{33}$	Holothurinosides H/H_1 Holothurin C? Cousteside A 17α-hydroxy impatienside A Marmoratoside B	Yes	No	P	(Caulier *et al.* 2013; Van Dyck *et al.* 2010a) (Elbandy *et al.* 2014) (Yuan *et al.* 2009b)
1465.7	1442	$C_{67}H_{110}O_{33}$	Argusides B/C	No	Yes	P	(Liu, BS *et al.* 2008b)
1475.7	1452	$C_{68}H_{108}O_{33}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014a)

$[M + Na]^+$ *m/z*	MW	Formula	Compound Name	Body wall	Viscera	Novel (N)/ Published (P)	References
		$C_{65}H_{112}O_{35}$					
1477.7	1454	$C_{68}H_{110}O_{33}$ $C_{65}H_{114}O_{35}$	Lessoniosides A/B/C/D/E Unidentified	Yes	Yes	P	(Bahrami & Franco 2015)
1479.7	1456	$C_{67}H_{108}O_{34}$	Holothurinosides I/I_1	No	Yes	P	(Van Dyck *et al.* 2010a)
1481.7	1458	$C_{66}H_{106}O_{35}$ $C_{67}H_{110}O_{34}$	Unidentified	Yes	Yes	N	(Bahrami *et al.* 2014b)
1489.7	1466	$C_{68}H_{106}O_{34}$	Unidentified	Yes	No	N	-
1491.5	1468	$C_{68}H_{108}O_{34}$	Unidentified	No	Yes	N	-
1493.7	1470	$C_{68}H_{110}O_{34}$ $C_{65}H_{114}O_{36}$	Unidentified	No	Yes	N	–
1495.7	1472	$C_{67}H_{108}O_{35}$	Holothurinoside K_1 Unidentified	No	Yes	P N	(Van Dyck *et al.* 2010b) –
1507.7	1484	$C_{69}H_{112}O_{34}$	25-acetoxy bivittoside D	Yes	Yes	P	(Yuan *et al.* 2009b)
1521.7	1498	$C_{69}H_{110}O_{35}$	Unidentified	Yes	Yes	N	-
1535.7	1412	$C_{69}H_{108}O_{36}$	Unidentified	Yes	No	N	-
1539.7	1416	$C_{69}H_{112}O_{36}$	Unidentified	Yes	No	N	-
1591.7	1568	$C_{66}H_{120}O_{41}$	Unidentified	No	Yes	N	–

a * The composition was not measured through the ESI analysis.

5.5 Common saponins between the viscera and body wall

Over 89 saponin congeners were found in the body wall of which 54 saponin congeners have been reported previously. However, the comparison of saponins in the viscera and body wall of *H. lessoni*, showed a large number (around 80 saponins) are shared between the body wall and the viscera as summarised in Table 5.1. Holothurin A was the major saponins in both body wall and viscera Figure 5.11.

Even though the ions at *m/z* 1227.7 and 1229.5 were reported in both body wall and viscera as major glycosides, our results revealed a higher abundance of these saponins in the body wall than in the viscera (Figure 5.11). The other compounds which gave a more intense signal in the body wall sample than the viscera sample were the ions at *m/z* 1291.5 and 1199.6 which corresponded to an unidentified saponin and Arguside D, respectively. In contrast, the ion at *m/z* 1259.5 which corresponded to the sulphated isomeric compounds Holothurins A_3 and D (Bahrami *et al.* 2014a; Bahrami *et al.* 2014b; Bondoc *et al.* 2013; Dang *et al.* 2007), was more intense in the viscera compared to the body wall.

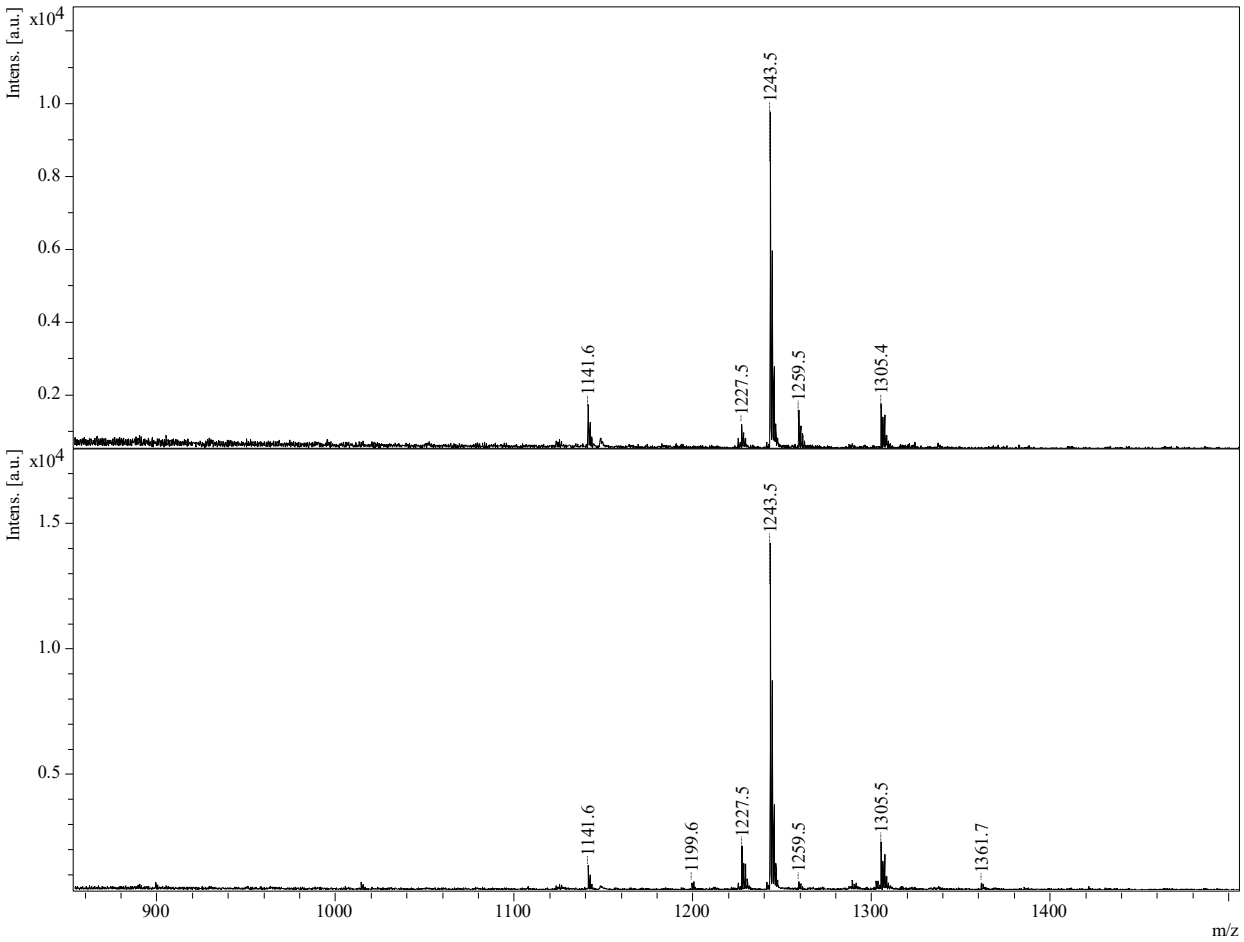

Figure 5.11. (+) MALDI spectra of butanolic saponin- enriched extract from viscera (top) and body wall (bottom) of *H. lessoni*.

Some saponin congeners including the ions detected at *m/z* 1123.5, 1125.5, 1141.5 1301.6, 1303.5, 1305.6 and 1307.5 were apparently found with similar intensities in both body wall and viscera. These findings suggested that saponins were generated in both body wall and viscera in various concentrations which proposed diverse function of saponins with different mechanisms of action. These data were in agreement with Van Dyck *et a*l. (2010a) who reported saponins originated from different cells for different purposes.

The saponin congeners identified in this species contained different key diagnostic peaks namely 493, 507, 523, 639 and so on. For instance the ion at 1305 was a novel pentasaccharide triterpene glycosides which contained the key diagnostic peaks at *m/z* 507.2 and 639.6 corresponded to $[MeGlc\text{-}Glc\text{-}Qui+Na]^+$ and $[MeGlc\text{-}Glc\text{-}Qui\text{-}Xyl+Na]^+$, respectively. Further it had an aglycone with

molecular weight of 486 Da.

A large number of identified saponins have been also reported in other species. For instance, Kitagawa *et al.* (1982) was the first to report the presence of 24-dehydroechinoside A or Scabraside A in the cuvierian tubules of the sea cucumber *Actinopyga agassizi* Selenka. Han *et al.* (2012) also found this compound in *H. scabra.* The structure of Scabraside A was also described in the sea cucumber *H. Scabra* using NMR and ESI techniques by Han *et al.* (2009a). Fuscocinerosides A/B/C and Pervicoside C were reported in the sea cucumber *Holothuria fuscocinerea* in which they differed in the lateral side chains of their aglycones (Zhang, S-Y *et al.* 2006a). Fuscocineroside A defined as an acetylated-sulphated tetraosides triterpene glycoside. Fuscocineroside C also reported in the *H. scabra* (Han *et al.* 2012). Bondoc *et al.* (2013) investigated saponin congeners in three species from Holothuriidae (*H. scabra* Jaeger 1833, *H. fuscocinerea* Jaeger 1833, and *H. impatiens* Forskal 1775). This group assigned peaks at 1227 for Fuscocinerosides B/C, 24-dehydroechinoside A or Scabraside A and another isomer.

Chanley *et al.* (1959) was the first to report the structure of Holothurin A in the sea cucumber *A. agassizi* Selenka. Later Kitagawa *et al.* (1979) described the structure of Holothurin A in the cuvierian tubules of sea cucumber *H. leucospilota* using spectroscopy methods.

Holothurin A_3, along with Holothurin A_4 were primarily isolated from the methanol extract of the sea cucumber *H. scabra* by Dang *et al.* (2007). This group indicated both Holothurins A_3 and A_4 as sulphated tetrasaccharide triterpene glycosides, contacting sulXyl, Qui, Glc and MeGlc with the ratio of 1:1:1:1, which were different in the lateral side of their aglycone moieties.

5.6 Unique saponins in the body wall

The integrated HPCPC-MS analysis indicated the presence of 35 new and 54 reported saponins in the body wall. Among those nine ions were found exclusively in the body wall compared to the viscera, were *m/z* 1069, 1103, 1189, 1459, 1461, 1463, 1489, 1535 and 1539. Most of them had

high molecular weights ranging from *m/z* 1400 to 1600. This result indicated epidermal or adjacent epidermal states for these saponins (the outer body wall epithelium directing sea water) as Caulier *et al.* (2013) reported the ion at *m/z* 1463 in the seawater surrounding *H. lessoni.* Over 30 saponin congeners were found exclusively in the viscera compared to the body wall. These saponins could be involved in the regulation of the reproductive systems, acting as natural emulsifiers and assisting absorption of food in digestive organs or playing defines mechanism (Bakus 1968; Mercier *et al.* 2009).

Mass spectrometry analysis revealed that saponin observed at *m/z* 1463.7, corresponding to Holothurinosides H/H_1, localised exclusively in the body wall, probably in the epidermis. This observation was consistent with the findings proposed by Caulier *et al.* (2013) and Van Dyck *et al.* (2011) for the body wall of *H. lessoni* and *H. forskali*, respectively. Caulier *et al.* (2013) reported the presence of this glycoside in water surrounding the animal which might release from the epidermis. Further, Van Dyck *et al.* (2011) stated this saponin congener localised in the epidermis of body wall. In addition, Van Dyck *et al.* (2010a) also indicated the presence of Holothurinosides H/H_1 in the cuvierian tubules of *H. forskali*, while cuvierian tubules were absent in *H. lessoni*. However, this ion was not detected in the viscera indicating a particular localisation of saponin which might be generated by further glycosylation of other saponins.

5.7 Distribution of saponin (body wall vs. viscera)

Some of saponins were reported in several genera. For instance, the ion at *m/z* 1141 which corresponds to Desholothurin A (synonymous with Nobiliside 2a) or Desholothurin A_1 (synonymous with Arguside E) were also reported in different species of sea cucumbers independently (Elbandy *et al.* 2014; Han *et al.* 2009b; Kitagawa *et al.* 1982; Rodriguez *et al.* 1991; Van Dyck *et al.* 2010a; Van Dyck *et al.* 2009). Desholothurin A was first detected in the sea cucumber *Actinopyga agassizi* Selenka (Kitagawa *et al.* 1982), however the structure of this triterpene glycoside was reconfirmed with a compound isolated from the sea cucumber *H. forskali* (Rodriguez *et al.* 1991).

Van Dyck and associates (2011) investigated the contribution and the precise localisation of saponins in the body wall of *H. forskali* by MALDI-MSI, and found specific localisation of saponin congeners within the body wall. They stated triterpene glycosides were mostly confined in the epidermis and mesothelium of the body wall and released when animal is stressed. This group also examined the secretion of saponins in the challenged and non-stressed holothuroids. Holothurinoside G (*m/z* 1449) was the only saponin detected in the seawater surrounding non-stressed holothuroids, originated from epidermis, while Holothurinosides C (*m/z* 1125) and F (*m/z* 1433), and Desholothurin A (*m/z* 1141) were secreted when the animals were stressed (Van Dyck *et al.* 2011). Further, they noted the presence of two saponins at *m/z* 1301 and 1317 (Holothurinosides M and L, respectively) in the water surroundings of stressed holothuroids, which stemmed from an internal organ such as the respiratory trees rather than the epidermis. Since stimulated sea cucumbers contract the body and discharge water from respiratory trees. They concluded that the ions at *m/z* 1125, 1141, 1301, 1317 and 1433 were stress-specific saponins, which could play more vital defensive roles. However, these glycosides were noted in both viscera and body wall of *H. lessoni.*

They reported saponins detected at *m/z* 1125 (Holothurinosides C/C_1), 1433 (Holothurinosides F/F_1) and 1449 (Holothurinosides G/G_1) present only in the epidermis, whereas saponins observed at *m/z* 1303 (Holothurinosides A/A_1) localised exclusively in the mesothelium, and saponins at *m/z* 1141 and 1287 were present in both epithelia of body wall of relaxed holothuroids. Saponin observed at *m/z* 1463 was mainly located in the epidermis, whereas those at 1479 showed no particular localisation (Van Dyck *et al.* 2011).

However they reported different localisation of some saponins in the body wall of stressed holothuroid compared to non-stressed animals. For instances, saponin ions observed at *m/z* 1287 and 1303 had completely disappeared in stressed specimens, indicating the near mesothelial localisation of these saponins, and ions at *m/z* 1141 were exclusively detected in the epidermis

(Van Dyck *et al.* 2011).

This group also reported that saponins were presented almost entirely in epithelial cells, with only one or two saponins observed in low concentration in the dermis. Some congeners were found solely in the mesothelium but most were observed in the epidermis. After a prolonged period of stress, mesothelial saponins were not observable on the tissue sections, suggesting release of them into the coelomic fluid. For epidermal saponins, Holothurinoside C appeared to be utilised or discharged outside of the epidermis. Their MALDI-MSI analysis of saponins from the cuvierian tubules showed that the prolonged stress situation modified Holothurinosides C/C_1 (*m/z* 1125) to Holothurinosides F/F_1 and H/H_1 (*m/z* 1433 and 1463, respectively), and Desholothurins A/A_1 (*m/z* 1141) to Holothurinosides G/G_1 and I/I_1 (*m/z* 1449 and 1479, respectively) (Van Dyck *et al.* 2011; Van Dyck *et al.* 2010a). This occurred by the addition of a disaccharide: either Qui-Glc or MeGlc-Glc. This modification, addition of a disaccharide, increased the saponins hydrophobicity and membranolysis (i.e. more toxic) (Kalinin, VI 2000). The natural concentration level of saponins released from stimulated body wall of sea cucumbers were reported to be low in water surrounding these species representing that these triterpene glycoside function as a chemical aposematic signal to alert predators of the noxiousness or, at least, the unpleasant taste of the holothuroid tissues rather than as deterrent (Camazine 1985; Eisner & Grant 1981).

Ions at *m/z* 1287 and 1303 localised in mesothelial or near mesothelial (the inner body wall epithelium toward the coelomic cavity), while saponins at *m/z* 11xx and 14xx had an epidermal or adjacent epidermal states (the outer body wall epithelium) (Van Dyck *et al.* 2011; Van Dyck *et al.* 2010a).

Van Dyck *et al.* (2010a) also studied the cuvierian tubules of *H. forskali* in both relaxed and stressed conditions by MALDI-MSI to determine the localisation of saponins. Likewise in the body wall they found eight major peaks at *m/z* 1125, 1141, 1287, 1303, 1433, 1449, 1463 and 1479 (Van Dyck *et al.* 2010a), and categorised them into three different groups, corresponding to the

isomeric saponins, which corresponded to different physiological states. Further they found saponin ions at *m/z* 1125 and 1141 in low concentrations exclusively in non-stimulated tissues. The second group, the most abundant saponins, noticed at *m/z* 1287 and 1303, was more localised in the connective tissue of both stimulated and non-stimulated individuals' tissues with the same concentration (expression level). They observed the third group of saponin ions at *m/z* 1433, 1449, 1463 and 1479 in the outer part of the connective tissue of stressed specimen. They stated that the third group (*m/z* 14xx) were stress-specific and might originate from the first group (*m/z* 11xx) via glycosylation modifications. They also reported that different cell populations corresponded to generate different set of saponins involving in a complex chemical defence mechanism (Van Dyck *et al.* 2010a). For instances, Holothurinosides A/A_1 (*m/z* 1303) and E/E_1 (*m/z* 1287) produced by the vacuolar cells, while the other congeners generated by the neurosecretory-like cells. All of above discussed findings supported our data in which some saponin congeners were exclusively localised in the viscera or the body wall, representing likely specific and particular biological functions of these substances while common congeners in the viscera and body wall might play the same role; general physio-chemical functions.

5.8 Bioactivity of sea cucumber fractions and saponins

5.8.1 Antifungal and antibacterial activities of purified saponins

Sea cucumbers have been used as traditional remedy to cure infection diseases. Previously studies have shown that some triterpene glycosides isolated from sea cucumber species possess antifungal activity (Kitagawa *et al.* 1981a). The antifungal activity of isobutanol-enriched saponin and HPCPC fractions from *H. lessoni* viscera and body wall were assessed against *F. pseudograminearum, P. irregulare* and *R. solani.* Our results revealed several tested saponin congeners (fractions) have strong antifungal activities against *F. pseudograminearum* and *R. solani*. The antifungal activities were defined by the diameter of the zone of inhibition, which was measured from the edges of the fungal growth through the centre of the disc. As a typical example,

antifungal activities of some of these saponins are shown in Figure 5.12. For instances, Fraction 2 (.Holothurin A= spot 9), Fraction 30 (Holothurin A and Intercedenside A = spot 15) and Fraction 32 (Holothurin A and ion at *m/z* 1205 = spot 16) showed significant antifungal activities.

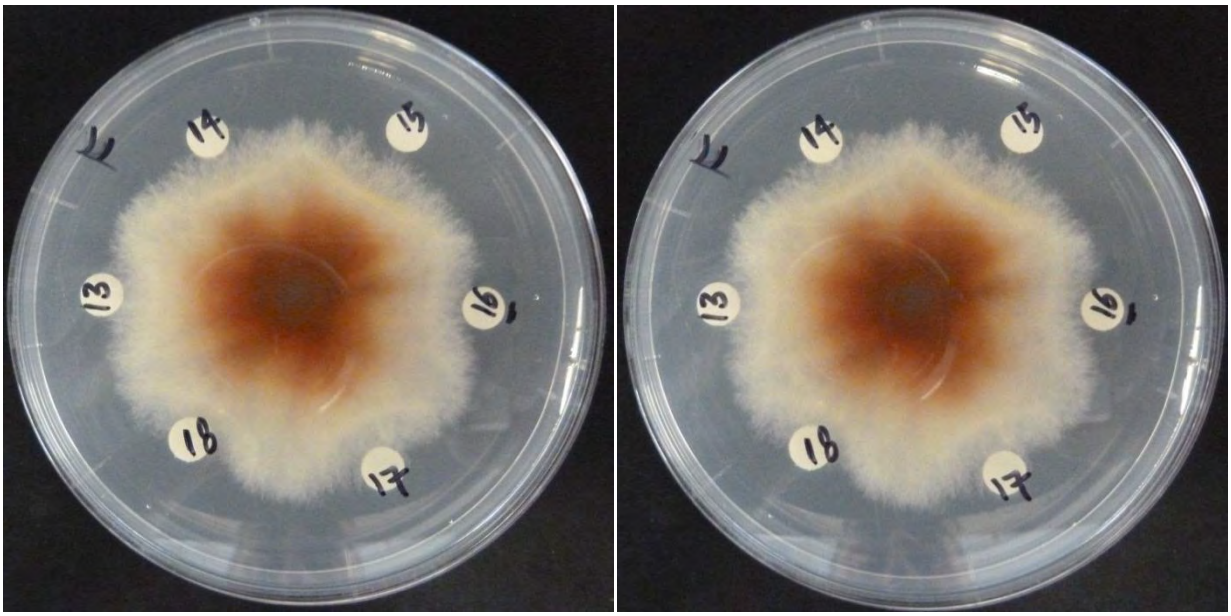

Figure 5.12. Antifungal activity of saponins isolated from body wall of *H. lessoni* against *Fusarium.* In this figure Holothurin A (spot 14), and Holothurin A and Intercedenside A (spot 15).

However, the examined triterpene glycosides had no effect on *P. irregulare*. Our data indicated that holothurian glycosides exhibit different activities against different fungi strains, which might be associated with the cellular structures of fungi. This data suggested that such discrepancy could be described by particularities of the chemical composition and structure of different types of cellular membranes. Therefore more studies are required to understand the mechanism of action of these secondary metabolites against fungi.

Our result suggested that saponins having a linear sugar moiety, a sulphate group and an acetoxy group in the structure possess high antifungal activity. For instance fractions which contained Holothurin A and/or Intercedenside A showed strong anti-fungal activity. It is noteworthy to mention that these compounds are sulphated compounds bearing a linear sugar residue.

In contrast, the examined saponins had no considerable effect or inhibited on resistant pathogen *S. aureus*, using the same concentration as used for the antifungal activity assay, however, vancomycin exhibited a strong activity (Figure 5.13). This observation was consistence with the antibacterial findings of sea cucumber extracts reported by Mokhlesi *et al.* (2012) and Kuznetsova *et al.* (1982). However, some studies reported antibacterial activity of sea cucumber crude extracts (Abraham *et al.* 2002; Park, SY *et al.* 2011), which might be associated with other chemical class such as AMPs.

5.8.2 Anti-oxidant activity of sea cucumber extracts

Further the antioxidant activities of different extracts of sea cucumber were evaluated using DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay. The DPPH assay was used to determine the intrinsic antioxidant activity using α-tocopherol as the standard. Human immortalized keratinocytes (HaCat cells) were chosen as the target cells. Preliminary results indicated that sea cucumber extracts possess high antioxidant activity. In addition, preliminary results showed that the water extract and isobutanol fractions possess the highest antioxidant activity. In summary, sea cucumber extracts tested in this experiment showed antioxidants activity comparable to other natural antioxidants.

5.9 Conclusion

A high diverse range of saponin congeners was identified in the body wall. This vast diversity could be associated with different roles of saponins including kairomones; as chemical communicates to attract symbionts, chemical defence mechanism; the most acceptable biological functions for these bioactive compounds, or aposematic signal; threatening potential predators of the unpalatability food. Saponins are considered as a defence mechanism, in which they are deleterious for most organisms, in sea cucumbers either based on adhesive defence mechanism or toxic mechanism (Caulier *et al.* 2011).

The results revealed that some saponins are organ specific. This specific localisation might be

attributed to a particular function of these congeners, which will require further studies to be carried out.

Sea cucumbers produce a diverse range of saponin congeners many of which were common between the body wall and the viscera. Nevertheless some of them were, specific to the viscera or the body wall. Further, the MS analyses also indicated that this sea cucumber species produced a mixture of common and unique saponin types. The most abundant ions observed under positive ion conditions were mainly sulphated compounds, which were common between the viscera and body wall. This study showed that saponins were synthesised in both viscera and body wall, but further studies are warranted to investigate the biosynthesis of these secondary metabolites to discover which cells are producing saponins.

In conclusion, our data revealed that there were differences in the distribution of saponins between the body wall and viscera, and showed a higher number of saponins for the viscera than the body wall, and highlighted some saponin congeners were found exclusively in the viscera. In contrast, some highly glycosylated saponins, such as ions at *m/z* 1461 and 1463, found only in the body wall which might act as kairomones or defence molecules. In fact the large sugar moieties linked to these saponins increase the water solubility of these molecules. This diverse range of saponins holds promises for biotechnological applications.

CHAPTER 6

SAPONIN PROFILE OF THE VISCERA OF THE SEA CUCUMBER *STICHOPUS HERMANNI*

This chapter addresses the distribution of saponins in the viscera (all internal organs) of the sea cucumber *Stichopus hermanni* Semper 1868, (Figure 6.1) in the class *(Holothuroidea),* and includes an introduction; a brief methods section including purification and MS analyses; the distribution of saponins; and their bioactivity.

Figure 6.1. *Stichopus hermanni* pictures from New Caledonia reefs (Photographed by Dr. Steven Purcell).

6.1 Introduction

Stichopus hermanni, commonly known as curryfish, is a representative of the family Stichopodidae, which has species of commercial importance, of the Order Aspidochirotida. Chemical investigation of *S. hermanni* dated back to the 1980s where its triterpene glycosides components were reported to exhibit antifungal activities (Kitagawa *et al.* 1981a; Kitagawa *et al.* 1981b). However, in China, *Apostichopus japonicus* Selenka (*Stichopus japonicus*) is considered as the best edible species among the members of this family with the highest economic value (Liao 1997).

Sea cucumbers belonging to the family Stichopodidae are usually consumed as a traditional tonic and culinary delicacy, and are popular for their medicinal characteristics, particularly in Asian countries. For instance in Malaysia, *S. variegatus* Semper, is widely utilized as a traditional remedy for hypertension, rheumatism, asthma, sinus, cuts, and burns (Fredalina *et al.* 1999). This species is consumed as a tonic and source of pharmacological agents in Asian countries.

The distribution of triterpene glycosides in more than 10 sea cucumbers species belonging to the family Stichopodidae has been reported. They included *Astichopus multifidus* (Stonik, VA *et al.* 1982b), *Apostichopus (=Stichopus) japonicas* (Kitagawa *et al.* 1978b; Maltsev *et al.* 1984), *P.*

(=Stichopus) californicus (Levin *et al.* 1986), *S. chloronotus* (Kitagawa *et al.* 1981a; Maltsev *et al.* 1983; Sharypov *et al.* 1981; Stonik, VA *et al.* 1982b; Stonik, VA *et al.* 1982c), *S. hermanni* (Kobayashi *et al.* 1991), *S. variegatrus* (Stonik, VA *et al.* 1982b; Stonik, VA *et al.* 1982c), *T. ananas* (Kobayashi *et al.* 1991; Stonik, VA *et al.* 1982a), *T. anax* (Kobayashi *et al.* 1991). All previous studies have investigated saponins from the body wall or cuvierian tubules.

Most of studies on distribution of saponins in the *Stichopus* genera have been conducted by *Kitagawa et al. (1981a)* and Stonik, V (1986) who investigated the distribution of saponin constituents in more than ten sea cucumber species belonging to different families. For instance, Kitagawa *et al.* (1978b) determined the full structure of two antifungal saponins named Holotoxins A and B from *Stichopus japonicus* (ma-namakio in Japanese) which were the first full structure elucidated of sea cucumber saponins. They had the same aglycones, but different sugar moieties.

So far, only one study has focused on the investigation of triterpene glycosides from the body wall of *S. hermanni.* Kobayashi *et al.* (1991) stated the existence of six triterpene glycosides in the body wall of *S. hermanni* at the 102nd Annual Meeting of the Pharmaceutical Society of Japan in 1982. This group reported Stichlorosides A_1, A_2, B_1, B_2, C_1, and C_2 from the body wall of *S. hermanni* and *Thelonota ananas* (baika-namako in Japanese) collected off Okinawa (Japan) (Kitagawa *et al.* 1981a; Kitagawa *et al.* 1981b; Kobayashi *et al.* 1991). These glycosides were first reported in the sea cucumber *S. chloronotus* by Kitagawa and co-workers (Kitagawa *et al.* 1981a; Kitagawa *et al.* 1981b). These authors also identified Stichlorosides A_1, B_1 and C_1 in the body wall of *Thelenota anax* (Kobayashi *et al.* 1991). All these glycosides were also identified in *Stichopus variegatus* by Stonik *et al* (Stonik, VA *et al.* 1982b; Stonik, VA *et al.* 1982c), but, they gave them different names i.e., Stichoposides C, D and E and their 25(26)-dehydro derivatives. Therefore, Stichlorosides A_1, B_1 and C_1 were previously reported in five different species of sea cucumbers, whereas Stichlorosides A_2, B_2, and C_2 were identified in four different species. However, none of these studies provided the accurate mass and MS/MS spectra of these compounds.

Kelecom *et al.* (1976) reported Thelothurins A and B from the sea cucumber *Thelonota ananas*, and suggested partial structures of both saponins. Later Kobayashi *et al.* (1991) reinvestigated this

extract to clarify the structural correlation of these glycosides with Stichlorosides, and found that this mixture comprised of Stichlorosides A_1, A_2, B_1, B_2, C_1, and C_2. They stated the structures of Thelothurins A and B must be revised.

Kitagawa *et al.* (1981b) also isolated triterpene glycoside Stichlorosides A_1, A_2, B_1, B_2, C_1, and C_2 from the body wall of the sea cucumber *Stichopus chloronotus* Brandt. They elucidated the structures of their aglycones named stichlorogenol (for A_1, B_1 and C_1), and dehydrostichlorogenol (for A_2, B_2, and C_2). These are the first examples of saponins isolated from sea cucumber having lanost-7-ene type aglycones. This group proposed the elemental compositions for these six antifungal triterpene glycosides though the proposed structures did not match with the molecular formulae.

This group also reported an aglycone with an elemental composition of $C_{30}H_{46}O_5$ as a major aglycone in this species and noticed that this new aglycone possesses a 7-ene moiety instead of a 9(11)-ene moiety which is more common among sea cucumbers aglycones. These authors documented the enzymatic digestion of Stichloroside A_1 and A_2 to liberate stichlorogenol ($C_{30}H_{48}O_4$), and dehydrostichlorogenol ($C_{30}H_{46}O_4$), respectively. The latter has an additional double bond at $\triangle^{25}$ (terminal methylene moiety) compared to stichlorogenol.

They concluded that stichlorogenol and dehydrostichlorogenol were genuine aglycones of Stichlorosides A_1, B_1, C_1, and A_2, B_2, C_2, respectively, which corresponded to 7-ene-23-ol acetylated analogues (Kitagawa *et al.* 1981b).

Stichloroside A_1 is a hexasaccharide containing Xyl, Glc and MeGlc in the ratio 2:2:2. The analysis also indicated the presence of an acetoxy group in the C-23 position. Alkaline treatment of this glycoside provided desacetylatedstichloroside A_1 (Kitagawa *et al.* 1981a). This group reported the enzymatic hydrolysis of A_1 yielded four hydrolysates including the genin stichlorogenol and three compounds with elemental compositions of $C_{35}H_{56}O_8$, $C_{48}H_{78}O_{18}{\cdot}2H_2O$ and $C_{53}H_{86}O_{22}{\cdot}2H_2O$. Stichloroside A_2 is a hexaglycoside of dehydrostichlorogenol having the same sugar moiety of that of A_1.

Stichlorosides A_1, B_1 and C_1 consisted of a γ-lactone moiety and a double bond in their aglycones while Stichlorosides A_2, B_2, and C_2 contained an additional double bound in the position C-25. Thao *et al.* (2014) also recently reported a new compound, Stichloroside F which has a similar aglycone as stichlorogenol having a 7-ene double bond (Kitagawa *et al.* 1981b).

This chapter addresses the purification and structure elucidation of several holostane glycosides including many new saponins along with multiple known compounds from the viscera of the sea cucumber *S. hermanni* using the same methods as described previously.

6.2 Methods

The methods were described in detail in previous chapters. Briefly, the ethanolic extract of *S. hermanni* was subjected to several standard partition purification steps followed by XAD-4 column chromatography. The dried eluate was then dissolved in water and partitioned with iso-butanol. The iso- butanol extract was further purified using HPCPC. Fractions were placed onto pre-coated silica gel TLC plates and compounds were visualized by spraying with 15% H_2SO_4 in EtOH and heating for 10 min. The high resolution mass spectra were acquired using Bruker Autoflex III Smartbeam (MALDI) and Waters Synapt HDMS mass spectrometers. Sodium ions were not introduced to the samples when the negative ion mode was conducted. Mass spectrometry analysis combined with the existing literature led to discovery of several known and new glycosides.

6.2.1 Bioactivity test

6.2.1.1. Antifungal activity assay (plug type diffusion assay)

The antifungal activities of the isobutanol-saponin enriched and HPCPC fractions (purified saponins) were tested against three strains including *Fusarium pseudograminearum, Pythium irregulare* and *Rhizoctonia solani* using a modified disc diffusion agar assay. The test fungi were grown on HPDA medium for 7 days, and a plug of the radial growth of each fungus was cut (0.5 x 0.5 cm cubes). The cubes were then placed onto the centre of a new HPDA plate and incubated at 27°C for 24 hours, or until the fungal growth surrounding the cube to 1.5 cm diameter. At this stage, 40 µl of the samples were spotted onto standard paper discs and air-dried. The six discs

were then placed onto the fungal growth plates about 1.5 cm from the edge and pressed into the agar using sterile tweezers. The plates were then re-incubated at 27°C and checked for inhibition zones every 24 hours for four days. The negative controls were methanol and plates of each fungus culture with tested samples, while Benomyl ® (50 μg/mL) was used as a positive control.

6.2.1.2. Antibacterial activity assay

Antibacterial activity of saponin extracts were examined against resistant pathogen *Staphylococcus aureus* using a typical agar diffusion assay. Antibiotic assay medium (AAM) was used for the antibacterial activity assay. The test culture was grown in tryptone soy broth (TSB) and incubated at 37°C for 18 – 22 hrs. The growth of the culture was evaluated by measuring the optical density (OD) using a Shimadzu UV-160A spectrophotometer at 600 nm (OD600 nm), and OD was adjusted to 0.2. The AAM was seeded with the culture (1% v/v) and dispensed into 9 cm petri dish plates at 25 ml/plate, and cut using a cork borer to make 10 wells (6 mm). Each well was then filled with 40 µl of samples and the plates incubated at 37°C for 18 – 24 hours. Vancomycin (0.25 μg/mL) was used as a positive control.

6.3 Structure characterisation of triterpene glycosides by MALDI- and ESI-MS

The saponin profile of the butanol –saponin-enriched fraction was revealed by MADLI-MS in the positive ion mode. The MALDI-MS spectrum of saponin congeners from the viscera of *S. hermanni* is shown in Figure 6.2.

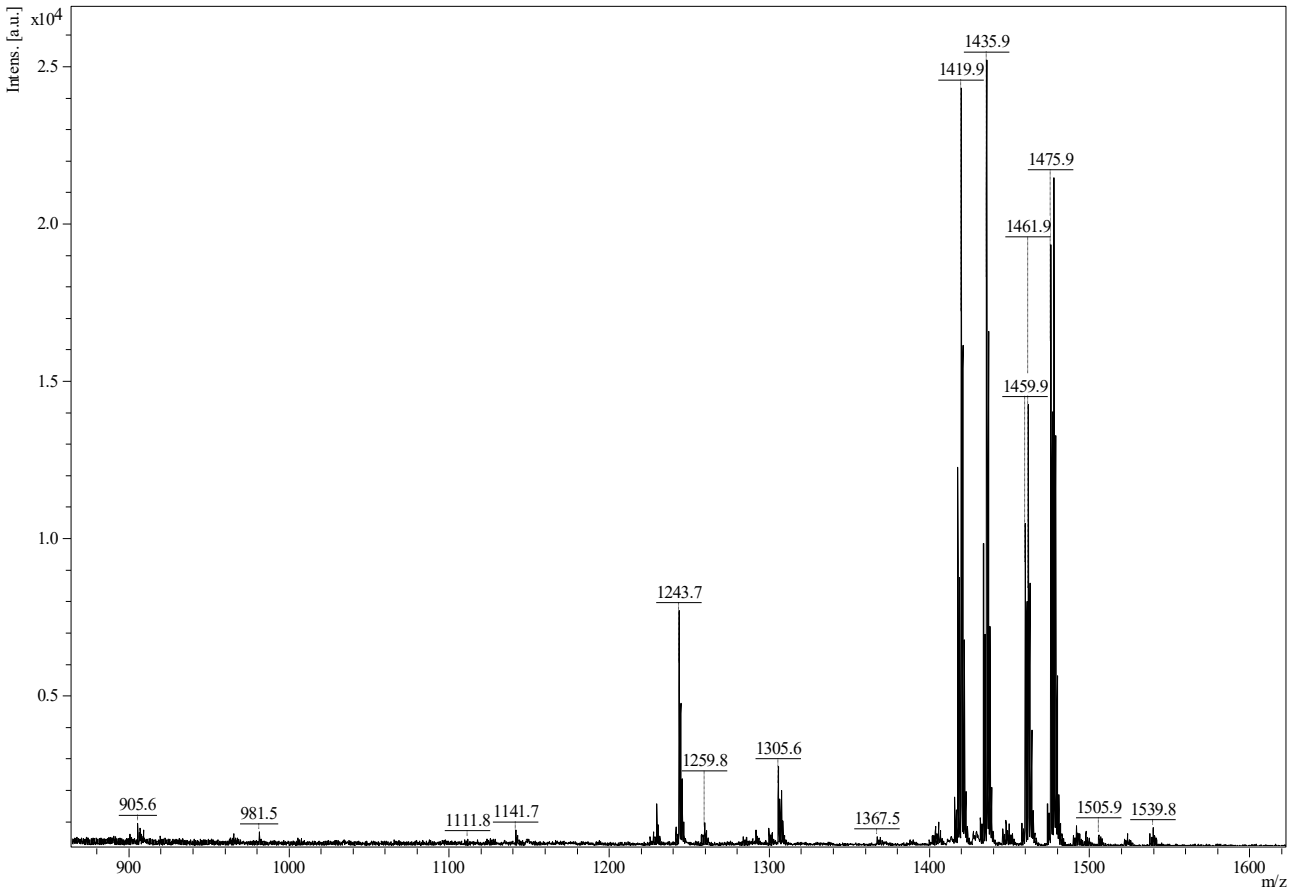

Figure 6.2. MALDI-MS spectrum of isobutanol-saponin enriched extract.
This spectrum is unique for this species.

The major triterpene glycosides in the viscera of *S. hermanni* (Stichopodidae, Aspidochirotida), were detected at *m/z* 1435.7, 1419.7, 1477.7, 1475.7, 1243.5, 1461.7, 1417.7, 1433.7, 1459.7, 1305.5 and 1307.5, respectively, in which each peak could represent at least one saponin congener, even though some peaks were identified as isomeric compounds. This analysis indicated the presence of several saponin classes. Further analysis revealed that they were mostly non-sulphated glycosides.

Similar analysis was also conducted on the pooled HPCPC Fractions by MALDI and ESI-MS. As a typical example, the positive ion mode MALDI- MS spectrum of Fraction 121 is demonstrated in Figure 6.3. The major peak at *m/z* 1435 corresponded to a new saponin which I have described latter on.

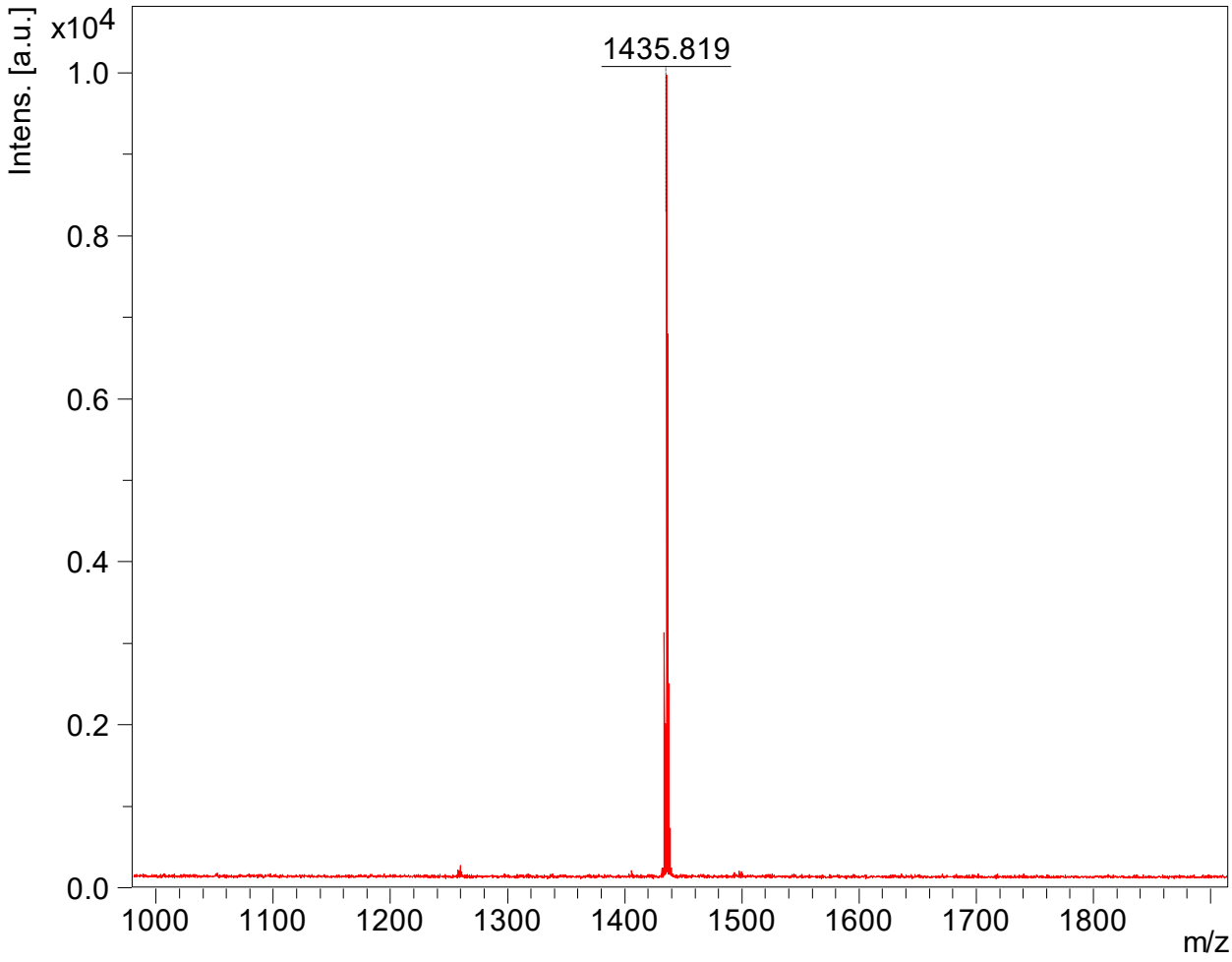

Figure 6.3. (+) MALDI-MS spectrum of Fraction 121.
The sole major peak at *m/z* 1435 corresponded to a new saponin.

This spectrum showed the presence of a single peak at *m/z* 1435 indicating that the HPCPC was an effective tool for purifying saponins.

MALDI-MS data was then corroborated by ESI-MS analysis. HPCPC fractions were analysed by ESI-MS in both positive and negative ion modes. As an example, for the analysis which was performed on Fraction 140 in the (+), "top spectrum", and (-) "bottom one" ion modes ESI-MS are shown in Figure 6.4. The positive ion mode ESI-MS exhibited ion peaks at *m/z* 1435 and 1433 $[M + Na]^+$ which had peaks at *m/z* 1411 and 1409 $[M - H]^-$ in the negative ion mode ESI-MS, respectively. As it is notable from this spectrum, most of saponin ions were identified as a pair, in which they differed by two hydrogen molecules; dihydro and dehydro- analogues.

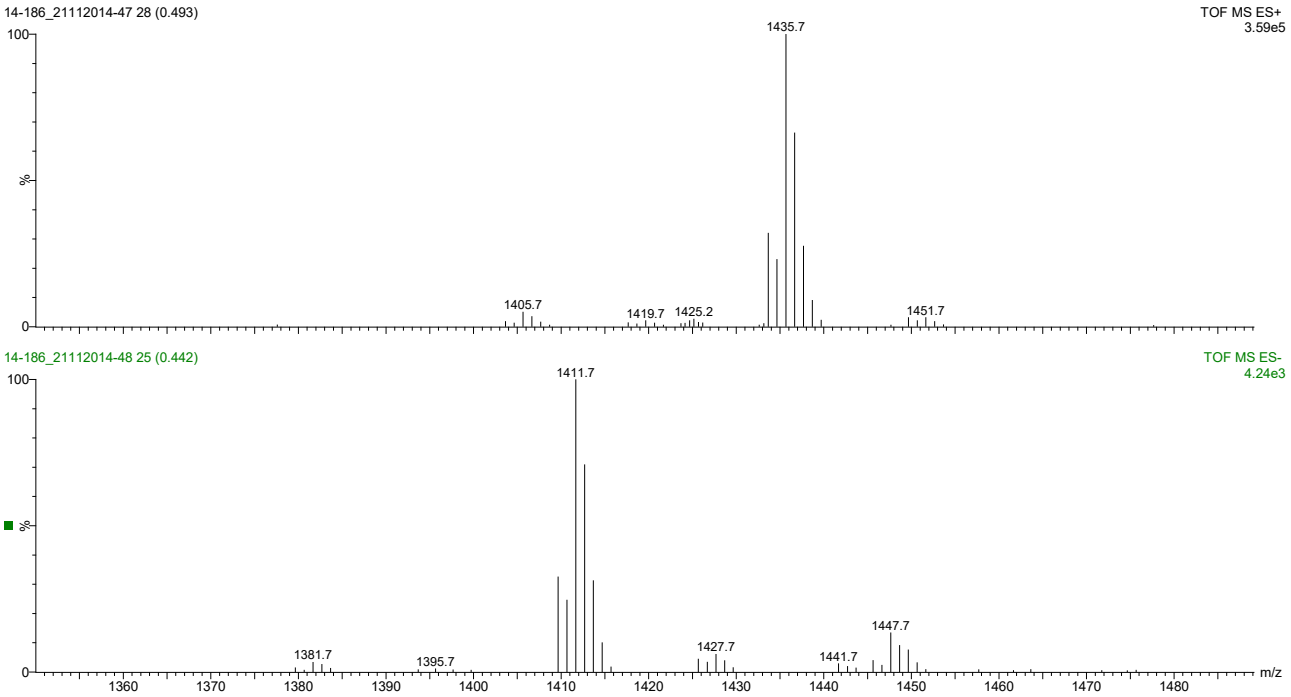

Figure 6.4. Saponin profile of Fraction 140 using ESI-MS in both positive (top) and negative (bottom) ion modes.

ESI data validated the MALDI analysis and proved that MS is reliable, robust and accurate technique for structure determination of saponins. The assignment of saponin ions were achieved by MALDI- and ESI-MS, and molecular formulae and elemental compositions were assigned by ESI-MS/MS as summarised in Table 6.1. This analysis revealed the presence of over 101 saponin congeners in the viscera of the *S. hermanni*. Some of these saponins were common among sea cucumbers belonging to this family, while some were unique to this species. Thus a diverse range of saponin congeners were discovered including sulphated, non-sulphated and acetylated. However, a large number of saponins were found to be acetylated. The chemical structures of some of identified saponins are shown in Figure 6.5.

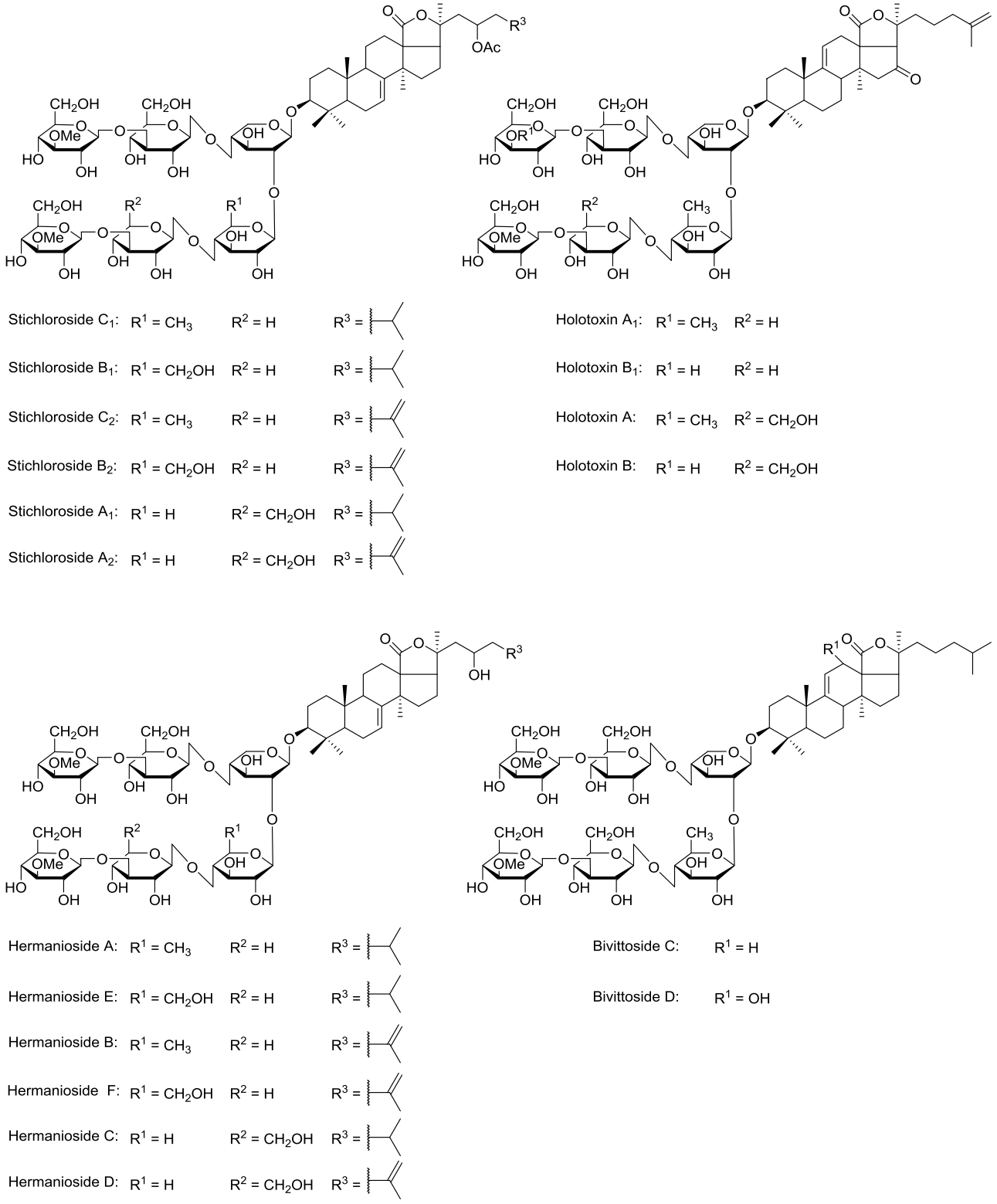

Figure 6.5. Chemical structures of some of identified saponins in the viscera of *S. hermanni*. The vast majority of saponins contained a glycone bearing a C-7(8) double bond.

6.3.1 Identification of saponin by negative-ion ESI-MS

MS/MS analyses were also performed in the negative ion mode on all compounds under experimental conditions similar to those used for positive ion mode. The identification of saponins

using the negative ion mode facilitates prediction of elemental composition as indicated by the presence of the number of Na atoms existing in the molecules in addition to confirm the data obtained from the positive ion mode. If the mass discrepancy of a given parent ion in the negative ion mode analysis was 46 u compared to that in the positive mode, it showed the presence of two Na atoms which indicates the present of sulphate groups in the molecule. If the mass (*m/z*) discrepancy of a given parent ion in the positive and negative modes was 24 u, it corroborated the presence of one Na atom indicating the presence of a non-sulphated saponin.

6.4 HPCPC purification of isobutanol saponin enriched extract

HPCPC proved to be an effective technique in overcoming challenges associated with purification of saponins due to their related chemical structures. Integration of mass spectrometry and counter current chromatography increase the efficiency of discovering novel saponins as a potential for novel therapeutics to improve people's well-being and health.

As Figure 6.2 indicates the saponin-enriched isobutanol fraction contained multiple saponin congeners. Thus, HPCPC was applied to purify saponin congeners as described previously. Briefly, Two hundred and forty milligrams of butanolic saponin extract was introduced into HPCPC and the apparatus was performed in the descending mode. One hundred and fifty three fractions were collected and monitored by TLC. The TLC profile of the butanolic extract demonstrated the presence of several bands, Figure 6.6. (lane 1), while the TLC pattern of HPCPC fractions indicated the presence of one band, and in some case two bands. As a typical example, the TLC profile of HPCPC Fractions 24-40 is shown in Figure 6.6.

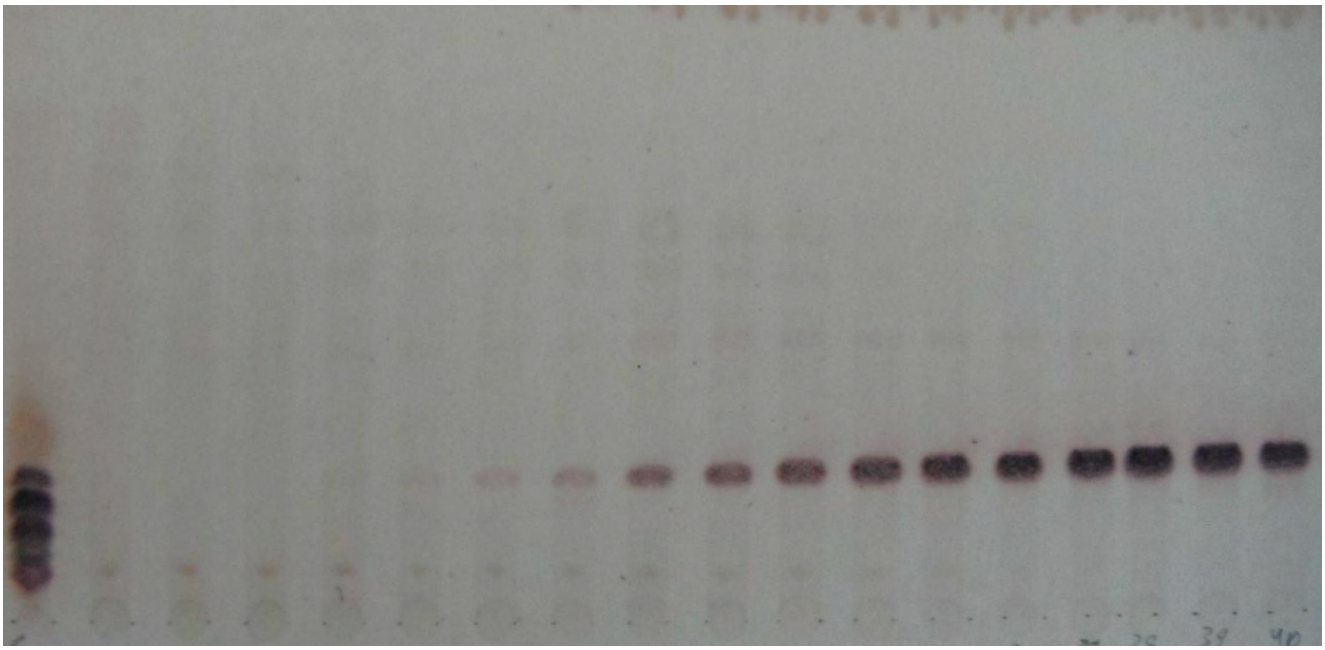

Figure 6.6. TLC profile of isobutanolic extract (lane 1) and purified HPCPC Fractions of the viscera of the *S. hermanni* using the lower phase of $CHCl_3$:MeOH:H_2O (7:13:8) system.
The numbers under each lane indicated the fraction number in the fraction collector.

All analyses conducted on saponin ions of the HPCPC fractions using MALDI-MS/(MS) and ESI-MS/(MS) are listed in Table 6.1. Our result revealed the presence of at least 41 new sulphated, non-sulphated and acetylated triterpene glycosides.

Table 6.1. Summary of saponin congeners identified from the viscera of *S. hermanni* by MALDI-ToF-MS^2 and ESI-MS^2.
This table illustrates the 41 novel identified compounds (N) along with the 60 known compounds (P). This table also shows some identical saponins, which have been given different names by different researchers in which they might be isomeric congeners.

$[M + Na]^+$ *m/z*	**MW**	**Formula**	**Compound name**	**Novel (N)/ Published (P)**	**References**
759.2	736	$C_{35}H_{53}NaO_{13}S$	Leucospilotaside C	P	(Han *et al.* 2008)
905.4	882	$C_{41}H_{63}NaO_{17}S$	Holothurin B_4	P	(Silchenko *et al.* 2005c)
			Holothurin B	P	(Elyakov *et al.* 1973; Kitagawa *et al.* 1978a)
			Nobiliside B	P	(Wu, J *et al.* 2006b)
907.4	884	$C_{41}H_{65}NaO_{17}S$	Holothurin B_2	P	(Silchenko *et al.* 2005c)
			Leucospilotaside B	P	(Han *et al.* 2010c)
1087.6	1064	$C_{52}H_{88}O_{22}$	Unidentified	N	-
1089.6	1066	$C_{52}H_{90}O_{22}$	Unidentified	N	-
1099.6	1076	$C_{52}H_{84}O_{23}$	Unidentified	N	-
1101.7	1078	$C_{52}H_{86}O_{23}$	Unidentified	N	-
1103.5	1080	$C_{52}H_{88}O_{23}$	Unidentified	N	-
1113.7	1090	$C_{53}H_{86}O_{23}$	Unidentified	N	-
1123.5	1100	$C_{54}H_{84}O_{23}$	Unidentified	N	-
1125.5	1102	$C_{54}H_{86}O_{23}$	Holothurinoside C	P	(Kitagawa *et al.* 1982;

[M + Na]$^+$ *m/z*	MW	Formula	Compound name	Novel (N)/ Published (P)	References
			Holothurinoside C_1		Rodriguez *et al.* 1991; Van Dyck *et al.* 2010a; Van Dyck *et al.* 2009)
1127.6	1104	$C_{54}H_{88}O_{23}$ $C_{53}H_{84}O_{24}$	Holothurinoside Y Holothurinoside X	P P	(Bahrami *et al.* 2014a)
1139.6	1116	$C_{55}H_{88}O_{23}$	Thelenotoside B	P	(Stonik, VA *et al.* 1982a)
1141.6	1118	$C_{54}H_{86}O_{24}$	Desholothurin A (Nobiliside 2a), Desholothurin A_1 (Arguside E)	P	(Elbandy *et al.* 2014; Kitagawa *et al.* 1982; Liu, BS *et al.* 2008a; Rodriguez *et al.* 1991; Van Dyck *et al.* 2009)
1205.5	1182	$C_{56}H_{78}O_{27}$ $C_{55}H_{83}NaO_{24}S$	Unidentified Unidentified	N N	- -
1207.5	1184	$C_{60}H_{80}O_{24}$ $C_{54}H_{81}NaO_{25}S$	Unidentified Intercedenside G	N P	- (Zou *et al.* 2005)
1209.5	1186	$C_{54}H_{83}NaO_{25}S$	Mollisoside A	P	(Moraes, G. *et al.* 2005)
1221.5	1198	$C_{56}H_{78}O_{28}$ $C_{55}H_{83}NaO_{25}S$	Unidentified Intercedenside A	N P	- (Zou *et al.* 2003)
1225.5	1202	$C_{54}H_{83}NaO_{26}S$	Unidentified	N	-
1227.5	1204	$C_{54}H_{85}NaO_{26}S$	Fuscocinerosides B/C, Scabraside A or 24–dehydroechinoside A	P	(Han *et al.* 2012; Han *et al.* 2009a; Han *et al.* 2009b; Kitagawa *et al.* 1982; Zhang, S-Y *et al.* 2006a)
		$C_{59}H_{96}O_{25}$	Holotoxin F	P	(Wang, Z *et al.* 2012b)
1229.5	1206	$C_{54}H_{87}NaO_{26}S$	Holothurin $A_{2,}$ Echinoside A	P	(Kalinin, V & Stonik 1982; Kitagawa *et al.* 1980; Kitagawa *et al.* 1985; Kobayashi *et al.* 1991; Oleinikova *et al.* 1982b)
1237.5	1214	$C_{56}H_{78}O_{29}$ $C_{55}H_{83}NaO_{26}S$	Unidentified Intercedenside H	N P	- (Zou *et al.* 2005)
1241.5	1218	$C_{59}H_{94}O_{26}$	Unidentified	N	-
1243.5	1220	$C_{54}H_{85}NaO_{27}S$	Holothurin A Scabraside B 17-Hydroxy fuscocineroside B, 25-Hydroxy fuscocinerosiden B,	P	(Bhatnagar *et al.* 1985; Elyakov *et al.* 1973; Han *et al.* 2009a; Kitagawa *et al.* 1982; Kitagawa *et al.* 1979; Kobayashi *et al.* 1991; Stonik, VA *et al.* 1979; Yuan, W *et al.* 2008)
		$C_{59}H_{96}O_{26}$	Unidentified	N	-
1245.5	1222	$C_{54}H_{87}NaO_{27}S$	Holothurin A_1 Holothurin A_4 Scabraside D	P	(Dang *et al.* 2007; Han *et al.* 2012; Han *et al.* 2009b; Oleinikova *et al.* 1982a)
		$C_{59}H_{98}O_{26}$	Unidentified	N	-
1257.5	1234	a*	Unidentified	N	-
1259.5	1236	$C_{54}H_{85}NaO_{28}S$	Holothurin A_3 Holothurin D	P	(Dang *et al.* 2007)
		$C_{59}H_{96}O_{27}$	Unidentified	N	-
		$C_{59}H_{96}O_{27}$	Variegatuside D	P	(Wang, X-H *et al.* 2014)

[M + Na]$^+$ *m/z*	MW	Formula	Compound name	Novel (N)/ Published (P)	References
1263.6	1240	$C_{59}H_{100}O_{27}$	Unidentified	N	-
1269.5	1246	$C_{60}H_{94}O_{27}$	Compound 8	P	(Kitagawa *et al.* 1978b)
1275.6	1252	$C_{59}H_{96}O_{28}$ $C_{60}H_{100}O_{27}$	Unidentified	N	-
1279.6	1256	$C_{59}H_{100}O_{28}$	Unidentified	N	-
1283.7	1260	$C_{61}H_{96}O_{27}$	Unidentified	N	-
1287.6	1264	$C_{60}H_{96}O_{28}$	Holothurinoside E, Holothurinoside E_1,	P	(Van Dyck *et al.* 2010a; Van Dyck *et al.* 2009)
			Holothurinoside O	P	(Bahrami *et al.* 2014b)
			Holothurinoside P	P	(Bahrami *et al.* 2014b)
			17-dehydroxyholothurioside A	P	(Elbandy *et al.* 2014; Sun, GQ *et al.* 2008)
1291.5	1268	a*	Unidentified	N	-
1299.5	1276	$C_{61}H_{96}O_{28}$	Unidentified	N	-
1301.6	1278	$C_{61}H_{98}O_{28}$ $C_{60}H_{94}O_{29}$	Holothurinoside M Unidentified	P N	(Van Dyck *et al.* 2011) -
1305.5	1282	$C_{60}H_{98}O_{29}$	Unidentified	N	-
1307.5	1284	a*	Unidentified	N	-
1401.7	1378	$C_{65}H_{102}O_{31}$	Holotoxin E Holotoxin B_1	P P	(Maltsev *et al.* 1984; Wang, Z *et al.* 2012b)
1403.7	1380	$C_{65}H_{104}O_{31}$	Parvimoside B Dihydro-Holotoxin B_1 Unidentified	P P N	(Iniguez-Martinez *et al.* 2005) -
1405.6	1382	$C_{65}H_{106}O_{31}$	Unidentified	N	-
1415.7	1392	$C_{66}H_{104}O_{31}$	Holotoxin A_1	P	(Maltsev *et al.* 1984)
1417.7	1394	$C_{66}H_{106}O_{31}$	Unidentified Cladoloside C_2	N P	- (Silchenko *et al.* 2013c)
1419.7	1396	$C_{66}H_{108}O_{31}$	Unidentified	N	-
1423.8	1398	$C_{66}H_{112}O_{31}$	Unidentified	N	-
1431.7	1408	$C_{66}H_{104}O_{32}$	Holotoxin B Holotoxin D	P P	(Kitagawa *et al.* 1978b) (Wang, Z *et al.* 2012b)
1433.7	1410	$C_{66}H_{106}O_{32}$	Unidentified Parvimoside A	N P	- (Iniguez-Martinez *et al.* 2005)
		$C_{67}H_{110}O_{31}$	Bivittoside C	P	(Kitagawa *et al.* 1989a)
1435.7	1412	$C_{66}H_{108}O_{32}$ $C_{66}H_{108}O_{32}$	Unidentified Unidentified Variegatusides E/F	N N P	- - (Wang, X-H *et al.* 2014)
1439.7	1416	$C_{66}H_{112}O_{32}$	Unidentified	N	-
1445.7	1422	$C_{67}H_{106}O_{32}$	Holotoxin A	P	(Kitagawa *et al.* 1978b)
1447.7	1424	$C_{67}H_{108}O_{32}$	Unidentified	N	-
			Impatienside A Marmoratoside A	P	(Sun, P *et al.* 2007; Yuan *et al.* 2009b)
1449.7	1426	$C_{67}H_{110}O_{32}$ $C_{66}H_{106}O_{33}$	Unidentified Bivittoside D, 25,26-Dihydroxy-holotoxin A_1	N P P	- (Kitagawa *et al.* 1989a; Kitigawa *et al.* 1981; Wang, Z *et al.* 2012a)

[M + Na]$^+$ *m/z*	MW	Formula	Compound name	Novel (N)/ Published (P)	References
1451.7	1428	$C_{66}H_{108}O_{33}$ $C_{67}H_{112}O_{32}$	Unidentified Unidentified	N N	- -
1453.7	1430	$C_{66}H_{110}O_{33}$	Unidentified	N	-
1455.7	1432	$C_{66}H_{112}O_{33}$	Unidentified	N	-
1457.7	1434	a*	Unidentified	N	-
1459.7	1436	$C_{68}H_{108}O_{32}$	Unidentified Unidentified Stichloroside C_2	N N P	- - (Kitagawa *et al.* 1981a)
1461.7	1438	$C_{68}H_{110}O_{32}$ $C_{64}H_{110}O_{35}$	Unidentified Unidentified Stichloroside C_1	N N P	- - (Kitagawa *et al.* 1981a)
1463.7	1440	$C_{67}H_{108}O_{33}$	Unidentified 17-hydroxyimpatien-side A Marmoratoside B	N P P	- (Yuan *et al.* 2009b)
1465.7	1442	$C_{67}H_{110}O_{33}$	Arguside B Arguside C	P	(Elbandy *et al.* 2014; Liu, BS *et al.* 2008b).
1475.6	1452	$C_{68}H_{108}O_{33}$ $C_{64}H_{108}O_{36}$	Unidentified Unidentified Stichloroside A_2	N N P	- - (Kitagawa *et al.* 1981a)
1477.7*	1454	$C_{68}H_{110}O_{33}$ $C_{64}H_{110}O_{36}$	Unidentified Unidentified Lessoniosides A/B/D Stichlorosides A_1/B_1	N N P P	- - (Bahrami & Franco 2015; Kitagawa *et al.* 1981a; Kitagawa *et al.* 1981b)
1481.7	1458	$C_{66}H_{106}O_{35}$	Unidentified	N	-
1491.7	1468	$C_{68}H_{108}O_{34}$ $C_{69}H_{112}O_{33}$	Unidentified	N	-
1493.7	1470	$C_{68}H_{110}O_{34}$ $C_{69}H_{114}O_{33}$	Unidentified	N	-
1497.7	1424	a*	Unidentified	N	-
1505.8	1482	$C_{69}H_{110}O_{34}$	Unidentified	N	-
1521.8	1498	$C_{69}H_{110}O_{35}$ $C_{71}H_{102}O_{34}$	Unidentified	N	-
1523.8	1500	$C_{69}H_{112}O_{35}$ $C_{71}H_{104}O_{34}$	Unidentified	N	-
1537.7	1514	$C_{69}H_{110}O_{36}$	Unidentified	N	-
1539.7	1516	$C_{69}H_{112}O_{36}$	Unidentified	N	-

a * The composition was not measured through the ESI analysis.

6.5 MALDI- and ESI-MS/MS analyses of saponins

All saponin ions were then subjected to MS^2 analysis using CID of which the glycoside undergone the sequential cleavage of the oligosaccharide chain converged to the aglycone part. Further using low kinetic energy in the CID to activate selected ion, no cross-ring cleavages of the core of the aglycone were expected. Nonetheless, some fragmentations of the lateral side chain of the

aglycone were likely to happen using low energy such as well-characterised McLafferty rearrangement (Demeyer *et al.* 2014). Based on that this low energy used here were able to fragment the acetoxy group linked to the lateral side chain or C-16 of the aglycone. In addition, the MS^2 analysis revealed the presence of 56 u (C_4H_8) and 98 u fragments corresponding to the cleavage in the side-chain of the aglycone. Apparently, fragmentations of the lateral chain of the aglycone were the first fragment of the selected parent ion. This conclusion was in agreement with observation by Demeyer *et al.* (2014). In this section the tandem mass spectrometry analysis of serval ions will be described as representative.

6.5.1 MALDI-MS/MS

As a typical example, the MS^2 fragmentation profile in the positive ion mode of the ion detected at *m/z* 1435 from Fraction 121 is illustrated in Figure 6.7. As can be seen in the spectrum, this compound had ions at 493 corresponding to the key diagnostic peak which discussed in previous chapters.

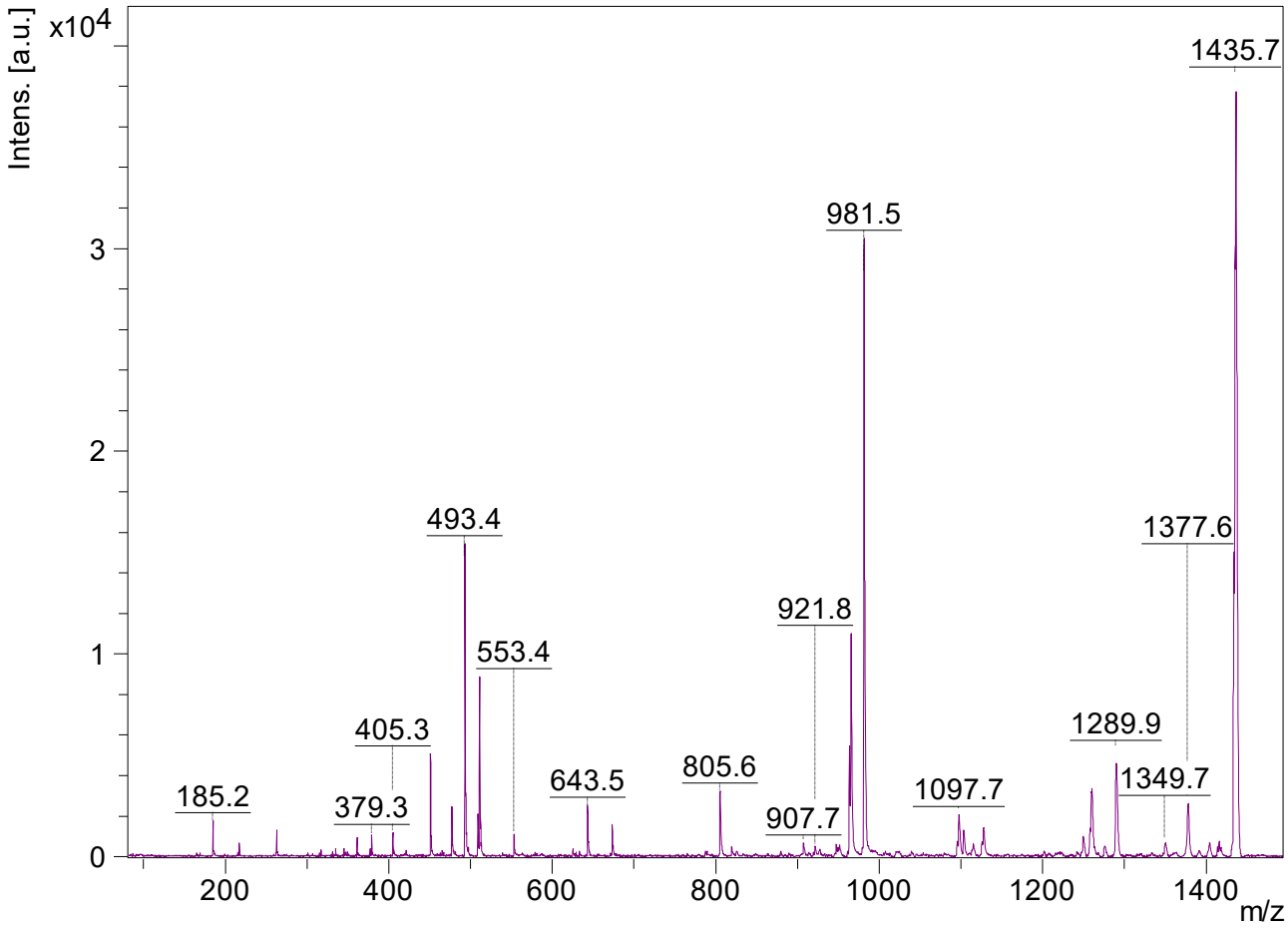

Figure 6.7. (+) MALDI-MS/MS fragmentation profile of the ion obsrved at *m/z* 1435. This analysis revealed that this glycoside composed of a hexasaccharide chain.

Our findings revealed that this triterpene glycoside contains six sugar residues. This deduction was established by fragment ion peaks at *m/z* 981, 805, 673, and 511 in the positive-ion mode ESI-MS^2 and MALDI-MS^2, corresponding to the sequential losses of AgI, MeGlc, Xyl, and Glc moieties, respectively. The key diagnostic ion at *m/z* 511 corresponded to [MeGlc-Glc-Xyl-H_2O+Na]$^+$. The ion at *m/z* 981 corresponded to sodiated entire sugar chain which generates by the loss of the aglycone. The MS^2 fingerprints of carbohydrate moieties of this glycoside were coincident with those of Stichlorosides B_1 and B_2 previously reported from *S. chloronotus*. The MS^2 analysis of the identified glycosides was shown to possess the same saccharide moiety; the sugar units in these triterpene glycosides were superimposable. Having a hexasaccharide chain is a characteristic feature for most of the major identified glycosides in this family.

6.5.2 ESI-MS/MS

All saponin ions observed in the ESI-MS spectrum of HPCPC fractions was also profiled by ESI-

MS^2. In the MS spectrum, most of these saponins were identified as pairs which differed by two hydrogen molecules; as dihydro and dehydro- derivatives. For example, the structure of the ions at *m/z* 1417 and 1419 will be discussed as follows.

The MS^2 ESI spectrum of the ion observed at *m/z* 1417 in the positive ion mode from Fraction 152 is shown in Figure 6.8. The MS^2 analysis revealed this isomeric glycoside had an aglycone similar to that of Stichloroside C_2 bearing two double bonds at $\triangle^{7(25)}$. This glycoside has a molecular formula $C_{66}H_{106}O_{31}$ for which we proposed the name Hermannioside B. The initial fragmentation of the lateral chain of the aglycone led to the loss of 56 u afforded the major ion at *m/z* 1361 corresponding to the elimination of C_4H_8 representing the existence of double bond in the side-chain.

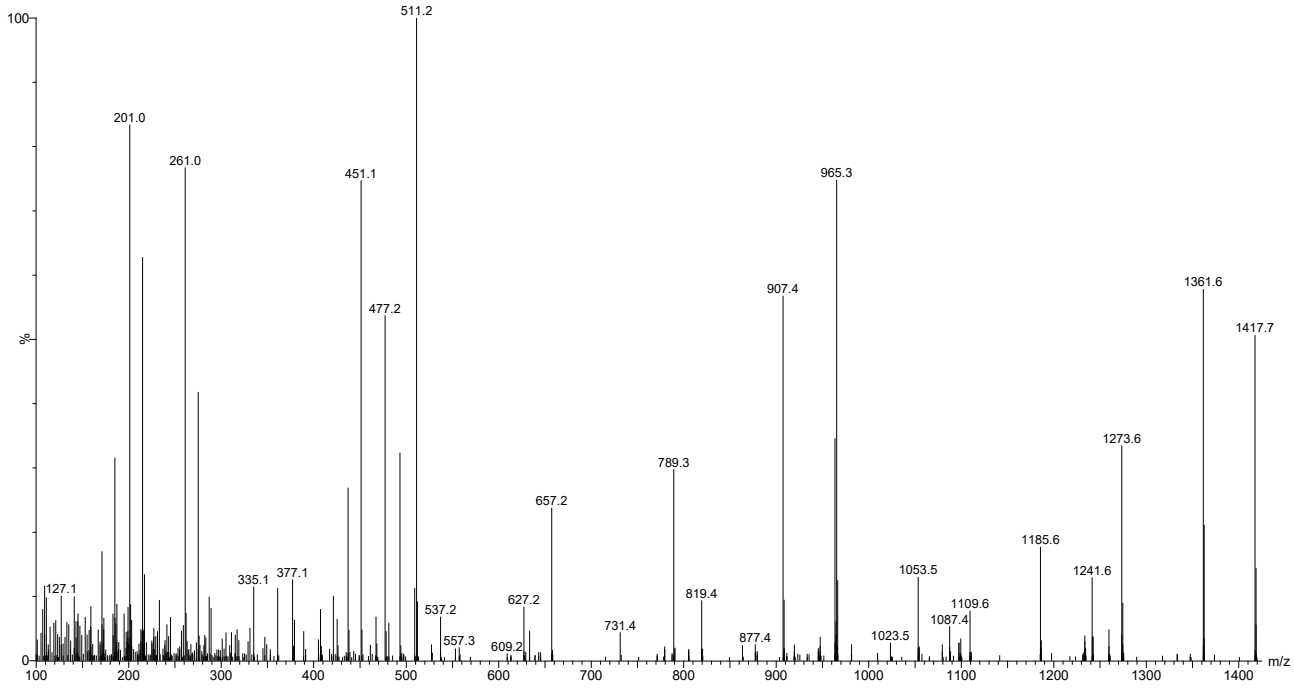

Figure 6.8. Fragmentation of the ion detected at *m/z* 1417 in the positive ion mode ESI-MS^2.

The sugar moiety of this novel compound consisted of six monosaccharide units, including Xyl:Qui:Glc:MeGlc in a 2:1:1:2 ratio, and was identical to those of Lessonioside A (Bahrami & Franco 2015), Stichloroside C_1 and Stichloroside C_2 (Kitagawa *et al.* 1981a). This glycoside contained the key diagnostic peaks at *m/z* 477, 493 and 511 corresponding to [MeGlc-Xyl-Qui+Na]$^+$, [MeGlc-Glc-Xyl+Na]$^+$ and [MeGlc-Glc-Xyl-H_2O+Na]$^+$, respectively. A major peak at 1361

was generated by the cleavage of the aglycone in the lateral side which led to a mass transition of 56 Da indicating the presence of a double bond in the lateral side.

Wang, Z *et al.* (2012a) also isolated Stichloroside C_1 and Holotoxin D_1 from *Stichopus japonicus*. The authors described a molecular formula of $C_{65}H_{102}O_{32}$ for Holotoxin D_1 with a *m/z* value of 1417.6 $[M+Na]^+$ in the positive ion mode. This group also reported 26-nor-25-oxo-holotoxin A_1 having identical mass and molecular formula as that of Holotoxin D_1, but different in chemical structures from the same species (Wang, Z *et al.* 2012b). However, the elucidated structure of the latter compound did not match with the proposed molecular formula, therefore the structure elucidation is under question. Even though these compounds had an identical mass to that of Cladoloside C_2 obtained from *Cladolabes schmeltzii* (Silchenko *et al.* 2013c), they possess different structures. New compound, Hermannioside B, showed a different MS fingerprint compared to these substances. Nonetheless, one of the isomer was found to be identical with Cladoloside C_2.

Another typical example was the ion detected at *m/z* 1419.7 in Fraction 152. This compound showed an *m/z* value of 1395.7 in the native mode ESI-MS representing lack of a sulphur group in the structure. The ESI-MS^2 spectrum of this ion in the positive ion mode is shown in Figure 6. 9. As can be seen this novel compound had very similar MS/MS profile to that of 1417.7. This analysis revealed that the sequence of sugar moieties in the carbohydrate chain was coincident with those for Hermannioside B (ion at 1417), but the difference was recognised in the aglycone moiety. In fact, this compound was defined as a hexaoside, having a nonlinear hexasaccharides chain consisting of Xyl:Qui:Glc:MeGlc in a 2:1:1:2 ratio for which we named Hermannioside A.

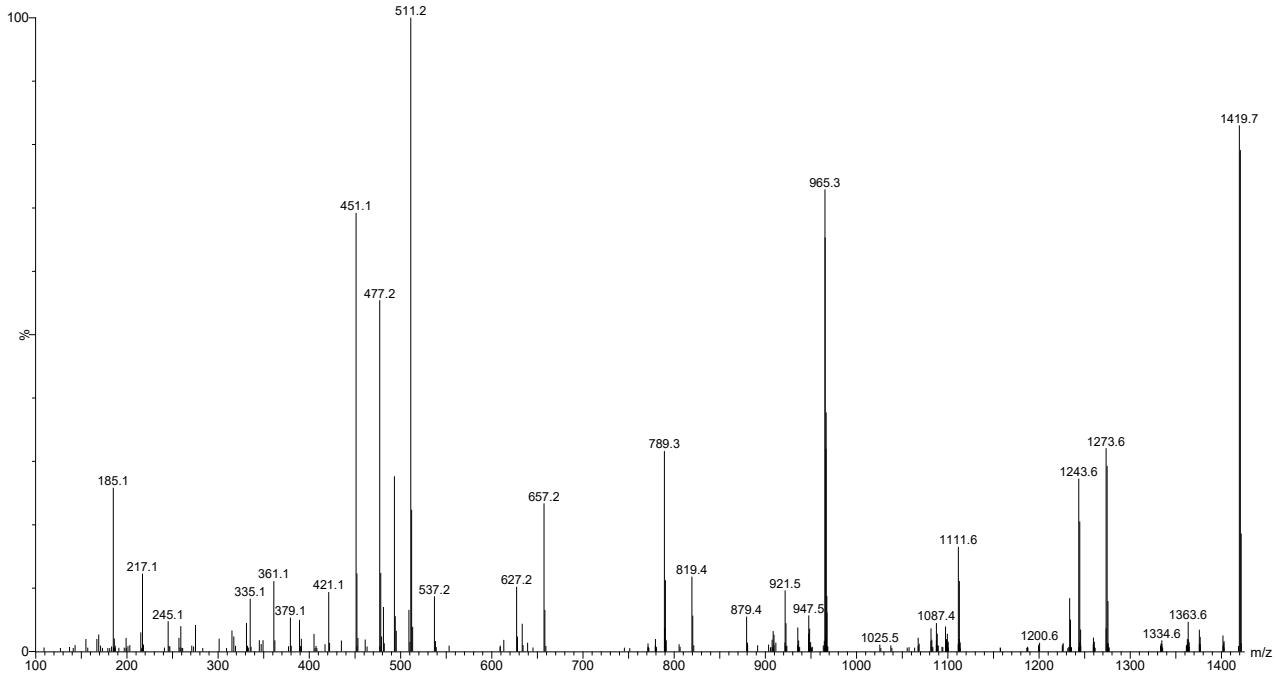

Figure 6.9. (+) ESI-MS/MS profile of the ion detected at *m/z* 1419.7 from Fraction 152. This analysis revealed a hexaoside sugar chain.

Comparing the aglycone part of 1419.7 with those published for Stichloroside C_1 from *Stichopus chloronotus* (Kitagawa *et al.* 1981a) demonstrated that the aglycone of the two compounds were almost identical, suggesting the presence of double bond at $\triangle^7$. However there was no acetoxy group in the C-23 position. This compound is the dihydro- analogue of the ion 1417.7.

6.6 Saponins distribution and diversity

The major triterpene glycosides from the viscera of *S. hermanni* (Stichopodidae, Aspidochirotida), were detected at *m/z* 1435.7, 1419.7, 1477.7, 1475.7, 1243.5, 1461.7, 1417.7, 1433.7, 1459.7, 1305.5 and 1307.5. In contrast to the major compounds detected in the *H. lessoni*, these compounds were non-sulphated, but mostly acetylated glycosides. These glycosides were very similar to glycosides of *Stichopus chloronotus* (Kitagawa *et al.* 1981a) and all other known glycosides from stichopodids, even though some were found to be identical between these species. Our results revealed that all major saponins isolated from *S. hermanni* possess hexasaccharides in their glycone moieties. This conclusion was consistence with published data on glycosides of the family Stichopodidae (Moraes, Greta *et al.* 2004). This species mostly produced triterpenoic oligoglycosides lacking a sulphate group in their sugar units. In fact having a

hexasaccharide chain increased the hydrophilic propriety of saponin congeners proposing they could be released in water surrounding sea cucumbers. The configurations of all sugar units in all previously known sea cucumber triterpene glycosides were D-configurations. Most of reported saponins from this species contained a holostane aglycone and characterised by the presence of a 7(8) double bond.

6.7 Major triterpene glycosides

The most abundant glycoside component from the viscera of sea cucumber *S. hermanni* was identified as the ion at *m/z* 1435. The positive ion mode ESI-MS exhibited an ion peak at *m/z* 1435 $[M + Na]^+$ which had a peak at *m/z* 1411 $[M-H]^-$ in the negative ion mode ESI-MS. Positive ion mode ESI-MS/MS of this ion from Fraction 146 is shown in Figure 6. 10. The positive ion mode ESI-MS^2 corroborated the MALDI-MS^2 data. Thus our finding revealed that this triterpene glycoside constructed of six sugar resides. The key diagnostic peak at *m/z* 511 corresponded to [MeGlc-Glc-Xyl-H_2O+Na]$^+$. The ion at *m/z* 981 corresponded to sodiated hydrated entire sugar chain. The MS^2 fingerprints of carbohydrate moieties were coincident with those of Stichlorosides B_1 and B_2 previously reported from *S. chloronotus*.

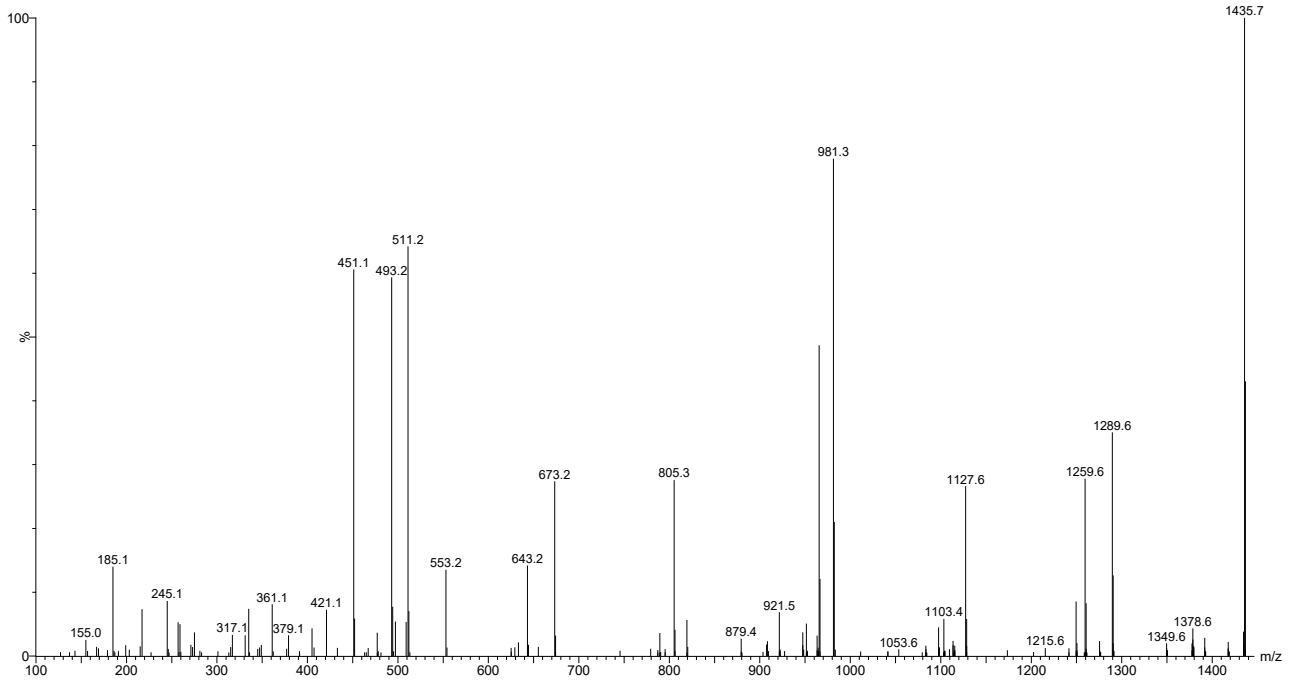

Figure 6.10. (+) ESI-MS^2 fragmentation pattern of ion detected at *m/z* 1435, the major saponin in the viscera of *S. hermanni*.
This hexaoside triterpene glycoside had identical sugar chain to those of Stichlorosides B_1 and B_2.

The aglycone of this triterpene glycoside was coincident with those of desacetylstichlorosides B_1 reported in the *S. chloronotus* (Kitagawa *et al.* 1981a), bearing a C-23 hydroxy group and a $\triangle^7$ double bond. The molecular formula of this compound was established as $C_{66}H_{108}O_{32}$ from the ion peak at *m/z* 1435 $[M + Na]^+$ in the positive ion mode ESI-MS and at *m/z* 1411 $[M - H]^+$ in the negative ion mode ESI-MS. Our results indicated the presence of an isomeric structure for this ions of which we proposed the names Hermanniosides C and E.

Wang *et al.* (2014) described the structures of Variegatusides E and F isolated from *S. variegatus* having identical molecular formulae and weights (1435 $[M + Na]^+$), varying from each other in the position of double bond in their aglycones. However, Variegatuside F had an identical structure with those reported for desacetylstichloroside B_1 isolated from the body wall of *Stichopus chloronotus* (Kitagawa *et al.* 1981a). Similar to those compounds reported in *S. chloronotus* by Kitagawa *et al.* (1981), these glycosides had no sulphate group in their sugar moieties.

The sugar moiety of Variegatuside E was reported to be identical to that of desacetylstichloroside B_1, while this group showed different ratio of monosaccharide residues for Variegatuside E compared to those reported in desacetylstichloroside B_1 (Wang, X-H *et al.* 2014). In addition, they

ascribed the presence of an olefinic bond at C9(11) position, whereas desacetystichloroside B_1 had been shown to have a C7(8) double bond instead (Kitagawa *et al.* 1981a).

We have also identified the ion at *m/z* 1433.7 among the major peaks. Our analysis revealed that this ion was dehydro- analogue of the ion at *m/z* 1435.7. This showed the identity of the sugar moieties of two saponins. However they differed from each other in their aglycone parts. Ion at 1433 had an extra double bond in $\triangle^{7\ (25)}$. The ESI-MS^2 fragmentation profile of this ion from Fraction is illustrated in Figure 6.11. The major peak at 1377 was generated by the loss of 56 u due to the cleavage of lateral side from the parent corresponding to C_4H_8 indicating the presence of a double bond in the side chain of aglycone.

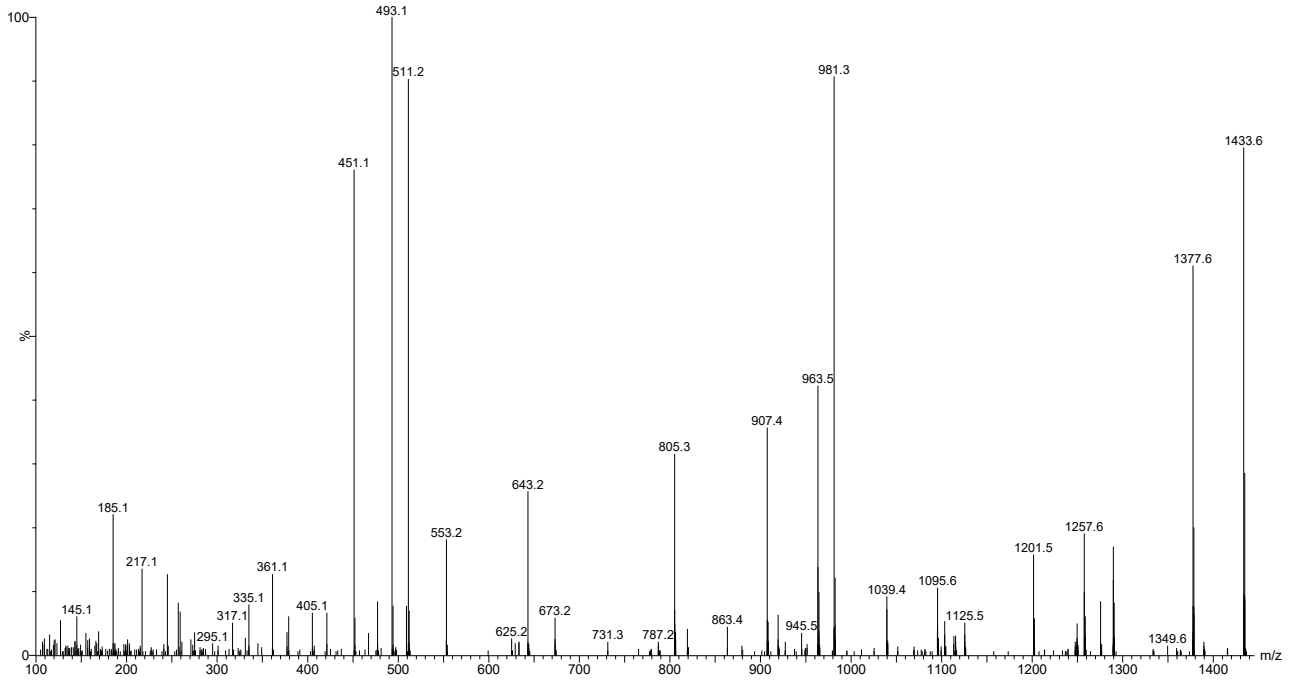

Figure 6.11. CID fragmentation profile of the isomeric ion observed at *m/z* 1433 in the positive ion mode of ESI.

The structures of Parvimosides A and B, isolated from the body wall of the sea cucumber *Stichopus parvimensis* (syn. *Parastichopus parvimensis*), were described by Iniguez-Martinez *et al.* (2005). They reported *m/z* values of 1410 ($C_{66}H_{106}O_{32}$) and 1380 ($C_{65}H_{104}O_{31}$) for Parvimosides A and B, respectively. Our analysis also revealed the occurrence of two peaks with identical *m/z* values of Parvimosides A and B. Even though the ion at *m/z* 1433 had the same molecular weight as Parvimoside A, their structures were found to be different for which we proposed the names

Hermaniosides D and F. Nevertheless one of the isomers was found to be as Parvimosides A. These glycosides were hexaosides like most of the major glycoside congeners from this family. For instance, the major glycosides from the *S. chloronotus* (Kitagawa *et al.* 1981a; Stonik, VA *et al.* 1982b), *S. variegatus* (Stonik, VA *et al.* 1982b) and *Astichopus multifidus* (Stonik, VA *et al.* 1982b) had the same structures of the carbohydrate moiety.

This group suggested that the structures of Parvimosides A and B were similar to Holotoxins B (Kitagawa *et al.* 1978b) and B_1 (Maltsev *et al.* 1984) respectively, isolated from *S. japonicus* with the difference being the absence of a double bond at C-25 position in their aglycones (Iniguez-Martinez *et al.* 2005). In fact these glycosides are dihydroholotoxins B and B_1, respectively. Parvimoside B had a similar structure to Parvimoside A, except the third sugar resides in the main chain is Xyl instead of Glc.

In addition, Holothurinosides F and F_1 (Van Dyck *et al.* 2009) were also found to have identical mass with this ion. However this ion had different structure compared to those, regardless of having the same molecular formula, described for Holothurinosides F and F_1. The structure of one of the isomers was identical with Bivittoside C (Kitigawa *et al.* 1981).

6.8 Common saponins

Elyakov *et al*. (1975) have clearly stated the specificity of glycoside from more than 40 species of sea cucumbers belong to the order Aspidochirotida (Elyakov *et al.* 1975; Elyakov *et al.* 1973). The specificity of triterpene glycosides for species, and genera even at the supergenus level of sea cucumbers is consistent (Moraes, Greta *et al.* 2004). Our analysis revealed the presence of several common saponins such as ions at 1415, 1417, 1459, 1461, 1475, and 1477 in this species which were corresponded to acetylated and non-acetylated compounds. Most of them were hexaosides bearing a $\triangle 7^{(8)}$ double bond. Some of these glycosides were found extensively in this family. Here we described the structure elucidation of some of these glycosides.

As was pointed out earlier in this chapter; the ion detected at *m/z* 1461.7 was among the major saponin congeners identified in this species. Our analysis revealed that this ion was not a single

compound. Indeed, this ion contained isomeric compounds. The efficient HPCPC purification technique applied in this study was able to isolate these isomeric compounds. For example the ion at *m/z* 1461.7 was detected in a few fractions such as Fractions 41 and 149. Our analysis discriminated between the structures of compounds associated with this ion in different fractions. As a typical example the ESI-MS/MS fragmentation pattern of the ion at *m/z* 1461.7 in the positive ion mode from Fraction 41 is shown in Figure 6.12. The consecutive MS experiment is discussed in detail below for stepwise the molecular structure elucidation of these compounds.

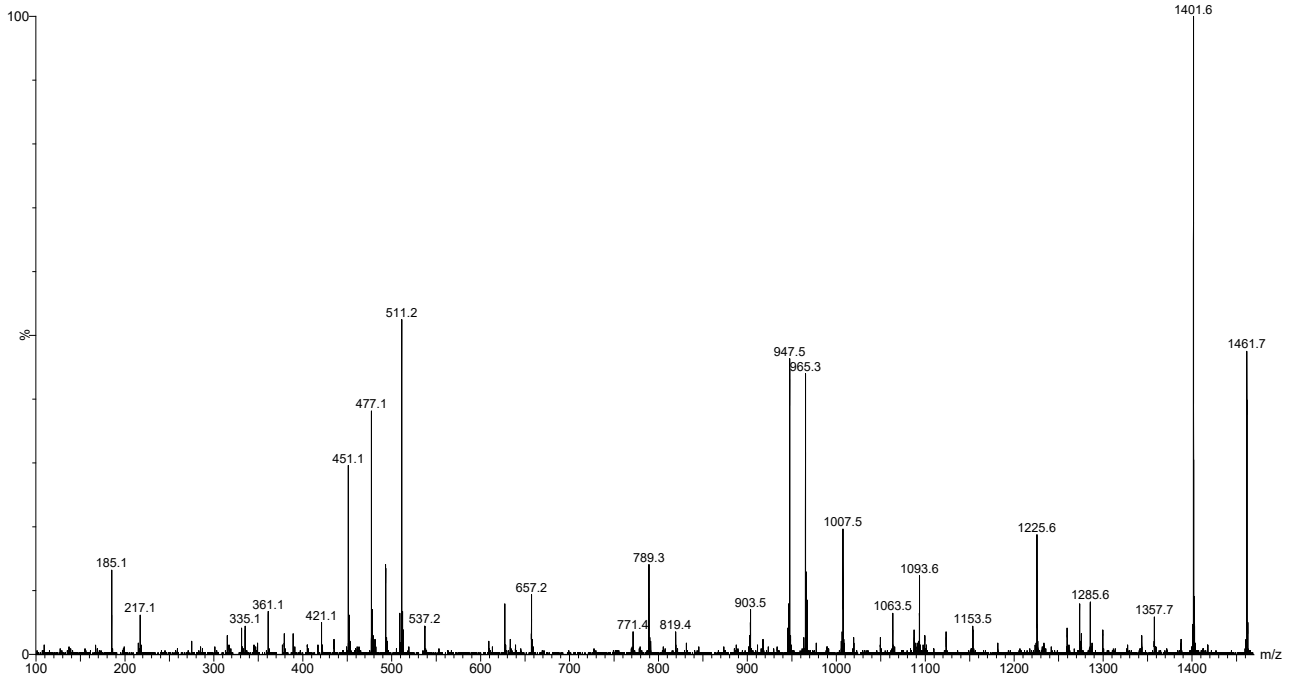

Figure 6.12. (+) ESI-MS/MS spectrum of the *m/z* 1461 ions observed from Fraction 41.
The peaks at *m/z* 477 and 537 corresponded to the sodiated deacetylated aglycone [DeAc Agl +Na]$^+$ and sodiated aglycone unit [Agl +Na]$^+$, respectively.

The procedure to elucidate the structure of saponin was described in detail in previous chapters. Briefly, the sequential losses of the deacetylated aglycone (DeAc Agl), acetic acid (AcOH), MeGlc, Xyl, Qui, Xyl and MeGlc residues followed by Glc afforded ion fragments at *m/z* 1007.5, 947.5, 771.4, 639.2, 493.1, 361.1 and 185.1, respectively. This successive of fragmentations demarcated the structure of compound to be identical with Stichloroside C_1 reported from *S. chloronotus* by Kitagawa *et al.* (1981a). Alternatively the consecutive losses of the AcOH and the DeAc Agl followed by the sequential losses of the sugar residues further corroborated the structure of Stichloroside C_1 in which was found to be a common saponin within species of the *Stichopus*

genus.

CID analysis revealed the presence of an acetoxy group in the structure of this compound. The prominent peak at 1401.7 was generated by the loss of a molecule of acetic acid corresponding to the presence of an acetoxy group (Bahrami & Franco 2015). The presence of acetoxy group was corroborated by the loss of an acetic acid molecule. Further, the major peak at *m/z* 477.1 corresponded to either the [DeAc Agl + Na]$^+$ or the key diagnostic ion [MeGlc-Xyl-Qui + Na]$^+$ (indeed it depends on which of the three independent fragmentation pathways first triggered), whereas the *m/z* 493 ion corresponded to the key sugar residues. This study was the first to describe the MS/MS spectra of Stichloroside C_1 and assigned its fragmentation pattern and confirmed the structure of these saponin congeners.

This spectrum showed the present of isomeric compound; however, in order to simplify it the structure of Stichloroside C_1 was only described here. The structure of other unidentified isomers which required further analysis will be published later.

A similar ion (1461.7) was also detected in the Fraction 149. Tandem mass spectrometry analysis of this ion demonstrated a different fragmentation profile compared to that of Stichloroside C_1. The ESI-MS^2 of this ion is shown in Figure 6.13.

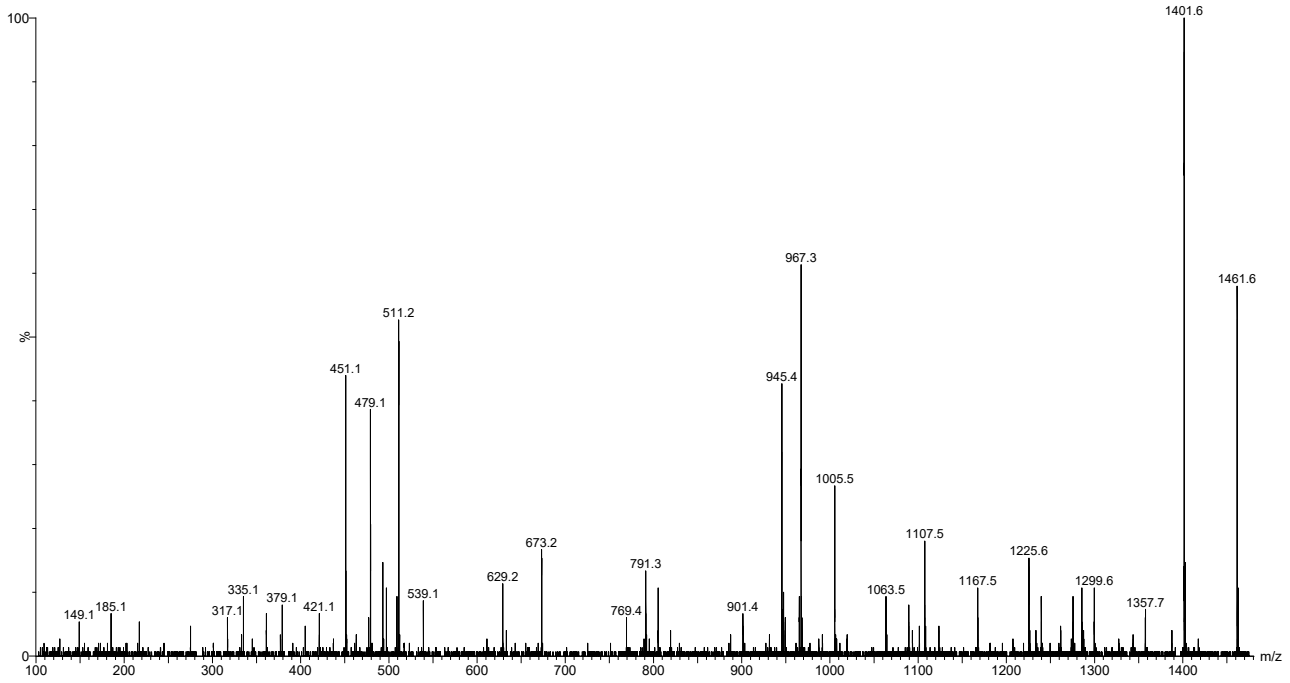

Figure 6.13. (+) ESI-MS/MS spectrum of the isomeric ion at *m/z* 1461.7 detected from Fraction 149. The major peak at *m/z* 479 and 539 corresponded to the nitrated DeAc AgI and nitrated AgI residues, respectively.

This analysis showed the presence of 2 extra hydrogen atoms in the aglycone of this novel acetylated compound compared to that of Stichloroside C_1 as the major peak at *m/z* 945.5 was corresponded to the loss of AgI of which indicated the mass of entire sugar chain. The ions at 479 and 539 were generated by the losses of nitrated DeAc AgI and nitrated AgI residues, respectively.

It is clear from the spectrum the sugar moiety of this hexasaccharide also varied from the sugar chain of Stichloroside C_1 even though both of them contained ions at 493 and 511 which corresponded to the diagnostic peaks.

The ion at *m/z* 1459 was also detected in Fraction 41. The positive ion mode ESI-MS/MS spectrum of the isomeric ion at *m/z* 1459 is illustrated in Figure 6. 14. Our analysis revealed that this compound is an acetylated compound bearing a hexasaccharide chain. The ion at *m/z* 1399.7 corresponded to the loss of an acetic acid.

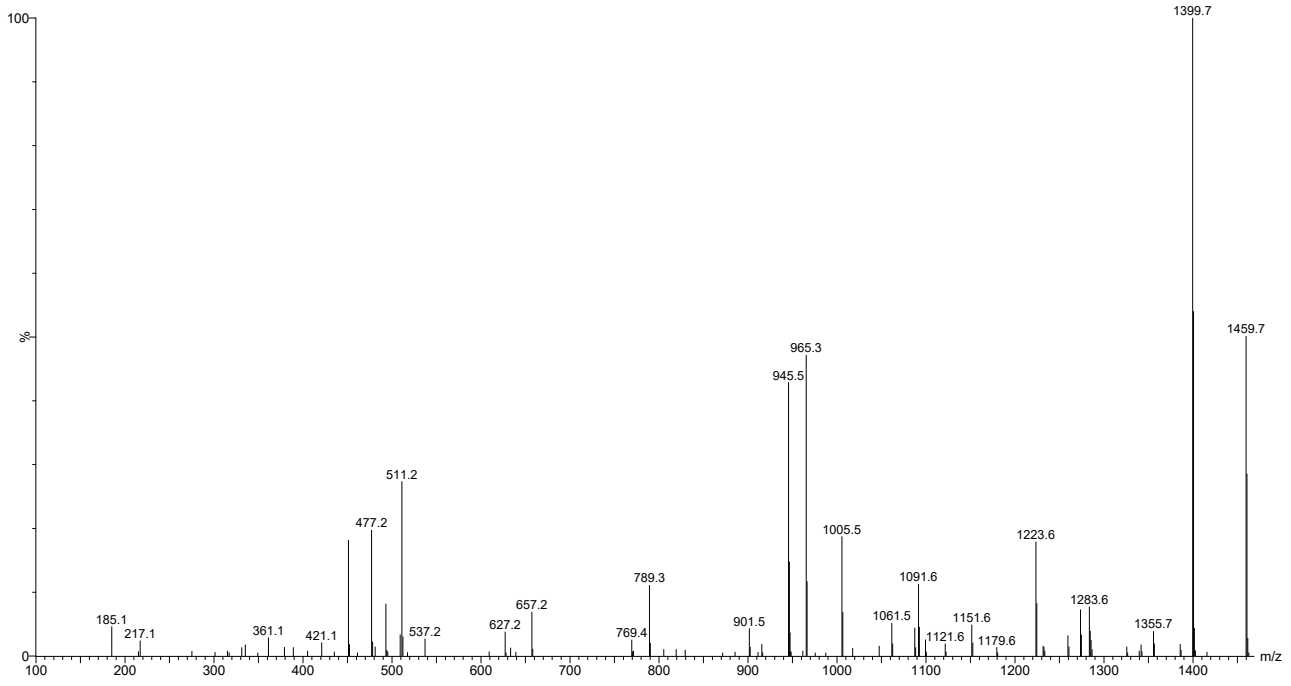

Figure 6.14. CID Fragmentation pattern of the ion detected at *m/z* 1459 in the positive ion mode ESI-MS^2.

The MS^2 fingerprints of carbohydrate moieties were coincident with those of Stichloroside C_2 previously reported from *S. chloronotus (Kitagawa et al. 1981a)*. Comparison between the aglycone moieties revealed that the ion at *m/z* 1459 differed from that of 1461 by the presence of an additional 25(26)-double bond. The aglycone part of this glycoside was coincident with those for the Stichloroside C_2 isolated from *S. chloronotus* with holostane type aglycone having 23-OAc,7(8)-25(26)-double bonds (Kitagawa *et al.* 1981a; Kitagawa *et al.* 1981b).

Another example of these common glycosides was the ion detected at *m/z* 1415.7 in the positive ion mode. The MS/MS spectrum of this glycoside is shown in Figure 6. 15. The major ion peak at *m/z* 961.5 produced by the loss of aglycone which corresponded to the whole sodiated sugar residues.

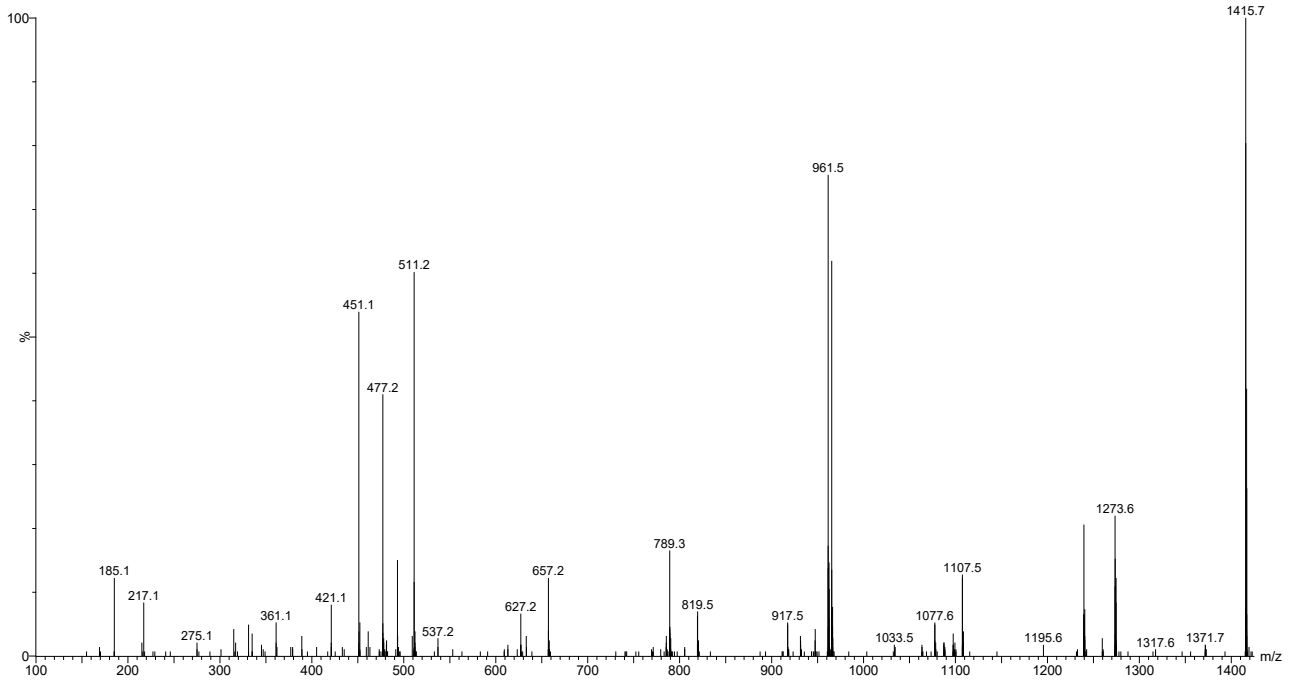

Figure 6.15. (+) ESI-MS/MS of the ion at *m/z* 1415.7. This analysis revealed the structure of Holotoxin A_1.

Mass spectrometry analysis indicated that the sugar part of this glycoside composed of six sugar residues which was identical with those reported for Holotoxin A_1 from *S. japonicus* (Maltsev *et al.* 1984) and Stichlorosides C_1/C_2 with a holostane type aglycone bearing double bonds at C-9 and C-25 positions (Kitagawa *et al.* 1978b; Maltsev *et al.* 1984).

Another common saponin was identified at *m/z* 1449.7 alongside the ion at 1447.7. Chemical analysis by tandem mass spectrometry of these ions revealed that both contained the key diagnostic peak at 507.2. By applying the same procedure the structure of the ion at *m/z* 1447 was found to be coincidence with Impatienside A previously reported from *Holothuria impatiens* (Sun, P *et al.* 2007). The positive ion mode ESI-MS^2 fragmentation of ion 1449.7 in Fraction 146 is shown in Figure 6.16.

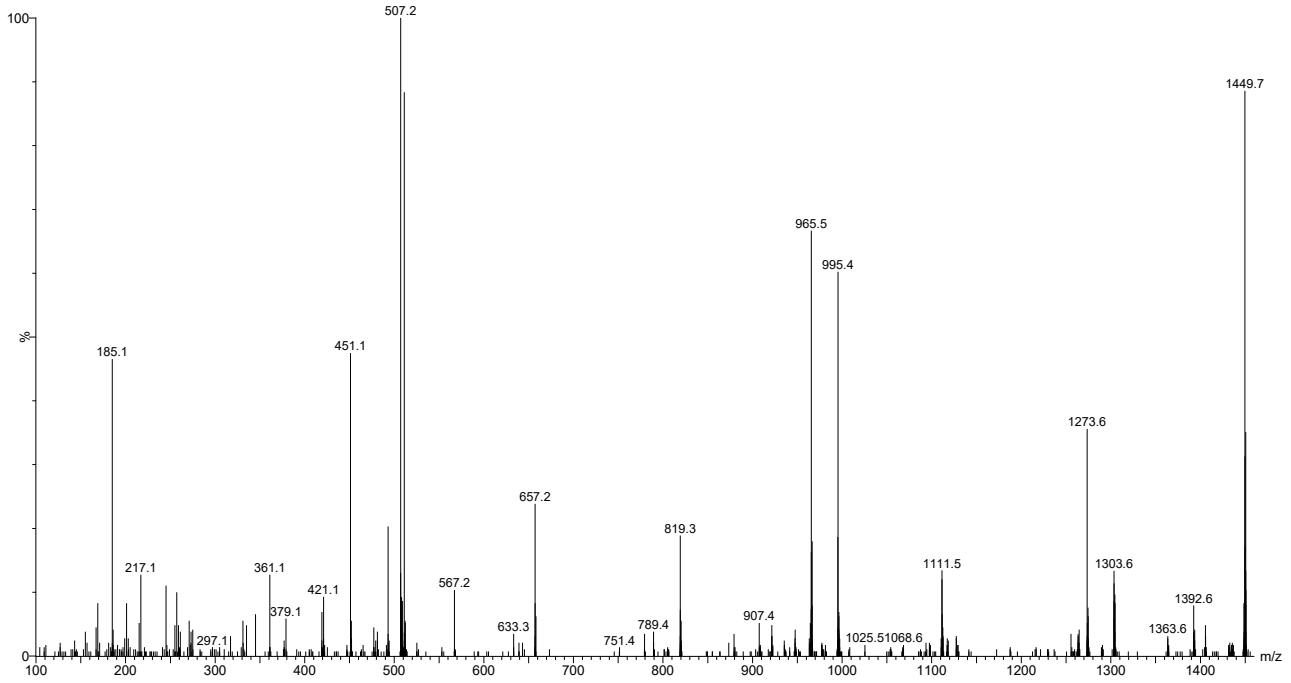

Figure 6.16. ESI-MS/MS of the ion at *m/z* 1449 in the positive ion mode. The peak at *m/z* 507 corresponded to $[MeGlc\text{-}Glc\text{-}Qui + Na]^+$.

This data revealed that the major ion at *m/z* 965.5 was generated by the sequential losses of MeGlc-Glc, and Qui. This saponin contained both 493 and 507 key diagnostic peaks. This hexaosides glycoside had the same sugar units as those reported for Bivittoside D from *Bohadschia bivittata* (Kitigawa *et al.* 1981). Our finding confirmed that this ion corresponded to Bivittoside D.

The structure of Holothurinosides G and G1 were described by Van Dyck *et al.* (2009) having the same molecular weight as Bivittoside D. In addition, Wang, Z *et al.* (2012a) also isolated a compound with *m/z* value of 1449.6 in the positive ion mode $[M+Na]^+$, known as 25,26-Dihyroxy-holotoxin A_1 from *S. japonicus*, having a molecular formula of $C_{66}H_{106}O_{33}$. Comparison of the MS/MS data of these compounds with Bivittoside D showed that they had different structures.

6.9 Unique saponins

While many identified saponin congeners were reported in other species, several saponins were specifically found in this species. Our analysis revealed the structure of several novel unique saponin congeners such as ions detected at *m/z* 1241, 1243 and 1405 from Fraction 149. This section will describe the structure elucidation of these glycosides as representative in details.

The positive ion mode ESI-MS/MS spectrum of the ion at *m/z* 1243 is illustrated in Figure 6. 17. This figure demonstrated two major peaks at 511.2 and 789.3 corresponding to the key diagnostic sugar moiety [MeGlc-Glc-Xyl-H_2O+Na]$^+$, and entire sodiated sugar residues, respectively.

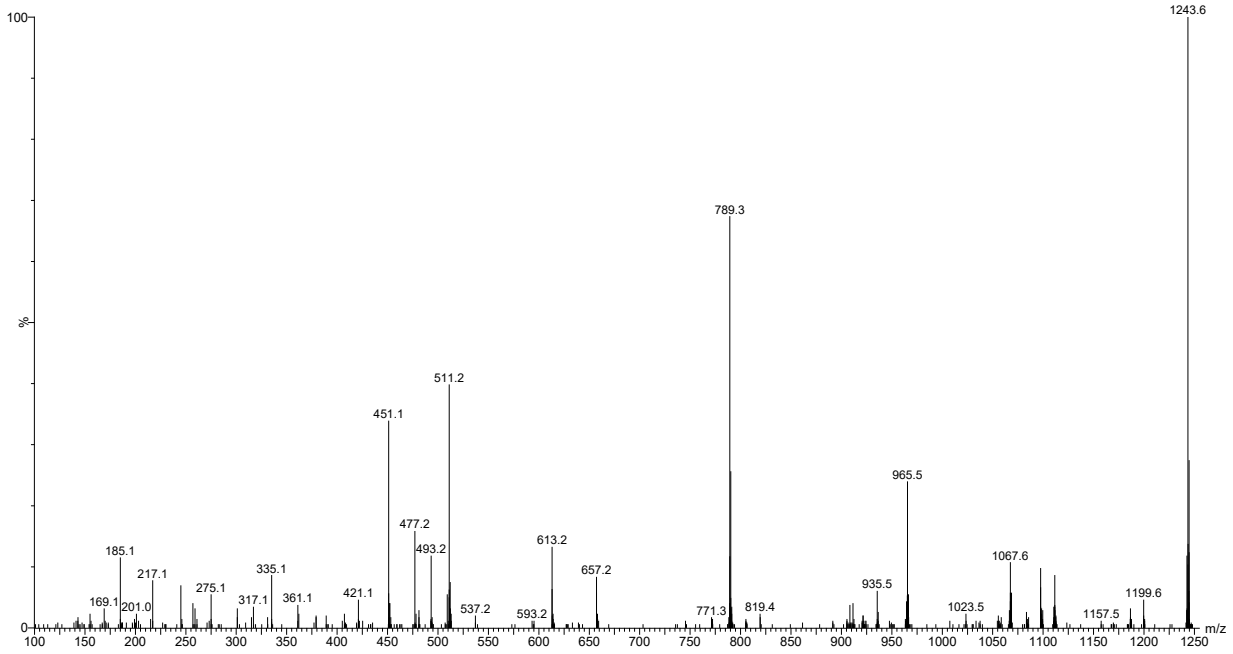

Figure 6.17. Tandem MS fingerprints of the ion at *m/z* 1243.6.
The dominant peak at 789.3 corresponded to the entire sugar residue which generated by the loss of the Agl.

Our analysis distinguished the structural differences between this compound and that of Holothurin A regardless of showing identical molecular mass. It is noteworthy that Holothurin A was found in Fraction 87. In contrast to Holothurin A, this non-sulphated compound comprised of five sugar residues contacting Xyl:Glc:Qui:MeGlc in a 2:1:1:1 ratio. In addition, MS/MS spectrum of this non-acetylated glycoside displayed ions at *m/z* 493 corresponding to the key diagnostic peak [MeGlc-Glc-Xyl+Na]$^+$. Whereas Holothurin A exhibited the ion at *m/z* 507 as a signal of [MeGlc-Glc-Qui+Na]$^+$. The aglycone moiety of this glycoside was coincident with those for Hermannioside A (1417.7) having a $\triangle^{7(8)}$ double bond.

The positive ion mode ESI-M/MS spectrum of the ion observed at 1241.6 was demonstrated in Figure 6.18. Based on mass spectrometry analysis this compound consisted of five sugar components identical with those of ion at 1243.6. The mass discrepancy between these two compounds indicating that the ion at *m/z* 1241 was dehydro analogue of the ion at 1243. Indeed,

this glycoside had two double bonds at $\triangle^{7(25)}$.

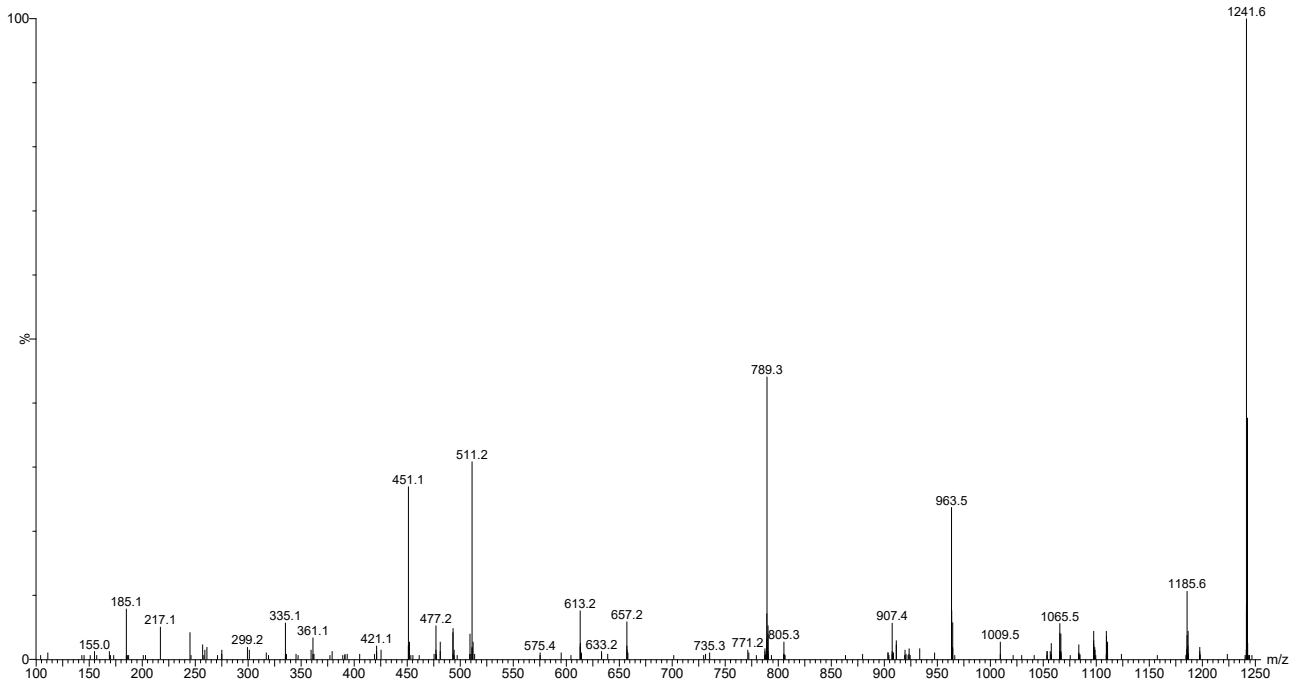

Figure 6.18. (+) ion mode ESI-MS/MS of the ion at 1241.6 from Fraction 149.
The prominent peaks at *m/z* 511 and 789 corresponded to the key diagnostic peak and entire sodiated sugar chain, respectively.

Figure 6.19 illustrated the structure of another novel compound detected at *m/z* 1405.7 from the same Fraction. The positive ion mode ESI-MS/MS spectrum displayed a major ion peak at 951.3 corresponding to the entire sodiated sugar moiety. This ion was also an isomeric compound. Our analysis revealed this glycoside was a hexasaccharide triterpene glycoside comprising of Xyl:Glc:MeGlc in a 3:1:2 ratio. The key diagnostic peaks at 463.1 and 511.2 corresponded to $[MeGlc\text{-}Xyl\text{-}Xyl+Na]^+$ and $[MeGlc\text{-}Glc\text{-}Xyl+Na]^+$, respectively.

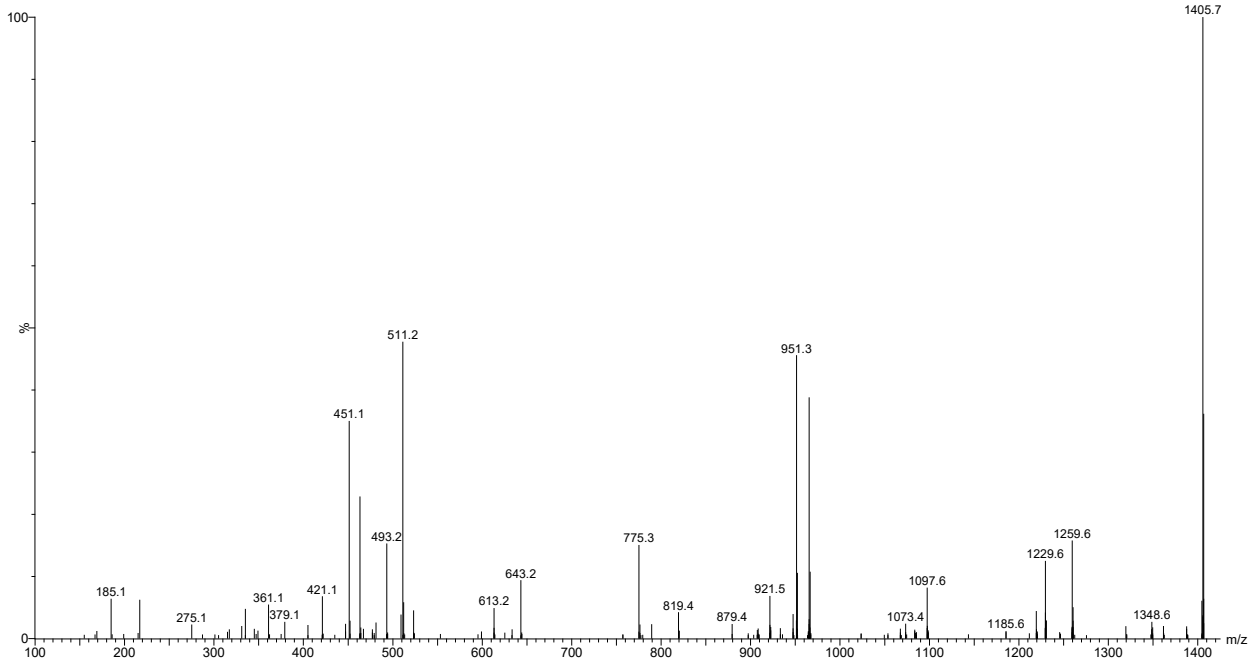

Figure 6.19. (+) ESI-MS^2 fragmentation of the ion observed at *m/z* 1405 in Fraction 149. CID generated the ion at *m/z* 477.2 corresponding to the sodiated Agl.

MS/MS analysis showed a similar aglycone with those in desacetylstichloroside C_1, bearing a double bond at C-7. Thus the mass spectrometry analysis determined the structure and molecular formula as $C_{65}H_{106}O_{31}$.

Another unique saponin was associated with the ion detected at *m/z* 1113.5 in Fraction 146. The positive ion mode ESI-MS^2 of this ion is shown in Figure 6. 20.

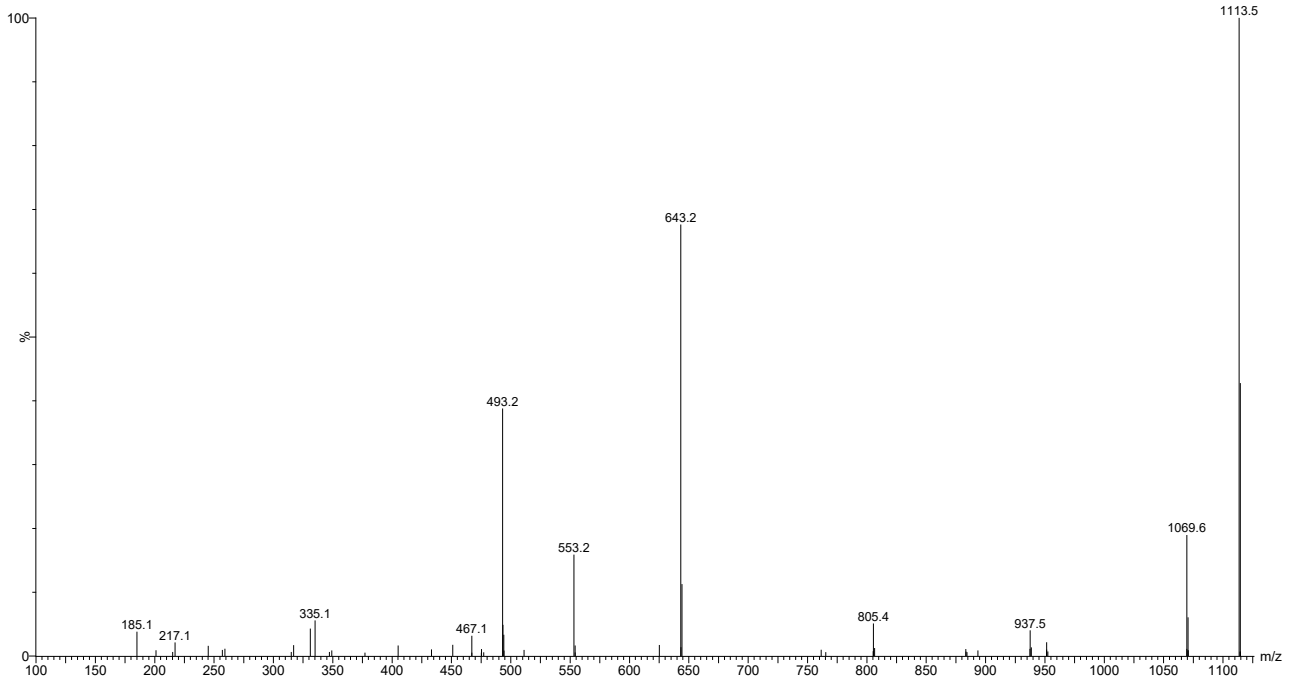

Figure 6.20. CID fingerprint of the ion observed at *m/z* 1113 in the ESI positive ion mode.
The abundant peak at 643 corresponded to the entire sugar residue.

The assignments of daughter ions have been conducted. The ion at *m/z* 643 stemmed from the parent ions by the loss of the aglycone which corresponded to the entire sugar moiety. The sequential losses of the aglycone, Xyl, MeGlc, Xyl followed by Glc yielded ion at 643, 493, 317 and 185, respectively. Alternatively, the consecutive elimination of MeGlc, Xyl, Glc and Xyl generated product ion were detected at 937, 805, 643 and 493. All of this analysis indicated that this glycoside comprised of a linear tetrasaccharide including Xyl, Glc and MeGlc in a 2:1:1 ratio. The major peak at *m/z* 1069 corresponded to the loss of CO_2.The aglycone of this compound was found to be identical with those reported to ion at *m/z* 1259. If fact the structure of this glycoside were the same as the ion at *m/z* 1259, except lacking the Qui residue in sugar residue.

6.10 Composition of glycoside fractions

As TLC and MALDI profiles have shown, major components were detected at around 1400 Da. They were mainly hexasaccharides. The most abundant glycoside was the ion at *m/z* 1435.

Moraes *et al.* (2004) categorised representatives of the family Stichopodidae into two groups on the basis of their glycoside compositions. The first group included species having Stichoposides and Thelenotosides and comprise *S. hermanni*, *S. variegatus, S. chloronotus*, *Astichopus*

multifidus, *T. ananas* and *T. anax*. The second one included species producing Holotoxins and contains *P. californicus* and *Apostichopus japonicus*. Nonetheless, our analysis could not confirm this observation as some common saponins such as Holotoxin A_1 were found in the viscera of *S. hermanni*. Further the major triterpene glycosides apparently were common among the species belonging to these genera. In other words no distinction was seen between these two categories.

6.11 Acetylated saponins

One of the modifications occurred in the structure of saponins is the addition of an acetoxy group to the aglycone moiety. The aglycone part of some of saponins identified from Stichopodidae family is characterised by the presence of a 7(8)-double bond in the holostane nucleus with a 23-acetoxy group. For instances, Stichoposides C, D, E, Stichlorosides A_1, B_1 and C_1 which were found in *Thelenota anax* and *Thelenota ananas* (Kobayashi et al. 1991). In this section we discussed the structure elucidation of two acetylated saponins, namely ions detected at *m/z* 1447.7 and 1475.7.

An ion with *m/z* value of 1447.7 was identified in Fraction 45. The positive ion mode ESI-MS/MS spectrum of this ion is shown in Figure 6.21. The most abundant fragment ion at *m/z* 1387.7 was generated by the loss of the acetoxy group from the aglycone. The aglycone of this new saponin was very similar to the aglycone of Stichlorosides A_1 having an acetoxy group at C-23 and a $\triangle^7$ double bond. Our analysis revealed that this glycoside comprised of six sugar residues including Xyl:Glc:MeGlc in the 3:1:2 ratio, respectively. The ion at *m/z* 951 corresponded to the entire sugar residue.

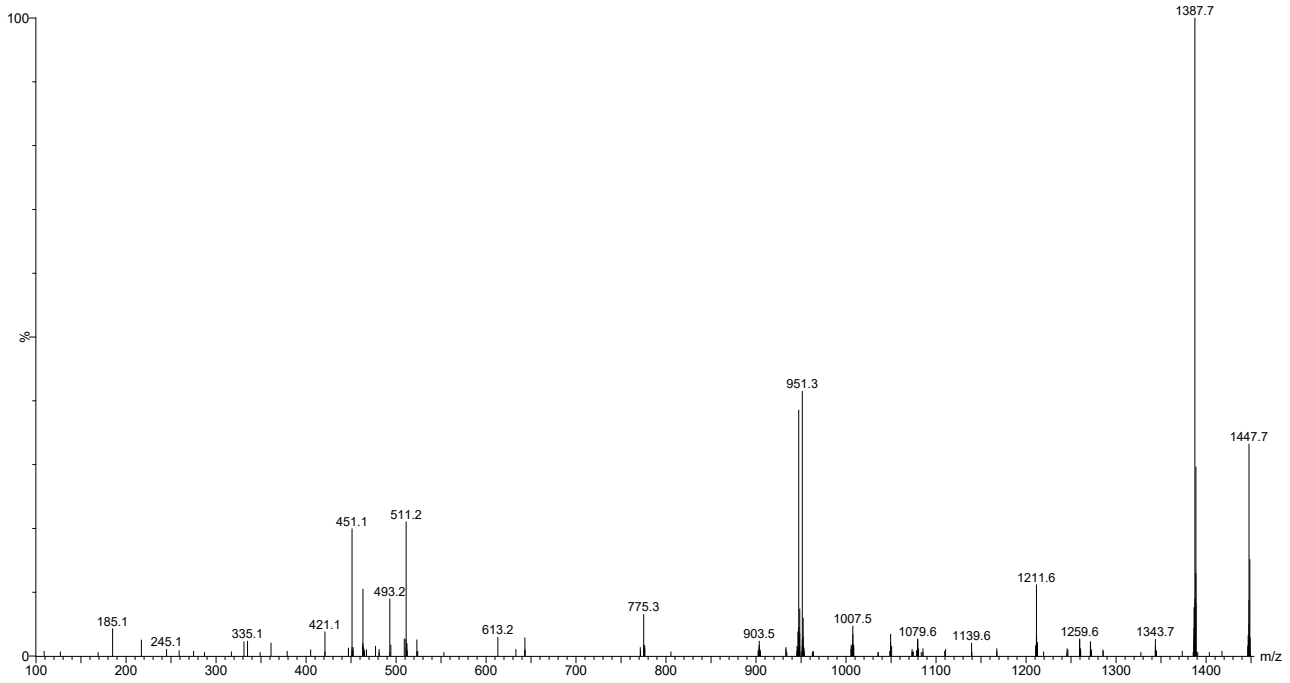

Figure 6.21. CID fragmentation profile of the ion at *m/z* 1447 from Fraction 45 in the positive mode of ESI.

Impatienside A (Sun, P *et al.* 2007) and (Marmoratoside A) (Yuan *et al.* 2009b) had also reported to have the same mass as the ion detected at *m/z* 1447 (which was also found in this species). However the structure of this acetylated glycoside was found to be different from those of Impatienside A (Marmoratoside A). As can be seen in the spectrum this novel compound contained the key diagnostic peaks at *m/z* 493 and 511, whereas Impatienside A was described to possess the ion at *m/z* 507 corresponding to $[MeGlc\text{-}Glc\text{-}Qui+Na]^+$ which was not seen in this new compound. Further this saponin had an acetoxy group which was absent in those glycosides.

Another example of acetylated saponin was the ion detected at *m/z* 1475.7. This ion was seen as a pair with the ion at *m/z* 1477.7. Our analysis revealed the presence of different isomers for this ion in different fractions. It is notable that we also identified different isomers associated with the ion at *m/z* 1477.7 in this species. As typical example, the ESI-MS^2 spectrum of the ion at 1475 in the positive ion mode from Fraction 66 is shown In Figure 6.22.

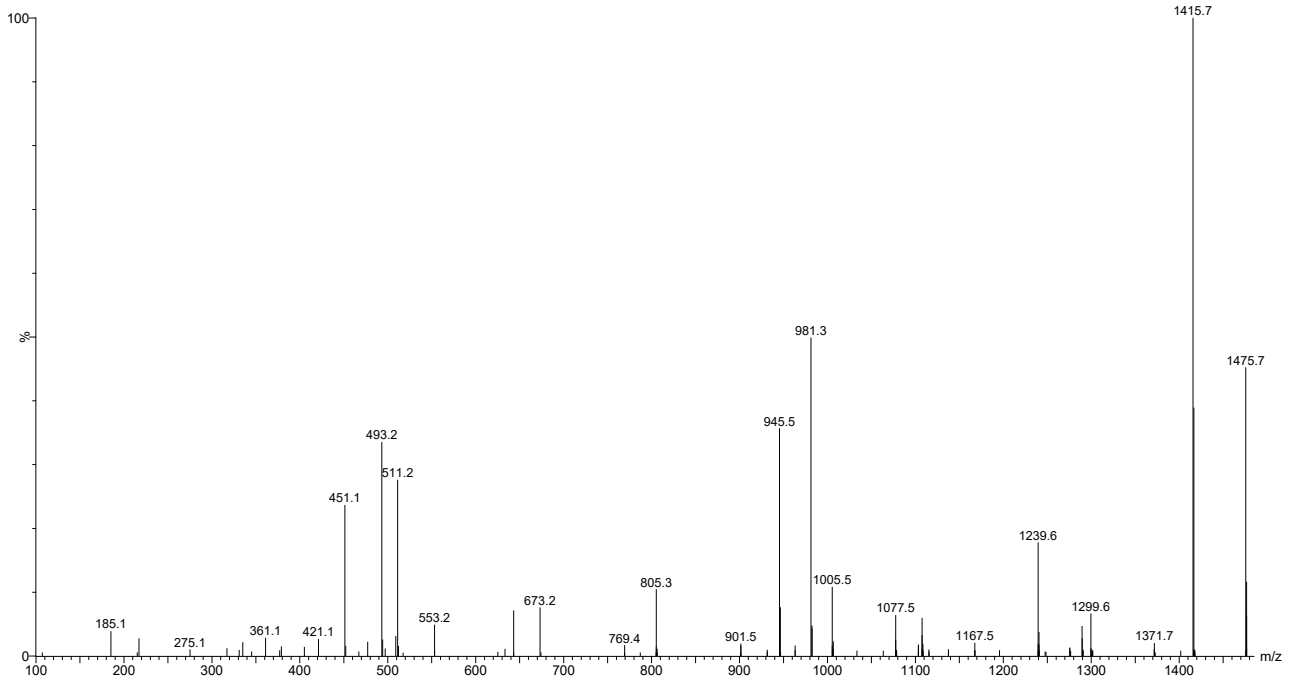

Figure 6.22. CID fingerprint of the ion at *m/z* 1475 using ESI in the positive ion mode from Fraction 66.

The presence of an acetoxy group was corroborated by the mass transition of 60 u corresponding to the loss of an acetic acid which generated the ion at 1415.7. The side chain of aglycone was thus comparable to that of dehydrostichlorogenol (Kitagawa *et al.* 1981a), a C-23-acetyoxy aglycone from the sea cucumber *S.* chloronotus in which had a $\triangle^7$ double bond.

The presence and sequence of six monosaccharide residues in the sugar moiety of the glycoside were deduced from MS/MS spectrum. The sugar moiety was identified as Xyl, Glc, Qui and MeGlc in a ratio of 2:1:1:2. The mass analysis corroborated that the sugar moieties of this glycoside were coincident with those of Stichloroside C_1 from *S. chloronotus* (Kitagawa *et al.* 1981a), indicating that these two triterpene glycoside had the same sugar chain (structure).

Stichlorosides C_2 and B_2 were also found in *S. variegatus* along with Stichoposides C and D (their 25(26)-dehydro derivatives) (Stonik, VA *et al.* 1982b; Stonik, VA *et al.* 1982c). Astichoposide C (Stichlorosides C_2) was found as the prominent glycoside in *Astichopus multifidus* (Stonik, VA *et al.* 1982b). Stonik, VA *et al.* (1982a) also stated Stichoposide C and its 25(26)-dehydro analogue in *T. ananas.* Stichlorosides C_1 and B_1 (Stichoposides C and D, respectively) were also reported in *S. chloronotus* (Stonik, VA *et al.* 1982b; Stonik, VA *et al.* 1982c).

Our analysis revealed the structure of Stichoposides A_1 and C in *S. hermanni*. Stichloroside A_1 (Stichoposide E) is a hexasaccharide with an aglycone identical to the aglycones of other Stichoposides and Thelenotosides. Stichloroside A_1 and its 25(26)-dehydro analogue (Stichloroside A_2) were also found in *S. chloronotous* and *S. variegatus*, respectively (Maltsev *et al.* 1983). Astichoposide C and Stichoposide C were isolated from *Astichopus multifidus* and *S. chloronotous*, respectively (Stonik, VA *et al.* 1982b). These hexaosides possess the same sugar moieties comprising of Glc:Xyl:Qui:MeGlc in a ratio of 1:2:1:2

Kitagawa *et al.* (1981a) have reported Stichlorosides A_1, B_1 and C_1 along with their 25(26)- dehydro derivatives from *S. chloronotus* specimens collected from the Japanese coast. It is notable that the same species collected off the Great Barrier Reef of Australia had of these glycosides without their 25(26)-dehydro analogues (Stonik, VA *et al.* 1982b; Stonik, VA *et al.* 1982c). The presence of Stichlorosides A_1, A_2, B_1 B_2, C_1 and C_2 have been also reported in the body wall of *S. hermanni* and *T. ananas* collected off Okinawa by Kitagawa *et al*. (Kobayashi *et al.* 1991). Stichlorosides C_1 (Stichoposide C), B_1 (Stichoposide D) and A_1 (Stichoposide E) were identified in *Thelenota anax* (Kobayashi *et al.* 1991).

Glycosides isolated from sea cucumber *H. lessoni* were significantly different from those found in *S. hermanni* such as Stichlorosides, Stichoposides and Thelenotosides, even though there were a few common saponins such as Lessoniosides, which are acetylated compounds similar to those found in the *S. hermanni*. The aglycones of saponins in *H. lessoni* possess a double bond at position 9(11), while those reported in *S. hermanni* mostly have a 7(8) double bond. Most of earlier reported glycosides from *Stichopus* possess a 23-acetoxy group in their aglycones while it was not reported in the aglycones of many saponins found in *Holothuria*. Most of triterpene glycosides identified in *S. hermanni* such as Stichoposides lack a sulphate group.

Stichoposide D was isolated from *S. chloronotus* and *S. variegatus* collected off the Great Barrier Reef by Stonik *et al*. (1982c), which is a hexasaccharide glycoside comprising of Xyl:Glc:MeGlc in a ratio of 2:2:2. It differs from Stichoposide C in sugar residues: it has a Glc instead of Qui. However the authors noticed the mixture of Stichoposide D and its $\triangle^{25}$-dihydro derivative in *S.*

variegatus (Stonik, VA *et al.* 1982c), which was also found in *S. hermanni*.

Stichoposide E was reported from *S. chloronotus* collected from the Great Barrier Reef as triterpene hexaosides containing Xyl:Glc:MeGlc in ratio of 2:2:2, which like other Stichoposides from this species had an aglycone with 23-acetoxy-holost- 7-en-3β-ol; identical with those found in Stichoposides C and D (Maltsev *et al.* 1983). On the basis of these facts, molecular formula for this compound has been ascribed to stichoposide E ($C_{68}H_{110}O_{33}$). Our analysis revealed the presence of most of described saponins in the viscera of *S. hermanni*.

6.12 Sulphated and non-sulphated saponin congeners

Although there were some common sulphated congeners such as Holothurins $A/A_3/D$ in this species, the large numbers of identified saponin were non-sulphated glycosides. In contrast, large portion of those were acetylated glycoside. These saponins were bearing a double bond at the $\triangle^{7(8)}$ or $\triangle^{9(11)}$ core of the aglycone. Majority of them had a 493 key diagnostic peak, while some had the ion 507 corresponding to the key diagnostic peak. For instances, most of ions detected in Fraction 87 including 1207.5, 1221.5, 1243.5, 1439.7 and 1453.7 demonstrated the ion at *m/z* 507.

Chemical analysis of ions by MS revealed the presence of different type of isomeric compounds with *m/z* value of 1259 including sulphated and non-sulphated. For instance the ion at *m/z* 1259 was detected in Fraction 111. Tandem mass spectrometry of this ion in the positive ion mode is shown in Figure 6. 23. As can be seen from the spectrum, the major peak at 789 was generated by the loss of the aglycone which indeed corresponded to the entire sugar chain.

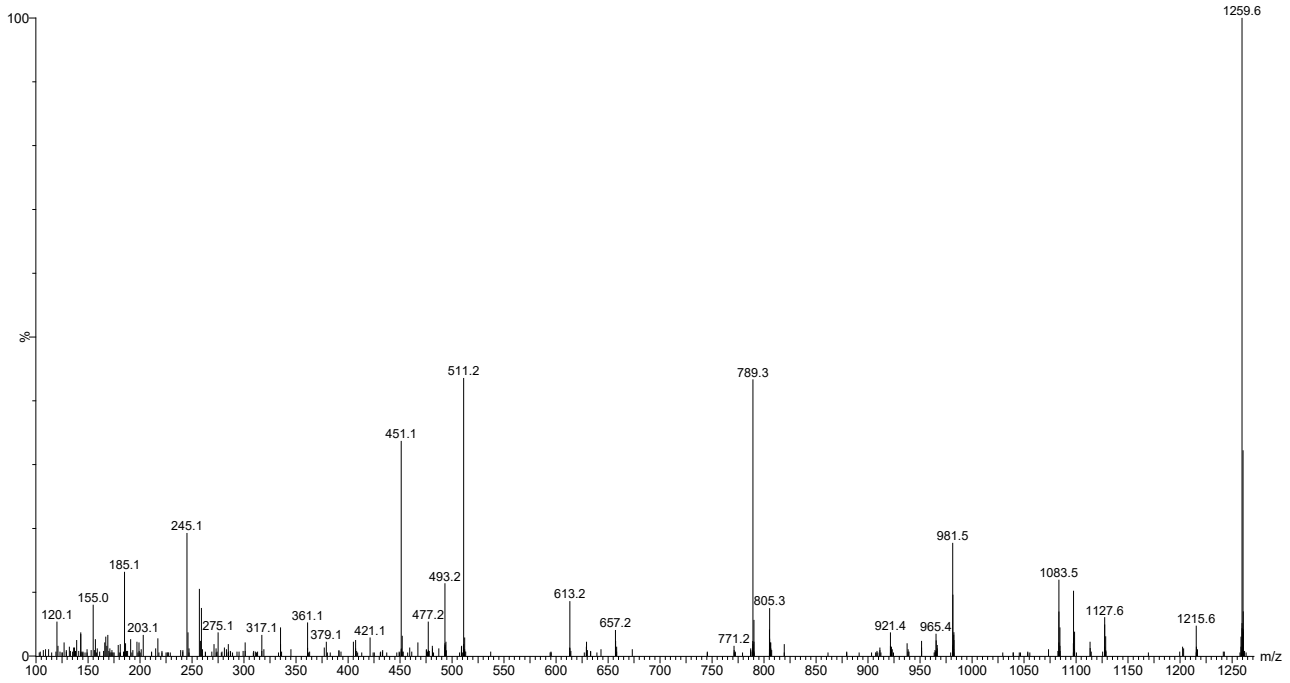

Figure 6.23. CID fragmentation patters of the ion at *m/z* 1259 in the positive ion mode ESI-MS^2. This glycoside contained the key diagnostic peaks at 493 and 511.

MS analysis revealed a structure of pentasaccharide glycoside for this ion. This conclusion was corroborated by fragment ion peaks at *m/z* 789, 657 and 511 in the positive-ion mode MS/MS, corresponding to the sequential losses of Agl, Xyl and Qui followed by the key diagnostic peak (511); i.e. MeGlc-Xyl-Glc moieties, respectively. Alternatively the consecutive elimination of MeGlc, Xyl, Glc, Xyl and Qui (hydrated) followed by the aglycone afforded ion peaks at 1083, 951, 789, 657 and 493, respectively. The aglycone of this glycoside had a C-7 double bond bearing a hydroxy group at C-23.

The spectrum indicated the structure of a new compound which had a different structure with those reported for Holothurins A_3 and D from *H. lessoni* (Bahrami *et al.* 2014a; Bahrami *et al.* 2014b). For instance this glycoside had no sulphate group and contained a different aglycone.

Indeed this ion was found to be an isomeric compound, in the second isomer Qui was replaced with a Glc and had different aglycone. The structures of six non-sulphated triterpene glycosides namely Variegatusides A-F were reported from *S. variegatus* by Wang, X-H *et al.* (2014). They stated the presence of a C-23 hydroxy group in their aglycone structures. They described the structures of Variegatuside D having a 8(9)-ene double bond with chemical formulae $C_{59}H_{96}O_{27}$

1259 $[M+Na]^+$ (Wang, X-H *et al.* 2014) which might produce by the loss of MeGlc from the ion at *m/z* 1435. The structure of this isomer was coincidence with Variegatuside D. In addition, it is noteworthy that Holothurins $A/A_3/D$ were also found in Fraction 95.

6.13 Taxonomical application of Saponins

Chemical and structural features of triterpene glycosides have been applied to determine taxonomic uncertainty in the class Holothuroidea. For instance, on the basis of the triterpene glycoside composition, the taxonomic status of *S. mollis* was revised and it was positioned in the new genus *Australostichopus* Levin (Moraes, Greta *et al.* 2004). These molecules are specific for different taxa, and they could be utilised for taxonomic classification and identification of genera and sometimes even species of sea cucumbers (Avilov, Sergey A. *et al.* 2004). For this reason, saponins have been applied for chemotaxonomy of sea cucumbers to solve taxonomic problems (Kalinin, VI *et al.* 2005). This method has been also applied to clarify the taxonomy of sea cucumbers belonging to the genus *Cucumaria* (Avilov, Sergey A. *et al.* 2004). Thus triterpene glycosides could be useful tools for taxonomic determination of sea cucumbers regardless of prefectures.

6.14 Bioactivity

6.14.1 Antifungal and antibacterial activities of purified saponins

The antifungal activity of isobutanol-enriched saponins and HPCPC fractions from *S. hermmani* viscera were evaluated against *F. pseudograminearum, P. irregulare* and *R. solani.* Our results revealed several tested saponin congeners (fractions) have strong antifungal activities against *F. pseudograminearum* and *R. solani*. As a typical example, antifungal activities of some of these saponins are illustrated in Figure 6.24. For instances, Fraction 95 (Holothurin A= spot 9), Fraction 41 (Novel saponins at *m/z* 1459 and 1461= spot 10) and Fraction 59 (Lessonioside A and ion at 1475 = spot 11) showed significant antifungal activities, while Fraction 105 (ions at 1435 and 1259= spot 8) and Fraction 117 (ion at 1435= spot 7) did not show antifungal activity.

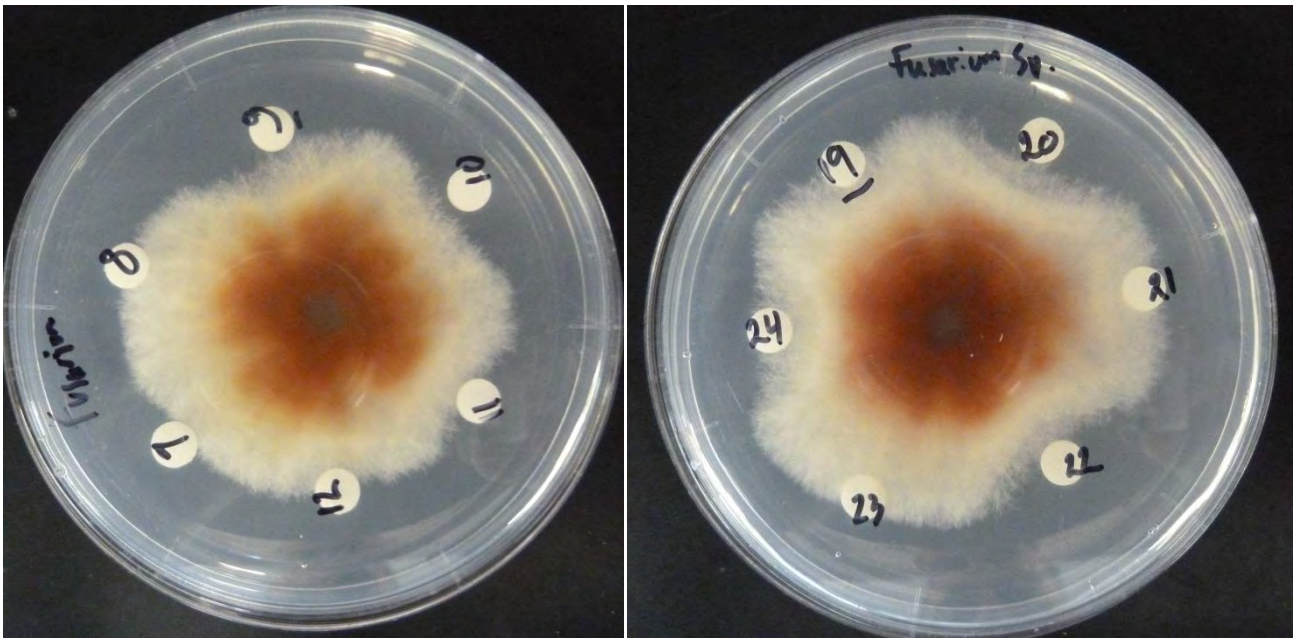

Figure 6.24. Antifungal activity of saponins isolated from *S. hermanni* viscera against *Fusarium.*
In this figure ions at *m/z* 1459 and 1461 (spot 10), Holothurin A (spot 9) show strong antifungal activity.

Several saponins were found to exhibit stronger cytotoxicity activity against *F. pseudograminearum* than *P. irregulare* and *R. solani.* Our results indicated that there are strong relationships between the chemical structures of saponins and their antifungal activity. Our limited results show that acetylated and mono-sulphated saponins usually exhibit higher antifungal activity compared to non-sulphated and non-acetylated compounds. For examples, Fractions 105 (ions at 1435 and 1259) and 117 (ion at 1435) (Figure 6.24) which were non-sulphated and non-acetylated compounds did not show any antifungal activity against tested fungi, whereas Holothurin A (sulphated) and acetylated compounds, spots 9 and 10 respectively, showed strong activity (Table 6.2).

Table 6.2. Antifungal activity of saponins from *S. hermanni* viscera; plug type diffusion assay, inhibition zone (diameter)

Saponins	***F. pseudograminearum*** (mm diameter)	***R. solani*** (mm diameter)	***P. irregulare*** (mm diameter)
Novel saponin at *m/z* 1435 (spot **7**)	0.0	0.0	0.0
Novel saponins at *m/z* 1435 and 1259 (spot **8**)	0.0	0.0	0.0
Holothurin A (spot **9**)	16	14	0.0
Novel compounds at 1461 and 1459 (spot **10)**	26	16	0.0
Lessoniosides A and B (spot **11**)	18	10	0.0

However, the examined triterpene glycosides had no effect on *P. irregulare* On the basis of the findings, holothurian glycosides showed different sensitivities against different fungi strains.

Our result, though with a limited number of samples, indicate that saponins, bearing a double bond at the lateral side of the aglycone, in particular $\triangle^{25}$, may have a role significant antifungal activity. This conclusion was consistent with the observation on antifungal activity of sea cucumber glycosides reported by Silchenko *et al.* (2012a). However, the presence of hydroxy groups in the lateral side of aglycone decreases the activity of glycosides. Silchenko *et al.* (2013a) also drew the same conclusion. They also stated that typicoside C_1 bearing a hydroxy group in the lateral side chain of the aglycone (C-22) did not exhibit an antifungal property (Silchenko *et al.* 2013a).

Antibacterial, antifungal cytotoxic properties of sea cucumber *S. hermanni* extracts were investigated against fungi and bacteria by Sarhadizadeh *et al.* (2014). They reported that the MeOH extract shows a strong antifungal activity. However they observed no inhibition zone towards the studied bacteria strains. This observation was in agreement with data reported on antifungal activity of aqueous fraction from *Bohadschia vitiensis* (Lakshmi *et al.* 2012). This group identified Bivittoside D as a major compound in the fraction. Kitagawa and co-workers also stated the strong antifungal properties of these major saponins isolated from the body wall of the sea cucumber *S. hermanni.*

In this study, a large number of saponins were found to contain holostane type aglycone with a $\triangle^{25}$ terminus double bond which would be expected to show strong growth inhibitory activity against different fungi. Therefore sea cucumber glycosides are found to be an interesting source of antifungal agents.

In contrast, the studied triterpene glycosides had no considerable effect or inhibited on resistant pathogen *S. aureus*, using the same concentrations as used for the antifungal activity assay. This observation was in good agreement with the antibacterial findings of sea cucumber extracts reported by Mokhlesi *et al.* (2012) and Kuznetsova *et al.* (1982). However, some studies reported antibacterial activity of sea cucumber crude extracts which might be associated with other chemical classes of compound (such as AMPs) other than saponins, or they might use a very high concentration of extracts.

6.15 Conclusion

This work presented the first study on the triterpene glycosidic contents of the viscera of the Australian sea cucumber *S. hermanni,* which revealed the presence of a large number (at least 101) of novel, new and known saponin congeners. It is noteworthy that most of them were reported for the first time from a *Stichopus* species. Notably, it was the first study of viscera of the family Stichopodidae, and has shown the most diverse and abundant saponins among all other representatives of this family studied so far. Based on the literature, other than the work that was conducted on the body wall of *S. hermanni* by Kitagawa *et al.* (1981a) no studies hitherto have been documented on this species to investigate saponins distribution.

Most of identified glycosides had an oxidised group in C-23 of the lateral chain of the aglycone and a 18(20)-lactone structure, and contained non-sulphated sugar residues. In contrast, large numbers of them were acetylated. Similar to most of sea cucumber triterpene glycosides, these glycosides had mainly either a $\Delta^{7(8)}$ or a $\Delta^{9(11)}$-double bond. All of these highlighted the chemical diversity of glycosides from sea cucumbers.

In addition to 60 triterpene glycosides already reported in the literature, 41 new molecules have

been identified. Some of them were exclusively found in this species. The exclusive saponins presented here deserve more attention and further investigation in terms of structure- activity relationship, biological roles and mode of actions. Further it showed that viscera are a rich source of this type of bioactive compound.

Most of saponin congeners in this species were isomeric compounds and most identified as a pair such as the ions at 1459 and 1461 or 1475 and 1477, representing dihydro and dehydro forms of congeners. The majority of major saponins were acetylated. Most sea cucumbers belonging to this family are appreciated for their commercial value and health benefits.

In conclusion, from the findings herein, these data suggested that, holothurian glycosides are potent antifungal compounds which have potential pharmaceutical and cosmeceutical applications. Scientific surveys showed that acetylated compounds possess higher cytotoxicity and anti-cancer activities compared to non-acetylated saponins. Since the viscera of *S. hermanni* have a high number of acetylated saponins, therefore it might be a good source of anti-cancer leads.

CHAPTER 7
CONCLUSION AND FUTURE DIRECTIONS

7.1 Summary of research

The Australian sea cucumber species, *Holothuria lessoni* and *Stichopus hermanni*, were found to produce a diverse range of saponin congeners which include isomeric compounds and glycosides that are sulphated, non-sulphated or acetylated. This study has focussed on the viscera which have proven to be a reservoir of multiple known and novel saponins, many of which are unique to each species. Furthermore, these major triterpene glycosides are common in the body wall and the viscera of *H. lessoni*, but there are some congeners found solely in the viscera or the body wall. The data also highlighted the presence of more saponins in the viscera than in the body wall. In contrast to *H. lessoni*, the majority of the saponins in *S. hermanni* were found to be acetylated, but with a lower prevalence of sulphated triterpene glycosides.

We have examined the viscera and body walls of 16 different sea cucumbers species, belonging to different families (data not shown), though we have described the saponin content in *H. lessoni* and *S. hermanni* in detail as representatives of two families to show how their saponin profiles are different. The MALDI analysis of the saponin congeners illustrates a unique saponin profile for the examined species suggesting the possible application of these secondary metabolites for the taxonomic classification of sea cucumber species.

7.2 Major findings of the project

The primary aims of this study were to isolate, purify and elucidate the structures of saponins in the viscera of Australian sea cucumber species. The hypotheses of this research have been proven, as sea cucumber viscera are a deep repository of saponins and contain a diverse range of novel saponins. A number of identified saponins have shown strong fungicidal, anti-oxidant, anti-viral and anti-cancer properties (Alsini thesis, 2014).

We have elucidated the structure of several novel saponins in the viscera of *H. lessoni*. Chapter two describes the structure of seven novel compounds; Holothurinoside O, Holothurinoside P, Holothurinoside Q, Holothurinoside R, Holothurinoside R_1, Holothurinoside S and Holothurinoside T in addition to six known compounds, including Holothurin A, Holothurinoside A, Holothurinoside A_1,

Holothurinoside E, Holothurinoside E_1 and 17-dehydroxy-holothurinoside A. Moreover, chapter three reports the structure elucidation of five novel saponins in the viscera of *H. lessoni*, including Holothurins D/E and Holothurinosides X/Y/Z, along with seven reported triterpene glycosides. Further chapter four outlies the structure elucidation of five new acetylated saponins, Lessoniosides A–E, along with two non-acetylated saponins Lessoniosides F and G.

As previous studies have focused mainly on the discovery of saponins from the body wall of sea cucumbers, only a few researchers have compared the saponin contents of the body wall with that of the individual internal organs such as cuvierian tubules and ovaries (Kitagawa *et al.* 1989a; Kitagawa *et al.* 1989b; Matsuno & Ishida 1969b; Van Dyck *et al.* 2010b). To our knowledge, no study has published the distribution of saponins from the viscera of sea cucumber species. Further, no study has documented the saponin profiles from the body wall of Australian sea cucumber species. Our extensive literature review has revealed that some identical saponins have been given different names, however, they could be isomeric compounds.

Previous studies have shown that saponins congeners are more concentrated (in terms of quantity number, type and intensity) in the Cuvierian tubules of some species than in the body walls (Kobayashi *et al.* 1991; Van Dyck *et al.* 2010b). Data suggests that saponin congeners might be involved in chemical defence mechanisms as they are deleterious to most of organisms. In contrast, some saponins are only found in the body wall, and some are also reported in the water surrounding the animals (Caulier *et al.* 2013) indicating sea cucumbers might use them to define their territories and/or also utilise them for chemical signalling.

There is evidence that sea cucumbers also utilise saponins as kairomones to attract symbionts such as crabs. Therefore saponins can also serve as allelochemicals. Having different kinds of biological functions might influence the localisation of saponins among the different organs.

It has been suggested that saponins may also have two regulatory roles during reproduction: (1) to prevent oocyte maturation and (2) to act as a mediator of gametogenesis (Kalinin, VI *et al.* 2008; Mercier *et al.* 2009). Saponins could be also responsible for other unknown functions which are

required to be investigated. The particular localisation of saponin might associate with their specific role. Therefore one could conclude that sea cucumbers take advantage of saponin diversity to deal with different conditions and in response to environmental stimuli.

7.3 Application of analytical techniques

Discovery of new medicine candidates from marine organisms offers structural diversity and novel characteristic activities (Blunt, JW *et al.* 2008, 2009; Mayer, AMS *et al.* 2011; Mayer, AMS *et al.* 2009). Nevertheless, together with these advantages, there are a number of drawbacks associated with the development of drugs from marine environments. These include the isolation and purification of bioactive substances of interest in sufficient amounts.

Purification of saponins is one of the biggest challenges due to their polarity as well as closely related chemical structures. However, advances in separation technologies such as HPCPC overcome the hurdles associated with the isolation and purification of saponins and potential development of new leads. Since HPCPC is an all-liquid technique the problem of irreversible adsorption of samples, which is a big challenge for most solid-phase chromatography techniques, is eliminated. In our studies the recovery was over 95%.

NMR spectroscopy can provide extensive structural information for saponins, but much larger quantities of high-purity samples are generally required. This would be more complicated with fractions containing more than one congener, especially if the NMR signals overlap, making their assignments more difficult. Moreover, the measurement of the absolute configuration of the sugar moieties of a saponin cannot be completely solved by NMR methods alone (Oleszek & Marston 2000). Therefore, the integration of HPCPC and MS techniques can provide an effective means to study the structural diversity of saponins, and enhance the efficiency of discovering novel saponins. In this study we used two different ionisation techniques, MALDI- and ESI-MS2, in order to validate our new structures.

Since the newly identified saponins clearly possess aglycone structures similar to that of other

previously established compounds we are confident that it is possible to elucidate the structure of these compounds based on MS analysis alone. Nevertheless, we acknowledge NMR analysis would be required to confirm the structure of the aglycones.

The structure of compounds were determined by investigating the consecutive losses of monosaccharides (MS/MS experiments), presenting of the key diagnostic peaks (MS/MS) and by knowledge of specific losses (56, 60, 98 or 100 u) due to McLafferty rearrangement at the lateral side chain of the aglycone and comparison the data with the existing literature.

MS^2 analysis has also identified the predominant fragment signal at *m/z* 593.2 results from $^{1,5}A_4$ cross-ring cleavage of the sulXyl residue (Bahrami *et al.* 2014b; Bondoc *et al.* 2013). Our results have also corroborated the cross-ring cleavages predominantly occurred in the sulphated compounds compared to non-sulphated glycosides. It is likely that the loss of sulphate group in the sugar moiety of saponins made them more susceptible for cross-ring cleavages.

7.4 Major triterpene glycosides

Our results have also revealed that all major saponins isolated from *S. hermanni* possess hexasaccharides in their glycone moieties. This conclusion is consistent with published data on glycosides of the family Stichopodidae (Moraes, Greta *et al.* 2004). In contrast, the major saponins in *H. lessoni* are predominately found to be sulphated compounds of which Holothurin A is found to be major triterpene glycoside in the viscera and the body wall of this species.

7.5 Acetylated saponins

Over 130 acetylated triterpene glycosidic saponins have been reported from sea cucumber species in which the majority are of the holostane type. One of the modifications occurred in the structure of saponins is the addition of an acetoxy group to the aglycone moiety which enhances the solubility of saponins in the water surrounding the species. Acetylated saponins are mainly reported in the family *Cucucmarridae*. However, the presence of acetylated saponins is only reported in a limited numbers of the genus *Holothuria*. Interestingly, our finding has highlighted the

presence of a number of acetylated saponins in the viscera and the body wall of *H. lessoni*.

In the *Holothuriidae* family, the acetoxy group is either located at C-16 of the aglycone core moiety such as in Arguside F and Lessonioside A (Bahrami & Franco 2015; Yuan *et al.* 2009a) and Nobiliside C (Wu, J *et al.* 2006b), or at the C-22, C23 or C-25 of the lateral chain, ie at C-25 of the Pervicosides A and D (Kitagawa *et al.* 1989b; Yuan *et al.* 2009a).

7.6 Sulphated and non-sulphated saponin congeners

This study has identified a large number of sulphated saponins in the examined species which is consistent with data reported by Van Dyck *et al.* (2010b) who found a large number of sulphated saponin in the Cuvierian tubules and the body wall of studied sea cucumber species. Triterpene glycosides from sea cucumbers possess complicated structures, and could be differentiated by several structural features. They include number and position of double bonds in the core and lateral side chain of the aglycone, number and position of sulphated groups in the sugar moieties, number and composition and sequence of saccharide residues in the saccharide chain, and the occurrence of hydroxy, epoxy, acetoxy and ketone groups in numerous positions of the aglycone, so on.

7.7 Future directions

This study revealed the presence of a large number of saponin congeners with high diversity in the viscera and body wall of sea cucumber species. Triterpene glycosides are well known for their biological properties (Aminin, Dmitry L. *et al.* 2014; Blunt, JW *et al.* 2015; Careaga & Maier 2015; Kim, SK & Himaya 2012; Kim, SK *et al.* 2012). Therefore the evaluation of the biological properties of identified saponins will shed light on the relationship between the structure of saponins and the biological activity.

Our preliminary data has indicated that the non-toxic dose of sea cucumbers saponin-rich extracts possess antiviral activity against dengue virus (Alsini 2014), in which inhibits binding and/or entry of vinous in addition to reduce the replication spread. This opens a window of research which

requires further investigation. As we have tested the saponin enriched fractions, it is difficult to speculate which saponin(s) is responsible for such activity, therefore, it is noteworthy to evaluate the activity of purified compounds.

The cytotoxicity of saponin-enriched fractions of sea cucumber species was investigated with non-small lung cancer A594 using the MTT and Crystal Violet assay. Our preliminary data has revealed the extracts possess cytotoxicity activity against the human lung cancer A549 cells in dose dependent manner (unpublished data), and also stimulated the apoptosis pathway. However, the purified compounds still need to be evaluated. A further study of anticancer properties of these substances against different cancer cell lines should be carried out to find out about the selectivity of saponins against cancerous cells.

Many Identified saponins showed strong antifungal activity against tested fungi. Further studies are warranted to discover the mode of action and the structure–activity relationships of these secondary metabolites. Our results indicated that sea cucumber viscera are good candidate for discovery of novel bioactive saponins.

Saponins can also stimulate the mammalian immune system, which has attracted the attention of scientists in their potential as vaccine adjuvants. Previous studies have reported that sea cucumber triterpene glycosides possess immunomodulatory property (Aminin, D. L. *et al.* 2010a; Aminin, Dmitry L. *et al.* 2014; Aminin, D. L. *et al.* 2010b; Lacaille-Dubois 2005). Further it has been documented that plant saponins have strong adjuvant properties in which most widely used saponin- based adjuvants are Quil A and its derivatives QS-21 (Sun, H-X *et al.* 2009), therefore, it is of value to study saponin- based adjuvants from sea cucumbers.

Our results have indicated that there are relationships between the chemical structures of saponins and their antifungal activity which is in agreement with data reported by Park, J-I *et al.* (2014). For instance, our finding has revealed that acetylated and mono-sulphated saponins usually exhibit higher antifungal activity compared to non-sulphated and non-acetylated compounds.

One of the alternative ways to overcome the problem associated with the purity of single saponins is using the combined saponin congeners as nutraceuticals (food supplements) rather than as pharmaceuticals, as they are often the first choice of cure by consumers (Straus 2000). However, the availability of sustainable raw products to meet demand is often the biggest obstacle to develop nutraceutical products (Benkendorff *et al.* 2009).

The distribution of saponins may vary in the viscera and the body wall in different seasons (Matsuno & Ishida 1969b). Therefore, collecting experimental specimen in different seasons may have an effect on the amount and type of saponins as Matsuno and Ishida (1969b) reported some internal organs such as gonads contain a large amount of saponin during breading seasons.

Further purified triterpene glucosides from Australian holothurians are biologically active towards fungi, virus, and cancer cell lines. These data highlight the potential development of new nutraceutical, pharmaceutical and/or cosmeceutical reagents for the prevention or the treatment of different diseases.

APPENDIX I: MEDIA RECIPES

Media Preparation: five media were used for the bioactivity assays, and their compositions are detailed below:

Potato Dextrose Agar (PDA)

PDA (Oxoid)	39g
R.O water	1,000 mL.

Half strength Potato Dextrose Agar (HPDA)

PDA (Oxoid)	19.5g
Agar (Sigma)	7.5 g
R.O water	1,000 mL.

Tryptone soya broth (TSB)

TSB (Oxoid)	30 g
R.O water	1,000 mL.

Tryptone soya agar (TSA)

TSA (Oxoid)	30 g
R.O water	1,000 mL.
Agar (Sigma)	15 g

Antibiotic assay medium no. 1 (AAM)

AAM (Oxoid)	27 g
R.O water	1,000 mL.

All media were autoclaved at 121°C for 35 minutes prior to use. Solid medium was poured into a 9 cm standard petri dish at 20 mL per plate.

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