Collagen-Stimulating Activity of Australian Sea Cucumber (*Holothuria atra)* Extracts on Human Skin Fibroblast Cells

A thesis submitted for the degree of Master Biotechnology Studies at Flinders University

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Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and 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 of the thesis.

Gintung Patantis

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Abbreviations

μL	Microliter
%	Percentage
ATTC	American Type Culture Collection
cm	Centimeter
CO_2	Carbon dioxide
CRL	Cell repository line
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Fetal bovine serum
$FeCl_3.7H_2O$	Ferric chloride hexahydrate
FRAP	Ferric reducing antioxidant power
g	Gram
GAE	Gallic acid equivalent
h	Hour
H_2O_2	Hydrogen peroxide
IMDM	Iscove's Modified Dulbecco's Medium
L	Liter
LC-DAD-MS	Liquid chromatography - PhotoDiode Array Detection - Mass Spectrometry
mba	Millibar
min	Minute
mL	Milliliter
mM	Millimolar
MMP-1	Matrix metalloproteinase-1
MMPs	Matrix metalloproteinases
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide
nm	Nanometer
^oC	Degree of Celsius
ORAC	Oxygen radical absorbance activity
PBS	Phosphate buffer saline
RO	Reverse osmosis
rpm	Revolutions per minutes
TGF-β	Transforming growth factor beta
TIMP-1	The tissue inhibitors of metalloproteinases-1
TLC	Thin-layer chromatography
TPTZ	Tripyridyltriazine
UK	The United Kingdom
USA	United States of America
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WW	Wet weight
x g	Relative centrifugal force

Abstract

Sea cucumbers (traded as *beche-de-mer*) have a long history as a functional food and tonic in Asia. Sea cucumbers contain a wide range of bioactive compounds, which have huge potential applications in the nutraceutical and pharmaceutical industries. The compounds detected so far include collagen, gelatin, saponins, fucoidan, and glucosaminoglycones. Saponins, major secondary metabolites in sea cucumbers, are reported to have antibacterial, antifungal, antiprotozoal, antiviral, antioxidant, mutagenic, hemolytic, immunomodulation, and neurotoxic activity. However, very few studies on the cosmetic application of sea cucumber saponins are currently available. The aim of this study was to determine the potential application of saponins-rich extracts of sea cucumbers for cosmeceutical products. We hypothesize that saponins-rich extracts from sea cucumber have anti-aging properties similar to saponins from ginseng that have been widely studied. As a first step, the extraction methods were optimized by using two solvents (water and 70% ethanol (70%EtOH)), and the conditions of shaking at 22ºC (room temperature) and 60ºC, and sonication at 22ºC. The safe dosages for the extracts were then determined by cytotoxicity assays against human skin fibroblast cells CRL 2076 using crystal violet staining. Anti-aging properties of the extracts were assessed by measuring collagen production in human skin fibroblast cells and by quantifying antioxidant capacity through estimation of Ferric Reducing Antioxidant Power (FRAP). The extracts, were characterized (total saponins, sugar, total phenolics and soluble protein contents) and fingerprinted using thin-layer chromatography (TLC). The yields of water extracts were significantly higher than 70%EtOH extracts. In addition, the 70%EtOH extracts from shaking at 60°C had significantly higher yields than other conditions; however the water extract was not significantly different. The safe dosages for sea cucumber extracts varied depending on extraction solvent and conditions and ranged from 2.5 - 10 mg/L extracts. At 2.5 mg/L concentration, the sea cucumber extracts increased the collagen production of human skin fibroblast cells up to 3.7-fold. Water extracts contained more antioxidant capacity compared to 70%EtOH extracts, however, the antioxidant capacities of the extracts were 100 times lower than common antioxidant sources such as green tea and grape. The chemical characterization of sea cucumber extracts contained 0.15 - 0.22 mg/ mg extract of saponins, 0.06 – 0.09 mg/ mg extract of sugar, 2.14 - 4.99μ /mg extract of phenolics and 0.03- 0.12 mg/ mg extract of soluble protein. TLC showed different distribution of compounds between water and 70%EtOH extracts with 70%EtOH extracts exhibiting a greater number of compounds. Results indicate strong anti-aging properties of sea cucumber extracts on human skin cells and confirm their potential application for anti-aging skin care products.

CHAPTER 1

INTRODUCTION

The need to keep a youthful appearance together with increasing income of communities in developing countries have led to the recent booming in the cosmetic industry (Brandt *et al*., 2011). Based on Allied Market research, the value of this industry is expected to reach US$390 billion in 2020 (Kerdudo *et al*., 2016). Skin care products constitute more than 60% of the total cosmetic production indicating their highest demand in the market (Brandt *et al.*, 2011). Synthetic ingredients used for cosmetic products have been reported to have many deleterious effects and hence they are becoming less popular among consumers in recent years. Therefore, development of new cosmetic ingredients from natural compounds that have better efficacy, quality and safety is gaining popularity in the industry (Kerdudo *et al*., 2016).

Sea cucumbers have a long history as a functional food and as traditional medicine in the south-east Asian region (Chen, 2005). As a medicine, sea cucumber or haishen (ginseng of the sea) is believed to preserve the blood, kidney, reproductive organs, reducing dryness of intestine, as well as a providing vital essence (Chen, 2003). Recent studies showed that sea cucumbers have huge potential application for medical and pharmaceutical purposes. These biopotencies were contributed by various sea cucumber biocompounds (Bordbar *et al*., 2011). Most especially the saponins group, which is a major sea cucumber secondary metabolite, has prolific bioactivities (Kim and Himaya, 2012). However, very few studies on the cosmetic application of sea cucumbers saponins are currently available particularly in the collagen stimulating production.

1.1. Morphological and taxonomical classification of sea cucumbers

Sea cucumbers belong to the phylum Echinodermata. Echinoderms are divided into five main classes, including Crinoidea (sea lilies and feather stars), Asteroidea (starfishes or sea stars), Ophiuroidea (basket stars and brittle stars), Echinoidea (sea urchins, sand dollars and sea biscuits) and Holothuroidea (sea cucumbers) (Matranga, 2005). Sea cucumbers are distinguished from other Echinoderms by leathery or soft body wall and the absence of a conspicuous skeleton (Pawson *et al*., 2010).

So far, more than 1600 species of sea cucumber are known and they are divided into six major orders and 200 genera (Pawson *et al*., 2010, Arnone *et al*., 2015). The order, genus and family classification of sea cucumbers are based on the surface and internal morphological features of the body, while the species level are based on the combinations of morphological characteristics of body and spicules. Furthermore, chemical fingerprinting properties of saponins (Bondoc *et al*., 2013) and molecular tools (Arndt and Smith, 1998) are used to classify the taxonomic levels of the sea cucumbers.

Sea cucumbers have special characteristics (Figure 1.1). The length of sea cucumbers is between a few centimeters to at least two meters. Most sea cucumbers have symmetrical bodies with mouths at the anterior and anuses at the posterior distal. These two ends are connected by intestines (Pawson *et al*., 2010). In addition, the mouth is surrounded by tentacles and used as one of the features of taxonomic classifications (Olivera-Castillo *et al*., 2013). Their body contains circular and radial longitudinal muscles. Ten or more ossicles form a calcareous ring surrounding the esophagus. The shape of the ring is also used in the classification of sea cucumbers (Figure 1.2) (Pawson *et al*., 2010).

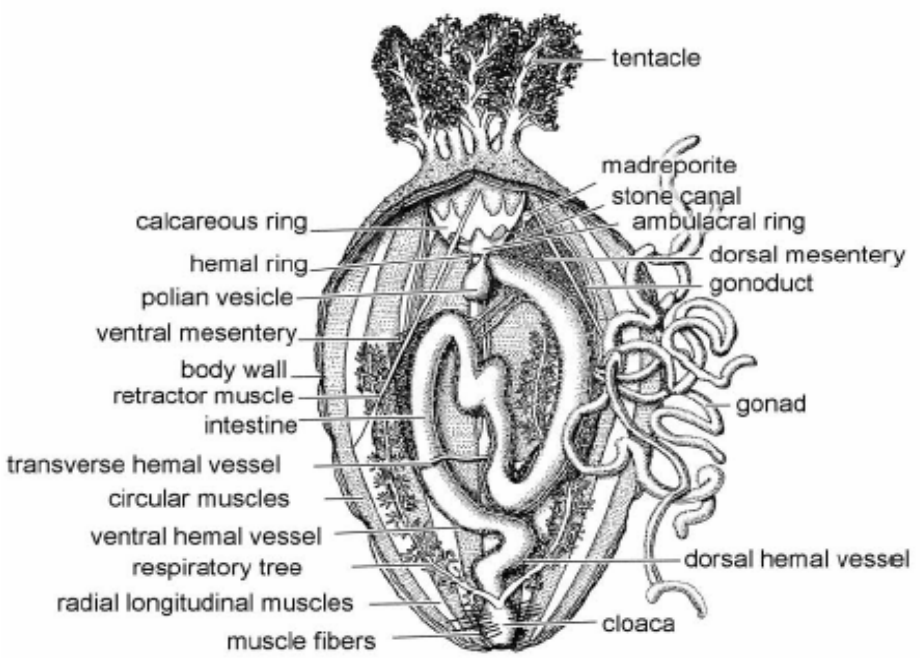


Figure 1.1. Anatomy of sea cucumber (Pawson *et al*., 2010)

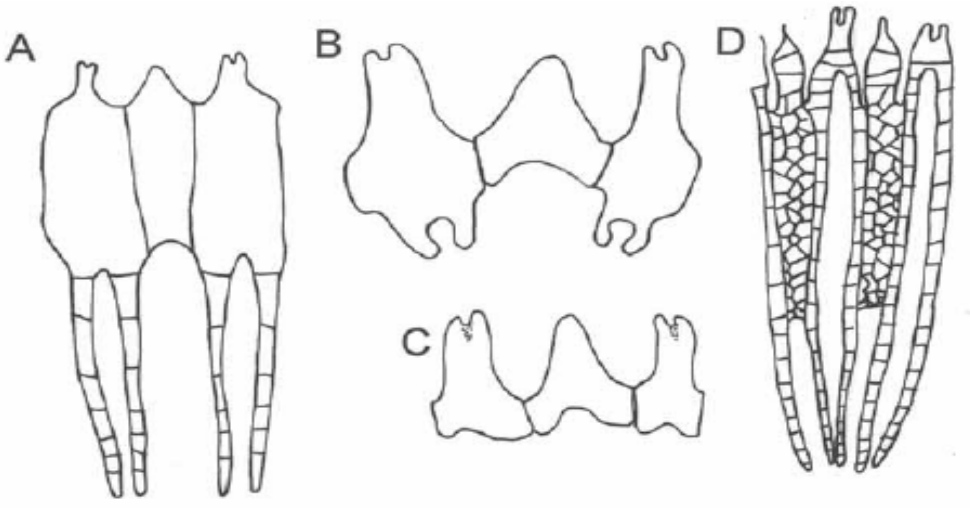


Figure 1.2. Examples of calcareous rings; A: medium posterior projections ring; B: short posterior projections ring; C, no posterior projections ring; D: tubular, long, complex, posterior projections ring (Pawson *et al*., 2010)

1.2. Distribution and habitat of sea cucumbers

Holothurians are distributed globally and inhabit various marine environments. They can be found from temperate ocean to tropical, but tropical oceans are the main habitat for many sea cucumbers. The order Aspidochirotida, which includes important

commercial sea cucumbers, are dominant in the tropics, while the Dendrochirotida with less commercial value is found in temperate areas (Purcell *et al*., 2010). Although some sea cucumbers can live at 66 m depths, the normal habitat of the sea cucumber is mainly less than 33 meters (Olivera-Castillo *et al*., 2013). Various environmental factors such as temperature, salinity, turbidity, nutrients and depth influence the sea cucumbers distribution (Lampe, 2013).

In addition, sea cucumbers can be easily found in various substrates of the seabed. Sand, rock and stony surfaces are important substrates for sea cucumbers, although, they can inhabit shell, clay and algae substrates. Large sea cucumbers are more likely to be found on rock and stone surfaces, while small sea cucumbers live in the holes or cracks of rocks or under rocks (Olivera-Castillo *et al*., 2013). Furthermore, some of sea cucumbers also live as epizooties on marine plant or invertebrate (Purcell *et al*., 2010). The habitat of sea cucumbers may be related to their function in ecology. Lampe (2013) reported that the sea cucumber is a bioturbator, as it recycles and processes debris and organic materials on the sea floor.

1.3. The sea cucumber industry

1.3.1. Commercial sea cucumbers

Sea cucumbers are an important trade commodity. Commonly known as *beche-de-mer* or gamat or haishen, sea cucumbers have been harvested and commercialized for over more than 1000 years (Hamel *et al*., 2001) in more than 70 countries worldwide (Purcell et al., 2012a). The global production of *beche-de-mer* is to supply demands from the Asian markets, especially China (Hamel *et al*., 2001). Up to date, around 60 species of sea cucumbers have been traded (Purcell et al., 2012a) out of more than 1600 known species (Arnone *et al*., 2015). The main source of *beche-de-mer* is wild capture

(Purcell *et al*., 2010) and just a few species are currently used in aquaculture. These species include *Apostichopus japonicus, Holothuria scabra, H. nobilis H. fuscogilva* (Chen, 2005).

The tropical species of sea cucumbers contribute more commercial *beche-de-mer* than the ones from the sub-tropics (Purcell et al., 2012a). Two families (Holothuriidae and Stichopodidae) from the order Aspidochirotida contain the most valuable sea cucumbers. These families include the genera Bohadschia, *Holothuria* (Holothuridae), *Actinopyga*, *Isostichopus*, *Stichopus*, *Parastichopus* and *Thelenota* (Stichopodidae) (Bruckner et al., 2003, Purcell et al., 2012a). In terms of commercial value, Chen (2005) classified China's sea cucumbers into several levels. The most valuable are *Apostichopus japonicus* or the prickly sea cucumbers. The second class is the genus *Holothuria* which includes *Holothuria scabra* (sandfish), *H. fuscogilva* (white teatfish), *H. nobilis* (black teatfish) and *H. moebii*. The next level is the genus *Stichopus* and *Thelenota*.

1.3.2. International trade of sea cucumbers

The international market of sea cucumber has grown since the 1980s. The main reason for this development is the re-joining of China into the world trade during that period (Hamel *et al*., 2001). The increasing demand of sea cucumbers is not only in the yield, but also in the type of species (Bordbar *et al*., 2011). As the main consumer of *beche-de-mer* products, the Asia-Pacific region also produces the highest number of sea cucumber with 20,000 to 40,000 tonnes per year. The second contributor is the Northern Hemisphere, with total production per year 9,000 tonnes. Africa regions and the Indian Ocean produce 2,000 to 2,500 tonnes per year, whereas the Caribbean and Latin America regions produce less than 1,000 tonnes (Toral-Granda *et al*., 2008).

The main producer of sea cucumber in Asia is China. During 2000 to 2005, China produced 10,000 tonnes dry sea cucumber per year (Choo, 2008) with more than 90% of the yields originating from aquaculture (Chen, 2005). Indonesia and Philippines shared around 47% of the world's Holothuroidea production with 2,572 tonnes per annum (wet weight). Japan produced 8,101 tonnes per year of *A. japonicus* in the period 2000 to 2005, which was the highest for temperate species (Choo, 2008).

Although China has a huge production of sea cucumbers, the local demand for this product exceeds this production. Therefore, China imports *beche-de-mer* products from other countries via Hong Kong SAR brokers (Chen, 2005). A total of 80% sea cucumber worldwide production is exported to China (Clarke, 2002). Singapore, Malaysia, Taiwan Province of China, Republic of Korea and Japan are the other big consumers of the sea cucumber products (Choo, 2008).

The price of the *beche-de-mer* product is varied and fluctuated. It depends on the species, grade and origin (Kinch *et al*., 2008). In around 2000, the price of *H. scabra* was US$ 50 per 100 kg (Hamel *et al*., 2001). While in 2007, the price of grade super large *H. scabra* from Papua New Guinea was US$ 60 per kg. Still from this country, the price of *H. whitmaei* was from US$ 9.10 to 30.30 per kg from the lowest grade to the highest (Kinch *et al*., 2008).

1.4. The potential of Australian sea cucumbers

1.4.1. Australian sea cucumbers

Australia has a huge diversity of sea cucumbers. Among the more than 1,600 species of sea cucumbers worldwide (Arnone *et al*., 2015), about 200 species inhabit Australian waters (Conand, 2004). This includes15 families and 62 genera (Rowe and Gates, 1995). Twenty commercial species are found in Australian waters. *Actinopyga*

echinites, A. mauritiana, A. miliaris, Holothuria atra, H. fuscogilva, H. scabra, H. whitmaei, Stichopus chloronotus, S. ocellatus, Thelenota ananas are the most common commercial sea cucumbers in Australia. The most diverse regions are the Torres Strait and Great Barrier Reef with 16 and 14 species respectively (Kinch *et al*., 2008).

Australia has a big production of *beche-de-mer*. Together with Melanesian countries; Australia is the largest exporter of this product in Western Central Pacific regions. In 2000, Australia exported around 150 tonnes of *beche-de-mer* including 146.5 to China Hong Kong SAR and 4.0 tonnes to Singapore (Kinch *et al*., 2008). The species contributing to the Australian production of *beche-de-mer* have changed over time. Analysis of the sea cucumber fishery in Great Barrier Reef from 1991 to 2011 showed the changing catch pattern of sea cucumbers. In the early stage, the high value teat fish (*H. whitmaei* and *H. fuscogilva*) dominated the production, while medium value species, burying blackfish (*A. spi*nea) and curry fish (*S. herrmanni*) became important in the later stage with 80% of the total production (Eriksson and Byrne, 2015).

1.4.2. The potential of *Holothuria atra*

H. atra or 'lollyfish' is one of the commercial sea cucumbers that inhibit Australian waters. It is found in the Coral Sea, Great Barrier Reef, Northern Territory and Western Australia (Kinch *et al*., 2008). In addition, as a commercial sea cucumber, *H. atra* product has been harvested and traded in at least 20 countries and island states in the western central Pacific region mainly for processed (cooked and dried) product as *beche-de-mer* or trepan (Purcell *et al*., 2012a). Recent studies show that *H. atra* contains bioactive compounds that have antioxidant and hepatoprotective activity against thioacetamide intoxication in rats (Esmat *et al*., 2013). Dakrory *et al*. (2015a) reported *H. atra* extract has an antioxidant role against nephrotoxicity induced by 7,

12-dimethylbenz (a) anthracene (DMBA) in male albino rats. *H. atra* extract has also been shown to have protective and curative effects against DMBA-induced hepatorenal diseases in rats (Dakrory *et al*., 2015b).

1.5. Sea cucumbers as functional foods and folk medicine

Sea cucumbers have a long history as a functional food in Asian cuisine. In China, the utilization of *beche-de-mer* is recorded since the Ming dynasty in 1368-1644 BC (Chen, 2005)). As a traditional medicine, sea cucumber is believed to maintain the blood, kidney, reproductive organs, reducing dryness as well as a vital essence. The other common utilizations are for mitigating weakness, constipation, impotency, and urination problems (Chen, 2003). Because of that, in China, sea cucumbers are more popular with the name haishen or 'ginseng of the sea'. Ginseng is a plant in the family Araliaceae that has similar bioactivity properties (Chen, 2005, Bordbar *et al.*, 2011). In addition, the functional properties of sea cucumbers make them luxurious seafood items (Fabinyi, 2012). Indigenous people in South East Asia have used water extracts of sea cucumbers as cures for cuts, peptic ulcers, chest pain, sores, inflammation and asthma (Taiyeb-Ali *et al*., 2003). A comprehensive review about sea cucumber as a functional food was published by Bordbar and others in 2011. They discussed high-value components and bioactive compounds of sea cucumber as potential sources for functional foods and nutraceuticals (Bordbar *et al*., 2011).

1.6. Bioactivity of sea cucumbers

Sea cucumber is a huge source of bioactive compounds with huge potential applications (Bordbar *et al*., 2011, Aminin *et al*., 2015, Shi *et al*., 2016). All body parts

of the sea cucumber contain metabolites with various functions and can be used as potential sources of nutraceutical, pharmaceutical and cosmeceutical products. The bioactive compounds in the body wall have been researched extensively because the wall is the main source of *beche-de-mer* (Hamel *et al*., 2001). Other parts of the sea cucumber including viscera (Bahrami *et al*., 2014) and eggs (Zhang *et al*., 2017) are also known as sources of bioactive compounds.

Review articles from Bordbar *et al*. (2011) and Shi *et al*. (2016) show that there are prolific biological and pharmacological properties of sea cucumber bioactive compounds, including anticancer, antitumor, anticoagulant, antimicrobial, antioxidant, antihyperlipidemic, anti-inflammatory, antihypertension and radioprotective, anti-angiogenic, and wound healing. These active compounds include triterpene glycosides (saponin), chondroitin sulfates, glycosaminoglycan (GAGs), acid mucopolysaccharide, sphingoid bases, glycolipids, fucosylated chondroitin sulfate, polysaccharides, sulfated polysaccharides, phospholipids, cerebrosides, phosphatidylcholines, sterols (glycosides and sulfates), phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids, collagen, gelatin, fucoidan, essential fatty acids and hydrolysates (Saito *et al*., 2002, Bordbar *et al*., 2011, Yu *et al*., 2013, Shi *et al*., 2016). Among these compounds, collagen and saponins have important roles. Collagen is known as the main constituent of sea cucumber body wall (Saito *et al*., 2002) while saponins are the most abundant secondary metabolites in sea cucumbers (Bahrami *et al*., 2014). In addition, sea cucumber saponins are known to have a wide range of bioactive properties (Caulier *et al*., 2011).

1.7. Saponins

Saponins are a group of metabolites that are found in terrestrial plants as well as in marine organisms, including soft corals, sea stars, sponges and sea cucumbers (Honey-Escandón *et al*., 2015). Saponins also are produced by bacteria (Elekofehinti, 2015). Saponins have surfactant properties and form foams when they are shaken in solution. It is that characteristic, that gives saponin its name coming from as the Latin word "sapo" meaning soap (Faizal and Geelen, 2013). Ecologically, saponins in the plant are related to the defense system from the pathogen and pest and for food quality (Francis *et al*., 2002). Saponins are used in the food industry as emulsifing, foaming, preservative and flavor agents (Güçlü-Üstündağ and Mazza, 2007). The potential applications of saponin for pharmaceutical purposes have been discussed in several review articles (Güçlü-Üstündağ and Mazza, 2007, Kim and Himaya, 2012, Netala *et al*., 2014).

Saponins are complex compounds that consist of two main parts, the sugar component (glycone) and non-sugar component (aglycone) (Figure 1.3) (Moghimipour and Handali, 2015). The complexity of their structure is due to the variety of aglycone structures, the nature of the side chains and the location of the attachment of glycone on the aglycone (Francis *et al*., 2002). Based on the aglycone structure, saponins are classified into steroidic aglycone and triterpenic aglycone saponins. The difference between steroidic and triterpenic backbones is that the former has 27 C atoms, while the latter has 30 C atoms (Moghimipour and Handali, 2015). Another classification is based on the number of the glycones that attach to the aglycones, they are divided into mono, di and tridesmosidic (Netala *et al*., 2014). The most common sugar attached to the aglycone are D-glucose, D-quinovose, D-glucuronic acid, D-galactose, Dgalacturonic acid, D- xylose, D-fucose, L-rhamnose and L-arabinose (Kim and

Himaya, 2012, Moghimipour and Handali, 2015). The type of structure influences the biological activities of the saponins (Kim and Himaya, 2012).

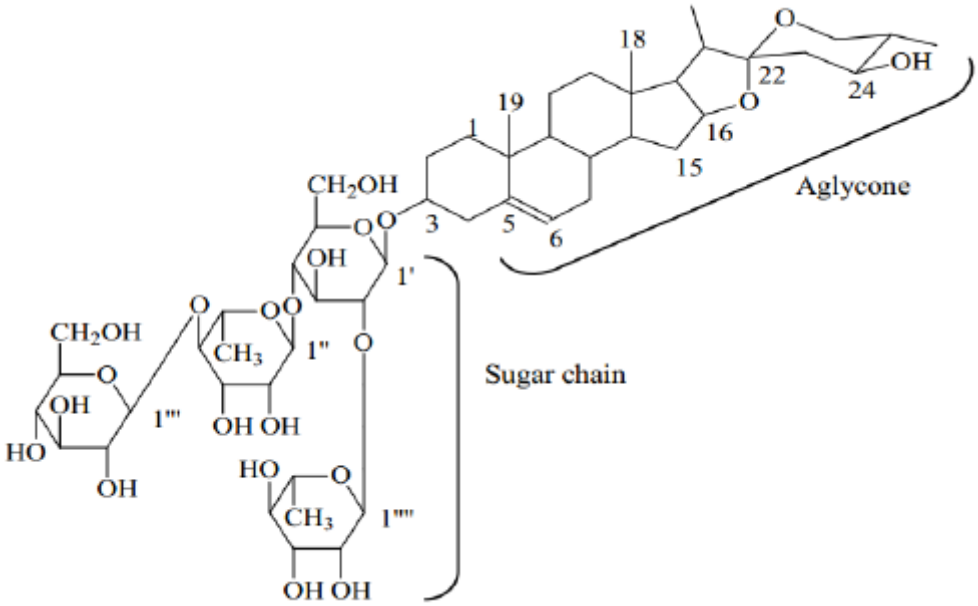


Figure 1.3. The structure of saponin (Moghimipour and Handali, 2015)

1.7.1. Sea cucumber saponins

The isolation of sea cucumber saponins was first reported by Nigrelli in 1952 and Yamanouchi in 1955 from different species (Honey-Escandón *et al*., 2015). Since then, more than 700 saponins have been isolated from sea cucumbers worldwide (Bahrami and Franco, 2015). Saponins are the most important and rich metabolites in sea cucumber (Kim and Himaya, 2012), in which the structure of holothurians saponins is mainly triterpenoid saponin (Kerr and Chen, 1995). The differences between plant and sea cucumber saponins are that in the sea cucumber the sulphate is only in one aglycone and the sugar chain has only one, while in the plant are in both aglycone and one and more sugar chains (Kalinin *et al*., 2005, Zhao, 2011).

Saponins in sea cucumbers are present in many organs. They are abundant in the body wall, cuvierian organs and gonad (Honey-Escandón *et al*., 2015). Recent research suggested that viscera of the sea cucumber *Holothuria lessoni* contain a higher

diversity and yield than body wall (Bahrami *et al*., 2014). Similar to terrestrial saponins, holothurian saponins were suggested to be a part of their defense mechanism. They also influence the reproduction process by organized of the oocyte, maintain for oocyte maturation and mediator of gametogenesis (Kalinin *et al*., 2008).

1.7.2. Extraction and isolation of saponins

The extraction and isolation of saponins can be done in several ways. The main steps of this process include grinding the material, soaking the material in a solvent and then further purification. The solvent, extraction conditions and properties of material are important factors that affect the efficiency and characteristics of the end product of saponins (Güçlü-Üstündağ and Mazza, 2007). In addition, utilization of additional treatments in the soaking step such as heat, ultrasound, microwave and pressure can assist the solvent extraction and have many benefits (Husni *et al*., 2009, Moghimipour and Handali, 2015).

The most common solvents for saponins extraction are alcohol (methanol, ethanol), water or a mixture of them. Other solvents also have been used to extract saponins such as alcoholic surfactant solutions, glycerine and solvent plus ammonia (Güçlü-Üstündağ and Mazza, 2007). While these independent solvent extractions are known as traditional methods, recent developments in extraction methodology increased their efficiency and made them more environmentally friendly by reducing time, solvent and waste without changing the output quality (Moghimipour and Handali, 2015). Microwave-assisted, ultrasound-assisted and accelerated solvent extractions are the modern technologies that are widely used for extraction. These additional treatments help to disrupt the structure of material and increase mass transfer of the solvent to the compound (Güçlü-Üstündağ and Mazza, 2007).

Furthermore, pressurized solvent extraction and heat reflux extraction, which use high temperature and pressure, are reported to increase the yield of saponins from sea cucumber (Husni *et al*., 2009). Another method is using super critical CO_2 that is considered to have benefits such as a freeing solvent in the product, easier removal of the solvent from the product and an oxygen free environment (Güçlü-Üstündağ and Mazza, 2007). Table 1.1 shows commonly used methods in the first step of extracting saponins, from ginseng and sea cucumber.

Table 1.1. Extraction methods of saponins with various parameters

Species	**Solvent**	**Temp °C**	**Other**	**References**
Ginseng	1. 55% ethanol 2. 88% ethanol	65-68 5-10	24 h stirring Overnight	Kang *et al*. (2009)
	1. Methanol	Hot		Sanada *et al*. (1974)
	1. Dichloromethane 2. 95% ethanol 3. Water	Boiled Boiled	60 min 90 min Sonicator 5 min	Lee *et al*. (2007)
	1. Water 2. 70% ammonium sulfate 3. 99% methanol	Boiled	60 min Reflux	Yokozawa *et al*. (2004)
	1. Water 2. Ultrafiltration	80	6 hours	Seol *et al*. (2014)
	1. 70%EtOH			Murthy and Paek (2016)
Stichopus japonicus	1. Water or 2. 70%EtOH	80	Pressurized solvent extraction (PSE) Heat reflux extraction (HRE)	Husni *et al*. (2009)
Holothurian arenicola	1. 70%EtOH 2. Methanol	 Boiled	Under reflux	El Nemr *et al*. (2012)
H. scabra *H. leucospilota* *S. chloronotus*	Water compare to Methanol			Althunibat *et al*. (2009)
H. axiloga	60% ethanol			Wei-Hua *et al*. (2008)

1.7.3. The potential applications of sea cucumber saponins

Holothurian saponins are reported to have a wide spectrum of potential applications and have been discussed in several review articles. The potential applications include cytotoxic and anticancer, antibacterial, antifungal, antiprotozoal, antiviral, antioxidant,

mutagenic, hemolytic, immunomodulation, neurotoxic, inhibition of Na^+, K^+ -ATPase, mitogenic, radioprotective and inhibition of root growth activities (Kalinin *et al*., 2008, Kim and Himaya, 2012, Aminin *et al*., 2015, Aminin, 2016). Among these bioactivities, anticancer and antiviral activities are the most widely studied (Kim and Himaya, 2012). However, the potential applications of sea cucumber saponins for cosmeceutical purposes are rarely reported.

The huge potential applications of sea cucumber saponins are through the membranotropic effects against cellular and model membranes. The saponins and Δ^5 and $\Delta^{5,7}$-sterols in the organism membrane formed single ion channels and large channels that are the principle of antifungal, antitumor, cytotoxic and hemolytic activities of sea cucumber saponins. This mechanism allows the increase of microviscosity and suppression of transport processes in the membranes (Augustin *et al*., 2011). Other properties of saponins also have key roles in the membranotropic activity such as an 18(20)-lactone, oxygen functional group, linear tetrasaccharide fragment, quinovose as the second monosaccharide and 3-Omethyl group (Kalinin *et al*., 2008).

1.8. Saponins for cosmeceutical applications

Sea cucumber has similar secondary metabolites to ginseng. Both of them contain a high abundance of saponins, even though the structures are different (Chu and Zhang, 2009, Kim and Himaya, 2012). The applications of ginseng saponins as cosmecutical ingredients have been already studied (Zhang *et al*., 2014, Hwang *et al*., 2015), whereas the study on sea cucumber saponins is still rare (Kim and Himaya, 2012). The studies of sea cucumber sapnins focused on a small part of cosmetic ingredient activities (antioxidant, antimicrobial, anti-inflammatory).

1.8.1. Cosmetics

Cosmetics have become an important need for human beings because of the good-looking concept. Good appearance is an important factor that influences human self-acceptance and socio-cultural relationship (Cipriani *et al*., 2016). Cosmetics are any materials that have the ability to clean, fragrant, increase the appearance, protect and preserve varied surface parts of the human body including skin, hair, nails, lips and also teeth. It can be used by rubbing, pouring, spraying or sprinkling the material to the body (de Castro, 2011). The demand of the cosmetic industry been increased over the past decade. Based on financial analysis report by Eurostaf Company, this industry is worth US$ 170 billion (Arora *et al*., 2012). Furthermore, Allied Market research is forecasting that by 2020, the industry would continually develop and would be worth US$ 390 billion (Kerdudo *et al*., 2016).

The new trends in the cosmetic industry are cosmeceuticals and nutricosmetics. Cosmeceuticals are the combination of cosmetic and pharmaceuticals, while nutricosmetics are cosmeceuticals in food form (Taofiq *et al*., 2016). Cosmeceutical products contain substances that affect the biological process in the parts of the body by affording essential nutrients for the body surfaces. These nutrients can increase the appearance, sparkle and health of the body (Hyde *et al*., 2010). Nutricosmetics are the newest development of skin care cosmetic products. Although has a similar purpose to improving the beauty of hair and skin, the application of the nutricosmetics is by eating the supplement (de Castro, 2011).

1.8.2. Skin aging

Many cosmetic products focus on skin protection. As the largest parts of the body, the skin prevents internal organs from environmental exposure as well as maintaining the depletion of water from the body (Kendall and Nicolaou, 2013). However, excessive exposure to the temperature, UV radiation, pollution, microbial and other external factors make the defense system in the skin weak and lead to changes in the skin structure. This phenomenon is known as skin aging (Taofiq *et al*., 2016). Changes in hormonal secretions are an undeniable mechanism from internal factor related to aging that also affects skin aging. It has a similar effect pattern to other internal organs (Papakonstantinou *et al*., 2012). UV radiation or photo aging agents, are reported especially as promoting photo-damage to the skin by changing the dermal extracellular matrix, resulting in wrinkles, weakening/roughening of the skin, spotted pigmentation of the skin, and thickening of the epidermal layer (Rittié and Fisher, 2002).

The mechanism of skin aging is related to the generation of reactive oxygen species (ROS) (Mukherjee *et al*., 2011, Leem, 2015, Tundis *et al*., 2015). It is described in Figure 4. Solar radiation is caught by skin layers. Melanin, as an absorber pigment in the skin, cannot absorb the excessive UV radiation more than its capacity. In addition, the antioxidant defense system that has the capacity to reduce free radical and ROS will become overwhelmed with a high level of UV. As a result, cellular components such as proteins, lipids, DNA and mitochondria are damaged (Pallela *et al*., 2010). Furthermore, accumulation of ROS generates activator protein-1 (AP-1) that is a transcription factor for collagen degradation by up regulating enzymes matrix metalloproteinases (MMPs). Overproductions of MMP-1, MPP-3 and MPP-9 lead to increased collagen breakdown (Leem, 2015). UV irradiation also causes reduction of collagen synthesis by down regulation of transforming growth factor-β (TGF-β), which

is a cytokine for collagen production promoter (Taofiq *et al*., 2016). Elastin and hyaluronic acid are other skin components that are influenced by ROS. It increases the production of elastase and hyaluroidase that promote elastin and hyaluronic acid degradation (Mukherjee *et al*., 2011). Therefore, the inhibitors of ROS, elastase, hyaluronidase, MMP enzymes can be potential cosmetic ingredients in the skin aging treatment (Taofiq *et al*., 2016).

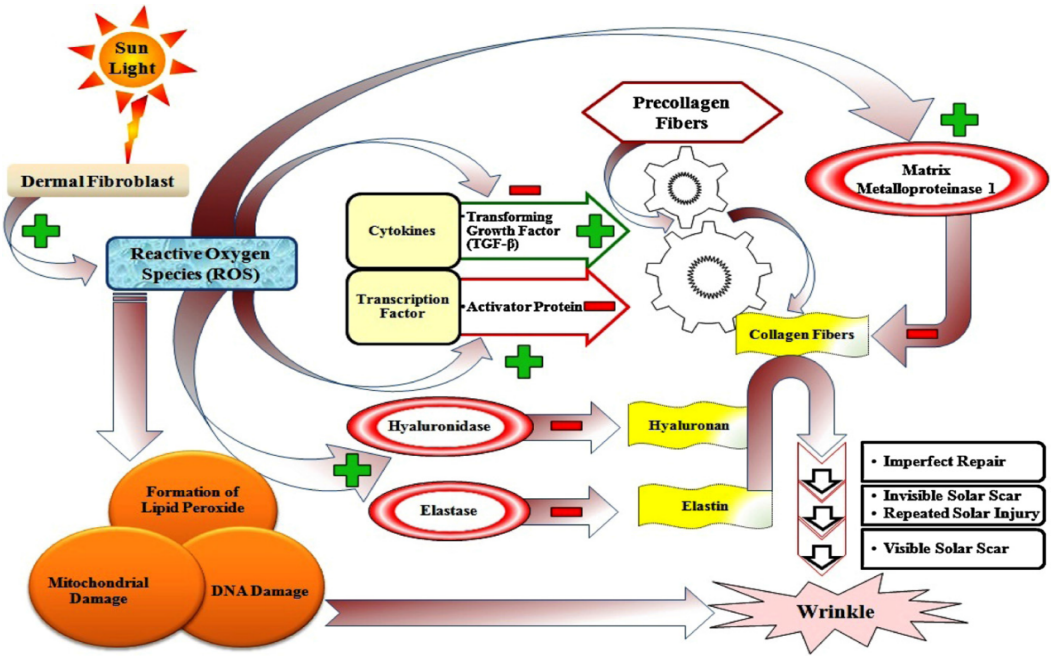


Figure 1.4. Mechanism of skin aging by UV radiation (+, increase; −, decrease). UV radiation induces ROS that damages lipids, DNA and mitochondria. It also up regulates MMPs via AP-1 resulting collagen degradation. In addition, ROS down regulates TGF-β, which is collagen production promoter. ROS also increases the levels of elastase and hyaluronidase that breakdown elastin and hyaluronic acid. The overall result changes in skin structures (Mukherjee *et al*., 2011).

1.8.3. Comparison of sea cucumber and ginseng saponins as cosmeceutical ingredients

The potency of cosmeceutical ingredients can be classified into several different activities. Those include skin whitening, MMP inhibition; anti-hyaluronidase, anti-tyrosinase, anti-collagenase, anti-elastase, UV and photo oxidative protection, antioxidant, anti-microbial, anti-inflammatory, anti-skin cancer and moisturizing and pigment agents (Wijesinghe and Jeon, 2011, Wang *et al*., 2015, Sanjeewa *et al*., 2016, Taofiq *et al*., 2016).

1.8.3.1. Anti-tyrosinase /Skin whitening

The skin's color is determined by melanin production. Melanin also protects skin from UV radiation, scavenging toxic drugs and chemicals (Sanjeewa *et al*., 2016). Overexposure to UV radiation produces an over generation of melanin in the skin, because of increasing tyrosinase activity. Tyrosinase converts tyrosine to eumelanin (brown-black pigment) (Taofiq *et al*., 2016). Ginseng saponins type ginsenoside F1 is reported to reduce up to 70% production of melanin in B16 melanoma cells (Yoo *et al*., 2011). In addition, picrionoside A from ginseng leaves have been reported to have anti-melanogenic effects (Lee *et al*., 2015). No such activity is yet to be reported in saponins from sea cucumbers. The activity is reported from glycoprotein fraction of sea cucumber byproducts in the dried cucumber production process (Kim *et al*., 2016).

1.8.3.2. MMPs inhibitor

MMPs (matrix metalloproteinases) are essential enzymes in the cellular process. they are involved in wrinkled skin formation by degrading collagen in the skin (Sanjeewa *et al*., 2016). Jung *et al*. (2006) described that ginseng saponins had the ability to suppress

MMP-9 through inhibition of the activator protein-1 and mitogen-activated protein kinase signaling mechanism. Furthermore, the supernatant of cells culture contained 20(R)-ginsenoside Rg3 from ginseng root was found contain less MMP-2 and MMP-9 concentration compared with control (Yue *et al*., 2006). Sea cucumber saponins also have the ability to inhibit MPPs. The sea cucumber *Pearsonothuria graeffei* was reported to contain Ds-echinoside A, a triterpene glycoside that exhibits inhibition of MPP-9 expression (Zhao *et al*., 2011).

1.8.3.3. Pro collagen and elastin production

Collagen and elastin are crucial elements of the skin and contribute to its elasticity, flexibility and strength. During the process of aging and UV exposure, the collagen and elastin levels decrease and result in the formation of wrinkles (Taofiq *et al*., 2016). Therefore, increasing the production of collagen and elastin and suppressing collagenase and elastase can inhibit wrinkle formation in the skin. Lee *et al*. (2007) and Song *et al*. (2012) reported that ginsenosides from ginseng increased type I collagen production. Furthermore, Meybeck *et al*. discovered that ginsenoside stimulated elastin synthesis (Meybeck *et al*., 1998). However, the functionality of saponins from sea cucumber in relation to stimulating collagen and elastin production has not been reported yet.

1.8.3.4. Anti-hyaluronidase

Hyaluronic acid is an important skin component besides collagen and elastin, which has roles as a skin rejuvenant, holding moisture, increasing viscosity and reducing the permeability of extracellular fluid (Taofiq *et al*., 2016). GinsenosideRH1 from *Panax ginseng* was known to have anti-hyaluronidase activity since it inhibited the activation

of nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and NF-kB in RAW 264.7 cells (Park *et al*., 2004).

1.8.3.5. UV protection

As mentioned above, UV radiation is one of the main causes of skin aging, which is also known as photo aging (Taofiq *et al*., 2016). Total saponins and ginsenoside Rb from red ginseng were identified to reduce skin thickness and wrinkle formation induced by UVB exposure via collagen production and/or the inhibition of MPPs (Kim *et al*., 2009). While no report from sea cucumber saponins is found; Sea cucumber glycosides, however, exhibited fix effect on mouse after radiation by increasing karyocytes of femoral bones and spleen cells (Aminin, 2016).

1.8.3.6. Antioxidant

All reactions in the cells produce free electrons known as free radicals and lead to the generation of ROS, reactive nitrogen species (RNS) and reactive sulfur species (RSS). Cells have a balanced mechanism of combating ROS called antioxidants, overproduction of ROS however cannot be overcome by this mechanism. Therefore, the body needs outer sources of antioxidants (Taofiq *et al*., 2016). Both ginseng and sea cucumber saponins were reported to have antioxidant activities. Chen *et al*. (2009) found that ginsenosides had scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical agent, while Husni *et al*. (2009) described that saponins from water extracts of body wall of sea cucumbers had scavenging activity against DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) radical cation (ABTS+).

1.8.3.7. Anti-microbial

Skin is a common habitat for microorganisms. The diversity and abundance of microbes in the skin depend on the age of individual and other environmental factors. Increasing the abundance of *Staphylococcus aureus* can lead to atopic dermatitis, which is a common skin disease (Taofiq *et al*., 2016). Various studies have reported anti-microbial properties of saponins from ginseng and sea cucumbers. Studies from Sung and (Sung and Lee, 2008) showed that a combination saponins and antibiotics had the synergistic or additive effect to the methicillin-resistant *Staphylococcus areus* (MRSA). Kaswandi *et al*. (2000) examined the antibacterial activity of sea cucumber saponins.

1.8.3.8. Anti-inflammatory

Inflammation is the complex cellular reaction in response to harmful stimuli to the vascular tissue by eliminating the causes of injury and initiating the healing mechanism in the tissue. One of the inflammatory stimuli is oxidative stress since it activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and AP-1 (Wijesinghe and Jeon, 2011). GinsenosideRH1 was reported to have an anti-inflammatory effect since it inhibited the activation of nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and NF-kB in RAW 264.7 cells (Park *et al*., 2004). Tissue fraction containing saponins from body wall of sea cucumber was also reported as useful for inflammation treatment in animal model assays (Collin, 1998).

The cosmeceutical activities of ginseng and sea cucumber saponins are summarized in Table 1.2. The saponins of ginseng have been more comprehensively studied than that of sea cucumbers.

Table 1.2. Summary of the cosmeceutical activity of ginseng and sea cucumber saponins (√ means the saponins activities have been reported)

Ingredients Activities	Saponins Ginseng	Saponins Sea cucumber	References
Skin whitening	√	√	Yoo *et al*. (2011) Lee *et al*. (2015) Kim *et al*. (2016)
MMPs inhibitor	√	√	Jung *et al*. (2006) Yue *et al*. (2006) Zhao *et al*. (2011)
Anti-hyaluronidase	√		Park *et al*. (2004)
Pro collagen production	√		Lee *et al*. (2007) Song *et al*. (2012)
Pro elastin production	√		Meybeck *et al*. (1998)
UV Protection	√		Kim *et al*. (2009)
Antioxidant	√	√	Chen *et al*. (2009) Husni *et al*. (2009)
Anti-inflammatory	√	√	Park *et al*. (2004) Collin (1998)
Antimicrobial	√	√	Sung and Lee (2008) Kaswandi *et al*. (2000)

1.9. Commercial cosmetics from sea cucumbers

Although there are few studies on the suitability of sea cucumber saponins for cosmetic purposes, many sea cucumbers based cosmetic products are currently available in the market. Skin N Sea Cucumber Mystery Cream is anti-wrinkle and whitening. The ingredient of the product is not explained (http://www.tradekorea.com). Haishen 80 Sea Cucumber Nutritive Fresh Cream is a hydrating moisturizer that claims to brighten skin and reduce the appearance of fine lines and wrinkles. This product contains 80% of sea cucumber extract (http://www.hikoreanfashion.com). Another product is Prime Youth Black Sea Cucumber Mask Sheet. All sea cucumber cosmetic products come from South Korea.

1.10. Aims and scope

The main purpose of this project is to determine the potential applications of sea cucumber extracts for skin care and cosmeceutical ingredients especially for anti-oxidant and anti-aging properties. The scope of the project includes:

1. Optimization of the extraction method of compounds from sea cucumbers.
2. Test the toxicity of these extracts by *in vitro* skin cell-based assays to establish the safe doses.
3. Screen the extracts for their functional characteristics, including antioxidant and 'anti-aging' actions (mainly on collagen stimulating assays).
4. Chemical characterizations of the extracts include, saponins, sugars, phenolics, protein content and TLC profile.

1.11. Hypothesis

The hypothesis of this study is that the sea cucumber extracts have potential applications for anti-aging similar to saponins from ginseng. In addition, the different extraction processes and solvents will produce different characteristics of extract which influence the bioactivity of the extracts.

1.12. Research significanc

The study of the development of functional ingredients from sea cucumbers for cosmeceutical products is the first attempt to determine the potential application of sea cucumbers for increasing collagen production. As a successful result, this study may promote the potential application of sea cucumber for cosmetic products ingredients. This research completes the potential applications of sea cucumber not only for

nutraceuticals and pharmaceuticals but also for cosmeceuticals. These varied applications will increase the sea cucumbers' demand which is mainly obtained from wild sources. Therefore, the conservation and aquaculture aspect of sea cucumber are also important factors that need to be considered.

CHAPTER 2

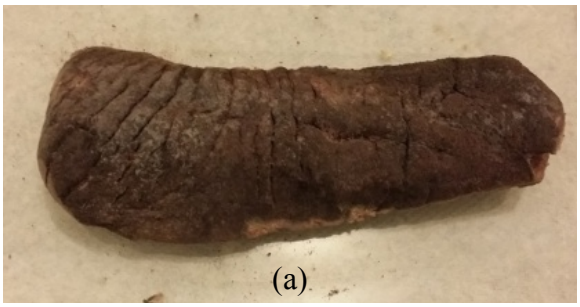
MATERIALS AND METHODS

2.1. Reagents

The reagents used in this study were purchased from Sigma-Aldrich (USA) unless stated otherwise (Appendix 1). The chemicals were of analytical grade. Water used in this study was high purity Milli-Q grade from the Milli-Q® Academic system (Millipore, USA).

2.2. Sea cucumber sample

Dried *Holothuria atra* body wall was used in this study (Figure 2.1). The sample was collected from Chanel Island, North Territory, around 2011 from previous studies and kept at -20°C. *H. atra* is one of the commercial species from Australian water. The body wall of this species mainly used for *beche-de-mer*. Recent studies show that the body wall of *H. atra* extract contains bioactive compounds that have antioxidant and hepatoprotective activity (Esmat *et al*., 2013). The dried samples were blended into powder using high speed blender (Blendtec classic 475, Blendtec, USA) and stored at -20°C before used.



(a)

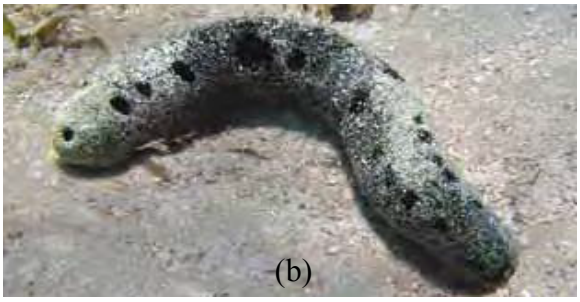


Figure 2.1. (a) Dried *H. atra* body wall sample collected from Chanel Island, North Territory used in this study; (b) Under water image of *H. atra* (Purcell *et al*., 2012b)

2.3. Extraction optimization procedures

Optimization of sea cucumber extracts was conducted at three different conditions, shaking at 22°C (room temperature), shaking at 60°C, and sonication at 22°C (Table 2.1). Two solvents, water and 70%EtOH (v/v) were used in the extraction process. The temperature at 22 and 60°C were chosen because they could compare the effectivity of the extraction processes between room temperature and high temperature conditions. In addition, it was reported that extraction using high temperature increased the yield of ginseng saponins (Yokozawa *et al*. 2004). The sea cucumber powder was extracted at the ratio 1:5 (w/v) for 30 min in 50 mL Falcone tubes. After 30 min extraction, the extract was centrifuged at 4.000 x g, 4°C for 10 min. The extractions were conducted four times and the supernatant was collected together. New solvent was added each extraction steps to eliminate the saturation level of solvent that will decrease the effectivity of extraction process. In the shaking at 22°C, the supernatant of each extraction was separated for determining the efficiency of extraction at each stage.

All extracts were dried using a freeze dryer (Virtis, USA), rotary evaporator (Rotavor R; Buchi Labortechnik AG, Switzerland) and centrivap concentrator (Labconco, USA). The water extracts were directly dried in the freeze dryer. 70%EtOH extracts were

concentrated by evaporating the ethanol in a rotary evaporator under vacuum (200 – 75 mba) at 45°C or centrivap concentrator for 8 – 9 h. Afterwards, the remaining solvents in the extracts were dried in the freeze dryer. The dried extracts were kept in -20°C until used. The yield of extracts from the sea cucumber powder was calculated as follows.

Equation 2.1

Extract yield (%)

$$= \frac{weight\ of\ dried\ extract}{weight\ of\ dried\ sea\ cucumber}\ x\ 100\%$$

Table 2.1. Extraction optimization of sea cucumber

Types of extraction	Temperature	Shaking	Apparatus
Shaking	22°C (room temperature)	90 rpm	Orbital Mixer Incubator (Ratek, Australia)
Shaking	60°C	90 rpm	Orbital Mixer Incubator (Ratek, Australia)
Sonication	22°C (room temperature)		Bransonic 12 (Emerson Industrial Automation, USA)

2.4. Cytotoxicity of *H. atra* extracts

The cytotoxicity assay was conducted using the crystal violet method. There are various methods that have been used in the cytotoxicity assay including 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), neutral red and crystal violet (Ishiyama *et al*., 1996). The mechanisms of these methods are different. MTT assay is based on the activity of mitochondrial dehydrogenase of the cells to convert yellow MTT to purple formazan (Mosmann, 1983, Young *et al*., 2005). While neutral red and crystal violet methods are primarily based on lysosome activity and staining of DNA-associated proteins, respectively. In the crystal violet assay, the

amount of dye taken by the cell monolayer can be quantified by a plate reader. Crystal violet assay is known as a simple method to obtain the relative density of cells. However, the staining of crystal violet dye to the DNA and the color that is formed are dependent on the pH of the solution (Vega-Avila and Pugsley, 2011). In addition, after the culture reaching confluence, the absorbance of the stained cells does not correspond to the viable cell numbers (Chiba *et al*., 1998). In this study, the crystal violet assay was modified from Mosmann (1983) and used to determine the concentration of extracts that are non- toxic to CRL 2076 skin fibroblast cells by measuring the cell viability.

2.4.1. Cell line and cell culture

Cell line CRL 2076 was used in the anti-skin aging assays. CRL 2076 is adherent skin fibroblast cells. Skin fibroblast constructs dermal skin, which is responsible for skin aging process. This skin mainly consisted of skin proteins and matrixes. The cells were obtained from the American Type Culture Collection (ATTC). ATCC-formulated Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, USA) was used to preserve or subculture the cells. Preparation of a complete growth medium was by adding 10% (v/v) Fetal Bovine Serum (FBS) (DKSH, Switzerland), 2.5 mL of 10,000 units/ mL Penicillin-Streptomycin Solution (Sigma-Aldrich, USA) and 5 mL of 200 mM L-glutamine solution (Sigma-Aldrich, USA) to make 500 mL IMDM medium. Every 6 to 8 d the cells were subcultured with change 50% of the medium every 3 to 4d.

Maintaining or subculturing the cells was conducted by aspirating the old medium and then washing with phosphate buffer saline (PBS) twice. In order to detach the cells from the flask, 1 mL of 0.25% (w/v) trypsin-ethylenediaminetetraacetic acid (EDTA)

solution was used. The flask, then incubated for 5 min at 37°C in 5% (v/v) CO_2 humidified incubator (MCA-18AIC, Japan) and 5 mL of IMDM was added to neutralize the trypsin. The cells were then centrifuged at 153 x g for 5 min and the supernatant was aspirated after centrifugation. The cells were resuspended in 1 mL of IMDM before the cell count. For the cell count, 50 µL of cell suspension was mixed with 50 µL of trypan blue solution and 10 µL of the mixture was transferred onto a hemocytometer. The number of viable cells (colorless) was recorded with the assistance of a microscope (Leitz, Germany) under 100x magnification. After calculating using Equation 2.2 and Equation 2.3, an appropriate volume of resuspended cells was added into 75 cm^3 culture flasks containing cells density of 3 x 10^5 cells. The prepared culture flask was then incubated at 37°C in a 5% (v/v) CO_2 humidified incubator for further use.

Equation 2.2

Cell concentration (cells/mL)

$$= \left(\frac{Total\ number\ of\ viable\ cells}{Total\ number\ of\ hemocytometer\ squares\ used}\right) x\ dilution\ factor\ x\ 10^4$$

Equation 2.3

Volume of cell suspension added into culture flask (mL)

$$= \frac{Sedling\ density}{Cells\ concentration}$$

2.4.2. Standard curve

A concentration of skin fibroblast cells CRL 2076 ranging from 0 to 40,000 cells/ well was set up in the crystal violet assay. A cell stock with a density of 0.8 x 10^6 cells/mL was prepared and then 100 µL of the stock cells suspension was added into a well containing 100 µL IMDM to give a final concentration of 40,000 cells/well. Six

replicates were used for each cell density. A serial dilution was performed by transferring 100 µL from the well with the highest density (40,000 cells/mL) to the next well. Therefore, cell densities of 40,000, 20,000, 10,000, 5,000, 2,500, 1,250 and 0 cells/mL were established for the standard curve. In order to avoid the evaporation of media, 100 µL of PBS was added into the wells surrounding the cells. The plate was then incubated at 37°C in 5% (v/v) CO_2 humidified incubator for 24 h.

2.4.3. Treatment plates

A density of 10,000 cells/ well was used in the treatment plate. For this purpose, 100 µL of 1 x 10^5 cells/ mL was added into 96 well flat-bottom microplates. The cells were then incubated for 24 h at 37°C in 5% (v/v) CO_2 humidified incubator. Both water and 70%EtOH sea cucumber extracts from shaking at 60°C and sonication were used. The concentrations of the extracts were 0, 2.5, 5, 7.5, 10 and 50 µg/mL and prepared using IMDM medium as a solvent. Included in the treatment were control solvents, which were 1% water and 1% ethanol in IMDM. Six replicates were set up for each concentration of extract and control. After 24 h incubation at 37°C in 5% (v/v) CO_2 humidified incubator, the medium from the plate was aspirated off and 100 µL of the diluted extract was introduced into the corresponding well. The plate was then incubated at 37°C in 5% (v/v) CO_2 humidified incubator for 48 and 72 h.

2.4.4. Crystal violet staining assay

After incubation for 24 (for standard curve), 48 and 72 h (for treatment), the cells were stained with crystal violet. This method is based on the binding of the dye to the DNA-associated proteins of the cells. The number of the dye binding is linear with the growth

or reduction rate of the cells (Ishiyama *et al*., 1996). Briefly, the medium was aspirated off from each well before the staining step. Into each well, 50 µL of 0.5% (w/v) crystal violet (0.5 g crystal violet dissolved in 100 mL of 50% (v/v) methanol) was added and incubated for 10 min. The dye was cleaned with RO water and then the plate was left to dry for 15 min to overnight at room temperature. The crystal violet was de-stained by adding 50 µL of 33% (v/v) acetic acid to each well. The plate was left for another 10 min. The absorbance was read at wavelength of 570 nm with 630 nm as a reference wavelength (µQuant, Bio-Tek Instruments, Inc., USA). Cell viability was determined using Equation 2.4.

Equation 2.4

Cell viability

$$= \frac{Treatment\ cells\ number}{Control\ cells\ number}\ x\ 100\%$$

2.5. Anti-aging properties of *H. atra* extracts

Determination of anti-aging properties of *H. atra* extracts was conducted by analyzing the antioxidant activity of these extracts and the effects of these extracts on collagen production of human skin fibroblast cells CRL 2076.

2.5.1. Antioxidant properties

Antioxidant properties of sea cucumber extracts were analyzed using Ferric Reducing Antioxidant Power (FRAP). This assay is based on the ability of antioxidants to reduce electron transfer (Fe^{3+} to Fe^{2+}) in the presence of tripyridyltriazine (TPTZ) and form blue Fe^{2+} - TPTZ complex (Benzie and Strain, 1996). The advantages of FRAP assay are simple, rapid, inexpensive, and strong and does not require specialized equipment.

While, the disadvantages are Fe^{2+}, a well-known prooxidant can react with hydrogen peroxide (H_2O_2) to produce hydroxyl radical, which is the most harmful free radical found *in vivo*. In addition, FRAP assay also cannot detect molecules that action by radical quenching (H transfer), particularly thiols and proteins (Alam *et al*., 2013).

The sea cucumber extracts were prepared in 10 mg/mL (w/v) concentration using MilliQ water. The stock reagents included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6- tris(2-pyridyl)-s-triazine) reagent in 40 mM HCl and 20 mM ferric chloride hexahydrate ($FeCl_3.7H_2O$). The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.7H_2O$ solution. The FRAP reagent was incubated for 10 min at 37°C before use. The extracts (10 µL) and 30 µL of milliQ water were reacted with 300 µL of the FRAP solution for another 4 min at 37°C. The colored products (ferrous tris (2-pyridyl)-s-triazine complex) were then read at 593 nm (µQuant Microplate Spectrophotometer, BioTek Instrument Inc., USA) against a blank consisting of 300 µL of the FRAP, 10 µL of sample solvent and 30 µL of milliQ water. As a standard curve was used $FeSO_4.7H_2O$ (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM). The results were expressed in mM equivalent $FeSO_4.7H_2O$ /mg of extract using Equation 2.5.

Equation 2.5

FRAP value (mM $FeSO_4.7H_2O$ equivalents/ mg extract)

$$= \left(\frac{absorbance\ at\ 593\ nm}{slope}\right) / extract\ concentration$$

2.5.2. Collagen production

2.5.2.1. Treatment method

CRL 2076 skin fibroblast cells were trypsinised and resuspended in complete IMDM as previously described. Cells were incubated at a density of 4 x 10^4 cells/0.5 mL in a 24-well plate and incubated at 37°C in 5% (v/v) CO_2 humidified incubator for 24 h to allow the cells to attach to the plate surface. The safe doses of the extracts from cytotoxicity tests were chosen for this assay. The extracts were diluted with IMDM and then 0.5 mL of these extracts were added to the corresponding wells of a 24-well flat bottom plate to achieve the final concentration of treatment. The medium was aspirated off before adding the sea cucumber extracts. The plate was incubated for 72 h at the same condition as described previously. After incubation, the cell supernatant was pipetted out and centrifuged at 12.000 x g for 20 min at 4°C. The cell-free supernatants were then saved and stored at -80°C until used in the bioassay.

2.5.2.2. Collagen assay

Collagen assay quantifies collagen in culture medium released by skin fibroblast cells. The collagen concentration in the cells was reported not significantly compared to in the media (Biocolor, 2011). The soluble collagen in the medium then analyzed using Sircol ™ Collagen Assay Kit (Biocolor, UK). This assay is based on a dye binding method that assesses the level of newly synthesized collagen production during cell growth and development. The method was based on the manufacture's protocol with some modifications. 50 µL of the sample was mixed with 500 µL of Sircol dye reagent and then shaken for 30 min. The mixture was centrifuged at 12,000 x g for 10 min and the supernatant was removed. The pellet was washed with 350 µL of ice-cold acid salt wash reagent and centrifuged at 12,000 x g for 10 min. The supernatant was removed

and 250 µL of alkali reagent was added to the pellet and vortexed to detach the dye from collagen. The solution was then left at room temperature for 10 min to allow the dye dissolved. The effect of sea cucumber treatment to the collagen release was measured at 540 nm. A standard curve from rat tail acid-soluble collagen type II with concentrations 0, 15.63, 31.25, 62.5, 125, 250 and 500 µg/mL was used as a standard. Collagen concentration was calculated using Equation 2.6 and expressed as µg/mL. A negative control (media treatment) was used as a blank to normalize the concentration of all treatment samples (Equation 2.7).

Equation 2.6

Soluble collagen concentration (µg/mL)

$$= \frac{Absorbance\ at\ 540}{Slope}$$

Equation 2.7

Soluble collagen concentration of treatment (CT) (µg/mL)

$$= (CS - CM) - (CE - CM)$$

CT: soluble collagen concentration of treatment
CS: soluble collagen concentration of sample
CE: soluble collagen concentration of extract
CM: soluble collagen concentration of media

2.6. Chemical characterisation of *H. atra* extracts

2.6.1. Saponins content

The estimation of the saponins content of sea cucumber was conducted using vanillin-sulfuric acid colorimetric test adopted from Bondoc *et al*. (2013) with some modifications. Saponins are oxidized by sulfuric acid and OH group at their C-3 position interact with vanillin to give chromogens. Another method is using a

hemolytic assay. The principle of this method is releasing oxy-hemoglobin which gives measurable color from the reaction of saponins and blood reagent (Cheok *et al*., 2014). The sulfuric acid-vanillin method has the limitation that it is not specific. Some compounds such as sterols and bile acids have a hydroxyl group in the 3-position and give chromophores when reacting with sulfuric acid and vanillin (Baccou *et al*., 1977). Sea cucumber extracts were prepared in 10 mg/mL (w/v) concentration using MilliQ water. The samples (20 µL) were added to 5% (w/v) vanillin in ethanol. The mixture then added with 500 µL of 72% Sulfuric acid. The solution was incubated at 60°C in a dry block heater (Ratex Instrument) for 10 min and cooled in cold water. Before reading absorbance at 540 nm (µQuant Microplate Spectrophotometer), the solution was vortexed. A serial concentration of Quillaja bark saponins (0, 0.5, 1, 2, 3, 4 and 5 mg/mL) was constructed for a standard curve. The saponin content was expressed as mg of Quillaja bark saponin equivalents per mg of extract and mg of Quillaja bark saponin equivalents per gram of dried sea cucumber using Equation 2.8 and Equation 2.9 equations respectively.

Equation 2.8.

Total saponin content (mg Quillaja bark saponin equivalents/ mg extract)

$$= \left(\frac{absorbance\ at\ 540\ nm}{slope}\right) / exract\ concentration$$

Equation 2.9.

Total saponin content (mg Quillaja bark saponin equivalents / gram dried sea cucumber)

$$= Total\ saponin\ content\ (mg/mg\ extract)\ X\ extract\ yield\ (\%)$$

2.6.2. Sugars content

The sugars content of *H. atra* extracts was determined by Anthrone-Sulfuric acid method based on Hansen and Møller (1975) with some modifications. Sulfuric acid hydrolyzed and digested sugars in the sample. The product interacted with anthrone producing color. This method is easy to conduct, requires only 0.1 to 1 pmol amounts of sugar and gives accurate results in a narrow concentration range (5- to l0-fold). However, this method has a large variation in molar absorptivities for various sugars (Gerchakov and Hatcher, 1972). In addition, compared to HPLC method, sulfuric acid – the anthrone method gives a slightly higher number of sugars (Irick *et al*., 1988).

Sea cucumber extracts were prepared in 0.5 mg/mL (w/v) concentration using MilliQ water. 0.5 mL of sample was reacted with 1 mL of 3% anthrone in sulfuric acid (w/v). The solution was incubated at 100°C for 5 min using oven (Conthervan) and cooled on ice for another 5 min. The absorbance was read at 620 nm (μQuant Microplate Spectrophotometer) against a blank. A 0.25 mg/mL of D-Glucose was diluted to make a standard curve. The sugar concentration was expressed as mg D-Glucose equivalents per mg of extract using Equation 2.10.

Equation 2.10.

Sugar content (mg D-Glucose equivalents/ mg extract)

$$= \left(\frac{absorbance\ at\ 620\ nm}{slope}\right) / extract\ concentration$$

2.6.3. Total phenolics content

The contents of total phenolics in sea cucumber extracts were analyzed based on Folin-Ciocalteu method described by (Singleton and Rossi, 1965) with some modifications. The Folin-Ciocalteu's reagent contains molybdenum and tungsten at oxidation state.

However, in the presence of phenolic compounds, molybdenum and tungsten form a blue complex. The color strength is linearly correlated to the concentration of the phenolic compounds. The Follin assay is easy to conduct and produces consistent estimates of the phenolics within a sample. However, there are some limitations of this method. Folin reagents may be unsuitable to compare of phenolic samples from different sources because the absorbance of the reagents is influenced by structural variation of phenolics. In addition, the presence of inhibitor metabolites may affect the result (Appel *et al*., 2001).

The sea cucumber extracts were prepared in 10 mg/mL (w/v) concentration using MilliQ water. 50 µL of Folin-Ciocalteu's reagent and 1 mL of MilliQ water were added to 200 µL of samples and mixed thoroughly. The mixture was incubated for 7 min at room temperature. To the mixture, 290 µL of sodium carbonate (200 g/L) was added and allowed to stand for 1 h at room temperature to allow the reaction to complete. The absorbance of the mixture was read at 760 nm (µQuant Microplate Spectrophotometer) against a blank. A standard curve was obtained using various concentrations of gallic acid (0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.8 and 0.1 g/L). Results were expressed as microgram (µg) of gallic acid equivalents (GAE) per mg of extract using Equation 2.11. To determine whether the total phenolics concentration and antioxidant capacity of sea cucumber extract have a relationship, the value of both properties was analyzed using regression method. One way ANOVA was used to determine significance level of this correlation.

Equation 2.11.

Total phenolics concentration (µg GAE/ mg extract)

$$= \frac{\left(\frac{absorbance\ at\ 760\ nm}{slope}\right) X\ 1000}{extract\ concentration}$$

2.6.4. Soluble protein

The total soluble protein in the sea cucumber extracts was analyzed using DC Protein Assay (BioRad, USA). This method is adopted from Lowry assay with some modifications to reduce the time of analysis. This method combines the reactions of peptide bonds and copper ions under alkaline environment to produce Cu+, which further react with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction) (Lowry et al., 1951). The protocol followed the manufactures instruction using Microplate Assay Protocol. Sea cucumber extracts were prepared at 10 mg/mL (w/v) concentration using MilliQ water. 5 µL of the sample was pipetted into 96-well microplates.25 µL of reagent A (an alkaline copper tartrate solution) and 200 µL of reagent B (a dilute Folin Reagent) were added to the sample. The plate was gently agitated to mix the reagents and left for 15 min. The absorbance was read at 750 nm (µQuant Microplate Spectrophotometer). A serial dilution (0 – 1.4 mg/mL) of Bovine Serum Albumin (BSA) was used as a standard curve. The soluble protein was expressed in mg per mg extract using Equation 2.12.

Equation 2.12.

The soluble protein yield (mg/mL) per extract

$$= \left(\frac{absorbance\ at\ 750\ nm}{slope}\right) / extract\ concentration$$

2.6.5. Thin-layer Chromatography (TLC) analysis

The fingerprinting of *H. atra* body wall extract was analyzed using TLC. TLC is a chromatographic method used to separate compounds from the mixture using a thin stationary phase and mobile phase. Mixed compounds will separate based on different affinities of compounds to the stationary and mobile phases. The different compounds

will move at different speeds. *H. atra* extracts were dissolved in 90% methanol at concentration of 50 mg/mL. A silica gel 60 F254 aluminium sheets (Merck #1.05554.0001) was used as a stationary phase. Fifteen microliters of the extract were loaded to the plate and developed using lower phase of chloroform: methanol: water (7: 13: 8) mobile phase system. The separated compounds were observed with UV light at 254 and 365 nm. The TLC plate was further stained using iodine vapor method (Wang and Benning, 2011). The TLC plate was put into sealed box container. A 250 mg of iodine was put in the container and left for approximately 20 min to let the iodine saturate and develope color in the plate. The Retention factor (Rf) of each compound was calculated using

Equation 2.13.

Equation 2.13.

$$Rf = \frac{Movement\ distance\ of\ compound}{Movement\ distance\ of\ solvent}$$

2.7. Correlation between chemical characteristics of extract and collagen production

The correlation between chemical profiles of extract and the increasing of collagen production of human skin fibroblast cells CRL 2076 was analyzed using a regression test and the significance level was found using one-way ANOVA. Four samples (shaking at 60°C and sonication at 22°C for both water and 70%EtOH extracts) were tested. The value of each extract's chemical characters including saponins, sugar, protein, phenolics and antioxidant content were transformed from extract treatment concentration (2.5 mg/L). The correlation coefficient (R^2) and the significant number (p) were described.

2.8. Statistical analysis

All measurements and analysis were conducted for at least three independent experiments except extraction optimization with only one independent experiment with three replicates. The results were averaged and presented as mean ± standard deviation (SD). Statistical analysis was performed with IBM SPSS 22 software. Significance variables were calculated using one-way ANOVA followed by Tukey's post-hoc test with a significance level 95% (p<0.05). The correlation analysis was measured with regression test.

CHAPTER 3

RESULTS

3.1. Extraction optimization of *H. atra*

The efficiency of each extraction stage was analyzed for the shaking at 22°C. The yield of extract of each extraction stage was measured and shown in Figure 3.1. At first extraction, the water solvent produced 7.58±0.18% extract yield, while the 70%EtOH produced10.88±0.23%. At the second extraction, water and 70%EtOH solvents gained 11.37±0.37% and 5.23±0.17% yield respectively. While, the third extraction took out 5.42±0.09% of yield for water and 2.75±0.07% of yield for 70%EtOH. The last extraction only produced below 2% for both solvents. The total extract yield of water and ethanol 79% were 26.36±0.15 and 19.81±0.21%, respectively. The extracts of shaking at 60°C and sonication at 22°C were collected together from the four extraction stages.

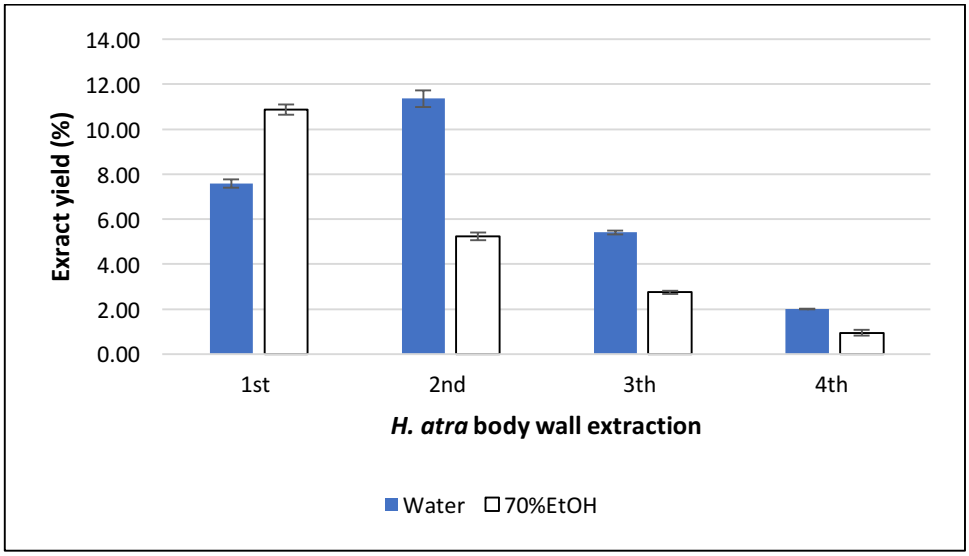


Figure 3.1. *H. atra* body wall extract yields from each stage of extraction of four sequence extractions in the shaking at 22°C. The sea cucumber powder was extracted at a ratio of 1:5 (w/v) for 30 min at 90 rpm. Values are expressed as mean ± SD (n=3). Values are calculated based on the dry weight of extract compared to dry weight of body wall multiplied by 100.

The yield of *H. atra* extract was different, depending on the types of extractions and the solvents (Figure 3.2). Generally, water extracts produced a higher yield compared to 70%EtOH extracts and this was significantly different (p<0.05). The yields are between 26±15% to 29±2.93% and 19±0.21% to 24±1.91% for water and 70%EtOH respectively. The yield of water extract was not significantly different between all extract conditions. The 70%EtOH extract yield from shaking at 60°C (24.10±1.57%), was however, significantly different (p<0.05) compare to shaking at 22°C and sonication. The two higher extract yield (shaking at 60°C and sonication 22°C) for both water and 70%EtOH extracts were further analyzed for cytotoxicity against human skin fibroblast cells CRL 2076.

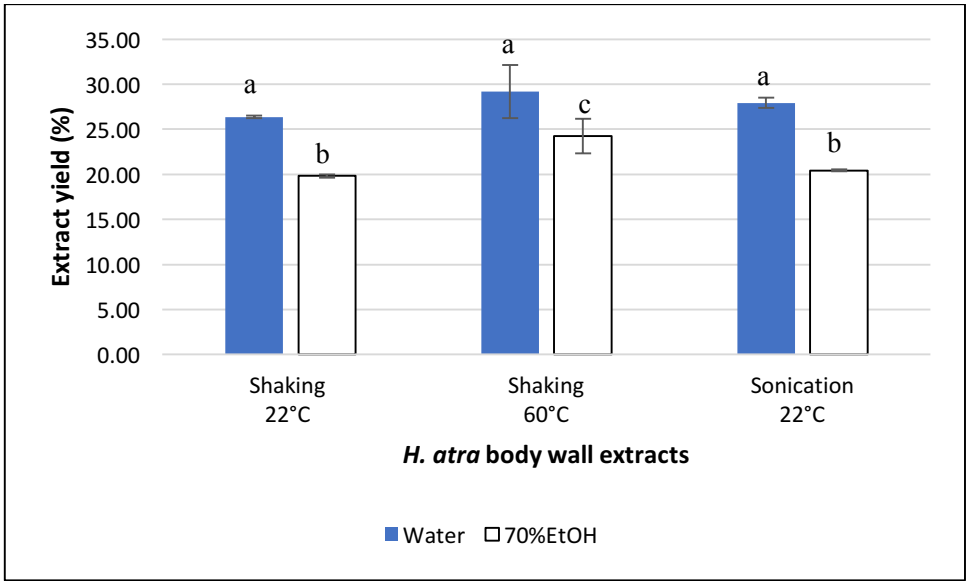
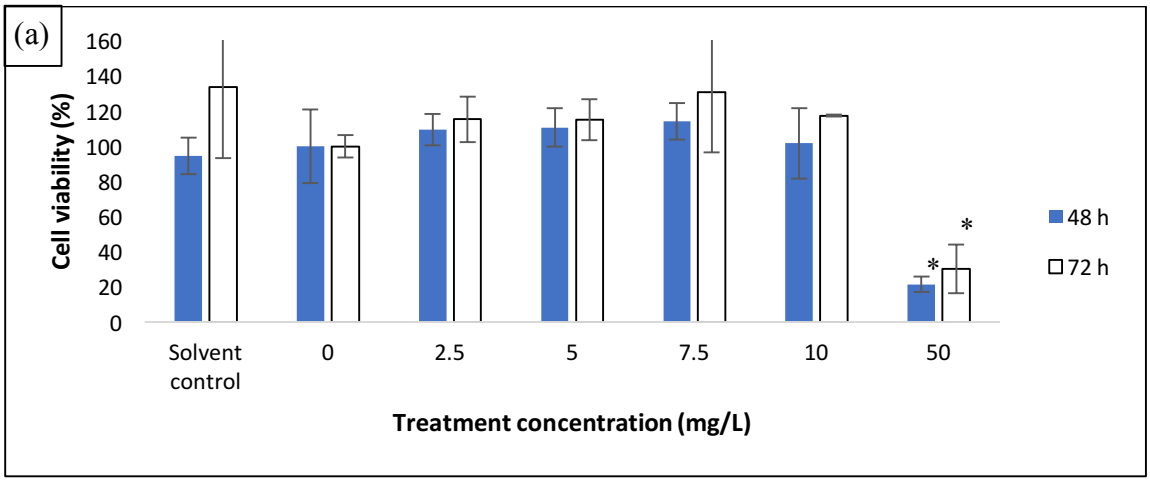
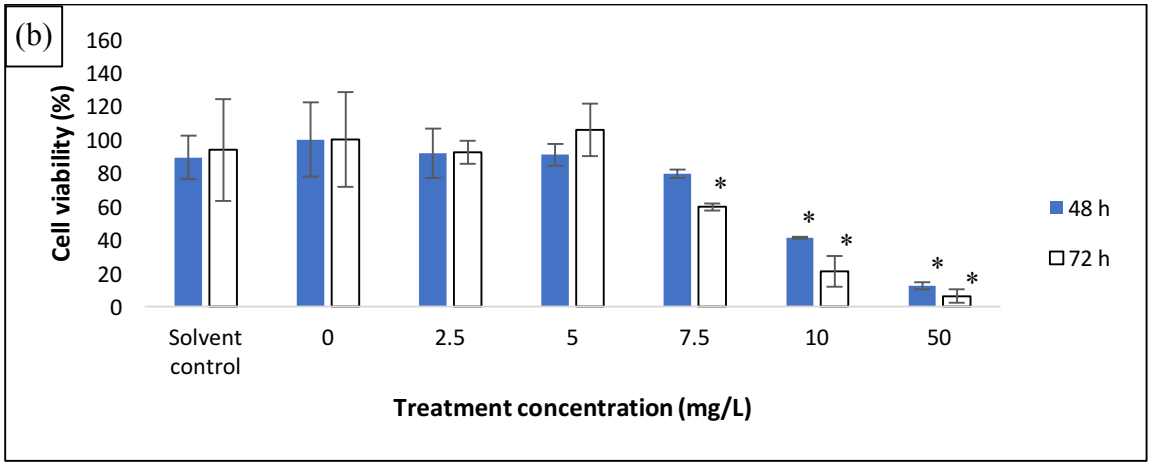


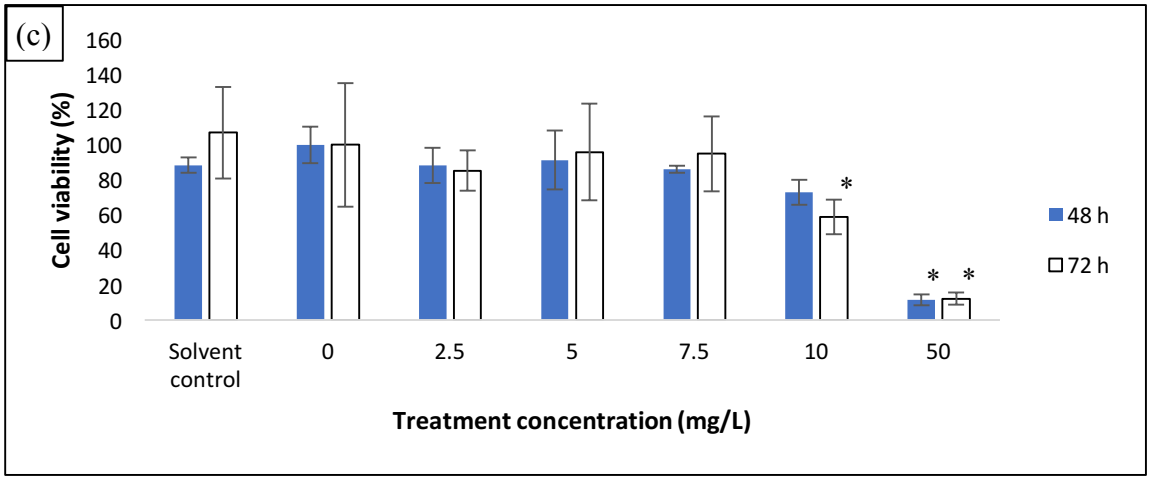
Figure 3.2. *H. atra* body wall extract yields from different extraction conditions and solvents. The sea cucumber powder was extracted at a ratio of 1:5 (w/v) for 30 min of each cycle extraction from a total of four extractions. Shaking extraction was conducted at 90 rpm and sonication with continuous sonication. Values are expressed as mean ± SD (n=3). Values are calculated based on the dry weight of extract compared to dry weight of body wall multiplied by 100. Values in the same solvent with different letters are significantly different at p< 0.05.

3.2. Cytotoxicity of *H. atra* extracts

Ingredients of cosmeceutical products should have no toxicity to the skin, therefore the cytotoxicity of sea cucumber extracts was analyzed against normal skin cells. Serial concentrations of extract from 0, 2.5, 5, 7.5, 10 and 50 mg/L were tested. The results are presented in Figure 3.3. The cytotoxicity of *H. atra* extracts was different depending on the extract conditions and solvents. The water extract of shaking at 60°C had no significant effect on the skin fibroblast cells up to 10 mg/L in both 48 and 72 h incubation (Figure 3.3a), while the 70%EtOH extract was up to 7.5 mg/L at 48 h and 5 mg/L at 72h (Figure 3.3b). A different result was obtained from sonication extracts. The water extract did not significantly inhibit the cells up to 7.5 mg//L. However at a concentration of10 mg/L, the extracts inhibited 20% and 30% of cells for 48 and 72h respectively (Figure 3.3c). Whereas, the 70%EtOH extract significantly reduced the fibroblast cells at 5 mg/L (Figure 3.3d). All solvent controls (1% water and 1% ethanol in IMDM media) did not have an effect on the skin fibroblast cells. The non-toxic concentration (2.5 mg/L) from all extracts was chosen to study anti-aging properties especially the collagen production.


(a)
Cell viability (%)
160
140
120
100
80
60
40
20
0
Solvent control
0
2.5
5
7.5
10
50
Treatment concentration (mg/L)
48 h
72 h
*
*
*


(b)
Cell viability (%)
160
140
120
100
80
60
40
20
0
Solvent control
0
2.5
5
7.5
10
50
Treatment concentration (mg/L)
48 h
72 h
*
*
*
*
*


(c)
Cell viability (%)
160
140
120
100
80
60
40
20
0
Solvent control
0
2.5
5
7.5
10
50
Treatment concentration (mg/L)
48 h
72 h
*
*
*

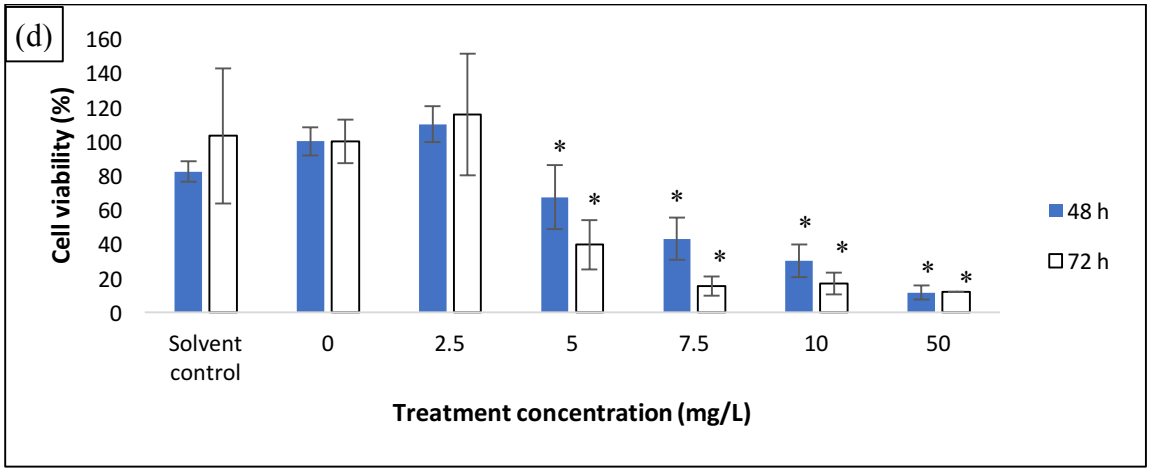


Figure 3.3. Cytotoxicity of *H. atra extracts* against human skin fibroblast cells CRL 2076. (a) Water extract of shaking at 60°C; (b) 70%EtOH extract of shaking at 60°C; (c) Water extract of sonication at 22°C; (d) 70%EtOH extract of sonication at 22°C. The concentrations of *H. atra* extracts were from 0 to 50 mg/L. 1% water or 1% ethanol were used as solvent controls for each extract. The cells were stained using crystal violet assay. Values are expressed as mean ±SD from three independent experiments. Cell viability was calculated based on cell number of extract treatment compared to the cell number of media control (0 mg/L treatment concentration) multiplied by 100. * $p<0.05$ compared to the control.

3.3. Anti-aging properties of *H. atra* extracts

Two different anti-aging properties of *H. atra* extracts were analyzed. Those properties are antioxidant activity and effect of extracts on the collagen production of human skin fibroblast cells.

3.3.1. Antioxidant properties

The antioxidant potential of *H. atra* extracts from all extraction conditions was compared. As is shown in Figure 3.4, overall water extracts had a significantly greater antioxidant potential than 70%EtOH extracts ($p<0.05$). Water extracts have antioxidant properties from 0.020±0.001 to 0.025±0.002 mM eq. $FeSO_4.7H_2O$/ mg extract while 70%EtOH extracts from 0.014±0.001 to 0.019±0.002 mM eq. $FeSO_4.7H_2O$/ mg extract. In the aqueous extract, the different extraction processes produced similar antioxidant

properties. While in 70%EtOH solvent, extracts of sonication (0.019±0.002 mM eq. $FeSO_4.7H_2O$/ mg extracts) were significantly different compared to shaking at 22°C (p<0.05) but not to shaking at 60°C and had the highest antioxidant potential among other ethanol extracts.

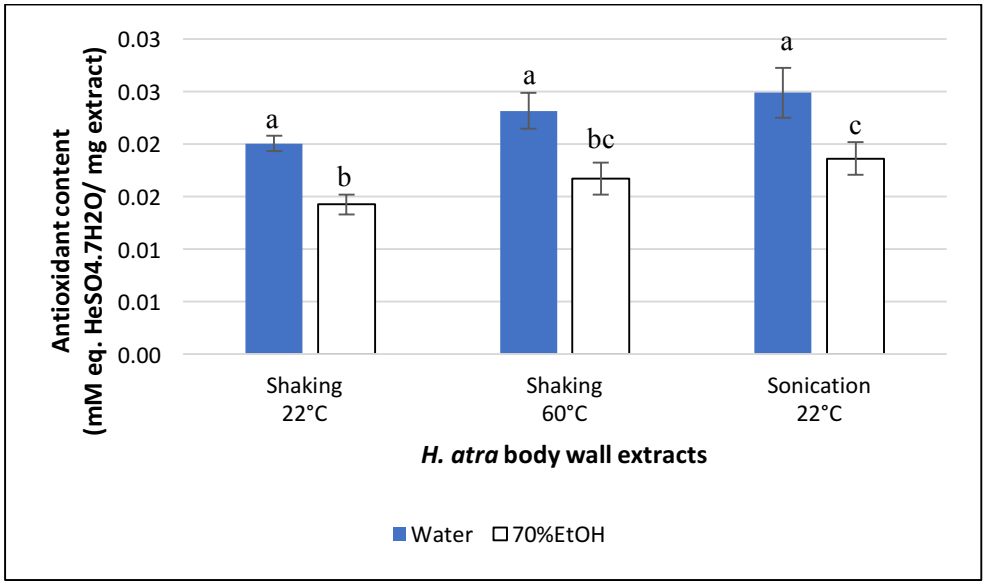


Figure 3.4. Antioxidant Activity of *H. atra* body wall extracts from different extraction conditions and solvents using FRAP assay. Values are expressed as mean ±SD from three independent experiments. Values in the same solvent with different letters are significantly different at p<0.05.

3.3.2. Effect of *H. atra* extracts on collagen production in skin fibroblast cell CRL 2076

H. atra extracts that were shaken at 60°C and sonicated at 22°C increased the collagen production of human skin fibroblast cells CRL 2076 (Figure 3.5). Water and 70%EtOH extracts from shaking at 60°C at 2.5 mg/L had a similar effect in enhancing the collagen production of cells. Both significantly enhanced (p<0.01) the collagen production of cells to 103.8±21.9 and 101.8±19.1 µg/mL respectively. Sonication extracts had a different effect on the collagen production. The sonication water extract increased production of collagen to 32.5±10.4 µg/mL, however, it was considered not

significant compared to the control (28.3±7.3). Whereas, treatment of the sonication 70%EtOH extract to skin fibroblast cell significantly accelerated (p<0.01) the collagen production to 105.4±10.1 µg/mL.

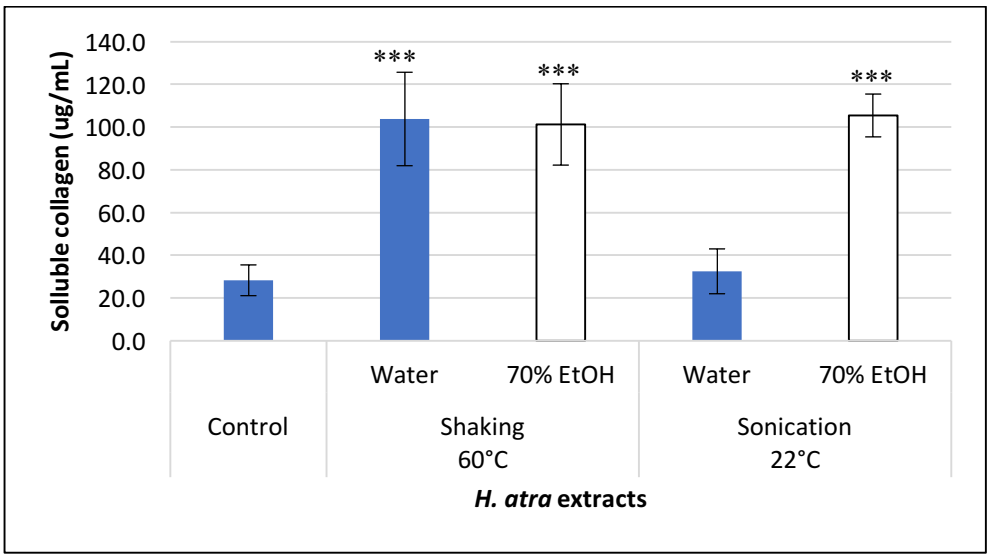


Figure 3.5. Effect of *H. atra extracts* to the collagen production of human skin fibroblast cells CRL 2076. The treatment concentration was 2.5 mg/L. Skin fibroblast cells culture without treatment were used as a control. Values are expressed as mean ±SD from four independent experiments. ***p<0.001 compared to the control.

3.4. Chemical characteristic of *H. atra* extracts

3.4.1. Saponins content

The saponins content of *H. atra* extracts were varied in response to the different extraction conditions and solvents, varying from 0.16±0.02 to 0.22±0.01 mg eq. plant saponins/ mg extract (Figure 3.6a). Shaking at 22°C and at 60°C produced similar saponins in water and 70%EtOH extract. Whereas, in sonication at 22°C, 70%EtOH extract (0.22±0.01 mg eq. plant saponins/ mg extract) contained significantly more saponins than in water extract (0.17±0.01 mg eq. plant saponins/ mg extract) (p<0.05). In addition, 70%EtOH extract from sonication was the highest saponins content and significantly different compared to 70%EtOH extract from shaking at 22°C (p<0.05).

The total saponins number per body wall weight was similar for all extraction conditions, except 70%EtOH from shaking at 22°C (Figure 3.6b). The number was from 0.046±0.002 to 0.053±0.004 mg/mg dried body wall and 0.031±0.003 mg/mg dried body wall for 70%EtOH from shaking at 22°C. It was a significantly lower compared to other extracts (p<0.05).

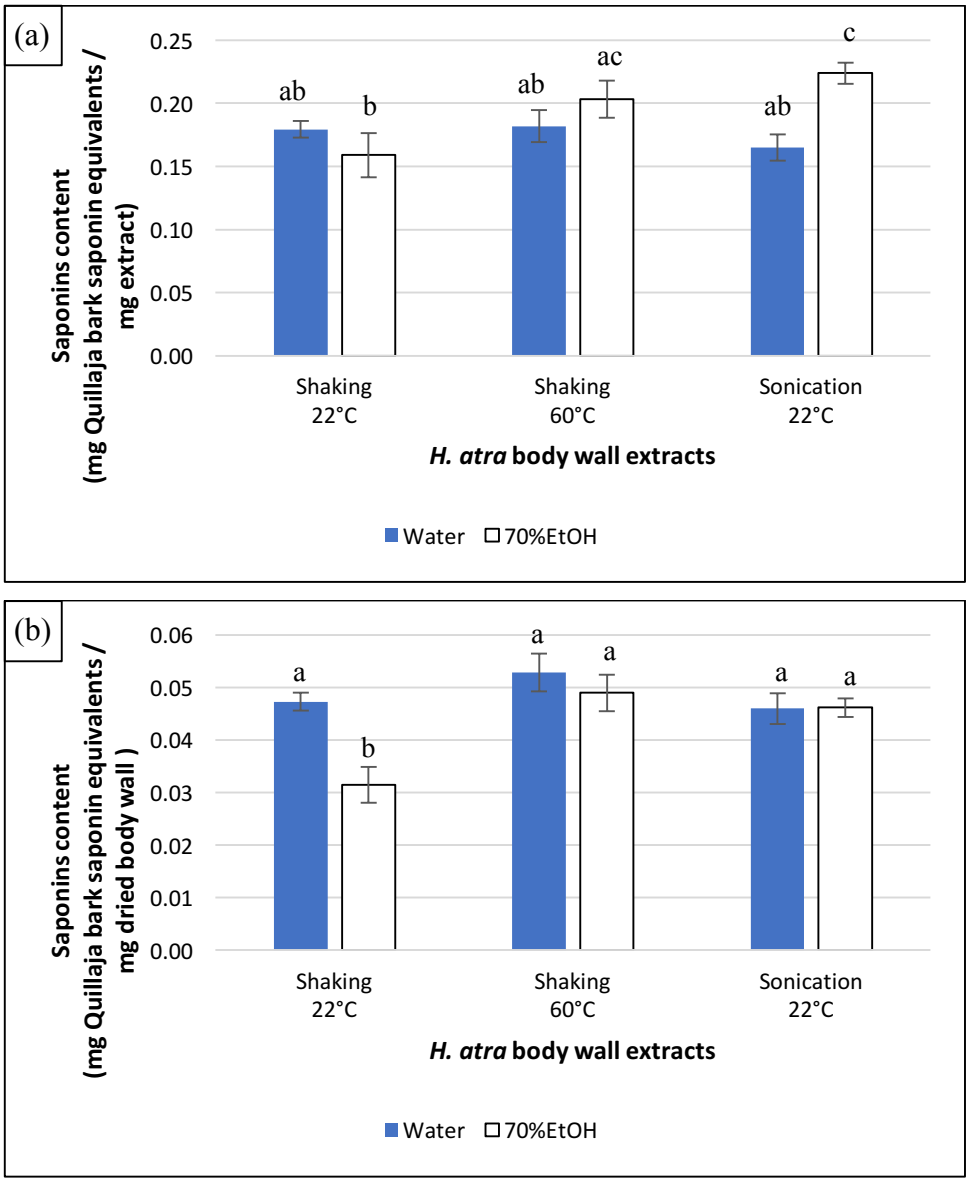


Figure 3.6. Total saponins content of *H. atra* body wall extracts from different extraction conditions and solvents using vanillin-sulfuric acid method. (a) Total saponins per mg extract and (b) Total saponins per mg dried body wall. Values are expressed as mean ± SD from three independent experiments. Values in the same solvent with different letters are significantly different at p< 0.05.

3.4.2. Sugars content

The sugars content of various *H. atra* extracts were analyzed. As shown in the Figure 3.7, generally the values were similar (0.062±0.008 – 0.091±0.009 mg eq. D-Glucose/ mg extract). Different extraction conditions and solvents did not significantly influence the sugar content of the extracts. Shaking at 60°C extracts contain 0.062±0.008 and 0.067±0.007 mg eq. D-Glucose/ mg extract for water and 70%EtOH respectively. While, shaking at 22°C extracts contain 0.081±0.003 and 0.091±0.009 mg eq. D-Glucose/ mg extract water and 70%EtOH respectively.

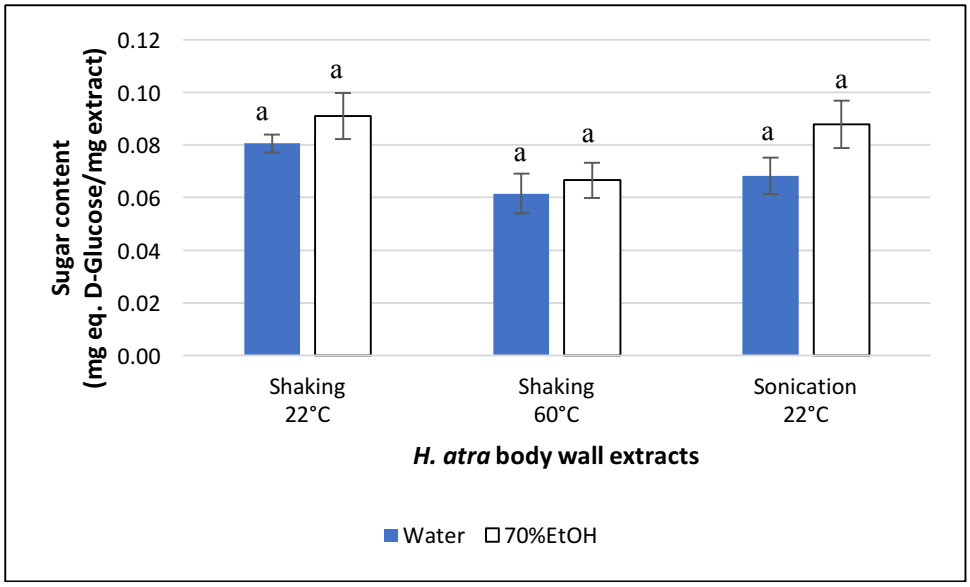


Figure 3.7. Sugars content of *H. atra* body wall extracts from different extraction conditions and solvents using anthrone-sulfuric acid method. Values are expressed as mean ± SD from three independent experiments. Values in the same solvent with different letters are significantly different at $p < 0.05$.

3.4.3. Total phenolics content

The total phenolics content of *H. atra* extracts from various extraction conditions was measured, and the results are shown in Figure 3.8. The average values of the total phenolics in extracts varied from 2.14±0.20 to 4.99±0.54 µg GAE/mg of extract, depending on solvents and extraction conditions. There was a significant difference of

the phenolics content between aqueous (3.60±0.44 µg – 4.99±0.54 µg GAE/mg of extract) and organic extracts (1.55±0.34 – 2.14±0.20 µg GAE/mg of extract) (p<0.05) in all different conditions. However, the types of extraction processes did not significantly affect the total phenolics content in both solvents. Shaking at 22°C contained phenolics 4.99±0.54 and 2.14±0.2 µg GAE/mg of extract for water and 70%EtOH solvents respectively. While, shaking at 60°C contained 3.60±0.44 and 1.55±0.29 µg GAE/ mg extract respectively.

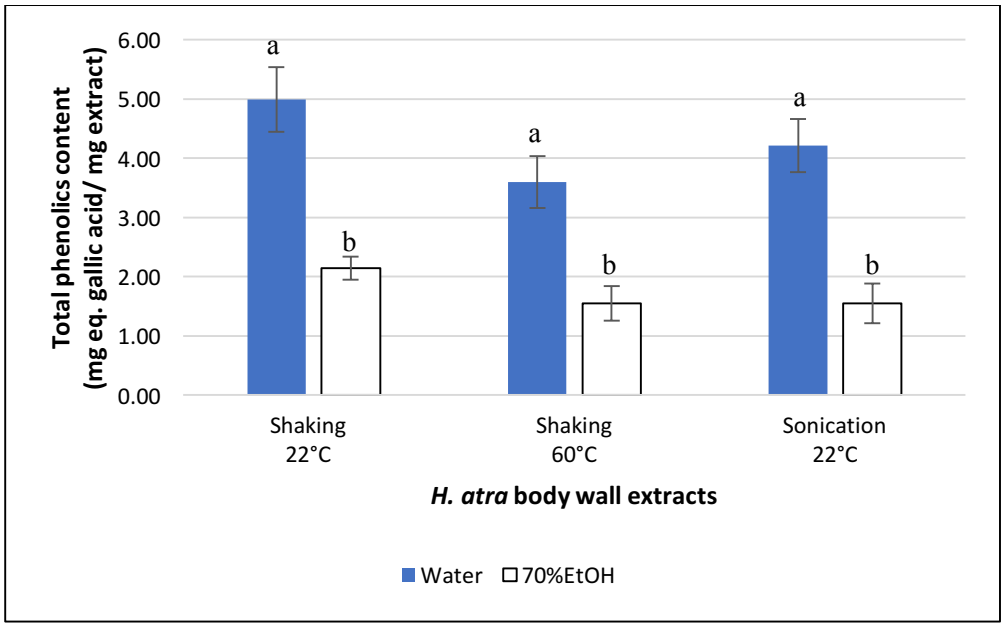


Figure 3.8. Total phenolics content of *H. atra* extracts from different extraction conditions and solvents using Folin-Ciocalteu method. Values are expressed as mean ±SD from three independent experiments. Values in the same solvent with different letters are significantly different at p<0.05.

3.4.4. Soluble protein

Soluble protein from different extraction methods is presented in Figure 3.9. Water extracts show significantly higher protein (0.109±0.011 – 0.122±0.014 mg/ mg extract) than ethanol extract (0.033±0.004 – 0.036±0.007 mg/ mg extract) with p<0.05. The

different extraction conditions, however, did not significantly affect the soluble protein of *H. atra* extracts in both water and 70%EtOH solvents.

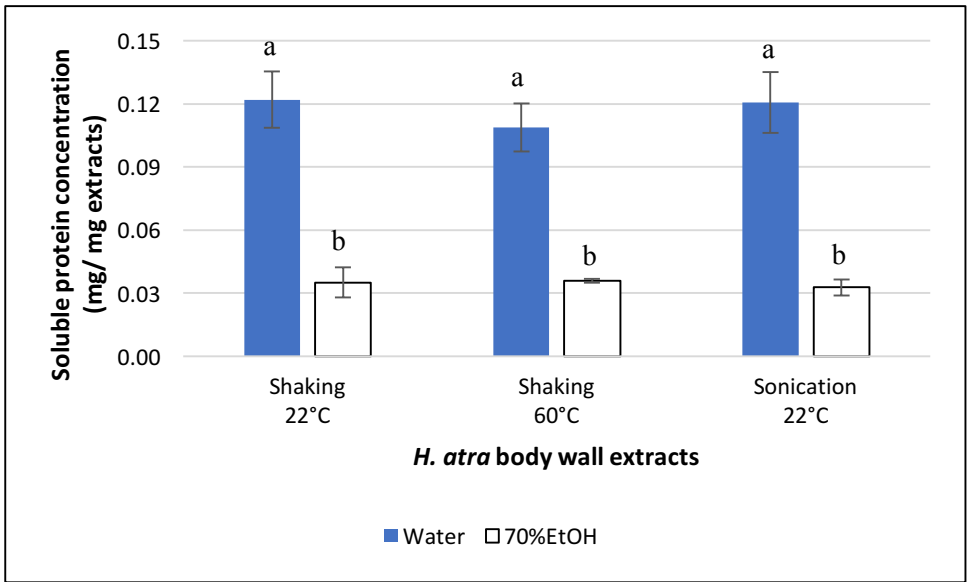


Figure 3.9. Protein content of *H. atra* extracts from different extraction conditions and solvents. Values are expressed as mean ± SD from three independent experiments. Values in the same solvent with different letters are significantly different at $p < 0.05$.

3.4.5. TLC analysis of active extracts

Fingerprinting analysis of *H. atra* extract using TLC revealed slightly different compounds between each extract (Figure 3.10 and Table 3.1). A total 13 compounds were observed. Most of the compounds were observed in UV 254 nm after staining with iodine (Figure 3.10a), while only small number compounds were observed in UV 365 nm (Figure 3.10b). 70%EtOH extracts from both shaking at 60°C (line 3) and sonication at 22°C (line 5) produced more compounds compare water extracts (line 2 and 4) with 11 and 10 out of 13 compounds respectively. Although they had similar compounds, sonication water extract (line 4) had less intensity in compound number 10 to 13 compared to compounds from shaking at 60°C water extract (line 2). 70%EtOH of both extracts (sonication and shaking at 60°C) have similar numbers, however, some

of the compounds have different retention time (Rf). 70%EtOH extract from shaking at 60°C (line 3) have compounds with Rf 0.22 and 0.52, while from sonication (line 5) have compounds with 0.45 and 0.58. The other compounds were similar to each other. Plant saponins (line 1) as a sample comparison did not show separated compounds both in UV and iodine staining.

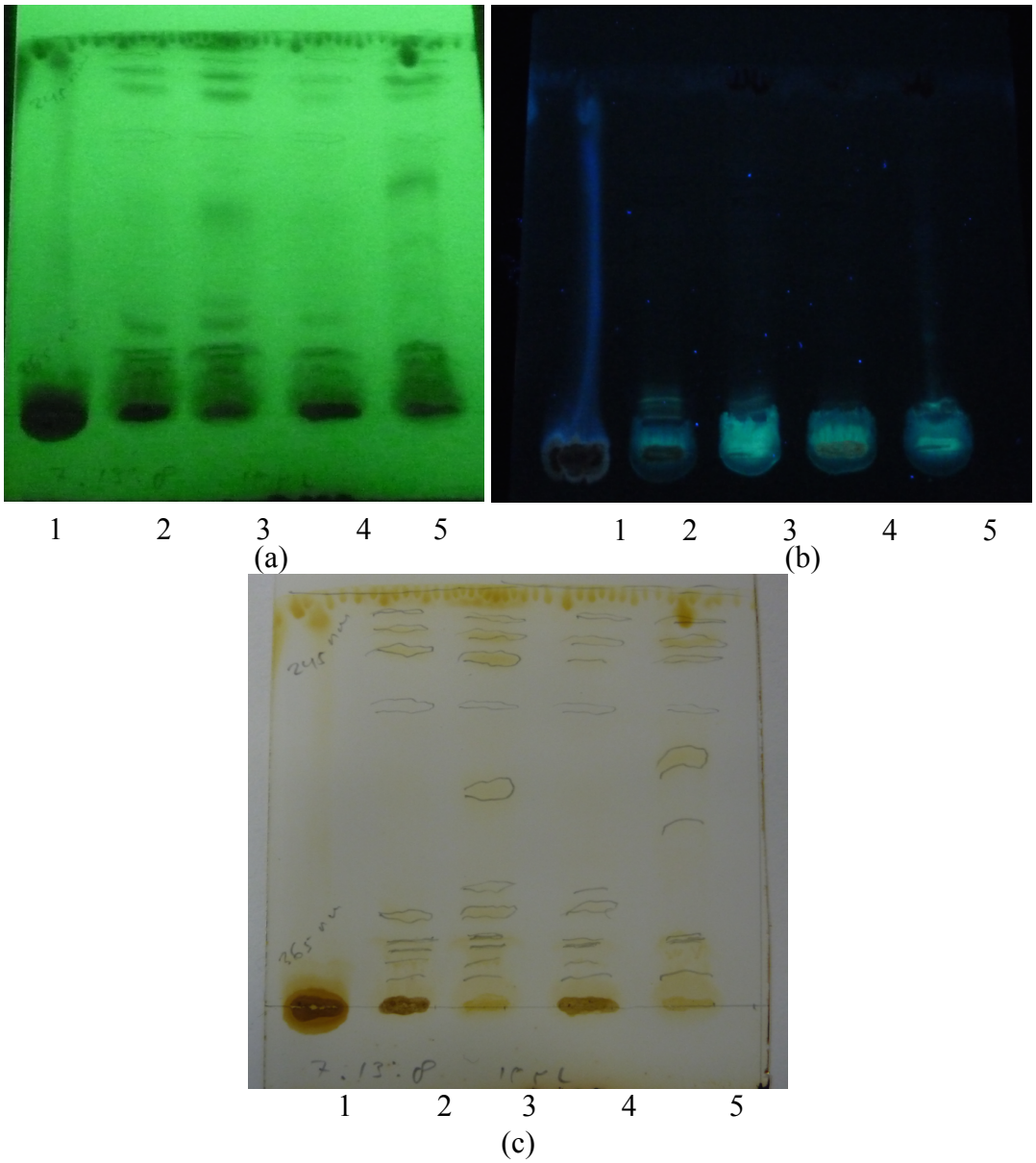


Figure 3.10. Thin layer chromatography (TLC) analysis of *H. atra* extract. (a) Observed using UV 254 nm; (b) Observed using UV 365 nm and (c) staining with iodine. Samples were dissolved using 90% methanol at concentration 50 mg/mL and running using the lower phase of $CHCl_3$-MeOH-H_2O (7:13:8) system. 1: Plant saponins; 2: Shaking 60°C water extract; 3: Shaking 60°C 70%EtOH extract; 4: Sonication at 22°C water extract and 5: Sonication at 22°C 70%EtOH extract

Table 3.1. The retention time of *H. atra* extract compounds from TLC analysis. 2: Shaking 60°C water extract; 3: Shaking 60°C 70%EtOH extract; 4: Sonication at 22°C water extract and 5: Sonication at 22°C 70%EtOH extract. (-) compound not found in the extract (+) compound found in the extract with different intensity.

Compound	Rf	Samples			
		2	**3**	**4**	**5**
1	0.06	++	++	++	++
2	0.10	++	++	++	++
3	0.14	++	++	++	++
4	0.16	++	++	++	++
5	0.22	++	++	++	-
6	0.29	-	+	-	+
7	0.45	-	-	-	+
8	0.52	-	+++	-	-
9	0.58	-	-	-	+++
10	0.73	++	++	+	++
11	0.84	++	++	+	++
12	0.90	++	++	+	++
13	0.94	++	++	+	++

CHAPTER 4

DISCUSSION

4.1. Water extraction produced higher yield compared to 70%EtOH extraction

Water and 70%EtOH were applied to extract compounds from the body wall of *H. atria*. The type of solvent is one of the important factors that influences the effectiveness of the extraction. The different polarity of solvents produce different secondary metabolites and bioactivity of the compounds (Cos *et al*., 2006). In terms of saponins extraction, alcohol (methanol, ethanol), water or a mixture of them is the most common solvent extraction. Other solvents also have been used to extract saponins such as alcoholic surfactant solutions, glycerine and glycyrrhizic acid solvent plus ammonia (Güçlü-Üstündağ and Mazza, 2007).

Water and ethanol were used in this study because they are known as non-toxic or generally recognized as safe (GRAS) solvents for extracting bioactive compounds for human application purposes (Husni *et al*., 2009). Furthermore, water and ethanol are considered as green solvents (Byrne *et al*., 2016). Green solvents are solvents that have minimal impact on the environment, health and safety (EHS) when used in the chemical production (Capello *et al*., 2007), including the net cumulative energy demand (CED) level of the solvent production. Furthermore, water and ethanol are also categorized as bio-based solvents because they are renewably sourced and produced from biomass on a large scale (Byrne *et al*., 2016).

The efficiency of each extraction processes in the shaking at 22°C was analyzed. Each extraction stage produced a different percentage of yield (Figure 3.1). In the first extraction, water produced less yield compared to the second extraction, while the yield from ethanol was higher in the first extraction. In the first extraction, most of the water

was absorbed by the *H. atra* powder, thus the volume of the supernatant after centrifugation was less than 5% (v/w). In contrast, 70%EtOH was absorbed, but after centrifugation the volume of the supernatant was similar from the initial solvent (5%). These results indicate that water was absorbed more by the sample than the ethanol. After the second extraction, water and 70%EtOH produced more than 70% and 80% of the total yield respectively. While, the last extraction only produced below 10% of total yield for both solvents. These results demonstrate that two stages of extractions were considered effective to produce extracts from sea cucumbers. Therefore, to reduce the time and energy, for further extraction processes (different sea cucumber species), the extraction process was only done until the second cycle with the similar total ratio of solvent (1: 20).

Water extracts produced more yield compared to 70%EtOH (Figure 3.2). The yields of *H. atra* extract were between 26 to 29% for water and 19% to 24% for 70%EtOH. This indicates that particles in the body wall of *H. atra* are more soluble in water than in 70%EtOH. This result was similar with the previous result from (Althunibat *et al*., 2009). They reported that aqueous extracts of body wall *H. scabra, H. leucospilota and Stichopus chloronotus* (4.03%, 4.29% and 5.25% respectively) were significantly higher than organic extracts (0.25%, 0.25% and 0.17% respectively). In addition, the extract yield of organic solvent in this study (19 - 24%) was similar to result from Nobsathian *et al*. (2017). They stated that using methanol to extract dried body wall of *H. atra* produced 19.74% of yield. Another study, however, showed that water and 70%EtOH-soluble matters extracted from the body wall of sea cucumber (*S. japonicus*) using heat reflux extraction and pressurized solvent extraction were not significantly different (Husni *et al*., 2009). The differences may be because of different sample and extraction process that were used.

Shaking at 60°C produced more yield in the 70%EtOH extract compared to shaking and sonication at 22°C. Increasing temperature to a certain degree was reported to enhance the efficiency of the extraction by increasing the solubility of the material and the diffusion coefficient of solvent (Spigno *et al*., 2007). Increasing extract yield of mushroom *Inonotus obliquus* was obtained by increasing the temperature of extraction (Hu *et al*., 2009). Similarly, sonication improved the efficiency of extraction process by increasing the rate of mass transfer and the penetration of solvent. In addition, sonication can also break biological cell walls, providing a way to release the content (Wang and Weller, 2006, Güçlü-Üstündağ and Mazza, 2007). However, in this study, sonication did not significantly increased the yield of extract in both solvents. This may be because of the different sample and sonication type that were used. Considering these result, this study was limited by the low replicate number in the experiment, which was only one experiment with three replicates. This was due to the limited number of *H. atra* samples available.

4.2. Different cytotoxicity effects of *H. atra* extracts prepared by different extraction conditions and solvents

Cytotoxicity assays are a common method to analyze the sensitivity of compounds to the living cells (Ishiyama *et al*., 1996). They are performed either to discover the highest activity of the compounds in the drugs discovery or to determine the side effect of the compounds to the cells. In this study, the cytotoxicity assay was to determine the safe dosage of the extract that will be applied to the skin cell and used crystal violet staining assay.

The cytotoxicity of *H. atra* extracts against human skin fibroblast cells CRL 2076 were different depending on the extraction conditions and solvents (Figure 3.3). EtOH

(70%) extracts from shaking at 60°C and sonication at 22°C reduced the viability of skin fibroblast cells up to 40% at concentration 7.5 and 5 mg/L respectively. While, water extracts were inhibited at concentration 50 and 10 mg/L respectively. At these concentrations, the extracts were considered toxic. López-García *et al*. (2014) divided the level of cytotoxicity to non-cytotoxicity (cell viability above 80%), weak (80%-60%), moderate (60% - 40%) and strong (below 40%).

These results demonstrate that 70%EtOH extract were more toxic than water extract. In addition, shaking at 60°C produced less toxic extract compared to sonication in both water and 70%EtOH solvents. The different effect of these extracts may be because different extraction conditions and solvents produced a different composition of compounds. All the extracts, however, showed no significant effect on the growth of skin fibroblast cells at treatment concentration 2.5 mg/L, therefore, this concentration is considered as a safe dosage and used for treating the collagen production assay.

4.3. Saponin and sugar contents of *H. atra* extracts have the highest correlation number to the cytotoxicity of human skin fibroblast cells

In order to understand which chemical characters have a responsibility to cytotoxicity of human skin fibroblast cells, each chemical property was analyzed the correlation using regression method. The significance (p<0.05) and correlation coefficient (R^2) numbers were observed. The results are shown in Table 4.1 the correlation graphs are in Appendix 9.

Table 4.1. Correlation between chemical characteristics of extract and cell viability of human skin fibroblast cells. The correlation analysis was conducted using a regression test and the significance level was found using one-way ANOVA with $n = 20$.

Chemical characteristic	Slope	Correlation	Significance
Saponins content	**y = -8.2686x + 91.084**	$\boldsymbol{R^2 = 0.44387}$	$\boldsymbol{p = 0.001}$
Sugar content	**y = -22.57x + 91.143**	$\boldsymbol{R^2 = 0.45116}$	$\boldsymbol{p = 0.001}$
Protein content	y = -11.173x + 79.577	$R^2 = 0.17722$	$p = 0.065$
Total phenolic content	y = -0.3674x + 82.127	$R^2 = 0.22712$	$p = 0.034$
Antioxidant properties	y = -68.858x + 88.603	$R^2 = 0.36407$	$p = 0.005$

Based on the correlation between chemical profiles and viability of *H. atria* extracts, saponin and sugar contents of the extracts have the highest correlation number and significance effect to viability of skin fibroblast cells CRL 2076. Both of them have a low negative correlation with R^2 = 0.44 and 0.45 respectively (Mukaka, 2012). Increasing saponin and sugar contents was inversely correlated with the cell viability effect of the *H. atra* extracts. Saponins are known have membranotropic effects against cellular and model membranes, resulting in saponins have a wide spectrum of biological activities. The saponins and Δ^5 and $\Delta^{5,7}$-sterols in the organism membrane form single ion channels and large channels. This mechanism allows the increase of microviscosity and suppression of transport processes in the membranes (Augustin *et al*., 2011). In addition, saponins have 18(20)-lactone, oxygen functional group, linear tetrasaccharide fragment, quinovose as the second monosaccharide and 3-Omethyl group that have key roles in the membranotropic activity (Kalinin *et al*., 2008). Furthermore, saponins have different mechanisms in the anticancer treatment, including inducement of apoptosis, stimulation of non-apoptotic cell death, autophagy, inhibition of angiogenesis, the disintegration of the cytoskeleton, inhibition of metastasis (Tian *et al*., 2013).

The cytotoxicity of sea cucumber extracts against skin fibroblast cells has had little study. Most research has focused on cancer cells. One study reported that glycoprotein fractions from the liquid extracts of boiled Korean sea cucumber *Stichopus japonicas* were non-toxic up to 5 mg/mL on the mouse melanoma cell line B16-F10 (Kim *et al*., 2016). This result was different compared to this study which had non-toxic concentrations between 2.5 – 10 mg/L. The differences may be because of the type of sea cucumber species, extracts preparation and the cells line. A study of *H. atra* extracts against cancer cells HeLa and MCF-7 showed that at a concentration of 0.78 mg/L, the extract inhibited the viability of cells to 40.58 and 30.22% respectively (Dhinakaran and Lipton, 2014).

4.4. Anti-aging properties of *H. atra* extract

4.4.1. Low antioxidant properties of *H. atra* extract

Exposure to excessive sunlight causes excessive ROS production in the body. To neutralise the high level of ROS in the body, endogenous antioxidant sources are needed. Antioxidants protect the human body from further damage caused by ROS such as DNA damage, skin inflammation, hyper pigmentation, stimulation of dermal fibroblast for expression of matrix metalloproteinase 1 (MMP-1) responsible for collagen degradation and a decrease in collagen synthesis thus resulting in a photo-aged skin effect (Taofiq *et al*., 2016). Therefore, antioxidants property is one of the important ingredients for skin aging products.

The antioxidant potential of sea cucumber has been widely reported (Zhong *et al*., 2007, Husni *et al*., 2009, Althunibat *et al*., 2009) including from *H. atra* (Esmat *et al*., 2013, Dhinakaran and Lipton, 2014). However, most of them were analysed using oxygen radical absorbance activity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)

method (Zhong *et al*., 2007, Esmat *et al*., 2013, Dhinakaran and Lipton, 2014). While in this study was analysed using FRAP assay. FRAP measures the activity of antioxidant substances to decrease electron transfer (Fe^{3+} to Fe^{2+}) in the presence of TPTZ and form blue Fe^{2+} - TPTZ complex (Benzie and Strain, 1996).

In this study, water extracts of *H. atra* have significantly higher antioxidant properties compare to 70%EtOH (Figure 3.4), indicating that the antioxidant compounds in *H. atra* were more soluble in water than in 70%EtOH. Similar results were reported by Althunibat *et al*. (2009) who used DPPH assay to analyse antioxidant properties from three different sea cucumbers. In addition, 70%EtOH extracts of shaking at 60°C and sonication had higher antioxidant activity compared to shaking at room temperature. A study by Zong *et al*. showed that processing dried sea cucumber using rehydration at 65°C for 48 hours produced greater antioxidant activity than fresh sea cucumber, even though the differences were not significant. However, the increase of antioxidant properties in processed sea cucumber may be caused by losing weight during rehydration process rather than directly increase the antioxidant activity (Zhong *et al*., 2007). In addition, sonication reported slightly improved antioxidant capacity of fucoidan from sea cucumber *Isostichopus badionotus*. Sonication treatment led to decrease the molecular weight of fucoidan without major destruction of the structure. The low molecular weight of fucoidants has higher antioxidant properties (Guo *et al*., 2014). A study of stability polyphenols and antioxidant activity of red grape, however, showed that grape treatment at temperature 60°C decreased the antioxidant yield around 4% compare to freeze drying treatment (Larrauri *et al*., 1997). The differences of the result may be because of the different sample and compound that extracted.

Antioxidant compounds in sea cucumbers are reported to protect the body from high levels of solar radiation and high oxygen from photosynthesis of algae symbionts.

These compounds came from their metabolites and exogenous sources such as food, including vitamins E, C, phenolic compounds and carotenoids (Zhong *et al*., 2007).

Sea cucumber has antioxidant potential; however, the value is lower than common antioxidant sources from plants. Using FRAP assay, green tea leaf has 3 mM/gram dried sample of antioxidant when extracted at 90°C for 15 minutes,100 times higher to the sea cumber extract in this study (0.03 mM/mg extract or 0.007 mM/ gram dried sample) (Langley-Evans, 2000). A similar result was reported by Mamelona *et al*. (2007). Using ORAC assay, extracts of sea cucumber *Cucumaria frondosa* from water, organic solvent and a mixture of them have lower antioxidant properties compare to strong antioxidant from grape seeds (15 - 85x), grape skin (18 – 100x) and medicinal plant leaves (20 – 114x). The summary of comparison antioxidant activities in this study and other sources is presented in Table 4.2.

In this study, the low antioxidant properties also may be come from the long storage of sample as the sample was obtained in 2011. Puupponen-Pimiä *et al*. (2003) reported that in many cases long term freezing storage of vegetables reduced the antioxidant properties up to 20%. Another possibility of the antioxidant reduction was the extraction process. Some studies reported that extraction of the antioxidant compound was conducted in dark condition to minimize the effect of light oxidation on the destruction of the compounds (Mamelona *et al.*, 2007, Althunibat *et al.*, 2009). While in this study, the extraction process was in normal light conditions.

Table 4.2. Comparison of antioxidant properties of *H. atra* and other antioxidant sources

Species	Antioxidant properties	Method	References
Sea cucumbers			
Holothuria atra	0.03 mM of Fe^{2+} equivalent/mg extract or 0.007 mM of Fe^{2+} equivalent/ g DW	FRAP	This study
Cucumaria frondosa	140- 800 µmol of Torlox equivalent/g DW	ORAC	Mamelona *et al*. (2007)
H. edulis	IC50 = 2.03 - 8.73	DPPH	Althunibat *et al*. (2013)
H. leucospilota	IC50 = 3.91 - 5.44	DPPH	Althunibat *et a*l. (2009)
H. scabra	IC50 = >10	DPPH	Althunibat *et al*. (2009)
Stichopus chloronotus	IC50 = 2.13 - >10	DPPH	Althunibat *et al*. (2009)
S. horrens	3.2 - 6.8 µmol of Torlox equivalent/g DW	DPPH	Husni *et al*. (2009)
S. horrens	IC50 = >10	DPPH	Althunibat *et al*. (2013)
Tea			
Black tea (*Camellia sinensis*)	0.54 mg of Torlox Equivalent/g DW	DPPH	Atoui *et al*. (2005)
Green tea (*Camellia sinensis*)	0.57 mg of Torlox Equivalent/g DW	DPPH	Atoui *et a*l. (2005)
Green tea	1.03 – 1.63 mM of Fe^{2+} equivalent/ g DW	FRAP	Rusak *et al*. (2008)
White tea	1.08 – 1.79 mM of Fe^{2+} equivalent/ g DW	FRAP	Rusak *et al*. (2008)
Green tea	1.03 – 1.63 mM of Fe^{2+} equivalent/ g DW	FRAP	Rusak *et al*. (2008)
Grape			
Black pearl grape	225.04 µM Torlox Equivalent/g DW	FRAP	Xu *et al*. (2010)
Purple	146.18 µM Torlox Equivalent/g DW	FRAP	Xu *et al*. (2010)
Green	4.95 µmol Fe^{2+} equivalent/ g WW	FRAP	Fu *et al*. (2011)
Red	6.70 µmol Fe^{2+} equivalent /g WW	FRAP	Fu *et al*. (2011)

DW: Dry weight
WW: Wet weight

4.4.2. Negligible correlations between antioxidant properties and phenolic content of *H. atra* extract

A regression test was applied to determine whether the antioxidant activity and the phenolics content of *H. atra* extracts are correlated. The result is shown in Figure 4.1.

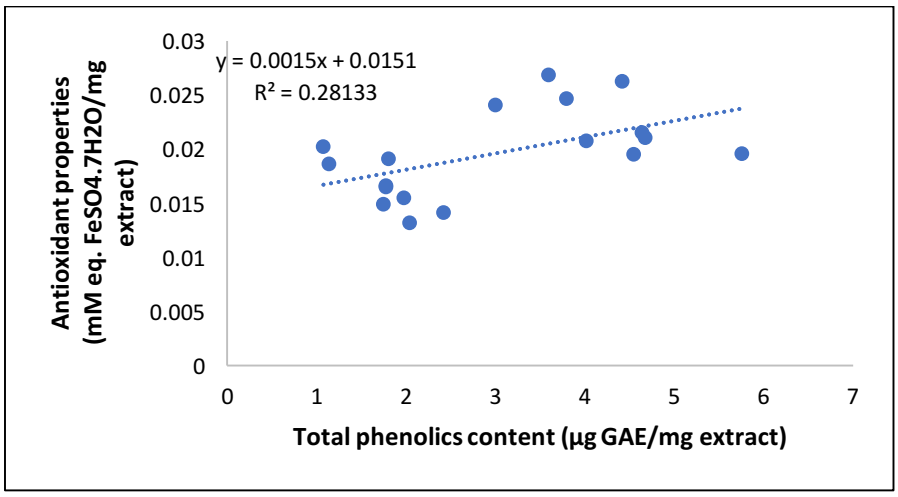


Figure 4.1. Correlation between total phenolics content and antioxidant properties of *H. atra* extracts. The correlation analysis was conducted using a regression test with $n = 18$.

There were weak correlations between antioxidant properties using the FRAP assay of the whole of extracts and their phenolics content. The level of correlation was negligible (R^2 = 0.28133) (Mukaka, 2012). This result demonstrated a weak contribution of phenolic compounds in antioxidant properties of *H. atra* extracts. This result is similar to studies of three Malaysian sea cucumbers where the correlation of antioxidant properties using DPPH and β-carotene bleaching methods and phenolic compounds were R^2 = 0.31 and 0.1 respectively (Althunibat *et al.*, 2009). They suggested that other compounds such as carotenoids, ascorbic acid, glutathione and gadusol may contribute to the antioxidant activity rather than phenolic compounds. However, a different result was obtained from the study of phenolic contents and antioxidant properties of the Atlantic sea cucumber, *Cucumaria frondosa* (Mamelona *et al.*, 2007). The correlation of phenolic compounds and antioxidant activity was different depending on the type of organs. Total phenolics of gonads and muscles had significant correlations to the ORAC values (R^2 = 0.79, p = 0.047 and R^2 = 0.92, p = 0.042 respectively). Whereas, digestive tract and respiratory apparatus had a low correlation. They authors suggested that the variation of the result might be because of

a difference in phenolic constituents between tissues. In addition, the contribution of individual phenols to anti-peroxyl radicals was varied among chemical species and depended upon their concentration (Mamelona *et al.*, 2007).

4.4.3. *H. atra* extracts significantly increased collagen production of human skin fibroblast CRL 2076

To the best of our knowledge, this study is the first attempt to determine the induction of collagen production using sea cucumber extracts. Collagen is a crucial element of the skin structure. More than 80% of extracellular matrix in connective tissue is composed of collagen (Song *et al*., 2012). In addition, collagen contributes to the elasticity, flexibility and strength of the skin. However, during the process of aging and UV exposure, the level of collagen decreases and results in the formation of wrinkles (Taofiq *et al*., 2016).

H. atra extracts significantly stimulated the collagen production of human skin fibroblast cells (Figure 3.5). Treatment of 2.5 mg/L of extract from shaking at 60°C and sonication at 22°C increased the collagen production 1.2 to 3.7 times. The varied activity of *H. atra* extracts may be because of different chemical profiles of the extracts. To understand which chemical characters have a responsibility to the stimulating collagen production of human skin fibroblast cells, the correlation of each chemical property was analyzed using regression method. The significance (p<0.05) and correlation coefficient (R^2) numbers were observed.

Table 4.3. Correlation between chemical characteristics of extract and collagen production. The analysis used a regression test and the significance level was found using one-way ANOVA with $n = 4$.

Chemical characteristic	Slope	Correlation	Significance
Saponins content	y = 329.66x - 59.335	$R^2 = 0.65448$	$p = 0.191$
Sugar content	y = 242.56x + 57.061	$R^2 = 0.07267$	$p = 0.739$
Protein content	y = -160.38x + 130.05	$R^2 = 0.51736$	$p = 0.279$
Total phenolic content	y = -5.7899x + 139.67	$R^2 = 0.59197$	$p = 0.230$
Antioxidant properties	y = -2030.7x + 205.94	$R^2 = 0.55211$	$p = 0.266$

Based on the correlation of the chemical characteristics of the extract (Table 4.3), saponins content had the highest correlation ($R^2 = 0.65448$) although this was not significant ($p = 0.191$). This result, however, was limited by the number of data that were used, which was only four data and at one concentration (2.5 mg/L). Saponins from plants especially from ginseng are known to stimulate the collagen production in the skin cells. Panax ginseng extract which contains more than 45% saponins induced collagen type I production in human skin fibroblast cells up to 1.6 times depending on the extract concentrations (0.1 - 1mg/mL) (Lee *et al*., 2007). Treatment of sun ginseng extract (2 - 50 mg/L), which contains high less-polar saponins to skin cells increased the collagen production approximately 50% (Song *et al*., 2012). Another triterpenoid saponins from the plant *Viola hondoensis* inducing the production of collagen expression in dermal fibroblast cells (Moon *et al*., 2004). Using pure saponin from *Viola hondoensis* (Acutoside a) at concentrations 0.1 to 10 μM increased the type I procollagen expression in the fibroblast cells culture up to 500%.

There are some suggested mechanisms of saponins-rich extracts that explain the increase of collagen production in skin cells. These processes include activation of Smad signaling and regulating MMP-1 and TIMP-1 expression in human dermal

fibroblasts (Lee *et al*., 2007, Song *et al*., 2012). Smad is an important transcription factor in the expression of type I collagen induced by TGF-β or sphingosine 1-phosphate. Smad2 phosphorylation is an initial molecular process of Smad signaling that can be activated by saponins treatment (Lee *et al*., 2007). Matrix metalloproteinases (MMPs) are a group of enzymes that are important for the destruction and transformation of the extracellular matrix. MMP-1 (collagenase-1), in particular, triggers the degradation of collagen molecules, leading to skin fragility, laxity, roughness, dryness, pigmentation, and wrinkle formation. In addition, an increase of MMP-1 has been observed in the dermis due to a loss in the tissue inhibitors of MMPs (TIMPs). Ginseng saponins are reported to suppress the MMP-1 protein level and increase TIMP-1 production in fibroblasts (Song *et al*., 2012). In this study, it is possible that sea cucumber extracts have both mechanism actions, therefore this need further analysis.

4.5. Chemical characteristic of *H. atra* extracts

4.5.1. More saponins content in 70%EtOH extracts

Saponins are the most important and rich metabolites in sea cucumber (Kim and Himaya, 2012). The saponins in sea cucumbers are present in many organs. It is abundant in the body wall, cuvierian organs and gonad (Honey-Escandón *et al*., 2015). Holoturian saponins were reported to have a wide spectrum of potential applications, including cytotoxic and anticancer, antibacterial, antifungal, antiprotozoal, antiviral, antioxidant, mutagenic, hemolytic, immunomodulation, neurotoxic, inhibition of Na^+,K^+ -ATPase, mitogenic, radioprotective and inhibition of root growth activities (Kalinin *et al*., 2008, Kim and Himaya, 2012, Aminin *et al*., 2015, Aminin, 2016). These applications are affected by the structure of saponins that led to membranotropic

activity (Augustin *et al*., 2011). The structure of saponins consists of two main parts, the sugar component (glycone) and non-sugar component (aglycone) (Moghimipour and Handali, 2015). Therefore, the analysis of saponins content is based on these structures. Sulfuric acid-vanillin assay is the most common spectrophotometric method in saponins quantification. The basic principle of this method is the reaction of oxidized triterpene saponins with vanillin using sulfuric acid and the reaction will produce color. In this study, the saponins content of *H. atra* extracts were between 0.159 to 0.224 mg eq./ mg extract or 0.031 to 0.052 mg eq./ mg dried body wall (Figure 3.6). Comparison of these results to other sea cucumber studies is difficult because of different methods and units that were used. For example, the butanolic phase of *H.* fuscocinerea, *H.* scabra and *H. impatiens* contained saponins from 0.10 to 0.18 mg eq./ mL body wall (Bondoc *et al*., 2013). Another study using hemolotic method reported that butanolic fraction *H. atra* extract had 0.973 mg eq./ gram wet weight body wall (Van Dyck *et al*., 2010).

In this study, sonication 70%EtOH extracts of *H. atra* body wall contained more saponins than water extracts. The results indicated that saponins from *H. atra* were more soluble in 70%EtOH than water. Similar results were obtained from the study of ginseng saponin. Using 50% ethanol, the yield of ginseng saponins from roots of *Panax quinquefolius* was higher compared to 65% aqueous glycerin extract (Gafner *et al*., 2004). In addition, extraction of ginseng saponins using microwave-assisted process produced the highest yield using 75% ethanol (1.44 – 1.92 times) among the different ethanol concentrations used (30, 45 and 60 %) (Kwon *et al*., 2003). In contrast, saponins from *Stichopus japonicas* was more soluble in water than 70%EtOH when extracted using heat reflux extraction and pressurized solvent extraction (Husni *et al*.,

2009). This result however was based on the yield of saponins that were extracted, not from colorimetric quantification.

70%EtOH extracts from sonication produced the highest saponins compared to other extraction conditions. The sonication-assisted extraction of saponins from ginseng roots was reported three times faster than the conventional extraction method. In addition, sonication can be conducted at a lower temperature which can avoid the degradation of the compound from high temperature (Lin *et al*., 2001). Another assisted extraction also increased the saponins content in the extract; a microwave-assisted process to extract ginseng saponins produced a slightly higher yield of saponin compare to the conventional method (Kwon *et al*., 2003).

4.5.2. Similar sugar contents in water and 70%EtOH extracts

Sugars are one type of carbohydrate or saccharides. Polysaccharides from sea cucumbers are reported to have various biological activities including anticoagulant activity, antitumor activity, immunomodulating activity, osteoclastogenesis inhibitory activity and neurosphere formation enhancing activity (Liu *et al*., 2012). Quantifying sugars content can be carried out using spectrophotometric and HPLC. Combining sulfuric acid with anthrone, phenol or orcinol is the most common spectrophotometric method for measuring sugar content (Monsigny *et al*., 1988). The sulfuric acid - anthrone method is based on the production of furfuraldehyde derivatives from hydrolysis of glycosidic bonds and dehydration of monomers by high temperature and a strong acid condition. The derivatives react with anthrone to develop color (Laurentin and Edwards, 2003).

The sugar contents of *H. atra* extracts in this study were from 0.062 to 0.091 mg/ mg extract (Figure 3.7). This result was higher with total carbohydrate of *H. atra* extract

using acetonitrile and 0.1% trifluoroacetic acid solvent which contain 0.018 mg/ mg extract (Esmat *et al*., 2013). These differences may be because of the solvent used. Water and 70%EtOH extracts contain similar sugars content in all extraction conditions. This indicates that sugars in *H. atra* extracts were dissolved in both water and 70%EtOH. This result was different than other studies. Hot water (62 ± 5°C) was used to extract polysaccharides from roots of valerian (*Valeriana officinalis*) rather than used room temperature water because of the greater efficiency (Hromadkova *et al*., 2002). In addition, ultrasound was reported to increase the yield of polysaccharides isolated from the ethanol-insoluble plant *Salvia officinalis* from 9.3 to 10% compared to traditional extraction (Hromadkova *et al*., 1999). In this case, may be because of the different samples and extraction time that was used, since in their study used 2 to 3 h extraction, while in this study used 30 minutes each cycle (Hromadkova *et al*., 1999, Hromadkova *et al*., 2002).

4.5.3. Low phenolic content of *H. atra* extract

The total phenolics content of *H. atra* extracts was calculated using Folin-Ciocalteau. The Folin-Cicocalteu reagent measures the ability of any substance to reduce phosphomolybdic and phosphotungstic acids, which are blue when reduced (Singleton and Rossi, 1965).

Phenolics compounds are known as one of the antioxidant agents (Zhong *et al.*, 2007). Sea cucumbers are known to contain phenolic compounds in their body. The compounds were reported to come from the food of sea cucumbers such as phytoplankton and macro-algae particles. These foods contain phenolics-rich compounds (Althunibat *et al.*, 2009). Findings of this study showed that the average values of total phenolics in *H. atra* extracts varied from 2.14 to 4.99 μg GAE/mg of

extract, depending on solvents and extraction conditions (Figure 3.8). The number was similar with total phenolic content from an aqueous and organic extract of *Stichopus horrens* and *H. edulis* which were 1.49 - 5.24 and 2.17 - 7.33 μg GAE/ mg extract respectively (Althunibat *et al*., 2013). If the number converted to mg GAE/ gram dried body wall (0.31 – 1.32 mg GAE/gram dried body wall), however, the result was lower than the phenolic content from *S. japonicas*. Using the same solvent, the phenolic content of this sea cucumber was 4.86 to 9.45 mg GAE/gram dried body wall (Husni *et al*., 2009). The phenolics content of *H. atra* extracts are low compared to common known phenolics sources such as tea and grape. Various type of tea contains phenolics compound from 110 to 405 mg GAE/ gram (Atoui *et al*., 2005, Rusak *et al*., 2008) and grape from 23 to 80 mg GAE/ gram (Xu *et al*., 2010, Fu *et al*., 2011). The summary of comparison of phenolics content of *H. atra* extracts with other published data is summarized in Table 4.4.

Water extracts of *H. atra* contained significantly higher phenolics than 70%EtOH extracts (Figure 3.8), indicating that most of the phenolics compounds in the *H. atra* extracts were hydrophilic compounds. A similar result was reported from Malaysian (Althunibat *et al*., 2009) and Antarctic sea cucumbers (Mamelona *et al*., 2007).

Different extraction processes had no effect on the phenolics content of water and 70%EtOH extracts of *H. atra*. Different results were obtained from the study of processing the sea cucumber *Cucumaria frondosa* at 65°C for 48 hours. The phenolic content of methanol extract reduced up to 75% (Zhong *et al*., 2007). From the study of the antioxidant activity of barley and malt, it is suggested that the reduction of phenolics content is caused by the changing of phenolic compounds bound; degradation of lignin which could cause to the release of phenolic acid derivatives; and/or the thermal degradation of the phenolic compounds (Maillard and Berset, 1995).

Another study reported that extraction of the fruit mengkudu (*Morinda citrifolia*) at 65°C for 80 minutes using 40%EtOH increases the concentration of phenolics compare to 25, 35, 45 and 55°C with same conditions (Thoo *et al*., 2010).

Table 4.4. Comparison of phenolics content of *H. atra* and other phenolic sources

Species	Total phenolics content		Reference
	(μg GAE/mg extract)	(mg GAE/g dried sample)	
Sea cucumber			
Holothuria atra	2.14 - 4.99	0.31 - 1.32	This study
Cucumaria frondosa		0.225 – 2.36	Mamelona *et al*. (2007)
H. edulis	2.17 - 7.33		Althunibat *et al*. (2013)
H. leucospilota	2.91 - 9.7		Althunibat *et al*. (2009)
H. scabra	1.53 - 4.85		Althunibat *et al*. (2009)
Stichopus chloronotus	1.66 - 8.27		Althunibat *et al*. (2009)
S. horrens	1.49 - 5.24		Althunibat *et al*. (2013)
S. japonicus	11.88 - 22.77	4.86 - 9.45	Husni *et al*. (2009)
Tea			
Black tea (*Camellia sinensis*)		282	Atoui *et al.* (2005)
Green tea (*Camellia sinensis*)		405	Atoui *et al*. (2005)
White tea		110 - 200	Rusak *et al*. (2008)
Green tea		140 - 200	Rusak *et al*. (2008)
Grape			
Black pearl grape		40.20	Xu *et al*. (2010)
Purple		27.56	Xu *et al.* (2010)
Green		23.20	Fu *et al.* (2011)
Red		80.28	Fu *et al*. (2011)

The phenolics group consist of various compounds. In this study these compounds were not analysed. However, in another study using HPLC analysis, the phenolic compounds of *H. atra* from 60:40 (v/v) acetonitrile and 0.1% trifluoroacetic acid extract were shown to contain chlorogenic acid, ascorbic acid, pyrogallol, rutin, coumaric acid and catechin. Chlorogenic acid was the major component with more than 92% of the total phenolic content (Esmat *et al*., 2013). While using Liquid

chromatography - PhotoDiode Array Detection - Mass Spectrometry (LC-DAD-MS) analysis, Chinese green tea was shown to contain gallocatechin 3-gallate, catechin dimmer, caffeine, epigallocatechin 3-methyl gallate, epicatechin 3-gallate, myricetin 3-glycoside, quercetin 3-glycoside, kaempferol 3-glycoside, kaempferol 3-rutinoside, ester of caffeic acid, ester of coumaric acid and xanthoxylin (Atoui *et al*., 2005).

4.5.4. More soluble protein in water extracts

Protein is the main component of sea cucumber after water. After sea cucumber processing, the protein content is between 10 to 70% depend on the processing techniques and type of species (Chang-Lee et al., 1989, Zhong et al., 2007). Proteins in sea cucumbers have beneficial activities. Sea cucumber gelatin hydrolysate reported has antihypertensive activity and decreased the serum triglyceride levels (Taboada *et al*., 2003, Zhao *et al.*, 2007). Glycoprotein fractions from liquid extracts of boiled sea cucumber have skin whitening and anti-corrugation activities (Kim *et al*., 2016). DC Protein Assay (BioRad, USA) which is adopted from the Lowry assay was used to measure protein of the *H. atra* extracts. This method combines the reactions of peptide bonds and copper ions under an alkaline environment to produce Cu+, which further reacts with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction) (Lowry *et al*., 1951).

The soluble protein content in *H. atra* extracts ranged from 0.033 to 0.122 mg/ mg extract depending on the solvents and condition of extractions (Figure 3.9). This number was lower compared to *H. atra* extract using acetonitrile and 0.1% trifluoroacetic acid solvent which contain 0.29 mg/ mg extract (Esmat *et al*., 2013). In addition, using 10% trichloracetic acid to precipitate the water-soluble protein of *Stichopus japonicus* obtained 23% of soluble protein (Tanikawa, 1955). These

differences may be because of the solvent and material that were used. Water extract had a significantly higher soluble protein than 70%EtOH, indicating that protein was more soluble in water rather than in 70%EtOH. A similar result was obtained from the study of antioxidant and antiproliferative of mushroom *Inonotus obliquus*. The soluble protein in water extracts was significantly higher (0.08 – 0.14 mg/ mg extract) than ethanol extract (not detected) (Hu *et al*., 2009).

Overall, the *H. atra* water and 70%EtOH extracts contained saponins ranging from 0.16 to 0.22 mg/ mg extract, sugars 0.62 - 0.91 mg/ mg extract and soluble protein 0.109 - 0.122 mg/ mg extract. *H. atra* extract using acetonitrile and 0.1% trifluoroacetic acid solvent contain 0.018 mg/ mg extract carbohydrate, 0.29 mg/ mg extract protein and 0.027 mg/ mg extract lipid (Esmat *et al*., 2013).

4.5.5. More compounds in 70%EtOH extracts

Chromatographic fingerprinting analysis has been widely used for standardized herbal medicine (Fan *et al*., 2006). The types of chromatography methods include high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), and thin-layer chromatography (TLC) (Xie *et al*., 2006). Although the TLC method is less sensitive than HPLC, it is adequate for comparing compound composition between different extracts. In addition, TLC is simple, easy to use and specific detection reagents are available (Sherma, 2000).

The 70%EtOH extracts contained more compounds than water extracts in both shaking at 60 and sonication at room temperature (Figure 3.10, Table 3.1). This result indicates that the *H. atra* extracts contain more ethanol soluble compounds than water soluble. In plant material, more bioactive compound groups were extracted by ethanol than water; ethanol extracts tannins, polyphenols, flavonol, terpenoids and alkaloids groups while

water extracts anthocyanins, tannins, saponins and terpenoids groups (Azmir *et al*., 2013). In both water and 70%EtOH extracts, there were some similar compounds (compounds number 1 to 4 and 10 to 13) detected in the TLC plate, indicating that these compounds are soluble in water and 70%EtOH. *Azmir et al*. (2013) stated that water and ethanol have similar ability to extract tannins and terpenoids compounds. In addition, 70%EtOH solvent contains 30% water, therefore the 30% water in that ethanol may extract these compounds.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1. Conclusion

The present study demonstrated the potential application of sea cucumber extracts for cosmetic ingredients. The extraction optimization showed that water extracts contained significantly higher of yield than 70%EtOH extracts. Shaking at 60°C produced more yields from 70%EtOH compared to shaking and sonication at 22°C, however these treatments in the water extract were not significantly different.

The safe dosages for sea cucumber extracts varied depending on extraction solvents and conditions and ranged from 2.5 – 10 mg/L extracts. At 2.5 mg/L concentration, the sea cucumber extracts increased the collagen production of human skin fibroblast cells up to 3.7-fold. Water extracts contained more antioxidant capacity and phenolics content compared to 70%EtOH, however, the antioxidant capacities and phenolics content of the extracts were 100 times lower than common antioxidant sources such as green tea and grape.

The chemical characterization of sea cucumber extracts contained 0.15 - 0.22 mg/ mg extract of saponins, 0.06 - 0.09 mg/ mg extract of sugars, 2.14 - 4.99μg /mg extract of phenolics and 0.03 - 0.12 mg/ mg extract of soluble protein. TLC showed a different distribution of compounds between water and 70%EtOH extracts with ethanol 70% extracts exhibiting a higher number of compounds (11 compounds compared to 9 compounds from water extracts). These results indicate strong anti-aging properties of sea cucumber extracts on human skin cells and confirm their potential application for anti-aging skin care product ingredients.

5.2. Future directions

The findings of this study support the potential application of sea cucumber extract for cosmetic application. However, there are some further studies that need to be done to complete this result. In treating skin fibroblast cells using different concentrations of extract in the collagen production it is important to understand the effect of the extract, i.e. whether the collagen increase is dose-dependent or independent. In addition, it is necessary to include a positive control in the treatment to understand which one is better in terms of increasing collagen production. Furthermore, extract purification and identification using TLC-MS and LC-MS are important to distinguish which compound(s) in the extract has influenced the collagen production. The increase of collagen in the cells can occur by different mechanisms, such as inhibiting of MMPs and up regulation of growth factor β, therefore it is important to determine which actions are effected by the sea cucumber extract.

Further studies focusing on the other cosmetic ingredient activities including skin whitening anti-hyaluronidase, pro elastin production and UV protection using different type of cells such as melanocyte and keratinocyte cells, are crucial to determine the total application of sea cucumber extract for cosmeceutical applications. Also, it is important to use different parts and species of sea cucumber in these analyses. Viscera, for example, is a byproduct of sea cucumber processing for food that is known contain more saponins than body wall. A further experiment in the scale-up of the extraction process and *in vivo* studies also can be a part of the clinical pipeline studies.

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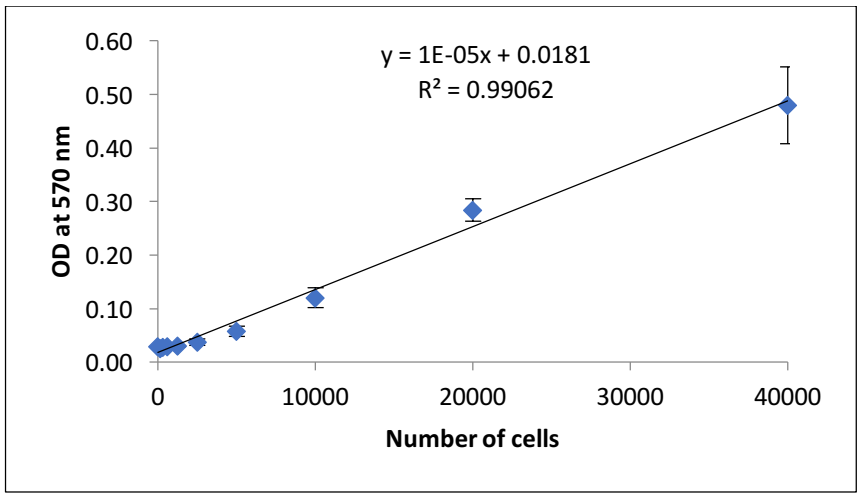
Appendices

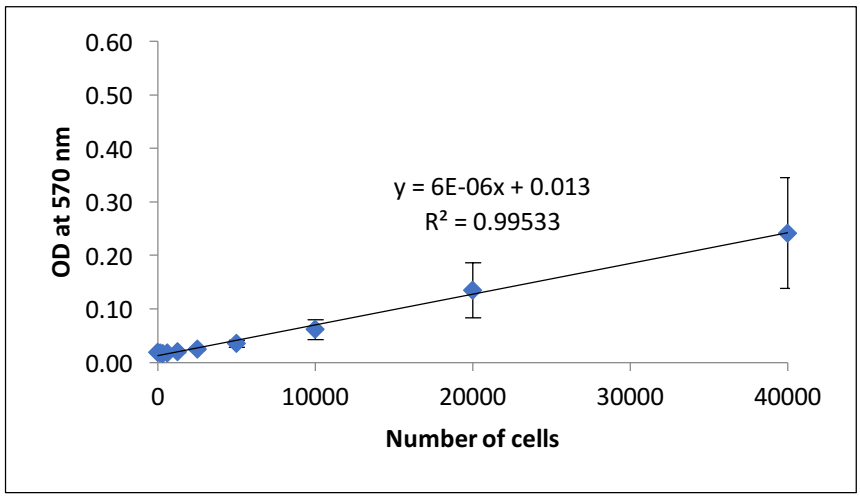
Appendix 1. List of chemical reagents

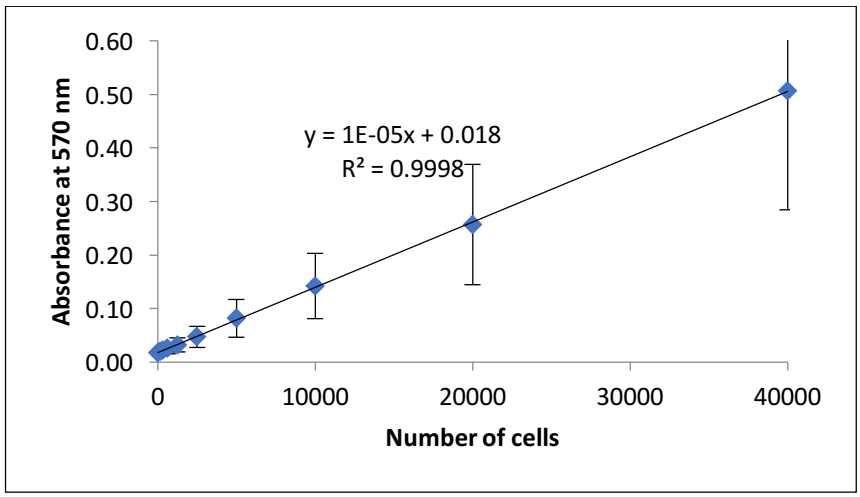
Assay	Chemical reagents	Brand	No Cat	CAS
Extraction optimization	Ethanol	Chem Supply	EL043	64-17-5
	MilliQ Water			
Cytotoxicity				
Cell culture	Iscove's Modified Dulbecco's Medium	Sigma-Aldrich	I3390	
	Fetal bovine serum	DKSH	001F	
	L-Glutamine solution	Sigma-Aldrich	G7513	56-85-9
	Penicillin-Streptomycin	Sigma-Aldrich	P4333	
Trypan blue	Trypan Blue	Sigma	T6146	72-57-1
	Sodium chloride	Sigma	S7653	7647-14-5
Crystal violet	Crystal Violet	Sigma	C0775	548-62-9
	Methanol	Merck	107018	67-56-1
	Acetic acid glacial	Chem Supply	AA221	64-19-7
Anti-aging properties				
FRAP	Tris(2,4,6)-(2-pyridyl)-s-triazine (TPTZ)	Sigma	T1253	3682-35-7
	Hydrochloric acid, fuming 37%	Merck	100317	7647-01-0
	Sodium acetate trihydrate	Sigma-Aldrich	S8625	6131-90-4
	Acetic acid glacial	Chem Supply	AA221	64-19-7
	Ferric chloride hexahydrate	BDH Analar	101104R	10025-77-1
	Ferrous sulfate heptahydrate	Sigma	F8263	7782-63-0
Total Phenolics	Folin-Ciocalteu's phenol reagent	Sigma-Aldrich	F9252	
	Sodium carbonate	Merck		497-19-8
	Gallic acid	Sigma	G-7384	149-91-7
Collagen	Sircol ™ Collagen Assay Kit	Biocolor, UK		
	Dye reagent			
	Alkali reagent			
	Referred standard			
	Acid-Salt wash reagent			
	Acid neutralising reagent			
	Collagen isolation and concentration			

Chemical characterizations				
Saponins	Saponin from quillaja bark	Sigma-Aldrich	S4521	8047-15-2
	Vanillin	Sigma	V2375	121-33-5
	Sulfuric acid 98%	Merck	112080	7664-93-9
	Ethanol	Chem Supply	EL043	64-17-5
Sugars	Anthrone	Sigma-Aldrich	319899	90-44-8
	Sulfuric acid 98%	Merck	112080	7664-93-9
	D-Glucose	Sigma	G8270	50-99-7
Protein	DC™ Protein Assay Kit II	Bio Rad	5000112	
	Protein Standard – BSA			
	Reagent A			
	Reagent B			
Fingerprinting analysis				
TLC	Silica gel 60 F254 aluminium sheets	Merck	1.05554.0001	
	Methanol	Chem Supply	MA004	67-56-1
	Chloroform	Merck	107024	67-66-3
	Iodine			
	MilliQ Water			

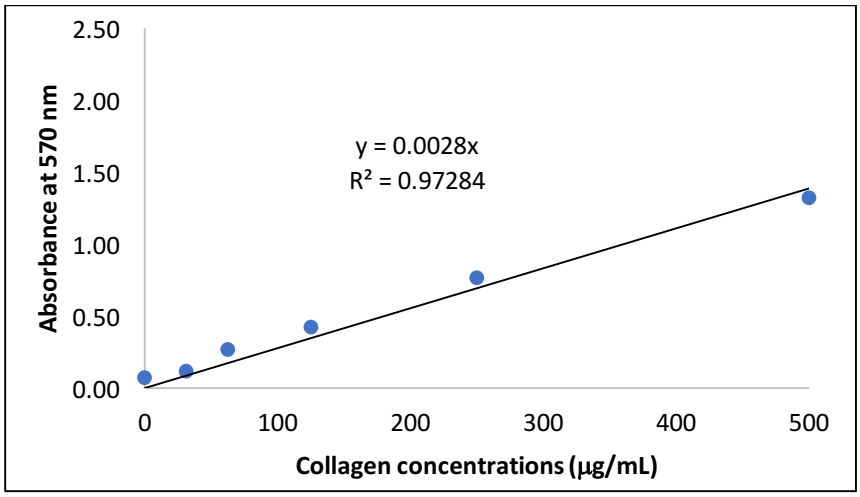
Appendix 2. Standard curves of crystal violet assay

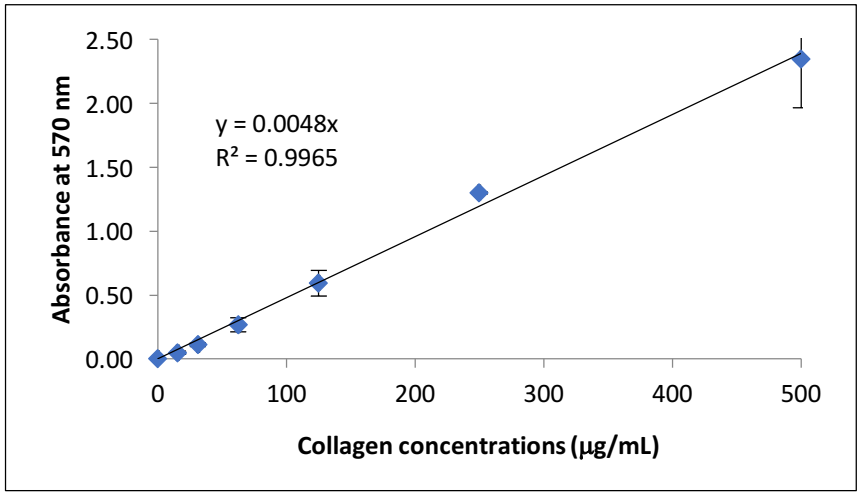




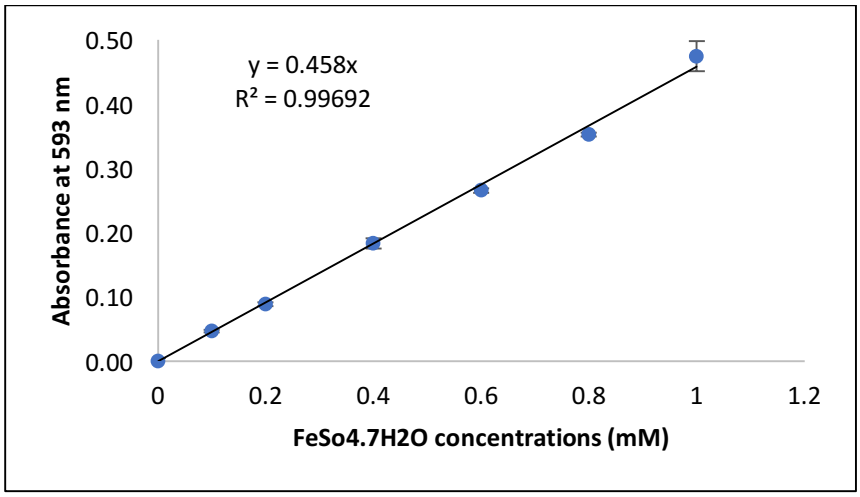


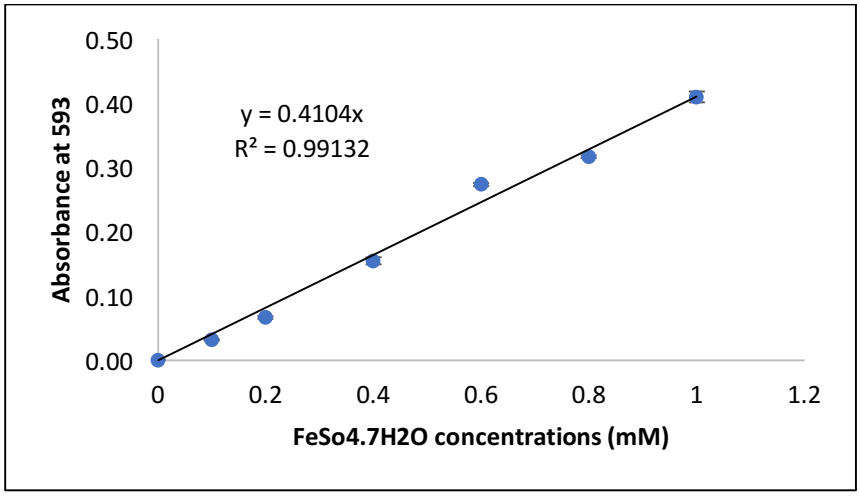
Appendix 3. Standard curves of collagen assay

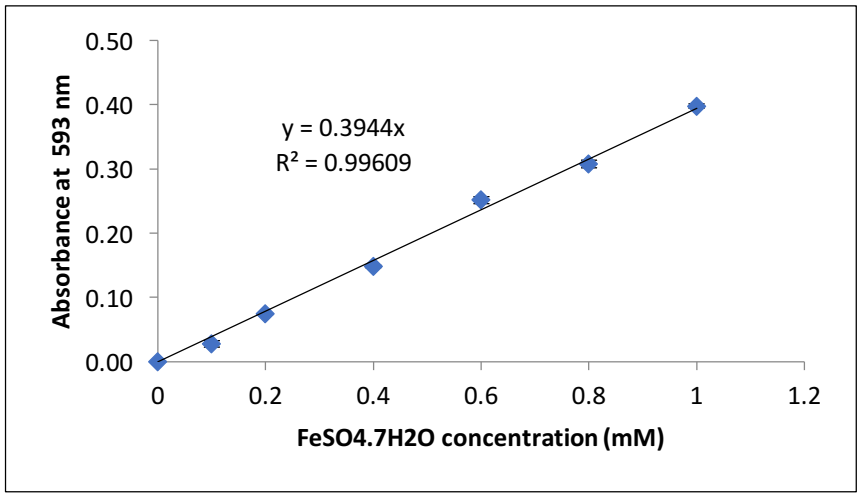




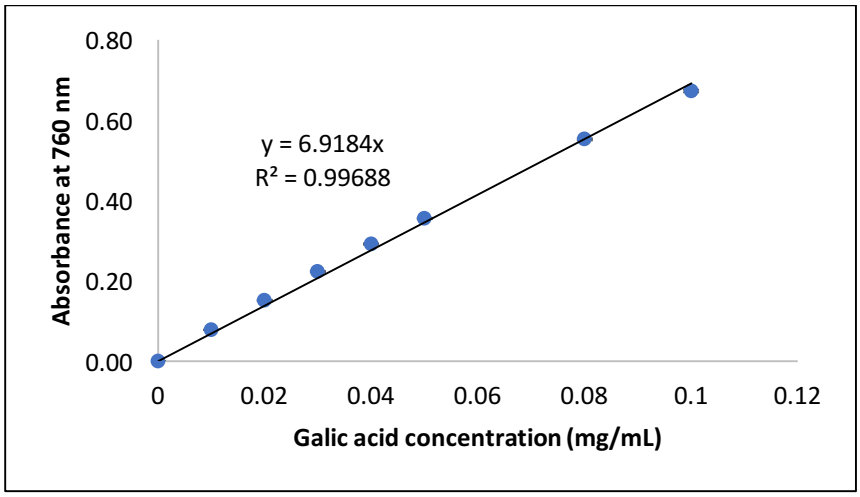
Appendix 4. Standard curves of FRAP assay

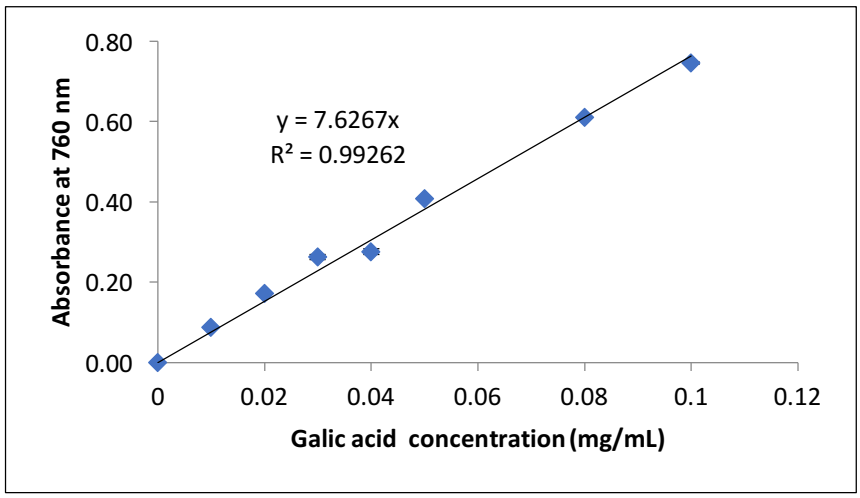


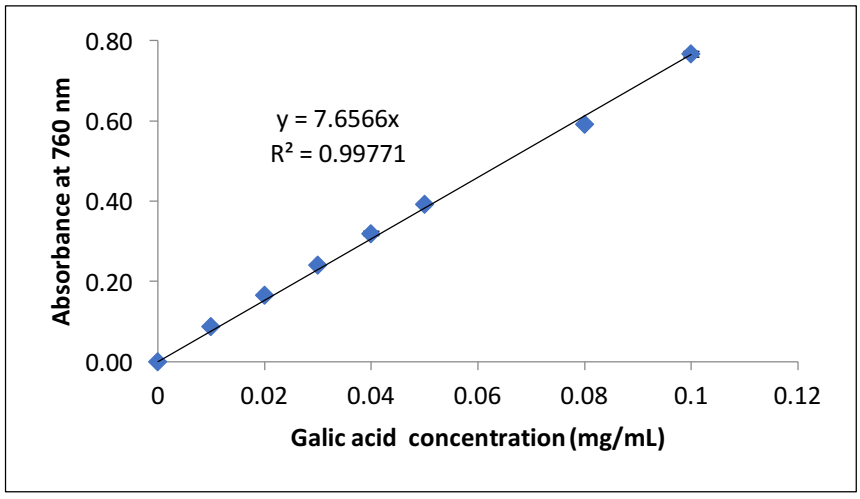




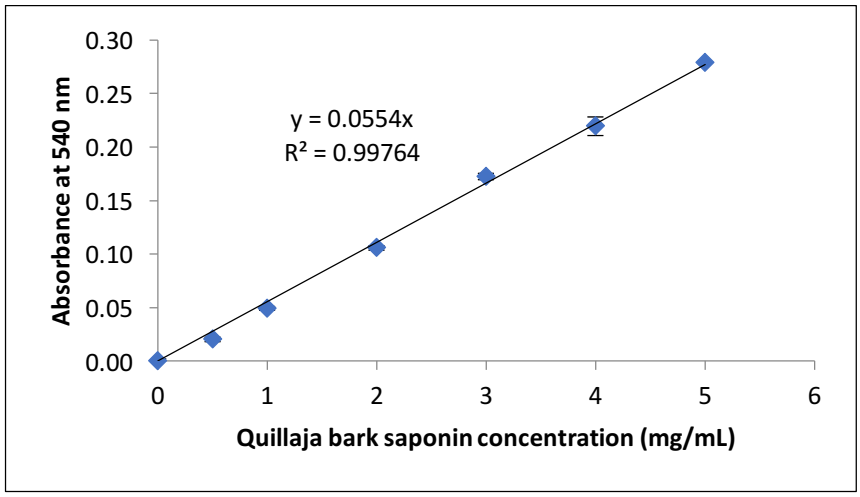
Appendix 5. Standard curves of total phenolics assay

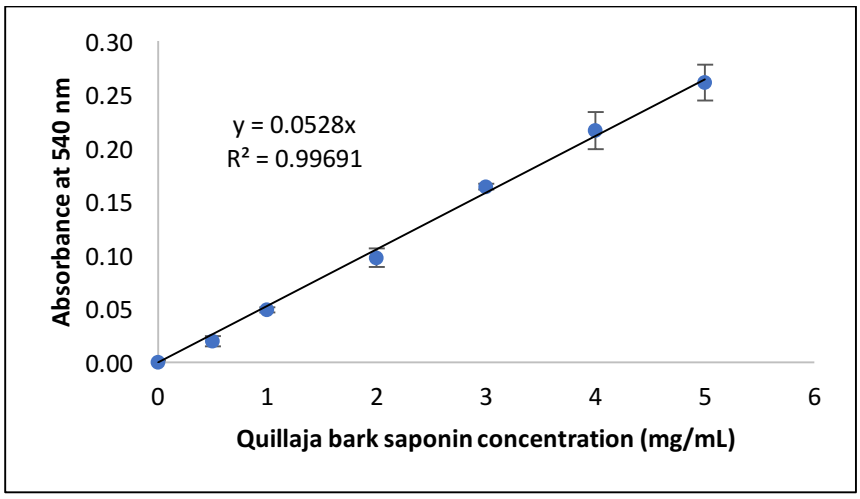


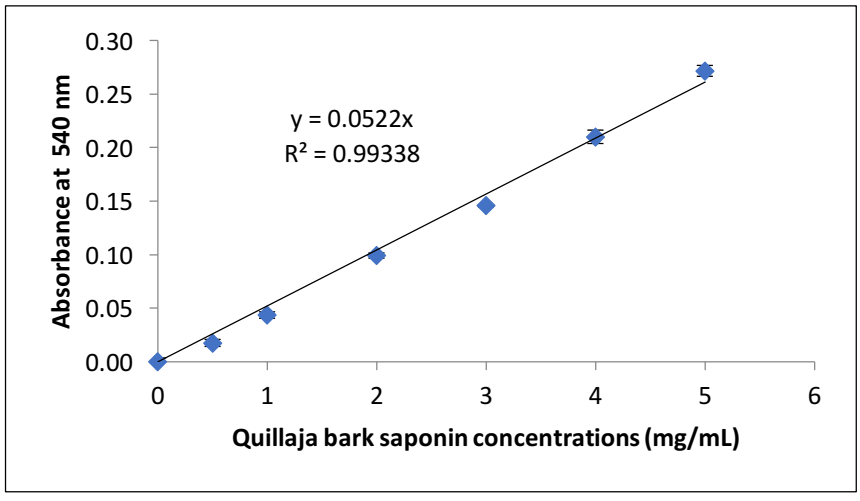




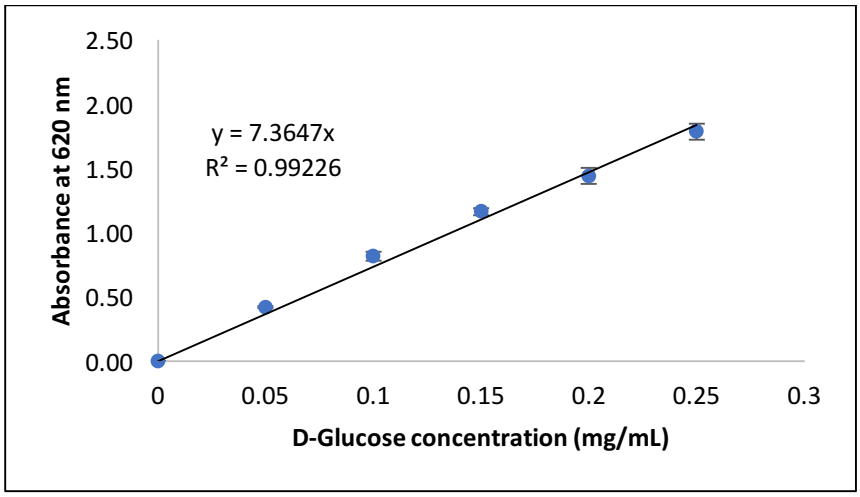
Appendix 6. Standard curves of saponins assay

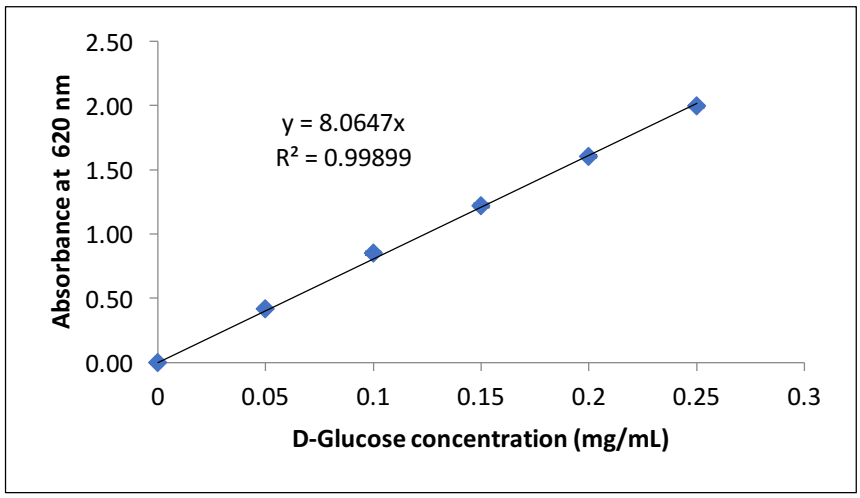


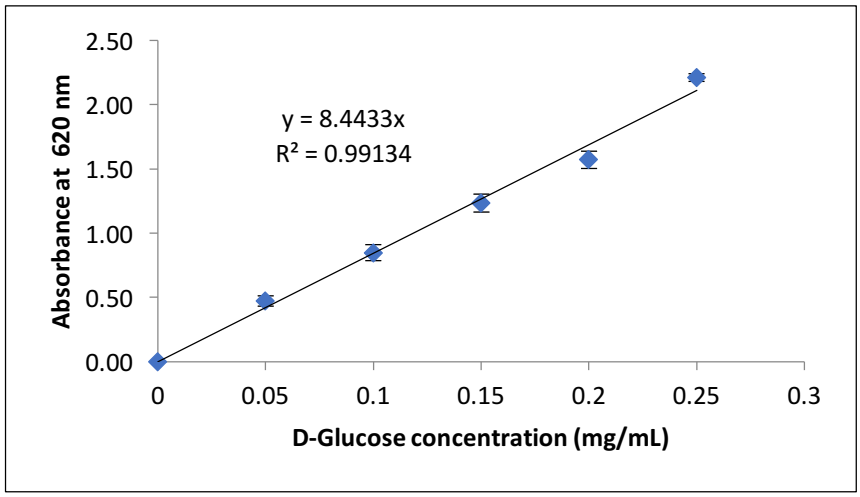




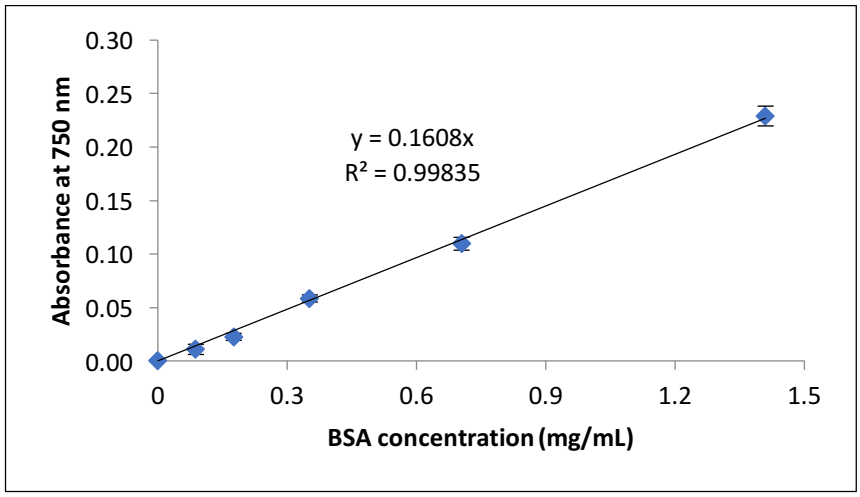
Appendix 7. Standard curves of sugars assay

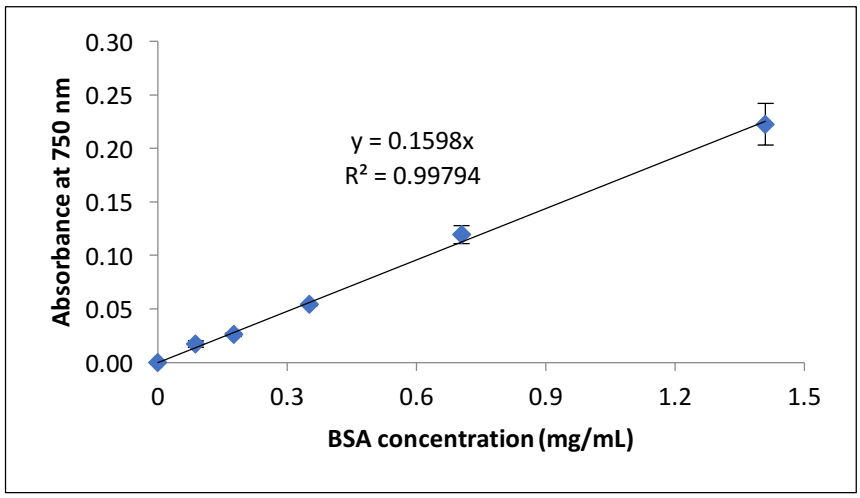


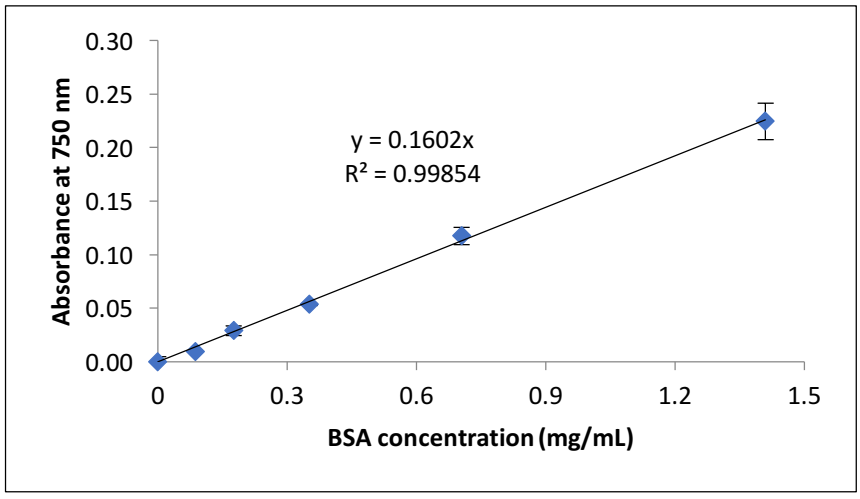




Appendix 8. Standard curves of soluble protein assay

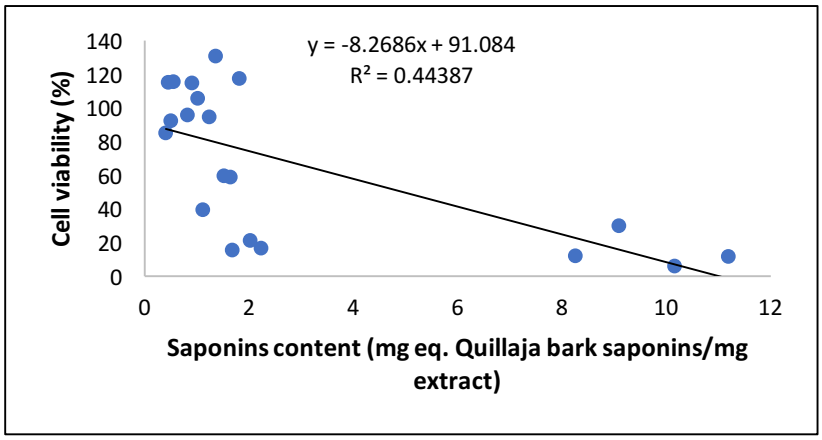




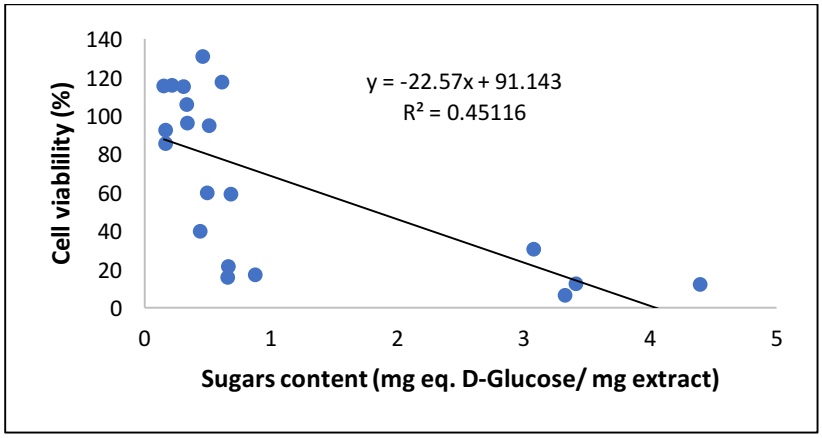


Appendix 9. Correlation between chemical characterizations and cytotoxicity of *H. atra* extract

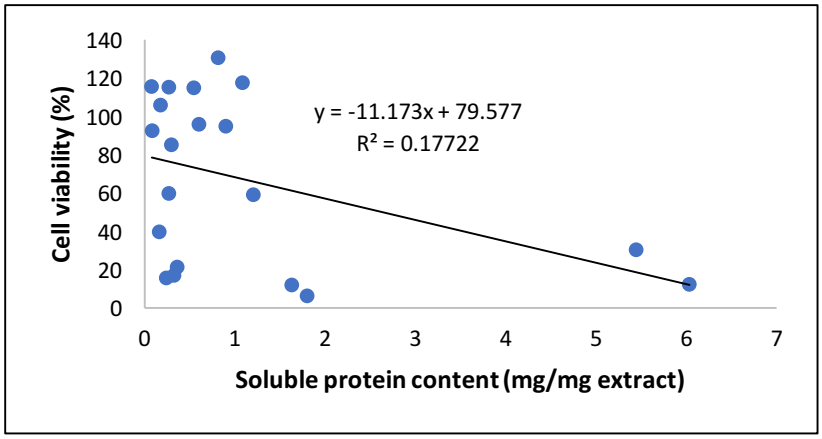
Saponins content



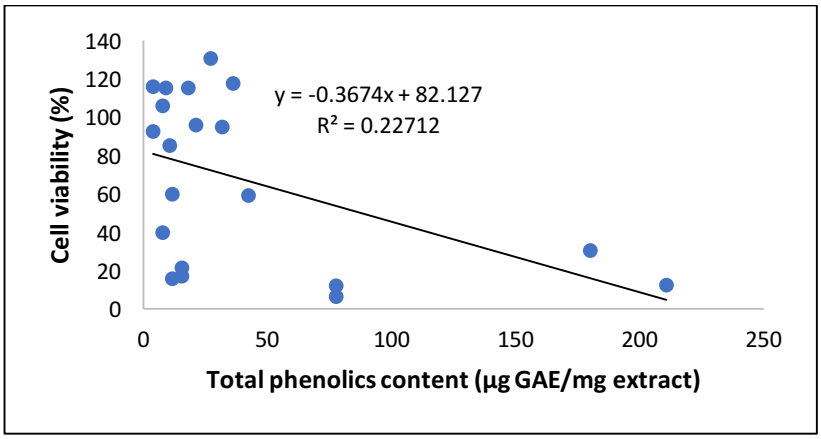
Sugars content



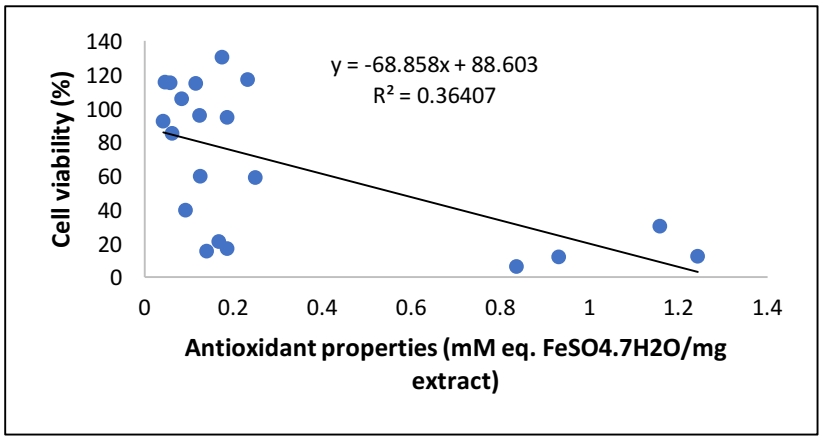
Soluble protein content



Total phenolics content

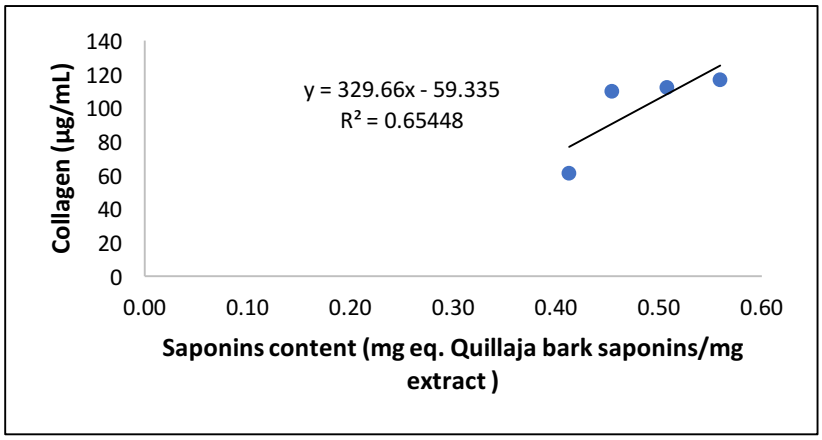


Antioxidant properties

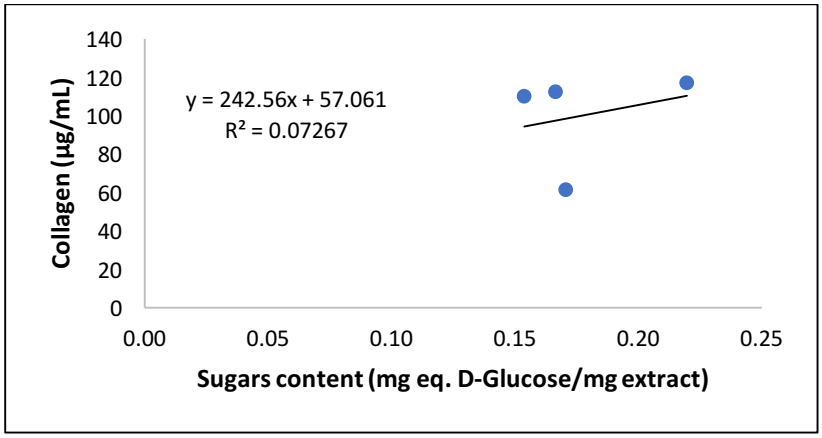


Appendix 10. Correlation between chemical characterizations of *H. atra* extract and collagen production

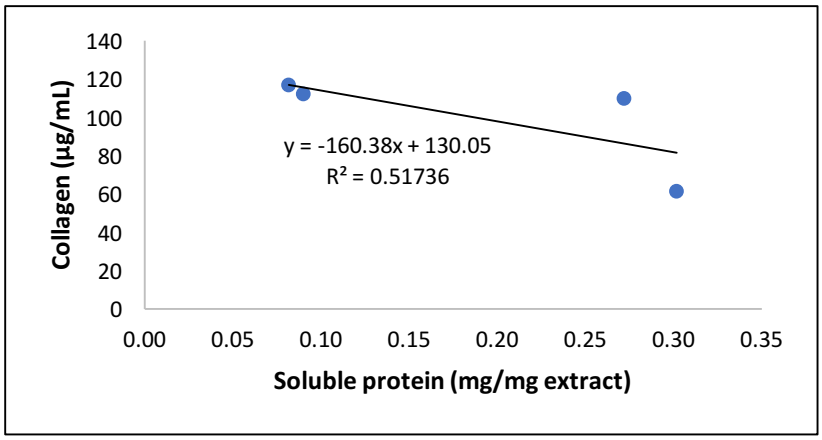
Saponins content



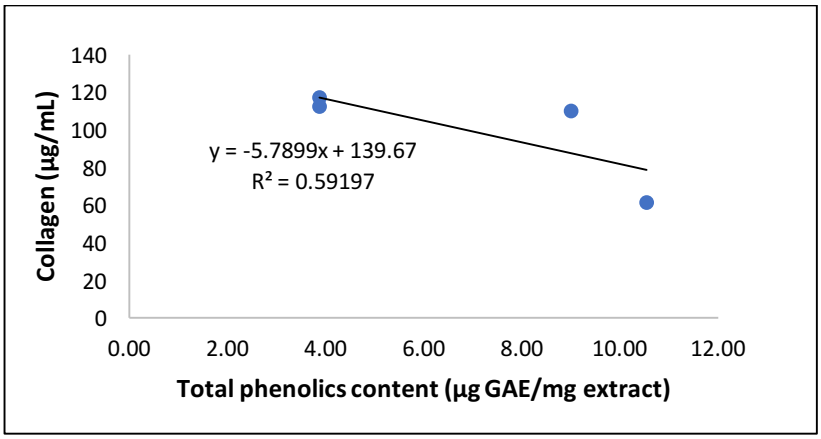
Sugars content



Soluble protein content



Total phenolics content



Antioxidant properties