Effect of High-pressure Processing (HPP) on Phenolics and their Antioxidant Activities of Atlantic Sea Cucumber (*Cucumaria frondosa*)

By

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Abstract

Sea cucumber belongs to the phylum Echinodermata and Holothuroidea class, which contains numerous bioactive compounds, including phenolics. In particular, body wall, aquapharyngeal bulb/ flower, and internal organs/ viscera are rich sources of phenolics. Sea cucumber is harvested mainly for its body wall and muscle bands. However, during sea cucumber processing, several visceral by-products are produced, including gonad, respiratory tract, and intestine, which are ultimately discarded as waste. These visceral by-products represent up to 50% of the sea cucumber biomass and are a rich source of bioactive compounds, mainly phenolics. The most common sea cucumber found in the North Atlantic region is the orange-footed sea cucumber (*Cucumaria frondosa,* Gunnerus, 1767). However, this species is under-explored for its bioactive compounds, especially its polyphenolic composition. Hence, this study investigated the free, esterified, and insoluble-bound phenolics from different commercial body parts (tentacles and body wall) and processing discards of sea cucumber (*C. frondosa*), as affected by high-pressure processing (HPP) pre-treatment. For that, sea cucumber was subjected to high-pressure (2000, 4000, and 6000 bar for 5, 10, and 15 min), followed by separation into the three aforementioned fractions. The contents of total phenolics and flavonoids were determined, and antioxidant activity reflected in scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), and hydroxyl radicals was monitored. The metal chelating ability of various phenolic fractions was also evaluated. Moreover, bioactivities such as inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission, LDL-cholesterol oxidation inhibition, antiglycation activity, and α-glucosidase activity were examined. Furthermore, thiobarbituric acid (TBA) assay in a fish-model system was performed. Compared to untreated samples, those treated with HPP exhibited significantly higher total phenolics,

flavonoids, and antioxidant activities. Treatment of 6000 bar for 10 min offered the optimal results. The free phenolic fraction was the predominant form present in all body parts examined. The highest amount of phenolics and antioxidant activity was observed in flower in the free phenolic fraction, whereas esterified and insoluble-bound phenolic fractions were more abundant in the body wall. Besides, in terms of bioactivities, all phenolic fractions showed strong inhibitory properties, mainly those that were in the free phenolic fraction. Additionally, 31, 23, and 20 phenolic compounds were identified from the flower, internal organs, and body wall, respectively, using UHPLC-QTOF-MS/MS. Phenolic acids, mainly *p*-coumaric acid, protocatechuic acid, hydroxygallic acid, and chlorogenic acid, and flavonoids, including quercetin and catechin, were the major compounds found in the different phenolic fractions of Atlantic sea cucumber. The highest amount of phenolic compounds of sea cucumber was observed as free phenolic in internal organs based on the UHPLC-MS analysis. Thus, this investigation enhances the fundamental understanding of the full utilization of Atlantic sea cucumber species and leads to the production of a multitude of value-added products.

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AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonate
AGEs	Advanced glycation end-products
ANOVA	Analysis of variance
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BW	Body wall
CD	Conjugated dienes
CE	Catechin equivalents
DFO	Department of Fisheries and Oceans
DHA	Docosahexaenoic acid
DMPO	5,5-Dimethyl-1-pyrroline-N-oxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
E	Esterified phenolic fraction
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
EPA	Eicosapentaenoic acid
ESI	Emulsification stability index
ET	Electron transfer

F	Free phenolic fraction
FAO	Food and Agriculture Organization
FCS	Fucosylated chondroitin sulfate
FL	Flower
FRAP	Ferric reducing antioxidant power
FTIR	Fourier-transform infrared spectroscopy
GAE	Gallic acid equivalents
GC	Gas chromatography
HAT	Hydrogen atom transfer
HPP	High-pressure processing
UHPLC-MS/MS	Ultra high-performance liquid chromatography-mass spectrometry/ mass spectrometry
IB	Insoluble-bound phenolic fraction
IO	Internal organs
LDL	Low-density lipoprotein
MALDI	Matrix-assisted laser desorption ionisation
MDA	Malondialdehyde
MPa	Megapascal
MS	Mass spectroscopy
MUFA	Monounsaturated fatty acids
NAFO	Northwest Atlantic Fisheries Organization
ORAC	Oxygen radical absorbance capacity
POD	Peroxidase

PPO	Polyphenol oxidase
PUFA	Polyunsaturated fatty acids
QTOF	Quadrupole time of flight
ROS	Reactive oxygen species
SET	Single electron transfer
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
TFC	Total flavonoid content
TOF	Time of flight
UHPLC	Ultra-high performance liquid chromatography

Chapter 1

Introduction and Overview

Sea cucumber, also known as holothurian, is considered a luxury and delicacy food item and has been used in traditional medicine in the Asian culture for decades (Hossain, Dave, & Shahidi, 2020a). This echinoderm possesses a wide range of bioactive compounds that show unique biological activities. As a result, sea cucumber has received greater attention due to its potential therapeutic benefits and as a marine food product. Especially, it has gained increasing interest due to its impressive nutritional profile, including low fat and high protein contents. This invertebrate contains numerous compounds, namely lipids (PUFAs), proteins (collagen and peptides), vitamins, polysaccharides (chondroitin sulfate), saponins, and phenolics, which exert unique biological and pharmacological properties such as antioxidant, anti-inflammatory, antimicrobial, anticancer, antithrombotic, and wound healing properties (Bordbar, Anwar, & Saari, 2011; Senadheera, Dave, & Shahidi, 2020). The body wall of sea cucumber is the main marketable food product, whereas some sea cucumber processing industries also sell the tentacles (flower), which are mainly found as a dried form in the market (Hossain et al., 2020a).

The most common sea cucumber distributed in the North Atlantic Ocean is *Cucumaria frondosa* (Gunnerus, 1767), orange-footed sea cucumber, which has been harvested in recent years for commercial purposes (Hossain et al., 2020a). In Newfoundland and Labrador, sea cucumber is harvested from Northwest and Southeast regions of the St. Pierre Bank (NAFO Division 3Ps), and the landed volume has gradually increased from 454 MT in 2003 to 5,579 MT in 2018, which was valued at $6.1 million (Gianasi et al., 2020). However, the main drawback that hinders the expansion of this highly marketable sea cucumber species is that a limited amount of information

is available on the potential utilization, resulting in it is still considered as a medium-grade product compared to other commercial sea cucumber species. Particularly, this species is under-explored for its bioactive compounds and full utilization. The body wall of this marine invertebrate is the major edible part; however, during sea cucumber processing, non-marketable portions, such as internal organs including gonad, respiratory tract, and intestine, are ultimately discarded as processing waste. During processing, these visceral by-products represent up to 50% of the sea cucumber biomass and are a rich source of fatty acids, amino acids, vitamins, and minerals, as well as carotenoids, and phenolics (Hossain et al., 2020a; Liu et al., 2020a; Senadheera, Dave, & Shahidi, 2021). Apart from this, commercial body parts such as the body wall and flower of Atlantic sea cucumber contain a wide range of bioactive compounds, including phenolics (Zhong, Khan, & Shahidi, 2007; Mamelona et al., 2007). Therefore, sustainable harvesting, processing, and utilization of this benthic marine invertebrate and its discards are crucial to increasing product quality and maintaining market demand. However, less information is available on the bioactive compounds (e.g., phenolics) of *C. frondosa* and its discards and their potential utilization as functional ingredients. Specifically, the chemical composition of phenolic compounds and their biological activity of any sea cucumber species are poorly known. Thus, there is a need to develop a promising bioprocessing strategy for the extraction, identification, and characterization of phenolic compounds in order to maximize the sustainability and economic viability of the industry.

Phenolic compounds are powerful natural antioxidants, which demonstrate multiple biological properties such as antithrombotic, anticoagulant, anticancer, anti-inflammatory, antitumor, and antidiabetic activities (Shahidi & Ambigaipalan, 2015). However, most of the phenolic-rich functional foods available in the marketplace are obtained from the terrestrial environment, while less attention has been paid to the marine environment even though it provides

many healthy foods due to its abundant and phenomenal biodiversity. Hence, much attention has been paid in recent years to upgrading seafood and its processing waste to value-added products by recovering its bioactive compounds, including phenolics. Phenolic compounds can be categorized into three groups based on their solubility in the extraction medium, namely free, esterified/ etherified, and insoluble-bound phenolics. Most of the free and esterified are localized in the vacuole of cells by weak chemical bonding, while insoluble-bound phenolics are attached with the cell wall components (e.g., structural proteins, cellulose, and hemicellulose) via covalent bonding (Yeo et al., 2021). Thus, there is a need to develop a promising bioprocessing strategy to extract and identify phenolics and their biological properties in order to improve their bioaccessibility and bioavailability. For instance, high-pressure processing (HPP) is a technique that affects the extraction efficacy with higher extraction yields in a shorter time while avoiding thermal degradation of their chemical structures and improving bioaccessibility (Rodríguez-Roque et al., 2015). Few of the recent studies have focused on the extraction and identification of phenolics from different species of sea cucumber. So far, only a couple of studies have been conducted on the phenolics of Atlantic sea cucumber, mainly on the total yield of phenolics and their antioxidant properties (Zhong, Khan, & Shahidi, 2007; Mamelona et al., 2007). However, to the best of our knowledge, there is no report available on identifying and quantifying individual phenolic compounds from the Atlantic sea cucumber. Moreover, no study has been done on the extraction of free, esterified, and insoluble-bound phenolics and their antioxidant activity from any species of sea cucumbers, including Atlantic sea cucumber.

Thus, the main objective of this study was to extract, identify, quantify, and characterize phenolic compounds using HPP as a pre-treatment and determine their bioactive potential of different commercial body parts of the Atlantic sea cucumber and its processing discards. The

central hypothesis of the present study was the HPP would increase the extraction of phenolic compounds and improve antioxidant activity of different body parts of sea cucumber compared to their untreated counterparts.

The specific objectives of this study are summarised below.

I. To extract free, esterified, and insoluble-bound phenolics from the Atlantic sea cucumber and its processing discards using HPP pre-treatment;
II. To optimize HPP parameters (2000, 4000, and 6000 bar for 5, 10, and 15 min);
III. To measure the effect of HPP on the antioxidant potential using radical scavenging activity and metal chelation ability of free, esterified, and insoluble-bound phenolics;
IV. To determine bioactivities such as inhibitory activities against LDL cholesterol oxidation and DNA strand breakage of sea cucumber phenolics; and
V. To identify and quantify free, esterified, and insoluble-bound phenolics in the Atlantic sea cucumber using ultrahigh performance liquid chromatography (UHPLC) coupled with a mass spectrometry (MS).

Chapter 2

Literature Review

2.1. Sea cucumber

2.1.1. History of sea cucumber: Traditional medicine to aquaculture

Sea cucumbers are marine invertebrates belonging to the class Holothuroidea and the phylum Echinodermata and are also called holothurians or holothuroid echinoderms. Sea cucumber has been consumed and utilized as a traditional medicine for centuries in Asia, particularly in China, where it has long been an important part of their culture and tradition (Purcell et al., 2013). Ancient Chinese texts refer to sea cucumber as "Hai Sheng" that translates to "ginseng of the sea", and has been harvested for human consumption since 200 BC (Yang, Hamel, & Mercier, 2015). One of the tales asserts that the first Emperor of China assigned one of his servants to search for the everlasting elixirs (immortals) in the three Fairy Mountains located at northeast China (Bohai Bay, Gulf of the Yellow Sea). The servant and his crew started their journey by a boat, and after drifting a very long time at sea, they did not discover the Fairy Mountains, the everlasting elixirs, or the immortals. After a long time sailing and searching, they ran out of food on the boat and had to land on an island where sea cucumbers were abundant. Several days after eating sea cucumbers, the servant became stronger; so, he started to eat sea cucumbers regularly and stayed on the island for over 50 years. At the age of 90 years, he still looked very young, healthy, and strong as well as being free from any diseases. Finally, he realized that the sea cucumbers were actually the “everlasting elixir” and assigned a crew to deliver sea cucumbers to the first Emperor that he was searching for many years. In another tale, over a thousand years ago, a fisherman (Hai Sheng) discovered his parents developed a very strange disease and could not recover for a long time. One day, Hai Sheng fell asleep, and in his dream, he saw that only a

creature in the sea could cure his parents. Several days after having meals with that creature (sea cucumber), his parents recovered completely from the strange disease, and he named it as "seagods" (Yang & Bai, 2015).

Over the years, beliefs have been recognized in modern biomedical research towards the potential medicinal benefits as well as multiple biological activities of sea cucumber. Sea cucumbers have been used as traditional medicine in Asian countries to cure hypertension, anemia, kidney problem, asthma, stomach ulcers, reproductive disorder, rheumatism, wound injuries, cuts and burns, joint pain, back pain, impotence, constipation, and so on. For example, boiled sea cucumber skin extracts have been consumed to treat asthma, hypertension, cuts, burns, and wound healing in Malaysia, whereas dry tablets prepared from sea cucumber body wall have been used for physiological and nutraceutical benefits in Asia and the USA (Fredalina et al., 1999; Hossain et al., 2020a; Taiyeb-Ali et al., 2003). Therefore, dry capsules made from the body wall, liquid extract prepared from whole sea cucumber, and extracts obtained from the skin of sea cucumber are popular as nutritional supplements in Asia (Southeast Asia). Interestingly, sea cucumber viscera from Pacific and Mediterranean species have been consumed as raw, boiled, or pickled forms. Additionally, sea cucumber-based components have found application in cosmetics, including face masks, facial hydrating gels, creams, and body lotions (Bordbar, Anwar, & Saari, 2011; Hossain et al., 2020a; Siahaan et al., 2017; Wen, Hu, & Fan, 2010).

Sea cucumber comprises very low levels of fat and cholesterol but a high content of protein when compared to other types of food. From a nutritional perspective, sea cucumber is an ideal tonic food and has an impressive nutritional profile such as carbohydrates (mainly polysaccharide), proteins (mostly collagen), lipids (mainly omega-3 and 6 fatty acids), minerals (mostly zinc, calcium, magnesium, and iron), and vitamins (mainly A, B1, B2, and B3) (Aminin et al., 2015;

Hossain et al., 2020a; Liu et al., 2017; Liu et al., 2019a; Senadheera, Dave, & Shahidi, 2021). However, the proximate composition of sea cucumber depends on the species, geographical location, seasonal variation, and feeding behavior (Xu, Zhang, & Wen, 2018). Generally, fresh sea cucumbers contain 82.0 to 92.6% moisture, 2.5 to 13.8% protein, 0.1 to 0.9% fat, 0.2 to 2.0% carbohydrate, and 1.5 to 4.3% ash (Bordbar et al., 2011). Moreover, proteins account for 40-60%, whereas total lipids account for 2-8% in sea cucumber dry matter. For example, the chemical composition and nutritional quality were determined from eight dried sea cucumbers and reported that the protein contents varied between 40.7 and 63.3% (Wen, Hu, Fan, 2010). Collagen is the main protein of sea cucumber, with up to 70% insoluble collagen fibrils present in the body wall (Saito et al., 2002; Senadheera, Dave, & Shahidi, 2020). Furthermore, glycine and glutamic acid are the two common amino acids in sea cucumber proteins. Especially, amino acid residues, including cysteine, histidine, asparagine, and glutamate in peptides are closely related to their metal chelating ability (Liu et al., 2019b). Unsaturated fatty acids can account for up to 70% of sea cucumber lipid; among them, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are dominant fatty acids, mainly in tropical sea cucumbers. Additionally, sea cucumber polysaccharides are fucosylated chondroitin sulfate (FCS), fucoidan, and fucan, which have the potential to ease arthritis and connective tissue disorders (Bordbar et al., 2011).

Sea cucumber contains several bioactive constituents, including glycosaminoglycans (Nagase et al., 1995; Xu, Zhang, & Wen, 2018), triterpene glycosides (saponins) (Aminin et al., 2010; Pangestuti & Arifin, 2017), chondroitin sulfate (Ustyuzhanina et al., 2018; Vieira, Mulloy, & Mourão, 1991), sulfated polysaccharides (Hossain et al., 2020a; Mourao, Pereira, & Pavao, 1996), phenolics (Mamelona et al., 2007; Zhong, Khan, & Shahidi, 2007), cerebrosides (Ming-Ping et al., 2012), lectins (Gowda, Goswami, & Khan, 2008), and sterols (glycosides and sulfates)

(Claereboudt et al., 2018). In particular, the body wall, which represents around 50% of the total body weight, is a rich source of polysaccharides and proteins (collagen), whereas flower and internal organs mostly contain carotenoids and phenolics (Oh et al., 2017; Pangestuti & Arifin, 2017). The bioactive compounds identified from different sea cucumbers possess various biological functions, including anticancer (Aminin et al., 2015; Janakiram, Mohammed, & Rao, 2015; Wargasetia, Permana, & Widodo, 2018), antitumor (Borsig et al., 2007), anticoagulant (Chen et al., 2011; Nagase et al., 1995), anti-inflammatory (Olivera-Castillo et al., 2018; Whitehouse & Fairlie, 1994), anti-angiogenic (Tian et al., 2005), anti-obesity (Tian et al., 2016), anti-hypertension (Dewi et al., 2020), antithrombotic (Liu et al., 2016a), antioxidant (Althunibat et al., 2009; Zou et al., 2016), antimicrobial (Darya et al., 2020; Ghadiri et al., 2018), and wound healing properties (Miguel-Ruiz & García-Arrarás, 2007). For instance, triterpene glycoside can induce apoptosis in cancer cells via different pathways, including Fas and Caspase-8 activation, decreasing BCL2 and increasing caspase 3, intracellular caspase cell death pathways, and increasing p21 and p16. Sea cucumber extract has cytotoxicity effects on many cancer cells, namely pancreatic cancer (AsPC-1 and S2013), lung cancer (LNM35, NCI-H460-Luc2, A549, and SPC-A4), prostate cancer (LNCaP and PC-3), colon cancer (Caco-2, CT26, HCT116, and DLD-1), and breast cancer (MCF7 and MDA-MB-435) (Wargasetia et al., 2018). For instance, Omran and Khedr (2015) extracted non-sulfated hexoside from *Holothuria polii* and reported that this glycoside showed cytotoxic effect against two tumor cell lines, MCF7 (breast adenocarcinoma cell line) and HCT116 (colon adenocarcinoma cell line). Furthermore, a fucan compound extracted from *Isostichopus badionotus* exhibited high antithrombotic and anticoagulant properties *in vitro* compared with heparin (Chen et al., 2012). FCS from *Cucumaria frondosa* can enhance glucose metabolism and insulin sensitivity via maintaining the crucial genes for phosphorylation, including

Ser473-PKB, p85-PI3K, and Thr308-PKB in PI3K/PKB pathways (Xu, Zhang, & Wen, 2018). Similarly, FCS was isolated from *Acaudina molpadioides*, and its impact on adipogenesis was examined *in vitro*. Results demonstrated that FCS could inhibit adipose (fat) tissue growth (Xu et al., 2015). Besides this, these biologically active compounds can be used to prepare cosmetics for the face, mouth, hand, nails, hair, feet, scalp, and other body parts due to their antimicrobial, anti-aging, photo-protective, anti-wrinkling, and moisture retention properties (Siahaan et al., 2017).

Over time, sea cucumber acquired the place among the most expensive seafood in the world due to its unique and enormous medicinal properties. Sea cucumbers are considered as a luxurious and nutritious food throughout Asia, especially they are served at the wedding, corporate, and banquet events (Gianasi et al., 2020; Purcell et al., 2014). Due to medicinal properties, dried sea cucumber, known as "bêche-de-mer", has been widely consumed in China, Japan, Malaysia, Korea, Indonesia, and Russia. Approximately 1500 species of sea cucumber have been documented so far, and around 100 of them are globally harvested commercially for human consumption (Mercier & Hamel, 2013). The most common commercial sea cucumber species found in the market is summarized in Table 2.1. Sea cucumbers are harvested all over the world, mainly in tropical regions. Commercially, sea cucumber body parts can be divided into three parts, namely body wall (major edible part), aquapharyngeal bulb/ tentacles/ flower, and viscera/ internal organs (processing discards). The price of the dried sea cucumber may vary from US$ 15 to 2,950/ kg (Mohsen &Yang, 2021). However, due to overfishing, many wild populations are overexploited throughout the world. As a result, aquaculture, restocking, and sea ranching have been started in a few countries, particularly in the shallow tropical waters of the Indo-Pacific and Asia (Brown et al., 2010; Mercier & Hamel, 2013; Pangestuti & Arifin, 2017). In order to supply the exponentially growing demand for sea cucumbers in the Asian markets, many species have been explored as

potential candidates for aquaculture. Traditionally, deposit-feeding species were initially focused on aquaculture production. Nevertheless, suspension-feeding species, such as Atlantic sea cucumber (*C. frondosa*), differs from cultivated sea cucumbers in several ways; mainly, it is a passive suspension-feeder, cold-water species, and produces large yolky eggs (Gianasi et al. 2020; Singh et al., 1998). Although aquaculture of the Atlantic sea cucumber stays mainly theoretical, *C. frondosa* has many positive points in favor of an aquaculture candidate. For example, *C. frondosa* diet contains mainly phytoplankton, which is supposed to be more nutritive than the deposit-feeding sea cucumbers. Moreover, large eggs may have better chances of survival during metamorphosis. However, the major challenge for being an aquaculture candidate is the slow growth rate of this species compared to its other commercial counterparts. It is estimated that *C. frondosa* may take up to 25 years to become an ideal size for marketing in the natural environment (Gianasi et al., 2020).

Table 2. 1. The most common commercial sea cucumber species in the market

Scientific name	Common name	Distribution (Country/ region)	Price (US$/ kg) dried	References
Apostichopus japonicus	Japanese sea cucumber and Bêche-de-mer japonaise	Northeast Asia (Japan, China, Korea, and Russian Federation)	970-2950	(Purcell, Samyn, & Conand, 2012)
Actinopyga mauritiana	Surf redfish and Holothurie brune des brisants	Indo-Pacific, South China Sea, Africa, and Hawaii	15-145	(Bruckner, Johnson, & Field, 2003)
Actinopyga palauensis	Deepwater blackfish and	East Australia	95-116	(Purcell, Samyn, &

	Panning's blackfish			Conand, 2012)
Cucumaria frondosa	Orange-footed sea cucumber, Atlantic sea cucumber Northern sea cucumber, Phenix sea cucumber, and Pumpkin	Eastern coast of Canada, southeast coast of New England, northern Europe, Scandinavia, and Barents Sea	30-330	(Hossain et al., 2020a)
Cucumaria japonica	Japanese cucumaria and Black sea cucumber	Yellow Sea, Honshu Island, and Pacific coast	N/A	(Purcell, Samyn, & Conand, 2012)
Holothuria atra	Lollyfish and Black lollyfish	Indo-Pacific, Red Sea, South-Pacific, South China Sea, and Persian Gulf	63-210	(Purcell, Samyn, & Conand, 2012)
Holothuria fuscogilva	White teatfish	South-Pacific, South East Asia, and Indian Ocean	128 – 274	(Bordbar et al., 2011)
Holothuria hilla	Tiger Tail, Mani-mani, Bat-tuli, and light-spotted sea cucumber	Indo-Pacific, South-Pacific, Persian Gulf, South China Sea, and Red Sea	3-20	(Purcell, Samyn, & Conand, 2012)
Holothuria nobilis	Black teatfish	Indo-Pacific, South Pacific, East Africa, Red sea, and Arabian Sea	100-140	(Bordbar et al., 2011)
Holothuria scabra	Sandfish	South-Pacific, South East Asia, and Indian Ocean	115-1668	(Bruckner, Johnson, & Field, 2003)
Isostichopus badionotus	Four-sided sea cucumber	Caribbean Sea, Mid Atlantic, and Western Africa	203-402	(Purcell, Samyn, &

				Conand, 2012)
Pearsonothuria graeffei	Blackspotted sea cucumber, Flowerfish, and orange fish	South-Pacific and South East Asia	2-5	(Purcell, Samyn, & Conand, 2012)
Stichopus japonicus	Japanese sea cucumber	North West pacific and Japan Coastal Areas	450-670	(Bordbar et al., 2011)
Thelenota ananas	Prickly redfish	South-Pacific	22-184	(Purcell, Samyn, & Conand, 2012)

2.1.2. Biology of sea cucumber

Sea cucumbers are ubiquitous and play vital roles in marine ecosystem as they assist in recycling nutrients and breaking detritus (Purcell et al., 2016; Sun et al., 2020). Sea cucumbers are deposit or suspension feeders, which consume and grind sediment, organic matters, or phytoplankton into finer particles. Suspension-feeding sea cucumbers regulate water quality by altering its pH and carbonate content, whereas deposit-feeding sea cucumbers modulate the stratification and stability of the sediment via bioturbation and ingestion (Massin, 1982; Uthicke, 2001). Sea cucumber, mainly *C. frondosa*, has a soft and cylindrical body with leathery skin and branched gonad. The mouth is surrounded by aquapharyngeal bulb/ tentacles/ flower, which can be pulled back inside the body. Generally, the body of sea cucumber is roughly cylindrical, containing five rows of tube feet on the skin, known as "podia". The most common commercial sea cucumbers belong to the taxonomic order of Aspidochirotida and Dendrochirotida. Most species are present as 1:1 sex ratio, and it is quite difficult to differentiate males from females by their exterior appearance. Usually, sea cucumbers release sperms and unfertilized eggs (oocytes)

directly into the water column. Female sea cucumber can release up to millions of oocytes at a time, where spermatozoa (sperm cells) need to swim to discover and fertilize these oocytes (Mercier & Hamel, 2013; Purcell et al., 2010). Reproductive cycles of sea cucumbers depend on the type of species, geographical location, and environmental condition. For example, the spawning activity of most tropical sea cucumbers tends to have a peak at the beginning of the summer (Mercier & Hamel, 2009). Moreover, some commercial species can spawn in the winter (e.g., *Holothuria whitmaei*), whereas fewer species prefer to spawn throughout the whole year (e.g., *Holothuria scabrain* and *Isosticopus fuscusin*). Furthermore, a few species spawn once a year; *Cucumaria frondosa* typically spawns in the spring or early summer (Sun, Hamel, & Mercier, 2018).

2.1.3. Sea cucumber fishery

Sea cucumbers are fished globally, mainly in tropical regions. Sea cucumber fisheries depend on the fishery history, ecological attributes of species, socio-economic structures, and modes of exploitation (Purcell, Samyn, & Conand, 2012). Sea cucumber fisheries are often small-scale fisheries in coastal areas of developing countries, and are commonly industrialized in developed countries. In small-scale fisheries, sea cucumbers are mainly harvested by wading or skin diving in shallow waters, whereas modern fishing gears and larger boats are used for fishing in developed countries. However, sea cucumber fisheries have a poor history of sustainability, and many have collapsed because of overfishing. Sea cucumber fisheries refer to as “boom and bust”, and their stocks are at risk from over-exploitation due to improper management (Gianasi et al., 2020; Uthicke, Welch, & Benzie, 2004).

Harvesting of temperate sea cucumbers in the northern hemisphere includes *C. japonica*, *P. parvimensis*, and *P. californicus* on the Pacific coast and *C. frondosa* in the North Atlantic

(Hamel & Mercier, 2008). Among them, *C. frondosa* is mainly harvested for human consumption on a significant scale and exported globally. The commercial exploitation of sea cucumbers in the temperate region started a few decades ago, which exists in the US, Canada, Russian Federation, Iceland, and Scandinavia (Purcell et al., 2010). In 1990, the first experimental fishery for *C. frondosa* was developed in the state of Maine (USA) and then expanded into the Atlantic provinces of Canada during the 1990s. In 1994, Newfoundland and Labrador (NL) started showing interest in sea cucumber when a survey was done on southern Newfoundland (St. Pierre Bank) and discovered the potential of sea cucumber fishery in the Northwest Atlantic Fisheries Organization (NAFO) Subdivision 3Ps (Hamel & Mercier, 2008). In the Canadian portion of the St. Pierre Bank, there are two main sea cucumber concentrations, namely northwest and southwest of the French Economic Zone. The commercial fishery has mainly targeted within the northwest area (Western Bed) of the resource distribution. Initially, 8 exploratory licenses were issued for harvesting in 2003, and it is increased up to 32 in 2017. In 2003, the landed amount of sea cucumber was allocated 454 tonnes in NL, then it was increased to 612, 907, 2242, and 5600 tonnes in 2005, 2010, 2013, and 2018, respectively (Pantin et al., 2018). In Atlantic Canada, commercial harvest of *C. frondosa* was carried out using scallop chain sweeps or light urchin drags. Later, a specifically designed sea cucumber drag was used in Newfoundland as a standardized fishing gear, which significantly reduced the amount of by-catch. In 2004, a New Emerging Fisheries Policy was established on the St. Pierre Bank for the sea cucumber drag fishery (DFO, 2017; Gianasi et al., 2020; Hamel & Mercier, 2008; Pantin et al., 2018; Purcell et al., 2010).

2.1.4. *Cucumaria frondosa*: Description, distribution, and growth

The most common sea cucumber found in the east coast of Canada is *C. frondosa*, which is also known as orange-footed sea cucumber (Gianasi et al., 2020). *C. frondosa* is a Dendrochirotida that feeds on planktonic particles suspended in the water column, mainly phytoplankton, zooplankton, and organic matter, by spreading out their tentacles. It has a tube-shaped elongated body with leathery skin, which looks like a cucumber. The worm-like body is extremely flexible, where the mouth is surrounded by aquapharyngeal bulb/ tentacles at one end and an anus at the other (Figure 2.1). These tentacles are used for feeding phytoplankton, mainly diatoms and organic detritus (Hamel & Mercier, 1998). Moreover, the body wall consists of an external epithelium, and its thickness is around 3 mm without muscle bands, depends on the harvesting time and location. The body wall is the major commercial body part of this species, accounting for up to 50% of the total body weight. The most common body wall colors are dark- and grey-brown; however, yellowish, pinkish, and reddish colors can be found in some populations (Montgomery et al., 2019). On the other hand, internal organs include gonad, respiratory tract, and intestine, which represent up to 50% or more of the sea cucumber body weight.

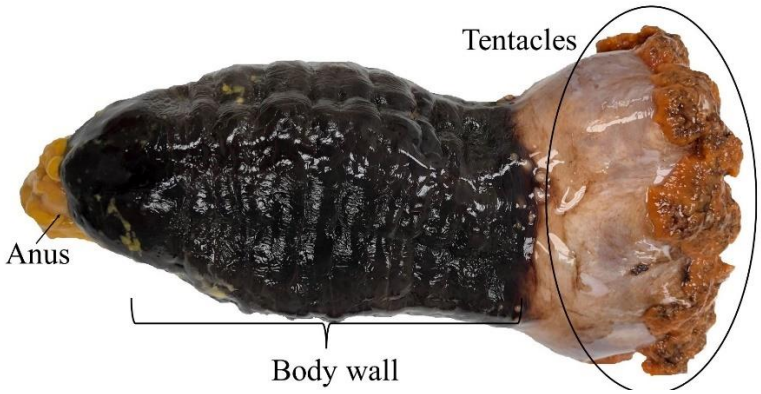


Figure 2. 1. Body parts of *Cucumaria frondosa* (Source: Hossain et al., 2020a).

Atlantic sea cucumber is widely distributed in the North Atlantic and Arctic Ocean, including the Norwegian and Barents Sea along the coast of the Russian Federation. In the Atlantic provinces of Canada, *C. frondosa* is mainly harvested in St. Lawrence Estuary, Bay of Fundy, Nova Scotia, Avalon Peninsula, and St. Pierre bank. This sea cucumber prefers rocky (corals and seaweeds), sandy, or mixed substrates (stone, gravel, sand, and shells) with strong currents, and it is abundant in depths from 30 to 300 m at densities of up to 50 individuals/ m^2 (Gianasi et al., 2020; Singh et al., 1998). The growth rate of this species is slow in comparison to other commercial species. The average growth rate is 0.2 cm per month, and it takes about two years at a size of 3.5 cm to display sexual maturity and 5.5 years to reach a length of 12 cm. The fastest growth rate is observed during spring/ summer, and that is 0.5–0.6 cm per month. It has been reported that the *C. frondosa* can grow up to 20 cm (500 g) close to shore and up to 50 cm (1.5 - 2 kg) in deep offshore waters (Hamel & Mercier, 1996; Nelson, MacDonald, & Robinson, 2012). On average, the maximum length, width, and weight are 40–50 cm, 10–15 cm, and 100 to 500 g, respectively (Hossain et al., 2020a).

2.1.5. *Cucumaria frondosa*: Processing technique

The quality and market value of sea cucumber products depend on the species, location, weight, size, body wall thickness, and processing techniques (Hossain et al., 2020a). Therefore, handling and storage must be performed appropriately to prevent damage to the body wall, which is the main marketable product. Handling and holding of sea cucumbers are quite difficult after harvesting as they can autolyze themselves upon removal from water or under stress. During autolysis, the texture and organoleptic properties of the body wall/ inner dermis could degrade through protein breakdown, including endogenous proteases (Gianasi, Hamel, & Mercier, 2016; Hossain et al., 2020a). Autolysis occurs in in response to external environment, including high salt

concentration, UV light exposure, nutrient deficiency, and elevated temperature, among others. Metalloproteinase is the main endogenous protease that triggers the autolysis process in the body wall of sea cucumber and leading to severe quality deterioration during cultivation, handling, storage, processing, and transportation (Liu et al., 2018a; Liu et al., 2020b). Moreover, the body wall could be deteriorated due to the hydrolysis of collagen, the main compound of the body wall, when the digestive tract secretes different enzymes, including trypsin, chymotrypsin, and cathepsin, after harvesting (Yan et al., 2014). The color, flavor, odor, and texture of the final products can be altered due to the development of new compounds through lipid oxidation, protein breakdown, and enzyme secretion. Among them, texture property is an important characteristic of the final products, mainly body wall, as it is directly affected to the market price (Liu et al., 2020c). Traditionally, sea cucumber is placed in fresh water mixed with ice and/ or sea salt after harvesting. However, sea salt and ice may damage the quality of sea cucumber body wall during transportation due to direct contact. Gianasia, Hamel, and Mercier (2016) reported that a mix of cold seawater with freshwater ice as the most efficient cooling medium for transportation. In contrast, the addition of salt yielded the highest rates of mortality and skin necrosis.

The market demand for the final products depends on the way they are processed. The market demand for processed products is very high; however, a limited amount of raw sea cucumber is eaten worldwide. In Korea, Japan, Hong Kong, Taiwan, and Singapore, the body wall and internal organs are sometimes eaten raw, boiled, or pickled forms with vinegar and soya sauce (Purcell, Samyn, & Conand, 2012). Sea cucumber processing may include various steps of eviscerating, cleaning, freezing, cooking, drying, and packaging. Generally, Atlantic sea cucumbers are cut longitudinally and eviscerated, followed by drying, and sold as dried body wall (*beche-de-mer* or *trepang*) (Couillard, Maltais, & Belley, 2021). The processing of Atlantic sea

cucumber maintains somewhat different techniques compared to the protocols followed for the tropical and temperate sea cucumbers (Gianasi et al., 2020). The most common *C. frondosa* products are cocoon cut (skin with meat, dry or frozen), butterfly cut (skin with or without meat, dry or frozen), raw meat (frozen), and sliced skin (frozen/ dried) (Figure 2.2). Some industries separate muscle bands and aquapharyngeal bulbs (labeled 'flowers') from the body wall and sell them as fresh, frozen, or dried forms, whereas internal organs are considered as processing discards. During butterfly cut, sea cucumbers are cut longitudinally and remove internal organs with a scraping tool. As a result, sea cucumber is "unrolled", which provides it a butterfly-like shape. However, cocoon cut can be obtained by cutting the tentacles to make an opening for eviscerating. A specially designed cleaning tool or vacuum is used for gutting. Cocoon cut receives a higher value in the Asian market compared to the longitudinal cut (butterfly cut) because of its attractive tubular shape (Hossain et al., 2020a).

Figure 2. 2. Butterfly cut and cocoon cut sea cucumber (Source: Hossain et al., 2020a).

2.1.6. *Cucumaria frondosa*: Landing, commercial products, and trade market

The sea cucumber capture statistics are generally provided based on the live weight of animals; however, some Asian countries record the data based on the dry weight. According to the FAO (Food and Agriculture Organization) global statistics report on sea cucumber, the volume of sea cucumber catches increases gradually worldwide. Figure 2.3 shows how the volume of sea cucumbers catch (in tonnes) has increased worldwide since 2000 (FAO, 2020). However, some countries report their catches by large groups of species; thus, there is a lack of official data regarding individual species. Therefore, recording the statistics for a particular species often underestimates the real production.

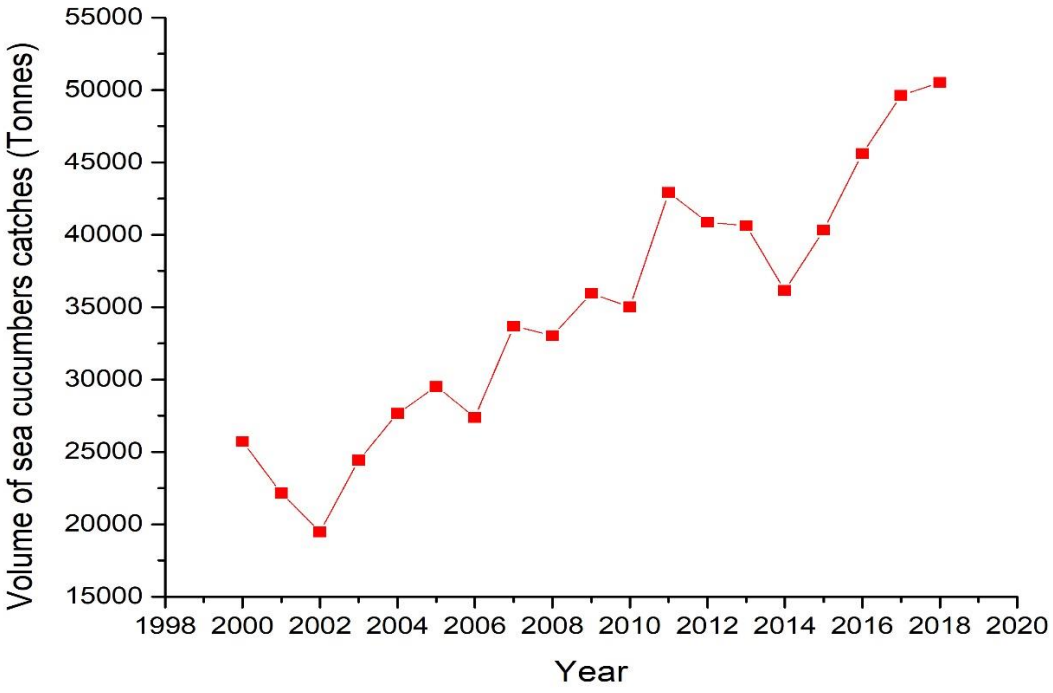


Figure 2. 3. Volume of sea cucumbers catches worldwide (tonnes- live weight)

In Canada, the major sea cucumber landing provinces are Newfoundland and Labrador (NL), Nova Scotia (NS), and Quebec as well as British Columbia (BC), which targets a different species (*Apostichopus californicus*). The Department of Fisheries and Oceans (DFO) of Canada reported that the volume of sea cucumber catch has doubled over the last decade in the Atlantic and Pacific coasts (Figure 2.4) (DFO, 2021). Particularly, the volume was expanded over eight times in NL from 2008 to 2019 (698 to 5,987 metric tonnes), which the highest capture landings, followed by the NS and BC in 2019. In 2019, NL contributed 52.28% (5,987 metric tonnes) of the total sea cucumber landings in Canada and accounted for $9 million in revenue. The total Canadian landing boosted from 4,516 to 11,450 metric tonnes from 2008 to 2019. Additionally, from 2008 to 2019, the total Canadian landed value was increased from $4 to $26.5 million (Figure 2.5).

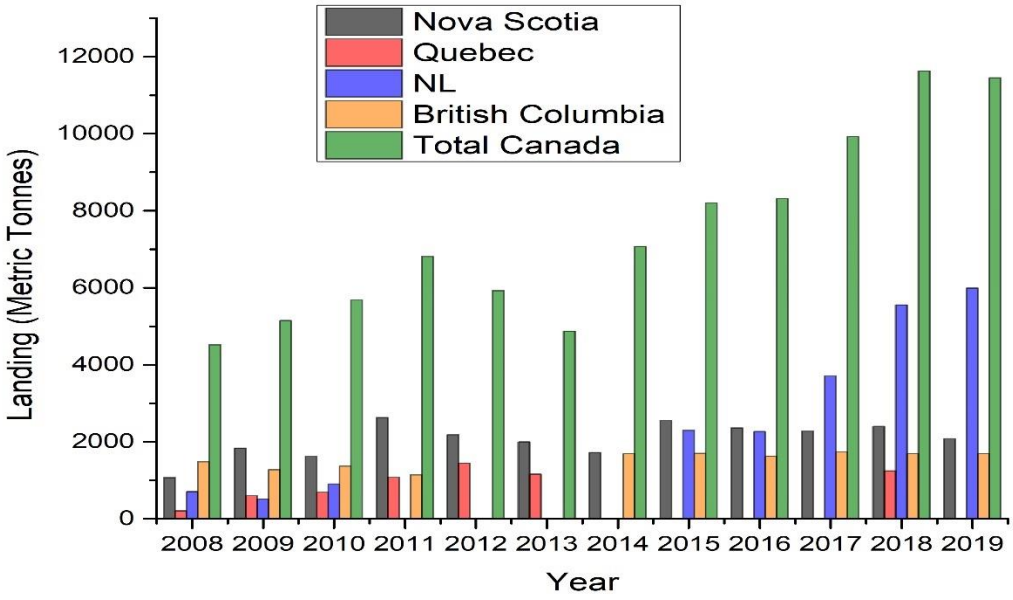


Figure 2. 4. Volume of sea cucumbers catches in Atlantic and Pacific Coasts (metric tonnes, live weight) (Source: Hossain et al., 2020a).

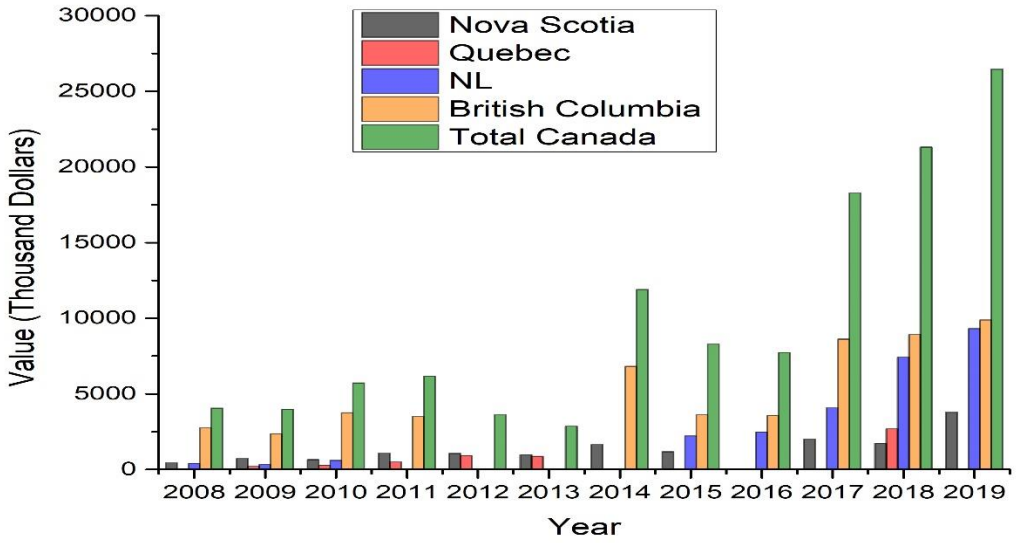


Figure 2. 5. Landed value of sea cucumber in the Atlantic and Pacific Coasts (thousand dollars, C$) (Source: Hossain et al., 2020a).

Sea cucumber is considered a luxury food in Asian culture and has become a traditional part of Chinese cuisine, mainly during the Chinese New Year, weddings, banquets, hungry ghost festivals, and corporate events (Gianasi et al., 2020; Purcell et al., 2014). Due to the high market demand, *C. frondosa* is sold in the Asian market as a dried, frozen, cooked-dried, cooked-salted, and cooked-salted-dried form. The processing discards, including gut materials, are considered as by-products or sometimes used to produce fish meal (Tripoteau et al., 2015). Dried body wall (*beche-de-mer or trepang*) and internal muscle bands are the major commercial products. Around 99% of the global trade comes in the dried products (Brown et al., 2010; Louw & Bűrgener, 2020). Approximately 90% of trade takes place in the Asian markets, including China, Taiwan, Hong Kong, Singapore, Japan, Korea, Malaysia, and Indonesia. Although *C. frondosa* is smaller in size, thinner in body wall thickness, and irregular in shape after drying compared to other commercial species, the market demand has increased due to its rich nutritional profiles. However, *C. frondosa*

is still considered a medium-grade species and yielded much lower prices compared to other sea cucumbers in the world market (Hair, Pickering, & Mills, 2012; Hossain et al., 2020a; Hamel & Mercier, 2008). The value of *C. frondosa* products (i.e., dried products) depends on the body wall thickness, skin color, and processing integrity, and the price varies from US$ 20 to 400 per kilogram (Hossain et al., 2020a). The most common *C. frondosa* products found in the market are shown in Table 2.2.

Table 2. 2. Common *Cucumaria frondosa* products in the market (Source: Hossain et al., 2020a).

Product name	Brand/manufacturer	Price (US$)
PEACE PAVILION dried sea cucumber	Peace Pavilion	$150.00/ lb
Canadian wild freshly dried sea cucumber	Northbay Foods	$135.00/ lb
Dried Canada wild sea cucumber	Arctica Food	$98.00/ lb
Dried sea cucumber- butterfly cut/whole cut	Atlantic Sea Cucumber	$55.00-75.00/ lb
Arctic dried sea cucumber	Naturally North	$65.00/ lb
Sea cucumber liquid extract	HawaiiPharm	$20.00/oz
Dried *Cucumaria frondosa* flower	Northbay foods	$15.00/ lb
Canadian sea cucumber (whole/split east coast)	SEACOO	N/A

2.1.7. *Cucumaria frondosa*: Bioactive compounds and their biological activities

Sea cucumbers are one of the potential marine invertebrates for food, essential nutrients, and bioactive compounds. Particularly, *C. frondosa* has a notable nutritional profile with significant content of protein, carbohydrate, minerals, and vitamins as well as a low fat and cholesterol level (Gajdosechova et al., 2020). It has been reported that the fresh whole *C. frondosa*

contains approximately 90.5% moisture, 5.5% protein, 3.5% ash, 1.5% carbohydrate, and 0.8% lipid. Moreover, protein from fresh *C. frondosa* comprises a high content of glutamic acid, leucine, lysine, glycine, and asparagine, along with a considerable amount of arginine, alanine, proline, and valine. Furthermore, it contains a high content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Zhong, Khan, & Shahidi, 2007). However, the viscera of *C. frondosa* is a rich source of polyunsaturated fatty acids (PUFA, around 44%), mainly EPA and DHA, and a good source of monounsaturated fatty acids (MUFA, about 30%) (Gianasi et al., 2017). Similarly, Liu et al. (2021) reported that the fresh and dried viscera had the similar fatty acid composition, with high percentage of PUFA (~31%), mostly EPA (~28%). Additionally, viscera contain a significant number of essential elements, including Fe, Cu, Zn, K, Mn, As, Na, Mg, Se, Ca, and Ni, and vitamins, mainly niacin, alpha-tocopherol, riboflavin, pantothenic acid, folates, and thiamine (Mamelona, Saint-Louis, & Pelletier, 2010).

Atlantic sea cucumber has gained much attention from researchers due to its bioactive compounds and beneficial effect on human health and potential therapeutic uses. The bioactive compounds identified from this species are quite diverse and include polysaccharides (fucosylated chondroitin sulfate, FCS), saponins (triterpene glycosides), cerebrosides, carotenoids, phenolic compounds, and other biologically active compounds (Bordbar et al., 2011; Hossain et al., 2020a; Khotimchenko, 2018; Ustyuzhanina et al., 2017). The main beneficial compounds of *C. frondosa* and their biological activities are shown in Figure 2.6. The body wall of *C. frondosa* contains most of the bioactive compounds and nutrients, including FCS, glycosides, fucoidan, cerebrosides, phenolics, protein (collagen and bioactive peptide), omega 3, vitamins, and minerals, whereas tentacles and viscera comprise phenolics, amino acids, omega 3 fatty acids, vitamins, and minerals (Hossain et al., 2020a). For example, the body wall of this species is a rich source of acidic

polysaccharides, mainly sulfated polysaccharides (FCS). FCS and fucan are the two types of polysaccharides identified in *C. frondosa*. FCS is a unique glycosaminoglycan identified from *C. frondosa*, and its biological activities depend on the degree of sulfation, position of a sulfate group, and arrangement of branches along the backbone (Hu et al., 2016; Ustyuzhanina et al., 2017). Moreover, Kale et al. (2013) identified the monosaccharides composition of Northern sea cucumber as N-acetylgalactosamine, N-acetylglucosamine, N-acetylneuraminic acid, glucuronic acid, glucose, mannose, fucose, and galactose. On the other hand, saponins are triterpene glycosides, and are known as holothurin/ frondoside A when they are identified from sea cucumber. The major saponin in *C. frondosa* is frondoside A, and it also comprises other triterpene glycosides such as frondoside B, frondoside C, frondoside A2-1, frondoside A2-2, frondoside A2-3, and frondoside A2-4 (Hossain et al., 2020a). Some studies have reported that sea cucumber can shoot sticky threads, mainly saponins, as a chemical means of defense against pathogens, predators, and competitors. However, they remain prey for some highly categorized predators such as big mollusks (Claereboudt et al., 2018). Furthermore, *C. frondosa* is a good source of carotenoids as they eat algae and other carotenoid-rich phytoplankton. Zakharenko et al. (2020) identified 14 carotenoids from *C. frondosa* using supercritical CO_2 extraction and found that the major components of carotenoids were cucumariaxanthin, canthaxanthin, cucumariaxanthin B. lutein, and β-carotene. Additionally, different body parts of Northern sea cucumber, mainly tentacles and viscera, contain considerable amounts of phenolic compounds, mostly free phenolics, with moderate antioxidant properties. Phenolic acids are the major phenolics found in *C. frondosa*, and this could be due to the absorption of phenolics from phytoplankton by this species (Hossain et al., 2020b; Mamelona et al., 2007; Zhong, Khan, & Shahidi, 2007).

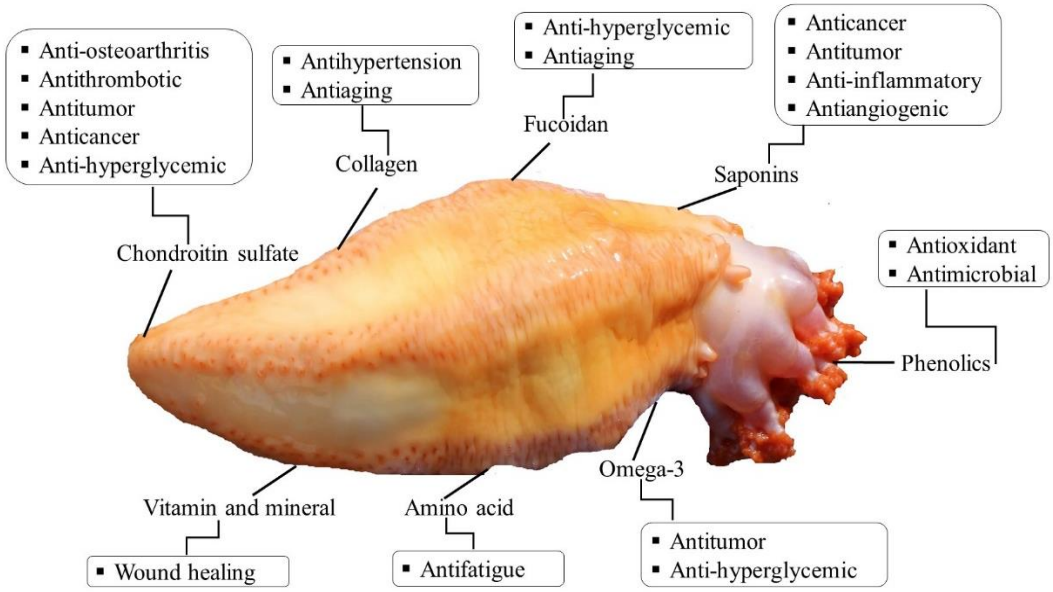


Figure 2. 6. Beneficial compounds of Atlantic sea cucumber and their beneficial effect on human health (Source: Hossain et al., 2020a).

As discussed in earlier sections, sea cucumbers have long been considered as a traditional food and medicine in Asian countries. Various bioactive compounds have been reported in sea cucumber, and their functions are mainly species-specific and closely related to the bioactive compounds. For example, *C. frondosa* has the potential to show anticancer, antithrombotic, anticoagulant, anti-angiogenic, anti-inflammatory, antihypertension, antitumor, antimicrobial, antifungal, and antioxidant properties (Hossain et al., 2020a). Particularly, frondoside A of *C. frondosa* has been studied by many researchers due to its potential anticancer property. For example, the effect of frondoside A with a combination of gemcitabine on pancreatic cancer was investigated and found that the combination was more effective than the gemcitabine alone (Al Shemaili et al., 2014). Moreover, FCS and other frondosides identified from this species demonstrate inhibitory activity against pancreatic cancer, lung cancer, colon cancer, and breast cancer (Al Marzouqi et al., 2011; Al Shemaili et al., 2014; Attoub et al., 2013; Janakiram et al.,

2010; Liu et al., 2016b). For instance, Liu et al. (2016a) prepared low-molecular-weight FCS from *C. frondosa* and found that the FCS significantly inhibited the growth of Lewis lung carcinoma through the activation of caspase-3 activity. Furthermore, fucoidan from *C. frondosa* exhibits anti-hyperglycemic, antithrombotic, and anticoagulant properties (Hu et al., 2016; Wang et al., 2016a). Additionally, frondoside A, frondoside C, frondanol A5, and FCS demonstrate antitumor activities, whereas FCS has the potential in showing antithrombotic and anticoagulant activities (Hossain et al., 2020a; Liu et al., 2016a). Liu et al. (2016b) determined the effect of FCS, isolated from *C. frondosa*, on the antithrombotic and anticoagulant activities *in vitro* and *in vivo*. It was found that the FCS exhibited antithrombotic and anticoagulant activities, especially its depolymerized fragment. Besides, EPA enriched phosphatidylcholine, fucoidan, and FCS from *C. frondosa* exhibit notable anti-hyperglycemic properties (Hu et al., 2014; Wang et al., 2016a). For example, EPA enriched phosphatidylcholine demonstrated anti-hyperglycemic activities via up-regulating phosphatidylinositol 3 kinase/ protein kinase B signal pathway mediated by insulin (Hu et al., 2014). On the other hand, phenolics and protein hydrolysates of *C. frondosa* shows strong antioxidant and antimicrobial activities (Tripoteau et al., 2015). Senadheera et al. (2021) prepared protein hydrolysates from body wall, flower, and internal organs of *C. frondosa* using different enzymes such as Alcalase, Corolase, and Flavourzyme. Result suggested that the hydrolysates prepared with the combination of Alcalase and Flavourzyme had the maximum radical scavenging activity against ABTS and DPPH radicals.

2.2. Phenolics

2.2.1. Chemistry and classification

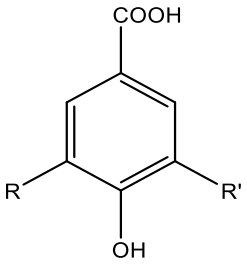
Phenolic compounds are secondary metabolites of plants, which can also be found in seaweeds and marine invertebrates. Phenolics are powerful antioxidants that contain one or more aromatic rings carrying one or more hydroxyl groups. Phenolic compounds are abundant in fruits, vegetables, spices, herbs, algae, microalgae, teas, and industrial wastes, among others. For example, seaweeds such as brown seaweeds are a good source of phlorotannins, whereas green and red seaweeds are rich in phenolics acids, flavonoids, phenolic terpenoids, and bromophenols (Cotas et al., 2020). Moreover, phenolic compounds play a key role in protecting plants by engaging in defense mechanisms against ultraviolet radiation and pathogen attacks. Phenolics involve in the plants for growth regulation and are also responsible for the color, flavor, bitterness, and astringency of foods. For example, phenolics are responsible for providing the bitter taste of unripe fruits and vegetables because of their interaction with salivary glycoprotein (Alara, Abdurahman, & Ukaegbu, 2021). Phenolics in plants are mainly derived from phenylalanine, and in some cases, tyrosine. The formation of *trans*-cinnamic acid from phenylalanine is catalyzed by phenylalanine ammonia-lyase (PAL), whereas *p*-hydroxycinnamic acid from tyrosine is catalyzed by tyrosine ammonia-lyase (TAL) (Shahidi & Ambigaipalan, 2015). Phenolics can be categorized into different groups: phenolic acids, flavonoids, tannins, stilbenes, lignans, and coumarins (Shahidi et al., 2019). Phenolic acids are a major type of phenolic compounds and usually occur in various conjugated forms than the free type. Phenolic acids consist of hydroxycinnamic acids (C6–C3), mainly caffeic, ferulic, *p*-coumaric, and sinapic acids, and hydroxybenzoic acids (C6–C1), mostly vanillic, syringic, ellagic, *p*-hydroxybenzoic, and protocatechuic acids (Figure 8). Based on the substitution and functional groups, such as methoxy and hydroxyl groups, the

differentiation occurs among the individual phenolic acids. Flavonoids are a group of more than 4,000 phenolic compounds and are composed of 2 aromatic rings (A and B) linked by a three-ring structure (C) in the C6–C3–C6 form. According to the different substitution patterns, such as hydroxyl and methoxy groups, flavonoids can be classified into different groups, mainly flavones, flavanones, flavanonols, flavonols, flavanols, isoflavones, and anthocyanidins (Figure 2.7).

The commonly encountered flavonoids which are widely distributed in fruits and vegetables are catechin, quercetin, naringenin, daidzein, and cyanidin glycoside. However, anthocyanins play an important role in providing color in plants, especially fruits and vegetables, and these colors can change depending on the pH, being red in acidic, blue to violet in neutral, and yellow to green in alkaline solutions. Moreover, depending on the chemical structure, tannins can be classified into two classes of hydrolyzable (e.g., ellagitannins) and condensed (e.g., proanthocyanidins). Hydrolyzable tannins contain a central glucose core, esterified to gallic or ellagic acid, while the condensed tannins are oligomers and polymers of flavonoids. Furthermore, stilbenes possess a carbon skeleton of C6-C2-C6, which includes resveratrol. Based on the level of polymerization, stilbene can be categorized into several groups, mainly monomers, dimers, trimers, tetramers, and hexamers. Besides, coumarins are referred to as benzopyrone with the basic skeleton of C6-C3 (Huang, Cai, & Zhang, 2010; Shahidi & Ambigaipalan, 2015; Shahidi & Yeo, 2018; Shahidi et al., 2019).

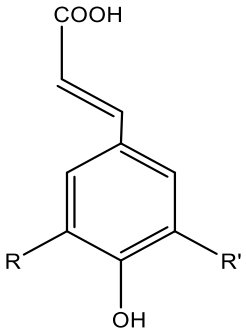
Phenolic acids

Benzoic acid derivatives



R = R' = H; *p*-Hydroxybenzoic acid
R = OH, R' = H; Protocatechuic acid
R = OCH_3, R' = H; Vanillic acid
R = R' = OH; Gallic acid
R = R' = OCH_3; Syringic acid

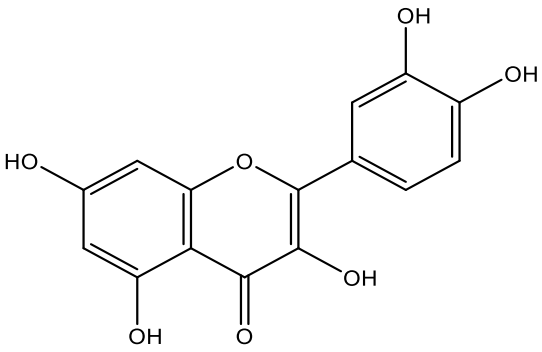
Cinnamic acid derivatives



R = R' = H; *p*-Coumaric acid
R = OH, R' = H; Caffeic acid
R = OCH_3, R' = H; Ferulic acid
R = R' = OCH_3; Sinapic acid

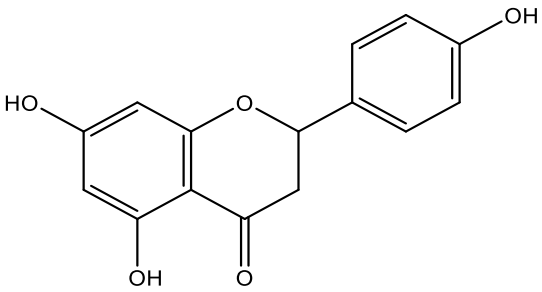
Flavonoids

Flavonols



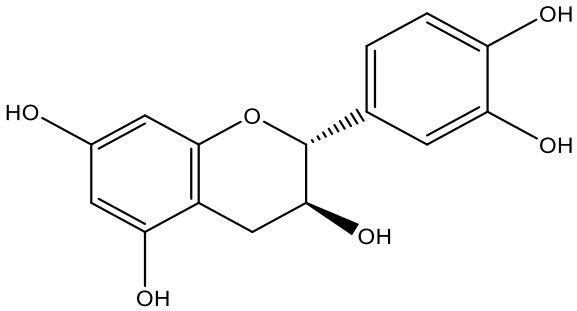
Quercetin

Flavanones



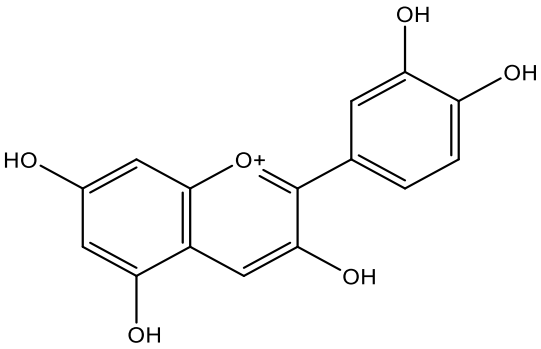
Naringenin

Flavan-3-ols



Catechin

Anthocyanidins



Cyanidin

Figure 2. 7. Chemical structures of common phenolic acids and flavonoids

Based on the distribution in nature and location in plants, phenolics can be classified into free, soluble esters, etherified (glycosylated), and insoluble-bound fractions. Soluble phenolic compounds are free phenolics, which do not form a chemical bond with other molecules, whereas insoluble-bound phenolic compounds are covalently bound to the cell wall substances, mainly arabinoxylans, cellulose, pectin, and structural proteins by ether, ester, and C-C bonds (Figure 2.8). Moreover, soluble phenolics include esters that are linked through the ester bond with glucuronic acid and fatty acids (Shahidi & Yeo, 2016). For instance, hydroxyl groups of cell wall substances can form ester bonds with the carboxyl group of phenolic acids (e.g., cinnamic and benzoic acids) and can also create ether bonds with the hydroxyl groups of phenolic compounds. Additionally, C-C bonds can form between a carbon atom of cell wall substances and a carbon atom of phenolic compounds (McLusky et al., 1999). Generally, insoluble phenolics include high molecular weight compounds (e.g., phenolic acids and tannins) and soluble phenolic compounds consist of low and medium molecular weight compounds (e.g., simple phenol, tannins, and flavonoids). Usually, insoluble-bound phenolics are localized in the cell wall substances through covalent bonds, whereas soluble phenolics are trapped by weak interaction with other components and localized in the vacuole of plant cells. Insoluble-bound phenolics present a comparatively high amount (20–60%) in plants compared to the soluble phenolics and are not extracted using an extraction medium since they are bound covalently to the insoluble macromolecules. Therefore, soluble phenolic compounds can be extracted by using organic solvents, whereas enzymatic, acid, or alkaline hydrolysis is used to break the chemical bond in order to extract insoluble-bound phenolic compounds (Shahidi & Yeo, 2018; Yeo & Shahidi, 2017).

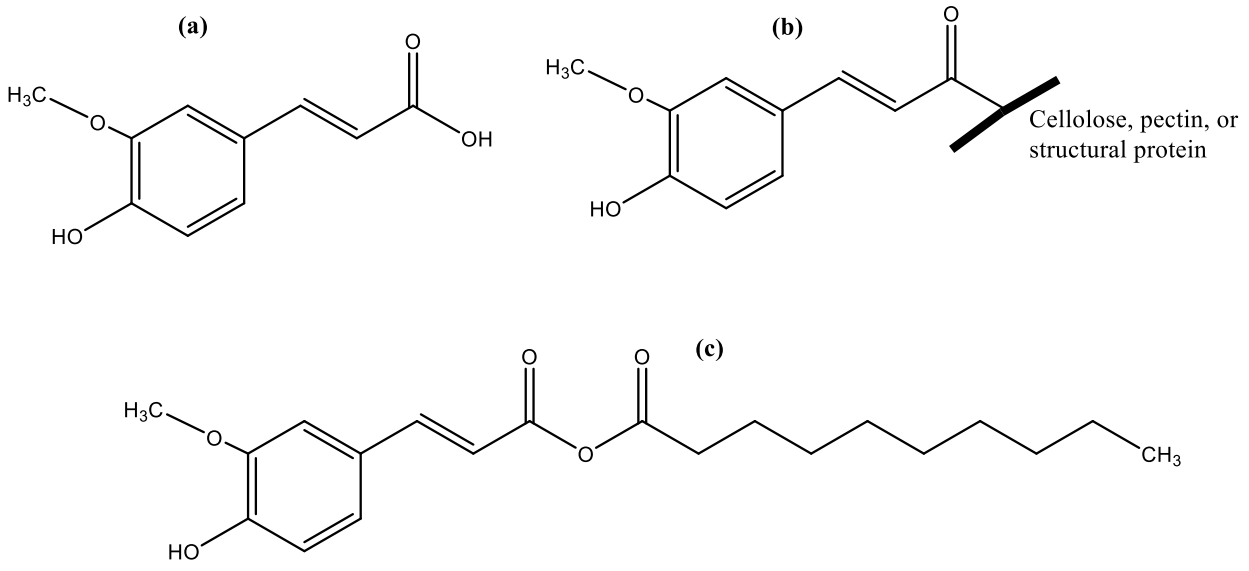


Figure 2. 8. Free (a), bound (b), and ester form (c) of ferulic acid

2.2.2. Antioxidant and antimicrobial activities of phenolics compounds

Biologically active compounds such as phenolics are widely used for functional food processing, and nowadays, extra attention has been paid to procure beneficial antioxidants from natural sources with an aim to protect the human body from many chronic diseases. Antioxidants are substances that preserve food by inhibiting oxidation mechanisms and protect the human body against free radicals. Phenolics are powerful antioxidants, which demonstrate protection against degenerative diseases, mainly cardiovascular diseases, cancers, and neurodegenerative diseases (Xiao et al., 2013). This is because phenolic compounds have the potential to protect the human body against damage by reactive oxygen species (ROS). Moreover, phenolics have the ability to act as antioxidants to prevent platelet aggregation, low-density lipoprotein (LDL) oxidation, DNA oxidation, and red blood cell damage (Shahidi & Ambigaipalan, 2015). For instance, green tea is a rich source of catechins, which can delay DNA oxidation, LDL cholesterol oxidation, and

propagating of lipid peroxyl radicals *in vitro* (Zhong & Shahidi, 2012). Moreover, methyl gallate and gallic acid from *Givotia rottleriformis* shows inhibitory activity against the hepatitis C virus (HCV) and reduces the growth of human epidermoid carcinoma (A431) skin cancer cells (Hsu et al., 2015; Kamatham et al., 2015). Methyl gallate also exhibits anti-viral and anti-bacterial properties, whereas ferulic acid has inhibitory activity against cardiovascular disease, diabetes, cancer, and neurodegenerative disease (Choi et al., 2008; Narasimhan et al., 2015). Similarly, flavonoids, mainly rutin, quercetin, naringin, and hesperidin, inhibit NF-kB activation, inflammation, and apoptosis, which may be initiated by cisplatin (anticancer drug). Besides, quercetin and kaempferol have a great potential for the prevention of cardiovascular diseases, cancer (mainly lung cancer and breast cancer), and cognitive malfunction (Shahidi & Yeo, 2016). Additionally, tannins could be used as hypoglycemic agents as they have the potential to manage and prevent diabetes mellitus (Ajebli & Eddouks, 2019).

On the other hand, due to the lipid oxidation, the quality attributes of food, including flavor, color, and texture, are deteriorated, which ultimately decreases the shelf life and nutritional value of food. Thus, antioxidants are widely used to control the rate and extent of lipid oxidation in foods. For instance, phenolics from mint leaves could retard the oxidation process of cooked ground meat during the storage at 4°C and maintain the quality up to 14 days (Brown, John, & Shahidi, 2019). One of the main assays to measure the degree of lipid oxidation is the thiobarbituric acid reactive substances (TBARS) test, which is used to determine the secondary oxidation products, including aldehydes, ketones, alcohols, hydrocarbons, and epoxy components, and these are believed in producing rancid flavors and aromas (Shahidi, 1998). Autoxidation is one of the main pathways of lipid oxidation, where polyunsaturated fatty acids (PUFA) are involved in a free radical chain reaction under heat, light, metal ions, or enzymes such as lipoxygenase. Synthetic

antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) have been used as antioxidants in foods to prevent oxidation and off-flavor development. Nevertheless, due to the carcinogenic and toxicity characteristics of some synthetic antioxidants, researchers have shown much attention towards natural antioxidants (Balasundram, Sundram, & Samman, 2006; Shahidi, 2015).

Phenolics can act as antioxidants in several ways. The main mechanisms of antioxidants to neutralize free radicals are hydrogen atom transfer (HAT), single electron transfer (SET), metal chelation, and reducing power. Therefore, the effectiveness of antioxidants is mainly dependent on the number and arrangement of the hydroxyl groups in the molecules of interest (Shahidi & Naczk, 2004). For example, phenolic antioxidants can donate hydrogen atoms from the hydroxyl groups to lipid radicals in order to neutralize the oxidation reaction. Interestingly, antioxidant radical does not involve in further oxidation, as it is stabilized by resonance. In contrast, the hydrogen atoms of phenolics can react with ROS, which breaks the cycle of the generation of new radicals. Phenolics with specific structures can also chelate metal ions (e.g., ferrous and copper), where metal ions can no longer act as an initiator of lipid oxidation due to the formation of a complex between the metal ions and antioxidants. Besides, synergistic effects can be observed between the phenolics and other antioxidants such as α-tocopherol, β-carotene, and ascorbic acid (Pereira et al., 2009; Shahidi & Zhong, 2015). There are numerous techniques that are available for determining antioxidant activity, including radical scavenging assays that include SET (e.g., ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay) and HAT (e.g., total radical trapping antioxidant parameter (TRAP) and oxygen radical absorbance capacity (ORAC)) mechanisms (Shahidi & Ho,

2007). However, each assay has a different mechanism of action, resulting in providing varied antioxidant potential for the same sample (Shahidi & Hossain, 2018).

Phenolic compounds were observed to have antimicrobial activity against bacteria, viruses, and fungi. For example, 6,6′-bieckol (phlorotanin) obtained from *Ecklonia cava* has the potential to inhibits HIV-1 induced cell fusion, cytopathogenic effect, and cell lysis effect (Cotas et al., 2020). However, the mode of action of antimicrobial activity of phenolics is not yet fully determined. It has been reported that phenolics may alter the function of the genetic material, react with the cell membrane, or inactivate essential enzymes of microorganisms (Martillanes et al., 2017). For instance, condensed tannins may modify the permeability of cell membranes and even inactivate the metabolism by binding to enzymes. Moreover, flavonoids may link to soluble proteins of the cell wall to form complex, while phenolic acids have been demonstrated to disrupt the cell membrane integrity (Bouarab-Chibane et al., 2019). Apart from this, antimicrobial properties are related to the chelating properties of phenolics, as the metal ions are essential for microbial growth. Additionally, essential oils containing phenolics may cause functional damage to the bacterial cell membrane, inhibiting bacterial growth (Shahidi & Hossain, 2018).

2.2.3. Applications of phenolics compounds

Due to antioxidant, antimicrobial, and coloring properties, phenolic compounds have received significant attention from several industries, especially from the food, pharmaceutical, cosmetics, packing, and textile industries. In the food industry, phenolics can be used as a food preservative to inhibit the oxidation process and microbial growth of food products. For example, phenolic extracts obtained from *Litchi chinensis* Sonn. pericarp showed similar inhibitory activity against lipid oxidation of sheep meat nuggets to that of BHT (Das et al., 2016). Moreover, pomegranate peel extract was effective in inhibiting of lipid oxidation of beef meatballs and

preventing the growth of psychrophilic, mesophilic, lactic acid bacteria in Pacific white shrimp during storage (Martillanes et al., 2017). Furthermore, phenolics can be used in vegetable oils to improve their oxidative stability and frying characteristics. Rubió et al. (2012) found that the oxidative stability of olive oil enriched with phenolics (mainly flavonoids) was 10 h higher than the control oil during frying. However, only a limited number of phenolics are legislated by the regulatory authorities, including EFSA (European Food Safety Authority) and FDA (U.S. Food and Drug Administration), to use as food additives. The most common approved additives obtained from phenolics are ferulic acid (allowed in Japan), catechin (allowed in the US, EU, and China, E300), and anthocyanins (allowed by EFSA, E163) (Albuquerque et al., 2021). Particularly, anthocyanins have numerous applications in the food, cosmetic, and pharmaceutical industries due to their wide range of colors and therapeutic properties. For instance, Swer et al. (2019) found that incorporation of anthocyanin in yogurt, syrup, and hard-boiled candy, maintained the stable color and improved sensory attributes. In contrast, during the Maillard reaction, advanced glycation end-products (AGEs) can be generated, which can lead to the risk of chronic diseases, including diabetes. Therefore, phenolics can prevent the formation of AGEs by preventing the production of free radicals (Hu, Wang, & Shahidi, 2020; Wang et al., 2016b). The antiglycation ability of 16 selected phenolic compounds was determined, and it was found that the homoprotocatechuic acid, ferulic acid, quercetin, catechin, and 8-*O*-methylurolithin A had higher activity compared to the other selected phenolic compounds (Piwowar et al., 2019). On the other hand, Seapolynol™ (Botamedi Inc, Seoul, South Korea) is a nutritional supplement produced from the phenolic compounds (phlorotannins mainly dieckol) of brown algae that have been legislated by the European Food Safety Authority. This supplement demonstrated promising results as a cardioprotective, anti-hyperlipidemic, and antidiabetic agent (Ahn et al., 2017; Yeo et al., 2012).

Similarly, phlorotannins have been applied to produce various supplements in order to control cardiovascular diseases. The supplements containing phlorotannins are IdAlg™ (Bio Serae, Bram, Aude, France), HealSea™ (Diana Naturals, Rennes, Brittany, France), and Seanol™ (LiveChem Jeju Si, Jeju-do, South Korea and Simple Health, Maitland, Florida, USA) (Cotas et al., 2020).

Phenolics can be used in active food packaging and edible films/ coating due to their antioxidant and antimicrobial properties. Phenolics, mainly essential oils, can be incorporated into packaging materials by dispersing in the packaging polymer, coating or dipping, and encapsulation by microemulsions and nano-emulsions (Garavand et al., 2021). For example, Sáez et al. (2020) analyzed the effect of alginate coating enriched with tannins, tannic acid, and quebracho tannin on the quality characteristics of rainbow trout during cold storage. It was found that the coating significantly reduced the microbial counts and lipid oxidation compared to control. Moreover, phenolics have been used as bioactive compounds in cosmeceutical products, such as skincare and anti-aging products, owing to their ability to absorb ultraviolet radiation as they contain chromophores in their structures. Especially, quercetin, hydroxycinnamic acids, and resveratrol have shown a sun protection factor (SPF) varying from 7 to 30 (Albuquerque et al., 2021). Hydroxycinnamic acids, namely *p*-coumaric and protocatechuic, as well as *p*-hydroxybenzoic acids, were used to prepare semi-solid base cream, and found that these compounds had higher stability over a 6-month period and exhibited anti-inflammatory, antimicrobial, and anti-tyrosinase activities (Taofiq et al., 2019). Moreover, due to the antioxidant, anti-inflammatory, and antimicrobial effects of seaweed (*Saccharina japonica*) phenolics, its extract is already used to develop facial masks in South Korea (dasima extract, Natural Solutions) (Cotas et al., 2020). Similarly, the most common commercial products that are produced from seaweed phenolics (mainly phlorotannins) are UV screening (Helioguard®365, Switzerland), anti-aging agent

(ECKLEXT® BG, Japan), Aethic Sôvée® (AETHIC®, UK), *Fucus vesiculosus* extract (Bladderwrack Extract, Natural Solution, South Korea), and *Ulva compressa* extract (Green Confertii Extract, Natural Solution, USA) (Cotas et al., 2020; Morais et al., 2021).

The demand for natural dyes in the textile industry has also grown rapidly, and phenolics could serve as a potential candidate to fulfill this requirement due to their high biodegradability. For example, dyes extracted from oak (*Quercus* sp.) bark and tea leaves are mainly rich in ellagitannin, gallotannin, quercetin-3-*O*-glucoside, and quercetin, which exhibit UV protection and antimicrobial activity when applied on silk and cotton (Bonet-Aracil et al., 2016; Jia et al., 2017). Apart from these, the application of phenolics in coal, oil, gas, and explosive industries are also noteworthy. Particularly, phenolic resins, including bisphenol A, are used in appliance, wood, and construction commodities for numerous functions. Bisphenol A is a raw material to produce epoxy resins and polycarbonate plastics. Besides, chlorophenols are commonly used as antiseptics, herbicides, pesticides, and disinfectants. However, some of the phenolic compounds, including chlorophenols, bisphenol A, chlorocatechols, and nitrophenols, have been identified as being toxic (Anku et al., 2017; Igbinosa et al., 2013).

2.2.4. Phenolics in sea cucumber and their antioxidant activity

Plant and marine-based phenolic compounds are receiving increase attention due to their potential health benefits and multiple biological activities. Most of the phenolics have been researched from the terrestrial environment, while less attention has been paid to the marine environment even though it provides many healthy foods due to its abundant and phenomenal biodiversity. Sea cucumber is one of the marine invertebrates that can be investigated as a possible source of phenolic compounds. These marine invertebrates are a significant source of phenolics, showing moderate antioxidant activity even though they are considered as animal species. This

could be due to the absorption of phenolics from phytoplankton, which are the main food sources for sea cucumbers. Phytoplankton is a rich source of phenolic compounds, including phenolic acids, flavonoids, and tannins (Hossain et al., 2020a; Zhong et al., 2007). Various species of sea cucumber have different levels of phenolic compounds and varied antioxidant activities. This might be due to the different geographic locations, food habits, and harvesting times. Therefore, suspension-feeding species may have more phenolics compared to the deposit-feeding species. Table 2.3 shows the phenolic compounds of different sea cucumbers and their antioxidant activities.

Table 2. 3. Phenolics in sea cucumber and their antioxidant activity

Species	TPC (mg GAE/ g)	TFC (mg RE/ g)	DPPH (µmol TE/ g)	Identified compounds	References
Holothuria forskali	NA	NA	NA	Quinic acid, gallic acid, caffeic acid, syringic acid, *trans* ferulic acid, *o*-coumaric acid, rosmarinic acid, and salvianolic acid	Telahigue et al., 2020
Holothuria forskali	3.19- 5.21	NA	NA	NA	García et al., 2019
Holothuria atra	NA	NA	NA	Chlorogenic acid, pyrogallol, rutin, coumaric acid, catechin, and ascorbic acid	Esmat et al., 2013
Holothuria atra	NA	NA	81- 94*	Chlorogenic acid, pyrogallol, rutin, coumaric acid, and ascorbic acid	Dakrory et al., 2015
Holothuria atra	Detected	Detected	NA	NA	Sukmiwati et al., 2019
Holothuria arenicola	NA	NA	85- 95*	Chlorogenic acid, pyrogallol, rutin, and coumaric acid	Fahmy, 2015
Holothuria scabra	20- 46.54	NA	NA	NA	Pranweerapaiboon et al., 2020
Holothuria scabra	0.03	NA	33.77*	3-Hydroxybenzaldehyde and 4-hydroxybenzaldehyde	Nobsathian et al., 2017
Holothuria scabra	1.53- 4.85	NA	NA	NA	Althunibat et al., 2009
Holothuria leucospilota	4.58	0.84		2,4-Bis(1,1-dimethylethyl)-phenol	Ceesay et al., 2019

Holothuria leucospilota	2.91- 9.7	NA	3.91- 5.44**	NA	Althunibat et al., 2009
Holothuria tubulosa	NA	NA	NA	Epicatechin, 2,5-dihydroxybenzoic acid, ellagic acid, gallic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid, rutin, naringin, and quercetin	Alper & Günes, 2020
Cucumaria frondosa	0.88- 1.08	NA	4.51- 7.48	NA	Zhong et al., 2007
Cucumaria frondosa	0.22- 2.36	0.02- 0.59	NA	NA	Mamelona et al., 2007
Stichopus variegatus	10.55- 10.9	NA	1.67- 2.3**	NA	Ridhowati et al., 2018
Stichopus japonicas	13.6- 116.90	NA	NA	NA	Nguyen & Kim, 2015
Stichopus japonicus	18.65- 40.99	5.92- 30.38	3.2- 16.37	NA	Husni et al., 2009
Stichopus japonicus	3.53- 20.37	NA	NA	NA	Himaya et al., 2010
Stichopus chloronotus	1.66- 8.27	NA	2.13**	NA	Althunibat et al., 2009

Note: NA, not available; TPC, total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalents; RE, rutin equivalents; DPPH, DPPH radical scavenging capacity; * data expressed as %; and ** data expressed as IC_{50} in mg extract/ mL DPPH.

The most common phenolic compounds found in sea cucumber are gallic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, rutin, and pyrogallol (Figure 2.9). For instance, high-performance liquid chromatography coupled with a mass spectroscopy (HPLC-MS) was used to identify the phenolic compounds of *Holothuria forskali* extracts, and it was revealed that the phenolic compounds were mostly phenolic acids such as quinic, gallic, rosmarinic, and salvianolic acids. The same study reported that quinic acid was abundant in different body parts, including the digestive tract, muscle, body wall, gonad, and respiratory tree, whereas only gallic acid and caffeoylquinic acid were present in the gonad of this sea cucumber (Telahigue et al., 2020). Moreover, the body wall of *Holothuria atra* is a good source of chlorogenic acid (up to 92 wt%), and it also contains pyrogallol, coumaric acid, rutin, and catechin. These phenolics demonstrate strong antioxidant activity like DPPH radical scavenging activity and metal chelating activity. The presence of phenolics in the body wall could be due to the eating of phenolic-rich ingredients, mainly phytoplankton and particles derived from degrading marine macroalgae (Dakrory et al., 2015; Esmat et al., 2013). Similarly, chlorogenic acid is the major component (~90%) in the body wall of *Holothuria arenicola*, whereas pyrogallol, rutin, and coumaric acid are also identified from this species (Fahmy, 2015). However, Althunibat et al. (2009) compared the antioxidant activity of three Malaysian sea cucumber species (*Holothuria leucospilota*, *Holothuria scabra*, and *Stichopus chloronotus*), and reported that the extracts of *H. leucospilota* had higher TPC (9.7 mg GAE/ g), whereas a lower TPC was present in *H. scabra* (1.53 mg GAE/ g). *S. chloronotus* extracts showed a higher DPPH radical scavenging capacity compared to the other extracts. Likewise, methanol extracts of *H. scabra* was found to be a good source of phenolics dominated with 3- and 4-hydroxybenzaldehyde (Nobsathian et al., 2017). Besides, TPC and TFC were determined in the body wall of *H. leucospilota*, and contained 2,4-bis(1,1-dimethylethyl)-

phenol (Ceesay et al., 2019). Furthermore, phenolic compounds were identified and quantified using HPLC in the aqueous and methanolic extracts of *Holothuria tubulosa*. Based on the results, 13 and 12 phenolic compounds were identified from the aqueous and methanolic extracts, respectively, with main respective compounds being epicatechin (790.09 µg/ g extract) and 2,5-dihydroxybenzoic acid (153.89 µg/ g extract) (Alper & Güneş, 2020). In addition, TPC and TFC were determined from the water and 70% ethanol extracts of *Stichopus japonicus* body wall. Results suggested that the water extract had higher TPC and TFC, where TPC showed a significant correlation with the DPPH radical scavenging capacity (Husni et al., 2009). Similarly, TPC was determined from the various internal organ extracts (80% methanol, hexane, chloroform, ethyl acetate, butanol, and water) of *Stichopus japonicus* with the ethyl acetate being the best solvent to extract phenolics than the other solvents mentioned above (Nguyen & Kim, 2015).

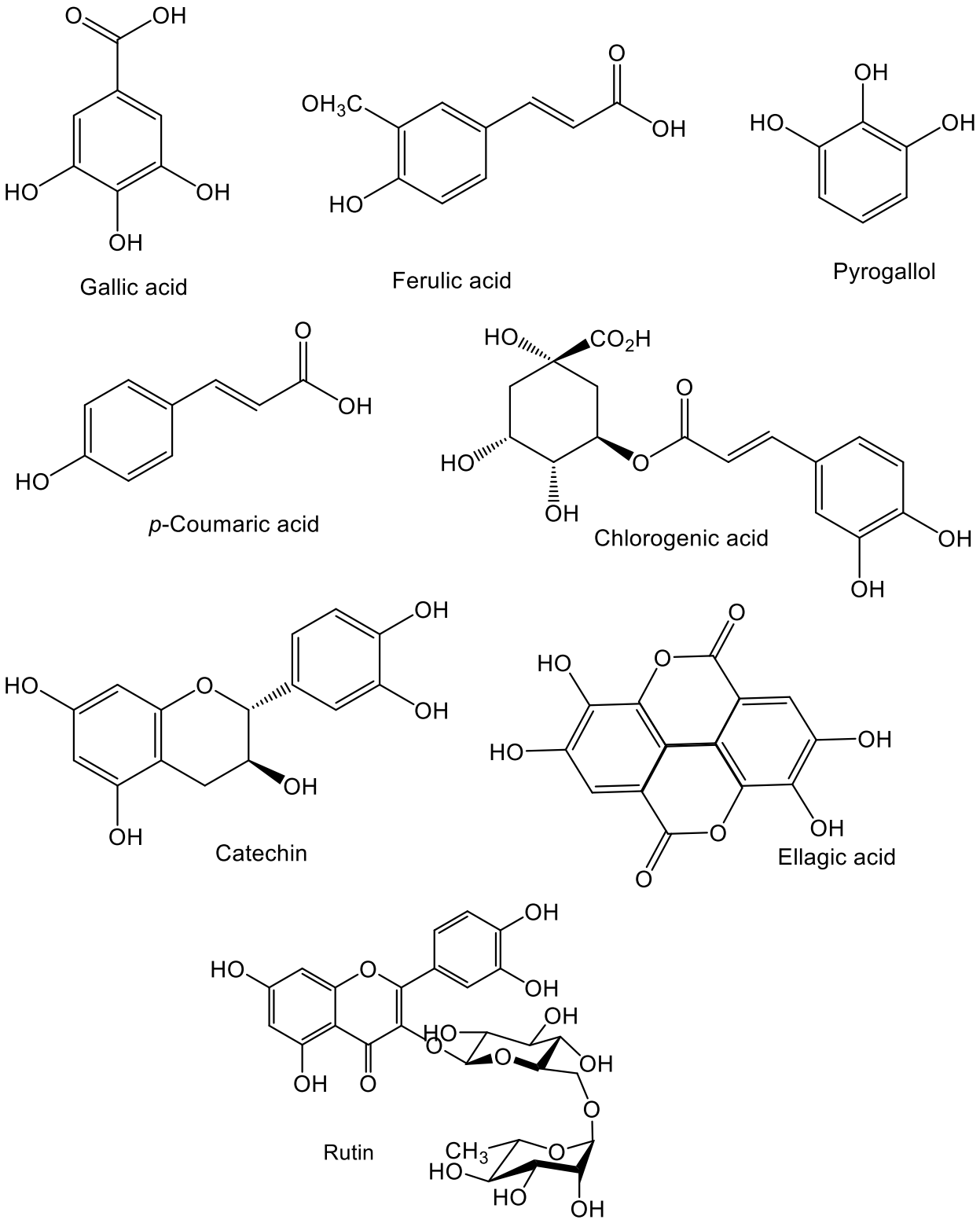


Figure 2. 9. Major phenolic compounds found in sea cucumber

The antioxidant activity, TPC, and TFC were determined in extracts from different body parts (digestive tract, gonad, muscle, and respiratory apparatus) of Atlantic sea cucumber, *Cucumaria frondosa*. The TPC varied from 22.5 to 236.0 mg GAE/ 100 g, while TFC ranged from 2.9 to 59.8 mg RE/ 100 g and ORAC values varied from 140 to 800 µmol TE/g. A higher TPC was also observed in the digestive tract when considering acetonitrile-rich fractions and ethyl acetate extracts, while maximum TFC was obtained from gonads using water-rich and acetonitrile-rich fractions. A good correlation existed between ORAC values and TFC in all extracts (Mamelona et al., 2007). Similarly, Mamelona and Pelletier (2010) determined the antioxidant activity (ORAC) of the viscera of *C. frondosa* using pressure liquid extraction (PLE) and found that the ethanol extracts had higher ORAC values compared to methanol, isopropanol, and water extracts at 60°C extraction temperature. Additionally, PLE allowed better extraction of TPC, total carotenoids, α-tocopherol using ethanol followed by isopropanol, methanol, and water. In another study, the antioxidant property of fresh and processed *C. frondosa* with/ without internal organs was evaluated. The processed (rehydrated) samples, mainly those with internal organs, exhibited higher ORAC and DPPPH radical scavenging activity, whereas fresh samples contained a significant amount of phenolics compared to their rehydrated counterparts (Zhong et al., 2007). Besides, the free, esterified, and insoluble-bound phenolics from different body parts (body wall, tentacles, and viscera) of *C. frondosa* were extracted using high-pressure processing (HPP). Results suggested that the HPP had a positive effect on the extraction of phenolics in which free fraction was the most predominant form of phenolics in all the selected body parts (Hossain, Dave, & Shahidi, 2020b).

2.2.5. Extraction of phenolics

Phenolics are secondary metabolites and can be present in the free aglycone, esters, and glycosides or aglycones form. Extraction of phenolics through inadequate procedures could change their recovery as the solubility and separation characteristics are influenced by their structural variations. Therefore, selecting an appropriate extraction procedure is very important in order to recover the targeted phenolic components. Traditional methods, including Soxhlet and maceration extraction, have been used for more than a century for the extraction of phenolics through solid-liquid/ liquid-liquid extraction. However, due to certain disadvantages, such as excessive consumption of energy, time, and solvents, researchers have been developing more cost-effective and greener methods for the extraction of phenolics. Nowadays, unconventional extraction methods, including microwave-assisted extraction (MAE), ultrasounds assisted extraction (UAE), subcritical water extraction (SWE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), enzyme-assisted extraction (EAE), and high hydrostatic pressure extraction (HHPE), have been used to extract phenolics due to their higher extraction efficiency (Table 2.4). For example, UAE generates cavitation of small bubbles due to the ultrasound waves, which accelerates the diffusion of phytochemicals from the material into the solvent. UAE is a simple and low-cost method that helps a rapid and better extraction of phenolics with reduced degradation of components compared to other methods of extraction. Moreover, UAE requires less solvent to accomplish complete recovery of phytochemicals (Alara et al., 2021). Different types of solvent have been used for extracting phenolics, such as polar (mainly water, methanol, ethanol, and propanol), dipolar aprotic (acetone and ethyl acetate), non-polar (n-hexane), and halogenated (chloroform) solvents. These solvents have different impacts on the extraction of phenolics, depending on their polarity (Zhang, Lin, & Ye, 2018). For example,

acetone has proven to be an effective solvent to extract both polar and non-polar phenolics from different samples. This is because acetone contains both polar (carbonyl group) and non-polar (two methyl groups) ends, which dissolve polar and non-polar substances, respectively.

Table 2. 4. Unconventional extraction methods and their applications

Extraction methods	Mechanisms	Major application	Advantages	Disadvantages	References
MAE	Use of microwave radiation energy to heat solute-solvent mixture	Extraction of monomeric phenolics (e.g., phenolic acids and flavonoids)	Less extraction time, low solvent volume, better accuracy, and higher yield	Less suitable for thermolabile compounds (e.g., anthocyanins)	Aires, 2017
UAE	Sonication to facilitate bubble cavitation, resulting tissue disruption	Extraction and recovery of natural products	Simple method, low cost, low solvent volume, low temperature, and better and faster extraction	High ultrasound waves may bring deleterious effects in active constituents	Shirzad et al., 2017
SFE	Extraction by supercritical fluids (e.g., CO_2) with modifiers	Extraction of phenolics and flavor compounds	Faster extraction, cutting down usage of	Low polarity CO_2 is suitable for the	Alara et al., 2021

			organic solvents, shorter time, and maximum degree of separation	extraction of non-polar components.	
PLE	Extraction with high temperature and pressure to enhance the solubility and desorption of compounds	Extraction of phenolics, mainly isoflavones and flavanones	Faster extraction, low solvent (e.g., water) volume, reduction of organic solvents, and better mass transfer	Low selectivity and need advanced instruments	Ameer, Shahbaz, & Kwon, 2017
EAE	Extraction with enzymes to degrade cell wall components into solvents	Phytochemicals (e.g., bound phenolics) that are bound to the plant structures (e.g., cell wall)	Eco-friendly methods and no use of organic solvents	Comparatively high cost	Alara et al., 2021
HHPE	Extraction with high pressure to break the plant structures,	Extraction and recovery of natural products	Higher extraction yields, shorter time, fewer impurities in the extraction solution, and minimal	High pressure may help to polymerize certain compounds with proteins	Jun, 2013

leading better extraction	thermal degradation on the chemical structures

Insoluble-bound phenolics are covalently bound to the cell wall matrix, and this bond can be disrupted by hydrolysis to extract phenolics. Generally, acid and alkaline hydrolysis are used to break the chemical bond to extract insoluble-bound phenolics. Nowadays, several other procedures, including enzymatic and microwave-assisted hydrolysis, have been used to release more phenolics. Acid hydrolysis is a convenient and simple extraction method, where 1–5% hydrochloric acid is used with water/ methanol. Nevertheless, phenolics can be degraded at low pH during the extraction process or storage (Shahidi & Yeo, 2016). Moreover, Fazary and Ju (2007) claimed that acid hydrolysis was insufficient to break the ester bonds properly; thus, alkali hydrolysis should be used for releasing the insoluble-bound phenolics. In alkali hydrolysis, a wide range of concentrations of sodium hydroxide (NaOH) is used to hydrolyze both ether and ester bonds. Generally, alkaline hydrolysis conducts at room temperature, resulting in a lower chance of degrading phenolics compared to acid hydrolysis (Krygier, Sosulski, & Hogge, 1982). On the other hand, enzymatic hydrolysis is an effective approach for the extraction of insoluble-bound phenolics, where carbohydrate-hydrolyzing enzymes such as amylase, pectinase, cellulase, hemicellulase, and glucanase are employed to break the cell wall matrix. As a result, solvents can easily penetrate into the samples, leading to better extraction. The enzymatic treatment minimizes the loss of phenolic and improves antioxidant activity (Shahidi & Yeo, 2016).

2.2.6. Pre-treatment for the extraction of phenolics

Various types of pre-treatments have been used to increase cell wall permeability in maximizing the extraction yield of phenolics. The extractability of plant phenolics depends mainly on the permeability of their tissues, as some of the compounds are located in chloroplasts, vacuoles, and apoplasts of plant cells (Fincan, 2015). The major resistance of molecular diffusion mostly comes from the adhering membranes and intact cell walls of plant structures. Thus, improving the permeability of membranes as well as cell walls is very crucial in improving the recovery of phenolics and antioxidant capacity (Zhao et al., 2014). The most common traditional pre-treatments are washing, drying, milling/ grinding, blanching, freezing, and thawing, which allow better extraction yields. For example, the milling/ grinding process reduces the particle size and increases the surface area, resulting in better penetration of solvents into the plant matrix. Psarrou et al. (2020) extracted phenolics from rosemary using grinding, immersion/ maceration, and pulsed electric fields (PEF) as pre-treatments, and found that the grinding increased the extraction rate, yield of TPC, and antioxidant activity, while maceration shortened the extraction time. Furthermore, these traditional pre-treatments help to inhibit microbial growth, inactive enzymes, maintain sensory qualities, and reduce the loss of bioactive compounds. Nowadays, many pre-treatments have been employed with modern technologies to assist the actual extraction process, and which can be classified into three methods, namely physical, chemical, and biological treatments. Physical methods mainly include mechanical disruption, pulsed electric field (PEF) application, high-pressure processing (HPP), and ultrasonic treatment, while chemical treatments consist of improving the inner- or outer-membrane permeabilization using different chemicals, mostly acids or bases (Aggarwal & Jain, 2019; Zhao et al., 2014). In the PEF, a moderate electric field is applied to increase the cell membrane permeability. The short processing time, low energy

consumption, and moderate temperature make PEF a potential pre-treatment to improve the recovery of phenolics. For example, the effect of PEE pre-treatment in ultrasound-assisted extraction of phenolics from thyme and rosemary by-products was determined and found that the PEF improved the recovery of phenolics and antioxidant properties (Tzima et al., 2021). Similarly, HPP can rupture the plant cell walls, resulting in better extraction. For instance, Prasad et al. (2010) discovered that high pressure doubled the recovery when phenolic compounds were extracted from longan fruit pericarp using high-pressure-assisted extraction. HPP has shown great potentials in the food industry as it can be used as a food processing technique as well as extraction method to extract bioactive ingredients. In contrast, various cell wall-degrading enzymes are used in biological treatment to break plant structures and weaken the overall internal resistance to transfer targeted compounds from the plant matrix to the external solution (Zhao et al., 2014). For example, enzymatic (cellulase and pectinase) pre-treatment was applied as a pre-treatment prior to ultrasound- and microwave-assisted extraction to extract phenolics from grape pomace. It was observed that the treatment of enzymes accelerated the release of intracellular biomolecules; thus, improved the recovery of phenolics (Drevelegka & Goula, 2020).

2.2.6.1. Pre-treatment: High-pressure processing (HPP)

High-pressure processing (HPP) has shown great potential in the food industry, where a product is processed under very high pressure. HPP is a non-thermal process and is useful to preserve foods containing heat-sensitive components. In 1990, the first HPP-based foods (jams, jellies, and sauces) were launched to the Japanese market by the Meidi-ya (Rastogi et al., 2007). Over the past decades, consumers are looking for convenient, ready-to-eat (RTE), and natural healthy foods, and HPP is a potential technique to satisfy these demands. So far, HPP has been

employed in many commercial products, including seafood, fish, cooked meats, dairy products, vegetables, and fruit juices, which are available on the contemporary food market. HPP is also used for separating the meat from seafood, mainly lobster, oyster, clam, crab, and fish, which can extend their shelf life and maintain better quality. HPP is a type of cold pasteurization, meaning it does not require any heating system, which can be conducted at room temperature. As a result, HPP preserves sensory and nutritional characteristics of compounds such as anthocyanin which are responsible for food color, that may alter upon heat treatment. The process is isostatic, which enables instant and uniform transmittance of pressure throughout the system, regardless of the food shape or size. HPP retains food shape, even at high pressures, due to the pressure transfers evenly throughout the samples. During this process, a little change occurs in temperature with the increase of pressure, where temperature elevates around 3°C per 100 MPa. HPP maintains the highest quality of foods and makes foods taste better by improving moisture retention, and hence juiciness inside the food. Moreover, it increases revenue and profit, decreases labor costs, and creates new products. HPP increases the shelf life of foods by inactivating food-borne pathogens, mostly *Salmonella*, *Listeria*, and *Vibrio* (Jun, 2013; Rastogi et al., 2007; Zhao, Zhang, & Zhang, 2017).

Apart from these benefits, HPP ranging from 1000 to 8000 bar is considered as an alternative method to extract bioactive compounds from different sources due to higher extraction yields, less time consumption, minimal heat requirement, and minimum thermal degradation of the chemical structure of biologically active compounds (Jun, 2013). HPP helps to inactivate enzymes because it is responsible for partial unfolding of proteins during the treatment and upon the release of pressure. This is because HPP modifies only non-covalent bonds, such as hydrogen, hydrophobic, and ionic bonds, resulting in the unfolding of proteins (Saikaewa et al., 2018). HPP could inactivate the residual enzymatic activity of polyphenol oxidase (PPO) and peroxidase

(POD) of fruits and vegetables, which are linked to the enzymatic degradation of anthocyanins, causing browning (da Silveira et al., 2019). Similarly, HPP helps to inactivate a variety of enzymes that are responsible for meat or fish spoilage (Pita-Calvo et al., 2018). At high pressures (i.e., 300–600 MPa), vegetative cells such as bacteria, yeast, and molds can be inactivated due to the permeabilization/ puncturing of the cell membrane. Generally, gram-negative bacteria are more vulnerable under HPP compared to gram-positive bacteria (Rastogi et al., 2007). Moreover, the structure of low molecular-weight compounds with covalent bonds such as volatile compounds, vitamins, pigments, flavors, and antioxidants, are not affected by HPP (i.e., 1000–10000 bar), while HPP can alter the structure of high-molecular-weight compounds like carbohydrates and proteins (Patras et al., 2009; Saikaewa et al., 2018). For example, the effect of HPP (100-600 MPa) on the molecular structure of flavonoids and lycopene was studied, and it was found that it did not destroy the structures of these components (Xi, 2006). Therefore, HPP is a novel method to extract bioactive compounds due to the faster diffusion and cell disruption, which improves higher accessibility of the solvent and leads to better extraction. For example, HPP causes cell damage that helps maximizing extractability of phenolic compounds from cell wall (Zhao, Zhang, & Zhang, 2017). Moreover, during HPP, the solubility and cell permeability increase according to the phase behavior and mass transfer theories (Altuner et al., 2012). Furthermore, HPP can also cause distraction of salt bridges and hydrophobic bonds, and deprotonation of charged groups, resulting in denaturation of proteins (Barbosa-Canovas et al., 1998). Therefore, more solvent can enter into the cell by disrupting the cellular walls and hydrophobic bonds which causes a higher yield of extraction. Usually, high pressure used in two ways such as high pressure as a pre-treatment or a novel extraction method, and the effectiveness of HPP mainly depends on pressure (1000–8000 bar), holding time (2.5-30 min), and temperature (10-60°C) (Jun, 2013). For instance,

carrot snacks pretreated by using HPP before vacuum frying and found to maintain phenolic content and antioxidant activity throughout the storage period (Albertos et al., 2016). Likewise, the effect of HPP pre-treatment (500 MPa for 10 min) on the phenolic profiles and antioxidant activities of oil palm fruits showed a notably increase in the total phenolic and flavonoid contents as well as antioxidant activities, particularly for insoluble-bound phenolics (Zhou et al., 2019).

2.3. Identification and quantification of phenolics

After their extraction, it is important to isolate and quantify the target phenolics in order to use them in various applications. In general, the recovery of phenolics depends on their sources, extraction and purification methods, storage conditions, particle size, and the presence of interfering compounds such as reducing substances (Shahidi & Naczk, 2004). The classical method for the measurement of phenolics is done by colorimetric techniques, mainly Folin–Ciocalteu method. This is the most widely used spectrophotometric technique to measure the total phenolic content (TPC) in a plant matrix. In this method, a blue chromophore complex (phosphomolybdic/ phosphotungstic acid) is formed, based on the chemical reduction of phenolics in an alkaline solution, that can be measured using a UV-Vis spectrophotometer (760-765 nm) (Aires, 2017). This is a rapid method to measure TPC with a low cost and is easy to perform; however, its results are limited. This is because of the ability of Folin's reagent to react with non-phenolic reducing substances, including pigments, ascorbic acid, reducing sugar, aromatic amines, and nitrogen-containing compounds (e.g., hydroxylamine and guanidine), and thus, evaluate the total reducing capacity and not just the TPC (Blainski, Lopes, & de Mello, 2013; Ikawa et al., 2003). Similarly, total flavonoid content can be measured through a spectrophotometric technique, whereas proanthocyanidins can be measured using vanillin and 4-(dimethylamino)-cinnamaldehyde (DMCA), bovine serum albumin (BSA), and butanol-HCl assays. Moreover, the

spectrophotometric method is widely used to determine the anthocyanin content, as the color of this pigment changes with the pH, which can be measured at 490 to 550 nm. However, all these spectrophotometric techniques can overestimate the total content of phenolics, and do not provide the exact content of individual phenolic compounds (Aires, 2017; Cotas et al., 2020).

Nowadays, the identification of phenolics is conducted through different chromatographic methods, namely liquid chromatography (LC), column chromatography, gas chromatography (GC), ion-exchange chromatography (IEC), affinity chromatography (AC), and thin-layer chromatography (TLC). These chromatographic techniques provide a better separation, purification, identification, quantification, isolation, and characterization of individual phenolics. In these methods, compounds are separated based on their molecular characteristics when they pass through two phases: stationary (solid, liquid, or gel) and mobile (liquid or gaseous) phases. The LC can be classified as high-performance liquid chromatography (HPLC), reverse-phase liquid chromatography (RPLC), and size exclusion liquid chromatography (SEC). Among them, HPLC is used widely to characterize phenolics which contains a sampler, pumps, and a detector (e.g., photodiode array (PDA) and UV-Vis). Each component detected has a particular retention time (RT), which can be analyzed through a digital processor (Aires, 2017; Cotas et al., 2020). In the couple of decades, the coupling of chromatography with mass spectrometry (MS) has become a popular trend to characterize a wide range of phenolic compounds. For example, LC-MS or HPLC-MS method allows a more accurate characterization of phenolics, which is based on the fragmentation of each separated compound (Ge, Li, & Lisak, 2020). The separation of fragments or molecules occurs based on their mass-to-charge (m/z) ratio through an electromagnetic field. Generally, LC-MS is composed of three parts, namely the ion source (converts samples into ions), the mass analyzer (separates masses based on their m/z), and the detector (measures the detected

signals). Similarly, the coupling of gas chromatography with mass spectrometry (GC-MS), ultrahigh performance liquid chromatography-electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS), matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and quantitative nuclear magnetic resonance (qNMR) have been used to identify and quantify of phenolics (Aires, 2017; Cotas et al., 2020). Therefore, possible methodologies to measure the phenolics could be pre-treatment, extraction, purification, identification, quantification, and characterization (Figure 2.10).

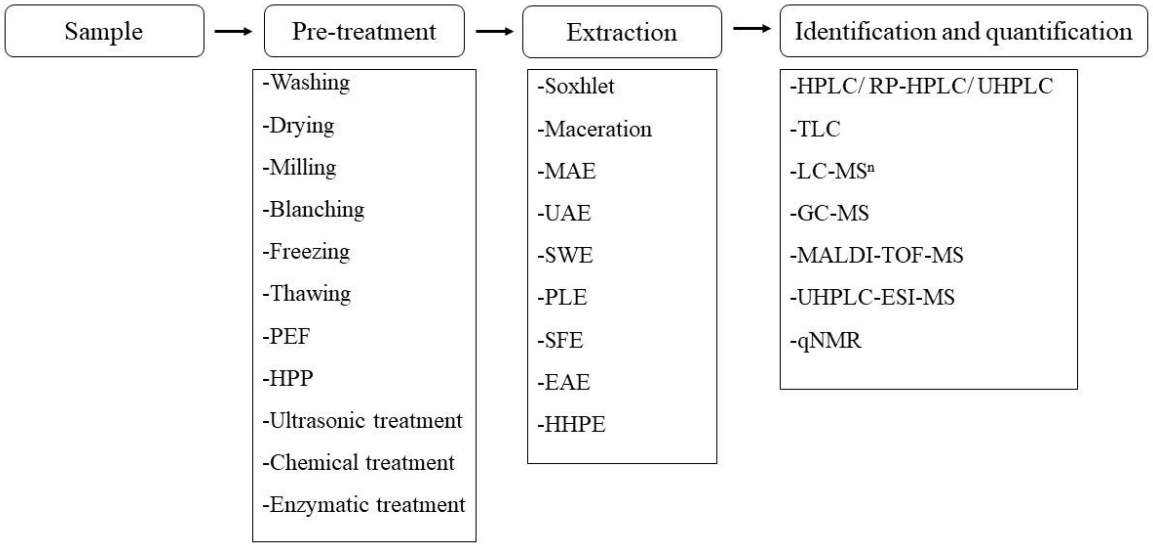


Figure 2. 10. Schematic representation of possible methodologies to measure phenolics

Chapter 3

Materials and Methods

3.1. Chemicals and reagents

The following chemicals were used for different analysis in the present study. The compounds 2,2′-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), hydrogen peroxide, 5,5-dimethyl-l-pyrroline *N*-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), caffeic acid, gallic acid, sinapic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, cinnamic acid, protocatechuic acid, vanillic acid, ferulic acid, syringic acid, ellagic acid, chlorogenic acid, quercetin, catechin, ferrous sulfate, human LDL cholesterol, supercoiled plasmid pBR322 DNA, agarose, tris acetate, bromophenol blue, glycerol, and Folin Ciocalteu's phenol reagent were purchased from the Sigma-Aldrich Ltd. (Oakville, ON, Canada). Mono- and dibasic potassium phosphates, potassium persulfate, and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2 carboxylic acid) were obtained from Fisher Scientific Co. (Nepean, ON, Canada). All other solvents and chemicals were analytical or chromatographic grade, and purchased from the Fisher Scientific Co. (Nepean, ON, Canada) and Sigma-Aldrich Ltd. (Oakville, ON, Canada).

3.2. Methods

3.2.1. Sample preparation

Atlantic sea cucumbers (*Cucumaria frondosa*) were collected from the Northwest and Southeast zones of the St. Pierre Bank (NAFO Division 3Ps), NL, Canada. The live sea cucumbers were placed in seawater with ice and transported to the food processing pilot plant (Marine Institute, St. John's, NL). After that, sea cucumbers were selected for analysis based on their weight (180-200 g). Sea cucumbers were gutted longitudinally using a fish filleting knife to separate their internal organs (gonads, respiratory tracts, and intestines) from the body walls and flowers. The body wall was then chopped into small pieces, and dissected samples were labeled as body wall (BW), flower (FL), and internal organs (IO). After that, control samples were left without any pre-treatment and transferred to a -18°C freezer for further analysis, whereas the samples for the high-pressure processing (HPP) pre-treatment were placed in plastic Ziploc bags. After freezing, both HPP-treated and untreated samples were dried using a Labconco FreeZone benchtop freeze dryer (Labconco, Kansas City, MO) for 72 h to remove the moisture. This is because, Zhong et al. (2007) concluded that the rehydrated *C. frondosa* had a higher antioxidant activity than the fresh one. The dried samples were ground and sieved to obtain powder with particle size of ≤1 mm. Ground samples were defatted with hexane (1:5 w/v, 6 min, 3X) at room temperature, and then air-dried and stored at -20°C. Figures 3.1 and 3.2 summarize the overall sample preparation and experimental design of the present study.

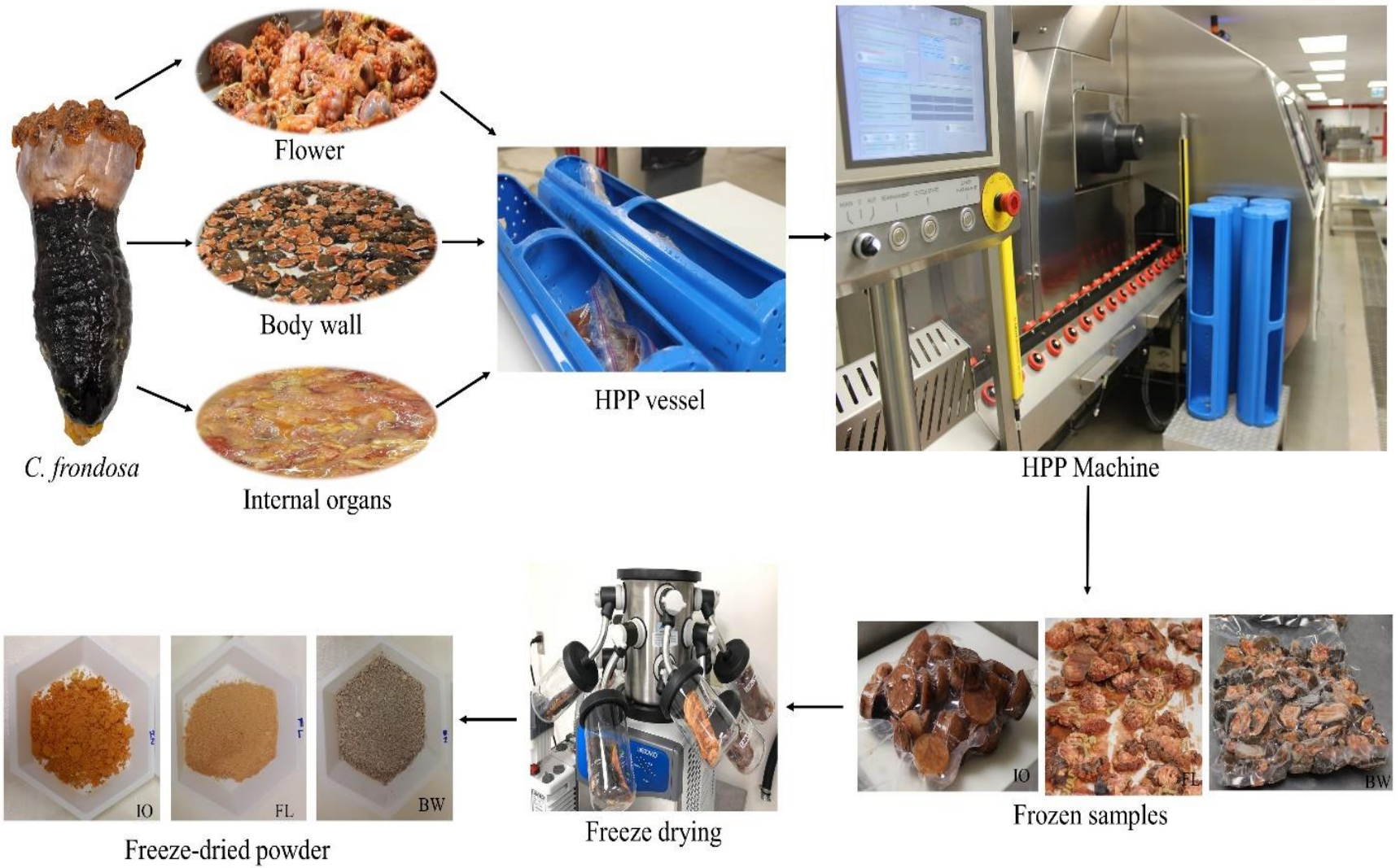


Figure 3. 1. Sea cucumber sample preparation

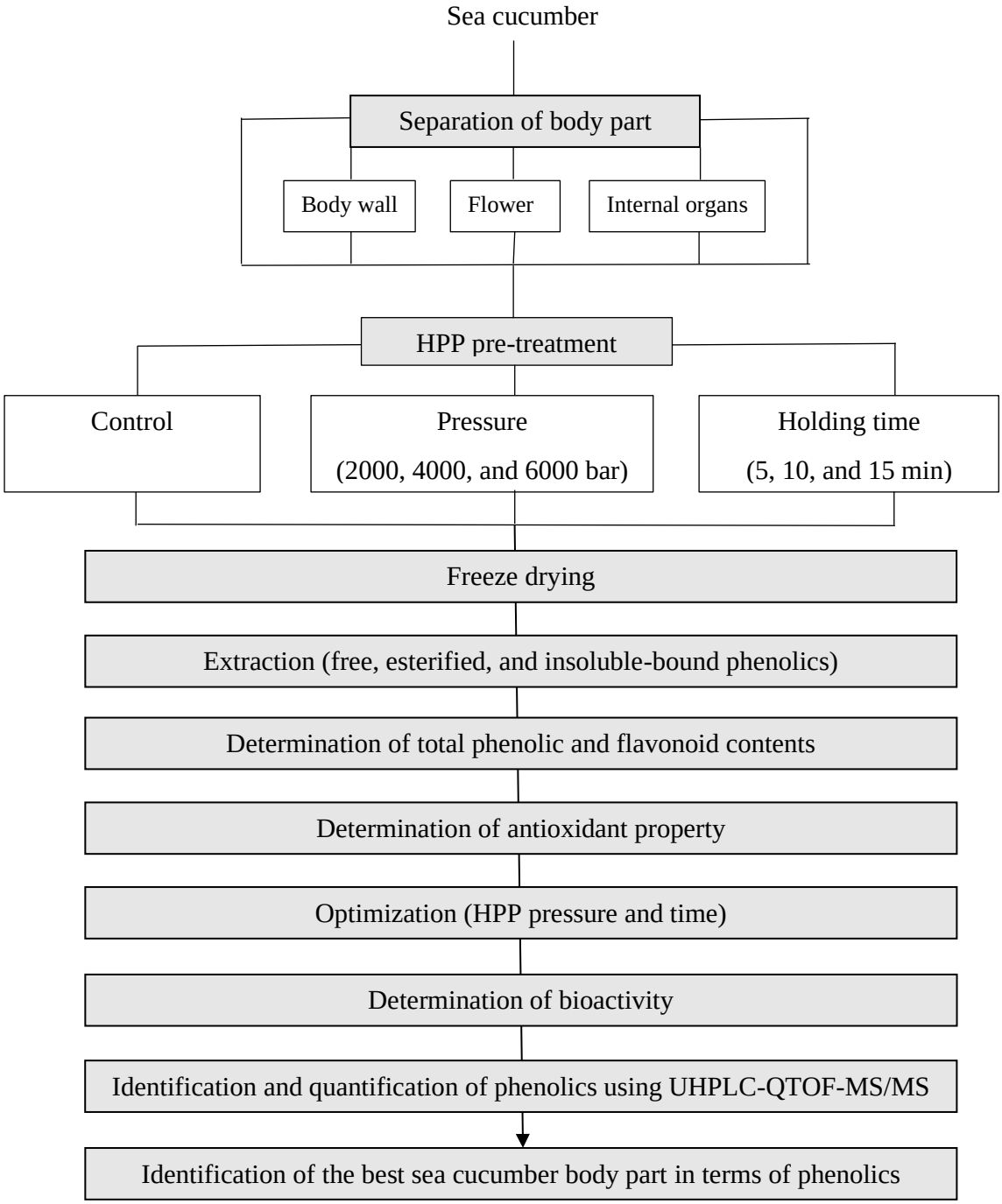


Figure 3. 2. The flowchart for the extraction of phenolics from HPP-treated and untreated sea cucumber and their antioxidant activity and bioactivity.

3.2.2. HPP pre-treatment

For the HPP pre-treatment, sea cucumber samples, including body wall, flower, and internal organs, were packaged in plastic Ziploc bags separately and sealed. Each bag contained around 200 g of body wall, flower, or internal organs. The sealed samples were placed into HPP vessels, and then they were placed into a pilot-scale NC Wave 6000/55 HPP equipment (Hyperbaric, Burgos, Spain). The volume and inside diameter of the pressure vessel were 55 L and 200 mm, respectively. After that, samples were subjected to the pressure of 2000 (200 MPa), 4000 (400 MPa), and 6000 (600 MPa) bar and holding time of 5, 10, and 15 min. All the treatments were conducted at ambient temperature (20°C) using water as a pressure-transmitting medium (Figure 3.3). Due to compression, the temperature increases by 2-3°C/ 1000 bar; however, this should not affect the extraction process when the pressure reaches 6000 bar. Following HPP treatment, the pressure was quickly released, and samples were taken out from the vessels. After that, samples were transferred to a -18°C freezer, followed by freeze drying.

Figure 3. 3. HPP pre-treatment (4000 bar for 5 min)

3.2.3. Extraction of free, esterified, and insoluble-bound phenolics

Free phenolic compounds of sea cucumber samples were extracted by ultrasound-assisted extraction method, and insoluble-bound phenolics were extracted by alkaline hydrolysis as described by Ambigaipalan, de Camargo, and Shahidi (2016) with slight modification. For free phenolics, the defatted treated and untreated samples were mixed with 70% acetone (1:10, v/v) and kept in an ultrasonic water bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA, USA) at 30°C for 15 min. The extraction was repeated two more times, and the sonicated slurry was centrifuged for 5 min at 5,000 g (Thermo Scientific™ Sorvall LYNX 6000 Superspeed Centrifuge, Thermo Fisher Scientific, Pittsburgh, PA, USA). The combined supernatants were evaporated using a rotary evaporator (R-300, Buchi, Flawil, Switzerland) at 40°C till all the solvent was removed, and the remaining aqueous suspension was acidified (pH 2.0) with 6 M HCl. Free phenolics were extracted with diethyl ether/ ethyl acetate (1:1, v/v) five times using a separatory funnel. The organic phase was evaporated to dryness under vacuum at 40°C and dissolved in 10 mL 80% HPLC grade methanol. The reconstituted extracts were stored at -20°C for further analyses.

The remaining water phase after the extraction of free phenolic was hydrolyzed with an equal volume of 4 M NaOH for 4 h under a nitrogen atmosphere to extract the esterified phenolics. The suspension was acidified (pH 2.0) with 6 M HCl, and then centrifuged and extracted the esterified phenolics with equal volumes of ethyl acetate/ diethyl ether (1:1, v/v), as detailed above. After evaporation, samples were reconstituted in 10 mL HPLC grade methanol and stored at -20°C for subsequent analyses.

The meal (solid) residue obtained from the centrifugation of free phenolics was used for the extraction of insoluble-bound phenolics. The samples were hydrolyzed under a nitrogen

environment, and the resulting slurry was acidified, as described above. The insoluble-bound phenolics were extracted with equal volumes of diethyl ether and ethyl acetate (1:1, v/v), followed by evaporation and reconstitution in 10 mL methanol (HPLC grade). Samples were stored at -20°C for subsequent analysis of phenolic content, antioxidant and biological activities, and phenolics profile of sea cucumber.

3.2.3.1.1. Determination of total phenolic content (TPC)

The total phenolic contents of each sea cucumber extract obtained from treated and untreated BW, FL, and IO were determined using the method described by Singleton and Rossi (1965) with some modifications. Briefly, 0.5 mL of sea cucumber extracts dissolved in methanol was added to 0.5 mL of 10% Folin–Ciocalteu's phenol reagent in the flask. After 5 min, the reaction mixture was subsequently neutralized by the addition of 1 mL of 7.5% sodium carbonate (Na_2CO_3), and the final mixture was then allowed to stand at room temperature in the dark for 2 h after adding 8 mL of distilled water. The absorbance was measured at 765 nm using a UV-visible spectrophotometer (HP 8452 A diode array spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). The total phenolic content was calculated using a standard curve consisted of gallic acid (GA) between the concentration arrays of 0-200 mg/ kg, and the results were expressed as milligram of gallic acid equivalents (GAE) per 100 grams of sample.

3.2.3.1.2. Determination of total flavonoid content (TFC)

The total flavonoid contents of each sea cucumber extract were determined using the method of Chandrasekara and Shahidi (2010) with slight modifications. Concisely, 1 mL sample was added to 4 mL of distilled water in a test tube and mixed with 0.3 mL of a 5% sodium nitrite ($NaNO_2$) and kept for 6 min. After that, 0.3 mL of 10% aluminum chloride ($AlCl_3$) was added, followed by the mixing of 2 mL of 1 M sodium hydroxide (NaOH) and 2.4 mL of distilled water.

After 15 min, the absorbance was read at 510 nm using a UV-visible spectrophotometer, and TFC was calculated and expressed as milligram catechin equivalents (mg CE) per 100 grams of sample.

3.2.3.2. Antioxidant activity of phenolic extracts

Figure 3.4 summarizes the experimental design for the antioxidant activity assays conducted for sea cucumber phenolic extracts.

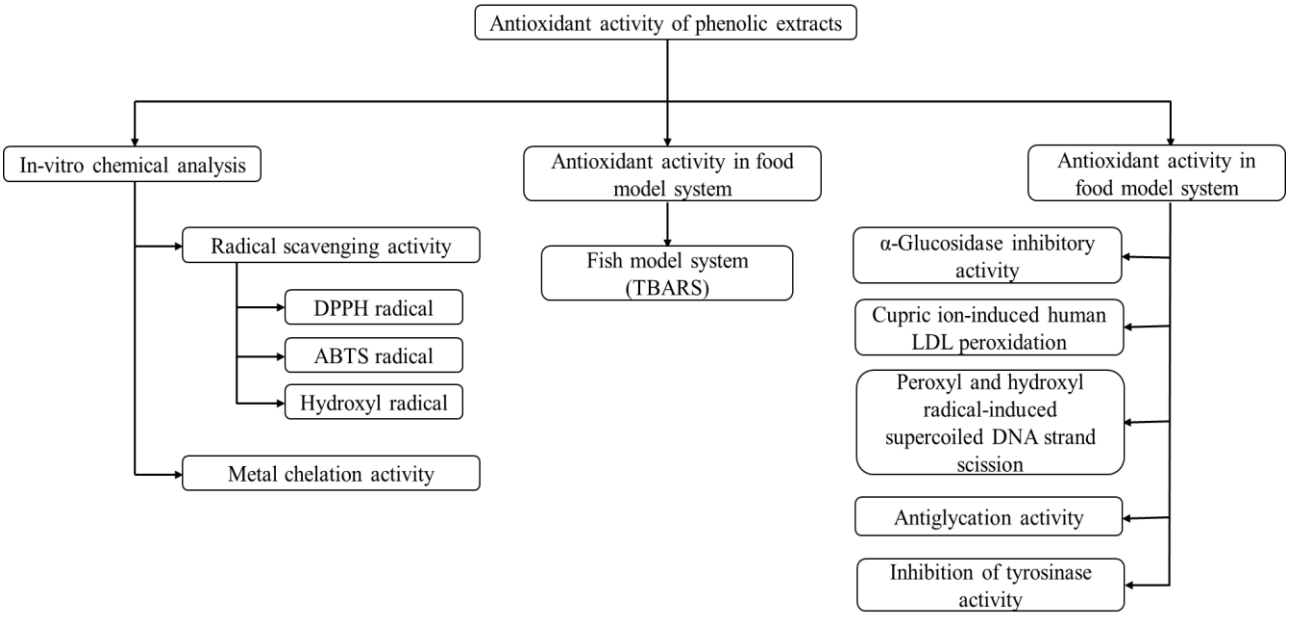


Figure 3. 4. Experimental design for antioxidant analysis of sea cucumber phenolic extracts.

3.2.3.2.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical becomes colorless or pale yellow when neutralized (Figure 3.5). The DPPH radical scavenging activity was determined using a Bruker-E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker BioSpin Co., Billerica, MA, USA) based on the method described by Shahidi, Liyana-Pathirana, and Wall (2006) with some modifications. Briefly, 0.1 mL of free, esterified, and insoluble bound phenolic extracts in methanol (1 mL extract/ 5 mL methanol) was mixed with 3.9 mL of a methanolic mixture of DPPH (0.30 mM). After that, the solution was shaken and allowed to keep in the dark for 30 min at room temperature. The absorbance was measured at 510 nm using an EPR spectrophotometer. The DPPH radical scavenging activity was expressed as mg of Trolox equivalents (TE) per 100 grams of sample. The antioxidant activity (%) of the samples was determined according to the following formula:

$$\% \text{ Inhibition of DPPH} = [(A_{control} - A_{sample})/ (A_{control})] \times 100$$

where $A_{control}$ is the absorbance of DPPH solution without the sample.

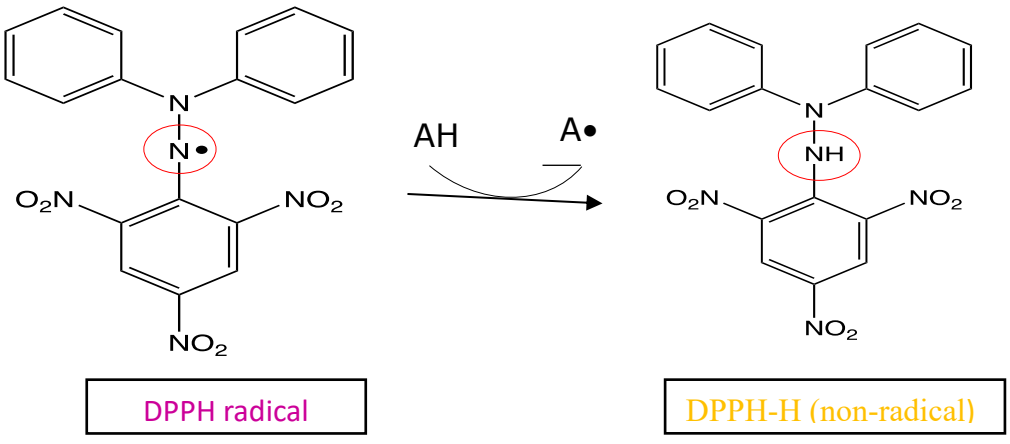


Figure 3. 5. Changes in chemical structure and color of DPPH radicals after reduction with antioxidants.

3.2.3.2.2. 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The ABTS radical-scavenging activity was determined as explained by Hossain, Moon, and Kim (2018) with some modifications. Briefly, 7.4 mM ABTS solution and 2.6 mM potassium persulfate ($K_2S_2O_8$) solution were mixed in equal volume and placed in the dark at room temperature for 12 h. The ABTS stock solution was diluted by adding ethanol (ABTS: ethanol; 1: 24) to obtain an absorbance of 1.5 ± 0.02 at 734 nm using a spectrophotometer. A fresh ABTS mixture was prepared for each assay. Finally, 0.3 mL of free, esterified, and insoluble bound phenolic extracts were added with 2.7 mL of the ABTS solution, and the absorbance was read after 8 min at 734 nm. The ABTS radical scavenging activity was expressed as mg of Trolox equivalents (TE) per 100 grams of sample. The scavenging activity was measured as a percentage (%) according to the following formula:

Scavenging activity % = 100- [(absorbance of sample/ absorbance of control) × 100]

3.2.3.2.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using the procedure of Chandrasekara and Shahidi (2011a) with slight modifications. Briefly, methanol was removed from sea cucumber extracts using nitrogen gas, followed by dissolving in 75 mM phosphate buffer (pH 7.2). Sea cucumber extract (200 µL) in 75 mM phosphate buffer was added with hydrogen peroxide (200 µL, 10 mM), $FeSO_4$ (200 µL, 10 mM), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 400 µL, 17.6 mM). After 4 min, samples were injected into an electron paramagnetic resonance (EPR) spectrometer, and their spectra were recorded. The hydroxyl radical scavenging activity was expressed as mg of Trolox equivalents (TE) per 100 grams of sample. Hydroxyl radical scavenging activity was calculated using the following equation:

Hydroxyl radical scavenging capacity (%) =

[(EPR signal intensity for control- EPR signal intensity for sample)/ EPR signal intensity for control] ×100

3.2.3.2.4. Metal chelation activity

Metal chelation activity of sea cucumber extracts was evaluated by the method of Ambigaipalan, Al-Khalifa, and Shahidi (2015) with slight modifications. Sea cucumber extract in methanol (0.4 mL) was added with 0.5 mL of 0.2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. Then mixed with distilled water (2.9 mL) and kept at room temperature for 12 min after vortexing. The absorbance was measured at 562 nm using a spectrophotometer. A standard curve was prepared using Na_3EDTA (trisodium salt of ethylenediaminetetraacetic acid), and the results were expressed as mg of EDTA equivalents per 100 grams of sample. The metal ion chelating ability (%) was calculated using the following equation:

Metal chelating activity (%) = [1- (absorbance of sample/ absorbance of control)] ×100

3.2.4. Optimization of HPP parameters

The HPP parameters (time and pressure) were optimized based on the content of total phenolics, total flavonoids, and antioxidant activities, the latter included DPPH, ABTS, and hydroxyl radical scavenging activities as well as metal chelation property. For each assay, a total 91 samples (3 phenolic fractions × 3 body parts × 3 pressures × 3 holding times) were analyzed. After that, the bioactivity was determined, and individual phenolic compounds were identified and quantified from the optimized sample preparations. Additionally, untreated samples were compared with their HPP-treated (optimized) counterparts.

3.2.5. Antioxidant activity of phenolic extracts in food and biological model systems

Different antioxidants such as phenolic compounds have been used in food industries to enhance the oxidative stability of food. Moreover, antioxidants can also prevent/ control certain diseases related to oxidative stress, including pulmonary dysfunction, cancer, skin lesions, inflammatory disorders, atherosclerosis, and so on, by retarding oxidation (Shahidi & Ambigaipalan, 2015). Therefore, antioxidant activity of phenolics extracted from sea cucumber was determined in food model system (TBARS assay) and biological model system (α-glucosidase inhibitory activity, cupric ion-induced human low-density lipoprotein (LDL) peroxidation, peroxyl and hydroxyl radical-induced supercoiled DNA strand scission, and antiglycation activity).

3.2.5.1. Antioxidant activity in a fish model system (thiobarbituric acid reactive substances, TBARS)

A fish model system was prepared according to the method of Wettasinghe and Shahidi (1999) to determine the antioxidant activity of phenolic extracts. Freshly ground Atlantic salmon (40 g) was mixed with 10 mL of distilled water in Mason jars, followed by the addition of 8 mg phenolic extracts (200 ppm). Butylated hydroxytoluene (BHT) was used as a positive control, whereas no phenolic extract was used for the negative control. The fish samples were mixed and cooked for 45 min in a water bath at 80°C with gentle stirring every 5 min. The samples were cooled at room temperature, and then transferred into plastic bags and stored for 14 days at 4°C (Figure 3.6).

Fish samples were randomly taken out according to a designated time interval (0, 3, 6, 9, 12, and 15 days) for the analysis of TBARS as described by Shahidi and Hong (1991) with slight modification. Briefly, 2.5 mL trichloroacetic acid (10%, w/v) was mixed to 1 g fish sample,

followed by vortexing for 2 min. After that, 2.5 mL 2-thiobarbituric acid (TBA, 0.02 M) reagent was mixed and centrifuged at 3,000 g for 10 min before filtering through a Whatman No. 3 filter paper. The samples were then cooked at 95°C for 40 min in a water bath and cooled at room temperature. A standard curve was constructed using 1,1,3,3-tetramethoxypropane, a precursor of malondialdehyde (MDA). The absorbance of the pink TBA-MDA adducts was read at 532 nm, and the results were expressed as mg MDA equivalents per kilogram of sample.

Figure 3. 6. Preparation of fish samples for the TBARS test.

3.2.5.2. α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity was measured as described by Liu et al. (2011) with some modifications. Phenolic extracts (10 µL) and α-glucosidase (10 U/ mL, 5 µL) were dissolved in 620 µL of sodium phosphate buffer (20 mM, pH 6.8) in Eppendorf tubes. After incubation at 37°C for 20 min, the mixture was mixed with 10 µL of 10 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution to initiate the reaction, and then incubated at 37ºC for another 20 min. After incubation, the reaction was terminated by the addition of 650 µL of 1 M sodium carbonate (Na_2CO_3), and the absorbance was read at 410 nm using a UV-visible spectrophotometer. A control without sample was also prepared, and the α-glucosidase inhibition rate (%) was calculated using the following equation:

$$\alpha\text{-Glucosidase inhibition (\%)} = [(A_{control} - A_{sample})/ (A_{control})] \times 100$$

3.2.5.3. Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation

The inhibitory effect of sea cucumber phenolic extracts on cupric ion-induced human LDL peroxidation was measured according to the method of Ambigaipalan and Shahidi (2015) with slight modification. Briefly, 5 mg/ mL of human LDL was dialyzed in 10 mM phosphate buffer (PBS, pH 7.4, 0.15 M NaCl) at 4°C under a nitrogen blanket in the dark for 12 h. Then, 0.1 mL sea cucumber extract dissolved in phosphate buffer (0.1 mg/ mL) was mixed with diluted 0.8 mL LDL cholesterol (0.04 mg LDL/ mL), followed by pre-incubation at 37°C for 15 min. After that, a solution of cupric sulfate (0.1 mL, 100 µM) was added to initiate the oxidation reaction, followed by incubation at 37°C for 22 h. The formation of conjugated dienes (CD) from the oxidation of LDL cholesterol was measured at 234 nm using a diode array spectrophotometer with 3 h intervals until the end of the incubation period. The blanks were run for each sample by replacing LDL and

$CuSO_4$ with the PBS for background correction. The inhibitory effect of the phenolic extracts on the formation of conjugated dienes was calculated using the following equation:

$$\% \text{ Inhibition CD} = [(A_{control}-A_{sample})/ (A_{control}-A_{native\ LDL})] \times 100$$

where $A_{control}$ is the absorbance of LDL with $CuSO_4$ and PBS; A_{sample} is the absorbance of LDL with $CuSO_4$ and sample; and $A_{native\ LDL}$ is the absorbance of LDL and PBS only.

3.2.5.4. Inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission

Peroxyl and hydroxyl radical-induced supercoiled plasmid DNA strand scission inhibitory activity of sea cucumber phenolic extracts was determined according to the method of Ambigaipalan and Shahidi (2015). At first, supercoiled pBR322 plasmid DNA (50 µg/ mL) was dissolved in 0.5 mM phosphate buffer (PBS, pH 7.4) and then mixed with PBS (2 µL), pBR322 DNA (50 µg/ mL, 2 µL), 4 µL of 7 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and phenolic extracts (0.1 mg/ mL, 2 µL) to determine their inhibitory activity against peroxyl radical induced oxidation. Similarly, 2 µL of $FeSO_4$ (0.5 mM), 2 µL of H_2O_2 (0.5 mM), 2 µL phenolic extracts (6 mg/ mL), 2 µL PBS (0.1 M), and 2 µL DNA (50 µg/ mL) were added to produce hydroxyl radical. After incubation at 37°C for 1 h, 1 µL of the loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, and 50% glycerol) was added to the reaction mixture. A control (DNA, PBS, and free radical generating reagents such as H_2O_2, $FeSO_4$, or AAPH) and a blank (DNA and PBS) was prepared for each set of tests. The mixture (10 µL) was then loaded onto 0.7% agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer (40 mM Tris-acetate containing 1 mM EDTA, pH 8.5), followed by the addition of SYBR safe (5 µL) into agarose gel solution (50 mL) as a gel stain. Gel electrophoresis was performed in a horizontal submarine gel electrophoresis apparatus (Owl Separation Systems Inc., Portsmouth, NH, USA) at 80 V for 90 min. The intensity

(area %) of bands was determined using the Alpha-Imager gel documentation system (Cell Biosciences, Santa Clara, CA, USA) to visualize the bands under transillumination of UV light. The retention of supercoiled DNA strand (%) was evaluated using the following equation:

DNA retention (%) =

(Area of supercoiled DNA with oxidative radical and extract/ Area of supercoiled DNA in control) × 100

3.2.5.5. Determination of antiglycation activity

Antiglycation activity of phenolic extracts was evaluated using the bovine serum albumin (BSA) assay described by Wang et al. (2016b) with some modifications. Advanced glycation end products (AGEs) are the results of glycation, which can be determined by examining their fluorescence intensity. For that, 500 µL BSA (2 mg/ mL) was incubated with 400 µL D-glucose (33 mM) and 100 µL phenolic extracts in 0.1 M phosphate buffer (PBS, pH 7) at 37°C for 7 days. PBS and 1 mM aminoguanidine (a typical inhibition agent of AGEs) were used as a control and positive control, respectively. After incubation, 100 µL sample solution was placed in the 96-well plate, and the level of AGEs was recorded by monitoring fluorescence intensity using a microplate reader (Gen5™ Microplate Reader, BioTek Instruments, Winooski, VT, USA). The excitation wavelength was 355/40 nm, whereas the emission wavelength was 405/10 nm. The inhibition percentage (%) was evaluated using the following equation:

$$\% \text{ Inhibition} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

3.2.6. Identification and quantification of phenolics using UHPLC-QTOF-MS/MS

Phenolic compounds in the free, esterified, and insoluble-bound fractions of treated and untreated sea cucumber body wall, flower, and internal organs were identified according to the

method described by Ambigaipalan, de Camargo, and Shahidi (2016) with some modifications. In this study, ultra high-performance liquid chromatography equipped with quadrupole time of fight and mass spectrometer (UHPLC-QTOF-MS/MS) was used to characterize phenolic compounds. The identification and quantification were conducted using an Agilent 1290 UHPLC system equipped with a binary pump (G4220A), an autosampler (G4226A), and a system controller linked to a OpenLab software (Agilent Technologies, Palo Alto, CA, USA). Separation of phenolics was done using Synergi™ Fusion LC-18 column (50 x 2 mm, 4 µm; Torrance, CA, USA), followed by detection using a diode array detector (DAD, G4212A) at 280 nm. The mobile phase was prepared with 0.1% formic acid in water (eluent A) and 0.1% formic acid in methanol (eluent B). The elution gradient was performed at 0 min, 100% A; 5 min, 90% A; 35 min, 85% A; 45 min, 60% A; 50 min, 60% A; and 55-65 min, 100% A, and the flow rate was 0.4 mL/ min. The samples were filtered using a 0.2 µm syringe filter (Thermo Scientific, Rockwood, TN, USA), and the injection volume was 5.0 µL. The phenolic acids and flavonoids were determined at 280 nm.

HPLC-MS/MS analysis was conducted using a triple TOF 5600 system (AB SCIEX, Redwood City, CA, USA) through the electrospray ionization (ESI) ionization source in the negative ion mode. The mass scan range was set from m/z 100 to 2000. The data was acquired and analyzed with PeakView® software (AB SCIEX, Redwood City, CA, USA). The quasi-molecular weights, mass errors, and isotope patterns were obtained through the XIC manager tool in Master View® (AB SCIEX, CA, USA). Phenolic compounds were identified by comparing their retention times and ion fragmentation patterns of authentic standards, including caffeic acid, gallic acid, sinapic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, cinnamic acid, protocatechuic acid, vanillic acid, ferulic acid, syringic acid, ellagic acid, chlorogenic acid, quercetin, and catechin, under the same conditions as the samples. Other compounds with no standards were tentatively identified

using mass spectrometry (MS^n), UV spectral, and literature data. The quantification of phenolic compounds was carried out using their corresponding standard components. For compounds with no standards, quantification was done using their corresponding aglycones. The results of quantification were expressed as mg/ 100g of samples.

3.2.8. Statistical analysis

Statistical analysis was carried out among different HPP treatments (pressure and time), types of phenolics (free, esterified, and insoluble-bound), and sea cucumber body parts (body wall, flower, and internal organs). The data were represented as mean ± standard deviation of triplicate measurements. The significance of differences in phenolic content, antioxidant activity, bioactivity, and individual phenolic content between treatments were analyzed by using one way analysis of variance (ANOVA), and means were compared by Tukey's HSD test ($P < 0.05$) using the IBM SPSS 27.0 for Windows (SPSS Inc., Chicago, IL, USA). Pearson's correlation coefficients were used to assess the linear relationship between phenolic contents and antioxidant assays.

Chapter 4

Results and Discussions

Effect of high-pressure processing (HPP) on the extraction of free, esterified, and insoluble-bound phenolics extracted from Atlantic sea cucumber

4.1. Introduction

Sea cucumber has been fished mainly for food and used locally in traditional medications, especially in the Asian culture. As a result, there has been a growing interest in research to identify biologically active compounds and maximize their yields responsible for therapeutic purposes. In particular, sea cucumber is well known for demonstrating various biological and pharmacological properties, including antioxidant, anticancer, anticoagulant, antithrombotic, and antimicrobial effects (Bordbar et al., 2011). The components responsible for the biological activities have been reported to be glycosaminoglycans, triterpene glycosides (saponins), chondroitin sulfate, fucoidans, proteins (peptides), and other secondary metabolites, including phenolics (Alper & Günes, 2020). However, a limited number of studies have reported on the phenolic compounds of sea cucumber and their antioxidant activity. These studies have been conducted on the total yield of phenolic compounds and their antioxidant activities (Mamelona et al., 2007; Zhong, Khan, & Shahidi, 2007). Moreover, no study has yet been conducted on the individual phenolic profile of sea cucumber, especially Atlantic sea cucumber.

Based on their solubility in the extraction medium, phenolic compounds can be divided into two groups, namely soluble (free and esterified/ etherified) phenolics and insoluble-bound phenolics. Esterified/ etherified phenolics generally occur as simple esters and as phenolic glycosides, while free phenolics are present as phenolic aglycones (Shahidi & Peng, 2018).

Usually, free and esterified phenolics are trapped by weak interaction with other compounds in the vacuole, whereas insoluble-bound phenolics are bound through covalent bonds in the plant cell wall matrices, resulting in low bioaccessibility (Li et al., 2012). Since insoluble-bound phenolics are covalently bound to the insoluble macromolecules, they are not absorbed in the small intestine, ending in the large intestine (colon). In there, they get fermented by several colon microbiotas. In contrast, free and esterified phenolics can be absorbed in the digestive tract; however, their efficacy is dependent on their bioavailability and bioaccessibility in the biological system (Yeo & Shahidi, 2017). Nevertheless, less than 10% of phenolic compounds could get through the small intestinal epithelium into the plasma, and the remaining 90% are directly transferred to the colon (Shahidi & Yeo, 2018). As most of the phenolics are present in food in the form of glycosides, esters, or polymers; thus, they need to be hydrolyzed by intestinal enzymes or catabolized by the colonic microorganisms before absorption (Shahidi et al., 2019). Even free phenolic compounds could be partially blocked by certain plant matrices, including cell walls (Zhou et al., 2019). Therefore, it is important to examine both soluble and insoluble phenolics in order to increase extractability and bioaccessibility.

Extraction of phenolic compounds using high-pressure processing (HPP) has been attracting increased attention as an effective pre-treatment or extraction method. Several studies have stated that HPP increases the content of phenolic compounds and antioxidant activity of plant-based foods. The main advantages of HPP over other extraction methods are less thermal degradation, oxidation, and hydrolysis of bioactive compounds. Especially, HPP is an ideal extraction method for thermo-sensitive compounds (e.g., anthocyanins) since it can be operated at room temperature (Moreira et al., 2020). HPP is also categorized as an environmentally friendly technique and has been applied to extract phenolic acids, flavonoids, anthocyanins, carotenoids,

and ginsenosides, among others, from different foods and food products. For example, da Silveira et al. (2019) found that the HPP treatment (500 MPa for 5 min) increased the total phenolic compounds and antioxidant activity (ORAC value) of açaí (*Euterpe oleracea*) juice and preserved the anthocyanins up to 40% compared to thermal pasteurization (85°C for 1 min). Nowadays, HPP is considered as an effective means to enhance extractability and bioaccessibility of biologically active components like other treatments, including air explosion, fermentation, and enzymatic hydrolysis, due to its ability to change the microstructure or natural matrix of foods (De Ancos et al., 2020). For example, Zhou et al. (2019) stated that the high-pressure pre-treatment improves the bioaccessibility of insoluble-bound phenolics that were extracted from oil palm fruits. Additionally, Rodríguez-Roque et al. (2015) indicated that the bioaccessibility of phenolics increased more than 30% in fruit-juice-based beverages upon HPP compared to untreated samples. This is because HPP may not only improve the extractability of phenolics but also increase the intestinal absorption by modifying the structures of phenolics, mainly those are bound to other macromolecules. Apart from this, HPP can control certain enzymatic reactions (e.g., polyphenol oxidase, PPO) and inhibit the growth of vegetative cells such as yeast and mold (Rastogi et al., 2007). As a result, HPP is considered as an alternative to thermal treatment with minimal impacts on nutritional, functional, and organoleptic properties.

To best of our knowledge, no study has been conducted on free, esterified, and insoluble bound phenolics of any sea cucumber species, including *C. frondosa*. Moreover, there is no report on the extraction of phenolics using HPP from any species of sea cucumber and its commercial body parts. Therefore, the aim of this study was to extract free, esterified, and insoluble bound phenolics from different commercial body parts (body wall, flower, and internal organs) of *C.*

frondosa using HPP as a pre-treatment. Also, to optimize the HPP parameters (pressure and holding time) in extracting phenolic compounds from the Atlantic sea cucumber.

4.2. Effects of high pressure on the total phenolic content (TPC)

The TPC of free, esterified, and insoluble-bound phenolics in HPP-treated sea cucumbers (body wall, flower, and internal organs) was determined using Folin−Ciocalteu's assay; results are shown in Figure 4.1. The selected pressure for the HPP was 2000 (200 MPa), 4000 (400 MPa), and 6000 (600 MPa) bar and had a positive effect on the TPC. With the increasing of HPP pressure, the TPC increased gradually in the free, esterified, and insoluble-bound phenolics, regardless of the sea cucumber body parts. For example, the TPC of free phenolics in the body wall increased from 170.78 to 220.69 mg gallic acid equivalents (GAE)/ 100 g of sample when the pressure was increased from 2000 to 6000 bar for 10 min. Whereas, these values for the flower and internal organs were increased from 178.73 to 241.38 and 175.76 to 227.87 mg GAE/ 100 g, respectively. Similarly, TPC increased by 29.07, 34.36, and 34.68% for body wall, flower, and internal organs in the esterified fraction, respectively, when the pressure was increased from 2000 to 6000 bar for 10 min. The free phenolic fraction was the predominant form present in all body parts examined. The highest amount of TPC was observed in the free phenolic fraction of the body wall treated at 6000 bar for 10 and 15 min. However, no significant difference ($p > 0.05$) was found between 10 and 15 min of HPP. Similar trends were noticed in the esterified and insoluble-bound phenolics of body wall, where 6000 bar demonstrated the highest TPC. Previous studies have reported that the HPP significantly increased the extractability of phenolic compounds compared to untreated samples. For instance, Andrés, Villanueva, and Tenorio, (2016) reported that the HPP (450 and 600 MPa) significantly improved the extractability of phenolics from smoothies during refrigerated storage compared to untreated samples. This could be due to the improvement of

solvent penetration into samples through the disruption of the cell membranes by HPP, which may increase the mass transfer and permeability, and therefore, improving the extraction (Casquete et al., 2014; Zhao, Zhang, & Zhang, 2017). Based on the mass transfer theory (mass transfer = pressure/ resistance), HPP increases the permeability (Shouqin, Jun, & Changzheng, 2005). The increase of phenolics content upon HPP has also been reported by other authors (da Silveira et al., 2019; Patras et al., 2009; Prasad et al., 2010). For example, Altuner et al. (2012) stated that the increase of high pressure could improve the solubility of phenolic compounds according to the phase behavior theory. As a result, the content of phenolics extracted from *Maclura pomifera* fruits was 2.24 times higher at 500 MPa than at 250 MPa. A similar trend was observed in blackberry and strawberry purées, where HPP significantly increased the TPC by 15 and 9.31%, respectively, when the pressure was improved from 400 to 600 MPa (Patras et al., 2009). It has also been proposed that HPP may change the distribution or location of compounds inside the cell and rupture the chemical bonding between phenolics and compounds like carbohydrates or proteins, this facilitates their better extraction (da Silveira et al., 2019). Furthermore, Prasad et al. (2010) demonstrated that the amount of TPC improved notably with increasing of HPP pressure from 200 to 500 MPa, when phenolics were extracted from longan fruit pericarp. This can probably be attributed to the disruption of plant cell membranes and faster diffusion caused by HPP, resulting in availability of more phenolic compounds for extraction up to an equilibrium point.

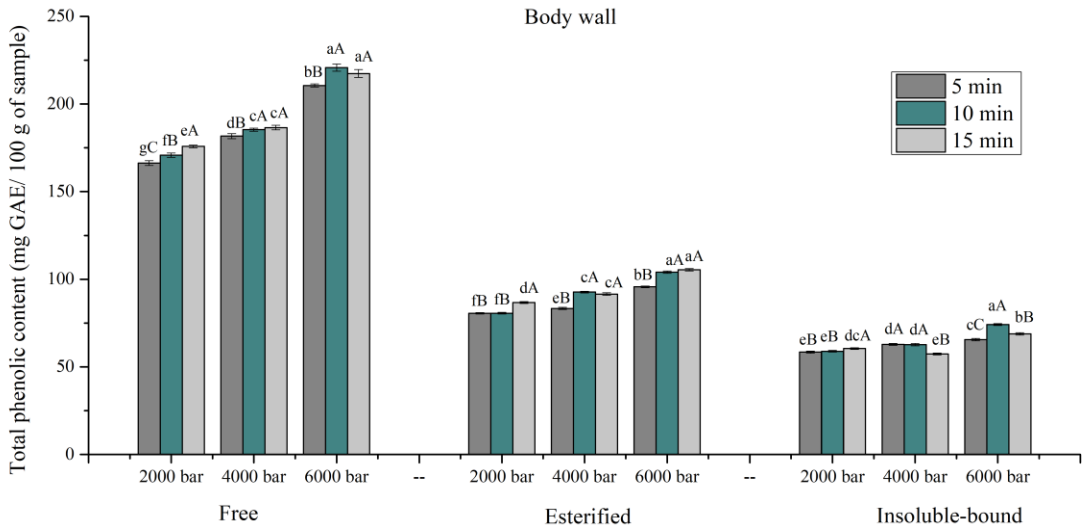
On the other hand, Zhou et al. (2019) reported that high-pressure pre-treatment (500 MPa for 10 min) significantly increased the TPC of free, esterified, and insoluble-bound phenolics of oil palm (*Elaeis guineensis*) fruits, mainly those of insoluble-bound phenolic fraction. A similar trend was observed in our study, where HPP had a positive effect on all the three different phenolic fractions, especially the free phenolic fraction. Most of the insoluble-bound phenolics are bound

to cell wall substances and other macromolecules, resulting in difficult extraction under normal conditions. Therefore, when HPP alter the inner physical structure of sea cucumber, more insoluble-bound phenolics could be released and extracted. However, in our study, HPP had a higher effect on the free phenolic fraction rather than the insoluble-bound phenolic fraction. Possible reasons for this trend could be the very low amount of insoluble-bound phenolics present in sea cucumber compared to the free phenolic fraction. Moreover, HPP improves the extraction of free phenolics as they could be partially blocked by some plant matrices, including cell walls (Zhou et al., 2019). Additionally, Saikaewa et al. (2018) stated that soluble phenolics are more vulnerable under HPP than the bound form as the high pressure has a limited effect on the covalent bonds. Likewise, Yeo and Shahidi (2017) found that the boiling treatment reduces the insoluble-bound phenolics of lentils, and this could be related to the formation of irreversible covalent bonds between liberated phenolics and other molecules like proteins.

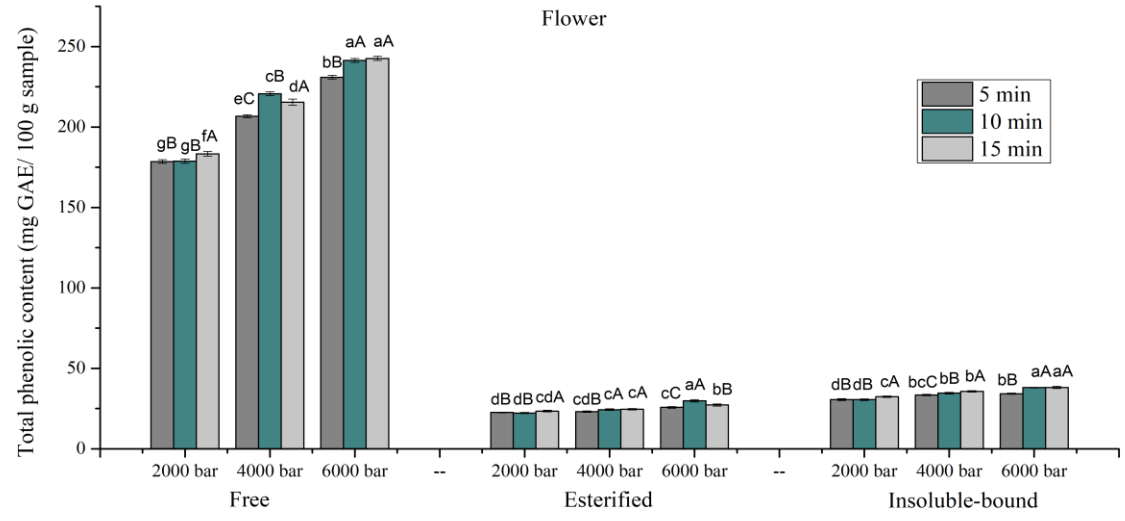
Similar trends were obtained for the flower and internal organs, where HPP pressure significantly ($p<0.05$) increased the content of TPC with increasing of pressure from 2000 to 6000 bar, mainly in the free phenolic fraction. For instance, the TPC of free phenolics increased from 178.73 to 241.38 mg GAE/ 100 g with raising of HPP pressure from 2000 to 6000 bar for 10 min. These results are in agreement with those published by Zuluaga et al. (2016); they reported that TPC increased by up to 36% after HPP at 400 MPa for 15 min. This could be due to the breaking down of the exine wall caused by pressure, leading to a higher extractability of phenolic compounds (Queiroz et al., 2010). However, HPP had a very limited effect on the esterified and insoluble-bound phenolics that were extracted from the flower, especially at 2000 and 4000 bar which afforded almost similar results. Likewise, the TPC of esterified and insoluble-bound phenolics of internal organs were nearly similar under different HPP pressures, mainly those

treated at 2000 and 4000 bar. However, to the best of our knowledge, no study has been done to extract bioactive compounds, including phenolics, using HPP from any species of sea cucumber, mainly *C. frondosa*. So far, only a study has examined the physicochemical properties and shelf-life of sea cucumber cultivated in the Bo Sea (Northern China) using HPP (550 MPa for 20 min). It was found that the HPP improved physicochemical characteristics, extended shelf-life, decreased the total volatile basic nitrogen (TVB-N) content, inactivated enzymes, increased protein contents, and protected mucopolysaccharide of sea cucumber (Xia, Liu, & Li, 2012). Besides, HPP (260 MPa for 3 minutes at room temperature) has been applied in the commercial processing of oysters by Motivatit Seafood Inc. (Houma, LA, USA), but it has not yet been documented for the processing of sea cucumber (Murchie et al., 2005).

[a]


Body wall
Total phenolic content (mg GAE/ 100 g of sample)
250
200
150
100
50
0
5 min
10 min
15 min
gC fB eA
dB cA cA
bB aA aA
fB fB dA
eB cA cA
bB aA aA
eB eB dcA
dA dA eB
cC aA bB
2000 bar
4000 bar
6000 bar
--
Free
Esterified
Insoluble-bound

[b]


Flower
Total phenolic content (mg GAE/ 100 g sample)
250
200
150
100
50
0
5 min
10 min
15 min
gB gB fA
eC cB dA
bB aA aA
dB dB cdA
cdB cA cA
cC aA bB
dB dB cA
bcC bB bA
bB aA aA
2000 bar
4000 bar
6000 bar
--
Free
Esterified
Insoluble-bound

[c]

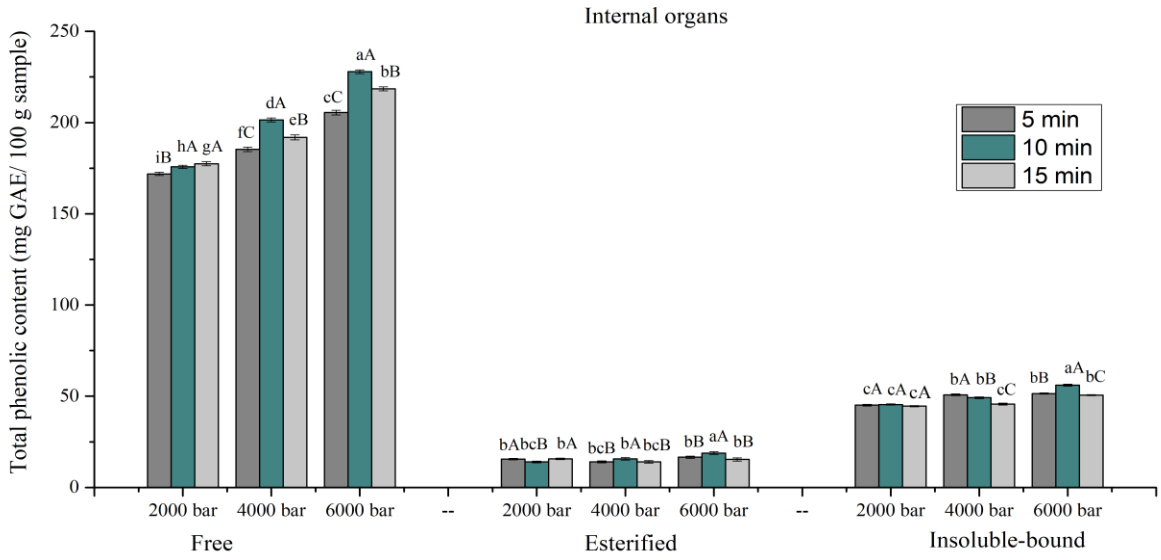


Figure 4. 1. Total phenolic content of HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg gallic acid equivalents (GAE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among HPP treatments. Different uppercase letters for the same pressure indicate significant differences ($p < 0.05$) among holding times.

4.3. Effects of pressure holding time on the total phenolic content (TPC)

The selected time for the HPP was 5, 10, and 15 min, and the results indicate that the TPC was influenced not only by the applied pressure but also by HPP holding time. An increase in time of HPP from 5 to 15 min along with the pressure from 2000 to 4000 bar resulted in an increase in TPC. For example, the TPC of body wall in the free fraction increased gradually with the increasing of time along with the pressure and then remained constant between 10 and 15 min at 6000 bar. Similar trends were detected for the esterified phenolic fraction in the body wall and free and insoluble-bound phenolic fractions in flower. At lower pressure levels, increasing HPP time led to lower TPC, whereas the opposite scenario was found at higher pressure levels. Similar results were found when HPP (200-400 MPa for 5-15 min) was applied to bee-pollen-based beverages, where TPC increased with treatment time and pressure, especially a longer period of holding time (Zuluaga et al., 2016). However, in our study, no significant difference was found between 10 and 15 min of holding time for the free and esterified phenolic fractions in the body wall and free and insoluble-bound fractions in flower at 6000 bar. Moreover, HPP holding time had very little effect on the TPC in 2000 and 4000 bar, regardless of the sea cucumber body parts. This could be due to the equilibrium of extraction was reached between 10 and 15 min of HPP, especially at the 6000 bar. Prasad et al. (2010) found that the HPP holding time (2.5 to 30 min) did not affect the extraction yield and TPC of longan fruit pericarp. Furthermore, based on the Pascal theory, pressure transfers instantly and uniformly throughout the whole material during the high-pressure treatment. Thus, pressure could easily reach to outside and inside of the cells in a very quick time, resulting in an equilibrium of extraction (Jun, 2013; Prasad et al., 2010).

However, the content of TPC decreased at 6000 bar for 15 min in the insoluble bound phenolic fraction of body wall, esterified fraction of flower, and all the three fractions of internal

organs (Figure 4.1). This decrease in TPC may be partially related to the oxidation of phenolics due to a longer holding time at high pressure. The loss of TPC could be associated with the oxidation of enzymes (polyphenol oxidase, PPO and peroxidase, POD), condensation reactions through covalent association with other phenolics, ionization, and polymerization of phenolics with proteins by HPP (Andrés et al., 2016; Saikaew et al., 2018). For example, Casquete et al. (2014) reported that the TPC of citrus peels decreased from 3 to 10 min of HPP time at 500 MPa, and which could be partly attributed to the enzymatic oxidation. Generally, HPP deactivates the activity of PPO and POD; however, these enzymes may partially be active under a long holding time with high pressure. It has been documented that the HPP (600 MPa for 10 min) deactivated 50% of the POD action in avocado slices (Woolf et al., 2013). This is because during HPP treatment, bioactive compounds are released due to the breakdown of cell walls and became more vulnerable to chemical and biochemical reactions under longer holding time than the bound form, favoring enzymatic degradation (da Silveira et al., 2019; Jesus et al., 2018; Saikaew et al., 2018).

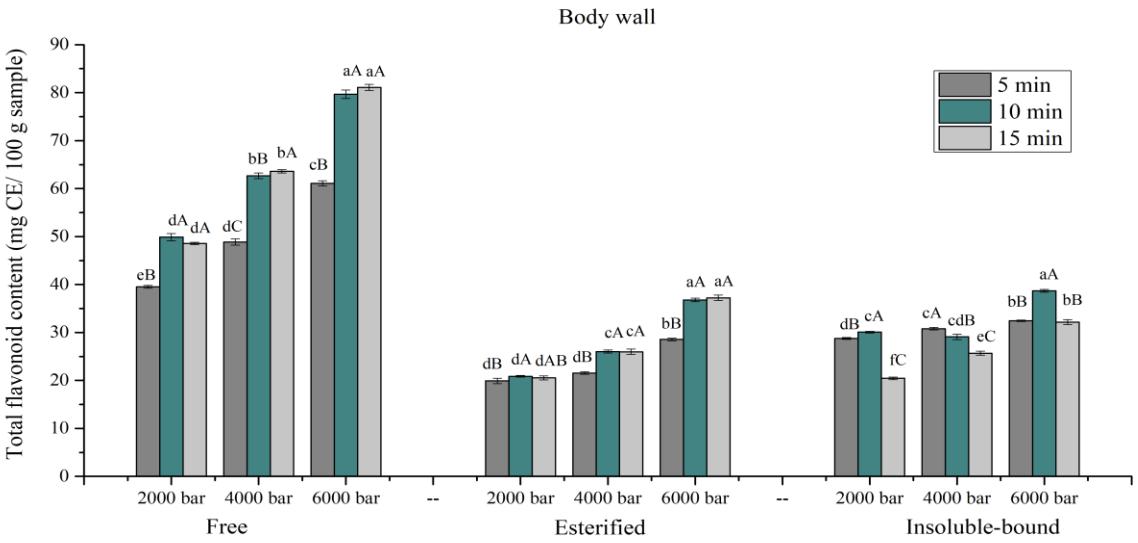
4.4. Effects of high pressure on the total flavonoid content (TFC)

The content of total flavonoid was determined in mg catechin equivalents (CE)/ 100 g, and the influence of HPP on changes in TFC is presented in Figure 4.2. It was found that the TFC increased with the increasing of HPP pressure, regardless of sea cucumber body parts and the types of phenolics, except for the esterified and insoluble-bound phenolics, where no significant difference was obtained between 2000 and 4000 bar in the internal organs. For example, the TFC of free phenolics in the body wall increased from 49.87 to 79.67 mg CE/ 100 g when the pressure was increased from 2000 to 6000 bar for 10 min. Whereas, these values for the flower and internal organs were 76.43.73 to 129.07 mg CE/ 100 g and 58.56.56 to 98.7 mg CE/ 100 g, respectively. Similarly, 28.66, 28.77, and 14.37% of TFC increased for body wall, flower, and internal organs

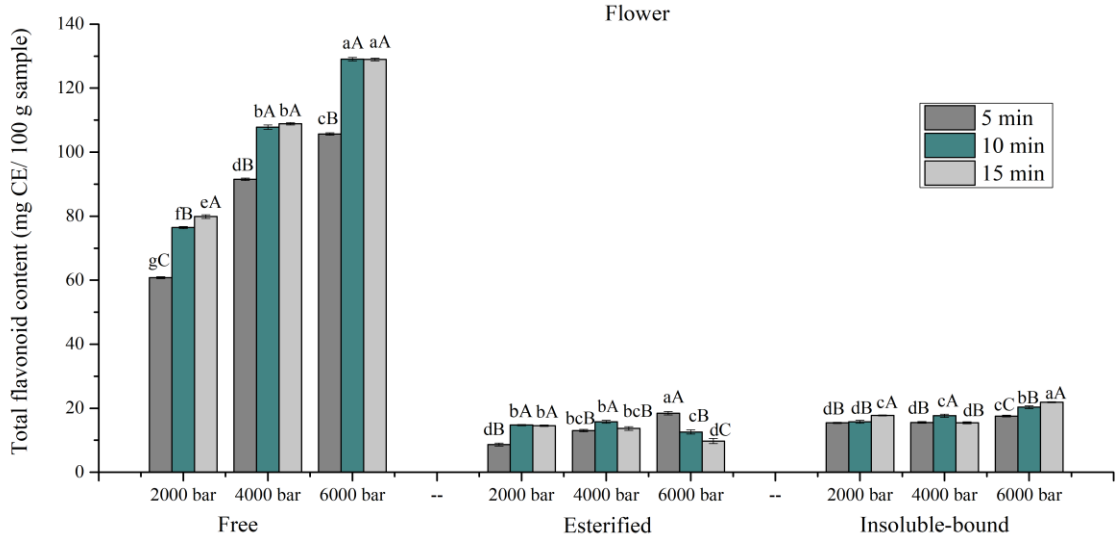
in the insoluble-bound fractions, respectively, when the pressure was increased from 2000 to 6000 bar for 10 min. The increase of TFC may be related to the breakdown of the cell wall structure of sea cucumber, and the inactivation of enzymes (e.g., PPO) related to the loss of phenolic substances due to high pressure (Rodríguez-Roque et al., 2015). Zhou et al. (2019) claimed that HPP pre-treatment (500 MPa for 10 min) significantly improved the TFC of all three different phenolic fractions of oil palm fruits, mainly those of insoluble-bound phenolics. Moreover, Albertos et al. (2016) found that vacuum fried carrot snacks pretreated with HPP (100 MPa for 2 min) had a significantly higher level of phenolics. This could be due to the disruption of tissue by HPP, favoring the release of phenolics during extraction.

However, HPP had a very limited effect on the TFC of esterified and insoluble-bound phenolics extracted from flower and internal organs, especially 2000 and 4000 bar provided almost similar results. Particularly, no significant difference was obtained between the 2000 and 4000 bar for esterified and insoluble-bound phenolics of internal organs, regardless of the holding time. This could be due to the minimal effect of low pressure (e.g., 2000 bar) on the bound phenolics. Our findings were in good agreement with those of De Ancos et al. (2020); they found a similar amount of TFC in fruit juice (Cara Cara) from different HPP pressures (200 and 400 MPa). A similar trend was obtained for tomato purée, where HPP (450, 550, and 650 MPa) did not affect the phenolics, regardless of holding time (Jeż et al., 2018). On the other hand, in our study, the highest amount of TFC was observed in flower in the free phenolic fraction, whereas esterified and insoluble-bound fractions were more abundant in the body wall.

[a]


Body wall
Total flavonoid content (mg CE/ 100 g sample)
5 min
10 min
15 min
2000 bar
4000 bar
6000 bar
Free
Esterified
Insoluble-bound

[b]


Flower
Total flavonoid content (mg CE/ 100 g sample)
5 min
10 min
15 min
2000 bar
4000 bar
6000 bar
Free
Esterified
Insoluble-bound

[c]

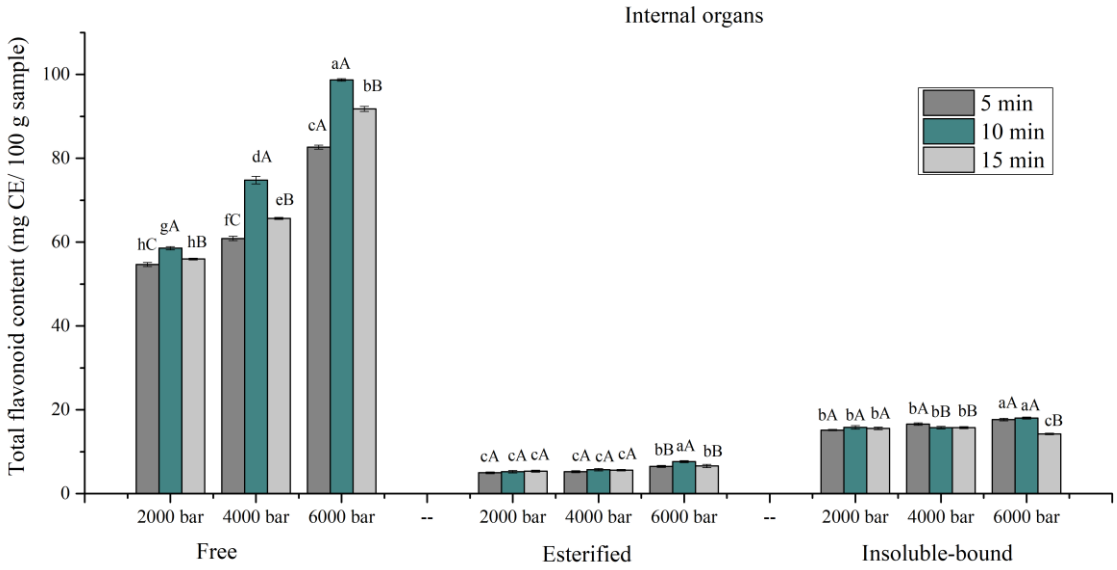


Figure 4. 2. Total flavonoid content of HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg catechin equivalents (CE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among HPP treatments. Different uppercase letters for the same pressure indicate significant differences ($p < 0.05$) among holding times.

4.5. Effects of pressure holding time on the total flavonoid content (TFC)

The selected time for the HPP was 5, 10, and 15 min, and the results indicate that the TFC was influenced not only by the applied pressure but also by HPP time (Figure 4.2). Our results showed that the TFC of the sea cucumber increased from 5 to 15 min along with the pressure, except insoluble-bound phenolics of body wall, where 15 min did not preserve the TFC. Moreover, no significant difference was found between 10 and 15 min of holding time for 6000 bar in free and esterified fractions of body wall. This could be due to the equilibrium of extraction was achieved between 10 and 15 min of HPP, especially at the 6000 bar. This trend gains support from the findings of Jeż et al. (2018); they reported that the HPP holding time (5, 10, and 15 min) did not change the content of phenolics of tomato purées. At lower pressure levels, increasing HPP time led to lower TFC contents, whereas the opposite trend was found at higher pressure levels. On the other hand, long holding time decreased the level of TFC for the internal organs. For instance, HPP holding time 15 min significantly reduced the TFC of free phenolics, regardless of HPP pressure. Similar trends were detected for the esterified and insoluble-bound phenolics, where longer HPP time decreased the TFC for 6000 bar. Similar results were observed by Saikaew et al. (2018), and they reported reduced TFC of waxy corn kernels at the longer HPP holding time. This loss of TFC could be partly related to the oxidation of phenolics due to a longer holding time (e.g., 15 min) at high pressure (e.g., 6000 bar). Generally, HPP deactivates the activity of PPO and POD; however, at high pressure and longer holding time, these enzymes may partially be active, resulting in oxidation (De Ancos et al., 2020; Queiroz et al., 2010; Zhao et al., 2016). Terefe et al. (2013) concluded that HPP (600 MPa for 5 min) decreased the phenolics of strawberry puree due to the enzymatic oxidation. Additionally, it has been reported that the HPP (600 MPa for 30 min)

activated the activity of PPO and POD of sweet potatoes but partially inhibited them in cocoyam and Peruvian carrot (Tribst et al., 2016).

4.6. Summary

HPP pressure and holding time had a significant effect on the TPC and TFC of all three different phenolic fractions, especially those were free phenolics. Particularly, 6000 bar showed significantly higher phenolics than the 2000 and 4000 bar, regardless of holding time. Similarly, the increasing trends were observed for the HPP holding time, mainly those were treated with 10 and 15 min at 6000 bar. Moreover, free phenolics were the most dominant phenolic fraction than the esterified and insoluble-bound phenolics, regardless of the sea cucumber body parts. Flower had the highest TPC and TFC in the free fraction, whereas esterified and insoluble-bound fractions were more abundant in the body wall. Thus, the results suggest that the HPP, mainly 6000 bar for 10 min, can be used as an effective approach for the extraction of phenolic compounds from different body parts of sea cucumber.

Chapter 5

Effect of high-pressure processing (HPP) on the antioxidant activity of sea cucumber phenolics

5.1. Introduction

Global demand for sea cucumber has been increasing rapidly due to its high nutritional profile and therapeutic effects. Therefore, there has been an increasing interest in research to identify the responsible compounds from sea cucumber, including the processing by-products, that show biological properties. Bioactive compounds such as chondroitin sulfate, saponins, and phenolics extracted from sea cucumber have been reported to have anticancer, antitumor, anti-inflammatory, antimicrobial, and antioxidant activities (Esmat et al., 2013). For instance, Pranweerapaiboon et al. (2020) found that the phenolic-rich ethyl acetate fraction of sea cucumber (*H. scabra*) extracts inhibited the synthesis of the pro-inflammatory cytokines, mainly inducible nitric oxide synthase (iNOS), nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and prostaglandin E_2 (PGE_2). Similarly, bioactive compounds (phenols, terpenoids, and saponins) were extracted from sea cucumber (*H. atra*) using methanol and it was found that the extracts had a higher antibacterial activity against *P. aeruginosa* (Sukmiwati et al., 2019). Recently, phenolics have received much attention owing to their role as antioxidants and scavengers of free radicals as well as reactive oxygen species (ROS). Sea cucumber (e.g., *C. frondosa*) phenolics, mainly flavonoids, have been demonstrated to possess antioxidant properties. These properties are mainly dependent on the body part of sea cucumber as well as the methods/ solvents used for their extraction (Husni et al., 2009). For example, methanolic extract of whole sea cucumber (*H. scabra*) demonstrated the highest DPPH radical scavenging activity (Nobsathian et al., 2017). Moreover, Hawa et al. (1999) concluded that sea cucumbers could be a potential

source of antioxidants in the future as they contain a wide range of antioxidant substances. Marine invertebrates, mainly sea cucumbers, are shielded against oxidative stress triggered by the exposure of UV radiation and deleterious ROS. This could be due to the presence of the endogenous metabolites and/or antioxidants in their soft skins (Dunlap, Shick, & Yamamoto, 1999; Safari & Yaghoubzadeh, 2020; Zhong et al., 2007). Therefore, sea cucumbers could act as a possible source of antioxidants. However, a limited number of studies have been reported on the antioxidant activity of phenolics obtained from sea cucumbers and their commercial body parts.

Recently, the effects of HPP on the retention of phenolics and antioxidant property of fruits and vegetables and their products have received much attention from researchers. It has been reported that the HPP could improve the bioaccessibility of phenolics and total antioxidant activity of food products (Cilla et al., 2012; da Silveira et al., 2019). This is because most phenolics (e.g., anthocyanins) are vulnerable under traditional thermal treatments, while HPP is a non-thermal process that can act as a substitute for heat-based processing methods. As a result, HPP has been applied to numerous commercial products on the premium food market, mainly fruit juices, vegetables, seafood, dairy products, and cooked meats, among others (Rastogi et al., 2007). Most published data have suggested that HPP preserves TPC and antioxidant property of fruit and vegetable products. However, HPP may negatively affect the antioxidant activity under certain conditions due to the oxidation of some phenolics at high pressure. Therefore, the objective of this study was to evaluate the antioxidant activity of different body parts of sea cucumber (*C. frondosa*) phenolics using HPP. To the best of our knowledge, this is the first study that focuses on the effect of HPP on the antioxidant property of any species of sea cucumber, especially Atlantic sea cucumber.

5.2. Effects of HPP on the DPPH radical scavenging capacity

The DPPH radical scavenging assay is often used to determine the antioxidant activity of phenolic compounds. The DPPH radical is a stable organic radical, which can be neutralized by antioxidants via electron and/or hydrogen atom transfer. The purple/ deep violet color of the DPPH radical becomes colorless/ pale yellow upon the reduction of its radical (Nwachukwu et al., 2021; Yeo & Shahidi, 2019). The efficacy of antioxidant capacity can be assessed by using electron paramagnetic resonance (EPR) spectroscopy or UV-Vis spectrometry at 517 nm.

In this study, we measured the DPPH radical scavenging activity in Trolox equivalents (TE). Trolox is a water-soluble analog of α-tocopherol which was used to determine the radical scavenging capacity of free, esterified, and insoluble-bound phenolics as presented in Table 5.1. When the pressure in HPP increased from 2000 to 6000 bars, the DPPH radical scavenging activity of flower and internal organs also increased, regardless of the type of phenolics. For example, the DPPH radical scavenging activity of free phenolic fraction in flower increased from 505.71 to 553.89 mg TE/ 100 g when the HPP pressure was raised from 2000 to 6000 bars for 10 min. In comparison, these values for the internal organs increased from 330.79 to 346.48 mg TE/ 100 g. Similar to the free phenolics, 13.5 and 8.8% of DPPH radical scavenging activity increased for flower and internal organs in the esterified fraction, respectively, when the pressure was increased from 2000 to 6000 bars for 10 min. The highest amount of activity was observed in the free phenolic fraction of flower among the three different fractions at 6000 bar for 10 min. Previous studies have reported that the HPP significantly increased the extractability of phenolic compounds and antioxidant activity due to the low temperature of HPP could prevent the oxidation of antioxidants. For instance, da Silveira et al. (2019) stated that the HPP increased the ORAC values from 632.42 to 841.66 μmol TE/ g when the pressure was increased from 450 to 500 MPa in açaí

juice. Similar results were reported by Paciulli et al. (2019) for blueberries treated at 600 MPa for 5 min. They showed that DPPH activity was higher than those in samples processed at 400 MPa for 1 min. The significantly higher DPPH radical scavenging activity in HPP-treated samples could be related to the higher extractability of antioxidant components (e.g., phenolics) due to high pressure. The antioxidant activity of phenolics is closely related to the type of phenolic components present in food and their synergistic effects (Chandrasekara & Shahidi, 2012a). Therefore, the increase of the DPPH radical scavenging activity of sea cucumber phenolics could be explained by the improvement of certain phenolic compounds by HPP. On the other hand, no significant ($p > 0.05$) difference was found in terms of DPPH radical scavenging activity among the HPP pressures for the body wall, regardless of holding time. Similar results were found by Queiroz et al. (2010) and Albertos et al. (2016), they reported that HPP did not change any antioxidant activity, regardless of HPP holding time. This could be related to the Folin-Ciocalteau method, which may measure other substances, such as proteins and reducing sugars, when determining total phenolic content. As a result, we could see the higher TPC upon HPP, but it may not increase the antioxidant activity (Moreira et al., 2020).

The selected time for the HPP was 5, 10, and 15 min, and the results indicated that the DPPH radical scavenging activity was also influenced by HPP holding time in addition to applied pressure. An increase in the time of HPP from 5 to 10 min for 6000 bar resulted in an increase in DPPH radical scavenging activity, followed by a decrease at 15 min in the free phenolic fraction of flower and internal organs. For instance, at 6000 bar, the DPPH radical scavenging activity of free phenolics in the flower was 515.78, 553.89, and 549.67 mg TE/ 100 g for 5, 10, and 15 min, respectively. Similar trends were obtained for esterified and insoluble-bound phenolics, where 6000 bar for 10 min offered the highest activity for flower and internal organs. However, HPP

holding time had a minimal effect on 2000 and 4000 bars. Therefore, we can conclude that the high pressure (e.g., 6000 bar) and long holding time (e.g., 10 min) preserve the DPPH radical scavenging activity of sea cucumber phenolics. Our results are in agreement with the findings of Paciulli et al. (2019), who reported that 600 MPa for 5 min had the highest DPPH radical scavenging activity compared to 600 MPa for 1 min, 400 MPa for 5 min, and 400 MPa for 1 min. The retention of the antioxidant property with the increase of holding time could be related to the better extraction of phenolics by HPP. However, the reduction of antioxidant activity at the longer holding time (e.g., 15 min) could be linked to poor inactivation of the enzymes (e.g., PPO and POD) responsible for the loss of phenolic compounds (Cao et al., 2010; Ferrari, Maresca, & Ciccarone, 2011; Rodríguez-Roque et al., 2015). On the other hand, HPP holding time did not have any effect on the antioxidant activity of body wall extracts, regardless of HPP pressure.

Table 5. 1. Effect of HPP on the DPPH radical scavenging activity (mg Trolox equivalents/ 100 g) of different sea cucumber body parts

Phenolics	Body part	Pressure (bar)	Time (min)		
			5	10	15
Free	Body wall	2000	235.65±1.35^{aA}	236.56±1.2^{aA}	236.67±0.8^{aA}
		4000	235.98±1.68^{aA}	237.56±0.9^{aA}	233.48±1.36^{aA}
		6000	236.54±0.92^{aA}	237.22±1.05^{aA}	235.57±1.25^{aA}
	Flower	2000	506.78±1.15^{aB}	505.71±1.35^{aC}	507.56±1.55^{aB}
		4000	507.45±0.94^{cB}	510.23±1.22^{aB}	510.89±1.85^{aB}
		6000	515.78±1.1^{cA}	553.89±1.25^{aA}	549.67±1.3^{bA}
	Internal organs	2000	330.98±0.9^{aC}	330.79±0.96^{aC}	326.45±1.16^{bA}
		4000	333.56±1.26^{aB}	333.67±1.18^{aB}	325.79±1.4^{bA}
		6000	336.67±1.34^{bA}	346.48±1.05^{aA}	310.76±1.12^{cB}
Esterified	Body wall	2000	170.98±0.32^{aA}	170.87±0.48^{aA}	171.67±0.35^{aA}
		4000	170.78±0.65^{aA}	171.56±0.46^{aA}	170.38±0.8^{aA}
		6000	171.78±0.5^{aA}	171.87±0.72^{aA}	172.49±0.65^{aA}
	Flower	2000	47.87±0.32^{bB}	49.89±0.38^{aB}	46.98±0.54^{bB}
		4000	48.65±0.45^{bB}	49.59±0.56^{aB}	46.67±0.48^{cB}
		6000	50.54±0.52^{bA}	54.65±0.75^{aA}	50.69±0.68^{bA}
	Internal organs	2000	39.78±0.45^{bA}	39.67±0.48^{bB}	40.86±0.45^{aB}
		4000	39.54±0.58^{bA}	40.69±0.66^{bB}	40.99±0.68^{bB}
		6000	40.56±0.65^{bA}	43.72±0.75^{aA}	42.65±0.87^{aA}
Insoluble-bound	Body wall	2000	91.65±0.58^{aA}	92.94±0.4^{aA}	92.31±0.45^{aA}
		4000	92.86±0.55^{aA}	94.09±0.63^{aA}	93.56±0.38^{aA}
		6000	93.57±0.68^{aA}	94.91±0.54^{aA}	92.87±0.65^{bA}
	Flower	2000	52.19±0.6^{aB}	51.67±0.52^{aC}	50.09±0.44^{bA}
		4000	51.98±0.42^{bB}	53.87±0.58^{aB}	51.56±0.45^{bA}
		6000	54.76±0.38^{bA}	58.65±0.25^{aA}	50.78±0.68^{cA}
	Internal organs	2000	66.18±0.45^{aB}	66.87±0.35^{aC}	67.49±0.38^{aB}
		4000	67.9±0.48^{bB}	69.59±0.48^{aB}	67.67±0.55^{bB}
		6000	69.12±0.32^{cA}	72.76±0.58^{aA}	71.67±0.25^{aA}

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among treatments. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) for each body part.

5.3. Effects of HPP on the ABTS radical scavenging ability

The ABTS radical scavenging test was proposed by Miller et al. (1993) and then modified by Re et al. (1999) and van den Berg et al. (2000). The ABTS radical scavenging activity assesses the ability of antioxidants to scavenge the stable radical cation ABTS•+, and the absorbance of the blue-green color can be measure at 734 nm. This assay works based on the hydrogen atom transfer (HAT) as well as single electron transfer (SET) mechanisms. Therefore, the ABTS•+ can be neutralized by antioxidants (e.g., phenolic compounds) as they can react with radical cations and terminate the radical chain reaction (Ambigaipalan et al., 2015; Shahidi & Zhong, 2015).

The ABTS radical scavenging activity of phenolics extracted from different body parts of sea cucumber, as Trolox equivalents (TE), is shown in Table 5.2. The HPP pressure on sea cucumber induced irregular changes in the ABTS radical scavenging activity of the analyzed extracts. The HPP pressure increased the ABTS radical scavenging activity of body wall slightly, regardless of the type of phenolics. For example, the ABTS radical scavenging activity increased from 465.98 to 470.14 mg TE/ 100 g in the body wall when the pressure was increased from 4000 to 6000 bars. However, at 2000 and 4000 bars, HPP had almost no effect on the antioxidant activity, regardless of type of phenolics. The increasing trend of antioxidant activity could be related to enhanced extractability of phenolics by HPP. Patras et al. (2009) stated that strawberry and blackberry purées treated at 600 MPa had a significantly higher antioxidant activity than the samples treated at 400 MPa. Similarly, Fernández Garcia, Butz, and Tauscher (2000) found that treatment of HPP at 600 MPa slightly increased the antioxidant activity (TEAC value) of apple juice. On the other hand, in our study, HPP pressure had no effect on free and esterified phenolics of flower and internal organs, whereas an increasing trend was found for insoluble-bound phenolics for the same body parts. Previous studies have also reported that HPP either improves

or does not impact the antioxidant property of treated foods. For example, Jeż et al. (2018) indicated that the DPPH radical scavenging activity of tomato purée was unaffected by different HPP pressures (450-650 MPa). Additionally, Zhou et al. (2019) claimed that the insoluble-bound phenolic fraction of oil palm fruits displayed the highest ABTS radical scavenging activity, followed by the esterified fraction.

In this study, the selected HPP holding time was 5, 10, and 15 min, and it was observed that time had nearly no effect on the ABTS radical scavenging activity, except the insoluble-bound phenolics of flower and internal organs, where 4000 and 6000 bars for 10 min provided the higher ($p< 0.05$) activity. For example, the ABTS radical scavenging activity was 91.57, 94.76, and 90.65 mg TE/ 100 g for 5, 10, and 15 min, respectively, in the insoluble-bound phenolic of internal organs treated at 6000 bar. The minimal effect of HPP holding time could be associated with the isostatic and quasi-instantaneous manner (i.e., pressure transfers instantly and uniformly) of pressure, which makes the holding time independent, regardless of sample shape or size (Pinela et al., 2018). For instance, Inada et al. (2017) discovered that HPP holding time (5-10 min) did not affect the antioxidant activity (FRAP, ORAC, and TEAC assays) of jabuticaba (*Myrciaria jaboticaba*) juice.

Table 5. 2. Effect of HPP on the ABTS radical scavenging activity (mg Trolox equivalents/ 100 g) of different sea cucumber body parts

Phenolics	Body part	Pressure (bar)	Time (min)		
			5	10	15
Free	Body wall	2000	465.34±1.35^{aC}	465.98±1.2^{aB}	466.76±0.8^{aB}
		4000	468.56±1.4^{aB}	470.54±0.9^{aA}	466.56±1.36^{bB}
		6000	471.45±0.9^{aA}	470.14±2.05^{aA}	470.45±1.25^{aA}
	Flower	2000	786.51±1.16^{aA}	786.42±1.35^{aA}	787.39±1.46^{aA}
		4000	786.76±0.9^{aA}	787.28±1.24^{aA}	786.45±1.85^{aA}
		6000	787.53±1.1^{aA}	787.45±3.25^{aA}	788.32±1.32^{aA}
	Internal organs	2000	563.67±0.9^{aA}	562.65±0.92^{aA}	563.21±1.15^{aA}
		4000	563.95±1.24^{aA}	563.65±1.15^{aA}	563.67±1.4^{aA}
		6000	564.32±1.48^{aA}	563.43±2.08^{aA}	558.43±1.14^{bB}
Esterified	Body wall	2000	160.05±0.32^{cB}	160.52±0.48^{bB}	161.46±0.35^{aB}
		4000	160.09±0.65^{bB}	162.34±0.45^{aA}	160.48±0.8^{bB}
		6000	162.38±0.5^{aA}	162.56±0.72^{aA}	163.76±0.65^{aA}
	Flower	2000	70.68±0.22^{bA}	71.43±0.38^{aA}	70.59±0.5^{aB}
		4000	70.68±0.45^{bA}	72.29±0.56^{aA}	71.89±0.48^{aA}
		6000	70.32±0.52^{bA}	72.56±1.75^{aA}	70.65±0.68^{aA}
	Internal organs	2000	74.38±0.44^{aB}	74.67±0.48^{aB}	75.07±0.45^{aA}
		4000	75.54±0.56^{bA}	76.87±0.68^{aA}	75.09±0.68^{bA}
		6000	75.43±0.65^{aAB}	76.54±0.75^{aA}	74.67±0.87^{bA}
Insoluble-bound	Body wall	2000	117.48±0.58^{aB}	117.05±0.4^{aB}	118.53±0.45^{aB}
		4000	119.43±0.54^{aA}	118.54±0.63^{aA}	117.54±0.38^{bB}
		6000	118.65±0.68^{aA}	118.65±0.58^{aA}	119.64±0.45^{aA}
	Flower	2000	73.11±0.3^{aB}	72.65±0.55^{aB}	72.07±0.42^{bB}
		4000	73.67±0.42^{bA}	75.54±0.58^{aA}	73.98±0.45^{bA}
		6000	74.06±0.38^{bA}	76.09±0.25^{aA}	73.67±0.68^{bA}
	Internal organs	2000	89.48±0.45^{aB}	89.32±0.35^{aB}	88.54±0.38^{bA}
		4000	90.39±0.48^{bB}	95.03±0.46^{aA}	89.67±0.55^{bA}
		6000	91.57±0.32^{bA}	94.76±0.58^{aA}	90.65±0.25^{bA}

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among treatments. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) for each body part.

5.4. Effects of HPP on the hydroxyl radical scavenging activity

The hydroxyl radical is considered the most reactive free radical and powerful oxidant that can be generated during lipid oxidation when hydroperoxide breaks down to alkoxyl radical. This radical can also be produced in the body via Fenton reaction in the mitochondria. Hydroxyl radicals are mainly found in biological systems and are engaged in the metabolism of cells by behaving as a signaling molecule. However, the excessive development of hydroxyl radicals may cause an imbalance between free radicals and antioxidants, which could stimulate cellular damage through oxidative stress. Therefore, preventing these excessive levels of hydroxyl radicals could inhibit the occurrence of various cellular disorders, including cardiovascular diseases, diabetes, and cancer. For example, supplying applicable concentrations of antioxidants (e.g., phenolics) through the diet could decrease the oxidative stress in the body cells (Shahidi & Yeo, 2020; Xie et al., 2019; You et al., 2010). The hydroxyl radical scavenging activity of antioxidants can be measured using EPR with the trapping of the spin adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Liang & Kitts, 2014).

In this study, the hydroxyl radical scavenging activity was determined in Trolox equivalents (TE), and the results are presented in Table 5.3. No statistically significant ($p > 0.05$) difference in hydroxyl radical scavenging activity was observed in the body wall irrespective of the HPP pressure, although a slight increase in the activity was found in all three phenolic fractions. This trend is in agreement with the finding of Tsikrika and Rai (2019), who reported no changes ($p > 0.05$) in the antioxidant activity (DPPH radical scavenging activity and FRAP) of Irish potato using HPP (6000 bar for 3 min), though a minor increase was noted in most of the HPP treated samples. This could be due to a combined impact of several components, acting either antagonistically or synergistically. On the other hand, the hydroxyl radical scavenging activity of

the flower increased significantly ($p < 0.05$) with increasing pressure from 2000 to 4000 bars, regardless of the type of phenolics. For instance, the hydroxyl radical scavenging activity was 1520.87, 1530.56, and 1541.32 mg TE/ 100 g for 2000, 4000, and 6000 bars, respectively, in the free fraction of flower. This can be attributed to the higher extraction of phenolic compounds during HPP. These results also agree with those found in longan fruit pericarp, where antioxidant activity (superoxide and DPPH radical scavenging activity) increased after HPP (200-500 MPa for 2.5-30 min) (Prasad et al., 2010). However, the HPP pressure induced irregular changes in the hydroxyl radical scavenging activity of the analyzed internal organs extracts. HPP pressure did not change the antioxidant activity of internal organs in the free fraction, whereas an increase in the insoluble-bound fraction was observed.

The HPP holding time had a significant effect on the hydroxyl radical scavenging activity of sea cucumber. HPP holding time did not change the antioxidant activity of body wall in all three phenolic fractions, regardless of HPP pressure. Jeż et al. (2018) stated HPP treatment (450-650 MPa for 5-15 min) did not change the FRAP value of tomato purée. On the other hand, the hydroxyl radical scavenging activity increased when the time was increased from 5 to 10 min for flower and decreased at 15 min. This could be due to the equilibrium of extraction reached at 10 min, or oxidation of phenolics occurring at a longer holding time. Saikaew et al. (2018) observed that the longer HPP holding time significantly decreased the antioxidant activities in the FRAP and ORAC assays of waxy corn extract. Moreover, Bisconsin-Junior et al. (2015) claimed that HPP treatment reduced antioxidant activity in orange juice, where time was the most vital factor influencing the reduction of antioxidant activity. The possible reason of reduction could be the presence of residual oxygen, which can oxidize certain phenolic compounds (Andrés et al., 2016).

Table 5. 3. Effect of HPP on the hydroxyl radical scavenging activity (mg Trolox equivalents/ 100 g) of different sea cucumber body parts

Phenolics	Body part	Pressure (bar)	Time (min)		
			5	10	15
Free	Body wall	2000	436.67±1.35^{aA}	436.39±1.2^{aA}	437.54±0.8^{aA}
		4000	435.56±1.4^{aA}	438.35±0.9^{aA}	436.28±1.35^{aA}
		6000	436.98±0.92^{aA}	438.68±1.55^{aA}	435.56±1.58^{aA}
	Flower	2000	1520.12±1.15^{aC}	1520.87±1.36^{aC}	1521.98±1.45^{aC}
		4000	1527.67±0.9^{bB}	1530.56±1.22^{aB}	1528.67±1.85^{abB}
		6000	1530.76±1.1^{bA}	1541.32±1.25^{aA}	1532.67±1.3^{bA}
	Internal organs	2000	596.69±0.94^{aA}	597.76±0.9^{aA}	597.67±1.15^{aA}
		4000	597.45±1.26^{aA}	596.78±1.16^{aA}	597.67±1.4^{aA}
		6000	596.67±1.35^{aA}	598.33±1.06^{aA}	598.99±1.1^{aA}
Esterified	Body wall	2000	406.12±0.32^{aA}	406.58±0.48^{aA}	407.56±0.35^{aA}
		4000	407.89±0.65^{aA}	407.89±0.45^{aA}	407.56±0.8^{aA}
		6000	406.78±0.5^{aA}	407.11±0.72^{aA}	407.67±0.65^{aA}
	Flower	2000	157.99±0.22^{aC}	156.33±0.38^{bC}	157.58±0.5^{aC}
		4000	160.65±0.45^{bB}	162.45±0.55^{aB}	160.12±0.48^{bB}
		6000	163.67±0.52^{cA}	178.86±0.75^{aA}	170.67±0.68^{bA}
	Internal organs	2000	130.94±0.45^{bB}	131.43±0.48^{bB}	132.78±0.45^{aA}
		4000	131.64±0.58^{aAB}	132.78±0.68^{aA}	131.89±0.68^{aA}
		6000	132.23±0.65^{aA}	132.37±0.75^{aA}	132.89±0.87^{aA}
Insoluble-bound	Body wall	2000	200.56±0.56^{aA}	200.67±0.4^{aA}	201.45±0.46^{aA}
		4000	200.68±0.55^{aA}	201.75±0.63^{aA}	200.93±0.38^{aA}
		6000	201.65±0.68^{aA}	201.67±0.58^{aA}	200.99±0.65^{aA}
	Flower	2000	177.54±0.6^{bC}	178.45±0.55^{bC}	180.76±0.42^{aC}
		4000	180.65±0.42^{bB}	180.45±0.58^{bB}	188.56±0.45^{aB}
		6000	191.43±0.38^{bA}	198.16±0.25^{aA}	190.67±0.68^{bA}
	Internal organs	2000	247.62±0.45^{aC}	248.98±0.35^{aB}	248.21±0.38^{aB}
		4000	249.12±0.48^{aB}	248.67±0.48^{aB}	248.54±0.54^{aB}
		6000	250.65±0.34^{bA}	254.35±0.58^{aA}	254.3±0.25^{aA}

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among treatments. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) for each body part.

5.5. Effects of HPP on the metal chelation activity

Metal ions, mainly iron, stimulate lipid oxidation by means of the Fenton reaction. They can act as prooxidants by breaking down lipid hydroperoxides into more reactive radicals (Klompong et al., 2007). Generally, antioxidants (e.g., phenolic compounds) form a coordinate complex with metal ions (e.g., Fe and Cu) and make them inaccessible for involving in lipid oxidation (Shahidi & Zhong, 2015). Metal chelators act as antioxidants by reducing the content of available metal ions to generate hydroxyl radicals by Fenton reactions as well as by scavenging ROS (Nwachukwu et al., 2021). Therefore, metal chelation activity can be evaluated when a complex is formed between antioxidant and metal ions, resulting in the loss of intensity of ferrozine-ferrous color complex (pink color), which can be measured at 562 nm.

In this study, we measured the metal chelation activity in ethylenediaminetetraacetic acid equivalents (EDTAE) to determine the chelating property of free, esterified, and insoluble-bound phenolics of sea cucumber as presented in Table 5.4. The HPP pressure increased the metal chelation activity of body wall, flower, and internal organs in the free and esterified phenolic fractions when the pressure was increased from 2000 to 6000 bars. However, 2000 and 4000 bars had almost a similar effect on the activity, whereas 6000 bar showed significantly ($p < 0.05$) higher values than their counterparts. For example, the metal chelation activity of free phenolics in flower was 25.19, 25.32, and 30.61 mg EDTAE/ 100 g for 2000, 4000, and 6000 bars, respectively, when the holding time was 10 min. The improvement of the antioxidant activity with the increase of HPP pressure could be linked to broken cells under pressure, as proposed by other authors (Andrés et al., 2016; Paciulli et al., 2019; Zuluaga et al., 2016). For instance, De Ancos et al. (2020) found that HPP treatment (200 and 400 MPa) significantly increased the FRAP value of orange juice. These results may be due to the higher extraction yield by HPP, causing higher antioxidant activity.

Moreover, Zhou et al. (2019) stated that FRAP values of phenolic compounds of oil palm fruit extracts increased with increasing HPP pressure, especially those of the insoluble-bound fraction. However, in this work, the free phenolic fraction had higher activity than the esterified and insoluble-bound fractions, possibly related to the amount and type of phenolics present in the free fraction.

Similar to the HPP pressure, the holding time had a significant effect on metal chelation activity of phenolic compounds obtained from sea cucumber. However, HPP holding time had a minimal effect on the HPP pressure of 2000 and 4000 bars, whereas 6000 bar for 10 and 15 min offered the highest results. For example, at 6000 bar, the metal chelation activity of free phenolics extracted from the body wall was 18.34, 21.67, and 20.54 mg EDTAE/ 100 g for 5, 10, and 15 min, respectively. Nevertheless, at 6000 bar, no significant ($p > 0.05$) difference was noted between 10 and 15 min of pre-treatment, except the flower in free and esterified fractions, where 10 min of holding time provided the higher activity. This could be due to the equilibrium of extraction reached at 10 min or oxidation of some phenolic compounds at high pressure and longer time (e.g., 6000 bar for 15 min). Apart from the oxidation of phenolics, the loss of antioxidant activity could be related to their association with proteins or ionization and condensation reactions via covalent bond formation with phenolic compounds at high pressure and/or longer holding time (Chakraborty et al., 2015; Tangwongchai, Ledward, & Ames, 2000; Wang et al., 2012). For instance, Saikaew et al. (2018) concluded that longer HPP holding time decreased the antioxidant activity (TEAC, FRAP, and ORAC assays) of waxy corn (*Zea mays* L. var. *ceratina*) kernels.

Table 5. 4. Effect of HPP on the metal chelation activity (mg ethylenediaminetetraacetic acid equivalents/ 100 g) of different sea cucumber body parts

Phenolics	Body part	Pressure (bar)	Time (min)		
			5	10	15
Free	Body wall	2000	16.29 ± 1.34^{aB}	16.39 ± 1.22^{aB}	17.25 ± 0.8^{aB}
		4000	16.45 ± 0.4^{bB}	18.34 ± 0.9^{aB}	18.43 ± 0.88^{aB}
		6000	18.34 ± 0.9^{bA}	21.67 ± 1.05^{aA}	20.54 ± 1.15^{aA}
	Flower	2000	24.09 ± 1.15^{aB}	25.19 ± 1.35^{aB}	25.28 ± 1.18^{aB}
		4000	24.47 ± 0.82^{aB}	25.32 ± 1.2^{aB}	23.45 ± 1.85^{aB}
		6000	26.45 ± 1.1^{bA}	30.61 ± 1.24^{aA}	27.34 ± 1.3^{bA}
	Internal organs	2000	17.78 ± 0.86^{bB}	19.69 ± 0.9^{aB}	20.56 ± 1.14^{aB}
		4000	18.48 ± 0.6^{bB}	21.39 ± 1.16^{aB}	20.28 ± 1.04^{aB}
		6000	20.48 ± 1.32^{bA}	25.67 ± 1.02^{aA}	25.45 ± 1.12^{aA}
Esterified	Body wall	2000	20.56 ± 0.72^{aA}	20.54 ± 0.78^{aA}	21.78 ± 0.85^{aA}
		4000	21.53 ± 0.95^{aA}	22.98 ± 0.65^{aA}	21.56 ± 0.88^{aA}
		6000	21.76 ± 0.5^{aA}	22.42 ± 0.7^{aA}	22.78 ± 0.65^{aA}
	Flower	2000	5.47 ± 0.22^{bC}	5.32 ± 0.38^{bC}	6.23 ± 0.5^{aC}
		4000	6.54 ± 0.45^{cB}	7.54 ± 0.54^{aB}	7.43 ± 0.28^{bB}
		6000	7.65 ± 0.52^{cA}	11.67 ± 0.75^{aA}	8.65 ± 0.68^{bA}
	Internal organs	2000	4.21 ± 0.45^{bC}	5.98 ± 0.42^{aC}	5.48 ± 0.38^{aB}
		4000	5.42 ± 0.32^{bB}	7.51 ± 0.64^{aB}	6.43 ± 0.62^{aB}
		6000	6.54 ± 0.65^{bA}	11.78 ± 0.72^{aA}	11.54 ± 0.82^{aA}
Insoluble-bound	Body wall	2000	4.68 ± 0.28^{aC}	4.28 ± 0.2^{bC}	4.32 ± 0.25^{aC}
		4000	5.38 ± 0.35^{bB}	6.41 ± 0.42^{aB}	5.49 ± 0.18^{bB}
		6000	6.43 ± 0.28^{bA}	9.24 ± 0.32^{aA}	9.54 ± 0.26^{aA}
	Flower	2000	2.7 ± 0.08^{aA}	2.7 ± 0.15^{aA}	2.71 ± 0.12^{aA}
		4000	2.71 ± 0.12^{aA}	2.73 ± 0.14^{aA}	2.75 ± 0.15^{aA}
		6000	2.72 ± 0.16^{aA}	2.73 ± 0.15^{aA}	2.74 ± 0.12^{aA}
	Internal organs	2000	6.18 ± 0.25^{aA}	6.28 ± 0.18^{aA}	6.46 ± 0.22^{aA}
		4000	6.39 ± 0.14^{aA}	6.58 ± 0.18^{aA}	6.43 ± 0.25^{aA}
		6000	6.41 ± 0.12^{aA}	6.27 ± 0.14^{aA}	6.46 ± 0.15^{aA}

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among treatments. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) for each body part.

5.6. Summary

HPP pressure and holding time had a positive significant effect on the antioxidant activity (DPPH, ABTS, and hydroxyl radical scavenging activity as well as metal chelation activity) of all three phenolic fractions, especially those of the free phenolics. In particular, 6000 bar showed significantly higher activity than the 2000 and 4000 bars, regardless of HPP holding time. Likewise, increasing trends were observed for the HPP holding time; mainly those treated for 10 and 15 min at 6000 bar. However, no significant difference ($p > 0.05$) was observed between 10 and 15 min of HPP treatment. Thus, HPP at 6000 bar for 10 min could be optimum for retaining the antioxidant activity of Atlantic sea cucumber. On the other hand, the highest antioxidant activity was observed in the free phenolic fraction compared to the esterified and insoluble-bound fractions, regardless of the sea cucumber body parts. The flower had the highest activity in all phenolic fractions, regardless of the type of assay used to determine the antioxidant activity. However, HPP did not result in similar levels of activity in each assay. On average, HPP increased the DPPH radical scavenging activity and metal chelation activity, while it had nearly no effect on the ABTS and hydroxyl radical scavenging activities. This could be due to the different mechanisms of action involved in each assay and hence, providing varied antioxidant properties. For instance, metal chelation is based on the ability of compounds to reduce the level of available metal ions to generate free radicals, whilst DPPH is a free-radical scavenging power. Prior, Wu, and Schaich (2005) stated that no single antioxidant activity assay could accurately assess all antioxidants in a complex or mixed system. Therefore, it has been recommended that of using at least two or three different methods to determine the antioxidant property for better reliability (Nwachukwu et al., 2021; Shahidi, 2015; Shahidi & Zhong, 2015).

Chapter 6

Phenolic compounds and antioxidant activity of HPP-treated and untreated sea cucumber

6.1. Introduction

The beneficial effects of marine bioactive components have long been known by researchers as well as consumers. One of the marine sources is sea cucumber, especially the Atlantic sea cucumber, which has not yet been sufficiently explored. Over the past decades, the bioactive compounds of sea cucumbers and their beneficial effects on human health as well as food preservation are being studied (García et el., 2019). These marine invertebrates have been used in traditional medicine and tonic food in many countries for centuries and have attracted increasing interest by the scientific community due to their wide range of biological activities, including anti-diabetic, anti-inflammatory, anticancer, anti-obesity, anti-atherosclerosis, antimicrobial, and immunomodulatory activities (Bordbar et al., 2011; Hossain et al. 2020a; Roggatz et al., 2018). For example, Himaya et al. (2010) concluded that phenolic compounds of sea cucumber (*S. japonicus*) had a potential anti-inflammatory activity, which could be used in formulating functional food. The biological activities of sea cucumbers can be attributed to their series of bioactive components, mainly chondroitin sulfates, saponins, cerebrosides, and phenolics (Janakiram et al., 2015; Oh et al., 2017). For instance, polysaccharides (Qin et al., 2018), protein hydrolysates (Senadheera et al., 2021; Yan, Tao, & Qin, 2016), and bioactive peptides (Safari &Yaghoubzadeh, 2020) obtained from sea cucumber have shown antioxidant activity. Moreover, phenolic compounds have been documented from different species of sea cucumber, but still, little information is available about the type of phenolics and their biological activities. For example, the total phenolic contents and antioxidant activities of Atlantic sea cucumber have been reported (Mamelona et al., 2007; Zhong et al., 2007); however, no study has yet been conducted on the

individual phenolic profile of this species. Furthermore, sea cucumber by-products, mainly viscera, represent up to 50% of its body weight and have the potential to show antioxidant activities (Mamelona et al., 2010). Therefore, there is a need to fill the information gap about the potential antioxidant activities of the bioactive compounds of Atlantic sea cucumber and its commercial body parts.

High-pressure processing (HPP) has been suggested as an alternative method to extract phenolic compounds due to the shorter extraction time and better extraction efficiency. HPP damages the cellular membrane, enhances mass transfer rate, and improves solvent permeability, resulting in higher extraction yield (De Ancos et al., 2019; Prasad et al., 2010). On the other hand, extraction of phenolic compounds using heat treatment may oxidize some of the compounds (e.g., anthocyanins), which can cause color changes in foods (Kim et al., 2012). To overcome this issue, HPP, a non-thermal process, can be used to preserve heat-sensitive compounds. Moreover, HPP can inactivate microbial growth and enzymatic action in foods due to the partial unfolding of proteins during the treatment and upon release of the pressure (Saikaew et al., 2018). Therefore, HPP could be an alternative way to increase the shelf life of fresh sea cucumber as well as the extraction efficacy of bioactive compounds (e.g., phenolic compounds). However, no information is available on the use of HPP in any species of sea cucumber. Thus, this study aims to investigate the changes of phenolic compounds and their antioxidant activity of Atlantic sea cucumber and its commercial body parts using HPP.

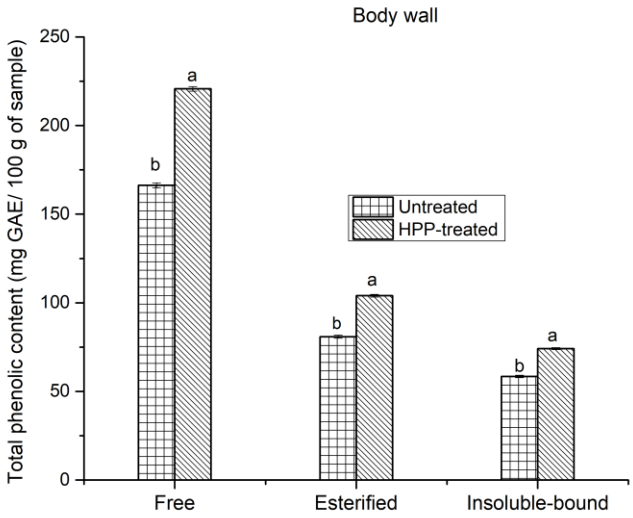
6.2. Total phenolic content (TPC)

Total phenolic content (TPC) was evaluated using Folin Ciocalteu's reagent, and the results were expressed as mg gallic acid equivalents (mg GAE)/ 100 g of sample. Figure 6.1 and Table 6.1 show the TPC of untreated and HPP-treated (6000 bar for 10 min) sea cucumber. It was found that the HPP treatment significantly ($p<0.05$) increased the TPC in all three phenolic fractions, regardless of sea cucumber body parts. For example, the TPC in the free, esterified, and insoluble-bound phenolics of untreated body wall was 166.27, 80.87, and 58.52 mg GAE/ 100 g, respectively, whereas these values increased to 220.69, 104.02, and 74.17 mg GAE/ 100 g by HPP, respectively. Similarly, HPP treatment significantly increased TPC in the free, esterified, and insoluble-bound phenolic fractions by 35.18, 31.29, and 24.65%, respectively, in flower in comparison to their untreated counterparts. Likewise, in internal organs, HPP treatment increased 32.59, 21.5, and 23.85% of TPC in the free, esterified, and insoluble-bound fractions, accordingly. The positive effect of HPP could be associated with the disruption of cell walls by high pressure, which increases the membrane permeability, favoring higher extraction yield (Vázquez-Gutiérrez et al., 2013). However, in this study, HPP had a higher effect on the free phenolic fraction than the esterified and insoluble-bound phenolic fractions. This could be due to the very low amount of esterified and insoluble-bound phenolics present in sea cucumber compared to the free phenolic fraction. Moreover, HPP may improve the extraction of free phenolics as they could be partially attached by some food matrices, which could easily be ruptured by HPP (Zhou et al., 2019). Furthermore, HPP may have a very minimal effect on covalent bonds, resulting in the lower extraction yield of insoluble-bound phenolics (Saikaewa et al., 2018).

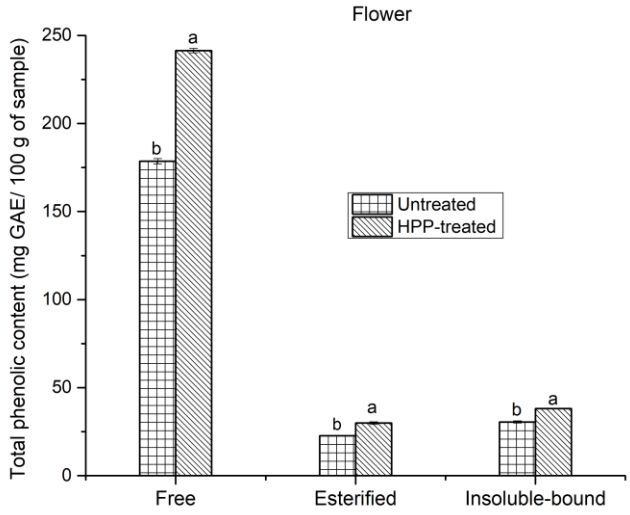
On the other hand, the highest amount of free phenolics was observed in flower, whereas the esterified and insoluble-bound phenolic fractions were abundant in the body wall. In contrast,

the TPC (free + esterified + insoluble-bound) was higher in the body wall, followed by the flower and internal organs. There are a couple of studies on phenolics of *C. frondosa* reporting that the Atlantic sea cucumber contains a considerable amount of phenolics. For example, Mamelona et al. (2007) reported that the TPC of *C. frondosa* internal organs (gonad, digestive tract, and respiratory apparatus) and muscles varied from 22.5 to 236 mg GAE/100 g dw, where the digestive tract showed a higher level of TPC. Similarly, Zhong et al. (2007) reported that fresh *C. frondosa* with or without internal organs had the higher TPC (100 to 108 mg GAE/ 100 g), than the rehydrated sea cucumber (30 to 80 mg GAE/ 100 g). Apart from this, TPC was also determined from other species of sea cucumber, including *H. forskali*, *H. atra*, *H. scabra*, *H. leucospilota*, *S. variegatus*, *S. japonicus*, and *S. chloronotus*, where TPC varied from 153 to 11690 mg GAE/ 100 g (Althunibat et al., 2009; García et al., 2019; Nguyen & Kim, 2015; Pranweerapaiboon et al., 2020; Ridhowati et al., 2018; Sukmiwati et al., 2019). For instance, García et al. (2019) stated that *H. forskali* extracts contained TPC of 319 to 521 mg GAE/ 100 g, which is aligned with our current study. The availability of phenolics in *C. frondosa* could be attributed to the presence of phenolic-rich material, including phytoplankton and particles obtained from degrading marine macroalgae, in their diet (Althunibat et al., 2009, Dakrory et al., 2015; Mamelona et al.,2007). This is because sea cucumber appears to absorb phenolic compounds from algal and/or microalgal sources, which are good sources of flavonoids, anthocyanidins, anthocyanins, and tannins (Zhong et al., 2007). Thus, phenolics in the tissue of sea cucumbers have the potential to serve as sources of antioxidants.

(a)


Body wall
Total phenolic content (mg GAE/ 100 g of sample)
250
200
150
100
50
0
a
b
a
b
a
b
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(b)


Flower
Total phenolic content (mg GAE/ 100 g of sample)
250
200
150
100
50
0
a
b
a
b
a
b
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(c)

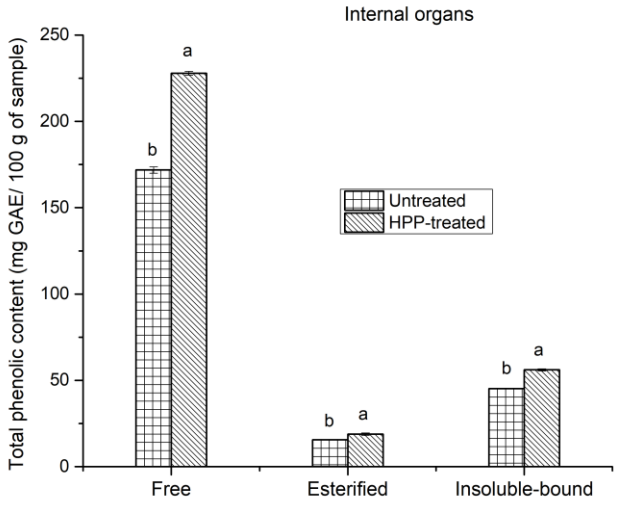


Figure 6. 1. Total phenolic content of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg gallic acid equivalents (GAE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

Table 6. 1. Total phenolic content (mg gallic acid equivalents/ 100 g) of untreated and HPP-treated sea cucumber.

Treatment	Phenolics	Body part		
		Body wall	Flower	Internal organs
Untreated	Free	166.27 ± 0.45^{cA}	178.56 ± 0.52^{aA}	171.86 ± 1.82^{bA}
	Esterified	80.87 ± 0.48^{aB}	22.75 ± 0.15^{bC}	15.53 ± 0.12^{cC}
	Insoluble-bound	58.52 ± 0.18^{aC}	30.54 ± 0.12^{cB}	45.28 ± 0.1^{bB}
	Total	305.66	231.85	232.67
HPP-treated	Free	220.69 ± 1.05^{cA}	241.38 ± 1.25^{aA}	227.87 ± 1.06^{bA}
	Esterified	104.02 ± 0.7^{aB}	29.87 ± 0.75^{bC}	18.87 ± 0.75^{cC}
	Insoluble-bound	74.17 ± 0.58^{aC}	38.07 ± 0.25^{cB}	56.08 ± 0.56^{bB}
	Total	398.88	309.31	302.82

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among different body parts. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among different phenolic fractions.

6.3. Total flavonoid content (TFC)

Total flavonoid content (TFC) of untreated and HPP-treated (6000 bar for 10 min) sea cucumber is reported as catechin equivalents (CE), and the results are shown in Figure 6.2 and Table 6.2. HPP-treated samples showed significantly ($p<0.05$) higher TFC than their untreated counterparts. For example, the TFC of untreated body wall in the free, esterified, and insoluble-bound fractions was 65.34, 28.55, and 28.78 mg CE/ 100 g, respectively, while these values for HPP-treated body wall were 79.67, 36.79, and 38.69 mg CE/ 100 g, respectively. Likewise, HPP pre-treatment resulted a significant increase of TFC in the free, esterified, and insoluble-bound phenolic fractions by 33.66, 54.29, and 29.17%, respectively, in flower in comparison to their untreated counterparts. Similarly, in internal organs, HPP pre-treatment increased TFC by 22.47, 50, and 17.65% in the free, esterified, and insoluble-bound fractions, respectively. This improvement in TFC could be due to the breaking down of cell wall matrices following HPP, which improves permeability (Zuluaga et al., 2016). However, in our study, HPP had a greater effect on the soluble phenolic than the insoluble phenolics. This could be due to the minimal effect of HPP on the covalent bonds, resulting in a lower impact on the insoluble phenolics (Saikaewa et al., 2018).

On the other hand, the highest TFC was observed in the free phenolics fraction of flower, whereas the esterified and insoluble-bound phenolics were abundant in the body wall. On the contrary, the TFC (free + esterified + insoluble-bound) was higher in flower, followed by body wall and internal organs. Therefore, it can be said that most of the sea cucumber (*C. frondosa*) phenolics are flavonoids and are mainly present in the flower. Our results are in agreement with those of Ceesay et al. (2019), who stated that sea cucumbers are likely to have flavonoids as they feed mainly on seaweeds, which are rich sources of catechins and flavonols. Moreover, Mamelona

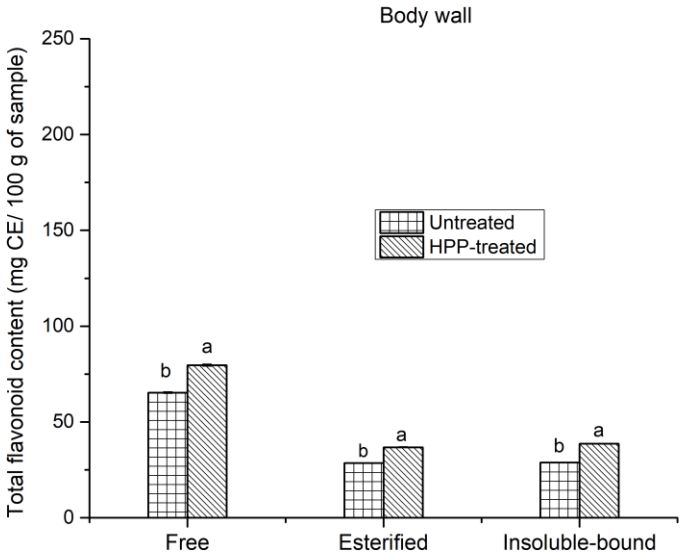
et al. (2007) found that the TFC of *C. frondosa* extracts varied from 2.9 to 59.8 mg of rutin equivalents (RE)/ 100 g, where gonads exhibited the maximum level of flavonoids. TFC was also determined from other species of sea cucumber (e.g., *H. leucospilota* and *S. japonicus*), and their content varied from 84 to 439 mg RE/ 100 g (Ceesay et al., 2019; Husni et al., 2009).

Table 6. 2. Total flavonoid content (mg catechin equivalents/ 100 g) of untreated and HPP-treated sea cucumber.

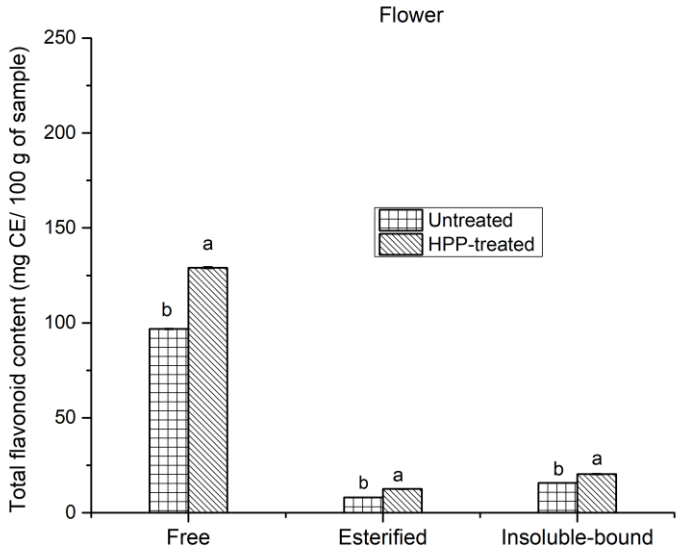
Treatment	Phenolics	Body part		
		Body wall	Flower	Internal organs
Untreated	Free	65.34 ± 0.42^{cA}	96.79 ± 0.38^{aA}	80.59 ± 0.56^{bA}
	Esterified	28.55 ± 0.12^{aB}	8.14 ± 0.15^{bC}	5.1 ± 0.1^{cC}
	Insoluble-bound	28.78 ± 0.22^{aB}	15.73 ± 0.18^{bB}	15.35 ± 0.24^{bB}
	Total	122.67	120.66	101.04
HPP-treated	Free	79.67 ± 0.48^{cA}	129.07 ± 0.55^{aA}	98.7 ± 0.32^{bA}
	Esterified	36.79 ± 0.36^{aC}	12.56 ± 0.65^{bC}	7.65 ± 0.25^{cC}
	Insoluble-bound	38.69 ± 0.28^{aB}	20.32 ± 0.42^{bB}	18.06 ± 0.24^{cB}
	Total	155.15	161.65	124.42

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among different body parts. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among different phenolic fractions.

(a)


Body wall
Total flavonoid content (mg CE/ 100 g of sample)
250
200
150
100
50
0
Untreated
HPP-treated
b
a
b
a
b
a
Free
Esterified
Insoluble-bound

(b)


Flower
Total flavonoid content (mg CE/ 100 g of sample)
250
200
150
100
50
0
Untreated
HPP-treated
b
a
b
a
b
a
Free
Esterified
Insoluble-bound

(c)

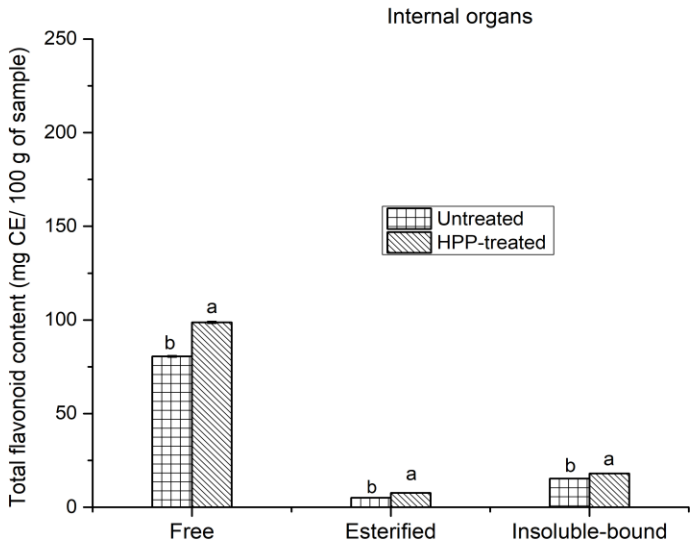


Figure 6. 2. Total flavonoid content of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg catechin equivalents (CE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

6.4. Investigation of antioxidant activities of phenolic compounds

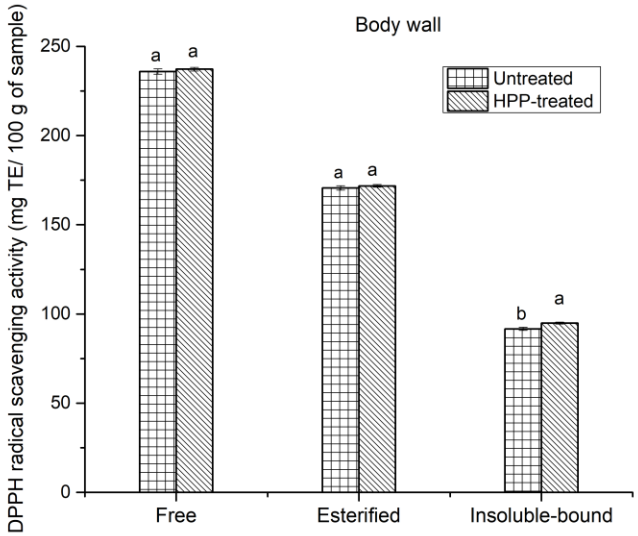
6.4.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was evaluated to assess the hydrogen atom or electron donating ability of phenolic compounds extracted from sea cucumber. DPPH radical scavenging activity of untreated and HPP-treated (6000 bar for 10 min) sea cucumber was determined in Trolox equivalents (TE), and the results are presented in Figure 6.3 and Table 6.3. According to the results, all phenolic fractions demonstrated strong DPPH radical scavenging activity, regardless of HPP treatment. However, HPP-treated flower and internal organs showed significantly ($p<0.05$) higher activity than their untreated counterparts. For instance, the DPPH radical scavenging activity of untreated flower in the free, esterified, and insoluble-bound fractions was 505.56, 47.67, and 50.87 mg TE/ 100 g, respectively, while these values for HPP-treated flower were 553.89, 54.65, and 58.65 mg TE/ 100 g, accordingly. Zhong et al. (2007) found that the phenolic-rich *C. frondosa* extracts had a strong DPPH radical scavenging activity, especially those from internal organs. Likewise, phenolic compounds were extracted from the internal organs of *C. frondosa,* and it was observed that the digestive tract had a higher ORAC value than the gonads and respiratory apparatus (Mamelona et al., 2007). DPPH radical scavenging activity of phenolics has also been determined from other species (e.g., *H. scabra, H. arenicola*, *H. atra*, *S. japonicus*, and *S. variegatus*) of sea cucumber and found to have potent antioxidant activity (Althunibat et al., 2009; Dakrory et al., 2015; Husni et al., 2009; Fahmy, 2015; Ridhowati et al., 2018; Nobsathian 2017). Moreover, HPP treatment rendered a significant increase of DPPH radical scavenging activity in the free, esterified, and insoluble-bound phenolic fractions by 4.81, 9.95, and 8.95%, respectively, in internal organs. Nevertheless, no significant ($P>0.05$) difference

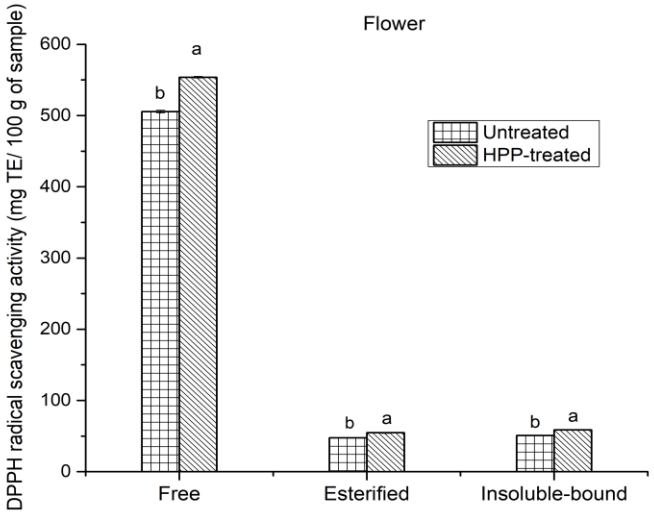
was observed between untreated and HPP-treated body wall in terms of DPPH radical scavenging activity; especially those in the free and esterified fractions.

The highest level of DPPH radical scavenging activity was observed in the free phenolic fraction of flower, followed by internal organs and body wall. Moreover, esterified and insoluble-bound fractions had a lower activity compared to the free phenolic fraction. Similarly, the total (free + esterified + insoluble-bound) DPPH radical scavenging activity was higher in flower, followed by internal organs and body wall, even though sum of the TPC was higher in the body wall. This might be due to the method used to determine TPC, where reducing substances (e.g., reducing sugars) may react with the Folin–Ciocalteu reagent, causing to increase in the TPC of body wall. Thus, although HPP increases the TPC of body wall, those composites may not only be due to phenolics compounds (Moreira et al., 2020; Zhao et al., 2016). Therefore, sea cucumber flower could be the most valuable body part in terms of antioxidant activity.

(a)


Body wall
DPPH radical scavenging activity (mg TE/ 100 g of sample)
250
200
150
100
50
0
a
a
a
a
b
a
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(b)


Flower
DPPH radical scavenging activity (mg TE/ 100 g of sample)
600
500
400
300
200
100
0
b
a
b
a
b
a
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(c)

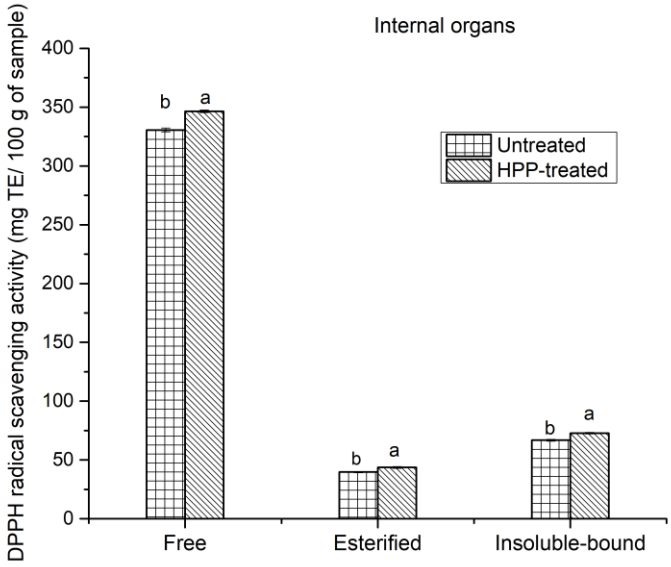


Figure 6. 3. DPPH radical scavenging activity of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg Trolox equivalents (TE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

Table 6. 3. DPPH radical scavenging activity (mg Trolox equivalents/ 100 g) of untreated and HP-treated sea cucumber.

Treatment	Phenolics	Body part		
		Body wall	Flower	Internal organs
Untreated	Free	235.89 ± 1.55^{cA}	505.56 ± 1.89^{aA}	330.56 ± 1.68^{bA}
	Esterified	170.67 ± 1.25^{aB}	47.67 ± 0.68^{bC}	39.76 ± 0.52^{cC}
	Insoluble-bound	91.67 ± 0.88^{aC}	50.87 ± 0.72^{cB}	66.78 ± 0.76^{bB}
	Total	498.23	604.1	437.1
HPP-treated	Free	237.22 ± 1.05^{cA}	553.89 ± 1.25^{aA}	346.48 ± 1.06^{bA}
	Esterified	171.87 ± 0.7^{aB}	54.65 ± 0.75^{bC}	43.72 ± 0.75^{cC}
	Insoluble-bound	94.91 ± 0.58^{aC}	58.65 ± 0.25^{cB}	72.76 ± 0.58^{bB}
	Total	504	667.19	462.96

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among different body parts. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among different phenolic fractions.

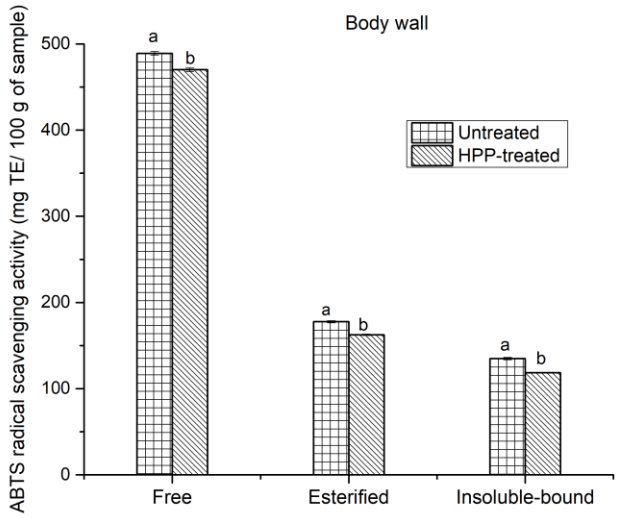
6.4.2. ABTS radical scavenging activity

The ABTS radical scavenging activity of untreated and HPP-treated (6000 bar for 10 min) sea cucumber was determined, and the results are presented in Figure 6.4 and Table 6.4 as Trolox equivalents (TE). It was found that the HPP significantly ($p<0.05$) decreased the ABTS radical scavenging activity of phenolics extracted from body wall and internal organs. For example, the ABTS radical scavenging activity of untreated body wall in the free, esterified, and insoluble-bound fractions was 489.14, 177.71, and 135.01 mg TE/ 100 g, respectively, while those for the HPP-treated sample were reduced to 470.14, 162.56, and 118.65 mg TE/ 100 g, respectively. Similarly, in the internal organs, HPP produced a minor but significant ($p<0.05$) decrease in the free, esterified, and insoluble-bound phenolic fractions by 4.03, 14.64, and 13.08%, respectively. The decrease of the ABTS radical scavenging activity after HPP could possibly be due to the oxidation of certain phenolic compounds or by the activation of detrimental food enzymes such as PPO or POD (De Ancos et al., 2020). However, the ABTS assay exhibited no significant ($p>0.05$) decrease in HPP-treated flower compared to untreated ones in the free and esterified phenolic fractions. Therefore, it can be said that the HPP preserves the ABTS radical scavenging activity of phenolics obtained from flower, mainly those were in soluble phenolics. This finding is aligned with the DPPH radical scavenging activity performed in the present study, where flower is most valuable sea cucumber body part in terms of antioxidant activity. However, DPPH radical-scavenging activity was lower than those of ABTS radical-scavenging activity at the same condition. It has been reported that the $ABTS^{+}$ have a greater scavenging property when compared with the DPPH radical from natural extracts (Hossain et al., 2018).

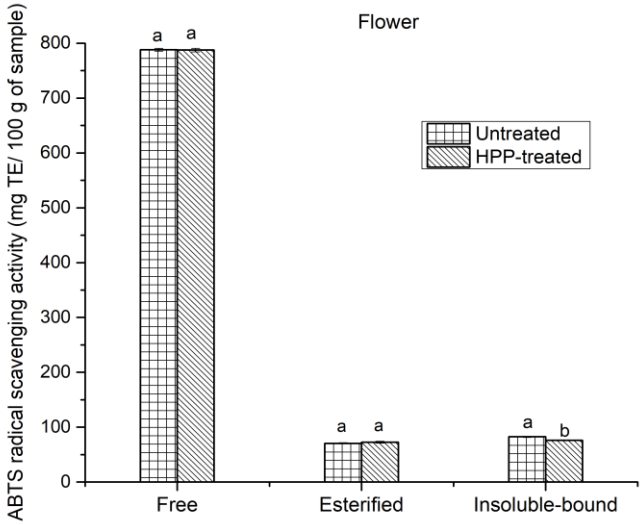
On the other hand, the highest amount of activity was observed in both treated and untreated flower in the free fraction. Likewise, the total (free + esterified + insoluble-bound) ABTS

radical scavenging activity was higher in flower, followed by body wall and internal organs, regardless of HPP treatment. There are no previous studies on the ABTS radical scavenging activity of phenolics extracted from *C. frondosa*; however, Husni et al. (2009) claimed that phenolic compounds of *S. japonicus* had a good correlation with the ABTS and DPPH radical scavenging activities.

(a)


Body wall
ABTS radical scavenging activity (mg TE/ 100 g of sample)
0
100
200
300
400
500
a
b
a
b
a
b
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(b)


Flower
ABTS radical scavenging activity (mg TE/ 100 g of sample)
0
100
200
300
400
500
600
700
800
a
a
a
a
a
b
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(c)

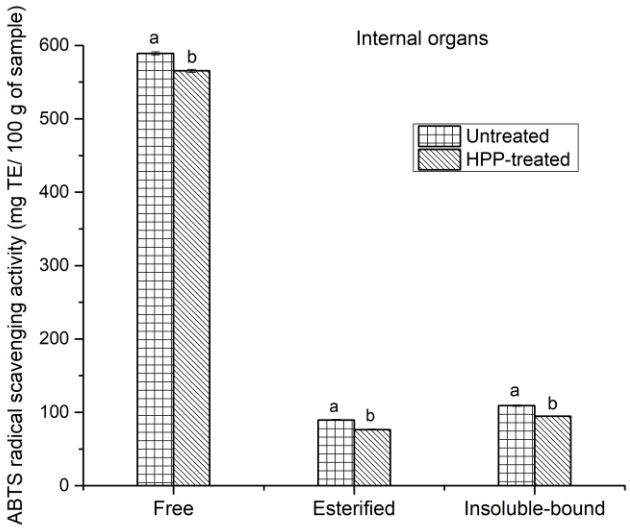


Figure 6. 4. ABTS radical scavenging activity of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg Trolox equivalents (TE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

Table 6. 4. ABTS radical scavenging activity (mg Trolox equivalents/ 100 g) of untreated and HP-treated sea cucumber.

Treatment	Phenolics	Body part		
		Body wall	Flower	Internal organs
Untreated	Free	489.14 ± 1.98^{cA}	788.13 ± 2.53^{aA}	589.18 ± 2.18^{bA}
	Esterified	177.71 ± 1.25^{aB}	70.6 ± 1.05^{cC}	89.67 ± 0.94^{bC}
	Insoluble-bound	135.01 ± 1.42^{aC}	82.56 ± 1.1^{cB}	109.03 ± 0.98^{bB}
	Total	801.86	941.29	787.88
HPP-treated	Free	470.14 ± 2.05^{cA}	787.45 ± 3.25^{aA}	565.43 ± 2.06^{bA}
	Esterified	162.56 ± 0.7^{aB}	72.56 ± 1.75^{cC}	76.54 ± 0.75^{bC}
	Insoluble-bound	118.65 ± 0.58^{aC}	76.09 ± 0.25^{cB}	94.76 ± 0.58^{bB}
	Total	751.35	936.1	736.73

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among different body parts. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among different phenolic fractions.

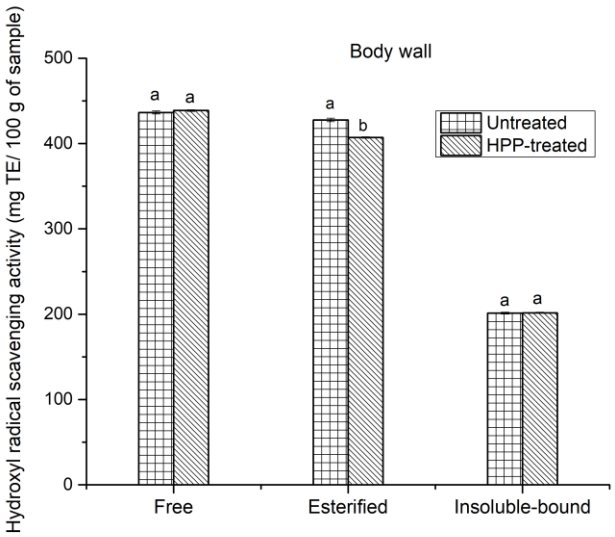
6.4.3. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of phenolics extracted from untreated and HPP-treated (6000 bar for 10 min) sea cucumber was determined, and results expressed as Trolox equivalents (TE) are shown in Figure 6.5 and Table 6.5. It was found that all phenolic fractions, regardless of HPP treatment, exhibited strong hydroxyl radical scavenging activity. However, HPP pre-treatment did not change the antioxidant activity of phenolics extracted from body wall in the free and insoluble-bound fractions, whereas a decrease in the esterified phenolic fraction was observed. Similarly, no significant ($p > 0.05$) difference was found between the untreated and HPP-treated internal organs in the free and esterified fraction, while HPP treatment increased the activity in the insoluble-bound fraction. In contrast, HPP treatment significantly improved the hydroxyl radical scavenging activity of phenolics obtained from flower in all three phenolic fractions. The increase of antioxidant activity could be associated with the higher content of phenolics obtained upon HPP treatment of flower than their untreated counterparts. The increase in the antioxidant activity after HPP has also been reported by other authors (Andrés et al., 2016; da Silveira et al., 2019; Patras et al., 2009). Thus, HPP could retain the antioxidant activity of the sea cucumber flower.

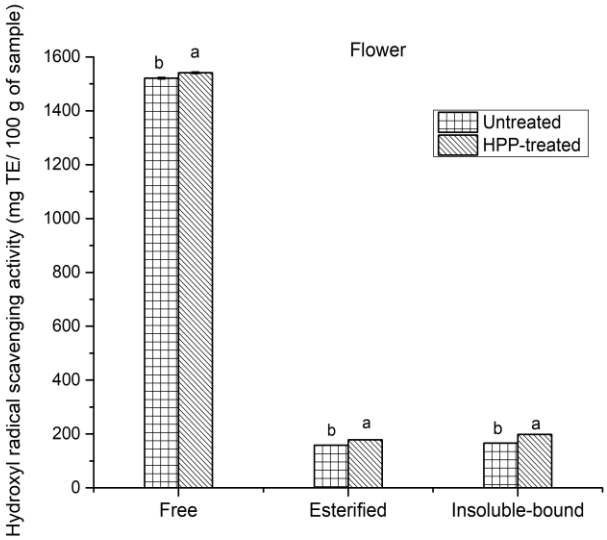
The highest amount of hydroxyl radical scavenging activity was recorded in the free fraction of HPP-treated flower (1541.32 mg TE/ 100 g), followed by internal organs (598.33 mg TE/ 100 g) and body wall (438.68 mg TE/ 100 g). Likewise, the total (free + esterified + insoluble-bound) hydroxyl radical scavenging activity was greater in the HPP-treated flower, followed by body wall and internal organs. To date, hydroxyl radical scavenging activity has not been determined from any species of sea cucumber phenolics; however, other antioxidant assays like

ORAC values have been evaluated for phenolics of *C. frondosa* which reported that internal organs had strong antioxidant activity (Mamelona et al., 2007; Zhong et al., 2007).

(a)



(b)



(c)

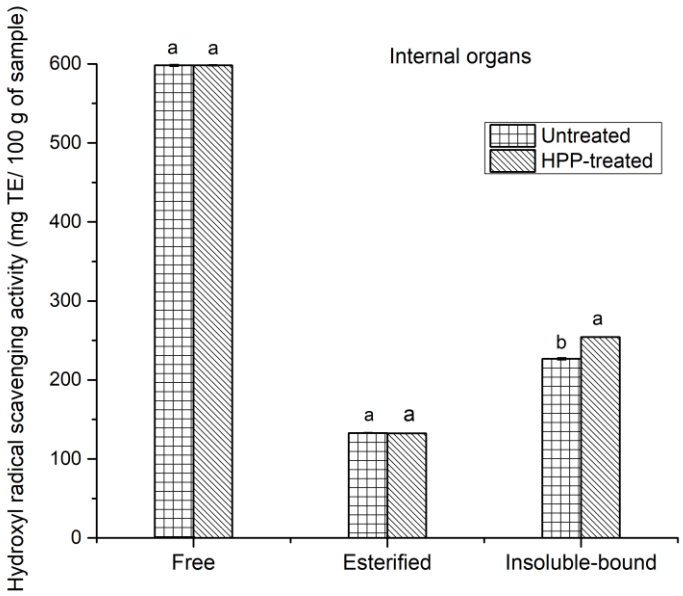


Figure 6. 5. Hydroxyl radical scavenging activity of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg Trolox equivalents (TE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

Table 6. 5. Hydroxyl radical scavenging activity (mg Trolox equivalents/ 100 g) of untreated and HP-treated sea cucumber.

Treatment	Phenolics	Body part		
		Body wall	Flower	Internal organs
Untreated	Free	436.45 ± 1.67^{cA}	1520.87 ± 4.26^{aA}	598.33 ± 1.58^{bA}
	Esterified	427.67 ± 2.08^{aB}	158.45 ± 1.08^{bC}	132.89 ± 0.82^{cC}
	Insoluble-bound	201.25 ± 1.16^{bC}	165.87 ± 1.58^{cB}	226.76 ± 1.5^{aB}
	Total	1065.37	1845.19	957.98
HPP-treated	Free	438.68 ± 1.05^{cA}	1541.32 ± 1.25^{aA}	598.33 ± 1.05^{bA}
	Esterified	407.11 ± 0.7^{aB}	178.86 ± 0.75^{bC}	132.37 ± 0.75^{cC}
	Insoluble-bound	201.67 ± 0.58^{bC}	198.16 ± 0.25^{cB}	254.3 ± 0.58^{aB}
	Total	1047.46	1918.34	985

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among different body parts. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among different phenolic fractions.

6.4.4. Metal chelation activity

The metal chelation activity was evaluated to determine the chelating property of free, esterified, and insoluble-bound phenolics of different sea cucumber body parts as presented in Figure 6.6 and Table 6.6. The results were expressed as ethylenediaminetetraacetic acid (EDTA) equivalents (EDTAE). It was found that HPP treatment (6000 bar for 10 min) significantly ($p<0.05$) increased the metal chelation activity in all three phenolic fractions, regardless of sea cucumber body parts. For example, the metal chelation activity in the free, esterified, and insoluble-bound phenolics of untreated internal organs was 18.68, 4.09, and 6.45 mg EDTAE/ 100 g, respectively, whereas these values increased to 25.67, 11.78, and 7.27 mg EDTAE/ 100 g upon HPP treatment. Similarly, in the body wall, HPP treatment generated a significant increase of metal chelation activity in the free and insoluble-bound phenolic fractions, whereas no change was observed in the esterified fraction in comparison to their untreated counterparts. Moreover, HPP-treated flower had higher activity in the soluble phenolics, while no significant difference was found in the insoluble phenolics. The increasing trend of antioxidant activity could be associated with higher TPC in all three phenolic fractions, mainly in the free fraction. Previous studies have also claimed that the HPP increased antioxidant activity of various foods, including fruit purées and juices (da Silveira et al., 2019; Patras et al., 2009; Zhou et al., 2019).

The highest metal chelation activity was recorded in the free fraction of flower (30.61 mg EDTAE/ 100 g), followed by internal organs (25.67 mg EDTAE/ 100 g) and body wall (21.67 mg EDTAE/ 100 g). However, the total (free + esterified + insoluble-bound) metal chelation activity was higher in the body wall compared to other body parts. Thus, sea cucumber body wall is rich in its metal chelation activity, even though flower in the free fraction showed greater activity. Previous studies have reported that phenolics extracted from sea cucumbers show strong

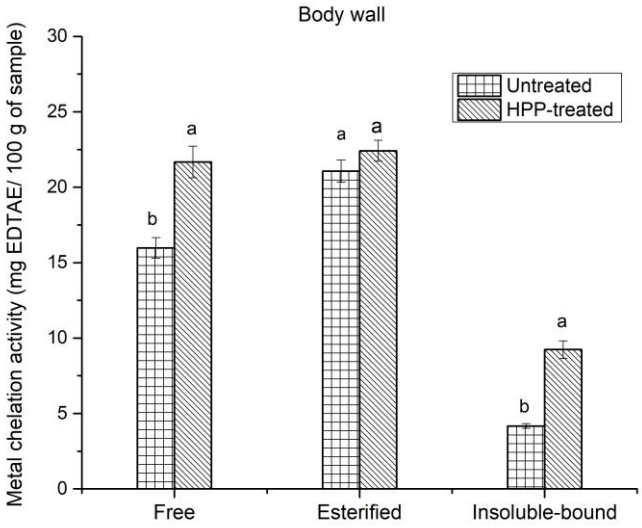
antioxidant activity such as radical scavenging activity and ORAC (Husni et al., 2009; Mamelona et al., 2007; Zhong et al., 2007). However, no study has been performed on the metal chelation activity of sea cucumber extracts, mainly phenolic extract.

Table 6. 6. Metal chelation activity (mg EDTA equivalents/ 100 g) of untreated and HP-treated sea cucumber.

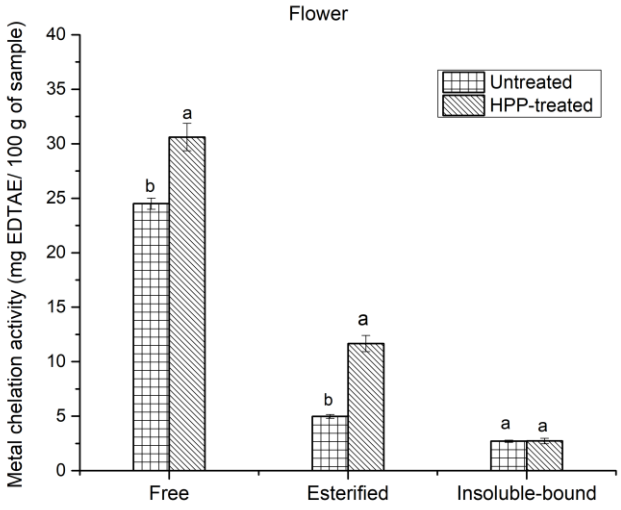
Treatment	Phenolics	Body part		
		Body wall	Flower	Internal organs
Untreated	Free	15.98 ± 0.68^{cB}	24.5 ± 0.52^{aA}	18.68 ± 0.28^{bA}
	Esterified	21.07 ± 0.74^{aA}	4.98 ± 0.18^{bC}	4.09 ± 0.2^{cC}
	Insoluble-bound	4.17 ± 0.16^{bC}	2.71 ± 0.1^{cB}	6.45 ± 0.42^{aB}
	Total	41.22	32.19	29.22
HPP-treated	Free	21.67 ± 1.05^{cA}	30.61 ± 1.25^{aA}	25.67 ± 1.05^{bA}
	Esterified	22.42 ± 0.7^{aA}	11.67 ± 0.75^{bB}	11.78 ± 0.75^{bB}
	Insoluble-bound	9.24 ± 0.58^{aB}	2.73 ± 0.25^{cC}	7.27 ± 0.58^{bC}
	Total	53.33	45.01	44.72

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters in the same row indicate significant differences ($p< 0.05$) among different body parts. Different uppercase letters in the same column indicate significant differences ($p< 0.05$) among different phenolic fractions.

(a)


Body wall
Metal chelation activity (mg EDTAE/ 100 g of sample)
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(b)


Flower
Metal chelation activity (mg EDTAE/ 100 g of sample)
Untreated
HPP-treated
Free
Esterified
Insoluble-bound

(c)

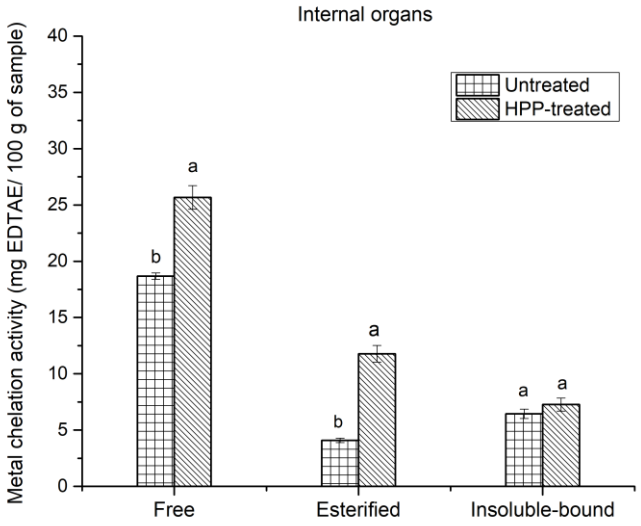


Figure 6. 6. Metal chelation activity of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber in mg EDTA equivalents (EDTAE) per 100 g of sample.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

6.5. Correlations between antioxidant activity versus TPC and TFC

Correlation coefficients describe the strength and direction of two variables that tend to change together. In other words, the correlation coefficient assesses if the two variables change proportionally to one another. Tables 6.7 and 6.8 show the correlation coefficient (r^2) of untreated and HPP-treated (6000 bar for 10 min) sea cucumber. In Table 6.7, regardless of HPP treatment, both TPC and TFC in all the sea cucumber body parts had a strong positive correlation with DPPH and ABTS radical scavenging activities, which suggests that phenolic compounds are the major components exhibiting antioxidant activity. However, HPP-treated samples had slightly higher r^2 values than the untreated counterparts, which indicates that HPP treatment enhanced antioxidant activity. Moreover, free and esterified phenolic fractions had a strong positive correlation than the insoluble-bound fraction, meaning that soluble phenolics are abundant in the Atlantic sea cucumber. Furthermore, TFC also showed a strong positive correlation with both the DPPH and ABTS radical scavenging activities, indicating that most of the sea cucumber phenolics are flavonoids. These results are in agreement with those of Ceesay et al. (2019), Husni et al. (2009), Mamelona et al. (2007), and Zhong et al. (2007), who noticed that sea cucumbers are likely to have higher flavonoids as they feed mainly on seaweeds which are rich in flavonoids. However, hydroxyl radical scavenging and metal chelation activities indicated a lower correlation compared to the other two methods, meaning that these assays are probably not the most suitable for determining the antioxidant activity of sea cucumber. In particular, a negative correlation was observed between the TFC and hydroxyl radical scavenging as well as metal chelation activities in the insoluble-bound fraction. Generally, variations among assays determining antioxidant activity could be explained by their specific mechanism of actions. Therefore, the compounds

responsible for antioxidant activity of sea cucumber could be due to the presence of phenolics, which improves upon HPP treatment.

In Table 6.8, regardless of sea cucumber body parts, both TPC and TFC in all three phenolic fractions positively correlated with the DPPH and ABTS radical scavenging activities, suggesting that these assays are the most sensitive approaches to evaluate the antioxidant activity of sea cucumber. Our results support the previous findings that sea cucumber phenolics are positively correlated with antioxidant activity (Mamelona et al., 2007; Husni et al., 2009). However, hydroxyl radical and metal chelation activities had a lower correlation with the TPC and TFC compared to the other two assays. Zhong et al. (2007) found no correlation between TPC and radical scavenging capacity of phenolics obtained from *C. frondosa*, and suggested that other compounds such as carotenoids, vitamin E, and terpenoids could also influence the antioxidant activity. Furthermore, TPC and TFC of flower and internal organs had a higher correlation with the antioxidant assays compared to the body wall, suggesting that flower and internal organs of sea cucumber are a good source of phenolics. These findings are in agreement with other reports which stated that phenolics are abundant in sea cucumber flower and internal organs, while the body wall is a good source of proteins and polysaccharides (e.g., chondroitin sulfate) (Bordbar et al., 2011; Hossain et al., 2020a).

Table 6. 7. Correlation coefficients (r^2) among total phenolics (TPC), total flavonoids (TFC), and antioxidant activities of different phenolic fractions of untreated and HPP-treated sea cucumber.

Treatment	Content	Phenolic fraction	Correlation coefficients (r^2)			
			DPPH radical	ABTS radical	Hydroxyl radical	Metal chelation
Untreated	TPC	Free	0.89	0.8	0.84	0.88
		Esterified	0.80	0.86	0.89	0.89
		Insoluble-bound	0.82	0.84	0.63	0.41
	TFC	Free	0.90	0.88	0.73	0.88
		Esterified	0.89	0.9	0.89	0.79
		Insoluble-bound	0.83	0.85	0.06	-0.15
HPP-treated	TPC	Free	0.89	0.82	0.89	0.79
		Esterified	0.85	0.88	0.79	0.85
		Insoluble-bound	0.87	0.81	0.81	0.77
	TFC	Free	0.92	0.91	0.89	0.89
		Esterified	0.91	0.89	0.89	0.88
		Insoluble-bound	0.87	0.85	-0.99	0.66

Table 6. 8. Correlation coefficients (r^2) among total phenolics (TPC), total flavonoids (TFC), and antioxidant activity of different sea cucumber body parts.

Treatment	Content	Body part	Correlation coefficients (r^2)			
			DPPH radical	ABTS radical	Hydroxyl radical	Metal chelation
Untreated	TPC	Body wall	0.82	0.79	0.68	0.41
		Flower	0.88	0.89	0.87	0.79
		Internal organs	0.81	0.88	0.81	0.79
	TFC	Body wall	0.83	0.79	0.52	0.21
		Flower	0.89	0.89	0.89	0.68
		Internal organs	0.86	0.81	0.77	0.7
HPP-treated	TPC	Body wall	0.82	0.8	0.74	0.61
		Flower	0.89	0.89	0.79	0.83
		Internal organs	0.83	0.81	0.88	0.81
	TFC	Body wall	0.81	0.82	0.57	0.42
		Flower	0.89	0.89	0.89	0.82
		Internal organs	0.89	0.88	0.78	0.74

6.6. Summary

The results in the present study suggest that HPP pre-treatment significantly enhances the TPC and TFC in the free, esterified, and insoluble-bound phenolic fractions of Atlantic sea cucumber. Among the three different phenolic fractions, the free phenolic fraction had the highest TPC and TFC and showed strong antioxidant activities, regardless of HPP pre-treatment. The highest amount of TPC and TFC was observed in the flower in the free phenolic fraction, followed by internal organs and body wall. In contrast, the TPC (free + esterified + insoluble-bound phenolics) was higher in the body wall, while the TFC was higher in flower, suggesting that sea cucumber phenolics are mostly flavonoids and are present in the flower.

The results here suggest that the antioxidant activity does not result in a similar way when determined using different assays. The HPP positively affected the DPPH radical scavenging and metal chelation activities, while the opposite scenario was observed in the ABTS radical scavenging activity of phenolics extracted from sea cucumber. Moreover, HPP had mixed effects on the hydroxyl radical scavenging activity. This is because every assay has a unique mechanism of action and behaves differently. It has been suggested that a single antioxidant method does not always display all antioxidants in a mixed or complex system (Shahidi, 2015; Shahidi & Zhong, 2015). On the other hand, the highest level of antioxidant activity was found in the free fraction of flower, followed by internal organs and body wall. On the contrary, the total (free + esterified + insoluble-bound) antioxidant activity was higher in flower, followed by internal organs and body wall, even though we have observed the TPC was higher in the body wall. Thus, although HPP increases the TPC of the body wall, those composites may not only be phenolic compounds and may include other compounds such as reducing sugars, proteins, vitamin E, and terpenoids, among others. This finding is also agreed with the correlation coefficient, where TPC and TFC of flower

had a strong correlation with the antioxidant assays compared to the body wall, suggesting that the flower of sea cucumber is the main source of phenolics. Consequently, HPP pre-treatment could be used as an effective approach to enhance the antioxidant activities and bioactivities of phenolic-rich extracts from sea cucumber, expanding the application areas and the economic value of this marine animal in the functional food and nutraceutical industry.

Chapter 7

Antioxidant activity of sea cucumber phenolics in food and biological model systems

7.1. Introduction

Lipid oxidation triggers the formation of off-flavor and generation of potentially toxic components, which decrease the nutritional value of foods. Autoxidation is the most common lipid oxidation pathway occurring in food, where free radicals result in further adverse consequences once consumed (Oh & Shahidi, 2018). Moreover, lipid oxidation can also occur in various cells in the body, hence initiating aging and a myriad of diseases like cardiovascular ailments, cancer, and immune system deficiencies (Rodriguez-Amaya & Shahidi, 2021). Thus, antioxidants, mainly phenolic compounds, have been used in improving oxidative stability of foods and preventing the oxidative stress in the human body. These antioxidants can hinder free radical chain reactions by donating an electron or a hydrogen atom to free radicals. Several studies have shown the therapeutic effect of phenolic compounds such as inhibition of formation of advanced glycation end-products (AGEs), low-density lipoprotein (LDL) oxidation, DNA oxidation, tyrosinase activity, α-glucosidase activity, and anti-hyperglycemic property, among others. In addition, phenolic compounds can also control the oxidation of meat, fish, or oily products during storage, which can be checked by evaluating their oxidation products (Oh, Ambigaipalan, & Shahidi, 2021; Shahidi & Ambigaipalan, 2015). Therefore, using extracts rich in phenolic compounds from natural sources in food and medicine has become increasingly popular in recent years.

Sea cucumber contains a number of health-promoting components, including phenolics, which are responsible for effective biological activities such as antioxidant, anti-inflammatory, anticancer, hepatoprotective, and antibacterial properties (Hossain et al., 2020a). For example, phenolics compounds of sea cucumbers, including *H. tubulosa, H. scabra, H. leucospilota, S.*

variegatus, and *S. chloronotus*, could serve as a good source for the development of anticancer agents, especially against colon, breast, cervical, and human non-small lung cancers (Alper & Güneş, 2020; Althunibat et al., 2009; Ridhowati et al., 2018). Moreover, Pranweerapaiboon et al. (2020) stated that phenolic-rich *H. scabra* extracts inhibited pro-inflammatory cytokines synthesis, mainly inducible nitric oxide synthase (iNOS), nitric oxide (NO), interleukin-1β (IL-1β), prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α). Similarly, *S. japonicus* phenolics inhibited the production of PGE_2 and NO by controlling iNOS and cyclooxygenase-2 (COX-2) (Himaya, et al., 2010). Furthermore, phenolic-rich *H. atra* extract had good curative, hepatoprotective, and antioxidant activity against 7,12-dimethylbenz[a]anthracene (DMBA)-induced hepatorenal dysfunction in the rat model (Dakrory et al., 2015). Likewise, phenolic compounds (mainly chlorogenic acid, pyrogallol, catechin, rutin, pyrogallol, and coumaric acid) of sea cucumber (*H. atra*) demonstrated notable hepatoprotective activity against thioacetamide (TAA)-induced liver fibrosis in rats (Esmat et al., 2013). In addition, Fahmy (2015) reported the anticholestatic effects of phenolic compounds (mostly pyrogallol, rutin, chlorogenic acid, and coumaric acid) extracted from *H. arenicola*. Besides, phenolic-rich *H. atra* and *H. foskali* extracts showed antibacterial activity against *B. subtilis*, *B. cereus*, *E. coli*, *P. aeruginosa*, and *P. aeruginosa* (Sukmiwati et al., 2019; Telahigue et al., 2020). Furthermore, Nguyen and Kim (2015) stated that the α-glucosidase inhibitory activity of sea cucumber (*S. japonicas*) internal organs and suggested that this could be used in preventing of obesity and diabetes mellitus.

Antioxidant activities of phenolics such as ORAC and free radical scavenging activity of different species of sea cucumber have been determined. However, bioactivities such as inhibitory activity against DNA strand breakage, tyrosinase, α-glucosidase, formation of AGEs, and oxidation of LDL cholesterol of sea cucumber phenolics have not yet been studied, especially for

the free, esterified, and insoluble-bound phenolic fractions. Thus, the aim of this study was to evaluate the antioxidant potential of phenolics obtained from Atlantic sea cucumber in food and biological model systems.

7.2. Antioxidant activity in a fish model system (thiobarbituric acid reactive substances, TBARS)

The thiobarbituric acid (TBA) assay was used to determine the antioxidant activity of sea cucumber phenolics in a fish (Atlantic salmon) model system. The secondary oxidation products such as aldehydes and ketones are involved in the development off-flavor, causing quality deterioration of muscle foods (Shahidi & Zhong, 2015). Therefore, TBA assay is used to determine these secondary lipid oxidation products (malonaldehyde, MDA) and related compounds using the spectrophotometric method. The TBA reagent reacts with the MDA and other TBA reactive substances (TBARS) in samples and develops an MDA-TBA adduct, among other. This adduct produces pink color, which can be measured at 530-535 nm using a spectrophotometer (Shahidi, 1998).

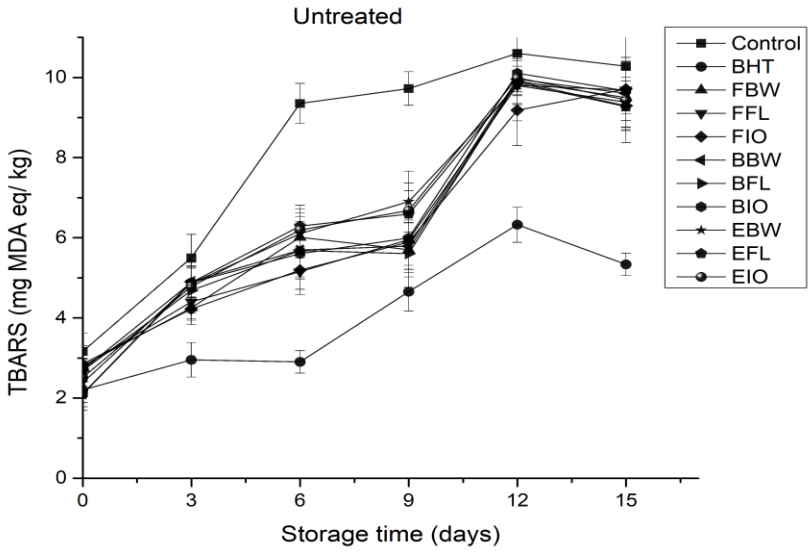
In this study, TBARS was calculated as MDA equivalents, and the results are shown in Figure 7.1 for untreated and HPP-treated samples. The samples treated with butylated hydroxytoluene (BHT), synthetic antioxidant, are considered as a positive control, whereas samples prepared without any sea cucumber phenolics are categorized as a control. As shown in Figure 7.1, samples treated with phenolics obtained from untreated sea cucumber demonstrated an increasing trend of TBARS during cold storage (4°C) for 15 days. Similar trends were observed for the HPP-treated sea cucumber, where the lowest TBARS values were observed at the beginning of the storage after cooking (day 0). Overall, no significant difference was found between samples

treated with phenolics extracted from untreated and HPP-treated samples. From day 0 to 15, all samples displayed higher and lower TBARS values for the control and positive control, respectively. Meanwhile, soluble phenolics of sea cucumber exhibited a better inhibition of the formation of TBARS when compared with the insoluble-bound phenolics. The TBARS values for all treated samples with phenolic extracts were below 7 mg MDA eq/ kg up to 9 days of storage and then increased to around 10 mg MDA eq/ kg at the end of storage. Generally, TBARS value below 8, 5, and 3 mg MDA eq/ kg is considered suitable for human consumption, good quality, and perfect quality, respectively, for fish and fish products (Socaciu, Semeniuc, & Vodnar, 2018). However, in our study, the TBARS for the control sample reached 9.31 mg MDA eq/ kg on the 6^{th} day of storage, while the sample treated with BHT maintained the TBARS value below 6.32 mg MDA eq/ kg throughout the storage. Therefore, it can be concluded that the sample treated with sea cucumber phenolics maintained the quality of Atlantic salmon up to 9 days of storage, while the control sample was unacceptable for human consumption after 3 days of storage. Our results are in accordance with those of Castro et al. (2019) and Goulas et al. (2019) who reported that polyphenolic coating/ extract maintained the TBARS values of Atlantic salmon in an acceptable limit up to 17 days of storage period.

The sample treated with sea cucumber body wall, flower, and internal organs extracts in the free phenolic fraction, on the other hand, showed a higher inhibitory activity up to 9 days. This could possibly be due to the radical scavenging activities and metal chelating ability of phenolics obtained from different body parts of sea cucumber. However, the TBARS values for all samples, including samples treated with phenolics, control, and positive control (treated with BHT), remained constant or started reducing after 12 days of storage. The reduction of TBARS values does not indicate lower oxidation occurs after 12 days; however, it may be due to the reaction of

secondary oxidation products with other compounds (e.g., amine), resulting in lower TBARS values. Moreover, microbial attack increases with storage time which reduces the content of substrates that can react with the TBA reagent. As a result, TBARS values start to decline after certain periods due to the formation of less color complex with the TBA reagent (Liu et al., 2018b; Kim et al., 2004). Thus, the results suggest that sea cucumber phenolics have the potential to use as natural antioxidants in order to inhibit lipid oxidation.

[a]



[b]

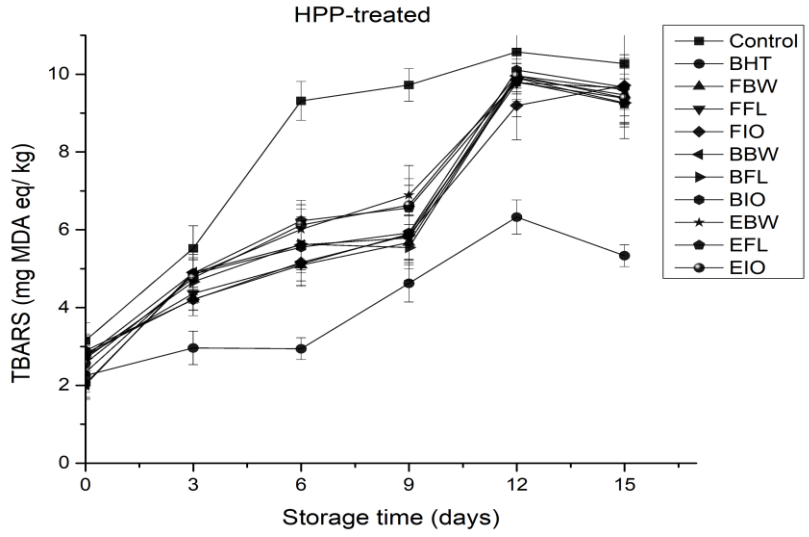


Figure 7. 1. TBARS values of untreated [a] and HPP-treated [b] sea cucumber phenolics in a fish model system. Data represent mean values for each sample ± standard deviation (n=3). Abbreviations are: BHT, butylated hydroxytoluene; F, free phenolics; B, insoluble-bound phenolics; E, esterified phenolics, BW, body wall; FL, flower; and IO, internal organs.

7.3. α-Glucosidase inhibitory activity of sea cucumber

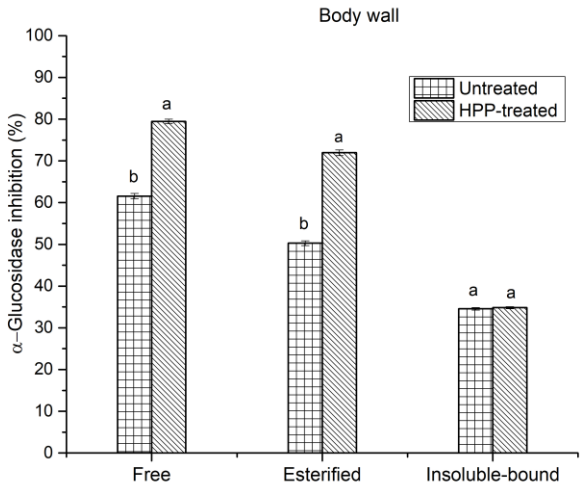
α-Glucosidase hydrolyzes the dietary carbohydrates into simple sugars, hence enabling their gastrointestinal absorption. This digestive enzyme is located on the epithelium of the small intestine and is involved in the hydrolysis of nonreducing 1,4-linked α-D-glucose residues of sugars and releases α-D-glucose. Due to the build-up of sugar in the blood instead of being used for energy, type-2 diabetes may occur when the body does not utilize the insulin it makes, or the pancreas does not generate enough insulin (Rahman, de Camargo, & Shahidi, 2017). In particular, the release of insulin increases due to the high concentration of sugar present in the bloodstream in order to absorb the excess amount. However, insulin resistance can occur due to chronic high blood sugar, leading to type-2 diabetes and triggering severe complications such as stroke, heart disease, and diabetic retinopathy (Yeo & Shahidi, 2020). Therefore, type-2 diabetes can be prevented by controlling the sugar content in the bloodstream. As a result, inhibition of α-glucosidase activity is one of the main pathways for controlling/ preventing type-2 diabetes since the enzyme generates more sugar and raises sugar levels in the bloodstream.

The α-glucosidase inhibitory activity of phenolics in HPP-treated and untreated sea cucumber was assessed, and the results are presented in Figure 7.2. According to the results, soluble phenolics in HPP-treated sea cucumber demonstrated a higher α-glucosidase inhibitory activity than the untreated counterparts, whereas HPP did not have any effect on the insoluble-bound phenolics. For example, the α-glucosidase activity of the HPP-treated free phenolic fraction was 79.44, 79.69, and 80.17% for body wall, flower, and internal organs, respectively, whereas these percentages decreased to 61.56, 78.23, and 64.32%, respectively for the untreated counterparts. Similarly, α-glucosidase activity increased by 43.09, 59.21, and 28.94% for body wall, flower, and internal organs, respectively, in the esterified phenolic fraction upon HPP. The

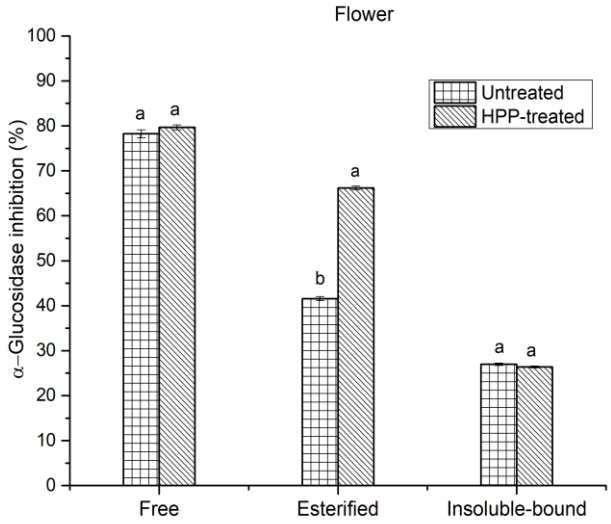
inhibition of α-glucosidase property and TPC were positively correlated (r^2= 0.78), indicating that the amount of phenolics present in each fraction could contribute to the inhibitory activity. Lu et al. (2021) stated that HPP-treated lemon (*C. limon*) flavedo had a higher inhibitory rate against carbohydrate hydrolyzing enzymes (e.g., α-amylase and α-glucosidase) than the samples treated with ultrasound-assisted extraction. This could be related to the release of functional compounds, such as phenolic compounds, from cell wall upon HPP that inhibits the activity of carbohydrate hydrolyzing enzymes.

On the other hand, the highest and similar activity was observed in the free phenolic fraction of flower, body wall, and internal organs, while the opposite scenario was seen in the insoluble bound phenolic fraction. The actual mechanism of phenolic compounds in order to inhibit the α-glucosidase enzyme is still unknown. However, phenolic compounds, mainly their hydroxyl groups, may bind with this enzyme via hydrogen bonding and delay glucose absorption (Rahman et al., 2017). α-Glucosidase inhibitory activity of phenolics has not been determined from any species of sea cucumber; however, solvent fractions obtained from the internal organs of *S. japonicas* showed strong α-glucosidase inhibitory activity, which could be used for the prevention of obesity and type-2 diabetes (Nguyen & Kim, 2015).

[a]


Body wall
α–Glucosidase inhibition (%)
Untreated
HPP-treated
Free
Esterified
Insoluble-bound
a
b
a
b
a
a

[b]


Flower
α–Glucosidase inhibition (%)
Untreated
HPP-treated
Free
Esterified
Insoluble-bound
a
a
b
a
a
a

[c]

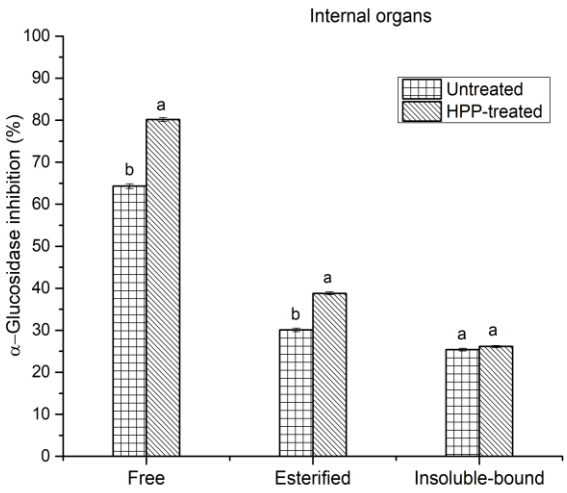


Figure 7. 2. α-Glucosidase inhibitory activity of untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

7.4. Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation

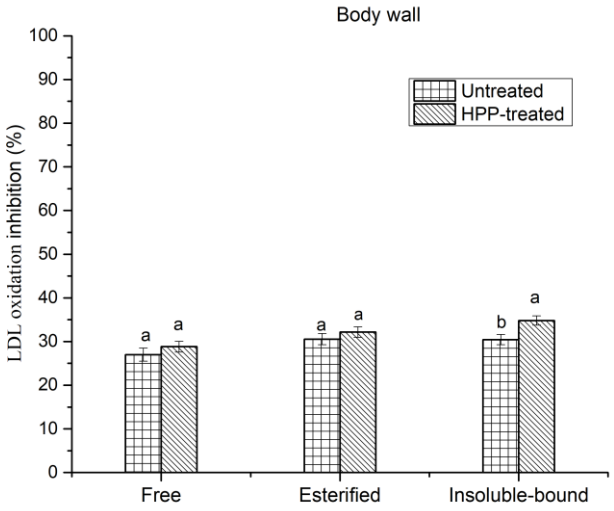
LDL cholesterol is known as a "bad cholesterol" and is mainly composed of cholesterols, phospholipids, and triacylglycerols (TAGs), among others. LDL plays a major role in transporting cholesterol and TAGs to cells in the human body by acting as a shuttle. LDL is susceptible to oxidation due to the presence of lipid components. As a result, oxidation of LDL is believed to be one of the main risk factors in developing atherosclerosis, major reason for cardiovascular disease (CVD) development. This could occur via narrowing of blood vessels and ultimately blocking the bloodstream when cholesterol gets oxidized in the bloodstream and deposits inside the arterial wall (Chandrasekara & Shahidi, 2011a). Therefore, preventing the oxidation of LDL cholesterol could be an effective way to control CVD. The oxidation of LDL is initiated by the action of metal ions or reactive oxygen species (ROS). Moreover, enzymes such as xanthine oxidase and 15-lipoxygenase could be responsible for the oxidation of LDL-cholesterol by producing lipid hydroperoxides from polyunsaturated fatty acids (PUFAs) (Rahman et al., 2017).

In the present study, copper-induced LDL oxidation was initiated by copper sulphate, and the oxidative susceptibility of LDL was determined by monitoring the formation of conjugated dienes (CD, primary oxidation product) using a spectrophotometer at 234 nm (Figure 7.3). The increase in CD is associated with the oxidation of phospholipids and TAGs (Kittiphattanabawon et al., 2013). According to the results, a near constant absorbance was reached at 8 hr of incubation and this was used to calculate the oxidation of LDL cholesterol. Moreover, the absorbance of the control (LDL cholesterol without phenolics) increased gradually throughout the incubation period, which indicates the formation of oxidation products, mainly CD. No significant ($p>0.05$) difference was found between HPP-treated and untreated samples except for the flower in the free

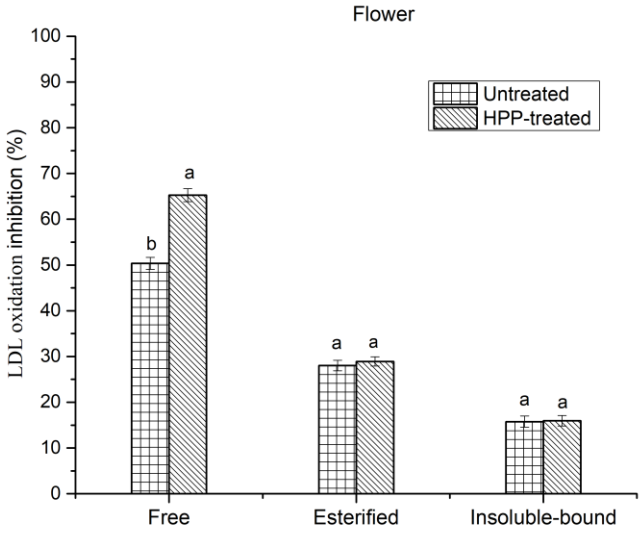
phenolic fraction and body wall in the insoluble-bound phenolic fraction, where HPP-treated samples showed higher inhibitory activity than their untreated counterparts.

Inhibition of LDL cholesterol oxidation of free phenolic fraction ranged from ~29 to ~66% for the HPP treated samples, whereas that for their untreated counterparts was ~27 to ~51%. A higher inhibition was obtained from the free phenolic fraction of the flower, while the lowest was for the insoluble-bound phenolic fraction of the internal organs. Several studies have shown that phenolic compounds have the potential to inhibit LDL cholesterol oxidation (Ambigaipalan et al., 2016; Oh, Ambigaipalan, & Shahidi, 2021; Rahman et al., 2017). This could be due to the free radical scavenging activity as well as the chelating ability of phenolics, which can remove cupric ions from the LDL cholesterol (Chandrasekara & Shahidi, 2012b). The LDL oxidation inhibition activity and TPC of HPP-treated sea cucumber were moderately correlated (R^2= 0.62), suggesting that LDL oxidation inhibition activity depended on both the quantity of phenolic compounds as well as the type of phenolics present. Therefore, these findings indicate that phenolics of sea cucumber may serve as antioxidants in minimizing LDL cholesterol oxidation.

[a]


Body wall
Untreated
HPP-treated
LDL oxidation inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
a
a
a
a
b
a
Free
Esterified
Insoluble-bound

[b]


Flower
Untreated
HPP-treated
LDL oxidation inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
b
a
a
a
a
a
Free
Esterified
Insoluble-bound

[c]

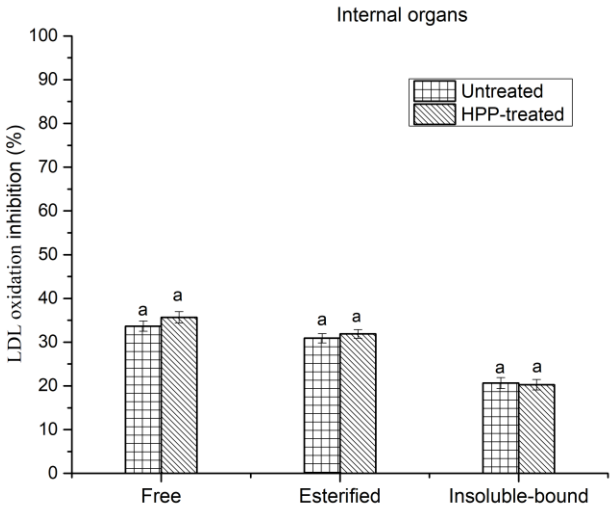


Figure 7. 3. Inhibition against human LDL cholesterol oxidation by untreated and HPP-treated body wall [a], flower [b], and internal organs [c] of sea cucumber.

Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters for the same phenolic fraction indicate significant differences ($p < 0.05$) among treatments.

7.5. Inhibition of hydroxyl and peroxyl radical-induced supercoiled DNA strand scission

DNA damage by free radicals could lead to cell mutation, which causes several pathological conditions such as aging and cancer. DNA damage may occur upon the action of ROS and it includes base modification, DNA strand breakage, production of base-free sites, protein-DNA crosslinks, and chromosomal arrangements (Chandrasekara & Shahidi, 2011b). Free radicals can damage the DNA at both the nucleotide bases and phosphate backbone. ROS generates continuously at the mitochondria via Fenton's reaction and involves in the metabolism of cells in the body. For instance, hydroxyl radicals can be produced from the degradation of hydrogen peroxide and interaction between hydrogen peroxide and superoxide. However, a high concentration of ROS may lead to oxidative stress, which is responsible for the oxidation of DNA (Chandrasekara, Daugelaite, & Shahidi, 2018). Therefore, it is important to prevent hydroxyl radical- and peroxyl radical-induced DNA scission to avoid killing the living cells. In this study, the inhibitory activity of phenolic compounds against free radicals induced DNA strand scission was determined in supercoiled plasmid DNA. Due to the oxidation of DNA induced by free radicals, the supercoiled DNA could change its shape to nicked open circular and linear forms. Thus, the inhibitory property of phenolics can be evaluated by monitoring the conformation of nicked DNA and intact DNA strand fractions using agarose gel electrophoresis. Usually, supercoiled DNA (intact DNA) could pass through the agarose gel network easily, while linear/ nicked open forms of DNA (oxidized DNA) showed restricted movements (Ambigaipalan & Shahidi, 2015; Shahidi & Zhong 2015).

In the present study, hydrogen peroxide and Fe^{2+} were used to produce hydroxyl radical, whereas AAPH was used to generate peroxyl radical. As presented in Figure 7.4, supercoiled plasmid DNA in control (DNA, buffer, and AAPH) was completely oxidized and formed open

circular DNA by peroxyl radicals. However, blank (DNA and buffer) and samples (DNA, buffer, AAPH, and phenolic extracts) nearly maintained their intact forms and moved more rapidly through an agarose gel network. Areas of these bands were used to determine the inhibitory activity of sea cucumber phenolic extracts. It was found that almost all three phenolic fractions obtained from HPP-treated sea cucumber showed strong inhibitory activity against hydroxyl radical- and peroxyl radical-induced DNA scission, regardless of body parts (Figure 7.5). However, the inhibitory activity against peroxyl radical was slightly higher than the hydroxyl radical. For example, the inhibition of DNA scission induced by peroxyl radical ranged from ~83 to 91%, while this value was ~73 to 79% for hydroxyl radical in the free phenolic fraction. These observations are in accordance with the finding of Chandrasekara and Shahidi (2011b), who claimed that peroxyl radicals had 2-3 times higher inhibitory activity than that of hydroxyl radicals. Due to the different half-life of each free radical, the differences between obtained values for peroxyl radical and hydroxyl activities can be observed. For instance, peroxyl radical has a comparatively prolonged half-life and has a greater affinity to diffuse into cells when compared to the extremely reactive hydroxyl radical (Kittiphattanabawon et al., 2013). Moreover, the inhibitory effect of peroxyl radical-induced DNA oxidation was varied from ~77 to ~86% in body wall, ~71 to ~91% in flower, and ~74 to ~91% in internal organs. The higher inhibitory activity was obtained by the free phenolic fraction, followed by insoluble-bound and esterified phenolic fractions of sea cucumber. Even though insoluble-bound phenolic fractions were not as efficient as their free counterparts regarding TPC and antioxidant activity, they demonstrated greater antioxidant properties in terms of inhibiting DNA oxidation. This could be the type of phenolic compound presence in a particular phenolic fraction and shows antioxidant property in biological systems (Ambigaipalan et al., 2016). There were no similar reports documented in the literature for sea

cucumber phenolic extracts in terms of hydroxyl and peroxyl radical-induced DNA strand scission. Nevertheless, phenolic extracts from many sources have been applied to hinder free radical-induced DNA strand scission and found that phenolic compounds had the potential to inhibit the oxidation of DNA (Chandrasekara & Shahidi, 2011b; Madhujith & Shahidi 2007; Rahman et al., 2017; Yeo et al., 2021). Therefore, the protective effect of sea cucumber extracts is possibly due to the scavenging and metal chelation activities of phenolics. In particular, the correlation coefficient between the inhibition of hydroxyl radical-induced DNA scission and hydroxyl radical scavenging activity is 0.69. It has been reported that the correlation coefficient between 0.7 to 0.9 could be defined as being highly positive (Mukaka, 2012). Therefore, phenolic compounds extracted from sea cucumber may serve as a good source of food ingredients for the prevention of the oxidation of DNA.

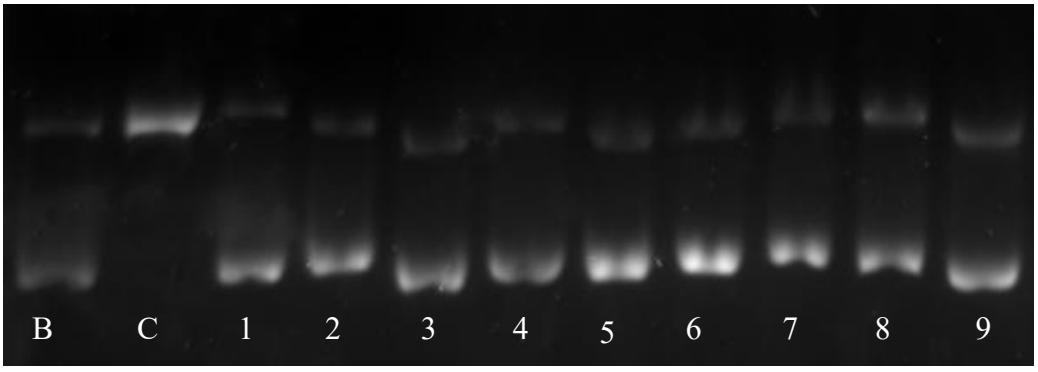
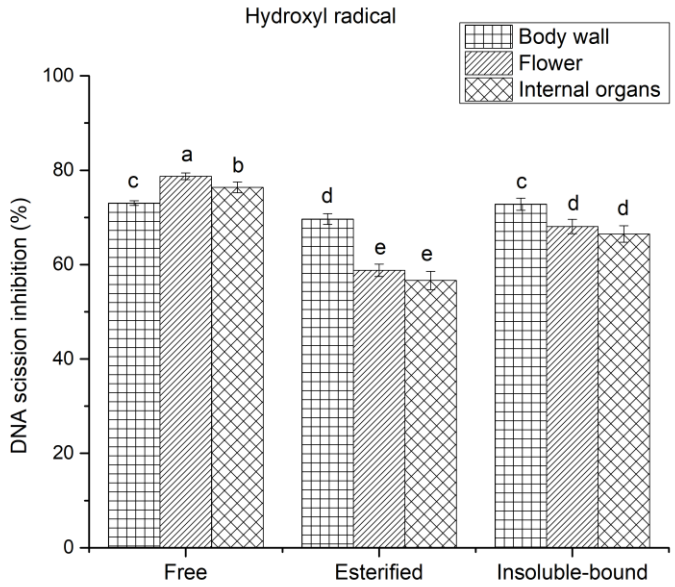


Figure 7. 4. Agarose gel electrophoresis of inhibition of peroxyl radical. Lanes: blank (B); control (C); free-body wall (1); free-flower (2); free-internal organs (3); esterified-body wall (4); esterified-flower (5); esterified-internal organs (6); insoluble-bound-body wall (7); insoluble-bound-flower (8); and insoluble-bound-internal organs (9).

[a]



[b]

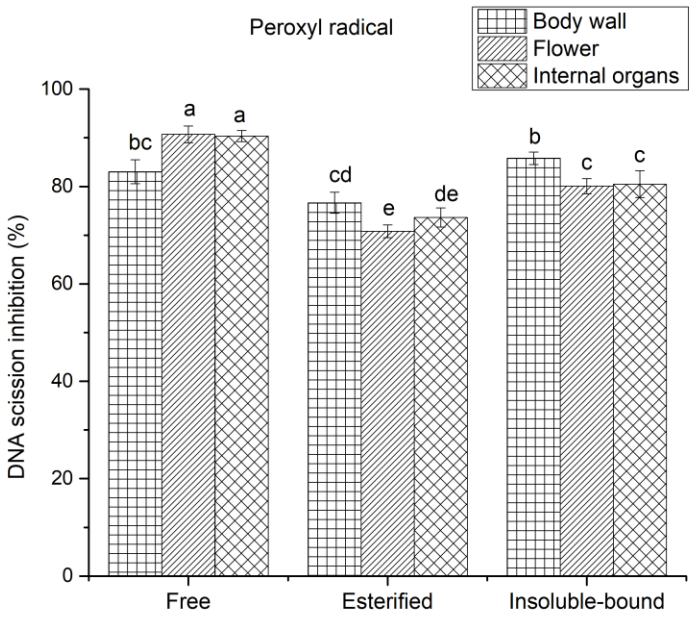


Figure 7. 5. Inhibition of hydroxyl [a] and peroxyl [b] radical induced DNA scission by HPP-treated sea cucumber phenolics. Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters indicate significant differences ($p < 0.05$) among treatments.

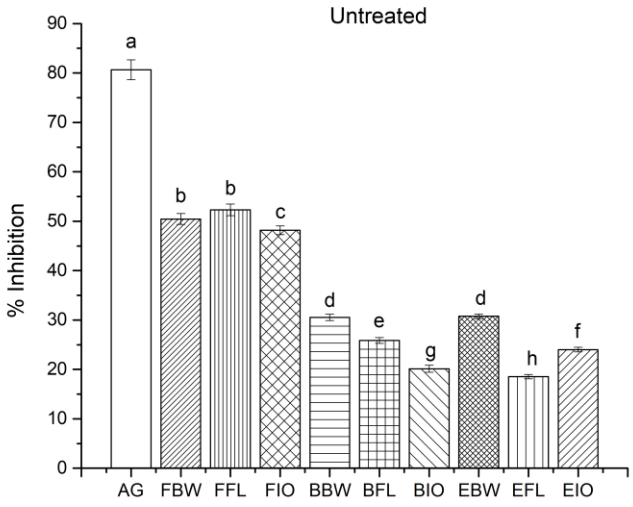
7.6. Antiglycation activity of sea cucumber phenolics

Advanced glycation end products (AGEs) are formed through nonenzymatic reaction of the carbonyl group of reducing sugars or other aldehydes that arise from lipid oxidation with proteins. AGEs are generated in the advanced stage of Maillard reaction when the intermediate components form an unstable Schiff base adduct which rearranges to a comparatively stable Amadori product (Hu, Wang, & Shahidi, 2020; Ramkissoon et al., 2013). AGEs are a group of complex and heterogeneous biomolecules, including fluorescent pentosidine, crossline, pyralline, glyoxal (GO), methylglyoxal (MGO), carboxyethyllysine (CEL), and non-fluorescent carboxymethyllysine (CML), generated in cells during oxidation of glucose and proteins/ lipids (Piwowar et al., 2019). During food processing, heating, and storage, AGEs can be generated; however, excessive accumulation of AGEs has been linked to many ailments, including diabetes, cardiovascular disease, neuropathy, retinopathy, atherosclerosis, nephropathy, aging, Alzheimer, and some types of cancer (Chen, Virk, & Chen, 2016; Khan et al., 2020). Therefore, formation of AGEs can be inhibited by blocking sugar attachment, breaking sugar-protein cross-links, and scavenging free radicals and carbonyls generated from lipid or sugar oxidation. Phenolic compounds have been reported to have significant inhibitory activity against glycation, possibly due to their ability to scavenge free radicals, chelate metal ions, and trap reactive carbonyl species through adduct formation (Wang et al., 2016b).

The antiglycation activity of phenolics in HPP-treated and untreated sea cucumber was assessed, and the results are presented in Figure 7.6. According to the results, phenolics extracted from HPP-treated sea cucumber demonstrated a higher inhibitory activity compared to their untreated counterparts, except for esterified phenolic fractions of flower and internal organs. A higher inhibitory activity was obtained for the free phenolic fraction, followed by insoluble-bound

and esterified fractions. For example, the antiglycation activity of HPP-treated free phenolic fraction was 63.54, 74.76, and 68.23% for body wall, flower, and internal organs, respectively, whereas for their untreated counterparts these values decreased to 50.45, 52.29, and 48.17%, respectively. Moreover, HPP-treated flower showed a higher activity in the free phenolic fraction than the other body parts, regardless of the phenolic fraction. However, the higher anti-glycation effect (80.65%) was observed by aminoguanidine (positive control), a typical inhibition agent of AGEs. These observations are in accordance with the inhibitory activity of phenolic-rich kiwiberry (*A. arguta*) extract, where HPP treatment significantly increased (almost two-fold) the anti-glycation potential compared to untreated samples (Błaszczak et al., 2021). Moreover, phenolic extracts have been employed to control the formation of AGEs, which exhibited antiglycation activity (Di Sotto et al., 2019; Wang et al., 2016b; Yeh et al., 2017; Zhang, Chen, & Wang, 2014). For instance, Zhang et al. (2014) reported that phenol-rich extract of *Punica granatum* could inhibit the formation of AGEs due to its antioxidant properties. Therefore, the inhibitory activity of sea cucumber phenolics might be related to the phenolics' free radical scavenging and metal chelation activities. Moreover, it was found that the inhibitory activity against the formation of AGEs was positively correlated with hydroxyl radical scavenging (R^2= 0.78) and metal chelation (R^2= 0.92) activities. Thus, these results show that sea cucumber with its phenolic compounds may serve as a good source of food ingredients for minimizing the formation of AGEs.

[a]



[b]

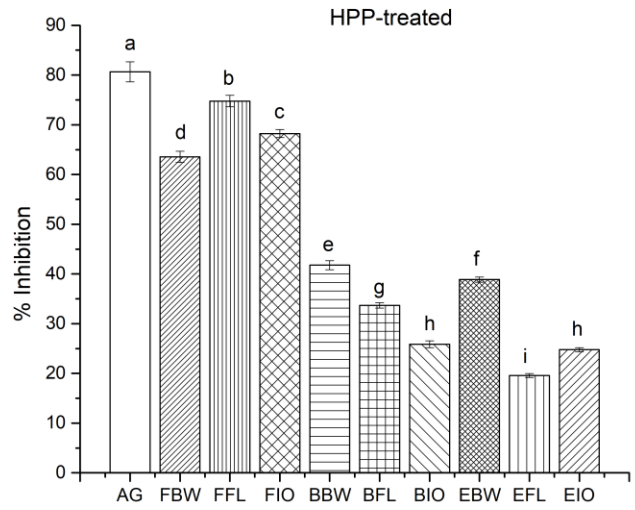


Figure 7. 6. Antiglycation activity of untreated [a] and HPP-treated [b] sea cucumber. Data represent mean values for each sample ± standard deviation (n=3). Different lowercase letters indicate significant differences ($p<0.05$) among treatments. Abbreviations are: AG, aminoguanidine; F, free; B, insoluble-bound; E, esterified, BW, body wall; FL, flower; and IO, internal organs.

7.7. Summary

The HPP pre-treatment significantly enhanced the antioxidant activity in food and biological systems. Hence, HPP pre-treatment could be an effective approach to improve the antioxidant activity and bioactivity of phenolic compounds of sea cucumber. Among the three different phenolic fractions, the free phenolic fraction had the highest bioactivities, regardless of HPP pre-treatment. However, the insoluble-bound phenolic fraction also showed strong biological activities even though the level of TPC was lower in that fraction. Thus, it can be concluded that biological activities do not depend fully on the quantity of phenolics present but on the type of phenolics present. Moreover, the highest biological activities were observed in the free phenolic fraction of flower, followed by internal organs and body wall. Additionally, biological activities were positively correlated with TPC and antioxidant activities, indicating that sea cucumber phenolics may be used as possible antioxidants in food and biological systems.

Chapter 8

Identification and quantification of phenolics in sea cucumber

8.1. Introduction

Sea cucumbers are considered as a delicacy and luxury food item in the Asian culture. Sea cucumbers are highly marketable echinoderms that contain a wide range of bioactive compounds, including phenolics, which have the potential in producing nutraceutical and pharmaceutical products (Dvoretsky & Dvoretsky, 2021). Phenolic compounds have a myriad of bioactivities, including antioxidants properties that can prevent, treat, or cure diseases like coronary heart disease and carcinogenesis. Studies have indicated that regular consumption of phenolic-rich foods can prevent the risk of type 2 diabetes, gastrointestinal cancers, cardiovascular diseases, and other disorders (Rahman, de Camargo, & Shahidi, 2018). The occurrence of phenolic compounds in sea cucumber could be due to their absorption from phytoplankton, which are the main food sources for sea cucumbers, mainly Atlantic sea cucumber. Phytoplankton is a rich source of phenolic compounds, including phenolic acids, flavonoids, and tannins (Hossain et al., 2020a; Zhong et al., 2007). The most common diatoms found in the sea cucumber diets are *C. gracilis, C. muelleri, C. calcitrans, D. euchlaia, I. galbana, P. tricornutum, R. salina, S. costatum* and *T. chuii*, which contain numerous bioactive compounds, including phenolics and carotenoids (Mercier & Hamel, 2013). For example, *I. galbana* and *Chaetoceros* sp. have been found to have shown strong antioxidant activity due to the presence of phenolic compounds (Goh, Yusoff, & Loh, 2010; Matos et al., 2019).

Various species of sea cucumber have different levels of phenolic compounds with varied antioxidant properties. This could be due to the different geographic locations, food habits, and harvesting times. Therefore, suspension-feeding species may have more phenolics compared to

deposit-feeding species. The most common phenolic compounds found in sea cucumbers, mainly *H. atra* and *H. tubulosa*, are gallic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, rutin, and pyrogallol. For example, high-performance liquid chromatography (HPLC) was used to identify the phenolic compounds of *H. atra* and found that the most common phenolics were chlorogenic acid (80.34%), followed by *p*-coumaric acid (2.43%), pyrogallol (2.25%), and rutin (0.82%) (Dakrory et al., 2015). Similarly, HPLC was used to identify phenolic compounds of *H. tubulosa*, and the major compounds were epicatechin (43.38%), ellagic acid (30.67%), and gallic acid (11.30%) (Alper & Günes, 2020). To date, no attempt has been made to identify and quantify phenolic compounds from Atlantic sea cucumber (*C. frondosa*), though this species contains a considerable amount of total phenolic with strong antioxidant activity. Therefore, the aim of this study was to investigate profiles of soluble and insoluble-bound phenolics present in Atlantic sea cucumber using UHPLC-QTOF-MS/MS.

8.2. Identification of phenolic compounds in HPP-treated and untreated sea cucumber body parts

8.2.1. Internal organs

Phenolic compounds were identified using UHPLC-QTOF-MS/MS by comparing their UV retention times (RT), observed $[M-H]^-$, and ion fragmentation patterns of authentic standards, including caffeic acid, gallic acid, sinapic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, cinnamic acid, protocatechuic acid, vanillic acid, ferulic acid, syringic acid, ellagic acid, chlorogenic acid, quercetin, and catechin. Other compounds with no standards were tentatively identified using mass spectrometry (MS^n), UV spectral, and literature data. For that, UV spectra and mass spectrometry of reference compounds were studied, and then fragmentation patterns were analyzed. After that,

a full mass scan was accomplished for samples, and unknown compounds were identified based on their RT, observed $[M-H]^-$, and MS^n data. HPLC chromatogram was captured by using a diode array detector (DAD) and the results for free phenolic fraction of HPP-treated internal organs are shown in Figure 8.1 and Table 8.1.

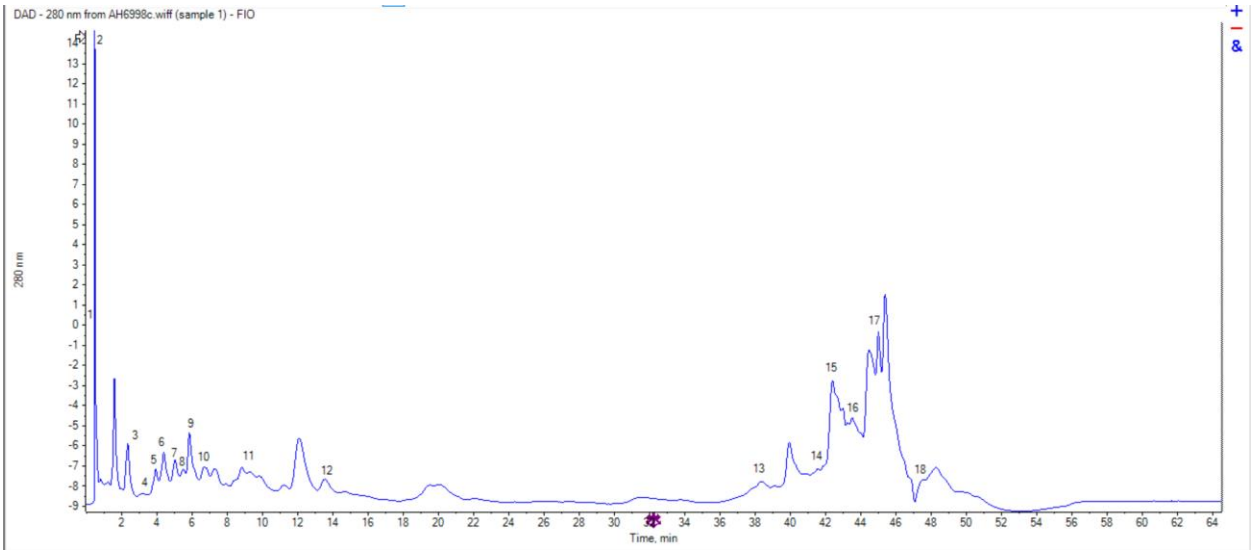


Figure 8. 1. Representative UHPLC chromatogram of free phenolic fraction of sea cucumber internal organs.

Table 8. 1. List of phenolic compounds identified from the free fraction of sea cucumber internal organs

C#	Compounds	$[M-H]^-$ (*m/z*)	RT (min)-UV	MS^2 ion fragments
1	Homovanillic acid	181	0.37	181, 137
2	Gallic acid	169	0.43	125
3	Protocatechuic acid	153	2.5	109
4	*p*-Hydroxybenzoic acid	137	3.52	121
5	Vanillic acid	167	4.56	105, 108, 121, 123
6	Catechin	289	4.99	203, 245
7	*p*-Hydroxybenzaldehyde	121	5.06	92
8	Chlorogenic acid	353	5.32	179, 191, 207
9	Syringic acid	197	5.36	109, 123, 153, 163, 179
10	*p*-Coumaric acid	163	6.78	119
11	Sinapinic acid	223	9.17	175, 179, 208
12	Hydroxygallic acid	187	13.5	125, 169
13	Quercetin	301	38.28	121, 151, 179, 255, 257, 273
14	Leachianol F	471	41.65	121, 153, 287, 349, 453
15	Cinnamic acid	147	42.17	103, 131, 135
16	Scopoletin	191	43.15	147
17	Caffeoyl glucoside	387	45.08	341
18	Ellagic acid	301	47.86	229, 257

Abbreviations are: RT, retention time; and C#, compound number

This study identified 18, 14, and 16 phenolic compounds in the free, esterified, and insoluble-bound phenolic fractions of HPP-treated internal organs, respectively. In contrast, 14, 11, and 13 compounds were identified from their untreated counterparts, respectively (Table 8.2). Consequently, considering these results along with the TPC and TFC, the contents of phenolic compounds were maximized when HPP was used as a pre-treatment. These trends are in agreement with those of Zhou et al. (2019), who were able to identify more phenolic compounds from different fractions of HPP-treated oil palm fruits than their untreated counterparts. Moreover, these results are in accordance with our previous data (section 6), where free phenolic fraction had a higher content of phenolic with strong antioxidant activity. Interestingly, a significant number of phenolic compounds were identified from the insoluble-bound phenolic fraction even though we observed a lower TPC for that fraction compared to the soluble phenolic fraction. Among these phenolic compounds, *p*-coumaric acid, gallic acid, hydroxygallic acid, syringic acid, ellagic acid, catechin, quercetin, and *p*-hydroxybenzaldehyde were found in all fractions, regardless of HPP treatment. However, caffeic acid was found only in the insoluble-bound phenolic fraction, while caffeoyl glucoside was available in the free phenolic fraction.

Compounds 1-7, 11, 13-15, and 18-19 were identified by comparing their retention times UV spectral data, and product ions with corresponding reference compounds (Table 8.2). Compound 1 exhibited a molecular ion $[M-H]^-$ at *m/z* 137 with ion fragmentation in MS^2 of m/z 121, which is that of *p*-hydroxybenzoic acid (Chandrasekara & Shahidi, 2011). Compound 2 showed $[M-H]^-$ at *m/z* 147, gave a product ion at 103, 131, and 135, which is the characteristic of cinnamic acid. The presence of *p*-hydroxybenzoic acid and cinnamic acid has previously been reported in sea cucumber (*H. tubulosa*) (Alper & Günes, 2020). Similarly, protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, and caffeic acid gave deprotonated ions in their respective

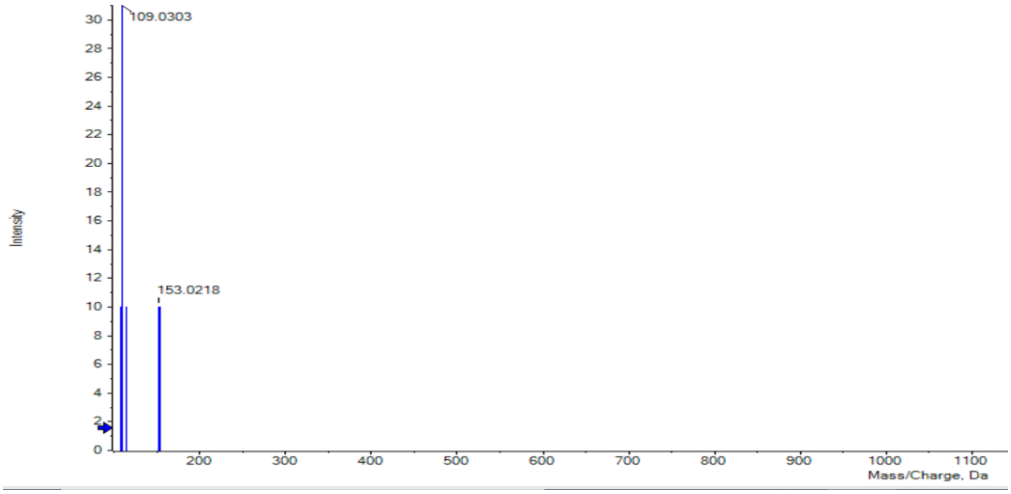
MS spectra at *m/z* 153, 163, 167, 169, and 179, respectively (Figure 8.2). Protocatechuic acid and *p*-coumaric acid gave a product ion at 109 and 119, respectively, in MS^2 due to the loss of a carboxyl moiety $[M-H-CO_2]^-$, whereas vanillic acid demonstrated MS^2 product ions at *m/z* 105, 108, 121, and 123, where 123 corresponded to $[M-H-CO_2]^-$ (Ambigaipalan et al., 2016). Likewise, gallic acid and caffeic acid showed their MS^2 fragmentation ions at *m/z* 125 and 135, respectively, due to the loss of a carboxyl moiety (Rahman et al., 2018). The presence of vanillic acid, gallic acid, and caffeic acid has previously been documented in sea cucumber (*H. tubulosa* and *H. forskali*) (Alper & Günes, 2020; Telahigue et al., 2020). Moreover, *p*-coumaric acid has been identified from different species of sea cucumber, including *H. tubulosa, H. atra, H. Arenicola,* and *H. forskali* (Alper & Günes, 2020; Dakrory et al., 2015; Esmat et al., 2013; Fahmy, 2015; Telahigue et al., 2020). Molecular ion $[M-H]^-$ at *m/z* 193 and product ion at *m/z* 178 in MS^2 due to the loss of $[M-H-CH_3]$, which matches with the fragmentation pattern of ferulic acid (Zhang et al., 2013). Syringic acid $[M-H]^-$ at *m/z* 197 showed MS^2 product ions at *m/z* 109, 123, 153, 163, and 179, where 153 corresponded to the loss of carboxyl group (Goufo et al., 2020). Sinapic acid was discovered with an observed $[M-H]^-$ *m/z* at 223, where the MS^2 spectrum was 175, 179 (loss of carboxyl moiety), and 208, matching the characteristics of sinapic acid (Zhong et al., 2020). Furthermore, ellagic acid was identified due to its $[M-H]^-$ *m/z* 301 and MS^2 fragments *m/z* 229 and 257 according to the retention time and MS data of the reference compound. The existence of syringic acid and ellagic acid has previously been reported in *H. forskali* and *H. tubulosa,* respectively (Alper & Günes, 2020; Telahigue et al., 2020).

Table 8. 2. List of phenolic compounds identified from HPP-treated and untreated sea cucumber internal organs

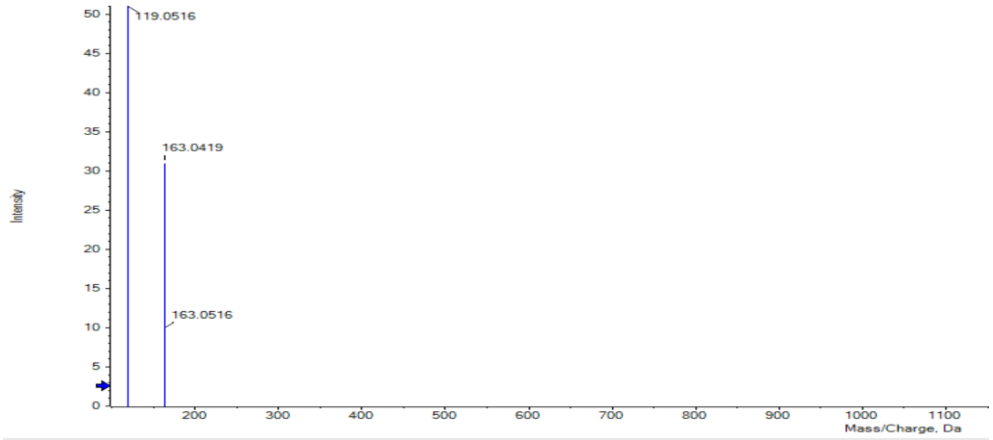
C#	Compounds	[M −H]$^-$ (*m/z*)	RT (min)-UV	MS^2 ion fragments	HPP-treated			Untreated		
					F	E	IB	F	E	IB
1	*p*-Hydroxybenzoic acid+	137	3.52	121	*	*		*	*	
2	Cinnamic acid+	147	42.17	103, 131, 135	*	*	*	*		*
3	Protocatechuic acid+	153	2.5	109	*		*	*	*	*
4	*p*-Coumaric acid+	163	6.78	119	*	*	*	*	*	*
5	Vanillic acid+	167	4.56	105, 108, 121, 123	*	*	*		*	*
6	Gallic acid+	169	0.43	125	*	*	*	*	*	*
7	Caffeic acid+	179	5.15	135			*			*
8	Homovanillic acid	181	0.37	181, 137	*					
9	Hydroxygallic acid	187	13.5	125, 169	*	*	*	*	*	*
10	Ferulic acid+	193	46.69	161, 178			*			
11	Syringic acid+	197	5.36	109, 123, 153, 163, 179	*	*	*	*	*	*
12	*p*-Coumaroyl glycolic acid	221	42.29	147, 163, 179		*	*			
13	Sinapinic acid+	223	9.17	175, 179, 208	*	*		*		
14	Ellagic acid+	301	47.86	229, 257	*	*	*	*	*	*
15	Chlorogenic acid+	353	5.32	179, 191, 207	*		*	*		*
16	Caffeoyl glucoside	387	45.08	341	*			*		
17	Chicoric acid	473	44.2	311, 293, 219, 179, 135			*			
18	Catechin+	289	4.99	203, 245	*	*	*	*	*	*
19	Quercetin+	301	38.28	121, 151, 179, 255, 257, 273	*	*	*	*	*	*
20	*p*-Hydroxybenzaldehyde	121	5.06	92	*	*	*	*	*	*
21	*p*-Hydroxycoumarin	161	2.07	105, 121		*				
22	Scopoletin	191	43.15	147	*					
23	Leachianol F	471	41.65	121, 153, 287, 349, 453	*					
Total number of compounds					*18*	*14*	*16*	*14*	*11*	*13*

Abbreviations are: RT, retention time; F, free; E, esterified; and IB, insoluble-bound. * Indicates the presence of the compound in the fraction. + Identified with authentic standard.

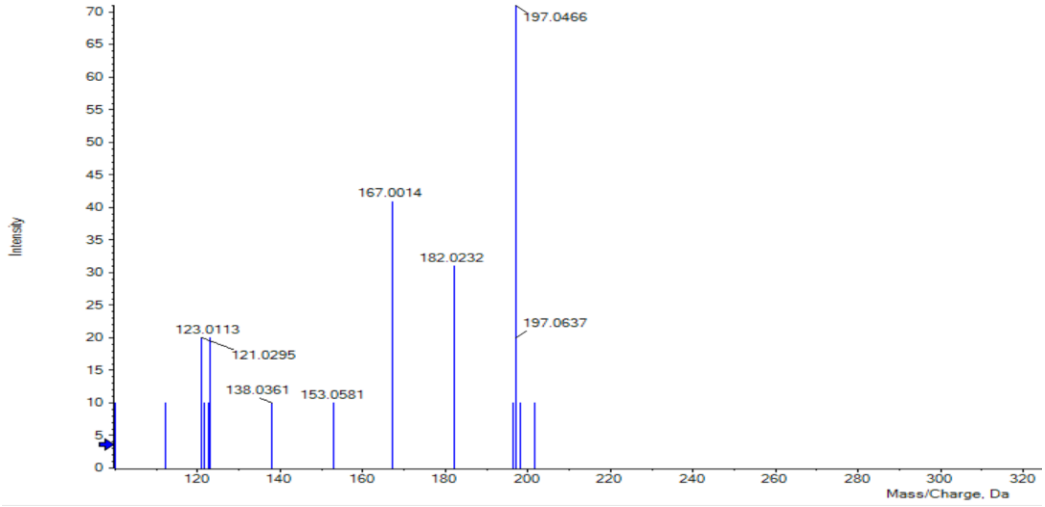
[a]


109.0303
153.0218
Intensity
Mass/Charge, Da

[b]


119.0516
163.0419
163.0516
Intensity
Mass/Charge, Da

[c]



[d]

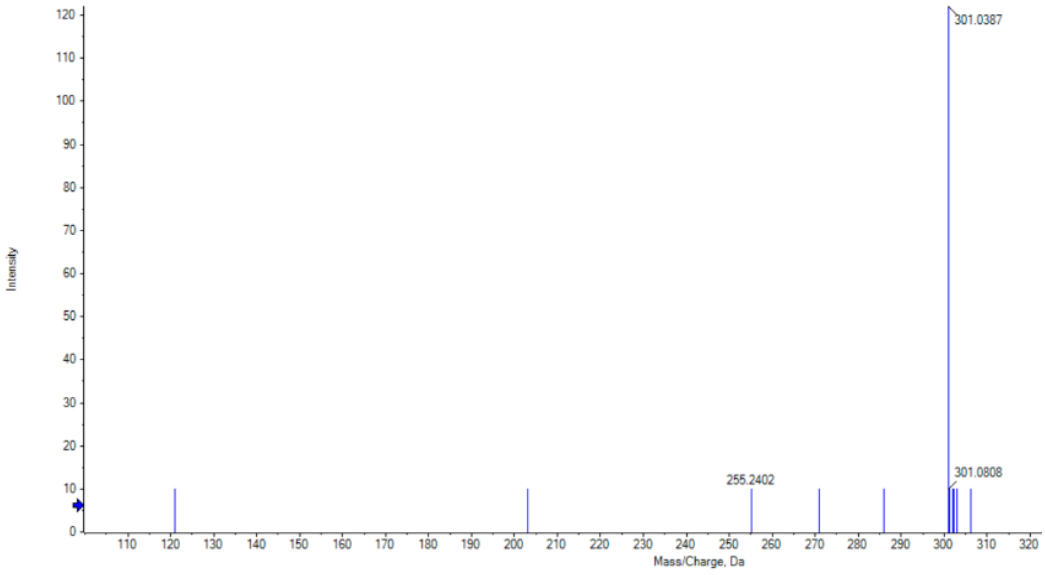


Figure 8. 2. MS/MS spectra of protocatechuic acid [a], *p*-coumaric acid [b], syringic acid [c], and quercetin [d].

Chlorogenic acid was identified based on the molecular ion at *m/z* 353 with an MS^2 value of *m/z* 191, showing loss of hexose $[M-H-162]^-$ (Rahman et al., 2018). It has been reported that the chlorogenic acid is abundant in sea cucumber and has been identified from various species of sea cucumber such as *H. atra, H. Arenicola,* and *H. tubulosa* (Alper & Günes, 2020; Dakrory et al., 2015; Esmat et al., 2013; Fahmy, 2015). The fragmentation of $[M-H]^-$ at *m/z* 289 was *m/z* 203 and 245, where m/z 245 fragment matched to the decarboxylation of catechin (Ambigaipalan et al., 2016). Quercetin showed a deprotonated ion at 301 and product ions at 121, 151, 179, 255, 257, and 273, which was confirmed by using a quercetin standard (Rahman et al., 2018). The presence of catechin and quercetin has previously been recorded in sea cucumbers such as *H. atra* and *H. tubulosa*, respectively (Alper & Günes, 2020; Esmat et al., 2013). The compounds which were tentatively identified according to the literature data are homovanillic acid, hydroxygallic acid, *p*-coumaroyl glycolic acid, caffeoyl glucoside, chicoric acid, *p*-hydroxybenzaldehyde, *p*-hydroxycoumarin, scopoletin, and leachianol F. Among them, only *p*-hydroxybenzaldehyde has been reported for *H. scabra* (Nobsathian et al., 2017); however, to the best of our knowledge, other compounds have not yet been reported in sea cucumber. Homovanillic acid, with a deprotonated ion at 181, showed loss of carboxyl group and gave a product ion at 137, which matches with the fragmentation pattern of a previous study (Grouzmann, Centeno, & Eugster, 2018). Hydroxygallic acid, deprotonated ions at *m/z* 187, was tentatively identified based on its MS^2 ion fragmentation at *m/z* 125 and 169 due to the loss of H_2O and CO_2 $[M-H-18-44]^-$ as well as water $[M-H-18]^-$, respectively. Hydroxygallic acid has previously been identified from pomegranate byproducts (Ambigaipalan et al., 2016). Moreover, *p*-coumaroyl glycolic acid was tentatively identified based on its MS^2 ion fragmentation at *m/z* 163, which was deprotonated at *m/z* 221 (Bashmil et al., 2021). Caffeoyl glucoside was deprotonated $[M-H]^-$ at *m/z* 387, which was tentatively identified based

on the product ion at *m/z* 341. Yeo and Shahidi (2020) proposed a deprotonated molecule at *m/z* 341 as being a caffeoyl glucoside of phenolics in lentil hulls. A deprotonated molecule $[M-H]^-$ at *m/z* 473 was found and tentatively identified as a chicoric acid based on the product ion at *m/z* 311, 293, 219, 179, and 135 (Goufo et al., 2020). *p*-hydroxybenzaldehyde, deprotonated at *m/z* 121, was tentatively identified based on its MS^2 ion fragmentation at *m/z* 92, indicating the loss of CHO (Zhong et al., 2020). Furthermore, *p*-hydroxycoumarin deprotonated $[M-H]^-$ at *m/z* 161 was tentatively identified based on the product ion at *m/z* 105 and 121, which was confirmed from National Center for Biotechnology Information (NCBI) data bank. A compound at *m/z* 191 along with a product ion at 147 was observed due to the loss of $[M-H-CO_2]^-$, tentatively identified as scopoletin, which is supported by the MS data in the literature (Zhong et al., 2020). Besides, leachianol F at *m/z* 471 was tentatively identified along with product ions at 121, 153, 287, 349, and 453 (Goufo et al., 2020).

8.2.2. Flower

In this study, 29, 16, and 9 phenolic compounds were identified from free, esterified, and insoluble-bound phenolic fractions of HPP-treated flower, respectively. All phenolic compounds identified in the HPP-treated samples were also detected in their untreated counterparts, except homovanillic acid, ferulic acid hexoside, rosmarinic acid, caffeoyl glucoside, myricetin, and sinapine (Table 8.3). However, the highest number of compounds were identified in the free phenolic fraction of flower, followed by esterified and insoluble-bound phenolic fractions. These results are in accordance with our previous data (section 6), where free phenolic fraction had a higher content of phenolics with strong antioxidant activity. Among these phenolic compounds, protocatechuic acid, *p*-coumaric acid, gallic acid, hydroxygallic acid, ellagic acid, catechin, and quercetin were found in all phenolic fractions, regardless of HPP treatment. However, *p*-hydroxybenzoic acid, ferulic acid, chlorogenic acid, chicoric acid, quercetin-3-*O*-arabinose, kaempferol 3-*O*-glucoside, epigallocatechin gallate, scopoletin, and fraxin were only found in the free phenolic fraction.

Phenolic compounds were identified by comparing their UV retention times, observed $[M-H]^-$, and ion fragmentation patterns of authentic standards. Other compounds with no standards were tentatively identified using mass spectrometry (MS^n), UV spectral, and literature data, as discussed in the section 8.2.1. According to Table 8.3, compounds 18 exhibited a molecular ion $[M-H]^-$ at *m/z* 355 with ion fragmentation in MS^2 of *m/z* 135, 175, 193, 217, and 236, which is the configuration of ferulic acid hexoside (Ambigaipalan et al., 2016). Homoveratric acid with a molecular ion $[M-H]^-$ at *m/z* 195 was tentatively identified based on its MS^2 data of 124, 163, 165, 181, which was confirmed from the National Institute of Standards and Technology (NIST) Chemistry WebBook. Compound 19 was tentatively identified with a molecular ion $[M-H]^-$ at

m/z 359 and its MS^2 data 133, 135, 161, 179, and 197 due to the loss of hexosyl group $[M-H-162]^-$ and two molecules of water $[M-H-162-H_2O-H_2O]^-$. Thus, this is the characteristic of rosmarinic acid, which is matched with literature data (Chen et al., 2005). The presence of rosmarinic acid has previously been reported in sea cucumber (*H. forskali*) (Telahigue et al., 2020). Compound 24 with a molecular ion $[M-H]^-$ at *m/z* 317 and a product ion 179 was tentatively identified as myricetin by Rahman et al. (2018). Quercetin-3-*O*-arabinose with MS^2 data of 311, 283, and 179 was tentatively identified according to the published data (Goufo et al., 2020). Compound 26 had an *m/z* 447 $[M-H]^-$ ion dissociating to yield a product ion at *m/z* 285 that suggests the loss of a glucose moiety (162 Da), which is the characteristic of kaempferol 3-*O*-glucoside (He et al., 2011). Compound 27 with a molecular ion $[M-H]^-$ at *m/z* 457 and product ion 331, 305, 261, 289, 221, and 219 was tentatively identified as epigallocatechin gallate by Goufo et al. (2020). Compound 30 had deprotonated ions at *m/z* 309, which were tentatively identified as sinapine according to the published data with MS^2 data of 175, 207, and 251 (Zhong et al., 2020). Compound 31 had an *m/z* 369 $[M-H]^-$ ion dissociating to yield product ion at *m/z* 207 that implying the loss of a glucose moiety (162 Da), which is the characteristic of fraxin (Goufo et al., 2020).

Table 8. 3. List of phenolic compounds identified from HPP-treated and untreated sea cucumber flower

C #	Compounds	[M −H]$^-$ (*m/z*)	RT (min)-UV	MS^2 ion fragments	HPP-treated			Untreated		
					F	E	IB	F	E	IB
1	*p*-Hydroxybenzoic acid+	137	3.52	121	*			*		
2	Cinnamic acid+	147	42.17	103, 131, 135	*	*		*	*	
3	Protocatechuic acid+	153	2.5	109	*	*	*	*	*	*
4	*p*-Coumaric acid+	163	6.78	119	*	*	*	*	*	*
5	Vanillic acid+	167	4.56	105, 108, 121, 123	*	*	*	*		
6	Gallic acid+	169	0.43	125	*	*	*	*	*	*
7	Caffeic acid+	179	5.15	135	*	*		*	*	
8	Homovanillic acid	181	0.37	181, 137	*					
9	Hydroxygallic acid	187	13.5	125, 169	*	*	*	*	*	*
10	Ferulic acid+	193	8.14	161, 178	*			*		
11	Isoferulic acid	193	46.69	133, 179	*	*		*		
12	Homoveratric acid	195	0.59	124, 163, 165, 181	*					
13	Syringic acid+	197	5.36	109, 123, 153, 163, 179	*	*	*	*	*	
14	*p*-Coumaroyl glycolic acid	221	42.29	147, 163, 179		*			*	
15	Sinapinic acid+	223	9.17	175, 179, 208	*	*		*		
16	Ellagic acid+	301	47.86	229, 257	*	*	*	*	*	*
17	Chlorogenic acid+	353	5.32	179, 191, 207	*			*		
18	Ferulic acid hexoside	355	39.98	135, 175, 193, 217, 236		*				
19	Rosmarinic acid	359	46.38	133, 135, 161, 179, 197, 359	*					
20	Caffeoyl glucoside	387	45.08	341	*					
21	Chicoric acid	473	44.2	311, 293, 219, 179, 135	*			*		
22	Catechin+	289	4.99	203, 245	*	*	*	*	*	*
23	Quercetin+	301	38.28	121, 151, 179, 255, 257, 273	*	*	*	*	*	*
24	Myricetin	317	50.61	179	*					
25	Quercetin-3-*O*-arabinose	433	44.58	311, 283, 179	*			*		
26	Kaempferol 3-*O*-glucoside	447	50.64	285	*			*		

27	Epigallocatechin gallate	457	45.07	331, 305, 261, 289, 221, 219	*			*			
28	*p*-Hydroxybenzaldehyde	121	5.06	92	*	*		*	*		
29	Scopoletin	191	43.15	147	*			*			
30	Sinapine	309	50.72	175, 207, 251	*						
31	Fraxin	369	41.19	207, 192	*			*			
Total number of compounds					*29*	*16*	*9*	*23*	*12*	*7*	

Abbreviations are: RT, retention time; F, free; E, esterified; and IB, insoluble-bound. * Indicates the presence of the compound in the fraction. + Identified with authentic standard.

8.2.3. Body wall

In this study, 17, 16, and 14 phenolic compounds were identified from free, esterified, and insoluble-bound phenolic fractions of HPP-treated body wall, respectively. In contrast, 13, 12, and 11 compounds were identified from untreated counterparts, respectively (Table 8.4). Compounds that were not detected in untreated counterparts were hydroxygallic acid, myricetin, phlorizin, and *p*-hydroxybenzaldehyde in the free phenolic fraction, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaroyl glycolic acid, and quercetin in the esterified phenolic fraction, and syringic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxycoumarin in the insoluble-bound phenolic fraction. However, the highest number of compounds were identified from the free phenolic fraction of body wall, followed by esterified and insoluble-bound phenolic fractions. Among these phenolic compounds, protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, isoferulic acid, ellagic acid, and catechin were found in all fractions, regardless of HPP treatment.

Phenolic compounds were identified by comparing their UV retention times, observed $[M-H]^-$, and ion fragmentation patterns of authentic standards. Other compounds with no standards were tentatively identified using mass spectrometry (MS^n), UV spectral, and literature data, as discussed in earlier sections. According to Table 8.4, phlorizin is the only compound that has not been identified from both internal organs and flower. Phlorizin exhibited a molecular ion $[M-H]^-$ at *m/z* 435 with ion fragmentation in MS^2 of *m/z* 167, 273, and 297, which was tentatively identified as reported by Mena et al. (2012).

Table 8. 4. List of phenolic compounds identified from HPP-treated and untreated sea cucumber body wall

C #	Compounds	[M −H]$^-$ (*m/z*)	RT (min)-UV	MS^2 ion fragments	HPP-treated			Untreated		
					F	E	IB	F	E	IB
1	*p*-Hydroxybenzoic acid+	137	3.52	121	*	*	*	*		*
2	Cinnamic acid+	147	42.17	103, 131, 135	*		*	*		*
3	Protocatechuic acid+	153	2.5	109	*	*	*	*	*	*
4	*p*-Coumaric acid+	163	6.78	119	*	*	*	*	*	*
5	Vanillic acid+	167	4.56	105, 108, 121, 123	*	*	*	*	*	*
6	Gallic acid+	169	0.43	125	*	*	*	*	*	*
7	Caffeic acid+	179	5.15	135		*				
8	Hydroxygallic acid	187	13.5	125, 169	*	*	*		*	*
9	Isoferulic acid	193	46.69	133, 179	*	*	*	*	*	*
10	Syringic acid+	197	5.36	109, 123, 153, 163, 179	*	*	*	*	*	
11	*p*-Coumaroyl glycolic acid	221	42.29	147, 163, 179	*	*		*		
12	Sinapinic acid+	223	9.17	175, 179, 208		*			*	
13	Ellagic acid+	301	47.86	229, 257	*	*	*	*	*	*
14	Catechin+	289	4.99	203, 245	*	*	*	*	*	*
15	Quercetin+	301	38.28	121, 151, 179, 255, 257, 273	*	*	*	*		*
16	Myricetin	317	50.61	179, 317	*					
17	Phlorizin	435	46.5	167, 273, 297	*					
18	Epigallocatechin gallate	457	45.07	331, 305, 261, 289, 221, 219	*			*		
19	*p*-Hydroxybenzaldehyde	121	5.06	92	*	*	*		*	
20	*p*-Hydroxycoumarin	161	2.07	105, 121		*	*		*	
Total number of compounds					*17*	*16*	*14*	*13*	*12*	*11*

Abbreviations are: RT, retention time; F, free; E, esterified; and IB, insoluble-bound. * Indicates the presence of the compound in the fraction. + Identified with authentic standard.

8.3. Quantification of phenolics in HPP-treated and untreated sea cucumber body parts

8.3.1. Internal organs

The quantification of phenolics was carried out by using the respective standard compounds. Moreover, the content of phenolic derivatives was calculated using their respective aglycones. The contents of phenolics in sea cucumber internal organs were determined by UHPLC, and the results are presented in Table 8.5. In the UHPLC analysis, the total content of phenolics, sum of all compounds quantified from free, esterified, and insoluble-bound phenolics, was 81.06 and 62.5 mg/ 100 g in the HPP-treated and untreated internal organs, respectively. Therefore, HPP treatment significantly increased the content of a certain phenolic compounds, while other compounds remain unchanged. For example, HPP treatment significantly increased the content of cinnamic acid, protocatechuic acid, gallic acid, hydroxygallic acid, sinapinic acid, and catechin in the free phenolic fraction, protocatechuic acid, *p*-coumaric acid, and catechin in the esterified phenolic fraction, and protocatechuic acid, *p*-coumaric acid, hydroxygallic acid, and chlorogenic acid in the esterified phenolic fraction. This phenomenon is in agreement with those of our previous findings in terms of TPC and TFC as well as literature data. For example, da Silveira et al. (2019) stated that HPP treatment at 500 MPa significantly increased the content of individual phenolic compounds of açaí juice compared to their untreated counterparts. Similarly, Zhou et al. (2019) reported that the height and area of phenolic compounds were significantly increased when oil palm fruits were treated with HPP. Furthermore, de Ancos et al. (2020) found a similar flavonoid profile in untreated and HPP-treated orange juice. This could be due to the disruption of cell walls upon HPP, causing a favorable condition to release phenolic compounds (Vázquez-Gutiérrez et al., 2013).

In the UHPLC analysis, the TPC of HPP-treated internal organs in free, esterified, and insoluble phenolic fraction was 44.58, 10.56, and 25.92 mg/ 100 g, respectively, while these contents for untreated counterparts were 33.97, 7.86, and 20.67 mg/ 100 g, respectively. Overall, all phenolic fractions showed a higher content of phenolic acids as well as flavonoids. In particular, the higher amount of cinnamic acid (3.24 mg/ 100g), protocatechuic acid (3.69 mg/ 100g), gallic acid (3.22 mg/ 100g), hydroxygallic acid (3.25 mg/ 100g), catechin (5.8 mg/ 100g), and quercetin (3.05 mg/ 100g) was observed in the free phenolic fraction, whereas protocatechuic acid (2.05 mg/ 100g), *p*-coumaric acid (2.88 mg/ 100g), and hydroxygallic acid (2.42 mg/ 100g) was abundant in the insoluble-bound fraction. These compounds have been reported to show strong bioactivities such as anticancer, anti-inflammatory, and antimicrobial effects (Yeo & Shahidi, 2020).

Table 8. 5. Quantification of phenolic compounds (mg/ 100 g) from HPP-treated and untreated sea cucumber internal organs

C#	Compounds	$[M-H]^-$ (*m/z*)	HPP-treated			Untreated		
			F	E	IB	F	E	IB
1	*p*-Hydroxybenzoic acid	137	1.4±0.03	0.49±0.08		1.35±0.03	0.46±0.06	
2	Cinnamic acid	147	3.24±0.28*		1.54±0.22	2.58±0.15		1.51±0.16
3	Protocatechuic acid	153	3.69±0.24*	1.39±0.1*	2.05±0.18*	2.68±0.26	1.12±0.08	1.46±0.08
4	*p*-Coumaric acid	163	2.89±0.08	1.38±0.05*	2.88±0.2*	2.81±0.01	1.11±0.1	1.8±0.12
5	Vanillic acid	167	1.37±0.12	0.73±0.1	0.95±0.06		0.7±0.06	0.89±0.1
6	Gallic acid	169	3.22±0.32*	0.89±0.12	1.55±0.15	2.48±0.18	0.85±0.1	1.51±0.22
7	Caffeic acid	179			1.01±0.09			1.02±0.16
8	Homovanillic acid	181	1.03±0.06					
9	Hydroxygallic acid	187	3.25±0.34*	0.53±0.04	2.42±0.18*	2.24±0.26	0.51±0.08	1.89±0.12
10	Isoferulic acid	193			0.76±0.15			
11	Syringic acid	197	2.55±0.1	0.34±0.1	1.34±0.05	2.5±0.08	0.34±0.05	1.3±0.08
12	*p*-Coumaroyl glycolic acid	221		0.25±0.1	0.66±0.18			
13	Sinapinic acid	223	2.5±0.18*	0.57±0.12		1.79±0.2		
14	Ellagic acid	301	1.66±0.15	0.54±0.15	2.13±0.28	1.61±0.1	0.51±0.12	2.1±0.24
15	Chlorogenic acid	353	2.47±0.18		3.06±0.08*	2.4±0.05		2.46±0.1
16	Caffeoyl glucoside	387	2.47±0.12			2.41±0.16		
17	Chicoric acid	473			0.73±0.16			
18	Catechin	289	5.8±0.58*	1.2±0.08*	2.33±0.32	4.38±0.36	0.85±0.1	2.33±0.16
19	Quercetin	301	3.05±0.32	0.73±0.18	1.7±0.2	3.02±0.14	0.7±0.06	1.66±0.1
20	*p*-Hydroxybenzaldehyde	121	1.81±0.08	0.72±0.06	0.81±0.18	1.75±0.1	0.71±0.12	0.75±0.08
21	*p*-Hydroxycoumarin	161		0.8±0.16				
22	Scopoletin	191	1.56±0.16					
23	Leachianol F	471	0.63±0.05					
Total			*44.58*	*10.56*	*25.92*	*33.97*	*7.86*	*20.67*
Total phenolic content (F+E+IB)				*81.06*			*62.5*	

All data represent the mean of triplicates. * Indicates significant differences ($p<0.05$) for the same phenolic fraction compared to untreated counterparts. F, free; E, esterified; and IB, insoluble-bound.

8.3.2. Flower

The content of phenolics in sea cucumber flower were determined by UHPLC, and the results are shown in Table 8.6. The contents of most of the identified phenolic compounds in three phenolic fractions of HPP-treated flower were not significantly increased when compared to their counterparts of non-treated samples. For instance, in free phenolic fraction, 9 out of 29 compounds showed a significantly higher content of phenolic by HPP treatment, and they are cinnamic acid, protocatechuic acid, gallic acid, syringic acid, sinapinic acid, ellagic acid, chlorogenic acid, chicoric acid, and catechin. Similarly, protocatechuic acid, *p*-coumaric acid, and quercetin in the esterified phenolic fraction and protocatechuic acid and quercetin in the insoluble-bound phenolic fraction had a higher content of phenolic under HPP pretreatment. The TPC obtained from UHPLC analysis in the free, esterified, and insoluble-bound fractions were 43.16, 18.16, and 11.54 mg/ 100 g, respectively, for the HPP-treated sample, while these contents reduced to 32.36, 13.68, and 8.87 mg/ 100 g, respectively, for the untreated counterparts. These results are in accordance with those of published data, as we discussed in the earlier section (section 6). According to the content of phenolic, the major phenolic compounds in the free phenolic fraction were phenolic acids and flavonoids such as protocatechuic acid (3.14 mg/ 100 g), ellagic acid (2.61 mg/ 100 g), chlorogenic acid (2.25), and catechin (3.37 mg/ 100 g), while the major compounds in the esterified and insoluble-bound phenolics were *p*-coumaric acid (2.08 mg/ 100 g) and protocatechuic acid (2.14 mg/ 100 g), respectively. Among them, chlorogenic acid, *p*-coumaric acid, ellagic acid, and catechin have been identified and quantified from different species of sea cucumber, where chlorogenic acid was the most abundant compound (Alper & Günes, 2020; Dakrory et al., 2015; Esmat et al., 2013; Fahmy, 2015).

Table 8. 6. Quantification of phenolic compounds (mg/ 100 g) from HPP-treated and untreated sea cucumber flower

C#	Compounds	$[M-H]^-$ (*m/z*)	HPP-treated			Untreated		
			F	E	IB	F	E	IB
1	*p*-Hydroxybenzoic acid	137	1.42±0.18			1.36±0.12		
2	Cinnamic acid	147	2.03±0.1*	1.32±0.16		1.4±0.14	1.26±0.1	
3	Protocatechuic acid	153	3.14±0.22*	1.63±0.12*	2.14±0.1*	2.4±0.16	1.01±0.08	1.63±0.12
4	*p*-Coumaric acid	163	1.34±0.1	2.08±0.1*	1.48±0.14	1.26±0.13	1.57±0.16	1.46±0.24
5	Vanillic acid	167	1.48±0.32	0.26±0.04	0.66±0.15	1.4±0.08		
6	Gallic acid	169	2.05±0.18*	2.46±0.34	1.63±0.26	1.39±0.06	2.48±0.24	1.6±0.08
7	Caffeic acid	179	1.19±0.2	0.72±0.1		1.19±0.16	0.7±0.15	
8	Homovanillic acid	181	0.97±0.15					
9	Hydroxygallic acid	187	1.03±0.08	1.79±0.2	1.64±0.18	1.02±0.1	1.71±0.16	1.55±0.24
10	Ferulic acid	193	0.97±0.16			0.96±0.2		
11	Isoferulic acid	193	1.01±0.08	0.73±0.16		1.01±0.12		
12	Homoveratric acid	195	1±0.15					
13	Syringic acid	197	1.34±0.08*	0.58±0.08	0.59±0.16	0.98±0.22	0.58±0.15	
14	*p*-Coumaroyl glycolic acid	221		0.73±0.15			0.7±0.1	
15	Sinapinic acid	223	1.76±0.14*	0.8±0.18		1.32±0.1		
16	Ellagic acid	301	2.61±0.18*	0.88±0.22	0.37±0.08	1.98±0.2	0.81±0.2	0.35±0.1
17	Chlorogenic acid	353	2.25±0.08*			1.74±0.18		
18	Ferulic acid hexoside	355		0.72±0.14				
19	Rosmarinic acid	359	1.05±0.26					
20	Caffeoyl glucoside	387	1.05±0.12					
21	Chicoric acid	473	2.32±0.16*			1.32±0.15		
22	Catechin	289	3.37±0.3*	1.6±0.3	1.35±0.22	2.58±0.15	1.54±0.25	1.26±0.2
23	Quercetin	301	1.34±0.06	1.04±0.15*	1.69±0.12*	1.29±0.18	0.51±0.22	1.02±0.06
24	Myricetin	317	0.2±0.04					
25	Quercetin-3-*O*-arabinose	433	1.34±0.16			1.27±0.22		

26	Kaempferol 3-*O*-glucoside	447	1.46±0.28			1.38±0.1			
27	Epigallocatechin gallate	457	1.05±0.06			1.01±0.08			
28	*p*-Hydroxybenzaldehyde	121	1.56±0.16	0.85±0.1		1.5±0.26	0.81±0.21		
29	Scopoletin	191	1.04±0.08			1.01±0.18			
30	Sinapine	309	0.97±0.12						
31	Fraxin	369	1.64±0.15			1.59±0.18			
Total			*43.97*	*18.16*	*11.54*	*32.36*	*13.68*	*8.87*	
Total phenolic content (F+E+IB)				*73.67*			*54.91*		

All data represent the mean of triplicates. * Indicates significant differences ($p<0.05$) for the same phenolic fraction compared to untreated counterparts. F, free; E, esterified; and IB, insoluble-bound.

8.3.3. Body wall

The contents of phenolics in the sea cucumber body wall were determined by UHPLC, and the results are shown in Table 8.7. It was found that the HPP treatment significantly increased some phenolic compounds that were identified from the body wall. For example, protocatechuic acid, *p*-coumaroyl glycolic acid, catechin, and epigallocatechin gallate in the free phenolic fraction, protocatechuic acid, *p*-coumaric acid, gallic acid, hydroxygallic acid, and catechin in the esterified phenolic fraction, and protocatechuic acid, catechin, and quercetin in the insoluble-bound phenolic fraction had a significantly higher content of phenolic in HPP-treated flower than the untreated counterparts. The TPC obtained from UHPLC analysis in the free, esterified, and insoluble-bound fractions was 31.62, 20.84, and 17.49 mg/ 100 g, respectively, for the HPP-treated samples, while these contents reduced to 24, 15.98, and 13.68 mg/ 100 g, respectively, for the untreated counterparts. In body wall, the major compounds were protocatechuic acid (3.47 mg/ 100 g), *p*-coumaroyl glycolic acid (3.14 mg/ 100 g), catechin (2.05 mg/ 100 g), and epigallocatechin gallate (4.09 mg/ 100 g) in the free phenolic fraction, protocatechuic acid (3.04 mg/ 100 g) and hydroxygallic acid (3.55 mg/ 100 g) in the esterified phenolic fraction, and protocatechuic acid (2.35 mg/ 100 g) in the esterified phenolic fraction. These compounds might contribute most to the antioxidant activity as reflected in DPPH radical scavenging activity, hydroxyl radical scavenging activity, and metal chelation ability of the tested extracts.

Table 8. 7. Quantification of phenolic compounds (mg/ 100 g) from HPP-treated and untreated sea cucumber body wall

C#	Compounds	$[M-H]^-$ (m/z)	HPP-treated			Untreated		
			F	E	IB	F	E	IB
1	*p*-Hydroxybenzoic acid	137	1.83±0.22	0.38±0.06	1.45±0.3	1.81±0.1		1.41±0.21
2	Cinnamic acid	147	1.55±0.2		1.35±0.14	1.47±0.08		1.3±0.1
3	Protocatechuic acid	153	3.47±0.12*	3.04±0.18*	2.35±0.12*	2.4±0.1	2.53±0.1	1.85±0.15
4	*p*-Coumaric acid	163	1.36±0.15	2.08±0.14*	1.67±0.2	1.3±0.2	1.48±0.12	1.61±0.18
5	Vanillic acid	167	1.45±0.21	1.01±0.2	0.58±0.08	1.38±0.16	1±0.21	0.57±0.12
6	Gallic acid	169	2.1±0.28	3.55±0.12*	1.69±0.22	2.01±0.24	2.96±0.18	1.64±0.06
7	Caffeic acid	179		0.7±0.16				
8	Hydroxygallic acid	187	0.7±0.06	2.45±0.22*	0.75±0.1		1.68±0.08	0.7±0.09
9	Isoferulic acid	193	1.49±0.16	0.87±0.2	1.04±0.15	1.42±0.1	0.81±0.14	1.01±0.2
10	Syringic acid	197	1.66±0.25	0.58±0.15	0.27±0.05	1.59±0.16	0.56±0.16	
11	*p*-Coumaroyl glycolic acid	221	3.14±0.24*	0.42±0.18		2.67±0.12		
12	Sinapinic acid	223		0.94±0.12			0.92±0.1	
13	Ellagic acid	301	1.76±0.15	1.39±0.21	1.4±0.2	1.7±0.08	1.35±0.2	1.4±0.16
14	Catechin	289	2.05±0.16*	1.37±0.06*	1.77±0.2*	1.27±0.2	0.99±0.15	1.21±0.1
15	Quercetin	301	1.56±0.06	0.27±0.04	1.52±0.12*	1.55±0.12		0.98±0.08
16	Myricetin	317	1.04±0.15					
17	Phlorizin	435	0.96±0.18					
18	Epigallocatechin gallate	457	4.09±0.21*			3.44±0.16		
19	*p*-Hydroxybenzaldehyde	121	1.39±0.25	0.87±0.15	0.78±0.15		0.8±0.16	
20	*p*-Hydroxycoumarin	161		0.93±0.1	0.86±0.18		0.93±0.08	
Total			*31.62*	*20.84*	*17.49*	*24*	*15.98*	*13.68*
Total phenolic content (F+E+IB)				*69.95*			*53.66*	

All data represent the mean of triplicates. * Indicates significant differences ($p<0.05$) for the same phenolic fraction compared to untreated counterparts. F, free; E, esterified; and IB, insoluble-bound.

In this study, a total of 33 different phenolic compounds were identified from the flower, internal organs, and body wall. Among them, to the best of our knowledge, 19 compounds were identified for the first time in any species of sea cumber. Most of the phenolic compounds were identified in the Atlantic sea cucumber are phenolic acids (protocatechuic acid, *p*-coumaric acid, vanillic acid, gallic acid, hydroxygallic acid, isoferulic acid, syringic acid, ellagic acid, and chlorogenic acid), flavonoids (catechin and quercetin), and *p*-hydroxybenzaldehyde (Figure 8.3). The distribution pattern of phenolic acids, flavonoids, and other polyphenols in HPP-treated internal organs, flower, and body wall of sea cucumber according to the UHPLC-QTOF-MS analysis is presented in Figure 8.4. Total phenolic acid and other polyphenol contents followed the same order internal organs > flower > body wall, whereas total flavonoid content followed the order internal organs > body wall > flower. The highest amount of phenolic compounds of sea cucumber existed as free phenolic in internal organs. In terms of phenolic fraction, total phenolic acid and flavonoid contents maintained the even order free > insoluble-bound > esterified phenolic, while other polyphenol content followed the order free > esterified > insoluble-bound phenolic. The TPC of free phenolic obtained from the Folin-Ciocalteau method was 220.69, 241.38, and 227.87 mg GAE/ 100 g for the HPP-treated body wall, flower, and internal organs, respectively, while the TPC obtained from UHPLC analysis for these body parts was 31.62, 43.97, and 44.58 mg/ 100 g, respectively. The discrepancies found in the present study could be due to the Folin−Ciocalteu's reagent may react with nonphenolic reducing constituents, including reducing sugars, vitamin C, amino acids, and other organic acids. Thus, individual phenolics should be identified and quantified using reliable methods, including UHPLC-MS.

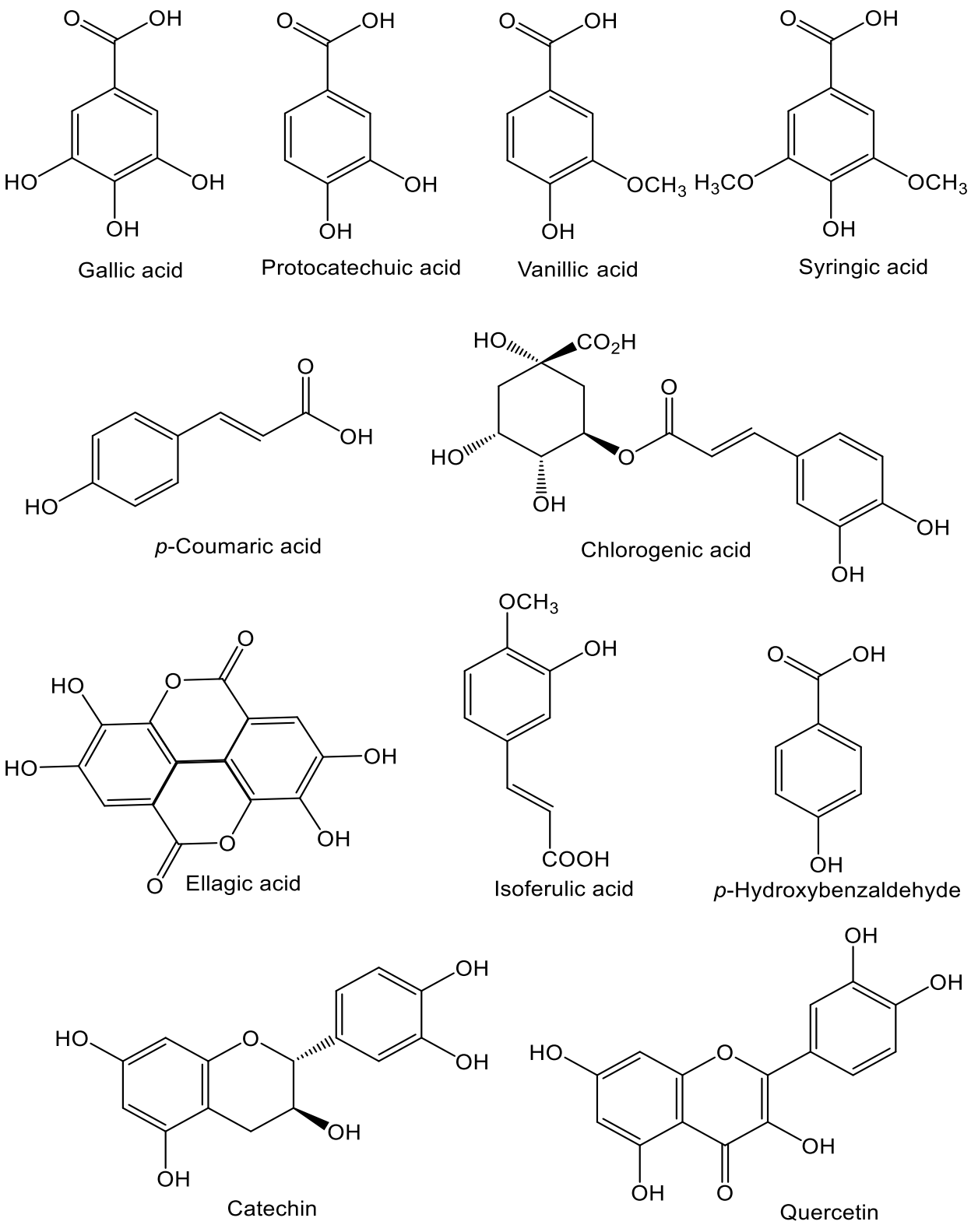


Figure 8. 3. Most common phenolic compounds found in Atlantic sea cucumber

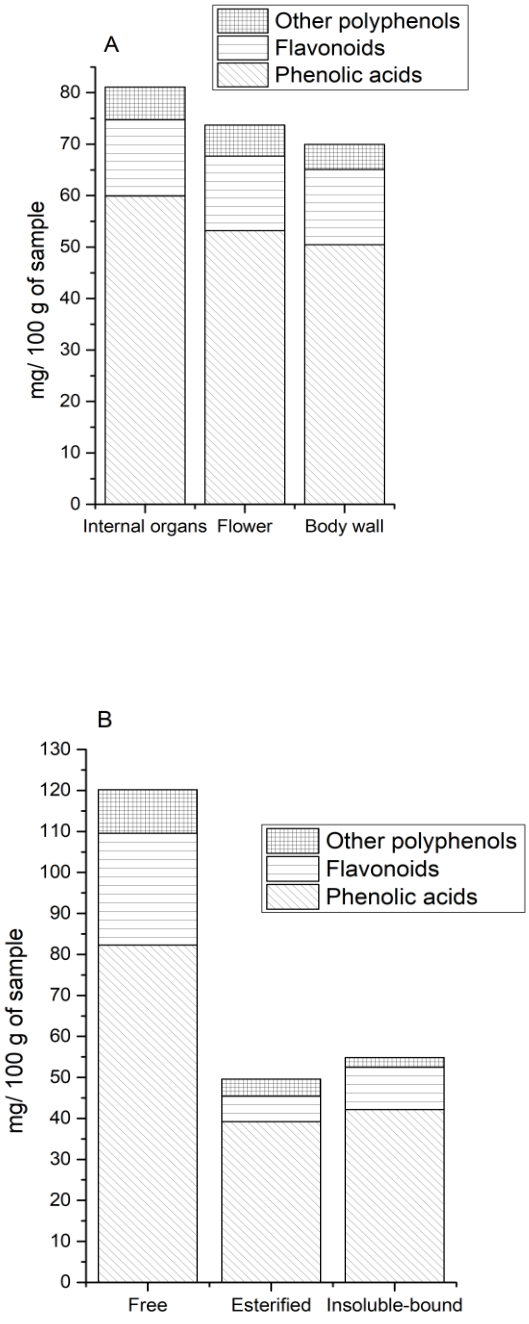


Figure 8. 4. Distribution pattern of phenolic acids, flavonoids, and other polyphenols (mg/ 100 g) in (A) sea cucumber internal organs, flower, and body wall and (B) as free, esterified, and insoluble-bound forms determined by UHPLC-QTOF-MS/MS.

8.4. Summary

Through UHPLC-QTOF-MS analysis, a reasonably varied profile of phenolic compounds was noticed in the free phenolic fraction compared to the esterified and insoluble-bound phenolic fractions, causing a higher antioxidant activity such as radical scavenging activities and metal chelation ability. In this study, a total of 31, 23, and 20 phenolic compounds were identified from the flower, internal organs, and body wall, respectively. The highest number of phenolic compounds were identified from HPP-treated flower in the free phenolic fraction, followed by HPP-treated internal organs and body wall in the free phenolic fraction. Up until now, no study has been conducted on the free, esterified, and insoluble-bound phenolics of sea cucumber, especially on the identification and quantification of the phenolic compounds from Atlantic sea cucumber. Therefore, this study offers valuable information that fulfills the gap in the existing literature in this and related research fields.

Chapter 9

Conclusion and Recommendations

This study evaluated free, esterified, and insoluble-bound phenolics from different commercial body parts, including body wall and flower, of Atlantic sea cucumber (*C. frondosa*) and its processing discards, the internal organs. Especially, the effect of food processing techniques such as high-pressure processing (HPP) on the antioxidant activities and bioactivities of sea cucumber was evaluated. HPP variables, namely pressure and holding time, had a significant effect on the total phenolic content (TPC) and total flavonoid (TFC) of all three phenolic fractions, especially those of the free phenolics. Particularly, 6000 bar pressure showed significantly higher phenolics than the 2000 and 4000 bar, regardless of HPP holding time. Similarly, an increasing trend was observed for the HPP holding time, mainly those treated for 10 and 15 min at 6000 bar. Thus, the results suggest that the HPP, mainly 6000 bar pressure for 10 min, can be used as an effective approach for the extraction of phenolic compounds from different body parts of sea cucumber. Moreover, this technique may also be employed to extract phenolics in other species of sea cucumber and other foods. Apart from this, free phenolics were the most dominant phenolic fraction compared with the esterified and insoluble-bound phenolics, regardless of the sea cucumber body parts. Flower had the highest TPC (241.38 mg GAE/ 100 g) and TFC (129.07 mg CE/ 100 g) in the free phenolic fraction, whereas esterified and insoluble-bound fractions were more abundant in the body wall. In contrast, the TPC (free + esterified + insoluble-bound phenolics) was higher in the body wall (398.88 mg GAE/ 100 g), while the TFC was higher in flower (161.65 mg CE/ 100 g), suggesting that sea cucumber phenolics are mostly present in the flower.

The HPP parameters, namely pressure and holding time, had a significant positive effect on the antioxidant activity (DPPH, ABTS, and hydroxyl radical scavenging activity as well as metal chelation activity) of all three phenolic fractions, especially those of the free phenolics. The flower had the highest activity in all phenolic fractions, regardless of the type of assay used to determine the antioxidant activity. However, HPP did not result in similar levels of activity in each assay. On average, HPP enhanced both the DPPH radical scavenging and metal chelation activities, while it had nearly no effect on hydroxyl radical scavenging activity and negative effect on ABTS radical scavenging activity. This could be due to the different mechanisms of action involved in each assay and hence, providing varied antioxidant properties. Therefore, at least two or three different assays should be used to determine the antioxidant property for better reliability. On the other hand, the highest level of antioxidant activity was found in the free phenolic fraction of the flower, followed by internal organs and body wall. This finding also agrees with the correlation coefficient, where TPC and TFC of flower showed a strong correlation with the antioxidant assays compared to the body wall, suggesting that the flower of sea cucumber is the main source of phenolics. Consequently, HPP pre-treatment could be used as an effective means to retain the antioxidant activities of phenolic-rich extracts from sea cucumber, expanding the potential application in the functional food and nutraceutical industry.

As antioxidants play a vital role in food preservation and health promotion, phenolic compounds were then assessed for their antioxidant efficacy in food and biological model systems. The HPP-treated samples showed better inhibitory activity against LDL cholesterol oxidation, DNA oxidation, α-glucosidase inhibition, formation of AGEs, and in the comminuted fish model system compared to their untreated counterparts. However, the insoluble-bound phenolic fraction showed strong biological activities even though the level of TPC was lower in that fraction. Thus,

it can be concluded that biological activities depend on the type of phenolics, not fully on the quantity of phenolics present. The efficacy in food and biological model systems of phenolic compounds revealed the potential of using sea cucumber phenolics as functional food ingredients and nutraceuticals for health promotion.

Lastly, phenolic profiles and their quantities in all phenolic fractions in sea cucumber were studied. Through UHPLC-QTOF-MS analysis, a relatively diverse spectrum of phenolics was found in the free phenolic fraction compared to the insoluble-bound phenolics. The highest number of phenolic compounds were identified in HPP-treated flower in the free phenolic fraction, followed by HPP-treated internal organs and body wall in the free phenolic fraction. Most of the phenolic compounds were identified as phenolic acids (mainly protocatechuic, *p*-coumaric, vanillic, gallic, hydroxygallic, isoferulic, syringic, ellagic, and chlorogenic acids), flavonoids (mostly catechin and quercetin), and *p*-hydroxybenzaldehyde. To the best of our knowledge, this is the only study where free, esterified, and insoluble-bound phenolics of sea cucumber were studied, especially on the identification and quantification of phenolics using UHPLC-MS. Thus, the present study provides valuable information that fills the existing gap in the available literature in this and related fields.

These results indicate that the exploration of phenolic compounds might reveal insights towards the discovery of new antioxidant components from *C. frondosa*. This investigation increases the fundamental understanding of the full utilization of Atlantic sea cucumber species and leads to the production of a multitude of value-added products. The current study will also provide a better understanding of the antioxidant properties of sea cucumber phenolics in different systems. The identification of phenolics could be an excellent way to increase the economic value of this marine animal due to its possible health benefits. However, further studies are needed to

isolate these compounds and evaluate their specific bioactivity. Confirmation of all these predictions needs experimental validation. *In vivo* analysis of phenolic compounds may further strengthen the findings from *in vitro* bioactive assays. Moreover, clinical evidence will accelerate the process of providing support for the health claims and commercializing of sea cucumber-derived phenolics as potential functional food or natural pharmaceutical ingredients. Furthermore, the economic analysis would assist in determining the applicability of sea cucumber phenolics in foods or supplements. In addition, this systematic approach would address both the environmental concerns and economical sustainability of sea cucumber industries through the full utilization of sea cucumber body parts, including discards.

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1. Hossain, A., Dave, D., & Shahidi, F. (2022). Phenolic compounds and antioxidant capacity of sea cucumber (*Cucumaria frondosa*) processing discards as affected by high-pressure processing (HPP). *Antioxidants*, 11, 1-19.
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3. Shahidi, F., & Hossain, A. (2018) Bioactives in spices, and spice oleoresins: Phytochemicals and their beneficial effects in food preservation and health promotion. *Journal of Food Bioactives*, 3, 1-68.

Manuscripts in preparation:

1. Effect of high-pressure processing (HPP) on phenolics of North Atlantic sea cucumber (*Cucumaria frondosa*)
2. Phenolic profiles of sea cucumber (*Cucumaria frondosa*) and their *in vitro* biological activities

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