

DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**BIO-INDUSTRIAL ACTIVE AGENTS FROM SEA
CUCUMBERS ALONG TURKISH COASTLINES**

by
Nazlı MERT

December, 2014
İZMİR

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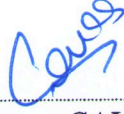
**A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of Dokuz Eylül University
in Partial Fulfillment of the Requirements for the Degree of Master of Science
in Biotechnology**

**by
Nazlı MERT**

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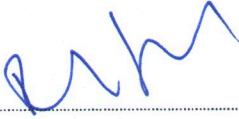
M.Sc THESIS EXAMINATION RESULT FORM

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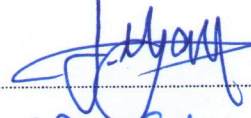
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Nazlı MERT

BIO-INDUSTRIAL ACTIVE AGENTS FROM SEA CUCUMBERS ALONG TURKISH COASTLINES

ABSTRACT

Sea cucumbers are member of phylum Echinodermata and class Holothuroidea. Holothurians are widely distributed in all of the marine ecosystems. *Holothuria tubulosa*, *H. sanctori*, *H. polii*, *H. mammata* and *Stichopus regalis* have wide distribution along Turkish coastline. They secrete important secondary metabolites called triterpene glycosides that have biotechnological properties such as anti-acne, hemolytic, cytotoxic, antimetastatic, anti-angiogenetic antifouling and wound healing. Although holothurians have vital biotechnological importance with their bioactive agents, the dried forms are exported to the far east countries with low costs in Turkey. In this thesis, qualitative and semi-quantitative experiments were carried out the determination of triterpene glycoside levels in sea cucumbers. The vitamin C, protein, carbohydrate, lipid contents and fatty acid composition were also studied. An adsorbent from lipid extracted residues of sea cucumber was developed against methylene blue. Antifouling performances of sea cucumbers' extract within model an antifouling paint were carried out. According to the results, the highest saponin concentration was observed in methanol-dichloromethane extract of *H. polii*. The adsorbent developed from lipid extracted residues showed more adsorption capacity against methylene blue compared to untreated sea cucumber biomass. The kinetic data supported pseudo-second order which means chemisorption. *In silico* tests showed that holothurinoside A and DCA had highest and lowest docking scores for cyanobacterial photosystem II, respectively. Although the most successful biocide was commercial biocide, zinc pyrion, in the antifouling tests, the extracts prepared with methanol and dichloromethane were also promising. In conclusion, these natural bioactive agents might contributed economy of the countries in the Mediterranean region. More researches are strongly recommend on these bioactive agents based on region and season.

Keywords: Adsorption, antifouling, *in silico*, saponin, sea cucumbers.

TÜRK KIYILARINDAKİ DENİZ HİYARLARINDAN BİYO-ENDÜSTRİYEL AKTİF AJANLAR

ÖZ

Deniz hıyarları Echinodermata şubesi ve Holothuroidea sınıfının üyeleridir. Holothurianlar tüm deniz ekosistemlerinde geniş yayılım göstermektedir. Türk kıyılarında bulunan türleri *Holothuria tubulosa*, *H. sanctori*, *H. polii*, *H. mammata* and *Stichopus regalis*'dir. Deniz hıyarları anti-akne, hemolitik, sitotoksik, antimetastatik, anti-angiogenetik, antifouling ve yara iyileştirme gibi biyoteknolojik öneme sahip triterpen glikozid olarak adlandırılan oldukça önemli sekonder metabolit salgılamaktadır. Deniz hıyarları biyoaktif ajanları ile biyoteknolojik açıdan öneme sahip olmasına rağmen kurutulmuş formları çok düşük fiyatlarla uzak doğu ülkelerine ihraç edilmektedir. Bu tezde deniz hıyarlarında bulunan triterpen glikozidlerin seviyelerini belirlemek için kalitatif ve yarı-kantitatif analizler yapılmıştır. Ayrıca C vitamini, protein, karbohidrat ve lipid içeriği ile yağ asidi kompozisyonu da çalışılmıştır. Metilen mavisi uzaklaştırmak amacı ile lipid ekstrakte edilmiş deniz hıyarı atıkları adsorban olarak geliştirilmeye çalışılmıştır. Ayrıca model antifouling boyalar kullanılarak deniz hıyarı ekstraktlarının antifouling performansı araştırılmıştır. Sonuçlara göre en yüksek saponin düzeyi *H. polii* metanol-diklorometan ekstraktında gözlenmiştir. Lipid ekstrakte edilerek geliştirilmiş adsorbanların işlem görmemiş deniz hıyarından elde edilen adsorbanlara kıyasla daha yüksek metilen mavisi adsorblama kapasitesine sahip olduğunu gösterilmiştir. Kinetik çalışmalar, adsorpsiyonun kemisorpsiyon anlamına gelen pseudo-second order kinetiğine uyduğunu göstermektedir. *In silico* testler siyanobakteriyal fotosistem II için doklama sonuçlarından en yüksek olan holothurinoside A ve en düşükünün DCA olduğunu göstermektedir. Antifouling testlerinde en başarılı biyosit ticari olan zinc pyrion olsa da metanol ve diklorometan ile hazırlanan ekstraktlar da umut vericidir. Sonuç olarak bu doğal kaynaklar Akdeniz bölgesindeki ülkelerin ekonomilerine katkı sağlayacaktır. Bu biyoaktif ajanlar ile ilgili sezon ve bölgeye dayalı daha çok araştırma önerilmektedir.

Anahtar kelimeler: Adsorpsiyon, antifouling, *in silico*, saponin, deniz hıyarı.

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CHAPTER ONE

INTRODUCTION

1.1 Biology and Ecology of Sea Cucumbers

Sea cucumbers *i.e* holothurins are slow-moving, ectothermic benthic marine invertebrate animals (Van Dyck, Gerbaux & Flammang, 2010; Bondoc, Lee, Cruz, Lebrilla & Juinio-Meñez, 2013; Zamora & Jeffs, 2012) which are the member of phylum Echinodermata (Spiny skin), class Holothuroidea (Chludil, Murray, Seldes & Maier, 2003; Zhou et al., 2014). Their predators are fishes, sea stars, gastropods and crustaceans (Francour, 1997; Bondoc et al., 2013). All around the world, there are about 1250 Holothurian species (Zhou et al., 2014; Han et al., 2009). Their habitats are from shallows to deep seas across the world (Bordbar, Anwar & Saari, 2011) and they can be easily found in the seashore (Chudhil et al., 2003) of the sandy or muddy layers (Hickman et al., 2008). *Holothuria* species are generally found near the seagrass (mostly around *Posidonia oceanica*) meadows (Kazanidis et al., 2010) (Figure 1.1).



Figure 1.1 *Holothuria tubulosa* around *Posidonia oceanica* meadow in Dikili, İzmir (The photo was taken by Levent Cavas).

Since the leathery body of the holothurians is elongated in the oral-aboral axis, they are quite different from other Echinodermata (Hickman et al., 2008). Furthermore, on the contrary of the other member of the phylum, their body generally out of spicules; they have tiny ossicles (Hickman et al., 2008). Some species of this class crawls on the sea floor and some of them hiding in the hollows (Hickman et al., 2008). On their body wall, along the ambulacra, there are circular and longitudinal muscles and in order to move, their tube feet distributed generally all over the ventral side, on the other hand, some tube feet homogenously located in five ambulacral areas (Hickman et al., 2008).

The coelomic cavity of holothurian has fluid which plays a role as hydrostatic skeleton and also has coelomocytes (Hickman et al., 2008; Kamenev, Dolmatov, Frolova & Khang, 2013). Their digestive system is posteriorly discharged to the muscular cloaca (Kamenev, Dolmatov, Frolova & Khang, 2013). They have two respiratory tubes for both respiration and excretion. For gas exchange; skin and tube feet are also of great importance. On the contrary to the other members of the phylum, the hemal system is quite developed in holothurians; water-vascular system of them lies free in coelom (Hickman et al., 2008, Kamenev, Dolmatov, Frolova & Khang, 2013). Although they have generally separate sexes, hermaphrodite holothurians can also be observed. They have just one gonad and this gonad form as tubules joined with gonoduct. Their free-swimming larvae called as auricularia (Hickman et al., 2008).

Holothurians are environment-engineers (Kazanidis et al., 2010); they are of great importance for marine ecosystem since they are both deposit and suspension feeders; they recycle the nutrients and stimulate microalgal growth to enhance the primary productivity (González-Wengüemert, Aydin & Conand, 2014; Kazanidis et al., 2010; Hickman et al., 2008). They also stir the sediments' upper layer (González-Wengüemert, Aydin & Conand, 2014), converts the wastes in nitrogenous compounds (Kazanidis et al., 2010) and their feces prevent the ocean acidification because of their high buffer capacity (Schneider, Silverman, Woolsey, Eriksson, Byrne, & Caldeira, 2011).

In East Asia countries, holothurians are of great importance because of their high nutrimental values. In Turkey, after collecting the holothurian species, frozen or dried portion of them have been exported to far east countries with low costs in which they are considered as a source of nutrimental and pharmaceutical compounds since two decades (Kazanidis et al., 2010). All around the world, the total number of exported sea cucumber was about 179 thousand tones in 2011 (Zhou et al., 2014). Reproduction dynamics of holothurians changes from region to region (Kazanidis et al., 2010).

Holothuria tubulosa, *H. sanctori*, *H. polii*, *H. mammata* and *Stichopus regalis* have wide distributed along Turkish coastlines (Figure 1.2).

Kingdom :	Animalia
Phylum:	Echinodermata
Classis :	Holothuroidea
Ordo:	Apodida
Ordo :	Elasipodida
Ordo :	Molpadiida
Ordo :	Dendrochirotida
Ordo :	Dactylochirotida
Ordo :	Aspidochirotida
Familia :	Holothuriidae
Genus:	<i>Holothuria</i>
Species:	<i>Holothuria tubulosa</i> Gmelin, 1791
	<i>Holothuria polii</i> Delle Chiaje, 1823
	<i>Holothuria sanctori</i> Delle Chiaje, 1823
	<i>Holothuria mammata</i> Grube, 1840

Figure 1.2 Taxonomy of holothurian in Turkey.

They have high beneficial nutrient profile, such as vitamins (Vitamin A, Vitamin B1, Vitamin B2 and Vitamin B3) and minerals (calcium, iron, magnesium and zinc) (Bordbar, Anwar & Saari, 2011). Matsuna & Tsushima (1995) reported that although some carotenoids (astaxanthin, canthaxanthin, phoenicoxanthin, β -carotene and β -echinenone) are common, some are (cucumariaxanthin A, B and C) indigenous to species. It was mentioned in the paper of Ibrahim (2012) that the flesh and coelomic fluid of *Holothuria scabra*, *Holothuria leucospilota* and *Holothuria atra* contain antibacterial carotenoids (xanthophyll, β -crptoxanthin and β -carotene).

Furthermore, it was reported in the review of Borbar, Anwar & Saari (2011) that holothurians have glycine, glutamic acid, aspartic acid, alanine, and arginine. Also, because of the presence of significant amount of threonine, tyrosine and phenylalanine, they have low lysine/arginine ratio together with high essential amino acid scores (Borbar, Anwar & Saari, 2011).

The risk of predation is high for holothurians, because they are slow-motioned. To protect themselves, they secrete some allelochemicals from their skin. These compounds are located in the body wall of sea cucumber where role as a physical barrier plays (Figure 1.3 a). The other important secondary metabolite source is cuvierian tubules where are located in the posterior of the animal (Figure 1.3 b). Expelled tubules lengthen into sticky white threats that may entangle potential predators (Bondoc et al., 2013).



Figure 1.3 The body wall (a) and the cuvierian tubules (b) of holothurian

1.1.1 *Holothuria polii*

Holothuria polii Delle Chiaje, 1823 (hereafter *H. polii*) is a common, white spot sea cucumber. The synonym of this species is *Muelleria parvula*. Their maximum length is 20 cm (Sealifebase, n.d). They are supported black with little white spots. They feed with organic substances. *H. polii* are both subtropical and benthic species. The region that they can be found is generally on hard flats and seagrass meadows. According to IUCN, *H. polii* is at least concern category (IUCN red list, n.d). They

are widely distributed in the Mediterranean sea (Algeria, Atlantic coast, Canary Islands, Croatia, France, Gulf of Suez (northern Red Sea), Italy, Spain and Turkey) from 0-250 m deep (Sealifebase, n.d).

1.1.2 *Holothuria tubulosa*

Holothuria tubulosa Gmelin, 1791 (hereafter *H. tubulosa*) is a tubular cylindrical sea cucumber with the length of 20-45 cm and diameter of 6 cm. Their colour change from pale brown to reddish brown with numerous conical papillae. In order to locomote, there are three lines of tube feet along their ventral side of the body. The tentacles on terminal side which are used for feeding are flattened and short. They are distributed from the Atlantic Ocean up to the Bay of Biscay and in the Mediterranean Sea, from surface to 100 m deep. Also they live on sandy flats (*Posidonia* or muddy rocks) (Despalatović, Grubelić, Šimunović, Antolić & Žuljević, 2004; European Marine-Life, n.d). The photograph of *H. tubulosa* and *H. polii* was given in Figure 1.4.



Figure 1.4 The holothurian samples a) *H. tubulosa* b) *H. polii*

1.2 Triterpene Glycosides

To protect themselves against external risks, organisms secrete some natural bioactive agents. These chemicals are called secondary metabolites (Sipkema, Franssen, Osinga, Tramper & Wijffels, 2005; Paul, Puglisi & Ritson-Williams, 2006; Acevedo et al., 2013). These chemically mediated agents perform some important ecological roles, for example protecting themselves against the predators, biofoulers and opportunistic pathogenic organisms and competing for the area and preventing overgrowth of epibionts (Acevedo et al., 2013).

Saponins *i.e* triterpene glycosides are rare in the animal kingdom (Kalinin, 2000) and they are the major secondary metabolites of Echinoderms, especially in the sea cucumbers (Holothuroidea), sponges and starfishes (Kerr & Chen, 1995; Schilenko et al., 2005). The first toxic fraction of sea cucumber, *Actionopyga agassizi*, from the Cuvierian tubules was investigated by Nigrelli (1952). Then, physiologically active components from *Holothuria lucospilota* were isolated and ichthyotoxic compounds from aqueous holothurian extracts were reported by Yamanouchi (1955). Nigrelli and Yamanouchi determined these natural bioactive molecules as “holothurin” (Kalinin, 2000).

These chemicals are released from their viscera, gonads, Cuvierian tubules and body walls (Bakus, 1968; Bakus, 1974; Matsuno & Ishida, 1969; Bondoc et al., 2013). Saponins of holothurians are characterized with a triterpenoid-structured portion and carbohydrate chains (Kerr & Chen, 1995). The structure of the triterpene glycosides are very complicated and the features of these molecules vary in the nature of the monosaccharide unit (position and number) and the property of glycone chain (number and the position of functional groups and double bonds) (Kalinin, 2000). The sea cucumber triterpene (4,4,14-trimethylsterol) oligoglycosides (or saponins) possess an aglycone and a glycone chain in their chemical compositions (Silchenko et al., 2012).

In holothurians, the lanostane-type aglycone chain belongs to three terpene units with 18(20)-lactone in their D ring (Silchenko et al., 2012). The structure of aglycone part of sea cucumber saponins is called holostane ($3\beta,20S$ -dihydroxy- 5α -lanostano-18,20-lactone) (Kalinin, 2000) (Figure 1.5). The triterpene part of sea cucumber triterpene glycosides have $\Delta^{9(11)}$ double bond (Kerr & Chen, 1995).

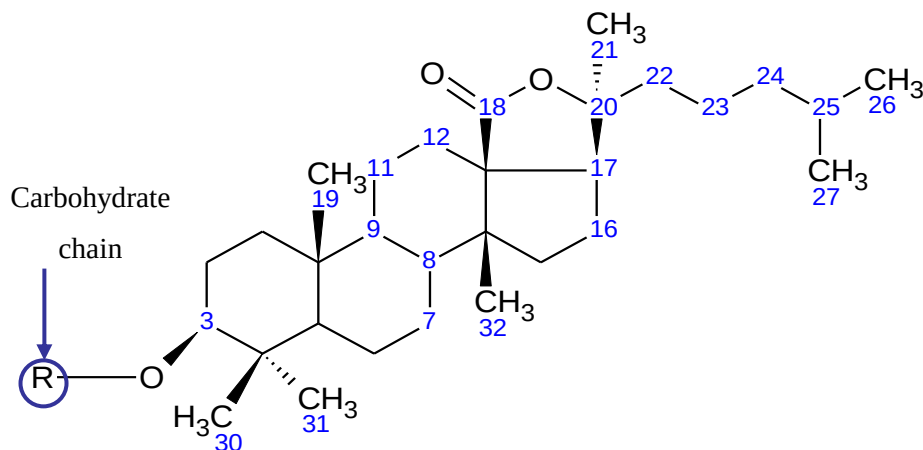


Figure 1.5 Holostane part of sea cucumber triterpene glycoside.

On the other hand, the glycone chain composes of 2 to 6 monosaccharide units (such as xylose, quinovose, glucose, 3-O methylglucose and 3-O methylquinovose) that linked to the C-3 of the holostanol skleton (Maier, 2008). Saponins from sea cucumber are mostly tetra or pentaosides (Maier, 2008) and they sometimes have sulfate group(s) on their carbohydrate units (Maier, 2008; Silchenko et al., 2012; Bondoc et al., 2013). Detailed structure of glycone chain of sea cucumber triterpene glycosides were given in Figure 1.6.

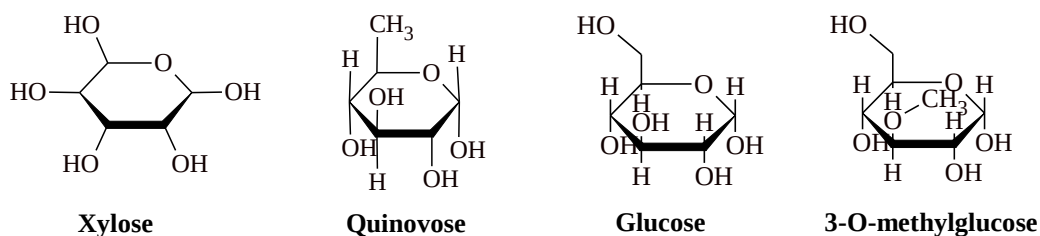


Figure 1.6 The structures of monosaccharide in glycone chain of sea cucumber triterpene glycosides

Kerr and Chen (1995) investigated the *in vivo* and *in vitro* biosynthesis of triterpene glycosides in sea cucumber. They reported that the precursor of sea cucumber triterpene glycosides is parkeol (lanost-9(11)-en-3 β -ol) because of the presence of $\Delta^{9(11)}$ double bond in their aglycone chain. In the study of Kerr and Chen (1995), it was reported that parkeol is the sole cyclization product of squalene in sea cucumbers, not of the isomeric lanosterol or cycloartenol. Both sea cucumber holothurins and sterols derived from parkeol with some enzymatic reactions and holothurins are synthesized in cuvier gland of sea cucumbers (Kerr & Chen, 1995) (Figure 1.7).

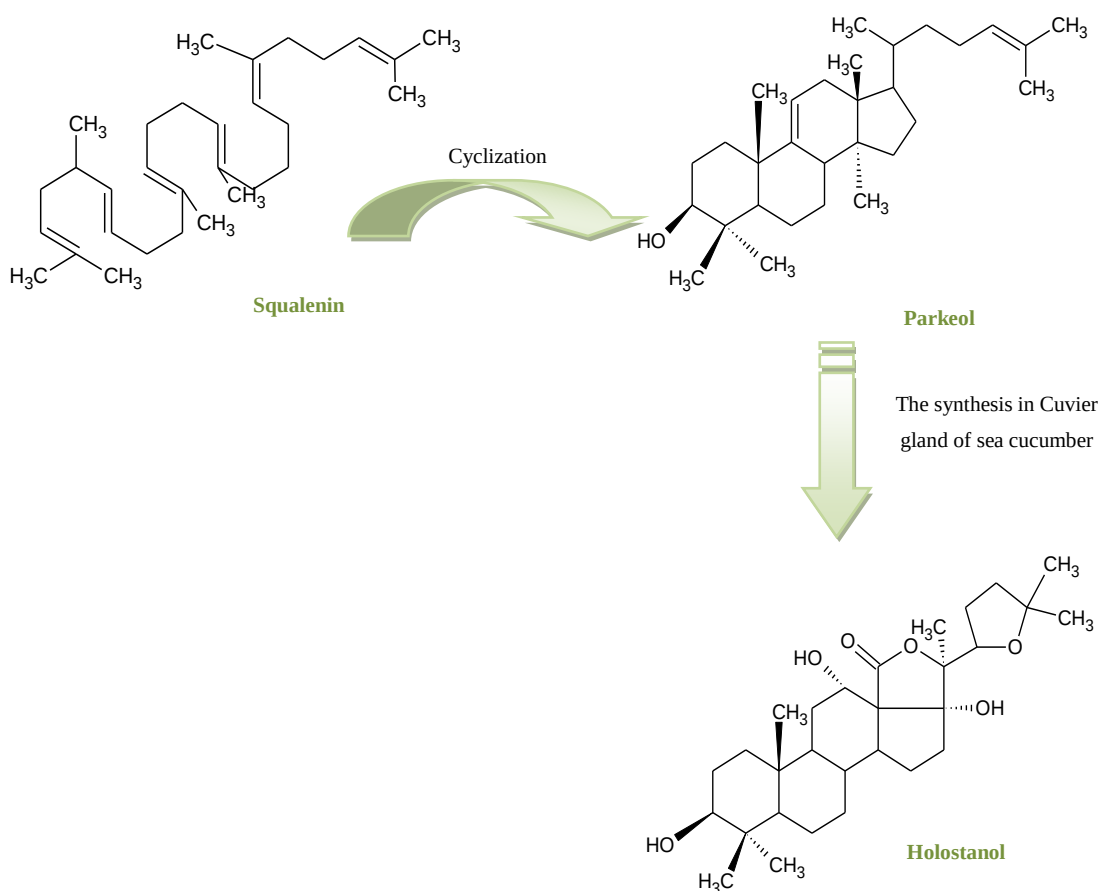


Figure 1.7 The biosynthesis of triterpene glycoside in sea cucumber (Kerr & Chen, 1995)

The triterpene saponins of holothurians are characteristic for subfamilies, genera and also species (Kalinin, Levin & Stonik, 1994; Stonik & Elyakov, 1988; Kalinin, 2000) (Table 1.1).

Table 1.1 List of triterpene glycosides in *Holothuria* genus

SPECIES	Name of triterpene glycoside	SPECIES	Name of triterpene glycoside	SPECIES	Name of triterpene glycoside
<i>Holothuria arenicola</i>	Holothurin A Holothurin B	<i>Holothuria fuscocinerea</i>	Holothurin A Holothurin B Fuscocineroside A Fuscocineroside B Fuscocineroside C Pervicoside C	<i>Holothuria nobilis</i>	Holothurin A Holothurin B Desholothurin A (Nobilisid 2a)
<i>Holothuria atra</i>	Holothurin A Holothurin A ₂ Holothurin B Holothurin B ₁ Holothurin B ₄	<i>Holothuria gracilis</i>	Holothurin A Holothurin A ₂ Holothurin B	<i>Holothuria pervicax</i>	Holothurin A Holothurin B Pervicoside A Pervicoside C
<i>Holothuria axiloga</i>	Holothurin A Holothurin A ₂ Holothurin B Axilogoside A	<i>Holothuria grisea</i>	Holothurin A Holothurin B 17-dehydroxy holothurinoside A	<i>Holothuria pulla</i>	Holothurin A Holothurin B
<i>Holothuria cinerascens</i>	Holothurin A	<i>Holothuria hilla</i>	Holothurin A Holothurin B Hillaside A Hillaside B	<i>Holothuria scabra</i>	Holothurin A Holothurin A ₂ Holothurin B 24-dehydro holothurin A ₂
<i>Holothuria coluber</i>	Holothurin A Holothurin B	<i>Holothuria impatiens</i>	Holothurin A Bivittoside C Bivittoside D Impatienside A	<i>Holothuria squanifera</i>	Holothurin A
<i>Holothuria cubana</i>	Holothurin A Holothurin B	<i>Holothuria leucospilota</i>	Holothurin A Holothurin B Holothurin B ₁ Holothurin B ₄ Fuscocineroside B Fuscocineroside C Desholothurin A (Nobilisid 2a) Leucospilotaside A Leucospilotaside B Leucospilotaside C Bivittoside C	<i>Holothuria surnamensis</i>	Holothurin A Holothurin B
<i>Holothuria difficilis</i>	Holothurin A Holothurin B	<i>Holothuria lubrica</i>	Holothurin A Holothurin B	<i>Holothuria tubulosa</i>	Holothurin A Holothurin B Holothurin B ₂ Holothurin B ₃ Holothurin B ₄
<i>Holothuria edulis</i>	Holothurin A Holothurin A ₂ Holothurin B	<i>Holothuria mexicana</i>	Holothurin A Holothurin B	<i>Holothuria polii</i>	Holothurin B ₂ Holothurin B ₃ Holothurin B ₄

Table 1.1 List of triterpene glycosides in *Holothuria* genus (continued)

<i>Holothuria forskalii</i>	Holothurinoside A Holothurinoside C Holothurinoside F Holothurinoside H Holothurinoside I Holothurinoside E ₁ Holothurinoside H ₁ Holothurinoside I ₁ Holothurinoside J ₁ Holothurinoside K ₁ Desholothurin A (Nobilisid 2a) Desholothurin A ₁ (Arguside E)	<i>Holothuria moebi</i>	Holothurin A	<i>Holothuria monacaria</i>	Holothurin A
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Kalinin (2000) reported that since sea cucumber triterpene glycosides consist of two different units (glycone and aglycone), each part of these molecules show many bioactivities. First, hydrophilicity is significant for deterrence of predators which all the polar structures of aglycone unit and sulfate can lead to this property. Because of its stereochemistry, lanostane part of the molecule plays formation of complex and hydrophilicity (Kalinin, 2000). Both 12 α -hydroxyl and 17 α -hydroxyl groups in triterpene glycosides cause hydrophilicity and membranolytic activity (Kitagawa, 1988; Lykhatskaya et al., 1991; Kalinin, 2000). The oxygene groups in 12 α -hydroxyl and 17 α -hydroxyl have effect on size of the molecules (Kalinin, 2000). 18(20)-lactone is of great importance on formation of membrane sterol complex (Kalinin et al., 1992; Kitagawa, 1988; Kalinin, 2000). The glycone parts of these molecules have formation and conservation property of the pores and channels, also they are of great importance on form and size of the molecules (Lykhatskaya et al., 1991; Kalinin, 2000). Xylose is the first monosaccharide that is linked to C-3 of the holostanol skleton (Maier, 2008) and shows formation of channel and pores and lysis. Xylose-linked 4-O-sulfate group has little membranotropic activity. The related scheme was given in Figure 1.8.

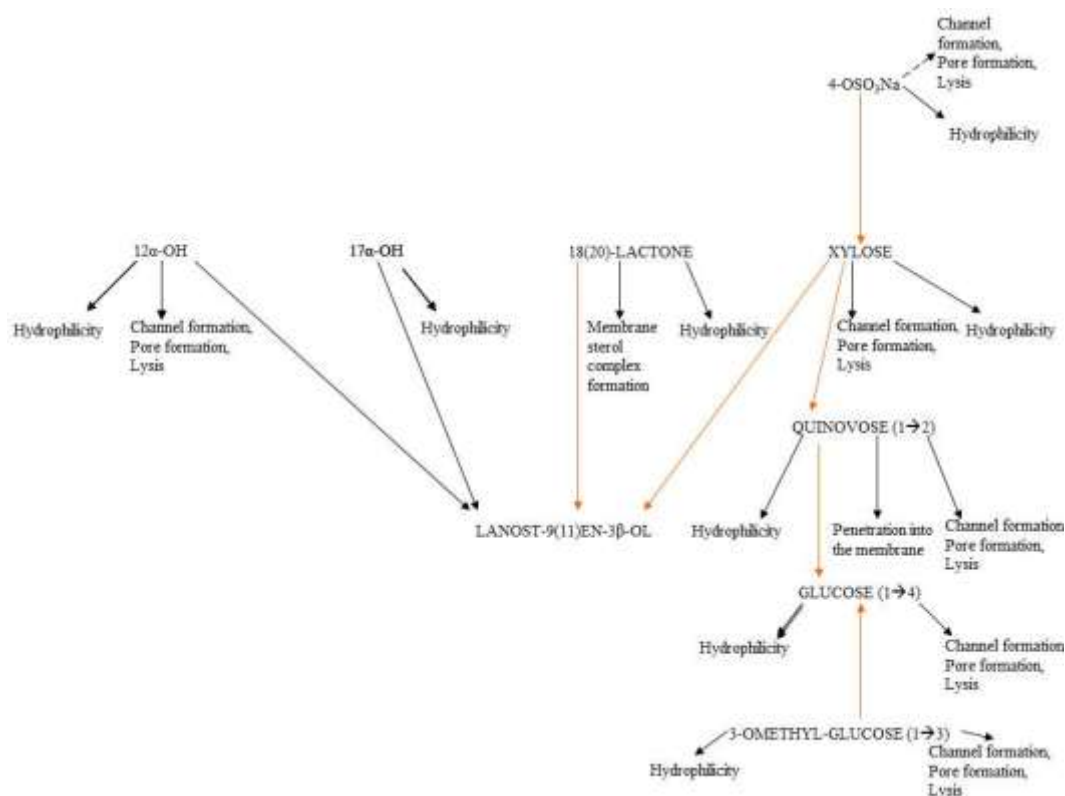


Figure 1.8 The biospecificity of each part of the triterpene glycoside in sea cucumber (Re-drawn from Kalinin, 2000)

1.3 The Biotechnological Importance of Sea Cucumber Extract

Since the glycone units of the molecule interact with the 5(6)-unsaturated sterols (especially cholesterol) of target cellular membranes unselectively, triterpene glycosides cause strong membranolytic activity (Kalinin, 2000). Thus, single-ion channels and large water-loaded pores can be observed (Kalinin, 2000). Such sterol-saponin interactions can cause loss of some ions, peptides and compounds in nucleotide pools, damage the ion hemostasis and osmolarity, cause cell death (Anisimov, 1987; Likhatskaya et al., 1985; Kalinin, Aminin, Avilov, Silchenko & Stonik, 2008; Aminin et al., 2010). The membranolytic effect of the glycone chain of sea cucumber triterpene glycosides can cause ichthyotoxicity, thus holothurians protect themselves from predators (Kalinin, 2000). Showing allelopathic activity, the glycosides of these molecules can protect the organisms from attachment of micro and macro foulers and opportunists (Kalinin, 2000)

Triterpene glycosides of holothurians show some industrial properties, especially they exhibit attractive feature on pharmacy and medicine. Because of the membranotropic effect of triterpene glycosides, they show antifungal, hemolytic and cytotoxic activity (Aminin et al., 2010; Kalinin, Aminin, Avilov, Silchenko & Stonik, 2008). Beside these, anti-angiogenetic (Zhang et al., 2009), antiproliferative and anticancer (Zhang et al., 2009; Careaga, Bueno, Muniain, Alché & Maier, 2009), antimetastatic (Attaub et al., 2013) anti-inflammatory (Aminin, Gorpenchenko, Bulgakov, Andryashchenko, Avilov & Kalinin, 2011), antifouling (Acevedo et al., 2013), antimicrobial (Omran & Allam, 2012), anti-inflammatory (Herencia et al., 1998) and antioxidant (Esmat, Said, Soliman, El-Masry & Badiea, 2013) properties of sea cucumber triterpene glycosides and extracts have so far been reported in scientific papers. An anti-acne property of sea cucumber extract is a novel industrial importance (Drugstore, n.d; Safi, n.d). In the review of Bordbar, Anwar & Saari (2011), it was indicated that sea cucumber extracts and triterpene glycosides have angiotensin converting enzyme inhibition, antihypertensive, antiviral, antitrombic effects. Furthermore, it was mentioned in the same review that these molecules and extracts show osteoarthritis and wound healing property (Bordbar, Anwar & Saari, 2011).

1.3.1 Adsorption

Removal of the undesirable substances from aqueous solution via solid material is defined as adsorption. It is used in many different areas such as decolorization and removal of nerve gases (Cengiz, 2007).

In adsorption phenomena, there are two crucial terms: adsorbent and adsorbate. Adsorbent is a solid material that adsorbs the layer of adsorbent from the solutions. The surface properties of the adsorbents (pore size distribution and specific surface area) are the most important parameter of this procedure because of the adsorption capacity. If the pore sizes are more than 50 nm, it could be defined as “macropores”. The term “mesopore” implies the pore size between 2-50 nm. The widths of the “micropores” are not exceed about 20 nm (Toth, 2002; Cengiz, 2007). Undesirable

molecules (adsorbates) bind onto an adsorbant either physically (physisorption) or chemically (chemisorption).

In the physical adsorption, van der Waals and electrostatic forces between the substrate and the molecules and atoms of adsorbate are existed, thus the other name of this process is van der Waal adsorption. There is a inverse correlation between the temperature and the adsorption. Enthalpy of physisorption is 1 kcal/mole and it is a reversible and fast process. Multilayer uptake is possible when the physical adsorption occurs (Cengiz, 2007).

The covalent or ionic bonds (chemical forces) lead to remove the adsorbate in the chemical adsorption, this process is also defined as activated adsorption. The correlation between the temperature and the adsorption is direct proportion; it needs high temperature to adsorb the material. 500-100 kcal/mole is the enthalpy, nevertheless it is an irreversible and variable process. Moreover, chemisorption is limited to one monolayer uptake (Cengiz, 2007).

The factors that affect the adsorption procedure are:

- Properties of adsorbent, adsorbate and solvent,
- Chemical features, structure and interactions,
- Pore properties,
- pH,
- Temperature etc. (Marczewska & Marczewski, 2002; Tsai, Lai & Hsien, 2003)

In order to optimize the temperature conditions for categorising the interactions, the points that the equilibrium solution concentrations keep stable are determined. In this MSc. thesis, the residue of lipid extracted and the untreated sea cucumber body walls were used for preparation of adsorbent.

1.3.2 Antifouling

Undesirable attachment and accumulation of mainly organic compounds, microorganisms, plants and animals to artificial surfaces immersed into seawater is defined as biological fouling or biofouling (Wahl, 1989; Clare, 1996). The first step of this natural and reversible process is adsorption of organic compounds (such as carbohydrates, polysaccharides, fatty acids) onto surface and formation of this nutrient-rich-layer occurs within a minute (Abarzua & Jakubowski, 1995). Accumulation of such organic compounds (nutrients) by electrostatic interactions, Brownian effect, van der Waals forces leads to generation of microorganism, thus biofilm formation can be observed (Callow & Fletcher, 1994; Abarzua & Jakubowski, 1995). Subsequent to formation of biofilm, bacterial colony adsorbed to this surface by physical forces and irreversible covalent interaction occurs between the molecules and the extracellular polymers of bacteria, thus bacterial population increase (Wahl, 1989; Abarzua & Jakubowski, 1995), micro (diatoms and protozoa) and macro foulers (balanus, sea worms, tunicates *i.e* animals and algae *i.e* plants) generate step by step on the artificial surface (Zahuranec, 1991; Clare et al., 1992; Abarzua & Jakubowski, 1995; Yebra et al., 2004; Almeida et al., 2007; Acevedo et al., 2013). For foulers, biofouling is an advantage for preventing from predators and for accessing to the nutrients (Flemming et al., 1996; Yebra et al., 2004).

In shipping industry, marine biofouling is of great importance for engineered marine structures, such as offshore platforms and ships (Acevedo et al., 2013). It causes not only increasing of the fuel and energy consumption of the vessels, rising of the ship's weight, expanding of the surface friction because of the reducing of surface smoothness, reducing maneuver capacity and decrease of the speed of the marine vehicle, but also the dry docking time and the man-power (Rascio, 2000; Acevedo et al., 2013). Wright (2009) reported that the cost of antifouling (AF) technology is approximately 4 billion US\$ per year. Traditional self-polishing type AF paints include toxic biocides. The most common of these biocides are chlorothalonil, dichlofluanid, diuron, econeal, irgarol 1051, kathon 5287, maneb,

TCMS pyridine, sea nine 211, TCMTB, thiram, zinc pyrithione, zineb and ziram (Thomas, 2001; Voulvoulis et al., 2002; Mert et al., 2014) (Figure 1.9).

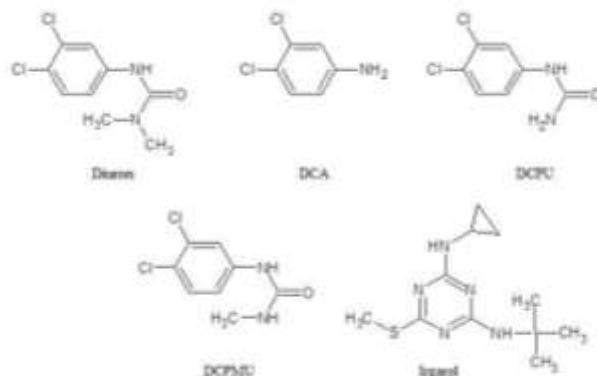


Figure 1.9 Some of toxic biocides

Diuron, irgarol 1051 and their degradation products have negative effects on marine photosynthetic organisms. Diuron and irgarol bind reversibly to D1 protein (QB site) of PSQ and block of the electron transfer in photosynthesis (Lam et al., 2005; Jones, 2005; Alyuruk & Cavas, 2013). The importance of research and development of eco-friendly natural products was underlined by current studies. Hellio et al., (2009), Acevedo et al., (2013), Perez et al., (2014) and Iyapparaj et al., (2014) reported the efficiency of extracted and/or isolated compounds (especially secondary metabolites) from organisms on antifouling technology. In this study, the antifouling properties of different extarcts of sea cucumbers were tested.

1.3.3 *In-silico* Evaluation

Molecular docking is a useful method to predict the orientation of the ligand on a receptor and to evaluate the binding affinity of these molecules. This computer-based interaction simulation method is defined as *in-silico* molecular docking. Recently, in order to predict the binding site and affinity of small molecules especially drugs on a protein based receptor, molecular docking studies are frequently used, therefore *in silico* analysis are of great importance on biological, chemical and pharmaceutical researches. *In silico* analysis are not only attractive on time consumption, but they also good at prediction of binding site and affinity of the ligand (such as drug) on the

receptor (generally protein) (Kar & Roy, 2010, 2012, 2013; Gundogdu-Hizliates, Alyuruk, Gocmenturk, Ergun & Cavas, 2014).

Recent studies show that there is a correlation between *in vitro* and *in silico* (Kar & Roy, 2013). Computer-based molecular docking studies are important alternatives to initial steps of the researches such as early-stage toxicity tests and experiments on living organisms (rabbits, mice, dogs etc.) (Stepnik, Malinowska & Rój, 2014).

Water: plastoquinone oxidoreductase, photosystem Q (B) protein 1 (Hereafter PSQ) is located in the photosynthetic thylakoid membrane. It is also a large homodimeric protein-cofactor complex (Guskov et al., 2009). Each monomers of PSQ consist of 20 subunits, 35 chlorophyll a and 12 carotenoid molecules, 25 integral lipids and 1 chloride. The position of chloride is crucial because of the proton-transfer reactions. For plastoquinol-plastoquinone exchange, there are also one third plastoquinone Q and a second plastoquinone-transfer channel (Guskov et al. 2009). The effect of toxic booster biocides and triterpene glycosides on PSQ was evaluated in this thesis.

1.4 The Aim of the Thesis

The aim of the thesis was to investigate the following experiments in the sea cucumbers along Turkish coastline:

- To characterize the triterpene glycosides
- To determine the levels of protein, carbohydrates, lipids, fatty acid composition, vitamin C, foam and froth
- To determine the moisture and ash level
- To determine the antifouling performances of sea cucumber extracts
- To determine the adsorption capacity of lipid extracted and the untreated sea cucumber body wall residues

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials and Instrument

In order to prepare extracts, technical grade ethanol, methanol, chloroform, n-hexane (Tekkim, 99%), analytical grade dichloromethane (Merck) and iso-butanol (Merck) were used. To remove the solvents in the extracts, rotary evaporator (Butchi Rotary evaporator, R3) was used. The extracts were lyophilized by using lyophilisator (Labconco Free Zone, 2.5 plus). The shaker (GFL 1092 Shaking Water Baths) was used for preparation of some extracts. Thin Layer Chromatography (TLC) determination was carried out on TLC silica gel 60 F₂₅₄ (20x20 cm, layer thickness 200 µm, Merck). As a standard saponin, *Quillaja* bark saponin (Sigma-Aldrich, S4521) was used. Acetic acid (Riedel de Haën) was used as a mobile phase for TLC. For colorimetric reactions, vanillin (Fluka; >98% for HPLC), sulfuric acid (Carlo Erba; 96%) analytical grade ethanol (Merck; for analysis) and 2,3,4-trimethoxy benzaldehyde (Merck) were used. In order to determine the protein level in sea cucumber body wall, commosie brilliant blue (Sigma, B5133), technical o-phosphoric acid (İron Kimya), bovine serum albumine (Sigma, 7906) and homogenizer (Heidolph) were used. Evaluation of the carbohydrate level in sea cucumber was carried out by using D (+) glucose 1-hydrate (Pancreac), D (+) galactose (Fluka), D (+) sucrose (Fluka), D (+) maltose (Fluka), D-fructose (Fluka) and phenol (Riedel de Haën). For HPLC determination of L-ascorbic acid (Sigma, A5960), Shimadzu Prominence LC-20A equipped with a DGU-20A5 solvent degasser, SPD-20A UV/VIS detector and CTO-10ASUP column oven were used. Inertsil C18 (3x150 mm) column was used. As a mobile phase, HPLC grade metaphosphoric acid (Merck) and Milli-Q water were applied. In order to determine the adsorption capacity of the sea cucumber body wall, Micro plate reader (ELx800, Biotek Instrurment, USA) was used.

2.2 Collection of Holothurians

Holothurians; *H. polii* Delle Chiaje, 1824 and *H. tubulosa* Gmelin, 1790 were collected from Dikili (Aegean Sea, Turkey) at a depth of 0-2 m (Figure 2.1). The official permission was taken from Republic of Turkey Ministry of Food, Agriculture and Livestock.

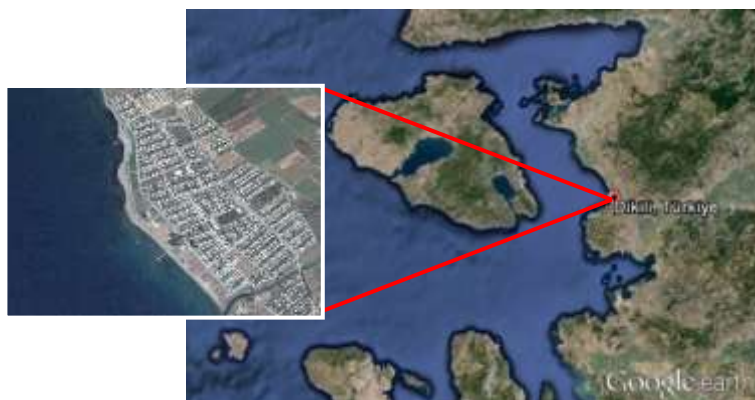


Figure 2.1 Google Earth image of Dikili Bay, İzmir coastline (39° 07' 15.38'' N and 26° 51' 11.94'' E) where Holothurian samples were collected.

The sea cucumbers were transported to the laboratory in the fresh-sea water and then they were killed by freezing at -17°C (Bondoc et al., 2013).

2.3 Preparation of the Extracts for Triterpene Glycosides

In order to prepare extracts for triterpene glycosides, different solvent and extraction systems were used.

2.3.1 Soxhlet Extraction

After freezing, holothurian samples were dried in the oven at 70°C for one day. 65 g of freeze-dried *H. polii* body-walls were cut into the small pieces and they were extracted with 200 mL ethanol by using soxhlet apparatus for 8 hours (Figure 2.2). The combined extracts were filtered with filter paper and concentrated to the dryness.



Figure 2.2 The a) dried, b) pinched *H. polii* samples and c) soxhlet apparatus.

The other extraction method was applied based on Bondoc et al. (2013) with some modifications. According to this, 20 g of freeze-dried *H. polii* body-walls were pinched into the small pieces and extracted with 250 mL 70% (v/v) ethanol by using soxhlet apparatus for 8 hours.

2.3.2 Maceration

To obtain sea cucumber triterpene glycosides, five different extraction methods were applied.

- Extraction of *Holothuria* sp.

500 g of dissected and dried mixed *Holothuria* sp. were extracted in 500 mL 70% (v/v) ethanol for 3 days.

- Extraction of *Holothuria tubulosa* with *Caulerpa cylindracea*

It was found two distinguishable sea cucumbers in the field trip. Invasive *Caulerpa cylindracea* was on the epidermis of *H. tubulosa*. The epidermal layer of the materials were penetrated by the rhizoids of *C. cylindracea*. It was very difficult to remove *C. cylindracea* from the surfaces (Figure 2.3).



Figure 2.3 The a) underwater b) dorsal view of *C. cylindracea* epibiosed *H. tubulosa*. (The photo “a” was taken by Levent Cavas)

100 g of *H. tubulosa* sample that was epibiosed with *C. cylindracea* was extracted in 35 mL 70% (v/v) ethanol for 3 days.

- Extraction of *H. tubulosa*

In order to evaluate the antifouling performance of *H. tubulosa* extract, 100 g of *H. tubulosa* sample was extracted in 100 mL 70% (v/v) ethanol for 3 days.

- Extraction of cuvierian tubules

For determination the total saponin level in *H. polii* samples, 25 g cuvierian tubules which was obtained from *H. polii* were extracted in 25 mL 70% (v/v) ethanol for 3 days.

- Aqueous meta-phosphoric acid extraction of *Holothuria* species

In order to determine the vitamin C content in both *H. polii* and *H. tubulosa*, the method of Rodriguez-Bernaldo de Quirós, Lopez-Hernandez and SirnaI-Lozano (2001) was used. According to this method, 2 g of both *H. polii* and *H. tubulosa* were added into 6 mL of aqueous 4.5% (w/v) metaphosphoric acid, vortexed for 2 minutes and centrifuged at 1100 rpm for 5 minutes. After removing of the supernatant, the

same extraction method was applied two times more, then all collected and pooled supernatants were diluted to 25 mL with Milli-Q water.

2.3.3 *N-Hexane, Dichloromethane, Chloroform and Butanol Extraction*

For determination of the saponin concentration in *Holothuria* species and for mass analysis, liquid-liquid extraction method of Bondoc et al (2013) was used. According to this method, 20 g freeze-dried sea cucumber samples were extracted with 250 mL 70% (v/v) ethanol. After extraction, the samples were filtered, evaporated by using rotary evaporator and lyophilized till dryness. The dried extract was dissolved in 90% methanol and extracted with n-hexane with the same volume. After that, water content of the hydromethanolic phase was adjusted to 20%, the sample was extracted with dichloromethane, water content of the hydromethanolic phase was adjusted to 40% and extracted with chloroform. After drying of the hydromethanolic phase, the fraction was desalted with methanol and the methanolic phase was evaporated. The dried material was diluted with water and extracted again with iso-butanol with the same volume (Figure 2.4). The iso-butanolic phase contained the semi-pure saponins. The semi-pure saponin contained iso-butanolic portion was diluted as 1/1000, 1/500, 1/250 and 1/100.



Figure 2.4 Liquid-liquid extraction

2.3.4 Methanol-Dichloromethane Extraction of Holothurians

In order to evaluate the antifouling performance of holothurians, methanol-dichloromethane extraction method of Acevedo et al. (2013) was used. According to this procedure, to obtain different polarity metabolites, 100 g wet tissues of *H. polii* and *H. tubulosa* samples were macerated with 100 mL methanol-dichloromethane (1:1) mixture at 20°C by shaking 120 rpm and after filtration, the same procedure was applied two times more. After filtration, each filtrate was evaporated to concentrate by using rotary evaporator. The pooled extracts for each sample were used in antifouling paints.

2.3.5 Lipid Extraction of Sea Cucumber

To determine the total lipid content in *Holothuria* species, 25 g of dissected and dried samples were extracted with 250 mL of n-hexane for 8 hours with soxhlet apparatus. After extraction procedure, the lipid extracted holothurian body wall wastes were dried and used as a methylene blue adsorbent.

2.4 Identification of Triterpene Glycosides by Thin Layer Chromatography

The ethanolic extracts were tested on TLC eluting with different solvent systems as mobile phase to obtain different fractions. The TLC plates were prepared as 4x8 cm and 8x8 cm. Briefly, the combination of the solvent systems were chloroform-ethanol-water (100:100:17); chloroform-methanol-water (65:35:10) and chloroform-methanol (65:35). As a positive standard, 5 mg/mL *Quillaja* bark saponin solution was tested on TLC eluting with chloroform-methanol-water (65:35:10); chloroform-ethanol-water (100:75:10) and chloroform-ethanol-water (100:100:17).

Because of the absence of UV absorbance, triterpene glycoside spots on TLC plates were visualized based on the procedure described by Olezsek et al. (2007). The ethanolic extracts of *H. polii* were subjected on TLC plates with different

solvent systems (as it mentioned above), then the fractions were visualized by using vanillin and analytical grade sulfuric acid. Briefly, solution I [5% ethanolic sulfuric acid (1000 μ L sulfuric acid was dissolved in 1900 μ L of ethanol)] and solution II [1% ethanolic vanillin (0.1 g vanillin was dissolved in 10 mL ethanol)] were prepared. The solution I was sprayed to the plate with 10 mL, then immediately the solution II was sprayed. After this application, the plate was heated for 2 minutes with hair dryer (Figure 2.5).



Figure 2.5 Application of vanillin-sulfuric acid on TLC.

2.5 Colorimetric Vanillin-Sulfuric Acid Assay

To determine the saponin concentration in sea cucumbers, the colorimetric vanillin-sulfuric acid assay was performed based on Hiai et al. (1976). According to this method, 800 mg vanillin was dissolved in 10 mL analytical grade ethanol. To obtain 72% (v/v) sulfuric acid 72 mL 96% sulfuric acid was added in 28 mL distilled water. As a blank, 0.5 mL water was put on a test tube and 0.5 mL 8% (w/v) vanillin-ethanol solution was added. Then 5 mL 72% (v/v) sulfuric acid solution was put on the test tube. The molecular structure of the aromatic aldehyde – vanillin- was given in Figure 2.6.

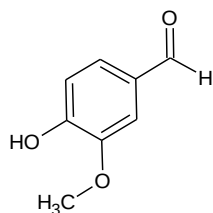


Figure 2.6 The molecular structure of vanillin

5 mg *Quillaja* bark saponin was dissolved in 1 mL water to obtain a stock solution and then this stock solution was serially diluted to 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL and 2.5 mg/mL. 0.5 mL sample was put on a test tube and 0.5 mL 8% (w/v) vanillin-ethanol solution was added. Then 5 mL 72% (v/v) sulfuric acid solution was put on the test tube. All tubes were mixed well and were put on ice bath for 1 minute. They were incubated in water bath at 60°C for 10 minutes, cooled and for all test tubes, spectrums were scanned and measured at 540 nm.

For detection of sea cucumber total saponins, 70% (v/v) ethanolic extract was used. The soxhlet extracts were serially diluted (1/2, 1/5, 1/10, 1/25 and 1/50). The macerated extracts were serially diluted (1/2, 1/5 and 1/10). After the reaction, spectrums were scanned for all test tubes. The absorbance of the extracts was measured at 540 nm.

2.6 Vanillin-Sulfuric Acid Colorimetric Test for Semi-Pure Saponins

The 70% (v/v) ethanolic soxhlet extract of *Holothuria* species was prepared according to Bondoc et al. (2013) with some modifications for vanillin-sulfuric acid colorimetric test for semi-pure saponins. After colorimetric reaction was obtained for all test tubes, the spectrum scanning was performed.

2.7 A New Method: 2,3,4-Trimethoxy Benzaldehyde-Sulfuric Acid Colorimetric Test for *Quillaja* Bark Saponins

As a new method for colorimetric test for *Quillaja* bark saponins, 2,3,4-trimethoxy benzaldehyde was used instead of vanillin. The molecular structure of 2,3,4-trimethoxy benzaldehyde was given in Figure 2.7.

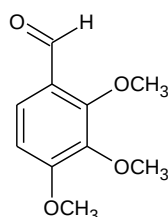


Figure 2.7 The molecular structure of 2,3,4-trimethoxy benzaldehyde

For this method, the procedure of Hiai et al. (1976) was used with some modifications. According to this new method, 800 mg 2,3,4-trimethoxy benzaldehyde was dissolved in 10 mL analytical grade ethanol. To obtain 72% (v/v) sulfuric acid, 72 mL 96% sulfuric acid was added in 28 mL distilled water. As a blank, 0.5 mL water was added to a test tube and 0.5 mL 8% (w/v) 2,3,4-trimethoxy benzaldehyde-ethanol solution was added. Then 5 mL 72% (v/v) sulfuric acid solution was put on the test tube. 5 mg *Quillaja* bark was dissolved in 1 mL water to obtain a stock solution, then this stock solution was dissolved to 1-4 mg/mL. 0.5 mL extract sample was put on a test tube and 0.5 mL 8% 2,3,4-trimethoxy benzaldehyde-ethanol solution was added. Then 5 mL 72% sulfuric acid solution was put on the test tube. All tubes were mixed well and were put on ice bath. They were incubated in water bath at 60°C for 10 minutes, cooled and then, the spectrum scanning was performed.

2.8 Mass Analysis

After visualization of the bands on TLC via spray solution, the bands were removed and then, they were dissolved in 1 mL ethanol and centrifuged (Figure 2.8). The supernatants were analysed by using UPLC-MS/MS. Iso-butanolic portion of n-

hexane, dichloromethane, chloroform and butanol extraction of holothurians were also analyzed by using MALDI-TOF/TOF and LC-MS/MS.

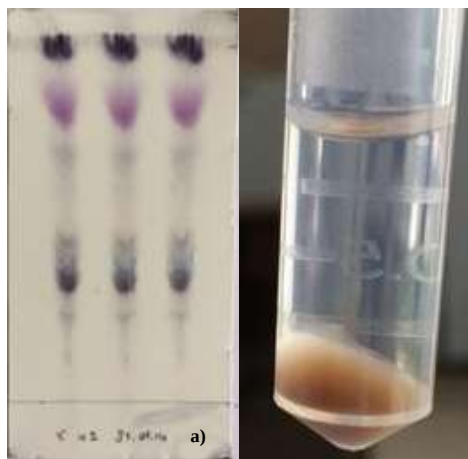


Figure 2.8 Scratched fractions a) from TLC plate b) dissolved in ethanol.

2.9 Determination of the Protein Level in Body Wall of Sea Cucumber

To estimate the protein concentration in the body wall of *Holothuria* species, the method of Bradford (1976) was used. According to this method, 25 mg coomassie brilliant blue was dissolved in 25 mL 95% ethanol and 25 mL ortho-phosphoric acid was added. The resulting solution was diluted to final volume was 250 mL.

To prepare the stock standard protein solution, 100 mg of bovine serum albumin was dissolved in 100 mL of distilled water. The aliquots were prepared as 100, 150, 200, 300 and 400 mg/L.

To determine the protein concentration of sea cucumber, 1 g of sea cucumber body wall was homogenized with 10 mL water by using homogenizer and centrifuged for 5 minutes at 8000 rpm. 100 μ L of the sample was dissolved in 900 μ L of the coomassie brilliant blue solution and the absorbance was measured at 595 nm.

2.10 Detection of Saponins Through Foam and Froth Test

The procedure of Wei (2011) was used to determine the foam level in sea cucumber body wall with some modifications. According to this method, the dried holothurians were prepared as a powder and 2 g of powder sea cucumber was boiled in 200 mL of distilled water for 1 hour on the heater. After water extraction, the sample was filtered with filter paper and 5 mL of distilled water was added to 10 mL of extract and the test tube was shaken vigorously for 30 seconds. Emulsion formation was evaluated as a positive.

For the froth test, the method of Wei (2011) was used with some modifications. In this method 2 g of dried powdered sea cucumber was extracted in 10 mL of distilled water for 5 minutes on the heater. After water extraction, the sample was filtered and 5 mL of water was added in 10 mL of extract and the test tube was shaken vigorously for 30 seconds. After waiting for 1 minute, honeycomb formation was observed.

2.11 Determination of Lipid Percentage in Sea Cucumber

To evaluate the lipid level in the body wall of *Holothuria* species, 25 g of dried materials (M_1) were put in the cartridge. After lipid extraction with n-hexane, the solvent was evaporated by using rotary evaporator and then, the waste was weighted (M_2). To calculate the percentage of lipids, the below formula was used:

$$\text{Lipid\%} = \left(\frac{M_2 - M_1}{M_1} \right) \times 100$$

(2.1)

M_1 = the initial weight of wet sea cucumber body wall

M_2 = the weight of the wet sea cucumber body wall after extraction

To determine the total fatty acid composition of *Holothuria* species, n-hexane extraction was carried out. The n-hexane extract was analyzed using GC-MS for fatty acid composition.

2.12 Carbohydrate Determination by using Phenol-Sulfuric Acid Reaction

To evaluate the carbohydrates in the sea cucumber extract, the method of Dubois et al. (1956) was used. According to this method, 2 mL of sugar solution (glucose, galactose, sucrose and maltose) and 0.05 mL of 80% phenol (w/v) was added into the test tube. Then, 5 mL of sulfuric acid was added into the tube rapidly and waited for 10 minutes. After shaking, the tubes were waited for 10 minutes in a water bath at 25°C to 30°C. The absorbances were measured at 490 nm for hexoses, 480 nm for pentoses and uronic acids.

As a blank, 2 mL of water and 0.05 mL of 80% phenol (w/v) was added into the test tube. Then, 5 mL of was added into the tube rapidly and waited for 10 minutes. The calibration curve was drawn between 0.5-50 mg/L.

2.13 Total Ash and Moisture Content

Total ash content of body wall of holothurian was estimated as mass difference between the initial weight and the samples waited at 600°C for 2 h.

To determine the moisture content of holothurian body wall, the sample was waited at 100°C for 1 h.

2.14 Determination of vitamin C Content in Holothurian by using HPLC

Determination of vitamin C level by using HPLC was carried out according to the method of Rodríguez-Bernaldo de Quirós et al. (2001). In this method, vitamin C was determined with an isocratic elution method with C18 column. As a mobile phase, Milli-Q water with meta-phosphoric acid at pH 2.2 was used. Flow rate,

column temperature, wavelength and analyze time were 1 mL/min, 25°C, 245 nm and 5 minutes, respectively.

The calibration curve was drawn at the range of 5 and 500 mg/L. To determine vitamin C content of sea cucumber, aqueous meta-phosphoric acid extraction method (Rodriguez-Bernaldo de Quirós et al., 2001) was used.

To determination of the detection limit (LOD), standard deviation of peak areas of solvent (water) in same retention time with ascorbic acid was calculated and the below formula was used (Mert et al., 2014).

$$LOD = \frac{3 \times STD}{S} \quad (2.2)$$

where STD is standard deviation and S is slope.

2.15 Adsorption Studies with Sea Cucumber Residues

After the extraction of dried sea cucumbers with ethanol by using soxhlet apparatus and lipid extraction, the residues of sea cucumbers were used as an adsorbent. According to this method, 0.5 g of sea cucumber residues were put on a bottle and 30 mL of methylene blue (hereafter MB) solution was added. The solution was shaken for 3 hours at 120 rpm at 30°C and the samples were taken for each 15 minutes. After the batch adsorption, the samples were centrifuged and absorbance was measured at 630 nm by using microplate reader.

2.15.1 FT-IR Spectrum of *H. polii* Body Wall

In order to determine the surface characteristics, untreated (hereafter UT) and lipid extracted (hereafter LE) *H. polii* body wall residues were scanned by FT-IR.

2.15.2 Adsorption Kinetics

In order to study kinetic regime of MB adsorption, two kinetic equations were used.

The pseudo-first order Lagergren equation (Lagergren, 1898):

$$\log(q_e - q) = \log q_e - \left(\left(\frac{k_1}{2.303} \right) \times t \right) \quad (2.3)$$

where

q_e ; amount of adsorbate (MB) adsorbed at equilibrium (mg/g),

q ; amount of adsorbate (MB) adsorbed at time (mg/g) and

k_1 ; rate constant (min^{-1}).

Another kinetic equation used in the present study is pseudo-second order (Ho & McKay, 2003):

$$\frac{t}{q} = \left(\frac{1}{k_2 \times q_2} \right)^2 + \left(\frac{1}{q_2} \right) \times t \quad (2.4)$$

where k_2 is the pseudo-second order rate constant (g/mg.min)

2.15.3 Adsorption Isotherms

In order to study isotherms of MB adsorption, Langmuir and Freundlich isotherm equations (Langmuir, 1918; Freundlich, 1906) were used:

$$\frac{1}{q} = \frac{1}{q_m} + \left(\frac{1}{b q_m} \right) \times \left(\frac{1}{C_e} \right) \quad (2.5)$$

$$\log q = \log K_f + \left(\frac{1}{n} \right) \ln C_e \quad (2.6)$$

where

q ; Adsorption at equilibrium (mg/g),
 q_m ; The maximum adsorption capacity (mg/g),
 b ; Langmuir constant (from adsorption capacity)
 C_e ; The concentration of MB at equilibrium (mg/L),
 K_f ; Adsorption capacity,
 $1/n$; Adsorption intensity (n_f)

By using different temperatures (30°C, 40°C, 50°C), the graphs for adsorption of MB on UT *H. polii* body walls and LE were drawn.

2.16 Antifouling Performance of Sea Cucumber Extract

The antifouling performances of the different extracts of sea cucumber, residues of extracted sea cucumber body walls, powder of dried sea cucumber body walls and a commercial antifouling paint were evaluated in a seawater immersion test for 1 month at Levent Marina, İzmir, Turkey. To prepare the self-polishing type antifouling paints, sea cucumber extracts were used as a biocide. The ingredient of the self-polishing type antifouling paint was given in Table 2.1.

Table 2.1 Paint composition of rosin based antifouling paints

Paint Component	Percent by weight (w/w)	
Rosin	34	39
Xylene	40	40
ZnO	11	12
Fiber	5	5
Biocide	6	-
CaCO ₃	4	4

An example of antifouling paint coated metal plate was given in Figure 2.9.



Figure 2.9 Antifouling paint coated metal plate.

To evaluate the antifouling performance, natural bioactive agents from sea cucumbers and a reference commercial biocide were tested. The paints were tested for a period of 30 days (from June 18 to July 17, 2014) at Levent Marina, İzmir, by comparing with paint free and rosin-based biocide free paints as references.

Methanol-dichloromethane extraction of *H. polii* and *H. tubulosa*, soxhlet extract of *H. polii*, macerated extract of *H. tubulosa*, *H. tubulosa* with *C. cylindracea*, powder of dried *H. polii* body wall and residue of soxhlet extracted *H. polii* were used as natural bioactive agents on antifouling performance. Zinc pyrion was used as a reference commercial biocide.

The painted steel panels were immersed into seawater at a depth of 50 cm for evaluation of the antifouling performance.

In the literature (Arimura, Hiyoshi, Nakamura & Tsuboi, 2004; Topcam, 2013), there are criteria to estimate the level of fouling on the panels. According to the literature, there are five levels of fouling. If there are no fouling organisms on the surface of the panels, it is evaluated as Level 1. Level 2 is defined as microfilm layer formation present on the surface. In the Level 3, microfilm layer is present and macrofouling is at the beginning state. If there are some micro- and macrofouling organisms on the surface of the panels, it is evaluated as Level 4. If the surface is

heavily covered by micro- and macrofoulers (>50%), level of the fouling is defined as level 5.

2.17 *In-silico* Analysis

X-Ray crystal structural coordinates of cyanobacterial photosystem II (PS II) were obtained from Protein Data Bank (Berman et al., 2000). For *in silico* analysis, 3BZ1 (pdb file of cyanobacterial photosystem II at 2.9-Å resolution) was used as template of macromolecules. *In silico* molecular docking experiments were carried out with PyRx-Pyron Prescription 0.8 Virtual screening tool and AutoDock Vina. To draw and the 3D structure optimization of the ligands, ACD/Labs Release: 11.0 version of freeware ChemSketch. For virtual screening and the determining the relevant residues with the molecule, AutoDock Tool 1.5.6 was used. To visualize the three-dimension version of cyanobacterial photosystem II (pdbid: 3BZ1), YASARA was used (Krieger, 2010). As a positive control, SDF format of Diuron, DCA, DCPMU, DCPU and Irgarol were retrieved from PubChem Compound Databank (CID: 3120, CID: 7257, CID: 71215388, CID: 91590). Comparison of binding sites of the chemicals and the positive control, AutoDock Vina, AutoDock Tool 1.5.6 (Trott and Olson, 2010) were performed. For energy minimizing and converting the PDB, SDF and MOL format to the PDBQT format PyRx-Pyron Prescription 0.8 Virtual screening tool was used. Nucleotide binding area that were revealed from UniProt and labeled on the macromolecule, dimension of the cubic geometry was obtained from this labeled area. According to this area, for 3BZ1, each dimension of the cubic geometry of X, Y and Z were set to 50, 62 and 43 Å, respectively. The central coordinates were determined as 33.7533, 53.3092 and 63.3225 for X, Y and Z, respectively. To obtain the best value of molecular docking, each round was performed triplicate.

CHAPTER THREE

RESULTS

3.1 Thin Layer Chromatography

After extraction procedure, triterpene glycosides were visualized with sulfuric acid (solution I) and vanillin (solution II) by using spraying apparatus (Figure 2.5). The results of this procedure were given in Figures 3.1 - 3.3. As a positive control, 0.5-10 mg/mL *Quillaja* bark saponin were applied on 8x8 cm TLC plates (Figure 3.4). For *Quillaja* bark saponins, mobile phase was chloroform-methanol-water-acetic acid (240:175:30:1).

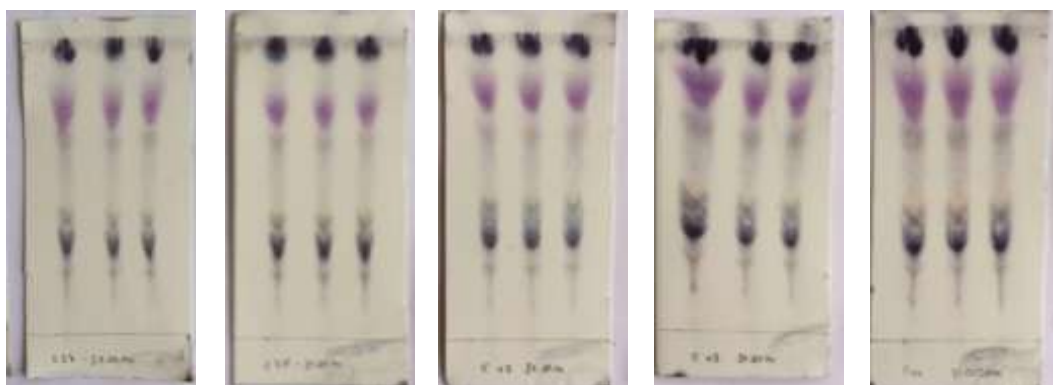


Figure 3.1 TLC of sea cucumber 100% ethanolic extract visualised with spray reagents (Mobile phase: chloroform-ethanol-water (100:100:17)).

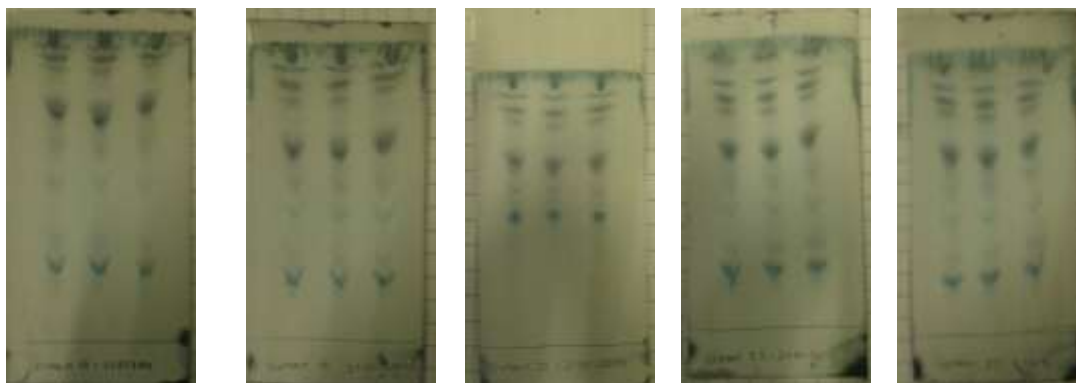


Figure 3.2 TLC of sea cucumber 100% ethanolic extract visualised with spray reagents (Mobile phase: chloroform-methanol-water (65:35:10)).

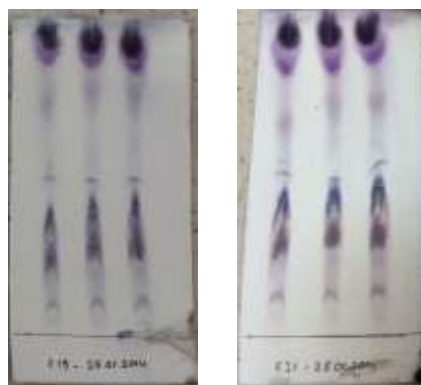


Figure 3.3 TLC of sea cucumber 100% ethanolic extract visualised with spray reagents (65:35)).

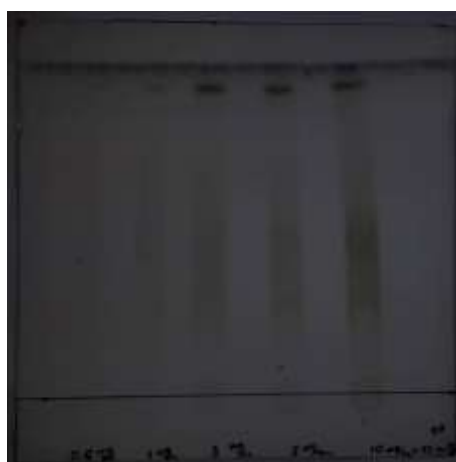


Figure 3.4 TLC of *Quillaja* bark saponin with different concentrations (0.5-10 mg/mL) (Mobile phase: chloroform-methanol-water-acetic acid (240:175:30:1)).

3.2 Colorimetric Assay

After incubation, blank was bitter yellow and *Quillaja* bark saponin-containing tubes were reddish color. The color gradient was observed (Figure 3.5).

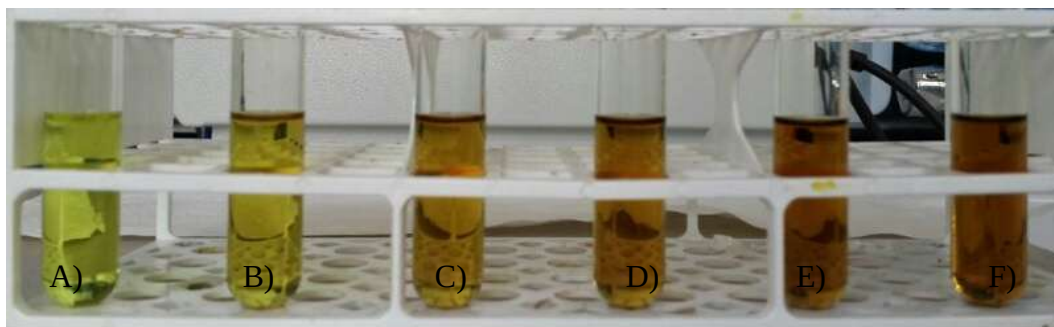


Figure 3.5 Vanillin-sulfuric acid colorimetric assay for *Quillaja* bark saponin. A: Blank B: 0.1 mg/mL; C: 0.5 mg/mL; D: 1 mg/mL; E: 2.5 mg/mL; F: 5 mg/mL *Quillaja* bark.

For calculation of the total saponin content in sea cucumber extracts, calibration curve was plotted with the range of 0.5 -5 mg/mL. According to the results, the R^2 value was determined as 0.9997 (Figure 3.6).

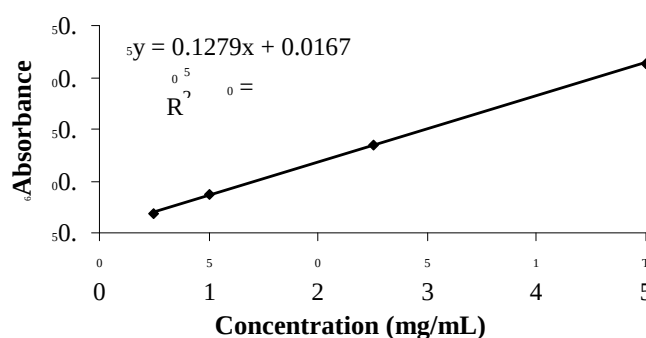


Figure 3.6 The calibration curve of *Quillaja* bark saponin at 540 nm.

The principle of vanillin-sulphuric acid method is based on converting the glycone chain to furfural. And it reacts with free $-OH$ in C-3 position of aglycone with the phenolic aldehyde –vanillin- to obtain chromogenic compound in an acidic environment (Hiai et al., 1976). The spectrums were given in Figure 3.7 - 3.10.

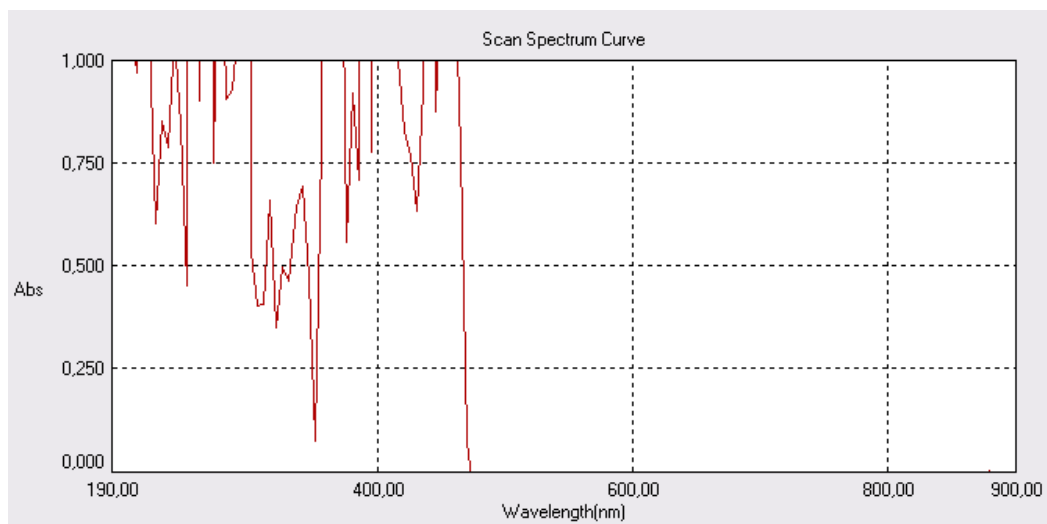


Figure 3.7 Spectrum of water blank (2.5 mL 72% sulfuric acid-vanillin solution + 250 μ L water).

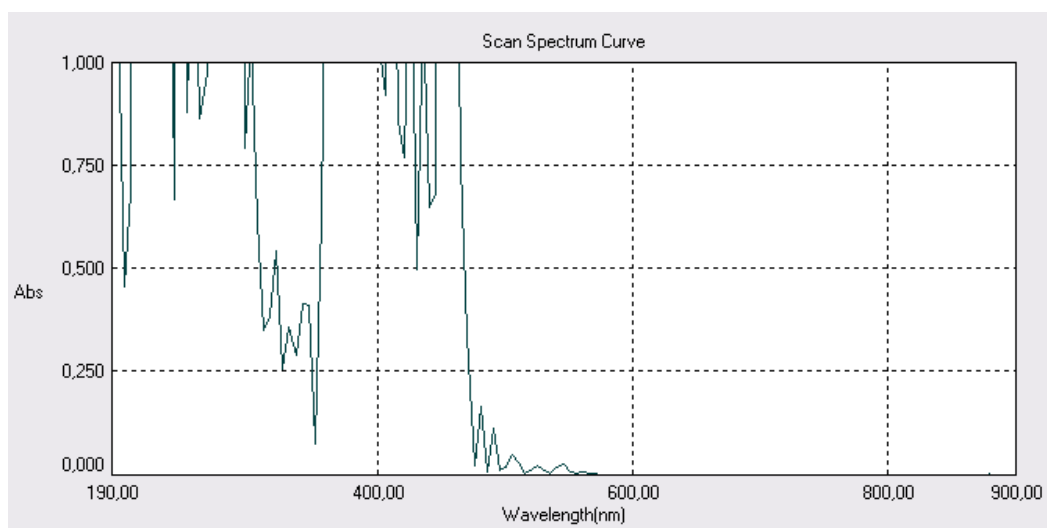


Figure 3.8 Spectrum of 0.5 mg/mL *Quillaja* bark (2.5 mL 72% sulfuric acid-vanillin solution + 250 μ L sample).

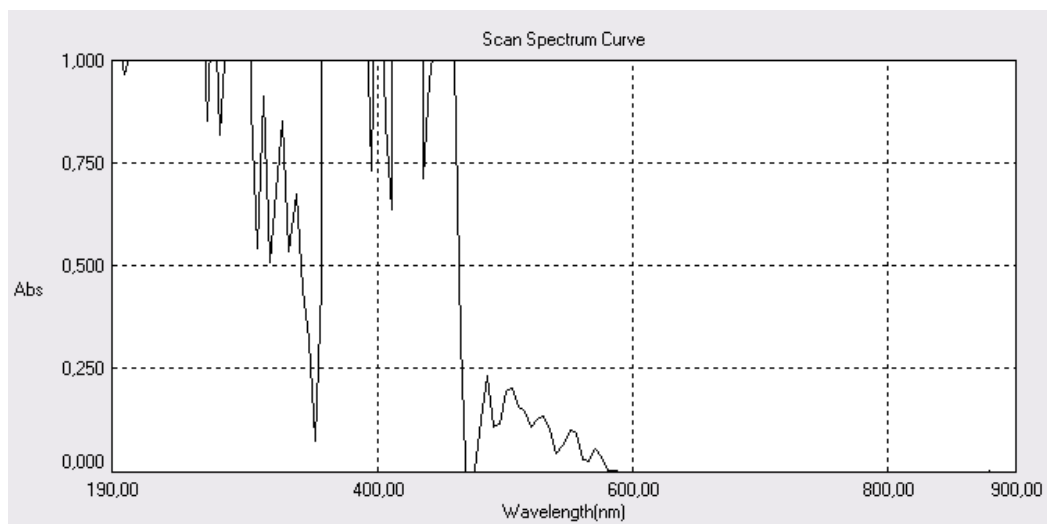


Figure 3.9 Spectrum of 5 mg/mL *Quillaja* bark (2.5 mL 72% sulfuric acid-vanillin solution + 250 μ L sample).

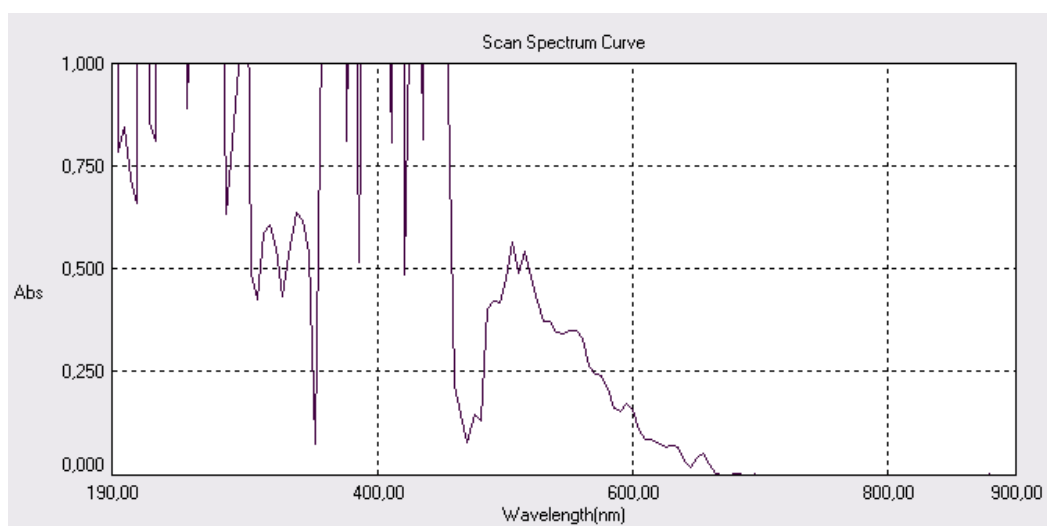


Figure 3.10 Spectrum of 40 mg/mL *Quillaja* bark (2.5 mL 72% sulfuric acid-vanillin solution + 250 μ L sample).

According to the spectrums, in Figure 3.7 there was no maximum absorbance, in following figures, the maximum absorbances were 420, 486 and 505 nm. The absorbance shift was observed when the concentration was increased.

3.3 Vanillin-Sulfuric Acid Colorimetric Test for Detection of Sea Cucumber Saponins

After incubation, blank was bitter yellow and extract-containing tubes were greenish color. The color gradient based on increasing concentration was observed (Figure 3.11). The spectrum was given in Figure 3.12.

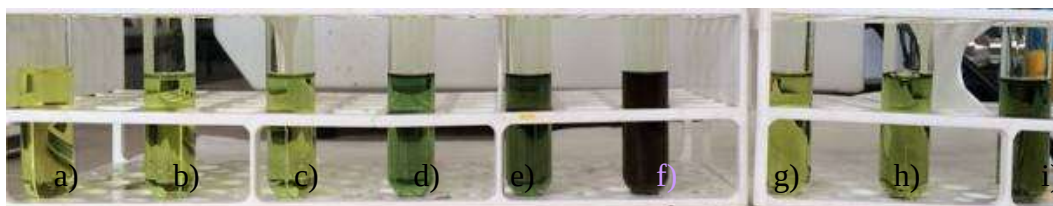


Figure 3.11 Vanillin-sulfuric acid colorimetric assay for 70% sea cucumber extracts. a: Blank b: 1/50 diluted 70% ethanolic soxhlet extract; c: 1/25 diluted 70% ethanolic soxhlet extract, d: 1/10 diluted 70% ethanolic soxhlet extract; e: 1/5 diluted 70% ethanolic soxhlet extract; f: 1/2 diluted 70% ethanolic soxhlet extract; g: 1/10 diluted 70% ethanolic macerated extract; h: 1/5 diluted 70% ethanolic macerated extract; i: 1/2 diluted 70% ethanolic macerated extract.

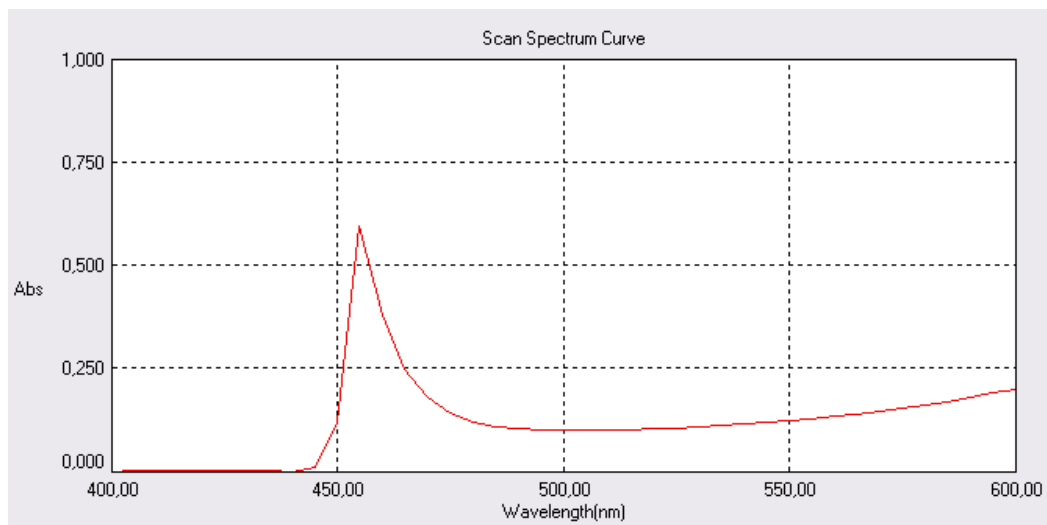


Figure 3.12 Spectrum of macerated extract.

In Figure 3.12, the maximum absorbance was shown at 455 nm. The reason of the absorbance shift could be explained with the structural difference between *Quillaja* bark and holothurian saponins.

The total saponin concentrations in holothurian extracts were given in Table 3.1.

Table 3.1 Total saponin concentrations of sea cucumber extracts

Sample	Saponin concentration (mg eq. plant saponins/mL body wall)	Saponin concentration (mg eq. plant saponins /g)
<i>H. polii</i> methanol/dichloromethane extract	2.989 ± 1.944	8.967 ± 1.282
<i>H. tubulosa</i> methanol/dichloromethane extract	2.634 ± 1.848	7.904 ± 1.318
<i>H. polii</i> (soxhlet)	1.339 ± 0.439	4.121 ± 1.350
<i>H. species</i> (macerated)	1.431 ± 0.235	1.431 ± 0.235
<i>H. tubulosa</i> (macerated)	1.019 ± 0.282	1.019 ± 0.282
<i>H. tubulosa</i> with <i>Caulerpa cylindracea</i> (macerated)	1.141 ± 0.250	0.399 ± 0.087
Cuvierian tubules (macerated)	2.085 ± 0.705	2.085 ± 0.705

The highest saponin concentrations were observed in the methanol/dichloromethane extract of *H. polii* and *H. tubulosa* is 8.967 ± 1.282 and 7.904 ± 1.318 mg eq. plant saponins/g body wall, respectively. The lowest saponin concentration was obtained in macerated extract of *H. tubulosa* sample with *C. cylindracea* (0.399 ± 0.087 mg eq. plant saponins/g body wall).

3.4 2,3,4-trimethoxy benzaldehyde-Sulfuric Acid Colorimetric Test for *Quillaja* Bark Saponins

After incubation, blank was orange and *Quillaja* bark saponin-containing tubes were reddish color. In Figure 3.13 the color gradient was observed.

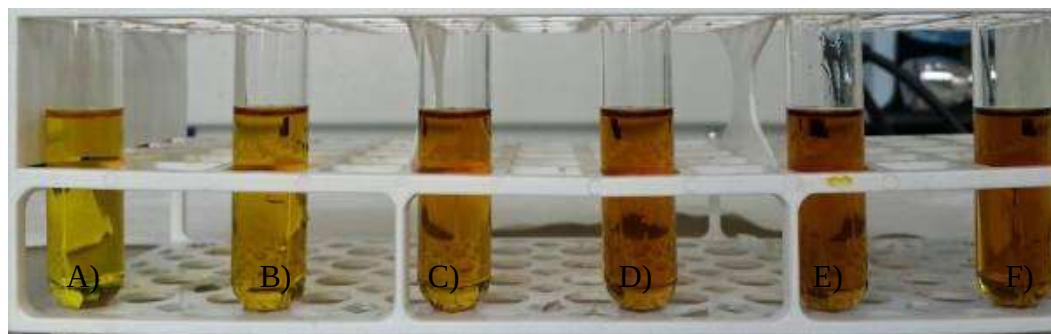


Figure 3.13 2,3,4-trimethoxy benzaldehyde-sulfuric acid colorimetric assay for *Quillaja* bark saponin. A: Blank B: 1 mg/mL; C: 2 mg/mL; D: 3 mg/mL; E: 4 mg/mL and F: 5 mg/mL *Quillaja* bark.

In Figure 3.14, the spectrum of the 2,3,4-trimethoxy benzaldehyde-saponin complex was given. According to this result, the maximum absorbance was at 440 nm.

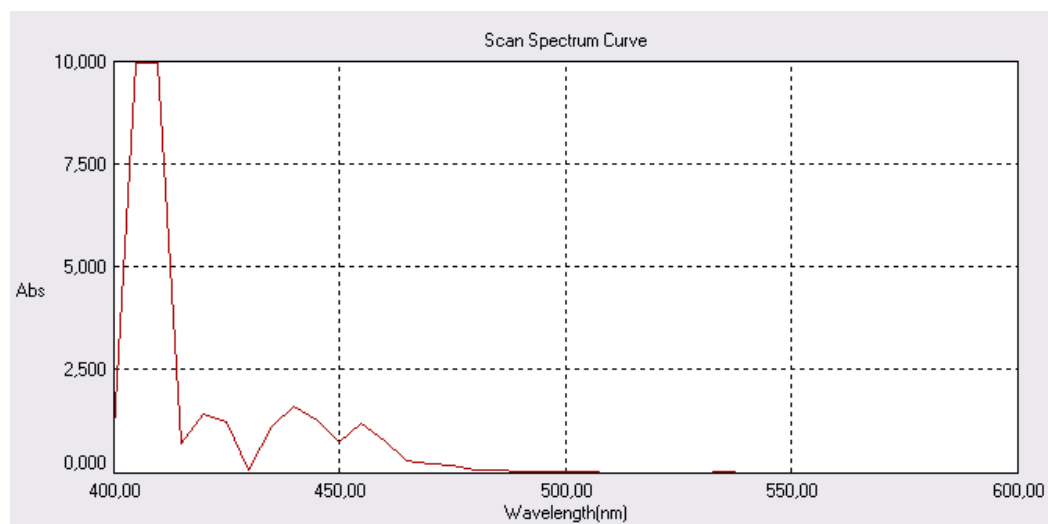


Figure 3.14 Spectrum of 1 mg/mL *Quillaja* bark.

3.5 Mass Analysis

The saponins found in *Holothuria* species were also characterized by using mass spectrometry. Two modes are used in the mass spectrometry: positive and negative. In positive ion mode, observed ions are associated with sodium coordinated species $[M-H+2Na]^+$ and $[M+Na]^+$. However, in the negative ion mode, deprotonated ions $[M-H]^-$ are observed (Bondoc et al., 2013).

The ethanolic extract were visualized on the TLC plates by using spray solution 1 and 2 which were mentioned materials and method part. The colours were well in line with current literature (Olezsek et al., 2007). The R_f values were obtained and then relevant spots were scratched from TLC (note that the scratched spots were not visualized with spray solutions). The removed spots were dissolved in ethanol and then centrifuged for removal of TLC's stationary phase. The supernatants were analyzed in UPLC-MS/MS.

We analyzed TLC spots with and without colour reagent. The results of colourful TLC spots were given in Figures 3.16 – 3.19. However, it was not possible to characterize the triterpene glycosides by using spectrums. Therefore, the spots scratched by using their R_fs were analyzed. According to the Van Dyck et al. (2010), the molecular peak of the holothurinoside A is 1303 *m/z* and the structure of the molecule was given in Figure 3.15. The peak at 1302.87687 *m/z* might be considered as holothurinoside A. When the Figures 3.20 - 3.22 were examined, we found a main peak at 1302 *m/z*. This peak could be holothurinoside A. In Figures 3.23 - 3.24 the MS/MS results at 750-1000 *m/z* were given.

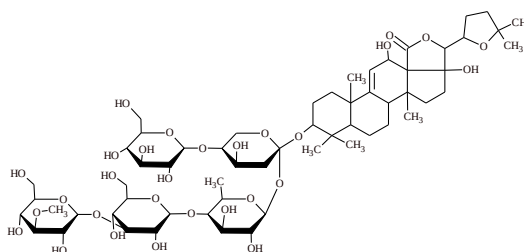


Figure 3.15 The structure of holothurinoside A

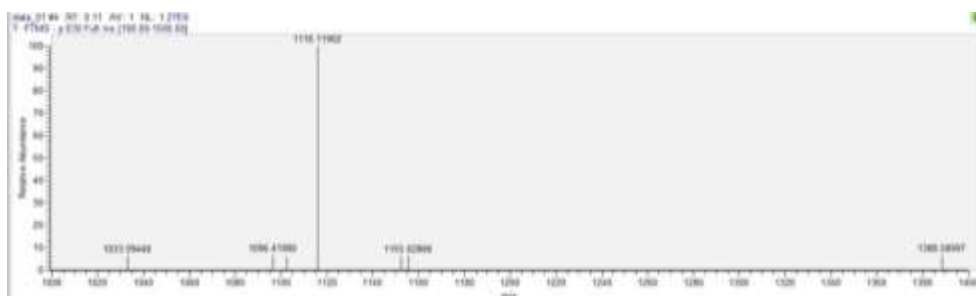


Figure 3.16 The mass spectrum of the vanillin-sulfuric acid applied and scratched TLC fraction at 1000-1400 *m/z* (R_f: 0.94).

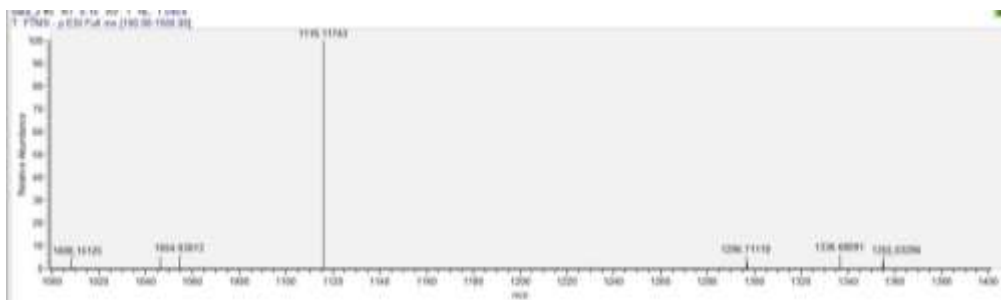


Figure 3.17 The mass spectrum of the vanillin-sulfuric acid applied and scratched TLC fraction at 1000-1400 m/z (Rf: 0.75).

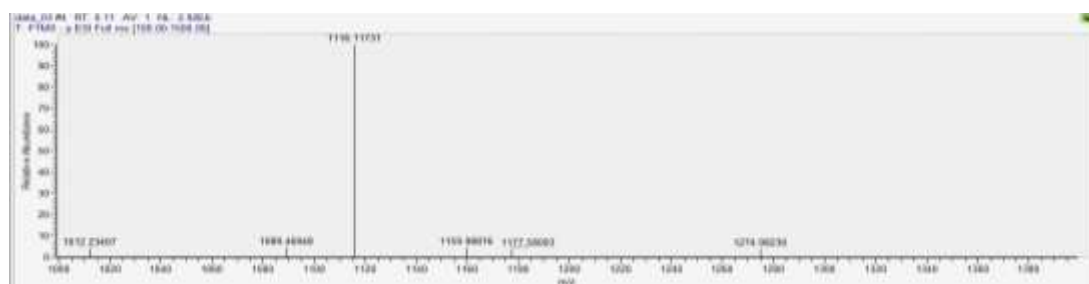


Figure 3.18 The mass spectrum of the vanillin-sulfuric acid applied and scratched TLC fraction at 1000-1400 m/z (Rf: 0.38)

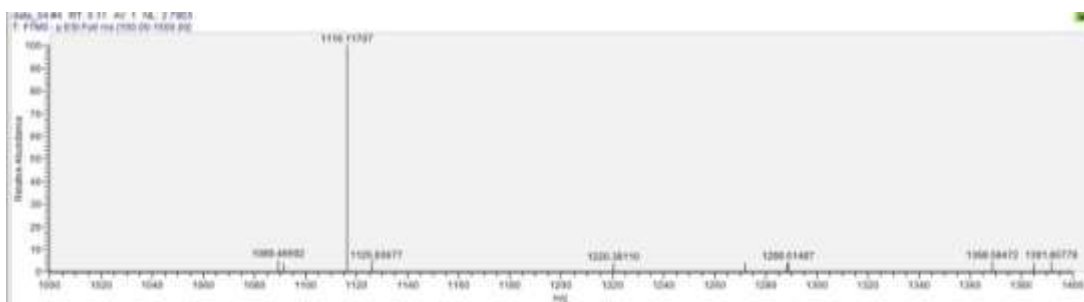


Figure 3.19 The mass spectrum of the all TLC fractions that was applied vanillin-sulfuric acid and scratched at 1000-1400 m/z.

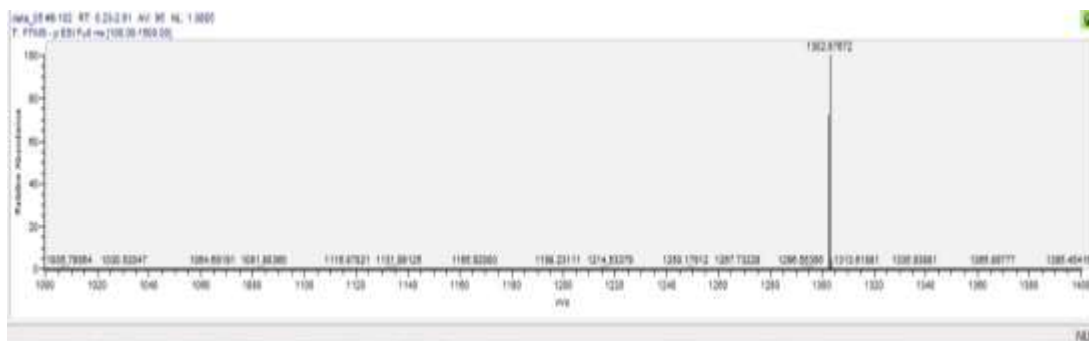


Figure 3.20 The mass spectrum of scratched TLC fraction at 1000-1400 m/z (Rf: 0.94)

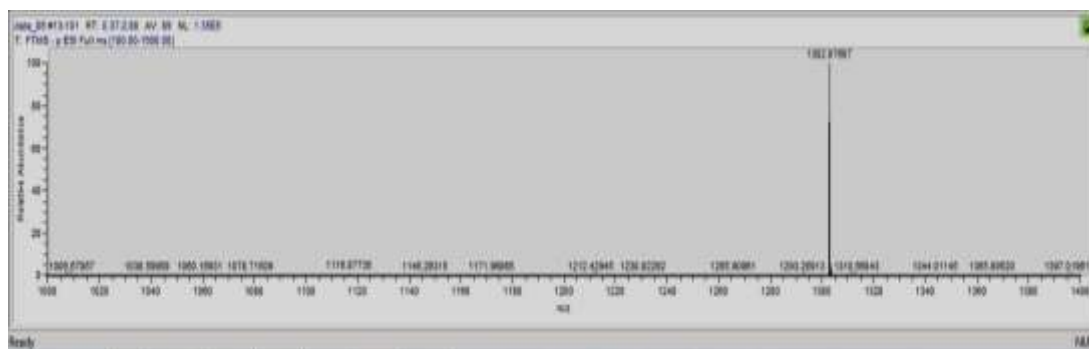


Figure 3.21 The mass spectrum of scratched TLC fraction at 1000-1400 m/z (Rf: 0.75)

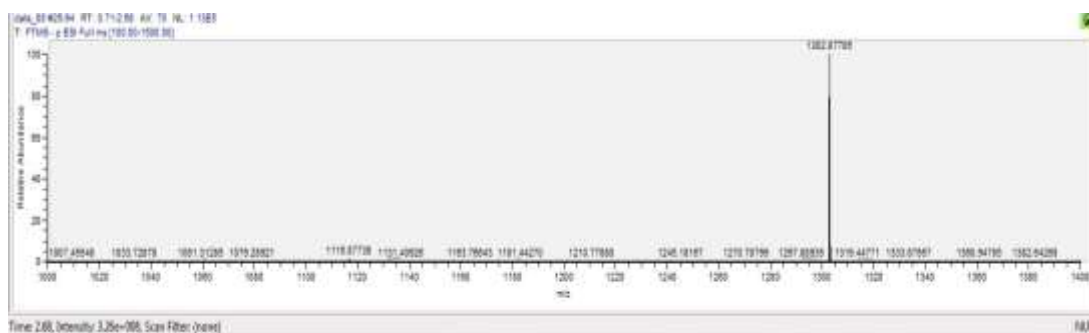


Figure 3.22 The mass spectrum of all TLC fractions that was scratched at 1000-1400 m/z .

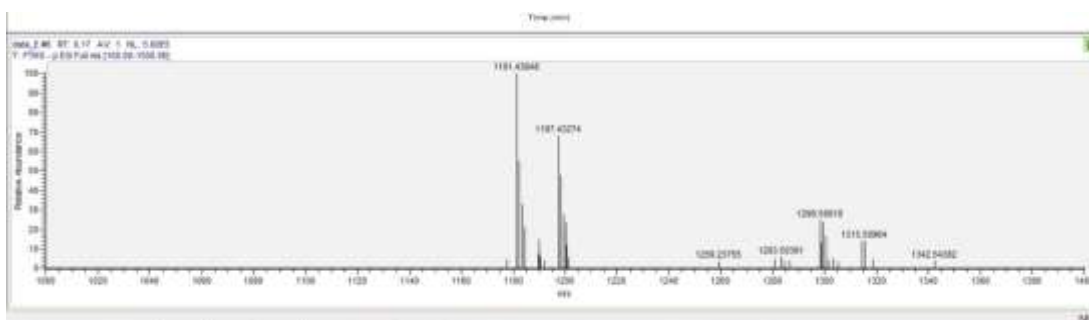


Figure 3.23 The mass spectrum of *H. polii* soxhlet extract at 1000-1400 m/z.

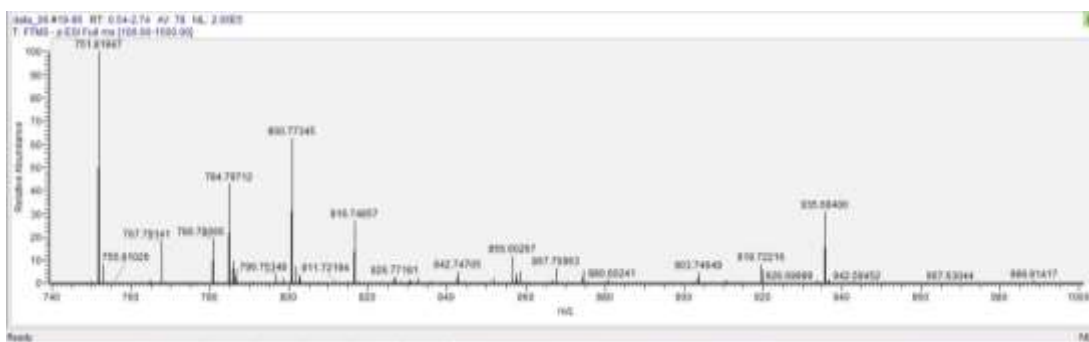


Figure 3.24 The mass spectrum of scratched TLC fraction at 750-1000 m/z (Rf: 0.75).

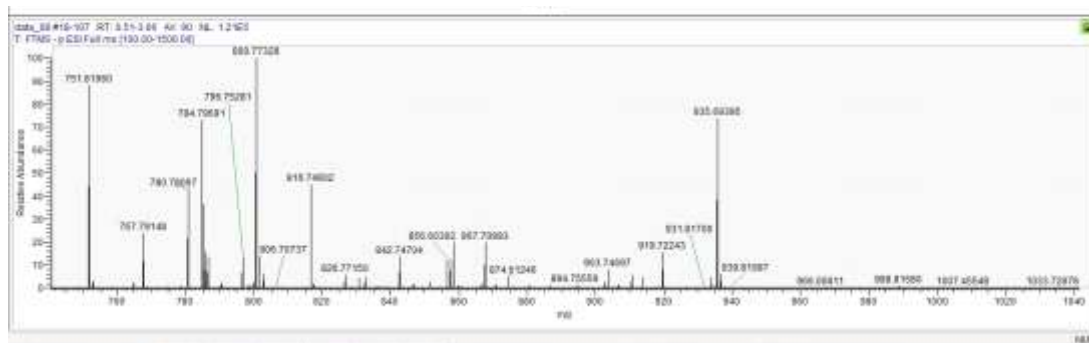


Figure 3.25 The mass spectrum of all TLC fractions that was scratched at 750-1000 m/z.

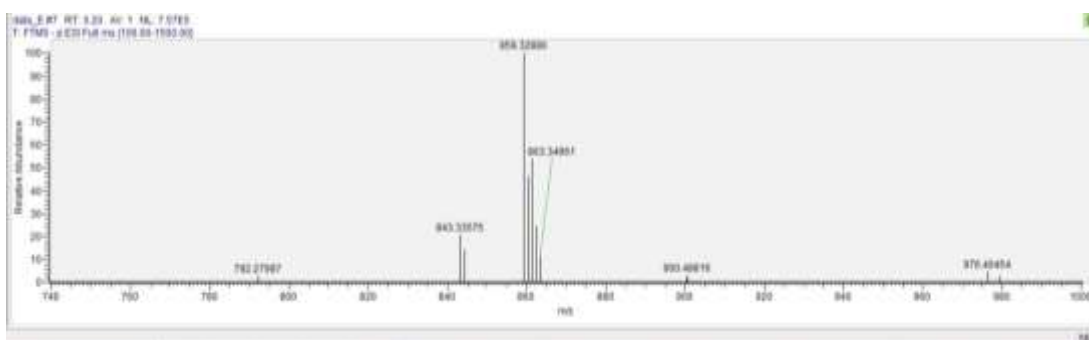


Figure 3.26 The mass spectrum of *H. polii* soxhlet extract at 750-1000 m/z.

Semi-purified iso-butanolic extract were prepared according to Bondoc et al., (2013). The structure of holothurine A was given in Figure 3.27.

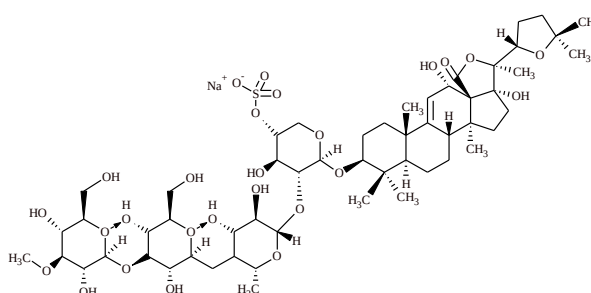


Figure 3.27 The structure of holothurine A

In the beginning of the evaluating mass experiments, the paper of Bondoc et al. (2013) was followed, especially their Table 2 was used to compare with our results. However, their Table 2 was not well in line with the results of this thesis. It was very interesting to note that they published a corrigendum in the same journal very recently (Bondoc et al., 2014) where they corrected their Table 2 for sulphated triterpene glycosides. However, the confusion in Table 2 is still valid because when

this table in the corrigendum was examined , there is no holothurin A. But in the previous version of the paper (Bondoc et al., 2013), they mentioned the existence of holothurin A. We think that correct m/z for holothurin A must be 1197 and we observed this peak in our spectrums. The mass spectrums (MALDI-TOF-MS in negative ion mode) of iso-butanolic and soxhlet extracts were given in Figures 3.28 – 3.31.

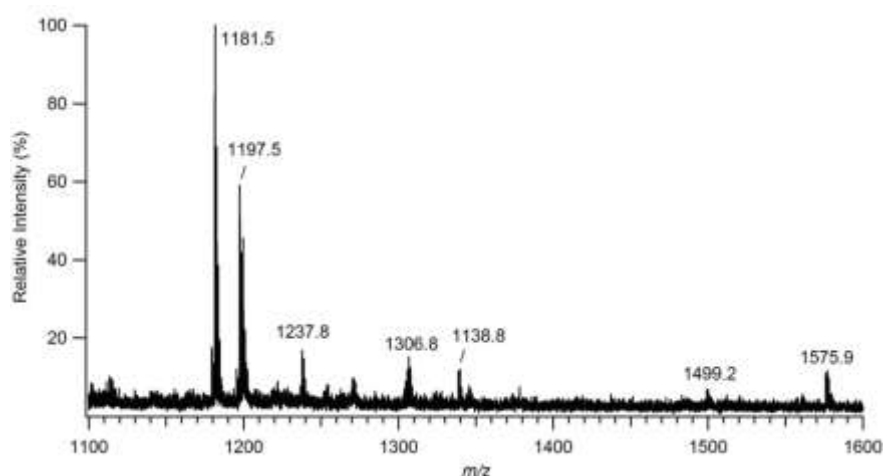


Figure 3.28 The mass spectrum of iso-butanolic portion of liquid-liquid extraction at 1100-1600 m/z .

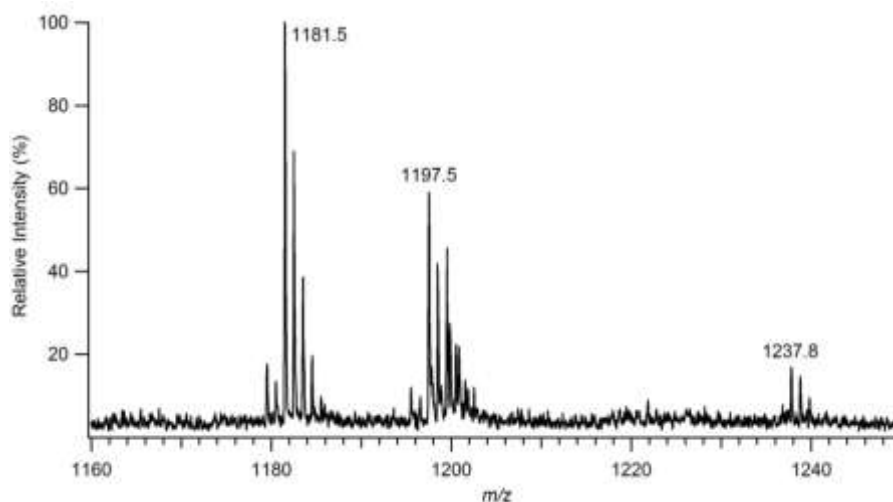


Figure 3.29 The mass spectrum of iso-butanolic portion of liquid-liquid extraction at 1160-1240 m/z .

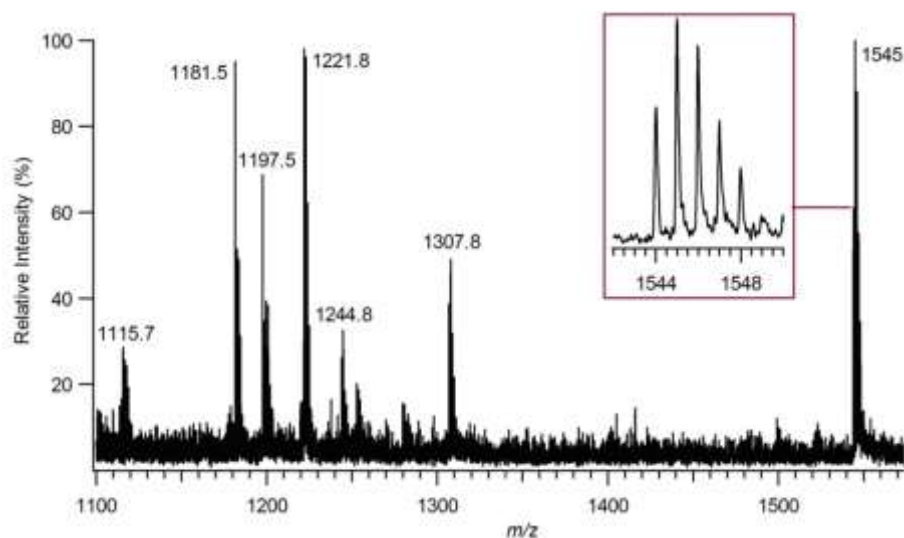


Figure 3.30 The mass spectrum of ethanolic extract of *H. polii* at 1100-1600 m/z.

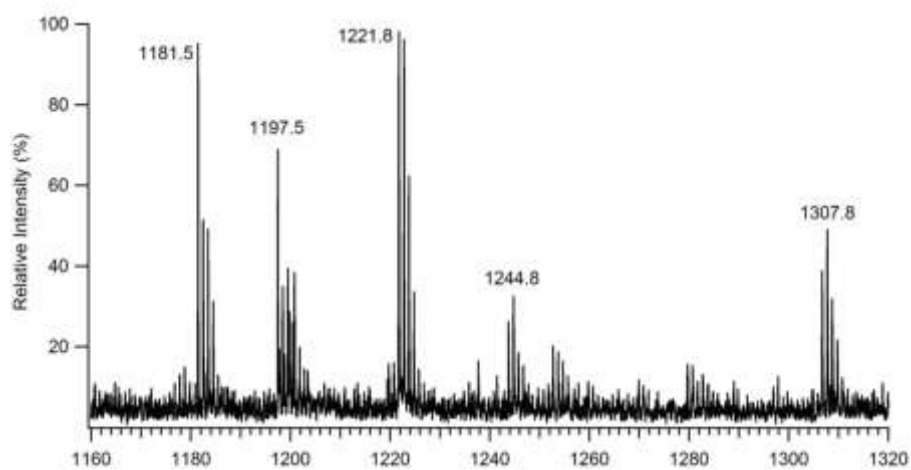


Figure 3.31 The mass spectrum of ethanolic extract of *H. polii* at 1160-1320 m/z.

In order to evaluate the molecule at 1221.8 m/z, tandem mass spectrum was evaluated. In Figure 3.32, the peak at 639.8 m/z might be the glycon part with Na^+ (Methyl glucose, glucose, qinovose, xylose and Na^+) of holothurin A. The similar explanation could be found in Bondoc et al. (2013). But the confusion is still valid in the table 2 of Bondoc et al., (2013) and Bondoc et al., (2014). A new erratum must be published by Bondoc and her colleagues.

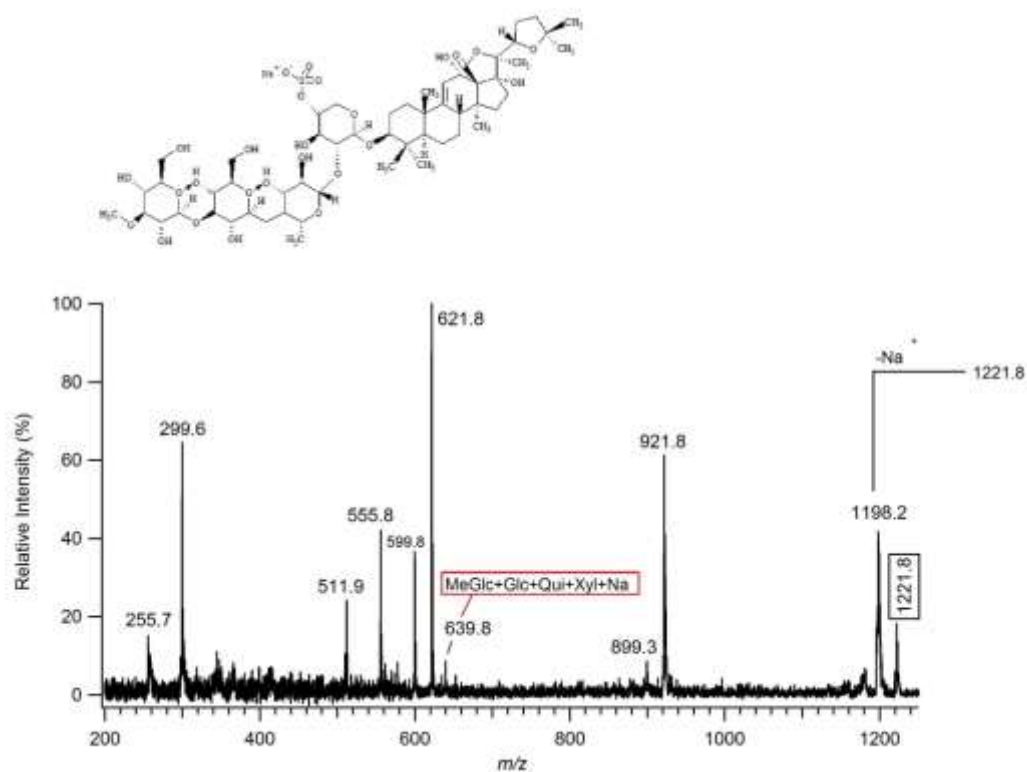


Figure 3.32 The MS-MS result of holothurin A (1221.8 m/z) at 200-1200 m/z.

Semi-purified butanolic extract was analyzed in LC/MS-MS. The related results were given in Figure 3.33.

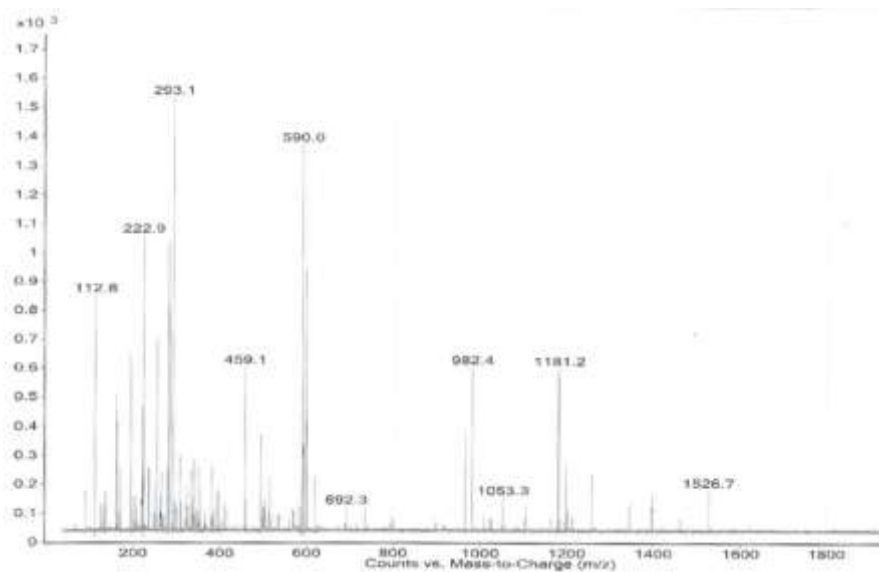


Figure 3.33 The LC/MS-MS result of iso-butabolic portion of liquid-liquid extraction at 200-1800 m/z.

According to the results of LC/MS-MS, unidentified triterpene glycosides were observed in iso-butabolic portion of liquid-liquid extraction at 200-1800 m/z. All mass spectra were given in Table 3.2.

Table 3.2 Mass spectrum results of sea cucumber extracts

Molecular Weight	Ion Mode	Fraction	Saponin Name
843.33575	+	70% soxhlet extract	Unidentified
859.32886	+	70% soxhlet extract	Unidentified
863.34961	+	70% soxhlet extract	Unidentified
900.48816	+	70% soxhlet extract	Unidentified
976.40454	+	70% soxhlet extract	Unidentified
1116.11743	+	TLC-vanillin-sulfuric acid	Unidentified
1181.43848	+	70% soxhlet extract	Unidentified
1197.43274	+	70% soxhlet extract	Unidentified
1302.87687	+	70% soxhlet extract scratched from TLC	Holothurinoside A
1181.5	-	Isobutanolic fraction	Unidentified
1197.5	-	Isobutanolic fraction	Holothurin A
1197.5	-	70% soxhlet extract	Holothurin A
1198.2	-	70% soxhlet extract	Holothurin A
1181.2	-	Isobutanolic fraction	Unidentified

3.6 Protein Level in Body Wall of Sea Cucumber

To determine the protein level by using Bradford method (1976), calibration curve was plotted with the absorbance versus concentration. The range of calibration curve was 100-500 mg/L and R^2 value was found as 0.994 (Figure 3.34).

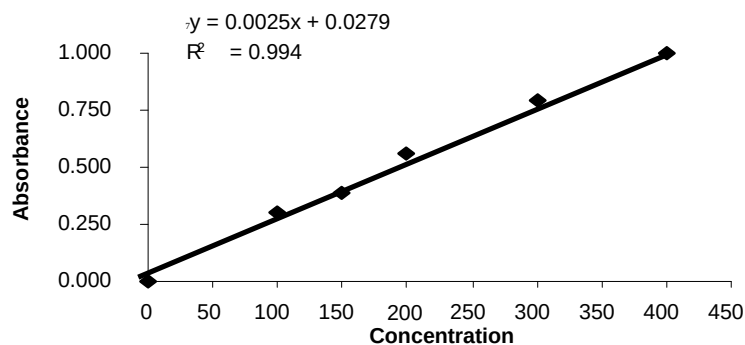


Figure 3.34 The calibration curve of Bradford method.

The protein concentration in the tissues of sea cucumber was estimated by using Bradford method (1976). Bovine serum albumin was used as a standard protein. To estimate the protein concentration in holothurian samples, the calibration curve in Figure 3.34 was used. The concentration of supernatant was 0.112 g wet weight/mL water. *H. polii* and *H. tubulosa* have 3.704 ± 1.147 and 5.126 ± 0.116 mg protein/g wet weight in their tissue, respectively.

3.7 Foam and Froth Test

As a bio-industrial activity, new bio-surfactant-like extracts were obtained. To get this property, *Quillaja* bark saponin was used as a positive control. The calibration curve was drawn in the range of 10-100 $\mu\text{g/mL}$ *Quillaja* bark saponin standard's foam height. The calibration curve was given in Figure 3.35. The foam test were given in Figure 3.36.

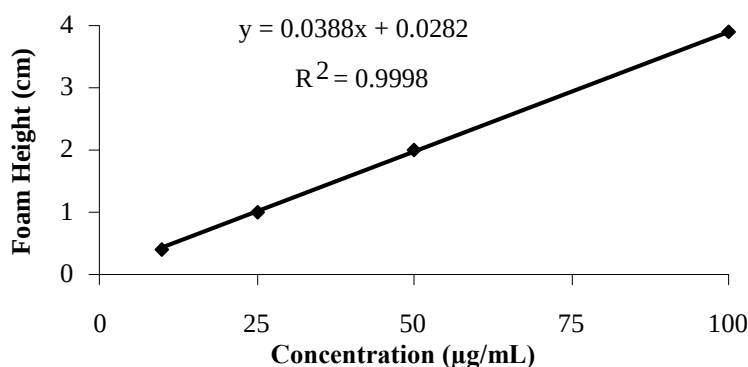


Figure 3.35 The calibration curve of *Quillaja* bark saponin

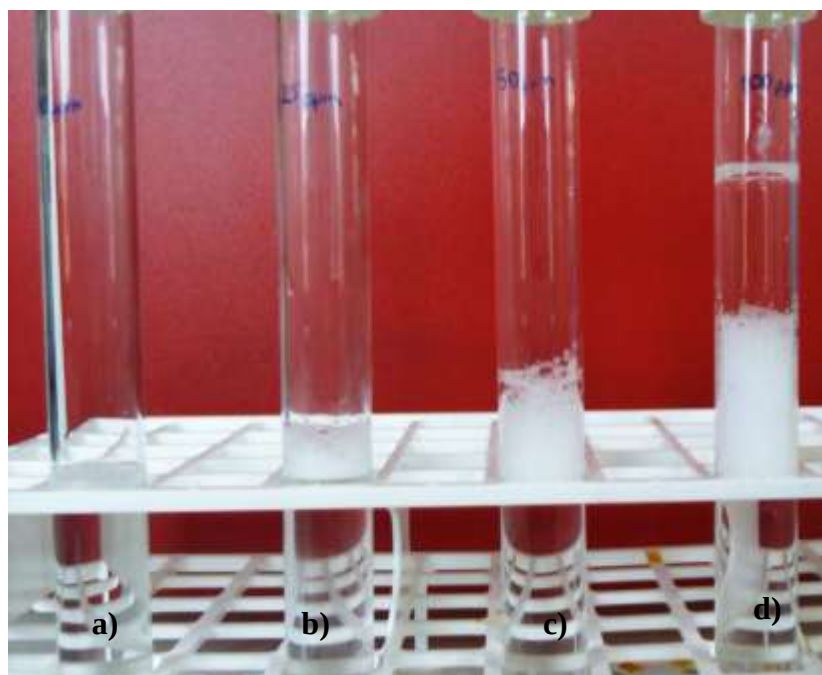


Figure 3.36 Foam test for *Quillja* bark saponin a) 10 µg/mL; b) 25 µg/mL; c) 50 µg/mL; d) 100 µg/mL.

The extracts were prepared as mentioned in section 2.3. According to the results, 92 µg/mL foam formation and 94 µg/mL float formation was observed.

3.8 Lipid Level and Fatty Acid Composition in Sea Cucumber

After 250 mL n-hexane extraction of 25 grams (M_1) dried *Holothuria* species to obtain fatty acids, the extracted body wall wastes (M_2) were measured. An example calculation was given below.

$$M_1: 25.0000 \text{ g}, M_2: 24.1434 \text{ g}$$

The lipid contents in dried *H. polii* and *H. tubulosa* were found as $8.30 \pm 0.2 \%$ $3.43 \pm 0.1 \%$, respectively.

In Table 3.3, the comparative fatty acid composition between *H. polii* and *H. tubulosa* was given. According to this table, the most abundant fatty acid is DHA (14.71%) in *H. polii* and stearic acid (22.00%) is in *H. tubulosa*.

Table 3.3 Fatty acid composition of *Holothuria* species (n.d, not detectable)

Percent (%)		
Fatty acid	<i>H. polii</i>	<i>H. tubulosa</i>
Others	20.35	n.d
Erusic acid (C22:1)	13.36	n.d
Palmitoleic acid (C16:1)	2.03	n.d
Heptadecanoic acid (C17:0)	n.d	0.96
Poly Unsaturated Fatty acids	n.d	n.d
DHA (C22:6)	14.71	n.d
Arachidonic acid (C20: 4n6)	n.d	5.08
cis-11, 14 Eicosadienoic acid (C20:2)	n.d	9.43
Linoleic acid (C18:2)	3.84	n.d
Linoleic acid (C18:3)	10.36	n.d
Mono Unsaturated Fatty acids	n.d	n.d
Eicosenoic acid (C20:01)	7.85	n.d
Oleic acid (C18:1)	5.28	4.99
Gondoic acid (C20:1)	n.d	3.40
Saturated Fatty acids	n.d	n.d
Lauric acid (C12:0)	1.25	8.79
Myristic acid (C14:0)	1.86	1.23
Palmitic acid (C16:0)	3.94	12.91
Stearic acid (C18:0)	6.35	22.00
Pentadecanoic acid (C15:0)	n.d	2.55
Tricosanoic acid (C23:0)	n.d	7.64
cis-5,8,11,14,17 Eicosapentaenoic acid (C20:)	<	19.00

3.9 Carbohydrate Level in Sea Cucumbers

In order to determine carbohydrate levels in sea cucumbers, Dubois, Gilles, Hamilton, Rebers and Smith (1956) was used. The calibration curves against sugar standards (D-glucose, D-fructose, D-sucrose, D-galactose and D-maltose) were drawn. The absorbences of hexoses, D-Glucose and D-Galactose, was measured at 490 nm and the absorbances of D-fructose, D-sucrose and D-maltose, pentose was measured at 480 nm. The calibration curve was plotted in the range of 5-50 mg/L (Figure 3.37).

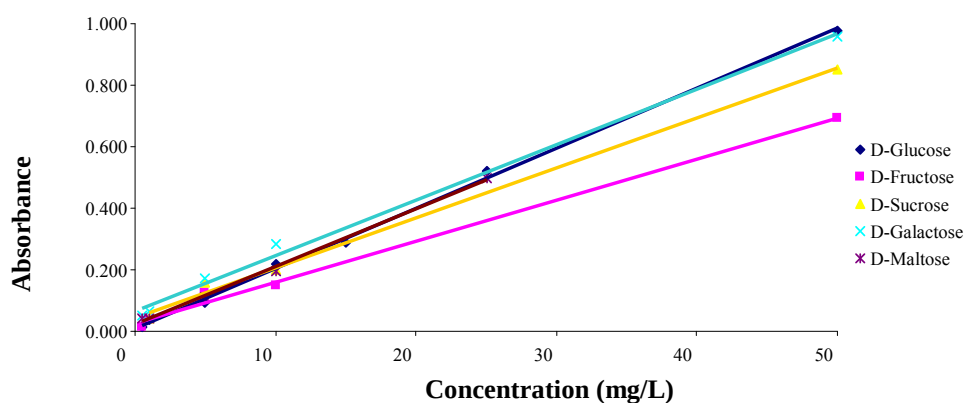


Figure 3.37 Calibration curve of sugar standards.

After calculating the levels of carbohydrates for all standards separately as mg/L, the results were normalised to concentration (mg/g wet weight). The related results were given in Table 3.4.

Table 3.4 The calculated carbohydrate concentration (mg/g wet weight) in sea cucumber extracts.

Samples	Concentration (mg/g wet weight)				
	D-glucose	D-fructose	D-sucrose	D-galactose	D-maltose
<i>H. polii</i> (water)	0.67 ± 0.47	0.85 ± 0.72	0.57 ± 0.65	0.41 ± 0.61	0.63 ± 0.51
<i>H. tubulosa</i> (water)	0.70 ± 0.25	0.90 ± 0.45	0.60 ± 0.47	0.47 ± 0.43	0.67 ± 0.30

According to the results, the highest carbohydrate concentration for all sugars was observed in *H. tubulosa*. These results show that the most abundant sugar is D-

fructose (0.90 ± 0.45 mg/g wet weight) for *H. tubulosa* and the least abundant carbohydrate is D-galactose (0.41 ± 0.61 mg/g wet weight) in *H. polii*.

3.10 Total Ash and Moisture Content

In order to evaluate the total ash content of sea cucumber, the initial (*I*) and end (*E*) weight (g) of the samples were determined after heat treatment at 600 °C. The difference between *I* and *E* (g) was evaluated. The ash contents in *H. polii* and *H. tubulosa* were found as 11.09 ± 2.86 % and 10.48 ± 1.87 %, respectively.

Total moisture content of *H. polii* and *H. tubulosa* was also determined. The moisture contents in *H. polii* and *H. tubulosa* were found as 80.64 ± 1.33 % and 83.20 ± 1.59 %, respectively.

3.11 Vitamin C Content in Sea Cucumber by Using HPLC

Determination of vitamin C content of holothurian samples were also studied. In order to determine the vitamin C level, RP-HPLC was used. The range of calibration curve was 50-400 mg/L. The calibration curve of L-ascorbic acid was given in Figure 3.38.

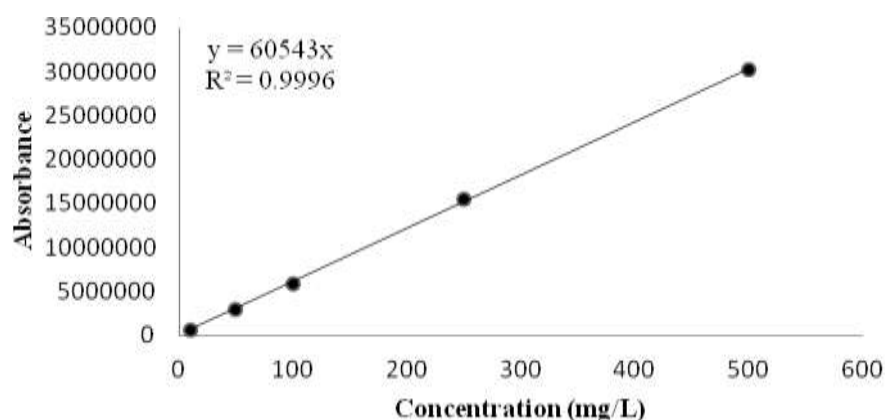


Figure 3.38 Calibration curve of ascorbic acid

The HPLC chromatogram of 50-400 mg/L ascorbic acid was given in Figure 3.39.

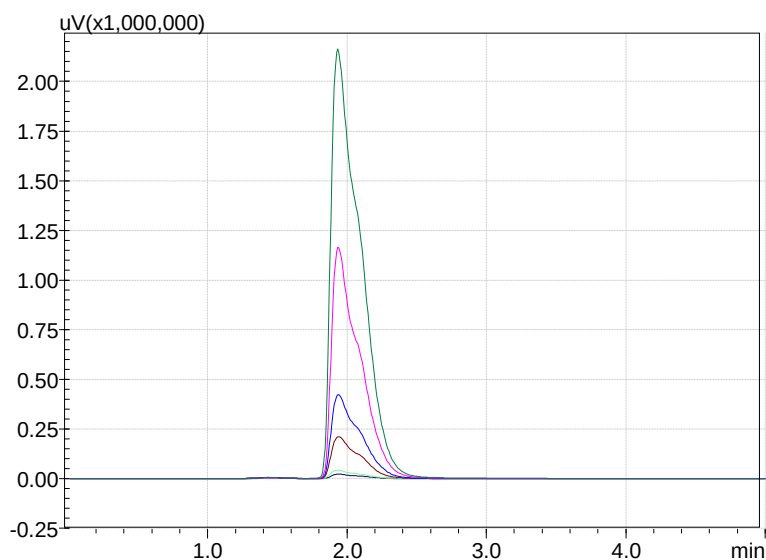


Figure 3.39 HPLC chromatogram comparison of ascorbic acid (Red: 50 mg/L; Blue: 100 mg/L; Pink: 200 mg/L; Green: 400 mg/L).

The level of vitamin C in holothurians was estimated by using calibration curve. *H. tubulosa* has 96.64 ± 2.10 mg/L and *H. polii* has 12.68 ± 0.24 mg/L vitamin C in their tissues. Limit of detection was also calculated as it was mentioned in section 2.14. According to the results, LOD is 0.064 mg/L.

3.12 Adsorption Studies with Sea Cucumber Wastes

3.12.1 FT-IR Spectrum

FT-IR spectra of the body wall of UT and LE *H. polii* were given in Figure 3.40 and 3.41, respectively.

According to the results, broad O-H stretch ($3389\text{--}3431\text{ cm}^{-1}$) and C-H bending ($1418\text{--}1423\text{ cm}^{-1}$) and sharp aromatic out of plane C-H bending (874 cm^{-1}) bands were observed for UT *H. polii* samples (Figure 3.40).

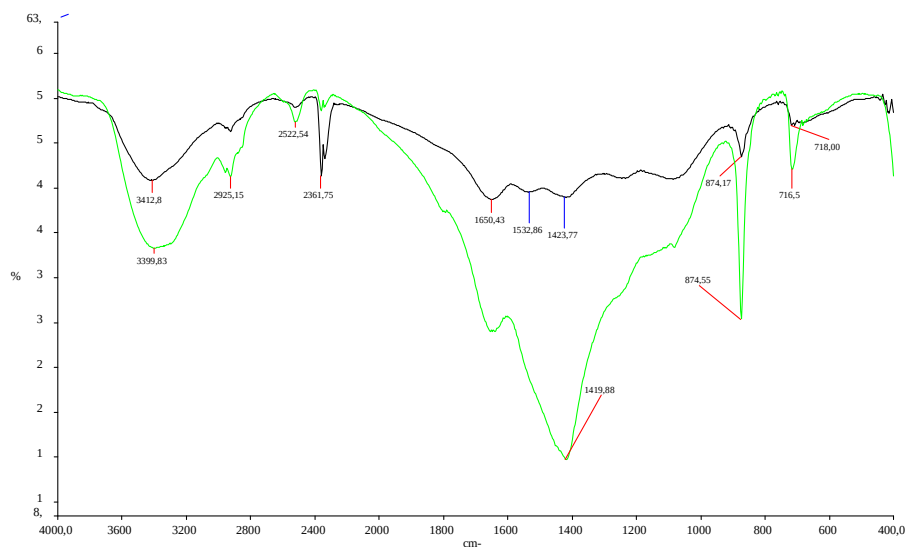


Figure 3.40 FT-IR spectrum of UT *H. polii* samples a) Before adsorption, b) 100 mg/L MB adsorbed sample.

For LE *H. polii* samples, broad O-H stretch ($3415\text{--}3429\text{ cm}^{-1}$), aliphatic C-H stretching ($2918\text{--}2924\text{ cm}^{-1}$), C-H bending ($1413\text{--}1418\text{ cm}^{-1}$), C-C stretching and C-O bends ($1244\text{--}1255\text{ cm}^{-1}$) were observed in Figure 3.41.

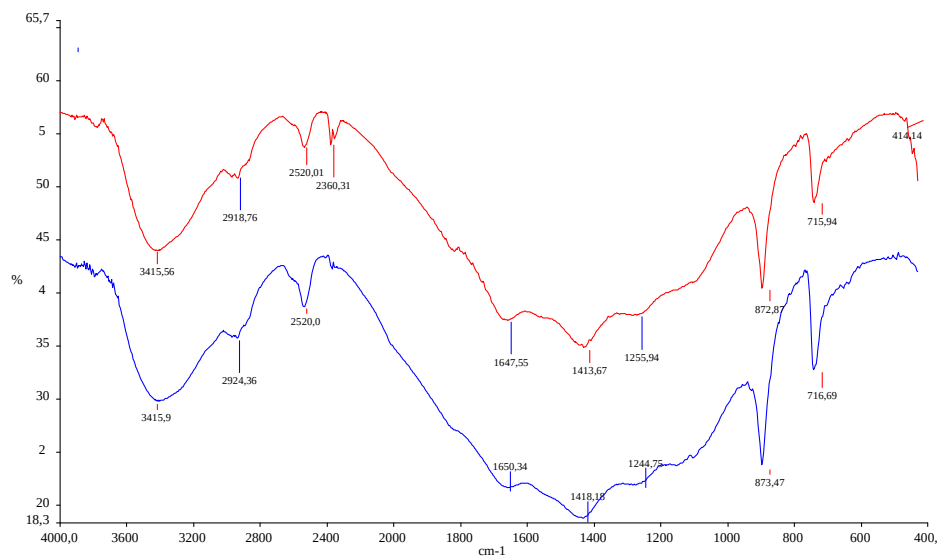


Figure 3.41 FT-IR spectrum of LE *H. polii* samples a) Before adsorption, b) 100 mg/L MB adsorbed sample.

3.12.2 Adsorption Kinetics

The adsorption kinetics data give fundamental information on the reaction rate between adsorbate and adsorbant. To study the adsorption kinetics, two different adsorbents were used: UT and LE *H. polii*. The data related to pseudo-first order and pseudo-second order adsorption kinetics were plotted to specify the adsorption process whether they are in physical or chemical nature.

3.12.2.1 Adsorption Kinetics of LE samples

For adsorption of 50 mg/L MB onto LE samples, pseudo-first and pseudo-second order kinetics were given in Figures 3.42 – 3.45. The R^2 values for pseudo-first order and pseudo-second order kinetics were 0.9722 and 0.9996, respectively. Therefore it might be classified as a pseudo-second order kinetic that means chemisorption at 30°C.

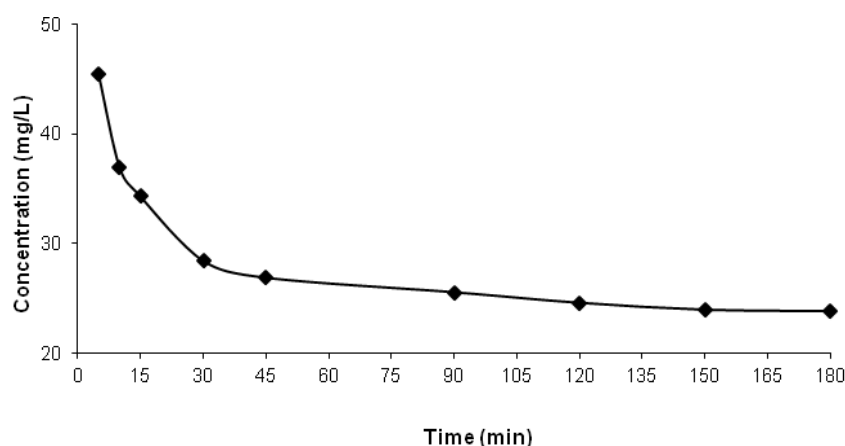


Figure 3.42 Removal of MB onto LE *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

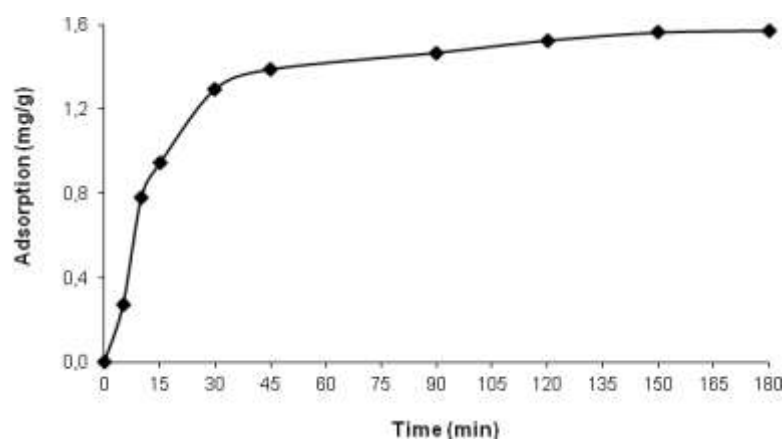


Figure 3.43 Adsorption kinetic of MB onto LE *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

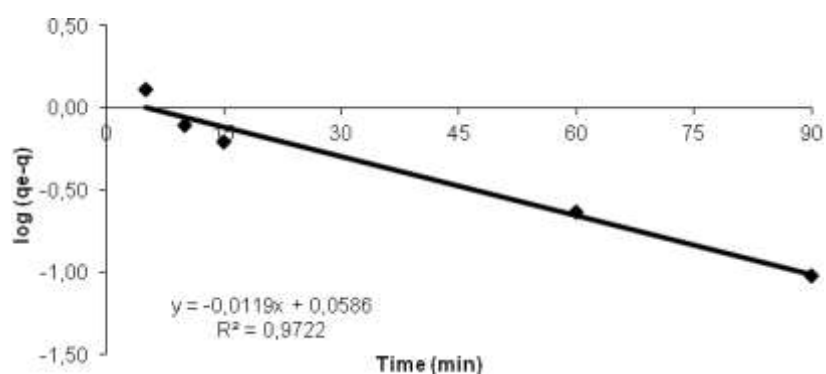


Figure 3.44 Pseudo-first order adsorption kinetics of MB by LE *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

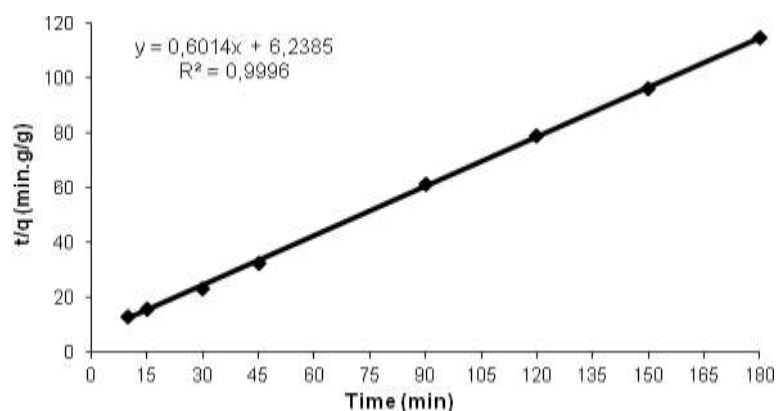


Figure 3.45 Pseudo-second order adsorption kinetics of MB by LE *H. polii* (MB concentration: 50 mg/L, temperature: 30°C)

For 100 mg/L MB adsorption onto LE *H. polii*, the plots were given in Figures 3.46 – 3.49. The R^2 values for pseudo-first order and pseudo-second order kinetics were 0.8774 and 0.9957, respectively. Therefore pseudo-second order kinetic (chemisorption) was fitted for the adsorption of 100 mg/L MB onto LE *H. polii*.

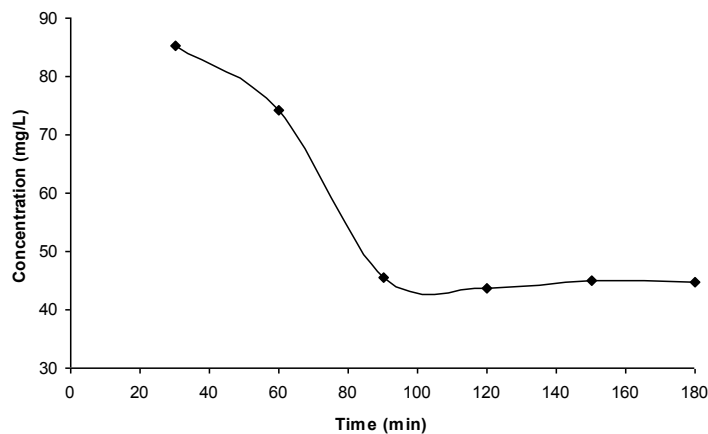


Figure 3.46 Removal of MB onto LE *H. polii* (MB concentration: 100 mg/L, temperature: 30°C)..

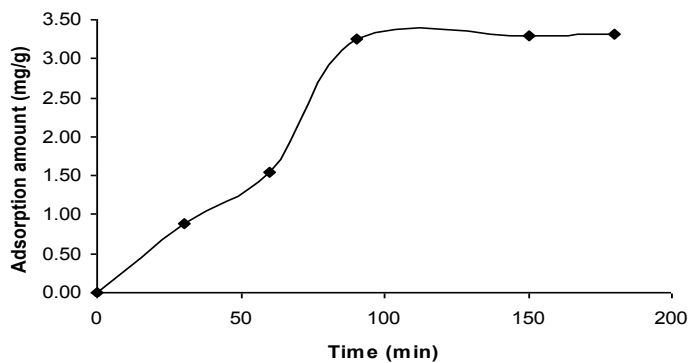


Figure 3.47 Adsorption kinetic of MB onto LE *H. polii* (MB concentration: 100 mg/L, temperature: 30°C).

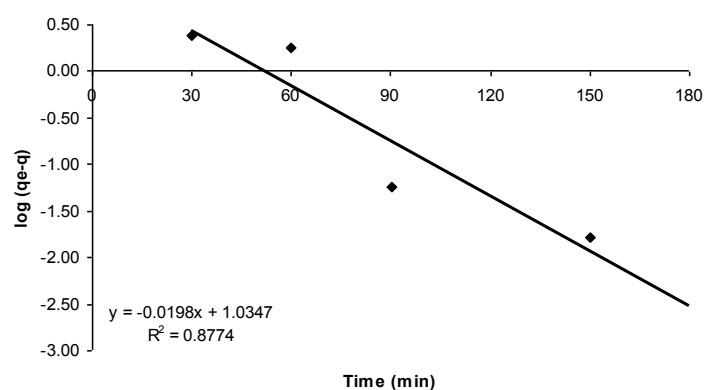


Figure 3.48 Pseudo-first order adsorption kinetics of MB by LE *H. polii* (MB concentration: 100 mg/L, temperature: 30°C).

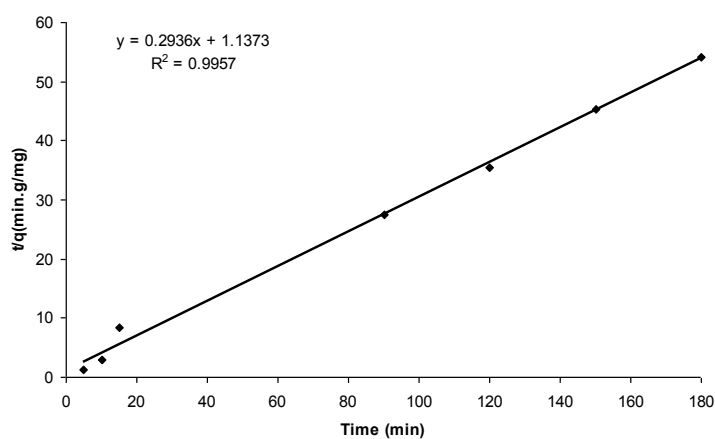


Figure 3.49 Pseudo-second order adsorption kinetics of MB by LE *H. polii* (MB concentration: 100 mg/L, temperature: 30°C).

After adsorption of 150 mg/L MB by LE samples, pseudo-first and pseudo-second order kinetics were plotted (Figures 3.50 - 3.53). The R^2 values for pseudo-first order and pseudo-second order kinetics were 0.9918 and 0.9924, respectively. Although the two values are quite same, the process seems as pseudo-second order kinetic.

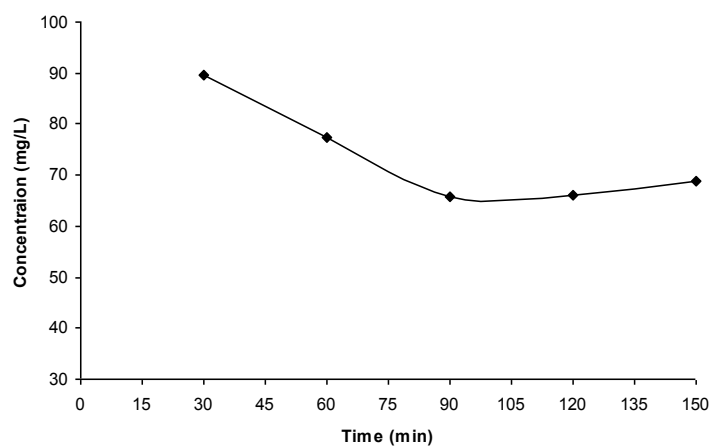


Figure 3.50 Removal of MB onto LE *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

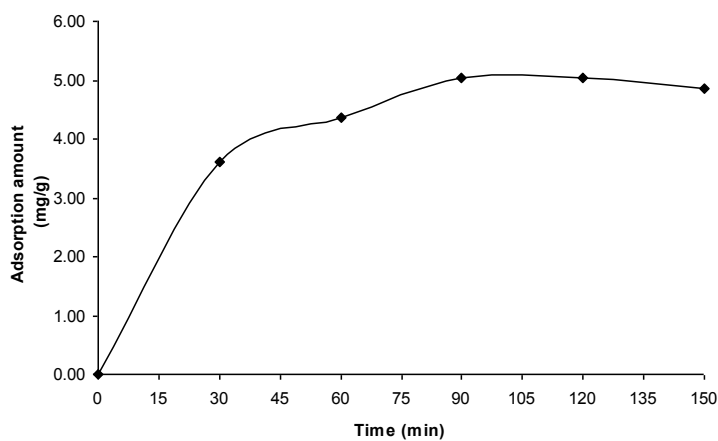


Figure 3.51 Adsorption kinetic of MB onto LE *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

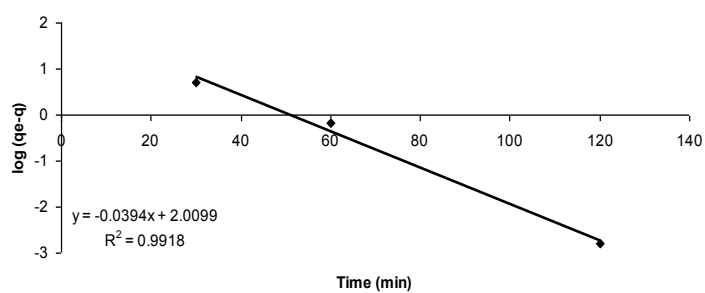


Figure 3.52 Pseudo-First order adsorption kinetics of MB by LE *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

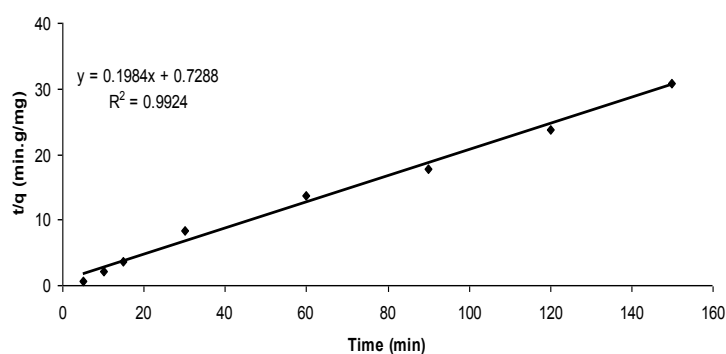


Figure 3.53 Pseudo-Second order adsorption kinetics of MB by LE *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

The plotted adsorption kinetics for 200 mg/L MB onto LE samples were given in Figures 3.54 - 3.57. The R^2 values for pseudo-first order and pseudo-second order kinetics were 0.9757 and 0.9999 for, respectively. The results were fitted well with the pseudo-second order kinetic and the adsorption was classified as chemisorption at 30°C.

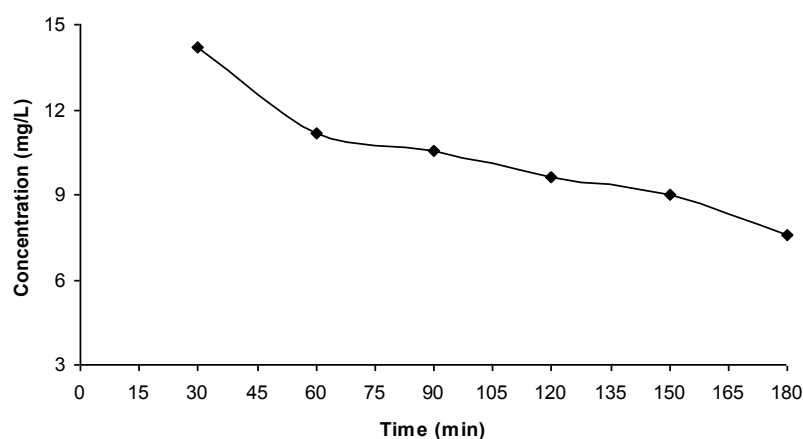


Figure 3.54 Removal of MB onto LE *H. polii* (MB concentration: 200 mg/L, temperature: 30°C).

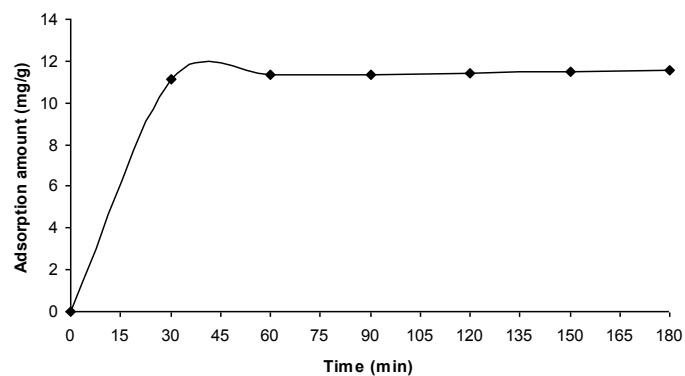


Figure 3.55 Adsorption kinetic of MB onto LE *H. polii* (MB concentration: 200 mg/L, temperature: 30°C).

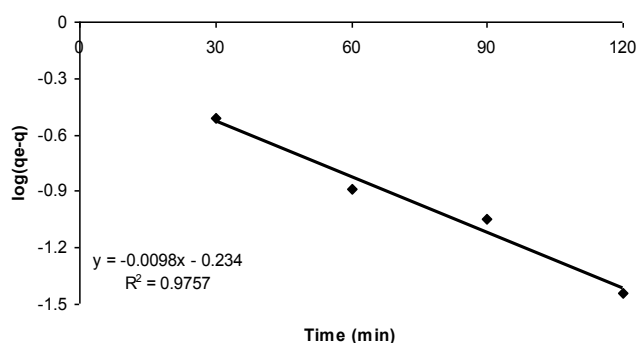


Figure 3.56 Pseudo-first order adsorption kinetics of MB by LE *H. polii* (MB concentration: 200 mg/L, temperature: 30°C).

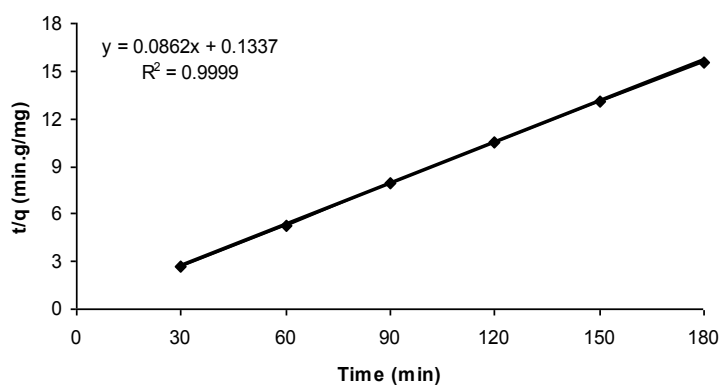


Figure 3.57 Pseudo-second order adsorption kinetics of MB by LE *H. polii* (MB concentration: 200 mg/L, temperature: 30°C)..

The adsorption (q ; mg/g) of MB onto LE *H. polii* at 30°C for all concentrations were given in Figure 3.58. The adsorption equilibrium time for 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L MB were 30, 90, 120 and 60 min, respectively.

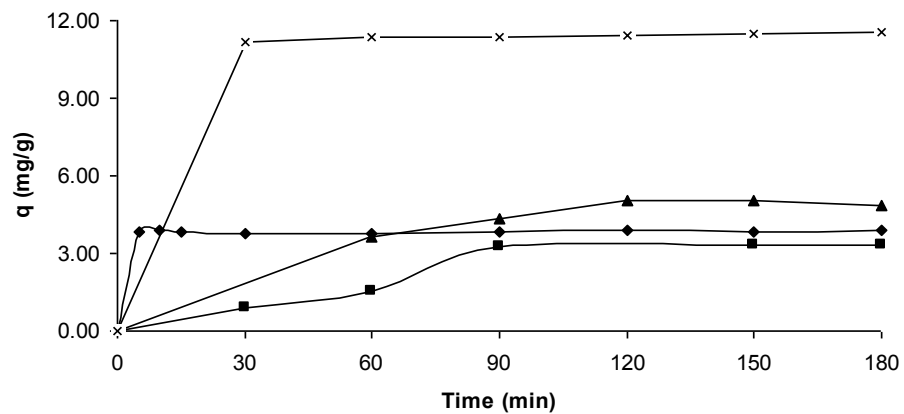


Figure 3.58 The adsorption kinetics of MB onto LE *H. polii* at different initial MB concentrations (-♦- 50 mg/L; -■- 100 mg/L; -▲- 150 mg/L; -x- 200 mg/L at 30 °C, 120 rpm).

3.12.2.2 Adsorption Kinetics of UT samples

Adsorption, pseudo-first order and pseudo-second order kinetics for 50 mg/L MB onto UT samples were plotted (Figures 3.59 – 3.62). The R^2 values for pseudo-first order and pseudo-second order kinetics were 0.977 and 0.9984, respectively. Adsorption at 30°C was classified as pseudo-second order kinetic.

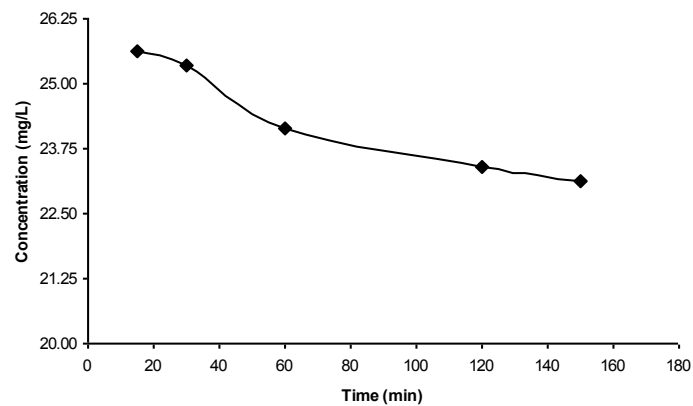


Figure 3.59 Removal of MB onto UT *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

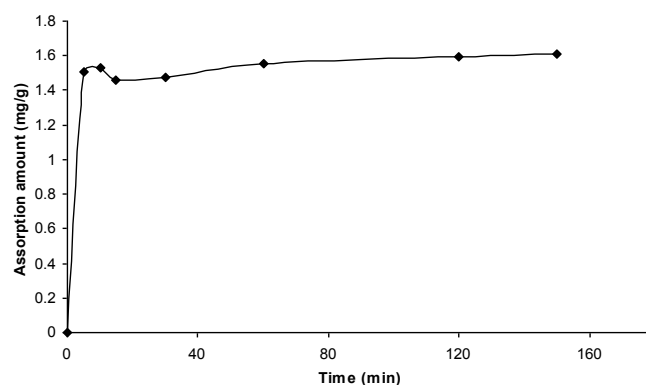


Figure 3.60 Adsorption kinetic of MB onto UT *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

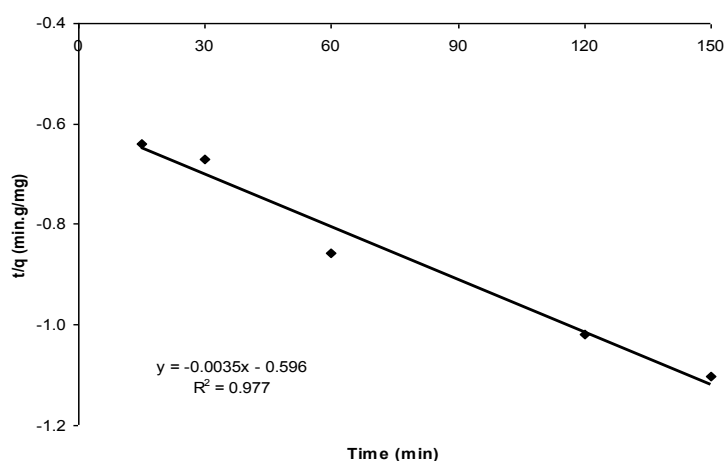


Figure 3.61 Pseudo-first-order adsorption kinetics of MB of UT *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

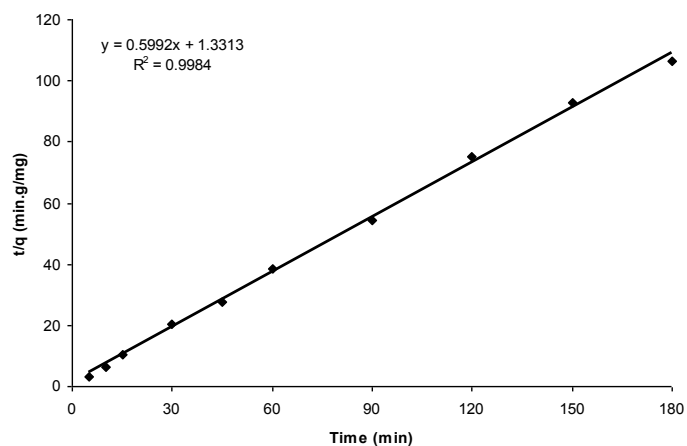


Figure 3.62 Pseudo-second-order adsorption kinetics of MB of UT *H. polii* (MB concentration: 50 mg/L, temperature: 30°C).

For 100 mg/L MB onto UT *H. polii* samples, the removal of MB, adsorption, pseudo-first order and pseudo-second kinetics were given in Figures 3.63 - 3.66. The R^2 value for pseudo-first order and pseudo-second order kinetics were 0.9567 and 0.9997 respectively. Thus the adsorption could be classified as pseudo-second order kinetic at 30°C.

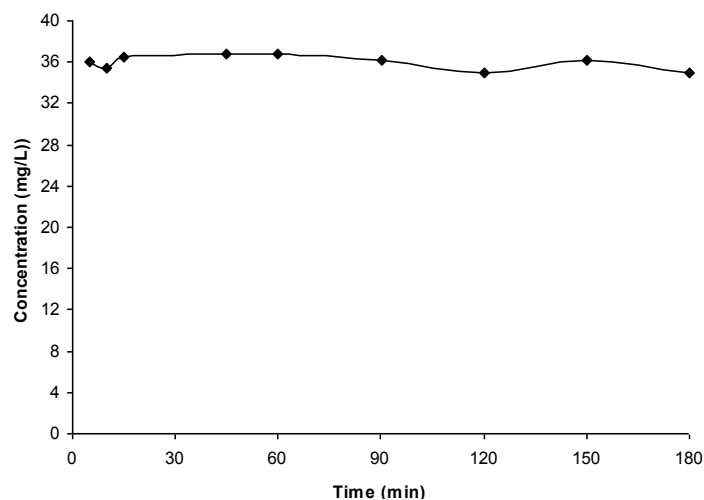


Figure 3.63 Removal of MB onto UT *H. polii* (MB concentration: 100 mg/L, temperature: 30°C).

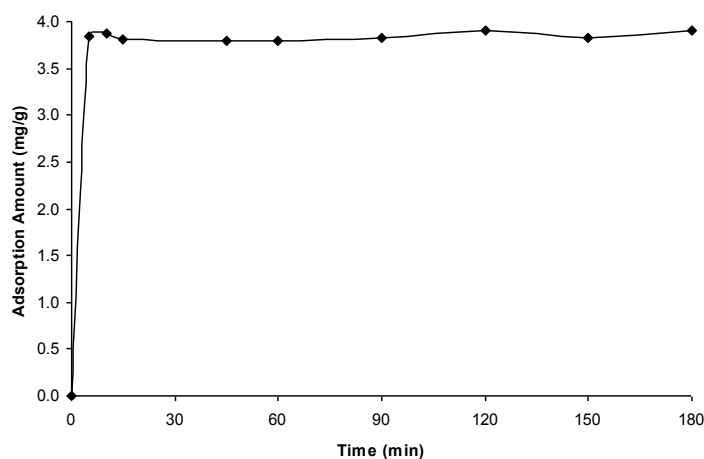


Figure 3.64 Adsorption kinetic of MB onto UT *H. polii* (MB concentration: 100 mg/L, temperature: 30°C).

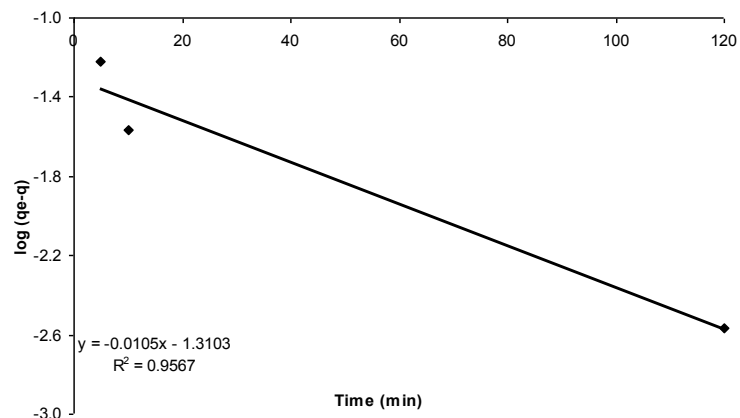


Figure 3.65 Pseudo-First order adsorption kinetics of MB by UT *H. polii* (MB concentration: 100 mg/L, temperature: 30°C)

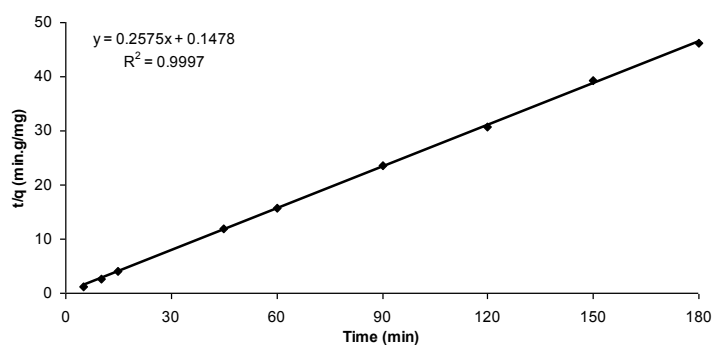


Figure 3.66 Pseudo-second order adsorption kinetics of MB by UT *H. polii* (MB concentration: 100 mg/L, temperature: 30°C).

The adsorption kinetics of 150 mg/L MB onto UT *H. polii* samples were given in Figures 3.67 – 3.70. According to the results, R^2 values for pseudo-first order and pseudo-second order kinetics were 0.9872 and 0.9951, respectively. Thus, the best-fit kinetic was pseudo-second order kinetic at 30°C.

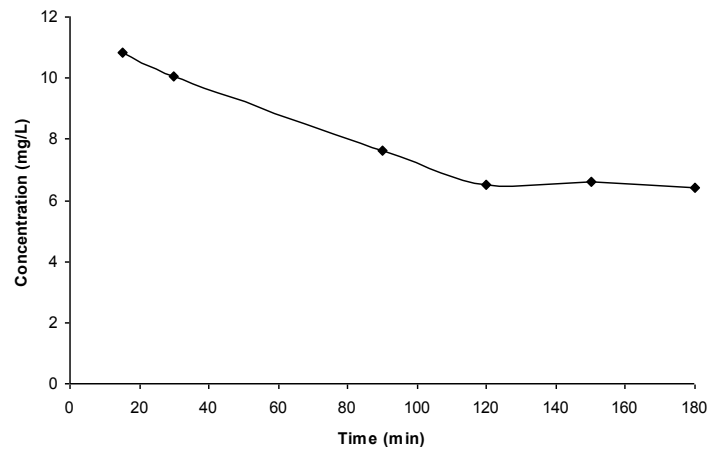


Figure 3.67 Removal of MB onto UT *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

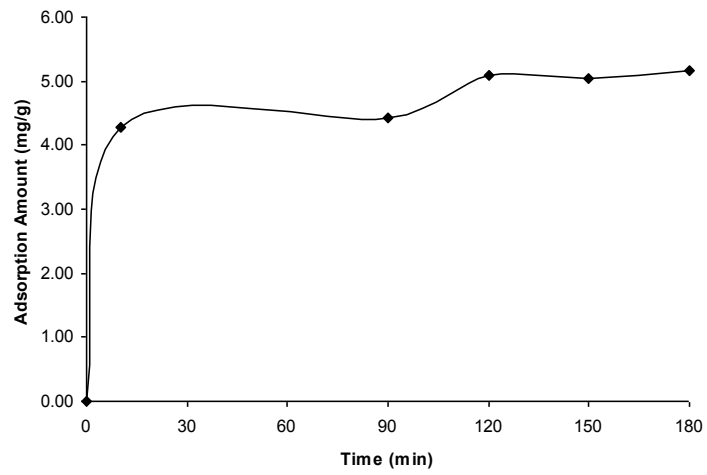


Figure 3.68 Adsorption kinetic of MB onto UT *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

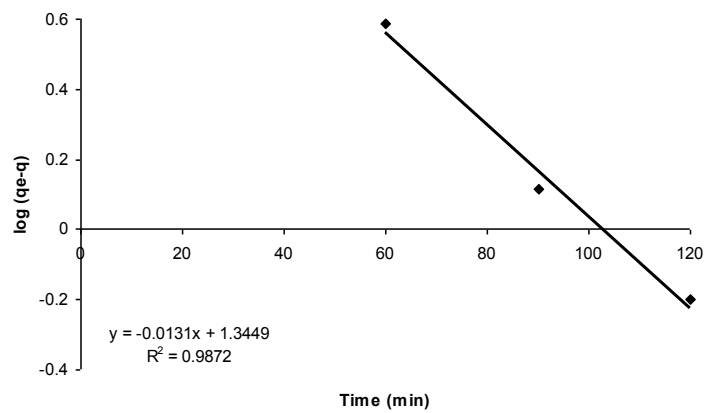


Figure 3.69 Pseudo-First order adsorption kinetics of MB by UT *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

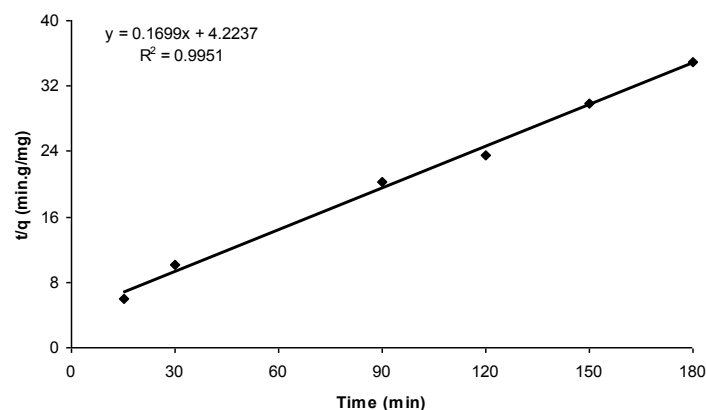


Figure 3.70 Pseudo-second order adsorption kinetics of MB by UT *H. polii* (MB concentration: 150 mg/L, temperature: 30°C).

The plotted adsorption kinetics for 200 mg/L MB onto UT samples were given in Figures 3.71 - 3.74. The R^2 values for pseudo-first order and pseudo-second order kinetics were 0.9751 and 0.9771, respectively. The results were fitted well with the pseudo-second order kinetic and adsorption was considered as chemisorption at 30°C.

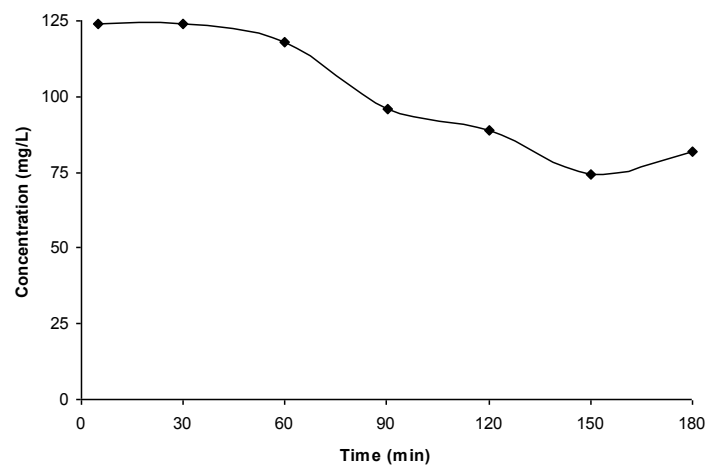


Figure 3.71 Removal of MB onto UT *H. polii* (MB concentration: 200 mg/L, temperature: 30°C).

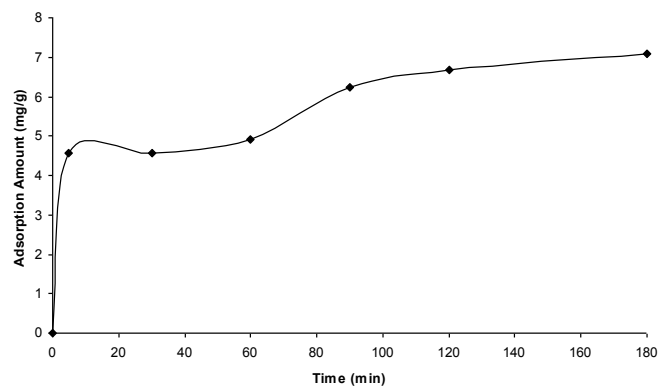


Figure 3.72 Adsorption kinetic of MB onto UT *H. polii* (MB concentration: 200 mg/L, temperature: 30°C).

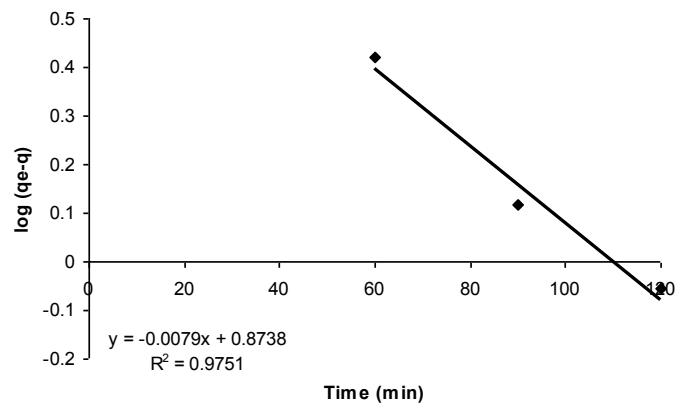


Figure 3.73 Pseudo-First order adsorption kinetics of MB by UT *H. polii* (MB concentration: 200 mg/L, temperature: 30°C).

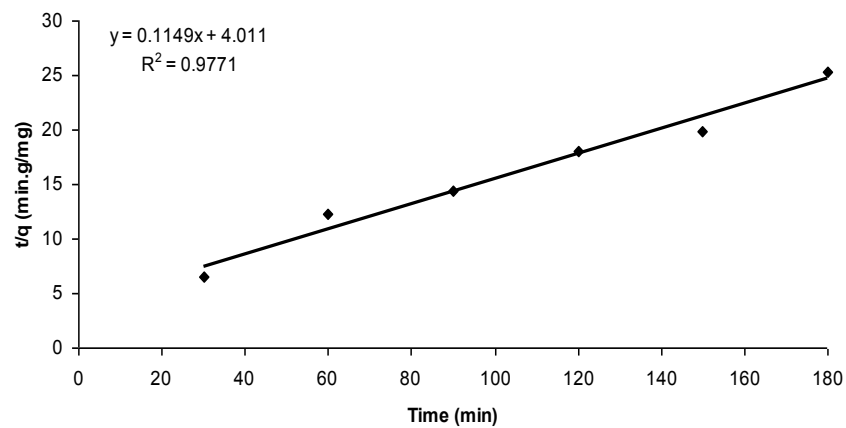


Figure 3.74 Pseudo-second order adsorption kinetics of MB by UT *H. polii* (MB concentration: 200 mg/L, temperature: 30°C)

The adsorption of MB onto UT *H. polii* for all concentrations were given in Figure 3.75. The adsorption equilibrium time for 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L MB onto UT *H. polii* were 60, 60, 120 and 120 min, respectively.

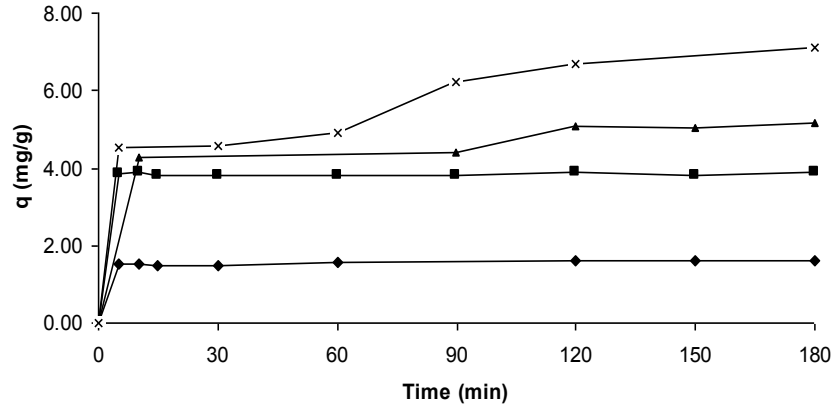


Figure 3.75 The adsorption kinetics of MB onto UT *H. polii* at different initial MB concentrations (♦- 50 mg/L; ■- 100 mg/L; ▲- 150 mg/L; x- 200 mg/L, at 30 °C, 120 rpm).

For both adsorbents, $q_{e, \text{exp}}$ (mg/g), pseudo-first order, $q_{e, \text{cal}}$ (mg/g), R^2) and pseudo-second order, $q_{e, \text{cal}}$ constants were determined, the results were given in Table 3.5. The Table 3.5 reveals that $q_{e, \text{exp}}$ values for LE samples are 1.563, 3.500, 5.040 and 11.458 for 50, 100, 150 and 200 mg/L MB, respectively and $q_{e, \text{exp}}$ for UT samples values are 1.690, 3.900, 5.730 and 7.550 for 50, 100, 150 and 200 mg/L MB, respectively. Also, the R^2 values of pseudo-second order kinetic were higher than those of pseudo-first order kinetics. Moreover, $q_{q, \text{exp}}$ values of LE samples were higher compared to other concentrations, especially when the MB concentration was 200 mg/L. Thus, it might have been thought as pseudo-second order kinetic, chemisorption, and LE samples are better for MB adsorption.

Table 3.5 Pseudo first and pseudo second order rate constants at 30°C different initial MB concentrations for the adsorption of methylene blue onto UT and LE *H. polii*

Type of Adsorbent	C_i (mg/L)	$q_{e, \text{exp}}$ (mg/g)	Pseudo-first-order rate constants			Pseudo-second-order rate constants		
			k_1 (1/min)	$q_{e, \text{cal}}$ (mg/g)	R^2	k_2 (g/mgmin)	$q_{e, \text{cal}}$ (mg/g)	R^2
LE <i>H. polii</i>	100	3.500	2.383	0.955	0.877	0.076	3.406	0.996
	150	5.040	0.090	102.094	0.991	0.054	5.051	0.992
	200	11.458	0.023	0.583	0.976	0.056	11.628	0.999
UT <i>H. polii</i>	50	1.690	1.373	0.992	0.977	0.270	1.669	0.998
	100	3.900	0.024	0.049	0.9567	0.449	3.883	0.997
	150	5.730	0.030	22.080	0.987	0.007	5.917	0.995
	200	7.550	0.016	7.464	0.975	0.005	6.944	0.977

3.12.3 Adsorption Isotherms

In order to determine the biosorption mechanism between *H. polii* and MB (monolayer or multilayer), two different isotherms (Langmuir and Freundlich' model) were also carried out. The graphs for adsorption on MB on UT and LE *H. polii* at different temperatures (30 °C, 40 °C, 50 °C) were drawn by plotting $\log C_e$ versus $\log q_e$ to determine Langmuir isotherm and were given at Figures 3.76 and 3.77, respectively.

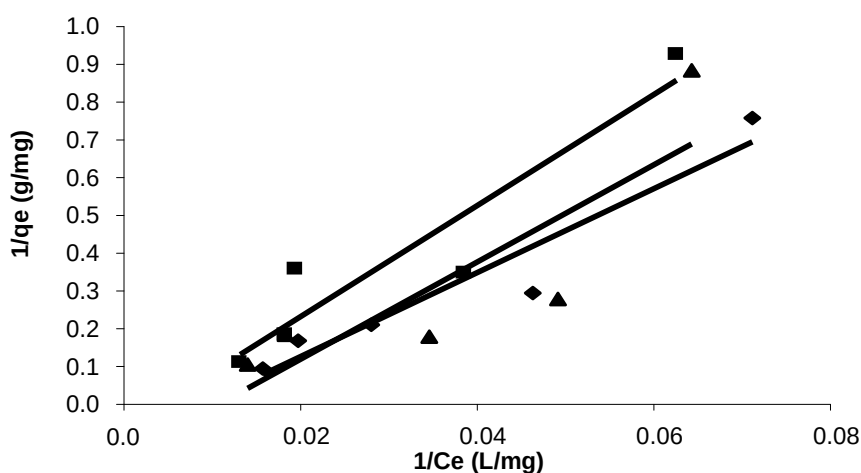


Figure 3.76 Langmuir isotherm plots for adsorption of MB onto UT *H. polii* (-♦- 30 °C; -■- 40°C; -▲- 50 °C).

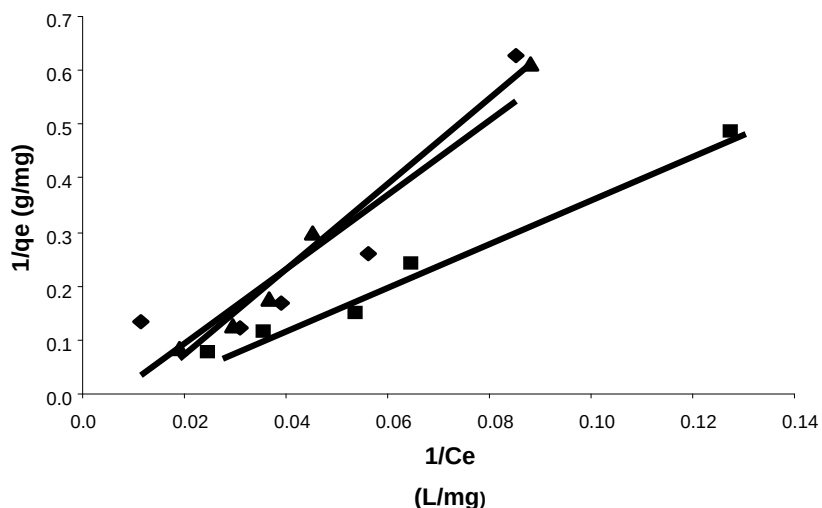


Figure 3.77 Langmuir isotherm plots for adsorption of MB onto LE *H. polii* body wall wastes (-♦- 30 °C; -■- 40°C; -▲- 50 °C)

The graphs for adsorption of MB onto UT and LE *H. polii* at different temperatures (30 °C, 40 °C, 50 °C) were drawn by plotting $\log C_e$ versus $\log q_e$ to determine Freundlich isotherm and the data were given at Figures 3.78 and 3.79, respectively.

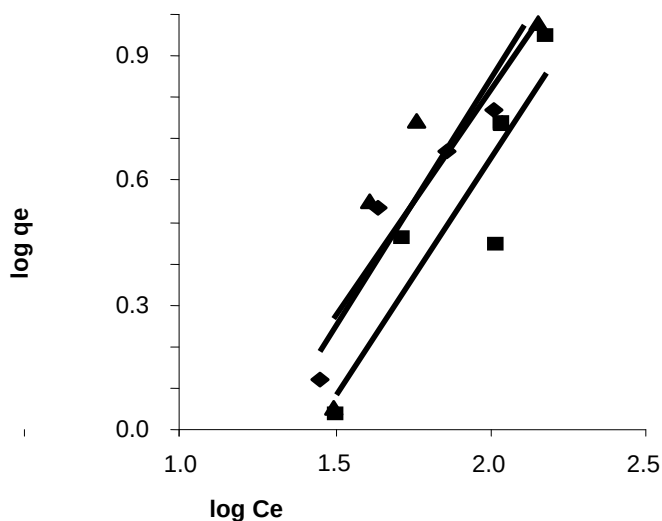


Figure 3.78 Freundlich isotherm plots for adsorption of MB onto UT *H. polii* body walls (-♦- 30°C; -■- 40°C; -▲- 50 °C).

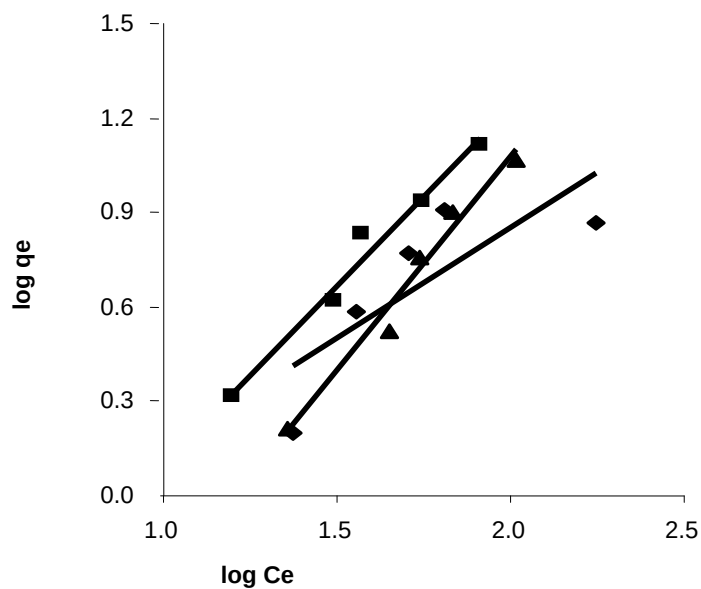


Figure 3.79 Freundlich isotherm plots for adsorption of MB onto LE *H. polii* body wall wastes (-♦- 30 °C; -■- 40°C; -▲- 50 °C).

Langmuir and Freundlich constants were given in Table 3.6.

Table 3.6 Langmuir and Freundlich constants for the adsorption of MB onto LE and UT *H. polii*

Type of Adsorbent	Temperature °C	Langmuir constants			Freundlich constants			
		q_m (mg/g)	b (L/mg)	R^2	K_f	n	$1/n$	R^2
LE <i>H. polii</i>	30	12.63	0.010	0.998	0.29	1.54	0.65	0.999
	40	43.67	0.002	0.956	0.09	0.89	1.13	0.976
	50	-	-	0.978	0.02	0.74	1.35	0.976
UT <i>H. polii</i>	30	-	-	0.999	0.04	0.79	1.27	0.998
	40	-	-	0.960	0.02	0.79	1.27	0.969
	50	-	-	0.887	0.03	0.89	1.12	0.836

The results of isotherm studies were well in line with the Freundlich models, therefore adsorption of MB onto LE *H. polii* was considered as a multilayer adsorption (Table 3.6). $1/n$ values are known as heterogeneity factor.

3.13 Antifouling Performance

In order to evaluate the antifouling performance of the sea cucumber extract, the plates were prepared and immersed into the water for a month (from June 18 to July 17, 2014 at Levent Marine, İzmir, Turkey). According to the results, all of the plates were non-homogenously covered by micro-slime layer. The uncoated plates (Figure 3.80), biocide-free plates (Figure 3.89) and anticorrosive coated plates (Figure 3.90) were highly covered by macro-fouling organisms. According to the results, the best antifouling performance was observed for zinc pyron including paint (Figure 3.88). The all photos related to testing period were given in Figures 3.80 – 3.90. All of the test panels were evaluated from level I to level V according to the fouling criteria. The assessment of fouling grades was given in Table 3.7.

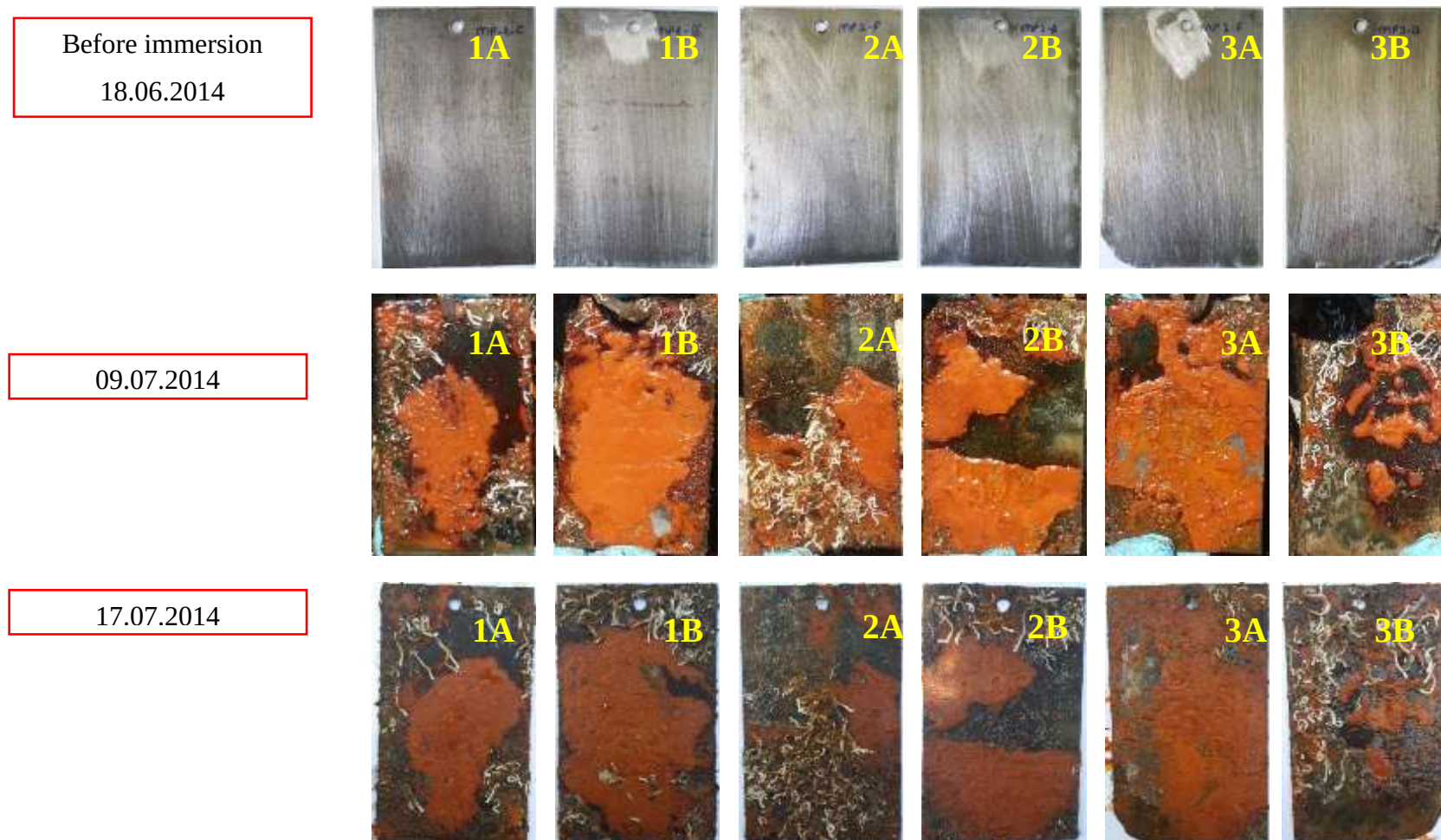


Figure 3.80 Uncoated test plates (A: front, B: back, the plates were studied in triplicate).

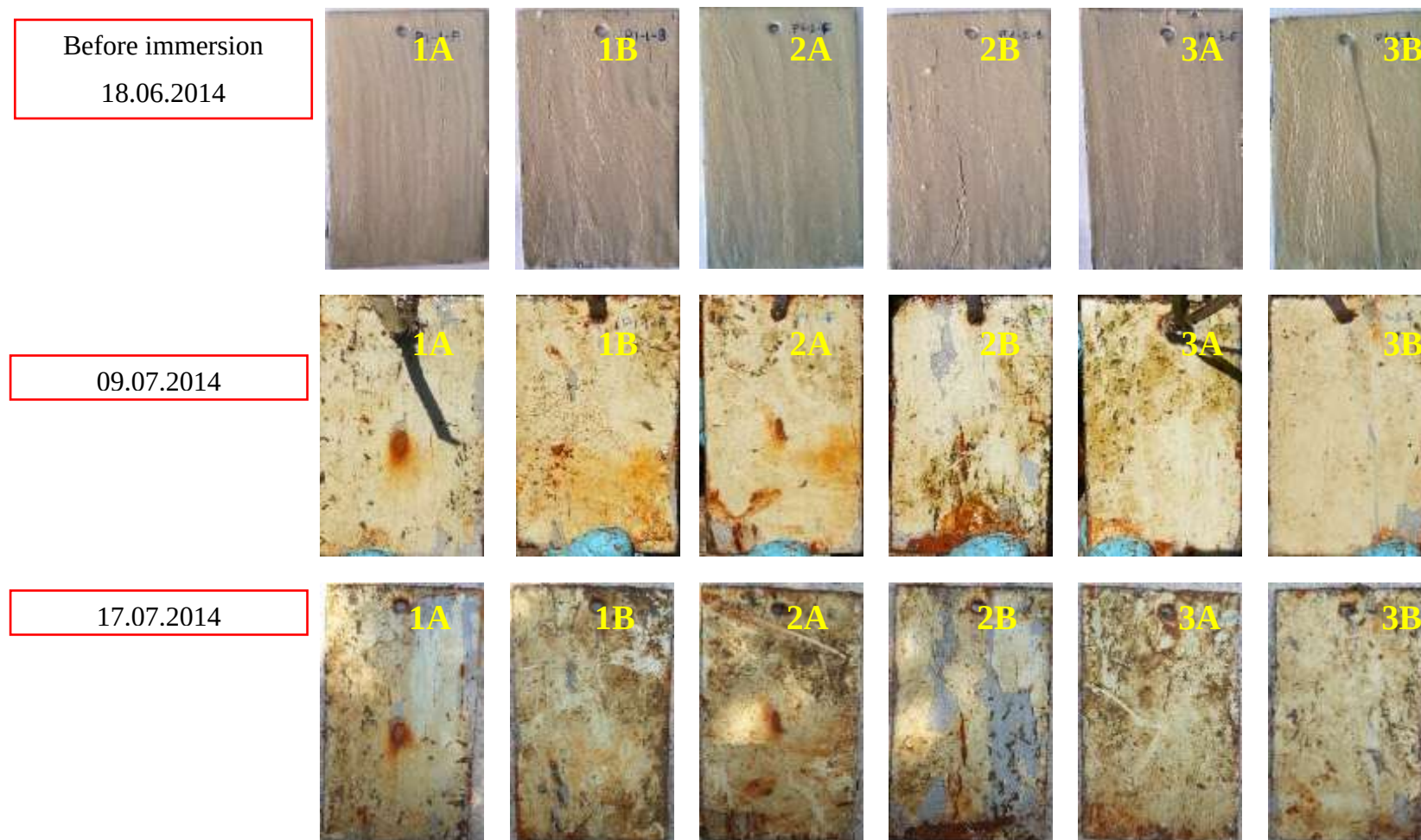


Figure 3.81 The test plates including *Holothuria polii* MeOH-Dichloromethane extract (A: front, B: back, the plates were studied in triplicate).

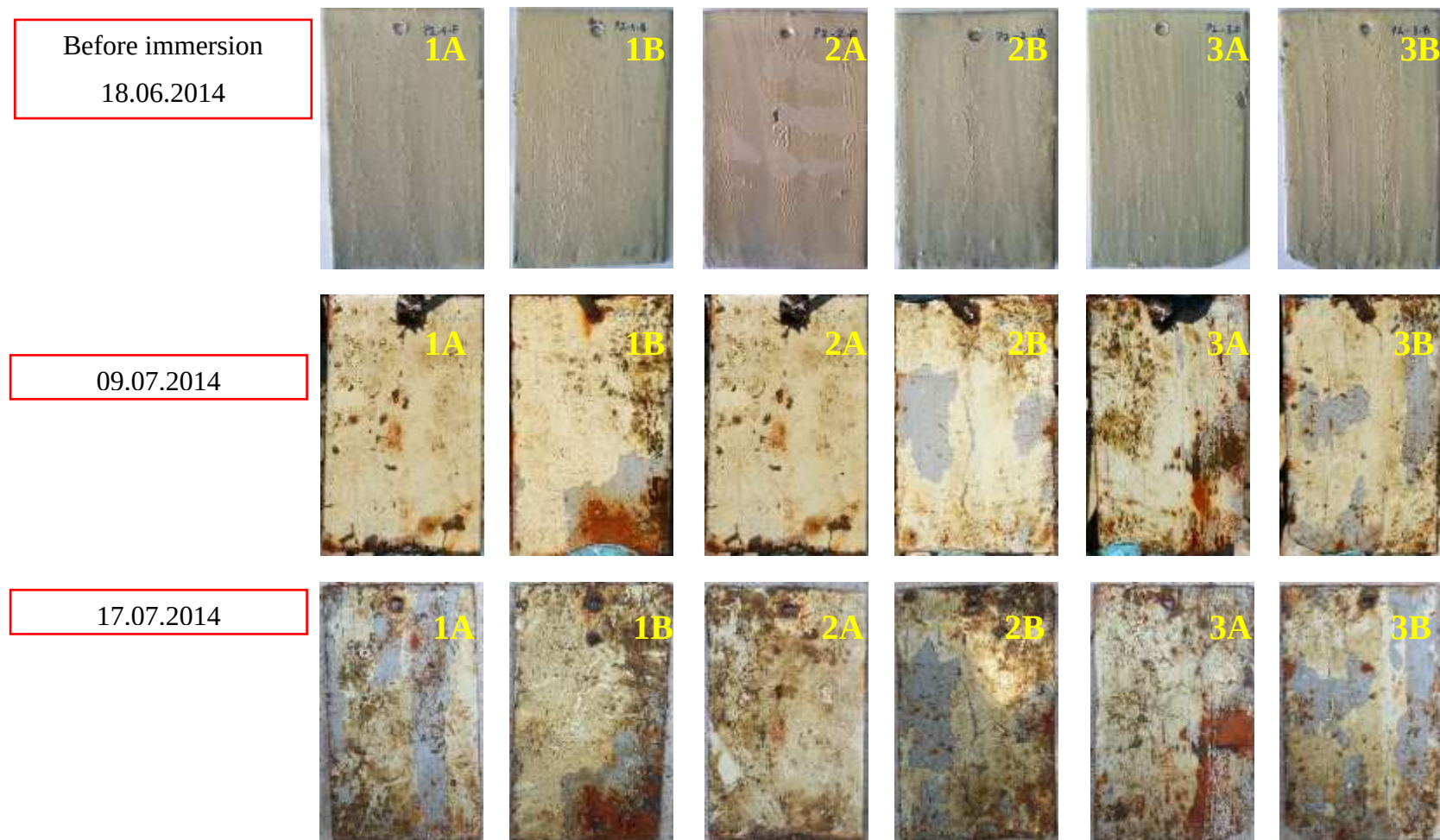


Figure 3.82 The test plates including *Holothuria tubulosa* MeOH-Dichloromethane extract (A: front, B: back, the plates were studied in triplicate).

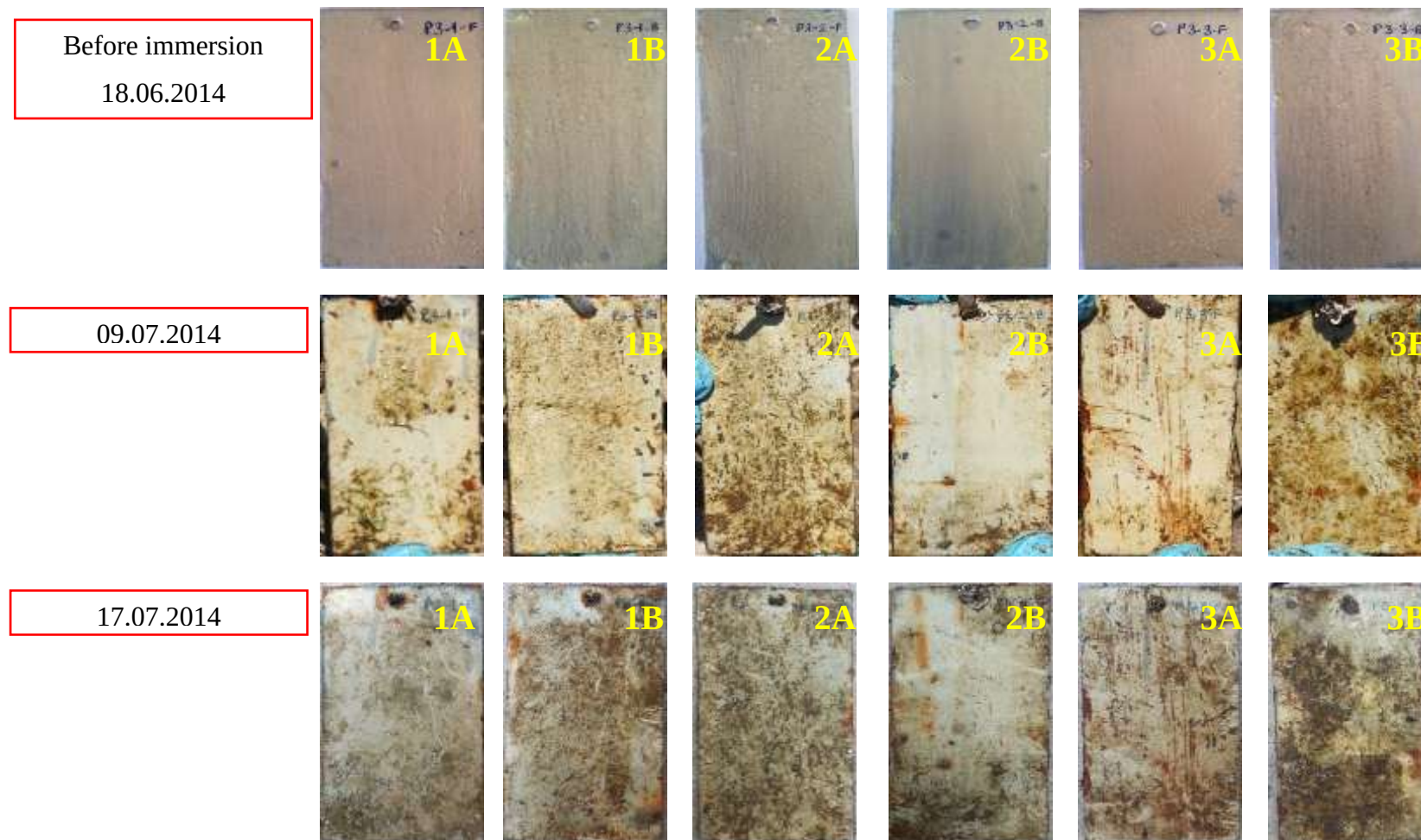


Figure 3.83 The test plates including 70% EtOH Soxhlet extract of *H. polii* (A: front, B: back, the plates were studied in triplicate).

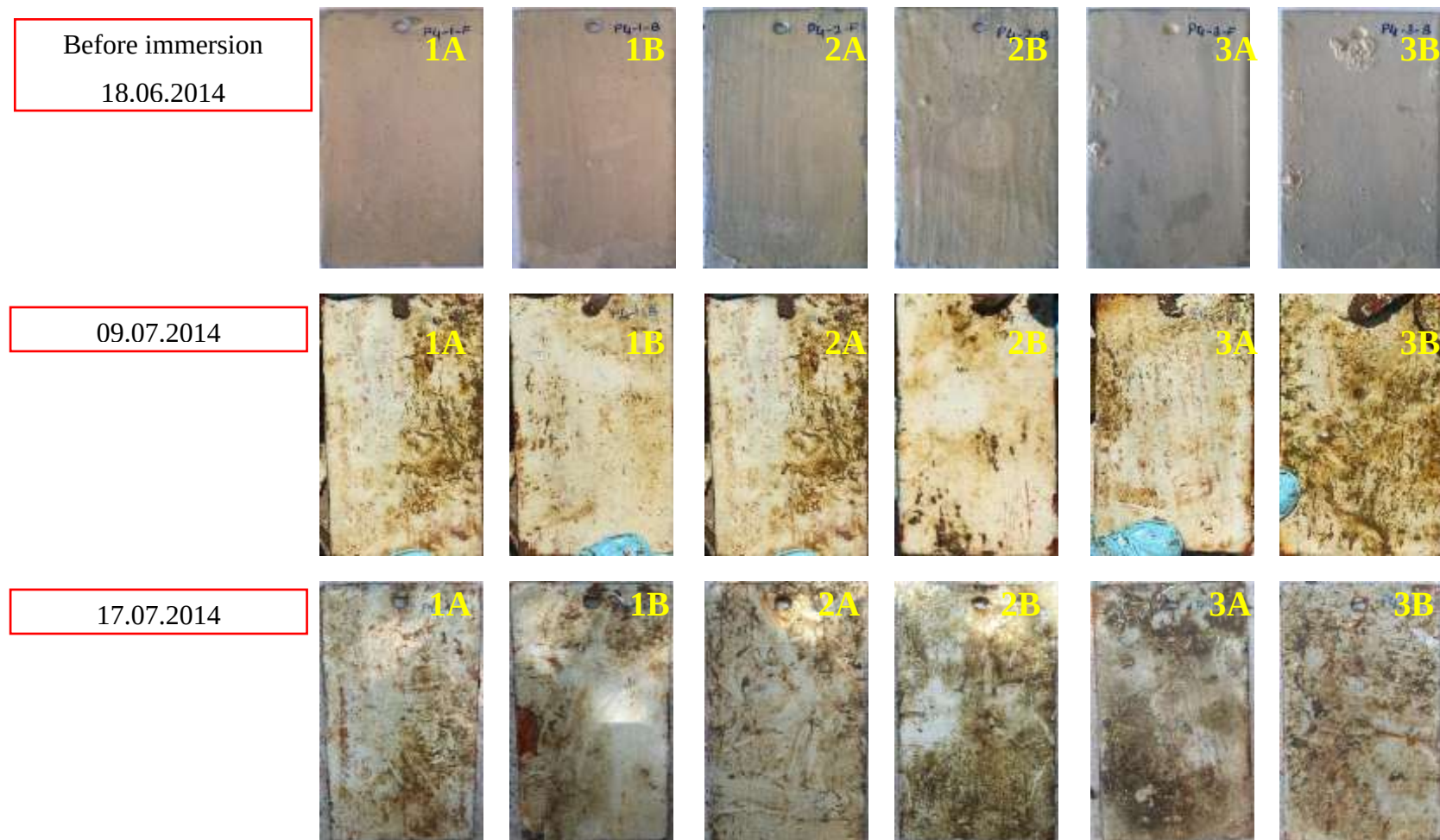


Figure 3.84 The test plates including 70% EtOH macerated extract of *H. tubulosa* (A: front, B: back, the plates were studied in triplicate).

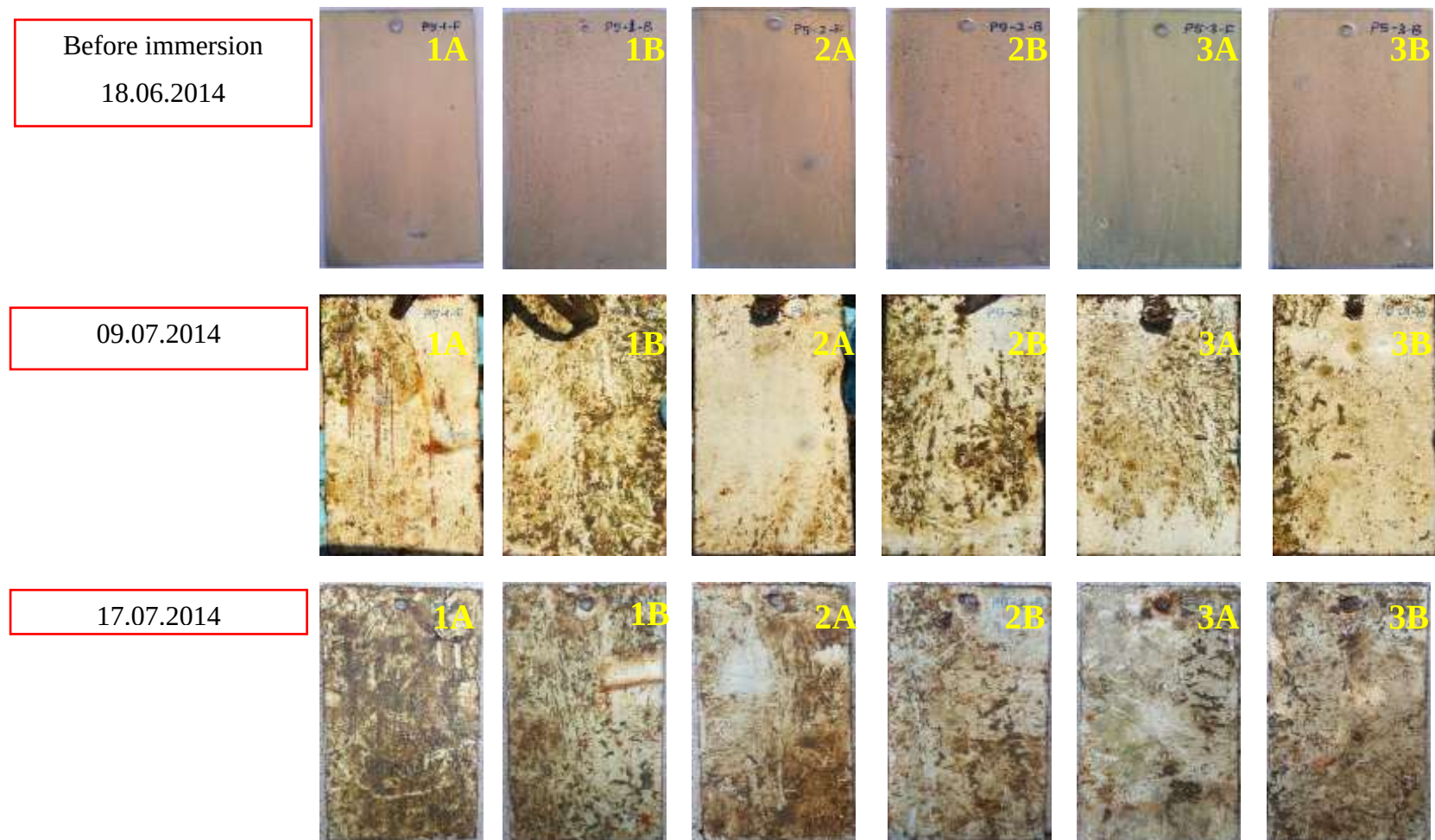


Figure 3.85 The test plates including 70% EtOH macerated extract of *H. tubulosa* with *C. cylindracea* (A: front, B: back, the plates were studied in triplicate).

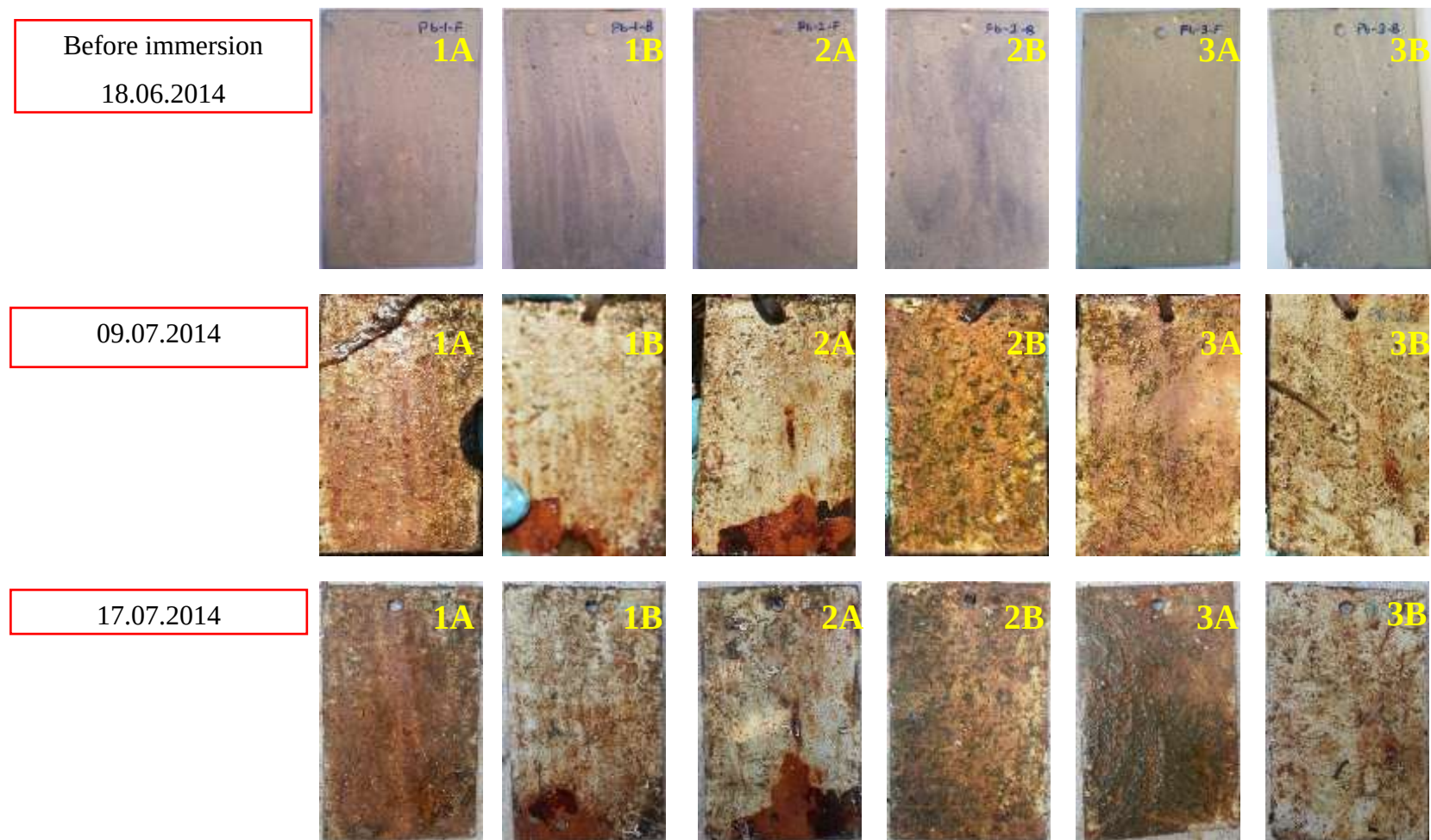


Figure 3.86 The test plates including *Holothuria polii* powder(A: front, B: back, the plates were studied in triplicate).

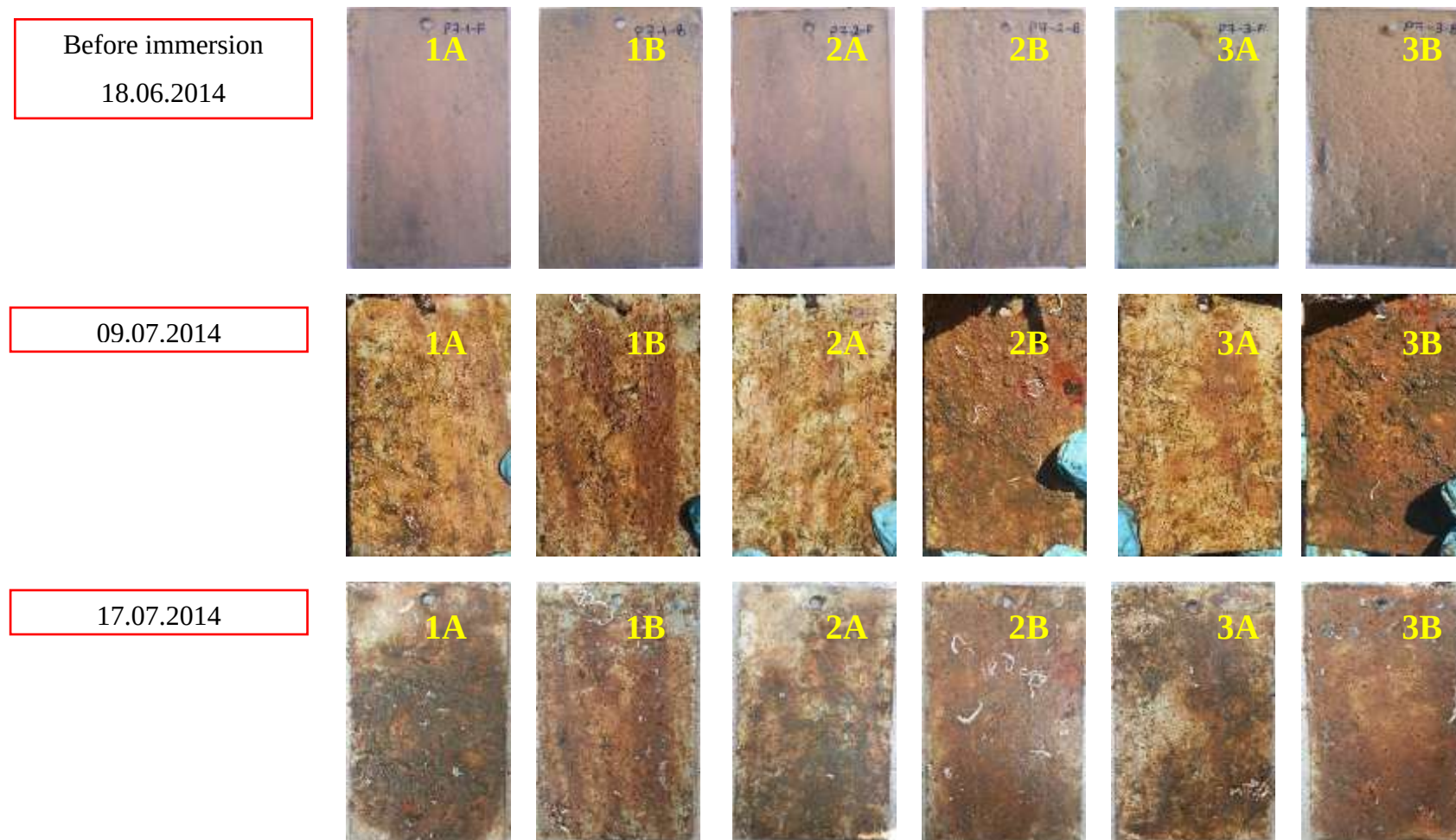


Figure 3.87 The test plates including powder of *Holothuria polii* waste (A: front, B: back, the plates were studied in triplicate).

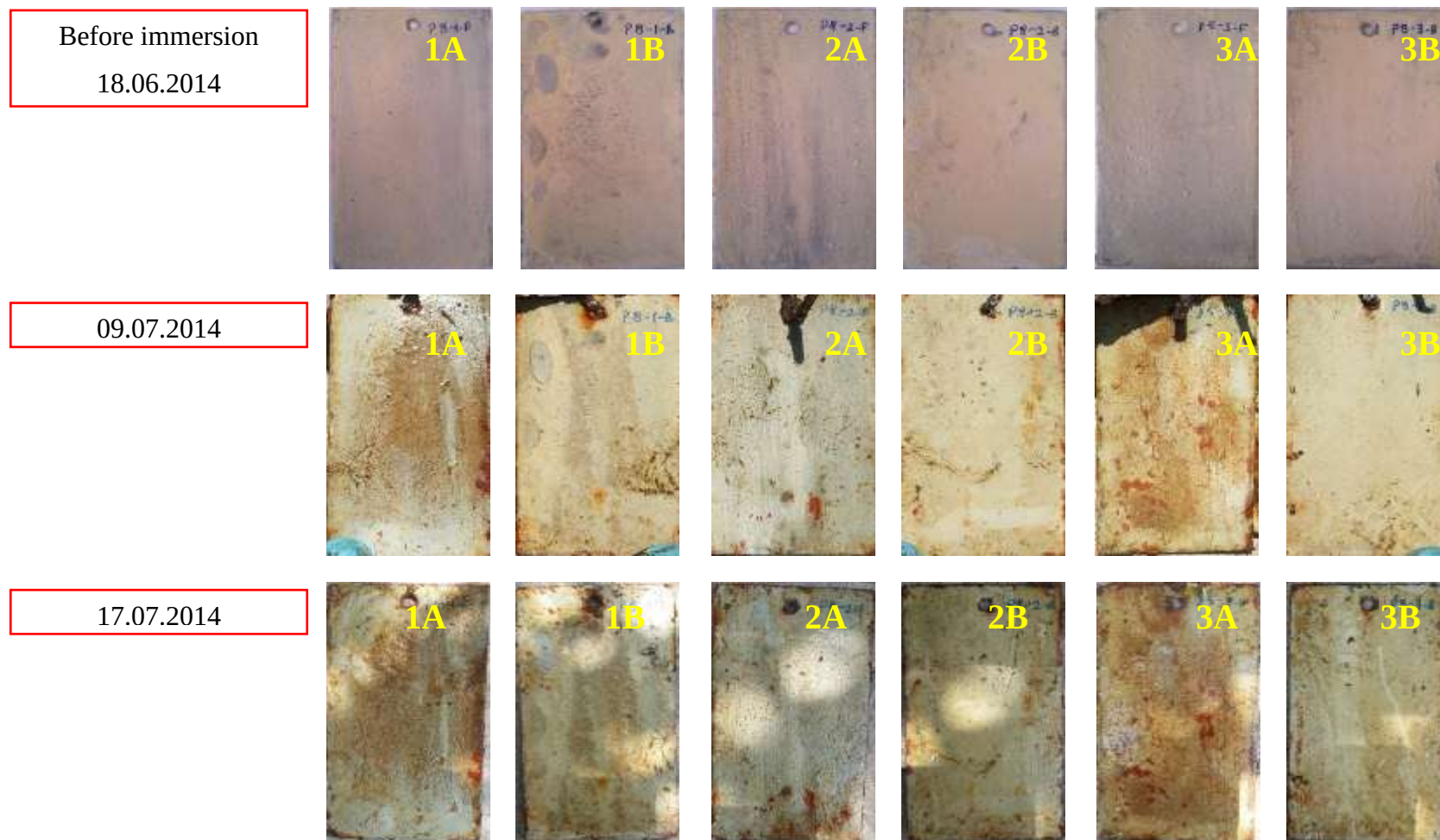


Figure 3.88 The test plates including zinc pyrron (A: front, B: back, the plates were studied in triplicate).

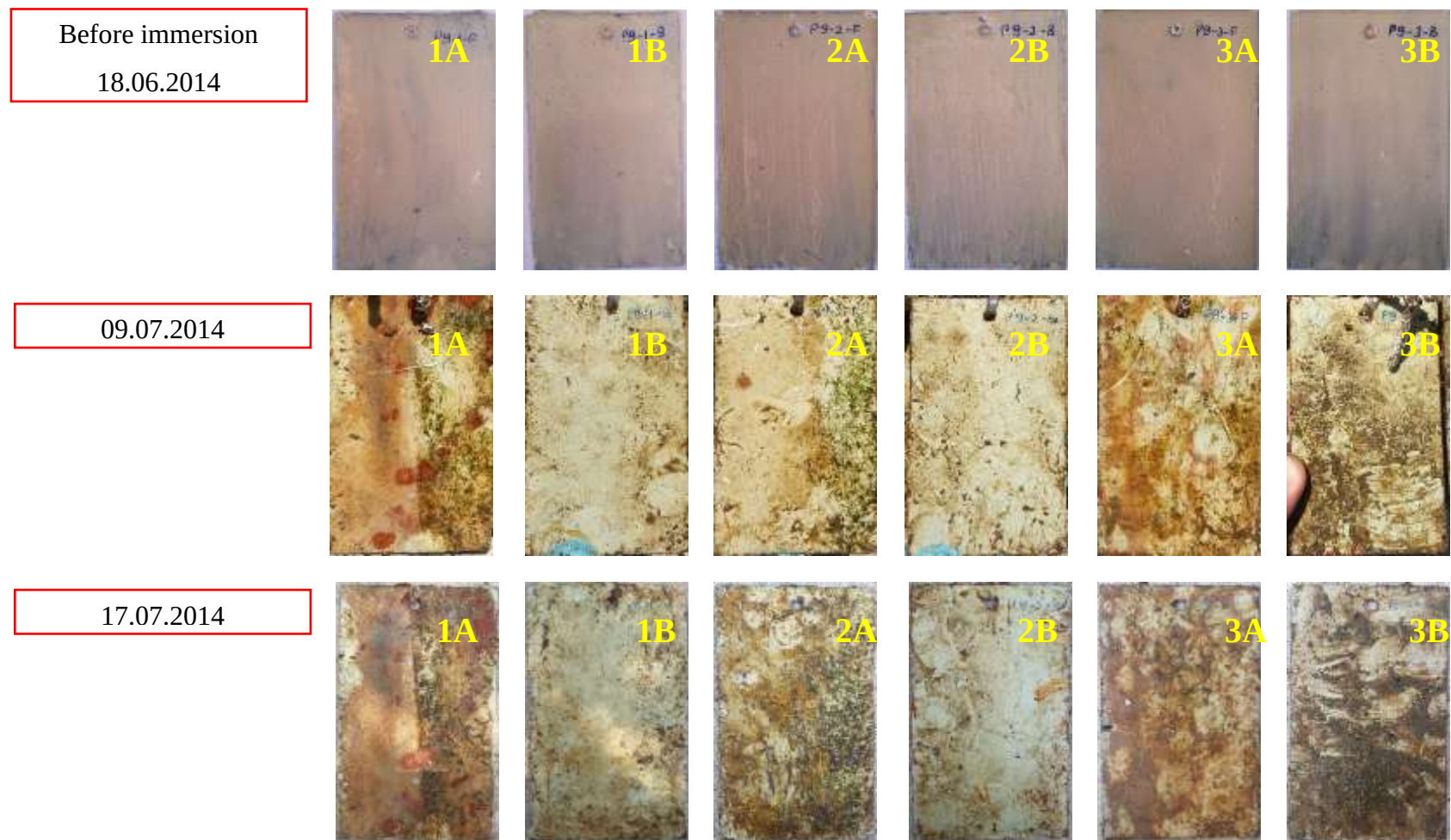


Figure 3.89 Biocide free paint applied test plates(A: front, B: back, the plates were studied in triplicate).

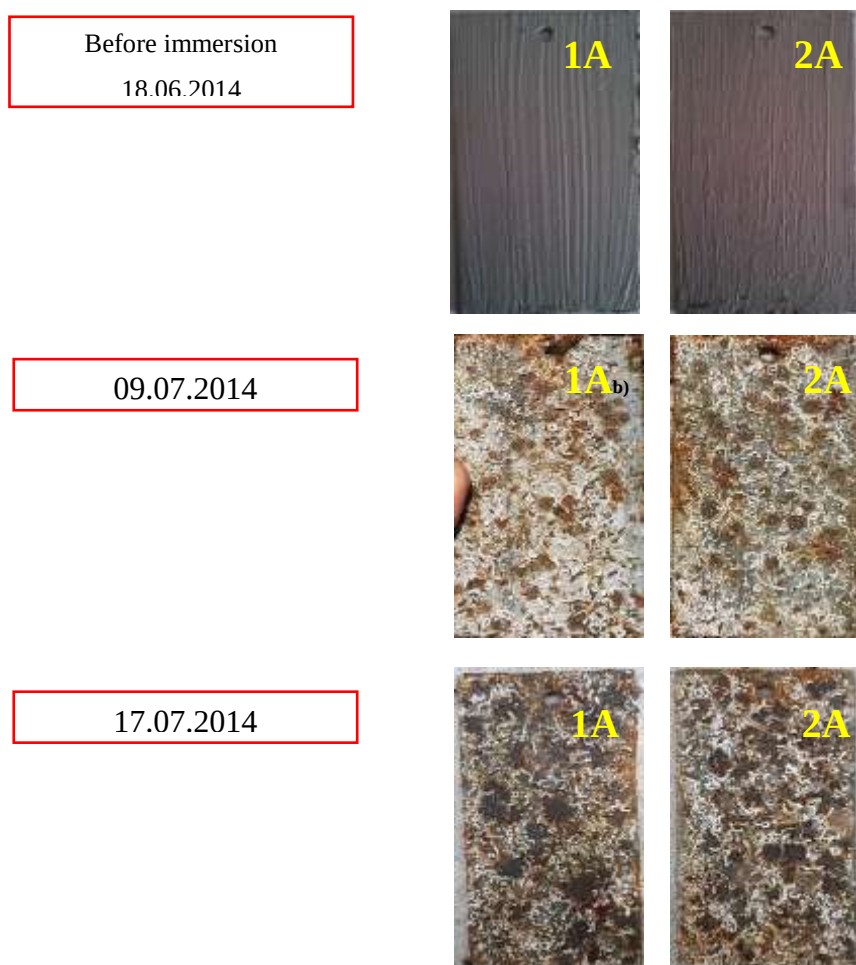


Figure 3.90 Anticorrosive paint applied test plates (A: front, the plates were studied in duplicate).

Table 3.7 The assessment of fouling grades on the surface of antifouling paint test panels

Plate Name	Field Test (30Days)	
	Side 1	Side 2
Paint Free Panel	V	V
MeOH-dichloromethane extract of <i>H. polii</i>	II	II
MeOH-Dichloromethane extract of <i>H. tubulosa</i>	II	II
Soxhlet extract with 70% EtOH of <i>H. polii</i>	III	III
Maceration with 70% EtOH of <i>Holothuria tubulosa</i>	III	III
Maceration with 70% EtOH of <i>Holothuria tubulosa</i> with <i>C. cylindracea</i>	IV	IV
<i>Holothuria polii</i> powder	III	III
<i>Holothuria polii</i> waste powder	IV	IV
Zinc pyrion	I	I
Biocide free	IV	IV
Anticorrosive	V	V

The non-homogenously micro-slime layer was observed on all of the plates. There were micro and macro organisms on the positive control. Although the best antifouling performance was observed for zinc pyrion, the extracts that were prepared according to Acevedo et al. (2013) had also good antifouling property.

3.14 *In-silico* Analysis

In order to visualize the 3D version of cyanobacterial photosystem II, YASARA was used. The molecules were visualized on ribbon model and some molecules which contain extra units deleted to obtain rigid form. In Figure 3.91 3D version of 3BZ1 was given.

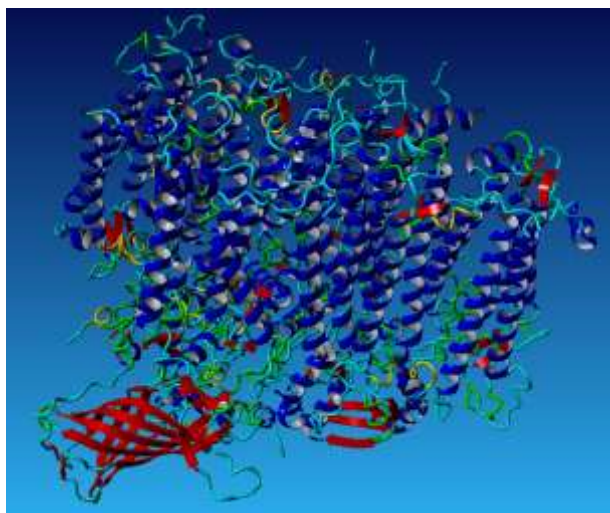


Figure 3.91 Yasara view of the 3BZ1.

The molecular docking results revealed that the scores of holothurinoside A higher than the positive control, diuron, DCA, DCPMU, DCPU and irgarol for cyanobacterial photosystem II (3BZ1). The highest docking score was -6.65 ± 0.75 kcal mol⁻¹ (holothurinoside A) and the lowest docking score was -5.30 ± 0.36 kcal mol⁻¹ (DCA) for 3BZ1. There is no affinity difference between holothurin A and holothurinoside A. The related results were given in Table 3.8.

Table 3.8 Molecular docking results obtained with AutoDock Vina 1.1.2 for diuron, DCA, DCPMU, DCPU, Irgarol, holothurin A and holothurinoside A on photosystem II crystal structures of cyanobacteria (PDB ID: 3BZ1).

Molecules	PDB ID: 3BZ1
	Vina score (kcal mol ⁻¹)
<i>Diuron</i>	-5.81 ± 0.30
<i>DCA</i>	-5.30 ± 0.36
<i>DCPMU</i>	-5.73 ± 0.30
<i>DCPU</i>	-5.93 ± 0.23
<i>Irgarol</i>	-6.34 ± 0.33
<i>Holothurin A</i>	-6.63 ± 0.84
<i>Holothurinoside A</i>	-6.65 ± 0.75

After docking studies, the conformations of the molecules were determined by AutoDock Tools. Both holothurin A and holothurinoside A (Pink molecules in

Figures 3.92 – 3.93) show high interaction with transmembrane proteins that are found in the position of 192-218 and 269-289 (green ball-stick formation in Figures 3.92 – 3.93) of the PSQ chain.



Figure 3.92 Docked holothurin A to cyanobacterial PSQ

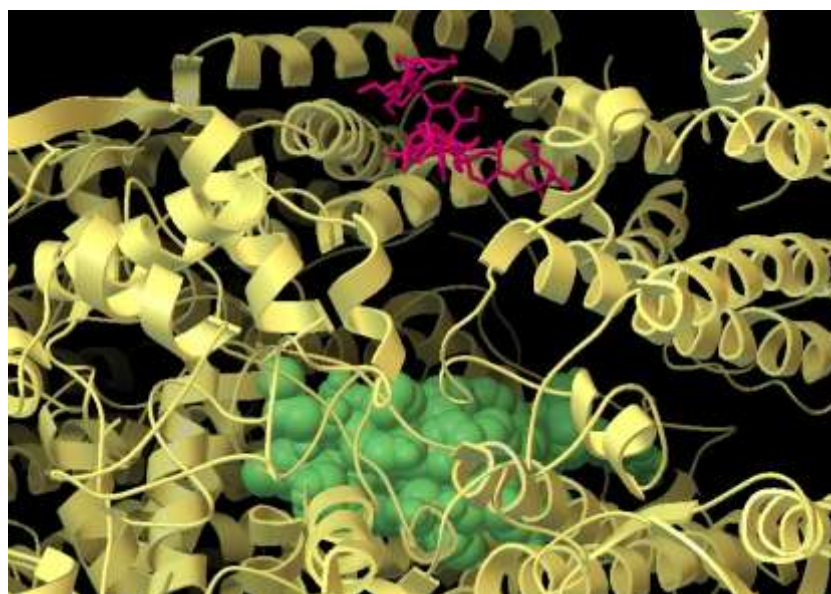


Figure 3.93 Docked holothurinoside A to cyanobacterial PSQ

CHAPTER FOUR

DISCUSSION AND CONCLUSION

In this study, sea cucumbers collected from İzmir coastline were used as a natural bio-active agent source.

In the literature about color reactions of saponins and sapogenins, Hiai, Oura and Nakajima (1976) reported that -OH group in C-3 position of the aglycone portion of triterpene glycoside reacts with vanillin and is converted to a chromogen in an acidic environment. Therefore, all of the saponins show different absorbance. But in the literature, most of the saponin determination studies were carried out at 540-544 nm. According to the results of this study, there was no maximum absorbance of triterpene saponins at 540-544 nm. In order to be well in line with the literature, all of the measurements related to determination of saponin content in holothurians were studied at 540 nm in this thesis. The saponins in *H. polii* and *H. tubulosa* qualitatively and semi-quantitatively were determined by means TLC and spectrophotometry .

Since holothurians are used as a nutrimental source, the carbohydrate, protein, lipid and vitamin contents of these animals are important. In this thesis, the level of protein was determined as 3.704 ± 1.147 mg/g wet weight and 5.126 ± 0.116 mg/g wet weight in *H. polii* and *H. tubulosa*, respectively. The difference between two species could be caused from the chemical composition of their body wall. In the study of Aydın et al. (2011), the protein level in sea cucumber body wall was measured as $8.66 \pm 1.20\%$ and $8.82 \pm 0.30\%$ in *H. polii* and *H. tubulosa*, respectively. On the other hand, the study of Sicuro et al. (2012), the crude protein percentages were 44.58 and 36.99 in *H. polii* and *H. tubulosa*, respectively. Since there had been not enough studies, the current papers in the literature were compared with the protein levels in this study. However, their measurement methods were different compared to this thesis. Bradford method in this thesis was selected because of its sensitivity compared to other methods.

Triterpene saponins which were obtained by water extraction of holothurians lead to 92 µg/mL foam formation and 94 µg/mL float formation.

Lipid contents of *H. polii* and *H. tubulosa* in the present study were found as 8.30 ± 0.20 % and 3.43 ± 0.10 %, respectively. When those values are compared with Sicuro et al. (2011) and Aydın et al. (2010), it could be said that our levels were higher than those of them. Sicuro et al. (2011) found the lipid contents of *H. tubulosa* and *H. polii* as 0.71 ± 0.12 % and 0.55 ± 0.12 %, respectively. Aydın et al. (2010) reported the *H. polii* and *H. tubulosa* samples as 0.15 ± 0.04 % and 0.18 ± 0.05 %, respectively. In the concept of this thesis, the fatty acid composition of sea cucumbers are also measured. The highest concentration of fatty acid in *H. polii* and *H. tubulosa* were found as DHA (17.71 %) and stearic acid (22.00 %), respectively. Since DHA and stearic acid are essential fatty acids, sea cucumbers along Turkish coastlines might be evaluated as food sources. Aydın et al. (2010) reported that the abundant fatty acid is C20:4n6 (20.36 ± 0.35 % and 11.20 ± 0.36 % for *H. tubulosa* and *H. polii*, respectively). Also, Sicuro et al. (2012) reported that the abundant fatty acid was C20:3 n:3 and it was found for *H. tubulosa* as 20.55 ± 4.52 % and for *H. polii* as 18.09 ± 1.76 %. The reason of these differences could be explained with seasonal and also regional changes (Chang-Lee et al., 1989). Carbohydrates were also studied in this thesis. The maximum sugar concentrations (D-fructose) in *H. polii* and *H. tubulosa* were found as 0.85 ± 0.72 mg/g wet weight and 0.90 ± 0.45 mg/g wet weight, respectively. On the other hand, minimum sugar concentrations (D-galactose) in *H. polii* and *H. tubulosa* were 0.41 ± 0.61 mg/g wet weight and 0.47 ± 0.43 mg/g wet weight, respectively.

The ash levels in *H. polii* and *H. tubulosa* were found as 11.09 ± 2.86 % and 10.48 ± 1.87 %, respectively. Also, in this study, moisture levels were found as 83.20 ± 1.59 % and 80.64 ± 1.33 % for *H. tubulosa* and *H. polii*, respectively. According to the literature, (Sicuro et al., 2011), the ash content of *H. tubulosa* and *H. polii* was found as 46.43 ± 0.51 % and 48.22 ± 1.09 %, respectively. Aydın et al. (2011) found the ash content as 5.15 ± 0.6 % and 7.85 ± 0.9 % for *H. tubulosa* and *H. polii*, respectively.

According to the antifouling results, all of the plates were non-homogenously covered by micro-slime layer. The metal plates without any coating were covered by micro and macro organisms. In addition, corrosion was observed in these plates. The best antifouling performance was observed in the plates including commercial biocide, zinc pyrion. Although all antifouling paints that contain sea cucumber extract were successful, it was seen that the mechanical strength of the paints are not high. In order to prevent this, the ratio of the fiber could be highered. The antifouling performance of extract of genus *Holothuria* was carried out by some researchers in the literature (Selvin and Lipton, 2002, 2004; Acevedo et al., 2013). The research of Selvin and Lipton (2002 and 2004) reveals that at a concentration of 4.2 mg/mL methanol extract of *H. scabra* prevents the foot adherence of *Patella vulgate*. In an another paper, it was reported that dichloromethane/methanol extract of *H. glaberrima* had very effective against fouling in Santa Marta (Colombia) and Mar del Plata (Argentina) (Acevedo et al., 2013). However, they did not consider the saponin contents in their extracts. This present study reveals that saponins might be related with the fouling protection. We found a direct correlation between total saponin level and AF performance of extracts.

In-silico analysis showed that triterpene glycosides from sea cucumbers might have affected the PSQ. In the literature, there is no published paper related to *in-silico* molecular modeling of biosides on PSQ. Since no algae on the surface of the plates was observed, the reason of absence of marine macro foulers (algae) could be associated with the inhibition of the PSQ by sea cucumber triterpene glycosides.

In conclusion Turkey is surrounded by three different sea ecosystems; Black sea, Aegean Sea and Mediterranean Sea. These ecosystems contain very important marine organisms with different features. They could be evaluated in blue biotechnology (Mert & Cavas, 2013). Marine biotechnology currently is a hot topic and European Union supported project calls are announced under Horizon 2020. Sea cucumbers could be investigated for their bioactive agents dependent on seasonal, regional changes etc. In this thesis, limited bioactive agents from limited seasons and regions were investigated. More researches are strongly recommended to explore

novel bioactive agents. I never recommend collection of these species for just exporting their dried forms. I recommend to explore their high value bioactive components. In collection studies, their population size must be controlled. Therefore, collection without official permission of Republic of Turkey Ministry of Food, Agriculture and Livestock must be prevented to protect the population of sea cucumbers along Turkish coastlines.

REFERENCES

- Abarzua, S., & Jakubowski, S. (1995). Biotechnological investigation for the prevention of biofouling. I. Biological and biochemical principles for the prevention of biofouling. *Marine Ecology Progress Series*, 123, 301-312.
- Acevedo, M. S., Puentes, C., Carreño, K., León, J. G., Stupak, M., García, M. et al. (2013). Antifouling paints based on marine natural products from Colombian Caribbean. *International Biodeterioration & Biodegradation*, 83, 97-104.
- Almeida, E., Diamantino, T. C., & Sousa, O. (2007). Marine paints: the particular case of antifouling paints. *Progress in Organic Coatings* 59, 2-20.
- Alyuruk, H. & Cavas, L. (2013). Toxicities of diuron and irgarol on the hatchability and early stage development of *Artemia salina*. *Turkish Journal of Biology*, 37, 151-157.
- Aminin, D. L., Chaykina, E. L., Agafonova, I. G., Avilov, S. A., Kalinin, V. I. & Stonik, V. A. (2010). Antitumor activity of the immunomodulatory lead cumaside. *International Immunopharmacology*, 10, 648-654.
- Aminin, D. L., Gorpenchenko, T. Y., Bulgakov, V. P., Andryjashchenko, P. V., Avilov, S. A., & Kalinin, V. I. (2011). Triterpene glycoside Cucumarioside A₂-2 from sea cucumber stimulates mouse immune cell adhesion, spreading and motility. *Journal of Medicinal Food*, 14 (6), 594-600.
- Anisimov, M. M. (1987). Triterpene glycosides and structure-functional properties of membranes. *Biologicheskie Nauki*, 49-63.
- Arimura, H., Hiyoshi, S., Nakamura, N., & Tsuboi, M. (2004). (Poly)oxyalkylene block silyl ester copolymer, antifouling coating composition, antifouling coating formed from antifouling coating composition, antifouling method using

antifouling coating composition and hull or underwater structure covered with antifouling coating. US Patent (6828030).

Attaub, S., Arafat, K., Géaude, A., Al Sultan, M. A., Bracke, M., Collin, P. et al. (2013). Frondoside A suppressive effects on lung cancer survival, tumor growth, angiogenesis, invasion, and metastasis. *Plos One*, 8 (1), e53087.

Aydın, M. (2008). The commercial sea cucumber fishery in Turkey. *SPC Beche de Mer Information Bulletin*, 28, 40-41.

Aydın, M., Sevgili, H., Tufan, B., Emre, Y. & Köse, S. (2011). Proximate composition and fatty acid profile of three different fresh and dried commercial sea cucumbers from Turkey. *International Journal of Food Science and Technology*, 46, 500–508.

Bakus, G.J. (1968). Defensive mechanisms and ecology of some tropical holothurians. *Marine Biology*, 2, 23–32.

Bay-Nouailhat A. (September 2006). *Description of Holothuria tubulosa*. Retrieved August 31, 2014, from <http://www.european-marine-life.org/30/holothuria-tubulosa.php>.

Bakus, G.J. (1974). Toxicity in holothurians: A geographical pattern. *Biotropica*, 6, 229–236.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., et al. (2000). The protein data bank. *Nucleic Acid Research*, 28, 235-242.

Bondoc, K .G. V., Lee, H., Cruz, L. J., Lebrilla, C. B., & Juinio-Meñez, A. (2013). Chemical fingerprinting and phylogenetic mapping of saponin congeners from three tropical holothurian sea cucumbers. *Comparative Biochemistry and Physiology, Part B*, 166 (3-4), 182-193.

- Bondoc, K .G. V., Lee, H., Cruz, L. J., Lebrilla, C. B., & Juinio-Meñez, A. (2014). Corrigendum to “Chemical fingerprinting and phylogenetic mapping of saponin congeners from three tropical holothurian sea cucumbers” [Comp. Biochem. Physiol. B 166 (3–4) (2013) 182–193]. *Comparative Biochemistry and Physiology, Part B*, 166 (3-4), 182-193.
- Bordbar, S., Anwar, F., & Saari, N. (2011). High-value components and bioactives from sea cucumbers for functional foods-A review. *Marine Drugs*, 9, 1761-1805.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–54.
- Callow, M. E., & Fletcher, R. L. (1994). The influence of low surface energy materials on bioadhesion: a review. *International Biodeterioration & Biodegradation* 34, 333-348.
- Careaga, V. P., Bueno, C., Muniain, C., Alché, L., & Maier, M. S. (2009). Antiproliferative, cytotoxic and hemolytic activities of a triterpene glycoside from *Psolus patagonicus* and its desulfated analog. *Chemotherapy*, 55, 60-68.
- Cengiz, S. (2007). *Immobilization of catalase onto clays*. MSc Thesis, Dokuz Eylül University, İzmir.
- Chang-Lee, M. V., Price, R. J., & Lampila, L. E. (1989). Effect of processing on proximate composition and mineral content of sea cucumbers (*Parastichopus* spp.). *Journal of Food Science*, 54, 567–572.
- Chludil, H. D., Murray, A. P., Seldes, A. M., & Maier, M. S. (2003). Biologically active triterpene glycosides from sea cucumbers (Holothuroidea, Echinodermata). *Studies in Natural Products Chemistry*, 28, 587-615.

- Clare, A. S., Rittschof, D., Gerhart, D. J., & Maki, J. S. (1992). Molecular approaches to nontoxic antifouling. *Invertebrate Reproduction and Development* 22, 67-76.
- Clare, A. S. (1996). Marine natural product antifoulants: status and potential. *Biofouling*, 9, 211-229.
- Despalatović, M., Grubelić, I., Šimunović, A., Antolić, B., & Žuljević, A. (2004). Reproductive biology of the holothurian *Holothuria tubulosa* (Echinodermata) in the Adriatic Sea. *Journal of the Marine Biological Association of the UK*, 84, 409-414.
- Drugstore (n.d). *Omojo Acne & Tissue Support-Sea Cucumber_Serum*. Retrieved August 26, 2014, from <http://www.drugstore.com/omojo-acne-and-tissue-support-sea-cucumber-serum/qxp416703>
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350-356.
- Esmat, A. Y., Said, M. M., Soliman, A. A., El-Masry, K. S. H. & Badiea, E. A. (2013). Bioactive compounds, antioxidant potential and hepatoprotective activity of sea cucumber (*Holothuria atra*) against thioacetamide intoxication in rats. *Nutrition*, 29, 258-267.
- European Marine-Life*, (n.d). Retrieved August 30, 2014, from <http://www.european-marine-life.org/30/photo-holothuria-tubulosa-b57-19.php>
- Flemming, H. C., Griebel, T., & Schaule, G. (1996). Antifouling strategies in technical systems - a short review. *Water Science and Technology*, 34, 517-524.

- Francour, P. (1997). Predation on holothurians: A literature review. *Invertebrate Biology*, 116, 52–60.
- Freundlich, H. (1906) Over the adsorption in the solution. *The Journal of Physical Chemistry*, 57, 385–470
- González-Wengüemert, M., Aydin, M., & Conand, C. (2014). Assesment of sea cucumber populations from the Aegean Sea (Turkey): First insights to sustainable management of new fisheries. *Ocean & Coastal Management*, 92, 87-94.
- Gundogdu-Hizliates C., Alyuruk H., Gocmenturk M., Ergun M.Y., & Cavas L. (2014). Synthesis of new ibuprofen derivatives with their *in silico* and *in vitro* cyclooxygenase-2 inhibitions. *Bioorganic Chemistry*, 52, 8-15.
- Guskov, A., Kern, J., Gabdulkhakov, A., Broser, M., Zouni, A., & Saenger, W. (2009). Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. *Nature Structural & Molecular Biology*, 16 (3), 334-42.
- Han, H., Yi, Y. H., Li, L., Liu, B. S., Pan, M. X., Yan, B., & Wang, X. H. (2009). Triterpene glycosides from sea cucumber *Holothuria leucospilota*. *Chinese Journal of Natural Medicines*, 7 (5), 0346-0350.
- Hellio, C., Maréchal, J., Da Gama, B., Pereira, R., & Clare, A. (2009). Natural marine products with antifouling activities. In: Hellio, C., Yebra, D. (Eds.), *Advances in marine antifouling coatings and technologies*. Woodhead Publishing Ltd & CRC Press LLC, Cambridge, UK, 572-622.
- Herencia, F., Ubeda, a., Ferrándiz, M. L., Terencio, M. C., Alcaraz, M. J., García-Carrascosa, M., et al. (1998). Anti-inflammatory activiy in mice of extracts from Mediterranean marine invertebtares. *Pharmacology Letters*, 62 (9), 115-120.

- Hiai, S., Oura, H., & Nakajimi, T. (1976). Color reaction of some sapogenins and saponins with vanillin and sulfuric acid. *Planta Medica*, 29, 116-122.
- Hickman, C. P., Roberts, L. S., Keen, S. L., Larson, A., I'Anson, H., & Eisenhour, D. J. (2008). *Integrated principles of zoology* (14th ed.) (469-496). New York: McGraw-Hill International Edition.
- Ho, Y. S., & McKay, G. (2003). Sorption of dyes and copper ions onto biosorbents. *Process Biochemistry*, 38, 1047-1061.
- Ibrahim, H. A. H. (2012). Antibacterial carotenoids of three *Holothuria* species in Hurghada, Egypt. *Egyptian Journal of Aquatic Research*, 38, 185-194.
- IUCN Red List (n.d) *Holothuria polii*. Retrieved August 31, 2014, from <http://www.iucnredlist.org/details/180295/0>.
- Iyapparaj, P., Revathi, P., Ramasubburayan, R., Prakash, S., Palavesam, A., Immanuel, G., Anantharaman, P., Sutreau, A. & Hellio, C. (2014). Antifouling and toxic properties of the bioactive metabolites from the seagrasses *Syringodium isoetifolium* and *Cymodocea serrulata*. *Ecotoxicology and Environmental Safety* 103, 54–60.
- Jones, R. (2005). The ecotoxicological effects of Photosystem II herbicides on corals. *Marine Pollution Bulletin*, 51, 495-506.
- Kalinin, V. I. (2000). System-theoretical (Holistic) approach to the modelling of structural-functional relationships of biomolecules and their evolution: an example of triterpene glycosides from sea cucumbers (Echinodermata, Holothurioidea). *Journal of Theoretical Biology*, 206, 151-168.
- Kalinin, V. I., Aminin, D. L., Avilov, S. A., Silchenko, A. S., & Stonik, V. A. (2008). Triterpene glycosides from sea cucumbers (Holothurioidae,

- Echinodermata), biological activities and functions. (In: Atta-ur-Rahman, ed.). *Studies in Natural Product Chemistry*, 35, 135-196.
- Kalinin, V. I., Levin, V. S., & Stonik, V. A. (1994). *The chemical morphology: Triterpene glycosides of sea cucumbers (Holothurioidea, Echinodermata)*. Vladivostok: Dalnauka.
- Kalinin, V. I., Volkova, O. V., Likhatskaya, G. N., Prokofieva, N. G., Agafonova, I. G., Anisimov, M. M., et al. (1992). Hemolytic activity of triterpene glycosides from Cucumariidae family holothurians and evolution of this group of toxins. *Journal of Natural Toxins*, 1, 17-30.
- Kamenev, Y. O., Dolmatov, I. Y., Frolova, L. T., & Khang, N. A. (2013). The morphology of the digestive tract and respiratory organs of the holothurian *Cladolabes schmeltzii* (Holothuroidea, Dendrochirotida). *Tissue and Cell*, 45, 126-139.
- Kar, S., & Roy, K. (2010). Predictive toxicology using QSAR: A perspective. *Journal of the Indian Chemical Society*, 87, 1455–1515.
- Kar, S., & Roy, K. (2012). Risk assessment for ecotoxicity of pharmaceuticals-an emerging issue. *Expert Opinion on Drug Safety*, 11, 235–274.
- Kar, S., & Roy, K. (2013). First report on predictive chemometric modeling, 3D-toxicophore mapping and *in silico* screening of *in vitro* basal cytotoxicity of diverse organic chemicals. *Toxicology in vitro*, 27, 597–608.
- Kazanidis, G., Antoniadou, C., Lolas, A. P., Neofitou, N., Vafidis, D., Chintiroglou, C., et al. (2010). Population dynamics and reproduction of *Holothuria tubulosa* (Holothuroidea: Echinodermata) in Aegean Sea. *Journal of Marine Biological Association of the United Kingdom*, 90 (5), 895-901..

- Kerr, R. G., & Chen, Z. (1995). In vivo and in vitro biosynthesis of saponins in sea cucumbers. *Journal of Natural Products*, 58 (2), 172-176.
- Kitagawa, I. (1988). Research of biologically active marine natural products. *Yakugaku Zasshi*, 108, 398-416.
- Krieger, E. (2010). *Yasara*. Retrieved November 24, 2014 from <http://www.yasara.org>
- Lagergren, S. (1898). Zur theorie der sogenannten adsorption gelöster stoffe, *Kungliga Svenska Vetenskapsakademiens. Handlingar*, 24, 1-39.
- Lam, K. H., Cai, Z., Wai, H. Y., Tsang, V. W. H., Lam, M. H. W., Cheung, R. Y. H., Yu, H. X. & Lam, P. K. S. (2005). Identification of a new Irgarol-1051 related s-triazine species in coastal waters. *Environmental Pollution*, 136, 221-230.
- Langmuir, I. (1918). The adsorption of gases on plane surfaes of glass, mica and platinum. *Journal of the American Chemical Society*, 40,1361–1403
- Lykhatskaya, G. N., Yarovaja, T. P, Rudnev, V. S, Popov, A. M., Anisimov, M. M., & Rovin, Y. G. (1985). Formation of a complex of triterpene glycoside holothurine A with cholesterol in model membranes. *Biophysika*, 30, 358–359.
- Lykhatskaya, G. N., Popov, A. M., Agafonova, I. G., Oleinikova, G. K., Kalinin, V. I., Kuznetsova, T. A, et al. (1991). Influence of holothurins and their genins on a proliferation of cells and the properties of models and biological membranes. In: *All-Union Conference Biological Active Substances of Hydrobionts New Drug, Medical and Technical Preparations*. Abstracts, 98. Vladivostok.
- Maier, M. S. (2008). Biological activities of sulfated glycosides from echinoderms. *Studies in Natural Products Chemistry*, 35, 311-354.

- Marczewska, A. D., & Marczewski, A. W. (2002). Effect of adsorbate structure on adsorption from solutions. *Applied Surface Science*, 196, 264-272
- Matsuno, T., & Ishida, T. (1969). Distribution and seasonal variation of toxic principles of sea cucumber (*Holothuria leucospilota* Brandt). *Experientia*, 25, 1261.
- Matsuno, T., & Tsushima, M. (1995). Comparative biochemical studies of carotenoids in sea cucumbers. *Comparative Biochemistry and Physiology*, 11, 597-605.
- Mert N., & Cavas L., (2013). *Blue biotechnology: The pharmaceutical potential of algae from Turkish coastline. Sağlıkta Inovasyon: Üniversiteler Sanayiye Çalıştayı*. Dokuz Eylül University, İzmir.
- Mert, N., Topcam, G., & Cavas, L. (2014). RP-HPLC optimization of Econeal by using artificial neural networks and its antifouling performance on the Turkish coastline. *Progress in Organic Coatings*, 77, 627– 635.
- Nigrelli, R. F. (1952). The effects of holothurin on fish and mice with Sarcoma 180. *Zoologica*, 37, 89-90.
- Olesek, W., Kapusta, I., & Stochmal, A. (2007). TLC of Triterpenes(Including Saponins). *Hajos/Thin Layer Chromatography in Phytochemistry*, 46772_C020, 519-542.
- Omran, N. E. E., & Allam, N. G. (2012). Screening of microbial contamination and antimicrobial activity of sea cucumber *Holothuria poii*. *Toxicology and Industrial Health*, 29 (10), 944-954.
- Paul, V., Puglisi, M., & Ritson-Williams, R. (2006). Marine chemical ecology. *Natural Product Report*, 23, 153-180.

- Perez, M., García, M., Sánchez, M., Stupak, M., Mazzuca, M., Palermo, JA., Blustein, G. (2014). Effect of secochiliolide acid isolated from the Patagonian shrub *Nardophyllum bryoides* as active component in antifouling paints. *International Biodeterioration & Biodegradation*, 89, 37-44.
- Rascio, V. J. D. (2000). Antifouling coatings: Where do we go from here. *Corrosion Reviews*, 18, 133-154.
- Rodriguez-Bernaldo de Quirós, A., Lopez-Hernandez, J. & SirnaI-Lozano, J. (2001). Determination of vitamin C in sea urchin: Comparison of two HPLC methods. *Chromatographia Supplement*, 53, 246-249.
- Safi, (n.d). Retrieved August 26, 2014, from <http://safi.com.my/web-en/skin-care/natural/spot-cream.php>
- Schneider, K., Silverman, Woolsey, E., Eriksson, H., Byrne, M., & Caldeira, K. (2011). Potential influence of sea cucumbers on coral reef CaCO₃ budget: a case study at One Tree Reef. *Journal of Geophysical Research*, 116, G04032.
- Sealifebase (n.d). *Holothuria polii*. Retrieved August 31, 2014 from <http://www.sealifebase.ca/summary/Holothuria-poli.html>
- Selvin, J., & Lipton, A.P. (2002). Development of a rapid ‘mollusc foot adherence bioassay’ for detecting potent antifouling bioactive compounds. *Current Science*, 83, 735-737.
- Selvin, J., & Lipton, A.P. (2004). Antifouling activity of bioactive substances extracted from *Holothuria scabra*. *Hydrobiologia*, 513, 251–253.
- Sicuro, B., Piccinno, M., Gai, F., Abete, M. C., Danieli, A., Dapra, F., et al. (2012). Food quality and safety of Mediterranean sea cucumbers *Holothuria tubulosa*

and *Holothuria polii* in Southern Adriatic Sea. *Asian Journal of Animal and Veterinary Advances*, 7, 851-859.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjashchenko, P. V., Dmitrenok, P. S., Kalinin, V. I., et al. (2012). 3 β -O-glycosylated 16 β -acetoxy-9 β -H-lonosta-7,24-diene-3 β ,18,20 β -triol, an intermediate metabolite from the sea cucumber *Eupentacta fraudatrix* and its biosynthetic significance. *Biochemical Systematics and Ecology*, 44, 53-60.

Silchenko, A. S., Stonik, V. A., Avilov, S. A., Kalinin, V. I., Kalinovsky, A. I., Zaharenko, A. M., et al. (2005). Holothurins B₂, B₃ and B₄, new triterpene glycosides from Mediterranean sea cucumbers of the genus *Holothuria*. *Journal of Natural Products*, 68 (4), 564-567.

Sipkema, D., Franssen, M., Osinga, R., Tramper, J., & Wijffels, R. (2005). Marine sponges as pharmacy. *Marine Biotechnology*, 7, 142-162.

Stepnik, K. E., Malinowska, I., & Rój, E. (2014). *In vitro* and *in silico* determination of oral, jejunal and Caco-2 human absorption of fatty acids and polyphenols. Micellar liquid chromatography. *Talanta*, 130, 265-273.

Stonik, V. A., & Elyakov, G. B. (1988). Secondary metabolites from echinoderms as chemotaxonomic markers. In: *Bioorganic marine chemistry* (Scheuer, P. J., ed.), 2, 43-86. Berlin: Springer-Verlag.

Thomas, K. V., Fileman, T. W., Readman, J. W., & Waldock, M. J. (2001). Antifouling paint booster herbicides in the UK coastal environment and the potential risks of biological effects. *Marine Pollution Bulletin*, 42, 677-688.

Topcam, G. (2013). *Seasonal changes of the zosteric acid content in Zostera noltii detritus*. MSc Thesis, Dokuz Eylül University, İzmir.

- Toth, J. (2002). Adsorption isotherms. *Encyclopedia of Surface And Colloid Science*, 212-224.
- Trott, O., & Olson, A.J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *Journal of Computational Chemistry*, 31, 455-461.
- Tsai, W., Lai, C. W., & Hsien, K. J. (2003). Effect of particle size of activated clay on the adsorption of paraquat from aqueous solution. *Journal of Colloid and Interface Science*, 263, 29-24.
- Wahl, M. (1989). Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Marine Ecology Progress Series*, 58, 175-189.
- Wei, S. E. (2011). *Isolation and determination of anti-nutritional compounds from root and shells of peanut (Arachis hypogaea)*. BSc Thesis, Universiti Tunku Abdul Rahman, Selangor, Malesia.
- Wright, T. (2009). *Marine coatings market*. Retrived November 24, 2014, from. http://www.coatingsworld.com/issues/2009-05/view_features/marine-coatings-market-2009-05-07-09-18-13/
- Van Dyck, S., Gerbaux, P., & Flammang., P. (2010). Qualitative and quantitative saponin contents in five sea cucumbers from the Indian Ocean. *Marine Drugs*, 8 (1), 173-189.
- Voulvoulis, N., Scrimshaw, M. D. & Lester, J. N. (2002). Partitioning of selected antifouling biocides in the aquatic environment. *Marine Environmental Research* 53, 1-16.
- Yamanouchi, T. (1955). On the poisonous substance contained in holothurians. *Publications of the Seto Marine Biological Laboratory*, 4 (2-3), 183-203.

- Yebra, D. M., Kiil, S., & Dam-Johansen, K. (2004). Antifouling technology past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50, 75-104.
- Zahuranec, B. J. (1991). *The United States Navy and research on bioactive substances from the sea*, In Thompson MF, 1991.
- Zamora, L. N., & Jeffs, A. G. (2012). Feeding, metabolism and growth in response to temperature in juveniles of Australasian sea cucumber, *Australostichopus mollis*. *Aquaculture*, 358-359, 92-97.
- Zhang, W., Lu, Y., Xu, B., Wu, J., Zhang, L., Gao, M., et al. (2009). Acidic mucopolysaccharide from *Holothuria leucospilota* has antitumor effect by inhibiting angiogenesis and tumor cell invasion in vivo and in vitro. *Cancer Biology & Therapy*, 8 (15), 1489-1499.
- Zhou, D. Y., Chang, X. N., Bao, S. S., Song, L., Zhu, B. W., Dong, X. P., et al. (2014). Purification and partial characterisation of cathepsin L-like proteinase from sea cucumber (*Stichopus japonicus*) and its tissue distribution in body wall. *Food Chemistry*, 158, 192-199.