Studies on biochemical and functional properties of saponins derived from starfish collected in Japan

Doctoral Dissertation

STUDIES ON BIOCHEMICAL AND FUNCTIONAL PROPERTIES OF SAPONINS DERIVED FROM STARFISH COLLECTED IN JAPAN

September 2016

Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

Doctoral Course of Applied Marine Biosciences

SHARMIN MST FARHANA

Doctoral Dissertation

STUDIES ON BIOCHEMICAL AND FUNCTIONAL PROPERTIES OF SAPONINS DERIVED FROM STARFISH COLLECTED IN JAPAN

September 2016

Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

Doctoral Course of Applied Marine Biosciences

SHARMIN MST FARHANA

［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

専　攻 Major	応用生命科学	氏　名 Name	SHARMIN MST FARHANA
論文題目 Title	Studies on biochemical and functional properties of saponins derived from starfish collected in Japan （日本沿岸産ヒトデ類サポニンの生化学的特性および機能に関する研究）		

Starfish are known as by-catch products in the worldwide fishing industry. Large starfish originally inhabited the coast of northeast Asia including Japan, Korea, China, and Russia. Starfish have the ability to consume a wide range of food source including mussels, scallops, clams, and some seabed fish, which causes considerable decline in the number of commercial shellfish. On the other hand, starfish are considered as an extremely rich source of biological active compounds, including saponin. Saponins are secondary metabolites and common in a large number of plants. It has been found that many different kinds of saponin derived from plants have possessed several biological activities. However, as to the biological properties of starfish saponin, very few studies have been carried out until now. Therefore, it is necessary to clarify the biochemical and functional properties of saponin among different species of starfish for the advanced utilization of starfish resources.

The variety of starfish species possesses different composition of active components. Therefore, first of all, species identification of starfish used in this study was carried out by using DNA-based method and then biochemical and functional properties of crude saponin fraction derived from starfish were studied. The profile of crude saponin was analyzed by thin layer chromatography (TLC) and compared with two other plant saponin (quillaja bark and tea seed). The common Japanese starfish, *Asterias amurensis* (Kihitode), *Luidia quinaria* (Sunahitode), *Astropecten scoparius* (Momijigai), and *Patiria pectinifera* (Itomakihitode) were used as materials. Since starfish were identified by the partial sequence of 16S rRNA region in mitochondrial DNA gene, it was confirmed that 16S rRNA marker was useful and applicable to identify starfish species. The composition, number of spot, and polarity of crude saponin detected by TLC analysis showed that there are any differences among starfish species, suggesting that these differences affect various characteristics and functions of each saponin. Antifungal and antibacterial activities of starfish saponin were evaluated with six fungal and four bacterial pathogens. The crude saponin extracted from four starfish *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera* exhibited predominant growth inhibitory activity against six human fungal pathogens. Crude saponin from *A. amurensis* showed the lowest minimum inhibitory concentrations (MIC) effect among starfish species against *Aspergillus flavus and Trichophyton mentagrophytes* with the values of 31.2 and 41.6 μg/mL, respectively. In contrast, crude

saponin from *A. amurensis* was slightly active against bacteria such as *Escherichia coli* and *Edwardsiella tarda* with the MIC values of 250 and 125 μg/mL, respectively. Hemolytic activity against 2% mouse erythrocytes was found in crude saponin of all the starfish and the highest activity was observed in *A. amurensis*. Cholesterol-binding ability was relatively higher in *A. amurensis* and *L. quinaria* with the values of 34.3 and 31.7 %, respectively, than in *A. scoparius* and *P. pectinifera*. From these results, it was suggested that any biological activities of starfish saponin depend on their composition.

In order to isolate single active saponin component, *A. amurensis* crude saponin was subjected onto silica gel column chromatography. Seven saponins fractions (Fr. A to G) were obtained after 1st step of fractionation. Remarkable antifungal and cholesterol-binding ability were observed in Fr. D, E, F, and G. The result showed two major differences in the number of discrete spots on TLC, after detection of saponin. Briefly, Fr. D to G exhibited high polar components compared with Fr. A to C. It was found that those fractions (Fr. D to G) having high polar components possess high antifungal and cholesterol-binding ability, suggesting that the activity may be related to their polarity. The active fractions (Fr. D, E, and F) were subjected to further chromatography and four single sub fractions (Fr. $D_{3\text{-}1}$, $E_{6\text{-}1}$, F_2, and $F_{3\text{-}3}$) having antifungal activity and cholesterol-binding ability were obtained. The isolated compounds were analyzed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOFMS) and then the structural property was identified. As the results from MALDI-TOFMS, three unknown saponins on the ion at *m/z* 1153.9 (Fr. $D_{3\text{-}1}$), 1165.9 (Fr. $E_{6\text{-}1}$), and 1251 (Fr. F_2), and thornasteroside A which is one of well-known saponins at *m/z* 1289 and 1303 ($F_{3\text{-}3}$) were clarified.

The results obtained in this study showed that all the starfish do not possess the same characteristics for biochemical and functional properties and the differences partially due to the characteristics of saponin. It also suggests that the biological activity (antifungal, hemolytic, and cholesterol-binding ability) of starfish saponin is affected by many factors such as the aglycone, number, position, and chemical structure of sugar side-chains. In addition, since the compounds $D_{3\text{-}1}$ and $F_{3\text{-}3}$ (thornasteroside A) among the fractionated active compounds exhibited the highest antifungal activity against screened filamentous and dermatophyte fungi and on the other hand, compounds $E_{6\text{-}1}$ and F_2 did not showed obvious activity against the same fungi, the existence of various active saponin depending on the structure of compound is thought to be very interesting knowledge for the advanced use of the starfish. As conclusion, from the findings on starfish saponin in this study, it was seemed that starfish have several useful functional properties which represented by antifungal and cholesterol-binding ability and become promising materials for food and pharmaceutical industries. For the efficient utilization of starfish, further analysis of starfish saponin including unknown saponin will be demanded.

Table of Contents

Chapter 1

General introduction and literature review

Starfish are marine invertebrate Echinoderms belonging to the class of Asteroidea and have several significant ecological roles in marine ecosystem. Starfish is one of the by-catch products by bottom trawling method which is reducing the work efficiency to the fisheries. In addition, starfish consumes a wide range of food source including mussels, scallops, clams, and some seabed fish and causes considerable decline to commercial shellfish. In recent years, a large number of different starfish species has been observed in the coastal areas of Japan especially in Hokkaido about 17,000 t/year. However, most of the starfish have been got rid of by fire destruction without any utilization.

On the other hand, it was also reported that starfish are an extremely rich source of biological active compounds. However, there are no effective methods to utilize these resources at industrial scale. This problem leads to a necessity to study the biochemical and functional properties of starfish active components, particularly saponin which exhibit a wide spectrum of biological activities. Several report about the isolation of the bioactive components from various seaweeds have been published. However, little attention has been paid to the exploration from marine animals (Hwang et al., 2014). Recently, marine animal's starfish have been identified as a source of new pharmacological agents because of their variety of species. Therefore, it is necessary to clarify the biological properties of saponin among different species of starfish for the advanced utilization of starfish resources.

A series of experiments were done for evaluating and utilizing of starfish resources. For conduct of these experiments and summarization of the results, a thorough study was done based

on different reviews and literatures in this chapter.

1.1 Starfish

Starfish have a central disc and five arms, though some species have a larger number of arms, and about 1,500~2,000 species of starfish were found in all the world's oceans. They are sometimes collected as curios, used in design or as logos, and cultured in aquarium. Starfish is well-known around the worldwide fishing industry as by-catch products. By-catch products are caught unintentionally while catching certain target species such as a different species, wrong sex, undersize, or juvenile individuals of the target species. According to one report recently published from Denmark (Fomsgaard et al., 2014), it was estimated that almost 80% of by-catch products were starfish (Fig. 1-1). Starfish are also caught by bottom trawling and gill net method and create a great problem to fishery. The large starfish originally inhabit the coast of northeast Asia including Japan, Korea, China, and Russia (Hwang et al., 2014). However, in the early 1990s, it has spread in the coastal area of Australia and Tasmania due to their wide habitat tolerance ability (Summerson et al., 2007) and has been identified as an invasive species in marine environment. The Asteroides occupy several significant ecological roles. Starfish, such as the ochre starfish (*Pisaster ochraceus*) and the reef starfish (*Stichaster australis*) have become widely known as the keystone species in the ecological concept. In recent years, the large outbreak of starfish causes significant loss of the marine ecosystem and fishing gears (Global invasive species database, 2016). Furthermore, the waste of them results in serious environmental pollution. In addition, it has an ability to consume a wide range of food sources including mussels, scallops, clams, and some seabed fish which causes considerable damage to commercial shellfish. The outbreaks of venomous starfish crown-of-thorns is also significantly

responsible for destroying the coral reefs. In Japan, the outbreak of starfish was observed from last decades, especially in the coastal area of Hokkaido (Ishii et al., 2006). Until now, any methods to control the large outbreak of starfish has been yet reported. In Hokkaido, it was reported that every year 17,000 tons of starfish are collected from coastal area and after fire destruction they remain without any utilization (Shah et al., 2013; Ishii et al., 2006). The huge amount of starfish ash causes environment pollution. However, Hwang et al. (2014) mentioned that a thorough investigation of these large outbreak starfish might be provided an effective way of reducing the environmental and economic burden of those species.

On the other hand, starfish are considered as one of the potential marine resources. *Oreaster reticulates, Luidia senegalensis* and *Echinaster* sp. have been used as traditional medicine for thousands of years in China and north-eastern Brazil to treat asthma, bronchitis, diabetes, and heart and stomach diseases (Alves and Rosa, 2007; Alves and Alves, 2011). A considerable number of world publications have drawn attention to the possibility of using starfish. Starfish are using in some feed meal as a protein supplementary and the waste of starfish are also using in agriculture sector as a predator and plant growth enhancer (Ishii et al., 2006). However, there are no effective methods to utilize these resources at industrial scale. Recently, it has been reported that starfish contain many biological active substances. Several secondary metabolites and molecules such as glycosylceramide, ceramide, cerebrosides (Inagaki et al., 2006; Ishii et al., 2006), and steroidal glycosides (Hwang et al., 2011; Ishizaki et al., 1997) have been identified from starfish.

1.2 Saponin

Saponin is a class of chemical compounds found in particular abundance in various plant

species. Saponin is naturally occurring surface active glycoside, which possess amphiphilic character originating from lipophilic aglycone (sapogenin) and hydrophilic sugar moieties (Fig.1-2) (Francis et al., 2001; Bottger et al., 2012). Saponin exhibits characteristics such as a strong foaming power in aqueous solution, from which the name saponin was derived. The aglycone usually consists of either steroidal or triterpenoidal structure in nature and both types are derived from a 27 or 30 carbon skeleton (Fig. 1-3) (Haralampidis et al., 2002; Vincken et al., 2007). Most of known saponins are monodesmosidic, which means that only one position of the aglycone is glycosylated (Fig. 1-4). The sugar moiety is attached by an ether linkage to the C3 hydroxyl group occurring in the majority of sapogenins (Augustin et al., 2011; Vincken et al., 2007). Saponin can possess from one or more straight or branched sugar moieties such as glucose, galactose, pentose, and xylose which are linked to a hydrophobic aglycone (sapogenin) (Oakenfull and Sidhu 1989; Ishizaki et al., 1997). The sugar chain contains from one to several monosaccharide residues (Summerson et al., 2007). Saponins are known to distribute widely in plants, but also in lower marine animals including echinoderms (mainly starfish and sea cucumbers) and sponges (Fig. 1-2) (Nishiyama et al., 1987; Kubanek et al., 2002). As pointed out by Xiao and Yu (2013), any of these biological activities are likely to be a part of self-defense mechanism of starfish.

1.2.1 Plant saponin

Saponins occur constitutively in many wild plants and cultivated crops. Plant saponin can be classified as triterpenoid or steroid saponin (Fig. 1-3). Steroidal saponins have been detected in oats, tomato seeds, alliums, asparagus, yam, yucca, fenugreek, ginseng, capsicum peppers, and aubergine, whereas triterpenoid saponins have been found in many legumes and also in alliums, tea, spinach, sugar beet, liquorice, sunflower, ginseng, horse chestnut, and quinoa. Their main

role is protection of organisms from harmful effects of pathogens, including anti-fungal, anti-viral, and anti-bacterial characteristics (Francis et al., 2001; Bottger et al., 2012; Podolak et al., 2010; Augustin et al., 2011; Vincken et al., 2007; Riguera, 1997; Fuchs et al., 2009). *Yucca schidigera* is one of the most common commercial sources of steroid saponins detected in many legumes such as soybeans, beans, peas, lucerne, etc. and also in alliums, tea, spinach, sugar beet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. *Quillaja saponaria* is a tree native to the Andes region, and the bark was peeled off and extracted with water by the indigenous peoples as a shampooing agent. Usually, saponins are considered to act as a defense against microbial and predatory attacks on plants (Francis et al., 2001). Plant saponins have been widely used in different industry such as food, medicine, pesticide, and animal feed (Palazon et al., 2006; Wang et al., 2005; Ibanoglu and Ibanoglu, 2000).

1.2.2 Marine animal saponin

In the animal kingdom, saponins have been found in the exclusively marine phylum Echinodermata and particularly in species of the classes Holothuroidea, sea cucumber, and Asteroidea, starfish (Kumar et al., 2007; Chludil et al., 2002). From last few decades marine organisms became top ranks for identification of new bioactive component due to its vast diversity. The secondary metabolites from marine organisms such as echinoderms, sponges, and ascidians are characterized by a remarkable diversity of chemical structures and physiological activities (Podolak et al., 2010; Vincken et al., 2007; Riguera, 1997). The metabolites of starfish can be subdivided into three main groups: asterosaponins, cyclic steroidal glycosides, and glycosides of polyhydroxylated steroids, and possess various kind of biological activity (Suh et al., 2011).

1.3 Biological properties of saponin

Several biological activity assays for saponins have been reported in many literatures (Jackson and Shaw, 1959; Van Atta et al., 1961; Coulson and Davis, 1962). The biological activities of saponin depend on their chemical structure. Structural variability occurs in aglycone type, carbon number, position, and chemical composition (number, shape, sequence, and type of monosaccharides) of sugar side chains, type, and number of functional groups attached to the aglycone nucleus (Potter et al., 1993). A similar diversity of effects of plant or animals saponin is reported by Francis et al. (2001) and it is likely that the range of potential biological activities is as diverse as the chemical structures of the compounds themselves. Saponins are considered to act as a defense against microbial and predatory attacks on plants (Francis et al. 2001). Though Takechi et al. (1999) pointed out that synthetic steroid saponin show both antifungal and hemolytic activity, in many cases, hemolytic triterpenoid saponin show little antifungal activity. It should be noted that hemolytic and antibacterial activity of saponin is affected by some factors such as the aglycone, number, position, and chemical structure of sugar side chains (Rakhimov et al., 1996). While many plant saponins extracted from tea seed, yucca, and quillaja bark have been widely used in the pharmaceutical, cosmetic, and food industries, the saponin from starfish are not yet utilized at an industrial scale. Therefore, antifungal, antibacterial, hemolytic, and cholesterol-binding ability of starfish saponins were investigated in this study.

1.3.1 Antifungal and antibacterial activity

The problem of antibiotic resistance has been observed during last decade and remains an interesting and important area of research (Wallace, 2004). Several drug discovery projects have screened echinoderms for antibiotic activities. Many saponins including yucca (Hussain and Cheeke, 1995; Tanaka et al., 1996), quillaja (Sen et al., 1998), and ginseng (Mahato et al., 1988)

have antimicrobial activity and are considered as a part of plants' defense systems. However, all saponins don't have same antimicrobial activity. Antifungal and antibacterial activities of saponin vary depending on the strains of the fungi and bacteria. Some saponins such as ivy saponin (Cioaca et al., 1978), spirostanol saponin (Okunji et al., 1990), and yucca saponin (Cheeke, 1998; Cheeke and Otero, 2005) show higher antimicrobial activity for gram-positive bacteria (*Staphylococcus aureus*) than gram-negative bacteria (*Escherichia coli*) at the same concentration. Moreover, extraction methods and fat content have important effects on the antibacterial activity of the resultant plant extracts. The mode of action against gram-positive and gram-negative bacteria is not clear yet. An early study by Rinehart et al. (1981) showed that antimicrobial activity was present in 43% of 83 unidentified species of echinoderms. Asterosaponin from starfish (Andersson et al., 1989) and triterpenoid saponins from Holothuroidea class of marine echinoderm animals (Samoilov and Girshovich, 1980) were also found to possess any antimicrobial activities. The antifungal activity of saponin depends on the ability to form complexes with sterols, which results in increased membrane permeability and leakage of cell contents (Morrissey and Osbourn, 1999) or the induction of programmed cell death in sensitive fungal cells (Ito et al., 2007). Many reports on the possibility of usage of saponin, as natural fungicides, have been published (Hostettmann and Marston, 1955; Olezek, 1996). Saponins obtained from different plants and marine animals exhibited antibacterial activity with different efficiencies, suggested that saponins from various sources differ in their biological activity depending on their different chemical structures and extraction procedure (Sen et al., 1998). Despite this fact, an antimicrobial agent from a variety of sources including the marine environments is needed to study.

1.3.2 Hemolytic activity

Saponins are known to have hemolytic characteristics as one of the biological activities. However, the hemolytic activity of saponin inducing toxicity in most animals is a major drawback for their clinical development (Liu et al., 2013) and has been used by researchers to follow the isolation of saponin. It is the simplest and fastest bioassay employed to detect and quantify some saponins in plant materials (Authi et al., 1988; El Izzi et al., 1992; Khalil and EI-Adawy, 1994; Onning and Asp, 1995; Oleszek, 1988; Onning et al., 1996; Choi et al., 2001; Menin et al., 2001; Plock et al., 2001). Quillaja bark saponin (Jenkins and Atwal, 1994), roots of *Platycodon grandiflorum* (Sun et al., 2011), *Cicer arietinum* L. (Hashim, 2014), Guar meal (Hassan et al., 2010), and synthetic triterpenoid or steroid saponins (Takechi et al., 1999) have hemolytic activity. However, all saponins have not similar hemolytic activity. For example, it has been known that soybean saponin shows lower hemolytic activity against chicken erythrocyte (Hassan et al., 2010) and saponin extracts from alfalfa root possess higher hemolytic activity than those from alfalfa leaves (Shany et al., 1970).

The exact mechanism of the hemolysis by saponin is not clearly understood. However, it was found to be correlated with their amphiphilic properties (Melzig et al., 2001). On the other hand, Winter (1994) pointed out a different mechanism that saponin interacted with the water channels aquaporin resulting in an increase of the water transport inside the cells, and then induced the hemolysis of erythrocytes. Numerous studies have proven that hemolytic activity of saponin is attributed to the affinity with their aglycone moiety to sterols within membranes, particularly cholesterol (Gee et al., 1993). Saponins possessing two or more sugar side chains show less hemolytic activity than saponin containing one sugar side chain (Mahato et al., 1988; Woldemichael and Wink, 2001). The hemolytic activity varied depending on the sugar units attached to the hydroxyl group of the aglycone (Anisimov et al., 1980; Mahato et al., 1988). The

stereochemistry of the saponin as related to side chain composition and length appear to be very important in conferring activity on the saponin molecule (Gee et al., 1998).

It is well-known that hemolytic activity of saponin increases with decreasing numbers of polar groups on the aglycone moiety (Namba et al., 1973). The active groups on the aglycone and the acylation of saponin affect hemolytic activity. Hemolytic assays have also been developed for detecting saponin in drugs or plant extracts based on their ability to rupture erythrocytes. Steroid saponins show both antifungal and hemolytic activity, but in many causes hemolytic triterpenoid saponins show little antifungal activity (Takechi et al., 1999).

1.3.3 Cholesterol-binding ability

Cardiovascular disease (CVD) is one of the most common causes of mortality in the United States, Europe, and most parts of Asia (Braunwald, 1997). According to a report published by World Health Organization, it was estimated that by 2020 about 25 million deaths of people will be occurred by CVD in worldwide (WHO, 2003). Hypocholesterolemia is a major risk factor for the progression and development of atherosclerosis (Prasad and Kalra, 1993). During the past four decades, many reports have focused on how to decrease plasma lipid concentrations and the absorption of fat in the intestinal tract to reduce diet-related chronic diseases. Therefore, medicine obtained from natural products for lowering plasma cholesterol and risk of CVD has gained wide acceptance by the general public (Ching et al., 2013).

A number of studies reported that saponins from *Platycodon grandiflorum* (Zhao et al., 2008), *Dioscorea* spp. (Son et al., 2007), *Panax notoginseng* (Zhang et al., 2008), and Karaya root (Afrose et al., 2009) have hypocholesterolemic effects, and the search for new saponin sources continues. The hypocholesterolemic action of saponin is thought to be mediated in part by its binding of cholesterol and bile acids in the intestinal lumen, thus enhancing the excretion of

these steroids in the feces (Sidhu and Oakenfull, 1986). A considerable number of scientific studies have reported that saponin has the ability to reduce the low density lipoprotein (LDL)-cholesterol in the serum of some animal such as rats, gerbils, and also human (Potter et al., 1993; Harris et al., 1997). It has been found that different kinds of saponin derived from plants have an ability to form a complex with cholesterol in the plasma membrane; this complex destroys the structural integrity of the cell membrane and increases its permeability (Ohtsuki, 1984). The evidence is extremely interesting not only in understanding the complex formation between cholesterol and saponin, but also in the industrial use of starfish saponin. However, until now, few studies have investigated the cholesterol-binding ability of starfish saponin.

1.4 Common Japanese starfish species

About 1,500~2,000 species of starfish have been found in worldwide ocean and about 300 species have been identified along in the coasts of Japan. Among them, *Asterias amurensis* (Kihitode) is known as the Northern Pacific starfish and habitat in all the Japanese coast such as the Tokyo bay, Sendai bay, Ise bay, Hakata bay, Kobe bay, and Ariake bay. *Luidia quinaria* (Sunahitode) is a predator and found in shallow water on soft sediments. In Ise bay which is located in central Japan, it was living throughout the oxygen-poor waters of the bay, while another starfish, *Astropecten scoparius* (Momijigai) tended to occupy a separate zone near the mouth of the bay. *Patiria pectinifera* (Itomakihitode) is native to the Sea of Japan and the Yellow Sea. *P. pectinifera* has been used as a model organism in developmental biology. The advantages of this organism are that it is easy to collect and maintain in the laboratory. The classification and the appearance of these starfish are shown in Fig. 1-5 and 1-6, respectively. These four species have also been identified feeding on the scallops and clams culture industry. During last decades,

the abnormal outbreak of these starfish was observed in different costal area of Japan and caused severe problems in shell fish industry. Therefore, it is necessary to study the biological properties of these common Japanese starfish species for advancing utilization of industrial scale. In this research, biochemical and functional properties of four starfish, *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera* were investigated and then compared among species.

1.5 **Research objectives and hypothesis of this thesis**

The previous information which was reviewed in this chapter indices that starfish is one of the most promising sources. In recent years, it has attached much more attention due to its large scale outbreak in different costal area of Japan, which causes severe damage in terms of economy to the fishery and aquaculture grounds for shellfish. However, nowadays, most attention to saponin from starfish has been given to their potential in pharmaceutical properties (Kicha et al., 2003; Ivanchina et al., 2012; Robin et al., 2013). In particular, the anti-cancer and anti-microbial properties of plant saponin secondary metabolites have been widely investigated. Development of anti-microbial, hemolytic, and cholesterol-binding compounds from starfish saponin is also an important area of research for the advanced utilization of starfish resources. The main aim of this study is to identify and characterize saponin from different Japanese starfish and to understand the biological activities of these compounds. It is hoped that the findings of this study on starfish saponin will provide the future development of novel functional metabolites.

The specific objectives for this thesis are to:

1) Characterize the saponin component in various starfish species.

2) Determine functional properties of starfish saponins with antimicrobial, hemolytic and cholesterol-binding ability among different species.

3) Clarify the relationship between function and structure of starfish saponin.

The hypotheses of this research are as follows:

1) Since saponin content in plant depends on some factors such as the provenances and geographical location of plant, I hypothesize that there is a possibility of variation in saponin content among starfish species.

2) Saponins have the ability to form complexes with sterols, which results in increased membrane permeability and leakage of cell contents (Morrissey and Osbourn, 1999) or the induction of programmed cell death (Ito et al., 2007). Starfish saponin may also exhibit significant biological properties such as antimicrobial, antifungal, hemolytic, and cholesterol-binding abilities. These properties are extremely interesting not only in understanding the complex formation between fungi membrane and saponin or cholesterol and saponin, but also in the industrial use of starfish saponin.

3) Fractionation, characterization, and identification of the active components of starfish saponin can provide the relationship between functions and structure information on the bio-utilization of those by-catch products.

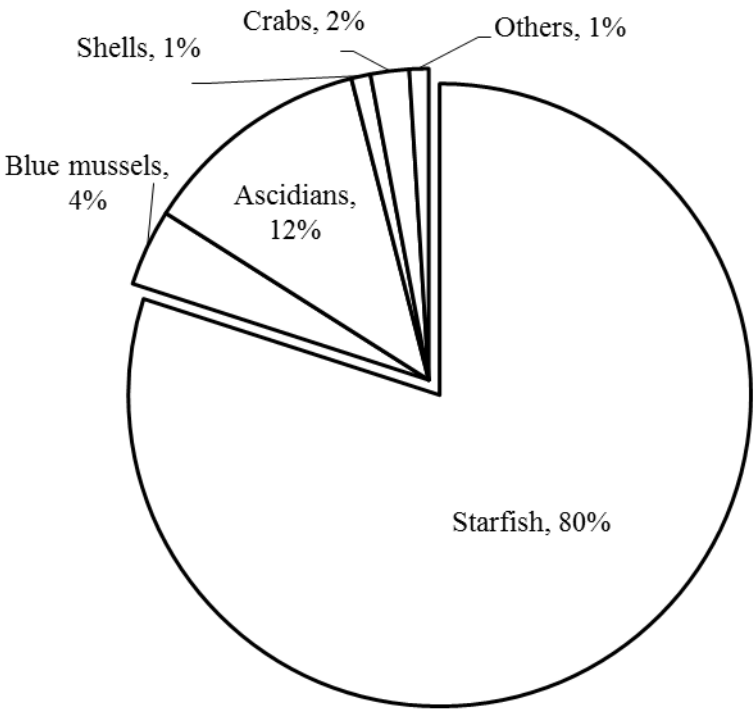
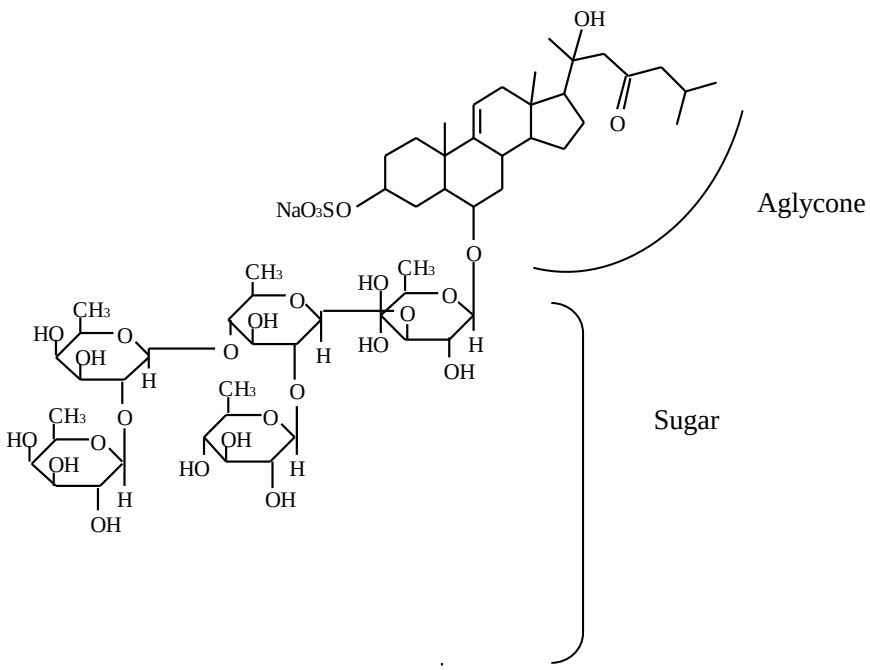


Fig. 1-1 By-catch product reported by DTU Aqua, Technical University of Denmark.

This data was cited from the unpublished report by Fomsgaard et al. (2014).

(a)



(b)

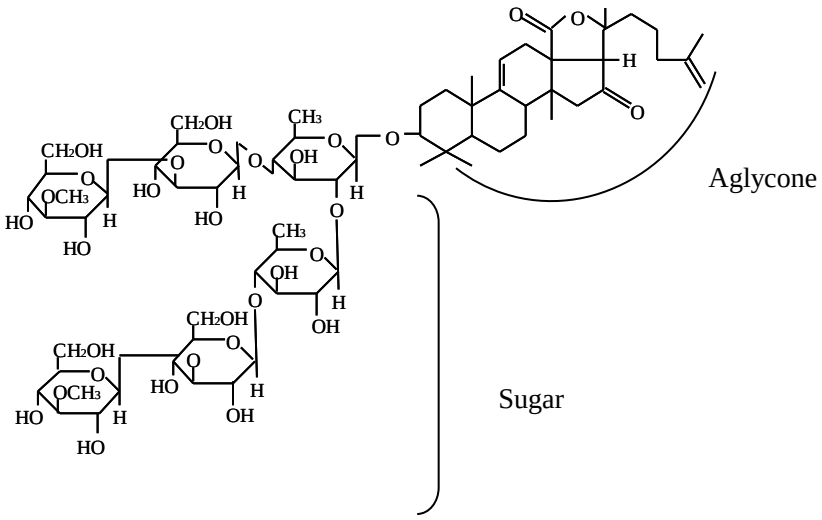
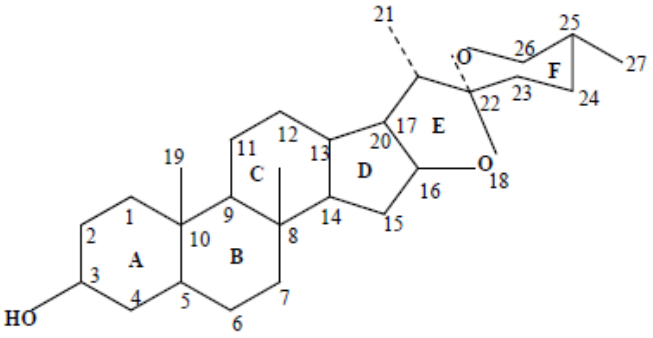


Fig. 1-2 Structures of saponins isolated from starfish and sea cucumber.

(a), ovarian asterosaponin-1; (b), holotoxin-A.

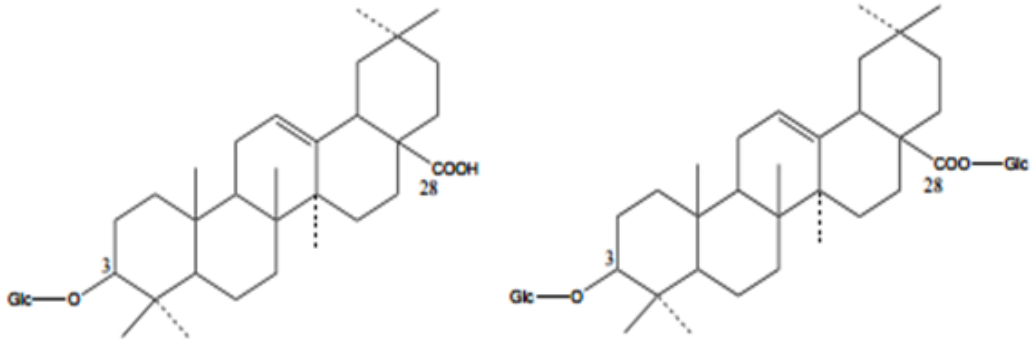


Steroid class

Steroid alkaloid class

Triterpenoid class

Fig. 1-3 Skeletal types of aglycone found in the three principal classes of saponin.



Monodesmosidic Bidesmosidic

Fig. 1-4 Monodesmosidic and bidesmosidic saponins.

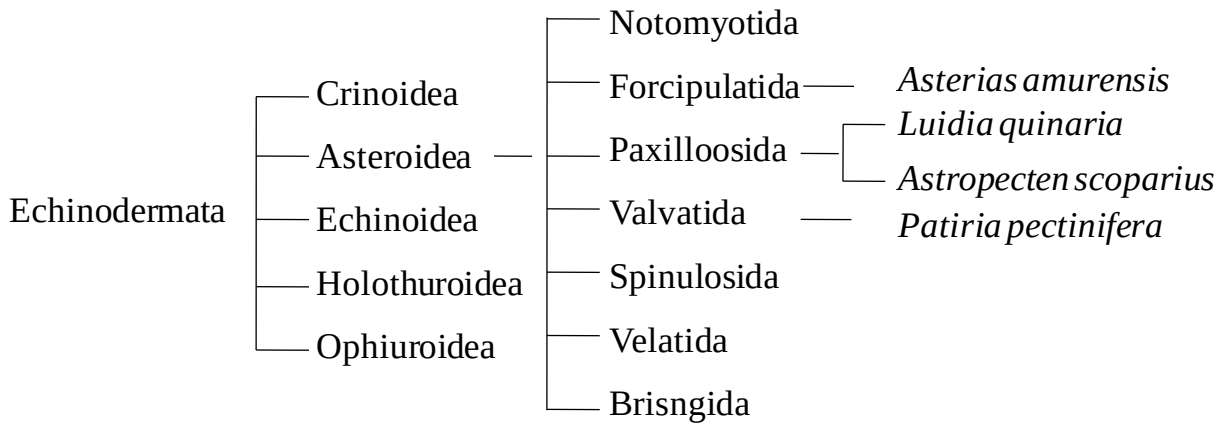


Fig. 1-5 Classification of Asteroidea.

A)

B)

C)

D)

Fig. 1-6 Starfish used in this study.

A), *Asterias amurensis* (Kihitode); B), *Luidia quinaria* (Sunahitode); C), *Astropecten scoparius* (Momijigai); and D), *Patiria pectinifera* (Itomakihitode).

Chapter 2

Functional properties of crude saponin derived from several Japanese starfish

2.1 Introduction

In the globalized trading system, invasion of exotic species has become a major concern in the marine environment since the number of human-mediated introduction has increased (Ruiz et al., 2000). Many studies researching marine invertebrates to determine the therapeutic potential of their bioactive materials have been showing very promising results. Starfish are marine invertebrate and possesses many useful pharmacological and biological activities. Various biological active compounds and molecules have recently been identified from starfish such as glycosylceramide, steroidal glycosides, ceramide, and cerebrosides (Inagaki et al., 2006; Ishii et al., 2006; Suh et al., 2011). Saponins are a group of natural plant glycosides, characterized by their strong foam-forming properties in aqueous solution that occur in a wide range of plant species (Osbourn, 1996). The presence of saponin has been reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anti-cancer properties (Man et al., 2010) and have also been commonly employed some other sector in medical practice for their potential health benefits. In addition, many plant saponins have been isolated and exhibit broad spectrum of biological uses, such as anti-cancer, anti-inflammatory, ion channel blocking, immune stimulating, antifungal, and anti-thrombotic property (Balandrin, 1996; Lacalle-Dubois and Wagner, 2000; Harmatha, 2000). Furthermore, it has been found that many different kinds of saponin derived from plants have the ability to bind to cholesterol (Potter et al., 1993). Saponin forms a complex with cholesterol in the plasma membrane; this complex destroys the structural integrity of the cell membrane and increases its permeability (Okano et

al., 1985). This property is extremely interesting not only in understanding the complex formation between cholesterol and saponin, but also in the industrial use of starfish saponin. A number of starfish have been identified in Japan. It has not been elucidated yet whether they have saponin with certain functionality such as antifungal, antibacterial, hemolytic, or cholesterol-binding ability.

Therefore, it is necessary to clarify the biological properties of saponin among different species of starfish for the advanced utilization of starfish resources. In this chapter, the antifungal and antibacterial activities of crude saponin extracted from four starfish were determined against six human fungal and four bacterial pathogens, respectively and then hemolytic activity of them was examined against mouse erythrocyte. In addition, cholesterol-binding ability of crude starfish saponin was also investigated.

2.2 Materials and Methods

2.2.1 Biological materials

The common Japanese starfish, *Asterias amurensis*, *Luidia quinaria*, *Astropecten scoparius*, and *Patiria pectinifera* were collected from the coast of Kobe, Hyogo prefecture, Japan in February 2014. They were immediately brought to the laboratory in ice. The starfish were washed thoroughly with tap water to remove other seabed fish and subsequently stored at -60°C until use.

2.2.2 Species identification based on DNA analysis

Genomic DNA was extracted from tube feet of starfish sample by using Quick gene-810 (Kurabo, Tokyo, Japan) as recommended by the manufacturer. The DNA concentration (ng/μL) was measured by a Biospec Nano (Shimadzu Corporation, Tokyo, Japan). A partial region of the mitochondrial 16S rRNA gene was amplified by the conventional polymerase chain reaction (PCR) using universal primers (16SarL, 5’-CGCCTGTTTATCAAAAACAT-3’

and 16SbrH, 5'-CCGGTCGAAACTCAGATCACGT-3'). Briefly, the reaction mixture for PCR was carried out in the 50 µL volume containing 5 µL (50 ng) of genomic DNA, 4 µL of dNTP (2.5 mM each), 5 µL of 10 × Ex Taq buffer, 0.4 µL of Ex Taq DNA polymerase (Takara Shuzo, Japan) (5 U/µL), and 1.5 µL of 20 µM of each primer, 16SarL and 16SbrH. PCR amplification was performed with Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 30 cycles consisting of denaturation at 98°C for 10 s, annealing at 53°C for 30 s and extension at 72°C for 60 s. The amplified PCR products were run in 1.2% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). The gel was run at 100 V for 30 min and visualized using LAS-4000 mini documentation system (Fujifilm Cooperation, Tokyo, Japan). The PCR samples were sequenced with BigDye® terminator V3.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the obtained sequence data were analyzed by SeqEd Version 1.0.3 (Perkin Elmer, Foster City, CA, USA) software. The sequences were subjected to blast search with national center for biotechnology information (NCBI) data base. These sequences were then aligned using the default settings in ClustalW package in the MEGA 6 software (Tamura et al., 2013).

2.2.3 Analysis of inorganic components

The micronutrient contents of each starfish were determined. Briefly, 1 g of sample was dissolved in a furnace and diluted to 200 mL with 1N HCl, and the diluted solution was analyzed using combustion thermal conductivity (CTC) and inductively coupled plasma spectrophotometer (ICP) method. Moisture content was determined with minced starfish sample by drying in an oven at a temperature of 105°C for 5 h.

2.2.4 Preparation of crude saponin

The crude saponin was extracted according to method described by Yasumoto et al. (1966). Crude saponin preparation methods with various solvents are shown in Fig. 2-1.

Briefly, one kilogram of starfish was cut into small pieces and then minced using a food grinder (Kitchen Aid, St. Joseph, MI, USA). The extraction was carried out with 3 L of methanol and repeated twice using each 2 L of methanol. The extract was filtered through a Whatman filter paper No. 2. The filtrate was concentrated up to 250 mL with a rotary evaporator (EYELA, Tokyo, Japan) under reduced pressure at 45 ºC. The concentrate was stirred with an equal volume of water (250 mL) and defatted with 250 mL benzene. After being freed from benzene, pH of the extract was adjusted to 3 with 1N HCl and then neutralized using 1N NaOH. The extract was dialyzed through an ultra-filtration membrane (MWCO: 1000, Millipore-amicon, Billerica, MA, USA) and then partitioned with *n*-butanol three times. After the *n*-butanol extract was concentrated up to 150 mL, three volumes of diethyl ether and a half volume of water were added. Finally, the aqueous layer was lyophilized to give as crude saponin.

2.2.5 Thin layer chromatography (TLC) analysis

The chromatographic profiles of the crude saponin were analyzed by thin layer chromatography (TLC). TLC analysis was performed on precoated silica gel 60 F_{254} plates (10 × 20 cm, Merck, Darmstadt, Germany). The mobile phase was a mixture of chloroform: methanol: water (65:35:10, lower phase). Quillaja bark and tea seed saponin were obtained from Wako Pure Chemical industry (Osaka, Japan) and Sigma Aldrich (St. Louis, MO, USA), respectively and were used for comparison with starfish saponin. The crude saponin (20 mg/mL) was properly diluted with distilled water before use. An aliquot of 4 µL (containing of 80 µg) of crude saponin sample was applied to a TLC plate. The chromatography was run in a chamber to a distance of 15 cm from the origin. The developed plates were air-dried and sprayed with freshly prepared solution with 50% sulfuric acid and 1% cinnamaldehyde in ethanol (after drying, plate was stained with acetic anhydride: sulfuric acid, 12:1), respectively, followed by heating at 110 ºC for 10 min. The movement of each separating spot

of the extract was expressed by its retention factor (R_f) using the following formula:

Retention factor (R_f) = Distance travelled by the compound/ Distance travelled by the solvent

2.3 Measurement of functional properties

2.3.1 Antifungal activity

Antifungal activity was determined with the two yeasts *Saccharomyces cerevisiae* (JCM 2194) and *Rhodotorula glutinis* (JCM 8173), the three filamentous fungi such as *Aspergillus flavus* (JCM 12721), *Cladosporium sphaerospermum* (NBRC 4460), and *Fusarium oxysporum* (NBRC 5942), and the dermatophyte fungi *Trichophyton mentagrophytes* (NBRC 32410). Pathogens were obtained from the Japan Collection of Microorganisms (JCM) and NITE Biological Resource Center (NBRC). Cultures were maintained on malt agar at 4 °C and sub cultured every month. Yeast fungi were grown in potato dextrose broth (PDA, 2.4 % in dH_2O) and cultivated on potato dextrose agar, DAIGO (3.9%). Filamentous fungi were grown in phosphate buffer (PB) (PB; 0.1 M KH_2PO_4 and 0.1M $Na_2HPO_4 \cdot 12H_2O$, pH 7.0) and cultivated on potato dextrose agar, DAIGO (3.9%). Similarly, dermatophytes fungi were grown in 707 broth (Bacto trypton 5 g, bacto yeast extract 3 g, $MgSO_4 \cdot 7H_2O$ 1 g in 1000 mL dH_2O, pH 7.0) and cultivated on sabouraud agar (4% glucose, 1% bactotrypton, 2% agar, pH 6.0 with 1 N HCl). The culture condition and incubation temperature are shown in Table 2-1. Agar and liquid broths were sterilized at 121ºC for 20 min.

2.3.2 Antibacterial activity

Antibacterial activity was performed with four bacterial strains such as *Vibrio parahemolyticus* (NBRC 12711), *Escherichia coli* (JCM 109), *Edwardsiella tarda* (GCT 03825T), and *Lactococcus garvieae* (JCM 8735). Pathogens were obtained from the Japan Collection of Microorganisms (JCM), NITE Biological Resource Center (NBRC), and Gifu Type Culture (GTC). *V. parahemolyticus* was grown in tryptic soy broth (TSB, 4% with 3%

NaCl) and cultivated on tryptic soy agar. *E. tarda* and *L. garvieae* were grown in broth culture (TSB, 4%) and agar medium (TSA, 4%). On the other hand, *E.coli* was followed by LB (Luria-Bertani) broth and LB agar (polypeptone 1%, yeast extract 0.5%, glucose 0.1%, agar 1.5%, thymin 1 mg/mL, 5N NaOH 150 µL). The culture condition and incubation temperature are shown in Table 2-2. Agar and liquid broths were sterilized at 121ºC for 20 min.

Disc diffusion assay

Antifungal and antibacterial activities were evaluated with disc diffusion method described by Reinheimer and Demkow, (l990). One hundred microliters of cultured fungi was uniformly smeared on an agar plate by a smear loop. Then, 8 mm diameter sterilized paper disc (Toyo Roshi Ltd., Tokyo, Japan) was loaded with 70 µL of the crude saponin sample at two concentrations of 5 and 10 mg/mL and then placed on agar plate. Quillaja bark and tea seed saponins were used for the positive control and distilled water for the negative control, respectively. A clear zone with a diameter was taken as antifungal and antibacterial activities. All analyses were carried out in triplicate and in two separate experiments. The mean value of each experiment was considered for further data analysis.

Determination of minimum inhibitory concentration (MIC)

The fungal and bacterial strains were grown on potato dextrose agar, sabouraud agar and tryptic soy agar, respectively. After incubation, fungal and bacterial growths were suspended in normal saline. The minimum inhibitory concentration (MIC) values for each crude saponin were determined through micro-dilution assay following a method described by Kumar et al. (2007) with minor modification. Briefly, 96-well plates were prepared by having 190 µL broth and 190 µL sterilized H_2O into each well. A 20 µL crude saponin initially prepared (at the concentration of 10 mg/mL) and added into the first well so that the concentration of the crude saponin became 500 µg/mL. From here the solution was transferred into eight

consecutive wells and then 10 µL inocula were added and kept in an incubator at 27 and 30°C. The bacterial and fungal suspensions were adjusted with broth to a concentration of 1.6-1.9×10^{5-7} cfu (colony forming units) /mL and 1.0–5.0×10^{3} spore/mL, respectively and stored at 4°C incubator depending on the bacterial and fungal species. A control was maintained with only culture medium and bacterial and fungal cells. Values obtained for crude saponin was compared with the values from the control and the difference is considered as growth inhibition activity. MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms and experiments were in triplicates and repeated three times.

2.3.3 Hemolytic activity

Mouse blood was obtained from the Japanese Biological Center (Tokyo, Japan). Hemolytic activity was determined according to the method described by Charles et al. (2009) with slight modifications. Briefly, 2 mL of aliquot of blood were washed three times with phosphate buffer saline (PBS) solution (0.15 M NaCl-0.01M Tris-HCl, pH 7.0) by centrifugation at 1,090 ×*g* for 5 min at 4 ºC. Washed erythrocytes were suspended in the PBS solution to obtain a concentration of 2%. Then, 0.5 mL of erythrocytes and 1 mL of PBS solution were mixed with 0.5 mL diluents containing 1, 5, 10, 50, 100, 500, and 1,000 µg/mL of individual crude starfish saponin in PBS solution. The mixtures were incubated for 30 min at 37 ºC and centrifuged at 270 ×*g* for 5 min. A volume of 1.5 mL PBS and distilled water were used as minimal and maximal hemolytic controls, respectively. After centrifugation, the presence of a suspension of a uniform red color was considered to indicate hemolysis, and a button formation in the bottom of the wells constituted a lack of hemolysis. A volume of 250 µL of each supernatant was transferred to a 96-well flat bottom micro plate, and the absorbance at 540 nm was measured with a micro plate reader (680 Microplate readers, BIO-RAD, Tokyo, Japan). The experiment was done in triplicate, and each sample was transferred three times into a 96-well micro plate. The concentrations that induced hemolysis of 50% of

erythrocytes hemolysis (HC_{50}) in the different crude saponin was calculated. All experiments were done in triplicate for the analysis of each HC_{50} and expressed as mean ± standard deviation (SD).

2.3.4 Cholesterol-binding ability

Cholesterol-binding ability was determined with the $FeCl_3$-H_2SO_4 method reported by Courchaine et al. (1956). Briefly, 2.5 g of $FeCl_3$ was dissolve in 100 mL of H_3PO_4 (iron solution), and 8 mL of the iron solution was diluted with 100 mL of H_2SO_4 (color solution). Cholesterol standard was obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Cholesterol stock solution (1 mg/3 mL) was prepared and properly diluted with 100% acetic acid before use. Standard curves were obtained by regression analysis using five cholesterol standards with 0.1, 0.2, 0.3, 0.4, and 0.5 mg/3 mL for cholesterol quantification. Two milliliters of color solution were added to the standard solution, and the solution was kept at room temperature for 30 min. The 0.3 mg/mL cholesterol standard was diluted with an equal volume of acetone: ethanol (1:1). Extracted crude saponin (10 mg/mL) were diluted with distilled water, and then mixed with 1 mL of cholesterol standard solution. The mixture was incubated at 37 ºC for 5 h and then centrifuged at 350 ×*g* for 5 min. The supernatant was discarded and 4 mL of acetone was added to suspend precipitate. The procedure was repeated and then 3 mL of acetic acid and 4 mL of color solution were added in the tube. The mixture was allowed to stand for 30 min at room temperature. Absorbance was measured at 550 nm. Cholesterol ester (%) was calculated using the following equation:

Cholesterol esters (%) = (cholesterol esters/ free cholesterol) x 100

2.3.5 Data analysis

Statistical analyses were performed using SPSS software (SPSS 16.0, IBM, USA). Data are expressed as mean ± SD. Data were compared with one way analysis of variance (ANOVA). Significant differences were determined by Tukey's test at $p < 0.05$ level.

2.4 Results

2.4.1 Species identification of starfish used in this study

First of all, species identification of starfish was done based on their phenotypic description including external structural appearance and morphological characteristics. Subsequently, species identification of four starfish by DNA-based method was carried out by a direct DNA sequencing analysis. Partial nucleotide sequence data of 16S rRNA gene was compared with those in NCBI gene data base. Figure 2-2 shows aligned DNA sequences of the amplified partial 16S rRNA region from the samples. From the results of the alignment with the estimated species, it was found that the partial sequences of the PCR products from four samples were almost identical with those of *A. amurensis* (Kihitode, 98.1%), *L. quinaria* (Sunahitode, 99.20%), *A. scoparius* (Momijigai, 99.17%), and *P. pectinifera* (Itomakihitode, 99.06%). Thus, it was confirmed that identification of starfish is enabled by using the nucleotide sequence encoding 16S rRNA gene of mtDNA.

2.4.2 Analysis of inorganic components

The results of moisture and micronutrient contents of four starfish *A. amurensis, L. quinaria, A. scoparius,* and *P. pectinfera* are shown in Table 2-3. The Cu content was found in *P. pectinfera* with the value of 10.0 µg/g and followed by *A. scoparius.* The As content was found similar in *A. amurensis* and *P. pectinfera* with the value of 2.10 µg/g and Cd content was higher in *P. pectinfera* with the value of 1.70 µg/g. Likewise, high level of Ca content was observed in *A. scoparius* with 16.30% of dry weight in the inorganic analysis coupled and relative high level of C content was found in *P. pectinfera* with 9.00% dry weight. Furthermore, the water content was observed higher in *L. quinaria* with 64.20% followed by *A. amurensis.* The results of Table 2-3 showed that there are no great differences on the micronutrient content among starfish species.

2.4.3 Yield of crude saponin

According to the saponin extraction procedure in Fig. 2-1, the water extracts obtained from four different starfish were lyophilized. The highest amount of crude saponin was obtained from *A. amurensis* with the value of 7.07 g/kg followed by *A. scoparius* (1.66 g/kg), and *P. pectinifera* (1.47 g/kg). The lowest amount of crude saponin was obtained from *L. quinaria* with the value of 1.25 g/kg yield.

2.4.4 Thin layer chromatography (TLC) analysis

The composition of crude saponin was analyzed by TLC. The results showed differences in the number of discrete spots on TLC, after detection of saponin with 50% sulfuric and 1% cinnamaldehyde staining solutions (Fig. 2-3). Based on the chemical structure, saponin consist two parts, a hydrophobic nucleus (sapogenin and aglycone) which is attaches with different length of sugar (glycone) chains in nature. Sulfuric acid was commonly used for detection of saponin and made a reaction to sugar part, and produced red, purple, yellow, and brown color band. On the other hand, cinnamaldehyde made a reaction to aglycone part and produced green and violet color band. Figure 2-3(a) show the chromatograms of *A. amurensis*. *A. amurensis* crude saponin consisted of several components, with R_f values ranging from 0.31 to 0.82. Figure 2-3(b) show the profiles of crude saponin from *L. quinaria*, *A. scoparius*, and *P. pectinifera*. The R_f values of *L. quinaria*, *A. scoparius*, and *P. pectinifera* were within the range of 0.19 to 0.53. TLC profiles of crude saponin from *L. quinaria*, *A. scoparius*, and *P. pectinifera* shown in Fig. 2-3(b) were quite different from those of crude saponin from *A. amurensis* shown in Fig. 2-3(a). Figure 2-3(c) show the chromatograms of plant saponin derived from quillaja bark and tea seed. Quillaja bark saponin gave several fractions, with R_f values ranging from 0.12 to 0.42, whereas tea seed saponin had R_f values ranging from 0.22 to 0.90 in 50% sulfuric acid and 1% cinnamaldehyde staining solutions. It was thought that the TLC profiles of crude saponin from *L. quinaria*, *A. scoparius*, and *P. pectinifera* were

similar to that of quillaja bark saponin and TLC profiles from *A. amurensis* was similar to that of tea seed saponin.

2.4.5 Antifungal activity

The antifungal spectra of the crude saponin from four starfish against six fungi are presented in Table 2-4 and Fig. 2-4 to 2-9 (as supplementary information). *A. amurensis* and *L. quinaria* exhibited predominant growth inhibitory activity against all the human fungal pathogens tested. Growth inhibitory activity of *A. amurensis* was in the range of 13.9–15.0 mm and 14.4–25.5 mm with amounts of 5 and 10 mg, respectively. The strongest antifungal activity was observed in *A. amurensis* saponin against dermatophyte fungi *T. mentagrophytes*, at 10 mg (Table 2-4 and Fig. 2-9). *A. scoparius* saponin exhibited the highest activity against *A. flavus* with growth inhibitory activity of 17.6 mm (at 10 mg). Remarkable antifungal activity was exhibited by plant saponin from tea seed against *S. cerevisiae* and *T. mentagrophytes* with growth inhibitory activity of 24.1 and 23.1 mm, respectively. Quillaja bark saponin was slightly active only against *S. cerevisiae*.

The crude saponin of *A. amurensis* showed significant antifungal activity against *A. flavus*, *R. glutinis*, *F. oxysporum*, and *T. mentagrophytes* with the MIC value of 31.2, 50.0, and 41.6 µg/mL, respectively (Table 2-5). *L. quinaria* and *A. scoparius* were most active against *T. mentagrophytes* and *A. flavus* with the MIC value of 50.0 µg/mL (Table 2-5). The crude saponin from *P. pectinifera* was most active against *A. flavus* and *F. oxysporum* with the MIC value of 41.6 µg/mL.

2.4.6 Antibacterial activity

The antibacterial activity of starfish crude saponin was tested against four bacterial pathogens. From the results shown in Table 2-6, it was found that *A. amurensis* was slightly active against *E. coli* and *E. tarda*. On the other hand, there was no zone of inhibition in *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera* against *V. parahemolyticus* and *L.*

garvieae.

The crude saponin that showed antibacterial activity in this assay was subjected to the MIC evaluation and the results are presented in Table 2-7. The minimum inhibitory concentrations of crude saponin were determined by serial broth dilution method and *A. amurensis* showed slight inhibitory activity against *E. coli* and *E. tarda* with the MIC value of 250 and 125 μg/mL (Table 2-7).

2.4.7 Hemolytic activity

Hemolytic activity of starfish saponin was evaluated using mouse erythrocytes. Positive control (100 % hemolysis) by distilled water and negative control (0% hemolysis) by PBS were used as standard references. The activity was expressed as the concentrations inducing 50% of erythrocytes hemolysis (HC_{50}) and HC_{50} was determined at the concentrations of 10-100 μg/mL. Results of hemolytic activity showed that starfish crude saponin exhibited the maximum hemolytic activity at 50 to1,000 μg/mL concentration (Fig. 2-10). The HC_{50} values indicate that starfish crude saponin of *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera*, induced strong hemolysis against mouse erythrocyte with HC_{50} values of 19.3 ± 2.08, 22.7 ± 3.21, 23.0 ± 4.00, and 32.7 ± 4.72 μg/mL, respectively. Furthermore, the HC_{50} values of plant saponins (18.9 ± 4.0 μg/mL for quillaja bark and 26.3 ± 4.5 μg/mL for tea seed) were not significantly different from those of starfish saponins such as *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera.*

2.4.8 Cholesterol-binding ability

The cholesterol-binding ability of crude starfish saponin was evaluated with the $FeCl_3$-H_2SO_4 method, and the percentage of cholesterol-binding ability was shown in Fig. 2-11. The cholesterol-binding ability was observed in the saponin of the two starfish of *A. amurensis* and *L. quinaria* with the values of 34.3 and 31.6%, respectively. On the other hand, crude saponin from two starfish *A. scoparius* and *P. pectinifera* showed lower cholesterol-binding

ability with the values of 29.8 and 28.7%. The cholesterol-binding ability was equivalent to that of plant saponins in quillaja bark and tea seed. There was no significant difference observed between starfish and plant saponin.

2.5 Discussion

In this study, the partial 16S rRNA sequences of mitochondrial gene of *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera* were investigated to identify starfish species. Mitochondrial gene order has been demonstrator to be one of the most useful methods for molecular identification (Matsubara et al., 2005). Previous studies have reported that the rapid diversification and adaptation to modern environments may conceal the primitive status of asteroid groups (Blake, 1987; 1988). Therefore, it was hypothesized that the universal primer (16SarL and 16SbrH) could be amplified the partial region of *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera*. As a result, partial 16S rRNA region of all the starfish used in this study could be amplified by using the universal primers (data not shown). PCR products of *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera* had a length of approximately 500 bp (Fig. 2-2).

The micronutrient results (Table 2-3) showed that there were no great differences in the micronutrient contents among species. However, Cd, Cu, and As contents had species-specificity. Ca and P are minerals that have an important role to the development and maintenance of the skeleton, together with many other physiologic functions in the body. Ca content was found to be higher in *A. scoparius* with 16.30% followed by *L. quinaria* with 10.60%. Moisture content of the starfish *L. quinaria* was detected as 64.2%, which was slightly fewer than the starfish of *A. planci* with the value of 67.7 to 69.1% by weight (Luo et al., 2011). When formulating diets for animals (pigs, chicken, and fish) or fertilizer for agriculture sector, it is necessary to consider an appropriate amount of micronutrient content

since excess or deficiency in one of the minerals will cause impaired utilization of the other (Gonzalez-Vega et al., 2013). Living starfish contain on average 1.65% N and 0.15% P of wet weight. Therefore, the removal of 10,000 t of starfish, would directly remove approximately 165 t N and 15 t P from the coastal area (Petersen et al., 2014). Nitrogen is considered to be the most important nutrient, and plants absorb more than any other elements. Thus, starfish has a great possibility to use as plant growth enhancer.

The high yield of crude saponin content in *A. amurensis* was obtained with 7.07 g/kg, compared with other species, whereas Park et al. (2009) reported that the content of crude saponin from *A. amurensis* was 0.009 g/kg. It is well known that saponin content varies from the season and size of species (Yasumoto et al., 1966). The yield variations of the crude saponin content from different starfish were observed with some differences in this study.

The qualitative analysis using TLC plays an important role in the study of saponin. Based on the analysis of the chromatogram and their retention factor of saponin, it was observed that there are several differences between starfish and plant saponin. The composition, number of spot, and polarity of crude saponin detected by TLC analysis also showed the differences among starfish species. Raphaela et al. (2014) reported that the polarity of saponin varied because of the sugar units linked to its structure, suggesting that this difference may affect various characteristics and function of each saponin. The common method for detection of saponin is color reactions (Muetzel et al., 2003; Kerem et al., 2005). The detection by 1% cinnamaldehyde staining solution showed clear well defined spots (Fig. 2-3B. These chromatograms could be preserved at room temperature without any further change in the background up to a month, which was an added advantage. Crude saponin from starfish presented less polar compounds represented by a purple spot on the top of the TLC plate stained with 50% sulfuric acid (Fig. 2-3A). On the other hand, only polar components were detected by 1% cinnamaldehyde stained solution. TLC has the advantage of speed of

analysis and comparison of many samples simultaneously, versatility of supports, solvent systems, and detection reagents (Stahl, 1969). Consequently, determination of the purity and the identity of the substances by their physical constants (melting point) is difficult to achieve (Miyase et al., 1994).

Saponins are a very diverse group of compounds with variations of sapogenin, the sugar length, sugar linkage, and the substituent on sugars (Hostettmann and Marston, 1995). The chemical and pharmacological properties of plant saponin have been published in sufficient literature. However, very few information on starfish saponins are available. Therefore, in this study, the antifungal, antibacterial, hemolytic, and cholesterol-binding ability of crude saponin extracted from four different starfish (*A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera*) were examined and then compared with two other common commercial plant saponin (quillaja bark and tea seed) which are widely used at industrial scale.

The present study revealed that all of starfish saponins have antifungal activity. Crude saponin extracted from *A. amurensis* showed predominant growth against *S. cerevisiae*, *R. glutinis*, *A. flavus*, and *T. mentagrophytes* (Table 2-4). Choi et al. (1999) noted that the methanol and water extracts of *P. pectinifera* were sensitive to *A. niger* and *T. mentagropytes*. The report supports this study that crude saponin obtained from *P. pectinifera* exhibited highest activity against *A. flavus* and *T. mentagrophytes*. Recently, Suguna et al. (2014) reported that the highest antifungal activity found in *n*-butanol extract of *L. maculate* against *T. mentagrophytes* was 21.0 ± 1.00 mm. However, in this study, crude saponin from *L. quinaria* showed the zone inhibition activity of 14.3 ± 1.15 mm against *T. mentagrophytes* with an amount of 10 mg. The difference in antifungal activity of saponin from *L. maculate* and *L. quinaria* against *T. mentagrophytes* suggested that saponin from various species, extracted using different procedures, differ in their biological activities (Sen et al., 1998).

Antibacterial activity of saponin also differs among the bacterial strains and by the extraction procedure of saponin. Extraction methods and fat content have important effects on the antibacterial activity of the resultant saponin (Hasan et al., 2010). The antibacterial results showed that the crude saponin extracted from *A. amurensis* had slight antibacterial activity against *E. coli* and *E. tarda*. There were no active in the saponin of two starfish, *A. scoparius* and *P. pectinifera*. On the other hand, all the four starfish crude saponin showed antifungal activity against six different human fungal pathogens. The mode of action of antibacterial and antifungal activity of saponin is not yet clear.

Saponins are known to exhibit hemolytic activity (Xie et al., 2008) which is strongly interrelated with the nature of both the aglycone and sugar side chains (Oda et al., 2000). Results of hemolytic activity assays showed that *A. amurensis* exhibited strong activity against mouse erythrocytes. The present results support the findings that Imamichi and Yokoyama. (2013) reported that crude extract from pyloric caeca of *A. amurensis* showed high hemolytic activity in rabbit erythrocytes. This study agrees with Choi et al. (2001) who reported that all saponin not have hemolytic activity. This is most likely because of different compositions of saponin causing different activity.

Cholesterol-binding ability was also determined in this study as one of the other functionality. The complex formation between cholesterol and plant saponin has been investigated in the past few decades. However, the mechanism remains unclear what happened to the cholesterol in the membrane after binding by the saponin (Stefan and Matthias, 2013). The result in this study indicates that saponin of the starfish *A. amurensis* and *L. quinaria* had high cholesterol-binding ability similar to that of tea seed saponin (Fig. 2-11). Matsui et al. (2006) noted that tea seed saponin has a significant effect to reduce serum cholesterol. Although a number of studies proved that plant saponin such as soybean, quillaja, and tea seed have cholesterol-binding ability in serum (Southon et al., 1988; Matsuura, 2001),

there was no information on cholesterol-binding ability of starfish saponin. The results of this study are the first report on cholesterol-binding ability of starfish saponin. The study proves that starfish saponin has the ability to bind cholesterol with the same efficiency as plant saponin, suggesting that starfish saponin could be used as a cholesterol lowering agent.

These four series (antifungal, antibacterial, hemolytic, and cholesterol-binding ability) of data in this study clearly showed that the biological activity of saponin varied in their composition of saponin. The crude saponin of starfish species, *A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera* had good inhibitory selectivity on the tested fungi and cholesterol-binding ability with advancing the hemolytic activity, suggesting the occurrence of diversity of saponin components and the difference in functionality among starfish species. The specific mode of action for their biological activity of saponin is not yet clear. However, it seemed that the antifungal, antibacterial, hemolytic, and cholesterol-binding ability of saponin may be affected by many factors such as the aglycone, number, position, and chemical structure of sugar side-chains (Raphaela et al., 2014). It is noteworthy that the functionality of each different starfish saponin had great individual variability.

2.6 Conclusion

Starfish saponin possessed various biological activities. In this study, four functional properties such as antifungal, antibacterial activity, hemolytic, and cholesterol-binding ability were investigated in the Japanese starfish saponin. The antifungal activity result differed among starfish. The crude saponin extracted from starfish demonstrated the difference composition of saponin which was confirmed by the functional properties result as well as TLC analysis. These functional properties results indicated that starfish are a good resource for obtaining the biological active substances for antifungal and cholesterol-binding effects.

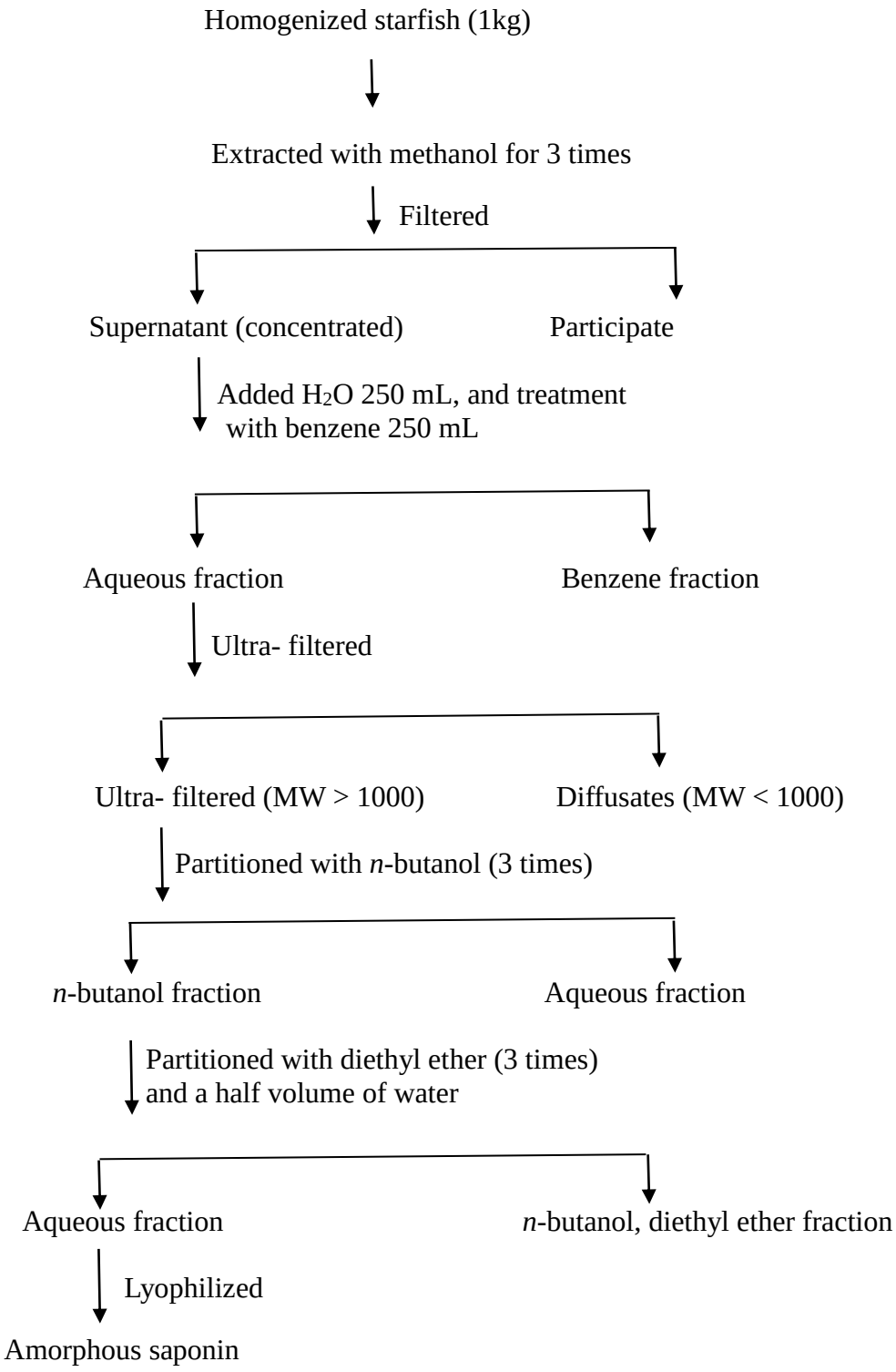


Fig. 2-1 Extraction procedure for crude saponin from starfish by using several organic solvents.

A)

```
Sample A          GAACTCTCCAAAAAAATTACGCTGTTATCCCTGCGGTAACTTATTCCTTTGCTCGCTATC  60
Asterias amurensis GAACTCTCCAAAAAAATTACGNTGTTAT--CTGCGGTAACTTATTCCTTTGCTCGCTATC  58
                  ********************** ******   ******************************

Sample A          TAGCGGATCACTTTATTTTAAAATGGTTATTTTTTATGTTTTTAGCGGAGGCTTTTTATA 120
Asterias amurensis TAGCGGATCACTTTATTTTAAAATGGTTATTTTTTATGTTTTTAGCGGAGGCTTTTTATA 118
                  ************************************************************

Sample A          CTCCGCGATTGCCCCAATCAAAGTTAGTATATTAAAAGGCTAAGAATAAAAAATTAGATT 180
Asterias amurensis CTCCGCGATTGCCCCAATCAAAGTTAGTATATTAAAAGGCTAAGAATAAAAAATTAGATT 178
                  ************************************************************

Sample A          TTATAAAATTTTTATAAGTTTAACATTAGATTTATTATTCTTTATTACCACTAATATTTT 240
Asterias amurensis TTATAAAATTTTTATAAGTTTAACATTAGATTTATTATTCTTTATTACCACTAATATTTT 238
                  ************************************************************

Sample A          TACTAAAGCTCGACAGGGTCTTCTCGTCCTACGAGTTTATTTCCGCTTCTTCACGAAAAT 300
Asterias amurensis TACTAAAGCTCGACAGGGTCTTCTCGTCCTACGAGTTTATTTCCGCTTCTTCACGAAAAT 298
                  ************************************************************

Sample A          ATAAAATTCAAGTTATAAGAAAGAGACAGCTTAACCCCAGTCTTGCCA-TTCATACCAGC 359
Asterias amurensis ATAAAATTCAACTTATAAGAAAGAGACAGCTTAACCCCAGTCTTGCCACTTCATACCAGC 358
                  ************ ************************************ ************

Sample A          CTCTATTTAAGAGGCAAATGATTATGCTACCTTTGCAC 397
Asterias amurensis CTCTATTTAAGAGGCAA-TGATTATGCTACCTTTGCAC 395
                  ****************** ********************
```

B)

```
Sample B          ---CTGCCCAGTGACTTAGTTAAACGGCCGCGGTATCTTGACCGTGCAAAGGTAGCATAA  57
Luidia quinaria   TGCCTGCCCAGTGACTTAGTTAAACGGCCGCGGTATCTTGACCGTGCAAAGGTAGCATAA  60
                     ************************************************************

Sample B          TCATTTGCCTCTTAAATAGAGGCTGGTATGAATGGCAAGACTGGGGTTAAGCTGTCTCTT 117
Luidia quinaria   TCATTTGCCTCTTAAATAGAGGCTGGTATGAATGGCAAGACTGGGGTTAAGCTGTCTCTT 120
                  ************************************************************

Sample B          TCTTATAACTTGAATTTTATATTTTCGTGAAGAAGCGGAAATAAACTCGTAGGACGAGAA 177
Luidia quinaria   TCTTATAACTTGAATTTTATATTTTCGTGAAGAAGCGGAAATAAACTCGTAGGACGAGAA 180
                  ************************************************************

Sample B          GACCCTGTCGAGCTTTAGTAAAAATATTAGTGGTAATAAAGAATAATAAATCTAATGTTA 237
Luidia quinaria   GACCCTGTCGAGCTTTAGTAAAAATATTAGTGGTAATAAAGAATAATAAATCTAATGTTA 240
                  ************************************************************

Sample B          AACTTATAAAAATTTTATAAAATCTAATTTTTTATTCTTAGCCTTTTAATATACTAACTT 297
Luidia quinaria   AACTTATAAAAATTTTATAAAATCTAATTTTTTATTCTTAGCCCTTTAATATACTAACTT 300
                  ********************************************** ***************

Sample B          TGATTGGGGCAATCGCGGAGTATAAAAATCCTCCGCTAAAAACATAAAAAA-TAACCATT 356
Luidia quinaria   TGATTGGGGCAATCGCGGAGTATAAAAAGCCTCCGCTAAAAACATAAAAAAATAACCATT 360
                  ****************************** ********************  ********

Sample B          TTAAAATAAAGTGATCCGCTACATAGCGAGCAAAGGAATAAGTTACCGCAGGGATAACAG 416
Luidia quinaria   TTAAAATAAAGTGATCCGCTAGATAGCGAGCAAAGGAATAAGTTACCGCAGGGATAACAG 420
                  ********************** ***************************************

Sample B          CGTAATTTTTTTGGAGAGTTCATATTGATAAAAAAGTTTGCGACCTCGATGTTGGATCGA 476
Luidia quinaria   CGTAATTTTTTTGGAGAGTTCATATTGATAAAAAAGTTTGCGACCTCGATGTTGGATCGA 480
                  ************************************************************

Sample B          GATTTCCTAGAGATGCAGCAGTCTCT 502
Luidia quinaria   GATTTCCTAGAGATGCAGCAGTCTCT 506
                  **************************
```

Fig. 2-2 Aligned nucleotide sequences of partial mitochondrial 16S ribosomal RNA gene from *A. amurensis* (A), *L. quinaria* (B), *A. scoparius* (C), and *P. pectinifera* (D*)* starfish species. The identical nucleotides were shown by dots.

C)

```
Sample C              CATTT-GCCTTTTAAATGGAGGCTAGTATGAATGGCAAGACGGGGGTTTAGCTGTCTCTC  59
Astropecten scoparius CGTTTTGCCTTTTAA-TGGAGGCTAGTATGAATGGCAAGACGGGGGTTTAGCTGTCTCTC  59
                      *  ***  *********  **********************************************

Sample C              TTTTAGGAGCTTGAATTTATTATCTTAGTGAAGAAACTAAGATAAAGTCGTAGGACGAGA 119
Astropecten scoparius TTTTAGGAGCTTGAATTTATTATCTTAGTGAAGAAACTAAGATAAGGTCGTAGGACGAGA 119
                      ********************************************  **************

Sample C              AGACCCTATCGAGCTTTAGCTATTAGTTAAAGTTAAGGGTTTTTTATTGTTAAGCTAATA 179
Astropecten scoparius AGACCCTATCGAGCTTTAGCTATTAGTTAAAGTTAAGGGTTTTTTATTGTTAAGCTAATA 179
                      ************************************************************

Sample C              AAGTTAAAAATTGATTTTACTTAATAATAAGAATAAACTGTTAATAAAGTGTTTTGGTTG 239
Astropecten scoparius AAGTTAAAAATTGATTTTACTTAATAATAAGAATAAACTGTTAATAAAGTGTTTTGGTTG 239
                      ************************************************************

Sample C              GGGCAACCACGGAGAAAAATCAACCTCCGGTTATAAAATAGAAAAAATTACTATTTTGTG 299
Astropecten scoparius GGGCAACCACGGAGAAAAATCAACCTCCGGTTATAAAATAGAAAAAATTACTATTTTGTG 299
                      ************************************************************

Sample C              AAATGTAAATTATTAAGAAGTGATCCACTGTGAAAGTGATCAAAGGAACAAGTTACCGTA 359
Astropecten scoparius AAATGTGAATTATTAAGAAGTGATCCACTGTGAAAGTGATCAAAGGAACAAGTTACCGTA 359
                      ******  ********************************************************

Sample C              GGGATAACAGCGTAATTTTTTTGGAGAGTTCATATTGATAAAAAAGTTTGCGACCTCGAT 419
Astropecten scoparius GGGATAACAGCGTAATTTTTTTGGAGAGTTCATATTGATAAAAAAGTTTGCGACCTCGAT 419
                      ************************************************************

Sample C              GTTGGATCGGGACTTCCAGGAGATGCAGCAGTTTCCAAGGGTTG---------------- 463
Astropecten scoparius GTTGGATCGGGACTTCCAGGAGATGCAGCAGTTTCCAAGGGTTGGTCTGTTCGACCATTA 479
                      ********************************************
```

D)

```
Sample D              TGCCTGCCCAGTGACTTAGTTAAACGGCCGCGGTATCTTGACCGTGCAAAGGTAGCATAA  60
Patiria pectinifera   TGCCTGCCCAGTGACTTAGTTAAACGGCCGCGGTATCTTGACCGTGCAAAGGTAGCATAA  60
                      ************************************************************

Sample D              TCATTTGCCTCTTAAATGGGGGCTGGTATGAATGGCAAGACGGGAATCAAGCTGTCTCTC 120
Patiria pectinifera   TCATTTGCCTCTTAAATGGGGGCTGGTATGAATGGCAAGACGGGAATCAAGCTGTCTCTC 120
                      ************************************************************

Saample D             CCTTATATCTTGAAGTTAATATTTCTGTGAAGAAGCAGAAATGAGATCGCAGGACGAGAA 180
Patiria pectinifera   CCTTATATCTTGAAGTTAATATTTCTGTGAAGAAGCAGAAATGAGATCGCAGGACGAGAA 180
                      ************************************************************

Sample D              GACCCTATCGAGCTTTAGTAGAAATAAGATATAA-CTTGTTAATAGTGTTTGTGCTGCTA 239
Patiria pectinifera   GACCCTATCGAGCTTTAGTAGAAATAAGATATAAGCTTGTTAATAGTGTTTGTGCTGCTA 240
                      ***********************************  *************************

Sample D              ATTCAATTGTTAAAAACAGTTCCTATAGTTTTACTGCTAAGAACATTAGTGTTGTATATA 299
Patiria pectinifera   ATTCAATTGTTAAAAACAGTTCCTATAGTTTTACTGCTAAAAACATTAGTGTTGTATATA 300
                      ******************************************  ******************

Sample D              TAATACTTAGGTTGGGGCAACCGCGGAGAATAATTACCCTCCGTTAATTTTATTGAAAAA 359
Patiria pectinifera   TAATACTTAGGTTGGGGCAACCGCGGAGAATAATTATCCTCCGTTAATTTTATTGAAAAA 360
                      ***********************************  *************************

Sample D              ATAATTATTTTTCAATATAAGATTTATTAAAAGAGTGATCCACTGAAATGGTGAGCAAAA 419
Patiria pectinifera   ATAATTATTTTTCAATATAAGATTTATTAAAAGAGTGATCCACTGAAATGGTGAGCAAAA 420
                      ********************************  ********************************

Sample D              GAATAAGTTACCGTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATTGATAAAAAA 479
Patiria pectinifera   GAATAAGTTACCGTAGGGATAACAGCGTAATTTTTTTGGAGAGTTCATATTGATAAAAAA 480
                      ************************************************************

Sample D              GTTTGCGACCTCGATGTTGGATCGGGATT-CCTGGGGATGCAGCAGTCCC-  528
Patiria pectinifera   GTTTGCGACCTCGATGTTGGATCGGGATTTCCTGGGGATGCAGCAGTTCCC  531
                      ********************************  *****************  **
```

Fig. 2-2 continued.

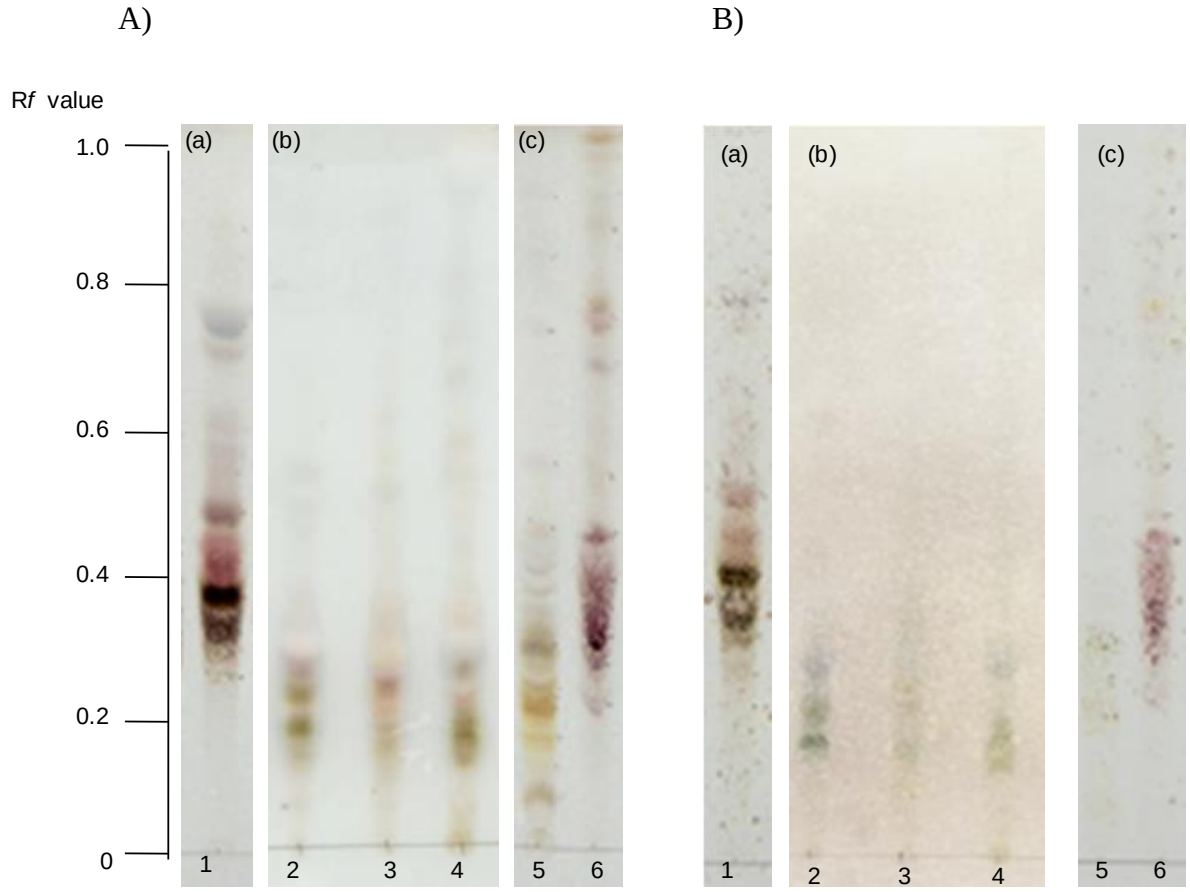


Fig. 2-3 Thin layer chromatography (TLC) pattern of obtained crude saponins.

Solvent, chloroform: methanol: water (65:35:10, lower phase), staining with 50% H_2SO_4 (A) and 1% cinnamaldehyde solution (B). 1, *A. amurensis*; 2, *L. quinaria*; 3, *A. scoparius*; and 4, *P. pectinifera*; 5, quillaja bark; 6, tea seed.

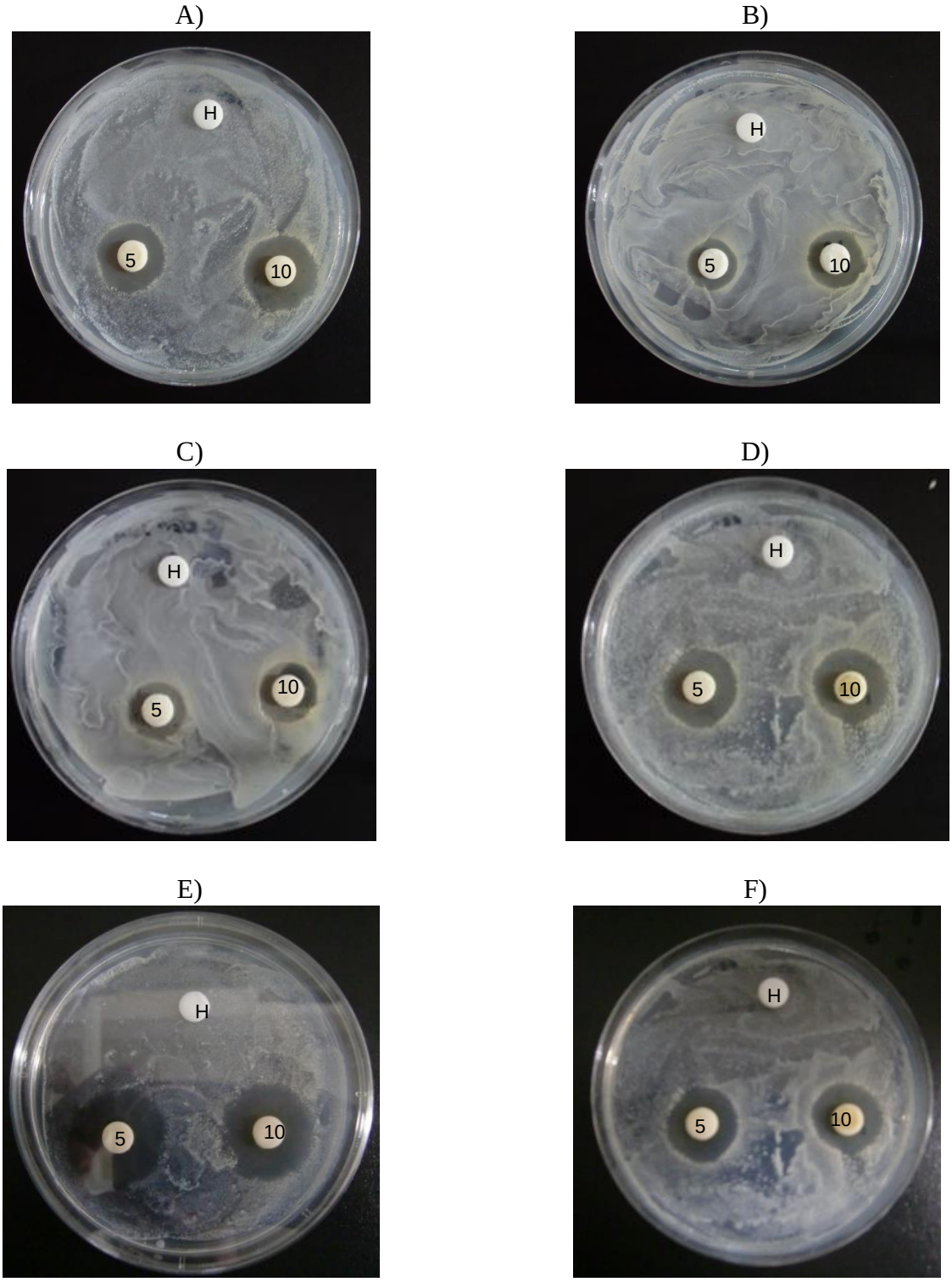


Fig. 2-4 Antifungal activity of starfish crude saponin against *S. cerevisiae* JCM 21974. (A), *A. amurensis*; (B), *L. quinaria*; (C), *A. scoparias*; (D), *P. pectenifera*; (E), Tea seed; (F), Quillija bark. H, sterilized water; 5, 5 mg of crude saponin; 10, 10 mg of crude saponin.

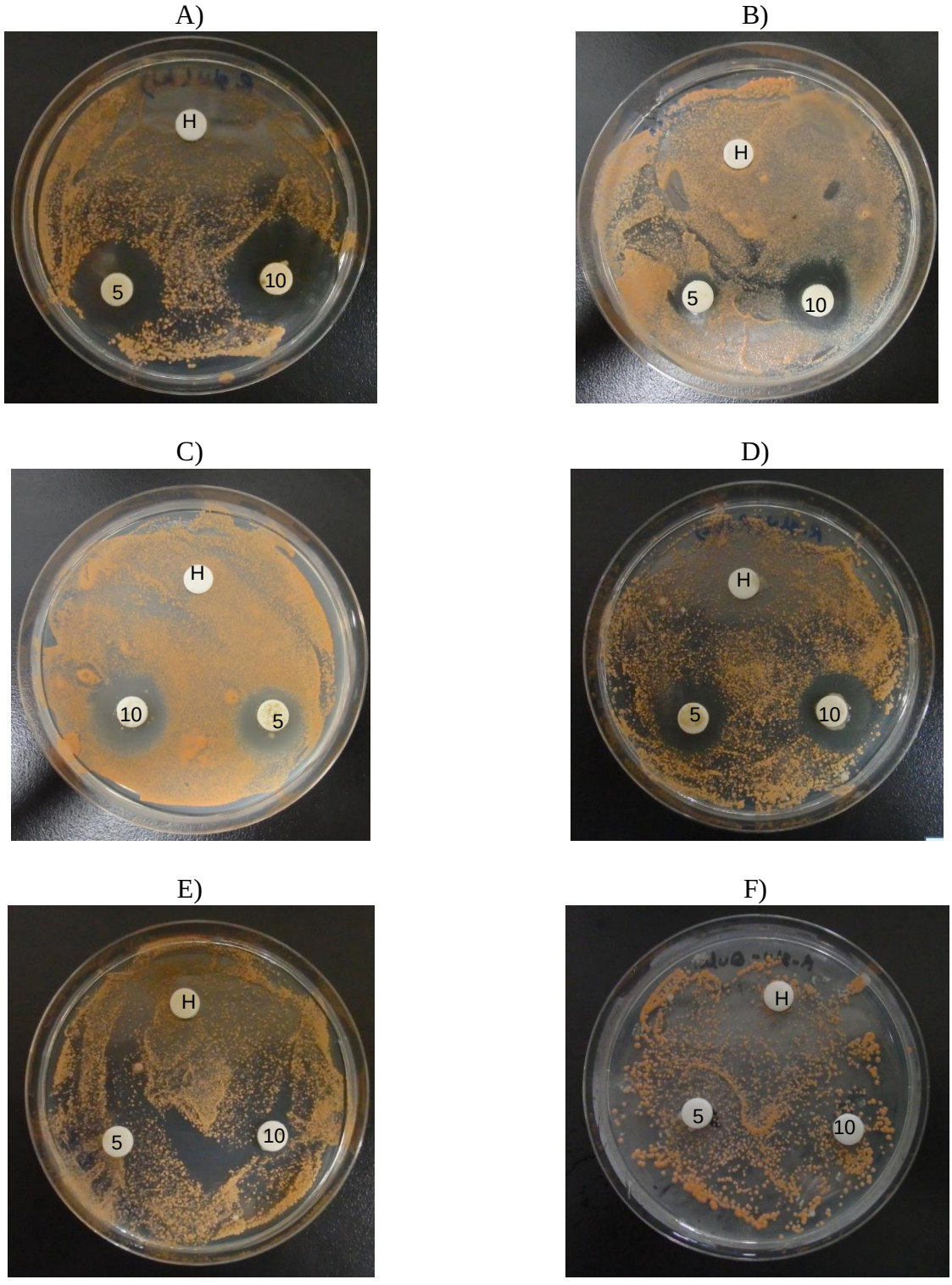


Fig. 2-5 Antifungal activity of starfish crude saponin against *R. glutinis* JCM 8173. (A), *A. amurensis*; (B), *L. quinaria*; (C), *A. scoparias*; (D), *P. pectenifera*; (E), Tea seed; (F), Quillija bark. H, sterilized water; 5, 5 mg of crude saponin; 10, 10 mg of crude saponin.

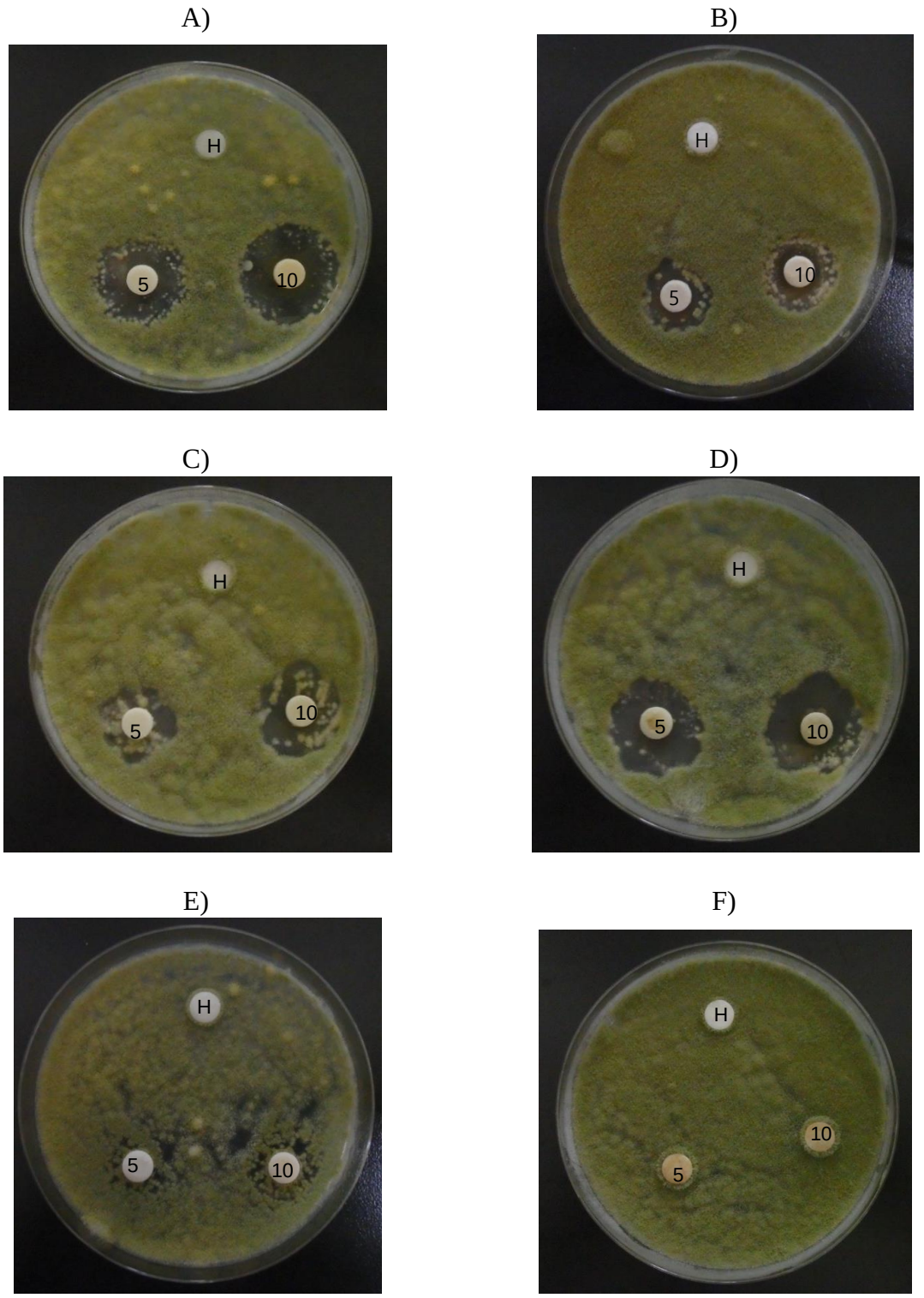


Fig. 2-6 Antifungal activity of starfish crude saponin against *A. flavus* JCM 12721. (A), *A. amurensis*; (B), *L. quinaria*; (C), *A. scoparias*; (D), *P. pectenifera*; (E), Tea seed; (F), Quillija bark. H, sterilized water; 5, 5 mg of crude saponin; 10, 10 mg of crude saponin.

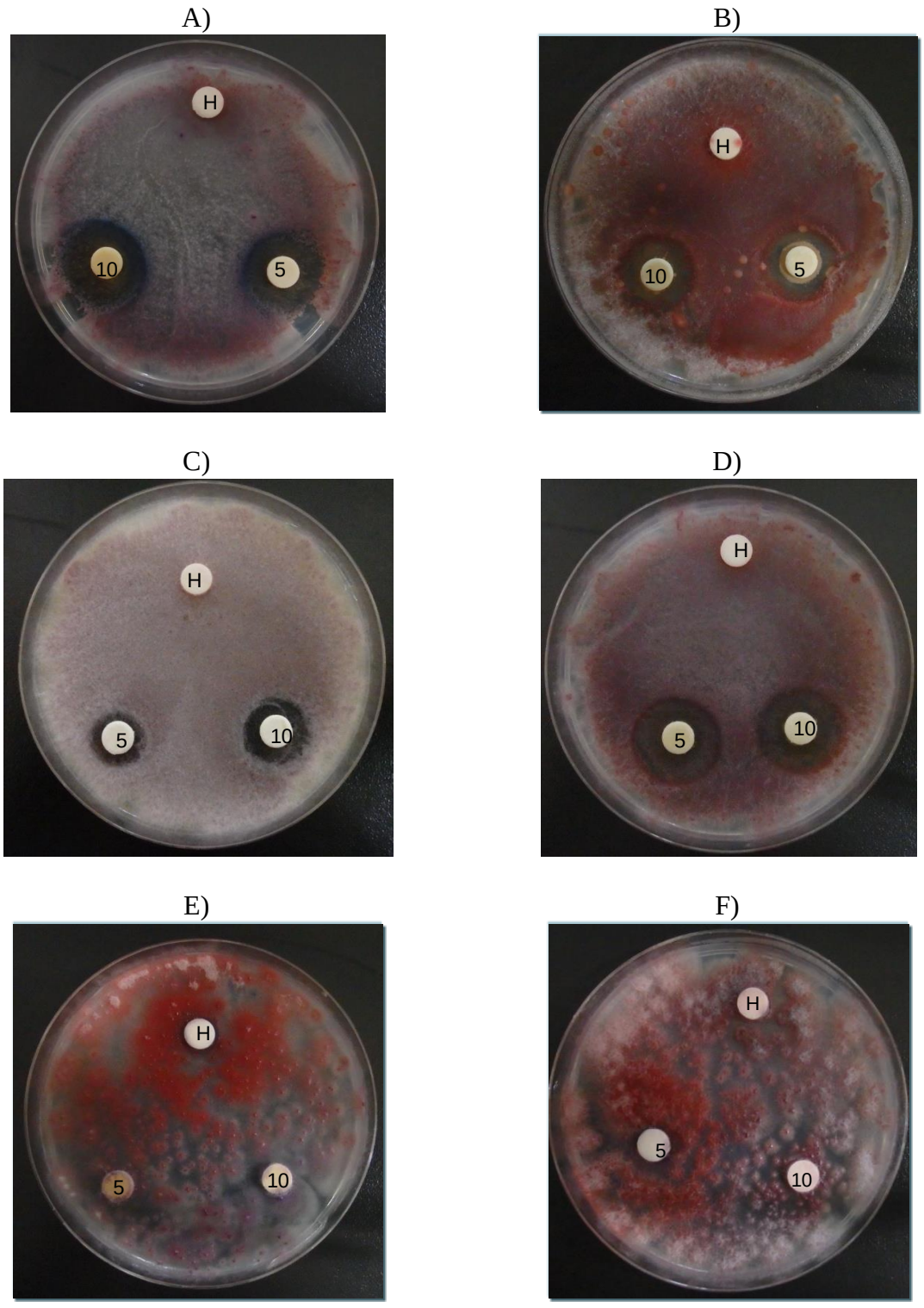


Fig. 2-7 Antifungal activity of starfish crude saponin against *F. oxysporum* NBRC 5942. (A), *A. amurensis*; (B), *L. quinaria*; (C), *A. scoparias*; (D), *P. pectenifera*; (E), Tea seed; (F), Quillija bark. H, sterilized water; 5, 5 mg of crude saponin; 10, 10 mg of crude saponin.

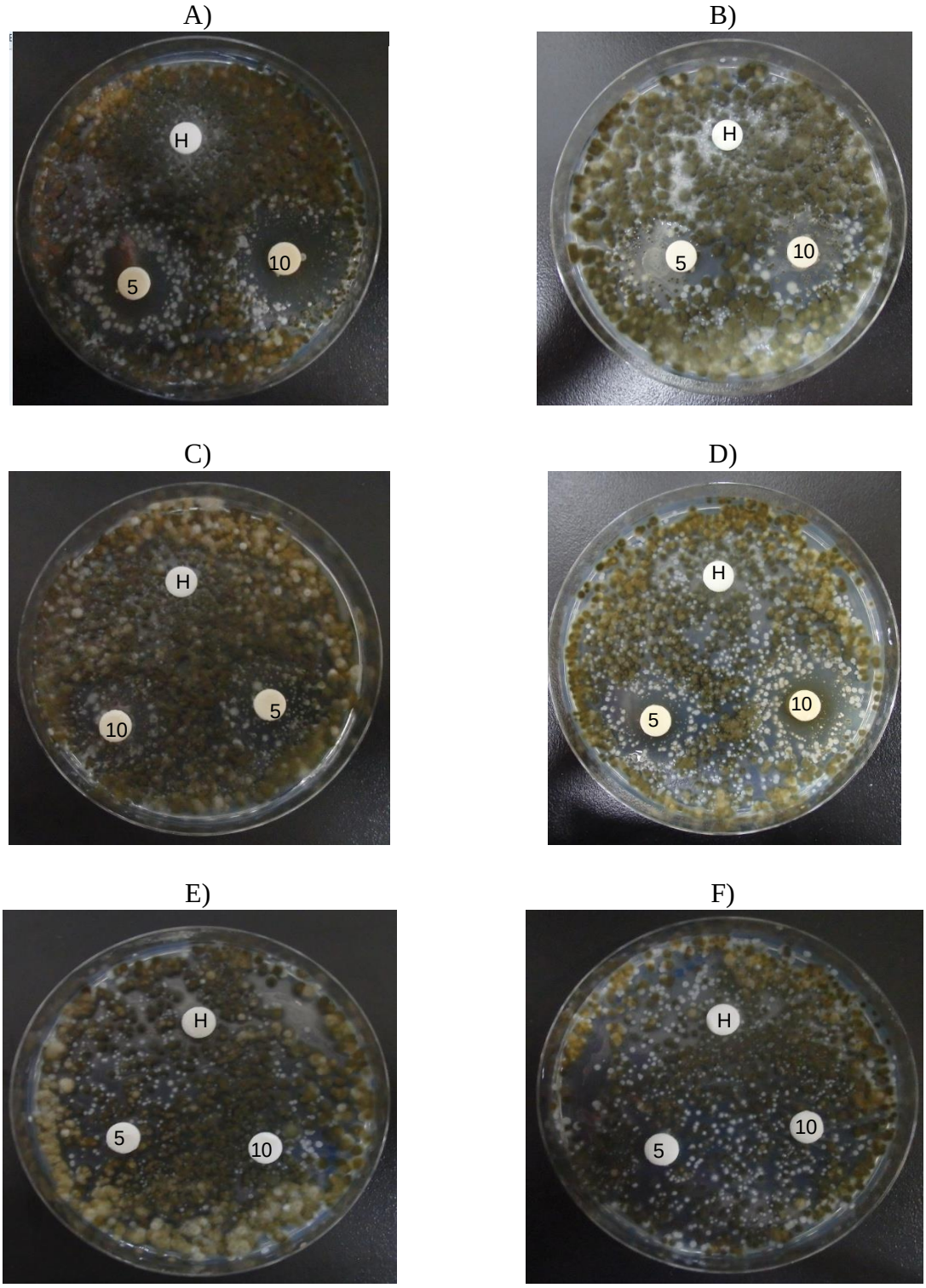


Fig. 2-8 Antifungal activity of starfish crude saponin against *C. sphaerospermum* NBRC 4460. (A), *A. amurensis*; (B), *L. quinaria*; (C), *A. scoparias*; (D), *P. pectenifera*; (E), Tea seed; (F), Quillija bark. H, sterilized water; 5, 5 mg of crude saponin; 10, 10 mg of crude saponin.

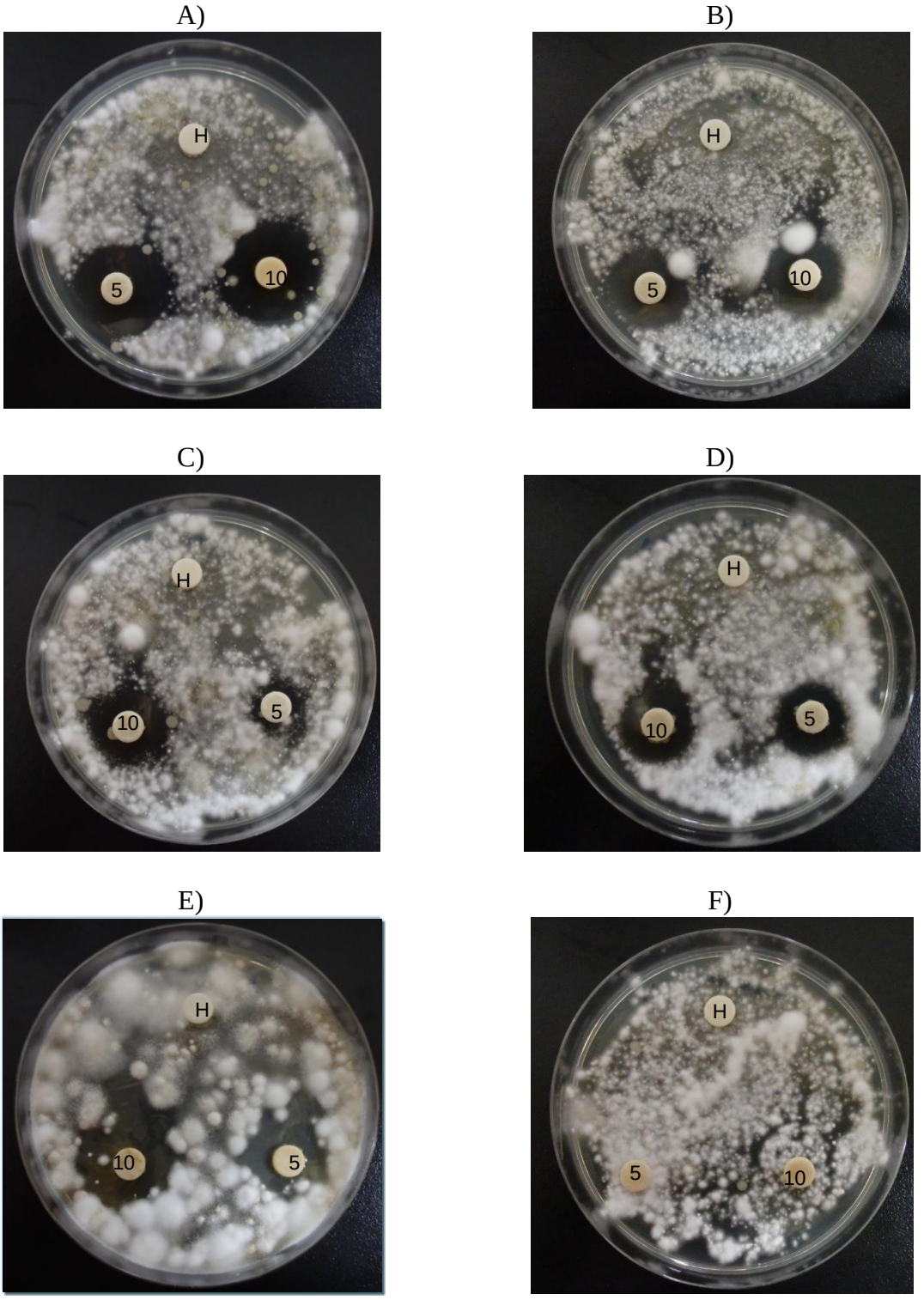


Fig. 2-9 Antifungal activity of starfish crude saponin against *T. mentagrophyte* NBRC 32410. (A), *A. amurensis*; (B), *L. quinaria*; (C), *A. scoparias*; (D), *P. pectenifera*; (E), Tea seed; (F), Quillija bark. H, sterilized water; 5, 5 mg of crude saponin; 10, 10 mg of crude saponin.

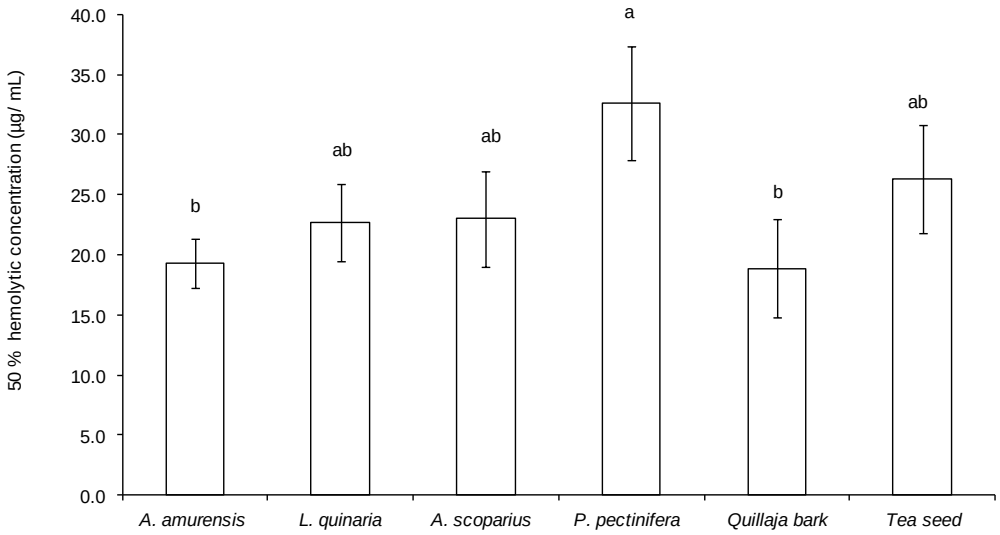


Fig. 2-10 Hemolytic activity of crude saponin from starfish against mouse erythrocytes. Hemolytic activity was expressed as 50% hemolytic concentration (HC_{50}) and compared using one-way ANOVA followed by the Tukey's test. Significant differences ($p < 0.05$) are represented by different letters over the columns.

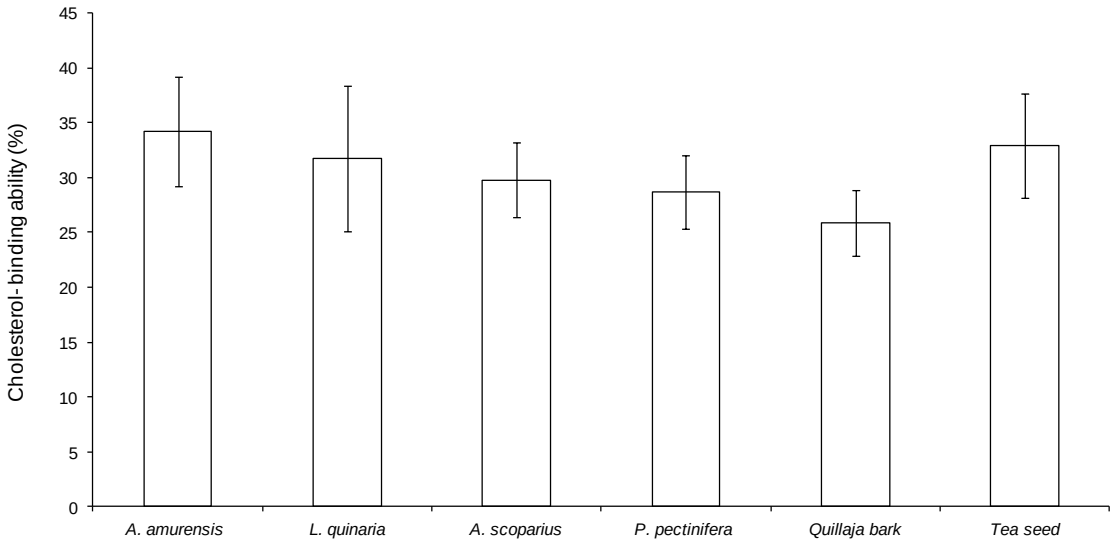


Fig. 2-11 Cholesterol-binding ability of crude saponin from starfish.

The results were compared using one-way ANOVA followed by the Tukey's test.

Significant differences ($p<0.05$) are represented by different letters over the colume.

Table 2-1 Strain name and culture condition of fungi used in this experiment

Fungal strain	Medium	Broth	Incubation time (hrs)	Incubation temperature (ºC)
Yeast				
Saccharomyces cerevisiae JCM 21974	PDA	PDB	24	30
Rhodotorula glutinis JCM 8173	PDA	PDB	48	30
Filamentous				
Aspergillus flavus JCM 12721	PDA	PB	48	27
Fusarium oxysporum NBRC 5442	PDA	PB	48	27
Cladosporium sphaerospermum NBRC 4460	PDA	PB	48	27
Dermatophyte				
Trichophytes mentagrophytes NBRC 32410	SA	707	48	28

PDA, potato dextrose agar; PDB, potato dextrose broth; PB, phosphate buffer; SA, sabouraud agar.

Table 2-2 Strain name and culture condition of bacteria used in this experiment

Bacterial strain	Medium	Broth	Incubation time(hrs)	Incubation temperature (ºC)
Vibrio parahemolyticus NBRC 12711	TSA	TSB	24	37
Escherichia coli JCM 109	LBA	LBB	24	37
Edwardsiella tarda GTC 03825T	TSA	TSB	24	37
Lactococcus garvieae JCM 8735	TSA	TSB	24	37

TSA, tryptic soy agar; TSB, tryptic soy broth; LBB, luria-bertani broth; LBA, luria-bertani agar.

Table 2-3 Micronutrient contents of four starfish collected from Kobe coast, Japan

Content	Unit	*A. amurensis*	*L. quinaria*	*A. scoparius*	*P. pectinfera*	Method
Water	%	63.90	64.20	50.00	58.90	105ºC
C	%	8.10	6.50	7.70	9.00	CTC
N	%	1.50	0.87	0.67	1.60	CTC
P	%	0.06	0.05	0.04	0.06	ICP
K	%	1.04	0.49	0.48	0.93	ICP
Na	%	0.32	0.25	0.28	0.34	ICP
Ca	%	9.40	10.60	16.30	10.40	ICP
Mg	%	0.60	0.78	1.50	1.00	ICP
Cd	μg/g	0.13	0.03	0.05	1.70	ICP
Cu	μg/g	2.10	2.50	4.90	10.00	ICP
Pb	μg/g	0.25	0.64	0.58	0.43	ICP
Hg	μg/g	0.03	0.02	0.01	0.06	AAS
As	μg/g	2.10	0.77	0.92	2.10	ICP

CTC, comblustion thermal conductivity method; ICP, inductively coupled plasma spectrophotometer method; AAS, atomic absorbtion spectrometer method.

Table 2-4 Antifungal activity of starfish crude saponin against six fungi with two different doses (5 and 10 mg)

	Zone of inhibition (mm)											
	S. cerevisiae		*R. glutinis*		*A. flavus*		*C. sphaerospermum*		*F. oxysporum*		*T. mentagrophytes*	
	5*	10*	5	10	5	10	5	10	5	10	5	10
Starfish												
A. amurensis	13.9 ± 1.72	23.4 ± 1.80	14.3 ± 0.84	23.5 ± 2.20	15.0 ± 0.71	24.8 ± 0.44	14.0 ± 0.44	14.4 ± 0.66	14.7 ± 1.25	14.7 ± 0.57	14.7 ± 1.00	25.5 ± 1.18
L. quinaria	14.0 ± 1.00	16.5 ± 0.95	10.0 ± 0.70	12.0 ± 1.00	13.7 ± 1.50	12.7 ± 1.15	14.0 ± 1.00	13.7 ± 0.57	14.3 ± 1.15	14.7 ± 0.57	14.1 ± 1.80	14.3 ± 1.15
A. scoparius	7.3 ± 0.21	9.3 ± 1.22	8.8 ± 0.91	13.0 ± 1.00	13.0 ± 0.51	17.6 ± 1.09	7.2 ± 1.59	9.8 ± 0.83	7.0 ± 2.05	9.2 ± 1.75	7.2 ± 0.74	14.7 ± 0.88
P. pectinifera	14.1 ± 0.87	15.3 ± 0.70	10.0 ± 0.58	14.8 ± 0.26	16.5 ± 0.75	17.9 ± 1.06	9.8 ± 1.00	9.3 ± 0.59	9.3 ± 1.18	16.2 ± 0.68	8.7 ± 1.52	15.0 ± 1.00
Plant												
Quillaja bark	9.9 ± 1.58	11.1 ± 1.27	-	-	-	-	-	-	-	-	-	-
Tea seed	21.1 ± 1.23	24.1 ± 1.23	7.2 ± 1.76	9.3 ± 1.87	-	-	-	-	-	-	22.0 ± 1.03	23.1 ± 1.76

Data are presented as mean ± standard deviation (SD) of 3 independent replicates in 2 separate experiments. -, no measurable zone. Crude saponin was added with amounts of 5 and 10 mg.

Table 2-5 Minimum inhibitory concentration (MIC) of four starfish crude saponin against six fungal strains

	MIC (μg/mL)					
	S. cerevisiae	*R. glutinis*	*A. flavus*	*F. oxysporum*	*C. sphaerospermum*	*T. mentagrophytes*
Starfish						
A. amurensis	62.5	50.0	31.2	50.0	83.3	41.6
L. quinaria	83.3	125.0	83.3	83.3	125.0	50.0
A. scoparius	83.3	83.3	50.0	83.3	83.3	125.0
P. pectinifera	62.5	83.3	41.6	41.6	83.3	50.0
Plant						
Quillaja bark	83.3	-	-	-	-	-
Tea seed	31.2	-	-	-	-	31.2

Data are presented 3 independent replicates. -, no measurable zone. Crude saponin was added with amount of 500 μg/mL.

Table 2-6 Antibacterial activity of starfish crude saponin against four bacterial strains with two different doses (5 and 10 mg)

	Zone of inhibition (mm)							
	V. parahemolyticus		*E. coli*		*E. tarda*		*L. garvieae*	
	5	10	5	10	5	10	5	10
Starfish								
A. amurensis	-	-	ND	+	+	+	-	-
L. quinaria	-	-	-	-	-	-	-	-
A. scoparius	-	-	-	-	-	-	-	-
P. pectinifera	-	-	-	-	-	-	-	-
Plant								
Quillaja bark	-	-	-	-	-	-	-	ND
Tea seed	-	-	-	-	-	-	-	-

Data are presented as mean ± standard deviation (SD) of 3 independent replicates in 2 separate experiments. -, no measurable zone; ND, not detected. Crude saponin was added with amounts of 5 and 10 mg.

Table 2-7 Minimum inhibitory concentration (MIC) of four starfish crude saponin against four bacterial strains

	MIC (μg/mL)			
	V. parahemolyticus	*E. coli*	*E. tarda*	*L. garvieae*
Starfish				
A. amurensis	-	250	125	-
L. quinaria	-	-	-	-
A. scoparius	-	-	-	-
P. pectinifera	-	-	-	-
Plant				
Quillaja bark	-	-	-	ND
Tea seed	-	-	-	-

Data are presented 3 independent replicates. -, no measurable zone; ND, not detected.
Crude saponin was added with amount of 500 μg/mL.

Chapter 3

Fractionation and the functional properties of *A. amurensis* crude saponin

3.1 Introduction

A. amurensis is a rich source of various classes of secondary metabolites including carotenoids, ceramides, and cerebrosides (Ishii et al., 2006 and 2007; Feng-Juan et al., 2013), and steroidal saponins (Hwang et al., 2011). Saponin consists of a sugar moiety such as glucose, galactose, pentoses, and xylose combined with a hydrophobic aglycone (sapogenin) (Ishizaki et al., 1997; Oakenfull and Sidhu, 1989). It is a complex and unique chemical structure molecule. Hence, the isolation and structure elucidation of saponin obtained from different sources is complicated due to the occurrence of many closely related substances (Francis et al., 2002). Thus, for many years, the complete characterization of saponin even from well-known saponin-containing plants was not achieved. In contrast, until to date saponin (asterosaponins) from starfish was isolated in few species (D'Auria et al., 1993). Chemical investigation of *A. amurensis* can be traced back to 1966 when its glycosidic components were reported to have biological activity (Yasumoto et al., 1966). However, the isolation techniques at that time were insufficient for separation and characterization of the active molecules. Subsequent research on the species led to the isolation and structural elucidation. Seven asterosaponins including Thornasteroside A, Versicoside A, Anasteroside B, Asteronyl pentaglycoside sulfate, and three steroidal glycoside were isolated from *A. amurensis* (Hwang et al., 2011; Hwang et al., 2014). These molecules possess the same asterosaponin type of aglycone, characterized by various functionalities (D'Auria et al., 1993; Hwang et al., 2014). It was suggested in some studies that asterosaponins play an important role to defense against predators. However, there is no specific method for isolation of single saponin component.

Therefore, in order to isolate single saponin component fractionation study was examined in this chapter. In Chapter 2, four starfish (*A. amurensis*, *L. quinaria*, *A. scoparius*, and *P. pectinifera*) crude saponin were extracted and investigated their antifungal, hemolytic, and cholesterol-binding ability. Since *A. amurensis* crude saponin possessed highest antifungal activity, study was conducted with this species. Firstly, crude saponin was fractionated by silica gel column and the obtained fractions were subjected to antifungal, hemolytic, and cholesterol-binding ability test as functional properties. Secondly, the active fractions were further purified by silica gel column chromatography and PR HPLC, and then functional properties were evaluated to identify active components from that species.

3.2 Materials and Methods

3.2.1 A. amurensis saponin fractionation

The crude saponin from *A. amurensis* was subjected to column chromatography [silica gel (200–300 mesh, Merck), column, 2.6 × 100 cm]. The separation was done with isocratic $CHCl_3$: MeOH: H_2O (65:35:10), lower phase. A flow rate was 5 mL/min and 10 mL of each fraction was collected using a fraction collector (SF-2120, ADVANTEC, Japan). Fraction was monitored by spectrophotometer at 208 nm and concentrated using a rotary evaporator (EYELA, CCA 1111, Japan) under reduced pressure at 45ºC and then lyophilized to give seven saponin fractions (Fr. A to G). Fraction D was rechromatographed onto silica gel column chromatography (column, 2.6 × 50 cm) using a stepwise gradient of $CHCl_3$: MeOH: H_2O (90:10:1, 80:20:5, 70:30:10, 65:35:10) to achieve three sub fractions (Fr. D_1 to D_3). Fraction D_3 showed antifungal activity was further purified by RP HPLC equipped with a Tosoh TSK gel, ODS-120A C18 (150× 4.6 mm, 5µm) column. Solvents were as follows: eluent A, MilliQ; eluent B, 100% methanol with a linier gradient (5 to 100%) over 90 min. The mobile phase flow rate was kept constantly at 0.5 mL/min and peaks were detected at

208 nm of UV detection to yield three subs Fr. D_{3-1} to D_{3-3}. On other hand, Fr. E was subjected to an YMC ODS -120A, C18 (300 × 6.0 mm, 5µm) column and eluted with isocratic 80% methanol, over 60 min, to yield six sub fractions (E_1 to E_6). Fraction E_6 was rechromatographed on the same condition and isolated sub Fr. E_{6-1}. Fraction F was subjected to a silica gel column (1.5 × 100 cm) and eluted with a stepwise gradient of $CHCl_3$:MeOH:H_2O (90:10:1, 80:20:5, 70:30:10, 65:35:10) to obtain three sub fractions (Fr. F_1 to F_3), and then sub Fr. F_3 was rechromatographed on a Tosoh TSK gel, ODS-120A C18 (150 × 4.6 mm, 5µm) column, eluted with a isocratic 80% methanol to yield three sub fractions (F_{3-1} to F_{3-3}).

3.2.2 Measurement of functional properties of fractionated saponin

Antifungal, hemolytic, and cholesterol-binding ability were determind with *A. amurensis* saponin fractions. Saponin fractions were added with an amount of 3 and 5 mg for antifungal activity and 250 µg/mL for determination of MIC. The methods were described in Chapter 2 (Materials and Methods section).

3.2.3 Data analysis

Statistical analyses were performed using SPSS software (SPSS 16.0, IBM, USA). Data are expressed as mean ± SD. Data were compared with one way analysis of variance (ANOVA). Significant differences were determined by Tukey's test at $p < 0.05$ level.

3.3 Results

3.3.1 TLC analysis of the fractionated saponin

Seven saponin fractions (Fr. A to G) were successfully eluted after 1st step of silica gel fractionation (Fig. 3-1). The chromatographic profiles of the saponin fractions were analyzed by TLC. The result showed two major differences in the number of discrete spots on TLC, after detection of saponin with 50% sulfuric acid and 1% cinnamaldehyde staining solutions

(Fig. 3-2). *A. amurensis* saponin Fr. A and B were detected with a relatively high R_f value ranging from 0.58 to 0.91 with low polar components. Fr. C contained a wide ranging of R_f value ranges from 0.25 to 0.76 on TLC. Furthermore, Fr. D to G contained relatively higher polor component with low R_f value ranging from 0.21 to 0.54. It was clearly observed from the results of 1% cinnamaldehyde staining solutions that each fraction contains more than three components. TLC profiles of saponin Fr. A to C were quite different from those of saponin Fr. D to G.

3.3.2 Antifungal activity of the fractionated saponin

The antifungal activity of *A. amurensis* seven saponin fractions against *S. cerevisiae*, *R. glutinis*, *A. flavus*, *F. oxysporum*, *C. sphaerospermum*, and *T. mentagrophytes* fungi were tested and the results were presented in Table 3-1 (also Figs. 3-3 and 3-4). Each saponin fraction was added on the paper disc at two different concentrations with 3 and 5 mg/mL. Digitonin saponin (Wako Pure Chemical industry, Osaka, Japan) was used for the positive control and distilled water was used for the negative control. The antifungal results showed a dose-dependent growth inhibitory activity against tested fungal pathogens. The maximum zone inhibition was observed in Fr. D to G with amount of 5 mg against *S. cerevisiae*, *R. glutinis*, *A. flavus*, *F. oxysporum*, and *C. sphaerospermum* in the range of 18.5 to 27.0 mm. The remarkable zone of inhibition was especially observed in Fr. F against *F. oxysporum* with 27.0 mm (5 mg). On the other hand, the dose-dependent fungal growth was not observed against *T. mentagrophytes* (Fig. 3-3). In contrast, the growth inhibitory activity was only found in digitonin saponin against *S. cerevisiae* and *T. mentagrophytes* with the ranges of 9.7 to 23.1 mm and no growth inhibitory activity observed against *R. glutinis*, *A. flavus*, and *F. oxysporum*. Furthermore, no growth inhibitory activity was observed in Fr. A to C against tested fungi both amounts of 3 and 5 mg.

The minimum inhibitory concentrations (MIC) of saponin fractions were determined by

serial broth dilution method. The fractions possessed antifungal activity were subjected to MIC analysis. Comparing the antifungal activity of crude saponin, *A. amurensis* saponin fractions showed significant activity against tested fungi. Fraction D showed strong inhibition activity with a lowest MIC value ranges from 15.6 to 25.0 µg/mL against *A. flavus*, *F. oxysporum*, *C. sphaerospermum*, and *T. mentagrophytes*. On the other hand, Fr. E and G showed moderate level of antifungal activity (Table 3-2).

3.3.3 Hemolytic activity of the fractionated saponin

Hemolytic activity of *A. amurensis* saponin was evaluated in mouse erythrocytes. Positive control (100% hemolysis) by distilled water and negative control (0% hemolysis) by PBS were used as standard references. The activity was expressed as the concentrations inducing 50% of erythrocyte hemolysis (HC_{50}) of the saponin fractions. The HC_{50} value was calculated in the range of 10 to 100 µg/mL. The HC_{50} value of *A. amurensis* saponin fractions (Fr. D to G) in mouse erythrocyte were with 32.3 ± 4.32, 36.2 ± 3.54, 38.5 ± 4.21, and 30.0 ± 2.61 µg/ mL, respectively (Fig. 3-5). On the other hand, Fr. A to C showed slightly less hemolytic activity with HC_{50} values of 41.5 ± 3.98, 42.6 ± 3.87, and 42.7 ± 4.21 µg/ mL, respectively. No significant difference between Fr. A to C and Fr. D to G was observed.

3.3.4 Cholesterol-binding ability of the fractionated saponin

Cholesterol-binding ability of *A. amurensis* saponin fractions were evaluated with $FeCl_3$-H_2SO_4 method and the percentage of cholesterol-binding ability were shown in Fig. 3-6. Fractions D and F showed the highest cholesterol-binding ability with the values of 32.8 and 31.9%, respectively. The cholesterol-binding ability of Fr. D and Fr. F was found to be similar to crude saponin with the values of 34.3%, respectively. Fractions E and G showed the cholesterol-binding ability with the values of 21.4 and 25.1%, respectively. On the other hand, the binding ability was significantly decreased ($p < 0.05$) in Fr. A, B, and C with the values of 1.5, 5.2, and 4.9 %, respectively.

3.3.5 Sub fractionation and functional properties

The functional properties of four active fractions obtained from *A. amurensis* (Fr. D, E, F, and G) showed predominant growth inhibitory activity and highest cholesterol-binding ability. Therefore, Fr. D, E, and F were selected for further purification. In this section, Fr. D, E, and F were fractionated and the obtained fraction was analyzed by TLC and then functional properties (antifungal and cholesterol-binding ability) were determined.

Fraction D was subjected to silica gel column to yield three sub fractions (Fig. 3-7A) named as Fr. D_1, D_2, and D_3. TLC results showed that sub Fr. D_3 contained one smear band having antifungal activity (Fig. 3-7B). Therefore, Fr. D_3 was subjected to a TSK gel ODS-120A C18 (150× 4.6 mm, 5µm) column eluted with linear gradient 5 to 100% methanol to yield three sub fractions Fr. D_{3-1}, D_{3-2}, and D_{3-3} (Fig. 3-7C). TLC results showed that Fr. D_{3-1} contained one band in both the staining solution 50% sulfuric acid and 1% cinnamaldehyde (Fig. 3-7D).

Fraction E was subjected to an YMC ODS-120A, C18 (300 × 6.0mm, 5µm) column eluted with 80% methanol to yield six sub fractions E_1 to E_6 (Fig. 3-8A). Pooled material was evaporated under reduced pressure to remove methanol, and then lyophilized. The TLC results showed that Fr. E_2 and E_6 contained one band with few smear part (Fig. 3-8B). Therefore, Fr. E_6 was subjected to the same column to yield one sub fraction (Fig. 3-8C), and TLC results showed that sub Fr. E_{6-1} contained one band in both the staining solution 50% sulfuric acid and 1% cinnamaldehyde (Fig. 3-8D).

In contrast, Fr. F was subjected to a silica gel column and eluted with stepwise gradient solution to yield three sub fractions Fr. F_1 to F_3 (Fig. 3-9A). TLC results revealed that sub Fr. F_2 and F_3 contained one smear band in both the staining solution 50% sulfuric acid and 1% cinnamaldehyde (Fig. 3-9B). Hence, sub Fr. F_3 was subjected to a TSK gel ODS (150× 4.6 mm, 5µm) column and eluted with 80% methanol to yield three sub fractions F_{3-1} to F_{3-3} (Fig.

3-9C). As a result, TLC pattern showed that sub Fr. F_{3-3} contained one band in both the staining solution (Fig. 3-9D).

Antifungal activity

The antifungal activity of the fractionated saponin components was determined with two different concentrations, with an amount of 3 mg for saponin fractions (Fr. D, E, and F) and with an amount of 1 mg for sub fractions. The maximum zone inhibition was observed against all filamentous fungi. The antifungal activity of Fr. D, sub Fr. D_3, and D_{3-1} was determined against *S. cerevisiae*, *R. glutinis*, *A. flavus*, *F. oxsyiporum*, and *C. sphaerospermum*. The higher growth inhibitory activity was obtained against *F. oxysporum* (29.7 mm) and *A. flavus* (27.0 mm) (Fig. 3-10). Next to this, a moderate level of antifungal activity was observed against *S. cerevisiae* (14.1 mm). The antifungal result of sub Fr. D_{3-1} revealed that the purified component possessed higher antifungal activity compared with Fr. D.

Nextly, antifungal activity of sub Fr. E_1 to E_6 was determined with the same fungi strain. No inhibition zone was observed in Fr. E_1 against tested fungi. The higher activity was observed in sub Fr. E_2 to E_6 against *S. cerevisia*, *R. glutinis*, *A. flavus*, and *F. oxysporum.* The purified sub Fr. E_{6-1} possessed high antifungal activity (Fig. 3-11).

Fraction F_2 and sub Fr. F_{3-3} also showed growth inhibition activity against tested fungi (Fig. 3-12). However, higher activity was observed in sub Fr. F_{3-3} compared with Fr. F_2. The present result revealed that all isolated fractions Fr. D_{3-1}, E_{6-1}, F_{3-3}, and F_2 showed higher antifungal activity against filamentous fungi compared with yeast.

The minimum inhibitory concentrations (MIC) of pure components were determined by serial broth dilution method (Table 3-3). Fractions D_{3-1} and F_{3-3} showed the maximum inhibitory activity against tested fungi, the MIC value ranges from 3.12~8.33 µg/mL. On the other hand, Fr. E_{6-1} was found sensitive against *A. flavus* and *T. mentagrophytes* with the MIC

value of 6.25 µg/mL.

Cholesterol-binding ability

The percentage of cholesterol-binding ability was calculated and the results are shown in Fig. 3-13. Digitonin saponin was used as a positive control. No significant difference was observed among digitonin saponin, sub Fr. D_3, and D_{3-1} with the values of 55.7, 57.1, and 56.4%, respectively. However, significant difference was observed between Fr. D and sub Fr. D_3 with the values of 32.8 and 57.1%. It was also observed that there was difference in cholesterol-binding ability to some extent between digitonin saponin and sub Fr. E_{6-1} with the values of 55.7 and 40.8% (Fig. 3-13). Faction F_2 and sub Fr. F_{3-3} showed the cholesterol-binding ability with the values of 28.9 and 55.1% (Fig. 3-14).

3.4 Discussion

A.amurensis saponin can be divided into three structural classes: sulfated steroidal glycosides (asterosaponin), steroidal cyclic glycosides, and polyhydroxylated steroidal glycosides (Minale et al., 1986). The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains, and the position of attachment of these moieties on the aglycone (Francis et al., 2002). Several researchers reported that *A. amurensis* saponin is highly complex structures which are frequently difficult to synthesize (Hwang et al., 2014) and also there are often vulnerable. Therefore, saponin obtained from *A. amurensis* was isolate and clarify the functional properties for the advanced utilization of starfish resources.

There are several strategies available for the isolation of saponin. *A. amurensis* saponin fractionation was carried out over a silica gel column chromatography with a gradient elution, and using RP HPLC Tosoh TSK gel, ODS-120A C18 and YMC ODS -120A, C18 columns. Repeated chromatography of the precipitate, led to the isolation of four pure saponin fractions.

After silica gel column fractionation, seven fractions were obtained (Fr. A to G). The antifungal results of Fr. D to G showed higher antifungal activity against tested six human fungal pathogens. Compared with yeast and filamentous fungi, *T. mentagrophytes* could be inhibited the fungal growth with 3 and 5 mg. A considerable number of scientific studies have reported that *T. mentagrophytes* proved to be quite sensitive to some plant saponin such as tea seed and also starfish saponin (Choi et al., 1999; Robin et al., 2013). The results demonstrated that the antifungal substances were present in *A. amurensis* saponin Fr. D, E, F, and G. The biological activity of saponin is more related to their carbohydrate moiety, although the involved mechanisms have not yet been characterized (Bouarab et al., 2002). The present data also support this notion. However, it is difficult to interpret the role of the carbohydrate moiety in the antifungal activity of starfish saponin.

Saponins have drawn in scientific attention due to their structural diversity and the significance of their adjuvant activity (Sun et al., 2011). The hemolytic activity results showed that the HC_{50} value for the seven saponin compounds were higher than crude saponin, suggesting that crude saponin was more potent than saponin fractions (Fig. 3-5). The cholesterol-binding results showed two major differences between Fr. A to C and Fr. D to G. The present results revealed that Fr. D to G showed different binding ability, due to different composition of saponin. It should be noted that different functional properties depend on defined molecular interactions between particular chemical groups of the saponin and their targets (Netala et al., 2015). The present finding also agrees with the finding that the components having higher polarity relatively (Fr. D to G) in *A. amurensis* saponin showed highest antifungal and cholesterol-binding ability. On the other hand, digitonin saponin proved that it has the ability to bind with cholesterol (Korchowiec et al., 2015). Therefore, digitonin saponin was selected as standard for comparison with starfish saponin, whether starfish saponin possessed the same cholesterol-binding ability. The results proved that

saponin fractions obtained from *A. amurensis* had the binding ability as same as digitonin saponin. Based on these experimental results (antifungal, hemolytic, and cholesterol-binding), Fr. D, E, F, and G exhibited strong activities compared with Fr. A, B, and C.

The purified sub Fr. $D_{3-1,}$ $E_{6-1,}$ $F_{2,}$ and F_{3-3} showed high antifungal and cholesterol-binding ability suggesting that pure component permeate cell membrane and leads to the formation of insoluble complexes on the cell membrane. Concerning the importance of the activity reported here, it is interesting to note that the isolated saponin could significantly inhibit the filamentous and dermatophytes fungi compared to yeast. The antifungal activity results of Fr. D_{3-1} showed comparatively higher than Fr. E_{6-1} and significant cholesterol-binding ability was also observed. In contrast, Fr. F_{3-3} revealed higher antifungal and cholesterol-binding ability compared with Fr. F. In this study, it was found that four saponin fractions possessed different functional properties. The compositional differences and aglycone structure of the saponin may play different roles in their antifungal and cholesterol-binding ability.

3.5 Conclusion

Four saponin fractions were isolated from *A. amurensis* (Fr. D_{3-1}, E_{6-1}, F_2, and F_{3-3}) and each fraction revealed different antifungal and cholesterol-binding ability. The present study suggests that antifungal substances present in *A. amurensis*, exhibit a specific inhibitory action on the growth and germination of fungi and possess distinctive chemical characteristics of saponin. This investigation has revealed that the marine organism *A. amurensis* produce structurally diverse secondary metabolites which could be of pharmaceutical interest. Finally, the results suggest that the starfish *A. amurensis* could be a potential marine resource for the antifungal and cholesterol-binding agents.

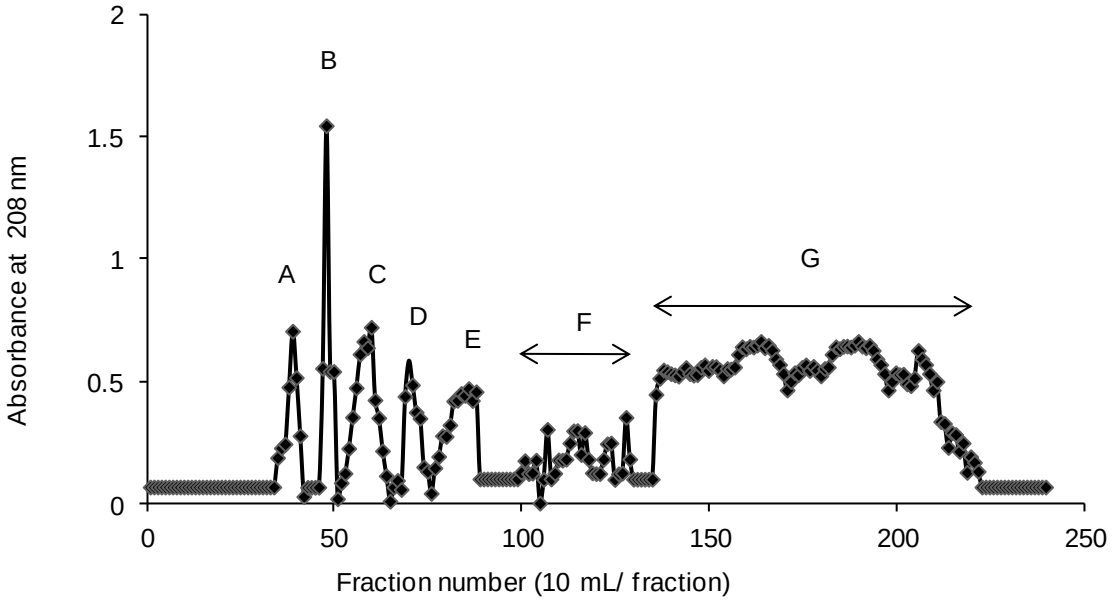


Fig. 3-1 Elution profile of *A. amurensis* crude saponin by Silica gel 60 (0.040-0.063 mm) column chromatography elution profile.

Column size: 2.6×100 cm

Eluent: $CHCl_3$:MeOH: H_2O (65:35:10, lower phase)

Flow rate: 5 mL/min

Fraction: 10 mL/fraction

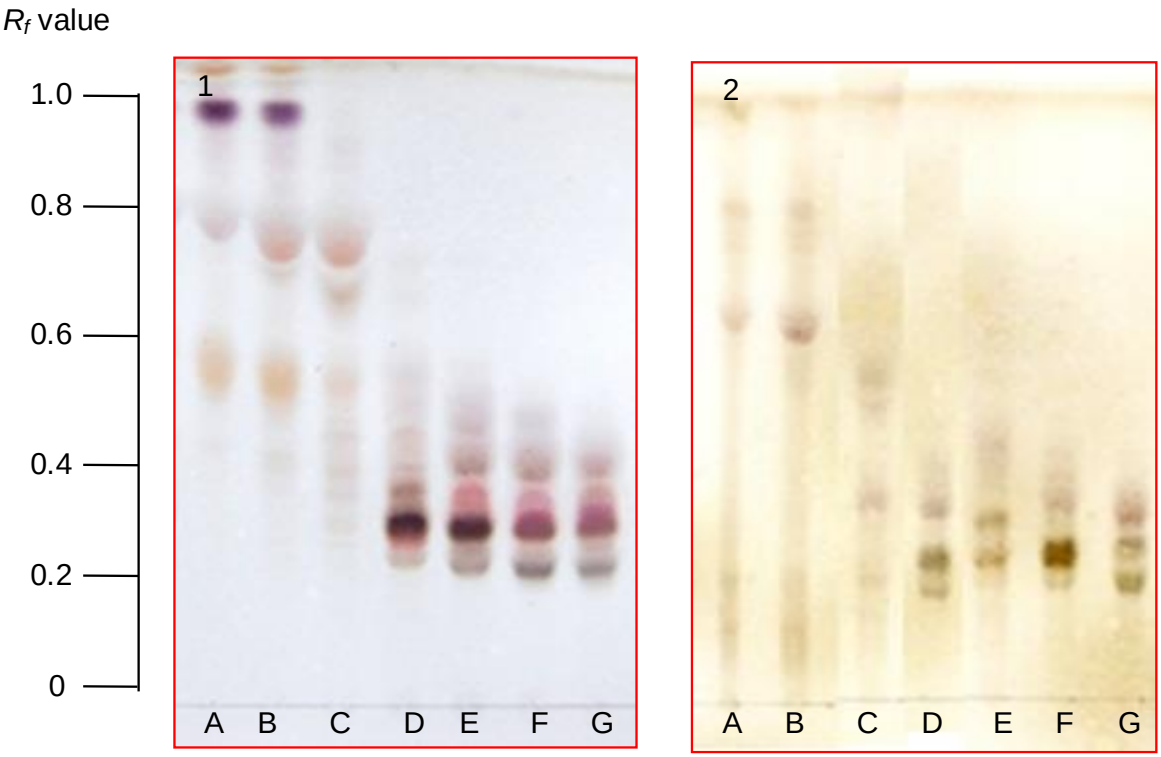
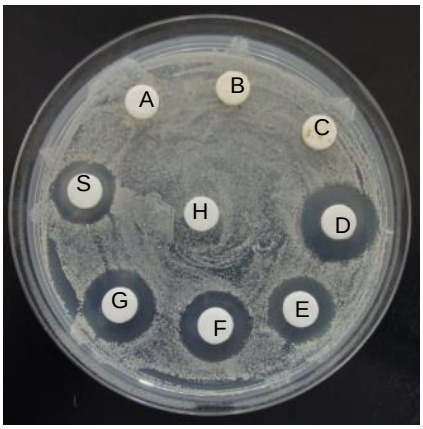
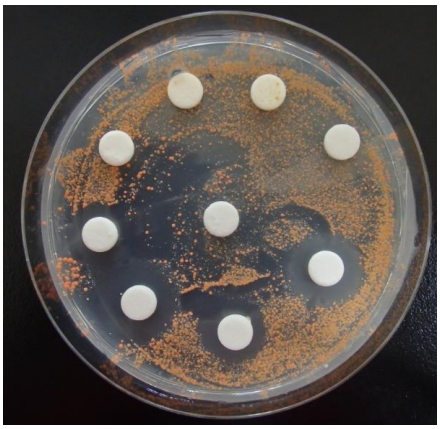


Fig. 3-2 TLC patterns obtained from *A. amurensis* saponin.

Each band on TLC was detected by 50% sulfuric acid (1) and 1% cinnamaldehyde (2). A to G were Fr. A to G.

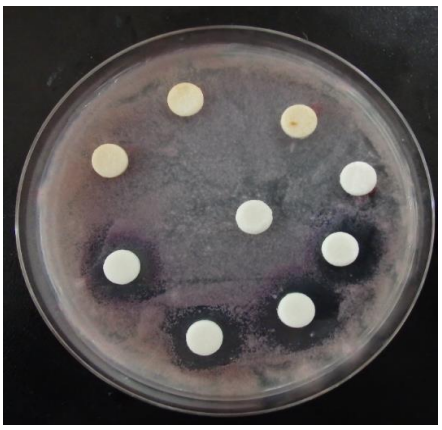


S. cerevisiae

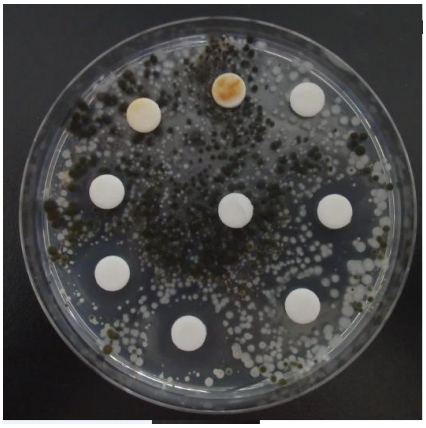


R. glutinis

A. flavus



F. oxysporum

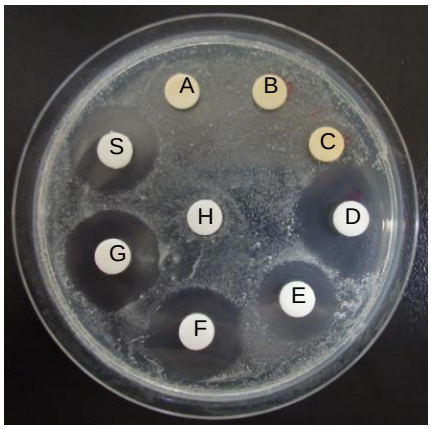


C. sphaerospermum

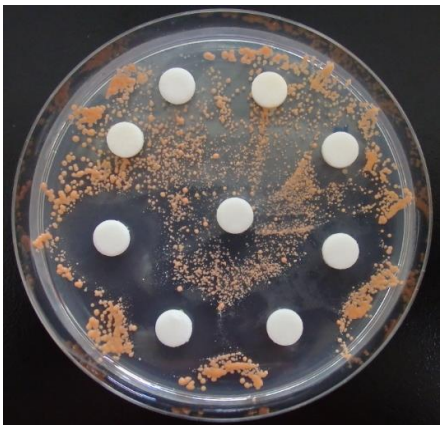
T. mentagrophytes

Fig. 3-3 Antifungal activity of *A. amurensis* saponin fractions.

S, digitonin saponin; H, dH_2O; A to G, Fr. A to G. 3 mg of each saponin fraction was added on paper disc.

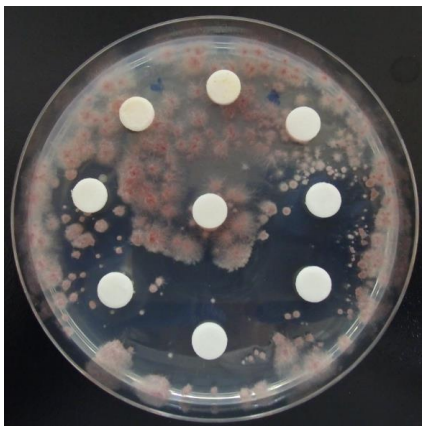


S. cerevisiae

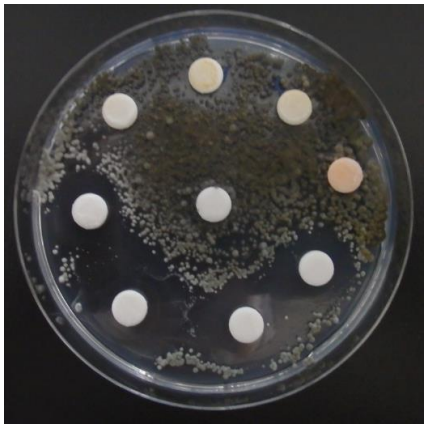


R. glutinis

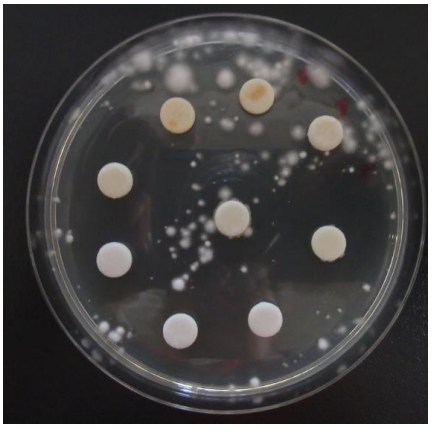
A. flavus



F. oxysporum



C. sphaerospermum



T. mentagrophytes

Fig. 3-4 Antifungal activity of *A. amurensis* saponin fractions.

S, digitonin saponin; H, dH_2O; A to G, Fr. A to G. 5 mg of each saponin fraction was added on paper disc.

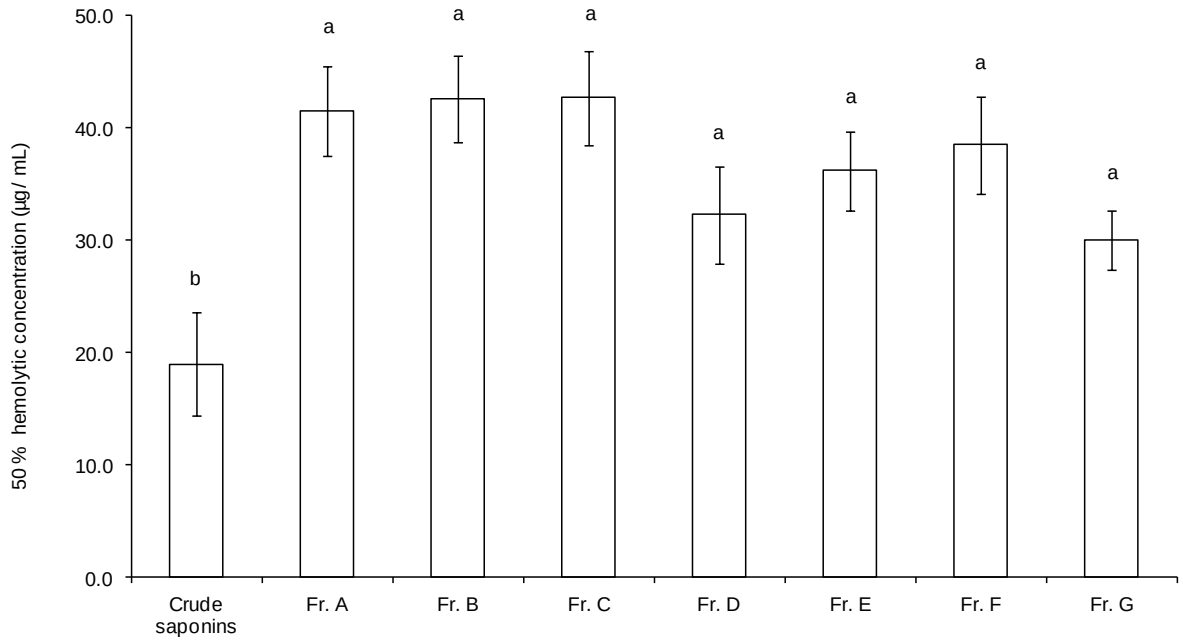


Fig. 3-5 Hemolytic activity of saponin fractions from *A. amurensis* against mouse erythrocytes.

Hemolytic activity was expressed as the concentrations of various fractions that induced hemolysis in 50% of mouse erythrocytes, and compared using one-way ANOVA followed by Tukey's test. Significant differences ($p < 0.05$) are represented by different letters over the column.

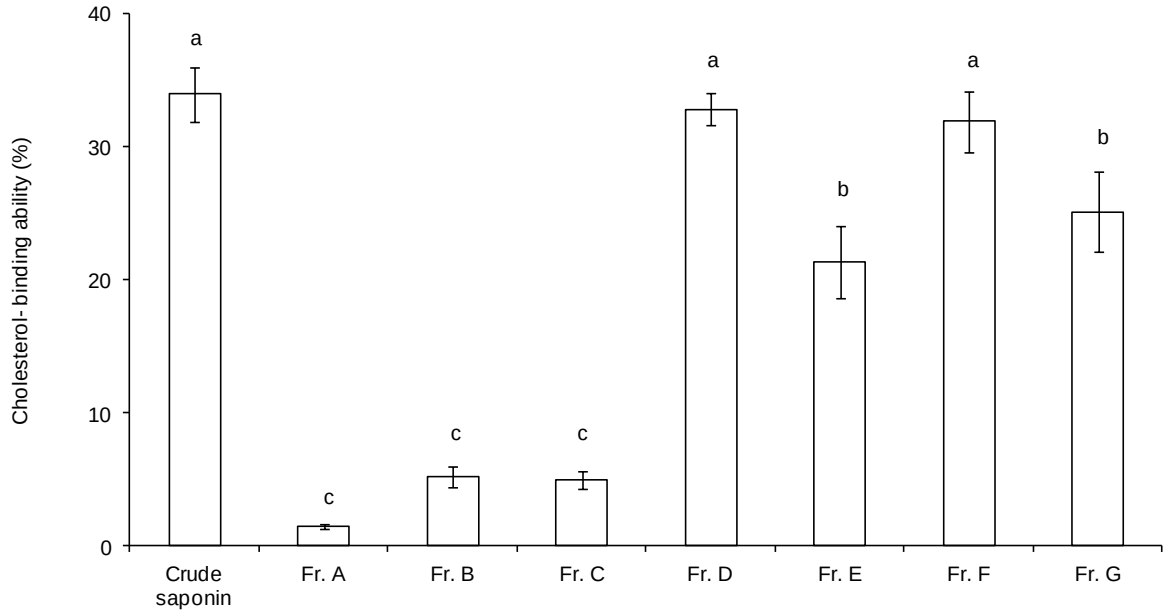


Fig. 3-6 Cholesterol-binding ability of *A. amurensis* saponin fractions.

The results are expressed as mean±SD (n=3). Values with different superscript letters (a, b and c) within a column are significantly different ($p < 0.05$).

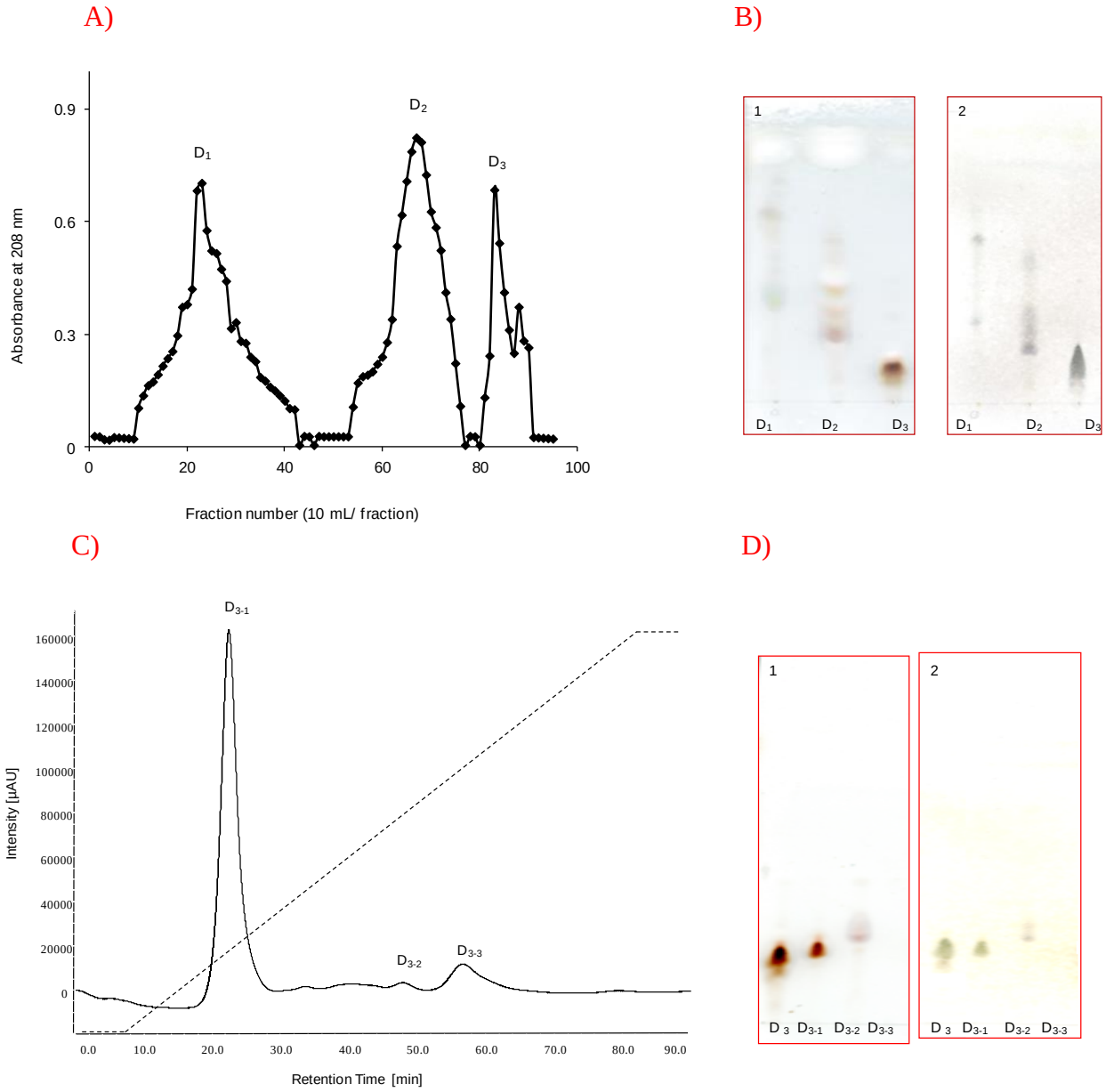


Fig. 3-7 Silica gel column elution profile (A) and TLC profiles (B) of Fr. D

Fr. D was eluted with stepwise gradient of $CHCl_3$:MeOH:H_2O (90:10:1, 80:20:5, 70:30:10, 65:35:10) to increase polarity. After the chromatography, sub Fr. D_3 was subjected to TSK gel ODS-120A, C18 HPLC column(C). TLC proliles of sub Fr. D_3 was shown in (D). Elution was performed with a linear gradient from 5-100% MeOH [A) MilliQ B) MeOH 100%] over 90 min, at a flow rate of 0.5 mL/min, at 208 nm (C). TLC profiles of sub Fr. D_3, each band on TLC plates were detected with 50% sulfuric acid (1) and 1% cinnamaldehyde (2), (D).

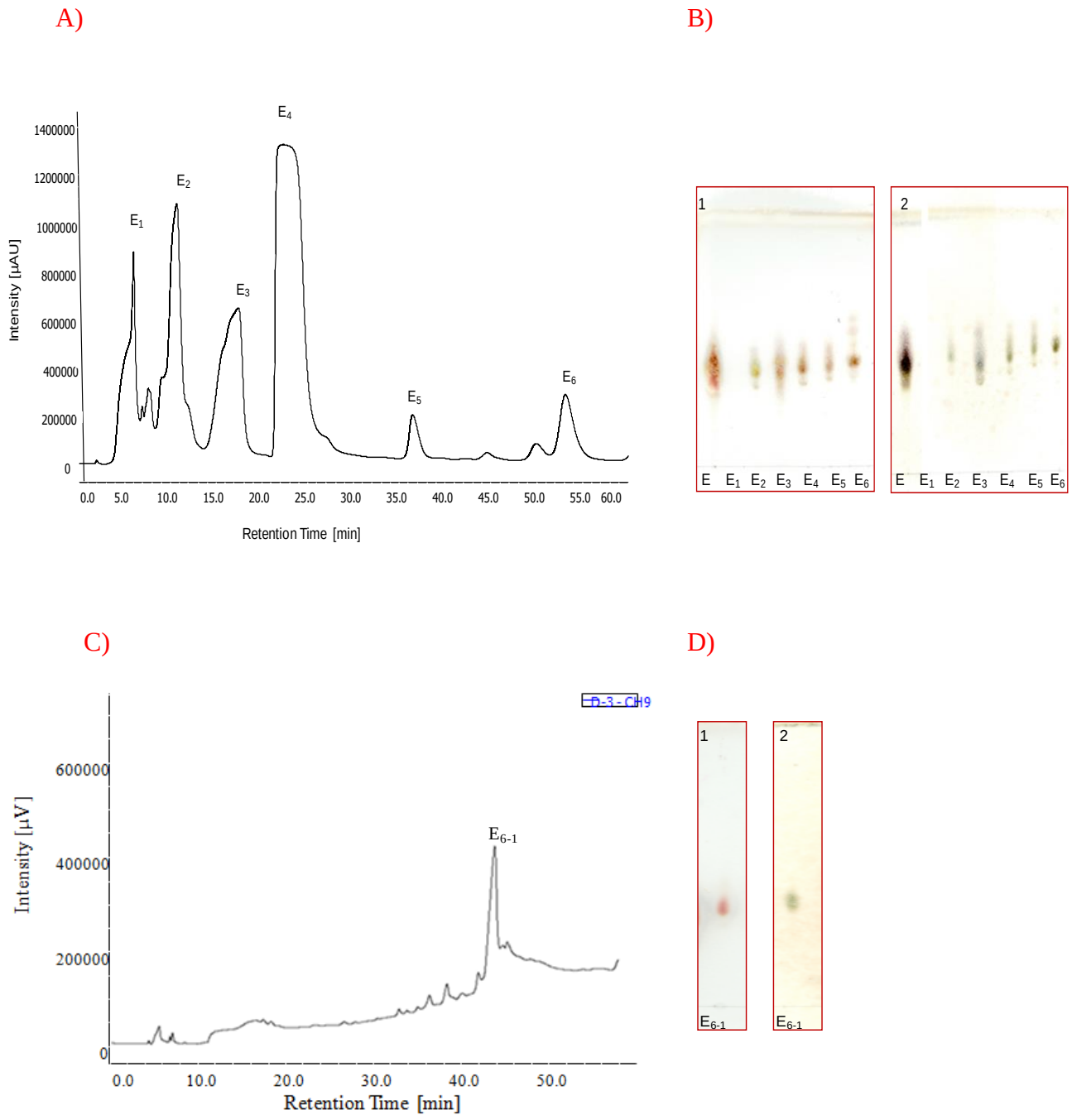


Fig. 3-8 Elution profile (A) by YMC ODS, 120A HPLC column and TLC profiles (B) of sub Fr. E.

Elution was performed with 80% methanol, over 60 min at a flow rate of 0.5 mL/min. (C) shows YMC ODS, 120A HPLC column elution profile of sub Fr. $E_{6.}$ (D) shows TLC profiles of Fr. $E_{6\text{-}1,}$ stained with 50% sulfuric acid (1) and 1% cinnamaldehyde (2), (D).

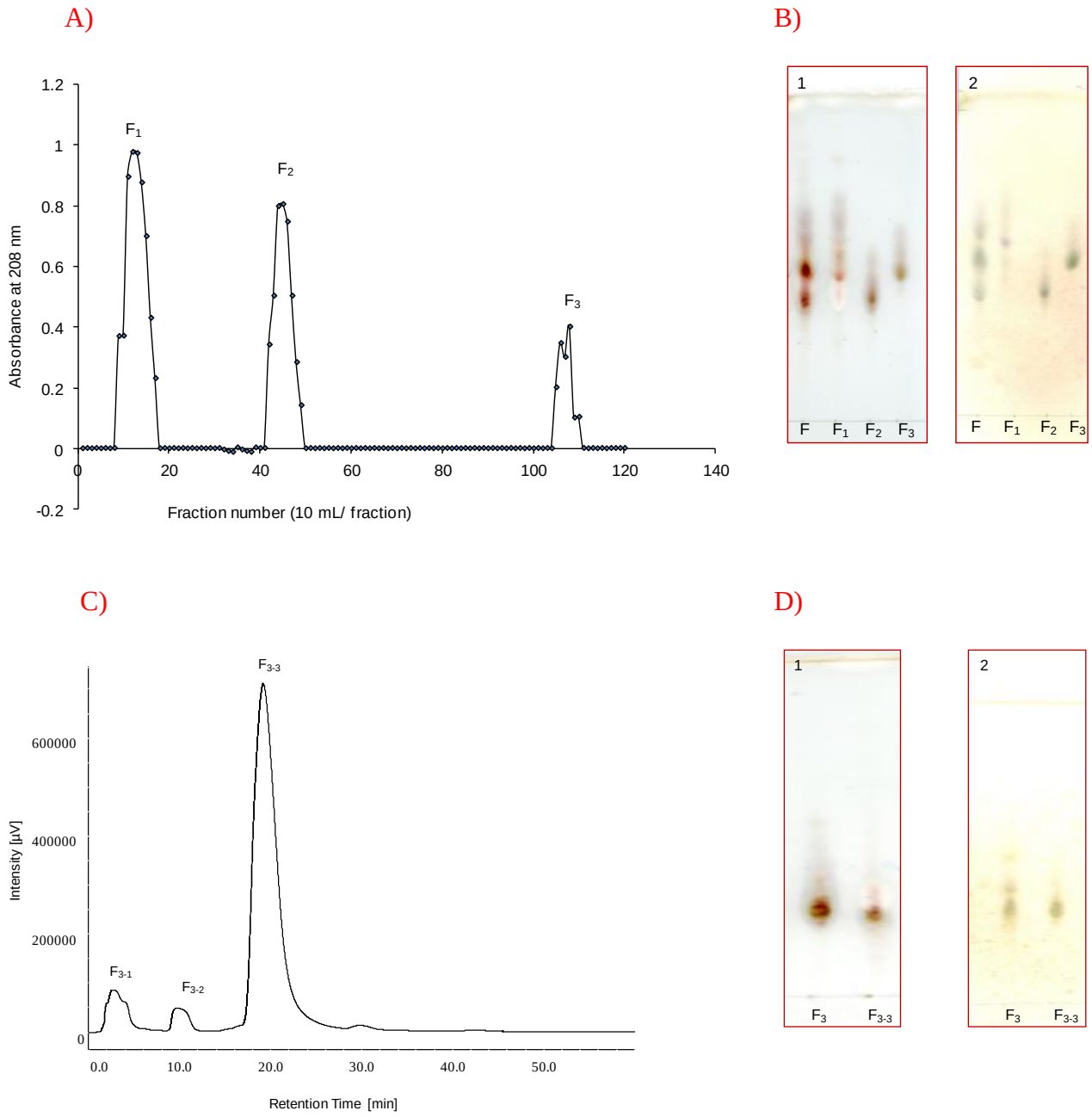


Fig. 3-9 Silica gel column elution profile (A) and TLC profiles (B) of Fr. F.

Fr. F was eluted with stepwise gradient of $CHCl_3$:MeOH:H_2O (90:10:1, 80:20:5, 70:30:10, 65:35:10). (C) shows TSK gel ODS -120A, C18 HPLC column elution profile of sub Fr. F_3. Elution was performed with 80% methanol, over 60 min, at a flow rate of 0.5 mL/min. (D) shows TLC profiles of sub Fr. F_3. Each band on TLC plates were detected with 50% sulfuric acid (1) and 1% cinnamaldehyde (2).

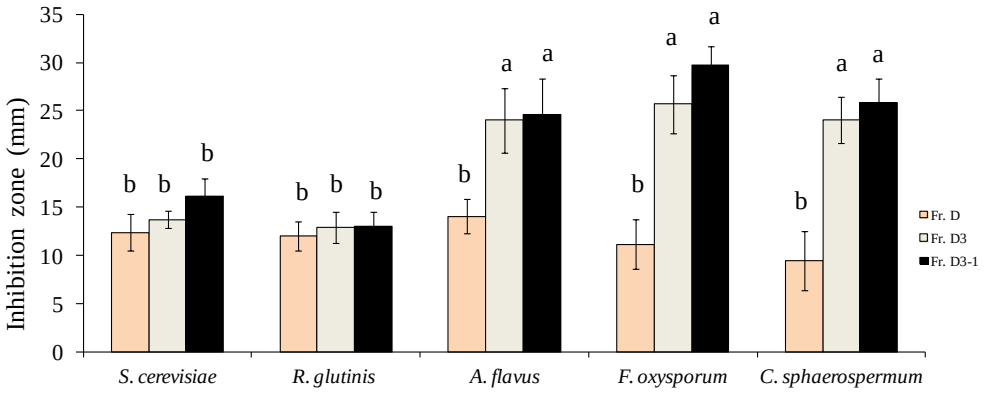


Fig. 3-10 Antifungal activity of *A. amurensis* saponin sub Fr. $D_{3\text{-}1}$.

Saponin fractions were added an amount of 1~3 mg (Fr. D, 1 mg and sub fractions 1mg). The results are expressed as mean±SD (n=3). Values with different superscript letters (a and b) within a column are significantly different ($p < 0.05$).

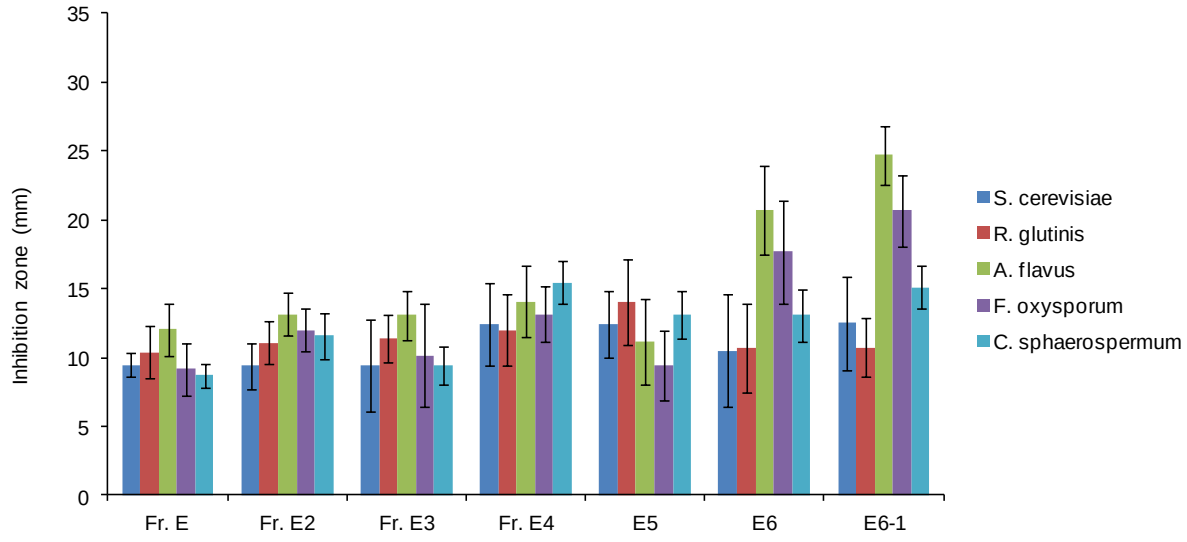


Fig. 3-11 Antifungal activity of Fr. E and its sub fractions.

Saponin fractions were added at 1~3 mg concentration (Fr. E, 3 and sub Fr. 1 mg).

The results are expressed as mean±SD (n=3).

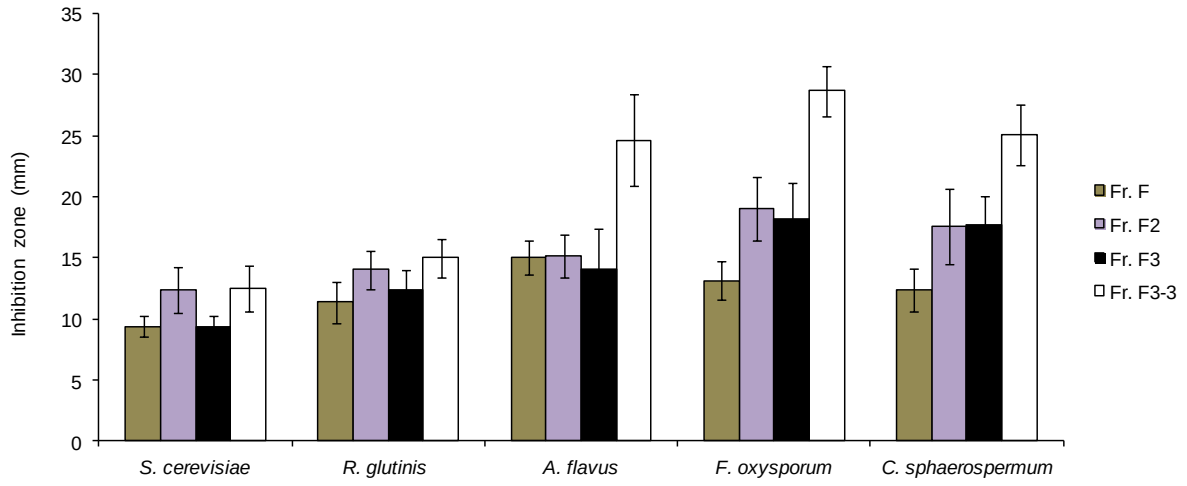


Fig. 3-12 Antifungal activity of Fr. F and its sub fractions.

Saponin fractions were added at 1~3 mg concentration (Fr. F, 3 and sub Fr. 1 mg).

The results are expressed as mean±SD (n=3).

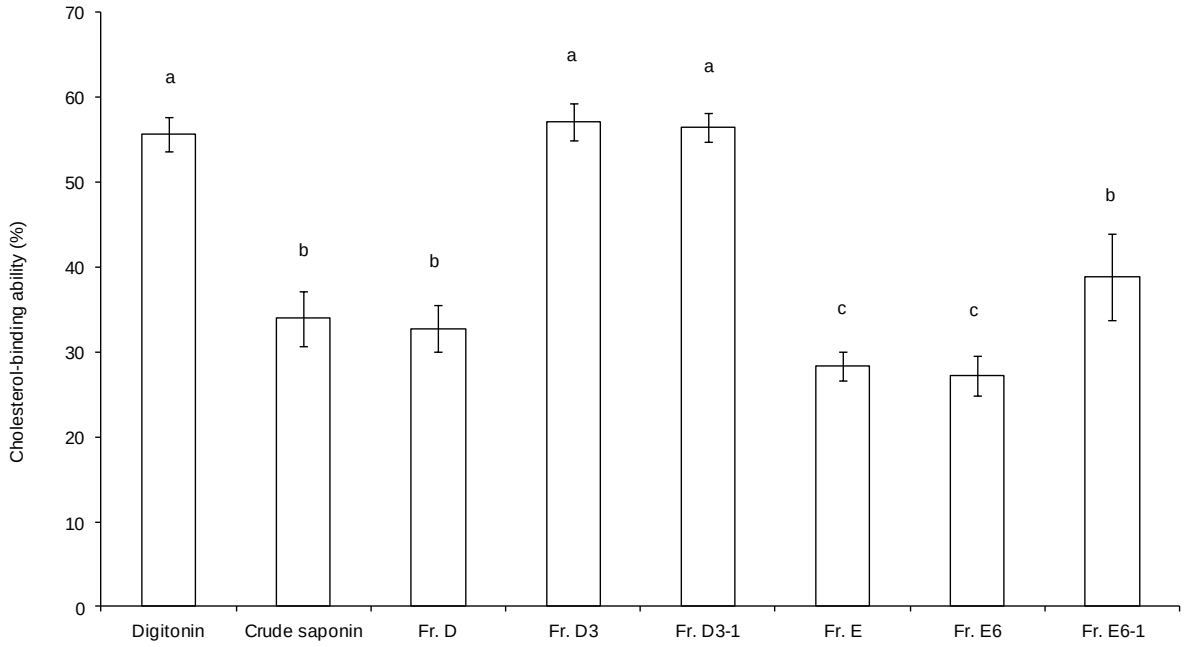


Fig. 3-13 Cholesterol-binding ability of Fr. D, E and their sub fractions.

The results were compared using one-way ANOVA followed by the Tukey's test. Significant differences ($p < 0.05$) are represented by different letters over the columns.

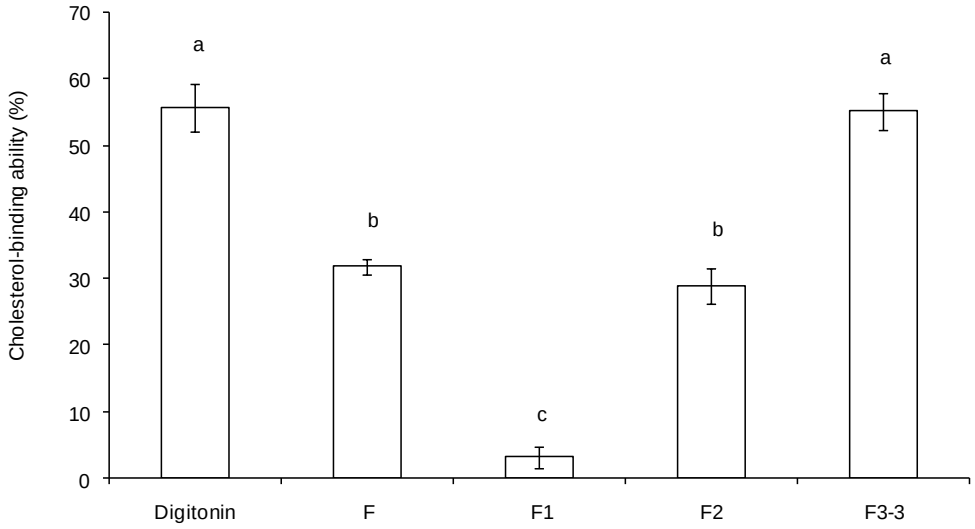


Fig. 3-14 Cholesterol-binding ability of Fr. F and its sub fractions.

The results were compared using one-way ANOVA followed by the Tukey's test. Significant differences ($p < 0.05$) are represented by different letters over the columns.

Table 3-1 Antifungal activity of *A. amurensis* saponin fractions

	Zone of inhibition (mm)															
	Fr. A		Fr. B		Fr. C		Fr. D		Fr. E		Fr. F		Fr. G		Digitonin	
	3	5	3	5	3	5	3	5	3	5	3	5	3	5	3	5
Yeast																
S. cerevisiae	-	-	-	-	-	-	14.4 ± 2.02	20.4 ± 1.80	12.3 ± 1.84	18.5 ± 2.20	15.0 ± 0.71	19.8 ± 1.04	14.0 ± 0.44	21.4 ± 2.16	9.7 ± 1.52	17.8 ± 2.13
R. glutinis	-	-	-	-	-	-	12.5 ± 1.50	22.5 ± 2.18	11.7 ± 2.25	23.7 ± 0.57	14.0 ± 1.00	20.0 ± 2.10	16.5 ± 0.95	22.0 ± 1.70	-	-
Filamentous fungi																
A. flavus	-	-	-	-	-	-	15.7 ± 2.07	21.3 ± 2.70	14.0 ± 1.58	22.8 ± 2.26	15.0 ± 2.51	21.6 ± 1.39	14.0 ± 1.00	25.7 ± 3.57	-	-
C. sphaerospermum	-	-	-	-	-	-	16.4 ± 3.05	23.9 ± 3.16	13.7 ± 1.52	25.0 ± 3.10	13.0 ± 3.10	24.7 ± 3.50	14.2 ± 1.59	19.8 ± 3.83	-	-
F. oxysporum,	-	-	-	-	-	-	14.3 ± 1.15	23.7 ± 2.57	9.8 ± 1.00	24.3 ± 2.59	10.8 ± 2.10	27.0 ± 2.05	11.2 ± 2.75	22.8 ± 2.91		-
Dermatophyte																
T. mentagrophytes	-	-	-	-	-	-	24.1 ± 3.23	26.1 ± 2.03	21.1 ± 2.03	25.1 ± 1.73	20.1 ± 3.13	24.1 ± 2.43	23.1 ± 1.23	27.1 ± 2.23	19.0 ± 1.03	23.1 ± 1.76

Data are presented as mean ± standard deviation (SD) of 3 independent replicates in 2 separate experiments.
-, no measurable zone. Saponin fractions were added with amounts of 3 and 5 mg.

Table 3-2 Minimum inhibitory concentration (MIC) of *A. amurensis* saponin fractions against six fungal strains

Saponin fractions	MIC (μg/mL)					
	S. cerevisiae	*R. glutinis*	*A. flavus*	*F. oxysporum*	*C. sphaerospermum*	*T. mentagrophytes*
Fr. D	25.0	25.0	15.6	15.6	15.6	17.8
Fr. E	31.5	41.6	20.8	62.5	62.5	31.2
Fr. F	31.2	41.6	31.2	41.6	31.2	31.2
Fr. G	31.5	41.6	20.8	20.8	25.0	25.0

Data are presented as 3 independent replicates. Saponin fractions were added with amount of 250 μg/mL.

Table 3-3 Minimum inhibitory concentration (MIC) of isolated saponin fractions against six fungal strains

Isolated saponin	MIC (μg/mL)					
	S. cerevisiae	*R. glutinis*	*A. flavus*	*F. oxysporum*	*C. sphaerospermum*	*T. mentagrophytes*
Fr. $D_{3\text{-}1}$	6.25	6.25	3.12	3.12	3.12	3.57
Fr. $E_{6\text{-}1}$	12.50	8.33	6.25	8.33	12.50	6.25
Fr. $F_{3\text{-}3}$	8.33	6.25	3.12	3.12	3.57	3.12
Fr. F_2	6.26	8.33	4.16	4.16	6.25	8.33

Data are presented as 3 independent replicates. Saponin sub fractions were added with amount of 50 μg/mL.

Chapter 4

Identification and characterization of *Asterias amurensis* saponin fractions

4.1 Introduction

The low molecular weight metabolites from starfish (class Asteroidea) are characterized by a remarkable diversity of polar steroids, including polyhydroxysteroids and related monosides and biosides as well as steroid oligoglycosides named as asterosaponins (Minale et al., 1993; Iorizzi et al., 2001; Stonik, 2001; Stonik et al., 2008; Dong et al., 2011; Ivanchina et al., 2011. These compounds are the predominant metabolites of starfish and include a broad variety of biological activities (Iorizzi et al., 2001). Saponins are very complex mixtures, whose separations into individual components are rather difficult. The task for saponin isolation was complicated not by only the occurrence of many closely related substances, but also by the fact that most of saponin lack a chromophore (Francis et al., 2002). Thus, for many years, the complete characterization of saponin from even well-known saponin-containing plants has not been achieved. The complete structure elucidation of these molecules (especially isomers) is demanded to determine structure-activity relationships, which can lead to the development of new compounds with commercial applications (Osbourn et al., 2011). Therefore, in order to overcome this problem, silica gel column chromatography and high performance liquid chromatography (HPLC) were employed to purify saponin in Chapter 3.

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) has become the preferred techniques for analysis of saponin. Mass spectrometry provides a highly sensitive platform for the analysis of saponin structures by generating product ions by the cleavage of the glycosidic bond. However, since the information is limited on the

sugar linkage pattern, site of attachment of sulfate groups, and the presence of sugar isomers by MS/MS analyzed, only a partial characterization of a saponin is possible.

Nuclear magnetic resonance (NMR) spectroscopy can also provide extensive structural information for saponin. However, larger quantities of high-purity samples are generally needed in NMR analysis. The less purified sample made complicated in the NMR signals, making their assignments more difficult. On the other hand, the measurement of the absolute configuration of the sugar moieties of a saponin can be completely solved by NMR methods (Kicha et al., 2011).

Based on the widespread interest in biological and structural significance on the expression of special activity of saponin component, the structural study was undertaken with the saponins of *A. amurensis* in Chapter 4. In Chapter 3, four saponin fractions (Fr. $D_{3\text{-}1}$, $E_{6\text{-}1}$, $F_{3\text{-}3}$, and F_2) were already isolated and their functional properties such as antifungal and cholesterol-binding ability were evaluated. Therefore, these saponin fractions were analyzed by mass spectrometry and then the entire ion at *m/z* was subjected to MS/MS analysis. The simplest fragmentation of the carbohydrate moiety of glycol conjugates and glycosides occurred during MS/MS analysis. The information regarding the sugar sequences and side chain was then analyzed. The presence of saponin ion (at *m/z*) was then identified based on their branches of sugar residue. In addition, the partial structure of sub Fr. F_2 was determined on the basis of 1D and 2D NMR spectral data of NMR.

4.2 Materials and methods

4.2.1 Materials

Four saponin fractions (Fr. $D_{3\text{-}1}$, $E_{6\text{-}1}$, $F_{3\text{-}3}$, and F_2) obtained from *A. amurensis* in Chapter 3 were used as sample.

4.2.2 MALDI-TOF/TOF mass spectrometry

MALDI-TOF/MS analysis was performed on a 4800 Plus MALDI-TOF/TOF analyzer (Applied Biosystems, Framingham, MA, USA). The instrument was calibrated using the AB Sciex Mass Standards Kit for Calibration of AB SCIEX TOF/TOF instruments. Data was firstly acquired in the MS positive-ion reflector mode at fixed laser energy of 4200 that provided both goo

d signal levels and mass resolution. The samples were placed onto a MALDI stainless steel MPT Anchor Chip TM 600/384 target plate (AB Sciex, Framingham, MA). All samples were prepared using a mixture of 2, 5-dihydroxybenzoic acid (DHB) as a matrix in the reflector mode (10 mg/mL in MilliQ water). The matrix solution (1 μL) and saponin fractions (1 μL, 100 μg/mL in methanol) were mixed and then spotted onto a stainless steel target and air-dried. For the recording of the single-stage MALDI-MS spectra, the quadrupole (rf-only mode) was set to pass ions between *m/z* 800 and *m/z* 2500 and all ions were transmitted into the pusher region of the time-of-flight analyzer where they were mass-analyzed with a 1-s integration time. A reflector interpretation method was created to run the MS/MS 1kv positive acquisition method in batch mode on all spots. The MS/MS was performed at 4200 fixed laser intensity on the 50 strongest precursor peaks discovered during the reflector mode acquisition.

4.2.3 NMR spectra

NMR experiment was performed on an AV600 (Buker, USA) with one-dimensional (1D) and two-dimensional (2D) NMR methods. 1D NMR method includes ^{1}H (600 MHz), ^{13}C (150 MHz), and DEPT, and 2D NMR method includes ^{1}H-^{1}H Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC, ^{1}H & ^{13}C), and Heteronuclear Multiple bond Correlation (HMBC, ^{1}H & ^{13}C neighbor). All NMR experiments were performed using

pyridine-d_5 (C_5D_5N) as the solvent. Chemical shifts were given on the δ scale and referenced by pyridine-d_5 as an internal standard. Data processing was carried out with Top Spin v3.2 program.

4.3 Results and discussion

Identification of the saponin compounds was attempted by soft ionization mass spectrum (MS) techniques including MALDI-TOF in the positive mode. Previous studies have reported that the fragment ion adducts of saponin provide valuable structural information about the feature of the aglycone and linkage site of the sugar residues (Cui et al., 2001). Therefore, the MS analyses were conducted by introducing sodium ions to the samples. All saponin detected in the positive ion mode spectra were predominantly singly charged sodium adducts of the molecules $[M + Na]^+$ (Liu et al., 2007; Kalinin et al., 2008). The main fragmentation of saponin generated by cleavage of the glycosidic bond yielded oligosaccharide and monosaccharide fragments (Liu et al., 2007).

The saponin fractions obtained from the *A. amurensis* were profiled using MALDI-TOF/MS. The prominence of the parent ions $[M + Na]^+$ in MS spectra also enables the analysis of saponin in fractions. The MALDI-TOF/MS results indicate that the saponin fractions are quite pure, which is consistent with the TLC data. As a representative example, the MALDI-TOF/MS of the saponin Fr. $D_{3\text{-}1}$ of *A. amurensis* is shown in Fig. 4-1(A). The fragmentation pattern of the sodiated compound at *m/z* 1153.9 $[M + Na]^+$ in successive MS experiments is discussed in detail below for stepwise elucidation of the molecular structure of these compounds. The MS/MS fragmentation pathways was shown in dots of the compound at *m/z* 1153.9 (Fr. $D_{3\text{-}1}$). First, the loss of ion products detected at *m/z* 1007, 845, 755, 609, and 463, respectively (Fig. 4-1(B)). Indeed, characteristic losses of 146 u, 162 u, 146 u, and 146 u can be associated to the presence

of quinovose or fucose (146 u), glucose, galactose, or DXHU (162 u), and fucose or quinovose (146 u) residues, respectively. The simultaneous loss of sugar units indicated characteristics of a branched sugar chain. Moreover, a loss of (72 u + 18 u) was observed between *m/z* 845 and *m/z* 755 fragmentations from sodiated saponin upon ionization. Fragment pattern and 755 fragment peak revealed the same type of sugar chain.

The same fragmentation behaviors have been observed from the positive spectra (Fig. 4-2(A)) of saponin with *m/z* 1165.9 (Fr. E_{6-1}). The possible fragmentation pathways were shown using dots (Fig. 4-2(B)). The losses of aglycone moieties generated ions at *m/z* 477 which correspond to the complete sugar components. The consecutive losses of side chain, sugar, and sulfate units generate signals were detected at *m/z* 1066, 1019, 769, 623, and 477, respectively. The losses of 146 u and 132 u can be associated to the presence of quinovose or fucose and xylose sugar residues, and the losses of 100 u and 118 u can be associated to the presence of side chain and sulfate units. In the chromatograms of Fr. D_{3-1} and $E_{6-1,}$ one component was identified at *m/z* 1137, and the ion was subjected to MS/MS analysis. However, the fragmentation patterns were not similar to saponin ion. Therefore, further study was not carried out with this ion at *m/z* 1137.

From the result shown in Fig. 4-3(A), it was newly found that the ions identified at *m/z* 1289 and 1303 in the analysis of Fr. F_{3-3} correspond to those of Thornasteroside A ($C_{56}H_{91}O_{28}SNa$) and steroidal glycoside ($C_{57}H_{93}O_{28}SNa$), respectively, which were elucidated by Hwang et al. (2014) and Hwang et al. (2011). It is well-known that Thornasteroside A is a typical example of steroidal pentaglycoside sulfate and one of the most widely distributed asterosaponin (Kitagawa and Kobayashi, 1978; Riccio et al., 1985; D'Auria et al., 1993). It has been isolated from 15 species of the three major orders of Asteroidea (Kitagawa and Kobayashi, 1978; Riccio et al., 1985;

D'Auria et al., 1993). However, the structural elucidation of asterosaponin in general is challenging, because they are highly complex with branched oligosaccharides. The MS/MS spectrum of the $[M + Na]^+$ ion *m/z* 1289 are shown in Fig. 4-3(B). The loss of 120 u (1289 to 1170) and 100 u (1289 to1190) can be associated to the presence of $NaSO_4H$ and aglycone side chain, respectively. The characteristic losses of 3 unit of 146 u can be associated to the presence of quinovose or fucose sugar residues and losses of 301 u (162 u + 132 u + 7 u) can be associated to the presence of galactose (162 u), and xylose (132 u) sugar residues. It has been reported that Thornasteroside A consisted of five sugar residues (Kitagawa and Kobayashi, 1978; Komori et al., 1983; Minale et al., 1985; Riccio et al., 1985; Findlay et al., 1987; Bruno et al., 1989; Iorizzi et al., 1993; Maier et al., 2007). The MS/MS data obtained from Fr. $F_{3\text{-}3}$ was consistent with the structure of Thornasteroside A (Fig. 4-4) which was reported by Hwang et al. (2014). The position of sugar residues was not confirmed by MS/MS analysis. However, the number of sugar residues, aglycone side chain, and the presence of $NaSO_4H$ can be assumed that the component might be Thornasteroside A (Fig. 4-4).

A similar analysis was carried out on the ion at *m/z* 1267 of Fr. F_2. As can be seen in the Fig. 4-5, the spectrum has a different pattern compared with Fr. $D_{3\text{-}1}$ and Fr. $E_{6\text{-}1}$. The ion at *m/z* 1267 was subjected to MS/MS analysis. However, the ion did not give the same fragmentation like other fractions (Fr. $D_{3\text{-}1}$, $E_{6\text{-}1}$, and $F_{3\text{-}3}$). Therefore, the ion at *m/z* 1267 was confirmed by negative-ion reflector mode. As a result, it was confirmed that *m/z* 1267 contained a sodium group by the presence of the losses of 24 ions. Since the spectra obtained from MALDI-TOF/TOF showed a different fragmentation pattern compared with that usually detected for saponin, the identification of saponin is a much more complex and challenging task, and also there is a lack of information concerning the fragmentation pattern of some asterosaponin in

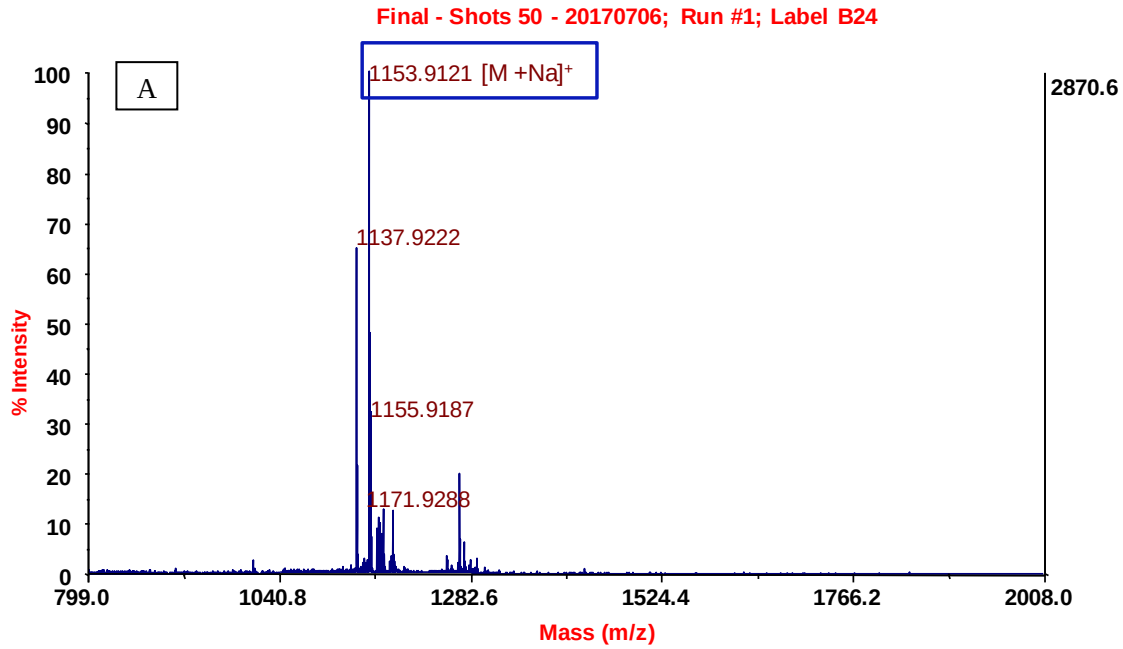
MALDI-TOF/TOF.

The ion was determined by MALDI-TOF/MS based on a pseudo-molecular ion at *m/z* 1267 $[M + Na]^+$ and subjected to structural analysis by NMR. The ^1H NMR spectrum suggested the aglycone of the component is typical cholestane-type steroid. The position of sugar residues and the side chain were determined as asterosaponin (Fig. 4-6). The sugar part was analyzed based on HMQC spectra. Five anomeric ^{13}C NMR signals at δc 104.5, 104.2, 102.3, 106.3, and 104.9 showed the presence of five sugar units. The sugar units was identified as two quinovose (-Qui), one galactose (-Glc), one fucose (-Fuc), and one xylose (Fig. 4-7). Analysis of HMBC data and the sugar residue allowed elucidation for the partial structure of sub Fr F_2 as asterosaponin (Fig. 4-8). All data were well corresponded with those of Thornasteroide A which was reported by Hwang et al. (2014). NMR data suggested both of compounds 1289 and 1267 (Fr. $F_{3\text{-}3}$ and F_2) have the same type of aglycone and same sugar members (Table 4-1). Five anomeric ^{13}C NMR signals and analysis of HMQC and HMBC NMR data (150 MHz, pyridine-d_5) allowed to elucidate for their partial structures as Thornasteroide A.

4.4 Conclusion

Four steroidal saponins were successfully isolated from the crude saponin extraction of *A. amurensis* in this chapter. Evidence from MALDI-TOF/MS and NMR suggested that the saponin component 1289 from Fr. $F_{3\text{-}3}$ and 1267 from Fr. F_2 corresponded with asterosaponin. The purity of *A. amurensis* fractions allowed mass spectrometry analysis and reveals the structure of isomeric compounds containing different aglycone and sugar residues at the ion of *m/z* 1153 and 1165. MS/MS data of Fr. $D_{3\text{-}1}$ at the ion of *m/z* 1153 was corresponded to that of deoxy- and desulfate-analogs of Thornasteroide A. However, the MS/MS data of Fr. $E_{6\text{-}1}$ was neither

correspond to Thornasteroide A nor the analog of Thornasteroide A. Therefore, more investigation is needed to idenfity the ion of *m/z* 1165 component. It can be concluded that the presence of a sulfate group in the sugar moiety of saponins made them more vulnerable to cross-ring cleavages by MS/MS analysis. Further investigations are certainly required to unambiguously identify those saponin components and to confirm the sugar units and the position of sugar residue.



MS/MS at *m/z* 1153

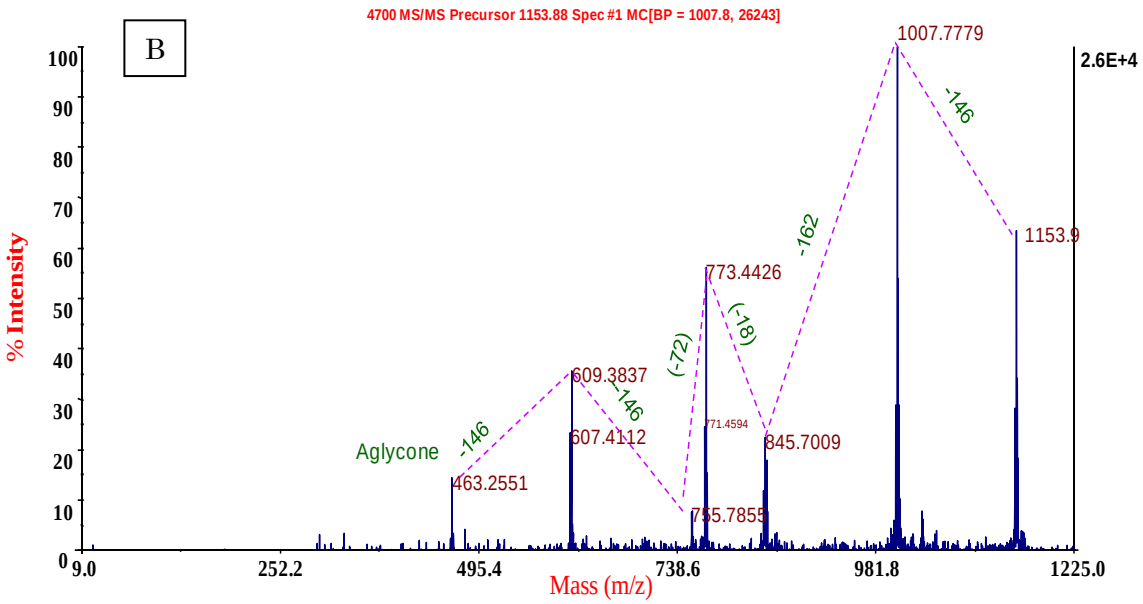
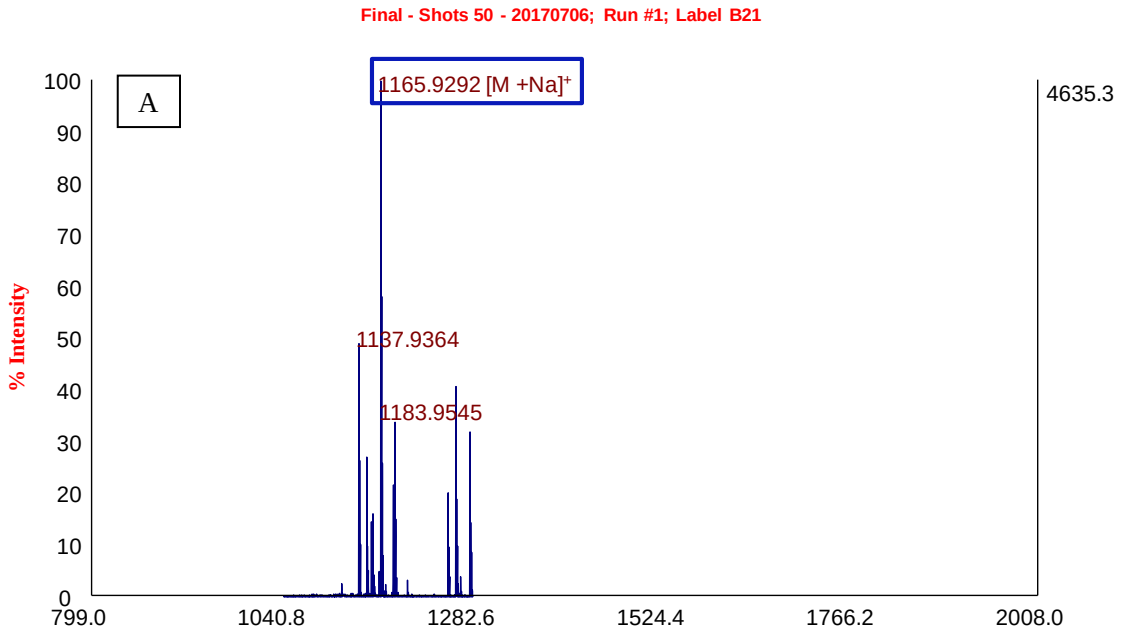


Fig. 4-1 MALDI-TOF mass spectrum of Fr. $D_{3\text{-}1}$(A).

The fragmentation of parent ion at *m/z* 1153.9 was analyzed by MS/MS. The consecutive losses of sulfate group and sugar units generated ion products detected at *m/z* 1007, 845, 755, 609, and 463 (B).



MS/MS at *m/z* 1165

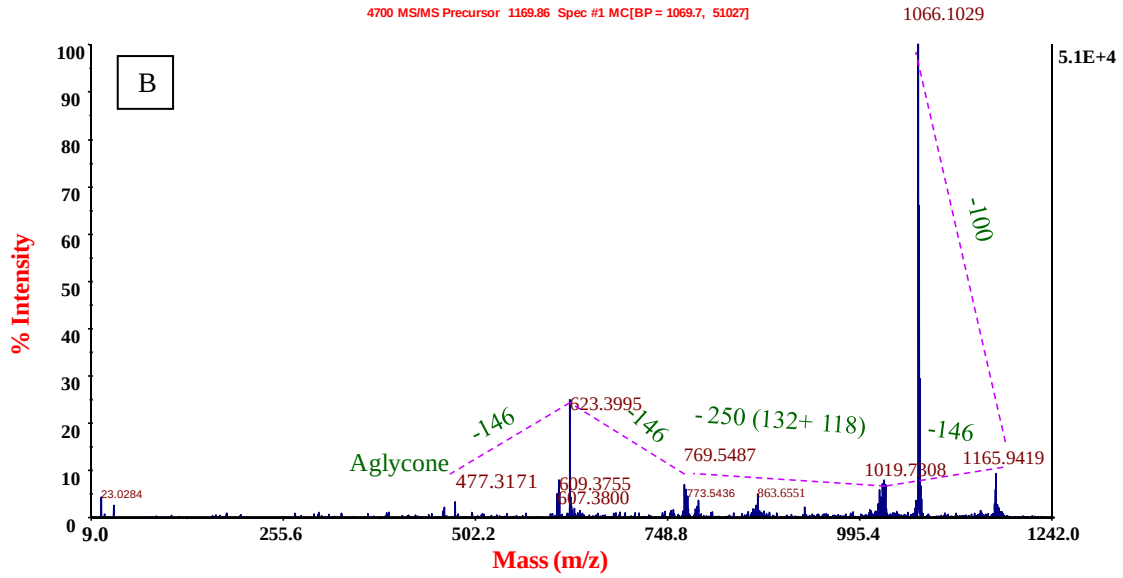
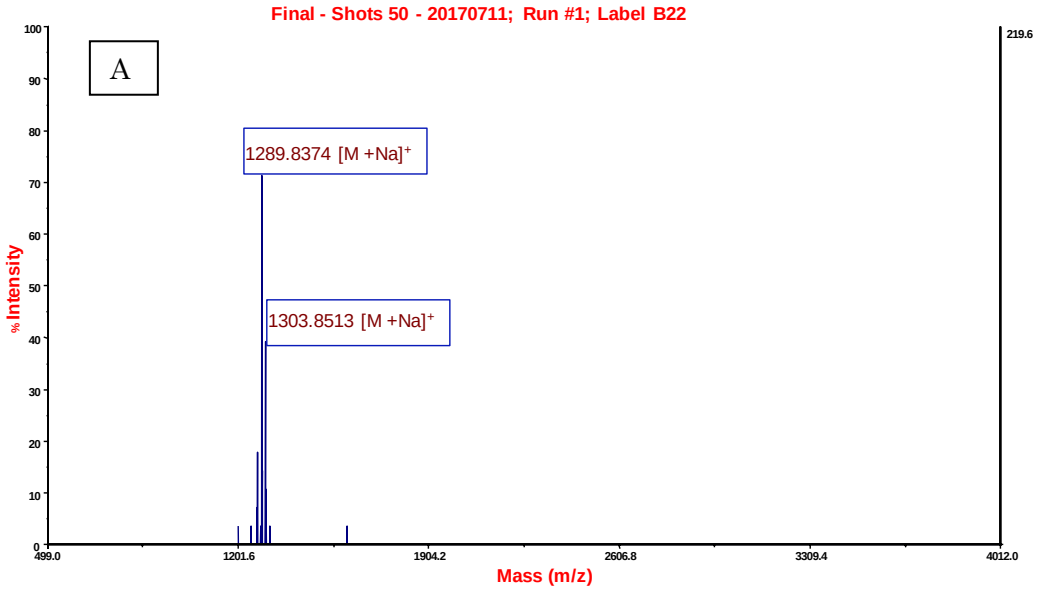


Fig. 4-2 MALDI-TOF mass spectrum of sub Fr. $E_{6\text{-}1}$(A).

The fragmentation of parent ion at *m/z* 1165.9 was analyzed by MS/MS. The consecutive losses of side chain and sugar units generated ion products detected at *m/z* 1066, 1019, 769, 623, and 477 (B).



MS/MS at *m/z* 1289

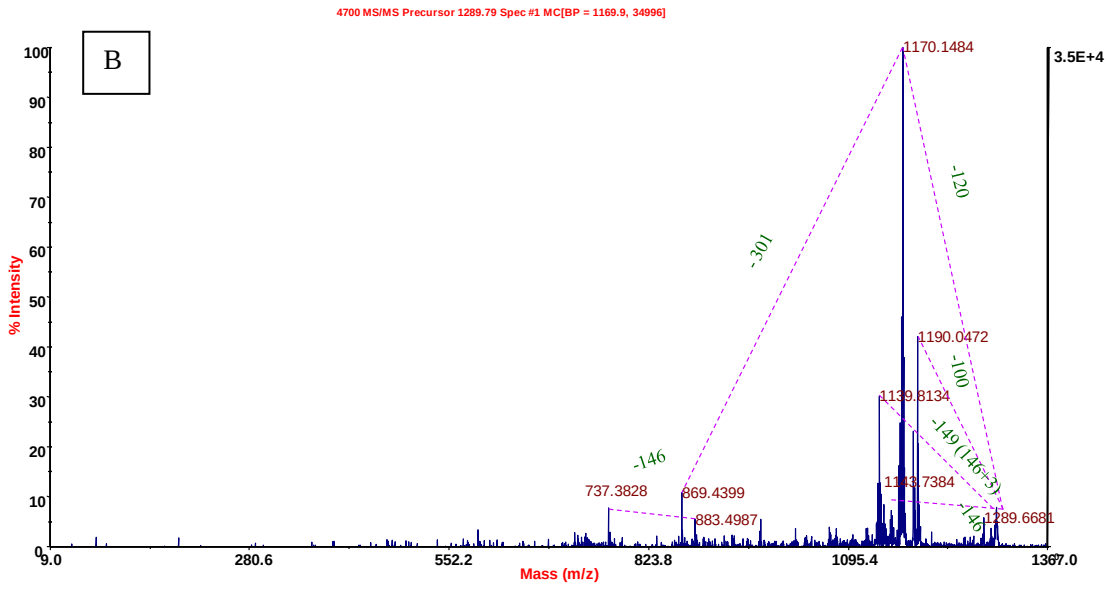
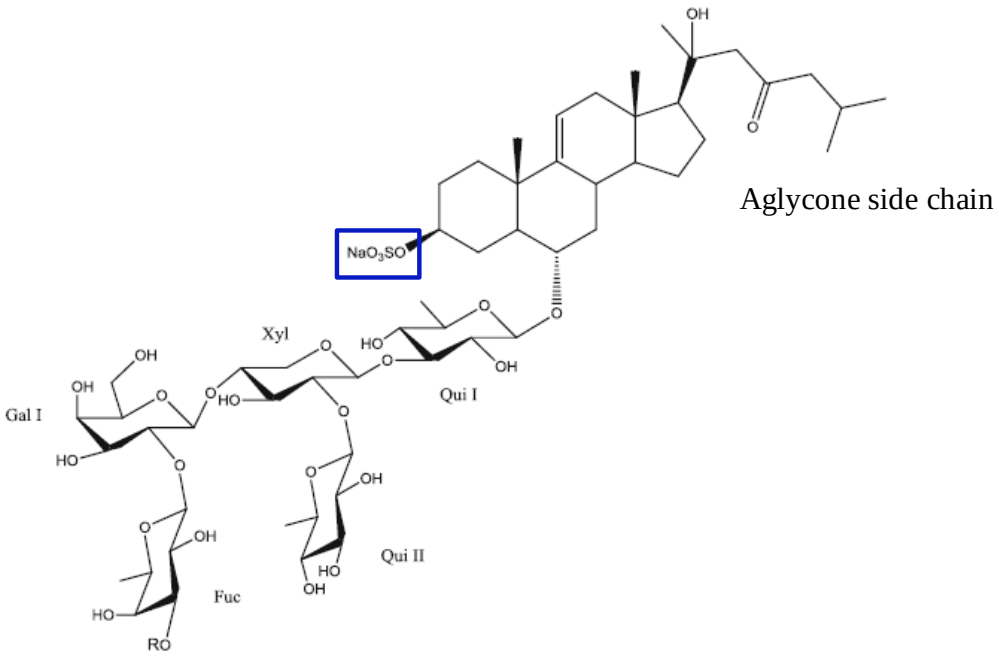


Fig. 4-3 MALDI-TOF mass spectrum of sub Fr. $F_{3\text{-}3}$ (A).

The fragmentation of parent ion at *m/z* 1289 was analyzed by MS/MS. The consecutive losses of side chain and sulfate group and sugar units generated ion products detected at *m/z* 1190, 1170, 1143, 1139, 869, and 737(B).



Sugar residues; 2 quinovose (-Qui), 1 galactose (-Glc), 1 xylose (Xyl) and 1 fucose (-Fuc).

Fig. 4-4 Thornasteroside A (1289, $C_{56}H_{91}O_{28}SNa$) isolated from *A. amurensis* (Hwang et al., 2014).

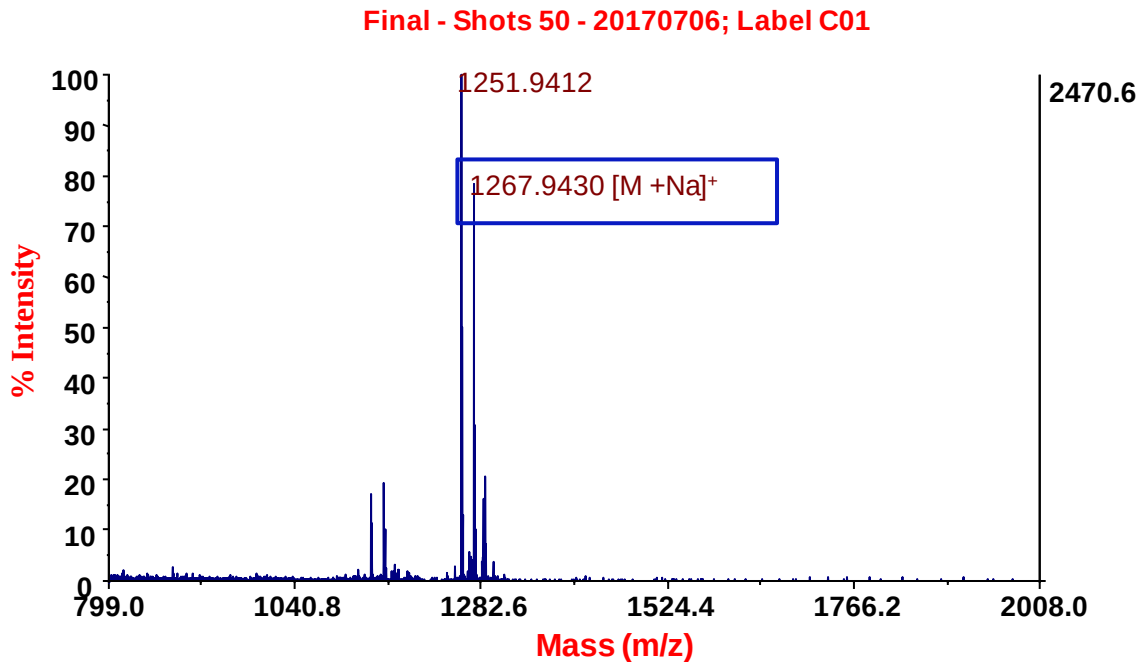


Fig. 4-5 MALDI-TOF mass spectrum of sub Fr. F_2.

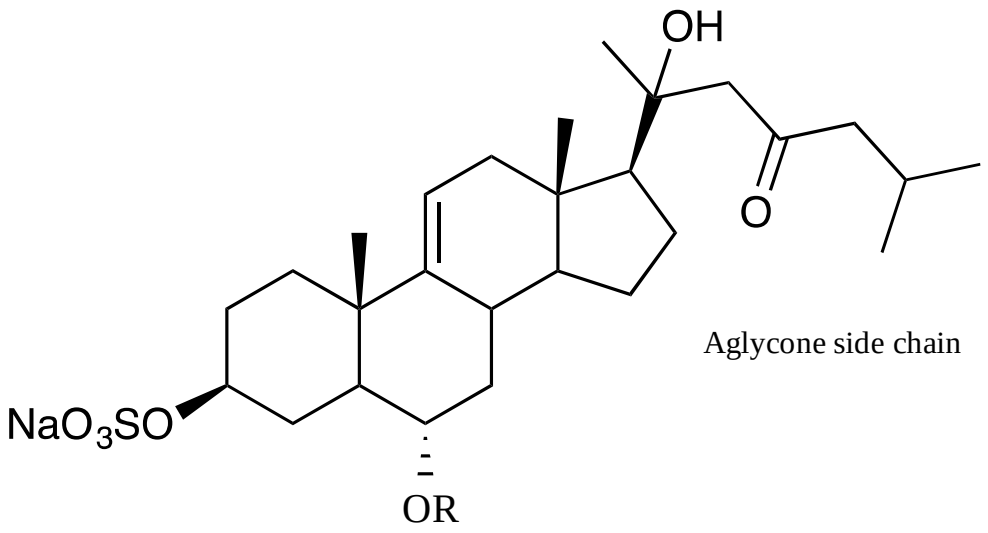


Fig. 4-6 Partial structure of asterosaponin isolated from *A. amurensis* saponin Fr. F_2.

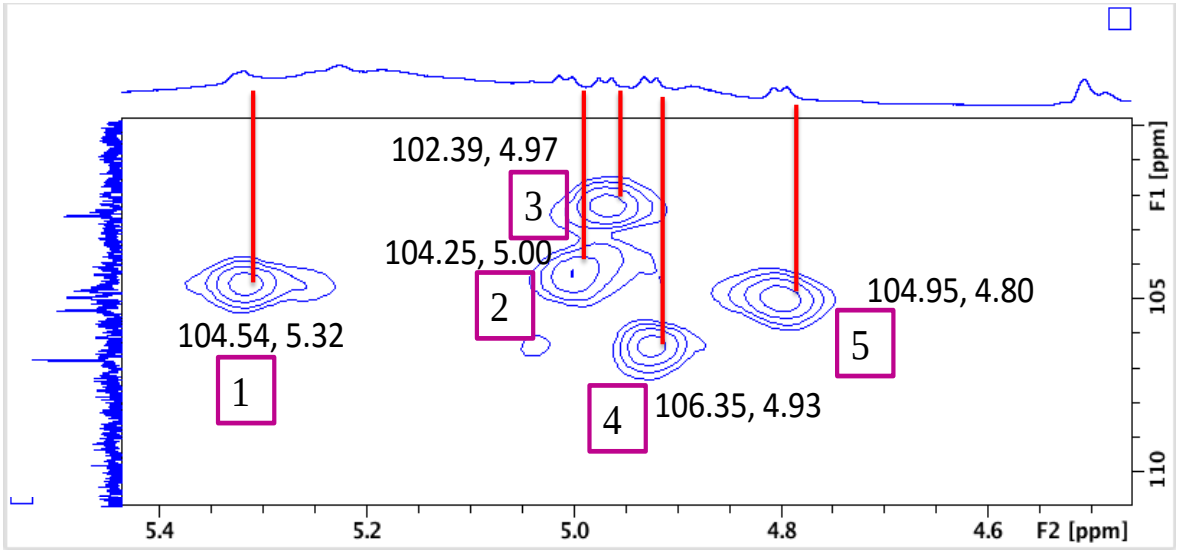


Fig. 4-7 HMQC spectrum of saponin sub Fr. F_2 (1267) in C_5D_5N of sugar residue.

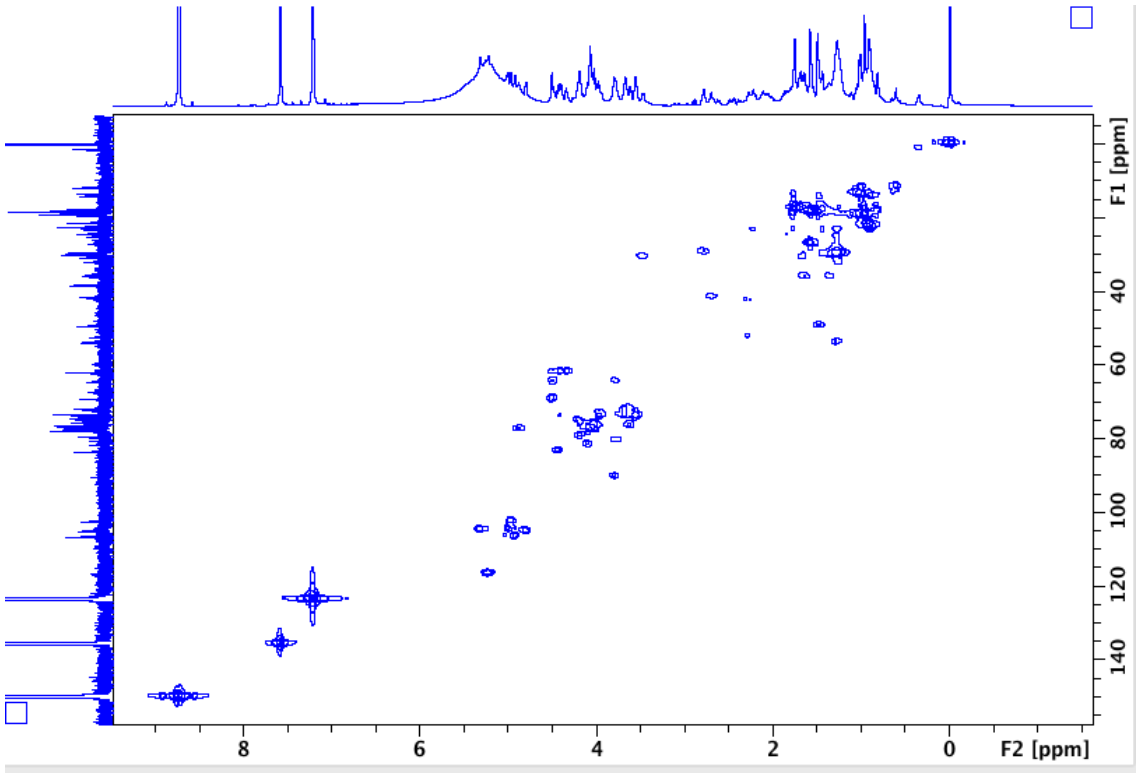


Fig. 4-8 HMQC spectrum of saponin sub Fr. F_2 (1267) in C_5D_5N.

Table 4-1 ^{1}H NMR data of Thronsosteroid A and Fr. F_2 (pyridine-d_5)

Position	Thronsosteroide A						Fr. F_2					
	δ_C and type		δ_H and coupling				δ_C and type		δ_H and coupling			
1	36.4	CH_2	1.39	m	1.65	m	36.0	CH_2	1.35	m	1.65	m
2	29.9	CH_2	1.90	m	2.82	mc	29.5	CH_2	1.87	m	2.78	m
3	77.9	CH	4.93	m			77.6	CH	4.89	br		
4	31.2	CH_2	1.71	m	3.51	br	30.8	CH_2	1.67	m	3.48	m
5	49.8	CH	1.50	m			49.4	CH	1.48	m		
6	80.9	CH	3.81	m			80.6	CH	3.79	m		
7	42.0	CH_2	1.31	m	2.72	m	41.6	CH_2	1.29	m	2.71	m
8	35.7	CH	2.11	m			35.3	CH	2.13	m		
9	145.8	C	–				145.6	C	-			
10	38.7	C	–				38.3	C	-			
11	117.1	CH	5.23				116.7	CH	5.23	d		
12	42.8	CH_2	2.05	br	2.28	m	42.4	CH_2	2.01	m	2.28	m
13	42.0	C	–				41.6	C				
14	54.4	CH	1.29	m			54.0	CH	1.27	m		
15	23.7	CH_2	1.67	m	2.26	m	23.3	CH_2	1.27	m	1.76	m
16	25.5	CH_2	1.26	m	1.82	m	25.2	CH_2	1.27	m	1.89	m
17	59.9	CH	1.67	m			59.9	CH	1.69	m		
18	14.0	CH_3	1.03	s			13.3	CH_3	1.025	s		
19	19.6	CH_3	0.97	s			19.2	CH_3	0.97	s		
20	74.1	C	–				73.8	C	-			
21	27.4	CH_3	1.61	s			27.1	CH_3	1.58	s		
22	55.3	CH_2	2.64	d	2.82	d	54.9	CH_2	2.51	d	2.87	d
23	212.1	C	–				215.4	C	-			
24	54.4	CH_2	2.40	dd	2.48	dd	52.4	CH_2	2.29	m	2.51	m
25	24.7	CH	2.24	m			24.7	CH	1.85	m		
26	23.0	CH_3	0.91	d			22.6	CH_3	0.91	d		
27	22.9	CH_3	0.91	d			22.5	CH_3	0.92	d		

Assignment based on DEPT, HMQC and HMBC NMR data (150 MHz, pyridine-d_5).
Data of sugar parts were not assignment.

Table 4-2 ^{13}C NMR data of Thronsosteroid A and Fr. F_2 (pyridine-d_5)

Position	Thronsosteroide A						Fr. F_2					
	δ_C and type		δ_H and coupling				δ_C and type		δ_H and coupling			
Fuc-1''''	107.8	CH	4.86	d			106.4	CH	4.93	d		
QuiI-1'	105.6	CH	4.83	d			105.0	CH	4.80	d		
QuiII-1'''	105.3	CH	5.34	d			104.5	CH	5.32	d		
Xyl-1''	104.9	CH	5.05	d			104.3	CH	5.00	d		
GalI-1''''	102.8	CH	5.00	d			102.4	CH	4.97	d		
QuiI-3'	90.5	CH	3.83	t			90.5	CH	3.80	m		
GalI-2''''	84.1	CH	4.49	m			83.5	CH	4.43	m		
Xyl-2''	82.0	CH	4.13	m			81.7	CH	4.10	m		
Xyl-4''	79.7	CH	4.23	m			79.5	CH	4.19	m		
GalI-5''''	77.4	CH	4.09	m			77.8	CH	4.03	m		
QuiII-3'''	77.2	CH	4.12	m			77.0	CH	4.07	m		
QuiII-2'''	76.6	CH	4.12	m			76.8	CH	3.62	m		
Xyl-3''	76.3	CH	4.22	m			76.7	CH	4.19	m		
QuiII-4'''	75.9	CH	4.08	m			76.5	CH	3.62	m		
Fuc-3''''	75.5	CH	4.01	t			76.2	CH	4.17	m		
GalI-3''''	75.4	CH	4.21	m			75.9	CH	4.04	m		
QuiI-4'	75.0	CH	3.58	m			75.5	CH	4.20	m		
QuiI-2'	74.5	CH	4.03	m			74.9	CH	4.21	m		
Fuc-2''''	74.3	CH	4.46	m			74.1	CH	3.97	m		
QuiII-5'''	74.0	CH	3.71	m			73.6	CH	3.67	m		
Fuc-4''''	73.0	CH	3.96	br			73.5	CH	3.67	m		
QuiI-5'	72.4	CH	3.72	m			72.6	CH	3.62	m		
Fuc-5''''	72.3	CH	3.64	m			72.1	CH	3.68	m		
GalI-4''''	69.7	CH	4.52	br			69.4	CH	4.50	m		
Xyl-5''	65.0	CH_2	3.83	m	4.51	m	64.6	CH_2	3.79	m	4.49	m
GalI-6''''	62.3	CH_2	4.37	dd	4.44	m	61.9	CH_2	4.33	m	4.40	m
QuiI-6'	18.9	CH_3	1.60	d			18.5	CH_3	1.49	m		
QuiII-6'''	18.3	CH_3	1.78	d			18.5	CH_3	1.70	m		
Fuc-6''''	17.6	CH_3	1.46	d			17.9	CH_3	1.75	m		

Assignment based on DEPT, HMQC and HMBC NMR data (150 MHz, pyridine-d_5).
Data of sugar parts were not assignment.

Chapter 5

General discussion and perspective for future study

5.1 Discussion

Saponins are a group of natural products mostly derived from plants. They show strong biological activity and have been used as herbal medicines for a long time (Ohtsuki, 1984). Certain marine animals including echinoderms, starfish, sea cucumber, and sponges, also produce saponin, most of which have a defensive role against infectious agents (Nishiyama et al.,1987; Kubanek et al., 2002; van Dyck et al., 2009). Marine organisms such as starfish have been found to produce a great diversity of novel bioactive secondary metabolites and be a potential source for new drug discovery.

Starfish saponin was extracted with different solvents such as methanol and *n*-butanol. It is well known that methanol is considered to be a universal solvent which is able to extract active components. It is common to collect steroidal glycosides from *n*-butanol extracts of entire animals of starfish (Kicha et al., 2011). The starfish was chopped in small pieces and homogenized with methanol. The extract was performed with *n*-butanol and then water, to afford sulfated steroidal glycosides (asterosaponins) from starfish (Kicha et al., 2011). These glycosides are also studied in the research of chemical constituents and biological activities of starfish, with considerable clinical interest, since they show several physiological, pharmacological, and immunological activities (Dong et al., 2011).

Saponins are classified as triterpene and steroidal types. Starfish saponin is identified as steroidal saponin. The four series of biological properties, antifungal, antibacterial, hemolytic, and cholesterol-binding ability were determined and the present data in this study clearly showed

that the biological activity of saponin varied in their composition of saponin. The action mechanisms of saponin may lie in damage to the membrane and leakage of cellular materials, ultimately leading to cell death (Mshvildadze et al., 2000). This activity has been documented in a number of saponins, and they show the damaging effects against a variety of fungi (Lalitha and Venkataraman, 1991) and rupture of the erythrocyte. It is thus assumed that the saponin components among starfish are not the same.

Saponins are complex mixtures composed of different types aglycones and carbohydrate fractions (the type, number and the binding site of monosaccharides) (Luciana et al., 2013). Saponin from *A. amurensis* was fractioned by silica gel column chromatography. The structural diversity of the saponin results in their physicochemical and biological properties (Oakenfull and Sidhu, 1989; Hostettmann and Marston, 1995). It is interesting that the saponin components having higher polarity relatively (Fr. D, E, F, and G in Chapter 3) were more active to the tested fungi cells than those having lower polarity (Fr. A, B, and C in Chapter 3). The antifungal activity, particularly for the saponin components having higher polarity (Fr. D to G), was more potent than the hemolytic activity. These observations indicate that the antifungal and hemolytic activity of these saponins components might be not correlated. It is known that saponin interactions with the cell membrane are dependent on the structure of the saponin as well as cell membrane (Francis et al., 2002). The increases in antifungal and cholesterol-binding ability of the saponins were significant especially in the case of the saponin components having higher polarity (Fr. D to G). These saponin components were approximately three fold more active than those having lower polarity (Fr. A to C). For the esterified saponins, the order of hemolytic activity in each series was not the same. It has been described that esterification of genin or aglycone in saponin molecule increases hemolytic activity because of reduction of polarity

(Chwalek et al., 2004). As a whole, the same phenomenon was observed in the present study, except for Fr. D to G that was active. In contrast, the cholesterol-binding ability is generally reduced after esterification for saponins fraction A to C. Therefore, it is worthwhile to further study these antifungal and cholesterol-binding compounds, and in a broad sense, the steroidal saponins of *A. amurensis* may contribute to explore the therapeutic potential of this important class of natural products as antifungal and cholesterol-lowering agents lead for pharmaceuticals and industrial use.

Starfish saponin can be categorized into three main groups: asterosaponins possessing a sulfated steroidal moiety, steroidal cyclic glycosides, and polyhydroxysteroidal saponin. Asterosaponins are well-known secondary metabolites of the common starfish *A. amurensis* in which they would be involved in chemical defense, digestion, and reproduction (Demeyer et al., 2014). Previous studies have identified seven different saponin congeners in the whole individuals of *A. amurensis* species (Hwang et al., 2011; Hwang et al., 2014). The asterosaponin family contains many different members (Marino et al., 1998; Tang et al., 2005; Cheng et al., 2006). According to the results, the presence of these anomeric configurations, anywhere in the sugar moiety, is unfavourable for both the antifungal and cholesterol-binding ability. The functional properties (antifungal activity) elucidated by four saponin components were not same. The fractions $D_{3\text{-}1}$ and $F_{3\text{-}3}$ (detected as Thronasteriside A) exhibited the higher antifungal activity. On the other hand, Fr. $E_{6\text{-}1}$ showed less activity with the exceptions of those possessing in the sugar of galactose group moiety. Meanwhile, saponin Fr. $D_{3\text{-}1}$, $F_{3\text{-}3}$, and F_2, including fucose and galactose groups of sugar residues, showed higher antifungal activity. These data led to assume that the presence of a certain degree of lipophilicity in the sugar moiety is essential for exhibiting

the higher activity as shown by Mimaki et al., 2001. With the same aglycone and length of sugar chain, the sugar linkage will determine the antifungal and cholesterol-binding ability.

The structural difference, for example, between Fr. F_2 and $E_{6\text{-}1}$ (with the difference of one mono- and di-saccharide residue), imposes a difference on their antifungal and cholesterol-binding ability. The selectivity and specificity of a particular saponin is apparently associated with the target at the cellular or molecular level, which is still unknown at this point. In the study on saponin structure-activity relationships, both the aglycone and the sugar moiety play an important role in the evaluation of biological activity (Takechi and Tanaka, 1995; Voutquenne et al., 2002; Chwalek et al., 2004). It also indicates that the functional properties of saponin may be depending on the position of sugar residue.

Based on the above results and discussion, it was suggested that starfish saponin can be used as antifungal and cholesterol-binding components. In conclusion, the results obtained in the present study showed that the crude saponin of starfish is an interesting source for antifungal compounds, and it could be used as a lead source in the development of the potent antifungal drugs. In addition, the environment and shellfish industry also be benefitted by removal of starfish from coastal areas.

5.2 Prospect for future studies

The study on biochemical and functional properties of starfish saponins has made remarkable progress owing to the present results. Starfish saponin exhibited significantly higher antifungal and cholesterol-binding ability. While plant saponins from tea seed and quillaja bark have been used at pharmaceutical, cosmetic, and food industry, the saponin from starfish are not yet utilized at industrial scale. However, properties of the rest are unknown and hence saponin risk to human or animal health remains to be assessed. In relation with these different biological functions,

several studies have suggested that each organ namely the aboral body wall, the oral body wall (including tube feet), the stomach, the pyloric caeca, and the gonads (Demeyer et al., 2014) would possess its own saponin mixture (Mackie et al., 1977; Voogt and Van Rheenen, 1982). Consequently, it is now difficult to discuss as to the distribution of saponin in this study. It is thus assumed that further research on distribution of saponin in starfish is necessary. Therefore, there are significant opportunities for extension of this research and application across a diverse range of marine animal, starfish.

Further research should be carried out based on the current findings in this study, e.g., to further characterize the isolated component, to investigate the chemical structure, and to clarify the relationship between function and structure. Plant saponins such as quillaja, soybean saponin are relevant to human nutrition because of their presence in several human food items and health products. It is anticipated that further studies could lead to promising applications of starfish saponins in food, or pharmaceutical industry.

References

Afrose, S., Hossain, M.S., Maki, T., Tsujii, H., 2009. Karaya root saponin exerts a hypocholesterolemic response in rats fed a high-cholesterol diet. Nut. Res. 29, 350-354.

Alves, R.R.N., Alves, H.N., 2011. The faunal drugstore: animal-based remedies used in traditional medicines in Latin America. J. Ethnobiol. Ethnomed. 7, 1-43.

Alves, R.R.N., Rosa, I.L., 2007. Zootherapy goes to town: the use of animal-based remedies in urban areas of NE and N Brazil. J. Ethnopharmacol. 113, 541-555.

Andersson, L.L., Bohlin, M. Iorizzi, R. Riccio, L. Minale, W. Moreno-Lopez., 1989. Biological activity of saponins and saponin-like compounds from starfish and brittle-stars. Toxicon 27, 179-188.

Anisimov, M.M., Prokofeva, N.G., Korotkikh, L.Y., Kapustina, I.I., Stonlik, V.A., 1980. Comparative study of cytotoxic activity of triterpene glycosides from marine organisms. Toxicol. 18, 221-223.

Augustin, J.M., Kuzina, V., Andersen, S.B., Bak, S., 2011. Molecular activities, biosynthesis and evolution of triterpenoid saponins, Phytochem. 72, 435-457.

Authi, K.S., Rao, G.H.R., Evenden, B.J., Crawford, N., 1988. Action of guanosine 50-(beta-thio) diphosphate on thrombin-induced activation and calcium mobilization in saponin-permeabilized and intact human platelets. Biochem. J. 255, 885-894.

Balandrin, M.F., 1996. Commercial utilization of plant-derived saponins: an overview of medicinal, pharmaceutical and industrial application. Saponins Used in Traditional and Modern Medicine. Waller, G.R., Yamasaki, K. eds., Plenum Press, New York, pp. 1-14.

Blake, D.B., 1987. A classification and phylogeny of post-palaeozoic sea stars (*Asteridea*:

Echinodermata). J. Nat. Hist. 21, 481-528.

Blake, D.B., 1988. Paxillosidans are not primitive asteroids: a hypothesis based on functional considerations. Echinoderm Biology. Burke, R.D. ed., Balkema, Rotterdam, pp. 309-314.

Bottger, S., Hofmann, K., Melzig, M.F., 2012. Saponins can perturb biologic membranes and reduce the surface tension of aqueous solutions: A correlation? Bioorg. Med. Chem. 20, 2822-2828.

Bouarab, K., Melton, R., Peart, J., Baulcombe, D., Osbourn, A., 2002. A saponin-detoxifying enzyme mediates suppression of plant defenses. Nature 418, 889-892.

Braunwald, M.D., 1997. Cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N. Engl. J. Med. 337, 1360-1369.

Bruno, I., Minale, L., Riccio, R., 1989. Starfish saponins, 38. Steroidal glycosides from the starfish *Pycnopodia helianthoides.* J. Nat. Prod. 52, 1022-1026.

Charles, G., Jean, L., Karl, G.L., Vakhtang, M., André, P., 2009. Haemolytic activity, cytotoxicity and membrane cell permeabilization of semi-synthetic and natural lupane- and oleanane-type saponins. Bioorg. Med. Chem. 17, 2002-2008.

Cheeke, P. R., 1998. Natural Toxicants in Feeds, Forages and Poisonous Plants. Upper Saddle River, Prentice-Hall, NJ.

Cheeke, P. R., Otero. R., 2005. Yucca, quillaja may have role in animal nutrition. Feedstuffs 3(Suppl.), 11-14.

Cheng, G., Zhang, X., Tang, H.F., Zhang, Y., Zhang, X.H., Cao, W.D., Gao, D.K., Wang, X.L., Jin, B.Q., 2006. Asterosaponin 1, acytostatic compound from the starfish *Culcita novaeguineae*, functions by inducing apoptosis in human glioblastoma U87MG cells. J. Neurooncol. 79, 235-241.

Ching, T.Y.C., Dillon, H., 2013. Major findings of the LOCHI study on children at 3 years of age and implications for audiological management. Int. J. Audiol. 52, 65-68.

Chludil, H.D., Seldes, A.M., Maier, M.S., 2002. Antifungal steroidal glycosides from the Patagonian starfish *Anasterias minuta*: Structure activity correlations. J. Nat. Prod. 65, 153-157.

Choi, D.H., Shin, S., Park, I.K., 1999. Characterization of antimicrobial agents extracted from *Asterina pectinifera*. Int. J. Antimicrob. Agents 11, 65-68.

Choi, S., Jung, S.Y., Kim, C.H., Kim, H.S., Rhim, H., Kim, S.C., Nah, S.Y., 2001. Effect of ginsenosides on voltage-dependent Ca2+ channel subtypes in bovine chromaffin cells. J. Ethnopharmacol. 74, 75-81.

Chwalek, M., Plé, K., Voutquenne-Nazabadioko, L., 2004. Synthesis and hemolytic activity of some hederagenin diglycosides, Chem. Pharm. Bull. 52, 965-971.

Cioaca, C., Margineanu, C., Cucu V., 1978. The saponins of *Hedera helix* with antibacterial activity. Pharmazie 33, 609-610.

Coulson, C.B., Davis, T., 1962. Saponin II. Fractionation and pharmacological properties of lucerne saponins. Possible relation of these to bloat. J. Sci. Food Agric. 13, 53-57.

Courchaine, A.J., Miller, W.H., Stein, D.B., 1959. Rapid semi micro procedure for estimating free and total cholesterol. Clin. Chem. 5, 609-614

Cui, M., Song, F., Liu, Z., Liu, S., 2001. Metal ion adducts in the structural analysis of ginsenosides by electrospray ionization with multi-stage mass spectrometry. Rapid Commun. Mass Spectrom. 15, 586-595.

D'Auria, M.V., Minale, L., Riccio, R., 1993. Polyoxygenated steroids of marine origin. Chem. Review 93, 1839-1895.

Demeyer, M., Winter, J., Caulier, G., Eeckhaut, I., Patrick, F., Pascal, G., 2014. Molecular diversity and body distribution of saponins in the sea star *Asterias rubens* by mass spectrometry. Comp. Biochem. Physiol. B 168, 1-11.

Dong, G., Xu, T.H., Yang, B., Lin, X.P., Zhou, X.F., Yang, X.W., Liu, Y.H., 2011. Chemical constituents and bioactivities of starfish. Chem. Biodivers. 8, 740-791.

El Izzi, A., Benie, T., Thieulant, M.L., Le Men-Olivier, L., Duval, J., 1992. Stimulation of LH release from cultured pituitary cells by saponins of *Petersianthus macrocarpus*: A permeabilizing effect. Planta Med. 58, 229-233.

Feng-Juan, W., Yong, X., Qing-Juan, T., Jie, X., Lei, D., Chang- Hu, X., Koretaro, T., Ming, W.Y., 2013. The protective effects of cerebrosides from sea cucumber and starfish on the oxidative damage in PC12 cells. J. Oleo Sci. 62, 717-727.

Findlay, J.A., Jaseja, M., Burnell, D.J., Brisson., J.R., 1987. Major saponins from the starfish *Asterias forbesi*. Complete structures by nuclear magnetic resonance methods. Can. J. Chem. 65, 1384-1391.

Fomsgaard, C., Saurel, C., Fitridge, I., Canal-Vergés, P., Petersen, J.K., 2014. Starfish by-products management for the mussel industry in Denmark. Aquaculture Europe 2014, unpublished data. https://www.was.org/easonline/documents/MeetingPresentations/AE2014/AE2014_0400.pdf.

Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199, 197-227.

Francis, G., Kerem, Z., Makkar, H.P.S., Becker, K., 2002. The biological action of saponins in animal systems: A review. Br. J. Nutr. 88, 587-605.

Fuchs, H., Bachran, D., Panjideh, H., Schellmann, N., Weng, A., Melzig, M.F., Sutherland, M.,

Bachran, C., 2009. Saponins as tool for improved targeted tumor therapies. Curr. Drug. Targets 10, 140-151.

Gee, J.M., Price, K.R., Ridout, G.L., Wortley, G.M., Hurrell, R.F., Johnson, I.T., 1998. Saponins of quinoa (*Chenopodium quinoa*): Effects of processing on their abundance in quinoa products and their biological effects on intestinal mucosal tissue. J. Sci. Food Agric. 63, 201-209.

Global Invasive Species Database, 2016. Species profile: *Asterias amurensis*; http://www.iucngisd.org/gisd/species.php?sc=82 "Accessed 17 May 2016."

Gonzalez-Vega, J.C., Walk, C.L., Liu, Y., Stein, H.H., 2013. Endogenous intestinal losses of calcium and true total tract digestibility of calcium in canola meal fed to growing pigs. J. Anim. Sci. 91, 4807-4816.

Haralampidis, K., Trojanowska, M., Osbourn., A.E., 2002. Biosynthesis of triterpenoid saponins in plants. Adv. Biochem. Eng. Biotechnol. 75, 31-49.

Harmatha, H., 2000. Chemo-ecological role of spirostanol saponins in the interaction between plant and insects. Saponins in Food, Feedstuffs and Medicinal Plants. Olezsek, W., Marston, A. eds., Springer International Publishing AG, Switzerland, pp. 129-141.

Harris, W.S., Dujovne, C.A., Windsor, S.L., Gerrond, L.L.C., Newton, F.A., Gelfand, R.A., 1997. Inhibiting cholesterol absorption with CP-88,818 (beta-tigogenin cellobioside; tiqueside): Studies in normal and hyperlipidemic subjects. J. Cardiovasc. Pharmacol. 30, 55-60.

Hashim, F.J., 2014. Hemolytic activity of *Cicer arietinum* l. Extracts by two extraction techniques. Asian J. Pharm. Clin. Res. 7, 141-145.

Hassan, S.M., Byrd, J.A., Cartwright, A.L., Bailey, C.A., 2010. Hemolytic and antimicrobial activities differ among saponin-rich extracts from guar, quillaja, yucca, and soybean.

Appl. Biochem. Biotechnol. 162, 1008-1017.

Hostettmann, K.A., Marston, A., 1995. Saponins. Chemistry and pharmacology of natural products. Cambridge University Press, Cambridge, United Kingdom, pp. 232-286.

Hussain, I., Cheeke., P.R., 1995. Effect of *Yucca scidigera* extract on rumen and blood profiles of steers fed concentrate- or roughage-based diets. Anim. Feed Sci. Tech. 51, 231-242.

Hwang, I.H., Kim, D.W., Kim, S.J., Min, B.S., Lee, S.H., Son, J.K., Kim, C.H., Chang, H.W., Na, M.K., 2011. Asterosaponins isolated from the starfish *Asterias amurensis*. Chem. Pharm. Bull. 59, 78-83.

Hwang, I.H., Kulkarni, R., Yang, H.M., Choo, S.J., Zhou, W., Lee, S.M., Jang, T.S., Jeong, G.S., Chang, H.W., Na, M.K., 2014. Complete NMR assignments of undegraded asterosaponins from *Asterias amurensis.* Arch. Pharm. Res. doi: 10.1007/s12272-014-0374-0379.

Ibanoglu, E., Ibanoglu, S., 2000. Foaming behavior of liquorice (*Glycyrrhiza glabra*) extract. Food Chem. 70, 333-336.

Imamichi, Y., Yokoyama, Y., 2013. Purification and characterization of a lectin from the starfish *Asterias amurensis.* Fish. Sci. doi: 10.1007/s12562-013-0667-9.

Inagaki, M., Ikeda, Y., Kawatake, S., Nakamura, K., Tanaka, M., Misawa, E., Yamada, M., Higuchi, R., 2006. Isolation and structure of four new ceramides from the starfish *Luidia maculata*. Chem. Pharm. Bull. 54, 1647-1649.

Iorizzi, M., De Marino, S., Zollo, F., 2001. Steroidal oligoglycosides from the Asteroidea. Curr. Org. Chem. 5, 951-973.

Ishii, T., Okino, T., Mino, Y., 2006. A ceramide and cerebroside from the starfish *Asterias amurensis* Luetken and their plant-growth promotion activities. J. Nat. Prod. 69, 1080-

1082.

Ishii, T., Okino, T., Mino, Y., Tamiya, H., Matsuda, F., 2007. Plant-growth regulators from common starfish (*Asterias amurensis* Luetken) waste. Plant Growth Regul. 52, 131-139.

Ishizaki, S., Iwase, M., Tanaka, M., 1997. Enhancing effect of starfish *Asterias amurensis* saponin upon thermal aggregation of actomyosin from walleye pollack meat. Fish. Sci. 63, 159-160.

Ito, S., Ihara, T., Tamura, H., Tanaka, S., Ikeda, T., Kajihara, H., Dissanayake, C., Abdel-Motaal, F.F., El-Sayed, M.A., 2007. α-Tomatine, the major saponin in tomato, induces programmed cell death mediated by reactive oxygen species in the fungal pathogen *Fusariu oxysporum*. FEBS Lett. 581, 3219-3224.

Ivanchina, N.V., Kalinovsky, A.I., Kicha, A.A., Malyarenko, T.V., Dmitrenok, P.S., Ermakova, S.P., Stonik, V.A., 2012. Two new asterosaponins from the far eastern starfish *Lethasterias fusca*. Nat. Prod. Commun. 7, 853-858.

Ivanchina, N.V., Kicha, A.A., Stonik, V.A., 2011. Steroid glycosides from marine organisms. Steroids 76, 425-454.

Jackson, H.D., Shaw, R.A., 1959. Chemical and biological properties of a respiratory inhibitor from alfalfa saponins. Arch. Biochem. Biophys. 84, 411-416.

Jenkins, K.J., Atwal, A.S., 1994. Effects of dietary saponins on fecal bile acids and neutral sterols, and availability of vitamins A and E in the chick. J. Nutr. Biochem. 5, 134-138.

Kalinin, V.I., Aminin, D.L., Avilov, S.A., Silchenko, A.S., Stonik, V.A., 2008. Triterpene glycosides from sea cucucmbers (Holothurioidea, Echinodermata). Biological activities and functions. Studies in Natural Products Chemistry. Atta-ur, R., ed. Elsevier, Amsterdam, The Netherlands 35, pp. 135-196.

Kerem, Z., German-Shashoua, H., Yarden, O., 2005. Microwave-assisted extraction of bioactive saponins from chickpea (*Cicer arietinum* L). J. Sci. Food Agri. 85, 406-412.

Khalil, A.H., EI-Adawy, T.A., 1994. Isolation, identification and toxicity of saponin from different legumes. Food Chem. 50, 197-201.

Kicha, A.A., Ivanchina, N.V., Stonik, V.A., 2003. Seasonal variations in the content of polyhydroxysteroids and related glycosides in the digestive tissues of the starfish *Patiria (=Asterina) pectinifera*. Comp. Biochem. Physiol. B 136, 897- 903.

Kicha, A.A., Kalinovsky, A.I., Ivanchina, N.V., Malyarenko, T.V., Dmitrenok, P.S., Ermakova, S.P., Stonik, V., 2011. Four new asterosaponins, hippasteriosides A-D, from the Far Eastern starfish *Hippasteria kurilensis*. Chem. Biodivers. 8, 166-175.

Kitagawa, I., Kobayashi, M., 1978. Saponin and sapogenol. XXVI. Steroidal saponins from the starfish *Acanthaster planci* L. (crown of thorns). (2). Structure of the major saponin thornasteroside A. Chem. Pharma. Bull. 26, 1864-1873.

Komori, T., Matsuo, J., Itakura, Y., Sakamoto, K., Ito, Y., Taguchi, S., Kawasaki, T., 1983. Biologically active glycosides from asteroidea, II. Steroid oligoglycosides from the starfish *Acanthaster planci* L., Isolation and structure of the oligoglycoside sulfates. Liebigs Ann. 24-36.

Korchowiec, B., Gorczyca, M., Wojszko, K., Janikowska, M., Henry, M., Rogalska, E., 2015. Impact of two different saponins on the organization of model lipid membranes. Biochim. Biophys. 1848, 1963-1973.

Kubanek, J., Whalen, K.E., Engel, S., Kelly, S.R., Henkel, T.P., Fenical, W., Pawlik, J.R., 2002. Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 1, 125–136.

Kumar, R., Kumar, A.C., Kumar, P.S., Lakshmia, V., 2007. Antifungal activity in triterpene glycosides from the sea cucumber *Actinopyga lecanoraq*. Bioorg. Med. Chem. Lett. 17, 4387–4391.

Lacalle-Dubois, M.A., Wagner, H., 2000. Bioactive saponins from plants: an update. Studies in Natural Products Chemistry Series. Atta-Ur-Rahman ed., vol. 21, Elsevier Science, Amsterdam, pp. 633-687.

Lalitha, T., Venkataraman, L.V., 1991. Antifungal activity and mode of action of saponins from Madhuca butyracea Macb. Indian J. Exp. Biol. 29, 558-562.

Liu, J., Yang, X., He, J., Xia, M., Xu, L., Yang, S., 2007. Structure analysis of triterpene saponins in *Polygala tenuifolia* by electrospray ionization ion trap multiple-stage mass spectrometry. J. Mass Spectrom. 42, 861-873.

Liu, Z., Gao, W., Jing, S., Zhang, Y., Man, S., Wang, Y., Zhang, J., Liu, C., 2013. Correlation among cytotoxicity, hemolytic activity and the composition of steroidal saponins from Paris L. J. Ethnopharm. 149, 422-430.

Luciana, M., Collins, P.F., Muetzel, R.L., Lim, K.O., 2013. Effects of alcohol use initiation on brain structure in typically developing adolescents. Am. J. Drug Alcohol Abuse. 39, 345-355.

Luo, P., Hu, C.Q., Xia, J.J., Ren, C.H., Jiang, X., 2011. Chemical constituent analysis of the crown-of-thorns starfish *Acanthaster planci* and potential utilization value of the starfish as feed ingredient for animals. Afr. J. Biotechnol. 10, 13610-13616.

Mackie, A.M., Singh, H., Owen, J., 1977. Studies on the distribution, biosynthesis and function of steroidal saponins in echinoderms. Comp. Biochem. Physiol. B 56, 9-14.

Mahato, S.B., Sudip, S.K., Poddar, G., 1988. Review article number 38: Triterpenoid saponins. Phytochem. 27, 3037-3067.

Maier, M.S., Centurio´n, R., Muniain, C., Haddad, R., Eberlin, M.N., 2007. Identification of sulfated steroidal glycosides from the starfish *Heliaster helianthus* by electrospray ionization mass spectrometry (UR-2177CP). Arkivoc 7, 301-309.

Man, S., Gao, W., Yanjun, Z., Huang, L., Liu, C., 2010. Chemical study and medical application of saponins as anti-cancer agents. Fitoterapia 81, 703-714.

Marino, S.D., Iorizzi, M., Palagiano, E., Zollo, F., Roussakis, C., 1998. Starfish saponins. 55. Isolation, structure elucidation, and biological activity of the steroid oligoglycosides from an Antarctic starfish of the family Asteriidae. J. Nat. Prod. 61, 1319-1327.

Matsubara, M., Komatsu, M., Araki, T., Asakawa, S., Yokobori, S., Watanabe, K., Wada, H., 2005. The phylogenetic status of Paxillosida (Asteroidea) based on complete mitochondrial DNA sequences. Mol. Phylogenet. Evol. 36, 598-605.

Matsui, Y., Kumagai, H., Masuda, H., 2006. Anti hypercholesterolemic activity of catechin-free saponin-rich extract from green tea leaves. Food Sci. Technol. Res. 12, 50-54.

Matsuura, M., 2001. Saponins in garlic as modifiers of the risk of cardiovascular disease. J. Nutr. 131, 1000-1005.

Melzig, M.F., Bader, G., Loose, R., 2001. Investigations of the mechanism of membrane activity of selected triterpenoid saponins. Plant. Med. 67, 43-48.

Menin, L., Panchichkina, M., Keriel, C., Olivares, J., Braun, U., Seppert, E.K., Saks, V.A., 2001. Macro compartmentation of total creatine in cardiomyocytes revisited. Mol. Cell. Biochem. 220, 149-159.

Mimaki, Y., Yokosuka, A., Kuroda, M., Sashida, Y., 2001. Cytotoxic activities and structure-

cytotoxic relationships of steroidal saponins. Biol. Pharm. Bull. 24, 286-289.

Minale, L., Riccio, R., Pizza, C., Zollo, F., 1986. Natural products and biological activities. A NAITO Foundation Symposium, Imura, H. Goto, T. Murachi, T. Nakajima, T. University of Tokyo Press and Elsevier Science Publishers, Tokyo, pp. 57-73.

Minale, L., Riccio, R., Zollo, F., 1993. Steroidal oligoglycosides and polyhydroxysteroids from echinoderms. Progress in the chemistry of organic natural products. Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, C.H., eds. Springer-Verlag, New York, pp. 75-308.

Miyase, T., Inose, Y., Veno, A., 1994. Studies on the constituents of Solidago virgaurea, Structures of solidago saponins, Chem. Pharm. Bull. Tokio 42, 617-624.

Morrissey, J.P., Osbourn, A.E., 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol. Mol. Biol. Rev. 63, 708-724.

Mshvildadze, V., Favel, A., Delmas, F., Elias, R., Faure, R., Decanosidze, Q., Kemertelidze, E., Balansard, G., 2000. Antifungal and antiprotozoal activities of saponins from *Hedera colchica*. Pharmazie 55, 325-326.

Muetzel, S., Hoffmann, E.M., Becker, K., 2003. Supplementation of barley straw with Sesbania pachycarpa leaves in vitro: Effects on fermentation variables and rumen microbial population structure quantified by ribosomal RNA-targeted probes. Br. J. Nutr. 89, 445-453.

Namba, T., Yoshsaki, M., Tomimori, T., Kobashi, K., Mitsui, K., Hase, J. 1973. Hemolytic and protective activity of ginseng saponins. Chem. Pharm. Bull. 21, 459-461.

Netala, V.R., Ghosh, S.B., Bobbu, P., Anitha, D., Tartte, V., 2015. Triterpenoid saponins: a review on biosynthesis, applications and mechanism of their action. Int. J. Pharm. Sci. 7,

24-28.

Nishiyama, I., Matsui, T., Hoshi, M., 1987. Purification of Co-ARIS, a cofactor for acrosome reaction-inducing substance, from the egg jelly of starfish. Dev. Growth Differ. 29, 161-169.

Oakenfull, D., Sidhu, G.S., 1989. Toxicants of Plant Origin Vol 11 Glycosides. Cheeke P, ed. CRC Press Inc, Florida, pp. 97-141.

Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., Yoshikawa, M., 2000. Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. Biol. Chem. 381, 67-74.

Ohtsuki, I., 1984. Saponin. Seitai-no-Kagaku 35, 493-494.

Okano, K., Ohkawa, N., Ikegami, S., 1985. Structure of ovarian asterosaponin-4, an inhibitor of spontaneous oocyte maturation from the starfish *Asterias amurensis*. Agric. Biol. Chem. 49, 2823-2826.

Okunji, C.O., Okeke, C.N., Gugnani, H.C., Iwu, M.M., 1990. An antifungal spirostanol saponin from fruit pulp of *Dracaena mannii*. Int. J. Crude Drug Res. 28, 193-199.

Oleszek, W., 1988. Solid phase extraction fractionation of alfalfa saponins. J. Sci. Food Agric. 44, 43-49.

Oleszek, W., 1996. Alfalfa saponins: Structure, biological, activity, and chemotaxonomy. Saponins used in food and agriculture. Waller, G. R. and Yamasaki, K. eds. Plenum Press, NY. pp. 155-170.

Onning, G., Asp, N.G., 1995. Effect of oat saponins on plasma and liver lipids in gerbils and rats. Br. J. Nutr. 73, 275-286.

Onning, G., Wang, Q., Wesrom, B., Asp, N.G., Karlsson, B.W., 1996. Influence of oat saponins

on intestinal permeability in vitro an in vitro in the rat. Br. J. Nutr. 74, 141-151.

Osboum, A.E., 1996. Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell 8, 1821-1831.

Osbourn, A., Goss, R.J.M., Field, R.A., 2011. The saponins-polar isoprenoids with important and diverse biological activities. Nat. Prod. Rep. 28, 1261-1268.

Palazon, J., Moyano, E., Bonfill, M., Osuna, L.T., Cusido, R.M., Pinol, M.T., 2006. Effect of organogenesis on steroidal saponin biosynthesis in calli cultures of *Ruscus aculeatus*. Fitoterapia 77, 216-220.

Park, H.Y., Kim, J.Y., Kim, H.J., Lee, G.H., Park, J.I., Lim, C.W., Kim, Y.K., Yoon, H.D., 2009. Insecticidal and repellent activities of crude saponin from the starfish *Asterias amurensis*. J. Fish. Sci. Technol. 12, 1-5.

Petersen, J.K., Hasler, B., Timmermann, K., Nielsen, P., Torring, D.B., Larsen, M.M., Holmer, M., 2014. Mussels as a tool for mitigation of nutrients in the marine environment. Marine Poll. Bull. 82, 137-143.

Plock, A., Sokolowska-Kohler, W, Presber, W., 2001. Application of flow cytometry and microscopical methods to characterize the effect of herbal drugs on *Leishmania spp.* Exp. Parasitol. 97, 141-153.

Podolak, I., Galanty, A., Sobolewska, D., 2010. Saponins as cytotoxic agents: a review. Phytochem. Rev. 9, 425-474.

Potter, S.M., Jimenez-Flores, R., Pollack, J., Lone, T.A., Berber-Jimenez, M.D., 1993. Protein saponin interaction and its influence on blood lipids. J. Agric. Food Chem. 41, 1287-1291.

Prasad, K., Kalra, J., 1993. Oxygen free radicals and hypercholesterolemic atherosclerosis: effect of vitamin E. Am. Heart. J. 125, 958-973.

Rakhimov, R.R., Benetis, N.P., Lund, A., Hwang, J.S., Prokofev, A.I., Lebedev. Y.S., 1996. Intramolecular and reorientation dynamics of bis (triphenylphosphine)-3, 6-di-tert-butyl-4, 5-dimethoxy-o-semiquinone complex of copper (I) in viscous media. Chem. Phys. Lett. 225, 156-162.

Raphaela, G.B., Carmen, L.Q., Ílio, M.J., Fernando, A.C., 2014. Fractionated extraction of saponins from Brazilian ginseng by sequential process using supercritical CO2, ethanol and water. J. Supercrit. Fluids 92, 272-281.

Reinheimer, J.A., Demkow, M.R., 1990. Candioti M C. Inhibition of coliform bacteria by lactic cultures. Aust. J. Dairy Technol. 45, 5-9.

Riccio, R., Greco, O.S., Minale, L., 1985. Starfish saponins, part 18. Steroidal glucoside sulfates from the starfish *Linckia laevigata*. J. Nat. Prod. 48, 97-101.

Riguera, R., 1997. Isolating bioactive compounds from marine organisms. J. Mar. Biotechnol. 5, 187-193.

Rinehart, J., Shaw, P.D. , Shield, L.S., Gloer, J.B., Harbour, G.C., Koker, M.E.S., Samain, D., Schwartz, R.E., Tymiak, A.A., Weller, D.L., Carter, G.T.,. Munro, M.H.G., Hughes, R.G.J., Renis, H.E., Swynenberg, E.G., Stringfellow, D.A., Vavra, J.J., Coats, J.H., Zurenko, G.E., Kuentzel, S.L., Li, L.H., Bakus, G.J., Brusca, R.C., Craft, L.L., Young, D.N., Connor, J.L., 1981. Marine natural products as sources of antivirus, antimicrobial and antineoplastic agents. Pure Appl. Chem. 53, 795-817.

Robin, J., Swati, S., Poonam, D., Madhvi, M., Dharmesh, K., Shalika, B., Harish, C., Neeraj, K., Shashi, B., Arvind, G., Ajit, K.S., Ashu, G., 2013. In vitro cytotoxicity, antimicrobial, and metal-chelating activity of triterpene saponins from tea seed grown in Kangra valley, India. Med. Chem. Res. 22, 4030-4038.

Robin, J., Swati, S., Poonam, D., Madhvi, M., Dharmesh, K., Shalika, B., Harish, C., Neeraj, K., Shashi, B., Arvind, G., Ajit, K.S., Ashu, G., 2013. In vitro cytotoxicity, antimicrobial, and metal-chelating activity of triterpene. Med. Chem. Res. DOI 10.1007/s00044-012-0404-4.

Ruiz, G.M., Rawlings, T.K., Dobbs, F.C., Drake, L.A., Mullady, T., Huq, A., Colwell, R.R., 2000. Global spread of microorganisms by ships. Nature 408, 49-50.

Samoilov, A.V., Girshovich. E.S., 1980. Antibiotic properties of the triterpene glycosides from marine animals of the class Holothuroidea. Antibiotic 25, 307-313.

Sen, S., Makkar, H.P.S., Becker, K., 1998. Alfalfa saponins and their implication in animal nutrition. J. Agric. Food Chem. 46, 131-140.

Shah, A.K.M., Kurihara1, H., Takahashi, K., 2013. Seasonal variations of lipid content and composition in starfish *Asterias amurensis* Lütken. Eurasian Chem-Technol. J. 15, 45-50.

Shany, S., Birk, Y., Gestetner, B., Bondi, A., 1970. Preparation, characterization and some properties of saponins from lucerne tops and roots. J. Sci. Food Agric. 21, 131-135.

Sidhu G.S., Oakenfull, D.G., 1986. A mechanism for the hypocholesterolaemic activity of saponins. Br. J. Nutr. 55, 643-649.

Son, I.S., Kim, J.H., Sohn, H.Y., Son, K.H., Kim, J.S., Kwon, C.S., 2007. Antioxidative and hypolipidemic effects of diosgenin, a steroidal saponin of Yam (Dioscorea spp.), on high cholesterol fed rats. Biosci. Biotechnol. Biochem. 71, 3063-3071.

Southon, S., Johnson, I.T., Gee, J.M., Price, K.R., 1988. The effect of gypsophylla saponins in the diet on mineral status and plasma cholesterol concentration in the rat. Br. J. Nutr. 59, 49-55.

Stahl, E., 1969. Thin layer chromatography - a laboratory handbook. Springer Verlag., Berlin.

Stefan, B., Matthias, F.M., 2013. The influence of saponins on cell membrane cholesterol. Bioorg. Med. Chem. 21, 7118-7124.

Stonik, V.A., 2001. Marine polar steroids. Russ. Chem. Rev. 70, 673-715.

Stonik, V.A., Ivanchina, N.V., Kicha, A.A., 2008. New polar steroids from starfish. J. Nat. Prod. Commun. 3, 1587-1610.

Suguna, A., Bragadeeswaran, S., Natarajan, E., Mohanraj, M., 2014. Antimicrobial properties of partially purified extract of starfish *Luidia maculata* (Muller & Troschel, 1842). Int. J. Nat. Prod. Res. 100-103.

Suh, S.J., Ko, H.K., Song, K.H., Kim, J.R., Kwon, K.M., Chang, Y.C., Lee, Y.C., Kim, D.S., Park, S.J., Yang, J.H., Son, J.K., Na, M.K., Chang, H.W., Kim, C.H., 2011. Ethylacetate fraction from Korean seaside starfish, *Asterias amurensis*, has an inhibitory effect on MMP-9 activity and expression and on migration behavior of TNF- induced human aortic smooth musclecells. Toxicol. In Vitro, 25, 767-773.

Summerson, R., Darbyshire, R., Lawrence, E., 2007. Invasive marine species range mapping. Bureau of Rural Sciences, Australian Government, p. 2.

Sun, H., Chen, L., Wang, J., Wang, K., Zhou, J., 2011. Structure–function relationship of the saponins from the roots of Platycodon grandiflorum for hemolytic and adjuvant activity. Int. Immunopharmacol. 11, 2047-2056.

Takechi, M., Matsunami, S., Nishizawa, J., Uno, C., Tanaka, Y., 1999. Hemolytic and antifungal activities of saponins or anti-ATPase and antiviral activities of cardiac glycosides. Planta. Med. 65, 585-586.

Takechi, M., Tanaka, Y., 1995. Haemolytic time course differences between steroid and triterpenoid saponins. Planta Med. 61, 76-77.

Tamura, K., Stecher, G., Peterson, D., Filipski, A, Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Tanaka, O., Tamura, Y., Masuda, H., Mizutani., K., 1996. Application of saponins in foods and cosmetics: Saponins of *Mohave yucca* and *Sapindus mukurossi*. Saponins used in food and agriculture. Waller, G. R. and Yamasaki, K. eds. Plenum Press, NY. pp. 1-11.

Tang, H.F., Yi, Y.H., Li, L., Sun, P., Zhang, S.Q., Zhao, Y.P., 2005. Bioactive asterosaponins from the starfish *Culcita novaeguineae*. J. Nat. Prod. 68, 337-341.

Van Atta, G.R., Guggolz, J., Thompson, C.R., 1961. Determination of saponin in alfalfa. J. Agric. Food Chem. 9, 77-79.

Van Etten, C.H., Mller, R.W., Wolff, I.A., Jones, Q., 1961. Amino acid composition of twenty seven selected seed meals. J. Agri. Food Chem. 9, 79-82.

Vincken, J.P., Heng, L., de Groot, A., Gruppen, H., 2007. Saponins, classification and occurrence in the plant kingdom, Phytochem. 68, 275-297.

Voogt, P.A., Van Rheenen, J.W.A., 1982. Carbohydrate content and composition of asterosaponins from different organs of the sea star *Asterias rubens*: relation to their haemolytic activity and implications for their biosynthesis. Comp. Biochem. Physiol. B 72, 683-688.

Voutquenne, L., Lavaud, C., Massiot, G., Le Men-Olivier, L., 2002. Structure-activity relationships of haemolytic saponins, Pharm. Biol. 40, 253-262.

Wallace, R.J., 2004. Antimicrobial properties of plant secondary metabolites. Proc. Nutr. Soc. 63, 621-629.

Wang, Z.W., Gu, M.Y., Li, G.Z., 2005. Surface properties of gleditsia saponin and synergisms of its binary system. J. Disper. Sci. Technol. 26, 341-347.

Winter, W.P., 1994. Mechanism of saponin induced red cell hemolysis: Evidence for the involvement of aquaporin CHIP28. Blood 84, 445.

Woldemichael, G.M., Wink, M., 2001. Identification and biological activities of triterpenoid saponins from *Chenopodium quinoa.* J. Agric. Food Chem. 49, 2327-2332.

World Health Organization, 2003. Shaping the future, Geneva, World Health Organization, World Health Report, 2003. http://www.who.int/whr/2003/en/whr03_en.pdf.

Xiao, G., Yu, B., 2013. Total synthesis of starfish saponin Goniopectenoside B. Chem. Eur. J. 19, 7708-7712.

Xie, Y., Yi-Ping, Y., Hong-Xiang, S., Duo, L., 2008. Contribution of glycidic moieties to haemolytic and adjuvant activity of platycodigenin-type saponins from the root of *Platycodo grandiflorum*. Vaccine 26, 3452-3460.

Yasumoto, T., Tanaka, M, Hashimoto, Y., 1966. Distribution of saponins in echinoderms. Bull. Jpn. Soc. Sci. Fish. 32, 673-676.

Zhao, H.L., Harding, S.V., Marinangeli, C.P., Kim, Y.S., Jones, P.J., 2008. Hypocholesterolemic and anti-obesity effects of saponins from Platycodon grandiflorum in hamsters fed atherogenic diets. J. Food Sci. 73, 195-200.

Zhang, Y.G., Zhang, H.G., Zhang, G.Y., Fan, J.S., Li, X.H., Liu, Y.H., 2008 Panax notoginseng saponins attenuate atherosclerosis in rats by regulating the blood lipid profile and anti-inflammatory action. Clin. Exp. Pharmacol. Physiol. 35, 1238-1244.

Acknowledgements

First and foremost, I thank the creator of the universe, Allah for His divine strength throughout the years in my studies, because without His divine guidance this would be impossible for me.

I would like to thank the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan, for providing me Monbukagakusho Scholarship (2013) and supporting my studies in Japan.

I am deeply grateful to my Ph.D supervisor Associate Professor Dr. Shoichiro Ishizaki for creating an opportunity in his lab and unique guidance, patience, and encouragement to complete this thesis work. With his dedication, passion, utmost guidance and discipline, I have learnt many unknown things that will be very much helpful for me to carry on future research and innovation.

I would also like to thank the members of my dissertation committee, Dr. Yuji Nagashima, Dr. Tomoyuki Koyama, and Dr. Hidehiro Kondo for all their suggestions and criticisms. I especially want to express my sincere gratitude to Professor Nagashima for his kind assistance whenever I needed academic advices and suggestions through my research period.

I would also express my gratitude to all my fellow members in the laboratory of marine biomaterial and functional biochemistry and also in the department who made my university life happy and memorable.

I am very much indebted to my entire family and my husband for their support, continued encouragement, and sacrifices throughout my Ph.D tenure and daily life. I especially want to thank my husband, Dr. Md. Sazedul Karim Sarker for his mental support and understanding. I also want to thank my beloved son Md. Saif Al Sarker and daughter Sabah Anum Sarker, for

their happiness and fulfilling my heart during sadness in challenging time, which is one of the most important inspiration and encouragement for my study and life in university.

I owe a lot to my parents, who encouraged and helped me at every stage of my personal and academic life, and longed to see this achievement come true, but I never had a chance to fulfill the primary duty to them and my family. Because of this, I wish to dedicate this thesis to my parents, my husband and my two angels.