Selection of Potential Antimicrobial Producing Fungi Isolated from Marine Organisms

Suraiya Manmana

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Microbiology
Prince of Songkla University
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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and it not being currently submitted in candidature for any degree.

…………..………………Signature

(Miss Suraiya Manmana)

Candidate

ชื่อวิทยานิพนธ์	การคัดเลือกเชื้อราที่มีศักยภาพในการสร้างสารต้านจุลินทรีย์ จากสิ่งมีชีวิตในทะเล
ผู้เขียน	นางสาวสุรัยยา หมานมานะ
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ปีการศึกษา	2561

บทคัดย่อ

การติดเชื้อจุลินทรีย์ดื้อยาปฏิชีวนะเพิ่มขึ้น ดังนั้นจึงจำเป็นที่ต้องหาแหล่งของยา ปฏิชีวนะใหม่เพื่อแก้ปัญหานี้ วัตถุประสงค์ของงานวิจัยนี้เพื่อคัดเลือกเชื้อราจากสิ่งมีชีวิตในทะเลที่ สร้างสารต้านจุลินทรีย์ แยกเชื้อราได้จำนวน 547 ไอโซเลทจากสิ่งมีชีวิตในทะเลจำนวน 17 ตัวอย่าง และคัดเลือกเชื้อราที่มีลักษณะทางสัณฐานวิทยาที่แตกต่างกันจำนวน 123 ไอโซเลทเพื่อนำไปสกัด ด้วยตัวทำละลายอินทรีย์ โดยเชื้อราแต่ละไอโซเลทได้สารสกัด 3 ส่วนคือ สารสกัดส่วนน้ำเลี้ยงเชื้อ ที่สกัดด้วยเอธิลอะซีเตท (BE) สารสกัดจากเส้นใยที่สกัดด้วยเอธิลอะซีเตท (CE) และ สารสกัดจาก เส้นใยที่สกัดด้วยเฮกเซน (CH) นำสารสกัดทั้งหมดมาทดสอบฤทธิ์ต้านจุลินทรีย์เบื้องต้นต่อ จุลินทรีย์ก่อโรค 9 สายพันธุ์ ด้วยวิธี colorimetric broth microdilution ที่ความเข้มข้น 200 ไมโครกรัมต่อมิลลิลิตร พบว่ามีสารสกัด 219 สาร (59.35%) จากเชื้อรา 109 ไอโซเลท (88.62%) ที่ สามารถต้านจุลินทรีย์ก่อโรคได้อย่างน้อย 1 ชนิด โดยมีฤทธิ์ต้านเชื้อ *Cryptococcus neoformans* ATCC 90112 (CN) มากที่สุด (35.77%) รองลงมาคือ *Staphylococcus aureus* ATCC 25923 (SA) (35.50%) methicillin-resistant *S. aureus* (MRSA) SK-1 (27.10%) *Microsporum gypseum* SH-MU4 (MG) (18.42%) *Candida albicans* ATCC90028 (CA) (10.02%) *Talaromyces marneffei* PSU-SKH1 (TM) (3.25%) *Acinetobacter baumannii* NPRC AB005 (AB005) (2.71%) *Pseudomonas aeruginosa* ATCC 27853 (PA) (1.89%) และ *Escherichia coli* ATCC25922 (EC) (1.08%) ตามลำดับ โดยมีค่าความเข้มข้นต่ำสุดที่ยับยั้งเชื้อ (minimum inhibitory concentration: MIC) และค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อแบคทีเรีย (minimum bactericidal concentration: MBC) หรือค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อรา (minimum fungicidal concentration: MFC) อยู่ในช่วง 2-200/ 16->200 ไมโครกรัมต่อมิลลิลิตรตามลำดับ ทำการจัดจำแนกเชื้อราที่มีฤทธิ์ในการต้านเชื้อจุลินทรีย์

ดี จำนวน 36 ไอโซเลท โดยใช้ลักษณะทางสัณฐานวิทยาและ/หรือ วิธีทางชีวโมเลกุล พบว่าเชื้อรา
จัดอยู่ใน 2 ไฟลัมคือ Ascomycota (35 ไอโซเลท) และ Basidiomycota (1 ไอโซเลท) รา
Ascomycota ที่มีฤทธิ์ต้านจุลินทรีย์ ประกอบด้วยจีนัส *Trichoderma* (10 isolates) *Aspergillus*
(8 isolates) *Penicillium* (8 isolates) *Letendraea* (2 isolates) *Cladosporium* (1 isolate) *Fusarium*
(1 isolate) *Pestalotiopis* (1 isolate) *Phaeosphaeriopsis* (1 isolate) และ *Trichothecium* (1 isolate)
มีเชื้อราเพียงหนึ่งไอโซเลทคือ *Schizophyllum commune* AMF238 ที่อยู่ในไฟลัม Basidiomycota
และมีเชื้อราจำนวน 2 ไอโซเลท ที่ไม่สามารถระบุสายพันธุ์ได้ สารสกัด AMF277BE ที่ได้จากเชื้อ
Aspergillus clavatonanicus AMF277 มีฤทธิ์กว้างในการยับยั้งเชื้อ SA, MRSA, EC, PA, AB005,
CN และ TM โดยมีค่า MIC เท่ากับ 32, 64, 64, 32, 200, 200 และ 200 ไมโครกรัมต่อมิลลิลิตร
ตามลำดับ สารสกัด AMF231CH จาก *Aspergillus unguis* AMF231 มีฤทธิ์ดีที่สุดในการยับยั้งเชื้อ
SA (MIC 4 µg/ml) และ MG (MIC 16 µg/ml) สารสกัด AMF198CH จากเชื้อ *Phaeosphaeriopsis*
musae AMF198 แสดงฤทธิ์ในการยับยั้งเชื้อ MRSA ดีที่สุด (MIC 16 µg/ml) สารสกัด AMF192CH
และ AMF192BE จากเชื้อรา *Trichothecium* sp. AMF192 แสดงฤทธิ์ต้านเชื้อราดีที่สุด โดยต้านเชื้อ
CA และ CN มีค่า MIC เท่ากับ 8 ไมโครกรัมต่อมิลลิลิตร และ 2 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ
การศึกษาผลของสารสกัดที่มีฤทธิ์ต้านจุลินทรีย์ที่ดีที่สุดต่อเซลล์ของจุลินทรีย์ก่อโรค โดยกล้อง
จุลทรรศน์อิเล็คตรอนชนิดส่องกราด พบว่าสารสกัดมีผลทำให้เซลล์ของเชื้อเปลี่ยนแปลงไป เซลล์
ผิดรูป เซลล์หด เซลล์เหี่ยว และเซลล์แตกเป็นรู ยิ่งไปกว่านั้นสารสกัด AMF222CE และ
AMF409BE จากเชื้อ *Trichoderma* spp. AMF222 และ AMF409 แสดงประสิทธิภาพต่ำในการ
ยับยั้งเชื้อ AB005 (MIC 200 µg/ml) สามารถเสริมฤทธิ์ของยา colistin ต่อเชื้อ AB005 เมื่อทดสอบ
ด้วยวิธี checkerboard แสดงค่า Fractional Inhibition Concentration index (FICI) อยู่ในช่วง 0.25-
0.5 และวิธี time-kill แสดงฤทธิ์ในการฆ่าเชื้อ โดยสามารถลดจำนวนเชื้อตั้งต้นลงได้มากกว่า 3 $\log_{10}$
CFU/ml ในชั่วโมงที่ 2 หลังจากบ่ม นอกจากนี้ยังได้นำสารสกัดจากเชื้อรามาทดสอบฤทธิ์ต้าน
ควอรัมเซนซิ่ง (Quorum sensing QS) เบื้องต้น โดยทดสอบการยับยั้งการสร้างสารสี violacein ของ
เชื้อ *Chromobacterium violaceum* DMST21761 ด้วยวิธี disk diffusion พบว่ามีสารสกัดจำนวนเพียง
4 สาร (AMF177BE, AMF199BE, AMF231BE, AMF480BE) แสดงฤทธิ์ต้าน QS ผลการศึกษา
ดังกล่าวแสดงให้เห็นว่าเชื้อราจากสิ่งมีชีวิตในทะเลเป็นแหล่งของสารต้านจุลินทรีย์ที่สำคัญ

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Author   Miss Suraiya Manmana

Major Program   Microbiology

Academic Year   2018

ABSTRACT

Infections caused by drug resistant microorganisms are increasing worldwide. There is a need to find new sources of antibiotics to combat this problem. The aim of this study was to select marine-derived fungi isolated from marine organisms that produce antimicrobial metabolites. Five hundred and forty-seven fungi were isolated from 17 marine organisms and 123 isolates were selected based on their different colony morphologies for chemical extraction. Three extracts were obtained from each isolate including broth ethyl acetate (BE), cell ethyl acetate (CE) and cell hexane (CH) extracts. All extracts were preliminarily screened for their antimicrobial activity using colorimetric broth microdilution methods at 200 µg/ml against nine human pathogens. The results demonstrated that 219 extracts (59.35%) from 109 isolates (88.62%) showed inhibitory activity against at least one test strain. Most of active extracts were effective against *Cryptococcus neoformans* ATCC 90112 (CN) (35.77%) followed by *Staphylococcus aureus* ATCC 25923 (SA) (35.50%), methicillin-resistant *S. aureus* (MRSA) SK-1 (27.10%), *Microsporum gypseum* SH-MU4 (MG) (18.42%), *Candida albicans* ATCC90028 (CA) (10.02%), *Talaromyces marneffei* PSU-SKH1 (TM) (3.25%), *Acinetobacter baumannii* NPRC AB005 (AB005) (2.71%), *Pseudomonas aeruginosa* ATCC 27853 (PA) (1.89%) and *Escherichia coli* ATCC25922 (EC) (1.08%), respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) values were varied in the range of 2-200 and 16->200 µg/ml., respectively. Thirty-six fungi presenting moderate to strong antimicrobial activity were identified based on morphological and/or molecular methods into two phyla, Ascomycota (35 isolates) and Basidiomycota (1 isolate). The active ascomycetous genera were *Trichoderma* (10 isolates), *Aspergillus* (8 isolates),

Penicillium (8 isolates), *Letendraea* (2 isolates), *Cladosporium* (1 isolate)*, Fusarium* (1 isolate), *Pestalotiopsis* (1 isolate), *Phaeosphaeriopsis* (1 isolate) and *Trichothecium* (1 isolate). Only one isolate, *Schizophyllum commune* AMF238 belonged to the phylum Basidiomycota and two isolates (AMF177 and AMF235) were unidentified. Broth ethyl acetate extracts from *Aspergillus clavatonanicus* AMF277 (AMF277BE) showed the broadest inhibitory activity against SA, MRSA, EC, PA, AB005, CN and TM with MIC values of 32, 64, 64, 32, 200, 200 and 200 µg/ml, respectively. AMF231CH from *Aspergillus unguis* AMF231 showed the strongest inhibitory activity against SA (MIC 4 µg/ml) and MG (MIC 16 µg/ml). AMF198CH from *Phaeosphaeriopsis musae* AMF198 was most active against MRSA (MIC 16 µg/ml). The mycelium and broth extracts of *Trichothecium* sp. AMF192 (AMF192CH and AMF192BE) displayed the most potential antifungal activity against CA and CN with MIC 8 µg/ml and 2 µg/ml, respectively. Electron microscopic observation of the treated cells with these active extracts showed morphological changes with deformation, collapsed, shrinkage and broken cell with holes. Furthermore, AMF222CE and AMF409BE from of *Trichoderma* spp. AMF222 and AMF409 presenting weak anti-AB (MIC 200 µg/ml) exhibited the synergistic effects with colistin against AB005. The checkerboard results showed fractional inhibitory concentration index (FICI) ranging from 0.25-0.5 and the time-kill assay revealed bactericidal activity ($> 3\log_{10}$ CFU/ml reduction) of these two extracts in combination with colistin after 2 h of incubation. In addition, the extracts were preliminarily determined for their ability to inhibit quorum sensing (QS) using violacein inhibition assay with *Chromobacterium violaceum* DMST21761 by a disk diffusion methods. Only four extracts (AMF177BE, AMF199BE, AMF231BE and AMF408BE) exhibited anti-QS activity. The results from this study can indicate that the marine-derived fungi are a potential source of antimicrobial active metabolites.

ACKNOWLEDGEMENTS

THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

Nowadays, infections caused by drug resistant microorganisms are increasing worldwide. There is a need to find new potential compounds to combat this problem. Terrestrial fungi are well known as a good source of bioactive natural products and various active metabolites have been found, lead to less discovery of new bioactive compounds. Thus, alternative approaches to control emerging of antimicrobial resistance are need. Marine habitats are among the well known natural resources receiving attention due to the availability of abundance of biologically active compounds producing microorganisms especially, bacteria and fungi. Marine-derived fungi have been recognized as one of the major sources for new compounds and secondary metabolites. The aim of this study was to select marine-derived fungi isolated from marine organisms that produce antimicrobial metabolites. The results showed that most of the fungal extracts exhibited antimicrobial activity against human pathogens, in particular against *Cryptococcus neoformans* and *Staphylococcus aureus*. In addition, the extracts exhibited anti-quorum sensing activity. Inhibition of bacterial quorum sensing has been suggested as a novel strategy for the prevention and control of bacterial infections. Furthermore, some fungal extracts exhibited the synergistic effects with colistin against *Acinetobacter baumannii* AB005 with fractional inhibitory concentration index (FICI) ranging from 0.25-0.5 and the time-kill assay showed bactericidal activity ($> 3\log_{10}$ CFU/ml reduction). *A. baumannii* is a major cause of hospital-acquired bacterial infections. It is able to develop resistance to multiple antibiotics and can cause serious infections in critically ill patients. Colistin is considered as one of the most important therapeutic options for treatment of *A. baumannii* infection. However, it can cause nephrotoxicity. The reduction of colistin dosage in fungal extract-colistin combination will decrease its toxicity. The results indicated that marine-derived fungi are a good source of antimicrobial metabolites against human pathogen that may be developed for further application.

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LIST OF ABBREVIATIONS AND SYMBOLS

^{o}C	=	Degree Celsius
pH	=	Potential of Hydrogen ion
rpm	=	Rounds per minute
h	=	Hour
CFU	=	Colony forming unit
μl	=	Microliter
ml	=	Milliliter
g	=	Gram
mg	=	Milligram
μg	=	Microgram
ng	=	Nanogram
cm	=	Centimeter
mm	=	Millimeter
nm	=	Nanometer
mM	=	Millimolar

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Infections caused by pathogenic microbes are problematic and lead to high mortality rate among infected patients. There are many commercially available antibiotics used for the treatment of microbial infections, but development of resistance among pathogenic microbes resulted in treatment failure. There is a need for an alternative approach to control emerging microbial infections (O'Neill, 2014).

Marine environment is among the well-known natural resources receiving attention worldwide due to the availability of the abundance of biologically active compounds. Marine microbes including bacteria and fungi have been recognized for their bioactive metabolites as therapeutic agents, cosmetics, and personal care products (Debbab *et al.*, 2010). The marine fungi play an important role in marine ecosystems and serve as decomposers, driving nutrient cycles in detritus environment, and symbionts (Richards *et al.*, 2012). In addition, compounds obtained from marine-derived fungi exhibited various biological properties including antibacterial, antiviral, antifungal, and anti-cancer properties (Bajpai, 2016; Hasan *et al.*, 2018, Preedanon *et al.*, 2016, Xu *et al.*, 2015; Youssef and Alahdal, 2018). Furthermore, Xu *et al.* (2015) reviewed research papers from January 2010 through March 2015 and found 116 new compounds with antifungal or antibacterial activities and 169 known antimicrobial compounds from marine-derived fungi mostly from algae, sponges and mangroves. Thus, fungi from marine organisms are the interesting source of new antimicrobial agents.

1.2 Reviews of the literature
1.2.1 Marine organisms
a) Ascidians or Tunicates

Ascidians or sea squirts belong to the phylum Chordata, class Ascidiacea. They are also known as tunicates. Ascidians are found in all oceans with

approximately 3000 described species. There are three main types including 1) solitarian ascidian, 2) social ascidians (group of individuals connected at their bases) and 3) compound ascidian or colonial ascidians (many small individuals called zooid formed colonies up in a gelatinous matrix with sharing external tunic) (Núñez-Pons *et al*., 2012).

Ascidians (Figure 1) have many mechanisms to prevent predation. The first line of protection by most ascidians is defensive chemistry, the high concentration of heavy metal such as vanadium (V) or sulfuric acid and hydrochloric acid in tunic bladder cells. Some ascidians avoid consumption by embedded the tunics with calcium carbonate spicules. However, the production of secondary metabolites is a common strategy too. Most of these physical and chemical defensive strategies block initial bacteriofilms, preventing biofouling epibiosis and infections. (Arumugam *et al*., 2017). Diverse fungal groups have been reported from various ascidians (Lopez-Legentil *et al*., 2015; Menezes *et al*., 2010). In addition, Ciavatta *et al*. (2013) isolated an alpha-pyrone derivative and a sesquiterpene having fungicidal activity against phytopathogenic fungi from a marine ascidian-associated fungus *Trichoderma harzianum* (NIO/BCC2000-51). Recently, Ivanets *et al*. (2018) reported four new indole-diterpene alkaloids, asperindoles A-D and a known *p*-terphenyl derivative from ascian-derived fungus *Aspergillus* sp. KMM4676. Asperindole A exhibited cytotoxic activity.
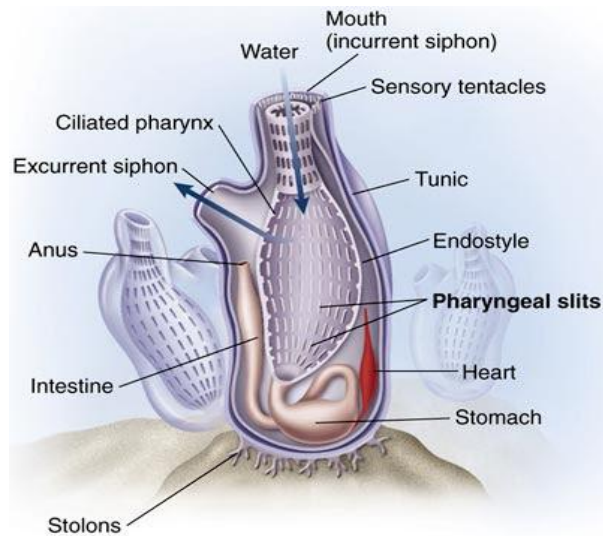
Figure 1 Anatomy of ascidian

Source: http://thefuhc.blogspot.com/2012/04/what-is-tunicate_18.htm (accessed 31 October 2018)

b)   Marine sponges

Sponges (phylum Porifera) are the simplest multicellular animals and the oldest living metazoans (Ćetković *et al.*, 2018). Marine sponges are filter-feeding organisms with an extremely effective and complex network of water-conducting channels and chambers lined with flagellated cell, the choanocytes (Figure 2). The knowledge of sponge diversity is still incomplete. To date, approximately 8500 species have been formally described (Moitinho-Silva *et al.*, 2017). They are classified into four classes: demosponges (Demospongiae), encrusting sponges (Homoscleromorpha), glass sponges (Hexactinellida) and calcareous sponges (Calcarea) (Bayari *et al.*, 2018).

Marine sponges are often found in symbiotic association with microorganisms. Sponge symbionts play important roles such as vitamin synthesis, bioactive compound production and biochemical transformations of nutrients or waste products. Furthermore, the porous structure and supported skeletal structure of sponges are become popular in bone tissue engineering and drug delivery studies (Granito *et al.*, 2017). Sponges harbored highly diverse and host-specific fungal communities (Menezes *et al.*, 2010; Nguyen and Thomas, 2018). Ebel (2012)

reviewed that sponge-associated fungi were a second good source of bioactive metabolites after algae. Various types of compounds including polyketides, sesquiterpenoids, meroditerpenes, chromanol derivatives, alkaloids, cyclopeptides, furan derivatives, decalin derivatives, secalonic acid and benzoic acid have been reported from sponge-associated fungi (Liu *et al*. 2018).
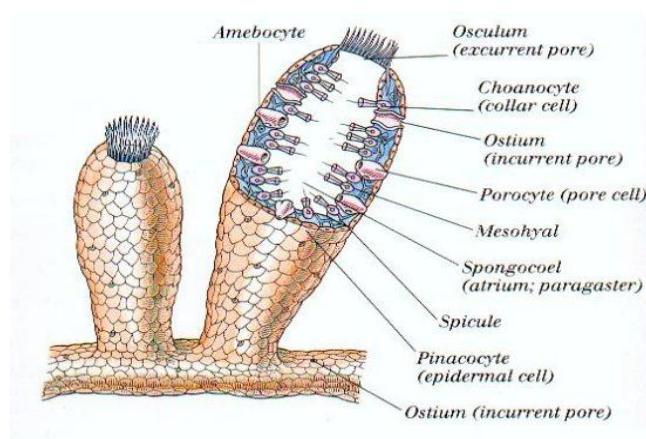
Figure 2 Marine sponge anatomy

Source: https://universe-review.ca/R10-33-anatomy01.htm (accessed 31 October 2018)

c) **Marine algae**

Marine algae are a large multicellular polyphyletic group of photosynthetic eukaryotic organisms in the kingdom Protista (Figure 3). There are about 9000 species of algae and they have been classified into three groups according to their composition of pigments as brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta) (Zhao *et al*., 2017). Algae have many types of life cycles and their sizes range from microscopic to giant algae. Algae play a major role in marine ecosystems as oxygen producers and as the base of food chain for aquatic life. Furthermore, seaweeds are an important source of biofuel and as source of food and pharmaceutical (Raja *et al*., 2013). Algae are also a major host of marine-derived fungi (Ebel, 2012; Ji and Wang, 2016).

Figure 3 Example of marine algae

Source: http://www.seaweed.ie/algae-l/ (accessed 31 October 2018)

d)   Corals

Corals are tiny invertebrate animals belonging to the phylum Cnidaria and the class Anthozoa. They are relatives of jellyfish and anemones (Rocha *et al*., 2011). The body of a coral consists of polyp (Figure 4). Coral can exist as individual polyps or in colonies and communities that contain hundreds to hundreds of thousands of polyps. Polyps get their nutrients from photosynthesis and from filter feeding. They are capable of reproducing both sexually (internal or external fertilization) and asexually (fragmentation, fission and budding). Corals can be classified into two groups by skeletons as hard coral and soft coral. Hard coral or stony coral produce a rigidcalcium carbonate skeletonin the crystallized form called aragonite with reef-building capabilities. Soft coral, including sea fans, may be present in a reef ecosystem but do not produce a rigid skeleton and do not form reefs (Hourigan *et al*., 2007). Recently, Cristianawati *et al*. (2017) found the antimicrobial capability of hard coral associated fungi against multidrug resistant (MDR) *Staphylococcus haemolyticus*.
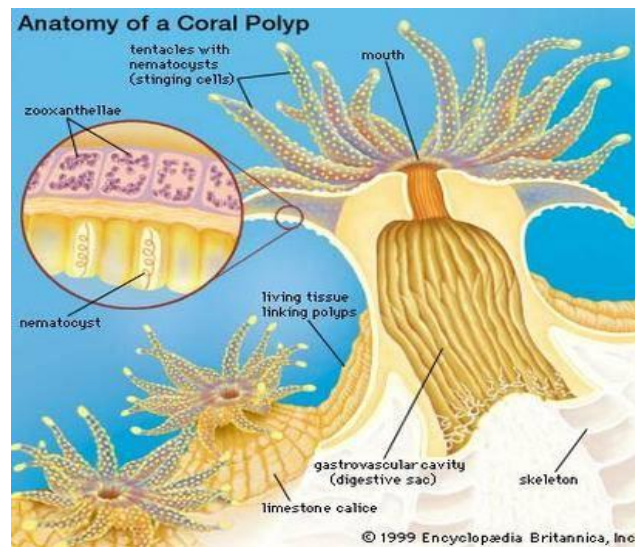
Figure 4 Structure and anatomy of coral

Source: https://www.britannica.com/science/polyp-zoology

e)   Sea cucumbers

Sea cucumbers are belonging to the phylum Echinodermata. They share a typical morphology, with a soft, cylindrical body, elongated from mouth to anus with a secondary bilateral symmetry (Figure 5). The alimentary canal is complete, the nervous system is not centralized and the reproductive system is simple. They can be found in all regions of the ocean (Bruckner, 2006). Sea cucumbers have been used in Asian traditional medicine and as food for a long time (Pangestuti and Arifin, 2018). They have many biological activities including antibacterial and antifungal inhibitory effects (Ghadiri *et al*., 2018). However, filamentous fungi still can be found from all organs of sea cucumbers (Pivkin, 2000).
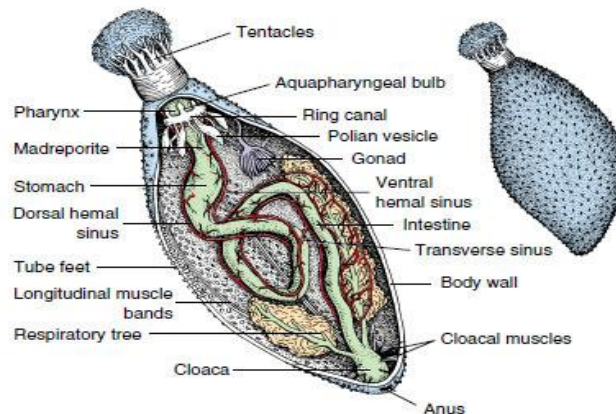
Figure 5 Anatomy of Sea cucumbers

Source: https://biocyclopedia.com/index/general_zoology/class_holothuroidea.php (accessed 31 October 2018)

f)   Bryozoans

Bryozoans are common known as moss animals. There are more than 6000 described living species both marine bryozoan and freshwater bryozoan. Bryozoans have many ranges in colony size from less than a millimeter to more than a meter. Most bryozoans live in colonies and are composed of many individuals. Each individual or zooid is enclosed a sheath of tissue with calcium carbonate (Figure 6). Some species resemble cnidarians and can be mistaken in the field for soft coral, hydrocorals and even scleractinian coral. Some species resemble hydroids. They are either rooted or free-living on rocks, shells and other hard substrates. Bryozoans are used as paleoenvironmental indicators. The high bryozoan diversity is an indicator of a healthy reef environment (Gordon, 2016). In addition, marine bryozoans also provided potential fungi as sources of new and bioactive compounds. For example, *Beauveria felina* EN-135 isolated from an unidentified marine bryozoan produced three new cyclohaxadepsipeptides of the isaridin class (isaridin G, desmethylisaridin G and desmethylisaridin C1). Desmethylisaridin C1 exhibited strong antibacterial activity against *Escherichia coli* with minimum inhibitory concentration (MIC) value of 8 µg/ml (Du *et al*., 2014).
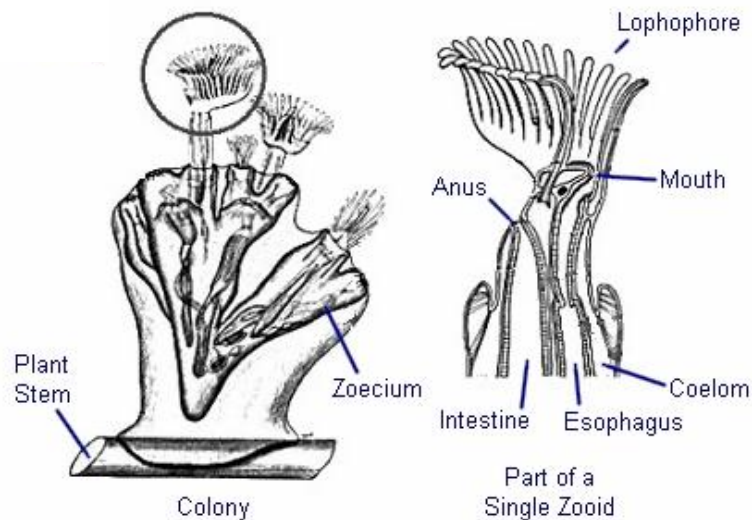
Figure 6 Bryozoan (Moss animal) anatomy

Source: https://njscuba.net/biology/sw_plant-like.php (accessed 31 October 2018)

g) **Sea fans or gorgonians**

Sea fans are invertebrate marine animals with cylindrical sessile form belonging to the phylum Cnidaria (Rocha *et al*., 2011). They live in compact colonies of many individual polyps. Each individual tiny polyp in the colonies has eight tentacles. The polyps form a plankton-catching net to ensnare prey which spread out their tentacles. A central internal skeleton, composed of a scleroprotein called gorgonin, supports all branches of the colony. The tissues are often colored in hues of red, orange or yellow with different branching patterns (Figure 7). Sea fans grow to about 60 cm. in height. It is closely related to coral and it can be found in all regions of the ocean (Rowley, 2018). Both healthy and diseased gorgonian sea fans harbored a diverse group of fungi (Phongpaichit *et al*., 2006; Preedanon *et al*., 2016, Toledo-Hernández *et al*., 2008; Zhang *et al*., 2012). However, these sea fan-derived fungi had a potential to produce antimicrobial metabolites (Preedanon *et al*., 2016).

Figure 7 Sea fans or gorgonians

Source: https://www.nsf.gov/news/mmg/mmg_disp.jsp?med_id=74649&from= (accessed 31 October 2018)

1.2.2   Marine-derived fungi

Kohlmeyer and Kohlmeyer (1979) have classified marine fungi into two groups, obligate marine fungi and facultative marine fungi, based on their ability to grow in the marine environment. The obligate marine fungi grow and sporulate in a marine or estuarine habitat while facultative marine fungi or marine-derived fungi are from freshwater and terrestrial milieus, but may also grow and sporulate in the marine environment. This study focused on marine-derived fungi.

1.2.2.1   Ecological roles of fungi in marine environment

Fungi play a major role in the decomposition of lignocellulose (dead plant tissue) and animal tissue. The degradation releases nutrients back into the ecosystem. The ecological roles of the terrestrial fungi have been described and thoroughly studied but the importance of fungi in marine ecosystem has been less studied (Bugni and Ireland, 2004). Some marine fungi have mutualistic symbiotic relationships with other organisms while others cause diseases of marine animals and plants.

Mutualistic symbiotic relationships between a host and a marine fungus occur in three forms including lichens, mycophycobioses and mycorrhizas. Lichens and mycophycobioses are important mutualistic associations with fungi and a number of algae. Mycophycobiosis is an obligate algal-fungal mutualistic association in which the fungal partner is immersed within the algae. In lichens, the fungi form the external structure surrounding an algae core. Mycorrhizae can be found in halophytic plants to enhance oxygen uptake and stress resistance to salt.

Although many studies revealed mutualistic symbioses between a fungus and a marine environment, other studies indicate that marine fungi can cause many diseases and infections. The studies involving mycopathogens of aquatic animals have been initiated due to the high incident of fungal diseases in wild population and aquaculture. Moreover, some marine fungi have been reported as pathogen of marine plants (*Hyde et al*., 1998) and animals (Ramaiah, 2006).

1.2.2.2 Distribution and habitats of marine fungi

Many studies reported that marine fungi obtained from marine environments mostly belonged to the phyla Ascomycota and Basidiomycota. Currently, some 530 species were described (Jones *et al*., 2009). In addition, Jones and Pang (2012) estimated number of marine fungi that there could be as many as 10,000 species. Nowadays, molecular techniques and advanced pyrosequencing techniques could reveal a great diversity of marine fungi.

Bugni and Ireland (2004) described the number of secondary metabolite producing fungi based on their marine source. This study found that sponges, algae and wood substrates were a good source of marine fungi (Figure 8).
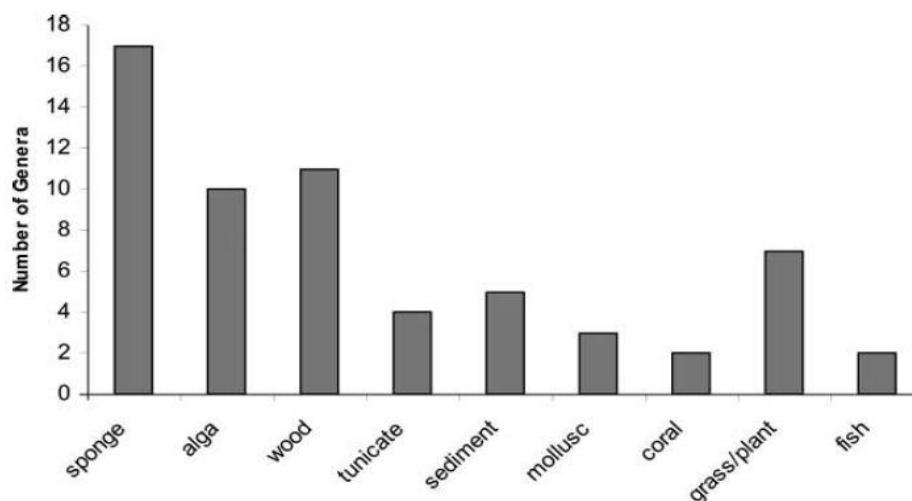
Figure 8 The number of fungal strains based on the marine source

Source: Bugni and Ireland (2004)

Additionally, Ebel (2012) revealed new compounds from marine-derived fungi sorted by source of the fungal strains as shown in Figure 9. Almost 80% of all new compounds from marine-derived fungi were obtained from living source while the notably non-living source was sediments. Algae are the predominant source for fungal diversity followed by sponges, sediments and mangrove habitats.
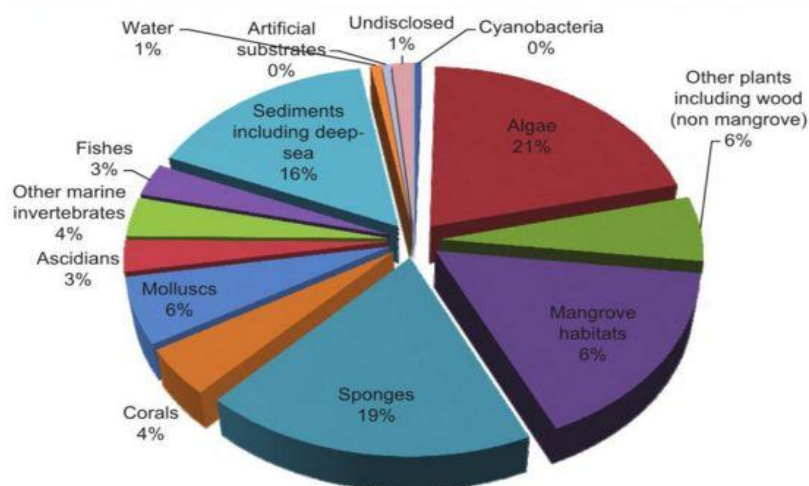
Figure 9 New compounds from marine-derived fungi divided by source of the fungal strains

Source: Ebel (2012)

1.2.2.3 Bioactive compounds of marine-derived fungi

Marine-derived fungi have been recognized as one of the major sources for biologically active secondary metabolites. The marine natural products are novel and their active compounds have potential pharmaceutical applications or economic value as cosmetics and fine chemicals (Bonugli-Santos *et al.*, 2015).

Since Alexander Fleming described the effects of penicillin derived from *Penicillium notatum* on bacteria in 1929, the bioactive compounds of fungi from terrestrial sources have been wildly studied. This led to less effective active secondary metabolite discovery. Thus, there is an urgent need to search for the new unique habitats, such as marine environments that harbor fungi with potential biosynthetic diversity.

Marine fungi provided many compounds with pharmacological properties such as antimicrobial, antiviral and antiprotozoal (Table 1). In addition, anticancer or cytotoxic agents against tumor cells have been isolated from various marine-derived fungi as shown in Table 2.

Table 1 Active compounds with antimicrobial activity obtained from marine-derived fungi

Compound	Fungal name	Source	Activity against
4′-Methoxyasperphenamte	*Aspergillus elegans*	*Sarcophyton* sp. (soft coral)	*S. epidermidis*
Asterrestide A	*Aspergillus terreus*	*Echinogorgia aurantiaca* (gorgonian)	Influenza virus strain H1N1 and H3N2
Ophiobolin U	*Aspergillus ustus*	*Codium fragile* (green algae)	*E. coli* (moderate activity)
Aspergillide D	*Aspergillus* sp.	*Melitodes squamata* (gorgonian)	H1N1 (moderate activity)

Table 1(Cont.) Active compounds with antimicrobial activity obtained from marine-derived fungi

Compound	Fungal name	Source	Activity
Yicathins C	*Aspergillus wentii*	*G.flabelliformis* (red algae)	*E. coli* and *Staphylococcus aureus*
Isochaetochromin B2 and ustilaginoidin D	*Metarhizium anisopliae*	Unidentified sponge	*Mycobacterium phlei*
Calcarides A-E	*Calcarisporium* sp.	Seawater	*S. epidermidis* and *Xanthomonascam pestris*
Penicillixanthone A	*Penicillium* sp.	*Dichotellagem-macea* (coral)	*M. luteus*, *P. nigrifaciens*, and *E.coli*
Didymellamide A	*Stagonosporopsis cucurbitacearum*	Unidentified sponge	*C. albicans* and *C. neoformans*
pestalochloride B	*Pestalotiopsis* sp.	*Sacophyton* sp. (soft coral)	*S. aureus* and *B. subtilis*
Penicifuran A	*Penicillium* sp.	Unidentified sponge	*S. albus*
Stachybotrin D	*Stachybotrys chartarum*	*Xestospongia testudinaris* (sponge)	Replication of HIV-I
Curvulamine	*Curvularia* sp.	*Argyrosomus argentatus* (fish)	*Veillonella parvula*, *Streptococcus* sp. and *Pepto-streptococcus* sp.
Asporyzin C	*Aspergillus oryzae*	*Heterosiphonia japonica* (red algae)	*E. coli*

Table 1(Cont.) Active compounds with antimicrobial activity obtained from marine-derived fungi

Compound	Fungal name	Source	Activity
Flavusid A-B	*Aspergillus flavus*	*Codium fragile* (green algae)	*S. aureus* and MRSA
Penicisteroid A	*Penicillium chrysogenum*	Unidentified red algae	*A. niger* and *Alternaria brassicae*
Isocyathisterol	*Aspergillus ustus*	*Codium fragile* (green algae)	*E.coli* and *S. aureus*
Methyl 6-acetyl-5,7,8-trihydroxy-4-methoxy-2-naphthoate	*Aspergillus tubingensis*	*Portunustrituber culatus* (crab)	*Vibrio anguillarum*
Austalides S-U	*Aspergillus aureolatus*	Unidentified sponge	Influenza virus A (H1N1)
Diorcinol	*Aspergillus sp.*	*Chondrillanu-cula* (sponge)	*S. epidermidis* and *S. aureus*
Asteltoxin E and F	*Aspergillus* sp.	Unidentified sponge	Influenza virus H3N2 and H1N1

Source: Blunt *et al*. (2018); Xu *et al*. (2015)

Table 2 Bioactive compounds with anticancer or cytotoxic activity obtained from marine-derived fungi

Compounds	Fungal	Source	Activity
Tricycloalternarene A	*Alternaria* sp.	*Callyspongia* sp. (sponge)	Inhibit NF-κB in murine RAW 264.7 macrophage cell line
Hydrogenated benzofurans			
Bicycloalternarene A-D			
Hydrogenated chromans			
Mono-cycloalternarene A			
Aspergillusidones D-F	*Aspergillus unguis*	Unidentified sponge	Inhibit aromatase, a therapeutic target for breast cancer treatment
Dankanstatin C	*Gymnascelladankaliensis*	*Halichondria japonica* (sponge)	Inhibit leukemia P388 cells.
2-(4-hydroxybenzoyl) quinazolin-4(3H)-one	*Paecilomyces oxalicum*	sediment	Inhibit human gastric cancer cell line SGC-7901
Penipacid A and E	*P. paneum*	Sediment	Inhibit human colon cancer RKO cells
Mariline A and A$_2$	*Stachylidium* sp.	*Callyspongia* cf. *C.flammea* (sponge)	Inhibit human leukocyte elastase (HLE)
Disydonol A and C	*Aspergillus* sp.	*Xestospongia testudinaria* (sponge)	Cytotoxic to HepG-2 and Caski human tumor cell line

Table 2 (Cont.) Bioactive compounds with anticancer or cytotoxic activity obtained from marine-derived fungi

Compounds	Fungal	Isolated from	Test cell line.
Asperterrestide A	*Aspergillus terreus*	*Echinogorgia aurantiaca* (gorgonian)	Cytotoxic to human carcinoma U937
Versiquinazolines A, B and F	*Aspergillus versicolor*	Unidentified gorgonian	Inhibit tumor cell line A549 (Lung adenocarcinoma) and A2780 (human ovarian cancer)
Chondrosterins K–M	*Chondrostereum* sp.	*Sarcophyton tortuosum* (soft coral)	Cytotoxicto human cancer cell lines; CNE1-2 (nasopharyngeal carcinoma)
Phenalenone	*Coniothyrium cereale*	*Enteromorpha* sp (algae)	Cytotoxic toward human tumor cell line: K562 (chronic myelogenous leukemia), U266 (multiple myeloma) and SKM1(myeloid leukemia)
Corynesidone A and Corynethers A	*Corynespora cassiicola*	Unidentified sponge	Cytotoxic to human promyelocytic leukemia (HL-60) and human cervical carcinoma (HeLa) cell lines

Source Blunt *et al*. (2018); Deshmukh *et al*. (2018)

1.2.3   Antimicrobial assays

1.2.3.1 Whole cell antimicrobial assay

In recent years, there has been a growing interest in the development of new antimicrobial agents from various sources to combat antibiotic resistance problems. So, a greater attention has been drawn to antimicrobial activity screening and evaluation of the methods (Balouiri *et al*., 2016). The gold standard guidelines or reference methods are approved and defined by various organizations such as the Clinical and Laboratory Standards Institute (CLSI), the International Organization for Standardization (ISO) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Schumacher *et al*., 2018). There are several well-known bioassays commonly used to evaluate the antimicrobial efficiency such as disk diffusion, well diffusion and agar or broth dilution methods.

A)   Diffusion methods

Agar disk diffusion method

Agar disk diffusion method was described in 1940s. It is the official methods used for routine antimicrobial susceptibility testing in many clinical microbial laboratories. This disk diffusion susceptibility method is the most practical and simple method. Currently, many well standardized methods have been approved for bacteria and yeasts. Moreover, this method has been well developed for testing fastidious bacteria such as streptococci, *Haemophilus* spp. and *Neisseria gonorrhoeae* using specific culture media, various incubation conditions and interpretive criteria for inhibition zone (Balouiri *et al*., 2016).

In general, the disk diffusion tests are performed by inoculating the test microorganisms on the agar surface. Then, filter paper disks (6 mm in diameter) containing the test compounds at a desired concentration are placed on the surface of the inoculated agar. The antimicrobial substances diffuse into the medium following the physical laws of nature. Thus, the depth of the agar medium is the most important factor in susceptibility testing system. Usually, the depth of the agar medium is recommended to be approximately 4 mm. The inhibitory activity is expressed as inhibition zone (Lorian, 1986). For standard antibiotic disk susceptibility test,

quantitative antibiogram results are categorized as susceptible, intermediate and resistant according to the size of inhibition zone. Its information also guides clinicians in the appropriate selection of initial treatment. However, the inhibition of bacterial growth does not refer to the bacterial death. This method cannot distinguish between bactericidal or bacteriostatic effects of antimicrobial agents (Balouiri *et al.*, 2016). In addition, this method is not suitable to determine the MIC, as it is difficult to exactly determine the amount of antimicrobial agents diffused into the agar medium. However, the advantages of this method over other methods are simplicity, the ability to test large numbers of microorganisms and antimicrobial agents, ease to interpret results provided and low cost. The disadvantages of this method are the lack of automation or mechanization of the test.

Antimicrobial gradient method (E-test)

The antimicrobial gradient method or E-test combines the principle of dilution and diffusion methods in order to determine the MIC value and perform similarly to the disk diffusion method. The paper strip containing exponential concentration gradient of antimicrobial agent is placed on surface of agar medium. After incubation, the MIC value can be read from the intersection of the strip and growth inhibition ellipse. Usually, the resulting growth pattern resembles a teardrop shape (Figure 10) (Schumacher *et al.*, 2018). Several studies showed a good correlation between the MIC values which obtained from broth dilution or agar dilution methods (Balouiri *et al.*, 2016). However, this test is costly.

Figure 10 Antimicrobial susceptibility tests using gradient method or E-test

Source:https://microbeonline.com/e-test-epsilometer-test-principle-purpose-procedure-results-and-interpretations (accessed 31 October 2018)

Other diffusion methods

The following diffusion based methods are used to screen for antimicrobial activity of natural product extracts and to investigate the antagonism between microorganisms in the microbiology research laboratories.

a)   **Agar well diffusion method (Balouiri *et al*., 2016)**

Agar well diffusion method is similar to disk diffusion method. It has been widely used to determine the antimicrobial effect of natural product extracts. After inoculation, a sterile cork borer (6-8 mm diameter) is punched into the well and then extract solution or antimicrobial agents at the desired concentration is added. The plates are incubated at optimal condition. The antimicrobial agent diffuses into an agar medium and causes inhibition of the microbial growth.

b)   **Agar plug diffusion method (Balouiri *et al*., 2016)**

Agar plug diffusion method is often used to determine the antagonism assay between microorganisms. The antibiotic producing strain is cultured on an agar medium. When it grows, it secretes antimicrobial substances into the agar medium. An agar containing antimicrobial substances is cut and placed on another agar surface plate inoculated with the test strain. The substances diffuse from the agar plug and the antimicrobial property is detected by the appearance of the inhibition zone around the agar plug.

c)   **Cross streak method (Balouiri *et al*., 2016)**

The microbial strain of interest is seeded by a single straight line through the center of the agar plate. After incubation time depending on the strain, the test microorganisms seeded by single streak perpendicular to the central streak. After reincubation, the antimicrobial interactions are analyzed by the determination of the inhibition zone size.

B)   **Dilution methods**

Dilution methods are the most appropriate ones for the determination of MIC values. Dilution methods, both agar and broth dilution, are used to

quantitatively measure the *in vitro* antimicrobial activity against pathogenic microorganism. MIC value is defined as the lowest concentration of antimicrobial agent that inhibits the visible growth of the tested microorganisms (Balouiri *et al*., 2016).

Broth dilution method

Broth dilution test is one of the most widely and commonly used method for antimicrobial susceptibility testing. This procedure based on preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium with a minimum volume of 1 to 2 ml for macrodilution or smaller volumes using 96 well microtiter plate (microdilution) (Balouiri *et al*., 2016). Mueller-Hinton broth (MHB) is recommended as the medium of choice for susceptibility testing of nonfastidious bacteria (CLSI, M07-A9). Then, antibiotic containing tubes are inoculated with bacterial suspension of 5 x 10^5 $\log_{10}$ CFU/ml. Following the overnight incubation under optimal condition, the bacterial growth in the test tubes is determined with unaided eyes as evidenced by turbidity. The lowest concentration of antimicrobial agent that inhibited growth represented the MIC. The precision of this method is considered to be $\pm$ 1 fold dilution, due in large part to the practice of manually preparing serial dilutions of the antibiotics (Reller *et al*., 2009).

The caution of this method is transferring of antimicrobial that avoid progressive dilution error. This step continued down to the next to last tube (Lorian, 1986). Moreover, the inoculum size, the incubation time, the type of culture medium and the inoculum preparation method can influence MIC values (Balouiri *et al*., 2016). The standard methods recommended by CLSI are shown in Table 3.

The main disadvantages of the macrodilution method are manual undertaking, the comparatively large amount of reagents and space required and risk of errors in the preparation of antimicrobial solutions for each test. Thus, the reproducibility and the economy of reagents and space that occurs due to the miniaturization and mechanization of the test by use of small and disposable plastic microdilution trays are the major advantages of the microdilution method (Balouiri *et al*., 2016; Garmana *et al*., 2014).

Agar dilution method

The agar dilution method involves the incorporation of varying desired concentration of the antimicrobial agent into a molten agar medium. Each plate contains a different concentration of antimicrobial agent, frequently using serial two-fold dilutions. The test microorganisms are applied on the agar surfaces with 32 to 60 different inocula to each plate. This method is suitable for both antibacterial and antifungal susceptibility testing. Moreover, this technique is often recommended as a standard method for fastidious bacteria. The lowest concentration of antimicrobial agent that completely inhibits growth under optimal condition is recorded as MIC value (Balouiri *et al*., 2016).

In general procedure, Mueller-Hinton agar (MHA) is the best recommended medium for routine susceptibility testing of nonfastidious bacteria. The agar containing antimicrobial agent should be used immediately or stored in sealed plastic bags at 2-8°C for up to five days for reference work or longer for routine test. The 2 µl of inoculum is placed onto the agar surface to obtain 3 mm pins. The final concentration of test microorganisms on agar surface will be approximately 10^4 CFU/spot (CLSI, M07-A9).

Nowadays, the commercial chromogenic agar media are available which enable faster CFU detection due to an early visible color response for susceptible strains. The advantages of the agar dilution method are simplicity, well-understood parameter and provide greater possibilities (Schumacher *et al*., 2018).

Table 3 Culture media, microbial inoculum size and incubation conditions for antimicrobial susceptibility testing methods as recommended by CLSI

Methods	Test microorganisms	Growth medium	Final inoculum size	Incubation temperature (^{o}C)	Incubation time (h)	Reference method
Disk-diffusion method	Bacteria	MHA	0.5 McFarland or 1-2x10^8 CFU/ml	35±2	16-18	M02
	Yeasts	MHA+GMB	0.5 McFarland or 1-5x10^6 CFU/ml	35±2	20-24	M44
	Molds	Non-supplement MHA	0.4-5x10^6 CFU/ml	-	-	M51
Broth macro-dilution	Bacteria	MHB	5x10^5 CFU/ml	35±2	20	M07
	Yeasts	RPMI 1640	0.5-2.5x10^3 CFU/ml	35	46-50	M27
	Molds	RPMI 1640	0.4-5x10^4 CFU/ml	35	48 for most fungi	M38
Broth micro-dilution	Bacteria	MHB	5x10^5 CFU/ml	35±2	20	M07
	Yeasts	RPMI 1640	0.5-2.5x10^3 CFU/ml	35	24-48	M27
	Molds	RPMI 1640	0.4-5x10^4 CFU/ml	35	48 for most fungi	M38
Agar dilution	Bacteria	MHA	10^4 CFU/spot	35±2	16-20	M07
Time-kill test	Bacteria	MHB	5x10^5 CFU/ml	35±2	0,4,18 and 24	M26

MHA = Mueller-Hinton agar   MHB = Mueller-Hinton broth   GMB = Glucose-methylene-blue   RPMI = Roswell Park Memorial Institute

Source: Balouiri *et al.*, 2016

1.2.3.2 Anti-quorum sensing

A)   Quorum sensing in bacteria

Quorum sensing (QS) is a mechanism of cell-cell communication in bacteria that regulates the expression of genes in population cell density. This mechanism operates through chemical signal molecules called autoinducers which produced and released by bacteria. Many species of bacteria use quorum sensing to coordinate gene expression (Miller and Bassler, 2001). At a threshold concentration, the signals activate a regulator that results in activation or repression of QS target gene. Usually, quorum sensing processes are beneficial when a group of bacteria acts together. For example, in the marine bacterium *Vibrio fischeri*, QS regulates luminescence in the squid light organ (Frederix and Downie, 2011). QS also controls processes including virulence and biofilm formation in pathogenic bacteria (Zhu *et al.*, 2002).

B)   Types of autoinducer molecules

At present, four types of autoinducer molecules have been reported, *N*-acyl homoserinelacton (AHL) molecules (autoinducer-1, AI-1), autoinducer peptides (AIP), boron-furan-derived signal molecules (autoinducer-2, AI-2), and miscellaneous quorum sensing molecules (Verbeke *et al.*, 2017).

a)   Autoinducer-1 (AI-1) (Kalia, 2013, Verbeke *et al.*, 2017)

AI-1 or AHLs based QS are the most common class of autoinducers used by Gram-negative bacteria. AHLs are synthesized by AHL-synthase gene such as LuxI homologues and distributed in and around the cells. When the concentration of AHL reaches minimal threshold, the AHL signal molecule binds with its specific receptor and activates the transcriptional regulator (LuxR homologues) of neighboring bacterial cell to trigger the expression of target genes. There are many forms of AHLs are shown in Figure 11. All AHLs consist of a homoserine lactone (HSL) ring and an acyl chain which are varied in size and composition on the third carbon with either oxo-or hydroxyl-substitution. AHLs serve not only as intraspecies communication molecules but also interspecies communication.
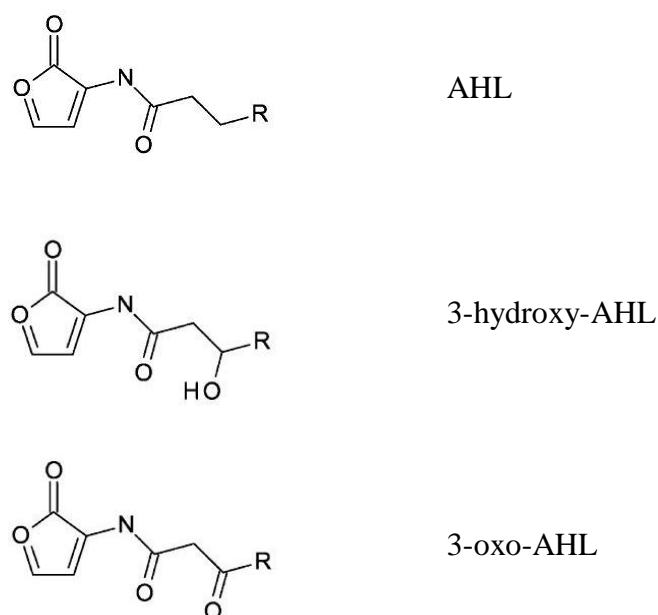
Figure 11 Different forms of QS signaling molecules in AHL based QS

b)   **Autoinducer peptides (AIP) (Kalia, 2013;Verbeke *et al*., 2017)**

Gram-positive bacteria regulate the QS signals through oligopeptide autoinducers which secreted into the extracellular environment. AIP are synthesized by bacterial ribosomes as pro-peptides and undergo posttranslational modifications during excretion to become activated. When a minimal threshold is reached, APIs bind to receptors leading to autophosphorylation and inactivation of intracellular response regulators through phosphor-transfer. These phosphorylated response regulators increase target gene expression. The quorum sensing mechanisms of these Gram-positive bacteria are different depending on the involved genes and receptor(s). For example, *Staphylococcus* species use the agr-quorum sensing system, *Streptococcus* species employ the ComX-quorum sensing system and *Bacillus* species use the Rap-quorum sensing system.

c)   **Boron-furan-derived signal molecules (autoinducer-2, AI-2) (Pereira *et al*., 2013)**

Autoinducer-2 (AI-2), a furanosyl borate diester or tetrahydroxy furan signaling molecules was first identified in *Vibrio harveyi* by Bassler *et al.* (1993). It was responsible for the induction of bioluminescence in *V. harveyi* cultures. The gene

responsible for AI-2 activity is called *luxS*. AI-2 is produced and recognized by many Gram-negative and Gram-positive bacteria such as *E. coli* (Lu *et al*., 2017), *Salmonella* spp. (Choi *et al*., 2007, 2012), *Pseudomonas aeruginosa* (Duan *et al*., 2003), *Bacillus cereus* (Auger *et al*., 2006), *S. aureus* (Zhao *et al*., 2010) and *Streptococcus pneumoniae* (Vidal *et al*., 2011). AI-2 acted as interspecies communication signal. AI-2 produced by *V. harveyi* co-cultured with *E. coli* affected gene expression in *E. coli*.

d)   Miscellaneous quorum sensing molecules

QS molecules other than the above three mentioned groups are included in this fourth group, such as fatty acid derivative, diffusible cellular factor produced by *Xanthomonas camprestis* (Barber *et al*., 1997), 3-hydroxypalmitic acid methyl ester from *Ralstonia solanacearum* (Flavier *et al*., 1997), diketopiperazines and cyclic peptides found in cell-free supernatants from *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* (Holden *et al*., 1999), 2-heptyl-3-hydroxy-4-quinolone from *P. aeruginosa* (Pesci *et al*. (1999) and (S)-3-hydroxytridecan-4-one produced by *Vibrio cholerae* (Higginns *et al*., 2007).

C)   Quorum sensing mechanism

The QS mechanism depends on the synthesis, release, and uptake of autoinducers (AIs) in the surrounding environment. The concentration of AIs is related to the density of bacterial population. The QS mechanisms of Gram-positive bacteria and Gram-negative bacteria are different. Gram-positive bacteria use peptides as a signaling molecules or AIs. Usually the precursor peptide AIs are modified and transported out of the cell via ATP-Binding Cassette (ABC) exporter complex. When the concentrations of these peptides are above a certain threshold, the sensor kinase protein will be activated and further phosphorylates the response regulator protein. The regulator protein binds to the target promoter that will lead to QS gene regulation. Gram-negative bacteria use small molecules as AIs, such as N-Acyl homoserine lactone and S-adenosyl methionine. The AIs are produced and freely diffuse out of the cell. When the concentration of AI reaches the certain threshold value, the positive feedback loop will be formed. These AIs will bind to their cognate receptor to form a

complex signaling network. Then these complexes bind to the target promoter that lead to QS gene regulation (Figure 12).
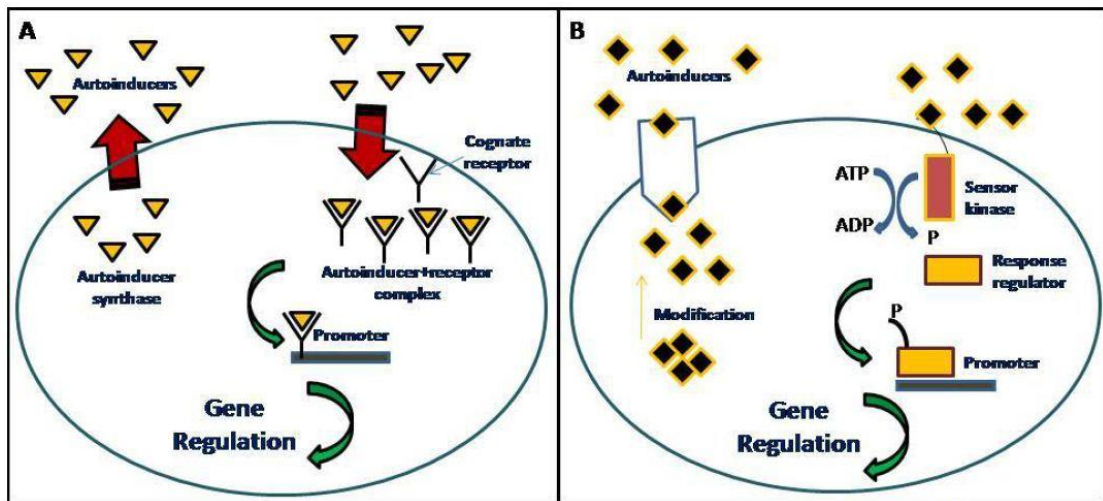
Figure 12 The quorum sensing molecule signaling network of Gram-negative bacteria (A) and Gram-positive bacteria (B)

Source: Koh *et al*. (2013)

D) **Quorum sensing controlled processes**

Quorum sensing systems regulate a wide range of physiological process in bacteria including bioluminescence, virulence gene expression, biofilm formation, pigment information, antibiotic production and formation and activity of many degradation enzymes (Rutherford and Bassler, 2012). It is an important process in bacteria related to virulence, pathogenicity and survival. Thus, inhibition of bacterial QS will lead to prevention and control of bacterial infections.

E) **Quorum sensing inhibition**

There are various mechanisms that can disrupt the QS process including reduction of AHL synthase activity, inhibition of the production of QS signal molecules, degradation of the AHL and mimicking the signal molecules primarily by using synthetic compounds as analogues of signal molecules. In addition, inhibition of QS signals by antibodies and decoy receptors have been suggested as a novel strategy for quorum sensing inhibition (Kalia, 2013).

a) **Bacterial reporters to detect QS signals and screening of QSI**

One of the most important strategies for the development of antimicrobial drugs which prevent or control bacterial pathogenicity-related to QS is to find a good detection system. Table 4 shows the list of biosensor strains that have been reported to provide sensitive, quantitative and real-time detection of QS signals.

Table 4 Microorganism strains used for the detection of quorum sensing signals

Microorganisms	Phenotype	Quorum sensing signal detected
Agrobacterium tumefaciens A136 [traI-lacZ fusion(pCF218)(pCF372)]	β-galactosidase activity	C6HSL to C14-HSL
A. tumefaciens strain NT1 (pDCI41E33 containing atraG::lacZ fusion)	β-galactosidase activity	AHLs with 3-oxo-, 3-hydroxy-, and 3-unsubstituted side chains of all lengths, (C6-C14HSL) with the exception of C4HSL
Chromobacterium violaceum strain CV026–CviR receptor	Violacein pigment production	Wide host range of AHLs
Escherichia coli plasmid carrying a luxCDABE cassette activated by AhyRI' receptor of *Aeromonas hydrophila* (pSB536)	Bioluminescence	C4HSL
E. coli plasmid carrying a luxCDABE cassette activated by AhyR receptor of *A. hydrophila* (pSB403)	Bioluminescence	Wide host range of AHLs
E. coli JM109 plasmid carrying a luxCDABE cassette activated by LuxR receptor of *Vibrio fischeri* (pSB401)	Bioluminescence	C6HSL
E. coli JM109 plasmid carrying a luxCDABE cassette activated by LasR receptor of *P. aeruginosa* (pSB1075)	Bioluminescence	C12HSL

Table 4 (Cont.) Microorganism strains used to detect quorum sensing signals

Microorganisms	Phenotype	Quorum sensing signal detected
E. coli JM109 plasmid carrying a luxCDABE cassette activated by RhlR receptor of *P. aeruginosa*	Bioluminescence	C4HSL
Pseudomonas aurofaciens strain 30-84I	Phenazine antibiotic production	C6HSL
Pseudomonas putida 117(pAS-C8)-CepR receptor	Green Fluorescent Protein	C8HSL
P. putida IsoF⁄gfp	Fluorescence	3OC12HSL
Serratia liquefaciens strain MG44	Biosurfactant production	C4HSL
S. liquefaciens strain PL10 — LuxAB reporter	Bioluminescence	C4HSL
Sinorhizobiummeliloti Rm41 sinI::lacZ (pJNSinR)	β-galactosidase activity	C16–C20HSL
Vibrio harveyi BB170 — LuxP receptor	Bioluminescence	AI-2
V. harveyi BB886 — LuxP receptor	Bioluminescence	AI-1

Source: Kalia (2013)

b)   **Quorum sensing inhibitors**

The ideal QS inhibitors are chemically stable and highly effective low-molecular-mass molecules exhibiting a high degree of specificity for the QS regulator without toxic side effects on either bacteria or an eventual eukaryotic host. There are some synthetic and natural compounds that act as QS inhibitors as shown in Table 5. They are able to interfere with QS-regulated behaviors and providing a potential treatment for infections associated with bacterial quorum sensing.

Table 5 Quorum sensing inhibitors from various sources

Quorum sensing inhibitor	Organism	Quorum sensing activity or targets
Plants		
Allium sativum (garlic) extract	*A. tumefaciens* NTL4	β-galactosidase
	C. violaceum	Violacein production
	P. aeruginosa	Alginate and elastase Biofilm formation
	P. aeruginosa strain IsoF/gfp	Fluorescence
Ananas comosus	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolytic protease, ealstase production and biofilm formation
Blueberry extracts	*C. violaceum*	Violacein production
Cinnamaldehyde and its derivative: 4-NO2-cinnamaldehyde	*Vibrio* spp.	AI-2-mediated QS — bioluminescence, Protease activity, pigment formation

Table 5 (Cont.) Quorum sensing inhibitors from various sources

Quorum sensing inhibitor	Organism	Quorum sensing activity or targets
Crown vetch	*C. violaceum* CV026	Violacein production
Grapefruit juice (furocoumarins)	*E. coli*	Biofilm formation
	P. aeruginosa	
	Salmonella Typhimurium	
Manilkara zapota	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolyticprotease, ealstase production and biofilmformation
Musa paradiciaca	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolyticprotease, ealstase production and biofilmformation
Ocimum sanctum	*C. violaceum*	Violacein production
	P. aeruginosa PAO1	Pyocyanin pigment, staphylolytic protease, ealstase production and biofilm formation
Passiflora incarnate (leaf)	*C. violaceum* CV0blu	Violacein production
Raspberry extracts	*C. violaceum*	Violacein production
Scorzonera sandrasica extract	*C. violaceum* ATCC12472 and CV026	Violacein production
	Erwinia caratovora	Carbapenem antibiotic production
Vanilla planifolia extract	*C. violaceum* CV026	Violacein production
Combretum albiflorum (bark)	*P. aeruginosa*	Biofilm formation and elastase

Table 5 (Cont.) Quorum sensing inhibitors from various sources

Quorum sensing inhibitor	Organism	Quorum sensing activity or targets
Salicyclic acid	*A. tumefaciens*	Stimulating AHL-lactonase expression
Vanillin	*Aeromonashydrophila*	Biofilm formation
Animal		
Mammalian paraoxonases	*P. aeruginosa*	Reducing 3OC12HSL dependent activity
Fungi		
Patulin and penicillic acid (*Penicillium* sp.)	*P. aeruginosa*	Biofilm
natural pigments (*Auricalaria auricular*)	*C. violaceum*	Violacein production
Marine fungi		
Kojic acid (*Alternaria* sp.)	*C. violaceum* CV017	Violacein production
	E. coli pSB401	Bioluminescence
Meleagrin (*Penicillium chrysogenium*)	*C. violaceum* CV017	Violacein production
Penicillium sp. SCS-KFD08	*C. violaceum* CV026	Violacein production
Marine bacteria		
Tyrosol and tyrosol acetate (*Oceannobacillus profundus*)	*C. violaceum* ATCC31532	Violacein production
Phenethylamide (*Halobacillus salinus* C42)	*C. violaceum* CV026	Competing N-acyl homoserine lactones for receptor binding
Bacillus cereus	*V. harveyi* BB120 and *C. violaceum* ATCC12472	Competing N-acyl homoserine lactones for receptor binding
Diketopiperazines (*Marinobacter* sp. SK-3)	*C. violaceum* CV017	Violacein production

Table 5 (Cont.) Quorum sensing inhibitors from various sources

Quorum sensing inhibitor	Organism	Quorum sensing activity or targets
	E.coli pSB401	Reducing the QS-dependent luminescence
Marine Cyanobacteria		
Tumonoic acid (*Blennothrix cantharidosmum*)	*V. harveyi*	Bioluminescence
Honaucins A-C (*Leptolyngbya crossbyana*)	*V. harveyi* BB120	Bioluminescence

Source: Kalia (2013)

1.2.3.3 Antimicrobial combination (Leekha *et al.*, 2011; Lorian, 1986)

Antimicrobial resistance is one of the most serious public health problems. The infectious diseases caused by resistant pathogens have remained a major cause of death and disability. Increasing of resistance pathogens makes many commonly used antibiotics have become less effective. One alternative approach to treat infectious diseases is a combination or synergistic therapy. This approach is useful and helpful for patients having serious infections with multiple drug resistant pathogens.

A)   Advantages of antimicrobial combination (Lorian, 1986)

There are many good reasons for the use of antimicrobial combination as described below:

a)   Decrease emergence of resistant strain

The emergence of resistant strains is the result of selective pressure from antimicrobial treatment. If the resistance mechanisms to two antimicrobial drugs are different, the opportunity of the strain to be resistant to both drugs is much lower than that to either one. The treatment of tuberculosis has been best demonstrated in this phenomenon, where treatment with multiple drugs clearly reduces the risk of infection with resistant pathogens. Streptomycin monotherapy yielded streptomycin resistance in 70% of tuberculosis cases after 120 days, whereas streptomycin and *para*-amino-salicylic acid (PAS) combination therapy yielded streptomycin resistance in at most 9% of cases.

b)   Decrease dose-related toxicity as a result of reduced dosage

The important goal of the use of antimicrobial combination has always been to reduce the dose of the potentially toxic antimicrobial agent while using the other agent to ensure a successful clinical outcome.

c)   Extend the antimicrobial spectrum for polymicrobial infection

Broad spectrum antibiotic is commonly used to treat polymicrobial infection. However, in some cases such as intra-abdominal infections caused by multiple microorganisms (Gram-positive cocci, Gram-negative bacilli and anaerobes) it may be necessary to treat with antimicrobial combination to combat each of the major pathogens.

d)   Increase killing activity

The *in vitro* studies showed that using antimicrobial combination will gain more effective inhibitory activity. The antimicrobial activity of synergistically combined drugs is greater than the sum of each individual drug. It is very useful in the treatment of serious infections that require rapid killing, such as the use of penicillin and gentamicin for endocarditis caused by *Enterococcus* spp. Another example is quinipristin-dalfopristin combined with other antimicrobial agents such as doxycycline could enhance killing activity against vancomycin-resistant enterococci (Eliopoulos and Wennersten, 2002; Kang and Rybak, 1997)

B)   Assessment of antimicrobial combination (Bollenbach, 2015; Lorian, 1986)

Combination of antimicrobial agents can result in three different forms of interactions, namely synergism, additivity or indifference and antagonism (Figure 13). Synergism is an interaction between two or more drugs that the combined effect is greater than the sum of the individual drugs, whereas additivity is equal to the sum of each drug and antagonism results in weaker activity.
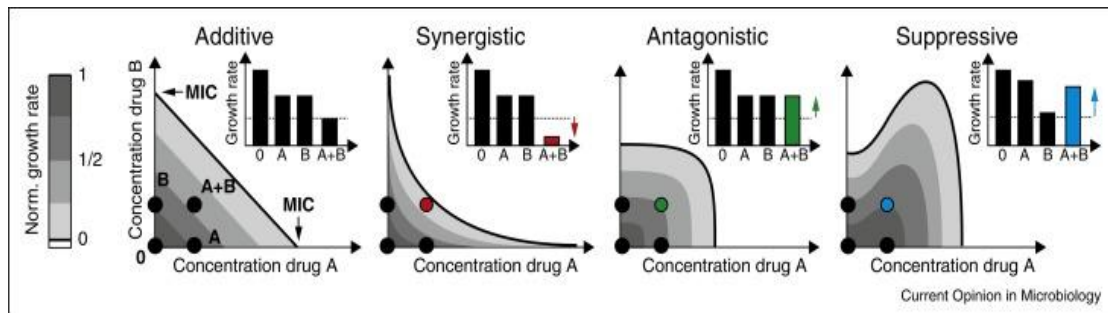
Figure 13 Drug interactions are defined by the shape of lines of equal effect in two-drug concentration space. Schematics showing growth rate (grayscale) and minimum inhibitory concentration (MIC) line (black, line of zero growth) in the two-dimensional concentration space of drugs A and B. The additive reference is given by linear interpolation of the MICs of the individual drugs. For synergistic and antagonistic drug interactions the MIC line lies below or above this additive expectation, respectively. Suppression is a hyper-antagonistic case in which drug A alleviates the effect of drug B. Insets: growth rates in the absence of drugs ('0'), and at fixed concentrations of drugs A and B individually and combined ('A+B'). The dashed horizontal line in insets indicates the additive expectation.

Source: Bollenbach (2015)

There are many methods that can be used to determine the interaction between the combined drugs including checkerboard method and time-kill assay.

C) Checkerboard method (Lorian, 1986)

Checkerboard is the most common technique used to determine antimicrobial combination *in vitro*. It is performed by multiple dilutions of the two antimicrobial agents being tested in concentrations equal to, above and below their MIC as shown in Figure 14. The checkerboard is more popular because it is easy to understand, the mathematics and interpretation of the results are simple and it can be performed using microdilution systems in the laboratory.

Figure 14 Concentrations of individual drug suggested to use in the checkerboard method

Interpretation of the results

        The fractional inhibitory concentration index (FICI) is a mathematical expression that most commonly used to define the results of antimicrobial combination. FICIs can be calculated using the following formula:

$$FICI = FIC_A + FIC_B = \frac{(A)}{MIC_A} + \frac{(B)}{MIC_B}$$

(A)   :   Concentration of drug A in combination

(B)   :   Concentration of drug B in combination

MIC_A :   MIC of drug A alone

MIC_B :   MIC of drug B alone

With this method, FICI is defined as (Sopirala *et al.*, 2010)

        Synergism   : FICI ≤ 0.5

        Additivity   : FICI $> 0.5\text{-}\leq 1$

        Indifference   : FICI $> 1\text{-}\leq 4$

        Antagonism   : FICI > 4

D)   Time-kill assay

A time kill curve assay is used to determine the efficacy of antimicrobial agent to the particular bacterial strains. The result can be expressed as the rate of killing which measuring the number of viable bacteria at various time intervals. At each time point (usually 0, 4, 8, 10, 12 and 24 hours of incubation), the viable cells are counted (Garmana *et al*., 2014). The procedure is recommended to test at several MIC concentrations and the test strain is grown in the presence and absence of the antimicrobial agent. The time-kill curve are plotted on semi-log graph paper with time on the x-axis and the number of viable cells on the y-axis (Lorian, 1986).

This method has been widely used to evaluate new drugs. The time-kill curve is used to study both concentration-dependent and time-dependent bactericidal activities of antimicrobial agents. Bactericidal activity is defined as ≥ 3 $\log_{10}$ CFU/ml reduction and bacteriostatic activity as < 3 $\log_{10}$ CFU/ml reduction as compared with the initial inoculum (Balouiri *et al*., 2016).

Furthermore, this method can be used to determine two or more drugs in combination. The synergy effect is defined as decrease of at least ≥ 2 $\log_{10}$ CFU/ml in combination compare with the most active single agent. Antagonism is defined as increase of at least ≥ 2 $\log_{10}$ CFU/ml in combination compare with the most active single agent (Akinyele *et al*., 2017; Sopirala *et al.,* 2010).

E)   Two or more antimicrobial combination

Nowadays, the treatment of the drug-resistant pathogens are often not restricted to the monotherapy of the most commonly used drugs, but generally extends to other antibiotic classes. One approach to combat antibiotic-resistant bacterial infection problems is the use of combination of two or more antimicrobial drugs. Combination therapy is often used in many areas of medicine such as HIV, tuberculosis and malarial treatments.

F)   Drug synergism between known antimicrobial agents and bioactive metabolites

At present, combination of natural active metabolites with antimicrobial potential and standard antibiotic is a novel concept to combat antimicrobial resistant pathogens. Extracts derived from natural sources play an important role in the treatment and prevention of human diseases. Several natural products exhibited synergistic activity with known antibiotics against a large panel of microorganism have been reported as shown in Table 6.

Table 6 List of natural products showing synergistic effect with standard antibiotics against a panel of pathogenic microorganisms

Natural products	Standard antibiotic	Microorganism	Reference
Usnic acid	Amikacin Etimicin Teicoplanin	MRSA	Zuo *et al*., 2018(a)
Chitosan	Ceflazidine	*P. aeruginosa* *S. aureus*	Muslim *et al*., 2018
Morusinol	Amikacin Streptomycin		
Kuwanon	Ciprofloxacin Etimicin Vancomycin	MRSA	Zuo *et al*., 2018(b)
Curcumin	Ceflazidine	*P. aeruginosa*	Roudashti *et al*., 2017
Persicariaodarata (DaunKesom) leaf extracts	Vancomycin	*S. aureus* *S. epidermidis* *S. pyogenes* *S. pneumoniae* *P. aeruginosa* *S.* Typhi	Ridzuan *et al*., 2017
Alpha-mongostin	Ceftazidime	*A. baumannii*	Pimchan *et al*., 2017

Table 6 (Cont.) List of natural products showing synergistic effect with standard antibiotics against a panel of pathogenic microorganisms

Natural products	Standard antibiotic	Microorganism	Reference
N-acetylcysteine	Tigecycline	*A. baumannii*	Feng *et al*., 2018
Melittin	Colistin	*A. baumannii*	Bardbari *et al*.,
	Imipenem		2018

1.2.4 Application of molecular method in fungal phylogeny, taxonomy and characterization

Traditionally, fungal identification and classification has largely been based on morphological characteristics. However, traditional identification methods are limited. It is relied on morphologies that are difficult to differentiate fungi especially at the species level and it needs personal experience background. At present, molecular methods are becoming more common. Mycologists apply a number of molecular techniques to identify individual fungal species and to organize fungi systematically. Nonetheless, morphological approaches still play an important role in fungal taxonomy. Several molecular techniques used for fungal identification are such as DNA GC content, restriction fragment length polymorphism (RFLP), random amplified polymeric DNA (RAPD) and DNA sequence based approach (Aslam *et al*., 2017).

1.2.4.1 PCR-based sequence analysis for phylogenetic tree

Polymerase Chain Reaction (PCR) is a powerful and most common molecular technique used to make multiple of copies of a specific sequence of DNA in only a few hours. PCR was first described by Kary B. Mullis in 1985. This process was applied and developed in many methods used for a wide range of genetic analysis (Singh *et al*., 2012). In the field of mycology, PCR technique is effectively used for fungal taxonomy and speciation, gene expression and cloning fungal mycobionts, plant pathogens, animal and human pathogens, mycotoxin producing fungi and many others. Ribosomal RNA genes are the most popular targets for the study of fungal

taxonomy because they are present in all microorganisms. Other protein-coding genes including RNA polymerase II (*rpb1 and rpb2*), elongation factor 1-α (*tef-1α*) and beta-tubulin genes can be used to determine the phylogenetic relationships among closely related fungi (Ge *et al*., 2014; Salgado-Salazar *et al*., 2012; Wen *et al*., 2016).

A)   The nuclear ribosomal DNA (Raja *et al*., 2017)

DNA sequencing of the ribosomal DNA has become of the most useful technique for fungal identification. The three regions of the rRNA gene comprising the large subunit (nrLSU-26S or 28S), small subunit (nrSSU-18S), and the entire internal transcribed spacer region (ITS1, 5.8S, ITS2) have been extensively used for the phylogenetic study in the kingdom Fungi. The 18S rRNA evolves the slowest evalution resulting in the lowest amount of variation among taxa. This data is useful for the differentiation among higher taxonomic levels such as family, order, class and phyla. The ITS evolves the fastest and exhibits the highest variation. The ITS region is valuable for species identification in fungi and commonly used in fungal taxonomy. The 28S rRNA gene provides a useful data at the intermediate taxonomic levels (family, genera). Moreover, the LSU region in combination with the ITS region is also useful for species identification. The diagrams of the rDNA regions and their primers are shown in Figures 15 and 16.
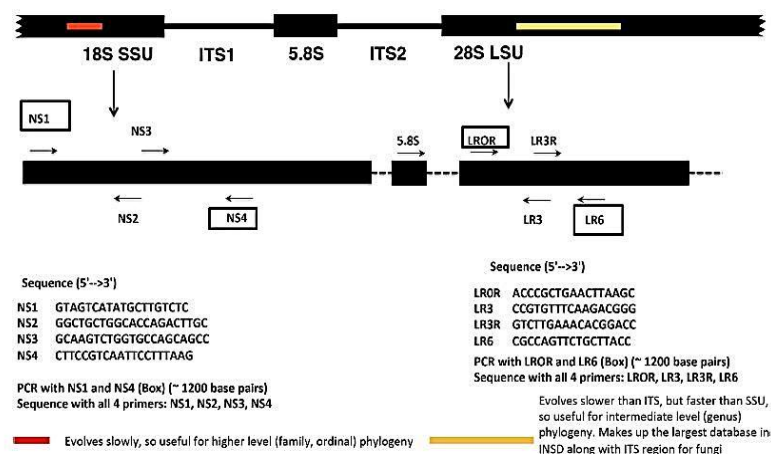
Figure 15 Diagram of the ribosomal DNA cluster of fungi and primers for amplification of small-subunit (SSU) and large-subunit (LSU) rDNA.

Source: Raja *et al*. (2017)
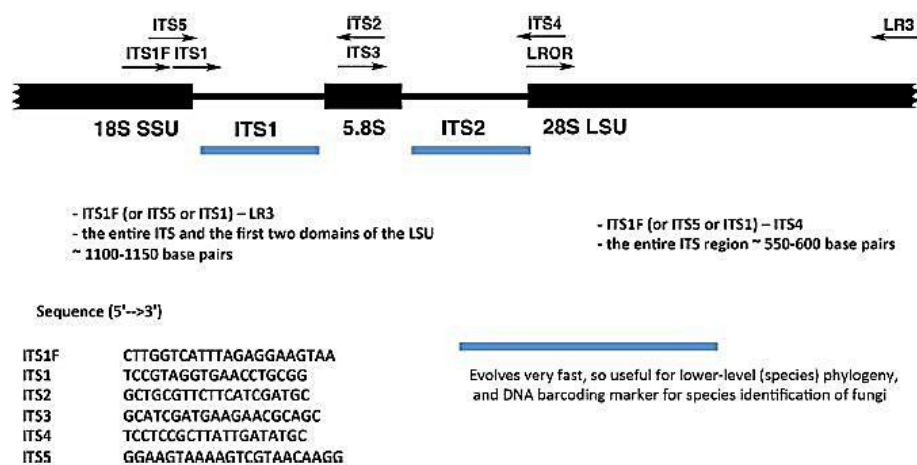
Figure 16 Diagram of ITS regions and their primers

Source: Raja *et al*. (2017)

B.) **PRC reaction components and conditions**

A PCR reaction is prepared by mixing several individual components including DNA template, primers, DNA polymerase, deoxyribonucleotide triphosphate (dNTPs) and magnesium ions and then adding sterile deionized water to achieve the desired concentration and volume of each reaction. The PCR sample volume is commonly performed in the range of 5 μl to 100 μl. The optimal concentrations of various components are listed in Table 7.

Table 7 Optimal concentration of each component for PCR amplification

Component	Optimal concentration
DNA template	1-10 ng genomic DNA
MgCl$_2$	0.5-5.0 mM
dNTPs	20-200 μl
Taq polymerase	0.5-2.5 units
Amplification buffer	1/10 final volume (buffer is supplied with 10X *Taq* polymerase)
Primers	0.1-10 μm
Sterile deionized water	To final volume
Final volume	5-100 μl

Source: Lorenz (2012)

The effects of each PCR component on the PCR results are as follows (Lorenz, 2012):

a) DNA template: The optimal quality of DNA may greatly benefit the PCR results. High purity DNA leads to increase yield of PCR reaction. The optimal concentration of DNA template is 10^4 to 10^7 molecules.

b) Magnesium salt (Mg^{2+}): PCR requires the magnesium to act as a cofactor during the reaction. Changing the magnesium concentration is the easiest reagent to manage the impact on the stringency of PCR. An insufficient concentration of Mg^{2+} will decrease the fidelity and specificity of DNA polymerase. Furthermore, magnesium concentration may affect the denaturation of the DNA template and primer annealing.

c) Deoxynucleotide 5′-triphosphate (dNTP): Inequivalent concentration of dNTP can cause problems for PCR. Low concentration of dNTP may increase the fidelity and specificity of the PCR reaction while higher dNTP concentration can inhibit the reaction.

d) DNA polymerase: *Taq* DNA polymerase is the first common heat-stable enzyme used for PCR. The excessive concentration of *Taq* DNA polymerase results in the accumulation of nonspecific PCR products while the lower concentrations give an insufficient amount of amplicon products.

e) PCR buffer: Buffer associated with DNA polymerase enzyme depends on types of DNA polymerase. Thus, choosing a suitable enzyme can be supportive for obtaining desired amplicon products. In typical standard PCR, the use of *Taq* DNA polymerase may be performed in 10 mM TrisHCl and 50 mM KCl.

f) Primer: Primers are the most important components of a good PCR assay. The target region on the DNA template is depending on the primer. Nowadays, a number of commercial or freeware primer design program and published primers are available. However, a high concentration of primers can increase the chance of unspecific priming while too low primer concentration affects the PCR yield.

g) Additive reagents: Several additive and enhancing reagents are added into the PCR reaction to improve PCR product yield and specificity of difficult targets in PCR amplification. Examples of enhancing reagents are shown in Table 8.

Table 8 Additive reagents used to enhance the PCR amplification

Additive reagents	Function	Recommended concentration
Betaine	Improvethe thermostability of DNA template	0.5 M-2.5 M
Bovine serum albumin (BSA)	Stabilize DNA polymerase	10-100 µg/ml
Dimethyl sulfoxide (DMSO)	Increase yield and disrupt base pairing	1-10%
Non-ionic detergents eg. Triton X-100, tween 20 or nonidet P-40 (NP-40)	Stabilize DNA polymerase	0.1-1%
Formamide	Increase amplification efficiency and disrupt base pairing	1.25-10%

Source: Lorenz (2012)

e)   The PCR thermocycling program (Clark and Russell, 2005)

PCR is based on using the ability of *Taq* polymerase to synthesize new strands of DNA complementary to the DNA template. The PCR process consists of three major steps with repeated cycles through several different temperatures as follows:

a) DNA denaturation: The first step of PCR cycle is to disrupt the hydrogen bonds between complementary bases of double stranded DNA (dsDNA) at high temperature (~90°C) for a minute or two. All dsDNA then become single stranded DNA (ssDNA).

b) DNA annealing: The temperature is lowered to 50°C to 60°C to promote primer binding to the complementary sequence on the template strands.

c) DNA extension: The final step is elongation of the primer. In this step, the temperature is increased to 72°C that is optimum for DNA polymerase to extend primer sequences from the 3' of each primer to the end of the amplicon.

The final phase of PCR cycle incorporates an extended elongation period to allow synthesis of many uncompleted amplicons to finish. Termination of the reaction is to cool down the mixture to $4^{\circ}C$. To increase the quantity of PCR products, the PCR thermal cycle should be repeated from step 1 to 3 over again for 25 to 35 times.

1.2.4.2 Basic methods for the detection of PCR products (Jenkins, 1994)

There are many different basic methods for the detection of PCR products such as the followings:

a) Direct visualization using ethidium bromide

Direct visualization using ethidium bromide is the easiest and most common method for detection of PCR products. Ethidium bromide is a fluorescent dye that intercalates between the DNA bases. It is used to visualize DNA band on agarose gel under a UV light at 260 nm. The size of PCR product can be estimated from the size of DNA marker (ladder). The disadvantages of this method are that the ethidium bromide can detect bands that contain approximately 5 ng of DNA or more and both specific and nonspecific DNA products will be stained. The expected DNA product and/ or excessive bands of the nonspecific product of the same size can lead to faulty interpretation of the results.

b) Incorporation of radioactive and nonradioactive labels

The direct visualization of amplified PCR products by incorporation of radioactive or nonradioactive labels is more sensitive than the ethidium bromide staining. Incorporation of specific labels is achieved by decreasing the number of non-labeled dNTPs. ^{32}P radiolabeled dNTP is commonly used while biotin and digoxigenin are nonradioactive labels. The PCR products labeled with radioisotopes are detected by autoradiography whereas nonradioactive labeled requires an enzyme assay with chromogenic substrates. The disadvantage of this method is the visualization of all PCR products including specific and nonspecific products. When compared with ethidium bromide, the number of nonspecific band is greater. Thus, the direct labeling of PRC products is not widely used.

c) Detection of specific PCR products using a DNA probe

The detection of specific PCR products using a DNA probe is highly recommended. The DNA probe (15-21 nucleotides in length) hybridizes specifically in the DNA target region. Consequently, only the specific amplified product will be visualized. The DNA probe can be radioactive or nonradioactive makers. The detection of PCR products using DNA probe increases sensitivity and accuracy more than the ethidium bromide staining. This method is widely used for detection of PCR products.

1.2.4.3 DNA sequencing and DNA sequence alignment

DNA sequencing is a method to detect the order of the four bases (adenine, guanine, cytosine, and thymine) in a strand of DNA. The DNA sequence alignment is the basic bioinformation tool for identification of the similar position between the biological sequences. This step could represent the evolutionary relationships between the sequence and homology can be inferred. This can be done using softwares such as Clustal W.

1.2.4.4 Phylogenetic tree reconstruction

A phylogeny is the evolutionary history of a group of organisms (or sequences).The phylogeny reconstruction is to describe evolutionary relationships in terms of relative recency of common ancestry. These relationships are represented as a branching diagram, or tree, with branches joined by nodes and leading to terminals at the tips of the tree (Hennig, 1966).

1.2.4.4.1 Method of analysis (Weiß and Göker, 2011)

Since DNA computing power and sequencing technology have highly advanced in the past few decades, molecular phylogenetic analysis has taken an important role in addressing various biological questions in particular evolutionary relationships among species and their phylogenetic positions (Yang *et al*., 2012). The concept of phylogenetic analysis is to reconstruct possible evolutionary relationships by extrapolating backward from a dataset derived from existing organisms (Waikagul

and Thaekham, 2014). In order to choose the best phylogenetic tree given a set of aligned sequences, a number of distinct methodologies have so far been proposed which may be roughly divided into three main categories. First, distance-based methods use sequence similarity to obtain an estimate of the pairwise evolutionary distance between taxa, and use those distances to construct or choose a topology. Second, parsimony-based methods seek the topology that minimizes the number of inferred evolutionary changes. Third, model-based methods (i.e., maximum likelihood and Bayesian techniques) use an explicit model of character evolution to identify topologies that are highly probable given the data (Zwickl, 2006). These methods are briefly described as follows:

A)   Distance-based methods

Distance-based methods are based on the distances which usually are estimates of the number of substitution events per nucleotide occurred between pairs of sequences, indicating the degree of similarity existing between them (Weiβ and Göker, 2010). Such distance is commonly used to construct the distance matrix between individual pairs of taxa. Thus, this method inferred a phylogenetic tree that best reflects the distances contained in a given distance matrix. Since the distance based methods have a great advantage in performing the phylogenetic analyses of the massive dataset in a short amount of time, they are still widely used for many purposes (Pardi and Gascuel, 2016).

Typically, there are two types of algorithms mainly involved in the distance-based methods including clustering-based algorithms (Unweighted pair group method with arithmetic mean (UPGMA) and Neighbor-Joining (NJ)) and optimality-based algorithms (Fitch-Margoliash and minimum evolution, Peng, 2007). Since the distance-based methods with clustering-based algorithms were employed to infer the phylogeny of the fungi encountered in this study, only UPGMA and NJ are herein described as follows.

a)   Unweighted pair group method with arithmetic mean (UPGMA)

UPGMA, described by Sokal and Michener in 1958 is the simplest hierarchical clustering approach to reconstruct a phylogenetic tree from a distance matrix giving a rooted tree (Weiβ and Göker, 2010).

Initially, the two operational taxonomic unit (OTU) assumed as A and B with the smallest genetic distance are clustered together to form a new clade where the branch length is a half of the distance between taxa A and B. The next clustering step is performed by creating a new distance matrix which includes OTU AB instead of A and B. The distances contained in this new distance matrix are obtained by using the formula as follow:

$$d_{(AB)X} = \frac{1}{2}\left(d_{AX} + d_{BX}\right)$$

where $d_{(AB)X}$ denotes the distance between OTU AB and taxon X, d_{AX} denotes the distance between taxa A and X while d_{BX} denotes the distance between taxa B and X. Thus the new distance matrix consists of the distances between OTU AB and all terminal taxa. The two taxa with smallest distance from this new distance matrix are then clustered where the branch length is calculated by dividing that smallest distance by 2. This process is repeated multiple times until only two OTUs are left. Since UPGMA assumes that the rate of evolution is constant over time and for all phylogenetic lineages (unweight), this method is currently rarely used.

b)   **Neighbor-Joining (NJ)**

For over a couple of decades, Neighbor-Joining method (NJ) has been introduced by Saitou and Nei (1987) for reconstructing phylogenetic trees from evolutionary distance data. This method is based on the principle of minimum evolution to find pairs of OTUs (neighbors) that minimize the total branch length at each stage of clustering of OTUs starting with a star-like tree. Since several methods are time consuming, the NJ has become a method of choice for large sets of taxa in which phylogenies with thousands of species or even more can be quickly inferred (Saitou and Nei, 1987; Weiβ and Göker, 2010). According to Saitou and Nei (1987), the phylogenetic tree reconstruction starts with an unrooted, bifurcating tree (star-like tree) which is produced under the assumption that there is no clustering of OTUs. The first topology is basically defined by successively joining a pair of neighbors (a pair of OTUs connected through a single interior node in the star-like tree) and producing a new pair of neighbors where the branch length is minimize based on the algorithm. The process is then iterated until a single node remains and the tree with unique final topologies is eventually generated (Saitou and Nei, 1987; Weiβ and Göker, 2010).

Since the NJ was published in 1987, this method has been modified for a multiple of times to reduce the running time as well as increase its capacity and accuracy. In 1997, the improved version of the NJ algorithm, BIONJ has been proposed and proven to be better than ordinary NJ in recovering the true topology with higher accuracy when the substitution rates are higher and vary among lineages (Gascuel, 1997). A few years later, Bruno *et al*. (2000) improved BIONJ by replacing the minimum-evolution criterion with a likelihood-based criterion resulting in even higher accuracy of the method. Desper and Gascuel (2002 and 2004) introduced a new balanced minimum evolution (BME) principle based on the balance of weighting scheme and also stated that their program FASTME implementing the BME principle has the great advantage of dramatically reducing the time consuming. A year after RapidNJ was proposed in order to speed up the search for pairs to be joined which can perform faster than the existing implementations such as QuickTree and QuickJoin (Simonsen *et al*., 2008), Wheeler (2009) introduced the new algorithm NINJA which can be applied to very large dataset containing 100,000 OTUs. So far many new as well as improved versions of algorithms related NJ have later been proposed such as FastJoin (Wang *et al*., 2012), Neighbor Joining Plus (NJ+, Płoński and Radomski, 2013), Fast NeighborNet (Porter, 2018), etc.

B)   Parsimony-based method

a)   Maximum parsimony

By the early 1970s the parsimony principle was already employed in botanical and zoological phylogenetics and systematics (Farris, 1970; Fitch, 1971). The aim of Maximum parsimony criterion is to seek the tree topology that requires the smallest number of substitution events to explain the observed alignment as shown in Figure 17 (Weiβ and Göker, 2011). Basically, only parsimony-informative sites are taken into account while all uninformative sites will be disregarded. Parsimony-uninformative sites include the constant sites where no change is required as well as any sites that all possible trees require an equal number of nucleotide changes. In Figure 18, the sites 5, 7 and 9 are informative while the remaining sites are not. The sites 1, 6 and 8 apparently are constant sites. On the other hand, all possible trees at sites 2, 3 and 4 need equal numbers of changes which are 1, 2 and 3 changes, respectively. These sites are thus considered to be uninformative. As long as

substitution rates do not vary too much between different branches, most parsimonious trees are good estimates of the phylogenetic relationship.
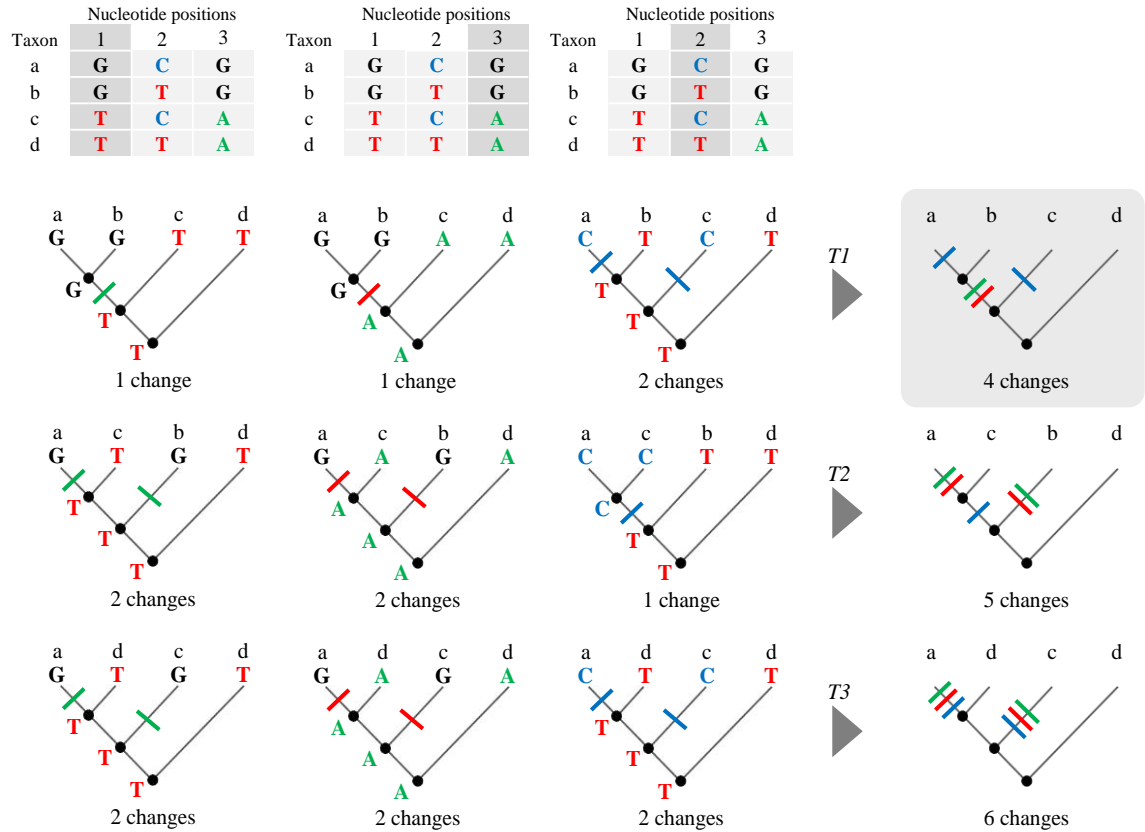
Figure 17 The maximum parsimony principle. For a given tree, the number of nucleotide changes along its branches is counted for each alignment column. Considering the topologies *T2* and *T3*, five and six nucleotide changes are needed, respectively while the topology *T1* requires only four changes. Thus, the topology *T1* is considered to be the most parsimonious tree for the given alignment.

Figure 18 An example of alignment dataset showing informative and uninformative sites

There are several approaches to evaluate how well a parsimonious tree explains the given dataset. Among them, the consistency index (CI) (Kluge and Farris, 1969) and the retention index (RI) (Farris, 1989) are widely employed in a parsimony framework. The CI indicates how a tree is consistent with a given dataset. The value of CI varies between 0–1. If CI is 1, it indicates that there is no homoplasy on the tree, thus a character perfectly fit to a tree and vice versa for the value of 0. Kluge and Farris (1969) defined the consistency index as

$$c = m/s$$

where m denotes the minimum number of change that the character (columns of an alignment) may need in any possible tree and s refers to the number of change in the character required parsimoniously by the considered tree, respectively.

According to Farris (1989), the amount of possible homoplasy occurred in a tree can also expressed as a retention index which is defined as

$$r = \frac{g-s}{g-m}$$

where g denotes the maximum number of change that the character may require on any possible tree. The retention index can be interpreted as same as the consistency index.

C)   Model-based methods

a)   Maximum Likelihood (ML)

Since the distance-and parsimony-based methods have tended to make an inconsistent estimate of the evolutionary tree when substitution rates in different lineages are high and sufficiently unequal, the model-based method has been considered to be more appropriate alternative to infer phylogeny of such cases (Felsenstein, 1981; Zwickl, 2006). The possibility of using the maximum likelihood criterion in molecular phylogenetic reconstruction was introduced and discussed by Edwards and Cavalli-Sforza in 1964 (Felsenstein, 2004; Weiβ and Göker, 2011). The likelihood computation was first applied to nucleotide sequences by Felsenstien (1981) who also provided a computer program which allows the phylogenetic inference from DNA (or RNA) sequences. Huelsenbeck and Crandall (1997) explained that the principle of maximum likelihood in phylogenetics is as simple as

the probability estimation of heads in a coin toss experiment and have stated that the main components of the approach consists of the data, a model describing the probability of the observed data and the maximum likelihood criterion. Basically, the likelihood estimate is calculated as the probability of the observed data under a given probabilistic model. Considering the coin flip experiment with the goal of estimating the probability of heads for the coin, the binomial distribution is assumed to be an appropriate probabilistic model of observing heads out of the coin. The likelihood is then computationally calculated using the formula for the binomial distribution in order to find the maximum likelihood estimate of the probability of the observed data. This concept can be simply applied to phylogenetics in which the observed data and probabilistic models are defined as sequence alignments and nucleotide substitution models, respectively. The maximum likelihood estimate of phylogeny is the tree with the greatest likelihood.

Weiß and Göker (2011) has pointed out that if the correct model of DNA substitution is used, then the probability of inferring the correct tree increases to infinity. Thus, the use of nucleotide substitution model has become more important for DNA-based phylogeny of organisms (Posada and Crandall, 2001). The DNA substitution models are considered as models of evolution which are used in phylogenetic analyses in order to describe the different probabilities of change from one nucleotide to another along the branches of a phylogenetic tree (Posoda and Crandall, 2001). The simplest model of DNA substitution is the Jukes Cantor (JC69) model which assumes that the rate of change from one nucleotide to another is the same for all possible changes (Jukes and Cantor, 1969). A number of nucleotide substitution models has later been developed such as F81 (Felsenstein, 1981), K80 (Kimura, 1980), HKY (Hasegawa *et al*., 1985), T92 (Tamura, 1992), TrN (Tamura and Nei, 1993), TPM (3-parameter model = K81, Kimura, 1981), TIM (transitional model, Posada, 2003), TVM (transversional model, Posada 2003), SYM (Zharkikh, 1994) and GTR (Tavaré, 1986). Any of these models can include invariable sites (+I), rate variation among site (+G) or both (+I+G). Since the use of a particular substitution model may change the outcome of the phylogenetic analysis, there have been many attempts to provide an effective tool to seek the best-fit model for a particular dataset. The model selection can be performed through statistical testing in

which different programs such as jModelTest (Posada, 2008), KAKUSAN (Tanabe, 2007) are readily available for all users. Among them, the jModelTest has so far been the most widely used (Santorum *et al*., 2014). Since the ModelTest was first proposed by Posada and Crandall (2001), it has been developed a lot over ten years which is currently well-known as jModelTest (Posada, 2009). The new version of the program, jModelTest 2 was introduced some years later where the set of candidate models has been expanded from 88 to 1,624 as well as another two heuristics have been implemented (Darriba *et al*., 2012). To avoid any configuration or execution issues and to dramatically reduce the time consumed of the analysis, the web server of the jModelTest 2 has been released and currently available (Santorum *et al*., 2014).

In the current study, the jModelTest was used to find the nucleotide substitution model that the most suits our dataset, its principle is herein briefly explained (for more details, see Posada and Crandall (2001) and Posada (2003 and 2009). The likelihood scores for the different models to a given dataset are initially computed on Phyml (Guindon and Gascuel, 2003) generated the base tree for individual model. These likelihood scores are used to select the best-fit model of nucleotide substitutions where the candidate models are ranked based on one of the three different methods including Akaike Information Criterion (AIC, Akaike, 1987), Bayesian Information Criterion (BIC, Schwarz, 1987) and a performance-based approach based on decision theory (DT, Minin *et al*., 2003). The smaller either the AIC or BIC, the better the fit of the model to the specific data (Posada and Crandall, 2001). The advantage of these two criteria is that they are capable of comparing both nested and nonnested models; nested model is the model that its parameters are a subset of the parameters in another model. It is still unclear if one of those criteria may take priority over another. However, Kuha (2004) has stated that the model selection should be carried out on both AIC and BIC and the best-fit one is referred to a model that favors by both of them. For the DT method, it selects models on the basis of the performance measurement as the expected branch length error resulting in slightly more accurate branch length estimates than those obtained by the Hierarchical Likelihood Ratio Test (hLRTs). The instruction of how to use jModelTest is available at http://evomics.org/learning/phylogenetics/jmodeltest.

Currently, many programs enabling the phylogenetic inference of datasets of DNA/RNA sequences under maximum likelihood are available including those that are distributed free of charge such as GARLI (Zwickl, 2006; Bazinet *et al*., 2014), IQ-TREE (Nguyen *et al*., 2014), PhyML (Guindon and Gascuel, 2003), RAxML (Stamatakis, 2006 and 2014) , TREE-PUZZLE (Schmidt *et al*., 2002), etc.

b)   Bayesian inference

Bayesian inference of phylogeny is based on a quantity called the posterior probability of a phylogenetic tree (the higher the posterior probability, the higher possibility to get the correct tree) which can be obtained on the basis of the Bayes's theorem where the prior probability of a tree is incorporated with the likelihood of an observed data (Huelsenbeck *et al*., 2001). Even though, this posterior probability seems to be easily formulated, it is impossible to do this analytically. Since Markov Chain Monte Carlo (MCMC) algorithms have allowed approximating the posterior probability of a tree, it has revolutionized Bayesian inference and been introduced to phylogenetics for over a couple of decades (Li, 1996; Mau and Newton, 1997; Mau *et al*., 1999; Rannala and Yang, 1996; Yang and Rannala, 1997). The concept of this algorithm is to generates a sample from the posterior probability which can be used to estimate the mean, the standard deviation of the posterior or even the whole posterior distribution instead of calculate the posterior distribution (Nascimento *et al*., 2017). Basically, MCMC generates a sequence of phylogenetic trees called a *Markov chain* from a particular given DNA/RNA sequence alignment using a stochastic mechanism under an assumed substitution model (Huelsenbeck *et al*., 2001; Huelsenbeck and Ronquist, 2001; Weiβ and Göker, 2011). The MCMC includes a few main steps as follows. A new state (a tree) of the xuchain is proposed using a stochastic mechanism. This state is subsequently either accepted or rejected based on the probability calculated with the Metropolis-Hastings algorithm (Huelsenbeck *et al*., 2001; Huelsenbeck and Ronquist, 2001). If the new state is accepted, the state of the chain is then updated. Otherwise the chain remains in the old state. This process of proposing and accepting/rejecting new state is repeated many thousands or millions of times. If the Markov chain is constructed properly, the proportion of the time any single tree is visited during the course of the chain is a valid approximation of its posterior probability which is defined as the MCMC branch support values. The

Bayesian MCMC analyses have become more popular and widely used because its ability to quickly infer phylogeny from large datasets. Recently, Nascimento *et al.* (2017) has provided a simple guide of how to use Bayesian MCMC in phylogenetic analyses as well as a list of Bayesian programs including BEAST (Bouckaert *et al.*, 2014; Drummond and Rambaut, 2007), MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist *et al.*, 2012), etc.

1.2.4.4.2   Evaluation of phylogenetic trees (Lesk, 2005)

Bootstrapping and jackknifing are statistical model testings that commonly used to evaluate and distinguish the reliability of estimated phylogenetic tree.

a)   Bootstrapping

This technique, was described by Felsenstein in 1985, is a well-known and common computational approach to phylogenetic tree. Bootstrapping is one of the most popular resampling methods used to assess the reliability of branches. The bootstrap alignment and bootstrap tree reconstruction are repeated a large number of time, and the resulting are stored. The bootstrap value that contains bipartition of the taxa in the tree is represented as percentages. A high value indicates well supported.

b)   Jackknifing

Jackknifing is a resampling method similar to bootstrapping analysis. It calculates by randomly deleting a certain percentage of columns from the original dataset. Typically 50% of the data columns are deleted (delete-half jackknife) and models deleting different percentages of columns have been proposed. The percentage that derived from phylogenetic tree which computes from jackknifing alignments is branch support values.

1.2.4.4.3   Phylogenetic tree reconstruction software

A number of available of phylogenetic tree construction softwares are available. Some of most widely used program such as MEGA (Kumar *et al.*, 2016), Clustal W and Clustal X (Larkin *et al.*, 2007), TREE-PUZZLE (Schmidt *et al.*, 2002) and PAUP (Swofford, 2002).

1.3   Objectives of this study

1. To isolate marine-derived fungi from marine organisms and to study their morphological characteristics.

2. To determine antimicrobial activity of extracts obtained from selected marine-derived fungi against human pathogens.

3. To identify potential antimicrobial producing fungi by molecular methods.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Collecting sites and marine organisms

Seventeen marine organisms including nine ascidians, two sponges, two bryozoans and each one of sea cucumber, coral, sea fan and alga (Figures 19 and 20) were collected near Phuket Coastal Fisheries Research and Development Center, Phuket province. There is no specific permission required for this location.
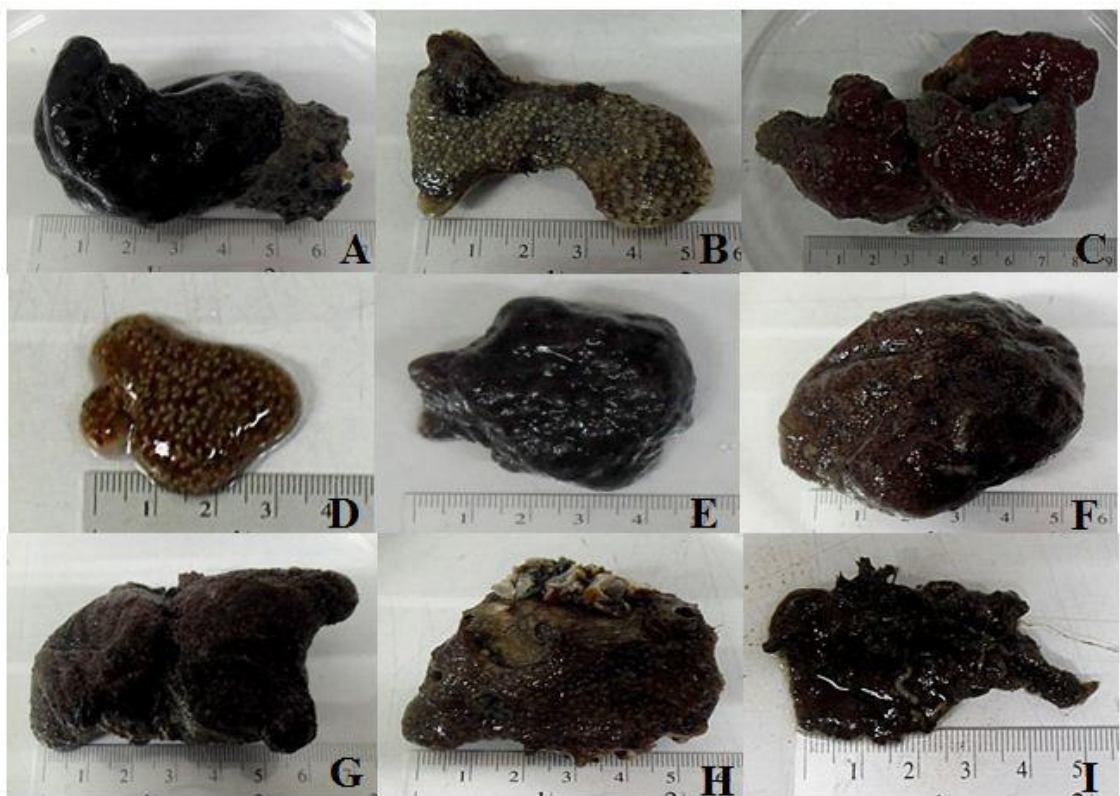
Figure 19 Marine ascidians collected for fungal isolation. (A) *Phallusia nigra*, (B) Family Botryllidae, (C) Family Pyuridae, (D) Family Didemnidae, and (E-I) Unidentified ascidians.
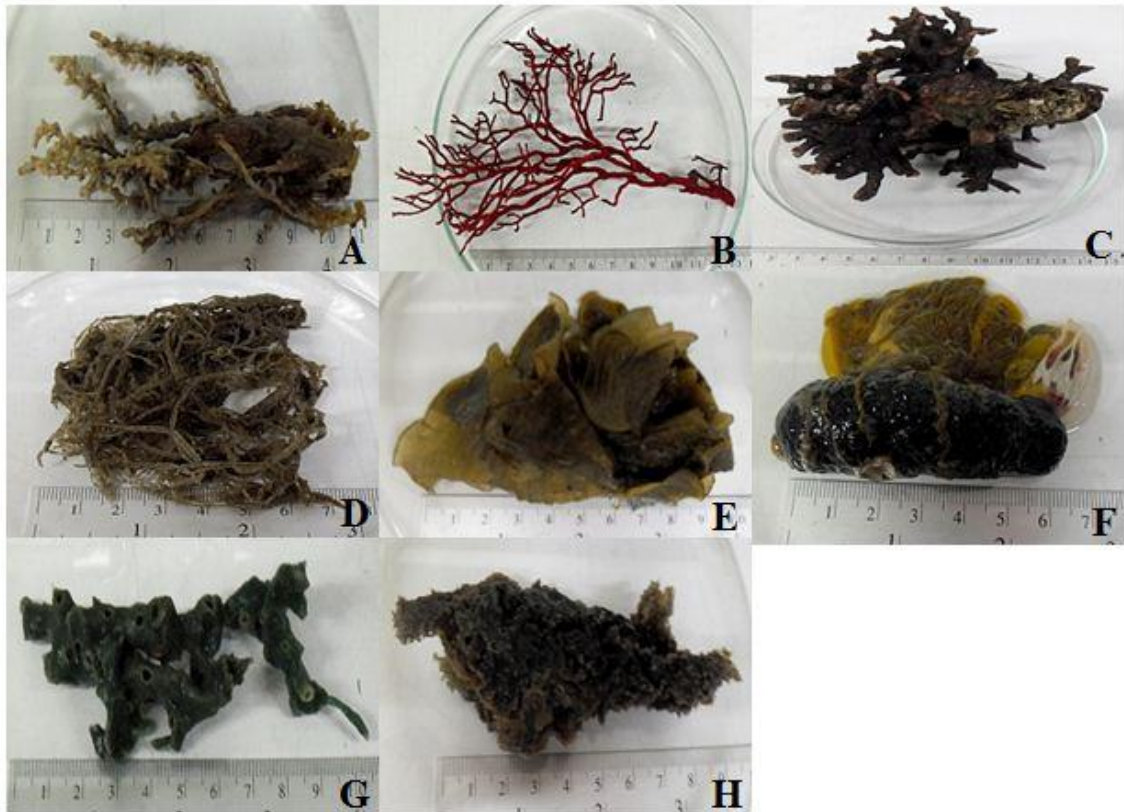
Figure 20 Marine organisms collected for fungal isolation. (A) Coral (*Carijoa riisei*), (B) Sea fan (*Menella* sp.), (C) Marine bryozoan *Schizoporella* sp., (D) *Amathia verticillatum,* (E) Brown algae (*Padina* sp.), (F) Unidentified sea cucumber and (G-H) unidentified sponge

2.1.2 Test microorganisms

2.1.2.1 Bacteria

- *Staphylococcus aureus* ATCC25923 (SA)
- Methicillin-resistant *Staphylococcus aureus* SK1 (MRSA SK1) clinical isolate obtained from the Pathology Department, Faculty of Medicine, Prince of Songkla University
- *Escherichia coli* ATCC25922 (EC)
- *Pseudomonas aeruginosa* ATCC27853 (PA)

- Multidrug-resistant *Acinetobacter baumannii* NPRC AB005 clinical isolate (AB005) obtained from the Natural Product Research Center of Excellence, Faculty of Science, Prince of Songkla University
- *Chromobacterium violaceum* DMST21761

2.1.2.2 Yeasts

- *Candida albicans* ATCC90028 (CA28)
- *Cryptococcus neoformans* ATCC90112 (CN12)

2.1.2.3 Filamentous fungi

- *Microsporum gypseum* SH-MU4 (MG) clinical isolate obtained from the Microbiology Department, Faculty of Medicine Siriraj Hospital, Mahidol University
- *Talaromyces marneffei* PSU-SKH1 (TM) clinical isolate obtained from the Pathology Department, Faculty of Medicine, Prince of Songkla University

2.1.3 Chemicals
- Lacto phenol cotton blue
- 0.85% Normal saline solution (NSS)
- Ethanol (commercial grade)
- Glycerol   (Merck)
- McFarland Standard   (Appendix)
- Dimethyl sulfoxide (DMSO)   (Fisher Chemical)
- D-glucose   (VWR Chemicals)
- Phosphate buffer saline (PBS) pH 7   (Appendix)
- 1.8% resazurin   (Appendix)

2.1.4 Media
- Half strength Seawater Potato Dextrose   (Appendix)
  agar
- 70% Seawater Potato Dextrose agar   (Appendix)
  (SPDA)

- Granulated agar	(Difco)
- Nutrient agar	(Difco)
- Nutrient broth	(Difco)
- Mueller-Hinton broth (MHB)	(Difco)
- Potato dextrose broth (PDB)	(Appendix)
- Rose-bengal chloramphenicol agar	(Merck)
- Sabouraud dextrose agar (SDA)	(Difco)
- Sabouraud dextrose broth (SDB)	(Difco)

2.1.5 Antibiotics

- Amphotericin B	(Sigma-Aldrich)
- Colistin	(Atlantic Laboratories Co., Ltd.)
- Clotrimazole	(Public Pharmaceutical Lab. Co., Ltd.)
- Gentamicin	(Nida Pharma Inc.)
- Penosep®	(M&H Manufacturing Co., Ltd.)
- Vancomycin	(Alkem Laboratories Ltd.)

2.1.6 Chemicals used for molecular analytical methods

- Agarose	(Vivantis)
- Chloroform	
- Isoamyl alcohol	
- CTAB lysis buffer	(Appendix)
- 50mM $MgCl_2$	(Invitrogen)
- 10X PCR buffer	(Invitrogen)
- 10mM DNTPs mix	(Invitrogen)
- *Taq* DNA polymerase	(Thermo Scientific)
- Absolute ethanol	
- 6X loading dye	(Appendix)
- TAE buffer (Tris Acetate EDTA buffer, pH 8)	(Appendix)
- EDTA (Ethylenediaminetetraacetic acid)	(Appendix)
- Tris-HCl pH8	(Appendix)

- 0.5 M NaOH   (Appendix)
- 7.5 M Ammonium acetate
- 70% ethanol
- Sea sand   (Sigma-Aldrich)
- GenepHlowTM Gel/PCR kit   (Geneaid Biotech Ltd.)

2.1.7 Instruments

- Incubator   (Gallenkamp)
- Light microscope   (Olympus CX31)
- Stereozoom microscope   (Olympus SZ-PT)
- Centrifuge   (Eppendorf)
- Gel electrophoresis machine   (Bio-Rad)
- Hemacytometer
- Automatic pipette   (Eppendorf)
- Multichannel automatic pipette   (Eppendorf)
- Freezer
- Heat block   (Labnet)
- Lyophilizer   (Labcondo)

2.2 Methods

2.2.1 Collection of marine organisms

Seventeen marine organisms were collected near Phuket Coastal Fisheries Research and Development Center, Phuket. The marine organisms including ascidians, sea cucumber, sponges, coral, gorgonian sea fan, bryozoan and brown alga. Collected samples were put in plastic bags with seawater and kept in an icebox, then brought back to the laboratory for fungal isolation.

2.2.2 Isolation of marine-derived fungi

Samples including ascidians, sea cucumber, gorgonian, sponges and bryozoan were washed with sterile distilled water, cut into 1x1 cm^2, and then transferred onto 70% seawater half strength potato dextrose agar (SPDA)

supplemented with 50 mg/L Penosep® (penicillin and streptomycin). Plates were incubated at 25°C for 7-10 days. Purified colonies were obtained using a hyphal tip isolation method. For soft coral, soft bodied bryozoan and algal samples, fungi were isolated according to Zhang *et al*. (2009). Samples were washed twice with sterile seawater and gently shaken with sterile glass beads in a shaking incubator at 60 rpm, for 10 minutes. The samples were washed twice with sterile seawater to remove temporarily and loosely living microorganisms. After that, the samples were transferred and rubbed on the surface of Rose Bengal Chloramphenicol (RBC) agar plate to inoculate epiphytic fungi. Afterwards, epiphytic fungi were removed by immersing these samples into 75% ethanol and dried with sterile paper towels. Then the samples were cut into 1x1 cm^2 pieces and cultured onto RBC plates to inoculate endophytic fungi. Plates were incubated at 25°C for 7-10 days to observe the mycelial growth and a hyphal tip isolation method was used to obtain the purified fungal colonies. The isolated fungi were inoculated onto 70% SPDA slant and in 20% glycerol at -80° C.

2.2.3 Cultivation of marine-derived fungi in broth medium for chemical extraction

Marine-derived fungal isolates were selected based on their different colonial morphologies. Each isolate was grown on SPDA at 25°C for 3-5 days, then the edge of colony was cut into 0.5x0.5 cm^2 with a sterile scapel. Five mycelial plugs were inoculated into 250 ml potato dextrose broth (PDB) at 25°C for 3 weeks. After incubation, the culture broth was filtered to separate the culture filtrate and fungal mycelium for chemical extraction with organic solvents (Phongpaichit *et al*., 2006).

2.2.4 Chemical extraction

The culture filtrate was extracted two times with ethyl acetate (EtOAc) in 1:1 ratio in separating funnel. The ethyl acetate extract was concentrated using a rotary vacuum evaporator under pressure at 40-45°C to obtain broth ethyl acetate extract (BE). The fungal mycelium was extracted by immersion in methanol (MeOH) for 7 days. The aqueous methanol layer was concentrated under reduced pressure by a rotary evaporator and water was added. The mixture was extracted with hexane two

times to obtain cell hexane (CH) extract after evaporation to dryness. Then, the aqueous residue layer was extracted with EtOAc two times to obtain cell ethyl acetate extract (CE) after evaporation to dryness (Figure 21).

Marine –derived fungi cultured in PBD

for 3 weeks

Culture filtrate

Fungal mycelia

Soaked with MeOH for 7 days

Extracted with EtOAc 2 times

Extracted with hexane 2 times

Concentrated by evaporation under reduced pressure at 45°C

Hexane layer

Aqueous layer

Broth **E**thyl acetate extract (BE)

Concentrated by evaporation under reduced pressure at 45°C

Extracted with EtOAc

2 times

Cell **H**exane extract

(CH)

Concentrated by evaporation under reduced pressure at 45°C

Cell **E**thyl acetate extract (CE)

Figure 21 Chemical extraction of fungal secondary metabolites

2.2.5 Preliminary antimicrobial activity testing at concentration of 200 μg/ml

2.2.5.1 Extract preparation

Extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (10 mg/ml) and kept at -20°C.

2.2.5.2 Inoculum preparation

S. aureus, MRSA, *E. coli*, *P. aeruginosa* and *A. baumannii* were cultured on nutrient agar (NA) and incubated at 35°C for 18-24 h. *C. albicans* and *C. neoformans* were cultured on Sabouraud dextrose agar (SDA) and incubated at 35°C, 18-24 h. for *C. albicans* and 25°C, 48 h. for *C. neoformans*. Three to five single colonies of each isolate were added into nutrient broth (NB) for bacteria and Sabouraud dextrose broth (SDB) for yeasts. Then the inoculum were shaken at 150 rpm., 35°C for 3-5 h. After incubation, bacteria and yeast suspensions were adjusted to equal the turbidity of 0.5 and 2 McFarland standard (MF) with sterile normal saline solution (NSS), respectively.

Filamentous fungi were grown on SDA at 25°C until sporulation. Sterile NSS were added and shaken gently to obtain spore suspension. The spore concentration was adjusted to 8 x10^{3} conidia/ml using a hemacytometer.

2.2.5.3 Antimicrobial activity testing

Extracts were determined of their antimicrobial activity by colorimetric broth microdilution methods at concentration of 200 μg/ml according to a modification of CLSI M7-A9 (CLSI, 2012) and Sarker *et al.* (2007), CLSI M27-A3 (CLSI, 2008a) and CLSI M38-A2 (CLSI, 2008b). Each extract (10 mg/ml) was diluted with MHB and SDB to obtain concentration of 1 mg/ml for the tests with bacteria and yeasts, respectively. 30 μl of extract samples were transferred to 96 well microtiter plates which contained 20μl of media and 50 μl of inoculum suspension (10^{6} log$_{10}$ CFU/ml) was added. The final concentration of extract was 200 μg/ml. Microtiter plates were incubated at 35°C for 15 h for bacteria and *C. albicans* and 25°C for 40 h for *C. neoformans*. Then, 30 μl of resazurin solution (1.8%) was added

to each well. The microtitle plates were re-incubated at the same temperatures for 3-5 h for bacteria and *C. albicans* and 8 h for *C. neoformans*. Each test was performed in triplicated.

Filamentous fungi were tested in the same manner as yeast. Conidia of fungi were adjusted to $8x10^3$ conidia/ml to use as inoculum. The microtiter plates were incubated at 25°C for 7 days. The mycelial growth was observed under the stereozoom microscope. No mycelial growth was recorded as a positive result.

In control experiment, standard antimicrobial agents (Table 9) were used as positive inhibitory controls. Media and inoculum in 1:1 ratio were used as growth control. DMSO was used as solvent control and medium broth only was used to check the sterility of the medium

Table 9 Standard antimicrobial agents used in this study

Antimicrobial agents	Concentration	Microorganism
Vancomycin	10 µg/ml	*S. aureus* and MRSA
Gentamicin	10 µg/ml	*E.coli* and *P. aeruginosa*,
Colistin	30 µg/ml	*A. buamannii*
Amphotericin B	10 µg/ml	*C. albicans*, *C. neoformans* and *T. marneffei*
Clotrimazole	32 µg/ml	*M. gypseum*

Interpretation of result: A blue or purple color indicated that extract can inhibit the test microorganisms (positive result). A pink color indicated that the extract cannot inhibit the test microorganisms (negative result). For filamentous fungi, fungal growth was observed under a stereozoom microscope.

The active extracts were further tested for their minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) or minimum fungicidal concentrations (MFC) according to CLSI guidelines.

2.2.6 Determination of MIC, MBC and MFC

The MIC of extracts was determination by colorimetric broth microdilution assays as in 2.2.5.3. The active extracts were diluted in triplicates using serial dilution method ranged from 0.25-128 µg/ml. The lowest concentration of the extracts that can inhibit the test microorganism was recorded as MIC.

For MBC and MFC determination, 5 µl of solution from each well from MIC and higher concentrations were dropped on nutrient agar (NA) for bacteria and SDA for yeast and filamentous fungi. The lowest concentration that showed complete absence of growth on agar was recorded as MBC for bacteria or MFC for yeast and filamentous fungi.

The MIC values were divided into 3 levels

Strong activity	MIC $\leq$ 8 µg/ml
Moderate activity	MIC= 16-64 µg/ml
Weak activity	MIC= 128-200 µg/ml

2.2.7 Study on the possible mechanisms of action of the active fungal extracts by a scanning electron microscopy (SEM)

The extracts presenting strong to moderate antimicrobial activity against each test microorganism were selected to study the possible mechanism of action by SEM. The test microorganism was treated with extract at four times its MIC values for 18 h. The tested cells were further treated in 2.5% glutaraldehyde in phosphate buffer solution (PBS) for 2 h and washed with PBS. After that, they were fixed with 1% osmium tetroxide for 1 h and then washed with water. A series of ethanol concentration in distilled water (50%, 70%, 80%, 90% and 100%) was used to dehydrate sample. The samples were dried using a critical point dryer, gold coated and scanned under SEM at the Scientific Equipment Center, Prince of Songkla University.

2.2.8   Quorum sensing inhibition (modification of Chenia, 2013)

The anti-quorum sensing potential of fungal extracts was determined using the disk diffusion assay at a concentration of 100 μg/disk. Blank disks (6 mm in diameter) were impregnated with 10 μl of extracts (10 mg/ml stock solution) and allowed to dry in the laminar air flow cabinet. *C. violaceum* DMST21761 were grown on Luria-Bertani (LB) agar at 35°C for 24 h and adjusted to the turbidity of 0.5 McFarland standard. *C. violaceum* were inoculated by swabbing thoroughly over the surface of Mueller-Hinton (MH) agar plate, then fungal extract containing disk were placed on the surface of the inoculated plate. The plates were incubated at 35°C for 18 h. Disks containing DMSO (1%), cinnamaldehyde (64 μg) and gentamicin (10 μg) were used as negative, positive and drug controls, respectively. Zone of inhibition were measured using a vernier caliper. Translucent zones represent inhibition of growth, while opaque zones indicated inhibition of violacein production. The fungal extracts causing inhibition zone greater than 10.00 mm. were selected for further study at concentration lower than 100 μg/disk (50, 25, 12.5 μg/disk).

2.2.9. Efficacy of the antibiotic combinations

2.2.9.1 Checkerboard method (Lorian, 1996)

The synergistic effect of extracts and colistin against *A. baumannii* NPRC005 was performed using a broth microdilution checkerboard method. Results were calculated mathematically and expressed in terms of a fractional inhibitory concentration index (FICI). Colistin and active extract were diluted with MHB to obtain 4 times their final concentrations of 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC and 2 MIC of each extract. 25 μl of extract and colistin were transferred to microtiter plate containing 50 μl of approximately 10^6 CFU/ml of *A. baumannii*a NPRC005 (Figure 22) and incubated at 35°C for 18 h. One well with no antibiotic and extract was used as a growth control in each plate. Resazurin dye was used to interpret result as in the MIC assay. The MIC of fungal extract alone and colistin alone and in combination were determined as the well in the microtiter plate with the lowest concentration at which there was no color change.

Figure 22 Checkerboard diagram of fungal extracts and colistin combination

The FICI was calculated using the following formula

$$FICI = FIC_A + FIC_B = \frac{(A)}{MIC_A} + \frac{(B)}{MIC_B}$$

(A)   Concentration of colistin in combination

(B)   Concentration of fungal extract in combination

MIC_A MIC of colistin alone

MIC_B MIC of fungal extract alone

Interpretation of the checkerboard results

Synergistic, additive, indefferenct, and antagonistic activities were defined by FICI of ≤ 0.5, > 0.5-≤1, > 1-≤ 4 and > 4, respectively.

2.2.9.2 Time-kill assay (Lorian, 1996)

The combinations with the lowest FICI results obtained from the checkerboard method were selected for further time-kill assay. The eppendorf containing MHB with extract-colistin combination were inoculated with *A. baumannii* NPRC005 and incubated at 35°C. The aliquots were removed at 0, 1, 2,

4, 6, 8, 10, 12, 16 and 24 h. and checked for total viable counts on nutrient agar. The amount of viable bacteria (CFU/ml) was enumerated after 18 h of incubation at 35°C. Media and inoculum (1:1 ratio) was used as growth control and DMSO as solvent control. Time-kill curves among viable colony counts (CFU/ml) and times were plotted.

Interpretation of the results

Bactericidal activity was defined as ≥ 3 $\log_{10}$ CFU/ml reduction and bacteriostatic activity as < 3 $\log_{10}$ CFU/ml reduction as compared with the initial inoculum. Synergy was defined as a ≥ 2 $\log_{10}$ CFU/ml decrease in colony count, indifference as a <2 $\log_{10}$ CFU/ml increase or decrease in colony count, and antagonism as a ≥ 2 $\log_{10}$ CFU/ml increase in colony count after 6 or 24 h with the combination compared with the most active drug alone (Sopirala *et al*., 2010).

Regrowth was defined as a >3 $\log_{10}$ CFU/ml decrease in colony count and a subsequent >2 $\log_{10}$ CFU/ml increase in colony count at 24 h (Bremmer *et al.,* 2016).

2.2.10 Identification of marine-derived fungi

Marine-derived fungi presenting antimicrobial activity were selected for identification using morphological and molecular characteristics.

2.2.10.1 Morphological identification

The active marine-derived fungi were cultured on SPDA at 25°C for 7 days. The macro-morphological characters were observed and recorded. Slide culture techniques were used to examine micro-morphological characters of fungi under a light microscopy. The fungal samples were stained with lactophenol cotton blue. Microscopic characters used for identification included sporulation, conidia shape, type of mycelia and other important characters according to keys (Domsch *et al*., 1993).

2.2.10.2 Molecular identification

2.2.10.2.1 Fungal growth for DNA extraction

Selected marine-derived fungi were cultivated in 50 ml PDB at 25°C, 200 rpm. for 3 days. The fermentation broth was filtered through sterile gauze. The fungal mycelia were washed with sterile distilled water ($\sim$ 60°C) and placed on a sterile paper towel to absorb water, then kept in microcentrifuge tube for further lyophilization. The lyophilized mycelia were stored at -20°C until use.

2.2.10.2.2 DNA extraction

a)   Quick and Dirty method (Wang *et al*., 1993)

The lyophilized mycelia were crushed with sterile sea sand and 0.5M NaOH ($\sim$500 µl) in a microcentrifuge tube. The microcentrifuge tube was centrifuged at 12,000 rpm., 25°C for 10 minutes. The supernatant was transferred to a new tube and diluted 1:20 with Tris-HCl pH8. The fungal DNA was kept at -20°C until use.

b)   CTAB method

The lyophilized mycelia were ground to a fine powder with sterile sea sand and 500 µl of CTAB lysis buffer. Then, the microcentifuge tube was incubated at 70°C for 30 minutes and centrifuged at 14,000 rpm., 25°C for 10 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed gently, and then centrifuged at 14000 rpm., 25°C for 10 minutes to separate the lipid and cellular debris into the organic phase (bottom) and DNA into an aqueous phase (top). The upper aqueous fraction was transferred to a new sterile tube and extracted two times with an equal volume of chloroform: isoamyl alcohol (24:1). The microtube was centrifuged at 14000 rpm., 25°C for 10 minutes to separate DNA and the upper layer phase was transferred to a new tube. After that a half volume of 7.5 M ammonium acetate and 2.5 times of the volume of cold absolute ethanol were added to the aqueous layer. Mixture was incubated at -20°C freezer for overnight and centrifuged at 14000 rpm., at 4°C for 10 minutes to precipitate DNA. The supernatant was discarded and the

pellet DNA at the bottom of the tube was washed in 500 µl of cold 75% ethanol. Then, the pellet DNA was allowed to air-dry at room temperature. The pellet DNA was resuspended with 50 µl of TAE buffer and stored at -20°C.

2.2.10.3 DNA amplification

The internal transcribted spacer (ITS) regions of the rDNA were amplified using universal primers, ITS4 and ITS5 (Table 10). The 50 µl of PCR mixture (Table 11) were performed using a Bio-Rad Thermal cycler. The PCR profiles are shown as follows:

The PCR profile for DNA amplication using ITS5/ITS4

Initial denaturation	94°C	2	min
Denaturation	94°C	1	min
Annealing	55°C	1	min	34 cycles
Extension	72°C	2	min
Final extension	72°C	2	min

Table 10 Universal and fungal specific primers used for DNA amplification and DNA sequencing

rDNA region	Primer	Direction	Sequence 5'-3'	Reference
ITS	ITS4	Reverse	TCCTCCGCTTATTGATATGC	White *et al*, 1990
	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	

Table 11 PCR mixture for DNA amplification

PCR mixtures	Stock concentration	Volume (µl)	Final concentration of 50 µl
Nanopure water		35.8	
PCR buffer	10X	5	1X
$MgCl_2$	25 mM	4	2.0 mM
dNTPs	10 mM	1	0.2 mM
Forward primer	10 µg/ml	1	0.2 µg/ml
Reverse primer	10 µg/ml	1	0.2µg/ml
Taq polymerase	5 units/µl	0.2	0.02 units/µl
DNA template	50-250ng	2	2-10 ng

2.2.10.4 DNA product determination

The quantity and quality of DNA product were examined in 1% agarose gel electrophoresis after stained with ethidium bromide. The gel was monitored under UV light transilluminator and photographed with gel documentation.

2.2.10.5 PCR product purification and DNA sequencing

PCR products were purified with GenepHlow^{TM} Gel/PCR kit. PCR products (90 µl) were mixed with 450 µl of Gel/PCR buffer. The PCR products were transferred to the DFH column in collection tube and centrifuged at 8,000 rpm. 30 seconds. The supernatant was discarded. Wash buffer (600 µl) was added to wash DNA and then centrifuged at 9,000 rpm for 1 minute. The supernatant was discarded. The DFH column was transferred to new 1.5 ml microcentrifuge tube. Elution buffer (30 µl) was added to elute DNA. The column was left for 10 minutes at room temperature then centrifuged at 13,500 rpm.1 minute to obtain the purified PCR product. The purified PCR products were sent to the Macrogen Inc. Korea for sequencing.

2.2.10.6 DNA sequences alignment and phylogenetic tree reconstruction

The consensus of forward and reverse sequences of each isolate was assembled using BioEdit V7.1.7 (Hall, 2012). Then sequences were compared against NCBI database by BLAST (Basic Local Alignment Search Tool) program on NCBI website. The selected closely related fungal species sequences obtained from NCBI were aligned using ClustalW (Thompson *et al*., 1994). Maximum parsimony analysis was used to create phylogenetic tree by PAUP* V4.0b10 (Swofford, 2002). The heuristic tree search with default options method was used for constructing parsimonious tree. Each clade of an observed tree was estimated confidence value using bootstrapping analysis.

CHAPTER 3

RESULTS

3.1 Number of isolated marine-derived fungi and their morphological identification

Marine organisms were collected from Phuket province in July, 2016 comprising ascidians (9 samples), sponges (2), bryozoans (2), a coral, a sea fan, a sea cucumber and an alga. A total of 547 fungal isolates were obtained from marine samples. Two hundred and eighty-two isolates were isolated from 9 ascidians and 15, 69, 29, 44, 89, and 19 isolates were obtained from sea cucumber, sponges, coral, sea fan, bryozoans and alga , respectively (Table 12).

One hundred and twenty-three fungal isolates were selected based on different colony morphological characteristics for chemical extraction. They were identified by their morphologies into 10 genera including *Aspergillus, Trichoderma*, *Penicillium*, *Cladosporium*, *Fusarium, Acremonium, Helminthosporium*, *Pestalotiopsis*, *Curvularia* and *Syncephalastrum*, and a group of unidentified fungi (Table 13).

Table 12 Number of isolated marine-derived fungi from marine organisms

Common name of marine organism	Scientific name	No. of isolated marine-derived fungi	Average no.
Ascidian	*Phallusia nigra*	43	
	Family Botryllidae	11	
	Family Pyuridae	43	
	Family Didemnidae	19	
	Unidentified ascidian #1	52	31.33
	Unidentified ascidian #2	26	
	Unidentified ascidian #3	44	
	Unidentified ascidian #4	26	

Table 12 (Cont.) Number of isolated marine-derived fungi from marine organisms

Common name of marine organism	Scientific name	No. of isolated marine-derived fungi	Average no.
	Unidentified ascidian #5	18	
Brown algae	*Padina* sp.	19	19
Bryozoan	*Schizoporella* sp.	25	44.5
	Amathia verticillatum	64	
Coral	*Carijoa riisei*	29	29
Sea cucumber	Unidentified sea cucumber	15	15
Sea fan	*Menella* sp.	44	44
sponge	Unidentified sponge #1	34	34.5
	Unidentified sponge #2	35	
Total		547	31.05

Table 13 Selected marine-derived fungi for chemical extraction

Number	Fungal genera	No. of selected fungi (%)
1	*Aspergillus*	30 (24.39)
2	*Trichoderma*	30 (24.39)
3	*Penicillium*	21 (17.07)
4	*Cladosporium*	5 (4.07)
5	*Fusarium*	3 (2.44)
6	*Acremonium*	1 (0.81)
7	*Helminthosporium*	1 (0.81)
8	*Pestalotiopsis*	1 (0.81)
9	*Curvularia*	1 (0.81)
10	*Syncephalastrum*	1 (0.81)
11	Unidentified fungi	29 (23.58)
Total		123 (100)

3.2 Preliminary antimicrobial screening

Three hundred and sixty-nine extracts from 123 isolates of selected marine-derived fungi were preliminary tested for their antimicrobial activity against nine human pathogens at a concentration of 200 µg/ml using colorimetric broth microdilution methods. Results are shown in Table 14. Two hundred and nineteen extracts (59.35%) presented inhibitory activity against at least one test strain. Most of active extracts were effective against *C. neoformans* ATCC 90112 (35.77%), *S. aureus* ATCC 25923 (35.50%), MRSA SK-1 (27.10%), *M. gypseum* SH-MU4 (18.42%), *C. albicans* ATCC 90028 (10.02%), *T. marneffei* PSU-SKH1 (3.25%), *A. baumannii* NPRC AB005 (2.71%), *P. aeruginosa* ATCC 27853 (1.89%) and *E. coli* ATCC 25922 (1.08%), respectively.

One hundred and nine out of 123 (88.62%) marine-derived fungi had antimicrobial activity. The majorities of the isolates were active against *C. neoformans* and *S. aureus* (69.72%), follow by MRSA (55.05%), *M. gypseum* (42.20%), *C. albicans* (23.85%), *T. marneffei* (9.17%), *A. baumannii* (9.17%), *P. aeruginosa* (5.50%) and *E. coli* (3.67%), respectively.

Table 14 Number of active extracts and active fungi tested at a concentration of 200 µg/ml against each test strain

No. of extracts	Test microorganisms									Total
	Bacteria					Yeast		Filamentous fungi		
	SA	MRSA	EC	PA	AB005	CA	CN	MG	TM	
Active extracts (%) n = 369	131 (35.50)	100 (27.10)	4 (1.08)	7 (1.89)	10 (2.71)	37 (10.02)	132 (35.77)	68 (18.42)	12 (3.25)	219 (59.35)
Active fungi (%) n= 123	76 (69.72)	60 (55.05)	4 (3.67)	6 (5.50)	10 (9.17)	26 (23.85)	76 (69.72)	46 (42.20)	10 (9.17)	109 (88.62)

SA = *Staphylococcus aureus* ATCC 25923   MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922   PA = *Pseudomonas aeruginosa* ATCC 27853

AB005= Multidrug-resistant *Acinetobacter baumannii* NPRC AB005   CA = *Candida albicans* ATCC 90028

CN = *Cryptococcus neoformans* ATCC 90112   MG = *Microsporum gypseum* SH-MU4

TM = *Talaromyces marneffei* PSU-SKH1

Three types of extracts, broth ethyl acetate (BE), cell ethyl acetate (CE) and cell hexane (CH) extracts were obtained from each fungus. The highest number of active extracts was the CH (37.33%), followed by BE (34.33%) and CE (28.34%) (Figure 23).

BE = broth ethyl acetate extracts; CE = cell ethyl acetate extracts; CH = cell hexane extracts

Figure 23 Types of active fungal extracts

Figure 24 shows the percentages of fungal extracts against each test strain. All types of extracts could inhibit all test strains except the CE extract had no activity against *E. coli*.
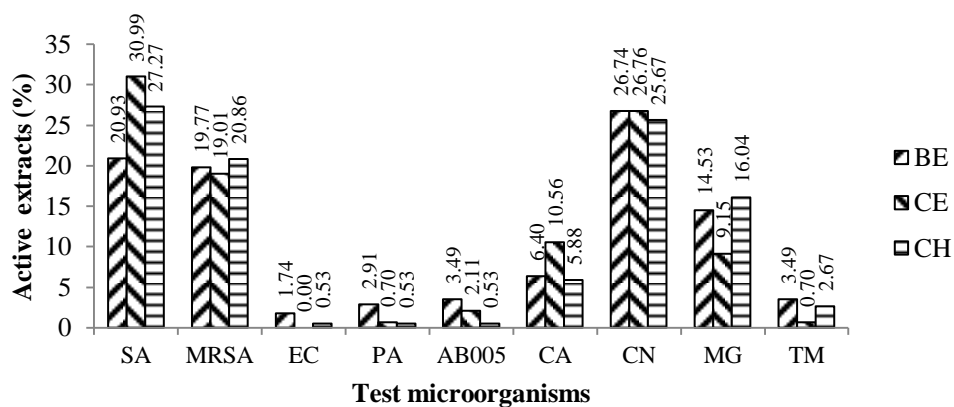
SA = *Staphylococcus aureus* ATCC 25923

EC = *Escherichia coli* ATCC 25922

AB005= Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

CN = *Cryptococcus neoformans* ATCC 90112

TM = *Talaromyces marneffei* PSU-SKH1

MRSA = Methicillin-resistant *S. aureus* SK1

PA = *Pseudomonas aeruginosa* ATCC 27853

CA = *Candida albicans* ATCC 90028

MG = *Microsporum gypseum* SH-MU4

Figure 24 Percentage of each type of active extracts against each test microorganism

Most of the extracts (43.84%) showed inhibitory activity against only one test strain. The rest inhibited 2, 3, 4, 5 and 6 test microorganisms with 21.00%, 12.79%, 12.79%, 5.94% and 3.65%, respectively (Figure 25).
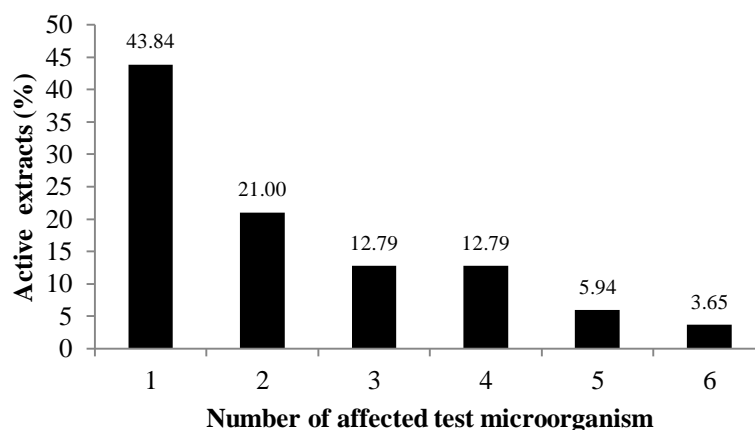
Figure 25 Number of susceptible test microorganisms inhibited by fungal extracts at a concentration of 200 µg/ml

3.3 Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) or minimum fungicidal concentrations (MFC)

Active extracts from the preliminary test were further determined for their MIC and MBC or MFC values using colorimetric broth microdilution methods at concentrations of 0.25-128 µg/ml. Results are shown in Table 15. The MIC/MBC or MFC ranged from 2-200/16->200 µg/ml. However, the range of MIC/MBC or MFC values were varied among each test microorganism.

Antimicrobial activity of the active extracts could be classified according to their MIC values as strong activity (MIC ≤8 µg/ml), moderate activity (MIC = 16-64 µg/ml) and weak activity (MIC = 128-200 µg/ml). In this study, most active extracts presented weak activity (62.6-90.91%), followed by moderate (8.33-35.88%) and only 0.76-2.70% had strong activity (Figure 26). The extracts with strong activity were AMF231CE and AMF231CH (against *S. aureus* with MIC 8 and 4 µg/ml, respectively), AMF192BE against *C. albicans* (MIC 8 µg/ml) and

AMF192CH that expressed the strongest activity against *C. neoformans* (MIC 2 µg/ml) (Table 16).

Table 15 MIC/MBC or MFC ranges of active extracts against each test microorganism

Test	MIC/MBC or MFC (µg/ml)								
	Bacteria					Yeast		Filamentous fungi	
	SA	MRSA	EC	PA	AB005	CA	CN	MG	TM
Active extracts	4-200/ 16->200	16-200/ 32->200	32-200/ 128->200	64-200/ 128->200	32-200/ 128->200	8-200/ 32->200	2-200/ 32->200	16-200/ 16->200	128-200/ 128->200
Vancomycin	0.5/0.5	0.5/0.5							
Gentamicin			0.5/1	0.5/1					
Colistin					16/16				
Amphotericin B						0.5/1	0.5/1		1/1
Clotrimazole								1/1	

SA = *Staphylococcus aureus* ATCC 25923   MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922   PA = *Pseudomonas aeruginosa* ATCC 27853

AB005= Multidrug-resistant *Acinetobacter baumannii* NPRC AB005   CA = *Candida albicans* ATCC 90028

CN = *Cryptococcus neoformans* ATCC 90112   MG = *Microsporum gypseum* SH-MU4

TM = *Talaromyces marneffei* PSU-SKH1

Table 16 Active extracts presenting strong (MIC ≤ 8 µg/ml) and moderate activity (MIC 16-64 µg/ml)

Active extracts	MIC/MBC or MFC (µg/ml.)								
	SA	MRSA	EC	PA	AB005	CA	CN	MG	TM
AMF3CH	200/>200	128/>200				128/200	32/200	32/128	200/>200
AMF7CH	64/>200	200/>200						128/128	
AMF18BE	64/>200	128/>200					200/>200	200/>200	
AMF18CE	16/128	128/128					200/>200		
AMF18CH	32/64	128/128					200/>200		
AMF21BE	64/>200	64/>200							
AMF46CH						200/200	64/200	64/64	128/128
AMF87BE	64/>200	128/128				200/>200	200/200	200/>200	
AMF89CE	64/64	128/128				200/>200	200/200		
AMF89CH	64/64	128/128					200/200		
AMF94CE	32/200								
AMF117CE	64/>200	128/>200				200/>200		128/>200	
AMF117CH	64/128	128/128					200/>200		
AMF127CE	200/>200						64/>200		
AMF127CH	200/>200						64/>200		
AMF131CE	32/>200	128/>200					200/>200		
AMF131CH	32/>200	128/128				200/>200	200/>200		
AMF144CH	64/128								
AMF169BE	64/128	64/128		200/>200	200/>200	200/>200		64/>200	
AMF169CE	64/>200	128/>200				128/>200	200/>200	128/>200	
AMF169CH	64/128	64/128				128/>200	200/>200		
AMF177CH	32/>200	128/128							

Table 16 (cont.) Active extracts presenting strong (MIC ≤ 8 µg/ml) and moderate activity (MIC 16-64 µg/ml)

Active extracts	MIC/MBC or MFC (µg/ml.)								
	SA	MRSA	EC	PA	AB005	CA	CN	MG	PM
AMF192BE						**8**/>200	16/200	128/128	200/>200
AMF192CE	200/>200	200/>200				200/>200	64/200	200/>200	
AMF192CH	200/200	64/128				16/64	**2**/32	32/64	200/>200
AMF198CE	64/128	64/64							
AMF198CH	16/32	16/32				128/>200	32/200	16/16	
AMF203BE	64/200	128/>200					200/>200		
AMF205BE	64/>200	128/>200					200/>200		
AMF205CE	64/>200	128/>200					200/>200		
AMF222BE	16/32	128/200					200/>200		128/128
AMF222CE	16/32	64/64			200/>200	200/>200	200/>200		
AMF229CH	64/64	200/>200							
AMF231BE	16/128	200/>200				200/>200		64/>200	
AMF231CE	**8**/16	200/>200				64/>200		16/128	
AMF231CH	**4**/16	200/>200				16/32		16/16	
AMF235CE	64/>200	128/128							
AMF238CE	200/>200	128/>200			32/>200	200/200	200/200	128/>200	
AMF273BE	32/128	128/>200					200/>200		
AMF273CE	32/64	128/128					200/>200	200/>200	
AMF273CH	128/200	128/128				200/200	200/200	64/>200	
AMF274BE	32/64	128/128				200/>200	64/>200	200/>200	200/200
AMF274CE	32/>200	128/128				32/>200	200/>200		200/200
AMF277BE	32/200	64/200	32/128	64/128	32/128		128/200		
AMF292BE	64/200	64/>200			200/>200		128/200	128/>200	200/>200
AMF347CH	64/>200	64/>200					128/>200	64/128	200/>200

Table 16 (cont.) Active extracts presenting strong (MIC ≤ 8 µg/ml) and moderate activity (MIC 16-64 µg/ml)

Active extracts	MIC/MBC or MFC (µg/ml.)								
	SA	MRSA	EC	PA	AB005	CA	CN	MG	PM
AMF360BE	32/64	200/>200				200/>200			
AMF360CE	16/32	200/>200							
AMF360CH	64/>200	200/>200						200/>200	
AMF366BE	64/>200	64/>200					200/>200		
AMF366CE	16/>200	32/>200							
AMF368CH	64/>200								
AMF409BE	16/32	64/128			200/>200		128/200		128/128
AMF409CE	32/200	200/>200					200/>200		
AMF413CH	128/>200	200/>200	200/>200					64/>200	
AMF420CE	128/>200	128/>200				16/32	128/200	64/64	
AMF420CH	128/>200	128/128				16/32	64/200	64/64	
AMF455CE	32/>200	200/>200					64/128		
AMF458CE	32/>200								

SA = *Staphylococcus aureus* ATCC 25923

EC = *Escherichia coli* ATCC 25922

AB005= Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

CN = *Cryptococcus neoformans* ATCC 90112
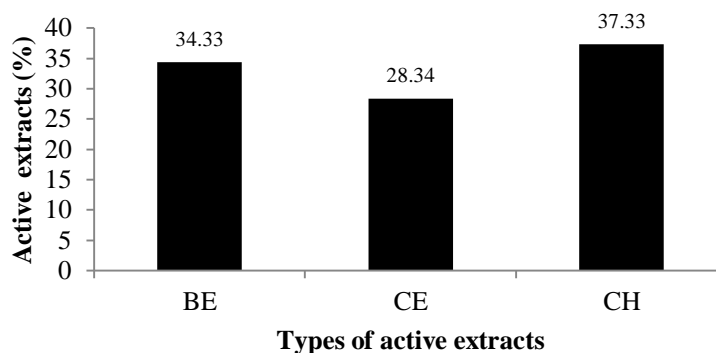
TM = *Talaromyces marneffei* PSU-SKH1

MRSA = Methicillin-resistant *S. aureus* SK1

PA = *Pseudomonas aeruginosa* ATCC 27853

CA = *Candida albicans* ATCC 90028

MG = *Microsporum gypseum* SH-MU4

Bold number = strong activity

NOTE: Results of all active isolates are shown in Table 1A in the eppendix
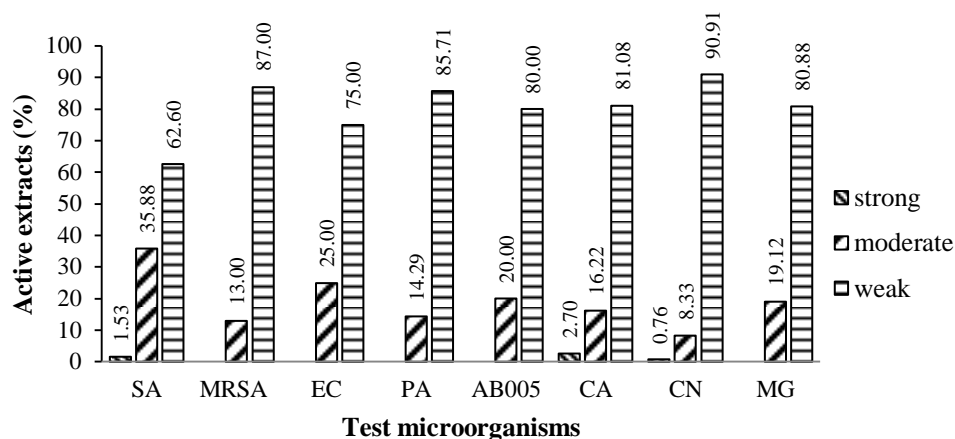
SA = *Staphylococcus aureus* ATCC 25923   MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922   PA = *Pseudomonas aeruginosa* ATCC 27853

AB005= Multidrug-resistant *Acinetobacter baumannii* NPRC AB005   CA = *Candida albicans* ATCC 90028

CN = *Cryptococcus neoformans* ATCC 90112   MG = *Microsporum gypseum* SH-MU4

Figure 26 Percentage of active extracts having strong (MIC<10 μg/ml), moderate (MIC 16-64 μg/ml) and weak (MIC 128-200 μg/ml) activity against each test microorganism

Antibacterial activity

Table 17 shows antimicrobial spectrum of active extracts from marine-derived fungi. One hundred and forty-three extracts (65.30%) exhibited antibacterial activity against at least one test strain of Gram-positive and Gram-negative bacteria with MIC/MBC in ranges of 4-200/16->200 µg/ml (Table 15). The potential extracts with strong activity were found against only Gram-positive bacteria, while moderate and weak activities were observed against Gram-negative bacteria (Figure 26) with MIC/MBC values ranging from 32-200/128->200 µg/ml (Table 15). AMF231CH displayed the greatest inhibitory activity against *S. aureus* ATCC 25923 (MIC/MBC 4/16 µg/ml). AMF198CH was the strongest effective extract against MRSA with MIC/MBC values of 16/32µg/ml. AMF277BE presented the broadest activity against all five tested bacteria with moderate activity. (Table 16)

Antifungal activity

One hundred and sixty-one extracts (73.52%) could inhibit yeasts and filamentous fungi with MIC/MFC values of 2-200/ 16->200µg/ml. The active extracts presented strong activity against the yeasts *C. neoformans* and *C. albicans* (MIC 2-8 µg/ml.) (Table 15). For filamentous fungi, the active extracts revealed only moderate and weak activities (MIC 16-200 µg/ml). AMF192CH showed the greatest activity against *C. neoformans* (MIC/MBC 2/32 µg/ml). AMF192BE showed the best anti-yeast activity against *C. albicans* and *C.neoformans* with MIC values of 8 and 16 µg/ml, respectively. AMF198CH, AMF231CE and AMF231CH were most effective against *M. gypseum* (MIC 16 µg/ml). *T. marneffei* was the least susceptible organism to these marine-derived fungal extracts. The best extracts against *T. marneffei* are AMF46CH, AMF222BE and AMF409 with MIC/MFC values of 128/128 µg/ml (Table 16).

In addition, the majority of the extracts (39.27%) had a broad spectrum of activity against both bacteria and fungi, 34.25% had only antifungal activity and 26.48% with only antibacterial activity. The results are shown in Table 16.

Table 17 Distribution of the antimicrobial spectrum of active extracts from marine derived fungi

% Active extracts	Activity	
	Antibacterial	Antifungal
26.48	←————————→	
34.25		←————————→
39.27	←——————————————————————→	

3.4 Study on possible mechanism of action of the active extracts by a scanning electron microscopy (SEM)

The active extracts with strong to moderate antimicrobial activity against each test microorganism were selected to study the possible mechanism of

action by SEM. The tested microorganisms were treated with extract at four times its MIC.

AMF231CH (MIC/MBC 4/16 µg/ml), AMF222BE (16/32) and AMF409BE (16/32) were tested against *S. aureus* ATCC25923 (SA) and the results are shown in Figure 27. Control cells treated with 1% DMSO exhibited normal round shape with smooth surface (Figure 27A), while broken and collapsed cells with cytoplasmic protrusion were observed in the cells treated with vancomycin (Figure 27B), AMF231CH (Figure 27C), AMF222BE (Figure 27D) and AMF409BE (Figure 27E).
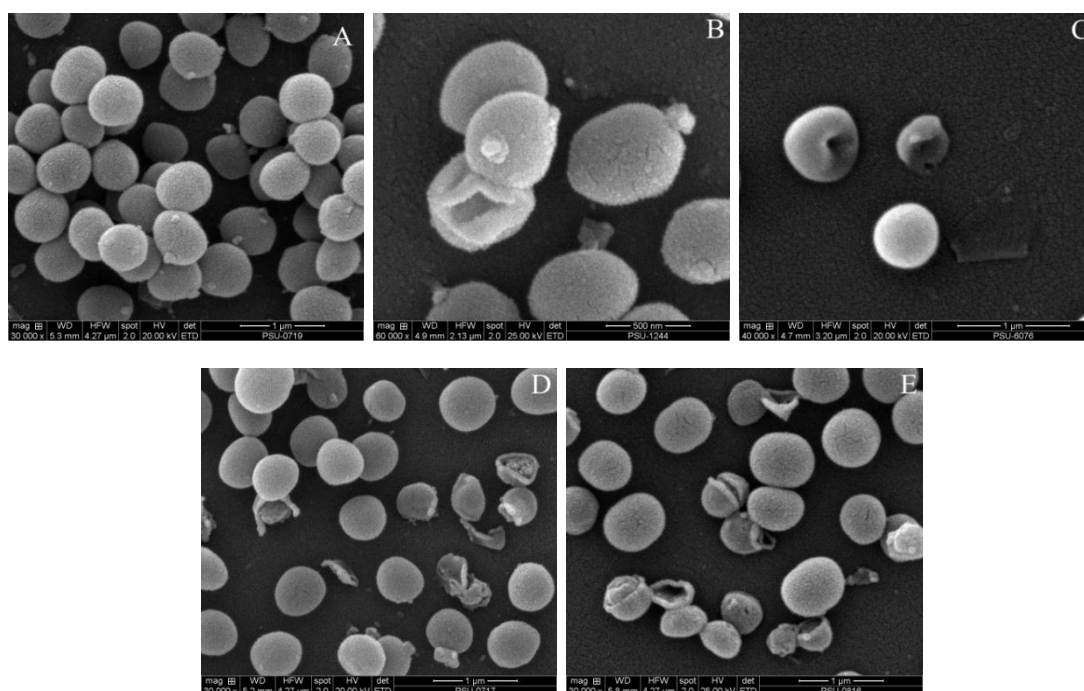
Figure 27 Scanning electron micrographs of *S. aureus* ATCC25923 (SA) after exposure with AMF231CH, AMF222BE and AMF409BE for 24 hours of incubation at 35°C

(A) SA + 1%DMSO   (B) SA+ 4MIC vancomycin   (C) SA+4MIC AMF231CH

(D) SA+ 4 MIC AMF222BE   (E) SA + 4MIC AMF409BE

AMF198CH was best active against both SA and MRSA with MIC/MBC values of 16/32 µg/ml. The SE micrographs of untreated cell showed intact cell with smooth cell surface as shown in Figure 28A (SA) and Figure 28D (MRSA). Figures 28C and 28F of the treated cells showed morphological

abnormalities with concave shape of SA and MRSA cells, respectively after exposure with 4MIC of AMF198CH as well as the cells treated with vancomycin (Figure 28B and Figure 28E).
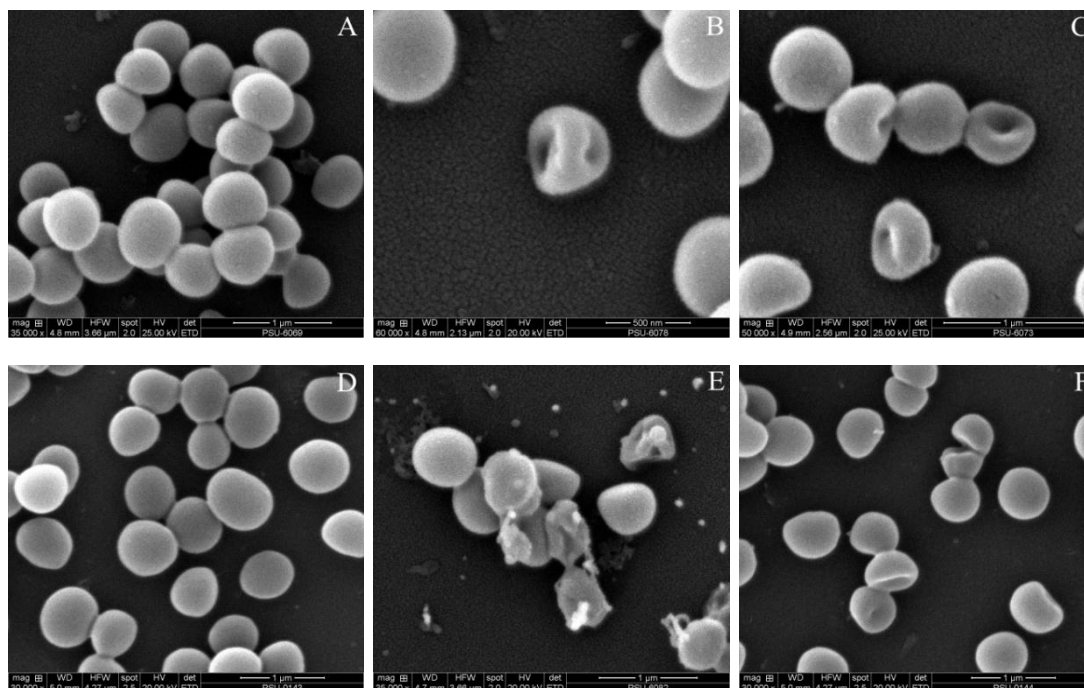
Figure 28 Scanning electron micrographs of *S.aureus* ATCC25923 (SA) and methicillin-resistant *S. aureus* (MRSA) SK1 after exposure with AMF198CH for 24 hours at 35°C

(A) SA + 1% DMSO   (B) SA+ 4MIC vancomycin   (C) SA+ 4MIC AMF198CH

(D) MRSA + 1% DMSO   (E) MRSA + 4MIC vancomycin   (F) MRSA + 4MIC AMF198CH

AMF277BE had the broadest inhibitory activity against all the tested bacteria. SEM images of all AMF277BE-treated cells exhibited deformed cells with open holes and deep craters or broken cells (Figures 29C, 29F, 29I and 29L), in particular *A. baumannii* cells showed serious deformation of cell shape (Figure 29O). *S. aureus* and MRSA treated with vancomycin showed lysed cell with opened pore and cytoplasmic protusion (Figures 29B and 29E) while *E. coli* and *P. aeruginosa* treated with gentamicin showed no morphological changes, except only rough surfaces and small protrusions (Figures 29H and 29K). For *A. baumannii*, colistin completely destroyed the cells leading to cell death. All control cells (1% DMSO) showed typical bacterial forms with smooth surfaces (Figures 29A, 29D, 29G, 29J and 29M).
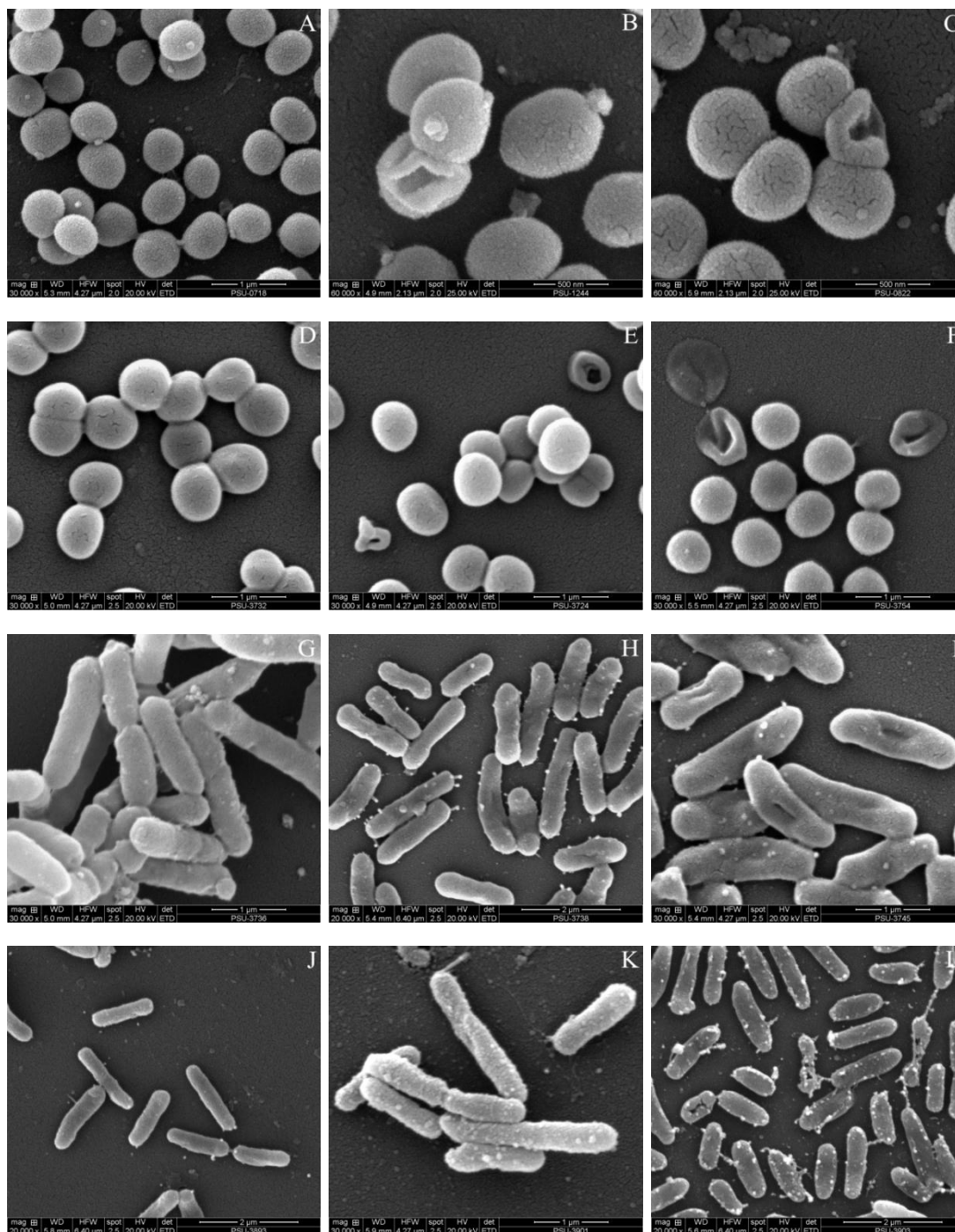
Figure 29 Scanning electron micrographs of *S. aureus* ATCC25923 (SA), MRSA SK1, *E. coli* ATCC25922 (EC), *P. aeruginosa* ATCC 27853 (PA) and *A. baumannii* NPRC005 (AB005) after exposure with AMF277BE for 24 hours at 35°C

(A) SA + 1% DMSO   (B) SA+ 4MIC vancomycin   (C) SA+ 4MIC AMF277BE
(D) MRSA + 1% DMSO   (E) MRSA+ 4MIC vancomycin   (F) MRSA+ 4MIC AMF277BE
(G) EC + 1% DMSO   (H) EC+ 4MIC gentamicin   (I) EC+ 4MIC AMF277BE
(J) PA + 1% DMSO   (K) PA+ 4MIC gentamicin   (L) PA+ 4MIC AMF277BE
(M) AB005 + 1% DMSO   (N) AB005+ 4MIC colistin   (O) AB005+ 4MIC AMF277BE
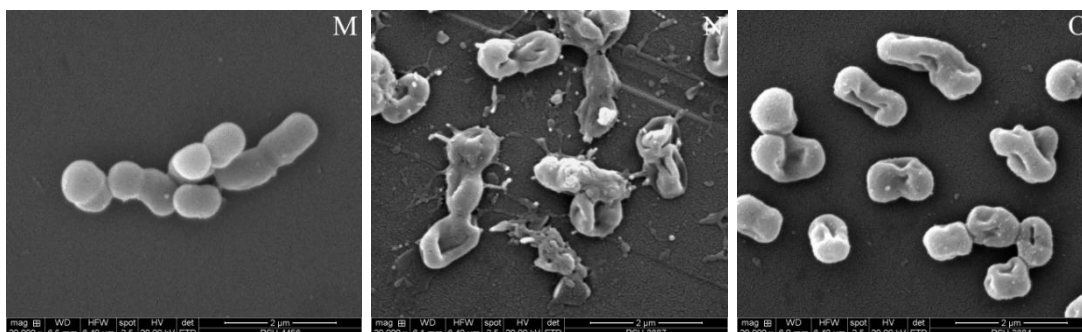
Figure 29 (Cont.) Scanning electron micrographs of *S. aureus* ATCC25923 (SA), MRSA SK1, *E. coli* ATCC25922 (EC), *P. aeruginosa* ATCC 27853 (PA) and *A. baumannii* NPRC005 (AB005) after exposure with AMF277BE for 24 hours at 35°C

(A) SA + 1% DMSO   (B) SA+ 4MIC vancomycin   (C) SA+ 4MIC AMF277BE

(D) MRSA + 1% DMSO   (E) MRSA+ 4MIC vancomycin   (F) MRSA+ 4MIC AMF277BE

(G) EC + 1% DMSO   (H) EC+ 4MIC gentamicin   (I) EC+ 4MIC AMF277BE

(J) PA + 1% DMSO   (K) PA+ 4MIC gentamicin   (L) PA+ 4MIC AMF277BE

(M) AB005 + 1% DMSO   (N) AB005+ 4MIC colistin   (O) AB005+ 4MIC AMF277BE

AMF192BE and AMF192CH gave the best results against *C. albicans* and *C. neoformans*. SE micrographs of *C. albicans* and *C. neoformans* are shown in Figure 30. *C. albicans* control cells (1% DMSO) had oval shaped with smooth surface (Figure 30A) and *C. neoformans* were round shaped (Figure 30E). Yeast cells treated with AMF192BE (Figure 30C and 30G) exhibited slightly morphological changes such as rough surface and cell shrinkage similar to *C. albicans* cells after exposure with amphotericin B (Figure 30B). In addition, cytoplasmic protrusions were also observed in daughter cells of *C. neoformans* treated with AMF192BE. *C. albicans* cell treated with AMF192CH (Figure 30D) showed deep evagination of the cell surface while *C. neoformans* cells were obviously flattened (Figure 30H). *C. neoformans* cells treated with amphotericin B showed broken cells with concave surface (Figure 30F).
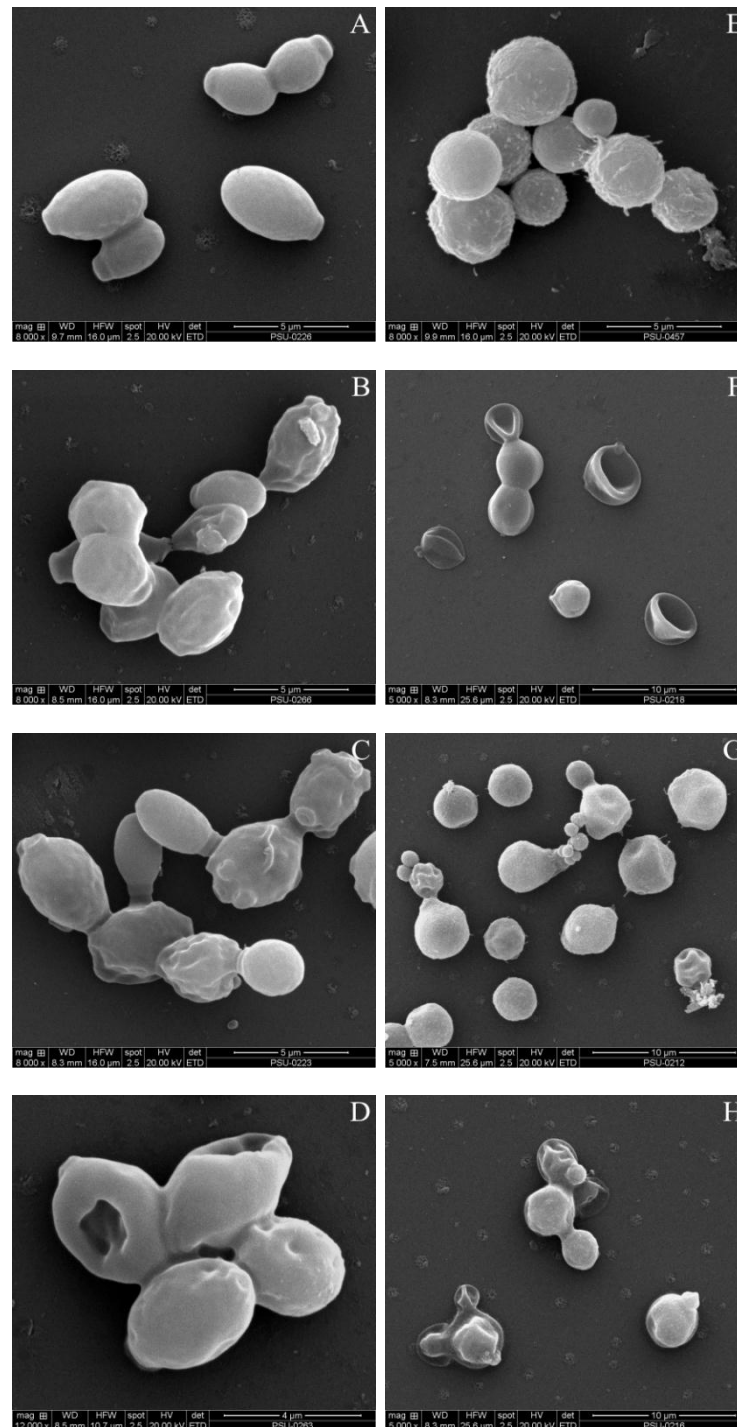
Figure 30 Scanning electron micrographs of *C. albicans* ATCC90028 (CA) and *C. neoformans* ATCC90112 (CN) after exposure with AMF192BE and AMF192CH for 24 hours at 25°C

(A) CA+ 1% DMSO (B) CA+ 4MIC Amphotericin B
(C) CA+ 4MIC AMF192BE (D) CA + 4MIC AMF192CH
(E) CN + 1% DMSO (F) CN+ 4MIC Amphotericin B
(G) CN+ 4MIC AMF192BE (H) CN+ 4MIC AMF192CH

In addition, AMF231CH was also tested with *M. gypseum*. SE micrographs of *M. gypseum* are shown in Figure 31. Only slightly morphological changes such as small holes were observed in cell treated with the extract (Figure 31C) whereas flatted and wrinkle mycelia were found in the clotrimazole treated cells (Figure 31B) as compared with the smooth surface control cells (1% DMSO) (Figure 31A).
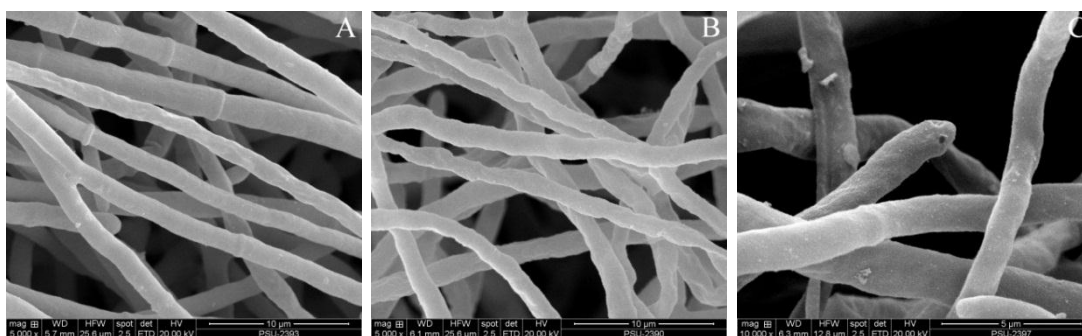
Figure 31 Scanning electron micrographs of *M. gypseum* SH-MU4 (MG) after exposure with AMF231CH for 3 days at 25°C

(A) MG+ 1% DMSO   (B) MG+ 4MIC clotrimazole   (C) MG+ 4MIC AMF231CH

3.5 Anti-quorum sensing activity

Three hundred and sixty-nine extracts from 123 selected marine-derived fungi were preliminary determined for their ability to inhibit quorum sensing using a disk diffusion assay at concentration of 100 µg/disk. Results are shown in Table 18 and Figure 32. Nine extracts (2.44%) obtained from nine fungal isolates presenting inhibitory activity with growth inhibition zones or violacein inhibition zones ranged from 6.35-19.85 mm. Six extracts showed only growth inhibition zone while three extracts including AMF177BE, AMF199BE and AMF231BE exhibited colorless pigment of violacein inhibition zones of 6.57, 7.80, and 6.50 mm, respectively.

Furthermore, extracts presenting growth inhibition zones from 10 mm. (AMF122BE, AMF210BE, AMF227BE and AMF480BE) were further tested at lower concentration (12.5-50 µg/disk). It was found that the growth inhibitory activity

was concentration-dependent as clearly seen with AMF277BE. One extract, AMF480BE exhibited violacein inhibition zone of 6.35 mm at concentration of 50 µg/disk. Therefore, four extracts (AMF177BE, AMF199BE, AMF231BE and AMF480BE) had anti-quorum sensing activity.

Table 18 Zone of inhibition of violacein production using *C. violaceum*

| Extracts | Concentration (µg/disk) | Inhibition zone | |
		Inhibition of growth (mm.)	Inhibition of violacein production (mm.)
AMF122BE	100	19.85	N
	50	8.50	N
	25	N	N
	12.5	N	N
AMF131BE	100	6.55	N
AMF177BE	100	N	6.57
AMF199BE	100	N	7.80
AMF210BE	100	10.01	N
	50	6.55	N
	25	N	N
	12.5	N	N
AMF231BE	100	N	6.50
AMF277BE	100	19.37	N
	50	16.3	N
	25	7.50	N
	12.5	6.50	N
AMF392BE	100	8.07	N
AMF480BE	100	11.10	N
	50	N	6.35
	25	N	N
	12.5	N	N
Cinnamaldehyde	64	8.34	1.11

BE = Broth ethyl acetate extract   CE = Cell ethyl acetate extract

CH = Cell hexane extract   N = No inhibition zone
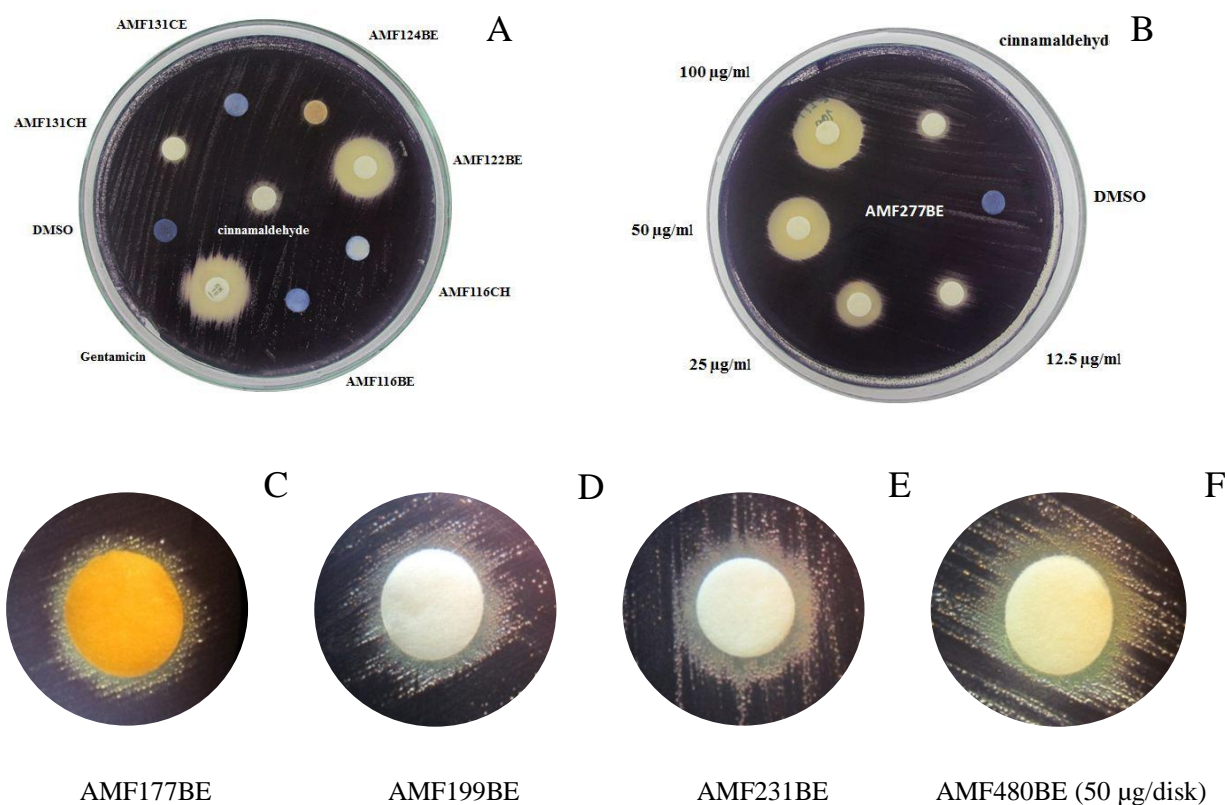
Figure 32 Anti-quorum sensing activities of fungal extracts using a disk diffusion assay against *C. violaceum*. violacein pigment production (A) 100 μg/disk and (B) 12.5-100 μg/disk. (C, D, E) Colorless pigment zones or violacein inhibition zones of AMF177BE, AMF199BE and AMF231BE, tested at 100 μg/disk, respectively and (F) AMF480BE (50 μg/disk)

3.6 Synergistic effects of extracts combined with colistin by a checkerboard assay against *A. baumannii* NPRC 005

Ten active extracts having anti-*A. baumannii* activity were determined for their synergistic effect in combination with colistin using a checkerboard method. Only two extracts including AMF222CE and AMF409BE showed synergistic effect (FICI ≤ 0.5). The results are shown in Table 19. Five sets of combinations of each extract showed synergistic activity (FICI 0.25-0.5). In addition, the concentration of AMF222CE (25 and 50 μg/ml) and AMF409BE (50 and 100 μg/ml) in combination can reduce the MIC of colistin to 8 times compared with the MIC of colistin alone.

Table 19 Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) of extracts combined with colistin against *A. baumannii* NPRC 005 showing synergistic activity

Extracts	MIC extract alone	MIC colistin alone	MIC in combination		FICI
			Extracts	Colistin	
AMF222CE	200	8	25	1	0.25
			12.5	2	0.3125
			50	1	0.375
			25	2	0.375
			50	2	0.5
AMF409BE	400	8	50	1	0.25
			25	2	0.3125
			100	1	0.375
			50	2	0.375
			100	2	0.5

Synergistic killing of fungal extracts and colistin by a time-kill assay

The best synergistic combinations between fungal extracts and colistin showing the lowest FICI (0.25) by a checkerboard method were further confirmed by a time-kill assay. Results are shown in Figures 33-35.

The effects of active extracts and colistin alone at their MIC concentrations on the viable counts of *A. baumannii* are presented in Figure 33. AMF222CE (200 µg/ml) slightly reduced the number of bacterial cells (>1 $\log_{10}$ CFU/ml) in 1-2 h and the bacteria regrowth was observed at 24 h. AMF409BE (400 µg/ml) revealed bacteriostatic activity with >2 $\log_{10}$ CFU/ml reduction in 1-8 h, followed by the regrowth of bacteria after 16 h of incubation. For colistin, the bactericidal effect (>3 $\log_{10}$ CFU/ml reduction) recorded after 2 to 16 h of incubation. However, bacterial regrowth close to initial number of inoculum was observed at 24 h.
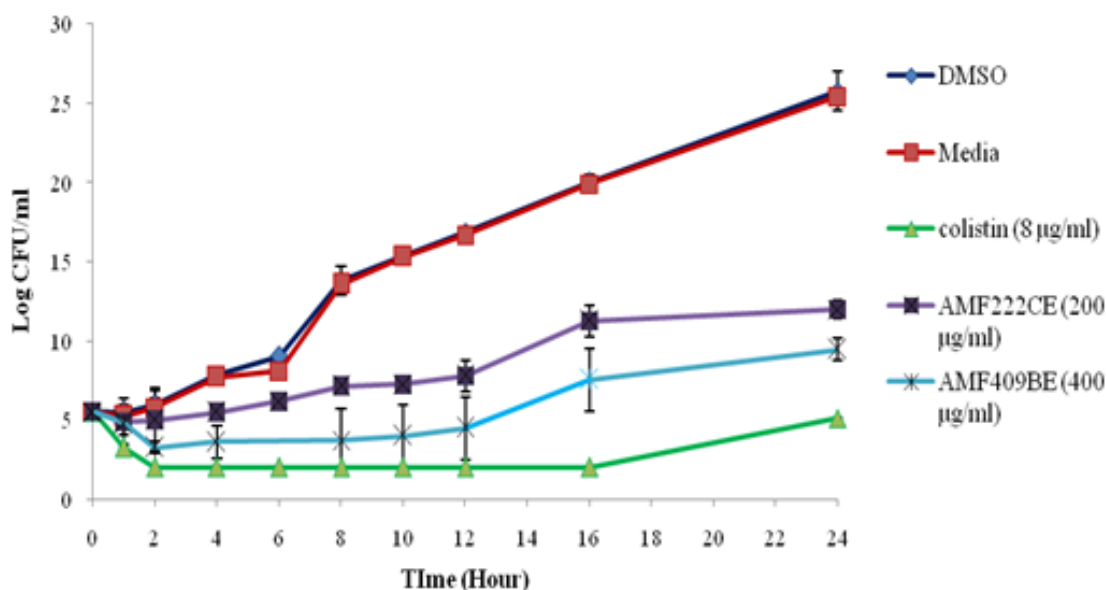
Figure 33 Time-kill curves at MIC concentrations of AMF222CE, AMF409BE and colistin against *A. baumannii* NPRC005

The synergistic effect of AMF222CE and colistin is presented in Figure 34. AMF222CE 25µg/ml in combination with colistin 1 µg/ml demonstrated >3 $\log_{10}$ CFU/ml reduction in number of viable cells (bactericidal activity) after 1-16 h of incubation as compared to the initial inoculum. It showed synergistic effect (>3 $\log_{10}$ decrease in the viable counts compared to the result from colistin, the most active drug alone.

The combination result of AMF409BE plus colistin (AMF409BE:colistin 50:1 µg/ml) in Figure 35 shows bactericidal effect after 1 to 24 h of incubation as well as the synergistic effect after the first hour of incubation (> $2\log_{10}$ CFU/ml reduction) up to 24 h as compared to colistin alone.

The time-kill assays confirmed the synergistic results of these two extracts in combination with colistin against *A. baumannii*.
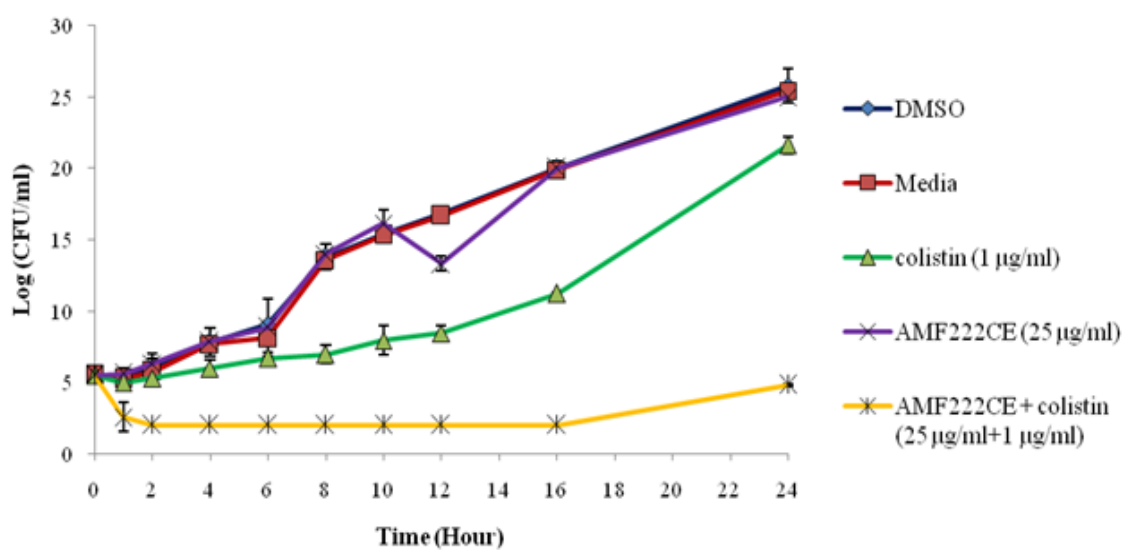
Figure 34 Time-kill curves of AMF222CE and colistin combination against *A. buamannii* NPRC 005.
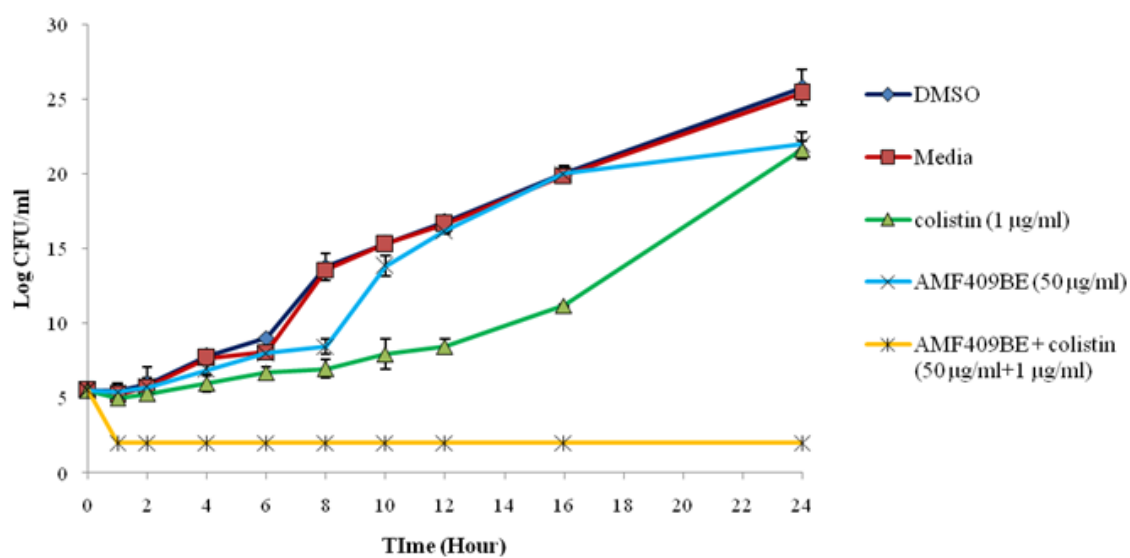
Figure 35 Time-kill curves of AMF409BE and colistin combination against *A. buamannii* NPRC005

3.7 Identification of marine-derived fungi presenting antimicrobial activity

Morphological identification

One hundred and nine out of 123 marine-derived fungal isolates presenting antimicrobial activity were identified using morphological characters as shown in Table 20. These fungi could be identified into 9 genera including *Trichoderma* (24.77%), *Aspergillus* (23.85%), *Penicillium* (18.35%), *Cladosporium* (4.59%), *Acremonium* (0.92%), *Fusarium* (0.92%), *Helminthosporium* (0.92%), *Pestalotiopsis* (0.92%), *Syncephalatum* (0.92%) and one group of unidentified fungi (22.02%). Most of the identifiable fungi sporulated on SPDA and were fast growing.

Table 20 Morphological identification of antimicrobial producing marine-derived fungi

Fungal genera	No. of isolate (%)
Trichoderma	27 (24.77%)
(AMF2, AMF3, AMF6, AMF7, AMF8, AMF11, AMF12, AMF14, AMF53, AMF94, AMF95, AMF124, AMF127, AMF130, AMF222, AMF274, AMF300, AMF304, AMF384, AMF409, AMF413, AMF417, AMF419, AMF455, AMF456, AMF458 and AMF475)	
Aspergillus	26 (23.85%)
(AMF21, AMF41, AMF46, AMF79, AMF122, AMF131, AMF160, AMF161, AMF169, AMF207, AMF210, AMF214, AMF227, AMF231, AMF241, AMF243, AMF248, AMF253, AMF271, AMF273, AMF277, AMF292, AMF294, AMF295, AMF322 and AMF480)	
Penicillium	
(AMF17, AMF18, AMF28, AMF30, AMF87, AMF89, AMF138, AMF203, AMF205, AMF228, AMF229, AMF242, AMF247, AMF285, AMF366, AMF376	

Table 20 (Cont.) Morphological identification of antimicrobial producing marine-derived fungi

Fungal genera	No. of isolate (%)
AMF398, AMF420, AMF426 and AMF450)	
Cladosporium	5 (4.59)
(AMF141, AMF172, AMF199, AMF358 and AMF360)	
Fusarium	3 (2.75)
(AMF344, AMF346 and AMF347)	
Acremonium	1 (0.92)
(AMF166)	
Helminthosporium	1 (0.92)
(AMF422)	
Pestalotiopsis	1 (0.92)
(AMF117)	
Syncephalastrum	1 (0.92)
(AMF143)	
Unidentified	24 (22.02)
(AMF45, AMF61, AMF115, AMF116, AMF119,	
AMF144, AMF177, AMF182, AMMF184, AMF185,	
AMF188, AMF192, AMF194, AMF198, AMF225,	
AMF235, AMF238, AMF244, AMF250, AMF290,	
AMF293, AMF323, AMF350 and AMF368)	
Total	**109 (100)**

The detailed characteristics of each genus are as follows:

Genus *Acremonium*

Taxonomic classification: Kingdom: Fungi, Phylum: Ascomycota, Order: Hypocreales, Family: Hypocreaceae

Morphological description:

Acremonium spp. are filamentous fungi with moderate to rapid growing, velvety to powdery in texture. The color of colony of AMF166 is white to pale pink on the surface. The reverse side is brownish yellow with soluble yellow pigment production throughout the agar medium. Microscopic features showed hyaline and septate hyphae. Simple erect phialides are formed directly on the hyphal tips. At the apex of each phialide, 1-celled hyaline, globose to cylindrical conidia are produced in cluster (Figure 36).

Fungal isolate: AMF166
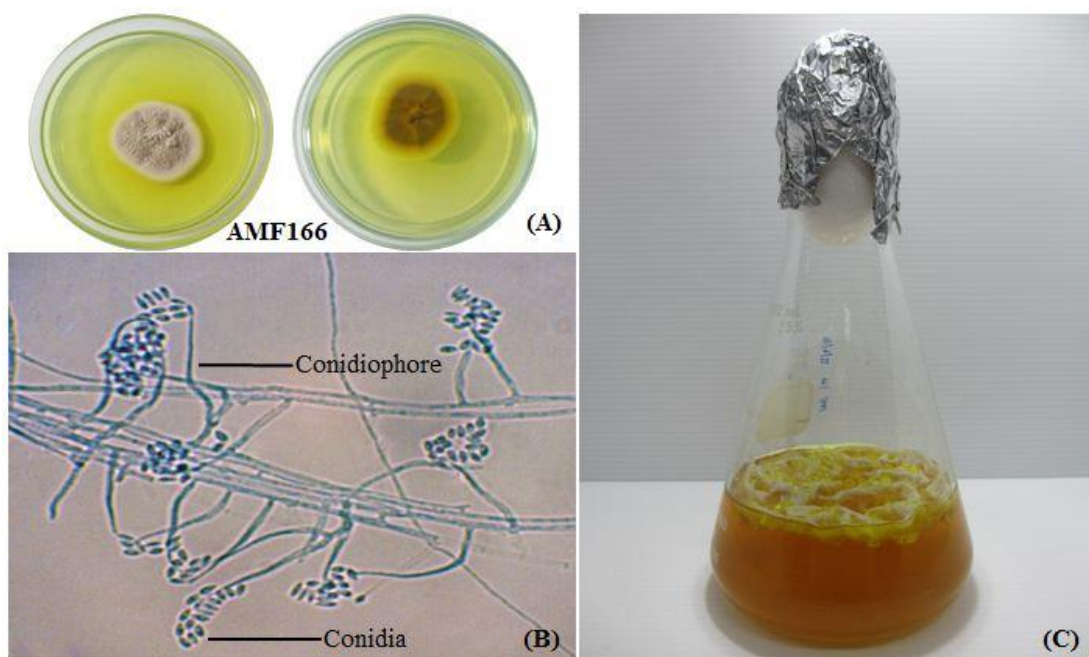
Figure 36 Morphological characteristic of *Acremonium* sp. AMF166

      (A) Left: Pale pink colony on SPDA 25°C (7 days) and right: the reverse

      (B) Conidia and conidiophores

      (C) Fermentation broth (PDB, 21 days)

Genus *Aspergillus*

Taxonomic classification:

Kingdom: Fungi, Phylum: Ascomycota, Class: Eurotiomycetes, Order: Eurotiales, Family: Trichocomaceae

Morphological description:

Aspergillus spp. are usually fast growing. The colonies are granular, in texture. *Aspergillus* group could be divided into 4 groups based on their surface color when grown on the SPDA medium as black, green, yellow and pale yellow. The reverse is uncolored to pale yellow in most of the isolates. Microscopic features showed the aspergillum-like spore-bearing structure, the most important structure of *Aspergillus* taxonomy, septate and hyaline hyphae. Conidiophores varied from short to long with smooth surface and foot cells. Conidial head shapes were varied from globose to subglobose (Figure 37).

Fungal isolates:

Black: AMF41, AMF160, AMF161, AMF248 and AMF277
Green: AMF21, AMF122, AMF131, AMF207, AMF210, AMF214, AMF227, AMF231, AMF241, AMF253, AMF271, AMF294, AMF295 and AMF322
Yellow: AMF79, AMF243, AMF273, AMF292 and AMF480
Pale yellow: AMF46 and AMF169
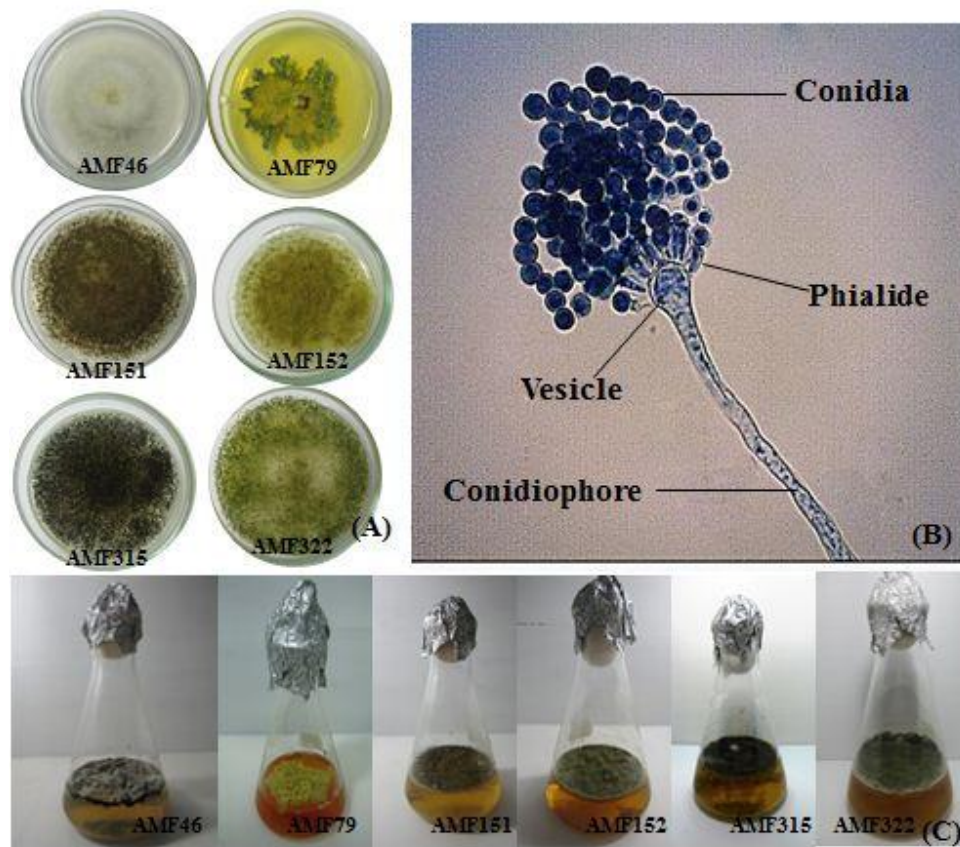
Figure 37 Morphological characteristics of *Aspergillus* spp.

(A) Colonies on SPDA 25°C (7 days) AMF46 (pale yellow); AMF79, AMF151, AMF152 (yellow); AMF315 (black) and AMF322 (green)

(B) Conidiophore, conidial head and conidia of *Aspergillus* sp. AMF370

(C) Fermentation broths (PDB, 21 days)

Genus *Cladosporium*

Taxonomic classification: Kingdom: Fungi Phylum: Ascomycota Class: Dothideomycetes Order: Capnodiales Family: Davidiellaceae

Morphological description:

Cladosporium is a dematiaceous fungus. Colonies are grayish brown to blackish brown, velvety or powdery in texture. They produce brown septate hyphae, erect and pigmented conidiophores. Conidia of *Cladosporium* are elliptical to cylindrical in shape, one to four celled in branch which are also known as shield shaped and have dark hila (Figure 38).

Fungal isolates: AMF141, AMF172, AMF199 and AMF358
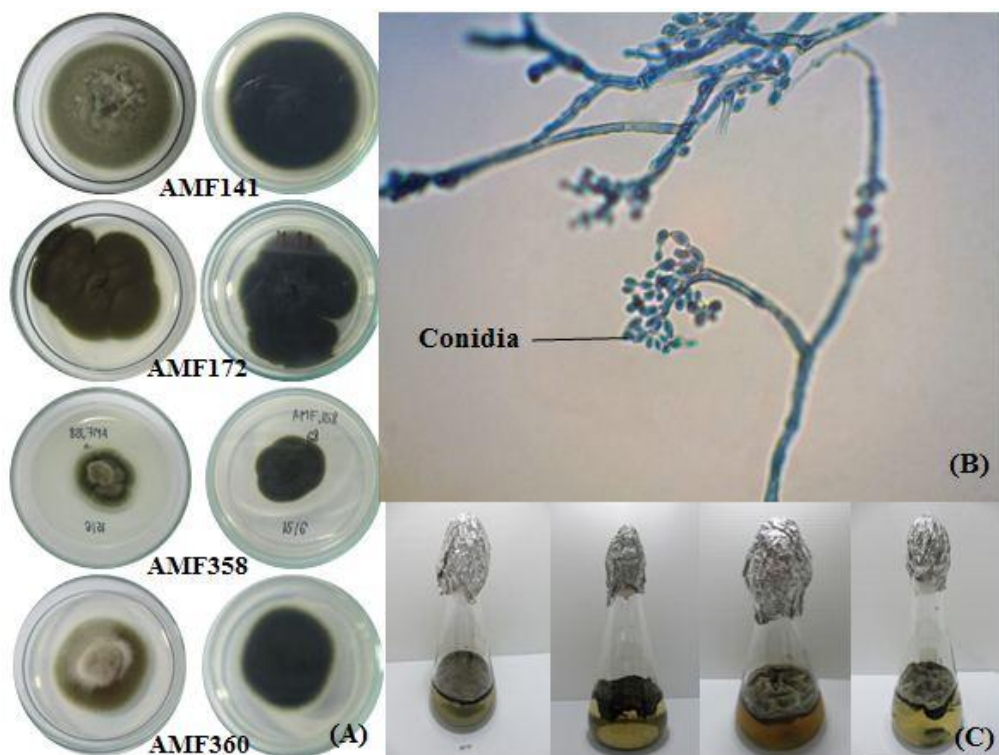
Figure 38 Morphological characteristics of *Cladosporium* spp.

(A) Left: colonies on SPDA 25°C (7 days) and right: the reverses

(B) Typical microscopic character of *Cladosporium* sp. AMF141

(C) Fermentation broths (PDB, 21 days)

Genus *Fusarium*

Taxonomic classification: Kingdom: Fungi, Phylum: Ascomycota, Class: Sordariomycetes, Order: Hypocreales, Family: Nectriaceae

Morphological description:

Fusarium spp. are fast growing with wooly to cottony aerial mycelia on PDA plate. The colony colors are white to pale yellow, to pale orange. They typically produce macro-and microconidia. Macroconidia presented 3-5 septate, sickle shaped or fusiform, slightly curved and stout. In this study, microconidia were not observed (Figure 39).

Fungal isolates: AMF334, AMF346 and AMF347
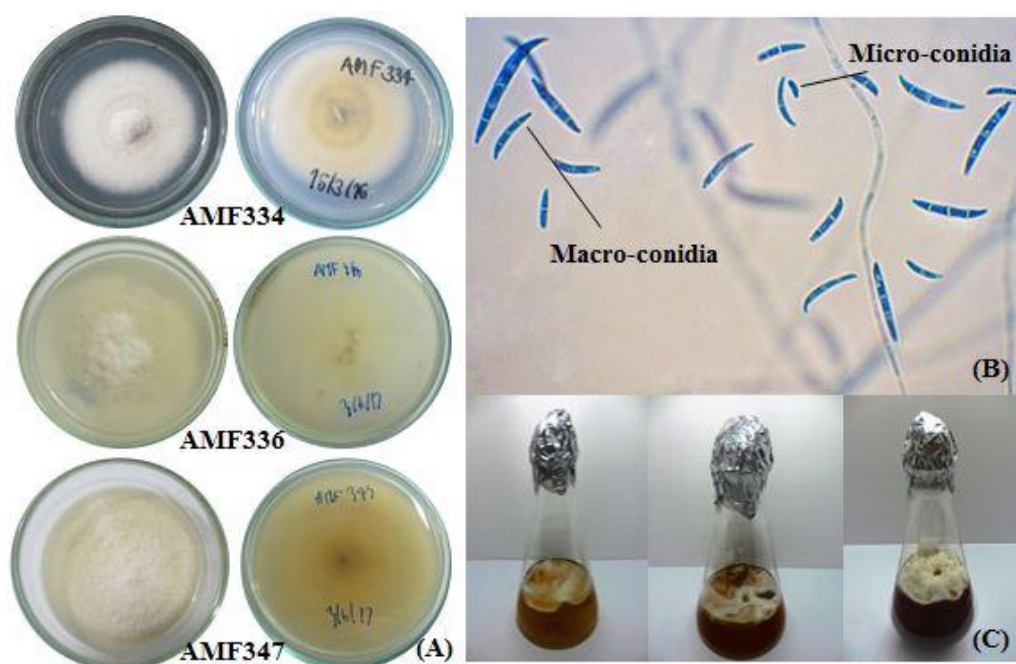
Figure 39 Morphological characteristics of *Fusarium* sp.

(A) Left: Colonies on SPDA 25°C (7 days) and right: the reverses

(B) Macroconidia and Microconidia of *Fusarium* sp. AMF347

(C) Fermentation broths (PBD, 21 days)

Genus *Helminthosporium*

Taxonomic classification: Kingdom: fungi, Phylum: Ascomycota, Class: Dothideomycetes, Order: Pleosporales, Family: Massarinaceae.

Morphological description:

Helminthosporium is a dematiaceous filamentous fungus. The texture is velvety to wooly. The colony color and the reverse are black. It produces brown septate hyphae. Conidiophores are brown to dark brown. Conidia are club shape, large with three or more cells. The conidia are located along the sides of the conidiophores (Figure 40).

Fungal isolates: AMF422
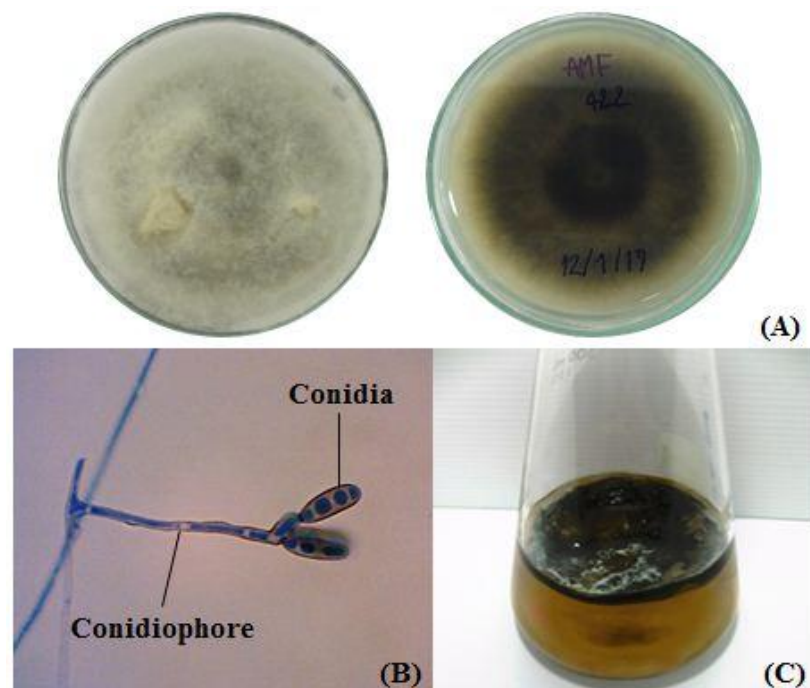
Figure 40 Morphological characteristics of *Helminthosporium* sp. AMA422

        (A) Left: colony on SPDA 25°C (7 days) and right: the reverse

        (B) Conidiophore and conidia

        (C) Fermentation broth (PDB, 21 days)

Genus *Penicillium*

Taxonomic classification: Kingdom: Fungi, Phylum: Ascomycota, Class: Euascomycetes, Order: Eurotiales, Family: Trichocomomaceae

Morphological description:

Penicillium spp. are moderate to rapid growing with velvety or woolly, green to deep green colonies. The microscopic features showed septate hyaline hyphae, branched or simple conidiophores, metulae, phialides and conidia. The metulae are secondary branches that from on conidiophores carry the flask-shaped phialides. Round conidia in chains at the tips of phialides are observed (Figure 41).

Fungal isolates: AMF17, AMF18, AMF28, AMF30, AMF87, AMF89, AMF138, AMF203, AMF205, AMF228, AMF229, AMF242, AMF247, AMF285, AMF366, AMF376, AMF398, AMF420, AMF426 and AMF450.
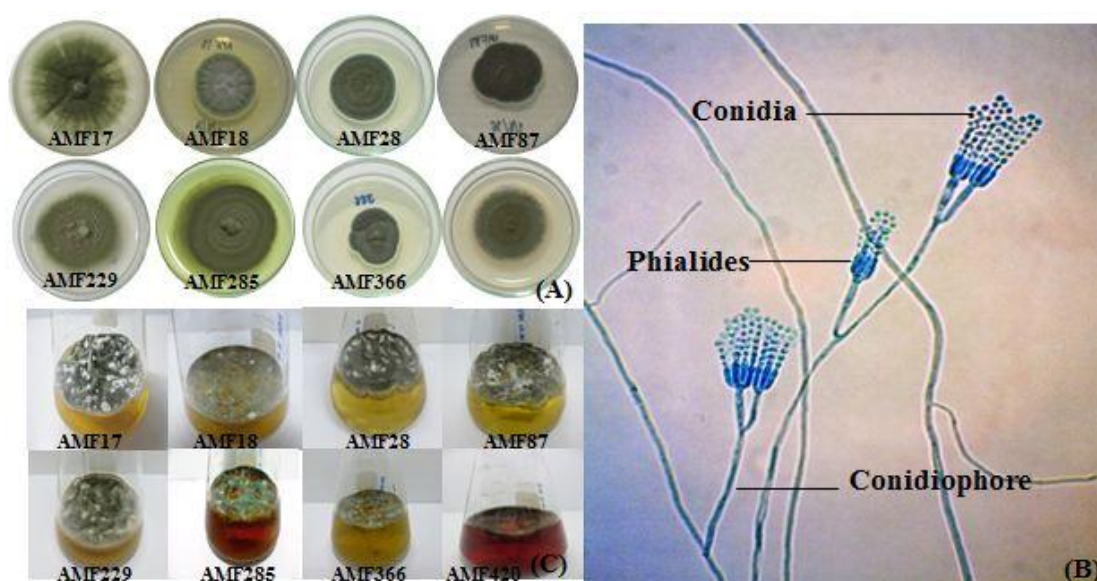
Figure 41 Morphological characteristic of *Penicillium* spp.

       (A) Colonies on SPDA 25°C (7 days)

       (B) Coniophores and conia of *Penicillium* sp. AMF28

       (C) Fermentation broths (PDB, 21 days)

Genus *Pestalotiopsis*

Taxonomic classification: Kingdom: Fungi, Phylum: Ascomycota, Class: Sordariomycetes, Order: Xylariales, Family: Amphisphaeriaceae

Morphological description:

Pestalotiopsis spp. have white fluffy aerial mycelia with black acervular conidiomata on the surface. The conidia are 5-celled with three brown median cells, straight to slightly curve. Both apical and basal cells are hyaline. The apical cell has two or more appendages or hairs (Figure 42).

Fungal isolates: AMF117
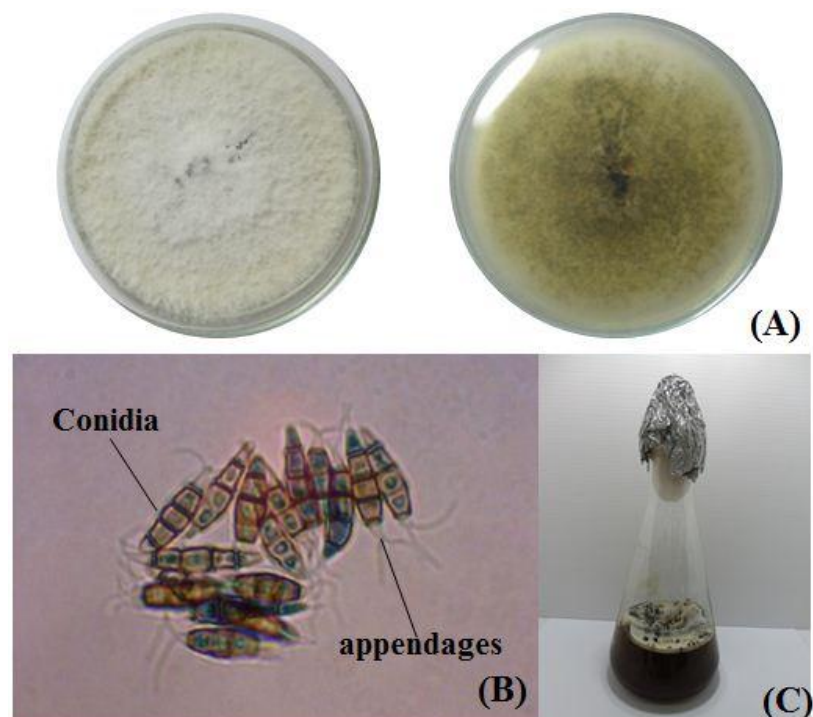
Figure 42 Morphological characteristic of *Pestalotiopsis* sp. AMF117

(A) Left: colony on SPDA 25°C (7 days) and right: the reverse

(B) Typical conidia

(C) Fermentation broth (21 days)

Genus *Syncephalastrum*

Taxonomic classification: Kingdom: Fungi, Phylum: Zygomycota, Class: Zygomycetes, Order: Mucorales, Family: Syncephalastraceae

Morphological description:

Syncephalastrum spp. are rapid growing, cottony to fluffy colonies. The color of colony initially white and turn to black. They produce non-septate hyphae and sympodial branching sporangiophores with terminal vesicles; finger-shaped or merosporangia. Each merosporangia contains a single row of 3-18 merosporangiospores, spherical to cylindrical in shape (Figure 43).

Fungal isolates: AMF143
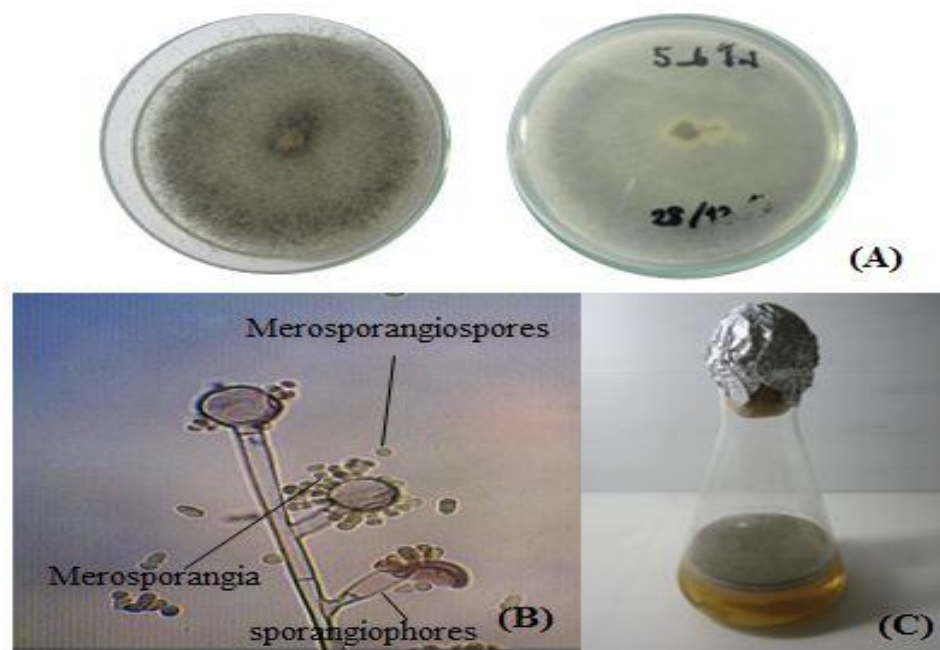
Figure 43 Morphological characteristic of *Syncephalastrum* sp. AMF143

(A) Left: colony on SPDA 25°C (7 days)

(B) Sporangiophores, merosporangia and merosporangiopores

(C) Fermentation broth (PDB, 21 days)

Genus *Trichoderma*

Taxonomic classification: Kingdom: Fungi, Phylum: Ascomycota, Class: Euascomycetes, Order: Hypocreales, Family: Hypocreaceae

Morphological description:

Trichoderma spp. are rapid growing, woolly colonies with white to patches green conidia. They produce hyaline septate hyphae, branched conidiophores, flask-shaped phialides and ovoid to globose conidia borne in terminal cluster (Figure 44).

Fungal isolates: AMF2, AMF3, AMF6, AMF7, AMF8, AMF11, AMD12, AMF14, AMF53, AMF94, AMF95, AMF124, AMF127, AMF130, AMF222, AMF274, AMF300, AMF304, AMF384, AMF409, AMF413, AMF417, AMF419, AMF455, AMF456, AMF458 and AMF475
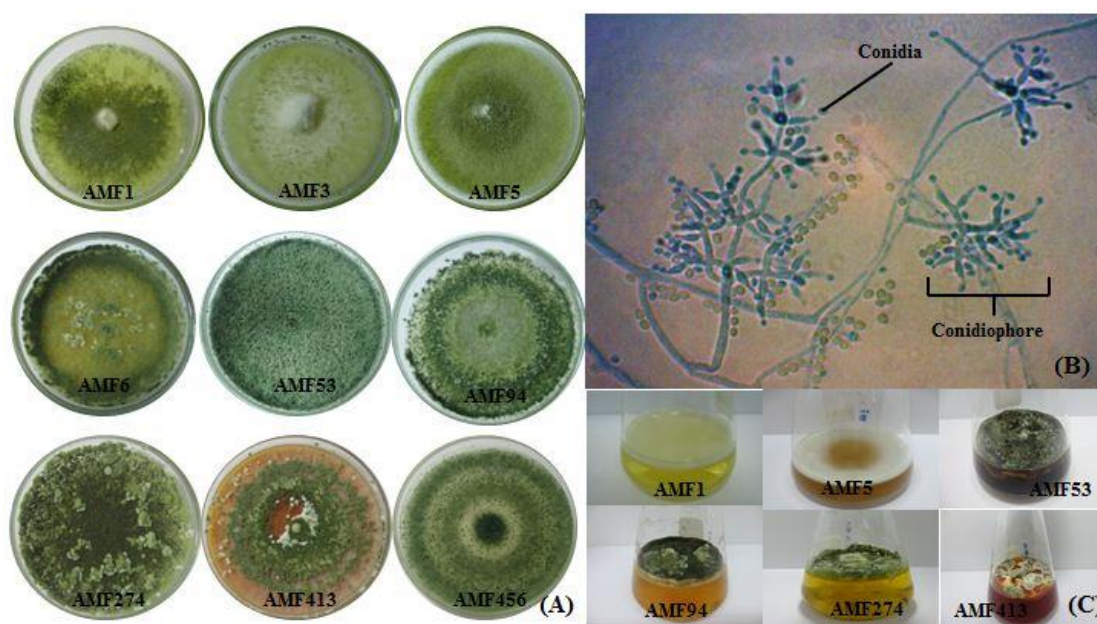
Figure 44 Morphological characteristic of *Trichoderma* spp.

(A) Colonies on SPDA 25°C (7 days)

(B) Conidiophores and conidia of *Trichoderma* sp. AMF93

(C) Fermentation broths (PDB, 21 days)

In addition, the fungi presenting the strong and moderate antimicrobial activity against each test strains and/or interesting NMR profiles were further identified by molecular method using phylogenetic analysis based mainly on ITS gene.

Molecular identification

Among 109 isolates of antimicrobial producing marine-derived fungi, extracts from 36 isolates exhibited moderate to strong activity (MIC $\leq$ 64 μg/ml). Sixteen active isolates that also showed interesting H^1 NMR profiles were further identified using molecular methods at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. In addition, two isolates including AMF222 and AMF409, of which their extracts showed synergistic effect with colistin against *A. baumannii* were also identified by molecular method in this study. Results are shown in Table 21.

The morphology-based species identification of 18 active marine-derived fungi was supported by ITS-based phylogenetic analysis where the maximum parsimony (MP) and the distance neighbor joining (NJ) were employed to reconstruct the phylogenetic trees. Their ITS sequences were compared with sequences obtained from the GenBank database. In some isolates, the LSU sequences were included in order to confirm their identification.

In this thesis, only the molecular identification of AMF222 and AMF409 was presented in detail. The blast search revealed that AMF222 and AMF409 belonged to the phylum Ascomycota, class Sordariomycetes, order Hypocreales and genus *Trichoderma*. The ex-type of *Trichoderma ghanense* and the strain BK36 were used as the outgroups. The ITS rRNA alignment consisted of 32 taxa and each comprised 532 total characters, 501 of which were constant, 9 were parsimony uninformative and 13 were parsimony informative. This yielded 1000 most parsimonious trees (MPTs) with tree length 26 steps, Consistency Index (CI) of 0.9615, Homoplasy Index (HI) of 0.0385, Retention Index (RI) of 0.9767 and Rescaled consistency index (RC) of 0.9392. MP analysis yielded 14 MPTs. The best tree estimated from K-H test is shown in Figure 45 while the distance neighbor

joining tree (NJ) is in Figure 46. The trees from MP and NJ analyses showed similar topologies.

The isolate AMF222 was clustered in the subclade B supported by 64% MP bootstrap value (Figure 45). It was found to be very close to *T. aethiopicum* (PPRC-J11 and type strain), *T. bissettii* (UTHSC 07-2998, 09-2160, 12-337 and 12-944) and *T. longibrachiatum* (ATCC201044, ATCC38586, UAMH-7955, UAMH-7956 and type strain). For isolate AMF409, the MP analysis placed it in the subclade A together with the ex-type of various strains of *Trichoderma orientale* with high MP bootstrap value (87%). Based on the NJ analysis (Figure 46), the isolates AMF409 and AMF222 were grouped in the subclade A (88% bootstrap value) and B (93% bootstrap value), respectively in which the topologies are nearly identical to the MP tree (Figure 46). The nucleotide-nucleotide search of ITS rDNA sequences of these two isolates were also performed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI, USA). The BLAST search with the sequence of AMF222 mostly hit sequences belonged to *T. aethiopicum*, *T. bissettii* and *T. longibrachiatum* with the percent of identity varying from 99.6% to 100% shown in Table 3A (in appendix) while the isolate AMF409 was identical to *T. orientale* TUB-F 837 (GenBank accession no.AY857230) with 100% identity (Table 4A in appendix). Thus, the ITS sequences revealed that the isolate AMF222 belonged to *Trichoderma* section *Longibrachiatum* while the isolate AMF409 could be identified as *T. orientale*.

Based on morphological and molecular identification, 36 marine-derived fungi presenting moderate to strong antimicrobial activity and/or interesting H^1 NMR profiles belonged to the two phyla, Ascomycota and Basidiomycota. Thirty-five isolates were in the Phylum Ascomycota comprising 9 genera including *Trichoderma* (10 isolates), *Aspergillus* (8 isolates), *Penicillium* (8 isolates), *Letendraea* (2 isolates), *Cladosporium* (1 isolate), *Fusarium* (1 isolate), *Pestalotiopsis* (1 isolate), *Phaeosphaeriopsis* (1 isolate) and *Trichothecium* (1 isolate). Whereas only one isolate, *Schizophyllum commune* AMF238 belonged to the phylum Basidiomycota, class Agaricomycetes and order Agaricales. Two isolates (AMF177 and AMF235) are unidentified.

In addition, 34 isolates that showed only interesting H^1 NMR profiles were also identified by molecular method at BIOTEC, Thailand. Results are shown in Table 2A (in the appendix). All of them belonged to the Ascomycota in 3 classes (Dothideomycetes, Eurotiomycetes, Sordariomycetes), 7 orders (Capnodiales, Pleosporales, Eurotiales, Hypocreales, Xylariales, Sordariales, and Glomerellales) and 19 genera.
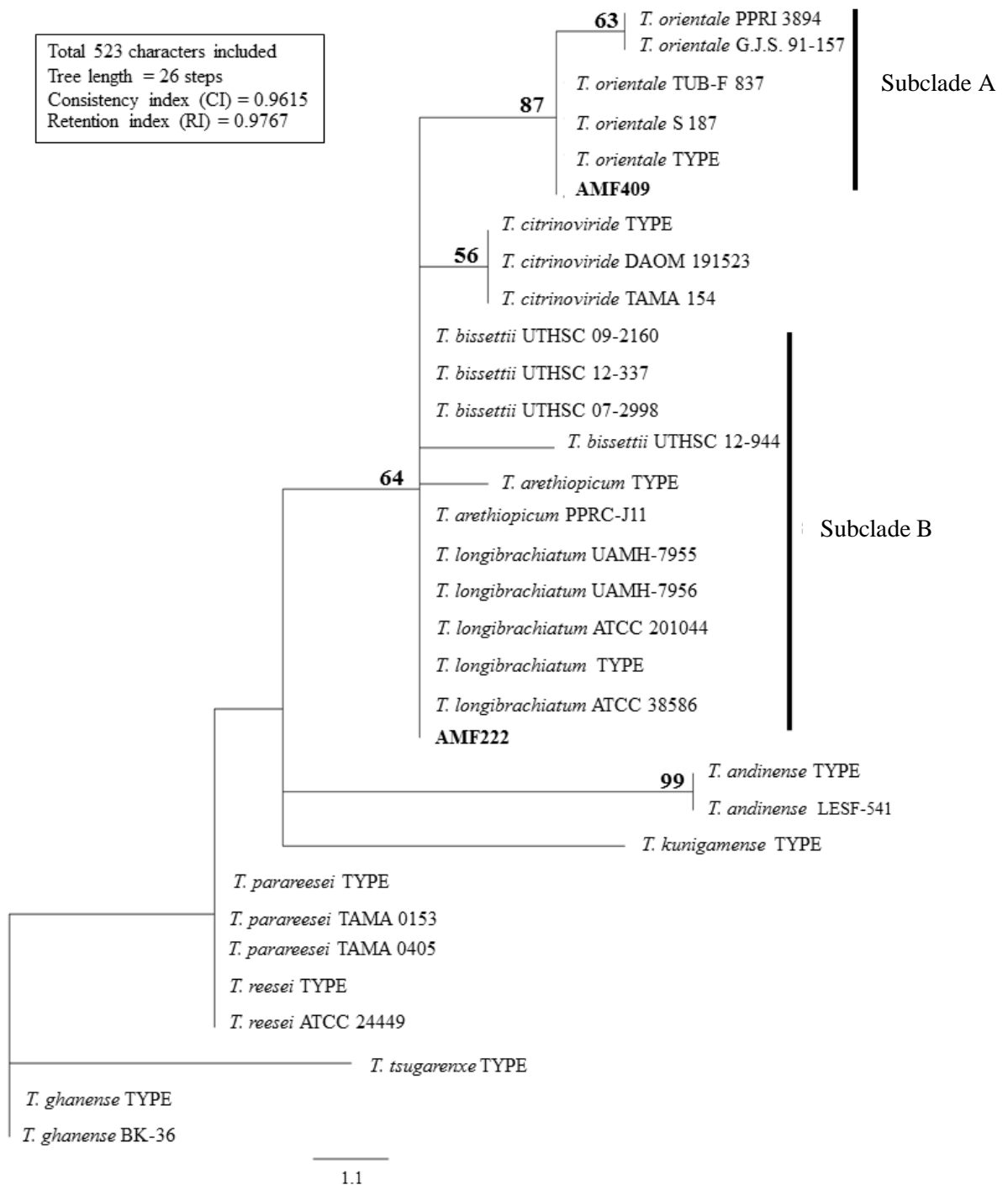
Figure 45 Phylogram obtained from ITS sequence analysis of marine-derived fungi, AMF222 and AMF409, generated from parsimony analysis. The number on each branch represents bootstrap values support (>50%) with 1000 replications. Length:length 26 steps, consistency index (CI): 0.9615, homoplasy index (HI): 0.0385, retention index (RI): 0.9767 and rescaled consistency index (RC): 0.9392.

NJ

63 | *T. orientale* PPRI 3894
| *T. orientale* G.J.S. 91-157
T. orientale TUB-F 837
T. orientale S 187
AMF409
T. orientale TYPE

} Subclade A

T. arethiopicum TYPE
T. bissettii UTHSC 12-944
T. longibrachiatum ATCC 201044
T. longibrachiatum TYPE
T. longibrachiatum ATCC 38586
AMF222
T. bissettii UTHSC 07-2998
T. arethiopicum PPRC-J11
T. longibrachiatum UAMH-7955
T. longibrachiatum UAMH-7956
T. bissettii UTHSC 12-337
T. bissettii UTHSC 09-2160

} Subclade B

T. citrinoviride TYPE
T. citrinoviride DAOM 191523
T. citrinoviride TAMA 154
T. andinense TYPE
T. andinense LESF-541
T. kunigamense TYPE
T. parareesei TYPE
T. parareesei TAMA 0153
T. parareesei TAMA 0405
T. reesei TYPE
T. reesei ATCC 24449
T. tsugarenxe TYPE
T. ghanense TYPE
T. ghanense BK-36

88
93
60
74
100
95
85

0.002

Figure 46 Phylogram obtained from ITS sequence analysis of marine-derived fungi, AMF222 and AMF409, generated from Neighbor-joining analysis.

Table 21 Identification of marine derived fungi presenting moderate to strong antimicrobial activity by molecular method

Phylum	Class	Order	AMF code*	BCC code	Morphological identification	Molecular identification	
						Expected species	Accession number (rDNA region)
Ascomycota	Dothideomycetes	Capnodiales	AMF360	86502	*Cladosporium* sp.	*Cladosporium sphaerospermum*	MH398545 (I)
							MH398557 (L)
		Pleosporales	AMF144	86498	Unidentified ascomycetes	*Letendraea helminthicola*	MH398541 (I)
							MH398554 (L)
			AMF368	86503	Unidentified ascomycetes	*Letendraea helminthicola*	MH398546 (I)
							MH398558 (L)
			AMF198	88127	Unidentified fungus	*Phaeosphaeriopsis musae*	MH997886 (I)
							MH997898 (L)
	Eurotiomycetes	Eurotiales	AMF21	-	*Aspergillus* sp.	ND	-
			AMF46	-	*Aspergillus* sp.	ND	-
			AMF273	-	*Aspergillus* sp.	ND	-
			AMF292	-	*Aspergillus* sp.	ND	-
			AMF169	86499	*Aspergillus* sp.	*Aspergillus candidus*	MH398542 (I)
							MH398555 (L)
			AMF277	84318	*Aspergillus* sp.	*Aspergillus clavatonanicus*	MF780709 (I)

Table 21 (Cont.) Identification of marine derived fungi presenting moderate to strong antimicrobial activity by molecular method

Phylum	Class	Order	AMF code*	BCC code	Morphological identification	Molecular identification	
						Expected species	Accession number (rDNA region)
			AMF131	84309	*Aspergillus* sp.	*Aspergillus fumigatus*	MF780706 (I)
							MF780712 (L)
			AMF231	84329	*Aspergillus* sp.	*Aspergillus unguis*	MF537637 (I)
			AMF87	-	*Penicillium* sp.	ND	-
			AMF203	-	*Penicillium* sp.	ND	-
			AMF205	-	*Penicillium* sp.	ND	-
			AMF229	-	*Penicillium* sp.	ND	-
			AMF366	-	*Penicillium* sp.	ND	-
			AMF420	-	*Penicillium* sp.	ND	-
			AMF18	84328	*Penicillium* sp.	*Penicillium citrinum*	MF537635 (I)
			AMF89	84308	*Penicillium* sp.	*Penicillium sclerotiorum*	MF537636 (I)
	Sordariomycetes	Hypocreales	AMF347	-	*Fusarium* sp.	ND	-
			AMF94	-	*Trichoderma* sp.	ND	-
			AMF127	-	*Trichoderma* sp.	ND	-
			AMF413	-	*Trichoderma* sp.	ND	-
			AMF455	-	*Trichoderma* sp.	ND	-
			AMF458	-	*Trichoderma* sp.	ND	-

Table 21 (Cont.) Identification of marine derived fungi presenting moderate to strong antimicrobial activity by molecular method

Phylum	Class	Order	AMF code*	BCC code	Morphological identification	Molecular identification	
						Expected species	Accession number (rDNA region)
			AMF274	84317	*Trichoderma* sp.	*Trichoderma longibrachiatum*	MF537639 (I)
			AMF222	-	*Trichoderma* sp.	*Trichoderma longibrachiatum*	MK448009
			AMF409	-	*Trichoderma* sp.	*Trichoderma orientale*	MK448010
			AMF3	86492	*Trichoderma* sp.	*Trichoderma reesei*	MH398538 (I) MH398551 (L)
			AMF7	86493	*Trichoderma* sp.	*Trichoderma reesei*	MH398534 (I) MH398531 (L)
			AMF192	86514	Unidentified fungus	*Trichothecium* sp	MH400414 (I) MH398571 (L)
		Xylariales	AMF117	86496	*Pestalotiopsis* sp.	*Pestalotiopsis* sp.	MH398536 (I) MH398533 (L)
Basiodiomycota Unidentified species	Agaricomycetes	Agaricales	AMF238	84313	Unidentified fungus	*Schizophyllum commune*	MF537638 (I)
			AMF177	-	Unidentified fungus	ND	-
			AMF235	-	Unidentified fungus	ND	-

BCC: BIOTEC culture collection   (I): ITS sequence   (L): LSU sequence   (BT): beta-tubulin sequence   ND= not done

* Source of the fungal strains shown in Table 5A in the appendrix

CHAPTER 4

DISCUSSION

4.1 Number of isolated marine-derived fungi and identification of active isolates

Five hundred and forty-seven isolates of marine-derived fungi were isolated from various marine organisms collected from Phuket province. The great numbers of yielded fungi were in the genera *Aspergillus*, *Penicillium* and *Trichoderma*. This result is similar to Menezes *et al*. (2010) who investigated the diversity of fungi isolated from marine invertebrates and one alga using the direct plating technique. The results revealed the majority of the obtained fungi are belonging to genera *Aspergillus*, *Penicillium*, *Trichoderma* and *Fusarium*. Members of these genera have been reported to have antimicrobial properties, anticancer and antioxidant activity (Farag *et al.,* 2016; Meng *et al*., 2015; Xu *et al*., 2015). In addition, marine derived fungi in this study have been commonly found in both terrestrial and marine habitats. Floods and winds may carry terrestrial fungi to marine environments (Bonugil-Santos *et al*., 2015). *Aspergillus* spp. and *Penicillium* spp. are a major group of active fungi which produce most of bioactive compounds (Bugni and Ireland, 2004; Imhoff, 2016).

Some investigators reported that sponges have yielded the greatest taxonomic fungal diversity (Baker *et al*., 2009; Imhoff, 2016). However, in this work the Bryozoan samples and a sea fan (*Menella* sp.) gave the highest diversity of filamentous fungi (44.5 and 44 average numbers of isolated fungi) followed by sponges (34.5). Abundance and distribution of marine-derived fungi depend on many factors such as substrates, ecology and phylogeny (Bonugil-Santos *et al*., 2015). The same types of sample from different locations may habour different numbers and groups of fungi. In this study, nine ascidian samples were collected. The numbers of isolated fungi varied from 11 to 64.

Traditional morphology-based fungal identification is based on comparative morphological features and the development of sexual and asexual

reproductive structures on solid media and culture conditions. However, the disadvantages of this method are time-consuming and the need of trained mycologists. In addition, using morphology alone in the identification of fungi to the species level is limited. Since DNA sequence-based phylogenetic analysis is a rapid, specific and accurate method, it is accepted to be a promising and effective tool for fungal classification as well as identification at species level. Additionally, this method provides phylogenetic relationships between fungi (Hibbett *et al*., 2016; Raja *et al*, 2017).

In this study, some selected active marine-derived fungi were first identified by morpholgy to the genus level and then further confirmed to the species levels by molecular technique based mainly on the ITS region. ITS is an official DNA barcoding for fungi. It evolves the fastest and exhibits the highest variation, thus it is valuable for species identification (Raja *et al*, 2017; Schoch *et al*, 2012). In some isolates (Table 21), the ITS sequence analysis alone was not efficient to identify them, the LSU region and other protein coding gene were also studied.

The identification results demonstrated that the active marine-derived fungi in this study were ascomycetes which were mostly distributed within three classes including Dothideomycetes, Eurotiomycetes and Sordariomycetes. There was only one isolate belonged to the Basidiomycota (Agaricomycetes). The result was correlated with several studies where most of the marine-derived fungi were found to be ascomycetes (Andreakis *et al*., 2015; Da Silva *et al*., 2008; Manohar and Raghukumar, 2013).

The colonies of AMF222 and AMF409 were wooly and rapid growing on PDA. The colony surface of AMF222 was dark green and the reverse was yellow with diffusible yellow pigment secreted into the medium. Whereas, AMF409 colony was green and the reverse was pale. Microscopic features of these two strains had similar characters including septate hyaline hyphae, branched conidiophores, flask-shaped phialides and green conidia, which could be primarily identified into the genus *Trichoderma*. Their ITS sequence data were used to reconstruct a maximum parsimony phylogenetic tree and distance neighbor joining (NJ) tree. Based on the

molecular results, AMF222 and AMF409 were well placed in the *Longibrachiatum* clade of *Trichoderma* (Hypocreales, Hypocreaceae; teleomorph: *Hypocrea*). AMF222 was identified as *Trichoderma* sp. section *Longibrachiatum*. The sequence alignment of AMF222 showed the close relationship with 3 species of *Trichoderma* including *T. aethiopicum*, *T. bissettii* and *T. longibrachiatum* with statistical support of 64% MP boostrap value and 93% NJ boostrap value and short branch length. AMF409 could be clearly identified as *T. orientale* with strong support of 87% MP and 88% NJ boostrap values.

Trichoderma species in the section *Longibrachiatum* are commonly found in soil and distributed worldwide. They are characterized by green conidia and rapid growth. The members of this section mostly have nearly identical morphological characters; typical branching patterns of the conidiophores and the production of yellow-green pigment (Bissett, 1984). *Longibrachiatum* clade comprises the most intensively studies *Trichoderma* species. Samuels *et al*. (2012) have revised 21 species in this section and eight species including *T. aethiopicum, T. capillare, T. flagellatum, T. gillesii, T. gracile, T. pinnatum, T. saturnisporopsis and T. solani* have been described. Recently, Sandoval-Dennis *et al*. (2014) studied phylogeny of the clinically relevant species of the emerging *Trichoderma* in this section and have described a new species named *T. bissettii.* Typically, a combination of morphological characters and molecular data are used for fungal identification. Since only the ITS region was used in the current study, AMF222 could only be classified as *Trichoderma* species in the section *Longbrachiatum*. ITS alone could not distinguish *Trichoderma* species in the section *Longbrachiatum*. This may indicate that the ITS sequences of those members are highly conserved and cannot be used to separate them apart (Druzhinina *et al*., 2008; Gazis *et al*., 2011, Samuels *et al*., 2012). Since AMF222 fell into the clade with three different species of *Trichoderma* (Figure 45 and 46), it is required further morphology-based species identification such as colony features on different media or different conditions in order to identify it to the species level. Samuels *et al.* (2012) have set up a synoptic key to 21 members of *Trichoderma* in the *Longibrachiatum* clade. According to this synoptic key, *T. aethiopicum* produces diffusable yellow pigment on PDA within 72 h at 25-35°C in darkness

whereas *T. longibrachiatum* releases diffusable olivaceous color pigment. In this work, AMF222 had diffusable olivaceous pigment. Thus, AMF222 can be identified as *T. longibrachiatum*. In addition, multigene phylogenetic analysis is also useful to clarify the taxonomy of *Trichoderma*. The most frequent loci used in multilocus sequence analysis are translation elongation factor 1 alpha (*Tef1*), endochitinase CHI 18-5 (*Chi18-5*), calmodulin (*cal1*) and actin 1 (*Act1*) genes (Druzhinina *et al.*, 2012; Sandoval-Denis *et al.*, 2014). Samuels *et al.* (2012) described eight new species from the members in this section using the multigene analyses including *Tef1, cal1* and *Chi 18-5* genes and Sandoval-Dennis *et al.* (2014) described *T. bissettii* sp. nov. and other species from clinical specimens belonging to the *Longibrachiatum* clade using different combination such as ITS, *Tef1* and *Chi18-5*. In addition, most of the isolates of *T. bissettii* were identified morphologically as *T. longibrachiatum* or *T. pseudokoningii* (Sandoval-Dennis *et al.* 2014).

Members of the section *Longibrachiatum* play important roles in many aspects. *T. reesei* is recognized as industrial cellulase producer (Druzhinina and Kubicek, 2017; Ellilä, *et al.*, 2017). Most of nosocomial infectious diseases in human are caused by *T. longibrachiatum* which has become an important opportunistic pathogen (Druzhinina *et al.*, 2012; Walsh *et al.*, 2004) followed by *T. citrinoviride*, *T. bissettii* and *T. orientale* (Sandoval-Dennis *et al.*, 2014). In addition, several members of this clade are able to produce a wide spectrum of bioactive compounds, especially the strains that were isolated from marine habitats (Gal-Hemed *et al.*, 2011; Paz *et al.*, 2010; Sun *et al.*, 2008). For examples, *T. saturnisporum* isolated from marine sponge *Dictyonella incisa* produce the novel compound, saturnispols A-H. Saturnispol F displayed remarkable antibacterial activity against vancomycin-resistant enterococci (VRE) with MIC range from 1.63-12.9 µg/ml (Meng *et al.*, 2018), *T. citrinoviride* obtained from marine brown alga synthesized trichocitrin and nafuredin which exhibited inhibition zone against *E. coli* 8.0 and 9.5 mm. (Liang *et al.*, 2016), Marine *T. citrinoviride* elaborated citrinoviric acid and penicillenol D with moderate cytotoxic acid on the human melanoma A-375 cell line (Zhang *et al.*, 2014). In this study, ten isolates of *Trichoderma* spp. including two isolates of

T. longibrachiatum (AMF222 and AMF274), one isolate of *T. orientale* (AMF409) and two isolates of *T. reesei* (AMF3 and AMF7) exhibited antimicrobial activity.

4.2 Antimicrobial activity of marine-derived fungal extracts

Since the discovery of penicillin, terrestrial environments have been a major source for new chemical structures, however there are only five different structure classes of antibacterial drugs from terrestrial microoganisms namely β-lactam, streptogramin, macrolide, tetracycline and cyclic lipopeptide (daptomycin). Recently, teixobactin belonging to a new class of antibiotics (macrocyclic depsipeptide) has been discovered by using a new method for culturing bacteria in soil (Ling *et al*., 2015). Hence, the search for new drugs and novel substances from other sources is needed. The marine environment is the largest one of the earth, with more than 70% of the earth's surface, and harbors an enormous number of different organisms. The oceans represent the resource of organisms with high biological and chemical diversity (Lindequist, 2016). Over the last five years from 2012 to 2017, many groups of marine natural products including alkaloids, terpenoids, peptides, halogenated compounds, polyketides, isocoumarins and nucleosides have been reported (Choudhary *et al*., 2017).

In this study, we focused on marine-derived fungi that produced antimicrobial metabolites. Three hundred and sixty-nine extracts from 123 marine-derived fungi were determined for antimicrobial activity against nine human pathogens. Approximately 60% of the selected fungi exhibited antimicrobial activity. 75% of active extracts presented antifungal activity. This trend is similar to the studies from Cuomo *et al.* (1995) and HÖller *et al.* (2004) who reported that the number of marine fungi had significantly higher antifungal activity than antibacterial properties. On the other hand, Suay *et al*., (2000) and Zainuddin *et al.* (2010) reported that the extracts from marine-derived fungi had more effective antibacterial activities.

Among the tested bacteria, Gram-positive bacteria (*S. aureus* ATCC25923 and MRSA SK-1) were more susceptible than the Gram-negative ones (*A. baumannii* NPRC AB005, *P. aeruginosa* ATCC27853 and *E.coli* ATC25922). This information was related to the significant differences in cell membrane structure

and composition between Gram-positive and Gram-negative bacteria. The major impact on the resistance of Gram-negative bacteria could be attributed to a thick layer of lipopolysaccharide outer membrane and selective barrier function of the cell membrane (Delcour, 2009). Moreover, several studies also found that bioactive natural products are more effective against Gram-positive bacteria than Gram-negative bacteria (Wu *et al*., 2018; Zhang *et al*., 2016).

Considering the type of extracts, cell hexane was the highest active extracts (CH), followed by the broth ethyl acetate (BE) and cell ethyl acetate (CE) extracts. This results suggest that active substances are retained in fungal mycelium as cell-bound components and some active compounds have been released into the broth medium. Active extracts may have low polarity and are better dissolved in hexane than in ethyl acetate (Kim and Mazza, 2006). This result is similar to Buatong *et al*., (2011) and Jeenkeawpieam *et al*. (2012) who investigated antimicrobial activity of endophytic fungi from mangrove plants and *Rhodomyrtus tomemtosa*. In contrast, Kuephadungphan *et al*. (2014) studied antimicrobial activity of invertebrate-pathogenic fungi in the genera *Akanthomyces* and *Gibellula* and found that CE extracts were the greatest active extracts, followed by CH and BE extracts. As the results, the different groups of fungi may provide different secondary metabolites.

Among the 219 active extracts, 59 extracts from 36 isolates presenting strong and moderate antimicrobial activity. The top three genera were *Trichoderma*, *Aspergillus* and *Penicillium*. Members of these genera have already been reported as a new source of new bioactive metabolites especially *Aspergillus* spp. and *Penicillium* spp. (Bugni and Ireland, 2004; Imhoff, 2016).

In this study, cell hexane extract from *Aspergillus unguis* AMF231 (AMF231CH) showed the strongest inhibitory activity against *S. aureus* (MIC 4 µg/ml) and *M. gypseum* (MIC 16 µg/ml). The results are correlated with Nielsen *et al*. (1999) who studied antibiotic activity obtained from *Emericella unguis* (anamorph: *Aspergillus unguis*) which isolated from marine organism. The extracts of *E. unguis* exhibited strong antibacterial activity against *S. aureus*. Furthermore, they found new depside, guisinol exhibiting potential anti-*S.aureus* activity, but did not inhibit the

growth of *Vibrio parahaemolyticus*. In another study, ethyl acetate extract of *A. unguis* isolated from sponge *Agelas* sp. exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa* and *C. albicans*. (El-Hady *et al.*, 2014). In addition, several study revealed that the active metabolites from *A. unguis* isolated from marine organism have pharmaceutical properties. Unguisins A and B exhibited antimicrobial activity against *S. aureus* and *Vibrio parahaemolyticus* (Malmstrøm, 1999). Aspergillusidones D-F and unguinol displayed antifungal activity (Sureram *et al.*, 2013).

Broth ethyl acetate extracts from *Aspergillus clavatonanicus* AMF277 (AMF277BE) showed the broadest inhibitory activity against *S. aureus*, MRSA, *E. coli*, *P. aeruginosa* and *A. baumannii* with MIC values of 32, 64, 32, 64 and 32 µg/ml. To the best of my knowledge, the activity of marine-derived *A. clavatonanicus* has not been reported. However, Zhang *et al*. (2008) studied the activity of *A. clavatonanicus*, an endophytic fungal strain from *Taxus mairei* and reported that clavatol and patulin from this fungus possessed inhibitory activity against several plant pathogenic fungi including *Botrytis cinerea*, *Didymella bryoniae*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum*. In addition, Mishra *et al*. (2017) found that an extract from *A. clavatonanicus* isolated from *Mirabilis jalapa* L. displayed antimicrobial potential against Gram-positive bacteria, Gram-negative bacteria and yeast. Furthermore, seven antibiotics including miconazole, ketoconazole, fluconazole, ampicillin, streptomycin, chloramphenicol and rifampicin were detected using UPLC-ESI-MS/MS. The detection of these antibiotics in the *A. clavatonanicus* extract supports the broad spectrum activity of AMF227BE in this study. Further investigation on chemical compositions of AMF222BE should be studied.

The mycelium and broth extracts of *Trichothecium* sp. AMF192 (AMF192 CH and 192BE) exhibited the most potential antifungal activity against *C. albicans* and *C. neoformans* with MIC 8 µg/ml and 2 µg/ml, respectively. This is the first report on antimicrobial activity of marine-derived *Trichothecium* sp. However, several secondary metabolites from terrestrial filamentous *Trichothecium* sp. such as rosoloactone from endophytic fungus *T. roseum* that exhibited anticancer activity *in vitro* (Zhou *et al*., 2017) and trichothecene mycotoxins (McCormick *et al*.,

2011) have been reported. Interestingly, guangomide A, previously isolated from an unidentified marine-derived fungus and exhibited weak antibacterial activity against *Staphylococcus epidermidis* and *Enterococcus durans*, was also isolated from *Trichothecium* sp. (Sy-Cordero *et al*., 2011).

AMF198CH from *Phaeosphaeriopsis musae* AMF198 was most active against MRSA (MIC 16 µg/ml). *P. musae* is commonly isolated from leaf spots of banana worldwide. It has been described as new species by Arzanlou and Crouse in 2006. Its anamorph is *Phaeoseptoria musae* (Azarlou and Crouse, 2006). However, the strains derived from marine habitat have also been isolated. In 2013, Zhang *et al*. reported for the first time on the potential antifungal activity of *P. musae* isolated from deep-sea sediment in the South China Sea against *Aspergillus vericolor*. However, only the inhibition zone of approximately 9 mm. was recorded. AMF198CH also exhibited moderate antifungal activity against *C. neoformans* (MIC 32 µg/ml) and *M. gypseum* (MIC 16 µg/ml). This isolate had potential antibacterial and antifungal activity.

The inhibitory activity of fungal extracts depends on various factors such as the fungal strains, the solvent used for extraction and the indicator strains (Synytsya *et al*., 2017). As mentioned earlier, ethyl acetate was used to extract metabolites from culture broths to obtain the BE and hexane and ethyl acetate were used for fungal mycelia (CH and CE). The antimicrobial metabolites distributed in all three types of extracts (37.33% CH, 34.33% BE and 28.34% CE). In some isolates such as *A. candidus* AMF169, *A. unguis* AMF231, *C. sphaerospermum* AMF360, *P. citrinum* AMF18 and *Trichothecium* sp. AMF192, all three types of extract had moderate antimicrobial activity. Therefore, more than one solvent should be used to recover all the active metabolites.

Fungi in the same genus or same species may produce the same major compounds. However, variations among the same species have also been reported. Klaiklay *et al*. (2016) reported two new compounds, asperlide and aspersidone from the soil-derived fungus *Aspergillus unguis* PSU-RSPG199. Later, Phainuphong *et al*. (2017, 2018) found nine new compounds from *A. unguis* PSU-RSPG204. These two

fungal isolates were from the same origin. In this study, many isolates of *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp. were selected based on their colony morphologies. The results in Table 16 show that two different strains of *Trichoderma reesei* isolated from the same ascidian host exhibited different antimicrobial results. Cell hexane extract of *T.reesei* AMF3 (AMF3CH) exhibited a broad inhibitory activity against *S. aureus*, MRSA and all tested fungi, in particular *C. neofarmans* and *M. gypseum* with MIC value of 32 µg/ml, while cell hexane extract of *T. reesei* AMF7 (AMF7CH) showed only moderate antibacterial activity against *S. aureus* (MIC 64 µg/ml) and weak activity against MRSA and *M. gypseum*. This result confirmed the strain-specific for fungal secondary metabolites production (Synytsya *et al*., 2017).

The results from this study can indicate that marine-derived fungi are a potential source of antimicrobial active metabolites. Furthermore, some active extracts will be further investigated for active constituents.

4.3 Study on possible mechanism of action of the active extracts by scanning electron microscopy (SEM)

The potential active fungal extracts comprising broth ethyl acetate and cell hexane extracts of *Trichothecium* sp. AMF192 (AMF192 BE and AMF192CH), CE extracts of *P. musae* AMF198 (AMF198CH) and *A unguis* AMF231 (AMF231CH) and BE extracts of *A. clavatonanicus* AMF277 (AMF277BE) *Trichoderma longibrachiatum* AMF222 (AMF222BE) and *T. orientale* AMF409 (AMF409BE) were studied on the possible modes of action by SEM and compared with standard drugs and untreated control cells. From the result, the treated cell revealed ultrastructural changes in the bacterial cells induced by the active extracts and antimicrobial drugs, while the control cells treated with 1% DMSO were well defined.

In Gram-positive bacteria, *S.aureus* treated with AMF222BE, AMF231CH and AMF409BE and *S. aureus* and MRSA treated with AMF198CH and AMF277BE, all extracts destroyed Gram-positive bacterial cells in the same manner as shown by vancomycin which revealed consistent morphological abnormalities such as pitted, wrinkled surfaces and protusions. This result indicated the major effect on

cell wall and cytoplasmic membrane. Vancomycin is a glycopeptide and especially effective against Gram-positive bacteria. It binds to D-Ala-D-Ala C terminus of pentapeptide resulting in blocking the production of peptidoglycan and inhibiting bacterial cell wall biosynthesis (Kim *et al.,* 2008; Lovering *et al.,* 2012). As previously mentioned, Gram-negative bacteria were more resistant to antimicrobial agents than the Gram-positive bacteria because of their outer membrane structure that acts as permeability barriers. Therefore, it is very difficult to find natural products against this group. In this study, AMF227BE showed the best activity against all the Gram-negative bacteria *E. coli* and *P. aeroginosa* treated with AMF277BE exhibited morphological alterations with open holes and deep craters or broken cells indicating its possible action on cell wall and cell membrane, whereas the cells treated with gentamicin showed little morphological changes with rough surfaces. Gentamicin is an aminoglycoside family that binds to the 16S rRNA component of 30S ribosome subunit leading to the inhibition of protein synthesis. The promoting tRNA mismatching can cause protein mistranslation and cell death (Kohanski *et al*., 2010). In addition, the SEM image of *A. baumannii* cells treated with AMF277BE was similar to colistin treated cells. Colistin is a cyclic polypeptide antibiotic that acts as detergent on cell membrane. It disrupts the bacterial outer cell membrane by competitively displaces divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids, leading to leakage of intracellular contents and bacterial death (Mohamed *et al.,* 2016). This result confirmed the possible action of AMF222BE on cell membrane.

For yeasts, AMF192BE and AMF192CH were moderately active against *C. albicans* and *C. neoformans*. The treated yeast cells with both extracts displayed cell deformations similar to the amphotericin B treated cells. Amphotericin B binds to sterol component, ergoterol, in the cell membrane and causes the changes in membrane permeability allowing leakage of intracellular components and ultimately cell death (Ghannoum and Rice, 1999; Mazu *et al*., 2016).

AMF231CH was best active against *M. gypseum*. The treated cell exhibited small holes in the cell, while the clotrimazole treated cells showed slightly morphological changes such as flatted and wrinkled mycelia. Clotrimazole is a broad

spectrum antifungal activity. It inhibits biosynthesis of the sterol of fungal cell membrane. Its action leads to increase membrane permeability and apparent disruption of enzyme systems bound to the membrane (Borgers, 1980). AMF231CH also caused holes in *S. aureus*. The SEM images confirmed that AMF231CH affected cell wall and cell membrane of both bacterial and fungal cells.

The SEM results revealed that the possible action of all active fungal extracts are on cell wall and/or cell membrane of the test microorganisms. The results are correlated with Supaphon *et al.,* (2013) who found that extracts from sea grass-derived endophytic fungi exhibited considerable morphological alterations including deformation, collapsed, shrinkage, rough surfaces and broken cells. The integrity of bacterial structures and cell wall are imperative in maintaining the vitality and the virulence of bacteria (Braga and Ricci, 2002).

Generally, extracts are a combination of various types of bioactive compounds. Marine-derived fungal strains have been reported to produce compounds with antimicrobial potential belonging to alkaloids, macrolides, terpenoids, peptide and other structure types (Choudhary *et al.*, 2017; Radjasa (2015). The mechanisms of action of secondary metabolites with medical potential associated with disruption of cell membrane and/or cell wall have been reported. Terpenes show activity against a wide range of organisms, bacteria, fungi, virus and protozoa. Most of terpenoides are lipophilic. They interact with biomembrane and membrane proteins. They can increase the fluidity and permeability of membrane that can lead to uncontrolled efflux of ions and metabolites and even to cell leakage, resulting in necrotic or apoptotic cell death (Putri *et al.*, 2015; Wink, 2015). Marine-derived *Aspergillus* sp. produced terpene compounds such as ophiobolin U, sydowic acid, sydonic acid and terretonin G (Elissawy *et al.*, 2015).

Many secondary metabolites are also reported to have antifungal activity. Phenolic compounds are one of the antifungal components in secondary metabolites. They can cause cell membrane disruption, inhibition of cell wall formation and inhibition of the mitochondria. They also damage lipids in the plasma membrane of microorganisms. The plasma membrane is semipermeable and serves to

control the transport of various metabolites into and out of cells. Disorder or damage of the plasma membrane can inhibit or impair the ability of the plasma membrane as an osmotic barrier and disrupt a number biosynthetic processes required in the membrane. Furthermore, nitrogen-containing compounds also exhibit an antifungal activity by disrupting cell membrane via inhibition of ergosterol biosynthesis (Freiesleben and Jäger, 2014).

However, the results from this study were only a preliminary study on possible mechanism of active fungal extracts. The true mechanism of active compounds should be further investigated.

4.4 Quorum sensing inhibition

The synthesis process of violacein purple pigment in *C. violaceum* is regulated by quorum sensing (QS) signal. Loss of purple pigmentation is indicative of inhibition of QS-controlled violacein production. Inhibition of violacein production using *C. violaceum* is widely used to assess the quorum sensing inhibitory activity (Chenia, 2013). The advantage of quorum sensing inhibition is neither kills the bacteria nor stops their growth and is less expected to develop resistance towards antibiotics (Abraham *et al.*, 2011). This test is a new alternative for antimicrobial discovery. In this study, only four extracts displayed anti-QS activity. AMF199BE, AMF231BE and AMF177BE at concentration 100 μg/disk inhibited violacein production, whereas AMF408BE showed inhibitory activity at 50 μg/disk. These results are similar to several studies regarding the inhibition of quorum sensing by natural products including marine-derived fungal extracts (Borges *et al.*, 2014; Burt *et al.*, 2014; Luís, *et al.*, 2016). Among them, only AMF231BE had antimicrobial activity against other tested microorganism (Table 16). Recently, secondary metabolites presenting anti-quorum sensing from marine-derived fungi were reported. Six compounds namely penicitor B, acelenes C-E and aspergillumarins A-B obtained from a fermentation broth of *Penicillium* sp. isolated from marine animal *Sipunculus nudus* exhibited strong anti-quorum sensing activity at 50 μg/well against *C. violaceum* CV026 by the well diffusion assay. At a non-inhibitory concentration, acelene E and aspergillumarins B at a concentration of 300 μM strongly reduced the

violacein production by up to 49.1% and 45.5%, respectively. In another study, kojic acid obtained from *Alternaria* sp. isolated from marine alga *Ulva pertusa* inhibited violacein production at MIC value of 239.25 µM. and reduced the bioluminescence of *E. coli* pSB401 at concentrations above 36 µM (Saurav *et al*., 2017). In addition, two mycotoxins, penicillic acid and patulin, produced by *Penicillium* sp. exhibited anti-quorum sensing activity of *P. aeruginosa* by regulating RhlR and LasR proteins. These proteins regulate virulence gene expression in *P. aeruginosa*. Furthermore, the study revealed that the *P. aeruginosa* biofilms formed in the presence of patulin were susceptible to tobramycin, while a significant tolerance was observed in control biofilms (Padder *et al*., 2018). Aspergillumarins A and B, kojic acid, penicillic acid, and patulin were also produced by some species of marine *Aspergillus* (Dong *et al*., 2010; Namikoshi *et al*., 2003; Li *et al*., 2012). AMF231BE and AMF408BE were from *Aspergillus* spp. and may contain these anti-QS compounds. Further investigation will be carried out.

Inhibition of quorum sensing system is one of potential targets for the control of bacterial biofilm infection. Wang *et al*. (2017) studied the potential anti-biofilm activity of marine-derived fungi against *S. aureus* biofilm. They found that emodin, citrinin, and secalonic acids B-D at the concentration of 12.5 µg/ml strongly reduced biofilm by ≥ 50%. The results from this study demonstrated that marine-derived fungi are important sources of quorum sensing inhibitors.

C. violaceum is a Gram-negative bacterium that widely distributed in natural environment. Recently, it has been reported as an environment opportunistic pathogen and also developed resistance to many antibiotics. (Batista and da Silva Neto, 2017; Yang and Li, 2011). In this study, five extracts, AMF122BE, AMF210BE, AMF277BE, AMF392BE and AMF408BE also inhibited the growth of *C. violaceum* (inhibition zones >8 mm, Table 18).

This study was the initial screening using the qualitative agar disk diffusion method. The quantitative assay of violacein production reduction in broth needs to be further determined.

4.5 Synergistic effects of extracts from marine-derived fungi and colistin against
A. baumannii

A. *baumannii* is a major cause of nosocomial pathogen worldwide. It can survive for long periods on hospital surfaces and medical equipment. In addition, it is able to develop resistance to multiple antibiotics and can cause serious infections in critically ill patients (Alsan and Klompas, 2010). Colistin is considered as one of the most important therapeutic options for treatment of *A. baumannii* infections. Colistin is a cationic cyclic polypeptide antibiotic belonging to the polymyxin antibiotics (polymyxin B and colistin). It is used for the treatment of several multidrug-resistant Gram-negative bacterial infections (Kassamali *et al*., 2015; Spapen *et al*., 2011). But unfortunately, the increase use of colistin has led to the emergence of resistance. Therefore, there is a need to develop alternative strategies to overcome this resistance problem. One alternative way is the combination therapy.

In this study, ten active extracts presenting anti-*A. baumannii* activity were further studied for their synergistic effect with colistin against *A. baumannii* NPRC005 using a broth microdilution checkerboard method. Two extracts, cell ethyl acetate extract from *T. longibrachiatum* AMF222 (AMF222CE) and broth ethyl acetate extract of *T. orientale* AMF409 (AMF409BE) exhibited the synergistic effects with FICI ranging from 0.25-0.5. The result correlated with many studies which determined the synergistic effects of colistin/ other old drugs combination (Soudeiha *et al*., 2017; Kaya *et al*., 2017; Wei and Yang, 2017), and colistin/ natural products combination (Boonyanugomol *et al*., 2017; Chi and Holo, 2018; Liktor-Busa *et al*., 2016). However, there is no report on the synergistic effect of colistin/ marine-derived fungal extracts against *A. baumannii*.

From the antimicrobial activity results, the MIC values of colistin against *A. baumannii* NPRC005 were in the range of 8-16 µg/ml. The susceptibility breakpoints of colistin for *A. baumannii* recommended by CLSI (2015) are MIC ≤ 2 µg/ml susceptible./ MIC ≥ 4 µg/ml resistant and MIC ≤ 2 µg/ml susceptible/ MIC >2 µg/ml resistant by EUCAST (2015). According to this breakpoint criteria, *A. baumannii* NPRC005 was resistant to colistin. After combination, AMF222BE

and AMF409BE effectively reduced the MIC of colistin in the synergistic combinations 4 to 8 times as compared with the MIC of colistin alone. These effective MIC of colistin in combination were below the susceptibility breakpoint. Therefore, these two extracts enhanced the susceptibility of *A. buamannii* to colistin. Furthermore, the toxicity of colistin is dose-dependent. Reducing colistin dosage will decrease its nephrotoxicity (Wei *et al*., 2017).

Subsequently, the combination concentrations with the lowest FICI result obtained from the checkerboard method were also confirmed by the time-kill assay. The colistin/ AMF222CE and AMF409BE combinations enhanced bactericidal activity of colistin (*A. baumannii*> 3 $\log_{10}$ CFU/ml reduction) after 2 h of incubation, whereas colistin and extracts alone at the same concentrations were unable to inhibit bacterial growth. This result correlated with Liu *et al* (2016) who studied the synergistic effect of meropenem combined with colistin against *A.baumannii*. The combination can decrease 3.8 $\log_{10}$ CFU/ml viable bacteria after 24 h. The results demonstrated that the synergistic effect of colistin in combination with these marine-derived fungal extracts against *A. baumannii* provided strong synergistic effects.

The studies on mechanism of action of synergistic combination of colistin and other drugs revealed that colistin can inhibit cell wall synthesis by decreasing the essential precursors and disrupting permeabilizing ability of bacterial outer membrane, permitting the entry of large hydrophobic molecules such as glycopeptides (Claeys *et al*., 2014; MacNair *et al*., 2018; Maifiah *et al*., 2017). From SEM studies, all active extracts tested showed possible effects on bacterial cell wall and cell membrane. These effects may enhance the activity of colistin resulting in cell death.

CHAPTER 5

CONCLUSIONS

A total of 547 isolates of marine-derived fungi were isolated from various marine organisms which collected from Phuket. The fungal isolation rate was 33 isolates/ organism. The highest fungal diversity was found in bryozoan. The great numbers of yielded fungi were in the genera *Aspergillus*, *Penicillium* and *Trichoderma*.

Selected marine-derived fungi were cultivated in PDB for 3 weeks at 25°C and extracted with ethyl acetate and hexane. Three types of extracts including broth ethyl acetate (BE), cell ethyl acetate (CE) and cell hexane (CH) extracts were obtained from each isolate. Fungal extracts were determined for their antimicrobial activity against nine human pathogens. The results showed that 219 out of 369 extracts (59.35%) exhibited inhibitory activity against a least one test strain. The extracts showed the most activity against *C. neoformans*. ATCC 90112 (35.77%) and *S. aureus* ATCC 25923 (35.50%) followed by MRSA SK-1 (27.10%), *M. gypseum* SH-MU4 (18.42%), *C. albicans* ATCC 90028 (10.02%), *T. marneffei* PSU-SKH1 (3.25%), *A. baumannii* NPRC AB005 (2.71%), *P. aeruginosa* ATCC 27853 (1.89%) and *E. coli* ATCC 25922 (1.08%). The MIC/MBC or MFC values ranged from 2-200/16->200 µg/ml.

Extracts showing strong inhibitory activity were further studied on possible mode of action by the SEM. The treated cells showed morphological changes and cell damage including deformation, collapsed, shrinkage, rough surfaces and broken cells when compared with untreated control cells (1% DMSO). The results illustrated that the extracts acted on cell membrane or cell wall of the test microorganisms leading to cell death.

In addition, the fungal extracts exhibited anti-quorum sensing activity at concentrations 50-100 µg/disk. Four extracts including AMF177BE, AMF199BE, AMF231BE and AMF480BE exhibited colorless pigment zone (violacein inhibition).

Extracts presenting anti-*A. baumannii* were determined for their synergistic effect in combination with colistin using a checkerboard method and time-kill assay. Two extracts, AMF222CE and AMF409BE, demonstrated synergistic effect with FICI ≤ 0.5. In time-kill assay, the combination of these extracts and colistin revealed > 3 $\log_{10}$ CFU/ml reduction in number of viable cells (bactericidal activity) compared with colistin alone. The time-kill assay results confirmed the checkerboard synergistic results of these extracts in combination with colistion against *A. baumannii*.

Morphological identification and phylogenetic analysis demonstrated that the 36 active marine-derived fungi presenting moderate to strong antimicrobial activity in this study belonged to two Phyla, Ascomycota and Basidiomycota. Thirty-five isolates were ascomycetes which were mostly distributed within three classes including Dothideomycetes, Eurotiomycetes and Sordariomycetes. Only one isolate belonged to the Basidiomycota (Agaricomycetes) and two isolates were unidentified.

The results from this study can be concluded that marine-derived fungi are a potential source of antimicrobial active metabolites.

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APPENDIX

Chemical

Normal saline solution (0.85% NSS)   100 ml

      NaCl   0.85g

      Distilled water   100 ml

      Autoclave at 121^oC/15 min, 15 pounds/ inch^2

McFarland Standard

No of McFarland	0.5	1	2	3	4	5	6	7	8	9	10
1% BaCl$_2$ (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
1% H$_2$SO$_4$ (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9.0
Approximate cell density (x10^8/ml)	1.5	3	6	9	12	15	18	21	24	27	30

Phosphate buffer solution (PBS) pH7

      0.2 M KH$_2$PO$_4$ 50 ml

      0.2 M NaOH   29.1 ml

     Adjust volume into 200 ml with distilled water and mix thoroughly.

1.8% Resazurin (stock solution)

      Resazurin   1.8 g

      Distilled water   100 ml

     Resazurin solution was sterilized via 0.45 µm Millipore filter and

     kept in the dark at 4^oC until used.

0.5 M NaOH   100 ml

      NaOH   2 g

      Distilled water   100 ml

1 M Tris-HCl pH8	100 ml

Tris-HCl (15.76 g.) was dissolved in distilled water, adjusted pH to 8 with NaOH solution and adjusted to final volume with distilled water.

50X TAE buffer	1000 ml
Tris base	242 g
0.5 M EDTA	100 ml
Glacial acetic acid	57.1 ml

Add distilled water to the final volume of 1000 ml and autoclave

6X Loading dye	250 ml
80% glycerol	93.6 ml
0.5 M EDTA	3 ml
Bromophenol blue	0.3 g
Xylene cyanol FF	0.3 g

Adjust with distilled water to final volume and mix thoroughly before storing at room temperature.

CTAB lysis buffer	100 ml
5 M NaCl	28 ml
10% CTAB	20 ml
5 M Tris-HCl	2 ml
0.5 M EDTA	5 ml

Add distilled water to the final volume of 100 ml and autoclave

5 M NaCl	100 ml
NaCl	29.22 g
Distilled water	100 ml

Autoclave at $121^{o}C$/15 min, 15 pounds/ $inch^{2}$

Media

Potato infusion	10 liters

> Boil 10 kilograms sliced unpeeled potatoes in 10 liters distilled water for 15 minutes. Filter through cheesecloth and collect effluent (potato infusion). Adjust to 10 liters with distilled water and Keep at -20°C until used.

Potato Dextrose Broth (PDB)	1000 ml
Potato infusion	200 ml
D-glucose	20 g
Distilled water	800 ml
70 % Seawater Potato Dextrose Agar (SPDA)	1000 ml
Potato infusion	200 ml
D-glucose	20 g
Agar	15 g
Seawater	700 ml
Distilled water	100 ml
Half strength Seawater Potato Dextrose Agar (HSPDA)	1000 ml
Potato infusion	100 ml
D-glucose	10 g
Agar	15 g
Seawater	700 ml
Distilled water	200 ml

*All media were autoclaved at 121°C/15 min, 15 pounds/inch^2

Table 1A Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF2CE	128/>200						128/>200		
AMF3CH	200/>200	128/>200				128/200	32/200	32/128	200/>200
AMF6BE	200/>200	128/>200							
AMF6CE	32/>200	128/>200					200/>200	200/>200	
AMF6CH	128/>200	128/128		200/>200			200/>200	128/>200	
AMF7CE	200/>200	200/>200							
AMF7CH	64/>200	200/>200						128/128	
AMF8CH								200/>200	
AMF11BE							200/>200		
AMF12BE							200/>200		
AMF12CE	128/>200						200/200		
AMF12CH	128/200	128/>200					200/200		
AMF14BE							200/200		
AMF14CE							200/>200		
AMF14CH							200/>200		
AMF17CE	128/>200							200/>200	
AMF17CH								200/>200	
AMF18BE	64/>200	128/>200					200/>200	200/>200	
AMF18CE	16/128	128/128					200/>200		
AMF18CH	32/64	128/128					200/>200		
AMF21BE	64/>200	64/>200							
AMF28BE	64/>200	128/>200					200/>200	200/>200	
AMF30BE	200/>200								

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF30CH	200/>200						200/>200	200/>200	
AMF41BE							200/>200		
AMF41CH							200/>200		
AMF45BE								200/>200	
AMF45CH	200/>200								
AMF46CH						200/200	64/200	64/64	128/128
AMF53BE						200/>200	128/>200		
AMF53CE						200/>200			
AMF61CE					200/>200				
AMF79CH	128/128	200/200			200/200				
AMF87BE	64/>200	128/128				200/>200	200/200	200/>200	
AMF87CE	128/>200								
AMF87CH	200/>200								
AMF89CE	64/64	128/128				200/>200	200/200		
AMF89CH	64/64	128/128					200/200		
AMF94CE	32/200								
AMF94CH		200/>200							
AMF95CE							200/>200		
AMF95CH							200/>200		
AMF115BE	128/>200	128/>200							
AMF116BE							128/>200		
AMF116CE	200/200								
AMF116CH	200/>200	200/>200					128/>200	128/>200	

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF117BE	200/200	128/>200					200/>200	200/200	
AMF117CE	64/>200	128/>200				200/>200		128/>200	
AMF117CH	64/128	128/128						200/>200	
AMF119BE							200/200		
AMF119CH	200/>200								
AMF122BE	128/>200	200/>200	200/>200	200/>200	128/128			200/>200	
AMF122CH	200/>200								
AMF124BE	200/>200	128/>200					200/>200	200/>200	
AMF124CE	128/>200	128/>200					200/>200	200/>200	
AMF124CH	200/>200	128/>200					32/>200	200/>200	
AMF127CE	200/>200						64/>200		
AMF127CH	200/>200						64/>200		
AMF130 CE							200/>200		
AMF131BE	200/>200	128/>200							
AMF131CE	32/>200	128/>200					200/>200		
AMF131CH	32/>200	128/128				200/>200	200/>200		
AMF138BE	128/>200	200/>200					200/>200	200/>200	
AMF141BE							200/200	200/>200	
AMF141CH	200/>200	200/>200							
AMF143CH	200/>200								
AMF144BE							200/200		
AMF144CH	64/128								
AMF160BE	200/>200	128/>200					200/>200		
AMF160CH							200/>200		

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF161BE						200/200	200/>200		
AMF161CE							200/>200		
AMF161CH	200/>200	128/>200					200/>200		
AMF166CH	200/>200						200/>200		
AMF169BE	64/128	64/128		200/>200	200/>200	200/>200		64/>200	
AMF169CE	64/>200	128/>200				128/>200	200/>200	128/>200	
AMF169CH	64/128	64/128				128/>200	200/>200		
AMF172CE	200/>200								
AMF172CH	200/>200								
AMF177BE	128/>200	128/128				200/>200	200/200	200/>200	
AMF177CE	128/>200	128/128							
AMF177CH	32/>200	128/128							
AMF182BE							200/>200		
AMF184CH	200/>200								
AMF185BE							200/>200	200/>200	
AMF185CH	200/>200								
AMF188CE								200/200	
AMF188CH							128/>200	128/200	
AMF192BE						8/>200	16/200	128/128	200/>200
AMF192CE	200/>200	200/>200				200/>200	64/200	200/>200	
AMF192CH	200/200	64/128				16/64	2/32	32/64	200/>200
AMF194CE	128/128								
AMF194CH	128/>200	200/>200							
AMF198BE								200/>200	

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF198CE	64/128	64/64							
AMF198CH	16/32	16/32				128/>200	32/200	16/16	
AMF199BE							200/>200	200/>200	
AMF203BE	64/200	128/>200					200/>200		
AMF203CH							200/>200		
AMF205BE	64/>200	128/>200					200/>200		
AMF205CE	64/>200	128/>200							
AMF205CH	128/>200	128/>200							
AMF207CE							200/>200		
AMF207CH						200/>200	200/200	200/>200	
AMF210BE							200/200		
AMF214CH							200/>200		
AMF222BE	16/32	128/200					200/>200		128/128
AMF222CE	16/32	64/64			200/>200	200/>200	200/>200		
AMF222CH						200/>200	200/200	128/128	
AMF225BE							200/>200		
AMF227BE		200/200					200/>200		
AMF227CE	200/>200	128/>200					200/>200		
AMF227CH		128/128					200/>200		
AMF228BE	128/200						200/ >200		
AMF228CH							200/ >200		
AMF229CH	64/64	200/>200							
AMF231BE	16/128	200/>200				200/>200		64/>200	
AMF 231CE	8/16	200/>200				64/>200		16/128	

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF231CH	4/16	200/>200				16/32		16/16	
AMF235CE	64/>200	128/128							
AMF235CH	128/>200								
AMF238BE							200/>200		
AMF238CE	200/>200	128/>200			32/>200	200/200	200/200	128/>200	
AMF241 BE						200/>200	200/>200		
AMF241 CE						200/>200	200/>200		
AMF241 CH							200/200		
AMF242BE							200/>200		
AMF242CE	200/>200								
AMF242CH							200/200		
AMF243CE							200/>200		
AMF243CH							200/>200		
AMF244CE							200/>200		
AMF244CH		200/>200					200/200	200/>200	
AMF247CE							200/>200		
AMF247CH							200/>200		
AMF248BE	200/>200							200/>200	
AMF248CE	128/200							200/>200	
AMF248CH	128/>200	200/>200							
AMF250CE							200/>200		
AMF250CH							200/>200		
AMF253BE							200/>200		
AMF253CE		200/>200							

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF253CH	128/>200								
AMF271BE	200/>200	128/>200		200/>200			200/>200		
AMF271CE	200/>200	128/>200							
AMF273BE	32/128	128/>200					200/>200		
AMF273CE	32/64	128/128					200/>200	200/>200	
AMF273CH	128/200	128/128				200/200	200/200	64/>200	
AMF274BE	32/64	128/128				200/>200	64/>200	200/>200	200/200
AMF274CE	32/>200	128/128				32/>200	200/>200		200/200
AMF274CH								200/>200	
AMF277BE	32/200	64/200	32/128	64/128	32/128		128/200		
AMF277CE							200/>200		
AMF277CH							200/200	128/>200	200/>200
AMF285CE	200/>200	200/>200							
AMF290CH		200/>200						200/>200	
AMF292BE	64/200	64/>200			200/>200		128/200	128/>200	200/200
AMF292CE	200/>200								
AMF292CH	200/>200	200/>200					128/>200	128/200	
AMF293CE						200/200			
AMF294BE				200/>200			200/>200	200/>200	
AMF294CE				200/>200		200/>200	200/>200	200/>200	
AMF294CH							200/>200		
AMF295BE	200/>200	200/>200					200/>200		
AMF295CE							200/>200		
AMF295CH							200/>200		

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF300BE							200/>200		
AMF300CE							200/>200		
AMF300CH	200/>200						200/200	200/>200	
AMF304CE						200/>200			
AMF322BE		128/>200			200/>200				
AMF322CE							200/>200		
AMF322CH	128/>200	128/128					200/>200		
AMF323CE							200/>200		
AMF323CH							200/>200		
AMF344CE							200/>200		
AMF346BE						200/>200			
AMF346CE	200/>200								
AMF346CH	128/>200	200/>200						200/>200	
AMF347BE	200/>200	200/>200							
AMF347CH	64/>200	64/>200					128/>200	64/128	200/>200
AMF350BE									200/>200
AMF350CH							200/>200		
AMF358CH							200/>200		
AMF360BE	32/64	200/>200				200/>200			
AMF360CE	16/32	200/>200							
AMF360CH	64/>200	200/>200						200/>200	
AMF366BE	64/>200	64/>200					200/>200		
AMF366CE	16/>200	32/>200							
AMF366CH	128/>200	128/128							

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF368CH	64/>200								
AMF376CE						200/>200	200/>200		
AMF376CH							200/200	200/>200	
AMF384BE	200/>200	200/>200							
AMF384CH	200/>200								
AMF398BE	128/>200	200/>200					200/>200	200/>200	
AMF409BE	16/32	64/128			200/>200		128/200		128/128
AMF409CE	32/200	200/>200					200/>200		
AMF409CH	128/128	128/128					200/200	200/>200	
AMF413BE								128/>200	
AMF413CH	128/>200	200/>200	200/>200					64/>200	
AMF417CE							128/>200		
AMF419CE							200/>200		
AMF420BE	128/200	128/128					200/>200	200/200	
AMF420CE	128/>200	128/>200				16/32	128/200	64/64	
AMF420CH	128/>200	128/128				16/32	64/200	64/64	
AMF422CH		200/>200							
AMF426CH	200/>200	200/>200							
AMF450BE	128/128	200/>200					200/>200	200/>200	
AMF450CE	200/>200								
AMF450CH							200/>200		
AMF455CE	32/>200	200/>200					64/128		
AMF455CH	200/>200								
AMF456BE	128/>200	200/>200						200/>200	

Table 1A (Cont.) Fungal extracts presenting antimicrobial activity

Active extracts	MIC/MBC or MFC (µg/ml)								
	SA	MRSA	EC	PA	AB 005	CA 90028	CN 90112	MG	TM
AMF456CE	200/>200								
AMF458CE	32/>200								
AMF475CE	128/>200								
AMF480BE			200/200						
AMF480CE	200/>200								

SA = *Staphylococcus aureus* ATCC 25923   MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922   PA = *Pseudomonas aeruginosa* ATCC 27853

AB005= Multidrug-resistant *Acinetobacter baumannii* NPRC AB005   CA = *Candida albicans* ATCC 90028

CN = *Cryptococcus neoformans* ATCC 90112   MG = *Microsporum gypseum* SH-MU4

TM = *Talaromyces marneffei* PSU-SKH1

Table 2A Identification of marine derived fungi presenting interesting H^1 NMR profile by molecular method obtained from BIOTEC

Phylum	Class	Order	AMF code	BCC code	region	Accession number	Expected species
Ascomycota	Dothideomycetes	Capnodiales	AMF141	86497	ITS	MH398540	*Cladosporium tenuissinum*
					LSU	MH398553	
		Pleosporales	AMF392	86504	ITS	MH398547	*Bipolaris* sp.
					LSU	MH398559	
			AMF116	88175	ITS	MK005256	*Montagnula* sp.
					LSU	MH997858	
			AMF200	88176	ITS	MK005257	*Neopyrenochaetatelephoni*
					LSU	MH997859	
			AMF188	86500	ITS	MH398543	*Periconia* sp.
					LSU	MH398556	
			AMF190	86513	ITS	MH400413	*Pleosporales* sp.
					LSU	MH398571	
			AMF350	84332	ITS	MF919624	*Pseudopithomyces maydicus*
					LSU	MF919633	
	Eurotiomycetes	Eurotiales	AMF41	84331	ITS	MF780703	*Aspergillus* sp.
					BT	MG003437	
			AMF79	84321	ITS	MF780705	*Aspergillus chevalieri*
			AMF243	84315	LSU	MF780713	*Aspergillus fumigatus*
			AMF315	86520	ITS	MH400418	*Aspergillus niger*
					LSU	MH398578	

Table 2A (Cont.) Identification of marine derived fungi presenting interesting H^1 NMR profile by molecular method obtained from BIOTEC

Phylum	Class	Order	AMF code	BCC code	Region	Accession number	Expected species
			AMF207	84312	LSU	MF919636	*Aspergillus nomius*
					BT	MG003438	
			AMF322	84320	ITS	MF780710	*Aspergillus pseudonomius*
			AMF293	86519	LSU	MH398577	*Eupenicillium* sp.
			AMF228	86501	ITS	MH398544	*Hyphodontia flavipora*
			AMF140	84310	ITS	MF780707	*Paecilomyces* sp.
			AMF30	86495	ITS	MF398535	*Penicillium citrinum*
					LSU	MH398532	
			AMF242	84314	LSU	MF919637	*Penicillium javanicum*
					BT	MG003439	
			AMF285	86516	ITS	MH400415	*Penicillium sclerotiorum*
					LSU	MH398574	
			AMF289	86517	ITS	MH400416	*Talaromyces* sp.
					LSU	MH398575	
			AMF247	84316	LSU	MF919632	*Talaromyces trachyspermus*
					BT	MG003440	
			AMF290	86518	ITS	MH400417	*Talaromyces verruculos*
					LSU	MH398576	
	Sordariomycetes	Hypocreales	AMF166	84311	ITS	MF780708	*Acremonium citrinum*

Table 2A (Cont.) Identification of marine derived fungi presenting interesting H[1] NMR profile by molecular method obtained from BIOTEC

Phylum	Class	Order	AMF code	BCC code	region	Accession number	Expected species
			AMF346	86521	ITS	MH398579	*Nectria ipomoeae*
					LSU	MH398579	
			AMF53	84307	ITS	MF780704	*Trichoderma asperellum*
					LSU	MF780711	
			AMF456	86505	ITS	MH398548	*Trichoderma asperellum*
					LSU	MH398560	
			AMF2	86491	ITS	MH398537	*Trichoderma harzianum*
					LSU	MH398550	
			AMF8	86494	ITS	MH398539	*Trichoderma harzinum*
					LSU	MH398552	
			AMF12	84306	ITS	MF537634	*Trichoderma harzianum*
			AMF474	86506	ITS	MH398549	*Trichoderma harzianum*
					LSU	MH398561	
			AMF300	84319	ITS	MF537640	*Trichoderma ressei*
		Xylariales	AMF45	84331	ITS	MF780703	*Pseudopestalotiopsis* sp.
		Sordariales	AMF115	86512	ITS	MH400412	*Pleosporales* sp.
					LSU	MH400412	
		Glomerellales	AMF194	86515	LSU	MH398573	*Plectosphaerella cucumerica*

BCC: BIOTEC culture collection   (I): ITS sequence   (L): LSU sequence   (BT): beta-tubulin sequence

Table 3A % sequence identity of AMF222

	AMF222	TB UTHSC 12-337	TB UTHSC 07-2998	TB UTHSC 09-2160	TA PPRC-J11	TL UAMH 7955	TL UAMH 7956	TL ATCC201044	TL TYPE	TL ATCC 38586
AMF222	**ID**	99.8	99.8	99.6	99.8	99.8	99.8	100	100	100
TB UTHSC 12-337	99.8	**ID**	100	99.8	100	100	100	99.8	99.8	99.8
TB UTHSC 07-2998	99.8	100	**ID**	99.8	100	100	100	99.8	99.8	99.8
TB UTHSC 09-2160	99.6	99.8	99.8	**ID**	99.8	99.8	99.8	99.6	99.6	99.6
TA PPRC-J11	99.8	100	100	99.8	**ID**	100	100	99.8	99.8	99.8
TL UAMH 7955	99.8	100	100	99.8	100	**ID**	100	99.8	99.8	99.8
TL UAMH 7956	99.8	100	100	99.8	100	100	**ID**	99.8	99.8	99.8
TL ATCC201044	100	99.8	99.8	99.6	99.8	99.8	99.8	**ID**	100	100
TL TYPE	100	99.8	99.8	99.6	99.8	99.8	99.8	100	**ID**	100
TL ATCC 38586	100	99.8	99.8	99.6	99.8	99.8	99.8	100	100	**ID**

TB= *Trichoderma bissettii* TA= *Trichoderma arethiopicum* TL= *Trichoderma longibrachiatum* ID= Identity

Table 4A % sequence identity of AMF409

	AMF409	*T. orientale* TUB F-837	*T. orientale* S187	*T. orientale* PPRI 3894	*T. orientale* TYPE	*T. orientale* G.J.S. 91-157
AMF409	**ID**	100	99.8	99.8	99.8	99.8
T. orientale TUB F-837	100	**ID**	99.8	99.8	99.8	99.8
T. orientale S187	99.8	99.8	**ID**	99.6	99.6	99.6
T. orientale PPRI 3894	99.8	99.8	99.6	**ID**	99.6	100
T. orientale TYPE	99.8	99.8	99.6	99.6	**ID**	99.6
T. orientale G.J.S. 91-157	99.8	99.8	99.6	100	99.6	**ID**

ID= Identity

Table 5A Marine-derived fungi code and isolation information

Fungal Code	Isolation Date	Isolation medium	Common name	Scientific name	Figure
AMF79-AMF121	10/07/15	SPDA+ antibiotic	Ascidian	*Phallusia nigra*	19A
AMF365-AMF375	21/07/15	SPDA+ antibiotic		Family Botryllidae	19B
AMF433-AMF446	16/09/15	SPDA+ antibiotic		Family Pyuridae	19C
AMF447-AMF465	16/09/15	SPDA+ antibiotic		Family Didemnidae	19D
AMF1-AMF52	21/07/15	SPDA+ antibiotic		Unidentified ascidian #1	19E
AMF53-AMF78	21/07/15	SPDA+ antibiotic		Unidentified ascidian #2	19F
AMF122-AMF165	10/07/15	SPDA+ antibiotic		Unidentified ascidian #3	19G
AMF376-AMF404	10/07/15	SPDA+ antibiotic		Unidentified ascidian #4	19H
AMF466-AMF480	16/09/15	SPDA+ antibiotic		Unidentified ascidian #5	19I
AMF210-AMF239	26/01/16	RBC	Coral	*Carijoa riisei*	20A
AMF166-AMF209	10/07/15	SPDA+ antibiotic	Sea fan	*Menella* sp.	20B
AMF322-AMF350	10/07/15	SPDA+ antibiotic	Bryozoan	*Schizoporella* sp.	20C
AMF259-AMF321	26/01/16	RBC		*Amathia verticillatum*	20D
AMF240-AMF258	26/01/16	RBC	Alga	*Padina* sp.	20E
AMF351-AMF364	10/07/15	SPDA+ antibiotic	Sea cucumber	Unidentified sea cucumber	20F
AMF405-AMF408	10/07/15	SPDA+ antibiotic	Sponge	Unidentified sponge #1	20G
AMF409-AMF432	10/07/15	SPDA+ antibiotic		Unidentified sponge #2	20H

SPDA+ antibiotic 70% = seawater half strength potato dextrose agar (SPDA) with penicillin and streptomycin   RBC = Rose Bengal Chloramphenicol agar
Collection Site: Phuket, Thailand; **Collection Date**: 9 July 2015

VITAE

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Educational Attainment

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Scholarship Awards during Enrolment

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- Centre of Excellence for Innovation in Chemistry (PERCH-CIC)

List of Publication or Proceeding

Manmana S., Phongpaichit S., Rukachaisirikul V. 2017. Antimicrobial Activity of Marine-derived *Trichoderma* spp. Isolated from Ascidians. Proceedings of the 3rd NIRC, the 45th National and 8th International Graduate Research Conference. Nakhon Ratchasima Rajabhat University, December 2-3, 2017. pp. 32-41.