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TITLE: Antagonistic and Antimycelial Growth Activities of Marine-Derived Fungi Against Plant Pathogenic Fungi and Secondary Metabolites of *Talaromyces trachyspermus*

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THESIS

ANTAGONISTIC AND ANTIMYCELIAL GROWTH ACTIVITIES OF MARINE-DERIVED FUNGI AGAINST PLANT PATHOGENIC FUNGI AND SECONDARY METABOLITES OF *TALAROMYCES TRACHYSPERMUS*

DECHA KUMLA

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Decha Kumla 2014: Antagonistic and Antimycelial Growth Activities of Marine-Derived Fungi Against Plant Pathogenic Fungi and Secondary Metabolites of *Talaromyces trachyspermus.* Master of Science (Plant Pathology), Major Field: Plant Pathology, Department of Plant Pathology. Thesis Advisor: Assistant Professor Tida Dethoup, Ph.D. 115 pages.

A total of 210 strains of marine-derived fungi were isolated from 36 samples of marine invertebrates (sponges and corals), collected from coral reefs at Similan Islands (Phang Nga Province), Lanta Islands **(**Krabi Province), and Kram Island (Chonburi Province), Thailand, and 36 species have been identified as *Arthrinium* sp., *Aspergillus candidus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus* spp., *Chaetomium* spp., *Cladosporium* spp., *Emericella nidulans*, *Emericella variecolor*, *Emericella* spp., *Eupenicillium* spp., *Fusarium solani*, *Fusarium* sp., *Hamigera* sp., *Humicola* sp., *Lasiodiphodia* spp., *Mucor hiemalis*, *Mucor* sp., *Neosartorya fischeri*, *Neosartorya pseudofischeri*, *Neosartorya* sp., *Paecilomyces lilacinus*, *Paecilomyces* spp., *Penicillium* spp., *Pestalotiopsis* spp., *Phoma* sp., *Phomopsis* spp., *Pseudoeurotium* sp., *Rhizopus* sp., *Scolecobasidium* sp., *Syncephalastrum* sp., *Talaromyces trachyspermus*, *Trichocladium* sp., *Trichoderma opacum*, *Trichoderma* spp., *Xylaria* spp. and sterile mycelia.

Among these marine-derived fungi, twelve isolates were subjected to preliminary screening for the antagonistic activity against ten plant pathogenic fungi by a dual culture. The results revealed that *E. variecolor* (KUFA 0103) and *N. pseudofischeri* (KUFA 0108) displayed relevant inhibitory activities against the mycelial growth of *Helminthosporium maydis*, while *E. variecolor* (KUFA 0103) exhibited also strong mycelial growth inhibition against *Alternaria brassicicola*. On the other hand, *E. nidulans* (KUFA 0102), *Emericella* spp. (KUFA 0104 and KUFA 0105), *N. pseudofischeri* (KUFA 0108) and *Neosartorya* sp. (KUFA 0109) displayed moderate inhibitory activity (50%) against *Colletotrichum capsici*, but were inactive against *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. Furthermore, *E. nidulans* (KUFA 0102), *Emericella* sp. (KUFA 0104), *N. pseudofischeri* (KUFA 0108) and *Neosartorya* sp. (KUFA 0109) were able to cause 50-60% inhibition of the mycelial growth of *Phytophthora palmivora*. However, all the marine-derived fungi tested were found to be inactive against the mycelial growth of *Pythium aphanidermatum* and the two Agonomycetous plant pathogenic fungi, *Rhizoctonia solani* and *Sclerotium rolfsii*.

In vitro antifungal activity evaluation of the EtOAc crude extracts of the culture of six marine-derived fungi against plant pathogenic fungi revealed that *T. trachyspermus* (KUFA 0021) extract was the most effective inhibitor of the mycelial growth in most of the plant pathogenic fungi. Moreover, the EtOAc crude extracts of *N. fischeri* (KUFA 0107), *Hamigera* sp. (KUFA 0106), *Pseudoeurotium* sp. (KUFA 0110), *N. pseudofischeri* (KUFA 0108) and *Emericella* sp. (KUFA 0104), displayed relevant antifungal properties against the selected plant pathogenic fungi. The EtOAc crude extract of *T. trachyspermus* (KUFA 0021) was found to completely inhibit the mycelial growth of *A. brassicicola*, *C. capsici*, *H. maydis*, *P. aphanidermatum*, *R. solani* and *S. rolfsii* at 1,000 ppm, and displayed total inhibition of mycelial growth on all plant pathogenic fungi at the highest concentration tested (10,000 ppm). Interestingly, this extract was still effective on the mycelial growth inhibition of *P. aphanidermatum* even at the concentration as low as 100 ppm. Chemical analysis of the EtOAc crude extract of the culture of *T. trachyspermus* (KUFA 0021) resulted in the isolation of, besides a new spiculisporic acid derivative, spiculisporic acid E and the new natural product 3-acetyl ergosterol 5, 8-endoperoxide, ergosta-4,6,8(14),22-tetraen-3-one, glaucanic acid and glauconic acid. All the compounds were tested for the antibacterial activity, and it was found that none of them was active against Gram-positive and Gram-negative bacteria, including multidrug-resistant strains and *Candida albicans*. Spiculisporic acid E, glaucanic acid and glauconic acid did not also show an *in vitro* growth inhibitory activity against the MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines. Moreover, spiculisporic acid E, glaucanic and glauconic acids were also found to be inactive on the mycelial growth inhibition of plant pathogenic fungi.

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Decha Kumla

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LIST OF ABBREVIATIONS

cm	=	centimeter
^{13}C NMR	=	carbon-13 Nuclear Magnetic Resonance
COSY	=	correlation spectroscopy (in NMR)
EtOAc	=	ethyl acetate
EtOH	=	ethanol
g	=	gram
GI	=	gause I agar
G1	=	colony radius of plant pathogenic fungi in the control
G2	=	colony radius of plant pathogenic fungi in the dual culture test
GPY	=	glucose peptone yeast extract agar
HMBC	=	heteronuclear multiple bond correlation spectroscopy
^{1}H NMR	=	proton nuclear magnetic resonance
HPLC	=	high performance liquid chromatography
HRMS	=	high resolution mass spectrometry
HSQC	=	heteronuclear single quantum coherence
IC_{50}	=	concentration of sample required to inhibit growth by 50%
KUFA	=	Kasetsart University Faculty of Agriculture
MEA	=	malt extract agar
ml	=	milliliter
µl	=	micrometer or micron
NaCl	=	sodium chloride
Na_2SO_4	=	sodium sulfate
nm	=	nanometer
NMR	=	nuclear magnetic resonance
No.	=	number
PDA	=	photodiode array
PDB	=	potato dextrose broth
Petrol	=	petroleum ether

LIST OF ABBREVIATIONS (Continued)

pH	=	potential of Hydrogen ion
ppm	=	parts per million
SEM	=	scanning electron microscopy
TLC	=	thin layer chromatography
UV	=	ultra violet

ANTAGONISTIC AND ANTIMYCELIAL GROWTH ACTIVITIES OF MARINE-DERIVED FUNGI AGAINST PLANT PATHOGENIC FUNGI AND SECONDARY METABOLITES OF *TALAROMYCES TRACHYSPERMUS*

INTRODUCTION

Marine invertebrates are the richest source of bioactive metabolites with potential for the development of new medicines and agrochemicals. They are also the major hosts of symbiotic microorganisms such as actinomyces, bacteria and fungi. Marine-derived fungi are often associated with marine organisms and substrata such as sponges, corals, tunicates, higher algae, sea grasses, mangroves, molluscs, woody substrates, driftwoods and sediments (Devarajan *et al*., 2002).

Several marine fungal species, as well as the products of their secondary metabolism, have been reported for their antibacterial and antifungal properties. For example, the new antifungal agent YM-202204, isolated from the culture broth of the marine fungus *Phoma* sp. Q60596, exhibited potent antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Talaroconvulutins B and C, isolated from the marine fungus *Talaromyces convolutes*, exhibited also relevant antifungal activity, inhibiting the growth of the human pathogenic fungi *A. fumigatus, A. niger* and *C. albicans* (Suzuki *et al*., 2000). From the marine fungus *Nigrospora* sp. PSU-F18, collected from *Annella* sp. at Similan Islands, Thailand, four new pyrones have been reported. Nigrosporapyrones A-D exhibited strong antibacterial activity against *Staphylococcus aureus* ATCC 25923 (Trisuwan *et al*., 2009).

Marine sponge-associated fungi have been also reported for their antagonistic activity against plant pathogenic fungi (Dethoup *et al*., 2009; Manoch *et al.*, 2009; Rongbian *et al.*, 2009; Buaruang *et al.*, 2010; Shen *et al.*, 2014). For example, the marine fungi *Emericella variecolor*, *Nodulisporium* sp., *Chaetomium globosum* and *Penicillium* sp. were found to inhibit the mycelial growth of *Alternaria alternata*,

Colletotrichum capcisi, *Fusarium oxysporum*, *Helminthosporium oryzae* and *Phytophthora palmivora* (Dethoup *et al*., 2009). In other study from our research group, *E.variecolor*, *Eurotium cristatum*, *Curvularia lunata*, *Cladobotyum varium* and *Acremonium* sp. extracts were also found to inhibit the mycelial growth of *Curvularia lunata Colletotrichum gloeosporioides*, *Rhizoctonia solani* and *Alternaria alternata* (Manoch *et al.*, 2009).

In addition to the chemical diversity and potential for the development of new drugs and agrochemicals, it is also relevant to emphasize the fungal diversity from marine invertebrates, namely those collected from Thai waters.

For example, Suetrong *et al*. (2007) reported several fungal strains from marine sources collected in Thailand coastal areas. Ten new records of marine fungi have been reported from driftwood and attached decaying mangrove wood collected from central, eastern and southern Thailand including *Aigialus* cf. *mangrovel*, *Dendryphiella arenaria*, *Lindra thallasiae*, *Mycosphaerella avicenniae*, *Manglicola guatemalensis*, *Patellaria* sp., *Pontoporiae* sp., *Sporomiella* sp., *Swampomyces aegyptiacus* and *Varicosporina prolifica.* However, there are several additional reports also focusing on marine-derived fungal diversity from marine invertebrates collected from Thai waters (Dethoup *et al*., 2009; Manoch *et al.*, 2009; Preedanon *et al*., 2009; Antia *et al.*, 2010; Buaruang *et al.*, 2010; Pinheiro *et al*., 2012; Prompanya *et al.*, 2014).

The main purpose of this study was the identification of marine invertebrate-associated fungi collected from Thai waters, as potential sources for the development of new antifungal agents to control phytopathogenic fungi. The preliminary antagonistic activity screening revealed the existence of several promising marine-derived fungi (Dual culture method), and a subsequent evaluation of the antifungal effect of EtOAc crude extracts (Dilution plate method) led us to the identification and selection of the most active extract for further chemical analysis. Additionally, we also characterized and identified marine invertebrate-associated fungi, collected from different locations.

OBJECTIVES

1. To isolate and identify marine invertebrate-associated fungi.

2. To study the *in vitro* antagonistic activity of selected marine-derived fungal extracts against ten species of plant pathogenic fungi.

3. To study the antifungal activity of crude extracts of six marine-derived fungi against ten species of plant pathogenic fungi *in vitro*.

4. To investigate the secondary metabolites from the marine-derived fungus *Talaromyces trachyspermus*.

LITERATURE REVIEW

1. Marine Invertebrate-Associated Fungi

Marine invertebrates are undoubtedly the richest source of bioactive metabolites, many of which revealing a great potential for the development of new drug candidates. Additionally, marine invertebrates are also hosts of symbiotic microorganisms such as fungi and bacteria, classified as a prolific source of biologically relevant secondary metabolites, being often the true metabolic producers. Symbionts can be located both intra- and extra-cellularly (Figure 1), and apparently, there is a specific habitat in the host sponge for some of their associated microflora. Extracellular symbionts are located on the outer layers of the host sponge as exosymbionts or in the mesophyll as endosymbionts, while intracellular or intranuclear symbionts permanently reside in host cells or nuclei (Lee *et al.*, 2001).

Marine sponge surfaces and internal tissues are more nutrient-rich than seawater and sediments, thus providing nutrient nourishment to their symbionts, in addition to physical protection. On the other hand, symbiotic microorganisms provide support in the nutritional assumption, beneficial to the host sponge either by intracellular digestion or by translocation of metabolites including nitrogen fixation, nitrification and photosynthesis.

Most of the identified marine fungal species have been previously reported also from terrestrial sources (Holler *et al*., 2000), however there are also several reports dealing with the association between fungi and marine macroorganisms. According to the kind of association with the host sponge, Li and Wang (2009) classified sponge-derived fungi into three groups comprising sponge-generalists, sponge-associations and sponge-specialists. On the other hand, Kohlmeyer and Kohlmeyer (1997) classified marine fungi into two distinct groups: “Obligate marine fungi” are those that grow and sporulate exclusively in a marine or estuarine habitat while “facultative marine fungi” are defined as “fungi from freshwater or terrestrial areas also able to grow in the natural marine environment”.

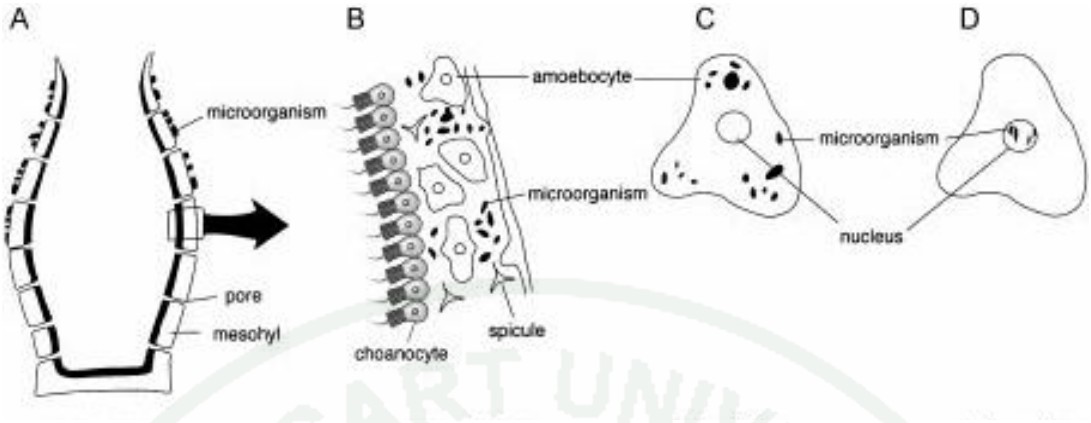


Figure 1 Schematic diagram of symbiotic relationships between sponges and microorganisms;
A. extracellular exosymbiosis B. extracellular endosymbiosis
C. intracellular symbiosis D. intranuclear symbiosis

Source: Lee *et al.* (2001)

2. Fungal Diversity from Marine Invertebrate-Associates

Despite the high biodiversity from tropical marine ecosystems, additional several reports are found in literature dealing with the characterization of fungal communities associated with invertebrates collected worldwide. In the following section an overview on reports published after the year of 2000 will be presented.

Holler *et al.* (2000) characterized the fungal communities isolated from sixteen marine sponges collected at different locations: *Biemma* sp., *Callyspongia* sp. and *Leucosolenia challenger* collected at the Pelorous Island, Great Barrier Reef, Australia; *Callyspongia vaginalis*, *Ectyplasia perox* and *Neofibularia nolitangere* collected at the Lauro Club Reef, Dominica; *Halichondria panacea, Myxilla incrustans, Leucosolenia* sp., and *Sycon* sp. collected at the Helgoland, Germany; *Ircinia oros* and *Ircinia variabilis* collected at Malta; *Aplysina aerophoba*, *Petrosia ficiformis* and *Oscarella lobularis* collected at the Tenerife, Spain. A total of eighty-nine fungal isolates were identified as belonging to 1) Ascomycota: *Chaetomium* sp.,

Emericella sp., *Emericellopsis* sp., *Eupenicillium* sp., *Eurotium* sp., *Leptosphaeria* sp., *Microascus* sp., *Monascus* sp., *Myxotrichum* sp., *Niesslia* sp., *Preussia* sp., *Sporormiella* sp. and *Talaromyces* sp.; 2) Zygomycota: *Mucor* sp. and *Syncephalastrum* sp.; 3) Mitosporic fungi: *Acremonium* sp., *Alternaria* sp., *Aplosporella* sp., *Arthrinium* sp., *Aspergillus* sp., *Asteromyces* sp., *Beauveria* sp., *Botrytis* sp., *Cladosporium* sp., *Coniothyrium* sp., *Doratomyces* sp., *Drechslera* sp., *Epicoccum* sp., *Fusarium* sp., *Geomyces* sp., *Geotrichum* sp., *Gliocladium* sp., *Gonatobotrys* sp., *Microsphaeropsis* sp., *Moniliella* sp., *Monochaetia* sp., *Myrothecium* sp., *Oidiodendron* sp., *Paecilomyces* sp., *Penicillium* sp., *Phialophorophoma* sp., *Phoma* sp., *Scolecobasidium* sp., *Scopulariopsis* sp., *Sporothrix* sp., *Stachybotrys* sp., *Stachylidium* sp., *Tolypocladium* sp., *Trichoderma* sp., *Ulocladium* sp., *Verticillium* sp. and thirty-seven additional strains of sterile mycelia.

A total of fifty fungal isolates were obtained from nine sponge species (*Subergorgia suberosa, S. mollis, Junceella* sp., *J. gemmacea, Ctenocella* sp., *C.* cf *umbraculum, Euplexaura* cf *pinnata* and two *Echinogorgia* sp.). The fungi species were identified as *Acremonium aculeatus, A. butryi, A. cervinus, A. ficuum, A. flavus, A. foetidus* var. *pallidus, A. furcatum, A. kangawensis, A. nutans, A. ochraceus, A. ornatus, A. pulverulentus, A. strictum, A. terricola, A. wentii, Chaetophoma* sp., *Cladosporium musae, C. sphaerospermum, Fusarium* sp., *Gliomastix cerealis, G. luzulae, G. murorum, Hymenula* sp., *Microascus triganosporus, Oidiodendron griseum, Penicillium brevicompactum, P. camemberti, P. canescens, P. citrinum, P. decumbens, P. frequentans, P. implicatum, P. janthinellum, P. lanoso, P. lilacinum, P. notatum, P. oxalicum, P. steckii, Phoma*-like, *Scolecobasidium humicola, Sporotrichum* sp., *Stibella* sp., *Trichoderma hamatum, T. harzianum, T. koningii, T. longibrachiatum, T. pseduokoningii, Tritirachium* sp., *Verticillium* sp. and *Virgaria* sp. (Koh *et al*., 2000).

From a marine coral identified as *Porites lutea,* collected at Lakshadweep islands, Arabian Sea, seven fungal species were reported and identified as *Acremonium* sp., *Aspergillus* sp., *Aureobasidium* sp., *Cladosporium* sp., *Chaetomium*

sp., *Fusarium* sp., *Labyrinthula* sp. (Ravindran *et al*., 2001). Later, Bringmann *et al.* (2002) reported the isolation of an *Emericella variecolor* strain isolated from the marine sponge *Haliclona valliculata*, collected at Secca di Capo di Fonze, Italy.

Morrison-Gardiner (2002) reported several marine fungi isolates including *Absidia* spp., *Alternaria* sp., *Aspergillus* sp., *Beauveria* spp., *Dreschlera* spp., *Humicola* spp., *Monilia* spp., *Oidiodendron* spp., *Penicillium* spp., *Pestalotiopsis* sp., *Phialophora* spp., *Phoma* spp., *Torulomyces* spp. and an unidentified fungus, from marine coral species collected at the Great Barrier Reef, Australia.

Twenty-seven genera of fungal isolates were obtained from an unidentified sponge collected in Australia, including *Absidia* spp., *Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Chrysosporium* sp., *Cirrenalia* sp., *Cladosporium* spp., *Curvularia* sp., *Cylindrocarpon* sp., *Dactlyosporium* sp., *Dreschlera* sp., *Epicoccum* sp., *Fusarium* sp., *Gaeumannomyces* sp., *Gonatobotryum* sp., *Humicola* spp., *Monilia* sp., *Mucor* spp., *Nigrospora* sp., *Pestolotiopsis* sp., *Penicillium* sp., *Torulomyces* spp., *Tritirachium* sp., *Verticillium* sp., *Wardomyces* sp., *Zalerion* spp. and *Zygosporium* spp. (Sarah, 2002).

Pivkin *et al*. (2005) reported a total of one hundred fungal species isolated from six marine sponges identified as *Amphilectus digitata*, *Halichondria panicea*, *Homaxinella subdola*, *Hymeniacidon assimilis*, *Myxilla incrustans* and *Phakettia cribrosa,* collected at Southern Coast of Sakhalin Island (Table 1).

Table 1 Fungal species isolated from marine sponge samples collected at Southern Coast of Sakhalin Island.

Sponge	Fungi
Amphilectus digitata	*Acremonium roseum, A. hyalinulum, Aspergillus fumigatus* var. *griseobrunneus, A. oryzae, A. varians, A. versicolor, Chaetomium globosum, Chaetomium* spp., *Cladosporium atroseptum, C. cladosporioides, C. brevicompactum, C. sphaerospermum, Eurotium repens, Penicillium adametzioides, P. aurantiogriseum, P. brevicompactum, P. camemberti, P. chrysogenum, P. corylophilum, P. janthinellum, P. simplicissimum, P. verrucosum* and *Periconia prolifica*
Halichondria panacea	*Acremonium fusidioides, A. minutisporum, Aspergillus chevalieri, A. flavus, A. oryzae, A. varians, A. versicolor, Ascotricha chartarum, Chaetomium globosum, Cladosporium atroseptum, Eupenicillium zonatum, Eurotium amstelodami, E. repens, Fusarium* sp., *Humicola fuscoatra, Microascus* spp., *Monodictys pelagica, Myceliophthora* sp., *Myrothecium roridum, Penicillium aurantiogriseum, P. brevicompactum, P. chrysogenum, P. citrinum, P. crustosum, P. olsonii, P. verrucosum, Pseudoeurotium zonatum, Periconia prolifica, Phoma* sp., *Trichoderma aureoviride* and sterile mycelium
Homaxinella subdola	*Acremonium fusidioides, A. strictum, Aspergillus granulosus, A. versicolor, Botryotrichum* sp., *Chaetomium globosum, Eurotium repens, Geomyces pannorum, Microascus singularis, Monodictys pelagica, Penicillium chrysogenum, Scopulariopsis candida, Stibella jappi* and sterile mycelium

Table 1 (Continued)

Sponge	Fungi
Hymeniacidon assimilis	*Acremonium fusidioides, A. roseum, Aspergillus flavus, A. speluneus, A. versicolor, Chaetomium aterrinum, C. globosum, Chaetomium* spp., *Eupenicillium crustaceum, Eurotium amstelodami, E. repens, Microascus brevicaulis, M. longirostris, M. singularis, Microascus* spp., *Penicillium aurantiogriseum, P. brevicompactum, P. camemberti. P. citrinum, P. corylophilum, P. janthinellum, P. paxilli, P. raistrickii, P. wortmannii, Pseudoeurotium zonatum, Pseudoeurotium* sp., *Scopulariopsis candida, Talaromyces helices, T. panasenkoi, Trichoderma koningii, T. aureoviride, T. viride, Trichoderma* sp. and sterile mycelium
Myxilla incrustans	*Aphanoascus aciculatus* and *Talaromyces panasenkoi*
Phakittia cribrosa	*Aspergillus speluneus, A. varians, Aspergillus* sp., *Chaetomium* spp., *Chrysosporium* sp., *Penicillium auratiogriseum, P. chrysogenum, P. citrinum, P. crustosum, P. raistrikii* and sterile mycelium

Source: Pivkin *et al*. (2005)

Toledo-Hernandez *et al*. (2007) reported the isolation of several fungal species from the sea fan *Gorgonia ventalina,* collected at San Juan, Puerto Rico. The identified species comprised *Aspergillus flavus, A. oryzae, A. niger, A. unguis, A. sydowii, A. ustus, Cladosporium sphaerocarpum, Gloeotinia tremulenta, Rhodotorula nymphae, Penicillium citrinum, P. citreonigrum, P. coffeae, P. steckii, Stachybortrys chartarum, Xylaria hypoxylon* and *Xylaria* sp.

Later, Toledo-Hernandez *et al*., (2008) identified several additional fungal strains from another *Gorgonia ventalina* sample, collected also in Puerto Rico. *Aspergillus aculeatus*, *A. flavus*, *A. oryzae*, *A. melleus*, *A. niger*, *A. foetidus*, *A. awamori*, *A. ochraceus*, *A. sydowii*, *A. tamari*, *A. terreus*, *A. unguis*, *A. ustus*, *A. versicolor*, *Candida* sp., *Chalaropsis* sp., *Cladosporium* sp., *C. cladosporioides*, *C. sphaerospermum*, *Davidiella tassiana*, *Gloetinia temulenta*, *Hypocrea lixii*, *Nectria* sp., *Nectria haematococca*, *Penicillium chrysogenum*, *P. citreonigrum*, *P. commune*, *P. minioluteum*, *P. citrinum*, *Pichia guillermondi*, *Rhodotorula nymphaeae*, *Stachybotrys chartarum*, *Stachybotrys chlorohalonata*, *Trichoderma harzianum*, *Tritirachium* sp. and *Xylaria hypoxylon*.

From the marine coral *Acropora formosa*, collected at Wistaria and Heron reefs, at Great Barrier Reef, Australia, seven fungal isolates were reported, being identified as *Alternaria* sp., *Aureobasidium pullulans*, *Cladosporium* sp., *Fusarium* sp., *Humicola fuscoatra*, *Penicillium citrinum* and *Phoma* sp. (Yarden *et al*., 2007).

In a study dealing with the identification of macroorganims associated microflora, fifty-seven fungi species were isolated from sediments, algae (*Sargassum cymosum*, *Padina* sp., *Caulerpa* sp. and an unidentified species), sponge (*Tedania ignis*) and a sea anemone (*Anemonia sargassensis*), collected at São Paulo, Brazil. Isolated fungi were identified by morphological and biochemical analyses and twenty-eight strains were isolated from sediments, twenty-one strains from marine algae, while *Penicillium* sp. was recorded from the sponge *Tedania ignis* and three additional fungal strains isolated from the sea anemone *Anemonia sargassensis*. The most abundant fungal genera were *Penicillium* sp., *Verticillium* sp., *Aspergillus* sp. and *Phoma* sp.

Karnat *et al*. (2008) reported several fungi species from a collection of invertebrates collected at Tamil Nadu, India. Invertebrate samples comprised *Sinularia kavarattiensis* (soft coral), *Spirastrella inconstans* var. *digitata* (sponge) and an unidentified coral. The isolated fungi were identified as *Acremonium butyri*, *A. fusidioides*, *Aspergillus terreus* group, *A. welllii* group and *Beauveria brongniartii*.

Taxonomic analysis of the associated microorganism communities isolated from marine sponges collected from New Zealand and Pulau Redang Marine Park, Malaysia, revealed several distinct fungal strains. While from the sponge samples collected in New Zealand, ninety-one fungal strains were isolated and identified as *Acremonium* sp., *Alternaria* sp., *Beauveria bassiana, Beauveria* sp., *Cladosporium* sp., *Dreschlera*-like sp., *Paecilomyces* sp., *Penicillium* sp., *Penicillium* spp., *Phoma* sp., *Spiromyces* sp., *Scopulariopsis* sp., *Verticillium* sp. and *Xylaria* sp., fifty-seven isolates were isolated from the sponge samples collected in Malaysia and identified as *Paecilomyces* sp., *Trichoderma* sp. and *Xylaria* sp. (Aline *et al.*, 2008).

From the marine sponge *Suberitis domuncula*, collected at the Northern Adriatic Sea near Rovinj, Croatia, eighty-one fungal strains were isolated, representing twenty genera including *Acremonium* sp., *Aspergillus ustus*, *Chaetomium* sp., *Cladosporium* sp., *Engyodontium album*, *Exophiala* sp., *Fusarium* sp., *Gliomastix* sp., *Nodulisporium* sp., *Paecilomyces* sp., *Penicillium* sp., *Petriella* sp., *Phialophora* sp., *Phoma* sp., *Scopulariopsis* sp., *Sporobolomyces* sp., *Stemphylium* sp., *Stilbella* sp., *Tolypocladium* sp., *Trichoderma* sp., as well as sterile mycelia (Proksch *et al*., 2008).

Silva *et al*. (2008) reported several filamentous fungi from the coral *Mussismilia hispida* collected from the northern coast in the state of São Paulo, Brazil. Isolated fungal strains were identified as *Aspergillus japonicus, A. sulphureus*, *Cladosporium cladosporioides, Cladosporium* sp., *Eutypella* sp., *Fusarium oxysporum, Khuskia oryzae, Mucor* sp., *Penicillium citrinum, P. sumatrense, Phoma* sp. and *Trichoderma* sp.

The study of two sponge samples collected at Oahu, Hawaii, revealed a total of twenty-five fungal isolates. While *Beauveria* sp., *Coniothyrium* sp., *Cytospora nitschkii, Hypocrea* sp., *Paraconiothyrium* sp., *Penicillium brevicompactum, Phoma* sp., *Plectosphaerella cucumerina, Stilbella* sp., *Stephanonectria* sp., *Trichoderma harzianum, Trichoderma* sp. and seven unidentified fungi were isolated from *Suberites zeteki*, the fungi species *Fusarium incarnatum, Fusicoccum* sp.,

Myrothecium cinctum, *Nectria* sp., *Nigrospora oryzae* and *Trichoderma* sp. were obtained from *Gelliodes fibrosa* (Wang *et al*., 2008).

Baker *et al*. (2009) reported the fungal associates from the marine sponge *Haliclona simulans* collected on the west coast of Ireland. Six different media were used including 1) malt extract agar, 2) glucose peptone agar, 3) carboxy methyl cellulose yeast extract agar, 4) malt extract, peptone, gellum gum, 5) glucose, peptone, gellum gum and 6) 10 g/l carboxy methyl cellulose, yeast extract, gellum gum. A total of eighty fungal strains were isolated from this sponge, namely thirteen isolates from malt extract, ten isolates from malt extract, peptone, gellum gum, twelve isolates from glucose peptone agar, ten isolates from glucose, peptone, gellum gum, eighteen isolates from carboxy methyl cellulose yeast extract agar and seventeen isolates from 10 g/l carboxy methyl cellulose, yeast extract, gellum gum. Molecular analysis of 18S rRNA region revealed nineteen different genotypes belonging to thirteen Orders including Polysporales, Agricomycotina, Mucorales, Saccharomycetales, Chaetothyriales, Helotiales, Eurotiales, Calosphaeriales, Chaetosphaeriales, Xylariales, Microascles, Hypocreales and Pleosporales.

Li and Wang (2009) reported the diversity of fungal associates from three marine sponges, *Gelliodes fibrosa*, *Haliclona caerulea* and *Mycale armata* collected at Hawaii. Seventeen fungal genera were isolated from *Gelliodes fibrosa* including *Aspergillus* sp., *Bartalinia* sp., *Bionectria* sp., *Bipolaris* sp., *Cochliobolus* sp., *Curvularia* sp., *Diaporthe* sp., *Eupenicillium* sp., *Fusarium* sp., *Fusacoccum* sp., *Hypocrea* sp., *Leptosphaerulina* sp., *Myrothecium* sp., *Nigrospora* sp., *Paraphaeosphaeria* sp., *Penicillium* sp. and *Trichoderma* sp. Six genera were isolated from *Mycale armata* including *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Eupenicillium* sp., *Lacazia* sp. and *Penicillium* sp., whereas eight genera were identified from *Haliclona caerulea* including *Ampelomyces* sp., *Aspergillus* sp., *Cladosporium* sp., *Didymella* sp., *Eupenicillium* sp., *Penicillium* sp., *Paraphaeosphaeria* sp. and *Tubercularia* sp.

During a study on the microbial symbionts from a marine sponge and a coral collected at China Sea, several fungal strains were identified. The fungal species

Apiospora montagnei, Aspergillus candidus, A. fumigatus, A. ochraceus, Candida parapsilosis, Cladosporium sp., *Davidiella tassiana, Didymocrea sadasivanii, Fusarium* sp., *Hypocrea koningii, Lentomitella cirrhosa, Marasmius alliaceus, Nigrospora oryzae, Paecilomyces lilacinus, Penicillium chrysogenum, P. purpurogenum, Pestalotiopsis guepinii, Rhizomucor pusillus* and *Scopulariopsis brevicaulis* were isolated from *Phakellia fusca*, while *A. versicolor, Davidiella tassiana, Fusarium* sp., *P. lilacinus, P. chrysogenum* and *P. pinophilum* were isolated from *Theonella swinhoei* (Li, 2009).

In a report from Rongbian *et al*. (2009) dealing with the marine sponge fungal associates, two sponges have been studied, *Haliclona angulate* and *Hymeniacidon* sp. The ITS gene analysis of the two filamentous fungi strains DQ25 and SC10, isolated from this sponges, showed greatest similarity to *Penicillium vinaceum* and *P. granulatum*, respectively.

From the Mediterranean sponge *Psammocinia* sp. collected at Israel, several fungi were identified, including *Acremonium implicatum, Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *A. sydowii, A. terreus, A. ustus, A. versicolor, Bionectria pseudochroleua, Chaetomium* sp., *Coprinellus* sp., *Cephalosporium* sp., *Cladosporium oxysporum, C. tenuissimum, Cochliobolus* sp., *Didymella* sp., *Dothideomycetes* sp., *Emericellopsis* sp., *Eupenicillium* sp., *Fusarium equiseti, F. proliferatum, F. solani, Gliomastix* sp., *Gymnoascus* sp., *Hypocrea orientali, H. atroviridis, Paraphaeosphaeria* sp., *Penicillium brevicompactum, P. chrysogenum, P. citrinum, P. crustosum, P. glabrum, P. implicatum, P. pinophilum, P. piscarium, P. steckii, Penicillium* sp., *Phoma leveillei, Phomopsis* sp., *Plectosphaerella* sp., *Preussia* sp., *Rhizopus* sp., *Stachybotrys* sp., *Trichoderma atroviride, T. harzianum, T. longibrachiatum, Trichoderma* sp., *Trichurus* sp. and *Verticillium* sp. (Paz *et al*., 2010).

Menezes *et al.* (2010) analysed three sponges, *Amphimedon viridis, Dragmacidum reticulate* and *Mycale laxissima*, collected at Sao Paulo State, Brazil. Eleven fungi genera were isolated from the sponge *Mycale laxissima* including *Aspergillus* sp., *Bionectria* sp., *Cladosporium* sp., *Cochliobolus* sp., *Fusarium* sp.,

Glomerella sp., *Penicillium* sp., *Phoma* sp., *Trichoderma* sp., *Verticillium* sp. and unidentified species, while Agaricales, *Aspergillus*, *Atheliales*, *Bionectria*, *Cladosporium*, *Cochliobolus*, *Fusarium*, *Glomerella*, *Penicillium*, *Rhizopus*, *Trichoderma* and several unidentified species were obtained from *Amphimedon viridis*. In addition, *Acremonium*, *Arthtiniun*, *Aspergillus*, *Botryosphaeria*, *Cochliobolus*, *Fusarium*, *Glomerella*, *Mucor*, *Nectria*, *Penicillium*, *Phoma*, *Polyporales*, *Rhizopus*, *Trichoderma* and several unidentified species, were isolated from *Dragmacidum reticulate.*

Aspergillus ustus was isolated from the marine sponge *Suberites domunculus* collected from the Adriatic Sea (Liu *et al*., 2011).

Wang *et al.* (2011) reported the diversity of marine fungi associated with the gorgonian coral *Echinogorgia rebekka*, collected from South China Sea. Fifty-three fungal isolates were obtained and identified as *Alternaria alternata*, *Aspergillus flavipes*, *A. versicolor*, *A. westerdijkiae*, *Aspergillus* sp., *Cladosporium cladosporioides*, *C. sphaerospermum*, *C. cucumerinum*, *C. uredinicola*, *Cladosporium* sp., *Hypocrea ixii*, *Nectria haematococca*, *Nigrospora* sp., *Penicillium chrysogenum*, *P. crustosum*, *P. glabrum* and *P. polonicum.*

From *Tethya aurantium*, a sponge collected at Mediterranean Sea, several fungal species were described including *Acremonium* sp., *Alternaria alternata*, *A. citri*, *Alternaria* sp., *Aspergillus granulosus*, *A. minutus*, *A. terreus*, *A. versicolor*, *Bartalinia robillardoides*, *Beauveria bassiana*, *Bionectria ochroleuca*, *B.* cf. *ochroleuca*, *B. rossmaniae*, *B. fuckeliana*, *Botryosphaeria* sp., *Cladosporium cladosporioides*, *C. sphaerospermum*, *Davidiella tassiana*, *Epicoccum nigrum*, *Eurotium chevalieri*, *Fusarium acuminatum*, *F. equiseti*, *F. oxysporum*, *Fusarium* sp., *Hypocrea lixii*, *Lewia infectoria*, *Mucor hiemalis*, *Paraphaeosphaeria* sp., *Paecilomyces lilacinus*, *Penicillium brevicompactum*, *P. canescens*, *P. chrysogenum*, *P. citreonigrum*, *P. commune*, *P. glabrum*, *P. roseopurpureum*, *P. sclerotiorum*, *P. virgatum*, *Petromyces alliaceus*, *Phialemonium obovatum*, *Phoma pomorum* var. *pomorum*, *Phoma* sp., *Pyrenochaeta cava*, *Verticillium* sp., *Volutella ciliate*,

Scopulariopsis brevicaulis, Septoria arundinacea, Trichoderma cerinum and *Trichoderma* sp. (Wiese *et al*., 2011).

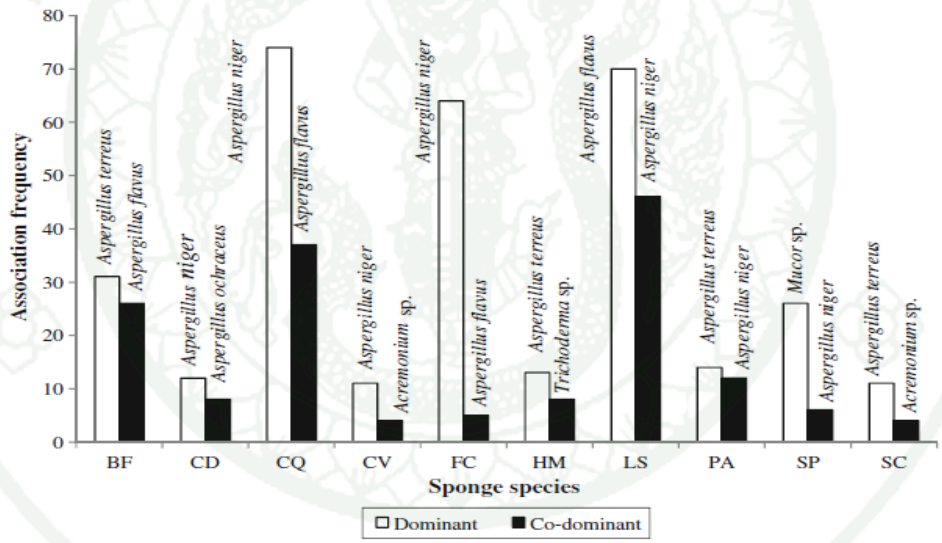
Miriam *et al.* (2012) studied the fungal associates from four marine sponges including *Axinella* cf. *corrugata*, *Dragmacidon reticulatum, Geodia corticostylifera* and *Mycale angulosa*. A total of four hundred and ten strains were recorded, one hundred and eleven strains isolated from *Axinella* cf. *corrugate,* one hundred and seventy-nine strains from *Mycale angulosa,* fourty from *Dragmacidon reticulatum* and eighty additional isolates from *Geodia corticostylifera*.

Raghukumar (2012) reported the marine fungi *Aspergillus restrictus, A. versicolor, A. versicolor, Asteromella* sp., *Bipolaris rostrate, Hormonema dematioides, Humicola alopallonella, Paecilomyces lilacinus, Penicillium restrictum, Phialophora bubaki, Pithomyces chartarum* and sterile mycelium isolated from seven marine corals including *Acropora hyacinthus, A. palifera, Acropora* sp., *Diploastrea heliopora, Goniastrea retiformis, G. australensis* and *Porites australensis* collected at Great Barrier Reef, Australia. While, *Acremonium* sp., *Aspergillus sydowii, Cladosporium sphaerospermum, Paecilomyces godlewski, Penicillium avellaneum, P. expansum* and *P. stoloniferum* were isolated from *Acropora palmate, Diploria labyrinthiformis, Meandrina meandrites, Montastrea annularis, Montastrea cavernosa* and *Porites porites* collected at Barbados, West Indies. The fungi genera *A. versicolor, Cladosporium sphaerospermum, Hormonema dematioides, P. restrictum, P. stoloniferum, Phialophora bubaki, Wallemia ichthyophaga* as well as sterile mycelium were isolated from the corals *Stylophora pistillata, Porites australensis, Porites* sp. and *Diploastrea heliopora* collected at Rarotonga, Cook Islands.

Thirunavukkarasu *et al.* (2012) reported the fungal associates from ten marine sponges including *Biemna fistulosa, Callyspongia diffusa, Cliona quadrata, C. viridis, Fasciospongia cavernosa, Haliclona madrepora, Lissodendoryx sinensis, Pseudosuberites andrewi, Sigmadocia pumila* and *Subritus carnosus* collected from Southern India. The fungi were identified as *Acremonium* sp., *Alternaria* sp., *Aspergillus candidus, A. flavus, A. fumigatus, A. niger, A. ochraceus, A. terreus,*

Aspergillus sp., *Chaetomium* sp, *Cladosporium* sp., *Curvularia tuberculate*, *Drechslera* sp., *Eurotium* sp., *Fusarium* sp., *Gliocladium* sp., *Humicola* sp., *Lasiodiplodia theobromae*, *Mucor* sp., *Penicillium* sp., *Phoma* sp., *Pseudogymnoascus* sp., *Sporormiella intermedia*, *Syncephalastrum* sp., *Thielaviopsis* sp., *Trichoderma* sp., *Tritirachium* sp. and sterile mycelium. The genus *Aspergillus*, representing fourteen species, was dominant and constituted 78.5% of the total isolates obtained from all the sponge samples. *Aspergillus niger* and *A. terreus* showed maximum association frequency in four sponge species, whereas *A. flavus* was found only in *Callyspongia diffusa* (Figure 2).

Recently, Subramani *et al.* (2013) reported a *Penicillium* sp. isolated from the marine sponge *Melophlus* sp., collected at Fiji Islands.



* BF = *Biemna fistulosa*, CD = *Callyspongia diffusa*, CQ = *Cliona quadrata*, CV = *Cliona viridis*, FC = *Fasciospongia cavernosa*, HM = *Haliclona madrepora*, LS = *Lissodendoryx sinensis*, PA = *Pseudosuberites andrewi*, SP = *Sigmadocia pumila* and SC = *Subritus carnosus*

Figure 2 Contribution by dominant and co-dominant fungi to the assemblages of fungal from marine sponge.

Source: Thirunavukkarasu *et al.* (2012)

2.1 Fungal Diversity from Marine Invertebrate-Associates collected in Thailand

Due to their physical properties as well as nutrient richness, marine sponges are hosts for a biologically diverse community of microorganisms, being classified as one of the richest sources of fungal diversity. Specifically, the tropical marine environment represents an extremely rich ecosystem as proved by several reports dealing with the identification of new fungal species isolated from Thailand coastal waters.

Dethoup *et al*. (2009) reported the isolation of several marine sponge-associated fungi from *Mycale armata, Haliclona* sp. and *Chalinula* sp. collected in the Gulf of Thailand, and they were identified as *Chaetomium globosum, C. minutum, Curvularia lunata, Emericella variecolor, Eupenicillium parvum, Menmoniella echinata, Nigrospora* sp., *Nodulisporium* sp., *Penicillium* sp. and *Speggazzinia tessarthra*. Later, *E. variecolor, Eurotium cristatum, C. lunata, Cladobotyum varium* and *Acremonium* sp. were also reported from the marine sponges *Clathria reinwardtii* and *Xestospongia testudinaria* collected from Samaesan Island, Chonburi province, Thailand (Manoch *et al.*, 2009).

Two *Nigrospora* sp. strains, F13 and PSU-F18, were isolated from sea fans collected from Thai waters. While strain F13 was identified from an unidentified gorgonian sea fan collected at the South of Thailand (Preedanon *et al*., 2009), strain PSU-F18 was isolated from an *Annela* sp., collected near Similan Islands (Trisuwan *et al*., 2009).

Antia *et al.* (2010) reported the isolation of a new *Aspergillus* sp. strain CRI322-03 from the marine sponge *Stylissa flabelliformis*, collected in Ton Sai Bay, Phi Phi Islands, Krabi, Province. Based on morphological characteristics and molecular phylogenetic analysis, the fungus was identified as *Aspergillus aculeatus*.

From the marine sponges *Clathria reinwardtii, Chalinula* sp., *Haliclona* sp., *Mycale armata* and *Xestospongia testudinaria*, collected at the Gulf of Thailand

near Ko Samaesan, Chonburi, seventy-nine isolates were identified belonging to twenty distinct genera. Twenty-three different species, mainly *Penicillium* spp. and *Phomopsis* sp., were identified.

Recently, a new *Aspergillus* sp., *A. similanensis* KUFA0013 was identified from the marine sponge *Rhabdermia* sp., collected from a coral reef at Similan Islands, Phang Nga, province. It was identified based on morphological features, including characteristic of ascospores, conidiogenesis and colonies, as well as by DNA sequence analysis (Prompanya *et al*., 2014).

3. Marine-Derived Fungi: An Established Source of Bioactive Secondary Metabolites

The unequivocal potential of fungi as a major source of new lead structures and bioactive metabolites is clearly stated by the several fungal metabolites that led to the development of chemicals and drugs used in Agriculture and Medicine. In fact, fungal metabolites revolutionized Medicine in the last century, leading to the development of several drugs, including antibacterial and cholesterol lowering agents.

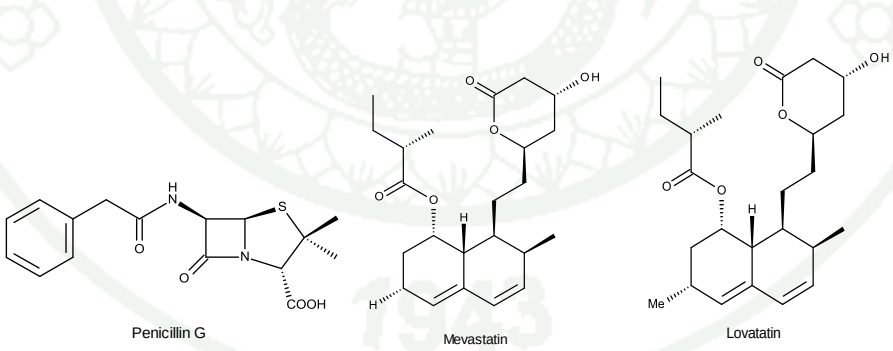


Figure 3 Structures of penicillin G, mevastatin and lovastatin.

The most successful finding was clearly the landmark discovery of the β-lactamic antibiotic penicillin G, isolated from *Penicillium notatum* (Walsh, 2004). Furthermore, also the top-selling class of cholesterol lowering agents, statins, correspond to synthetic analogues of mevastatin and lovastatin, initially reported from

P. citrinum (Endo *et al*., 1976), and *Monascus ruber* and *Aspergillus terreus*, respectively (Buckland *et al*., 1989; Negishi *et al*., 1986) (Figure 3).

Coincident with the focus on marine sources for the development of new drug leads, fungi from marine environment have been also identified as a prolific source of novel biologically active metabolites. Despite the special focus on macroorganisms such as sponges and tunicates, marine fungi represent a promising source of bioactive compounds with pharmaceutical potential, due to their ability of producing structurally unique secondary metabolites, consequence of the adaptation to the marine environment, as well as the sustainable production of secondary metabolites, especially by fermentation techniques (Xiong *et al*., 2013). Furthermore, recent reports provide strong evidence that some marine-derived drugs used in Medicine, supposedly produced by invertebrates, are in fact metabolic products from their associated microflora (Gerwick and Fenner, 2013; Simmons *et al*., 2008). Even though until 1992 only fifteen marine fungal metabolites have been reported (Fenical and Jensen, 1993), the following exponential focus on this source led to the identification of more than 1000 new natural fungal products (Rateb and Ebel, 2011).

Despite the absence of marine fungal metabolites in the current marine clinical pipeline, several candidates are now in pre-clinical development and are expected to advance clinical development soon (Bhatnagar and Kim, 2010; Gerwick and Fenner, 2013). The most recent and promising marine fungal metabolite, phenylahistidin (4), led to the development of the potent and selective tumor vascular disrupting agent plinabulin (NPI-2358) (5) (Figure 4). Phenylahistidin (4), originally isolated from the algicolous fungus *Aspergillus ustus*, displayed remarkable cytotoxicity against several human cancer cell lines leading to the development of two hundred synthetic analogues. Due to the promising activity of the synthetic analogue plinabulin (5), several clinical trials were performed, including a phase II clinical trial in combination with docetaxel in patients with non-small lung cancer.

Another promissing candidate in pre-clinical development refers to the antiviral and neuroprotective agent sorbicillactone (6) (Figure 4) recently qualified for human clinical trials. Originally isolated from the sponge-associated fungus

Penicillium chrysogenum, the bicyclic lactone displayed a highly selective and potential cytostatic activity against murine leukemic lymphoblasts (Bringmann *et al*., 2005; 2007). Also isolated from a marine sponge-derived fungus (*Scopulariopsis brevicaulis*), the cyclodepsipeptides scopularides A (7) and B (8) (Figure 4) were recently patented due to their potent cytotoxic activity against pancreatic and colon tumor cell lines (Yu *et al*., 2008).

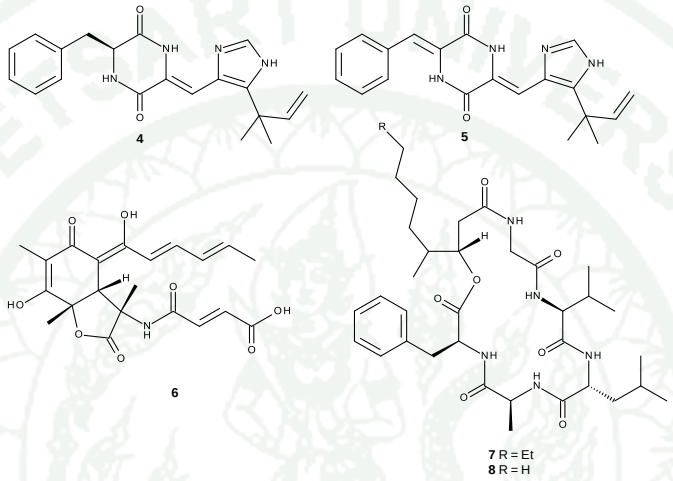


Figure 4 Examples of marine-derived fungal metabolites in pre-clinical development.

Thus, the increasing number of reports from the literature on the identification of new bioactive metabolites produced by marine fungi, as well as the several examples in preclinical development and new candidates with antagonistic activity against plant pathogens state the enormous potential of marine fungi as target for the development of new drugs and biocontrol agents for use in Agriculture.

4. An Overview on the Potential of the Genus *Talaromyces* as a Source of Bioactive Metabolites

The genus Talaromyces is widely distributed in the environment being recorded from soil, indoor and on organic materials undergoing decomposition (Samson and Pitt, 2000). Additionally, the genus attracted attention from chemists since it produces a wide variety of interesting bioactive compounds, such as

antibiotics. Despite the extensive review by Proksa (2010) on *Talaromyces flavus* secondary metabolites, several other species have been investigated for their secondary metabolites content, displaying a wide variety of structurally complex structures as well as relevant biological activities.

4.1 *Talaromyces bacillisporus*

Dethoup *et al*., (2006) reported the isolation of two oxyphenalenone dimers, bacillisporins D and E (Figure 5), from a culture of *T. bacillisporus* isolated from nonagricultural soil, collected in Kasetsart University, Bangkok.

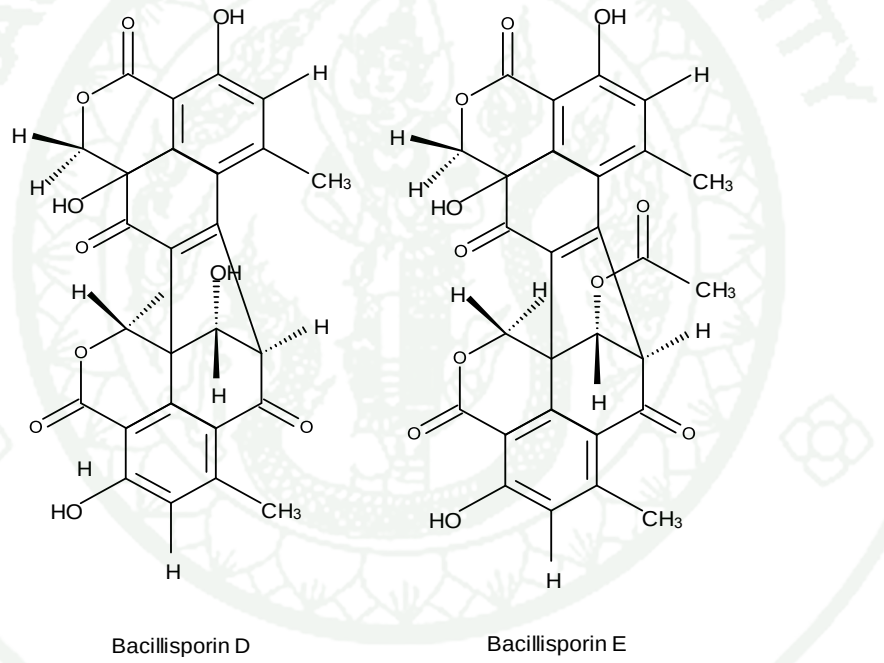


Figure 5 Structures of bacillisporins D and E.

4.2 *Talaromyces convolutes*

Four new tetramic acid derivatives, talaroconvolutins A-D (Figure 6) were isolated from the dichloromethane extract of the ascomata of *T. convolutus* Udagawa strain NE 76-1. Both talaroconvulutins B and C exhibited antifungal activity, inhibiting the growth of the human pathogenic fungi *Aspergillus fumigatus*,

A. niger and *Candida albicans*, however displaying weaker activity than the standard antifungal agent amphotericin B (Suzuki *et al*., 2000).

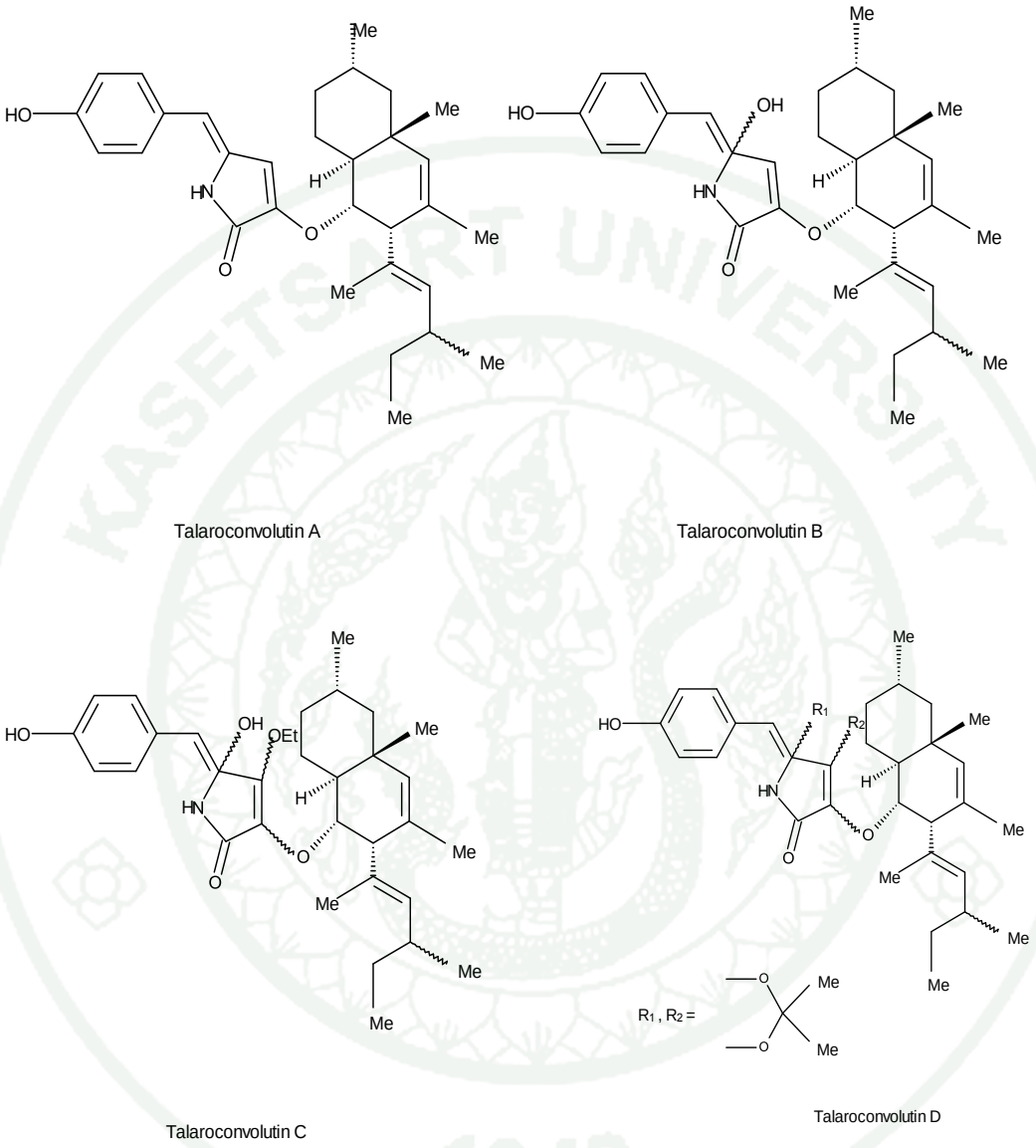


Figure 6 Structures of talaroconvolutins A-D.

4.3 *Talaromyces flavus*

Chemical analysis of the EtOAc extract of *T. flavus* strain BYDO7-13, isolated from a soil sample collected from Baiyangdian, Hebei, China, resulted in the isolation of six new polyesters, talapolyesters A-F (Figure 7). The new compounds were evaluated for their cytotoxic activity against five human tumor cell lines HL-60

(human promyelocytic leukemia), SMMC-7721 (hepatocellular carcinoma), A-549 (human lung carcinoma), MCF-7 (breast cancer) and SW480 (colon adenocarcinoma). Curiously, only the macrocyclic polyesters talapolyesters E-F exhibited significant activity against the five human tumor cell lines, with IC_{50} values ranging from 14.59 to 26.62 μM and 11.09 to 15.96 μM, respectively (He *et al*., 2014).

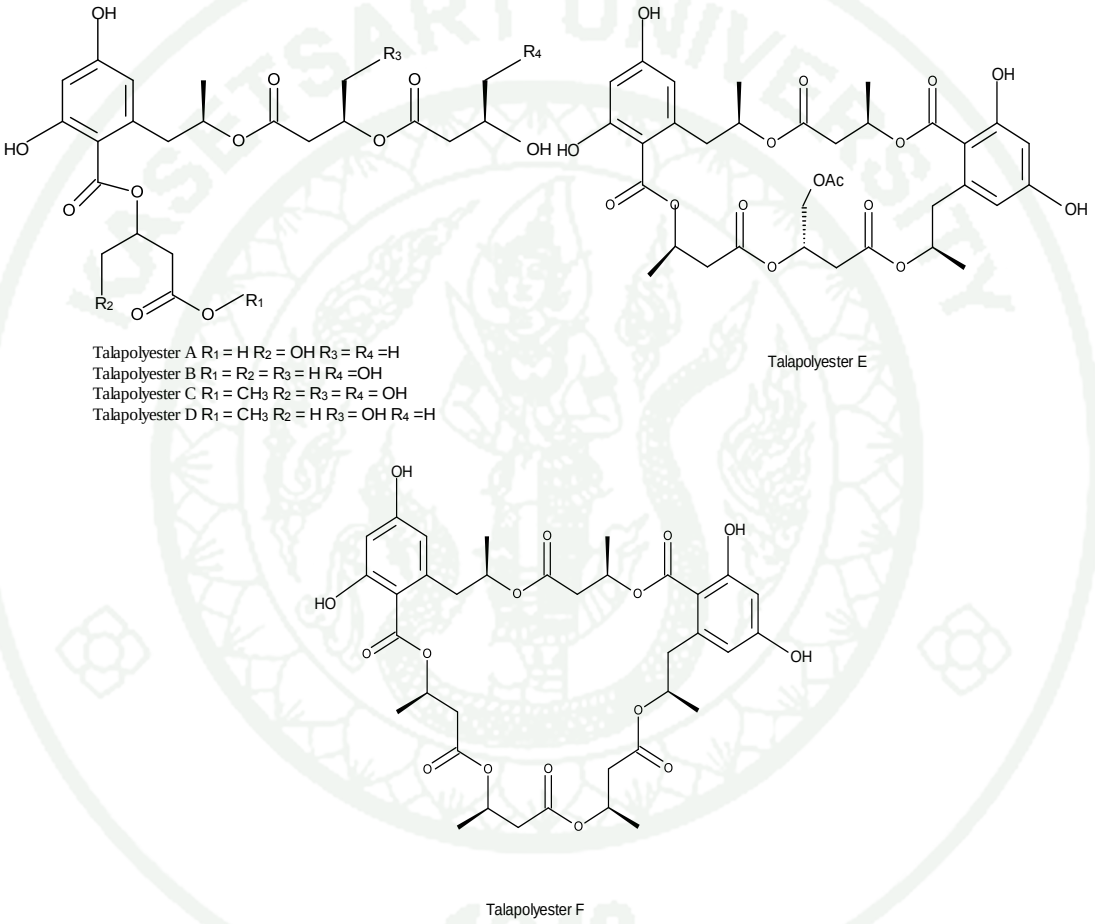


Figure 7 Structures of talapolyesters A-F.

4.4 *Talaromyces thailandiasis*

The chloroform extract of the culture of *T. thailandiasis* KPFC 3399 isolated from a soil sample collected in Trat Province, Southern Thailand, yielded two new merodrimanes, thailandolides A and B (Figure 8) (Dethoup *et al*., 2007).

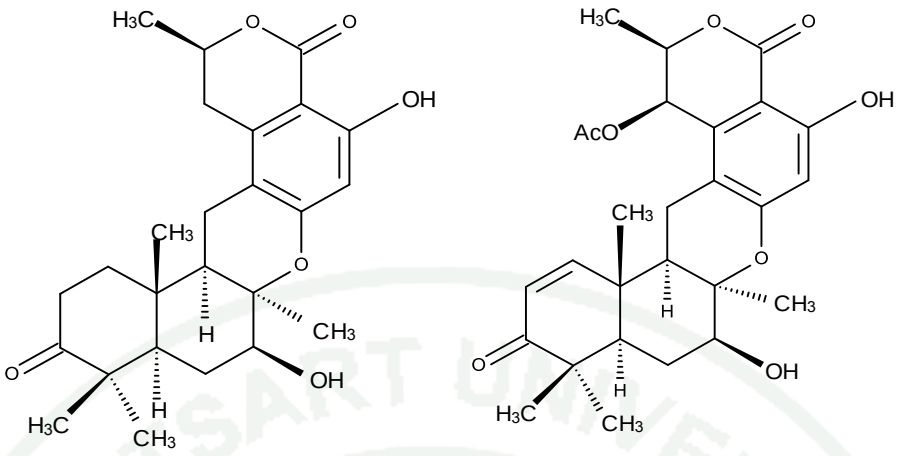
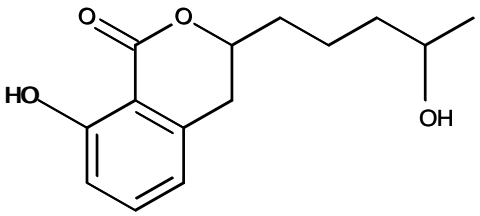


Figure 8 Structures of thailandolides A and B.

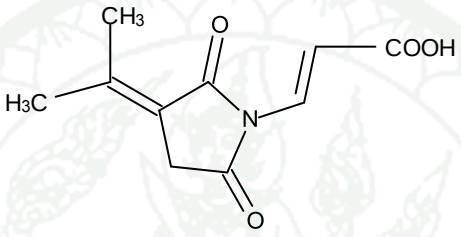
4.5 *Talaromyces verruculosus*

A culture of *T. verruculosus*, isolated in the rhizosphere soil of *Stellera chamaejasme* L. collected in the Qinling Mountains of Taibai, Shaanxi Province, China, was found to produce (-)-8-hydroxy-3-(4-hydroxypentyl)-3,4-dihydroisocoumarin (Figure 9) and (*E*)-3-(2,5-dioxo-3-(propan-2-ylidene)pyrrolidin-1-yl)acrylic acid (Figure 10). The *in vitro* antifungal activity, assayed by the growth rate method, revealed that the isocoumarin derivative (-)-8-hydroxy-3-(4-hydroxypentyl)-3,4-dihydroisocoumarin displayed strong activity against the phytopathogenic fungi *Alternaria solani*, *Valsa mali*, *Curvularia lunata* and *Botryosphaeria berengeriana*, while (*E*)-3-(2,5-dioxo-3-(propan-2-ylidene) pyrrolidin-1-yl)acrylic acid displayed only weak activity. Additionally, (-)-8-hydroxy-3-(4-hydroxypentyl)-3,4-dihydroisocoumarin exhibited also antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with MIC values of 2.5 and 5.0 μg / mL, respectively (Miao *et al*., 2012).



(-)-8-hydroxy-3-(4-hydroxypentyl)-3,4-dihydroisocoumarin

Figure 9 Structure of (-)-8-hydroxy-3-(4-hydroxypentyl)-3,4-dihydroisocoumarin.



(*E*)-3-(2,5-dioxo-3-(propan-2-ylidene) pyrrolidin-1-yl) acrylic acid

Figure10 Structure of (*E*)-3-(2,5-dioxo-3-(propan-2-ylidene)pyrrolidin-1-yl) acrylic acid.

4.6 *Talaromyces wortmannii*

From the EtOAc extract of a culture of *T. wortmannii*, isolated from a soil sample collected at Xishuangbanna, Yunnan Province, China, four new macrolides, wortmannilactones A-D (Figure 11), were reported. When tested against a panel of human cancer cell lines, including HCT-5 and HCT-115 (colon cancer), A549, MDA-MB-231 (breast cancer) and K562 (leucocythemia), the four lactones displayed moderate to weak cytotoxic activity, with IC_{50} values ranging from 28.7 to 130.5 μM (Dong *et al*., 2006).

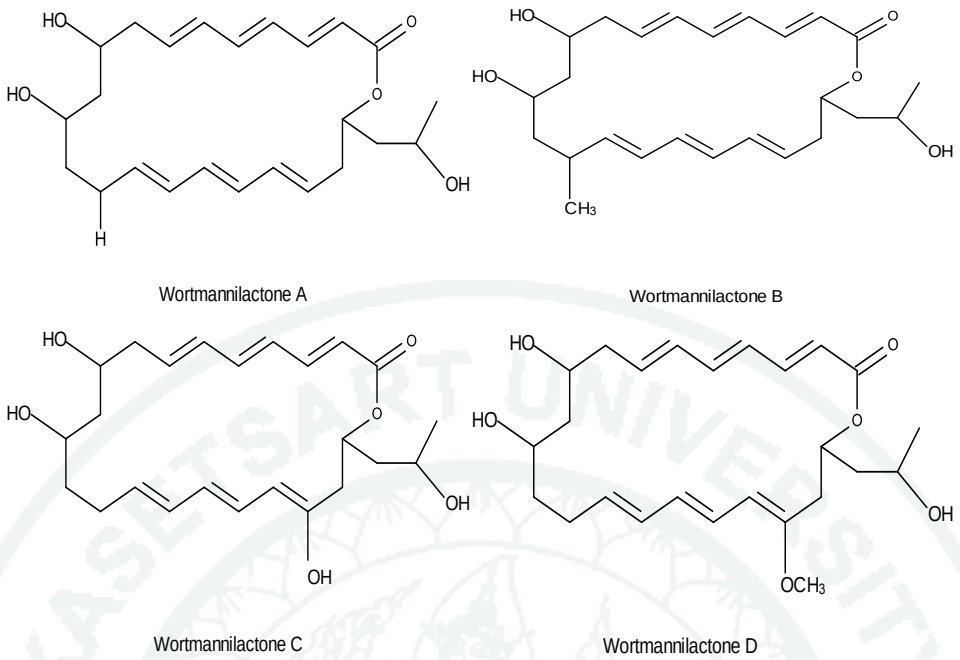


Figure 11 Structures of wortmannilactones A-D.

Two new cyclic peptides, talaromins A and B (Figure 12), were reported from an endophytic *T. wortmannii* strain, isolated from an *Aloe vera* sample, collected in Alexandria, Egypt. Talaromins A and B were tested for their cytotoxic activity against L5178Y mouse lymphoma cells and a broad spectrum of bacterial strains, but both peptides displayed no activity (Bara *et al*., 2013).

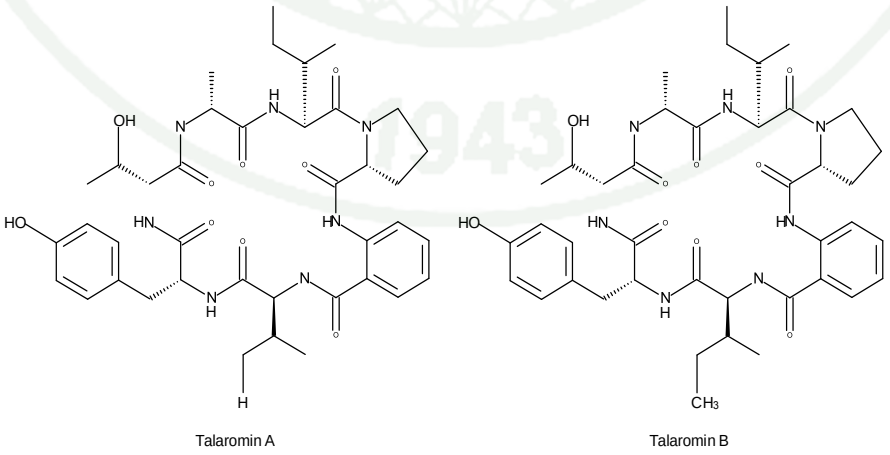


Figure 12 Structures of talaromins A and B.

4.7 Unspecified *Talaromyces* species

From an unspecified marine-derived *Talaromyces* sp. ka02k3, isolated from a seaweed collected in Kasai Rinkai Park, Tokyo, Japan, two novel azaphilone analogues, kasanosins A and B (Figure 13) were reported. Both azaphilones caused selective inhibitory activity of the DNA polymerases β and λ in family X of eukaryotic pols, with kasanosin A displaying significantly stronger inhibitory activity (Kimura *et al*., 2008). Later, an additional azaphilone analogue, kasanosin C (Figure 14), was also reported from an extract of *Talaromyces* sp. strain T1BF, isolated from the tissue of *Taxus yunnanensis*, collected at Kunming Botanic Garden, Chinese Academy of Sciences, Yunnan, China (Li *et al*., 2010).

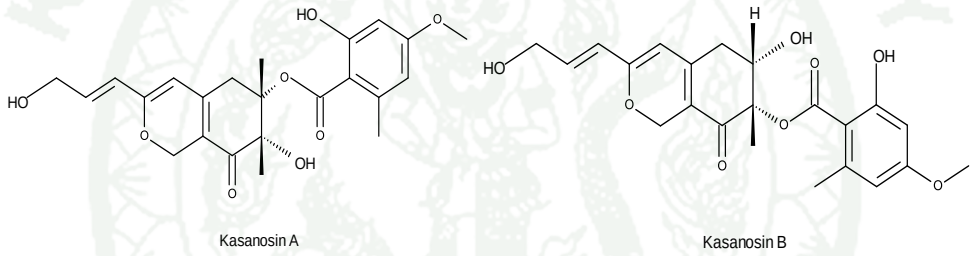


Figure 13 Structures of kasanosins A and B.

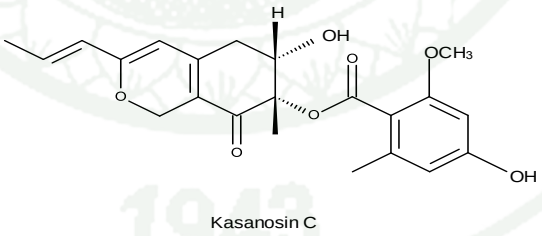


Figure 14 Structure of kasanosin C.

Recently, from the culture broth and mycelia of another marine-derived *Talaromyces* sp. (strain LF458), isolated from the sponge *Axinella verrucosa*, collected at Punta di Fetovaia, Isle of Elba, Italy, two novel spiroketals, talaromycesone A and B (Figure 15), as well as talaroxanthenone (Figure 16), a new isopentenyl xanthenone were reported. Both dimers, talaromycesone A and B, displayed remarkable antibacterial activities against the human pathogenic bacteria

Staphylococcus epidermitis and methicillin-resistant *Staphylococcus aureus* (MRSA), with IC_{50} values of 3.70 ± 0.13 and 17.36 ± 0.13 μM, and 5.48 ± 0.03 and 19.50 ± 1.25 μM, respectively. Additionally, talaromycesone A exhibited moderate acetylcholinesterase (AchE) inhibitory activity (IC_{50} = 7.49 ± 0.08 μM), but the most significant result was talaroxanthenone strong AchE inhibitory activity, with and IC_{50} = 1.61 ± 0.26 μM, more than tenfold stronger than huperzine. Furthermore, talaroxanthenone was also able to cause the inhibition of the enzyme phosphodiesterase (PDE-4B2), a key enzyme in inflammatory processes, with an IC_{50} value of 7.25 ± 0.17 μM (Wu *et al*., 2014).

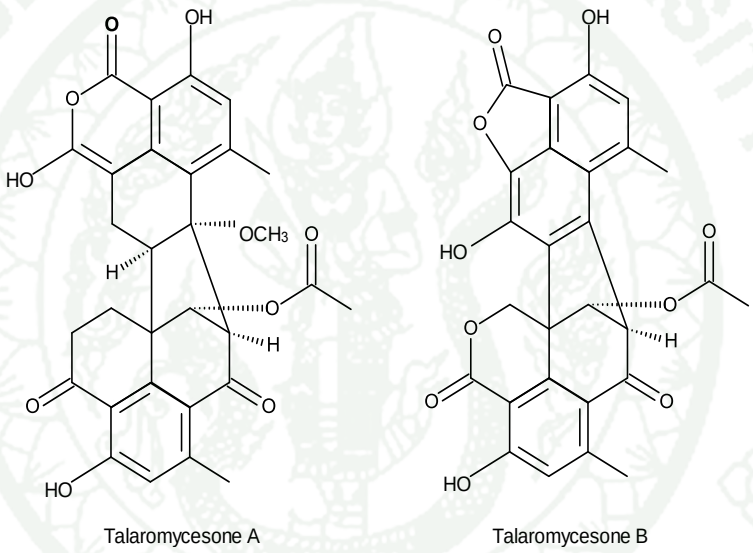


Figure 15 Structures of talaromycesone A and B.

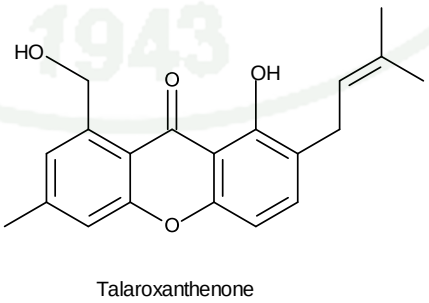


Figure 16 Structure of talaroxanthenone.

5. Marine Invertebrate-Associated Fungi Antagonistic Effect Against Plant Pathogens

The potential of fungi as producers of valuable bioactive metabolites is clearly revealed by several blockbuster drugs (Aly *et al*., 2011). However, the focus on marine-derived fungi as a source of new environmentally safe and easily biodegradable antimicrobial agents for use in agriculture is still scarce. However, there are a few promising reports dealing with the antifungal activity of fungi against phytopathogenic fungi.

Dethoup *et al*. (2009) reported the antagonistic activity of marine sponge-associated fungi *Mycale armata, Haliclona* sp. and *Chalinula* sp., collected from the Gulf of Thailand. Several fungal species including *Chaetomium globosum, C. minutum, Curvularia lunata, Emericella variecolor, Eupenicillium parvum, Menmoniella echinata, Nigrospora* sp., *Nodulisporium* sp., *Penicillium* sp. and *Speggazzinia tessarthra* were selected and tested for their antagonistic activity against ten plant pathogenic fungi (*Alternaria alternata, Colletotrichum capcisi, C. gloeosporioides, Fusarium oxysporum, Helminthosporium oryzae, Lasiodiplodia theobromae, Phytophthora palmivora, Pythium aphanidermatum, Rhizoctonia solani* and *Sclerotium rolfsii*). The results showed that all the selected marine sponge-associated fungi extracts could inhibit the mycelial growth of *A. alternata, C. capcisi*, *F. oxysporum, H. oryzae* and *Ph. palmivora*. While *E. variecolor, Nodulisporium* sp., *C. globosum* and *Penicillium* sp. effectively inhibited the mycelial growth of *C. gloeosporioides*, the remaining selected fungi extracts displayed only a moderate inhibition of the radial growth of this plant pathogen. Additionally, none of the fungi tested were able to inhibit the mycelial growth of *P. aphanidermatum, L. theobromae, R. solani* and *S. rolfsii*. In another study from the same research group, five sponge-associated fungi including *Emericella variecolor, Eurotium cristatum, Curvularia lunata, Cladobotyum varium* and *Acremonium* sp., were isolated from two marine sponges, *Clathria reinwardtii* and *Xestospongia testudinaria*, collected from Samaesan Island, Chonburi province, Thailand. The crude EtOAc extracts were evaluated for their antifungal activity against the same plant pathogens, and the results showed that the crude extracts from the selected marine-derived fungi were

effective against all phytopathogenic fungi tested. At the concentration of 10,000 ppm, *C. lunata* crude extract effectively inhibited (70-74%) the mycelium growth of *A. alternata*, *R. solani* and *C. gloeosporioides*, whereas *E. cristatum* crude extract was found to inhibit the mycelial growth (42-45%) of *Ph. palmivora, P. aphanidermatum, C. capcisi* and *S. rolfsii*. However, *E. variecolor* and *Acremonium* sp. crude extracts displayed only weak activity against *L. theobromae* and *F. oxysporum*. Finally, *C. varium* crude extract was found to be inactive against all the tested phytopathogenic fungi (Manoch *et al*., 2009).

Preedanon *et al*. (2009) reported the isolation of the marine-derived fungi *Nigrospora* sp. strain F13, from a gorgonian sea fan collected in Southern Thailand. The crude EtOAc extract from the culture broth of *Nigrospora* sp. F13 displayed strong antifungal activity against *Microsporum gypseum* (MIC 1 μg/ml) comparable to the standard drug miconazole. Moreover, chemical analysis of the extract led to the identification of dechlorogriseofulvin and chlorogriseofulvin derivatives also displaying antifungal activity against *M. gypseum* with MIC values of 2 and 32 μg/ml., respectively.

The crude extracts of the marine fungi *Penicillium vinaceum* strain DQ25 and *P. granulatum* strain SC10 were evaluated for their antifungal activity against several plant pathogenic fungi. Both extracts displayed activity against *Mucor miehei*, *Alternaria solani*, *Penicillium italicum* and *Fusarium oxysporum*, with *P. granulatum* strain SC10 crude extract exhibiting stronger activity than *P. vinaceum* strain DQ25 extract (Rongbian *et al*., 2009).

From the marine sponges *Clathria reinwardtii*, *Chalinula* sp., *Haliclona* sp., *Mycale armata* and *Xestospongia testudinaria*, collected at the Gulf of Thailand near Samaesan Island, Chonburi province, Thailand, seventy-nine fungal isolates were obtained. Four species (*Acremonium* sp., *Curvularia lunata, Emericella variecolor* and *Eurotium cristatum*) were selected and tested for their antifungal activity against six plant pathogenic fungi, namely *Alternaria alternata, Colletotrichum gloeosporioides, Fusarium oxysporum* f.sp. *lycopersici*, *Lasiodiplodia theobromae*, *Rhizoctonia solani* and *Sclerotium rolfsii*. The results showed that *C. lunata* crude

extract could inhibit 70-74% of mycelium growth of *A. alternata*, *C. gloeosporioides* and *R. solani* at the concentration of 10,000 ppm. *Acremonium* sp. crude extract inhibited the mycelium growth of *S. rolfsii*, *R. solani*, *F. oxysporum* f.sp. *lycopersici* and *C. gloeosporioides* at the concentration of 10,000 ppm. Additionally, *E. variecolor* and *E. cristatum* crude extracts were found to inhibit the mycelium growth of *R. solani* and *L. theobromae* causing 64 and 50% of inhibition, respectively (Buaruang *et al*., 2010).

Manilal *et al*. (2010) reported fourty-five marine fungal isolates from two marine sponges, *Fasciospongia cavernosa* and *Dendrilla nigra*, collected at southwest coast of India. Among the isolated fungi, fifteen strains displayed antibacterial activity against *Escherichia coli, Staphylococcus aureus* and *Staphylococcus epidermidis.* Moreover, three isolates of *Aspergillus clavatus* MFD15 exhibited complete antimicrobial activity against all pathogenic strains including Gram-positive and Gram-negative bacteria.

Several *Trichoderma* spp. were isolated from the Mediterranean sponge *Psammocinia* sp., and were evaluated for their antagonistic activity against three plant pathogenic fungi, *Botrytis cinerea* (BO5.10), *Rhizoctonia solani* (TP6) and *Alternaria alternata.* The results showed that all the tested fungi extracts displayed antagonistic activity on dual plate assay. *T. atroviride* and *T. asperelloides* effectively reduced the incidence of *R. solani* damping-off disease of beans and also induced defense responses in cucumber seedlings against *Pseudomonas syringae* pv. *lachrimans* (Hemed *et al*., 2011).

Vasanthabharathi and Jayalakshmi (2012) reported isolation of *Aspergillus flavipes, A. flavus, A. fumigatus, A. niger, A. terreus*, *Fusarium* sp., *Penicillium citrinum, Penicillium* spp., *Trichoderma virid* and *Trichoderma* sp. from the sponges *Callyspongia diffusa, Hyattella cribriformis, Sigmadocia carnosa* and *Spongia officininalis* var *ceylonensis,* collected at gulf of Mannar, Southeast coast of India. The fungal isolates were evaluated for their antibacterial activity, and the results showed that *P. citrinum* extract exhibited strong antibacterial activity against *Escherichia coli*, *Proteus mirabilus*, *Salmonella typhi*, *Salmonella paratyphi*, *Vibrio*

cholera, Klebsiella oxytoca, Klebsiella pneumonia, Staphylococcus aureus, Lactobacillus vulgaris and *Acidobacteria tumefaciens.* Additionally, *P. citrinum* extract displayed also strong antifungal activity against nine plant pathogenic fungi including, *Alternaria alternata, Botrytis cinerea, Cercospora theae, Fusarium udum, F. oxysporum, Macrophomina phaseolina, Poria hypolateritia, Phomopsis thae* and *Rhizoctonia solani.*

Recently, Shen *et al.* (2014) reported the antimicrobial activity of *Penicillium oxalicum* strain O312F against seven plant pathogenic fungi, *Alternaria solani, Colletotrichum graminicola, C. orbiculare, Fusarium graminearum, F. oxysporum, Pythium aphanidermatum* and *Rhizoctonia cerealis. P. oxalicum* crude extract displayed strong antifungal activity against *A. solani* and *F. graminearum.*

MATERIALS AND METHODS

Materials

1. Materials for collecting invertebrates samples

1.1 permanent markers
1.2 plastic bag
1.3 rubber bands
1.4 camera
1.5 paper notes

2. Laboratory Materials

2.1 forceps
2.2 fine needles
2.3 petri dishes
2.4 test tubes
2.5 beakers
2.6 agar media
2.7 electric scale
2.8 hot air oven
2.9 autoclave
2.10 alcohol lamp
2.11 glass slides and cover slips
2.12 70% and 95% ethyl alcohol
2.13 distilled water
2.14 lactophenol mounting media and emersion oil
2.15 thermometer
2.16 stereo microscope
2.17 light microscope
2.18 camera lucida

2.19 scanning electron microscope

3. Preservation

3.1 sterilized filter paper (Whatman No. 1 and No. 2)
3.2 plastic bags
3.3 aluminium foil
3.4 paper note
3.5 vials, size 1 dram
3.6 petri dishes

4. Media culture

4.1 potato dextrose agar (PDA)
4.2 malt extract agar (MEA)
4.3 glucose peptone yeast extract agar (GPY)
4.4 GI agar (GI)
4.5 potato dextrose broth (PDB)

5. Isolation and purification of secondary metabolites

5.1 distilled water
5.2 Petri dishes
5.3 Erlenmeyer flask size 250, 500, 1,000 and 2,000 ml.
5.4 cork borer
5.5 fitrate pump
5.6 paper filtrate Whatman No. 1
5.7 TLC aluminium sheets 20 x 20 cm silica gel 60 F_{254}, Merck
5.8 silica gel 60 F_{254} (0.063–0.200 mm), Merck for column chromatography
5.9 silica gel 60 F_{254}, Merck for thin layer chromatography
5.10 20 x 20 cm glass plates
5.11 sea sand
5.12 cotton

5.13 microcapillary pipettes, calibrated size 10 μl

5.14 vials, 4 dram

5.15 volumetric flask

5.16 hot plate

5.17 UV detector

5.18 aluminium foil

5.19 tank chamber

5.20 rotary evaporator (Buchi)

5.21 column chromatography

5.22 ethyl acetate (EtOAc)

5.23 chloroform ($CHCl_3$)

5.24 acetone (Me_2CO)

5.26 petroleum ether (Petrol)

5.27 methanol (CH_3OH)

5.28 formic acid (HCO_2H)

6. Structure elucidation of the compounds

6.1 Proton Nuclear Magnetic Resonance (^1H NMR)

6.2 Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR)

6.3 Correlation Spectroscopy (COSY)

6.4 Heteronuclear Single Quantum Coherence (HSQC)

6.5 Heteronuclear Multiple Bond Correlation (HMBC)

6.6 High Resolution Mass Spectrometry (HRMS)

Methods

1. Isolation and identification of marine fungi

1.1 Samples collection

The marine invertebrate samples were collected at coral reefs from different locations in Thailand by SCUBA diving at a depth of 10 metres during 2010-2011 (Table 2). The marine invertebrates were identified by Jamroen Buaruang, Division of Environmental Science, Faculty of Science, Ramkhamhaeng University, Bangkok. Subsequently, the samples were placed in plastic bags containing natural seawater and were stored in ice for later analysis.

Table 2 Marine invertebrate samples collected from various locations from 2010 to 2012.

Marine invertebrate	Location	Date of collection
Acanthella sp.*	Similan Islands, Phang Nga province	April 2010
Annella sp.*		
Cinachyrella sp.		
Dichotella sp.		
Halichondria sp.1		
Halichondria sp. 2		
Haliclona sp.		
Hymeraphia sp.		
Hyrtios erecta *		
Montipora aequituberculata		
Order Dendroceratida 1		
Order Dendroceratida 2		
Order Halicondrida *		
Pertrosia sp.		
Rhabdermia sp. 1		
Rhabdermia sp. 2		
Stylissa flabelliformis NO. 1		
Stylissa flabelliformis NO. 2		
Unidentified sea fan NO. 1		
Unidentified sea fan NO. 2		

Table 2 (Continued)

Marine invertebrate	Location	Date of collection
Unidentified sea fan NO. 3 *Xestospongia* sp.	Similan Islands**,** Phang Nga province	April 2010
Acanthogorgia sp. *Agelas* sp. *Axinyssa* sp. *Cinachyrella* sp. *Dendronephthya* sp. *Dysidea* sp. NO. 1 *Dysidea* sp. NO. 2 *Gorgonia* sp. *Halichondria* sp. NO. 1 *Halichondria* sp. NO. 2 *Hypnea* sp. *Petrosia* sp. Unidentified sponge	Lanta Islands**,** Krabi province	April 2011
Clathria reinwardtii	coral reef at Kram Island, Chonburi province	September 2011

* Illustrated

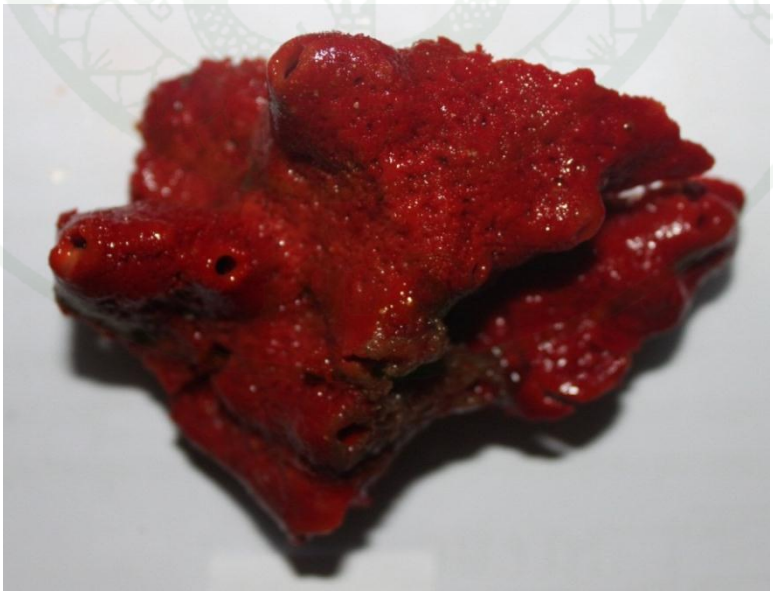


Figure 17 The marine sponge *Rhabdermia* sp., collected from Similan Islands, Phang Nga province.

Figure 18 The marine sponge *Stylisa flabelliformis*, collected from Similan Islands, Phang Nga province.

Figure 19 The marine sponge *Hyrtios erecta*, collected from Similan Islands, Phang Nga province.

Figure 20 The sea fan *Annella* sp., collected from Similan Islands, Phang Nga province.

Figure 21 The marine sponge Order Halicondrida, collected from Similan Islands, Phang Nga province.

Figure 22 The marine sponge *Acanthella* sp., collected from Similan Islands, Phang Nga province.

1.2 Isolation of fungi from marine invertebrates

The sample tissues were cut into pieces of 0.5 x 0.5 cm and placed on separate Petri-dishes containing one of the four isolation media namely, GPY, GI, Half PDA and MEA. All media contained 70% of sea water and streptomycin sulphate, then incubated at room temperature for 7 days. Hyphal tips were transfered onto PDA slant for further identification. The pure cultures were maintained at the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand in code KUFA.

1.3 Marine Fungi Taxonomic Identification

The identification of the fungi was based on morphological characteristics as observed from the growth pattern, color and texture. Meanwhile, colony characteristic were examined under a stereoscopic microscope and with the naked eye. Microscopic characteristics were thoroughly investigated on a slide preparation using sterile water and lactophenol as the mounting medium and they were examined

under a light microscope afterwards. The study of the ornamentation of ascospores was conducted using the scanning electron microscopy (SEM: SEOL JSM 6400).

2. *In vitro* antagonistic activity test of the marine-derived fungi against plant pathogenic fungi by dual culture method

Twelve isolates of marine fungi were selected to test for antagonistic activity against ten species of phytopathogenic fungi (Figure 23, Tables 3-4). The mycelium from the colony margin of selected marine fungi and the specific plant pathogenic fungi were cut with sterile cork borer (0.5 cm diam.) and placed on PDA as a dual culture, 7 cm apart. The Petri dishes plates were incubated at room temperature for 14 days. The inhibition levels were calculated by using the formula: G1-G2 / G1 x 100 where G1 = colony radius of plant pathogenic fungi in the control, and G2 = colony radius of plant pathogenic fungi in the dual culture test (Intana, 2003). Each treatment was performed with three replicates.

Table 3 The marine fungi isolated from marine sponges collected in Similan and Kram Islands, used for antagonistic test against plant pathogenic fungi.

Fungi	**KUFA**	**Marine invertebrates**
Emericella nidulans	KUFA 0101	*Halichondria* sp. 2
Emericella nidulans	KUFA 0102	*Hyrtios erecta*
Emericella variecolor	KUFA 0103	*Stylisa flabelliformis*
Emericella sp.	KUFA 0104	*Haliclona* sp.
Emericella sp.	KUFA 0105	*Dichotella* sp.
Hamigera sp.	KUFA 0106	*Halichondria* sp. 2
Neosartorya fischeri	KUFA 0107	Order Halicondrida
Neosartorya pseudofischeri	KUFA 0108	*Haliclona* sp.
Neosartorya sp.	KUFA 0109	*Pretosia* sp.
Pseudoeurotium sp.	KUFA 0110	*Dichotella* sp.
Xylaria sp.	KUFA 0111	*Haliclona* sp.
Talaromyces trachyspermus	KUFA 0021	*Clathria reinwardtii*

Table 4 Ten species of plant pathogenic fungi from various fruits and vegetables diseases used for antagonistic and antifungal activity tests.

Plant pathogenic fungi	**Diseases**	**Host plant**
Alternaria brassicicola	Leaf spot	*Brassica albograbra* (Chinese Kale)
Colletotrichum capsici (E.J. Butler & Bisby)	Chili anthracnose	*Capsicum annuum* (chili)
Colletotrichum gloeosporioides (Penz. & Sacc)	Anthractnose	*Pyrus pyrifolia* (pear)
Fusarium oxysporum (E.F. Sm. & Swingle)	Fusarium wilt	*Lycopersicon esculentum* (tomato)
Helminthosporium maydis (Y. Nisik. & C. Miyake)	Southern corn leaf blight	*Zea mays* (corn)
Lasiodiplodia theobromae ((Pat.) Griffon & Maubl)	Fruit rot	*Garcinia mangostana* (mangosteen)
Pythium aphanidermatum (Edson Fitzp)	Pythium root and stem rot	*Cucumis sativus* (cucumber)
Phytophthora palmivora (E.J. Butler)	Durian root rot	*Durio zibethinus* (durian)
Rhizoctonia solani (J.G. Kühn)	Sheath rot	*Oryza sativa* (rice)
Sclerotium rolfsii (Sacc)	Basal stem rot	*Vigna radiate*

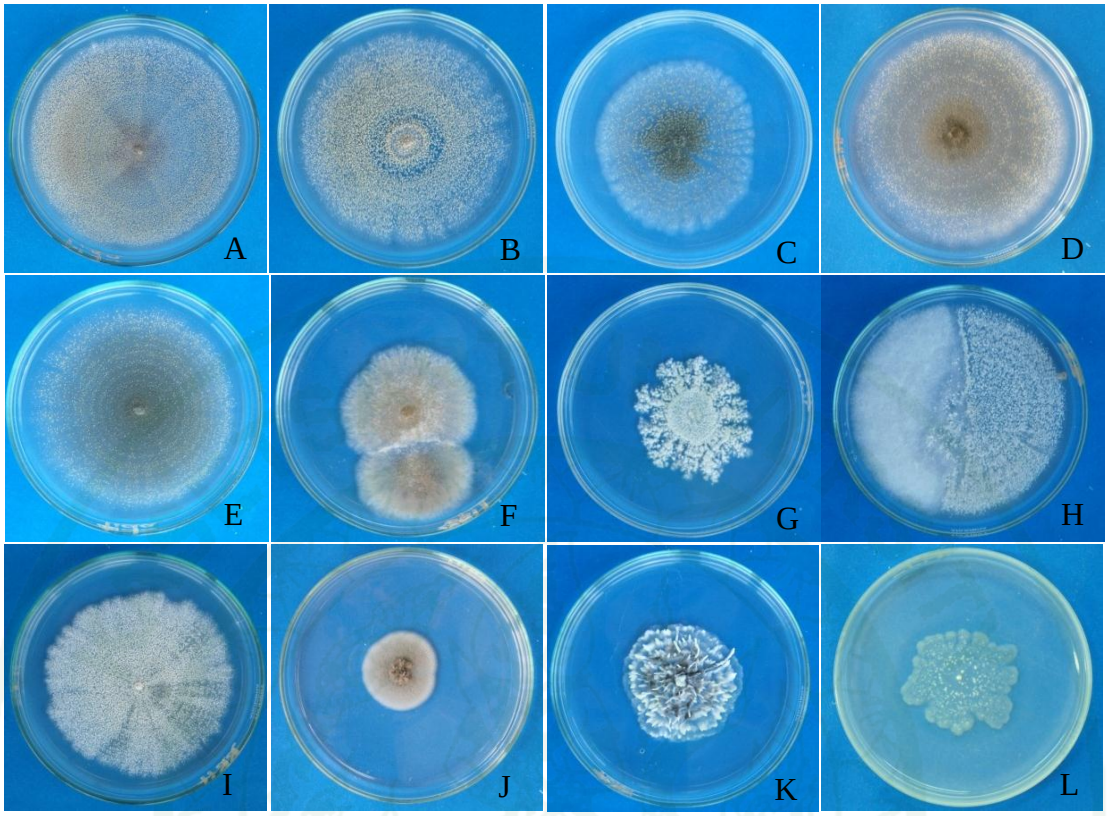


Figure 23 Coloies on PDA of selected marine-derived fungi for antagonistic activity test against plant pathogenic fungi using dual culture method;
A. *Emericella nidulans* KUFA 0101 B. *Emericella nidulans* KUFA 0102
C. *Emericella variecolor* KUFA 0103 D. *Emericella* sp. KUFA 0104
E. *Emericella* sp. KUFA 0105 F. *Hamigera* sp. KUFA 0106
G. *Neosartorya fischeri* KUFA 0107 H. *Neosartorya pseudofischeri*KUFA 0108
I. *Neosartorya* sp. KUFA 0109 J. *Pseudoeurotium* sp. KUFA 0110
K. *Xylaria* sp. KUFA 0111 L. *Talaromyces trachyspermus* KUFA 0021

3. Preparation of the marine fungi extract

Six species of marine-derived fungi, including *Emericella nidulans* (KUFA 0101), *Hamigera* sp. (KUFA 0106), *Neosartorya fischeri* (KUFA 0107) *Neosartorya pseudofischeri* (KUFA 0108), *Pseudoeurotium* sp. (KUFA 0110) and *Talaromyces trachyspermus* (KUFA 0021) were selected to evaluate for their antifungal activity against plant pathogenic fungi Each of the selected marine fungi was cultured in 500 ml Erlenmeyer flasks containing PDB 200 ml, and incubated on rotary shaker at 150 rpm for 7 days. Twenty-five 1,000 ml Erlenmeyer flasks, each containing 200 g cooked rice, were autoclaved at 121^{o}C for 15 minutes and then inoculated with approrimate 20 ml of mycelial suspension of each of the selected marine fungi. The inoculated flasks were then incubated at 28^{o}C for 30 days, after which 500 ml of ethyl acetate was added to each flask and macerated for three days. Filtration with the filter paper (Whatman No.1) to give the organic solutions which were combined and then evaporated under reduced pressure to furnish the crude ethyl acetate extracts of each fungus.

4. *In vitro* antifungal activity test of six marine fungi crude extracts against ten species of plant pathogenic fungi

Dilution plate method was used for the evaluation of the *in vitro* antimycelial growth of ten plant pathogenic fungi. One gram of each of the crude ethyl acetate extracts of *Emericella nidulans* (KUFA 0101), *Hamigera* sp. (KUFA 0106), *Neosartorya fischeri* (KUFA 0107) *N. pseudofischeri* (KUFA 0108), *Pseudoeurotium* sp. (KUFA 0110) and *Talaromyces trachyspermus* (KUFA 0021) was dissolved in 10 ml of sterile distilled water to prepare a stock solution of 100,000 ppm concentration. The stock solution was then serially diluted by sterile distilled water to four concentrations of 10, 100, 1,000 and 10,000 ppm. Each concentration of the crude fungal extracts was added to 9 ml of warm PDA, mixed, and poured into the Petri dishes. The mycelia of ten plant pathogenic fungi were cut with sterile cork borer and transferred to the PDA plates containing various concentrations of each crude extracts. All the Petri dishes were incubated at room temperature for 7 days. The PDA Petri dish void of the fungal crude extract was used as a control. The inhibition levels

were calculated using the formula: G1-G2 / G1 × 100, wher G1 = colony radius of the plant pathogenic fungi in the control, and G2 = colony radius of plant pathogenic fungi in the presence of the tested crude extract (Intana *et al.*, 2003). Each treatment was performed with three replications with complete randomized design.

5. Analytical secondary metabolites of *Talaromyces trachyspermus* (KUFA 0021)

5.1 General Experimental

5.1.1 Merck Si gel 60 (0.2-0.5 mm; 70-230 mesh) was used for column chromatography

5.1.2 Analytical and preparative TLC were performed on silica gel-60 (GF_{254}; Merck), 0.25 thickness. The plates were activated at 110^{o}C in the oven for 1 hour. All TLC plates were visualized under UV 254 nm or developed with iodine vapor.

5.1.3 Melting points were recorded on a Bock Monoscope and are uncorrected.

5.1.4 Rotations were determined on a Polax-2L instrument.

5.1.5 ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature in DMSO on a Bruker DRX instrument operating at 500 and 125 MHz respectively, ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were measured on a Bruker CxP spectrometer. The solvents used were deuterated chloroform (Merck) or hexadeuterated dimethylsulfoxide (Merck).

5.1.6 X-ray diffraction studies were performed with a Stoe IPOS image plant equipped with Mo Ka radiation. The structure was solved using SHELX 597 and refined with SHELXL 97. A perspective view of the molecule was obtained with ORTEP.

5.1.7 The solvents used were commercial grade of Vidrolab 2 which were distilled prior to use and analytical reagent grade of brand Merck and Lab-Scan.

5.1.8 All solvents were evaporated either by reduced pressure using “ Buchi rotary evaporator” or nitrogen gas.

5.1.9 The weight was measured on the balance Mettler AE 200.

5.2 Isolation and purification of the secondary metabolites from *Talaromyces trachyspermus* (KUFA 0021)

5.2.1 Fungus material

Talaromyces trachyspermus (KUFA 0021) was isolated from the marine sponge, *Clathria reianwardii*, which was collected from the coral reef at Kram Island, Chonburi, Thailand in September 2011. Tests, as well as taxonomical determination according to Stolk and Samson's description (Figure 24). The pure cultures were deposited as KUFA 0021 at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

5.2.2 Preparation of the crude extract

The mycelium plugs of *Talaromyces trachyspermus* (KUFA 0021) were transferred into 500 ml Erlenmeyer flasks containing 200 ml of PDB and incubated on a rotary shaker at 150 rpm for 1 week at 28°C for preparing mycelial suspension. Twenty-five 1,000 ml Erlenmeyer flasks, each containing 200 g cooked rice, were autoclaved at 121^{o}C for 15 minutes and then inoculated with 20 ml of mycelial suspension in each flask and incubated at 28^{o}C for 30 days. Then, 500 ml of ethyl acetate was added to each flask and macerated for 7 days. Filtration with the filter paper (Whatman No.1) to give the organic solutions which were combined and then evaporated under reduced pressure to furnish the crude ethyl acetate extract. Evaporation of the combined filtrates to a volume of 1,000 ml at reduced pressure followed by addition of anhydrous sodium solephate, filtration and evaporation of the filtrate at reduced pressure furnished 102.66 g of dark brown crude ethyl acetate extract.

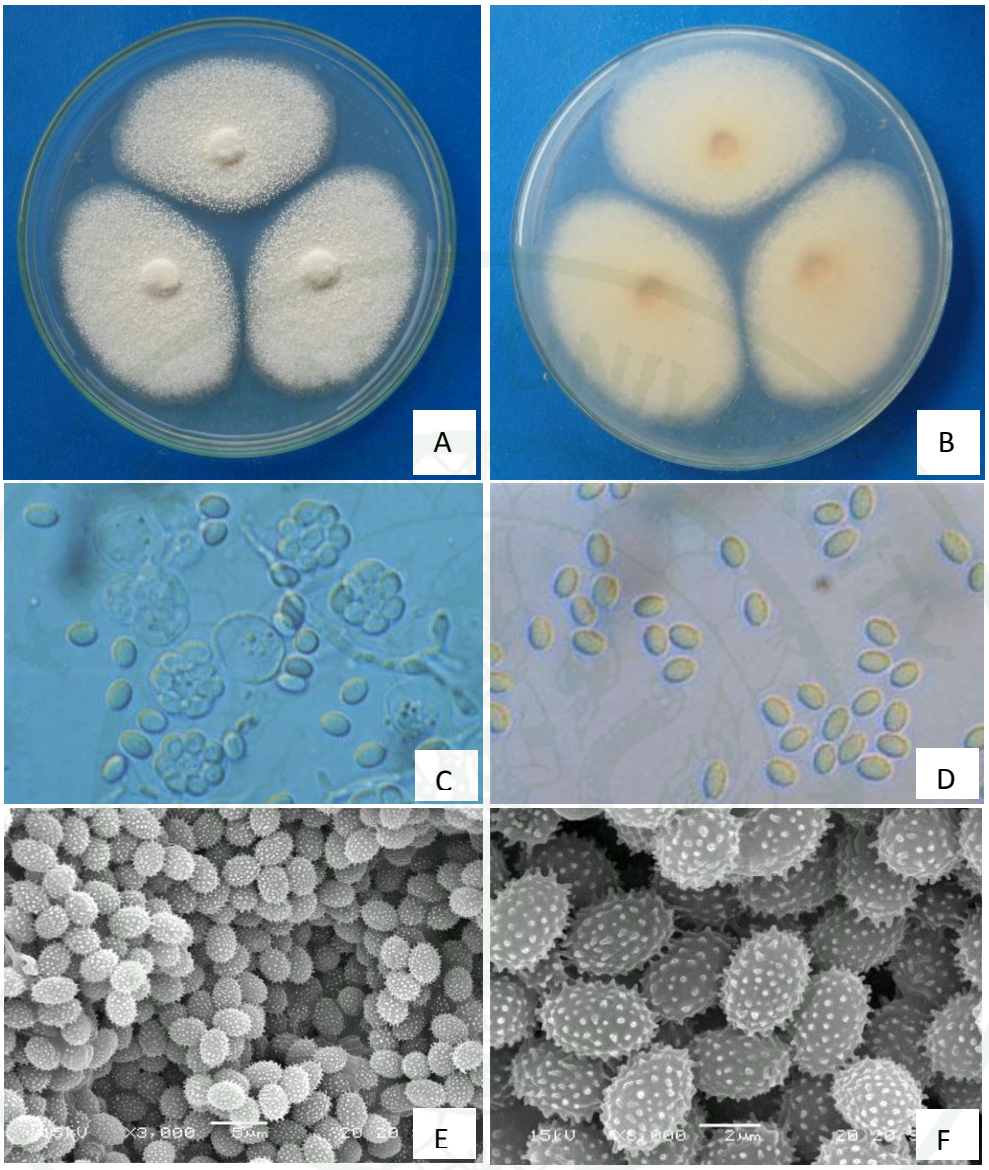


Figure 24 *Talaromyces trachyspermus* (Shear) Stolk & Samson (KUFA 0021) colony on PDA incubated for 14 days at 28 ^{o}C;
A. Obverse B. Reverse C. Ascus
D. Ascospores E. and F. ascospores (SEM)

5.2.3 Fractionation of the crude extract

The crude ethyl acetate extract (102.66 g) was dissolved in 500 ml of a 4:1 mixture of $CHCl_3$ and EtOAc and then washed with H_2O (3 x 500 ml). The organic layer was dried with anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give 94.5 g. The crude extract was applied to a column of silica gel (330 g) and eluted with petrol-$CHCl_3$, $CHCl_3$ and $CHCl_3$-Me_2CO, 250 ml fraction being collected as follows:

Fractions	**Eluents (ratio)**
1-59	Petrol : $CHCl_3$ (1:1)
60-130	Petrol : $CHCl_3$ (3:7)
131-329	Petrol : $CHCl_3$ (1:9)
330-403	$CHCl_3$: Me_2CO (9:1)
404-467	$CHCl_3$: Me_2CO (3:3)

The fractions were analyzed by analytical TLC and combined, according to their composition, as follows:

Fraction	**Weight (mg)**	**Isolated compounds**
1-8	2,4936	not purified
9-16	36.60	not purified
17-32	943.7	not purified
33-43	937.3	not purified
44-53	574.1	not purified
54-58	302.5	not purified
59-60	103.0	not purified
61-65	405.2	not purified
66-70	383.1	not purified
71-74	410.5	not purified

Fraction	Weight (mg)	Isolated compounds
75-79	255.8	glaucanic acid
80-84	487	glaucanic acid
85-88	175.9	glaucanic acid
89	47.9	glaucanic acid
90-92	133.4	glaucanic acid
93-101	413.8	glaucanic acid
102-107	164.4	glaucanic acid
108-130	1.2181	not purified
131-132	135.4	glauconic acid
133-136	631.3	glauconic acid
137-142	637.6	glauconic acid
143	148.2	glauconic acid
144-148	874.4	glauconic acid
149-153	748.5	glauconic acid
154-158	570.9	glauconic acid
159-163	451.5	glauconic acid
164-168	402.1	glauconic acid
169-173	362.3	glauconic acid
174-178	382.7	glauconic acid
179-188	719.6	glauconic acid
190-193	370.4	glauconic acid
194	57.0	glauconic acid
195	108.1	glauconic acid
196-201	420.5	glauconic acid
202-206	351.0	glauconic acid
207-210	344.8	glauconic acid
211-226	913.9	glauconic acid
227-242	785.7	glauconic acid
243-257	652.8	glauconic acid
258-290	1,576.3	glauconic acid

Fraction	Weight (mg)	Isolated compounds
291-331	1,296.5	glauconic acid
332	781.0	glauconic acid
333	4,343.8	not purified
334	101.1	spiculisporic acid E
335	1,255.5	spiculisporic acid E
336	662.4	spiculisporic acid E
337	1,009.5	spiculisporic acid E
338	1,563.4	spiculisporic acid E
339	825.9	spiculisporic acid E
340-345	3,006.4	spiculisporic acid E
346-350	2,780.5	spiculisporic acid E
351	415.1	spiculisporic acid E
352	365.5	spiculisporic acid E
353-358	2,770.5	spiculisporic acid E
359	436.8	spiculisporic acid E
360-364	1,816	spiculisporic acid E
365	357.2	spiculisporic acid E
366-369	934.2	spiculisporic acid E
370	203.3	spiculisporic acid E
371-375	1,618.9	spiculisporic acid E
376-380	780.0	spiculisporic acid E
381-385	736.9	spiculisporic acid E
386-390	303.7	spiculisporic acid E
391-395	255.0	spiculisporic acid E
396-400	226.7	spiculisporic acid E
401-406	261.4	spiculisporic acid E
407	4,149.9	not purified
408	4,273.4	not purified
409	2,921.7	not purified
410-420	9,675.6	not purified

Fraction	Weight (mg)	Isolated compounds
421-430	1,478.1	not purified
431-449	1,481.0	not purified
450-467	569.4	not purified

5.2.4 Isolation and purification of the compounds

Fractions 44-74 were combined (2.1784 g) and purified by TLC (Siliga gel, $CHCl_3$: petrol: EtAC: HCO_2H, 70: 25: 5: 0.01) to give ergosta-4, 6, 8(14), 22-tetraen-3-one.

Fractions 75-101 were combined (1.51 g) and crystallized in a mixture of petrol and $CHCl_3$ to give 1.12 g of glaucanic acid.

The mother liquor of fractions 75-101 were combined and purified on a silica gel column (14 g) and eluted with mixtures of petrol-$CHCl_3$ and $CHCl_3$-Me_2CO, wherein 100 ml sub-fractions were collected as follows:

Sub-fractions	Eluents (ratio)
1-51	petrol:$CHCl_3$ (1:1)
52-74	petrol:$CHCl_3$ (2:3)
75-89	petrol:$CHCl_3$ (3:7)
90-101	petrol:$CHCl_3$ (1:9)
102-109	$CHCl_3$-Me_2CO (9:1)
110-115	$CHCl_3$-Me_2CO (7:3)

Sub-fractions 15-57 were combined (59 mg) and purified by TLC (Si gel, $CHCl_3$: EtOAc: petrol: HCO_2H, 18:1:1:0.01) to give 9.0 mg of ergosta-4, 6, 8 (14), 22-tetraen-3-one.

Sub-fractions 58-101 were combined (151.6 mg) and purified by TLC (Si gel, $CHCl_3$: EtOAc: petrol: HCO_2H, 18:1:1:0.01) to give 18.5 mg of ergosta-4, 6, 8 (14), 22-tetraen-3-one and 7.2 mg of acetyl ergosterol 5, 8-endoperoxide.

Fractions 131-332 (13.72 g) were combined and crystallized in a mixture of petrol and $CHCl_3$ to give 12.23 g of glauconic acid.

Fractions 351-406 (11.48 g) were combined and crystallized in a mixture of $CHCl_3$ and Me_2CO to give 9.62 g of spiculisporic acid E.

5.3 Structure elucidation of the compounds

The structures of the compounds were established by spectroscopic methods (^{1}H, ^{13}C NMR, COSY, HSQC, HMBC, HRMS) as well as comparison of their NMR data with those in the literatures.

6. Place

The experiments of taxonomic study and antagonistic tests were conducted at Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok Province, Thailand. Scanning electron photomicrographs were examined at Scientific Equipment Centre, Biomolecular Sciences, Kasetsart University. The isolation, purification and structure elucidation of the secondary metabolites were conducted at Instituto de Ciencias Biomedicas de Abel Salazar (ICBAS), University of Porto, Portugal.

7. Duration

The study was carried out during October 2010 to October 2014.

RESULTS AND DISCUSSION

1. Fungal Diversity from Marine Invertebrates collected from Thai Waters

Two hundred and ten fungal isolates were isolated from thirty-six samples of marine invertebrates collected from different locations including Similan islands, Phang Nga province, Lanta Islands, Krabi province and Kram Island, Chonburi province. The fungal isolates were identified as belonging to thirty-six species including *Arthrinium* sp., *Aspergillus candidus*, *A. niger*, *A. terreus*, *Aspergillus* sp., *Chaetomium* sp., *Cladosporium* spp., *Emericella nidulans*, *E. variecolor*, *Emericella* spp., *Eupenicillium* spp., *Fusarium solani*, *Fusarium* sp., *Hamigera* sp., *Humicola* sp., *Lasiodiphodia* spp., *Mucor hiemalis*, *Mucor* sp., *Neosartorya fischeri*, *N. pseudofischeri*, *Neosartorya* sp., *Paecilomyces lilacinus*, *Paecilomyces* spp., *Penicillium* spp., *Pestalotiopsis* spp., *Phoma* sp., *Phomopsis* spp., *Pseudoeurotium* sp., *Rhizopus* sp., *Scolecobasidium* sp., *Syncephalastrum* sp., *Talaromyces trachyspermus*, *Trichocladium* spp., *Trichoderma opacum*, *Trichoderma* sp., *Xylaria* spp. and sterile mycelia. Sterile mycelia were evidently the most dominant fungal type isolated from all the marine samples followed by *Penicillium*, *Aspergillus*, *Phomopsis*, *Paecilomyces*, *Trichoderma* and *Pestalotiopsis*, respectively (Figure 25, Tables 5-6).

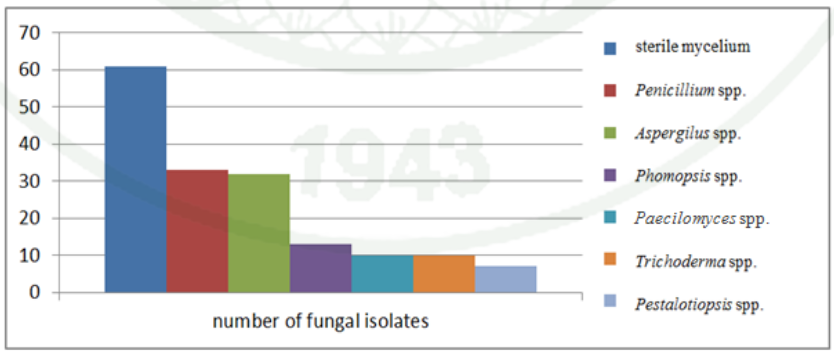


Figure 25 Occurrence of marine-derived fungi from marine invertebrate samples collected in Thailand.

Table 5 Fungal species isolated from marine invertebrates collected at Similan Islands, Phang Nga province, Thailand.

Fungi	Marine invertebrates																					
	A*	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
Arthrinium sp.														1								
Aspergillus candidus			1																			
Aspergillus niger	3																					
Aspergillus terreus			1																			
Aspergillus sp.		2	2				2				1					1					1	
Chaetomium sp.	1																					
Hamigera sp.						1																
Emericella nidulans						1			1													
Emericella variecolor																	1					
Emericella spp.				2	1	1			1													
Neosartorya fischeri													1									
Neosartorya pseudofischeri							1															
Neosartorya sp.														1								

Table 5 (Continued)

Fungi	Marine invertebrates																					
	A*	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
Paecilomyces lilacinus															1							
Paecilomyces spp.									3		1		2									
Penicillium spp.		2					1					1	1							2	2	2
Pestalotiopsis spp.										2		2										
Pseudoeurotium sp.				1																		
Xylaria spp.							1					1										
sterile mycelia	2	1	2	1	1		3	2	4	1	2	5	4	5	4		2	4	2		1	1
Total	6	5	6	4	2	3	8	2	9	3	4	9	8	7	5	1	3	4	2	2	4	3

*A = *Acanthella* sp. B = *Annella* sp., C = *Cinachyrella* sp. D = *Dichotella* sp.
E = *Halichondria* sp.1 F = *Halichondria* sp. 2 G = *Haliclona* sp. H = *Hymeraphia* sp.
I = *Hyrtios erecta* J = *Montipora aequituberculata* K = Order Dendroceratida 1 L = Order Dendroceratida 2
M = Order Halicondrida N = *Petrosia* sp. O = *Rhabdermia* sp. 1 P = *Rhabdermia* sp. 2
Q = *Stylissa flabelliformis* 1 R = *Stylissa flabelliformis* 2 S = unidentified sea fan 1 T = unidentified sea fan 2
U = unidentified sea fan 3 V = *Xestospongia* sp.

Table 6 Fungal species isolated from marine invertebrates collected at Lanta Islands, Krabi province, Thailand.

Fungi	Marine invertebrates												
	A*	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
Aspergillus niger		1				1	2	1		1	2	1	3
Apergillus terreus				1	1			1				1	1
Aspergillus sp.	1									1			
Cladosporium spp.										1		1	
Emericella variecolor							1						
Eupenicillium spp.		1			1					1	1		1
Fusarium solani										1	1		
Fusarium sp.										1			
Humicola sp.											1		
Lasiodiplodia spp.								1			1		
Mucor hiemalis													1
Mucor sp.													1
Paecilomyces spp.						1	1			1		1	
Penicillium spp.	1					2	3	2		4	3	2	5
Pestalotiopsis spp.			1			1	1						
Phoma sp.	1												
Phomopsis spp.	1			1	1	2	1	1	1	1		4	
Rhizopus sp.						1							
Scolecobasidium sp.						1							
Syncephalastrum sp.													1
Trichocladium sp.			1										
Trichoderma opacum		1										2	

Table 6 (continued)

Fungi	Marine invertebrates												
	A*	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
Trichoderma spp.	1						1	2		1		1	4
sterile mycelia	1		1			5				4	3	1	
Total	6	3	3	2	3	14	10	8	1	17	12	14	17

*A = *Acanthogorgia* sp. B = *Agelas* sp. C = *Axinyssa* sp.
D = *Cinachyrella* sp. E = *Dendronephthya* sp. F = *Dysidea* sp. 1
G = *Dysidea* sp. 2 H = *Gorgonia* sp. I = *Halichondria* sp.
J = *Halichondria* sp.2 K = *Hypnea* sp. L = *Petrosia* sp.
M = unidentified sponge

The higher fungal richness was obtained from the sponge *Halichondria* sp. and from an unidentified sponge, both harbouring seventeen fungal isolates, followed by *Petrosia* sp. and *Dysidea* sp.1, harbouring fourteen fungal isolates each. Curiously, these four fungal isolates were obtained from marine invertebrates collected from Lanta Islands, Krabi province. In fact, comparison among the fungal isolates from the same species of marine sponges collected from both locations (*Cinachyrella* sp., *Halichondria* sp. and *Petrosia* sp.), confirmed a tendency for a higher fungal richness in the samples collected from Lanta Islands, with exception to *Cynachyrella* sp. (Tables 5 and 6).

In addition to the referred species, isolated from several marine invertebrates collected from Similan Islands, Phang Nga province and Lanta Islands, Krabi province another fungal isolate identified as *Talaromyces trachyspermus*, was also isolated from *Clathria reianwardtii* collected from a coral reef at Kram Island, Chonburi province.

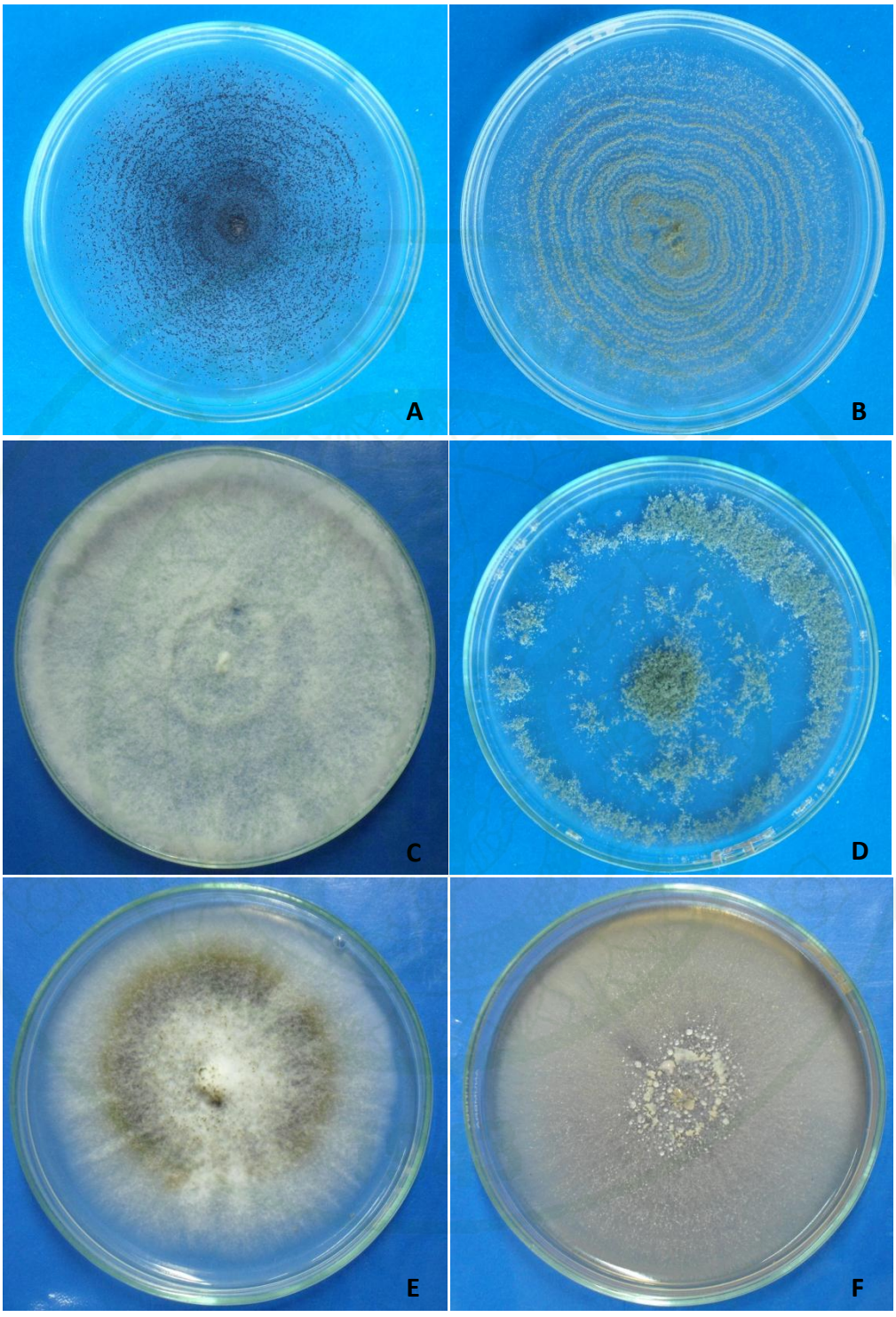


Figure 26 Colonies on PDA of marine-derived fungi, 7 days;

A. *Aspergillus niger* B. *Aspergillus* sp.

C. sterile mycelium D. *Trichoderma* sp.

E. *Phomopsis* sp. F. *Fusarium solani*

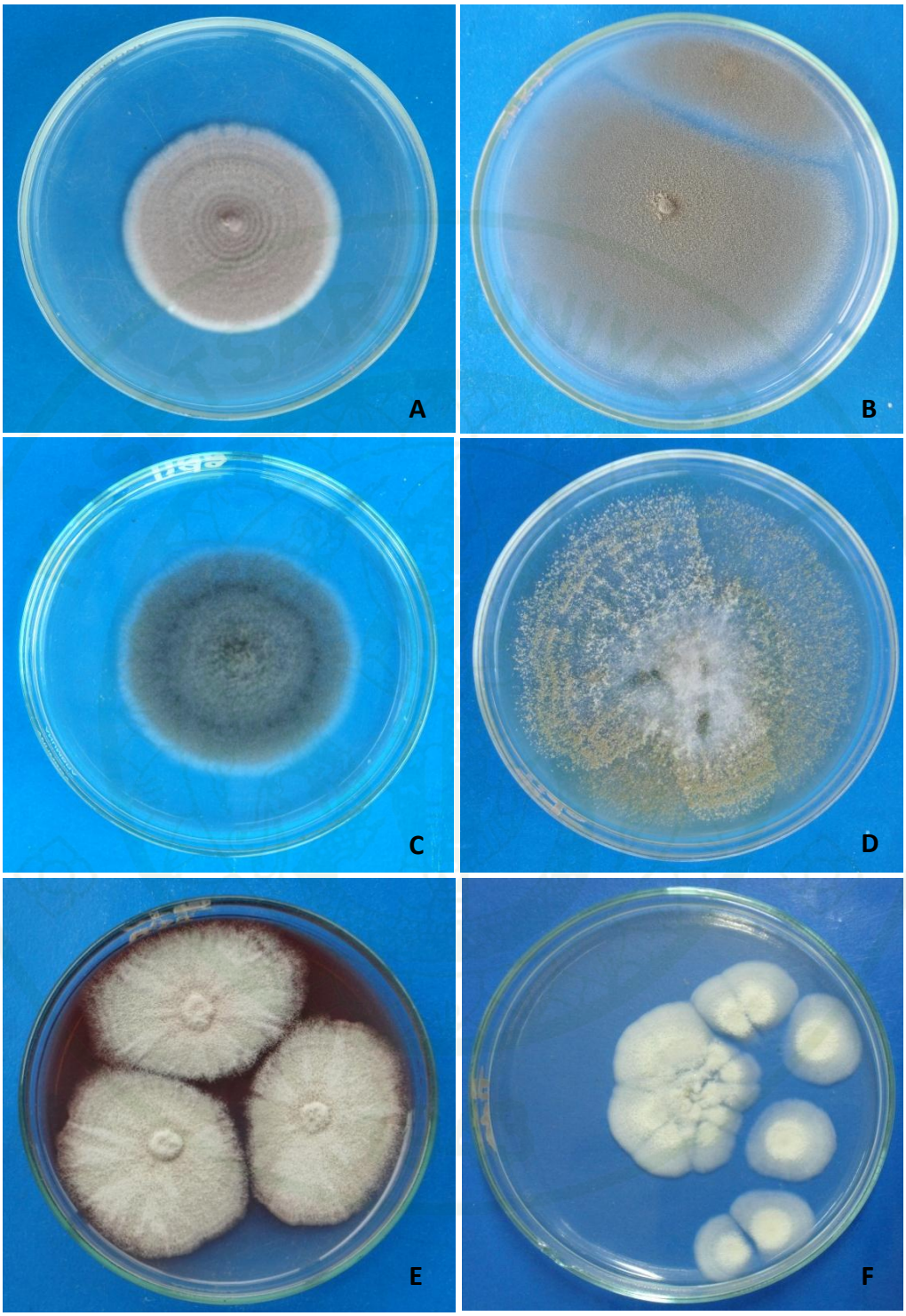


Figure 27 Colonies on PDA of marine-derived fungi, 7 days;

A. *Paecilomyces* spp. B. *Penicillium* spp.

C. *Cladosporium* spp. D. *Aspergillus* sp.

E. *Eupenicillium* sp. F. *Aspergillus candidus*

In the present study, most of the identified fungal species have been previously reported also from terrestrial sources. The most representative genus was identified as *Aspergillus*, being isolated from all the collected marine invertebrate samples. However, it is interesting to note that species referring to the sexual state of *Aspergillus* were also identified including *Neosartorya* and *Emericella*, both of which were isolated from sponges samples (Tables 6-7).

In addition to the genera *Aspergillus, Penicillium* and *Trichoderma* strains were also identified and classified as co-dominant fungi, being commonly found in association with marine vertebrate and invertebrate samples, collected from different locations. Curiously, *Aspergillus, Penicillium* and *Trichoderma* were found as the most representative fungal genera in other reports (Morrison-Gardiner, 2002; Li, 2009; Paz *et al*., 2010; Ding *et al*., 2011; Wiese *et al*., 2011; Thirunavukkarasu *et al*., 2012; Henríquez *et al*., 2014). On the other hand, several genera were isolated from a single host. Referring to the marine invertebrates collected from Similan Islands, Phang Nga province. *Arthrinium* was isolated only from *Pertrosia* sp., *Chaetomium* from *Acanthella* sp., *Hamigera* from *Halichondria* sp., and *Pseudoeurotium* from *Dichotella* sp. In addition, *Humicola* was also only isolated from *Hypnea* sp, *Phoma* from *Acanthogorgia* sp., *Rhizopus* from *Dysidea* sp., *Scolecobasidium* sp. from *Dysidea* sp. and *Syncephalastrum* sp. from an unidentified sponge, referring to the marine invertebrate samples collected from Lanta Islands, Krabi province. It was found from the present study that only three isolates of *Mucor* spp., *Rhizopus* sp. and *Syncephalastrum* sp. from an unidentified sponge were apparent. Unfortunately, the comparison between our results and previous reports may be problematic due to different sampling and isolation methods.

2. *In vitro* antagonistic activity evaluation of marine-derived fungi against plant pathogenic fungi

Twelve marine-derived fungi including *Emericella nidulans* (KUFA 0101), *E. nidulans* (KUFA 0102), *E. variecolor* (KUFA 0103), *Emericella* sp. (KUFA 0104), *Emericella* sp. (KUFA 0105), *Hamigera* sp. (KUFA 0106), *Neosartorya fischeri* (KUFA 0107), *N. pseudofischeri* (KUFA 0108), *Neosartorya* sp. (KUFA 0109),

Pseudoeurotium sp. (KUFA 0110), *Xylaria* sp. (KUFA 0111) and *Talaromyces trachyspermus* (KUFA 0021) were selected and tested for the antagonistic activity against ten plant pathogenic fungi belonging to 1) Agonomycetes (*Rhizoctonia solani* and *Sclerotium rolfsii*), 2) Coelomycetes (*Colletotrichum capsici*, *C. gloeosporioides* and *Lasiodiplodia theobromae*), 3) Hyphomycetes (*Alternaria brassicicola*, *Fusarium oxysporum* and *Helminthosporium maydis*) and 4) Oomycetes (*Pythium aphanidermatum* and *Phytophthora palmivora*).

The result on the mycelial growth inhibition for the two Agonomycetes plant pathogenic fungi (*R. solani* and *S. rolfsii*) revealed that *N. pseudofischeri* (KUFA 0108) and *Neosartorya* sp. (KUFA 0109) exhibited the highest antagonistic effect against *S. rolfsii* causing 47.47 and 36.29% mycelial growth inhibition, respectively. *Neosartorya pseudofischeri* (KUFA 0108) and *E. nidulans* (KUFA 0102) exhibited a weak antagonistic effect against *R. solani*, causing 37.03% and 32.22%, of mycelial growth inhibition, respectively (Figure 28). Additionally, both *Hamigera* sp. (KUFA 0106) and *Pseudoeurotium* sp. (KUFA 0110) did not display any antagonistic activity against *R. solani*, and half of the selected fungal species revealed to be inactive against *S. rolfsii* mycelial growth. Since the most active fungi species against the mycelial growth of the two Agonomycetes plant pathogenic fungi displayed only a weak effect, the selected marine-derived fungi did not prove to be effective against *R. solani* and *S. rolfsii* mycelial growth.

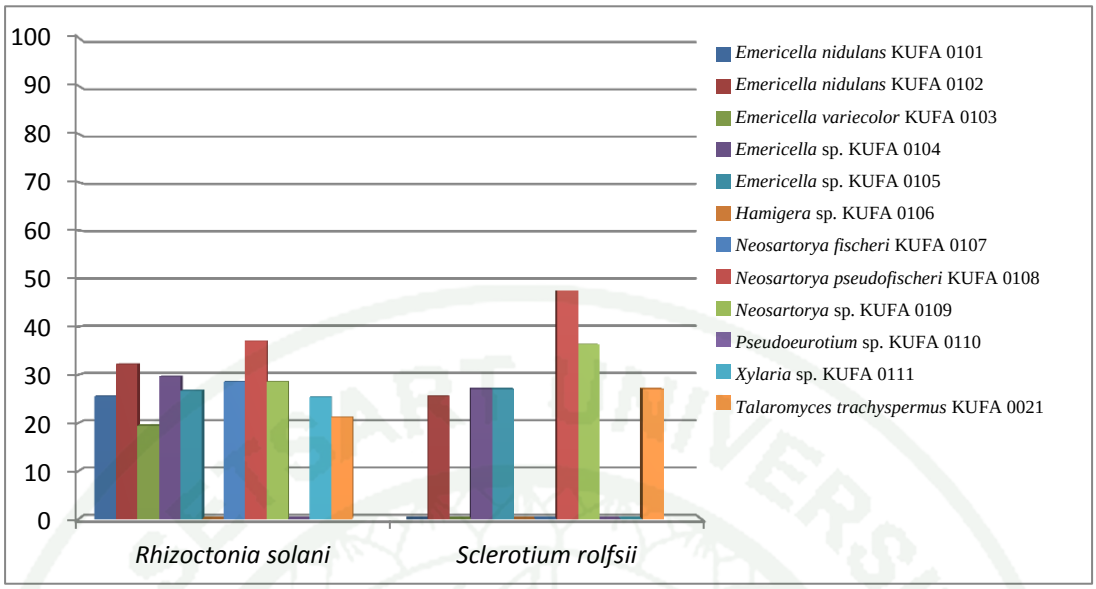


Figure 28 Percentage of inhibition on *Rhizoctonia solani* and *Sclerotium rolfsii* mycelial growth by twelve marine fungi on PDA as dual cultures.

The results on the antagonistic effect against *C. capsici*, *C. gloeosporioides* and *L. theobromae* revealed that *N. pseudofischeri* (KUFA 0108), *Emericella* sp. (KUFA 0105), *Neosartorya* sp. (KUFA 0109), *Emericella* sp. (KUFA 0104) and *E. nidulans* (KUFA 0102) displayed a moderate inhibitory effect on *C. capsici* mycelial growth, with inhibition values ranging from 50 to 60%. However, the selected fungi revealed only a weak antagonistic effect against the other *Colletotrichum* sp. The most active fungi were identified as *N. pseudofischeri* (KUFA 0108) and *Hamigera* sp. (KUFA 0106) leading to 49.25 and 45.81% of *C. gloeosporioides* mycelial growth inhibition. Additionally, none of the selected marine-derived fungi displayed relevant antagonistic activity against *L. theobromae*. In fact, five fungal species revealed no inhibitory effect on the mycelial growth (Figure 29).

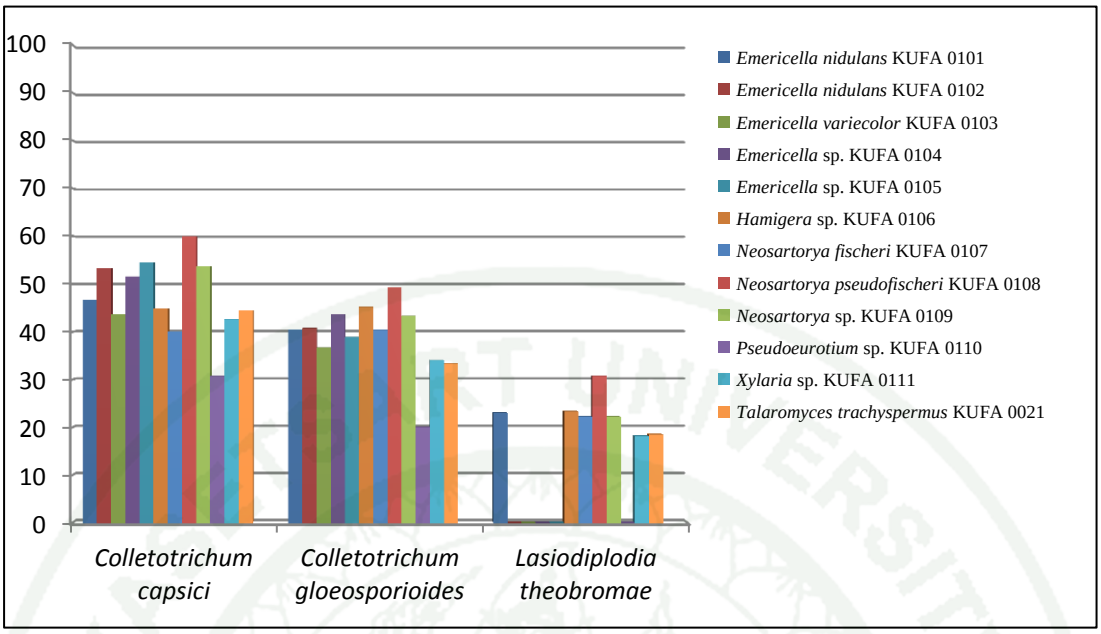


Figure 29 Percentage of inhibition on *Colletotrichum capsici*, *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* mycelial growth by twelve marine fungi on PDA as dual cultures.

Evaluation of the marine-derived fungi antagonistic effect against the three species from Hyphomycetes class revealed that more than half of the selected species displayed a moderate antifungal effect against *A. brassicola*. In fact, *E. nidulans* (KUFA 0101), *E. nidulans* (KUFA 0102), *Emericella* sp. (KUFA 0104), *Emericella* sp. (KUFA 0105), *Hamigera* sp. (KUFA 0106) and both *Neosartorya* sp. (KUFA 0108 and KUFA 0109) caused effective mycelial growth inhibition with values ranging from 50.74 to 67.03%. Most of the selected marine-derived fungi revealed also to be active on *H. maydis* mycelial growth inhibition. While *Pseudoeurotium* sp. (KUFA 0110) was identified as the most effective fungus causing 69.25% of mycelial growth inhibition, *E. nidulans* (KUFA 0101), *E. variecolor* (KUFA 0103), *N. fischeri* (KUFA 0107), *N. pseudofischeri* (KUFA 0108) and *Neosartorya* sp. (KUFA 0109) displayed moderate activity causing more than 60% of mycelial growth inhibition. Additionaly, *Hamigera* sp. (KUFA 0106) and *Xylaria* sp. (KUFA 0111) revealed to be moderately active causing more than 50% of mycelial growth inhibition. Curiously, also against *F. oxysporum* mycelial growth, *N. pseudofischeri* (KUFA

0108) and *Neosartorya* sp. (KUFA 0109) displayed the highest antifungal activity, both causing 55.18% of mycelial growth inhibition (Figure 30).

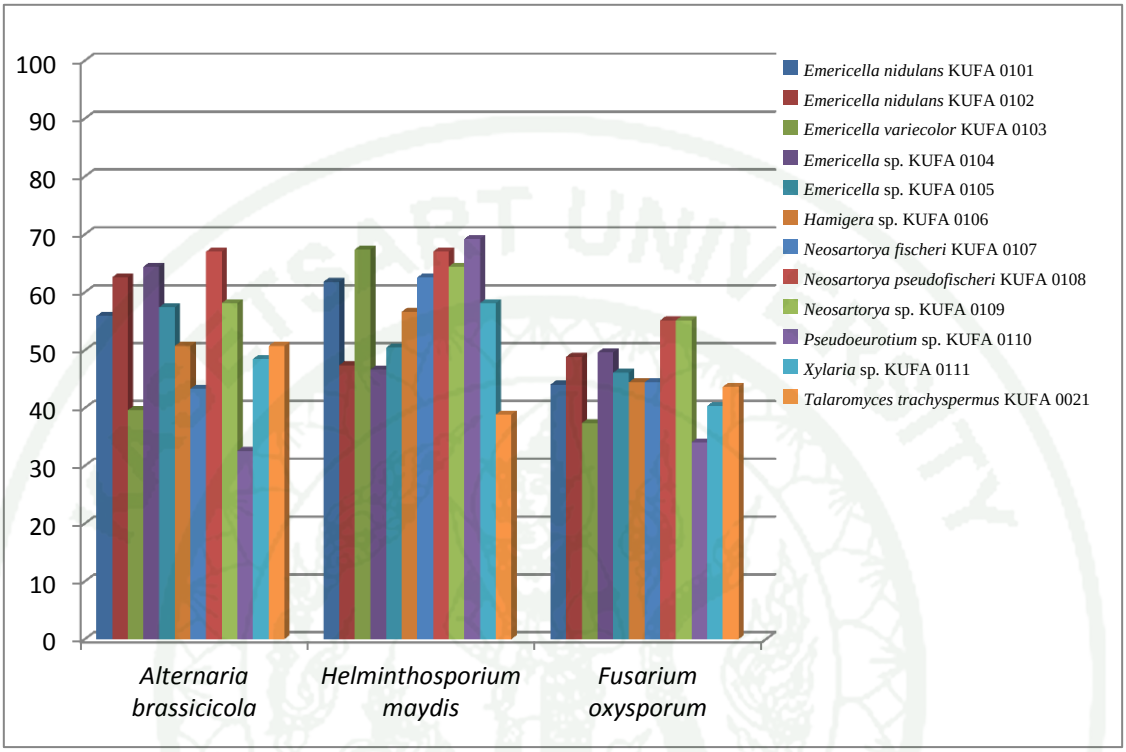


Figure 30 Percentage of inhibition on *Alternaria brassicicola*, *Helminthosporium maydis* and *Fusarium oxysporum* mycelial growth by twelve marine fungi on PDA as dual cultures.

The results from the antifungal activity against the two Oomycetes plant pathogenic fungi revealed that none of the selected marine-derived fungi could prevent *P. aphanidermatum* mycelial growth. However, both *E. nidulans* spp. (KUFA 0101 and KUFA 0102), *Emericella* sp. (KUFA 0104), *N. pseudofischeri* (KUFA 0108) and *Neosartorya* sp. (KUFA 0109) caused *P. palmivora* mycelial growth inhibition with values ranging from 52.29 to 60.00% (Figure 31).

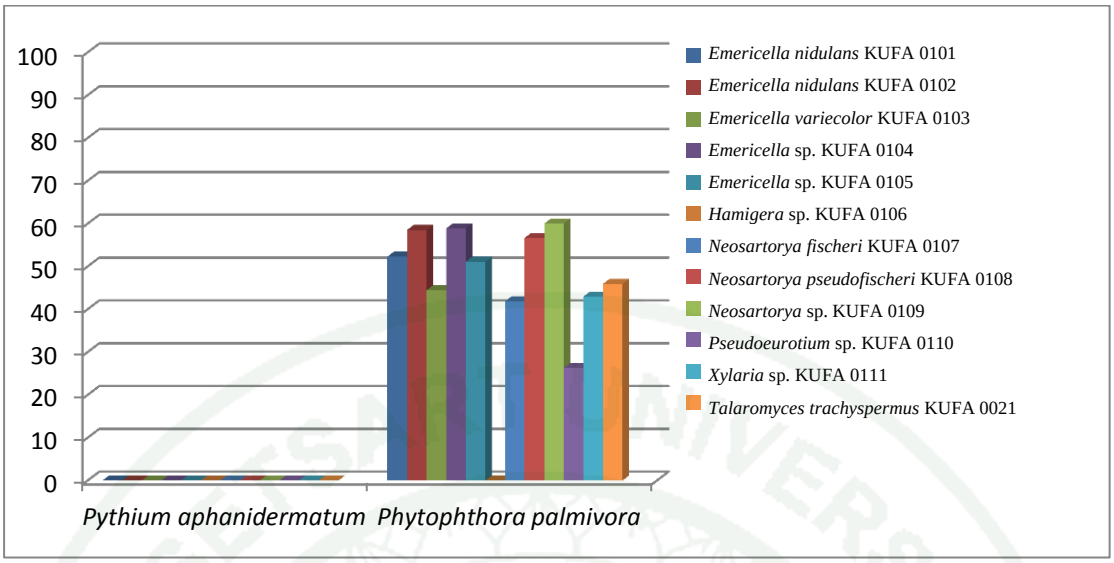


Figure 31 Percentage of inhibition on *Phytophthora palmivora* and *Pythium aphanidermatum* mycelial growth by twelve marine fungi on PDA as dual cultures.

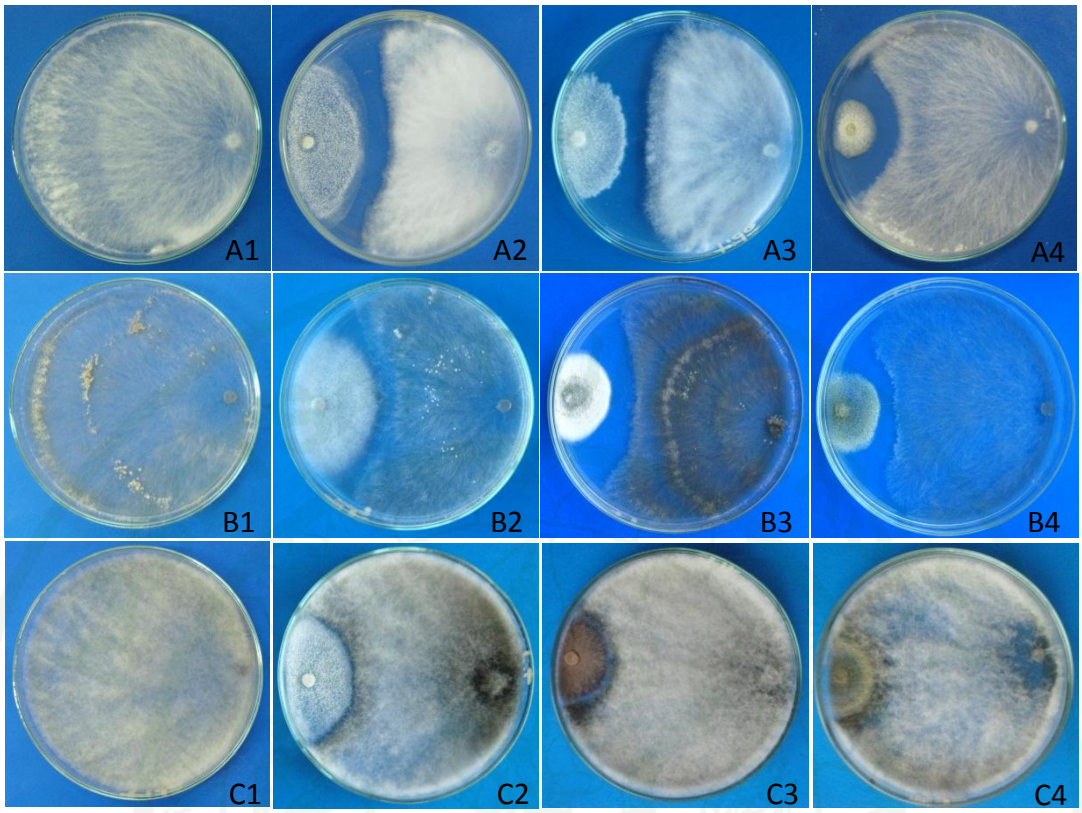


Figure 32 *In vitro* antagonistic test of marine fungi against *Sclerotium rolfsii* (A1-A4), *Rhizoctonia solani* (B1-B4) and *Lasiodiplodia theobromae* (C1-C4) as dual culture on PDA incubated 28 ^{o}C for 14 days;

A1. *Sclerotium rolfsii* (control)

A2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Sclerotium rolfsii*

A3. *Neosartorya* sp. (KUFA 0109) vs *Sclerotium rolfsii*

A4. *Talaromyces trachyspermus* (KUFA 0021) vs *Sclerotium rolfsii*

B1. *Rhizoctonia solani* (control)

B2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Rhizoctonia solani*

B3. *Emericella nidulans* (KUFA 0102) vs *Rhizoctonia solani*

B4. *Emericella* sp. (KUFA 0104) vs *Rhizoctonia solani*

C1. *Lasiodiplodia theobromae* (control)

C2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Lasiodiplodia theobromae*

C3. *Hamigera* sp. (KUFA 0106) vs *Lasiodiplodia theobromae*

C4. *Neosartorya* sp. (KUFA 0109) vs *Lasiodiplodia theobromae*

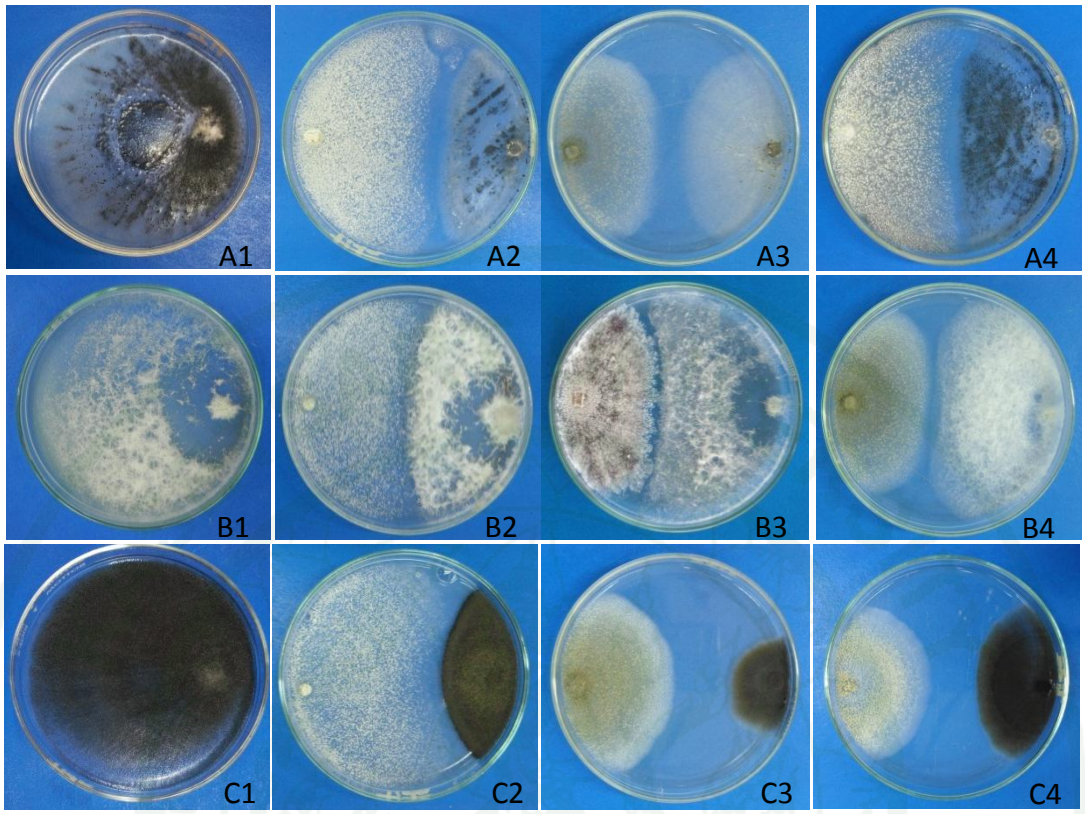


Figure 33 *In vitro* antagonistic test of marine fungi against *Colletotrichum capsici* (A1-A4), *Colletotrichum gloeosporioides* (B1-B4) and *Alternaria brassicicola* (C1-C4) as dual culture on PDA incubated 28 ^{o}C for 14 days;

A1. *Colletotrichum capsici* (control)

A2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Colletotrichum capsici*

A3. *Emericella* sp. (KUFA 0105) vs *Colletotrichum capsici*

A4. *Neosartorya* sp. (KUFA 0109) vs *Colletotrichum capsici*

B1. *Colletotrichum gloeosporioides* (control)

B2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Colletotrichum gloeosporioides*

B3. *Hamigera* sp. (KUFA 0106) vs *Colletotrichum gloeosporioides*

B4. *Emericella* sp. (KUFA 0104) vs *Colletotrichum gloeosporioides*

C1. *Alternaria brassicicola* (control)

C2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Alternaria brassicicola*

C3. *Emericella* sp. (KUFA 0104) vs *Alternaria brassicicola*

C4. *Emericella nidulans* (KUFA 0102) vs *Alternaria brassicicol*

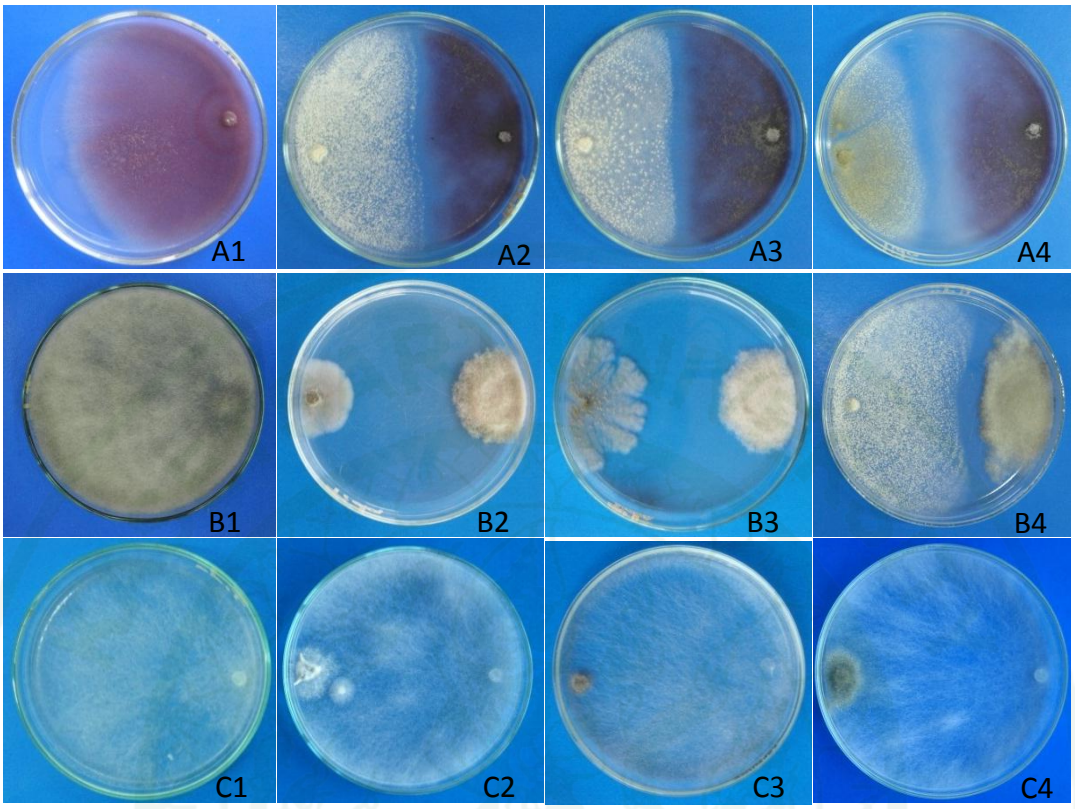


Figure 34 *In vitro* antagonistic test of marine fungi against *Fusarium oxysporum* (A1-A4), *Helminthosporium maydis* (B1-B4) and *Pythium aphanidermatum* (C1-C4) as dual culture on PDA incubated 28 ^{o}C for 14 days;

A1. *Fusarium oxysporum* (control)

A2. *Neosartorya pseudofischeri* (KUFA 0108) vs *Fusarium oxysporum*

A3. *Neosartorya* sp. (KUFA 0109) vs *Fusarium oxysporum*

A4. *Emericella* sp. (KUFA 0104) vs *Fusarium oxysporum*

B1. *Helminthosporium maydis* (control)

B2. *Pseudoeurotium* sp. (KUFA 0110) vs *Helminthosporium maydis*

B3. *Emericella varicolor* (KUFA 0103) vs *Helminthosporium maydis*

B4. *Neosartorya pseudofischeri* (KUFA 0108) vs *Helminthosporium maydis*

C1. *Pythium aphanidermatum* (Control)

C2. *Emericella* sp. (KUFA 0105) vs *Pythium aphanidermatum*

C3. *Xylaria* sp. (KUFA 0111) vs *Pythium aphanidermatum*

C4. *Pseudoeurotium* sp. (KUFA 0110) vs *Pythium aphanidermatum*

3. *In vitro* antimycelial growth activity evaluation of six marine fungi crude extracts against ten species of plant pathogenic fungi

The crude EtOAc extracts of *Emericella nidulans* (KUFA 0101), *Hamigera* sp. (KUFA 0106), *Neosartorya fischeri* (KUFA 0107) *Neosartorya pseudofischeri* (KUFA 0108), *Pseudoeurotium* sp. (KUFA 0110) and *Talaromyces trachyspermus* (KUFA 0021) were tested for their antifungal activity against ten plant pathogenic fungi, namely *Rhizoctonia solani*, *Sclerotium rolfsii*, *Colletotrichum capsici*, *C. gloeosporioides*, *Lasiodiplodia theobromae*, *Alternaria brassicicola*, *Fusarium oxysporum*, *Helminthosporium maydis*, *Pythium aphanidermatum* and *Phytophthora palmivora*. The effectiveness of the antifungal activity of the selected marine fungi was assessed based on the percentage of mycelial growth inhibition.

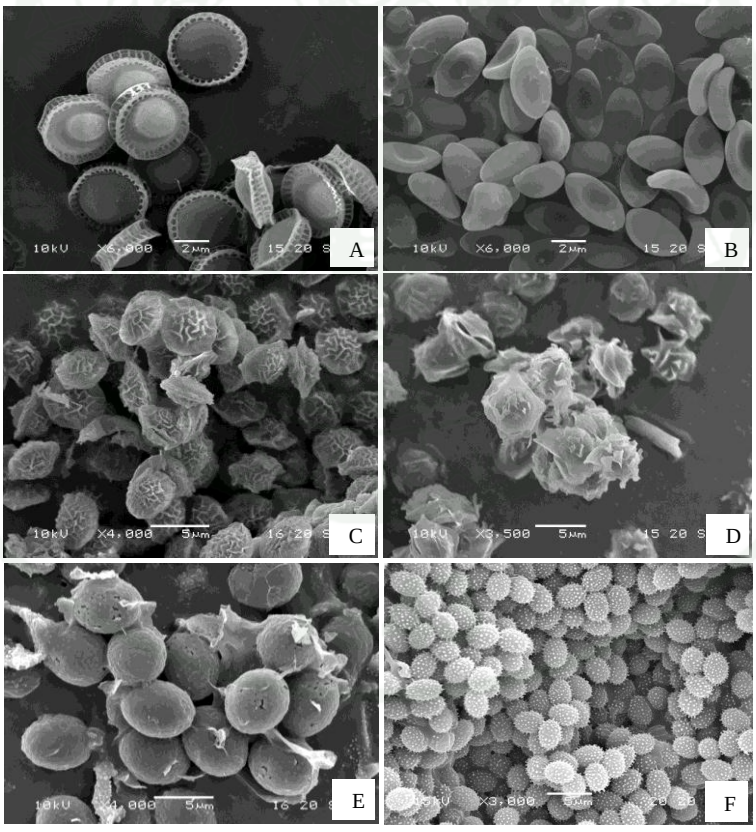


Figure 35 Scanning electron microscopes of ascospores of marine-derived fungi;

A. *Emericella nidulans* B. *Pseudoeurotium* sp.

C. *Neosartorya fischeri* D. *Neosartorya pseudofischeri*

E. *Hamigera* sp. F. *Talaromyces trachyspermus*

3.1 *Emericella nidulans* (KUFA 0101) crude extract antifungal activity evaluation against plant pathogenic fungi

Antifungal activity evaluation of *E. nidulans* (KUFA 0101) crude extract against the selected plant pathogenic fungi, revealed a relevant antagonistic effect at the highest concentration tested. At the concentration of 10,000 ppm, *E. nidulans* (KUFA 0101) EtOAc crude extract caused a complete inhibition on *Ph. palmivora* and *P. aphanidermatum* mycelial growth, as well as moderate effect against *R. solani* and *S. rolfsii*, leading to 57.4% of mycelial growth inhibition for both plant pathogens (Table 7).

Table 7 Inhibitory effect of *Emericella nidulans* (KUFA 0101) EtOAc crude extract on the mycelial growth of ten plant pathogenic fungi.

Plant pathogenic fungi	**% mycelial growth inhibition at concentrations (ppm)**				
	1	**10**	**100**	**1,000**	**10,000**
Rhizoctonia solani	0	0	0	0	57.40
Sclerotium rolfsii	8.59	0	0	24.44	57.40
Colletotrichum capsici	10.00	11.48	12.59	24.81	29.25
Colletotrichum gloeosporioides	0	0	0	11.11	32.22
Lasiodiplodia theobromae	0	0	0	19.63	31.48
Alternaria brassicicola	10.00	11.85	14.07	21.11	44.44
Fusarium oxysporum	0	0	0	0	17.40
Helminthosporium maydis	0	0	0	0	36.66
Pythium aphanidermatum	0	0	0	0	100
Phytophthora palmivora	0	0	0	0	100

3.2 *Hamigera* sp. (KUFA 0106) crude extract antifungal activity evaluation against plant pathogenic fungi

At the highest concentration tested (10,000 ppm), *Hamigera* sp. (KUFA 0106) crude extract displayed relevant antifungal activity against most of the tested plant pathogenic fungi, causing a complete mycelial growth inhibition of *R. solani*, *S. rolfsii*, *C. gloeosporioides*, *L. theobromae* and *P. aphanidermatum*, as well as a

moderate effect against the remaining plant pathogenic fungi, causing more than 50% of mycelial growth inhibition. Additionally, at the concentration of 1,000 ppm *Hamigera* sp. (KUFA 0106) crude extract displayed also a moderate inhibitory effect on the mycelial growth of both Agonomycetes, *R. solani* and *S. rolfsii*, causing 66.66 and 50.74% of mycelial growth inhibition, respectively (Table 8).

Table 8 Inhibitory effect of *Hamigera* sp. (KUFA 0106) EtOAc crude extract on the mycelial growth of ten plant pathogenic fungi.

Plant pathogenic fungi	% mycelial growth inhibition at concentrations (ppm)				
	1	**10**	**100**	**1,000**	**10,000**
Rhizoctonia solani	0	0	25.92	66.66	100
Sclerotium rolfsii	0	0	29.63	50.74	100
Colletotrichum capsici	22.22	16.66	28.14	39.25	72.59
Colletotrichum gloeosporioides	22.22	22.22	28.14	30.04	100
Lasiodiplodia theobromae	0	0	0	3.70	100
Alternaria brassicicola	15.55	19.25	25.55	43.33	66.66
Fusarium oxysporum	0	0	14.07	38.88	66.66
Helminthosporium maydis	21.11	27.77	16.66	23.70	62.96
Pythium aphanidermatum	0	0	0	0	100
Phytophthora palmivora	0	0	0	40.47	55.55

3.3 *Neosartorya fischeri* (KUFA 0107) crude extract antifungal activity evaluation against plant pathogenic fungi

Antifungal activity screening revealed that at the highest concentration tested, *N. fischeri* (KUFA 0107) EtOAc crude extract caused complete mycelial growth inhibition in all plant pathogens, except for *L. theobromae*. Additionally, at the concentration of 1,000 ppm, *N. fischeri* (KUFA 0107) crude extract displayed moderate antifungal activity against *S. rolfsii* and *P. aphanidermatum*, leading to 62.96 and 51.11% of mycelial growth inhibition, respectively (Table 9).

Table 9 Inhibitory effect of *Neosartorya fischeri* (KUFA 0107) EtOAc crude extract on the mycelial growth of ten plant pathogenic fungi.

Plant pathogenic fungi	% mycelial growth inhibition at concentrations (ppm)				
	1	**10**	**100**	**1,000**	**10,000**
Rhizoctonia solani	0	0	15.55	41.48	100
Sclerotium rolfsii	0	0	17.40	62.96	100
Colletotrichum capsici	0	0	9.63	18.51	100
Colletotrichum gloeosporioides	0	0	0	17.40	100
Lasiodiplodia theobromae	0	0	0	0	0
Alternaria brassicicola	0	13.33	11.11	34.81	100
Fusarium oxysporum	0	0	11.85	32.59	100
Helminthosporium maydis	0	0	0	0	100
Pythium aphanidermatum	0	0	0	51.11	100
Phytophthora palmivora	0	0	0	0	100

3.4 *Neosartorya pseudofischeri* (KUFA 0108) crude extract antifungal activity evaluation against plant pathogenic fungi

The results from the antagonistic effect evaluation of *N. pseudofischeri* (KUFA 0108) EtOAc crude extract against plant pathogenic fungi revealed a moderate to strong effect against all the plant pathogenic fungi at the highest concentration tested (10,000 ppm). The extract displayed strong antifungal activity against *C. gloeosporioides*, *L. theobromae* and *H. maydis*, leading to 92.59, 87.22 and 80.37% of mycelial growth inhibition, as well as a complete mycelial growth inhibition of *P. aphanidermatum* (Table 10). For the remaining plant pathogens, *N. pseudofischeri* (KUFA 0108) EtOAc crude extract revealed a moderate antifungal activity, causing more than 50% of mycelial growth inhibition.

Table 10 Inhibitory effect of *Neosartorya pseudofischeri* (KUFA 0108) EtOAc crude extract on the mycelial growth of ten plant pathogenic fungi.

Plant pathogenic fungi	% mycelial growth inhibition at concentrations (ppm)				
	1	**10**	**100**	**1,000**	**10,000**
Rhizoctonia solani	0	0	0	47.03	61.11
Sclerotium rolfsii	0	0	0	0	64.44
Colletotrichum capsici	16.66	15.18	22.22	37.22	73.70
Colletotrichum gloeosporioides	0	0	9.63	23.33	92.59
Lasiodiplodia theobromae	6.29	2.22	12.59	43.70	87.22
Alternaria brassicicola	29.25	38.14	40.37	49.44	77.77
Fusarium oxysporum	0	0	0	11.85	50.74
Helminthosporium maydis	9.25	14.81	25.0	42.77	80.37
Pythium aphanidermatum	0	0	0	5.55	100
Phytophthora palmivora	0	0	0	6.66	77.03

3.5 *Pseudoeurotium* sp. (KUFA 0110) crude extract antifungal activity evaluation against plant pathogenic fungi

Results from the antagonistic activity evaluation of *Pseudoeurotium* sp. (KUFA 0110) EtOAc crude extract revealed that at the concentration of 10,000 ppm, it caused a complete inhibition on the mycelial growth of four plant pathogenic fungi, namely *Ph. palmivora*, *P. aphanidermatum*, *S. rolfsii* and *R. solani*. *Pseudoeurotium* sp. (KUFA 0110) EtOAc crude extract displayed also a moderate inhibitory effect on the mycelial growth of *H. maydis* and *A. brassicicola* causing 54.01% inhibiton at the highest concentration tested (10,000 ppm), in both species (Table 11).

Table 11 Inhibitory effect of *Pseudoeurotium* sp. (KUFA 0110) EtOAc crude extract on the mycelial growth of ten plant pathogenic fungi.

Plant pathogenic fungi	% mycelial growth inhibition at concentrations (ppm)				
	1	**10**	**100**	**1,000**	**10,000**
Rhizoctonia solani	0	0	0	0	100
Sclerotium rolfsii	21.48	22.22	27.40	34.44	100
Colletotrichum capsici	0	0	0	17.40	31.11
Colletotrichum gloeosporioides	0	0	0	0	40.74
Lasiodiplodia theobromae	0	0	0	0	16.66
Alternaria brassicicola	17.77	17.03	32.22	41.85	54.01
Fusarium oxysporum	0	0	0	19.25	37.03
Helminthosporium maydis	10.00	24.07	32.22	41.85	54.01
Pythium aphanidermatum	0	0	0	0	100
Phytophthora palmivora	0	0	0	0	100

3.6 *Talaromyces trachyspermus* (KUFA 0021) crude extract antifungal activity evaluation against plant pathogenic fungi

Antifungal activity screening of *T. trachyspermus* (KUFA 0021) EtOAc crude extract revealed a complete mycelial growth inhibition in all plant pathogenic fungi, at the concentration of 10,000 ppm. Inhibition on the mycelial growth remained effective even at the concentration of 1,000 ppm, causing a complete mycelial growth inhibition of *R. solani*, *S. rolfsii*, *C. capsici*, *A. brassicicola*, *H. maydis* and *P. aphanidermatum*. Also at the concentration of 1,000 ppm, *T. trachyspermus* (KUFA 0021) EtOAc crude extract displayed moderate to strong antifungal activity against *C. gloeosporioides*, *L. theobromae* and *F. oxysporum*, with mycelial growth inhibition values ranging from 68.51 to 84.07%. Additionally, at the concentration as low as 100 ppm, the extract could moderately prevent the mycelial growth of *P. aphanidermatum*, causing 50.00% of inhibition (Table 12).

Table 12 Inhibitory effect of *Talaromyces trachyspermus* (KUFA 0021) EtOAc crude extract on the mycelial growth of ten plant pathogenic fungi.

Plant pathogenic fungi	% mycelial growth inhibition at concentrations (ppm)				
	1	**10**	**100**	**1,000**	**10,000**
Rhizoctonia solani	0	0	30.00	100	100
Sclerotium rolfsii	0	0	45.92	100	100
Colletotrichum capsici	0	0	13.33	100	100
Colletotrichum gloeosporioides	0	0	0	75.92	100
Lasiodiplodia theobromae	0	0	0	84.07	100
Alternaria brassicicola	17.77	15.53	27.77	100	100
Fusarium oxysporum	0	0	29.63	68.51	100
Helminthosporium maydis	0	6.66	21.48	100	100
Pythium aphanidermatum	0	0	50.00	100	100
Phytophthora palmivora	0	0	0	31.66	100

As previously referred, at the concentration of 10,000 ppm, *E. nidulans* (KUFA 0101) EtOAc crude extract displayed strong antifungal activity against some plant pathogenic fungi. Sibounnavong *et al*., (2009) have reported the antifungal activity of *E. nidulans* crude extract against *Fusarium* wilt pathogen. Additionally, some secondary metabolites with antifungal properties have been reported from *E. nidulans*, which may partially explain the crude extract activity against plant pathogenic fungi. *E. nidulans* isolate EN01 hexane and EtOAc extracts, yielded six compounds including epishamixanthone, shamixanthone, emericellin, ergosta-6, 22-diene-3-ol-5, 8-epidioxy-(3β-5α, 22E), sterigmatocystin and demethylsterigmatocystin, some of them displaying antifungal activity against plant pathogenic fungi (Moosophoon *et al*., 2006).

Analogously, the chemical analysis of *Hamigera avellanea* crude extract resulted in the isolation of (Z,Z)-N,N'-[1-[(4-hydroxy-phenyl)-methylene]-2-[(4-methoxy-phenyl)-methylene]-1,2-ethanediyl]-bis-for-mamide, which exhibited marginal activity against a variety of pathogenic fungi (*Pyricularia oryzae* and *Venturia inaequalis*) and bacteria (Adbel Rahim, 2011; Breinholt *et al*., 1996).

Eamvijarn *et al*. (2013) reported the antifungal activity of the crude extract of a *Neosartorya fischeri* strain, collected from a soil sample. Comparing to *N. fischeri* (KUFA 0107) and *N. pseudofischeri* (KUFA 0108) crude extracts, *N. fischeri* EtOAc extract revealed stronger inhibitory activity against the mycelial growth of phytopathogenic fungi, causing almost complete mycelial growth inhibition of *P. aphanidermatum* and *P. palmivora*, as well as strong to moderate antifungal effect against the majority of the plant pathogenic fungi, at the concentration of 1,000 ppm.

Analysis of the results clearly identified *T. trachyspermus* (KUFA 0021) EtOAc crude extract as the most active, leading to the complete mycelial growth inhibition of all the plant pathogenic fungi, at the highest concentration tested (10,000 ppm). Additionally, the extract also displayed moderate to strong antifungal activity, at the concentration of 1,000 ppm, against the majority of the selected plant pathogens.

Recently, *T. trachyspermus* antifungal activity of against *R. solani* was also reported. Comparing to the antifungal activity of *T. trachyspermus* (KUFA 0021), Sreeta *et al.* (2014) reported only a moderate effect, causing 50% of mycelial growth inhibition.

Despite the reports on the isolation of secondary metabolites from *Talaromyces trachyspermus* such as trachyspic acid, decylcitric acid and spiculisporic acid, there are no reports on the antifungal activity of metabolites isolated from the *Talaromyces* genus. So, taking this into account as well as the fact that from the selected marine-derived fungi, *Talaromyces trachyspermus* (KUFA 0021) EtOAc crude extract has been identified as the most active, we selected the EtOAc crude extract for further chemical analysis.

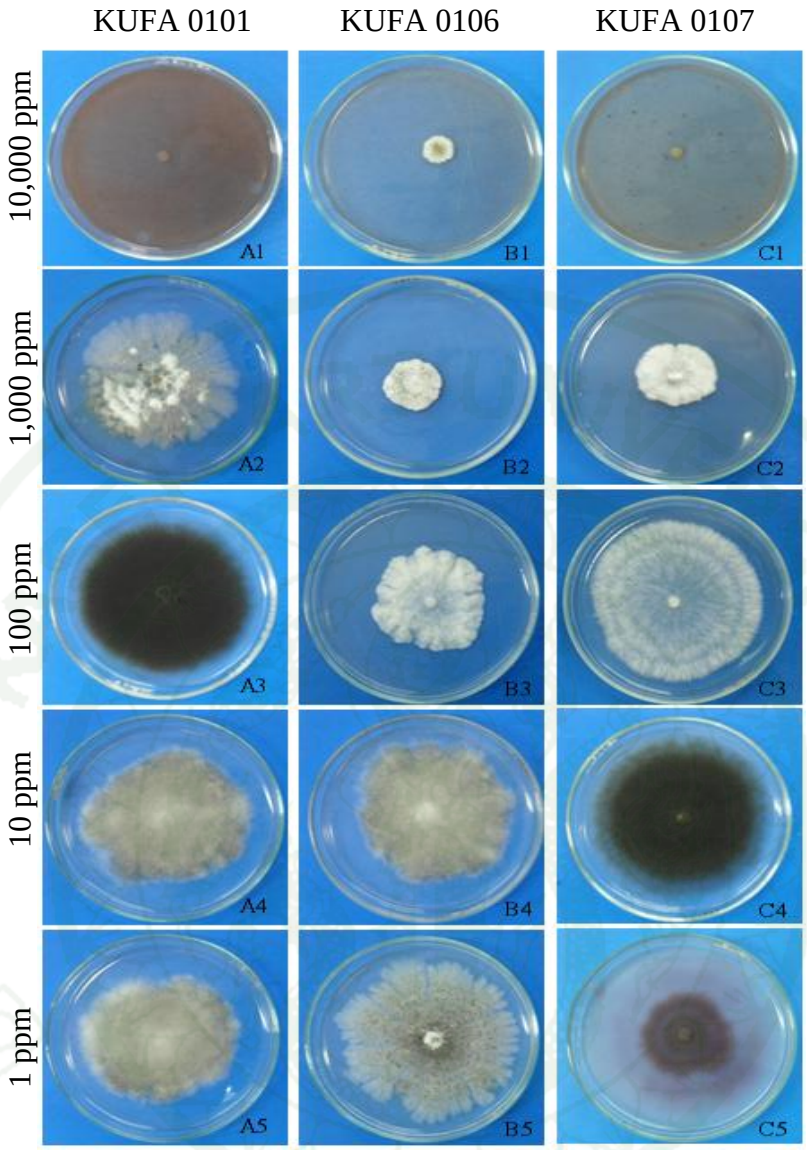


Figure 36 Antagonistic effect of EtOAc crude extracts of *Emericella nidulans* KUFA 0101 (A1-A5), *Hamigera* sp. KUFA 0106 (B1-B5) and *Neosartorya fischeri* KUFA 0107 (C1-C5) against plant pathogenic fungi on PDA at 28 ^{o}C for 7 days;

A1. *Pythium aphanidermatum* A2. *Colletotrichum capsici*
A3. *Alternaria brassicicola* A4-A5. *Helminthosporium maydis*
B1. *Lasiodiplodia theobromae* B2. *Rhizoctonia solani*
B3. *Sclerotium rolfsii* B4. *Helminthosporium maydis*
B5. *Colletotrichum capsici* C1. *Phytophthora palmivora*
C2-C3. *Sclerotium rolfsii* C4. *Alternaria brassicicola*
C5. *Fusarium oxysporum*

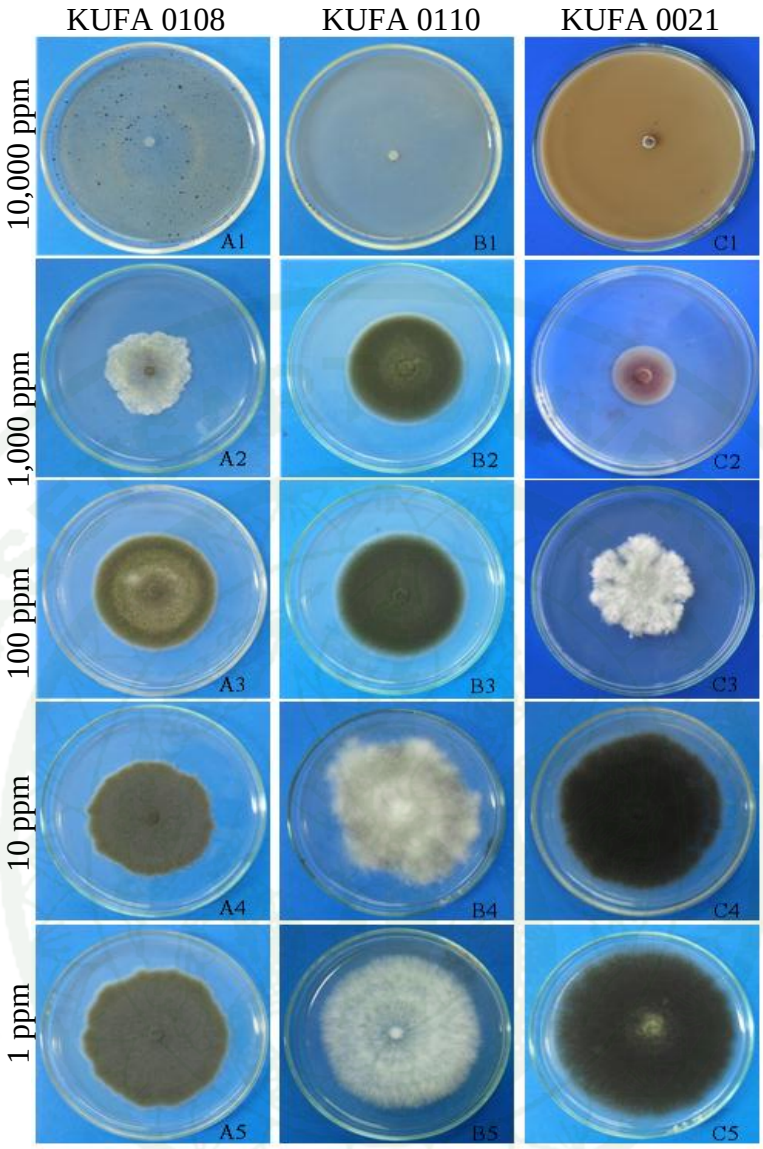


Figure 37 Antagonistic effect of EtOAc crude extracts of *Neosartorya pseudofischeri* KUFA 0108 (A1-A5), *Pseudoeurotium* sp. KUFA 0110 (B1-B5) and *Talaromyces trachyspermus* KUFA 0021 (C1-C5) against plant pathogenic fungi on PDA at 28 ^{o}C for 7 days;

A1. *Pythium aphanidermatum* A2. *Lasiodiplodia theobromae*
A3-A5. *Alternaria brassicicola* B1. *Sclerotium rolfsii*
B2-B3. *Alternaria brassicicola* B4. *Helminthosporium maydis*
B5. *Sclerotium rolfsii* C1. *Rhizoctonia solani*
C2. *Fusarium oxysporum* C3. *Sclerotium rolfsii*
C4-C5. *Alternaria brassicicola*

4. Isolation and purification of the secondary metabolites from *Talaromyces trachyspermus* (KUFA 0021)

The fungus *Talaromyces trachyspermus* KUFA 0021 was isolated from the marine sponge *Clathria reinwardtii*, collected from the coral reef from the Gulf of Thailand. The EtOAc extract of the culture of this fungus furnished, besides glaucanic (1a) and glauconic acids (1b) (Barton *et al*., 1962), a new spiculisporic acid derivative which we have named spiculisporic acid E (4), a new natural product 3-acetyl ergosterol 5, 8-endoperoxide (3) as well as ergosta-4, 6, 8 (14), 22-tetraen-3-one (2) (Figure 38). All the isolated compounds were tested for their antimicrobial activity against Gram positive (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633) and Gram negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacteria, *Candida albicans*, and multidrug-resistant isolates from the environment as well as for their *in vitro* growth inhibitory activity against the MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines by protein binding dye SRB method.

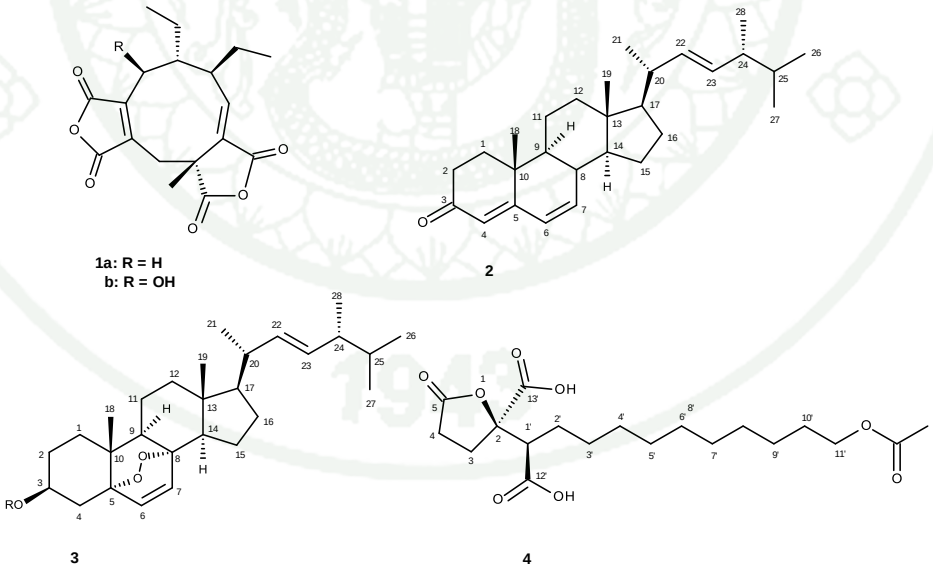


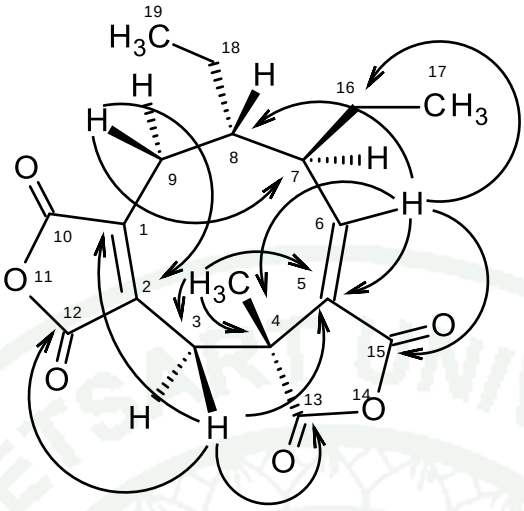
Figure 38 Secondary metabolites isolated from the culture of the marine-derived *Talaromyces trachyspermus* KUFA 0021.

Compound 1a was isolated as white crystal (Mp. 189-190 ºC) and its molecular formula $C_{18}H_{20}O_6$ was established on the basis of the (+)-HRESIMS *m/z* 333.1326 $[M+H]^+$, indicating nine degrees of unsaturation. The IR spectrum showed absorption bands for carbonyl (1835, 1767, 1671 cm^{-1}) and olefin (1652, 1449 cm^{-1}) groups. The ^{13}C NMR, DEPTs and HSQC spectra (Table 13) revealed the presence of four ester carbonyls (δ_C 173.7, 165.3, 164.5 and 163.7), three quaternary sp^2 (δ_C 148.4, 140.5, 131.8), one methine sp^2 (δ_C 150.1), one quaternary sp^3 (δ_C 48.5), two methine sp^3 (δ_C 48.1 and 43.9), four methylene sp^3 (δ_C 31.7, 28.4, 26.0 and 21.4) and three methyl (δ_C 20.3, 12.8 and 12.4) carbons. The ^1H NMR spectrum exhibited, besides a doublet at δ_H 6.99 (*J* = 12.2 Hz) of one olefinic proton (δ_C 150.1) and two doublets of geminally coupled protons at δ_H 3.27 (*J* = 13.6 Hz) and δ_H 2.69 (*J* = 11.5 Hz)(δ_C 31.7), two methyl triplets at 0.81 (*J* = 7.4 Hz, δ_C 12.8) and 1.08 (*J* = 7.3 Hz, δ_C 12.4), a methyl singlet at δ_H 1.50s (δ_C 20.3), a broad doublet at δ_H 2.88 (δ_C 28.4), a broad signal at δ_H 2.00 (δ_C 28.4) and multiplets at δ_H 2.09 (δ_C 43.9), 2.11 (δ_C 48.1), 1.87 (δ_C 21.4), 1.68 (δ_C 26.0), 1.51 (δ_C 26.0), 1.15 (δ_C 21.4). The COSY spectrum exhibited correlation of the olefinic proton at δ_H 6.99 d (*J* = 12.2, δ_C 150.1) to the multiplet at δ_H 2.09 (δ_C 43.9), of a broad doublet at δ_H 2.88 (*J* = 10.3 Hz, δ_C 24.8) to a broad signal at δ_H 2.00 (δ_C 24.8) and a multiplet at δ_H 2.11 (δ_C 48.1). The multiplet at δ_H 2.11 (δ_C 48.1) also gave cross peaks to multiplets at δ_H 1.87 (δ_C 21.4) and δ_H 1.15 (δ_C 21.4), which in turn, gave cross peaks to a methyl triplet at δ_H 1.08 (*J* = 7.3, δ_C 21.4). Similarly, the multiplets at δ_H 1.68 and 1.51 (δ_C 26.0) showed cross peaks to a multiplet at δ_H 2.09 (δ_C 43.9) and a methyl triplet at δ_H 0.81 (*J* = 7.4, δ_C 12.8). The COSY correlations confirmed the coupling systems of CH-6-CH-7-CH_2-16-CH_3-17 and CH_2-9-CH-8-CH_2-18-CH_3-19. That C-7 was connected to C-8 was corroborated by the HMBC cross peaks (Figure 39) of H-6 at δ_H 6.99 (*J* = 12.2 Hz) to C-8 (δ_C 48.1) and of H-9 (δ_H 2.88 d, *J* = 10.3 Hz). Moreover, H-6 also show HMBC cross peaks to the signals of the quaternary sp^2 carbon at δ_C131.8 (C-5), quaternary sp^3 carbon at δ_C 48.5 (C-4), methylene carbon at δ_C 26.0 (C-16), and a carbonyl at δ_C 163.7 (C-15). That the methyl group at δ_H1.50s (δ_C 20.3) was on C-4 was evidenced by the HMBC cross peaks of the methyl singlet at δ_H 1.50s to the carbons at δ_C 48.5 (C-4). Moreover, this methyl signal also gave cross peaks to C-5 and the methylene carbon at δ_C 31.7 (C-3). In turn, the methylene proton at δ_H 3.27, d (J = 13.6 Hz) exhibited cross peaks to the carbonyls at δ_C 173.7 (C-13), 165.3 (C-12) as well as to C-4, C-5,

and the quaternary sp^2 carbon at δ_C 140.5 (C-1). Taking these HMBC correlations into account, another coupling system of C-1 through C-6 was confirmed. That C-1 was connected to C-9 was substantiated by the HMBC correlations (Figure 39) of H-9 (δ_H 2.88 d, J = 10.3 Hz) to C-1 (δ_C 140.5) and C-2 (δ_C 148.4), thus forming a cyclononene portion. That the dihydrofuran-2, 5-dione was on C-4 and C-5, and the furan-2, 5-dione was on C-1 and C-2 was substantiated by the HMBC cross peaks of H-6 (δ_H 6.99) to C-15 (δ_C 163.7), of CH_3-20 (δ_H 1.50s) to C-13 (δ_C 173.7), as well as of H-3 (δ_H 3.27, d, J = 13.6) to C-13 (δ_C 173.7), C-12 (δ_C 1165.3).

Table 13 NMR data ($CDCl_3$, 500.13, 125. 77 MHz) of glaucanic acid (1a).

Position	δ_C, type	δ_H, (J in Hz)	COSY	HMBC
1	140.5, C	-	-	-
2	148.4, C	-	-	-
3a	31.7, CH_2	3.27,d (13.6)	H-3b	C-1, 2, 4,5, 12, 13
B		2.69, brd (11.5)	H3-a	-
4	48.5, C	-		C-4, 5, 6, 7, 8
5	131.8, C	-		
6	150.1, C	6.99, d (12.2)	H-7	C-4, 5, 7, 8 15, 16
7	43.9, CH	2.09, m	H-6, 8	C-6
8	48.1, CH	2.11, m	H-7, 9a, 9b	C-6
9a	28.2, CH_2	2.88, brd (10.3)	H-8, 9b	C-1, 2, 7, 8
B		2.00, br	H-8, 9a	-
10	164.5, CO	-	-	
12	165.3, CO	-	-	
13	173.7, CO	-	-	
15	163.7, CO	-	-	
16a	26.0, CH_2	1.68, m	H-7, 16b, 17	C-6, 7, 17
B		1.51, m	H-7, 16a, 17	C-6, 7, 17
17	12.8, CH_3	0.81, t (7.4)	H-16a, b	C-7, 16
18a	21.4, CH_3	1.87, m	H-8, 18b, 19	C-8, 9, 19
B		1.15, m	H-8, 18a, 19	C-8, 19
19	12.4, CH_3	1.08, t (7.3)	H-18a, b	C-8, 18
20	20.3, CH_3	1.50, s	-	C-3, 4, 5



1a

Figure 39 Key HMBC correlations of compound glauconic acid (1a).

Since compound 1a formed suitable crystal, the X-ray diffraction was carried out. X-ray analysis, as represented by the ORTEP view in Figure 40, not only confirmed the structure but also allowed us to determine the absolute configuration of C-4, C-7 and C-8 as 4*R*, 7*R* and 8*S*, respectively.

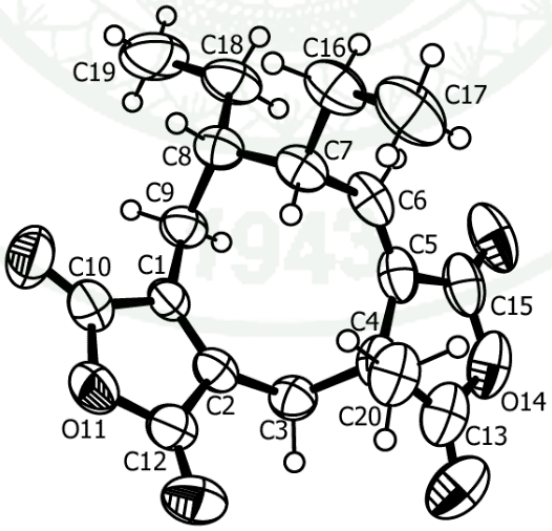
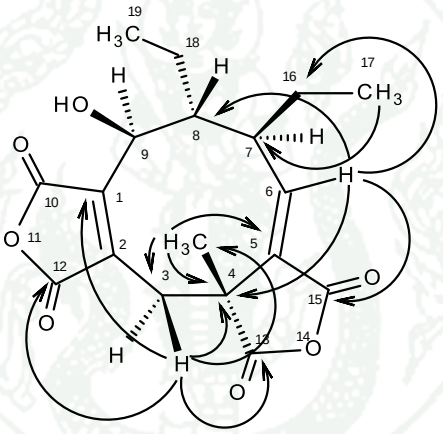


Figure 40 The ORTEP view of Key HMBC correlations of compound glauconic acid (1a).

Literature search revealed that the structure of 1a corresponded to that of glaucanic acid, a secondary metabolite previously reported from several fungal species including *Penicillium glaucum*, *Penicillium purpurogenum* and *Talaromyces atroroseus* (Baldwin *et al*., 1962; Natori *et al*., 1970; Frisvad *et al*., 2013).

Compound 1b was isolated as white crystal (Mp. 199-200ºC) and its molecular formula $C_{18}H_{20}O_7$ was established on the basis of the (+)-HRESIMS *m/z* 349.1291 $[M+H]^+$, indicating nine degrees of unsaturation. The IR spectrum showed absorption bands for hydroxyl (3575 cm^{-1}), carbonyl (1849, 1764 cm^{-1}), and olefin (1660, 1456cm^{-1}) groups. The general feature of the ^1H and ^{13}C NMR spectra of 1b closely resembled that of 1a (Table 14). The ^{13}C NMR, DEPTs and HSQC spectra (Table 15) revealed the presence of four ester carbonyls (δ_C 174.1, 164.6, 164.6 and 163.4), three quaternary sp^2 (δ_C 146.4, 143.2, 129.1), one methine sp^2 (δ_C 150.5), one quaternary sp^3 (δ_C 47.4), one oxygen bearing methine sp^3 (δ_C 65.4), two methine sp^3 (δ_C 52.9 and 37.7), three methylene sp^3 (δ_C 31.7, 27.7 and 19.5) and three methyl (δ_C 26.4, 13.0 and 12.4) carbons. The ^1H NMR spectrum exhibited, besides a doublet at δ_H 6.88 (*J* = 12.4 Hz) of one olefinic proton (δ_C 150.5) and a broad singlet at δ_H 5.09 (δ_C 65.4) of an oxymethine proton, two doublets of the germinally coupled methylene protons at δ_H 3.80 (*J* = 13.6 Hz, δ_C 31.7) and δ_H 3.44 (*J* = 13.4 Hz, δ_C 31.7), two triplets of the methyl protons at δ_H 1.15 (*J* = 7.3, δ_C 12.4) and δ_H 0.90 (*J* = 7.4, δ_C 13.0), a methyl singlet at δ_H 1.72 (δ_C 26.4), two multiplets at δ_H 1.64 (δ_C 19.5, 27.7) and δ_H 2.12m (δ_C 52.9) as well as a broad signal of one proton at δ_H 3.07 (δ_C 37.7). The COSY spectrum showed cross peaks of the signal of H-6 at δ_H 6.88 d, (*J* = 12.4 Hz) to signal of H-7 (δ_H 3.07), of the signal of H-7 to H-6 and H-16 (δ_H 1.64), of H-16 signal to CH_3-17 (δ_H 0.90, t, *J* = 7.4 Hz) signal, of H-8 signal (δ_H 2.12, m) to H-7 and H-18 (δ_H 1.64) signals, of H-18 signal to CH_3-19 (δ_H 1.15, *t*, *J* = 7.3) signal, and of H-9 signal (δ_H 5.09, brs) to the signal of H-8, thus confirming the coupling system of C-6 through C-17, and C-9 through C-19. This was confirmed by the HMBC crosspeaks of CH_3-17 to C-7 (δ_C 37.7) and C-16 (δ_C 27.7), of CH_3-19 to C-8 (δ_C 52.9) and C-18 (δ_C 19.5). That C-7 was connected to C-8 was evidenced by the HMBC cross peaks between H-16 signal (δ_H 1.64, m) to the signal of C-8 (δ_C 52.9). Furthermore, the HMBC spectrum also showed correlations of H-16 to the methine sp^2 carbon at δ_C 150.5 (H-6). In turn, H-6 also exhibited HMBC cross peaks to the quaternary carbon at δ_C 47.4

(C-4) and the carbonyl carbon at δ_C 163.4 (C-15) while the methylene protons at δ_H 3.80, d (J = 13.6, H-3) showed correlations to the quaternary sp^3 carbon at δ_C 47.4 (C-4), the quaternary sp^2 carbon at δ_C 143.2 (C-1), the carbonyl carbons at δ_C 164.6 (C-12) and δ_C 174.1 (C-13). Another H-3 signal (δ_H 3.44, d, J = 13.4, H-3) also gave HMBC cross peaks to C-4, C-5 (δ_C 129.1), C-1 and C-2 (δ_C 146.4) and the carbonyl carbon at δ_C 174.1 (C-13), thus confirming the coupling system of C-1 through C-6. That the methyl group at δ_H 1.72, brs (δ_C 26.4) was on C-4 was supported by the HMBC cross peaks of the broad singlet at δ_H 1.72 to C-3 (δ_C 31.7), C-4 (δ_C 47.4), C-5 (δ_C 129.1) and CO-13 (174.1) (Figure 41). Taking together the NMR data, the molecular formula and the degree of unsaturation, the structure of 1b should contain the cyclooctanonene ring, four carbonyls, and another two rings.



1b

Figure 41 Key HMBC correlations of compound glaucanic acid (1b).

Since 1b could be obtained as suitable crystals, its structure was also confirmed by X-ray crystallography. The ORTEP views of 1b (Figure 42) showed that the absolute configuration of C-4, C-7, C-8 and C-9 are 4*R*, 7*R*, 8*R* and 9*S*, respectively. The structure of 1b is compatible with that of glauconic acid, a fungal metabolites previously isolated from *Talaromyces trachyspermus*. (Moppett and Sutherland, 1966; Frisvad *et al.,* 1990; Samson *et al.,* 2011).

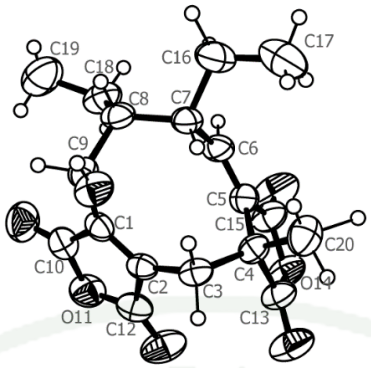


Figure 42 The ORTEP view of glaucanic acid (1b).

Table 14 Comparison of the ^{1}H and ^{13}C NMR data ($CDCl_3$, 500.13 MHz) of glaucanic acid (1a) and glauconic acid (1b).

	1a		1b	
Position	^{13}C ($\delta_{C,}$ type	^{1}H ($\delta_{H,}$ *J* in Hz)	^{13}C ($\delta_{C,}$ type)	^{1}H ($\delta_{H,}$ *J* in Hz)
1	140.5, C-		143.2, C-	
2	148.4, C-		146.4, C-	
3	31.7	3.27, d (13.6) 2.69, brd (11.5)	31.7, CH_2	3.80, d (13.6) 3.44, d (13.4)
4	48.5, -		47.4, C	-
5	131.8, C-		129.1, C-	
6	150.1, C6.99, d (12.2)		150.5, CH	6.88,d (12.4)
7	43.9, CH	2.09, m	37.7, CH	3.07, br
8	48.1, Ch	2.11, m	52.9, CH	2.12, m
9	28.2, CH_2	2.88, brd (10.3) 2.00,br	64.4, CH	5.09, brd
10	164.5, CO	-	164.6, CO	-
12	16.3, CO	-	164.6, CO	-
13	173.7, CO	-	174.1, CO	-
15	163.7, CO	-	163.4, CO	-
16	26.0, CH_2	1.68, m 1.51, m	27.7, CH_2	1.64, m
17	12.8, CH_3	0.81, t (7.4)	13.0, CH	0.90, t (7.4)
18	21.4, CH_2	1.87, m 1.15, m	19.5, CH_2	1.64, m
19	12.4, CH_3	1.08, t (7.3)	12.4, CH_3	1.15, t (7.3)
20	20.3, CH_3	1.50, s	26.4, CH_3	1.72, s

Table 15 NMR data ($CDCl_3$, 500.13, 125. 77 MHz) of glauconic acid (1b).

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
1	143.2, C-		-	-
2	146.4, C-		-	-
3a	31.7, CH_2	3.80, d (13.6)	H-3b	C-1, 4, 12, 13, 20
B		3.44, d (13.4)	H-3a	C-1, 2, 4, 5, 13
4	47.4, C	-		
5	129.1, C-			
6	150.5, CH	6.88, d (12.4)	H-7	C-4, 8, 16, 15
7	37.7, CH	3.07, br	H-6, 8, 16	-
8	52.9, CH	2.12, m	H-7, 9, 18	-
9	65.4, CH	5.09, brs	H-8	-
10	164.6, CO	-	-	-
12	164.6, CO	-	-	
13	174.1, CO	-	-	-
15	163.4, CO	-	-	-
16	27.7, CH_2	1.64, m	H-7, 17	C-6, 8, 17
17	13.0, CH_3	0.90, t (7.4)	H-16	C-7, 16
18	19.5, CH_2	1.64, m	H-8, 19	C-8, 7, 19
19	12.4, CH_3	1.15, t (7.3)	H-18	C-8, 18
20	20.3, CH_3	1.50, s	-	C-3, 4, 5, 13

Compound 2 was isolated as yellow viscous mass. The ^{13}C NMR spectrum showed twenty eight carbon signals which were categorized, by DEPTs and HSQC spectra (Table 16), as one conjugated ketone carbonyl (δ_C 199.6), three quaternary sp^2 (δ_C 164.5, 156.2, 124.4), five methine sp^2 (δ_C 135.0, 134.1, 132.5, 124.5, 123.0), two quaternary sp^3 (δ_C 44.0 and 36.8), five methine sp^3 (δ_C 33.1, 39.3, 42.9, 44.3, 55.7), six methylene sp^3 (δ_C 19.0, 25.4, 27.2, 34.1, 34.1, 35.6) and six methyl (δ_C 16.7, 17, 7, 18.9, 19.7, 20.0, 21.2) carbons. The ^1H NMR spectrum (Table 16), together with the HSQC spectrum, exhibited the signals of five olefinic protons at δ_H 5.74, s, (δ_C 123.0), 5.20, dd, *J* = 15.2, 8.1 Hz (δ_C 135.0), 5.26, dd, *J* = 15.2, 7.3 Hz (δ_C 132.5), 6.03, d, *J* = 9.5 (δ_C 124.5) and 6.61, d , *J* = 9.5 (δ_C 134.1). The COSY spectrum (Table 16) showed correlation between the olefic protons at δ_H 6.03, d, *J* = 9.5 (δ_C 124.5) and δ_H 6.61, d, *J* = 9.5 (δ_C 134.1) with a coupling constant value of 9.5 Hz, indicating the existence of a *cis*-double bond. The existence of the 4a, 7-dimethyl-2, 3, 4, 4a, 5, 6, 7-octahydrophenanthren-2-one moiety (Figure 43) was substantiated by

the HMBC (Table 17) cross peaks of the olefinic proton at δ_H 6.03, d, J = 9.5 (δ_C 124.5) to the carbons at δ_C 164.5, 124.4 and δ_C 36.8, of the olefinic proton at δ_H 6.61, d , J = 9.5 (δ_C 134.1) to the carbons at δ_C 164.5, 156.2, 24.4/124.5, 44.3, of the olefinic protons at δ_H 5.74, s, (δ_C 123.0) to the carbons at δ_C 124.5 36.8 and 34.1, of the methyl singlet at δ_H 1.00 (δ_C 16.7) to the carbons at δ_C 34.1, 36.8, 44.3, of the multiplet at δ_H 1.00 (δ_C 34.1) to the carbonyl carbon at δ_C 199.6, and of the methyl singlet at δ_H 0.96s (δ_C18.9) to the carbon at δ_C 35.6, 44.0 and 156.2.

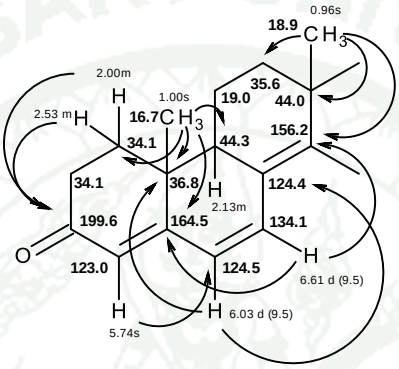


Figure 43 Structure of 4a, 7-dimethyl-2, 3, 4, 4a, 5, 6, 7 octahydrophenanthren-2-one moiety.

That this dimethyl octahydrophenanthrenone moiety was fused with the cyclopentane ring through C-7 and C-8 was supported by HMBC cross peak of the methyl singlet at δ_H 0.96s (δ_C18.9) to the carbon at δ_C 55.7 (Figure 44).

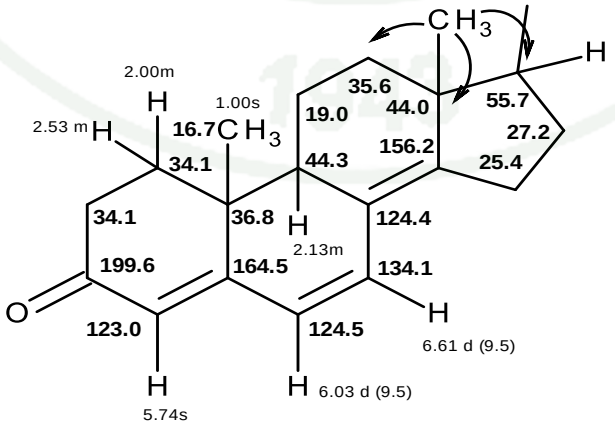


Figure 44 HMBC correlations of the cyclopentyl dimethyl octahydrophenanthrenone moiety.

Consequently, another portion of the molecule contained nine carbons, which comprised one disubstituted *trans*-double bond (δ_H 5.20 dd, *J* = 15.2, 8.1, δ_C 135.0 and δ_H 5.26 dd, *J* = 15.2, 7.3, δ_C 132.5), three methine sp^3 carbons (δ_H 1.47 m, δ_C 33.1, δ_H 2.14m, δ_C 39.3 and δ_H 1.89 m, δ_C 42.9, and four methyl (δ_H 1.06d, *J* = 6.7, δ_C 21.2; δ_H 0.93 d, *J* = 6.8, δ_C 17.7; δ_H 0.85 d, *J* = 6.8, δ_C 20.0, and δ_H 0.83 d, *J* = 6.8, δ_C 19.7). That this portion of the molecule was (3*E*)-5, 6-dimethyl-3-hepten-2-yl was evidenced by the COSY correlation between the olefinic protons with the coupling constant of 15.2 Hz, and by the HMBC cross peaks of the olefinic proton at δ_H 5.20 dd (*J* = 15.2, 8.1) to the carbon at δ_C 42.9, of the olefinic proton at δ_H 5.26 dd (*J* = 15.2, 7.3) to the carbon at δ_C 39.3, of the methyl doublet at δ_H 1.06 d, *J* = 6.7 (δ_C 21.2) to the carbons at δ_C 39.3, 135.0, of the methyl doublet at δ_H 0.93 d, *J* = 6.8 (δ_C 17.7) to the carbon at δ_C 33.1, 42.9, 132.5, of the methyl doublet at δ_H 0.85 d, *J* = 6.8 (δ_C 20.0) to the methyl carbon at δ_C 19.7, the carbons at δ_C 33.1 and δ_C 42.9, as well as of the methyl singlet at δ_H 0.83 d, *J* = 6.8 (δ_C 19.7) to the methyl carbon at δ_C 20.0, the carbons at δ_C 33.1 and δ_C 42.9.

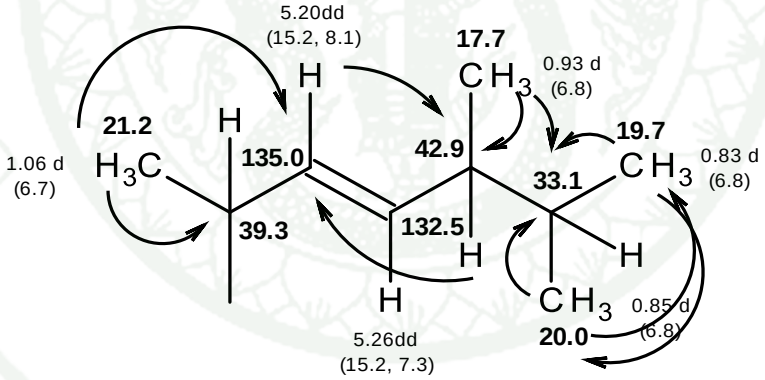


Figure 45 HMBC correlations of (3*E*)-5, 6-dimethyl-3-hepten-2-yl moiety.

That (3*E*)-5, 6-dimethyl-3-hepten-2-yl moiety (Figure 45) was connected to the dimethyl octahydrophenanthrenone moiety through the carbon at δ_C 39.3 of the former and the carbon at δ_C 55.7 of the latter was supported by the HMBC correlations of the methyl singlet at δ_H 1.06 d (*J* = 6.7) to the carbon at δ_H 55.7. (Figure 46) Thus, the structure of 2 is:

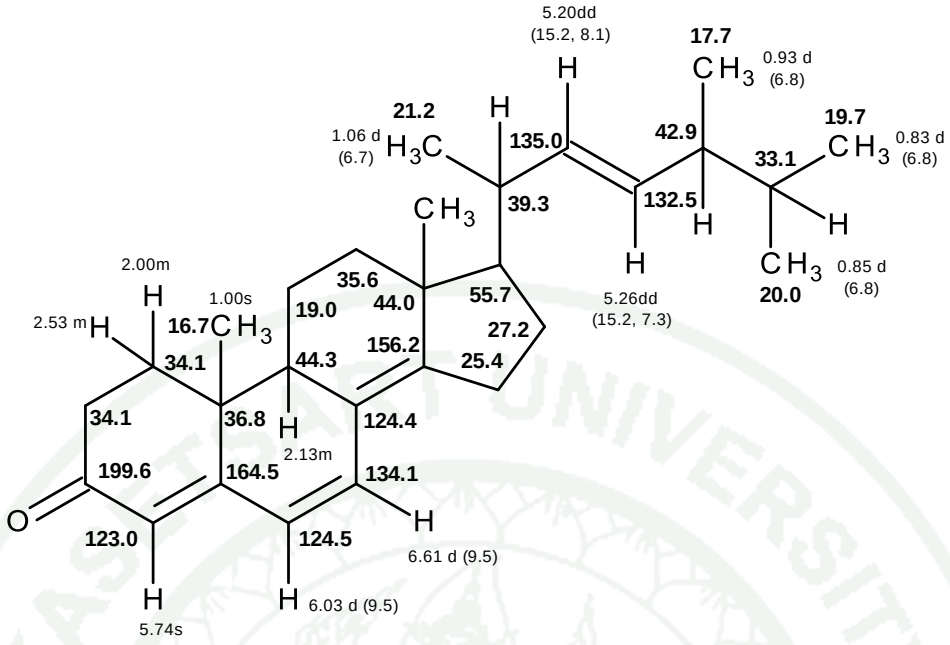
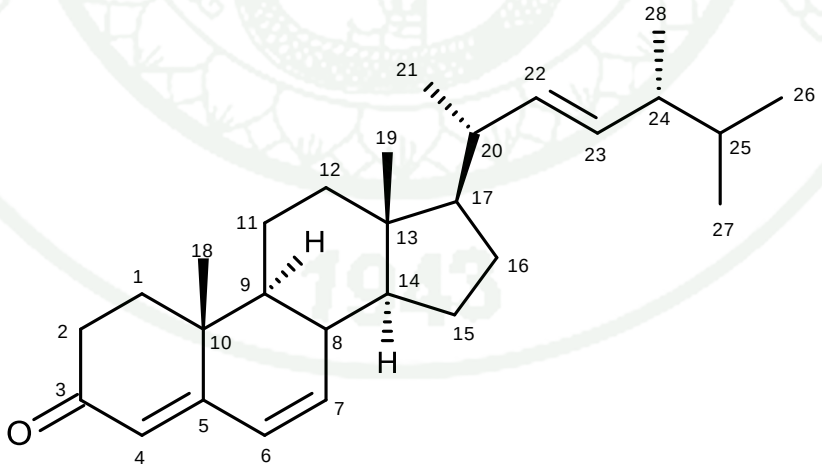


Figure 46 Planar structure of compound ergosta-4, 6, 8 (14), 22-tetraen-3-one (2).

The NMR data of compound 2 were compatible with ergosta-4, 6, 8 (14), 22-tetraen-3-one (Figure 47), previously isolated from the marine sponge *Dysidea herbacea* (Kobayashi *et al*., 1992).



2

Figure 47 The structure of ergosta-4, 6, 8 (14), 22-tetraen-3-one (2).

Table 16 NMR data ($CDCl_3$, 500.13, 125. 77 MHz) of ergosta-4, 6, 8 (14), 22-tetraen-3-one (2).

Position	$\delta_{C,}$ type	$\delta_{H,}$ (*J* in Hz)	COSY	HMBC
1	34.1, CH_2	2.00, m	-	CO-3
		2.53, m	-	CO-3
2	34.1, CH_2	1.79, m		
		2.46, m		
3	199.6, CO	-		
4	123.0, CH	5.74, s	-	C-2, 6, 10
5	164.5, C	-		
6	124.5, CH	6.03, d (9.5)	H-7	C-5, 8, 10
7	134.1, CH	6.61, d (9.5)	H-6	C-5, 8, 9, 14
8	124.4, C	-		
9	44.3, CH	2.12, m		
10	36.8, C	-		
11	19.0, CH_2	1.60, m		
		1.69, m		
12	35.6, CH_2	1.29, m		
		2.07, m		
13	44.0, C	-		
14	156.2, C	-		
15	25.4, CH_2	2.37, m		
		2.46, m		
16	27.2, CH_2	1.49, m		
		1.80, m		
17	55.7, CH	1.25, m		
18	18.9, CH_3	0.96, s	-	C-12, 13, 17
19	16.7, CH_3	1.00, s	-	C-1, 9, 10
20	39.3, CH	2.14, m	H-17, 21, 22	
21	21.2, CH_3	1.06, d (6.7)	H-20	C-17, 20, 22
22	135.0, CH	5.20, dd (15.2,	H-20, 23	
		8.1)	C-24	
23	132.5, CH	5.26, dd (15.2,	H-22, 24	
		7.3)	C-20	
24	42.9, CH	1.89, m		
25	31.1, CH	1.47, m		
26	19.7, CH_3	0.83, d (6.8)	H-25	C-24, 25, 27
27	20.0, CH_3	0.85, d (6.8)	H-25	C-24, 25, 26
28	17.7, CH_3	0.93, d (6.8)	H-24	C-23, 24, 25

Compound 3 was isolated as yellow viscous mass, and its molecular formula $C_{30}H_{46}O_4$ was established on the basis of the (+)-HRESIMS *m/z* 493.3297 $[M+Na]^+$, indicating eight degrees of unsaturation. The ^{13}C NMR, DEPTs and HSQC spectra (Table 17) revealed the presence of one ester carbonyl (δ_C 170.1), four methine sp^2 (δ_C 135.2, 135.1, 132.3, 130.9), two oxyquaternary sp^3 (δ_C 81.7, 79.4), one oxymethine sp^3 (δ_C 69.5), two quaternary sp^3 (δ_C 44.6, 37.0), six methine sp^3 (δ_C 56.2, 51.6, 51.0, 42.8, 39.7, 33.1), seven methylene sp^2 (δ_C 39.3, 34.3, 33.2, 28.6, 26.3, 23.4, 20.6) and seven methyl (δ_C 21.3, 20.9, 20.0, 19.6, 18.1, 17.6, 12.9) carbons. The general features of ^1H and ^{13}C NMR spectra of 3 closely resembled those of ergosterol 5, 8-endoperoxide (Kim *et al*., 2005), except for the presence of the acetyl group (δ_C 170.1; δ_C 21.3, δ_H 2.02, s). The COSY and HMBC correlations (Table 17) confirmed the presence of the β-acetoxyl group on C-3. Thus, the structure of compound 3 was established as 3-acetyl ergosterol 5,8-endoperoxide. Although 3-acetyl ergosterol 5,8-endoperoxide has been previously reported as acetylation product of ergosterol 5,8-endoperoxide isolated from *Ajuga remota* (Cantrell *et al*., 2001), it has never been isolated from any biological sources, and there was no previous report of its ^1H and ^{13}C NMR data.

Table 17 ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.47 MHz) and HMBC assignment for 3-acetyl ergosterol 5, 8-endoperoxide (3).

Position	$\delta_{C,}$ type	$\delta_{H,}$ (*J* in Hz)	COSY	HMBC
1	134.3, CH_2	1.97, m	H-2	C-3
		1.70, m		
2	26.3, CH_2	1.55, m	H-1, 3	
		1.95, m		
3	69.5, CH	4.98, m	H-2, 4	
4	33.2, CH_2	2.12, m	H-3	C-2, 3, 10
5	79.4, C	-		
6	135.1, CH	6.23, d (8.6)	H-7	C-4, 5, 7, 8, 10
7	130.9, CH	6.50, d (8.6)	H-6	C-5, 6, 8, 9
8	81.1, C	-		
9	51.0, CH	1.51, m	H-11	C-5, 10
10	37.0, C	-		
11	20.6, CH_2	1.40, m	H-9, 12	
		1.60, m		
12	39.3, CH_2	1.23, m	H-11	
		1.95, m		
13	44.6, C	-		
14	51.6, CH	1.51, m	H-15	
15	28.6, CH_2	1.33, m	H-14, 16	
		1.74, m		
16	23.4, CH_2	1.24, m	H-15	
		1.51, m		
17	56.2, CH	1.23, m	H-16, 20	
18	12.9, CH_3	0.81, s	C-13, 17	
19	18.1, CH_3	0.90, s	C-1, 8, 10	
20	39.7, CH	2.01, m	H-17, 21, 22	
21	19.6, CH_3	0.81, d (6.4)		C-17
22	132.3, CH	5.22, dd (15.2, 7.1)	H-20, 23	
23	135.2, CH	5.14, dd (15.2, 7.7)	H-20, 22	
24	42.8, CH	1.84, m	H-23, 25, 28	
25	33.1, CH	1.46, m	H-24, 26, 27	
26	20.0, CH_3	0.83, d (6.7)	H-25	C-24, 25
27	20.9, CH_3	1.00, d (6.6)	H-25	
28	17.6, CH_3	0.91, d (6.6)	H-24	C-22
OAc	170.1, CO	-		
	21.3, CH_3	2.02, s		CO (Ac)

Compound 4 was isolated as white amorphous solid (mp, 93-95ºC) and its molecular formula $C_{19}H_{30}O_8$ was established on the basis of the (+)-HRESIMS *m/z* 387.2019 $[M+H]^+$, indicating five degrees of unsaturation. The IR spectrum showed absorption bands for hydroxyl (3450cm^{-1}), carbonyl (1792, 1778, 1714 cm^{-1}), and alkyl (2921, 2852 cm^{-1}) groups. The ^{13}C NMR, DEPTs and HSQC spectra (Table 18) revealed the presence of four ester/carboxylic acid carbonyls (δ_C 177.7, 176.0, 174.6 and 172.1), one oxyquaternary sp^3 (δ_C 86.0), one methine sp^3 (δ_C 50.8), one oxymethylene sp^2 (δ_C 64.9), eleven methylene sp^2 (δ_C 22.7, 25.7, 27.3, 27.9, 28.4, 28.6, 29.0, 29.1, 29.2, 29.3, 29.5) and one methyl (δ_C 21.0) carbons. The ^{1}HNMR spectrum exhibited, besides a double doublet (*J* = 10.8, 2.7 Hz) of one methine proton at δ_H 3.05 and a triplet (*J* = 6.9) at δ_H 4.07, a broad singlet of the hydroxyl protons at δ_H 7.84, a methyl singlet at δ_H 2.07, multiplets of methylene protons at δ_H 2.62 (2H), 2.50 (2H), 1.83, 1.62 and a broad singlet of methylene protons at δ_H 1.26. Combining the degree of unsaturation and the ^{1}H and ^{13}C NMR data, it is clear that compound 4 must contain one ring, two esters and two carboxylic acids. The presence of a 5, 5-disubstituted dihydrofuran-2(3*H*)-one was substantiated by the COSY correlations of H-4 (δ_H 2.62, m) and H-3 (δ_H 2.50, m) as well as the HMBC correlations of H-4 to C-2 (δ_C 86.0), and H-3 to C-5 (δ_C 176.0). That the substituents on C-2 of the dihydrofuran-2(3*H*)-one moiety were a carboxyl and a carboxy methine groups was substantiated by the HMBC correlations of H-1' (3.05, dd, (*J* = 10.8, 2.7 Hz) to C-2 and C-13' (δ_C 177.7) as well as of H-3 to C-12' (δ_C 174.6). In turn, the carboxy methine group was linked to the alkyl chain was confirmed by the COSY correlations between H-1' and H-2' (δ_H 1.62, m, and 1.83, m; δ_C 27.3), as well as the HMBC correlation between H-3' (δ_H 1.62, m; δ_C 22.7) to C-1'. That the acetoxyl group (δ_H 2.07, δ_C 21.0; δ_C 172.1) was on C-11' was corroborated by the HMBC correlations of the methylene triplet at δ_H 4.07 (*J* = 6.9, H-11') and the methyl singlet at δ_H 2.07 to the carbonyl carbon at δ_C 172.1. Since H-11' also gave HMBC cross peak to the methylene carbon at δ_C 25.7, the acetylated alkyl side chain was proposed. Taking together the ^{1}H and ^{13}C NMR data, the COSY and HMBC correlations, and the molecular formula, the structure of 4 was identified as 2-[11-acetyloxy-1-carboxyundecyl]-5-oxotetrahydrofuran-2-carboxylic acid. 1-Carboxyalkyl-5-oxotetrahydrofuran-2-carboxylic acid derivatives are also known as spiculisporic acids. The stereochemistry of (-)-spiculisporic acid, firstly isolated from *Penicillium*

spiculisporum, was solved by enantioselective organocatalytic Mukaiyama-Michael reaction (Brown *et al*., 2003). Wang *et al.*, (2012) later reported isolation of three new spiculisporic acid analogues: spiculisporic acids B-D, and have tentatively established the absolute configuration of C-2 and C-1', by comparison of their ^{13}C chemical shift values with those of (-)-spiculisporic acid, as 2*S*, 1'*S*. However, the chemical shift values of C-2 (δ_C 86.0) and C-1' (δ_C 50.8) of compound 4 were much lower than those of C-2 (~ δ_C 88.0) and C-1' (~ δ_C 52) of (-)-spiculisporic acid, (-)-5-epi-spiculisporic acid, spiculisporic acids B-D (Shiozawa *et al*., 1995). This difference is probably due to the use of different solvents to obtain the ^{13}C NMR spectra. In the case of compound 2, its ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$, while those of (-)-spiculisporic acid, (-)-5-epi- spiculisporic acid and spiculisporic acids B-D were obtained in CD_3OD. Contrary to (-)-spiculisporic acid, 5-*epi*- spiculisporic acid (Goodwin, 2007), spiculisporic acids B-D (Wang *et al*., 2012), which are levorotatory, compound 4 is dextrorotatory. Interestingly, even though the absolute configuration of C-2 and C-1' of (-)-5-*epi*- spiculisporic acid (Goodwin, 2007), is 2*S*, 1'R, it is also rotatory. Thus, the absolute configuration of C-2 and C-1 of the γ-butanolide moiety of compound 4 is proposed to be enantiomer of (-)-spiculisporic acid, i. e. 2*R*, 1'*R*. Since compound 4 is a new compound, we have named it spiculisporic acid E.

Table 18 ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.47 MHz) and HMBC assignment for spiculisporic acid E (4).

Position	$\delta_{C,}$ type	$\delta_{H,}$ (*J* in Hz)	COSY	HMBC
2	86.0, C	-		
3	28.6, CH_2	2.50, m	H-2	C-2, 5
4	28.4, CH_2	2.62, m	H-3	C-2
5	176.0 CO	-		
1’	50.8, CH	3.05, dd (10.8, 2.7)	H-2’	C-2, 12’, 13’
2’	27.3, CH_2	1.62, m		H-1’
		1.83, m		H-1’, 3’
3’	22.7, CH_2	1.62, m		H-2’, 4’C-1’
4’	27.9, CH_2	1.26, m		
5’	29.5, CH_2	1.26, m		
6’	29.3, CH_2	1.26, m		
7’	29.2, CH_2	1.26, m		
8’	29.1, CH_2	1.26, m		
9’	25.7, CH_2	1.26, m		
10’	29.0, CH_2	1.62, m		
11’	64.9, CH_2	4.07, t (6.9)	H-10’	CO (OAc
12’	174.6, CO	-		
		7.84, brs		
13’	177.7, CO	-		
		7.84, br		
OAc	21.0, CH_3	2.07, s		CO (Ac
	172.1, CO	-		

Glaucanic acid, glauconic acid, spiculisporic acid E (4), were evaluated for their *in vitro* growth inhibitory activity on the MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines by protein binding dye SRB method (Eamvijarn *et al*., 2012) and none of them was active at the highest concentration tested (150 µM). Furthermore, glaucanic acid, glauconic acid, spiculisporic acid E (4) were evaluated, together with 3-Acetyl ergosterol 5, 8-endoperoxide (3) and ergosta-4, 6, 8 (14), 22-tetraen-3-one, for their antimicrobial activity against Gram positive (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633) and Gram negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacteria, *Candida albicans*, as well as

multidrug-resistant isolates from the environment (Gomes *et al*., 2014) and all of them were inactive at the highest concentration tested (256 µg/mL).

5. *In vitro* antifungal activity evaluation of *Talaromyces trachyspermus* (KUFA 0021) secondary metabolites against ten species of plant pathogenic fungi

Glaucanic acid, glauconic acid, as well as the new compound, spiculisporic acid E, were evaluated for their *in vitro* antifungal activity against ten plant pathogenic fungi, namely *Rhizoctonia solani*, *Sclerotium rolfsii*, *Colletotrichum capsici*, *C. gloeosporioides*, *Lasiodiplodia theobromae*, *Alternaria brassicicola*, *Fusarium oxysporum*, *Helminthosporium maydis*, *Pythium aphanidermatum* and *Phytophthora palmivora*, by the Paper disc method and none of the compounds was active at the highest concentration tested (10,000 ppm).

CONCLUSION

A total of two hundred and ten fungal isolates were isolated from thirty-six samples of marine invertebrates including *Arthrinium* sp., *Aspergillus candidus, A. niger, A. terreus, Aspergillus* sp., *Chaetomium* sp., *Cladosporium* sp., *Emericella nidulans, E. variecolor, Emericella* sp., *Eupenicillium* sp., *Fusarium solani, Fusarium* sp., *Hamigera* sp., *Humicola* sp., *Lasiodiphodia* sp., *Mucor hiemalis, Mucor* sp., *Neosartorya fischeri, N. pseudofischeri, Neosartorya* sp., *Paecilomyces lilacinus, Paecilomyces* sp., *Penicillium* sp., *Pestalotiopsis* sp., *Phoma* sp., *Phomopsis* sp., *Pseudoeurotium* sp., *Rhizopus* sp., *Scolecobasidium* sp., *Syncephalastrum* sp., *Talaromyces trachyspermus, Trichocladium* sp., *Trichoderma opacum, Trichoderma* sp., *Xylaria* sp. and sterile mycelium.

Preliminar screening of the antagonistic activity of twelve species of marine-derived fungi revealed relevant antifungal activity against plant pathogenic fungi. While most of the selected species showed only a marginal antifungal effect against the two Agonomycetes species (*Rhizoctonia solani* and *Sclerotium rolfsii*), *Colletotrichum gloeosporioides, Lasiodiplodia theobromae* and *Pythium aphanidermatum, Emericella nidulans* (KUFA 0101), *Hamigera* sp. (KUFA 0106), *Neosartorya fischeri* (KUFA 0107), *N. pseudofischeri* (KUFA 0108), *Pseudoeurotium* sp. (KUFA 0110), and *Talaromyces trachyspermus* (KUFA 0021) displayed relevant antifungal activity, specially against *Alternaria brassicicola* and *Helminthosporium maydis*, and also against *Colletotrichum capsici*, *Fusarium oxysporum* and *Phytophthora palmivora*. Due to the relevant antagonistic activity, these six fungal species were selected for further evaluation for the antifungal activity of their EtOAc crude extracts. Despite a strong antifungal activity of *N. fischeri* (KUFA 0107) and *Hamigera* sp. (KUFA 0106) EtOAc crude extracts, causing the inhibition of mycelial growth from some plant pathogenic fungi, even at lower concentration (1,000 ppm), *T. trachyspermus* (KUFA 0021) EtOAc crude extract was undoubtedly identified as the most active extract. *T. trachyspermus* (KUFA 0021) EtOAc crude extract caused a complete mycelial growth inhibition in all the tested plant pathogenic fungi at the highest concentration tested (10,000 ppm). Additionally, the antifungal activity

remained effective at a lower concentration (1,000 ppm), leading to the complete growth inhibition in half of the pathogens isolates. Even at 100 ppm, *T. trachyspermus* (KUFA 0021) crude extract displayed a moderate effect against *P. aphanidermatum*.

Due to *Talaromyces trachyspermus* (KUFA 0021) relevant antifungal properties, the chemical analysis of its EtOAc crude extract was performed, resulting in the isolation of a new spiculisporic acid derivative, spiculisporic acid E, a new natural product, 3-acetyl ergosterol 5, 8-endoperoxide, as well as three known compounds. The isolation of spiculisporic acid E as well as the new natural product, 3-acetyl ergosterol 5, 8-endoperoxide, indicates the potential of marine-derived fungi as source of new compounds with distinct chemical structures.

All the isolated compounds were tested for their antimicrobial activity against Gram positive and Gram negative bacteria was well as for their *in vitro* growth inhibitory activity against the MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines. All the compounds exhibited neither antibacterial nor growth inhibitory activity against the three human tumor cell lines.

Additionally, glaucanic and glauconic acids, as well as spiculisporic acid E, were also evaluated for their antifungal activity against ten plant pathogenic fungi, and none of the compounds were active. Despite the lack of activity of the tested compounds, *Talaromyces trachyspermus* (KUFA 0021) crude extract was identified as a potential source of metabolites with antifungal activity against plant pathogenic fungi, as stated by the results in this study.

Further studies on the isolation of the secondary metabolites of the active crude extracts of other marine-derived fungi as well as the evaluation of their effect on the mycelial growth of these plant pathogenic fungi are needed and will be performed in the future.

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APPENDIX

1. Culture media for isolating fungi

1.1. Glucose yeast extract peptone agar (GYP)

Glucose	1.0 g
Peptone	0.5 g
Yeast extract	0.1 g
Sterile seawater	1.0 L

1.2. Gause I (GI)

Starch	20 g
KNO_3	1.0 g
K_2HPO_4	0.5 g
$MgSO_4\,7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4$	0.01 g
Agar	15 g
Sterile seawater	1.0 L

1.3. Half Potato Dextrose Agar (Half PDA)

Potato	100 g
Dextrose	10 g
Agar	15 g
Sterile seawater	1.0 L

1.4. Malt Extract agar (MEA)

Malt extract	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	15.0 g
Sterile seawater	1,000 ml

2. Culture media for cultivating fungi

2.1. Potato Dextrose Agar (PDA)

Potato	200.0	g
Dextrose	20.0	g
Agar	15.0	g
Distilled water	1,000	ml

2.2. Potato Dextrose Broth (PDB)

Potato	200.0	g
Dextrose	20.0	g
Distilled water	1,000	ml

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PUBLICATIONS

1. Kumla, D., T. Dethoup, N. Singburaudum, J. Buaruang and A. Kijjoa. 2014. Fungi isolated from sponges, corals, sea fans and efficiency of the crude extracts of sponge-derived fungi against plant pathogenic fungi. pp. 530-537. *In* **The Proceedings of 52nd Kasetsart University Annual Conference.** February 4-7, 2014.

2. Kumla. D., T. Dethoup, S. Buttachon, N. Singburaudom, A.M.S. Silva and A. Kijjoa. 2014. Spiculisporic acid E, a New Spiculisporic Acid Derivative and Ergosterol Derivatives from the Marine-Sponge Associated Fungus *Talaromyces trachyspermus* (KUFA 0021). **Natural Product Communications** 9 (8): 1147-1150.