Biological and Chemical Investigations of Indonesian Marine-Derived Fungi and their Secondary Metabolites

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Dedicated to my parents
and to commemorate my late grandmother

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List of Abbreviations and Symbols

$[\alpha]_D$	specific rotation at the sodium D-line
1D	one-dimensional
2D	two-dimensional
ATCC	American type culture collection
AS	anisaldehyde-sulphuric acid
br	broad
C18	octadecyl
calc.	calculated
CC	column chromatography
CCDC	Cambridge crystallographic data centre
CD_3OD	deuterated methanol
COSY	correlation spectroscopy
CZ	Czapek-Dox
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DAD	diode array detector
DCM	dichloromethane
DEPT	distortionless excitation by polarization transfer
diam.	diameter
DMSO	dimethylsulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
dq	doublet of quartets
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
dt	doublet of triplets
EDTA	ethylene diamine tetraacetic acid
e.g.	*exempli gratia* (for example)
ELISA	enzyme linked immunosorbent assay
ESI	electrospray ionization
et. al.	*et alii* (and others)
EtOAc/EA	ethyl acetate
EtOH	ethanol
eV	electron volt
FCS	fetal calf serum
FTICR	Fourier transform ion cyclotron resonance
Fig(s).	figure(s)
h	hour
HBSS	Hank's balanced salt solution
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HSQC	heteronuclear single quantum correlation
HPLC	high performance liquid chromatography
HR-ESI-MS	high resolution-ESI-MS
Hz	hertz
IC_{50}	50% inhibitory concentration
i.e.	*id est* (that is)
INT	iodonitrotetrazolium chloride
IR	infrared

IZ	inhibition zone
J	coupling constant (in Hz)
m	multiplet
m/z	mass/charge
M^+	molecular ion
Me	methyl
MEA	malt extract agar
MeCN	acetonitrile
MeOH	methanol
µg	microgram
mg	milligram
MHz	megahertz
min	minute
ml	millilitre
µl	microlitre
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MW	molecular weight
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NRU	neutral red uptake
OD	optical density
PBS	phosphate buffered saline
PDA	potatoes dextrose agar
ppm	part per million
PTLC	preparative-TLC
q	quartet
qd	quartet of doublets
R_f	retention factor
ROESY	rotating frame nuclear Overhauser effect spectroscopy
RP-18	reversed phase C18
rpm	rotation per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
s	singlet
SBUG	Stammsamlung Biologie der Universität Greifswald
SD	standard deviation
sp.	species (singular)
SS	solvent system
δ	chemical shift (in ppm)
t	triplet
t_R	retention time
T	tesla
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
TSA	tryptic soy-bean agar
UV/Vis	ultraviolet/visible
v/v	volume per volume

1. INTRODUCTION

1.1. Marine Natural Products

Natural products have led to the excellent drugs for therapeutic purposes. In the period of 1989-1995 over 60% of the approved drugs and pre-NDA (New Drug Application) candidates were of natural origin. Drugs of natural origin have been classified as original natural products, semi-synthetically from natural products or synthetic products based on natural product models. Thereafter natural products played invaluable role in the drug discovery, particularly in the areas of cancer and infectious diseases. Of the 211 pre-NDA anticancer drug candidates (i.e., in preclinical or clinical development for the period above), 61% were the original natural products and about 4% of the candidates were marine-derived (Cragg *et al., 1*997).

The urgency of new antibiotic discovery becomes more serious since often no effective therapies are available and the development of resistance of many pathogens to currently used drugs (Cassell & Mekalanos, 2001; Nussbaum *et al.,* 2006; Saleem *et al.,* 2010). Moreover, bacteria can acquire drug resistance in a multitude of ways, thus to solve this problem is not a straightforward matter (Saleem *et al.,* 2010). A similar situation exists with the need to develop new cancer chemotherapeutic agents with activity against the disease-types still resistant to current therapies and to overcome the development of multidrug resistance, which is increasingly observed in the treatment of many tumors (Cragg *et al., 1*997).

Ironically, in the mid 1990s most large pharmaceutical companies have decreased the screening of natural products for drug discovery in favour of synthetic compound libraries (Lam, 2007). Moreover, some companies have terminated the activities of natural product-based drug discovery (Ortholan & Ganesan, 2004). New technologies such as high throughput screening could not activate the activities again, since the equipments were very costly and the updated data libraries were also not openly to access (Buss & Butler, 2004).

The stated phenomenon was happened for temporary short time. The facts proved that the chemical natural products are incredibly distinct from other medicines and continuously showing fascinating biological activity (Baker *et al.,* 2007; Labischinski & Ruebsamen-Waigmann, 2008). Currently, natural products are hence back to be rising desired in drug discovery. Some reviews demonstrated that natural products play a dominant role in the discovery of lead compounds for drugs development (Newman *et al.,*

2003; Koehn & Carter, 2005; Newman & Cragg, 2007; Saleem *et al.,* 2010). This trend is also precisely happened to marine natural products.

In December 2004 was the first drug from the sea Ziconotide to be approved in the United States for the treatment of pain. Two months later the drug was also approved by the European Commission for the treatment of severe, chronic pain in patients who require intrathecal analgesia. Ziconotide (ω-conotoxin MVIIA) is a peptide originally from a tropical marine cone snail. The drug is presently in the market under the trade name Prialt® (produced by Elan Pharmaceuticals). The second drug was Trabectedin (Ecteinascidin-743/ET-743) that is marketed under the trade name Yondelis® (PharmaMar/Johnson&Johnson/OrthoBiotech). Yondelis® was the first anticancer drug from the sea originally isolated from a tropical sea squirt *Ecteinascidia turbinata.* The drug also has been approved by the European Union in October 2007 for the treatment of soft tissue sarcoma (Molinski *et al.,* 2009; Mayer *et al.,* 2010). Moreover, both drugs are of the first members of new human drug classes (Butler, 2008).

1.2. Fungi as a Source of Natural Products

Fungi produce a vast range of secondary metabolites. Some of the metabolites are high-value products with pharmaceutical applications such as penicillins, a group of structurally related ß-lactam antibiotics isolated from *Penicillium chrysogenum*. Several non-ß-lactam antibiotics are also produced by fungi such as griseofulvin. Griseofulvin which is isolated from *Penicillium griseofulvum* has been used for several years to treat dermatophyte infections of the skin, nails and hair of humans. Some valuable secondary metabolites of fungal origin are listed in Table 1.1 (Deacon, 2006).

Table 1.1. Some valuable secondary metabolites produced commercially from fungi

Metabolite	Fungal source	Application
Penicillins	*P. chrysogenum*	Antibacterial
Cephalosporins	*Acremonium chrysogenum*	Antibacterial
Griseofulvin	*P. griseofulvum*	Antifungal
Fusidin	*Fusidium coccineum*	Antibacterial
Ciclosporins	*Tolypocladium* spp.	Immunosuppressants
Zearalenone	*Gibberella zeae*	Cattle growth promoter
Gibberellins	*Gibberella fujikuroi*	Plant hormone
Ergot alkaloids and related compounds	*Claviceps purpurea* and related fungi	Many effects including: antimigraine, vasoconstriction, vasodilation, antihypertension, anti-Parkinson, psychiatric disorders

Source: Deacon (2006)

Marine fungi are prolific resources of natural products (Liberra & Lindequist, 1995; Pietra, 1997; Jensen & Fenical, 2000; 2002; Ebel, 2010). However, the potential of marine fungi has only been investigated to a limited extent.

In recent years, marine fungi have been explored more intensely to obtain novel and biologically active compounds. Though compared to marine sponges and bacteria, marine fungi are still less explored. Nevertheless, success stories in marine fungi are quite significant. Cephalosporin C which was originally isolated the first time from *Cephalosporium acremonium* isolated from a sewage outlet off the Sardinian coast have played a key role to the reduction of infectious diseases and suffering of people throughout the world since last three decades (Demain & Elander, 1999). However, it was about incidental discovery and it took another 30 years until marine-derived fungi were investigated more systematically (Ebel, 2010).

1.2.1. Biology of Marine Fungi

Initially fungi were classified as 'cryptogamic plants' and separated into lichenized and non-lichenized groups. For many years fungi were recognized as belonging to kingdom plants until Whittaker's influential five-kingdom classification of living beings (Whittaker, 1969 *in* Kirk *et al*., 2008) and subsequent phylogenetic research has repeatedly confirmed that the fungi are a sister group to the animals rather than the plants.

The kingdom of fungi is the second largest group after insects and widely distributed in nature. Based on the observed ratio between flowering plant diversity and fungal diversity in countries where fungi have been sufficiently well studied, there are 1.5 million estimated fungal species (Hawksworth, 1991; 2001). They inhabit soils, the surface of mountain rocks and seawater (Feofilova, 2001). However, marine environment does not permit the development of large and fleshy fruiting bodies; therefore most of the fungi found in marine habitats are microscopic (Kohlmeyer & Kohlmeyer, 1979).

Marine fungi were from longtime as neglected resources. Unlike their related terrestrial fungi which were initially exploited, marine fungi have attracted great attention as considerable resources since the late 1980s (Ebel, 2010). The term marine fungi itself essentially do not represent a specific taxa, but are a group defined by their ecology. Most fungi isolated from marine samples are not proven to be obligate or facultative marine. Thus, the more general expression "marine-derived fungi" is used.

The generally accepted ecological definition of marine fungi is, "Obligate marine fungi are those which grow and sporulate exclusively in a marine or estuarine habitat; facultative marine fungi are those from freshwater or terrestrial milieus able to grow and possibly also to sporulate in the marine environment" (Kohlmeyer & Kohlmeyer, 1979). Accordingly, there is no taxonomic classification of marine fungi rather to a certain extent of ecological term.

Marine fungi comprise an estimated 1500 species, excluding those that form lichens (Hyde *et al.,* 1998). This number is far fewer when compared to the number of named and undescribed terrestrial fungi which was estimated 250,000 or more (Kohlmeyer & Kohlmeyer, 1979). So far less than 500 of filamentous higher marine fungi have been described and only 79 are associated with algae as parasites or symbionts, and 18 with animal hosts (Kohlmeyer & Volkmann-Kohlmeyer, 2003).

The distribution of marine fungi in the tropics has not been explored as thoroughly as in the temperate areas (Kohlmeyer, 1984; Blunt *et al.,* 2005; 2009). Nevertheless, inventory data for the marine fungi investigated in several tropical countries such as Thailand (Chaeprasert *et al.,* 2010), Palau Islands (Kohlmeyer, 1984; Chatmala *et al.,* 2004), Singapore (Lim & Tan, 1986; Sundari *et al.,* 2010), Brunei (Hyde, 1988), Malaysia (Jones & Hyde, 1988; Alias & Jones, 2000; Zainuddin *et al.,* 2010; Pang *et al.,* 2010) and Siargao Island, Philippines (Besitulo *et al*., 2002) are available. Many tropical regions have been largely unexplored, such as the Indonesian archipelago.

1.2.2. Biosynthesis of Marine Fungal Secondary Metabolites

Extreme conditions such as high salinity, low temperature, lightless and high pressure are supposed to be the inducer of the prolific active compounds of marine microorganisms. Their capabilities to produce unique and unusual secondary metabolites are possibly because of adaptation to a very distinct set of environmental pressure (Jensen & Fenical, 2002). It is also believed that the metabolites act as a chemical defense in competing for substrates (Gallo *et al*., 2004).

Secondary metabolites are defined as small organic molecules that derived from biosynthetic pathways which are not required for maintenance and growth of the respective organism. Besides their role in environmental adaptation, they often contribute to biological defense strategies. By co-cultivation of marine fungi with other microorganisms from the same ecosystem proved to be successful in activating silent gene clusters to produce bioactive secondary metabolites (Brakhage & Schroeckh, 2010). Small changes of cultivation parameters such as media composition were also possible to induce secondary metabolites production (Bode *et al.,* 2002; Calvo *et al*., 2002).

1.3. Marine Natural Products of Fungal Origin

The purpose of finding novel secondary metabolites is usually in order to obtain large numbers of fast growing isolates from marine sources (Kohlmeyer & Volkmann-Kohlmeyer, 2003). Interestingly, the corresponding chemistry was structurally diverse and related to that of terrestrial fungi (Höller *et al*., 2000). Marine environment that is different with freshwater condition is generally responsible for the production of diverse metabolites. Hence, optimization of physical and chemical factors such as salinity, temperature, pH and media components can sometimes be substantially increase the yield of bioactive compounds (Calvo *et al*., 2002).

Marine organisms are a fascinating source of novel and biologically active natural products. Over 14.000 new biologically active compounds have been identified from marine sources and at least 300 patents have been issued (Hunt & Vincent, 2006). Moreover, the number of reported secondary metabolites from marine fungi has steadily increased (Faulkner, 1997; 1998; 1999; 2000; 2001; Blunt *et al.,* 2003; 2004; 2005; 2006; 2007; 2008; 2009; 2010). In the last three years there were over 15 marine-derived secondary metabolites in human clinical trials (Saleem *et al*., 2007). This number increases rapidly since each year on average approximately 700 novel marine natural products have been published and of which 16–18% were of microbiological origin (Blunt *et al.,* 2010).

Fungi are one of the most significant groups of organisms to be exploited for drug discovery purposes. Especially Fungi Imperfecti have provided mankind with wide of different bioactive secondary metabolites such as β-lactam antibiotics, griseofulvin, cyclosporine A or lovastatin. In the last three years, most new natural products described in literature were isolated from fungi (Saleem *et al*., 2007).

Starting in the late 1980s quantities of new secondary metabolites from this long neglected source has been significantly increasing (Ebel, 2010). The highly developed and diverse secondary metabolites are the interesting factors of fungi. Marine fungi are still challenging to explore since only small parts of fungi that have been explored (less than 5% of about 1.5 Mio fungal strains).

The advantages of the investigation of marine fungi as of microorganisms when compared to macroorganisms are obvious, since biotechnological fermentations are possible without ecological exploitation and compounds can be reisolated after recultivation in large amounts which is nearly impossible for marine macroorganisms. Additionally, microorganisms can be easier manipulated genetically and straightforward scale-up of metabolite production (König & Wright, 1997; Stadler & Keller, 2008).

Sponges-derived natural products are presently the most investigated of marine natural products. Correspondingly, the study of natural products from sponges-associated microorganisms, namely bacteria and fungi are also plenty. Interestingly, fungal associates in sponges contribute 65.71% of compounds, almost double compared to the compounds produced by bacteria. In more detail, Ascomycota dominate the proportion of fungal producer by division (Fig. 1.1) (Thomas *et al.,* 2010).

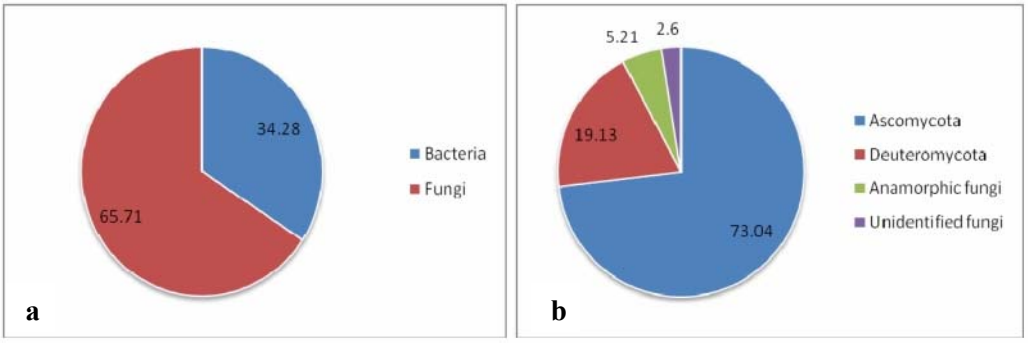


Fig. 1.1. Percentage distribution of compounds produced by (a) sponge-associated bacteria and fungi, (b) associated fungi-division wise (Thomas *et al.,* 2010)

1.3.1. Secondary Metabolites from Algicolous Fungi

Algae have been regarded as a valuable source for the isolation of fascinating marine fungi. Kohlmeyers stated that one third of all known higher marine fungi are associated with alga (Kohlmeyer & Kohlmeyer, 1979). Furthermore, the algicolous fungi may produce unusual and novel metabolites. It is not surprisingly since the fungi have to deal with their host as well as the marine environment.

Algal-fungal relationships were intensively investigated. There is a symbiotic association of fungi with algae in lichens where both the partners benefit. On the extreme end this association is called mycophycobiosis where an obligate symbiotic association exists between systemic fungi and marine macroalgae (Raghukumar, 2006). However, fungi are also potential as pathogens in algae (Johnson & Sparrow, 1961; Kohlmeyer & Kohlmeyer, 1979).

In the last decade secondary metabolites obtained from marine algicolous fungi have shown significant rising of the quantity and moreover diversity of the marine natural products (Blunt *et al.,* 2010). Some algae which have been reported to be hosts of some active compound-producing fungi are red algae *Liagora viscida* (Osterhage *et al.,* 2002), *Plocamium* sp. (Pontius *et al.,* 2008), *Acanthophora spicifera* (Greve *et al.,* 2008); green algae *Ulva* sp. (Osterhage *et al.,* 2000; Gamal-Eldeen *et al.,* 2009); *Ulva pertusa* (Cui *et al.,* 2010); brown algae *Rosenvingea* sp. (Cueto *et al.,* 2001), *Fucus vesiculosus* (Abdel-Lateff *et al.,* 2003) and *Sargassum horneri* (Nguyen *et al.,* 2007).

1.3.2. New Bioactive Natural Products from Marine-derived Fungi

There are several valuable reviews dealing with marine fungal metabolites (Liberra & Lindequist, 1995; Pietra, 1997; Bugni & Ireland, 2004; Bhadury *et al.,* 2006; Saleem *et al*., 2007; Ebel, 2010; Debbab *et al*., 2010). They covered new biologically active natural products of marine-derived fungi published until 2009. In addition, D.J. Faulkner has reviewed marine natural products (including metabolites from marine fungi) annually since 1984 to 2002 in Natural Product Reports. After his death in November 2002, this annual review has been continued by J.W. Blunt and his group.

Natural products isolated from marine fungi were generally classified into seven classes, namely polyketides, alkaloids, diketopiperazines, peptides, terpenoids, lipids, and shikimate-derived metabolites. Several review papers illustrated that polyketides dominate marine natural products of fungal origin (Ebel, 2010; Blunt *et al*., 2010).

Concerning to the previous review as mentioned before, in this currently report are listed new metabolites which have been published in January to September 2010.

1.3.2.1. Antimicrobial Compounds

Five new polyketide derivatives, 7-*O*-methylkoninginin D (**1**) and trichodermaketones A-D (**2**-**5**) were isolated from the marine-derived fungus *Trichoderma koningii*. The compounds were investigated for action against bacteria, fungi and for synergistic antifungal activity. Compound **2** showed synergistic antifungal activity against *Candida albicans* with 0.05 µg/ml ketoconazole (Song *et al.,* 2010).

Investigation for secondary metabolites of *Trichoderma* sp. 05FI48 which was isolated from an unidentified marine sponge revealed three new aminolipopeptides named trichoderins A (**6**), A1 (**7**) and B (**8**). The compounds were reported to be active against *Mycobacterium smegmatis, Mycobacterium bovis* BCG, and *Mycobacterium tuberculosis* H37Rv in both active and dormant states with MIC values in the range of 0.02-2.0 µg/ml (Pruksakorn *et al.,* 2010). The chemical structures of new antimicrobial compounds are presented in Fig. 1.2.

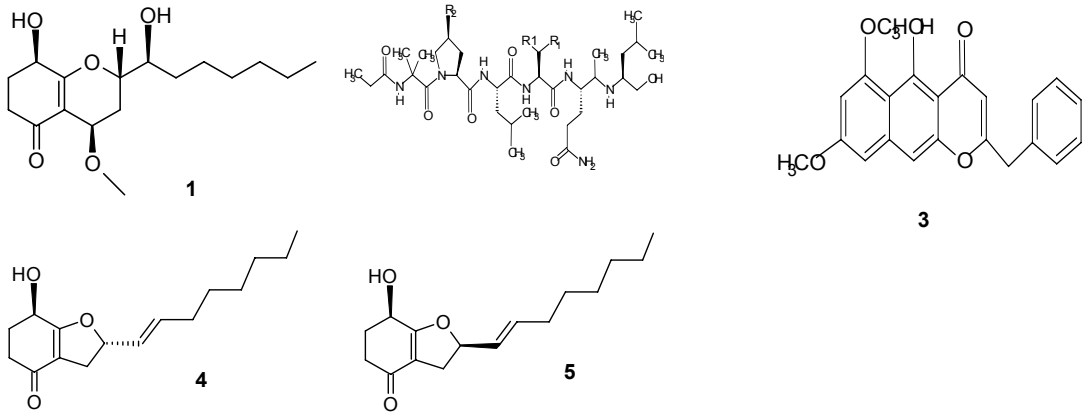


Fig. 1.2. New antimicrobial compounds isolated from *Trichoderma* spp.

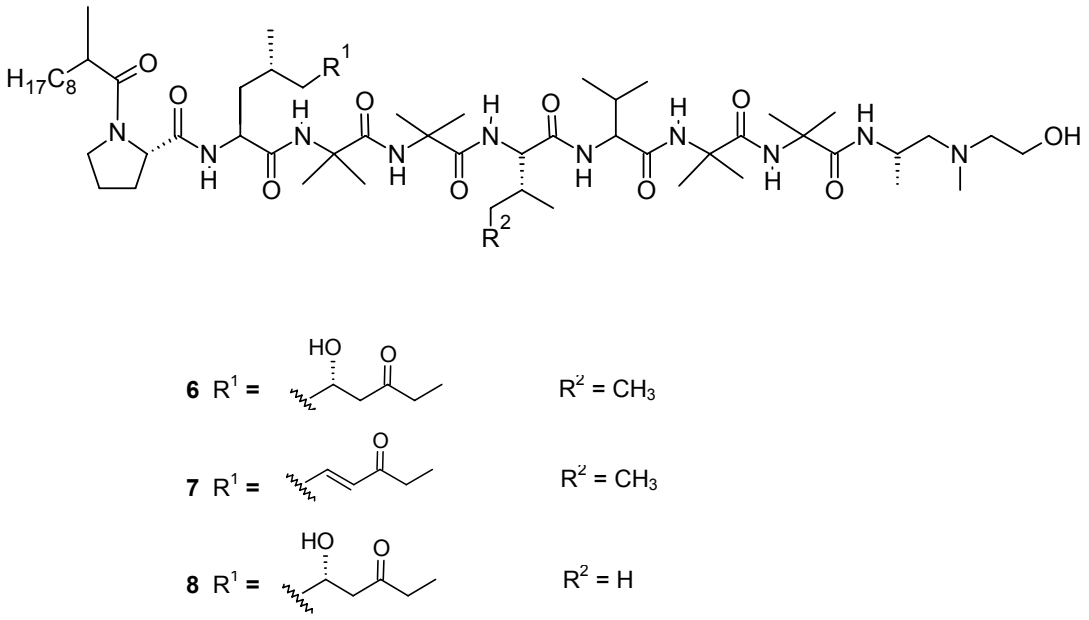


Fig. 1.2. New antimicrobial compounds isolated from *Trichoderma* spp. (*cont.*)

1.3.2.2. Cytotoxic Compounds

Azaspirofuran A (**9**), a new hetero-spirocyclic γ-lactam was isolated from the fermentation broth and mycelia of a marine-derived *Penicillium* sp. CNL-338 (Fig. 1.3). The compound showed cytotoxicity against A549 cell line with the IC_{50} value of 10 μM (Ren *et al*., 2010).

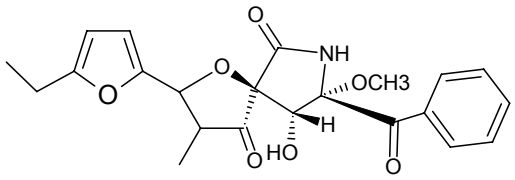


Fig. 1.3. Molecular structure of Azaspirofuran A (**9**)

A xanthone and five anthraquinones isolated from *Aspergillus versicolor* were found to be active in cytotoxicity test against five human tumor cell lines (A-549, SK-OV-3, SK-MEL-2, XF-498 and HCT-15). The compounds which were related to the aflatoxin biosynthesis in *Aspergillus* sp. were identified as sterigmatocystin (**10**), averantin (**11**), methyl averantin (**12)**, averufin (**13**), nidurufin (**14**) and versiconol (**15**) (Fig. 1.4). Compound **12** was the most active against the test cell lines with IC_{50} 0.41 to 1.17 μg/ml (Lee *et al.,* 2010).

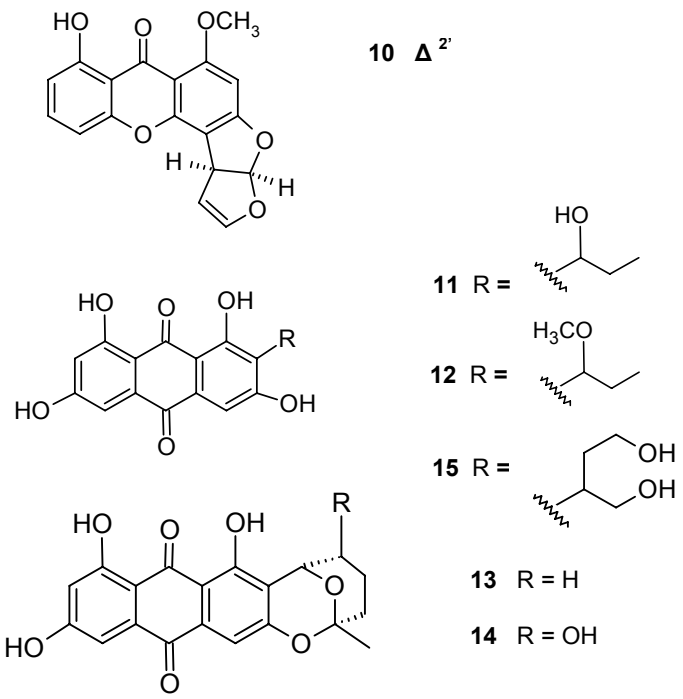


Fig. 1.4. A xanthone and five anthraquinones isolated from *A. versicolor*

A new cytotoxic compound, pyrolyl 4-quinolinone alkaloid (**16**) with an unprecedented ring system was isolated from the most productive mould *Penicillium* sp. The compound named penicinoline (Fig. 1.5) showed potent in vitro cytotoxicity toward 95-D and HepG2 cell lines with IC_{50} values of 0.57 and 6.5 μg/ml, respectively (Shao *et al.,* 2010).

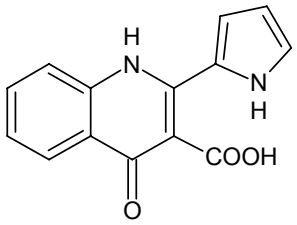


Fig. 1.5. A new pyrrolyl 4-quinolinone alkaloid (**16**) isolated from *Penicillium* sp.

Cytoglobosins A-G (**17**-**23**), new cytochalasan derivatives were isolated from the cultures of *Chaetomium globosum*, an endophytic fungus derived from marine green alga *Ulva pertusa.* The seven new fungal alkaloids (Fig. 1.6) exhibited cytotoxic activity against the A-549 tumor cell line (Cui *et al.* 2010).

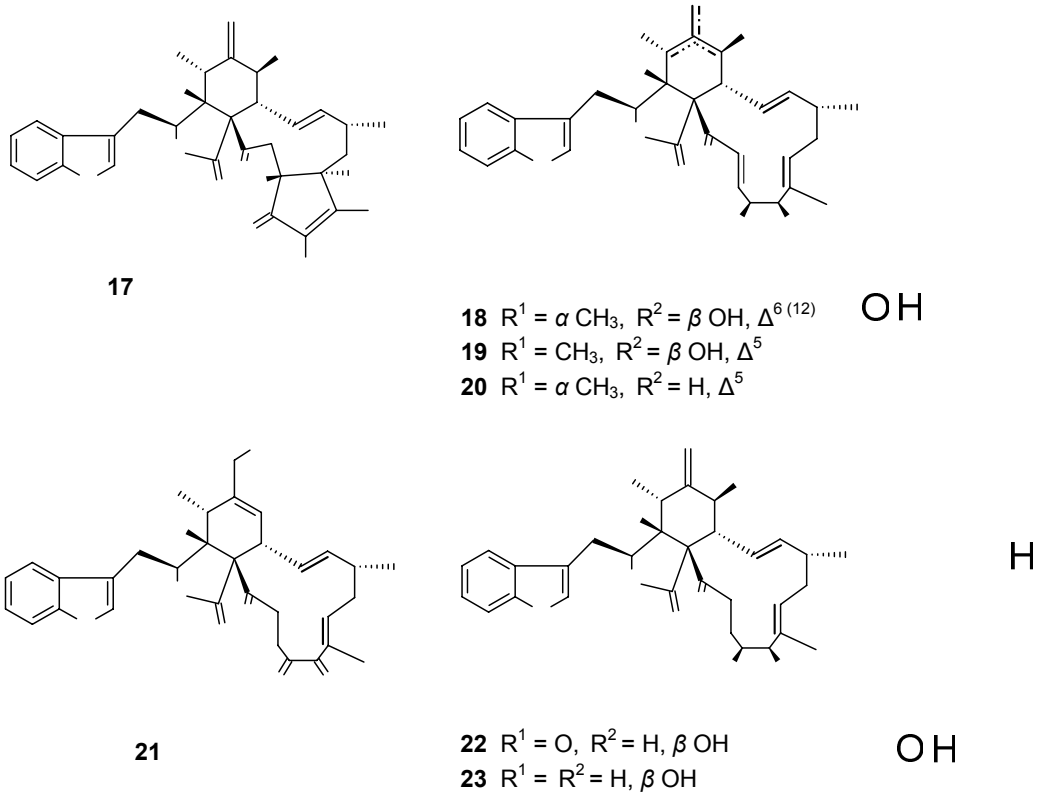


Fig. 1.6. Cytoglobosins A-G (**17-23**), new cytotoxic fungal alkaloids from *C. globosum*

Two new lactones, sonnerlactones 1 and 2 (**24**-**25**) have been isolated from a mangrove endophytic fungus Zh6-B1 from South China Sea. The two lactones (Fig. 1.7) showed cytotoxic activity against KV/MDR cell line and might possess beneficial therapeutic potential against drug-resistant tumors (Li *et al.*, 2010).

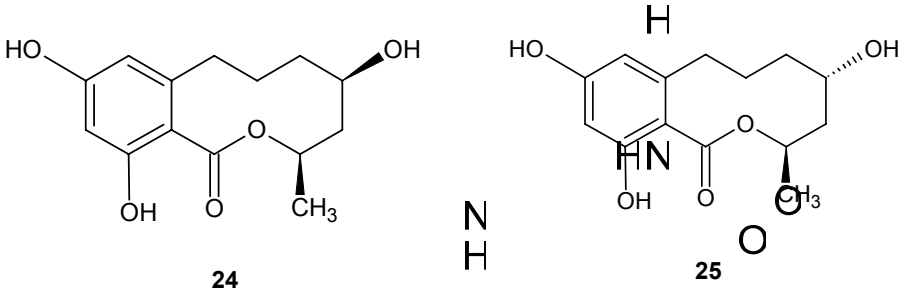


Fig. 1.7. Molecular structure of sonnerlactone 1 (**24**) and 2 (**25**)

Fig. 1.8 presents a new naphtha-γ-pyrone (**26)** and two new xanthone derivatives, 1,7-dihydroxy-2-methoxy-3-(3-methylbut-2-enyl)-9H-xanthen-9-one (**27**) and 1-hydroxy-4,7-dimethoxy-6-(3-oxobutyl)-9H-xanthen-9-one (**28**). Compound **26** isolated from a

mangrove endophytic fungus *Phomopsis* sp. ZSU-H26 exhibited cytotoxicity against HEp-2 and HepG2 cells (Huang *et al.,* 2010a). The two new xanthone derivatives were also reported by Huang *et al.,* (2010b). Preliminary pharmacological test showed that the compounds inhibited KB cells and KB_v200 cells.

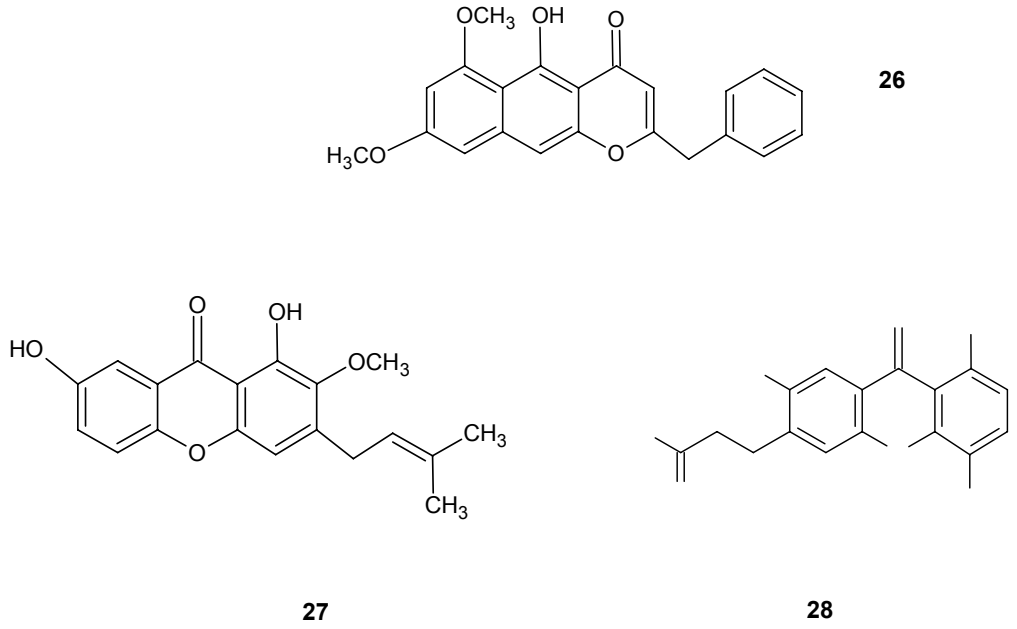


Fig. 1.8. A new naphtha-γ-pyrone (**26)** and two new xanthone derivatives (**27**-**28**) with cytotoxic activities

From *Penicillium expansum* 091006 isolated from mangrove plant *Excoecaria agallocha* yielded two new polyphenols containing both phenolic bisabolane sesquiterpenoid and diphenyl ether units, expansols A (**29**) and B (**30**) (Fig. 1.9), and two new phenolic bisabolane sesquiterpenoids. Compound **29** exhibited moderate cytotoxicity against HL-60 cell line with an IC_{50} value of 15.7 μM, and compound **30** inhibited the proliferation of A549 and HL-60 cells with IC_{50} values of 1.9 and 5.4 μM, respectively (Lu *et al.,* 2010).

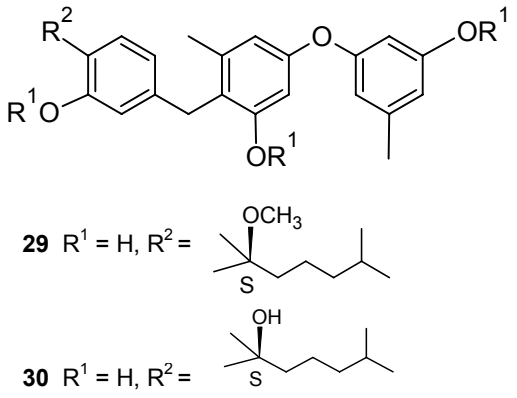


Fig. 1.9. Two new polyphenols expansols A (**29**) and B (**30**)

Fig. 1.10 presents two new compounds, hypochromins A (**31**) and B (**32**) isolated from EtOAc extracts of marine-derived *Hypocrea vinosa*. The compounds showed inhibitory activity toward tyrosine kinase with IC_{50} values of 58.7 μM and 18.0 μM, respectively. The compounds which have bis(naphtho-γ-pyrone) skeleton were also reported to have the potential as new antiangiogenic and antitumor molecules (Ohkawa *et al.*, 2010).

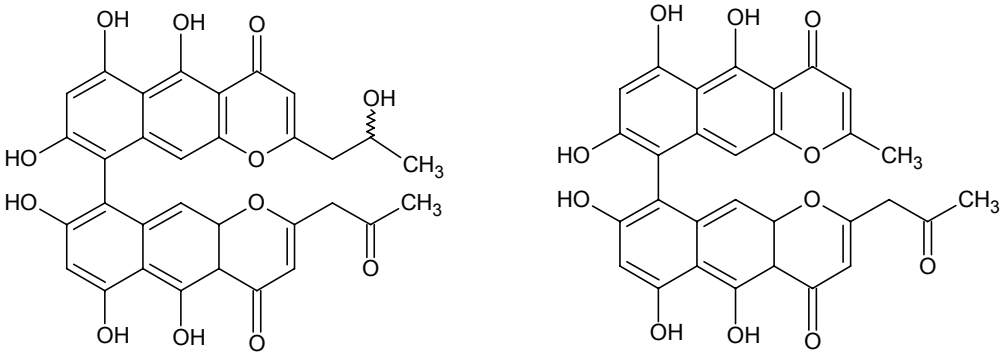


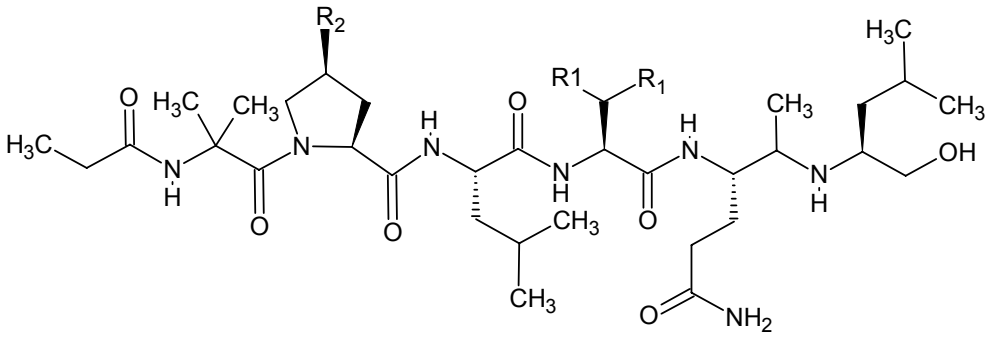
Fig. 1.10. Hypochromins A (**31**) and B (**32**) isolated from *Hypocrea vinosa*

1.3.2.3. Antiviral Compounds

The search for antiviral compounds from marine fungi has yielded some promising results such as equisetin and phomasetin which were isolated from *Fusarium heterosporum* and *Phoma* sp., respectively (Yasuhara-Bell & Lu, 2010). The compounds exhibited as inhibitors of HIV-1 integrase (Singh *et al.*, 1998). Sansalvamide A, a cyclic depsipeptide

isolated from *Fusarium* sp. was found to inhibit the topoisomerase of the pathogenic poxvirus *Molluscum contagiosum* (MCV) (Hwang *et al.,* 1999).

Halovirs A-E (**33-37**) (Fig. 1.11), a series of novel linear peptides isolated from *Scytidium* sp. showed potent antiviral activity against HSV-1 and HSV-2 (Rowley *et al.,* 2003). Stachyflin, a novel terpenoid isolated from *Stachybotrys* sp. RF-7260 shown antiviral activity against influenza A virus (H1N1) (Minagawa *et al.,* 2002a; 2002b). Recently, Watanabe *et al.,* (2010) has found the total synthesis of the compound.



33 $R_1 = CH_3$, $R_2 = OH$, n=12

34 $R_1 = H$, $R_2 = OH$, n=12

35 $R_1 = CH_3$, $R_2 = H$, n=12

36 $R_1 = CH_3$, $R_2 = OH$, n=10

37 $R_1 = CH_3$, $R_2 = H$, n=10

Fig. 1.11. Halovirs A-E (**33-37**) isolated from *Scytidium* sp.

1.3.2.4. Miscellaneous

From an unidentified marine-derived fungus isolated from brown alga *Ishige okamurae* in Japan, three novel diterpenes Phomactin I (**38**), 13-*epi*-Phomactin I (**39**) and Phomactin J (**40**) were isolated (Fig. 1.12) (Ishino *et al.,* 2010). Phomactins are reported to be active as platelet activating factor (PAF) antagonist (Sugano, 1996).

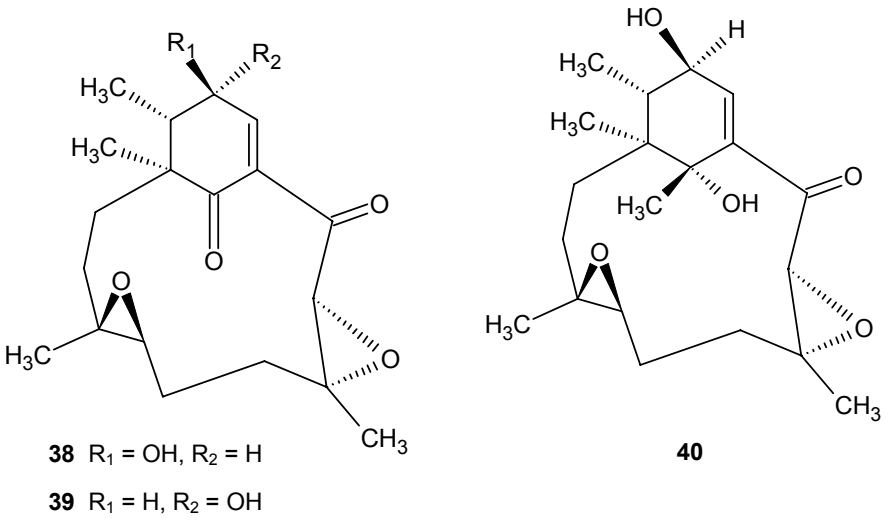


Fig. 1.12. Three novel diterpenes Phomactins I (**38**), 13-*epi*-Phomactin I (**39**) and Phomactin J (**40**)

Two new cyclohexene derivatives, nigrosporanenes A (**41**) and B (**42**) were isolated from the sea fan-derived fungus *Nigrospora* sp. PSU-F11. In the DPPH assay compounds **41** and **42** showed weak radical scavenging activity with IC_{50} values of 0.34 and 0.24 mg/ml, respectively. Compound **41** was also active in the cytotoxicity assay against MCF-7 and Vero cells with respective IC_{50} values of 9.37 and 5.42 µg/ml (Rukachaisirikul, 2010). Fig. 1.13 presents the chemical structures of nigrosporanenes.

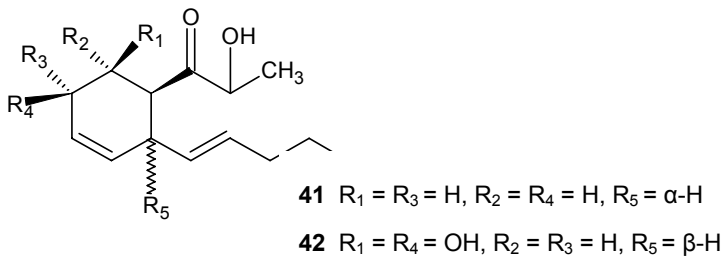


Fig. 1.13. Two new cyclohexene derivatives, nigrosporanenes A (**41**) and B (**42**)

In summary, in Table 1.2 are listed some new bioactive natural products from marine-derived fungi which were published in January-September 2010.

Table 1.2. Some new bioactive natural products from marine-derived fungi (published in January-September 2010)

Compounds	Chemical structure	Biological source	Activity	Reference(s)
Aspochalasins R-T	Alkaloid	*Spicaria elegans*	Cytotoxic	Lin *et al.*, 2010
Azaspirofuran A	γ-lactam	*Penicillium* sp.	Cytotoxic	Ren *et al*., 2010
Cryptosphaerolide	Sesquiterpenoid	*Cryptosphaeria* sp.	Cytotoxic	Oh *et al.*, 2010
Cytoglobosins A-G	Alkaloid	*Chaetomium globosum*	Cytotoxic	Cui *et al*., 2010
Expansols A-B	Polyphenol	*P. expansum*	Cytotoxic	Lu *et al*., 2010
Fusarnaphthoquinones A and B	Naphthoquinone	*Fusarium* sp.	Antifungal	Trisuwan *et al.*, 2010
Halovirs A-E	Peptide	*Scytidium* sp.	Antiviral	Rowley *et al.*, 2003; Watanabe *et al*., 2010
Hypochromins A-B	Naphtha-γ-pyrone	*Hypocrea vinosa*	Tyrosine kinase inhibitor	Ohkawa et al., 2010
Merulins A and C	Sesquiterpene	Endophytic fungus of *Xylocarpus granatum*	Cytotoxic	Chokpaiboon *et al*., 2010
Methyl averantin	Anthraquinone	*A. versicolor*	Cytotoxic	Lee *et al*., 2010a
Nigrosporanenes A-B	Cyclohexene	*Nigrospora sp.*	Antioxidant	Rukachaisirikul *et al*., 2010
Penicinoline	Alkaloid	*Penicillium sp.*	Cytotoxic	Shao *et al*., 2010
Phomactin I-J	Diterpene	Unidentified fungus	PAF antagonist	Ishino *et al*., 2010
Sonnerlactone 1-2	Lactone	Unidentified mangrove endophytic fungus	Cytotoxic	Li *et al*., 2010
Sterigmatocystin	Xanthone	*A. versicolor*	Cytotoxic	Lee *et al*., 2010b
Trichodermaketone A	Polyketide	*Trichoderma koningii*	Antifungal	Song *et al*., 2010
Trichodrins A-B	Aminolipopeptide	*Trichoderma* sp.	Antibiotic	Pruksakorn *et al.*, 2010

1.4. Aim and Scope of the Study

The aim of this study was to investigate the biological and chemical aspects of marine-derived fungi isolated from Indonesian marine habitats as well as their secondary metabolites. In order to accomplish this aim, the study was divided into three main parts as illustrated below.

1.4.1. Isolation and Cultivation of Marine-derived Fungi

Thirty two fungal strains were isolated from the red seaweeds, driftwoods, mollusc shells and sandy habitats. Morphological and biological screening afforded eleven fungal strains for further investigation of their secondary metabolites.

1.4.2. Biological Screening of Fungal Extracts

Marine fungal secondary metabolites which are considered as candidates for new drugs are those showing antibacterial, antifungal or cytototoxic activities. Therefore, this study was focused on the isolation of metabolites which showed antibacterial, antifungal or cytotoxic activities in preliminary bioassays study. Production of fungal bioactive compounds were induced by salinity and mixed culture with autoclaved *Staphylococcus aureus*.

1.4.3. Chemical and Pharmacological Investigations of Isolated Compounds

Bioassay-guided fractionation to purify the components responsible for the activity of the extracts was used to isolate the desired compounds. Further isolation steps were carried out on some selected strains according to their productivity and biological activity. Pure isolated compounds were then chemically elucidated with the help of collaborating scientists. The compounds were also tested in diverse bioassays.

2. MATERIALS AND METHODS

2.1. Biological Materials

Samples as fungal host were collected from Indonesian marine and mangroves area, namely in East and West Java, North Jakarta in April-May 2007 and from South Sulawesi in May 2008. The samples included driftwoods, mollusc shells, sand foam and algae. The algal samples were kindly provided by Dr. E. N. Zainuddin while the others were collected by the writer herself. Driftwood containing fruiting bodies is as presented in Fig. 2.1.

Fig. 2.1. Driftwood containing fruiting bodies collected from Indonesian sea water

Driftwood-derived fungi were obtained from spores that grown inside the fruiting body, while algal-derived fungi were isolated using surface sterilization method of small pieces of alga then incubated on Hagem agar medium (HA) at room temperature. Streptomycin or penicillin in first agar plates was added to prevent bacterial contaminants. For shells and sand foam-derived fungi, the direct incubation method was most commonly applied. The medium used for the direct incubation was PDA (potato dextrose agar) with sterile seawater. A pure culture of the fungi was prepared through repeated inoculation on agar plates.

The fungi were then grown in 50 ml flasks as pre-cultures and after 3-4 weeks of incubation, transferred into 3 liter cultures. After a period of incubation, both the mycelia and broth were extracted and subsequently screened for biological activity and those showing significant bioactivity were mass cultivated into two growth condition either with or without marine salt.

For identification purposes, fungi were cultivated using different media to allow the fungi to produce spores (Table 1, Appendix). The sporulated fungi were then kindly identified by Prof. Jan Kohlmeyer and Dr. Brigitte Volkmann-Kohlmeyer (Institute of Marine Sciences, University of North Carolina, USA), Dr. Peter Hoffmann (German Collection of Microorganisms and Cell Cultures-DSMZ Braunschweig, Germany), Dipl. -Biol. Beate Cuypers (RessourcenZentrum Marine Organismen-RZMO Greifswald, Germany) or PD Dr. Marc Stadler (InterMed Dortmund, Germany). All fungal cultures have been deposited at the Department of Pharmaceutical Biology, Institute of Pharmacy, Ernst-Moritz-Arndt University Greifswald, Germany.

2.1.1. Marine-derived Fungi (Excluding Marine Algicolous Fungi)

The fungi were isolated from driftwoods, sandy habitats and mollusc shells. All samples were collected during low tide period of the sea. The isolated fungi were initially grown on agar plates using PDA or HA medium, and subsequently cultivated on HA medium. Mass cultivation was carried out using liquid HA medium in Erlenmeyer flasks either with or without marine salt.

Composition of PDA medium is as follow (modified from Atlas, 2004):

Potatoes	300.0 g
Glucose	20.0 g
Agar	15.0 g
Seawater (ca. 30‰ salinity)	1000 ml

While HA medium consists of (Helmholz *et al*., 1999):

Ammonium succinate (self produced from 25% ammonia and succinic acid, VWR)	0.5 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeCl_3$ (1% solution)	0.5 ml
Glucose	5.0 g
Malt extract	5.0 g
Distilled water/Seawater (ca. 30‰ salinity)	1000 ml
pH (NaOH/HCl)	7.5

In addition, Czapek-Dox agar (CZ) and malt-extract agar (MEA) media were used particularly for sporulation of *Aspergillus* sp.

According to Klich (2002) CZ medium consists of:

Czapek concentrate*	10 ml
K_2HPO_4	1.0 g
Sucrose	30.0 g
Agar	17.5 g
Distilled water	1000 ml

*Composition of Czapek concentrate (with trace metals) is:

$NaNO_3$	30.0 g
KCl	5.0 g
$MgSO_4 \cdot 7H_2O$	5.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.05 g
Distilled water	100 ml

MEA medium consists of (Klich, 2002):

Malt extract	20.0 g
Peptone	1.0 g
Glucose	20.0 g
Agar	20.0 g
Distilled water	1000 ml

2.1.1.1. *Mycelium sterilium* KT03

The strain was isolated from driftwood TMK-1 that was collected from Malang, East Java. The fungus grew slowly and produced conidia since cultivated on HA medium. The mycelia of the strain are black and their spores consist of 2 cells (Fig. 2.2). However, the classification has not known yet and so that it is named by *Mycelium sterilium* with its internal number KT03.

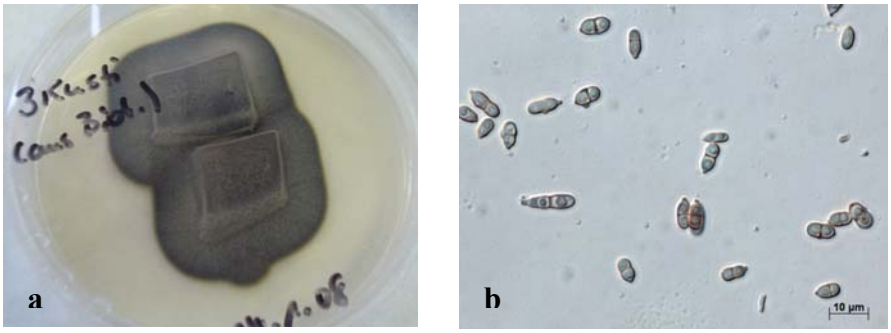


Fig. 2.2. KT03, (a). on HA medium, (b). Spores [1000-fold]

2.1.1.2. *Aspergillus* sp. KT13

The fungus was aseptically isolated from the surface of sandy habitat in Pelabuhanratu, West Java. The fungus was cultivated on an orbital shaker (120 rpm) for 3 weeks at room temperature using HA medium. Conidia of the fungus were produced when cultivated on typical media for *Aspergillus* and *Penicillium,* i.e. Czapek-Dox agar (CZ) and malt extract agar (MEA) media. Microscopic investigation indicated that the strain belongs to the genus *Aspergillus*. The fungus *Aspergillus* sp used in this study can be seen in Fig. 2.3.

According to Hawksworth *et al*. (1983) *Aspergillus* sp is classified as follow:

Phylum : Ascomycota
Class : Eurotiomycetes
Order : Eurotiales
Family : Trichocomaceae
Genus : *Aspergillus*
Species : *Aspergillus* sp.

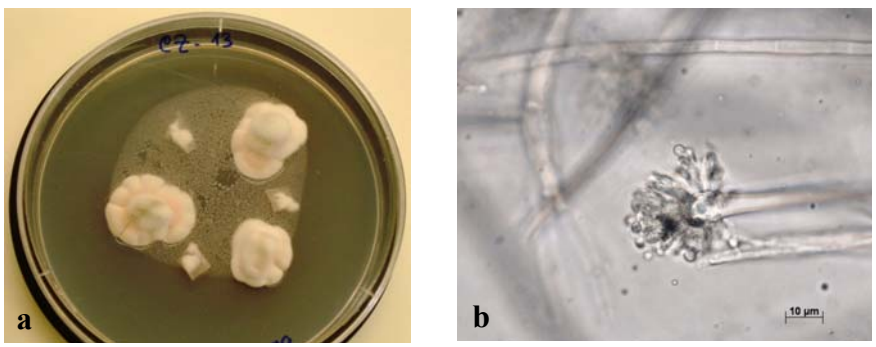
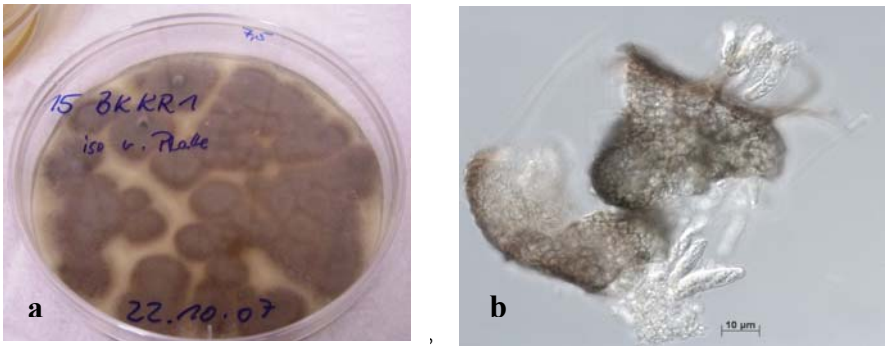


Fig. 2.3. *Aspergillus* sp. (a). on CZ medium, (b). Conidiophore [1000-fold]

2.1.1.3. Unidentified Fungus KT15

The fungus was aseptically isolated *in-situ* from mollusc shell BKKR-1 collected in Malang, East Java. Cultivation on corn meal agar allowed the fungus to produce ascospores. The certain classification has not accomplished yet, although it can be determined belongs to Ascomycota. The samples are as shown in Fig. 2.4.



2.1.1.4. *Mycelium sterilium* KT19

The fungus was isolated from the surface of sandy habitat in Malang, East Java. Cultivation on diverse media was not successfully to make the fungus sporulated. The fungus is presented in Fig. 2.5.

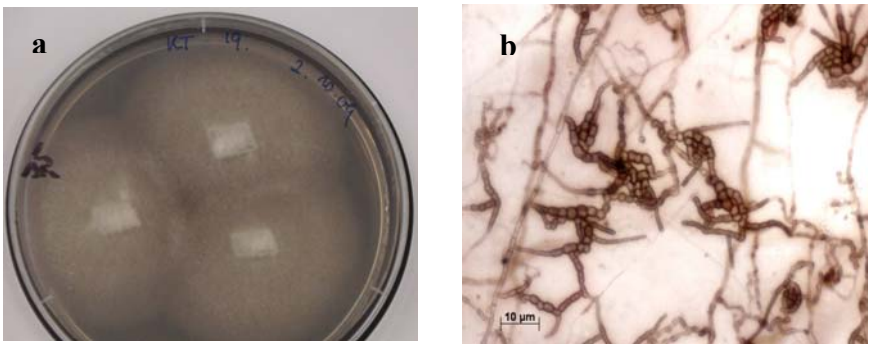


Fig. 2.5. KT19, (a). on HA agar medium, (b). Clamydospores [1000-fold]

2.1.1.5. *Lasiodiplodia theobromae* KT26

The fungus was isolated from driftwood BK-1 collected in Balekambang beach, East Java. Morphologically it was kindly identified by Dr. Peter Hoffmann as *Lasiodiplodia theobromae,* a plant pathogenic fungus particularly found in potatoes. The sample can be seen in Fig. 2.6.

Taxonomy of *L. theobromae* is described below (Hawksworth *et al*., 1983).

Phylum : Ascomycota

Class : Dothideomycetes

Order : Botryosphaeriales

Family : Botryosphaeriaceae

Genus : *Lasiodiplodia*

Species : *Lasiodiplodia theobromae*

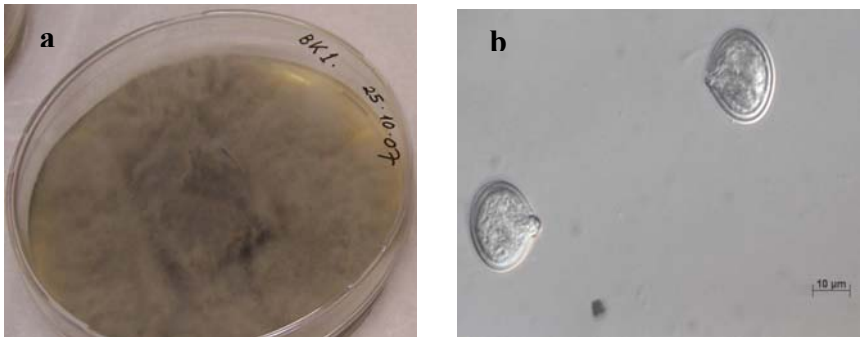


Fig. 2.6. *L. theobromae* KT26, (a). on HA medium, (b). Spores [1000-fold]

2.1.2. Marine Algicolous Fungi

All algal fungal hosts belong to Rhodophyta (red algae). They are commonly known as agar- or carrageenan-producing algae. The algal samples in the present study are presented in Fig. 2.7-11. Since we used surface sterilization method to isolate the fungi, therefore the isolated strains were classified as endophytic fungi. The isolated fungi were grown on agar plates using HA medium and subsequently cultivated in flasks either with or without marine salt.

Fig. 2.7. *Eucheuma edule* BNEE-1 collected from Bone, South Sulawesi as biological source of *Epicoccum nigrum* KT28

Fig. 2.8. *Eucheuma edule* TKEE-1 collected from Takalar, South Sulawesi as biological source of fungal strain KT29

Fig. 2.9. *Kappaphycus alvarezii* BRKA-1 collected from Barru, South Sulawesi as biological source of *Xylaria psidii* KT30 and fungal strain KT31

Fig. 2.10. *Gracilaria* sp. SGR-1 collected from South Sulawesi as biological source of fungal strain KT32

Fig. 2.11. *K. alvarezii* JNKA-1 collected from Jeneponto, South Sulawesi as biological source of *Coniothyrium* sp. KT33

The pure strains were cultivated on 200 ml medium in a 500 ml flask for 2-3 weeks. After optimum growth period, the culture was harvested and followed by extraction. The fungal biomass was separated from the culture broth, and the mycelia freeze-dried and subsequently extracted with DCM, methanol and water, while the broth volume was directly reduced by vacuum rotary vapour and afterwards extracted with ethyl acetate. The fungal extracts were subsequently screened for biological activity (antimicrobial, antioxidant and cytotoxic assays).

2.1.2.1. *Epicoccum nigrum* KT28

The fungus was isolated from red alga *Eucheuma edule* BNEE-1 collected in Bone, South Sulawesi. The fungal strain could be determined by Dipl. -Biol. Beate Cuypers rapidly as *Epicoccum nigrum* from their particular conidia. Morphologically it also can be

recognized from colour of the intense orange media with black spots of conidia when cultured for long time. This fungus grows cosmopolitan as mould. More detail picture can be seen in Fig. 2.12.

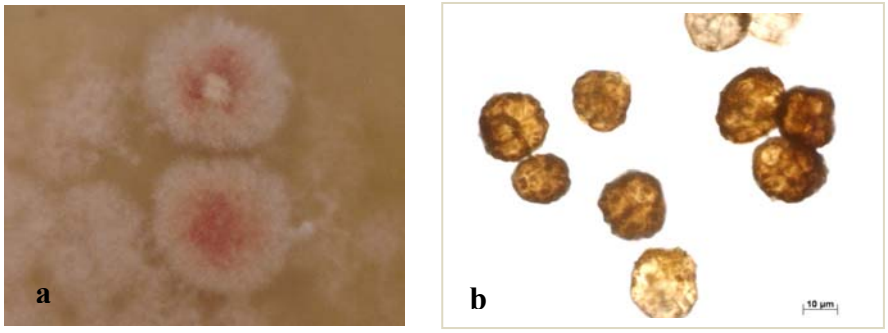


Fig. 2.12. *E. nigrum* KT28, (a). on HA medium, (b). Conidia [1000-fold]

Taxonomy of *E. nigrum* is as follow (Hawksworth *et al*., 1983).

Phylum : Ascomycota
Class : Dothideomycetes
Order : Pleosporales
Family : Pleosporaceae
Genus : *Epicoccum*
Species : *Epicoccum nigrum*

2.1.2.2. *Mycelium sterilium* KT29

The strain was isolated from red alga *Eucheuma edule* TKEE-1 collected in Takalar, South Sulawesi. The fungus was in vegetative status though cultivated in diverse media; therefore it could not be identified yet. The vegetative mycelia can be seen in Fig. 2.13.

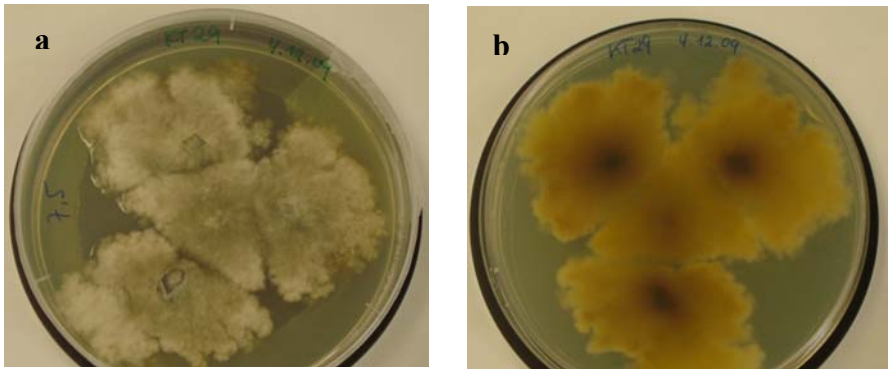


Fig. 2.13. KT29, (a) on HA medium, (b) reverse side

2.1.2.3. *Xylaria psidii* KT30

The fungus was isolated from red alga *Kappaphycus alvarezii* (later name of *Eucheuma cottonii*) BRKA-1 which was collected from Barru, South Sulawesi. Though cultivated in diverse media, the fungus was still in vegetative status; fortunately it could be identified from their specific stromata (Fig. 2.14). The identification was kindly performed by PD Dr. Marc Stadler.

Further identification using molecular technique revealed that the fungus belongs to *Xylaria psidii*. The molecular identification work was carried out by Dipl.-Biol. Fabienne Flessa at Department of Mycology, Bayreuth University, Germany.

Taxonomy of *Xylaria psidii* is as follow (Rogers *et al.,* 1992).

Phylum : Ascomycota
Class : Sordariomycetes
Order : Xylariales
Family : Xylariaceae
Genus : *Xylaria*
Species : *Xylaria psidii*

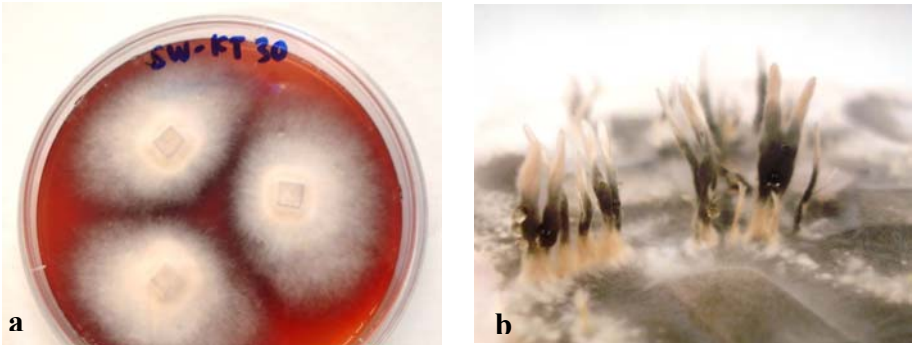


Fig. 2.14. *Xylaria psidii* KT30 (a) on malt-extract agar, (b) Stromata on rice agar medium

2.1.2.4. *Mycelium sterilium* KT31

The fungus was isolated from the same algal sample as KT30, *K. alvarezii* BRKA-1. Conidia were found after 3 weeks of cultivation on corn meal agar medium. The conidia were supposed to be anamorphic form of the strain, so that the certain classification could not be completed. The strain KT31 can be seen in Fig. 2.15.

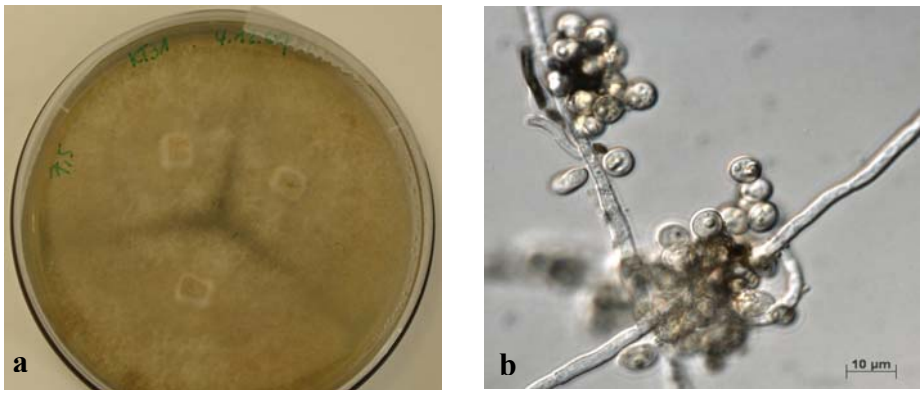


Fig. 2.15. KT31, (a) on HA medium,
(b) Conidia directly developed on mycelia [1000-fold]

2.1.2.5. *Mycelium sterilium* KT32

The sample was isolated from agar producer *Gracilaria* sp. SGR-1 which was collected also in South Sulawesi. The fungus produced conidia on corn meal agar or even on HA medium. The conidia were also supposed to be anamorphic form of the strain, so that the certain classification could not be accomplished yet. The fungus and the conidia can be seen in Fig. 2.16.

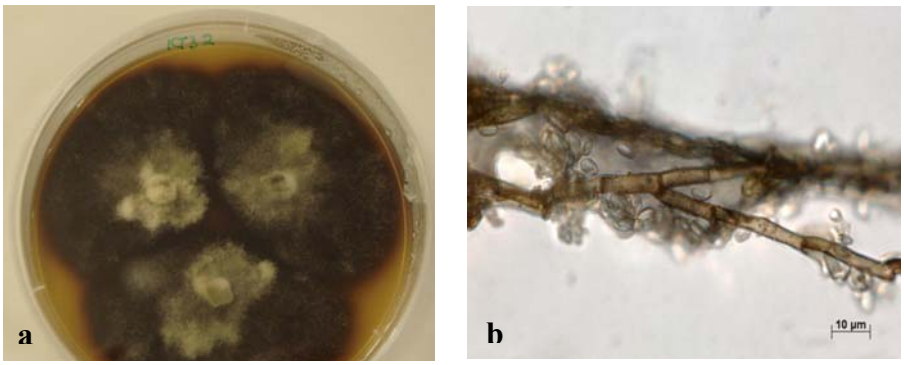


Fig. 2.16. KT32, (a) on HA medium,
(b) Conidia directly developed on mycelia [1000-fold]

2.1.2.6. *Coniothyrium* sp. KT33

The fungus was isolated from *K. alvarezii* JNKA-1 originally collected in Jeneponto, South Sulawesi. Spores developed in dark brown pycnidia were found on HA medium plates approximately after 2-3 weeks of cultivation (Fig. 2.17). Identification of the fungus was kindly done by Prof. Jan Kohlmeyer and Dr. Brigitte Volkmann-Kohlmeyer. According to the spores and morphological characteristics, the fungus was classified as *Coniothyrium* sp.

Taxonomy of *Coniothyrium* sp. is as follow (Hawksworth *et al*., 1983).

Phylum : Ascomycota

Class : Dothideomycetes

Order : Pleosporales

Family : Leptosphaeriaceae

Genus : *Coniothyrium*

Species : *Coniothyrium* sp.

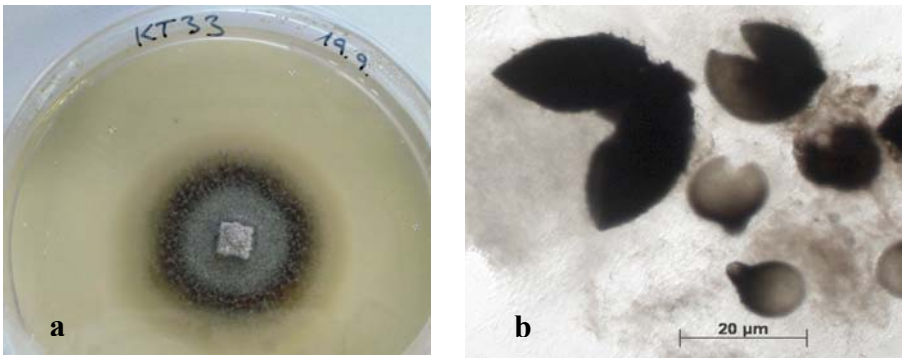


Fig. 2.17. *Coniothyrium* sp. KT33, (a) on HA medium, (b) Broken pycnidia and spores [400-fold]

2.2. Chemical Materials and Equipments

Several chemical materials and equipments used in the study are listed below. Solvents including dichloromethane, *n*-Hexane, acetone, ethanol and *iso*-propanol were purchased from Walter (Germany), while ethyl acetate and methanol from Gereso (Germany). They were all distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

Other general laboratory chemicals:

Glacial acetic acid	VWR
Hydrochloric acid	VWR
Formic acid	Merck
Concentrated sulfuric acid	Merck
Sodium hydroxide	Merck
Natrium sulfate	Merck
Ethyl formiate	Merck

Materials used for chromatography:

Precoated TLC plates (AluO, Silica Gel 60 F_{254}, layer thickness 0.25 mm	Merck
Precoated TLC plates (Glass), F_{254}, layer thickness 0.5 mm	Merck
Precoated TLC plates (AluO, RP-2, layer thickness 0.25 mm	Merck
SPE Cartridge C18	Chromabond®
Silica Gel 60, 0.015-0.040; 0.040-0.063 mm mesh size	Merck
Sephadex LH-20, 25-100 µm mesh size	Amersham Biosciences AB
HPLC Solvents:	
Methanol LiChroSolv HPLC	VWR
Acetonitrile LiChroSolv HPLC	Roth
Seasand p.a.	VWR
Aqua bidestillata (self prepared using SG Water-System Clear UV Plus)	Water preparation & Recycling GmbH

Equipments used:

Balances	BP61S-Sartorius
	KERN EW 420-3NM
Centrifuge	Universal 30F – Hettich
Drying oven	Binder
Fraction collector	BIO-RAD Model 2110
Freeze dryer	Christ® Loc-1m ALPHA 1-4
	Pfeiffer Vacuum
Extraction thimble	Schleicher & Schüll
Homogenizer	IKA®T25 digital ULTRA-TURRAX®
Hot plate	Heidolph MR3001K
Incubator	Memmert
Magnetic stirer	IKAMAG® REO
Microwave	Samsung
Miller	BRAUN
Shaker HS250 basic	IKA Labortechnik
Soxhlet apparatus	VWR International
Syringe	Hamilton 702N
Platform shaker	INNOVA 2100, New Brunswick Scientific Co.

pH-electrode	Mettler Delta 340
Rotary evaporator	Büchi Rotavapor R-114 Waterbath B-480 Vacuum System B-178
Sonicator	Transsonic 460-ELMA®
UV lamp	CAMAG
UV/Vis Spectrophotometer	SPECORD 50
Vacuum exsicator	Savant -Speed Vac® Plus SC110A Savant-Universal Vacuum System Plus with VaporNet®

HPLC equipments:

Pump	LC-10AT VP Shimadzu Liquid Chromatograph
HPLC program	Shimadzu System Controller SCL-10A VP
Detector	Diode Array Detector SPD-M10A
Column C-18	Lichrospher®
Printer	HP Deskjet 5550

2.3. Chromatographic Methods

2.3.1. Thin Layer Chromatography (TLC)

TLC was used to monitor the identity of each extracts and fractions, additionally to screen the qualitative purity of the isolated compound. It was also developed to optimize the solvent system that would be applied for column chromatography.

Analytical TLC was performed on precoated TLC plates with Si gel 60 F_{254} (0.2 mm, Merck) and RP-2 (0.2 mm, Merck) using diverse solvent systems for mostly semi-polar compounds. However, solvent system containing EtOAc:Acetone:*n*-Hexane:Formic acid (25:4:18:0.3, v/v) was mostly used, unless otherwise stated. The compounds were then detected by their UV absorbance at wavelength 254 and 366 nm and/or by spraying the TLC plates with spraying reagents followed by heating at 110 ^{o}C.

Solvent systems:

SS1	EtOAc:Acetone:*n*-Hexane:Formic acid (25:4:18:0.3, v/v)
SS2	DCM:EtOAc (65:35, v/v)

SS3	DCM:MeOH (10:1 to 4:1, v/v)
SS4	Toluene:Ethyl formiate: Formic acid (10:5:3, v/v)
SS5	Chloroform:MeOH (95:5, v/v)
SS6	EtOAc:Chloroform:MeOH (3:1.5:1, v/v)

Spraying reagents:

Anisaldehyde/H_2SO_4 Spray Reagent (DAB 10)	Merck
Dragendorff Reagent	Merck
Ninhydrin	Merck
$FeCl_3$ (1% Methanolic)	VWR
$AlCl_3$ (1% Methanolic)	VWR

2.3.2. Column Chromatography (CC)

Crude extracts were subjected to repeated separation through column chromatography using appropriate stationary phase and solvent system previously determined by TLC. Column chromatography was carried out on silica gel 60 (0.015-0.040 and 0.040-0.063 mm mesh size, Merck) and Sephadex LH-20 (25-100 µm mesh size, Amersham Biosciences). Further purification of the fractions was later performed on another CC, preparative TLC or semi-preparative HPLC using C-18 column.

The following separation systems were used:

a) Stationary Phase: Silica gel

Solvent systems:

SS1	EtOAc:Acetone:*n*-Hexane (25:4:18, v/v)
SS2	DCM: EtOAc (65:35, v/v)
SS3	*n*-Hexane:EtOAc (gradient)
SS4	DCM:EtOAc (gradient)
SS5	DCM:MeOH (10:1, v/v)
SS6	Chloroform:MeOH (95:5, v/v)

b) Stationary Phase: Sephadex LH-20

Solvent systems:

SS1	MeOH:H_2O (gradient)
SS2	MeOH
SS3	DCM:MeOH (10:1 to 4:1, v/v)

2.3.3. High Performance Liquid Chromatography (HPLC)

Analytical HPLC was used to identify the interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds. The gradient used started with 10:90 (MeOH:H_2O to 100% MeOH) in 35 min. Peaks were detected by UV-Vis diode array detector.

Semi-preparative HPLC was used for the isolation of pure compounds from fractions previously separated using column chromatography. Each injection consists of about 2-5 mg of the fraction dissolved in 1 ml of the solvent system. The solvent system consisting of MeOH or MeCN and aqua bidestillata was pumped through the column at a flow rate of 1 ml/min. The eluted peaks which were detected by the online UV detector were collected separately in round-bottom flasks.

2.4. Procedure for Extraction and Isolation of Secondary Metabolites

The fungal cultures were extracted both from culture broth and mycelia according to the scheme shown in Fig. 2.18. Furthermore, subsequent steps for isolation of secondary metabolites from each strain are presented in Figs. 2.19-24. Bioassay-guided approach was used to isolate the active compounds with *Bacillus subtilis* and *Vibrio anguillarum* as test organisms, unless otherwise stated.

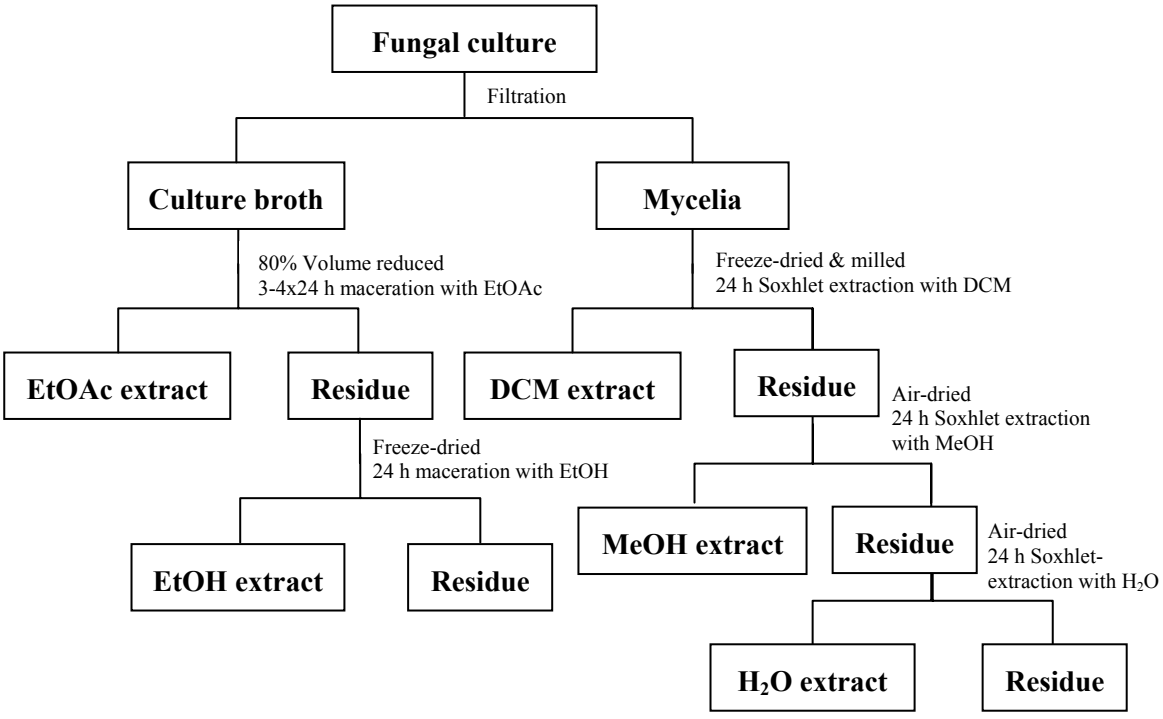


Fig. 2.18. Extraction scheme of fungal secondary metabolites

2.4.1. Isolation of Secondary Metabolites from *Aspergillus* sp. KT13

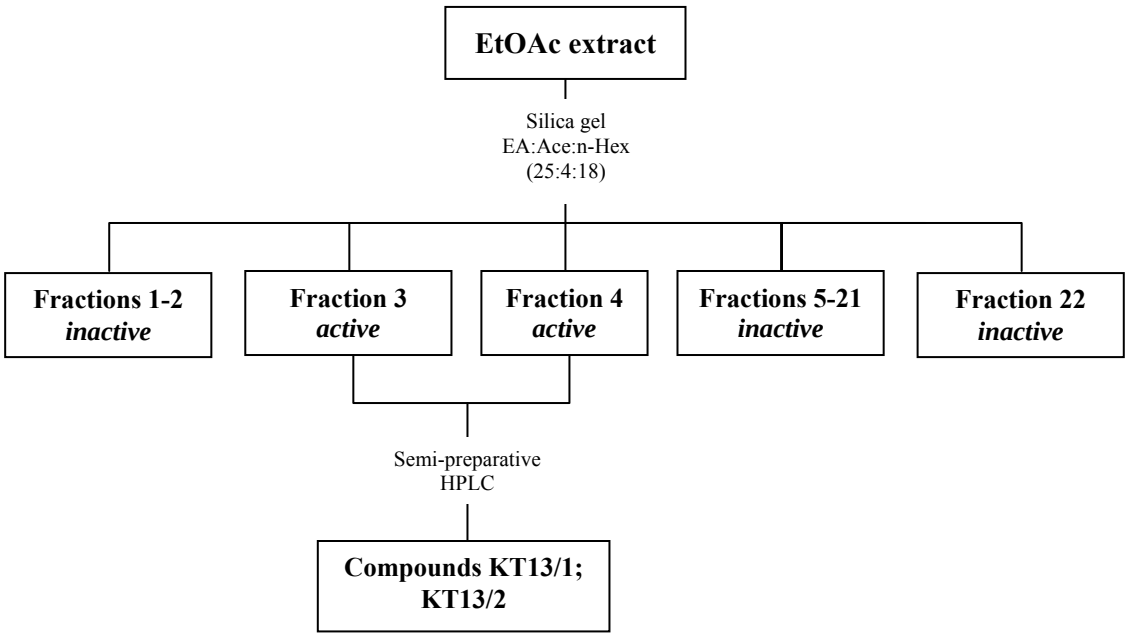


Fig. 2.19. Isolation scheme of secondary metabolites from *Aspergillus* sp. KT13

2.4.2. Isolation of Secondary Metabolites from KT19

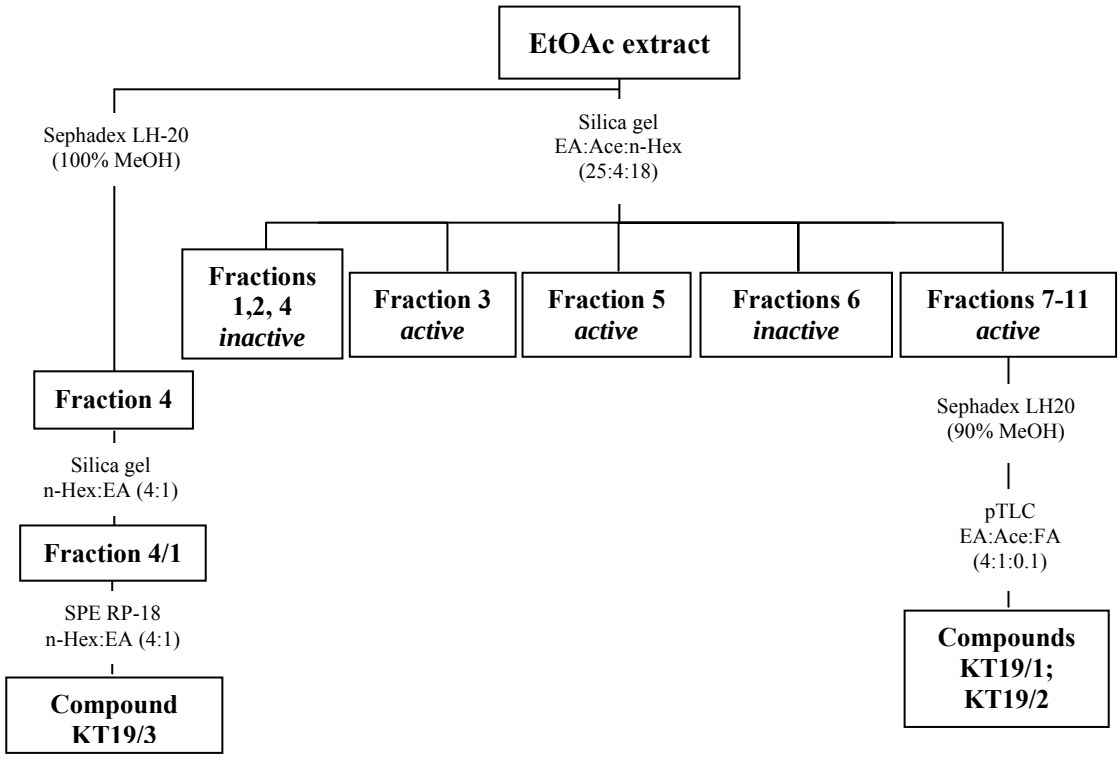


Fig. 2.20. Isolation scheme of secondary metabolites from KT19

2.4.3. Isolation of Secondary Metabolites from KT29

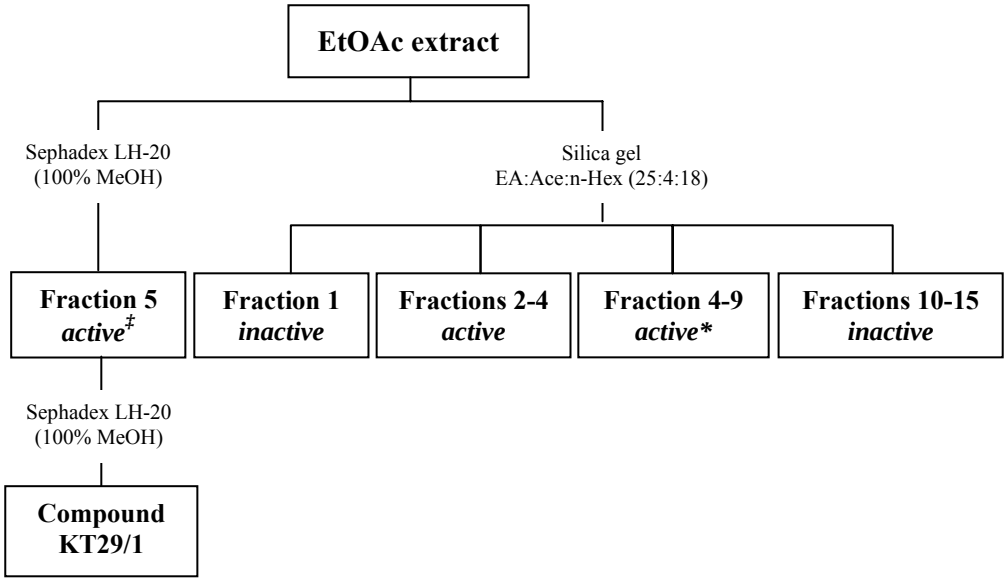


Fig. 2.21. Isolation scheme of secondary metabolites from KT29
(‡ active against *Cladosporium cucumerinum;* * weak active only against *V. anguillarum*)

2.4.4. Isolation of Secondary Metabolites from *Xylaria psidii* KT30

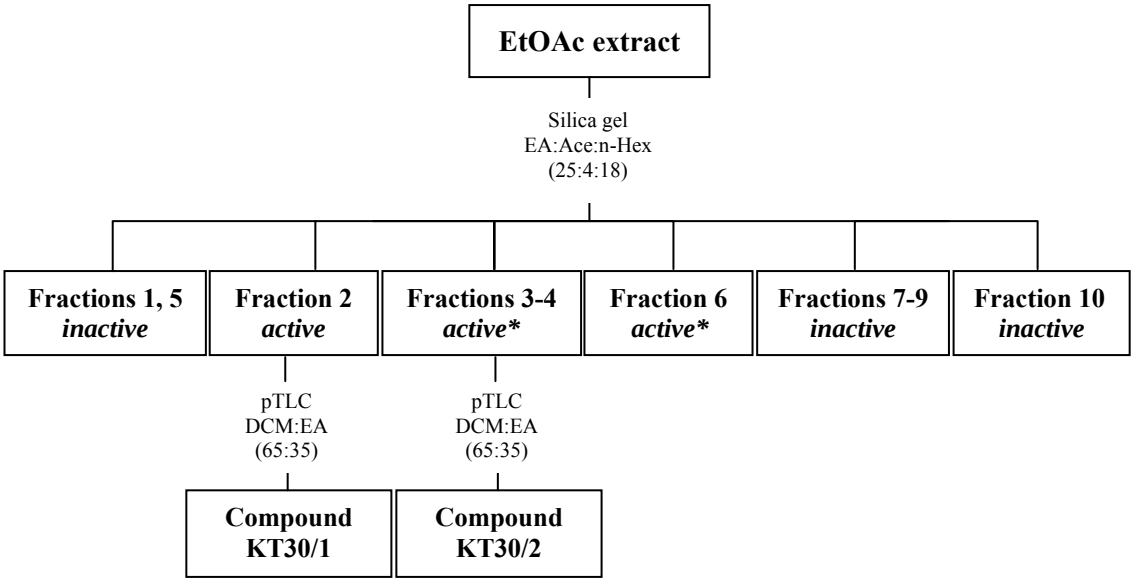


Fig. 2.22. Isolation scheme of secondary metabolites from *Xylaria psidii* KT30
(*weak active against *B. subtilis*)

2.4.5. Isolation of Secondary Metabolites from KT31

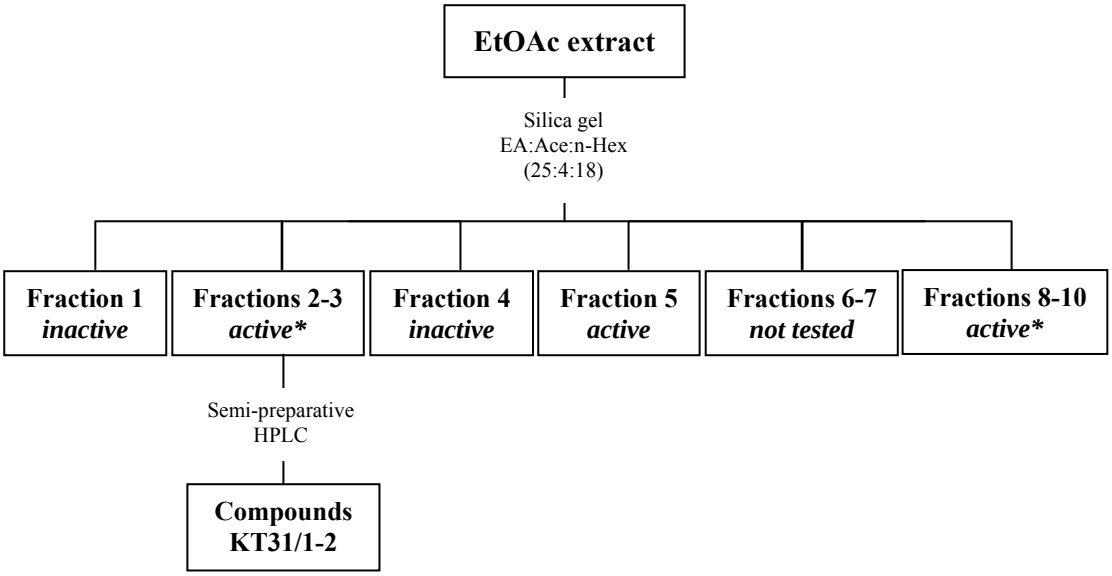


Fig. 2.23. Isolation scheme of secondary metabolites from KT31
(*active only against *V. anguillarum*)

2.4.6. Isolation of Secondary Metabolites from KT32

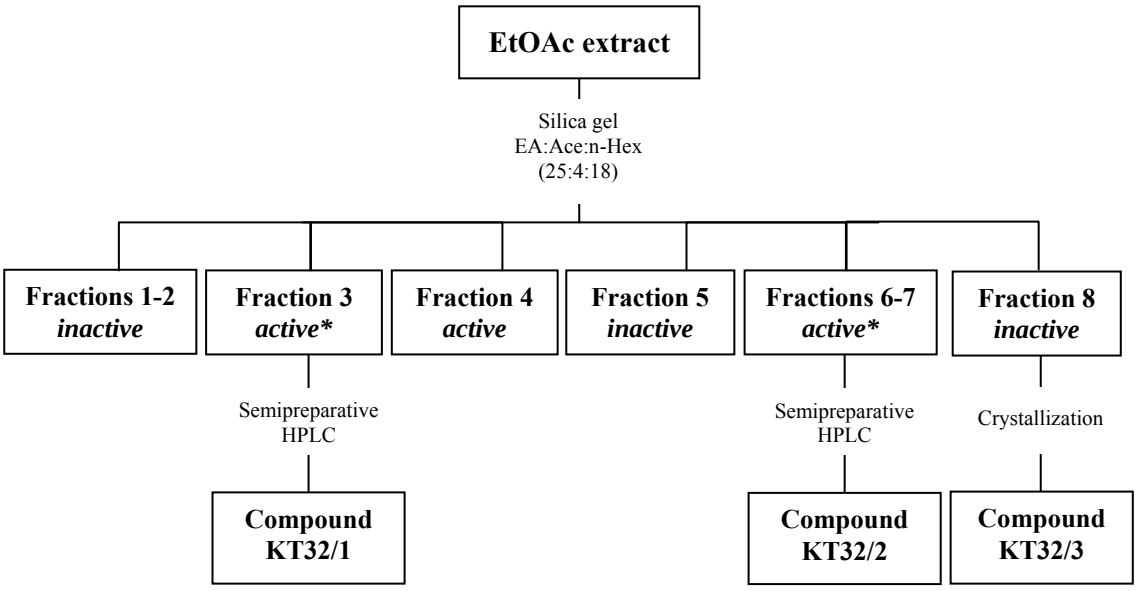


Fig. 2.24. Isolation scheme of secondary metabolites from KT32
(*weak active only against *V. anguillarum*)

2.5. Structure Elucidation

The purified compounds were mainly elucidated using one and two dimensional NMR techniques and various MS methods, while some crystalline compounds were elucidated using single X-ray crystallography. If necessary, additional parameters such as IR properties and melting point were determined. Literature searches were done using Chapman & Hall Natural Products on CD-ROM (2007) and Chemical Abstracts (Scifinder). Structures were designated as new, if they could not be found in Chemical Abstracts.

2.5.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

1D NMR spectra (^{1}H, ^{13}C) were recorded from a Varian Unity 400 at 400 MHz for ^{1}H, and at 100 MHz for ^{13}C NMR. 2D NMR spectra (HSQC, HMBC, COSY, ROESY) were recorded from a Varian Inova 600 at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts in ppm were referenced to the internal TMS (δ=0 ppm) for ^{1}H and CD_3OD (δ=49 ppm) for ^{13}C, respectively.

2.5.2. Mass Spectrometry (MS)

ESI Mass Spectra

Electrospray Ionization (ESI) mass spectra were measured from an API-150EX mass spectrometer (Applied Biosystems) with a turbo ion spray source.

HR-FTICR-MS Spectra

The high resolution positive ion ESI mass spectra were obtained from a Bruker Apex III 70 e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a 7.0 T superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 µl/h.

2.5.3. IR Spectra

Infrared (IR) spectra were measured on a Perkin Elmer System 2000 FT-IR or a Bruker Tensor 27 FTIR spectrophotometer equipped with Diamant-ATR. The wavenumber is indicated in cm^{-1}.

2.5.4. UV Spectra

UV spectra are detected using UV-VIS diode array detector SPD-M10A. Samples were diluted in MeOH or MeCN.

2.5.5. Optical Rotation

Optical rotations were performed with a JASCO DIP-1000 KYU digital polarimeter.

2.5.6. Melting Point

Melting points were measured using SMP II (Schorpp) and were uncorrected.

2.5.7. X-ray Crystallography

Single crystal X-ray analyses were measured at 100 K with a Saturn92 CCD detector mounted on a Micromax007 rotating anode X-ray source (Rigaku). Crystals were grown mostly from MeOH.

2.6. Bioassays

2.6.1. Antimicrobial Activity

Crude extracts, fractions and pure compounds were tested for activity against the following standard strains:

Gram positive bacteria

Bacillus subtilis ATCC 6051

Staphylococcus aureus ATCC 6538

Methicillin-resistant *S. aureus* (Northern Germany epidemic strain)

Staphylococcus epidermidis ATCC 1238

Gram negative bacteria

Escherichia coli ATCC 11229

Pseudomonas aeruginosa ATCC 22853

Aeromonas salmonicida ATCC 51413*

Vibrio anguillarum DSMZ 11323*

Yersinia ruckeri ATCC 29493*

*Fish pathogenic bacteria

Yeasts

Candida albicans ATCC 10231

Candida maltosa SBUG 700

The agar diffusion assay was performed according to Kirby-Bauer method (Boyle *et al*., 1973). Fish pathogenic bacteria were cultivated on TSA medium (BD-Difco™). Another bacteria and yeasts were cultivated on NA II medium (Merck).

For screening, aliquots of the test solution were applied to sterile filter-paper discs (6 mm diameter, Oxoid) to give a final disc loading concentration of 1 mg or 2 mg for crude extracts and various concentrations (300, 100, 50 and 25 µg/disc) for pure compounds. As positive controls, ampicillin (Merck) was used at concentration of 10, 50 and 100 µg/disc; gentamicin (Biochrom) at 10 µg/disc; oxytetracycline (Merck) at 30 µg/disc and nystatin-dihydrat (Roth) was used at concentrations of 50 and 100 µg/disc. The impregnated discs were placed on agar plates previously seeded with the selected test organisms, along with discs containing solvent blanks. The plates were incubated for 18-20 h at 37 °C and 26 °C for human and fish pathogenic bacteria, respectively. The antimicrobial activity was recorded as the clear zone of inhibition surrounding the disc at which the diameter was measured in mm. In order to improve the contrast of the inhibition zone, the plate was sprayed with INT (p-iodonitrotetrazolium chloride, SIGMA).

2.6.2. Determination of Minimum Inhibitory Concentration (MIC)

MIC of the isolated compounds was determined using broth dilution method with 96-wells microtiter plates. As test organisms *Bacillus subtilis, Staphylococcus aureus* and *Vibrio anguillarum* were used.

A suspension of bacteria was prepared by suspending a pin-head of the bacteria in 2 ml of sterile saline solution (0.9% NaCl in distilled water). Volume of 50 µl from this suspension was inoculated into 50 ml of sterile NB (nutrient broth) or TSB (trypticase soy broth, BD-Bacto™) for *V. anguillarum* and afterwards incubated overnight at 25 °C on water bath shaker at 120 rpm. For the test purposes the bacterial suspension was initially diluted 1000 times with the same media.

Composition of NB (Merck) is as follow:

Peptone from meat	5.0 g/l
Meat extract	3.0 g/l

The prepared product was then dissolved in distilled water at a concentration of 8 g/l and autoclaved at 121 °C for 20 min.

TSB (BD-Bacto™) consists of:

Pancreatic casein	17.0 g/l

Soya peptone	3.0 g/l
Dextrose	2.5 g/l
NaCl	5.0 g/l
Dikaliumphosphate	2.5 g/l

pH 7.3±0.2

The medium was prepared at a concentration of 8 g per liter distilled water and autoclaved at 121 ^{o}C for 20 min.

The start concentration of the compound was 2 mg/ml for crude extract and 300 µg/ml for pure compound then diluted into 2-fold serial number. Volume of 200 µl of test solutions was pipetted into the first row of the microtiter plate (A1-H1) in three replicates. All further wells except H12 were filled with 100 µl PBS buffer (pH 7.4). For the dilution steps, 100 µl of the first row was pipetted into the corresponding well of the second row and so on until the 11th row. Then 100 µl from the last row was discarded. Before taking for further dilution or the last dilution, the solution must be properly mixed. Finally 100 µl of bacterial suspension was added into the wells except the last row. Wells of A12-D12 were added with 100 µl of NB as media control (MC). E12-H12 wells were added with 100 µl of bacterial suspension as bacterial growth control (GC).

The microtiter plates were incubated for 24 h at 37 ^{o}C for *B. subtilis* and *S. aureus* and 26 ^{o}C for *V. anguillarum.* After incubation, 20 µl of INT was pipetted into each well and then incubated for 30 min. In order to determine the MIC, the extinction was measured at 550 nm using ELISA reader (Anthos Labtec HT2, Salzburg).

	1	2	3	4	5	6	7	8	9	10	11	12
A												MC
B												MC
C												MC
D												MC
E												GC
F												GC
G												GC
H												GC

Fig. 2.25. Microtiter plate for MIC test (MC: media control; GC: growth control)

2.6.3. Bioautograpic Assay

Bioautography can be considered as the most efficient assay for the detection of antimicrobial compounds in a complex mixture. The method allows the localization of

antimicrobial activity on a chromatogram (Hamburger & Cordell, 1987; Rios *et al.,* 1988). In this study agar overlay or immersion bioautography was used.

Amount of 500 µg of extract or 250 µg of fraction was applied on analytical TLC plate (AluO Si gel 60 F_{254}, 0.2 mm, Merck) and subsequently developed using appropriate solvent system. The TLC plates were dried properly and then put into the sterile Petri dish which was previously poured with 10 ml of agar medium as basic layer. Afterwards 20 ml of seeded agar medium (as described in **2.6.1.**) was applied onto the TLC plate. *B. subtilis, S. aureus* and *V. anguillarum* were used as test organisms.

The plates were incubated for 18-20 h at 37 ^{o}C for *B. subtilis* and *S. aureus*, while *V. anguillarum* at 26 ^{o}C. The active bands appeared as clear zones. INT was used to assist the contrast between the active bands and the background.

2.6.4. Fungicidal Activity against Phytopathogenic Fungus

Fungicidal activity was carried out by the method of Gottstein, a semiquantitative test that allows a reliable estimation of the activity of compounds with similar diffusion chararacteristics (Gottstein *et al.,* 1984). The phyopathogenic fungus *Cladosporium cucumerinum* Ell. et Arth. was used as test organism. The stock culture was cultivated in liquid medium using yeast nutrient solution.

Yeast nutrient solution consists of:

Mannitole	50.0 g
Saccharose	50.0 g
Succinic acid	5.4 g
Yeast extracts	3.0 g
KH_2PO_4	0.1 g
$MgSO_4 \cdot 7H_2O$	0.3 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$ZnSO_4 \cdot 7H_2O$	4.4 mg
H_2O	1000 ml
pH 5.4	

The antifungal test was performed by spraying hand-made TLC plates (glass plate, 20 x 20 cm, silica gel 60 HF_{254}, thickness 0.5 mm dried at 120 ^{o}C for 30 min in the oven) with conidia of *C. cucumerinum* (Gottstein *et al*., 1984). The crude extracts and pure compounds were loaded on the TLC plates using a microliter syringe. Test amounts for

crude extracts were 50 µg, 100 µg, 200 µg, 400 µg and 50 µg for pure compounds. Loaded samples give 10 mm diameter (area of 78.5 mm^2). Crude extracts were also chromatographed on TLC plates with ethyl acetate:chloroform:methanol (3:1.5:1, v/v). The dried plates were sprayed with 10 ml spore suspension of *C. cucumerinum* (spore density ca. 2.5 x 10^6 spores/ml) and dried at room temperature for about 15 min. The dried plates were then placed in a TLC chamber containing moistured filter paper and the fungus was cultivated in an incubator at 25 ^oC for two days.

2.6.5. Free Radical Scavenging Activity

The DPPH free radical scavenging assay is a simple and widely test used for screening of bioactive compounds. Qualitative measurement of radical scavenging properties was carried out in analytical TLC plate (AluO Si gel 60 F_{254}, 0.2 mm, Merck). Five microliter of 0.1% diluted extracts was applied as spot onto TLC plate. All extracts were diluted with ethanol. As positive control, 5 µl of 0.1% ascorbic acid (Sigma-Aldrich) was used and pure ethanol was used as negative control. After the solvents dried, the plates were then sprayed with DPPH-reagent (Fluka, Buch). The plates were subsequently incubated in the dark at 37 ^oC for about 30 min. The active extracts appeared as yellowish spots with the violet background.

The qualitatively active extracts were then carried out for quantitative assay using spectrometric method. The extracts with the concentration values of 500 µg/ml, 100 µg/ml, 50 µg/ml and 10 µg/ml were prepared from 1 mg/ml in glass tubes. Ascorbic acid was also prepared at the same concentration as positive control. Each tube was then added with 375 µl ethanol, 125 µl of DPPH reagent (0.05 % in ethanol) and 500 µl of samples. The solutions were mixed properly and after that incubated at room temperature in the dark for 30 min. The absorption was then measured at 517 nm (Molyneux, 2004).

The percentage of radical scavenger activity was determined according to the equation as follow:

$$\% \text{ radical scavenger activity} = 100 - \frac{A_{sample} \text{ x } 100}{A_{control}}$$

2.6.6. Cytotoxicity Assay

Cytotoxic activity was determined by Neutral Red Uptake Assay (NRU-Test) according to Bohrenfreund and Puerner (1985) with the cultivated human bladder

carcinoma cell line 5637 (ATCC HTB-9) used for the assay. Materials used in the present study are listed as follows.

Swelling buffer (pH 7.4):	20 mM Tris buffer substance (242 mg)
	1 mM $MgCl_2 \cdot 6H_2O$ (20 mg)
	0.5 mM $CaCl_2.6H_2O$ (11 mg)
	0.5 mM $CaCl_2.2H_2O$ (7 mg)
	Aqua bidestillata (add 100 ml)
Lysis buffer:	5% Benzalkonium chloride (0.5 g) in 3% Acetic acid (0.3 ml), add 10 ml aqua bidestillata
Trypsin/EDTA	
PBS/EDTA	
RPMI-Medium with 10% FCS	
NRU stock solution:	0.33 g NRU add 100 ml Aqua bidestillata
NRU-Solution for 1 plate:	120 µl NRU stock solution + 11.88 ml RPMI-Medium
Ethanol/Acetic acid:	1% Acetic acid, 59% EtOH and 42% Aqua dest.
Dilution of test substance:	250 µg/ml (end concentration in the well), dilute 1:2 ; possible: stock solution of 20 mg/ml in EtOH ($EtOH/H_2O$, DMSO) dilute with medium
Positive control	Etoposide
Solvent control	

For cytotoxicity assay, seeding of the test cells was previously prepared in 96-well plate (T25 or T75 cm^2). The old medium was decanted in the well plate and rinsed with 1 or 5 ml PBS/EDTA, shaked carefully for 30 s and the solution was decanted. The solution was added with 1 or 5 ml PBS/EDTA, incubated for 10 min at 37 °C and subsequently the solution was decanted. Volume of 1 or 3 ml Trypsin/EDTA was added into solution and then incubated for 3 min at 37 °C.

After incubation, 3 or 5 ml medium (with 10% FCS) was added and detached the cells from the bottom of the flask by rinsing transferred it into a centrifuge glass. The centrifuge was operated at 1000 U/min for 3 min and then decanted the supernatant carefully. Volume of 2 or 6 ml RPMI (with 10% FCS) was added and mixed well. From the prepared cell suspension, 20 µl of cell suspension was mixed with 460 µl of swelling buffer, incubated for 7 min at room temperature. Then 20 µl of lyses buffer was added and mixed well. The 10 µl of cells suspension was used for estimation the cell concentration

using a Bürker cell counter. The cell nuclei were counted from all 9 big fields (X) of the Bürker chamber and the number was calculated according to the formula below.

Cell number (Z) = X x 0.5 x 1111.1 x dilution factor (Y)

After the cell suspension was ready, subculture was prepared in new flasks T25 or T75. For one plate, 8 ml of RPMI medium with 0.2 Mio cells (2500 cells/well) was required. Volume of 100 µl cell suspensions was added into 2nd to 11th column; 2nd to 7th row (the well Cc to S; B to G as shown in Fig. 2.26), mixed well and then incubated for 24h.

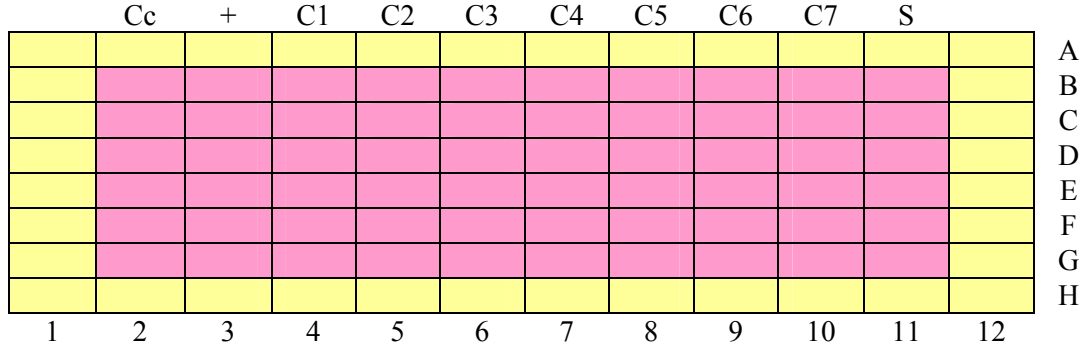


Fig. 2.26. The 96-well plate for cytotoxicity test
Cc: Cell control; +: positive control; C1: 100 µg test substance/ml medium; C2: $C1x2^{-1}$; C3: $C1x2^{-2}$ . . . C7: $C1x2^{-6}$; S: Solvent used to dissolve the substance as solvent control;
Without cells With cells

The medium of the seeding was then removed and add with new medium (with 10% FCS), 100 µl for well in the 3rd and 11th column; 150 µl for the others. Etoposide as positive control was added in volume of 50 µl in the 2nd column; 50 µl of solvent control in the 11th column and 150 µl of test substance (with the highest concentration) in the 4th column.

The test substance in the 4th column was mixed well and then diluted into 1:2 fold to afford the substance concentration of 1:2 (C2), 1:4 (C3), etc. by pipetted 150 µl of C1 to C2, C2 to C3 and so forth (C1-C7 as shown in Fig. 2.26). The cell culture microtiter plate was further incubated for 72 h at 37 °C. Aftter incubation, the cells were washed with 200 µl of HBSS and then added with new medium (with 10% FCS) containing NRU solution; incubated for 3 h at 37 °C. The supernatant of the cell culture was then removed carefully and rinsed the cells twice using 100 µl of HBSS. Volume of 200 µl EtOH/acetic acid was added into the wells and subsequently agitated for 45 min at room temperature. The OD of the cell culture was measured using ELISA reader at wavelength of 540 nm.

3. RESULTS

3.1. Isolation and Taxonomy of Fungal Strains

Thirty two of fungal strains have been isolated from marine area in Indonesia. Eleven of them were further cultivated and investigated. Five strains were isolated from driftwoods (KT03; KT26), sandy habitats (KT13; KT19) and mollusc shell (KT15), and these strains were in the present study further categorized as marine-derived fungi. Six strains isolated from marine algae were classified as marine algicolous fungi (KT28-33). Tables 3.1-2 list the fungal isolates.

3.1.1. Marine-derived Fungi

Three of the marine-derived fungi in this study were successfully collected using "direct" examination methods as described by Kohlmeyer and Kohlmeyer (1979). Materials collected were examined under the dissecting microscope for the presence of ascocarps, basidiocarps or pycnidia. Fruiting bodies were transferred with a needle to a microscope slide, torn apart in a drop of water to expose the spores, and carefully squeezed under a cover glass. The fungi from sandy habitats were collected using "indirect" methods or by incubation.

Table 3.1. Isolated marine-derived fungi (excluding marine algicolous fungi)

Strain	Reproductive structure	Classification and description
KT03	Conidia 2 cells spore (anamorphic form)	*Mycelium sterilium* Black mycelia; reverse black; mounting; growing slowly on solid media; spores developed on HA medium
KT13	Conidia from specialized conidiophores	*Aspergillus* sp. White mycelia on HA medium, orangish-white on CZ medium, greenish-white on MEA medium; reverse white-yellowish. Spores developed on CZ and MEA media.
KT15	Ascospores	Unidentified; Ascomycota Dark-brown mycelia; reverse black; fruiting bodies developed on cornmeal agar
KT19	Vegetative status	*Mycelium sterilium* Mycelia form 'sponge' structure; reverse brown; growing rapidly
KT26	Spores	*Lasiodiplodia theobromae* (identified by Dr. Peter Hoffmann) Black mycelia; reverse black; growing rapidly; spores developed on rice agar or rice-mill agar

3.1.2. Marine Algicolous Fungi

Fungi as sources of secondary metabolites in this study were mostly isolated from red seaweed (marine alga). Three strains were isolated from *Eucheuma cottonii* (later reclassified as *Kappaphycus alvarezii*), two strains from *Eucheuma edule* and one strain from *Gracilaria* sp. Surface sterilization followed by using antibiotic in culture media was the best way to acquire isolated pure strain from algae.

All fungal samples were cultivated using various media to induce sporulation. Finally, some fungi produced spores and then could be identified regarding to their morphological characteristics. Three isolates were identified as *Aspergillus* sp. (KT13), *Lasiodiplodia theobromae* (KT26) and *Epicoccum nigrum* (KT28). Two algicolous isolates were identified as *Xylaria psidii* (KT30) and *Coniothyrium* sp. (KT33). However, six strains are still in vegetative status even treated with varying media.

Table 3.2. Isolated marine algicolous fungi

Strain	Reproductive structure	Classification
KT28	Conidia	*Epicoccum nigrum* (identified by Dipl.-Biol. Beate Cuypers) Orangish-yellow mycelia; conidia formed black spots; reverse black; growing rapidly; spores developed on HA medium
KT29	Vegetative status	*Mycelium sterilium* Yellowish-white mycelia; reverse yellow
KT30	Stromata; vegetative status	*Xylaria psidii* (identified by PD Dr. Marc Stadler) Pink mycelia with red media on malt extract medium; white mycelia on HA medium; blackish-white stromata; stromata developed faster on rice agar medium
KT31	Conidia (anamorphic form)	*Mycelium sterilium* White mycelia; reverse yellowish; growing fast; conidia developed on HA medium
KT32	Conidia (anamorphic form)	*Mycelium sterilium* Black mycelia; reverse black; conidia developed directly on mycelia
KT33	Conidia in pycnidia	*Coniothyrium* sp. (identified by Drs. Jan Kohlmeyer & Brigitte Volkmann-Kohlmeyer) Black mycelia; reverse black; growing slowly; dark-brown pycnidia

Preliminary attempt was done to optimize the bioactives production using growth phase and salinity as variable factors. All strains produced more bioactive compounds (quantities or qualitatively) after about two-three weeks of cultivation in comparison with one week of cultivation. Moreover, some strains were more productive when cultivated

using media without salt addition or reversely. In parallel, the fungi were cultivated to obtain more amounts for further investigation and screened for their antimicrobial, radical scavenging and cytotoxic properties.

3.2. Growth Stage of Fungal Strains and Bioactives Production

Production of secondary metabolites in fungi fluctuates relating to their growth phase. In an attempt to determine the optimum fungal growth stage, the fungi were cultured in different periods and investigated for their mycelial biomass and bioactives production. Initially, five strains selected from all isolates were cultivated and screened for their bioactive properties. Then six other strains which were isolated from algae collected from coastal area of South Sulawesi were also screened.

3.2.1. Marine-derived Fungi

3.2.1.1. *Mycelium sterilium* KT03

Strain KT03 reached the stationary growth phase within 12 days of cultivation. The mycelial biomass of day 12 showed to be the highest biomass, namely 4.77 g/l or nine times more than the initial weight. At this period the pH value was 7.43 and continued to be neutral until 21 days of cultivation (Fig. 3.1).

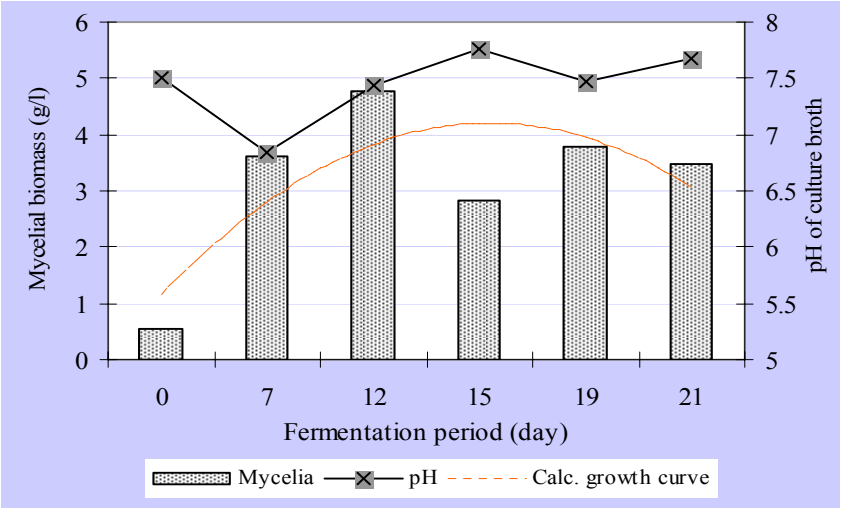


Fig. 3.1. Fungal growth and pH alteration during cultivation of KT03

In Fig. 3.2 the variation of yield and bioactivity of the EtOAc extracts of the fungus KT03 can be seen. The optimum yield after 12 to 19 days of cultivation was not significantly different, but the most active extracts against S*taphylococcus aureus* were obtained after 7 days of cultivation with inhibition zones (IZ) of 13.5 to 14 mm. According to these results the fungus was subsequently cultivated for two weeks.

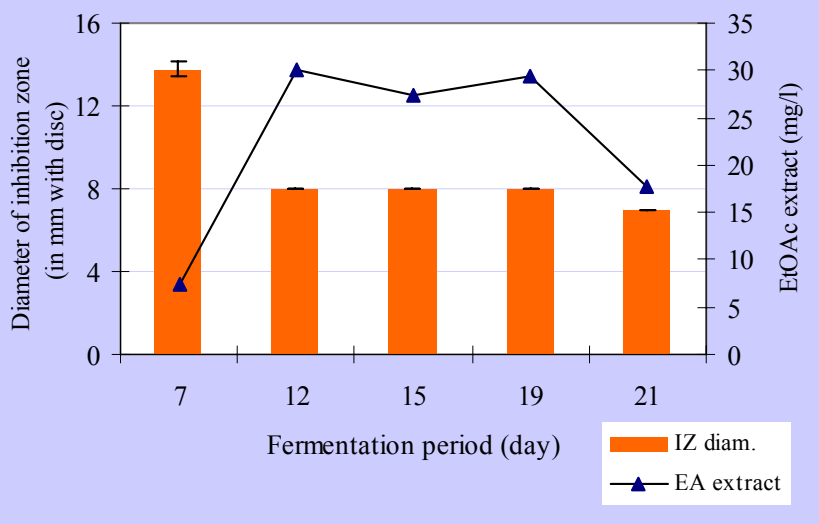


Fig. 3.2. EtOAc extract yield of KT03 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.1.2. *Aspergillus* sp. KT13

The growth curve of *Aspergillus* sp. KT13 can be seen in Fig. 3.3. Within 3 days of cultivation, the fungus was in exponential phase and on day 11 initiated in stationary phase until 21 days of fermentation. However, the maximum growth of the fungus was after 2 weeks of cultivation and decreased afterwards. The neutral pH value of 7.44 was reached during the stationary phase.

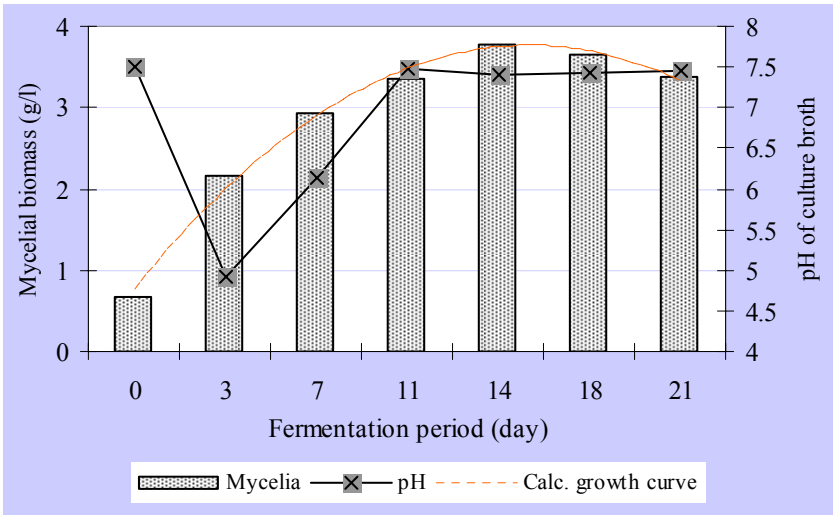


Fig. 3.3. Fungal growth and pH alteration during cultivation of *Aspergillus* sp. KT13

Fig. 3.4 presents the bioactivity of EtOAc extracts of *Aspergillus* sp. KT13 from different culture periods against *S. aureus*. It can be seen that the extract from day 7 cultivation showed medium activity (IZ 11-11.5 mm) as strong as the extract from day 21. From this result further cultivation was carried out for three weeks.

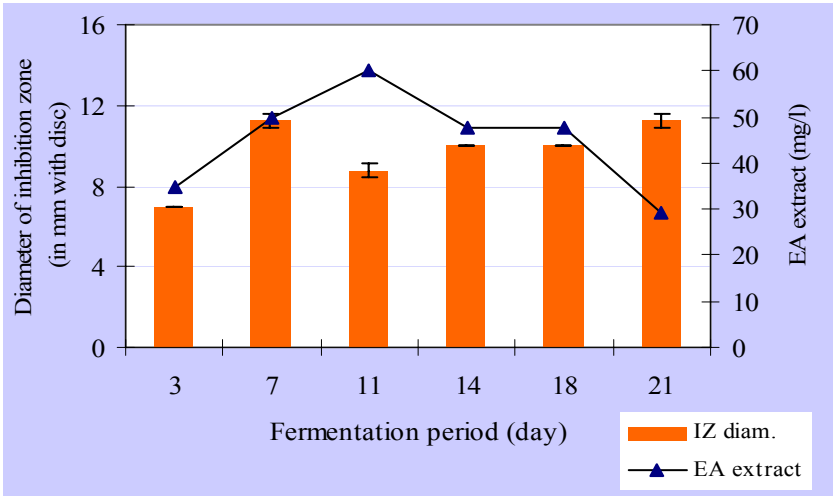


Fig. 3.4. EtOAc extract yield of *Aspergillus* sp. KT13 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.1.3. Unidentified Fungus KT15

Isolate KT15 grew rapidly in period of 6 days of cultivation and until day 14 it was in stationary phase (Fig. 3.5). After two weeks of cultivation the pH value had a tendency to be alkaline (up to 8.03) and the biomass showed that the fungus was currently in dead phase or autolytic phase.

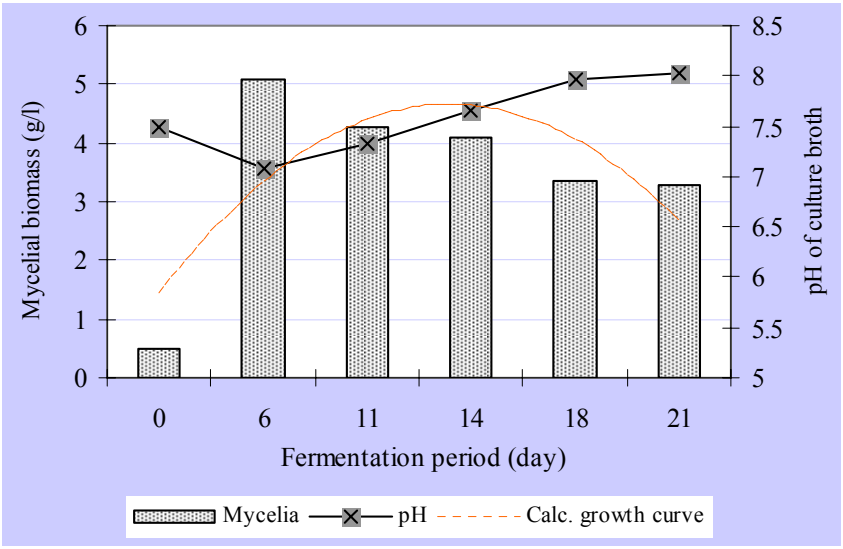


Fig. 3.5. Fungal growth and pH alteration during cultivation of KT15

Fig. 3.6 shows the correlation of EtOAc extract yield of KT15 and its antibacterial activity in different culture periods. The yield was produced mostly after 11 days of cultivation but the strongest antibacterial activity was reached after 6 days of cultivation with IZ values of 14-14.5 mm. Surprisingly, antibacterial activity of extract harvested on

day 14 increased again, while its yield was decreased. Therefore, the fungus was further cultivated for two weeks.

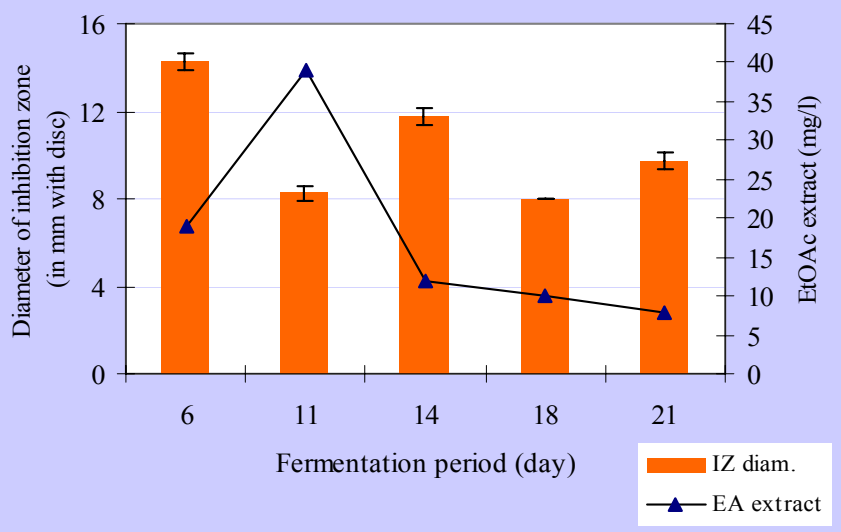


Fig. 3.6. EtOAc extract yield of KT15 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.1.4. *Mycelium sterilium* KT19

Fungal strain KT19 was in exponential phase until 6 days of cultivation and in period of one week afterwards it was in stationary phase. As can be seen in Fig. 3.7, the neutral pH culture of KT19 was relatively stable after 10 to 21 days of cultivation.

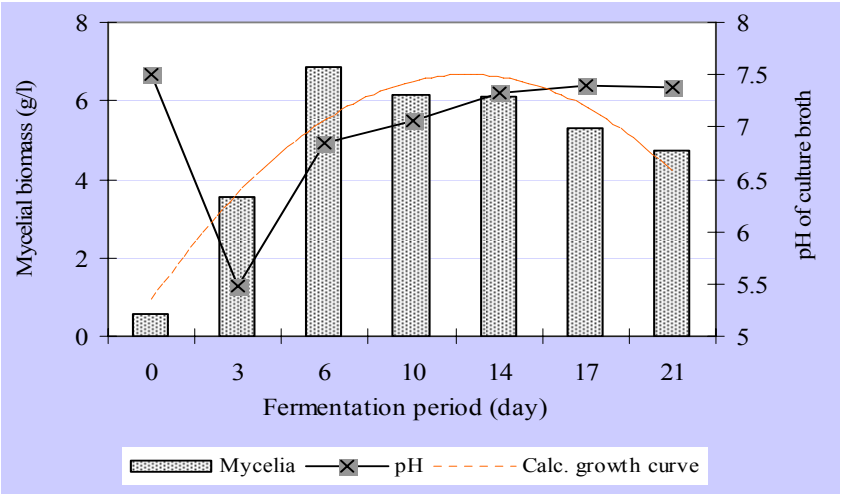


Fig. 3.7. Fungal growth and pH alteration during cultivation of KT19

Fig. 3.8 illustrates that the isolate KT19 produced metabolites highly after 6 days of cultivation as well as its strongest antibacterial activity. In period of 10 to 17 days the extract yield and the bioactivity against *S. aureus* were relatively stable. Therefore, for further cultivation the fungus was fermented for one week.

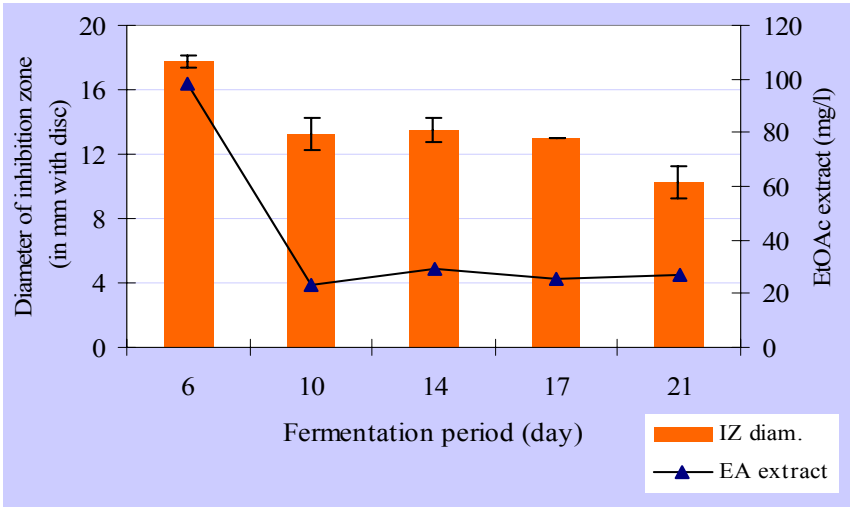


Fig. 3.8. EtOAc extract yield of KT19 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.2. Marine Algicolous Fungi

3.2.2.1. *Mycelium sterilium* KT29

Stationary growth phase of the fungus KT29 was relatively long, namely more than two weeks and after 25 days of cultivation the fungus initiated the death phase (Fig. 3.9). Starting on day 10 to 25 cultivation the pH value was nearly constant with values from 7 to 7.5. Fig. 3.10 demonstrates that the fungus produced bioactive metabolites also relatively in long period. The extract yielded after 21 days of cultivation showed the strongest bactericide effect against *S. aureus* with an IZ diameter of 12 mm. Therefore, the fungus was further fermented for three weeks.

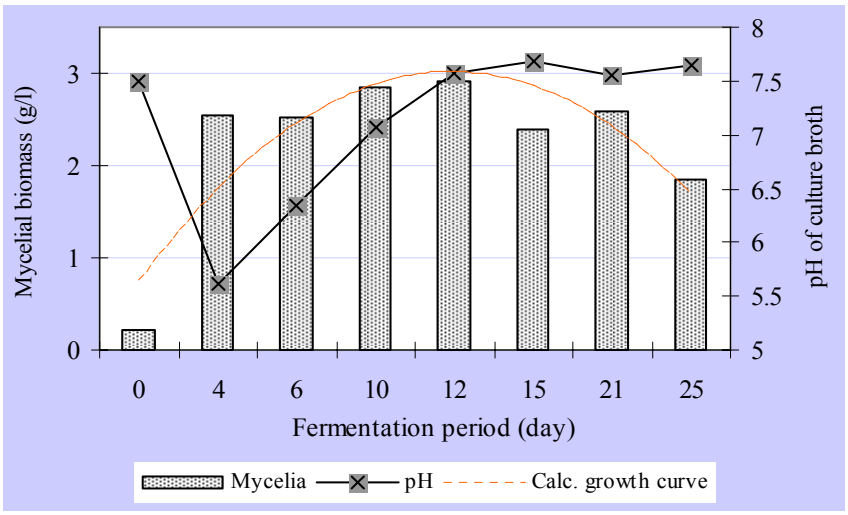


Fig. 3.9. Fungal growth and pH alteration during cultivation of KT29

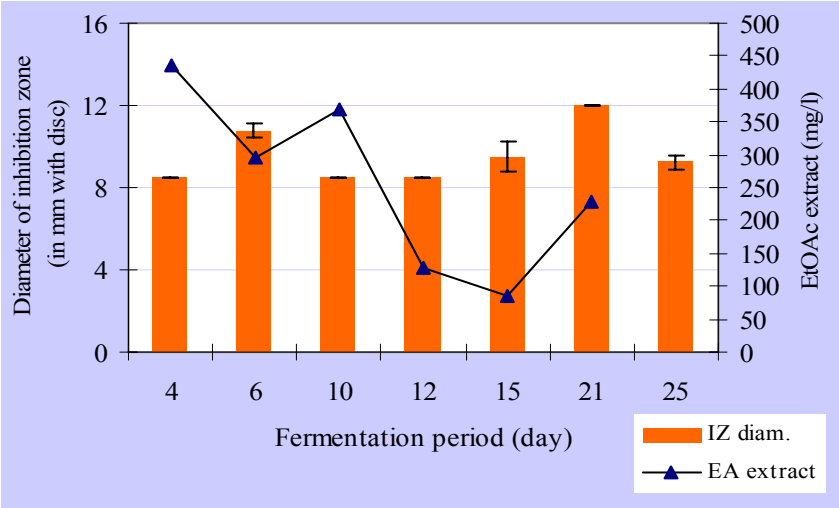


Fig. 3.10. EtOAc extract yield of KT29 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.2.2. *Xylaria psidii* KT30

The fungus *Xylaria psidii* KT30 grew rapidly in liquid medium. As shown in Fig. 3.11, its exponential growth phase happened for 3 days of cultivation and then decreased until 7 days of cultivation. After 3 weeks of cultivation the fungal growth was constantly in stationary phase. However, the mycelial biomass was only in the amount of 3.55±0.2 g/l. The pH value during 3 weeks of cultivation was relatively steady in the range of 6.6 to 7.3. Unlike the other fungi, the lag growth phase which is normally identical with low pH value in the present study has occurred earlier in the KT30. This condition can be seen from the growth curve of the fungus.

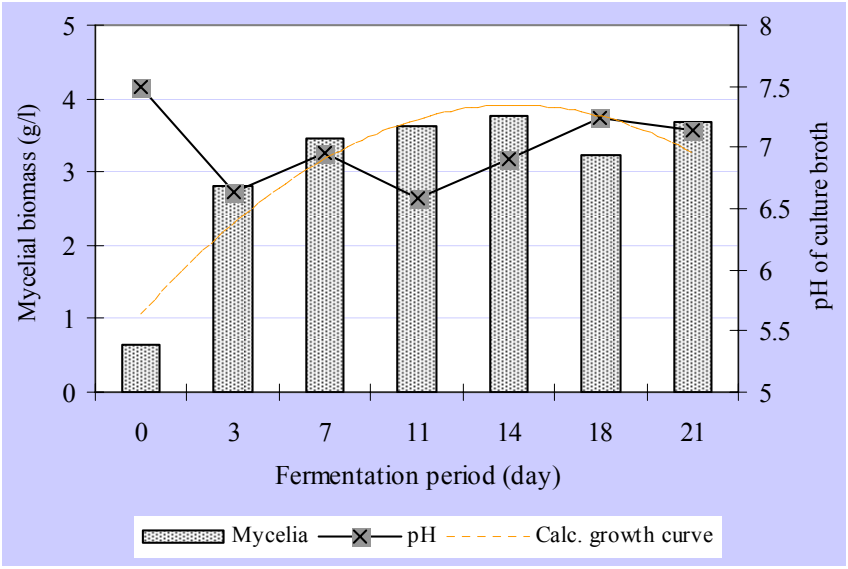


Fig. 3.11. Fungal growth and pH alteration during cultivation of *Xylaria psidii* KT30

Fig. 3.12 presents the antibacterial activity of EtOAc extract of *X. psidii* KT30 against *S. aureus*. The extract yielded from 3 days of cultivation showed the most active effect with IZ diameter of 12 mm. On the other hand, the extract yield was higher after 11 days of cultivation and decreased again afterwards. However, the bioactivity of the extract after 18 to 21 days of cultivation was higher than those from the extract from 11-day old culture. Therefore, the fungus was subsequently cultivated for about 18 days.

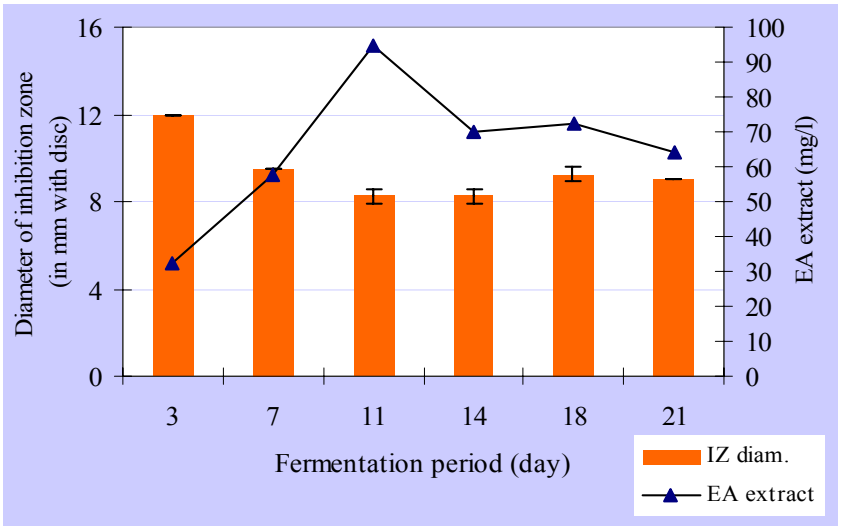


Fig. 3.12. EtOAc extract yield of *Xylaria psidii* KT30 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.2.3. *Mycelium sterilium* KT31

Strain KT31 grew exponentially within 6 days of cultivation and was in stationary phase for about 11 days afterwards. Surprisingly, on day 3 the fungal culture became red and this phenomenon occurred until the two following days. Starting from day 6 to 21 days of cultivation colour of the fungal culture was light brown. This result shows that the isolate KT31 produces red pigment in the liquid culture as strain KT30. Both isolates were isolated from the same specimen, red alga *Kappaphycus alvarezii*. Fig. 3.13 presents the fungi KT30 and KT31 cultured in liquid HA medium.

Fig. 3.13. Liquid cultures of 7-day old *X. psidii* KT30 and 3-day old KT31 in Hagem medium

Fig. 3.14 illustrates the correlation between fungal growth and its pH alteration during 21 days of cultivation. The neutral pH culture was reached after 10 days of cultivation and this value was subsequently stable over 21 days of cultivation.

As presented in Fig. 3.15, the isolate KT31 exhibited bactericide properties against various human pathogenic microbes. The yield extracted from day 6 culture broth showed the strongest activity against all of the test organisms. Highest activities were found against both *Staphylococcus aureus* strains, followed by *Staphylococcus epidermidis* and less activity against the pathogenic yeast *Candida albicans*. Interestingly, the antibacterial activity against *S. aureus* was relatively stable after 21 days of cultivation. However, the lowest yield of EtOAc extract was obtained after the three weeks of cultivation.

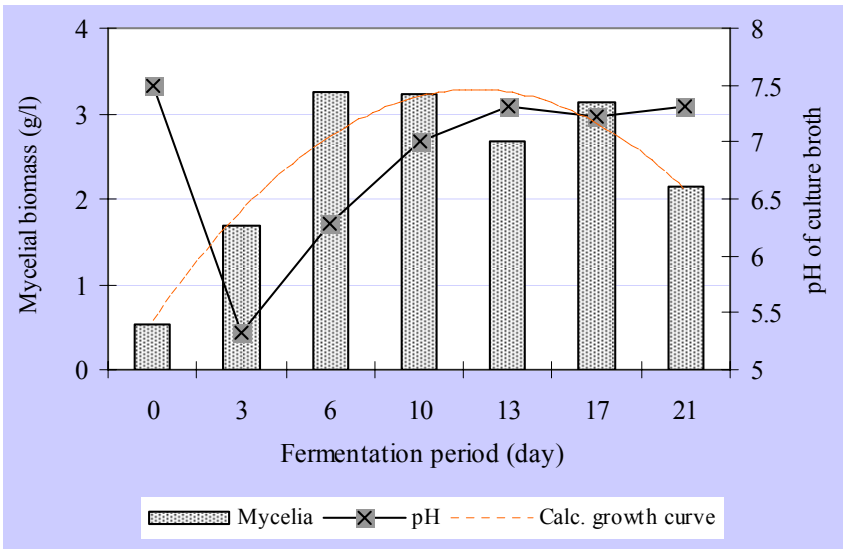


Fig. 3.14. Fungal growth and pH alteration during cultivation of KT31

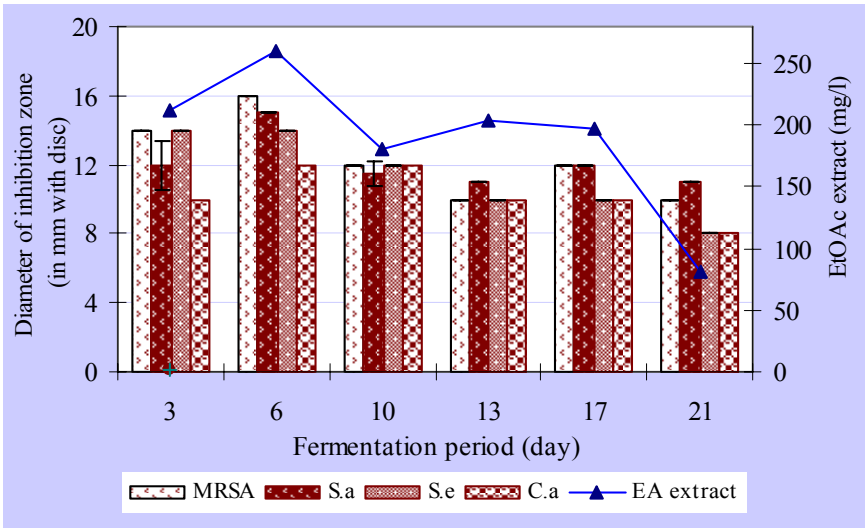


Fig. 3.15. EtOAc extract yield of KT31 in different culture periods and the bioactivity against two strains *S. aureus* (MRSA & S.a), *S. epidermidis* (S.e) and *C. albicans* (C.a) (1 mg/disc; n=2; Data are expressed as Mean±SD)

3.2.2.4. *Mycelium sterilium* KT32

The fungal growth of strain KT32 was in the stationary phase until 21 days of cultivation (Fig. 3.16). The highest mycelial biomass was obtained on day 10 with 3.51 g/l. Since the sixth day after cultivation, the pH value was about 7 to 7.5. The yield of EtOAc extract was also quite stable for 3 weeks of cultivation, as presented in Fig. 3.17. However, the antibacterial activity was decreased after 13 days of cultivation and unpredictably increased again on 21st day. Therefore, the fungus was subsequently cultivated for three weeks.

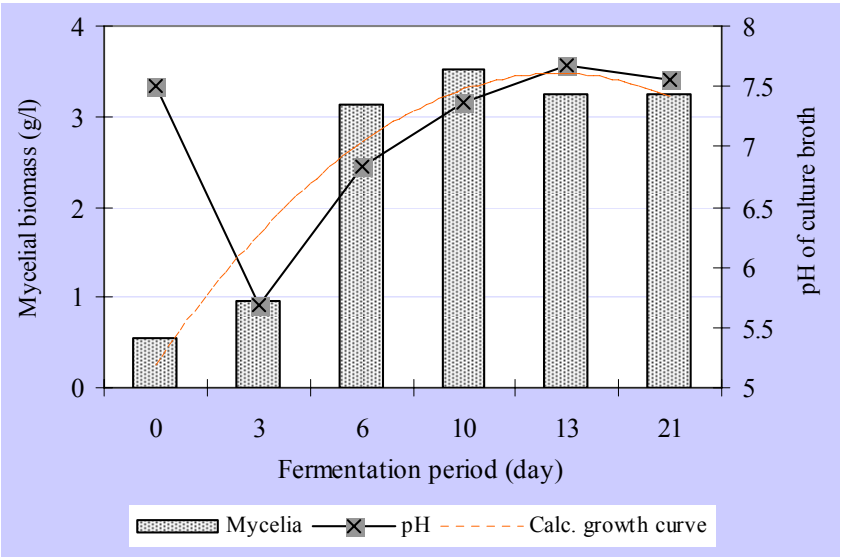


Fig. 3.16. Fungal growth and pH alteration during cultivation of KT32

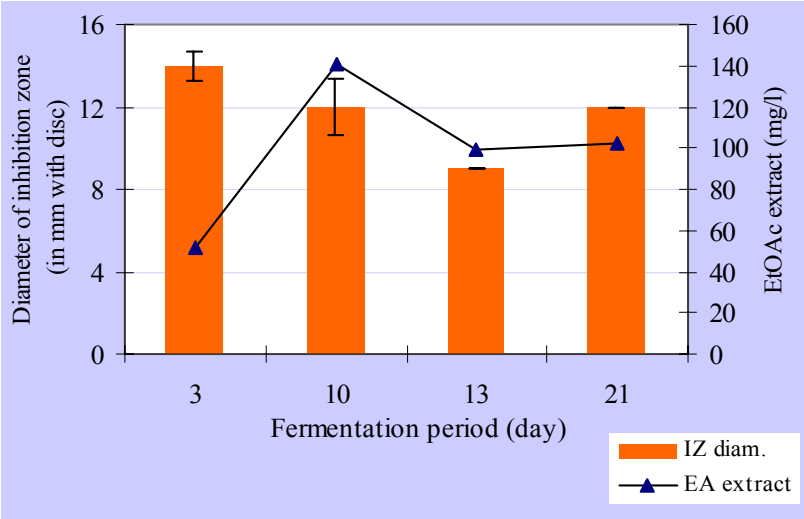


Fig. 3.17. EtOAc extract yield of KT32 in different culture periods and its bioactivity against *S. aureus* (2 mg/disc; n=2; Data are expressed as Mean±SD)

3.3. Biological Activity of Fungal Isolates

3.3.1. Antimicrobial Activity

Crude extracts isolated from culture broth and mycelia were tested against human- and fish pathogenic microbes. Ethyl acetate extracts isolated from the culture broth mostly exhibited potential activity against the test organisms, while ethanol extracts isolated from culture broth as well as dichloromethane-, methanol- and water extracts isolated from dried mycelia showed no activity except dichloromethane extracts of *Xylaria psidii* KT30 and strain KT31. Thus, further investigation was focused on ethyl acetate extracts.

Salinity is one of the environmental factors that affect fungal growth as well as production of secondary metabolites. In an attempt to select the best culture condition for bioactives production, the culture media used were modified either with or without marine salt. Further culture condition for each strain was then prepared according to these results.

3.3.1.1. Antibacterial Activity against Gram-positive Bacteria

Figs. 3.18-19 illustrate the antibacterial activity of EtOAc extracts of fungal culture broth against Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis.* Significantly to be seen in the figures, *Aspergillus* sp. KT13 was the most active fungus against both bacterial strains, with IZ in the range of 25 to 32 mm in average and followed by strains KT31, KT03, KT19, *Lasiodiplodia theobromae* KT26 and KT29 which were moderately active from both culture of each strain, freshwater and seawater cultures.

However, salt concentration influenced the activity of the fungal cultures. As can be seen in the figure, EtOAc extracts of KT19, *L. theobromae* KT26 and KT29 from seawater cultures showed more activity than those from the freshwater cultures and vice versa for the other strains. Significant effect of salinity was exhibited by the EtOAc extract of KT15. The extract which was isolated from seawater culture showed no activity against the test organisms.

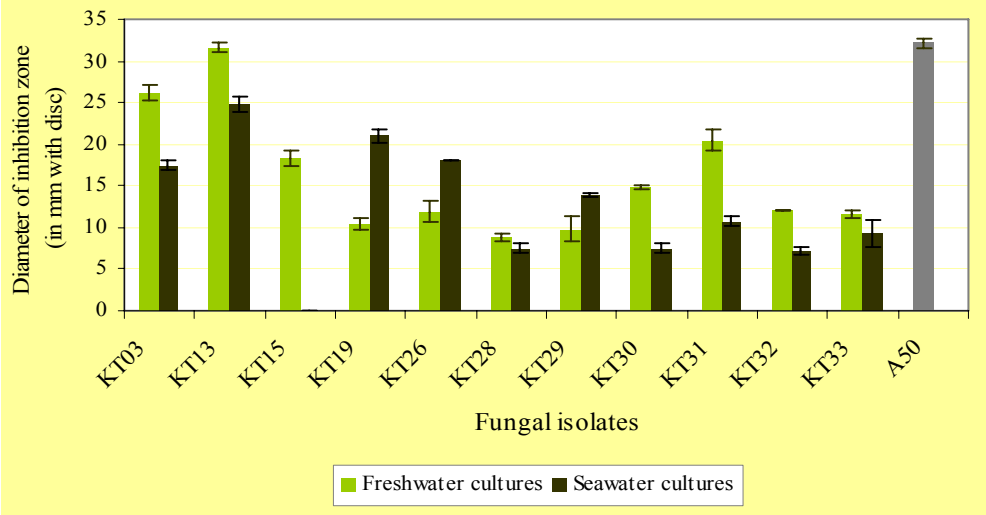


Fig. 3.18. Antibacterial activity of EtOAc extracts against *Staphylococcus aureus* (2 mg/disc; A50=Ampicillin 50 µg/disc; n=4; Data are expressed as Mean±SD)

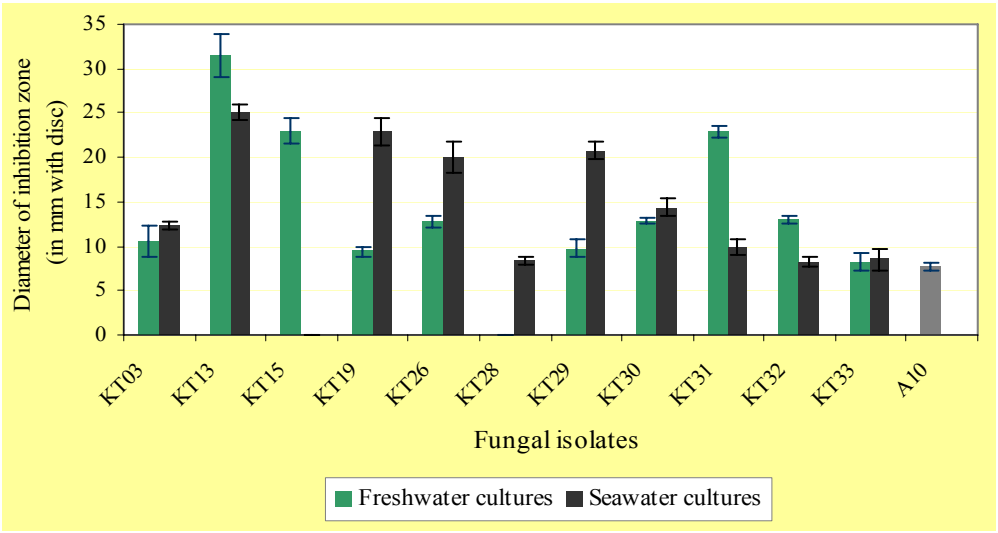


Fig. 3.19. Antibacterial activity of EtOAc extracts against *Bacillus subtilis* (2 mg/disc; A10=Ampicillin 10 µg/disc; n=4; Data are expressed as Mean±SD)

3.3.1.2. Antibacterial Activity against Gram-negative Bacteria

The bioactivity of the extracts against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* were not as high as against the Gram-positive bacteria. Two

strains, KT15 and *Coniothyrium* sp. KT33 showed no activity against the test organisms. As presented in Figs. 3.20-21, in general fungus KT19 possessed the most activity with IZ diameters in the range of 13-14.5 mm and 8-14 mm against *E. coli* and *P. aeruginosa*, respectively, followed by *Xylaria psidii* KT30, KT29 and KT03. The activity of KT19-extracts from freshwater and seawater cultures was similar. However, KT30 cultivated from freshwater medium was in fact the most active against *E. coli* and *P. aeruginosa* with IZ diameters of 22 and 13 mm, respectively. The strains KT03, KT19 and KT29 showed stronger activity when cultivated in seawater medium.

A fascinating result was also exhibited by *X. psidii* KT30 and fungus KT31. The EtOAc extracts of the fungi which were cultivated in seawater medium showed weak activity against *E. coli* with the corresponding IZ diameters of 9 and 11 mm, whereas the extracts of freshwater culture were strong active with IZ diameters of 22 and 18 mm, respectively. In addition, the extract of *X. psidii* KT30 from freshwater culture inhibited the growth of *P. aeruginosa* with an IZ of 13 mm while the extract of seawater culture showed no activity.

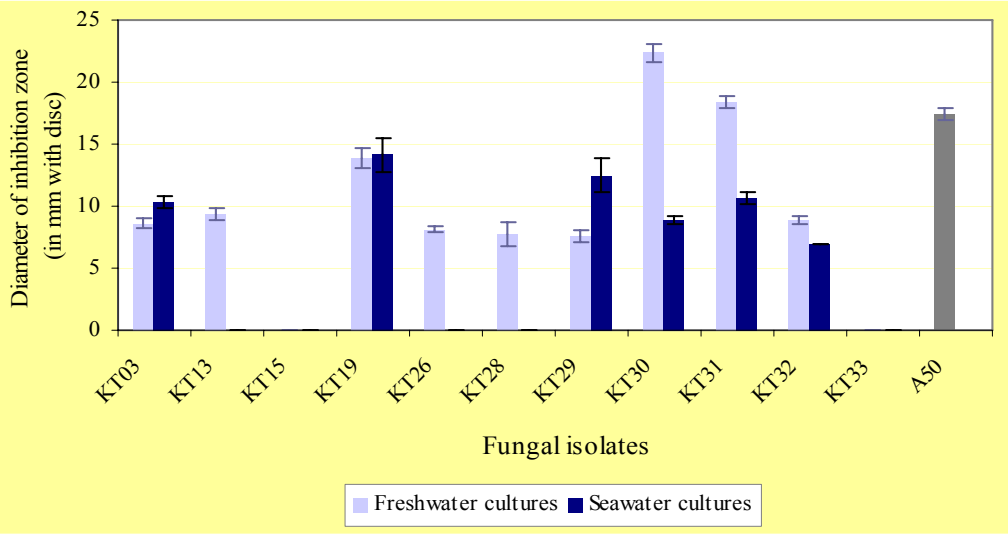


Fig. 3.20. Antibacterial activity of EtOAc extracts against *Escherichia coli* (2 mg/disc; A50=Ampicillin 50 µg/disc; n=4; Data are expressed as Mean±SD)

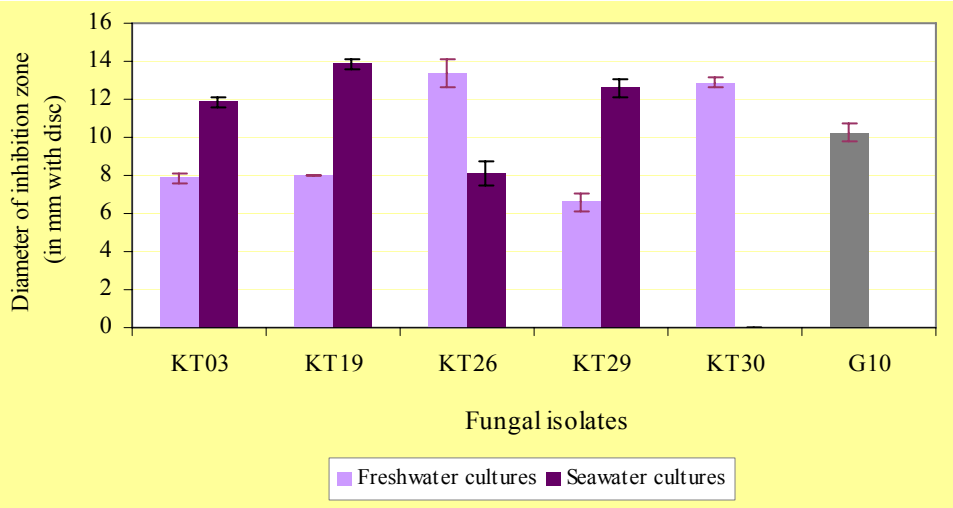


Fig. 3.21. Antibacterial activity of EtOAc extracts against *Pseudomonas aeruginosa* (2 mg/disc; G10=Gentamicin 10 µg/disc; n=4; Data are expressed as Mean±SD)

3.3.1.3. Antibacterial Activity against Fish Pathogenic Bacteria

The ethyl acetate extracts of culture broth as well as extracts of mycelial biomass were additionally tested against Gram-negative fish pathogenic bacteria *Vibrio anguillarum, Aeromonas salmonicida* and *Yersinia ruckeri.* These three bacteria were known as pathogen for marine and freshwater fish. The diseases caused by these microorganisms are commonly known as vibriosis, furunculosis and enteric redmouth disease, respectively.

Compared to the activity against Gram-negative human pathogenic bacteria, the related strains showed similar effects against fish pathogenic bacteria (see Figs. 3.20-21) especially against *V. anguillarum* and *A. salmonicida*. *Y. ruckeri* was the most resistant test organism (Figs. 3.22-24). As can be seen in Figs. 3.22-23, all of the ethyl acetate extracts isolated from freshwater fungal culture showed inhibition activity against the growth of *V. anguillarum* and *A. salmonicida*. Two strains KT15 and *Coniothyrium* sp. KT33 cultivated in seawater medium were inactive against the test organisms.

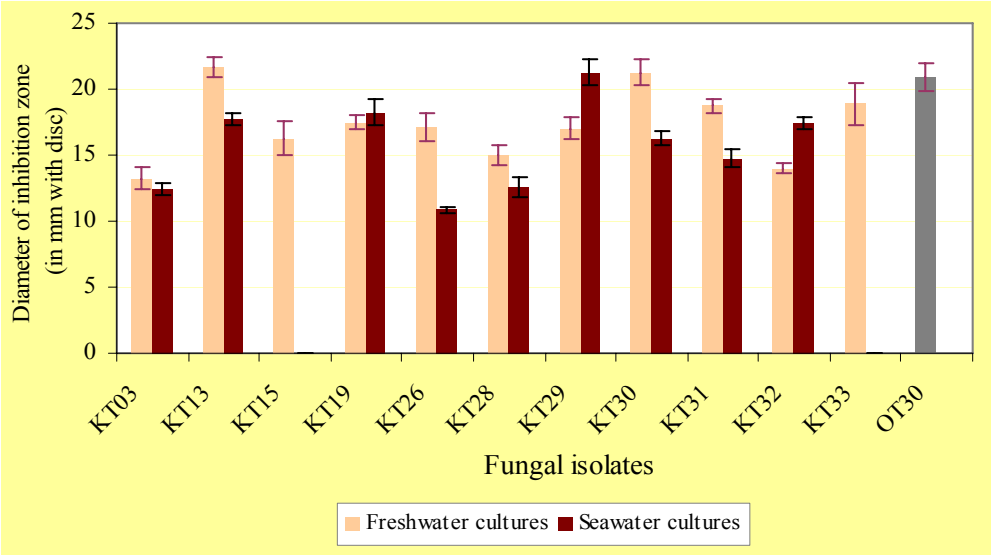


Fig. 3.22. Antibacterial activity of EtOAc extracts against *Vibrio anguillarum* (2 mg/disc; OT30=Oxytetracycline 30 µg/disc; n=4; Data are expressed as Mean±SD)

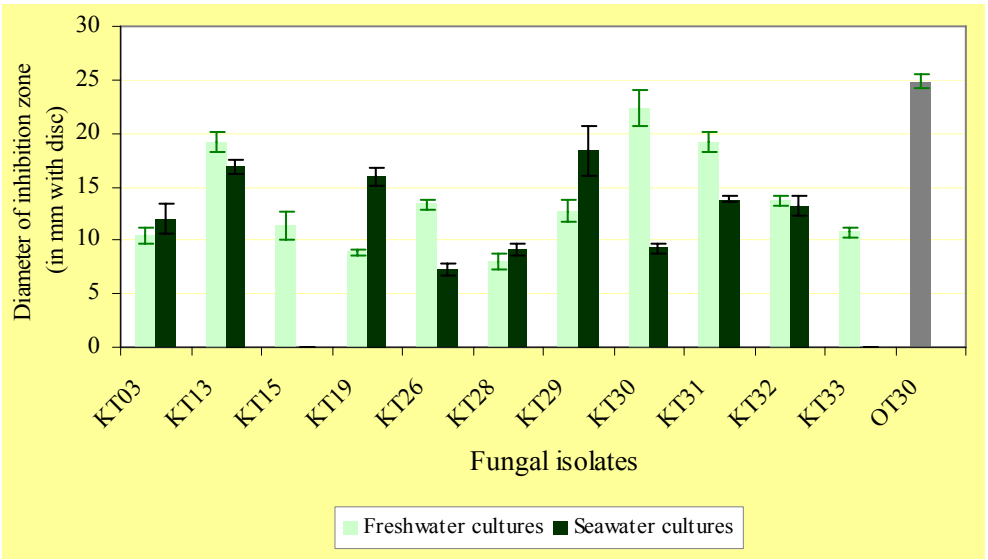


Fig. 3.23. Antibacterial activity of EtOAc extracts against *Aeromonas salmonicida* (2 mg/disc; OT30=Oxytetracycline 30 µg/disc; n=4; Data are expressed as Mean±SD)

Xylaria psidii KT30 exhibited the most active bactericide activities against most of the test organisms, followed by strain KT19. However, both fungal isolates showed different condition. *X. psidii* KT30 cultivated in freshwater medium produced more active compounds and vice versa for fungal strain KT19. Interestingly, both strains with the related mentioned condition showed similar effects on *Y. ruckeri.* Moreover, strains KT13, KT29 and KT31 presented similar activity.

In addition, Figs. 3.22-23 present that strains KT19 and KT29 showed strong antibacterial activity when cultivated in seawater medium. There were no significant effects

found for strains KT03 and KT32. Fig. 3.24 shows antibacterial activity of the ethyl acetate extracts against *Y. ruckeri.* Strains KT19 and *X. psidii* KT30 possessed strong bactericide effects against the test organism with IZ value of 25 mm. However, from two cultures with different salinity, only extracts of *X. psidii* KT30 showed antibacterial activity.

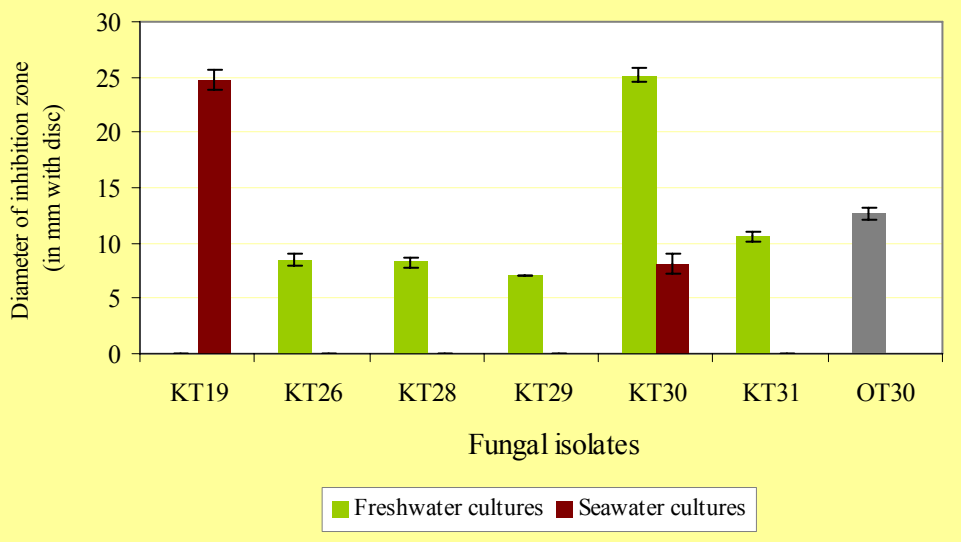


Fig. 3.24. Antibacterial activity of EtOAc extracts against *Yersinia ruckeri*
(2 mg/disc; OT30=Oxytetracycline 30 µg/disc; n=4; Data are expressed as Mean±SD)

3.3.1.4. Antifungal Activity against Yeast

Four strains exhibited antifungal properties against *Candida maltosa* (see Fig. 3.25). Surprisingly, DCM extracts of *Xylaria psidii* KT30 and KT31 showed antifungal activity, even not as strong as their EtOAc extracts. From Fig. 3.25 it can be seen that *X. psidii* KT30 was the most active against the test organism. It should be mentioned that both strains were originally isolated from the same sample red seaweed *Kappaphycus alvarezii.*

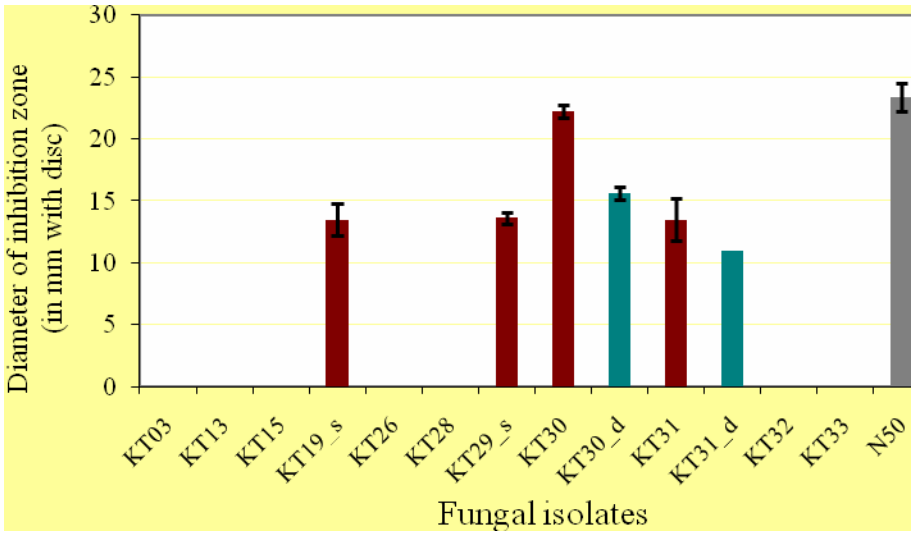


Fig. 3.25. Antifungal activity of EtOAc- and DCM extracts against *Candida maltosa*
(d: DCM extract; 2 mg/disc; N50=Nystatin 50 µg/disc; n=4;
Data are expressed as Mean±SD)

Strains KT19 and KT29 cultivated in seawater medium showed the same effect in producing antifungal compounds, as found for their activity against human and fish pathogenic bacteria (see parts **3.3.1.1 – 3).**

3.3.1.5. Fungicidal Activity against Phytopathogenic Fungus

Twenty one of EtOAc, DCM and MeOH extracts of the fungal culture broth were tested against plant pathogenic fungus *Cladosporium cucumerinum*. Most of EtOAc extracts showed fungicidal activity at 400 µg/spot. As positive control benomyl was used at amount of 80 ng/spot to afford inhibition area of 78.5 mm². Inhibition area of some fungal strains can be seen in Fig. 3.26. The figure expresses the larger of surface area illustrating the stronger activity. More detailed the surface area of inhibition zones of each active extract are listed in Table 3.3.

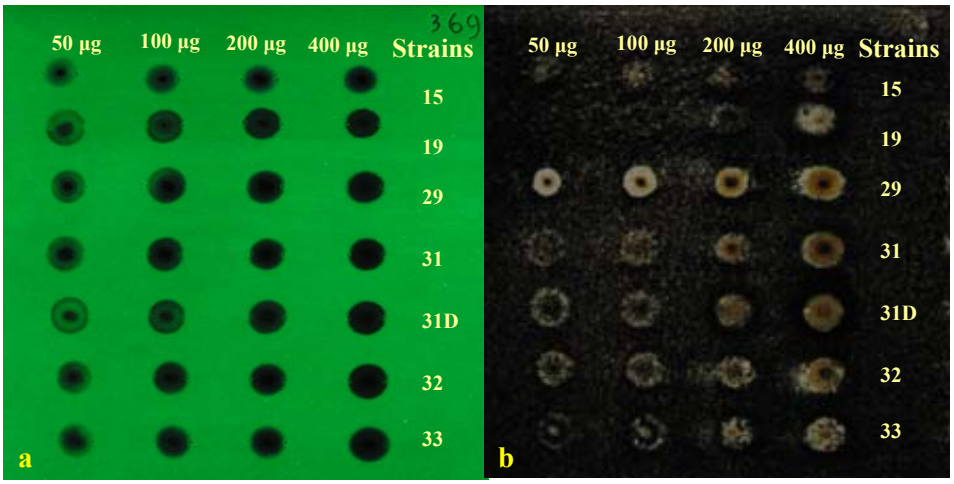


Fig. 3.26. Antifungal assay against *Cladosporium cucumerinum* on TLC plate, a. applied extracts on TLC plate before spraying with fungal spores (detected under 254 nm); b. after 2 days of incubation (inhibition zones showed antifungal activity)

Table 3.3. Inhibition area in mm² (non-growth of *Cladosporium cucumerinum* on silica plates) to indicate antifungal properties of EtOAc extracts of 11 fungal strains

Fungal Strain[a)]	Amount (µg/spot)			
	50	100	200	400
KT03	- [b)]	-	-	-
KT03s	-	-	-	-
KT13	-	-	-	-
KT13s	-	-	-	78.50
KT15	-	-	-	28.26
KT15s	-	-	-	-
KT19s	-	-	-	50.24
KT26	-	-	-	-
KT26s	-	-	-	-
KT29s	56.72	78.50	78.50	94.99
KT30	-	78.50 [c)]	19.63	78.50
KT31	-	-	28.26	94.99
KT32	-	-	78.50 [c)]	78.50
KT33	-	-	78.50 [c)]	78.50

Note: a) s : cultivated in seawater medium
b) no inhibition zone
c) no clear inhibition (fungistatic activity)

As presented in Table 3.3, ethyl acetate extract of fungus KT29 was the most active against the test organism. The extract inhibited the fungal growth up to the lowest amount used in this test, i.e. 50 µg/spot.

3.3.2. Antagonistic Study of Some Fungal Strains

The investigation of antifungal activity showed that isolates *Xylaria psidii* KT30 and KT31 possess considerable antifungal properties. Moreover, both strains were isolated from the same algal specimen. Therefore, dual cultures of both strains were carried out to observe the interaction between the two strains on agar plate. Fig. 3.27 shows the dual culture of *X. psidii* KT30 and KT31 on solid HA medium.

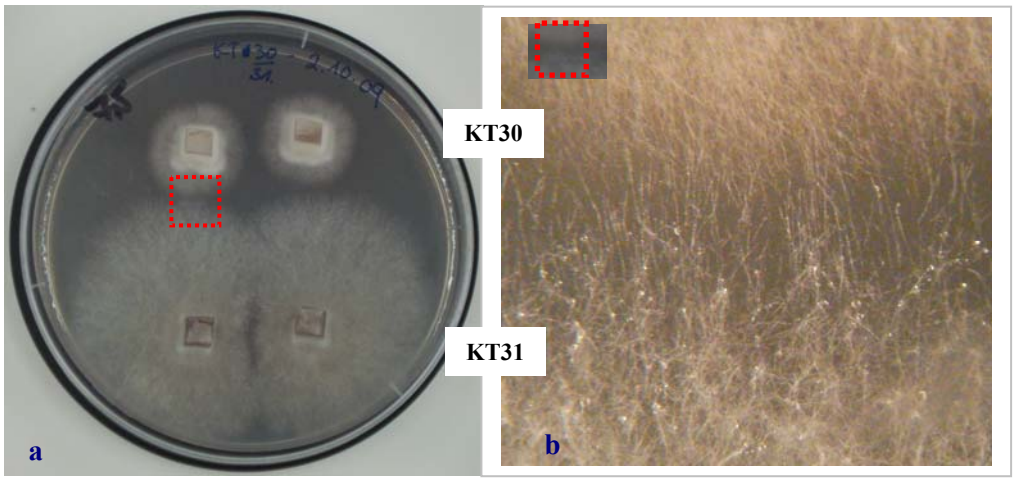


Fig. 3.27. a: Dual culture of 6-day old KT30 and KT31 on solid HA medium
b: Mycelial micrograph of the dual culture (magnification from the red box; 80-fold)

As presented in Fig. 3.27a, strain KT31 grows more rapidly than *X. psidii* KT30. Fig. 3.27b shows that there was no inhibition zone between the two cultures. However, as can be seen in Fig. 3.27b strain KT31 produced exudates (as water drops) which were excreted from the mycelia growing near to the mycelia of *X. psidii* KT30.

After 27 days of cultivation, the plate culture was full of mycelia. No inhibition zone was observed between the two strains, except the unusual performance of mycelia in the border of both cultures (Fig. 3.28a). Fig. 3.28b shows reverse plate of the dual culture which presents the difference of mycelial growth of KT30 in front of the mycelia of KT31. This observation showed that strain KT31 grew more expansive than *X. psidii* KT30.

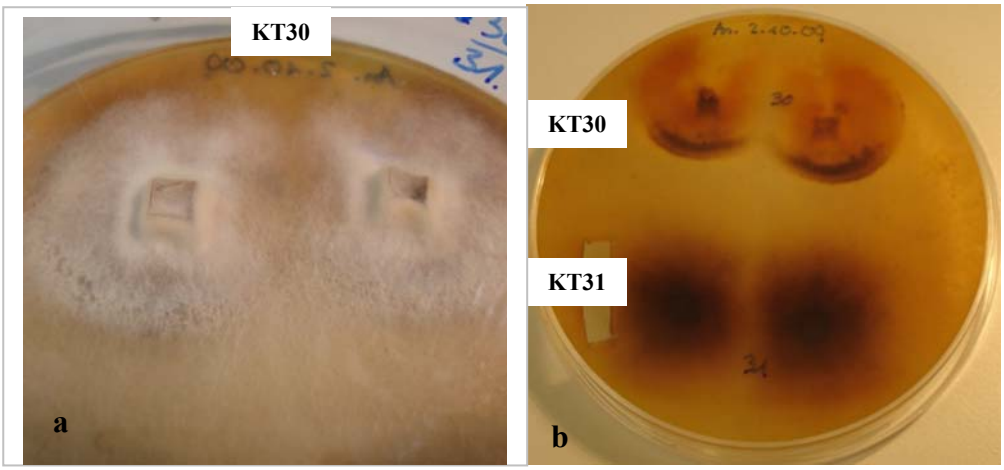


Fig. 3.28. a: 27-day old *X. psidii* KT30, b: Reverse dual culture of KT30 and KT31

Based on the result of dual culture of *X. psidii* KT30 and KT31, the fungus KT31 was then cultured together with two other fungal strains from our culture collection, KT19 and KT27. Both strains possessed strong antimicrobial properties. Fig. 3.29 exhibits the dual cultures of KT31 and strain KT19. The pictures show that no inhibition zone was observed.

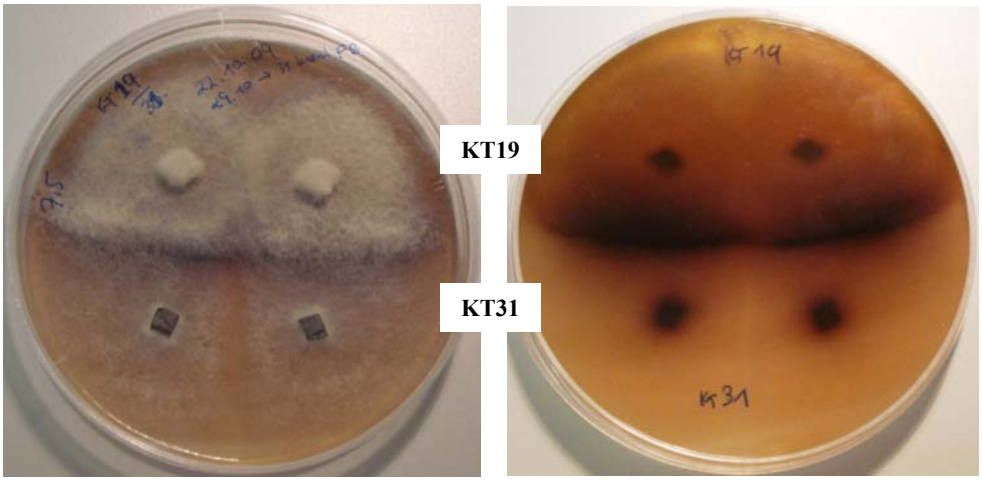


Fig. 3.29. Dual cultures of KT19 and KT31; 7-day old cultured on Hagem plate

In competition with strain KT27, the fungus KT31 also showed a strong survivability (Fig. 3.30). As can be seen in Fig. 3.30b, fungus KT27 produced chlamydospores and initiated to form fruiting bodies. In contrast, single culture of KT27 on the same medium grew well and no chlamydospores appeared on the same old culture. From this phenomenon can be concluded that KT27 produced chlamydospores as the response of the fungus to the strain KT31.

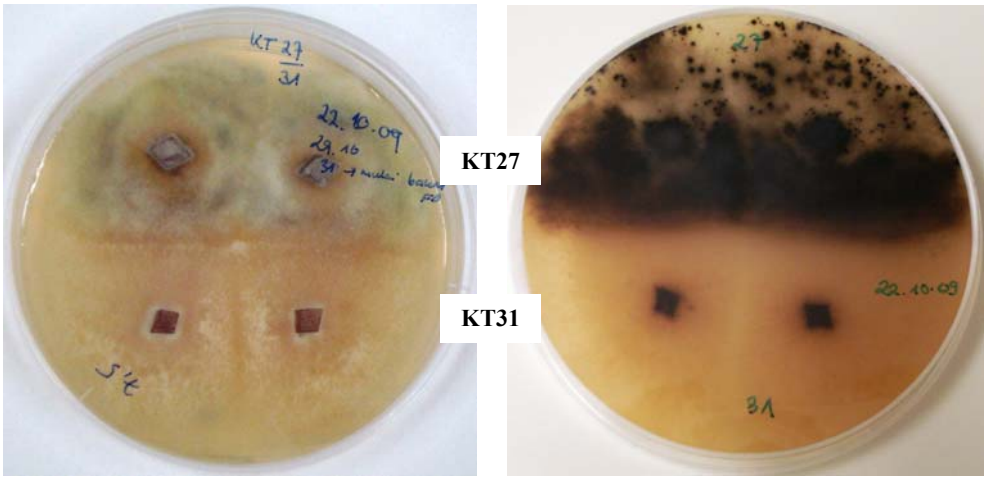


Fig. 3.30. Dual cultures of KT27 and KT31 on Hagem plate
(a: 7-day old culture, b: 39-day old culture)

3.3.3. Free Radical Scavenging Activity

Antioxidative properties of all samples was assessed using α,α-diphenyl-β-picrylhydrazyl (DPPH) for measuring radical scavenging activity. Five of 55 extracts were qualitatively active (some samples presented in Fig. 3.31). However, the most active extract, namely ethyl acetate extract of *Lasiodiplodia theobromae* KT26 showed only 39.7% DPPH radical scavenging effects at concentration of 1 mg/ml. According to this result, therefore, no further quantitative antioxidant test was carried out.

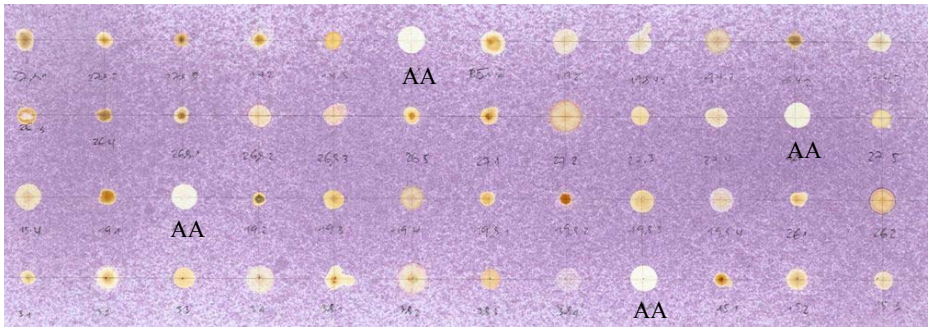


Fig. 3.31. DPPH radical scavenging test on TLC plate
(AA=Ascorbic acid as positive control)

3.3.4. Cytotoxic Activity

Ethyl acetate extracts of isolates *Xylaria psidii* KT30 and KT31 were found to be strong active in cytotoxic assay. Both strains exhibited IC_{50} values of 4 µg/ml and 1.5 µg/ml, respectively. In contrast, extracts yielded from seawater culture showed less active effects, i.e. 15 µg/ml and 14 µg/ml, correspondingly.

Interestingly, DCM extracts of both strains also possessed cytotoxic activity with the respective IC_{50} values of 8 and 5.5 µg/ml. These results show that DCM extracts of fungal mycelia cultured in freshwater media were more active compared to the ethyl acetate extracts of fungal broth from seawater cultures. Detail results are listed in Table 3.4.

Table 3.4. Cytotoxicity of EtOAc- and DCM extracts against 5637 cell line

Fungal strain	IC_{50} (µg/ml)		
	EtOAc extract (Freshwater)	EtOAc extract (Seawater)	DCM extract (Freshwater)
KT03	-[1)]	-	-
KT13	50	-	-
KT15	-	-	-
KT19	-	50	-
KT26	-	-	-
KT28	48	56	-
KT29	-	-	-
KT30	4	15	8
KT31	1.5	14	5.5
KT32	18	50	53.6
KT33	-	60	nt[2)]
Etoposide	0.35		

[1)] inactive
[2)] not tested

Table 3.4 also illustrates that the unidentified algicolous KT32 cultivated in seawater medium showed weak activity with an IC_{50} value of 50 µg/ml, while that from freshwater culture possessed medium activity with an IC_{50} value of 18 µg/ml. The fungal strains *Aspergillus* sp. KT13, KT19, *Epicoccum nigrum* KT28 and *Coniothyrium* sp. KT33 showed weak activity and the rest strains were inactive.

Ethyl acetate extracts of fungus KT31 collected from different culture periods were also tested for cytotoxic activity. The results showed that the most active extract was that isolated from 3 days of cultivation (3d) with an IC_{50} value of 4.27 µg/ml, followed by 10d with an IC_{50} value of 4.53 µg/ml. After 13 days of cultivation, the extract showed the lowest activity with an IC_{50} value of 11.75 µg/ml. Surprisingly, after 17 days of cultivation cytotoxicity of the extract increased again with an IC_{50} value of 5.95 µg/ml (Table 3.5).

Table 3.5. Cytotoxicity of EtOAc extracts of KT31 from different culture periods against 5637 cell line

Fermentation period (day)	IC_{50} (μg/ml)
3	4.27
6	5.42
10	4.53
13	11.75
17	5.95
21	8.23
Etoposide	0.35

3.4. Pigment Formation in *Xylaria psidii* KT30

Fungus *Xylaria psidii* KT30 was also observed for the pigment production in different culture media. The experiment was carried out according to the result of sporulation attempt of the fungus KT30. In several media formerly used for the sporulation investigation, the fungus produced red pigment in different intensities. The red pigment from the extract was detected at 518 nm.

The study found that the most favourable medium for the fungus to produce red pigment was solid malt-extract medium (SW) modified from Wright *et al.* (2003). From 3 culture plates of the fungus, pigment-containing methanol and water extracts in corresponding amounts of 1.90 and 0.62 g could be obtained.

However, the extracts which were methanol and water extracts showed no activity on antimicrobial assays, thus, no further investigation was undertaken. The plate cultures of *X. psidii* KT30 in different media are presented in Figs. 3.32-35.

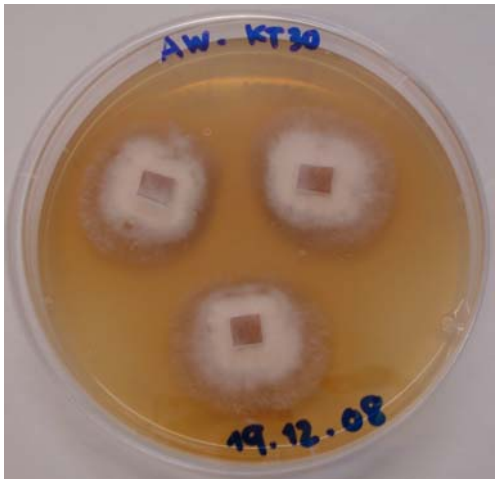


Fig. 3.32. 17-day old *X. psidii* KT30 cultured on yeast-extract medium (AW)

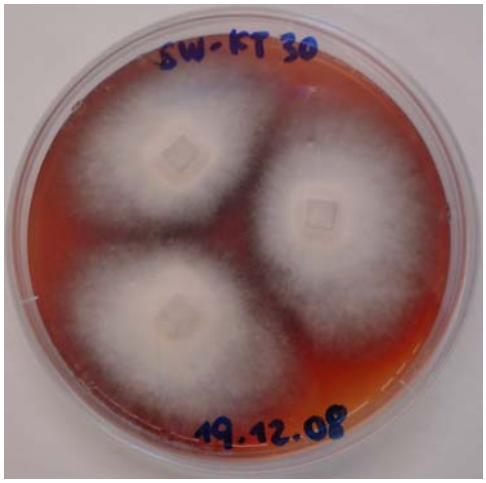


Fig. 3.33. 17-day old *X. psidii* KT30 cultured on malt-extract medium (SW)

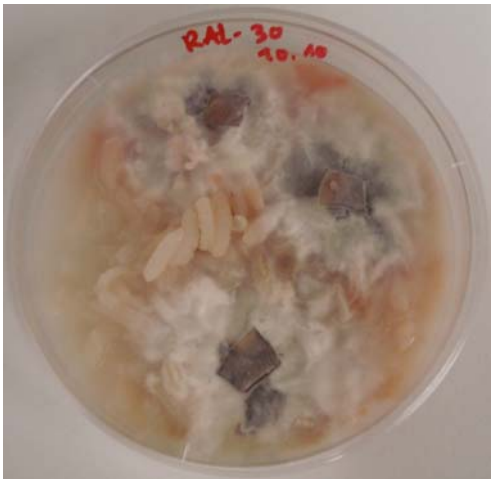


Fig. 3.34. 17-day old *X. psidii* KT30 cultured on rice agar medium

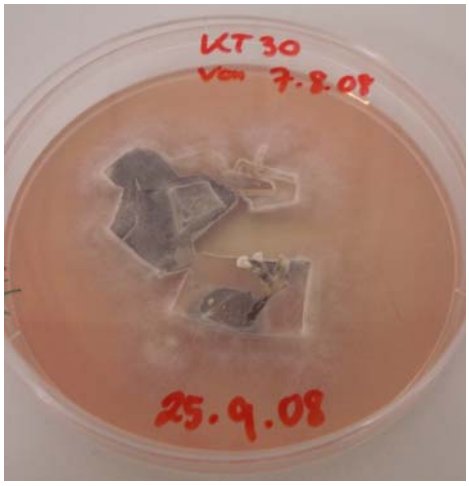


Fig. 3.35. 11-day old *X. psidii* KT30 cultured on Hagem medium

3.5. Production of Bioactive Metabolites in Strain KT31

3.5.1. Biosynthesis of Bioactive Compounds of KT31

Production of bioactive secondary metabolites is commonly diverse in microorganisms. Therefore, in the present study investigation of the correlation between growth stage and production of bioactive compounds was carried out to determine the optimum growth stage of the fungi, as described in part **3.2.** The optimum growth stage where organisms showed the optimal production of bioactive metabolites was chosen according to this study.

Investigation of the ethyl acetate extracts of KT31 which were harvested at different growth phases exhibited the dynamic of secondary metabolites production as presented in Fig. 3.36. In part **3.2.2.3** was illustrated that extract obtained after 6 days of cultivation (d6) showed the strongest antimicrobial activities.

Five bands with R_f values of 0.90, 0.76, 0.64, 0.56 and 0.26 were found to fluctuate according to the culture period, while the three others (R_f values of 0.40, 0.32 and 0.20) were present in all periods. However, results of the bioautographic assays of extracts d3-d13 showed that only two bands were active, i.e. at R_f 0.76 and 0.20 (see Figs. 3.36-37), while three and four bands were found to be active from extracts of d17 and d21, respectively (Fig. 3.38).

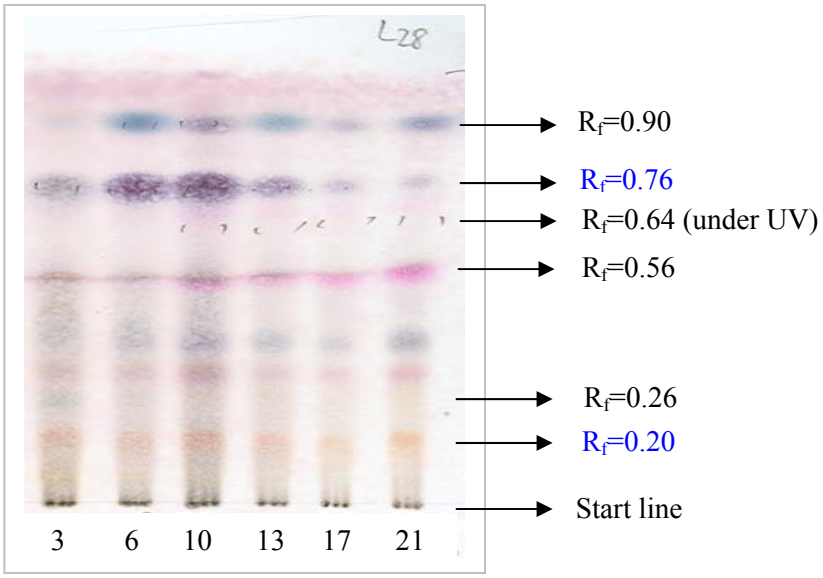


Fig. 3.36. TLC profiles of KT31-EtOAc extracts from different culture periods (3, 6, 10, 13, 17 and 21 days of cultivation; spraying with AS reagent)

Figs. 3.37-38 show interesting results of bioautographic assay of related extracts against *Vibrio anguillarum*. From the pictures can be seen that all extracts contain two main active components with R_f values of 0.20 and 0.76. As a complex mixture, extract of d6 was the most active against the test organisms (see part **3.2.2.3**). However, the results show that the single fraction of the active components of d10 and d13 exhibited higher activity.

Bioautography against *Bacillus subtilis* presented similar result. The activity of d13 was not as high as of d10 against *V. anguillarum.* Interestingly, extracts of d17 and d21 showed lower activity but in fact they produced more diverse active metabolites as can be seen in Fig. 3.39. For more detail, Fig. 3.40 presents the HPLC traces of ethyl acetate extracts of KT31 from different culture periods.

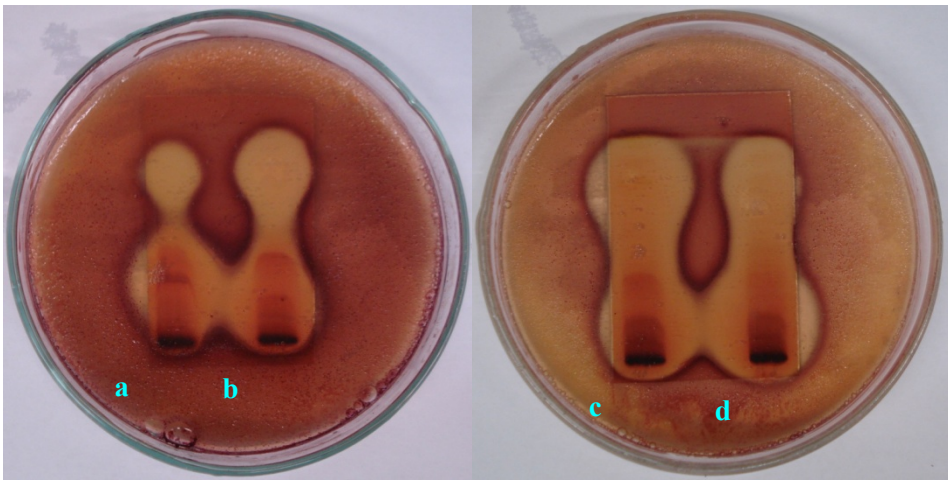


Fig. 3.37. Bioautograph of EtOAc extracts of KT31 from different culture periods against *V. anguillarum* (a: 3 days, b: 6 days, c: 10 days, d: 13 days of cultivation; 0.4 mg/line)

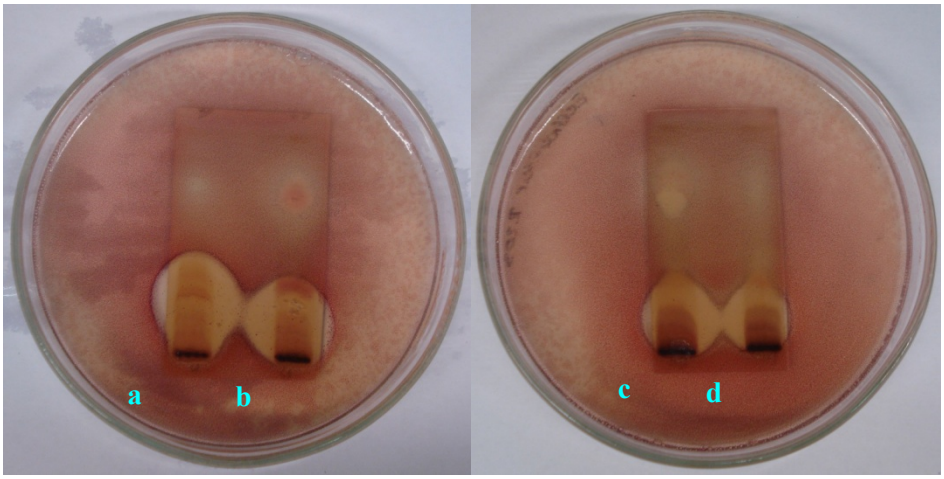


Fig. 3.38. Bioautograph of EtOAc extracts of KT31 from different culture periods against *B. subtilis* (a: 3 days, b: 6 days, c: 10 days, d: 13 days of cultivation; 0.4 mg/line)

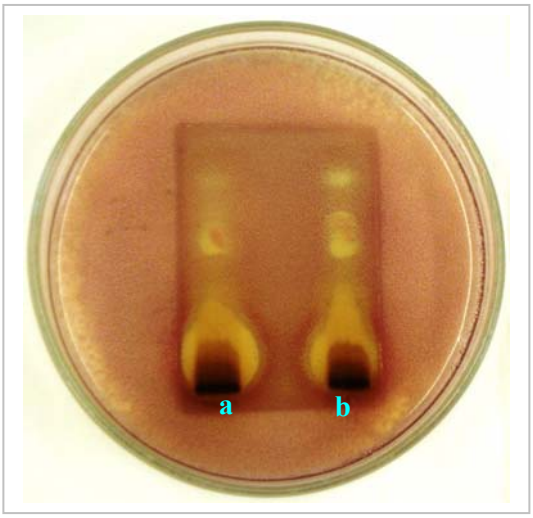


Fig. 3.39. Bioautograph of EtOAc extracts of KT31 from different culture periods against *B. subtilis* (a: 17 days, b: 21 days of cultivation; 0.4 mg/line)

Fig. 3.40 shows the HPLC analyses of the ethyl acetate extracts from different culture periods. From the HPLC traces 10 different peaks can be detected easily. Five of the peaks with t_R at 8, 11, 26, 31 and 40 min are obviously diverse according to the culture period. Three peaks at t_R 26, 31 and 40 min are prominent. However, the peak with t_R 31 min was significantly decreased after cultivation for 13 days and reversely with the peak at t_R 41 min.

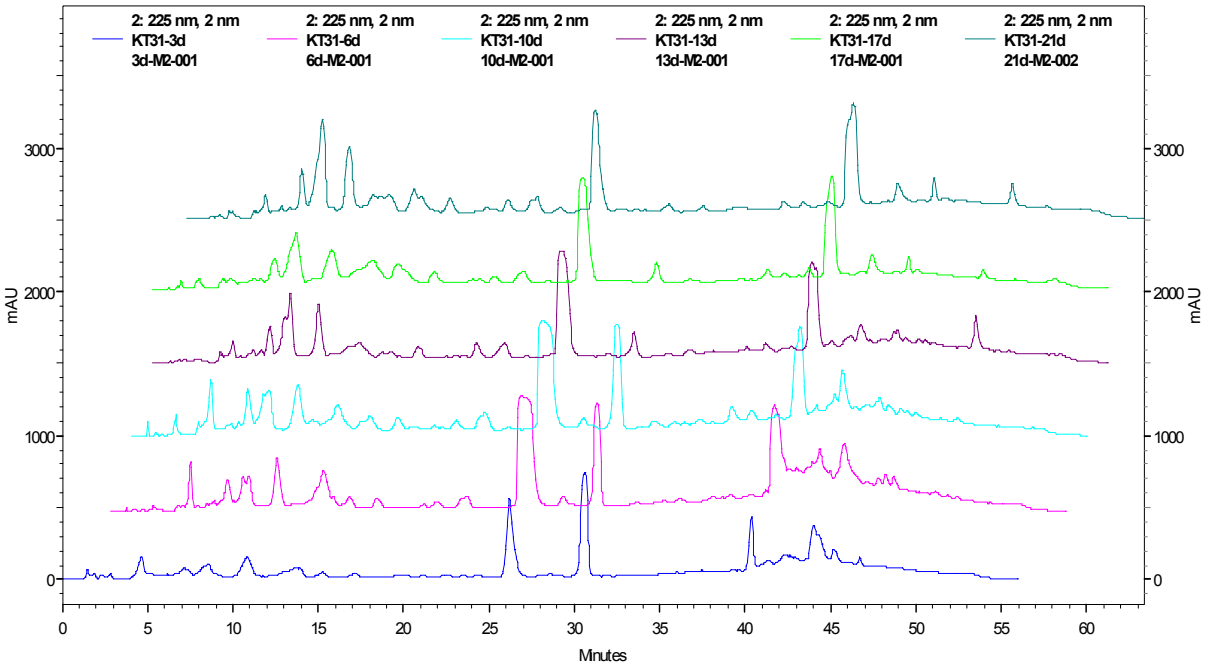


Fig. 3.40. HPLC traces of KT31-EtOAc extracts from different culture periods (3, 6, 10, 13, 17 and 21 days of cultivation; solvent gradient MeOH/H_2O)

3.5.2. Induction of Metabolites Production by Salinity

Extreme environmental conditions are supposed to play a key role in the production of secondary metabolites in living organisms. In order to survive under such condition they produce secondary metabolites either as biological weapon or for adaptation purposes. In the present study we used salinity as the designed extreme condition. Two different concentrations, 10% and 20% salinity were used to induce production of secondary metabolites particularly in KT31.

As presented in Fig. 3.41, extract of the fungal broth cultivated in freshwater medium showed strong activity against both Gram-positive bacteria, while extracts of the stimulated cultures were less active. Surprisingly, by comparing with the fungal growth in freshwater medium, the fungus grew two times more rapidly when cultivated in 10%

salinity. However, 20% salinity was too extreme for the fungus so that it could not grow well.

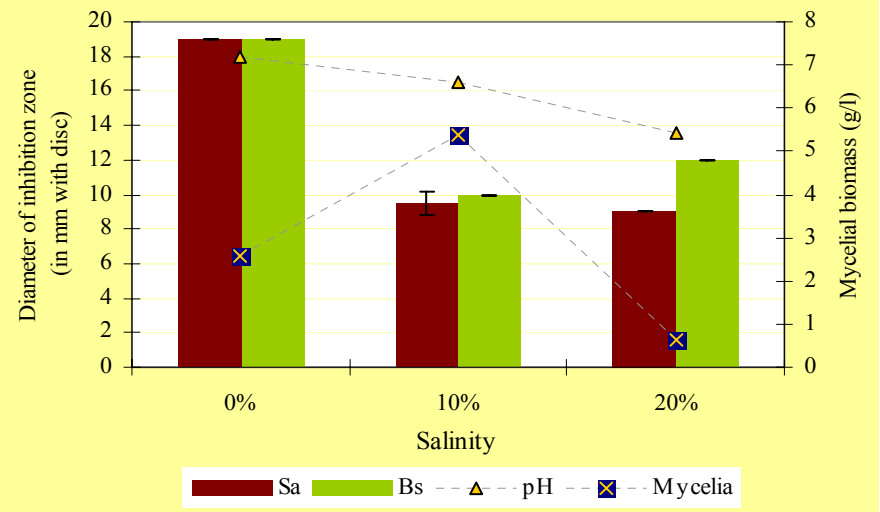


Fig. 3.41. Antibacterial activity of EtOAc extract from different culture salinity of KT31 against Gram-positive bacteria (21 days of cultivation; Sa: *S. aureus*, Bs: *B. subtilis*; 2 mg/disc; n=2, Data are expressed as Mean±SD)

Fig. 3.41 also presents that pH values of the cultures were affected by the salt concentration. The pH of fungus cultured in freshwater medium was 7.2, while the pH values in 10% and 20% salinity were 6.58 and 5.41, respectively.

In Fig. 3.42 HPLC traces of the ethyl acetate extracts isolated from culture broths with various salt concentrations can be seen. Five peaks at t_R 3, 7, 28, 31 27, 39 and 40 appear differently from the three cultures. The peak at t_R 3 min appears dominantly in the culture with 10% salinity, while the culture with 20% is dominated by the peak at t_R 39 min.

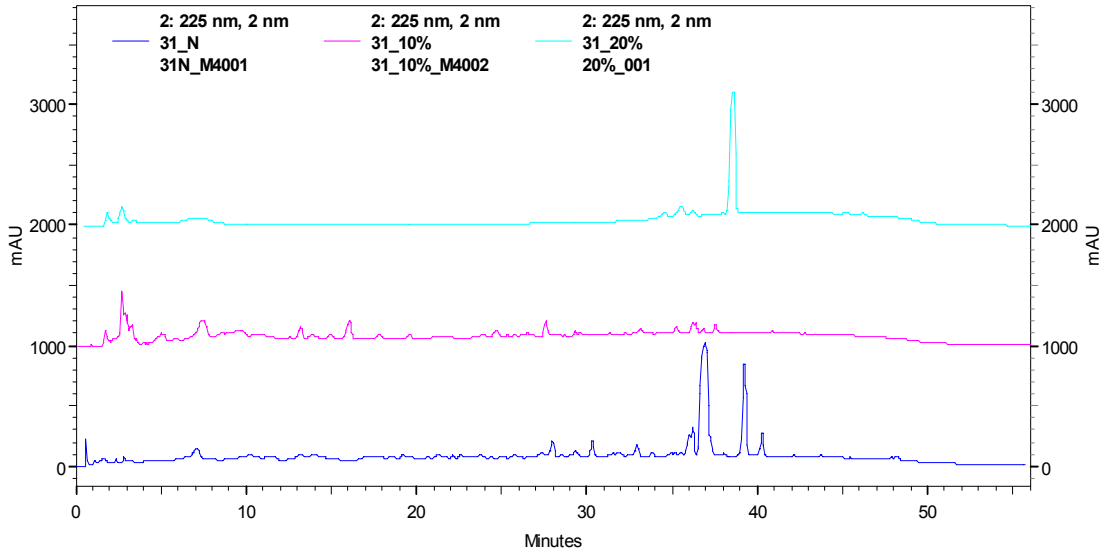


Fig. 3.42. HPLC traces of EtOAc extracts of KT31 cultivated with high salinity media (21 days of cultivation; N: freshwater medium, 10%: medium with 10% salinity, 20%: medium with 20% salinity; solvent gradient MeOH/H_2O)

3.5.3. Co-culture with Autoclaved *Staphylococcus aureus*

Fig. 3.43 presents the bioactivity of ethyl acetate extracts obtained from the fungal culture broth of KT31 which was co-cultivated with the autoclaved *Staphylococcus aureus* (5%, v/v). Two different treatments were applied, i.e. (1) the autoclaved *S. aureus* was added since day 0 cultivation (later written as d0), (2) the autoclaved *S. aureus* was added since day 3 cultivation (written as d3), and the single culture was used as a control (N). As shown in the figure, the antibacterial activity of the fungal extracts from mixed culture was increased. Interestingly, d0 showed higher activity with bacterial growth inhibition of 25 mm than d3 (IZ 21.5 mm) against *Bacillus subtilis*. However, no considerable difference was observed from d0 and d3 in their activities against *S. aureus* which showed inhibition zones of 23 mm and 22.5 mm, respectively.

In cytotoxic assays against carcinoma cell line 5637, the EtOAc extract of d0 also showed the highest activity than the other two cultures with an IC_{50} value of 1.88 µg/ml. Extracts of d3 and control showed cytotoxic activity with IC_{50} values of 4.34 and 3.19 µg/ml, respectively. This result confirmed that mixed culture of fungus KT31 with autoclaved *S. aureus* can be used to enhance the production of bioactive metabolites.

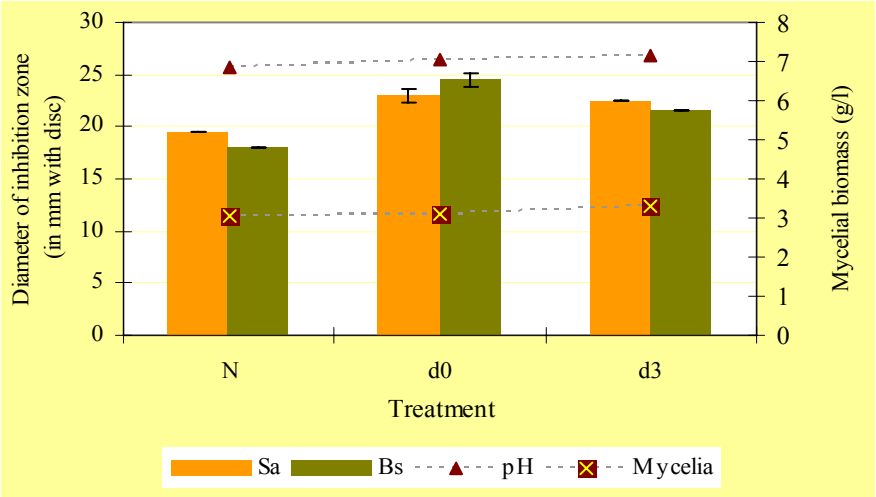


Fig. 3.43. Antibacterial activity of EtOAc extract from mixed culture of KT31 with autoclaved *S. aureus* against Gram-positive bacteria (21 days of cultivation; Sa: *S. aureus*, Bs: *B. subtilis*; N=single culture, d0=mixed since day 0, d3=mixed since day 3; 2 mg/disc; n=2, Data are expressed as Mean±SD)

HPLC traces of the extracts are illustrated in Fig. 3.44. There were eight peaks clearly presented from all extracts with t_R of 6, 11, 21, 26, 29, 32, 36 and 39 min. However, extract of single culture (N) showed roughly containing more complex mixtures. Five peaks with t_R of 6, 21, 29, 36 and 39 min are obviously different. Compared to the control, extract of d0 exhibited different intensities of the five mentioned peaks, while extract of d3 seemed to be relatively similar.

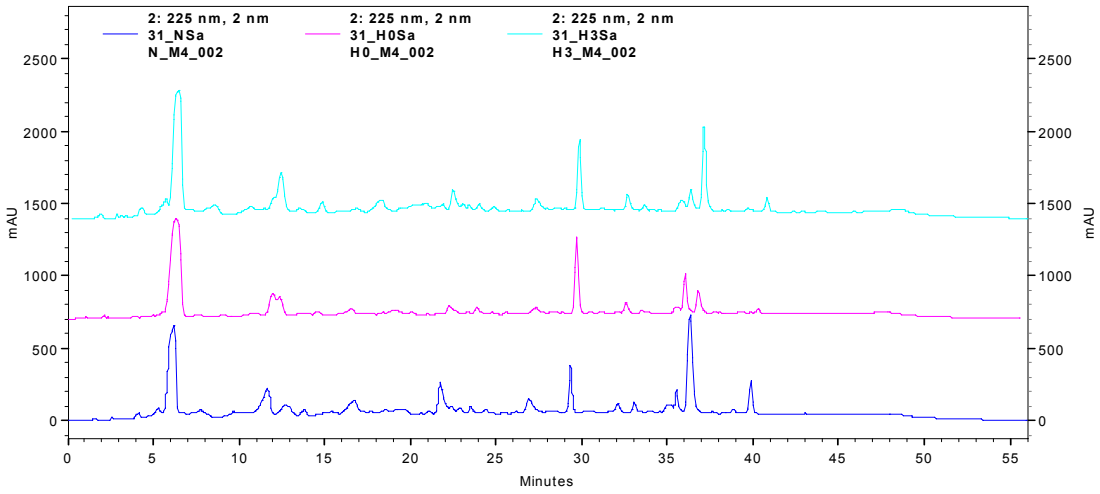


Fig. 3.44. HPLC traces of EtOAc extracts of KT31 co-cultivated with autoclaved *S. aureus* (21 days of cultivation; N: single culture, H0: mixed since day 0, H3: mixed since day 3; solvent gradient MeOH/H_2O)

3.6. Isolation of Bioactive Compounds from Marine-derived Fungi

From five fungal isolates, two strains were further investigated for their active secondary metabolites. Bioassay-guided fractionation was applied to obtain the targeted biologically active compounds. The isolation process and structure elucidation of isolated compounds are described as follows.

3.6.1. Isolated Compounds from *Aspergillus* sp. KT13

3.6.1.1. Bioautographic Assay

Solvent system containing ethyl acetate, acetone and *n*-Hexane (25:4:18) was used as mobile phase to separate the crude extract of *Aspergillus* sp. KT13 on TLC. Bands on TLC were detected under UV light at 254 nm and 366 nm as well as by spraying with anisaldehyde-sulphuric acid (AS). The obtained chromatogram is presented in Fig. 3.45.

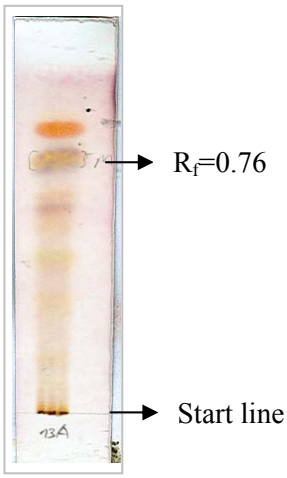


Fig. 3.45. TLC-profile of KT13-EtOAc extract
(0.5 mg/line; spraying with AS reagent)

In parallel, another TLC plate was not sprayed with AS reagent, but incubated on agar plate medium with *Bacillus subtilis* as test organism. After 20 h of incubation the active band was detected. The prominent band at R_f 0.76 exhibited antibacterial activity against the test organism. Subsequently, the mentioned solvent system was applied on further fractionation process to isolate the active components.

3.6.1.2. Fractionation and Purification

In an attempt to isolate the bioactive components, column chromatography was applied using silica gel (0.040-0.063 mm) as stationary phase and the appropriate solvent systems (SS) as described below were used as mobile phase.

- SS1 : EtOAc:Acetone:*n*-Hexane (25:4:18, v/v)
- SS2 : EtOAc:Acetone:*n*-Hexane (30:4:10, v/v)
- SS3 : EtOAc:Acetone:*n*-Hexane (25:4:18, v/v)
- SS4 : EtOAc:Acetone:*n*-Hexane (40:4:5, v/v)
- SS5 : EtOAc

Ethyl acetate extract of *Aspergillus* sp. KT13 (381 mg) was fractionated. According to the TLC analyses, collected fractions were then combined. In total 22 fractions were collected and subsequently screened for their biological activity against *Bacillus subtilis* representing human pathogenic bacteria and *Vibrio anguillarum* as fish pathogenic bacterium. The 300 µg/sheet of fraction F4 showed biological activity with inhibition zones of 10 mm and 12 mm against *B. subtilis* and *V. anguillarum,* respectively. Selected fractions were presented in Fig. 3.46.

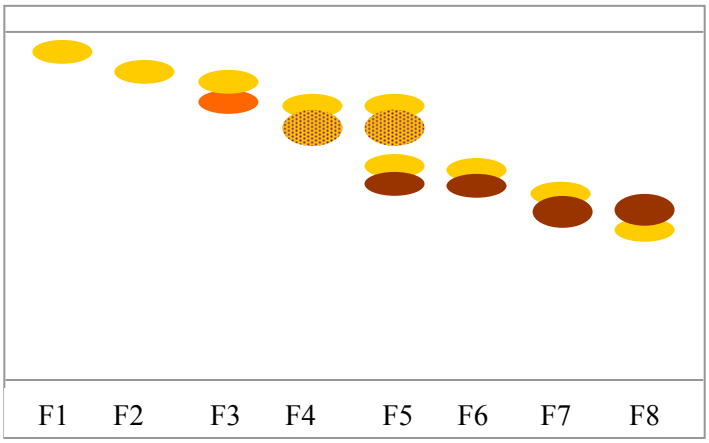


Fig. 3.46. TLC-chromatogram of collected fractions (F1-8) of KT13 (detected under UV light at 254 nm and 366 nm, and spraying with AS reagent)

F4 which contained the major bioactive components (21.5%) was further analyzed by HPLC. Based on the HPLC chromatogram of F4, semi preparative HPLC was carried out to separate the active compounds with mobile phase 50-70% of methanol/water in 5 min followed by another 5 min of 70% methanol and 70-90% methanol/water in 6 min.

The analytical HPLC with DAD detection revealed 8 subfractions F4.1-8 with two major components F4.4 (oily; 24.2 mg) and F4.5 (oily; 33 mg) at retention times of 11.95 and 12.53 min, respectively. The HPLC chromatogram can be seen in Fig. 3.47 as follow.

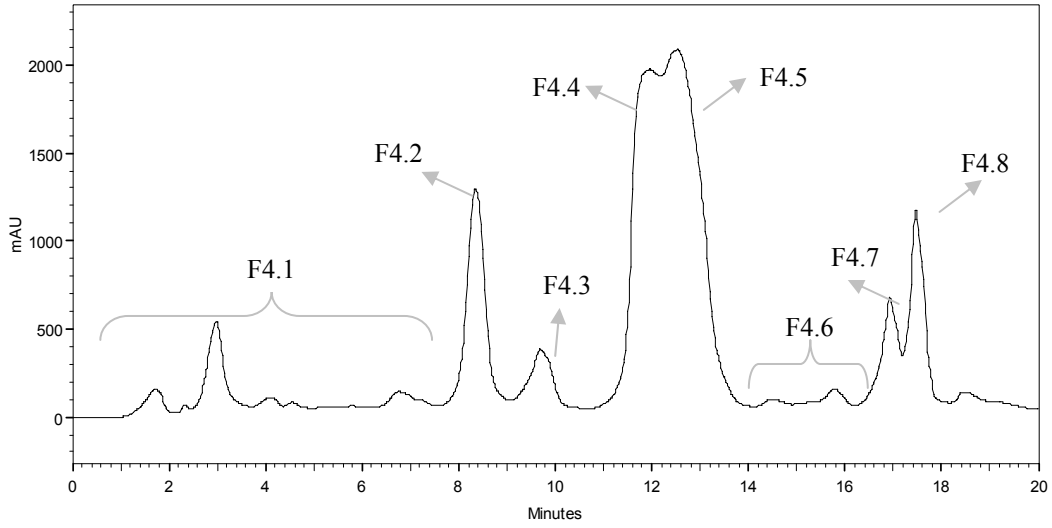


Fig. 3.47. HPLC trace of KT13/F4 detected at 254 nm

The entire subfractions were then assayed for their antibacterial properties against *B. subtilis* and *V. anguillarum.* The most active component was F4.8 with IZs of 9 mm and 23 mm against *B. subtilis* and *V. anguillarum,* respectively, followed by F4.7 with IZs of 7 mm and 20 mm; F4.5 with IZs of 6 mm and 15 mm; and F4.4 was active against *V. anguillarum* only with IZ of 10 mm. All other components were inactive.

F4.4 and F4.5 were obtained as light yellow oily substance. By considering its biological activities and the available amount, F4.4 and F4.5 were further investigated to elucidate their molecular structure. Fig. 3.48 presents HPLC chromatogram of F4.5 after semi preparative HPLC separation.

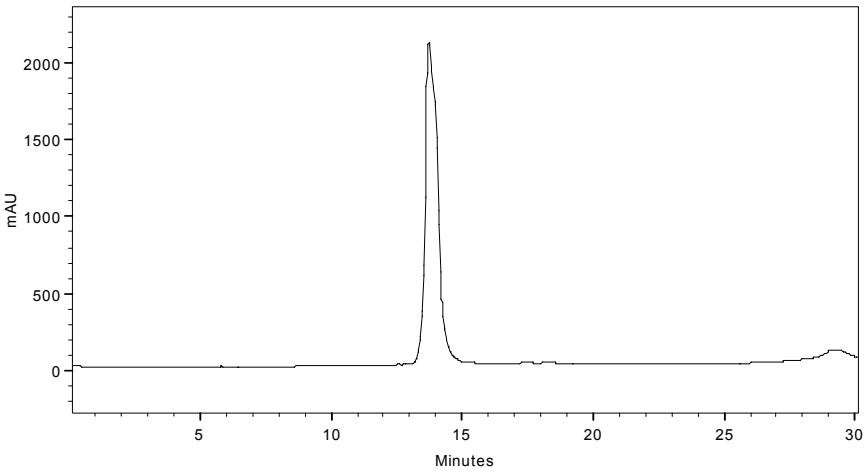


Fig. 3.48. HPLC chromatogram of KT13/F4.5 detected at 219 nm

Under UV detection F4.5 also exhibited the intense peaks at the wavelengths of 219, 241 and 298 nm. As can be seen in Fig. 3.49, however the most intense absorbance was measured at 219 nm.

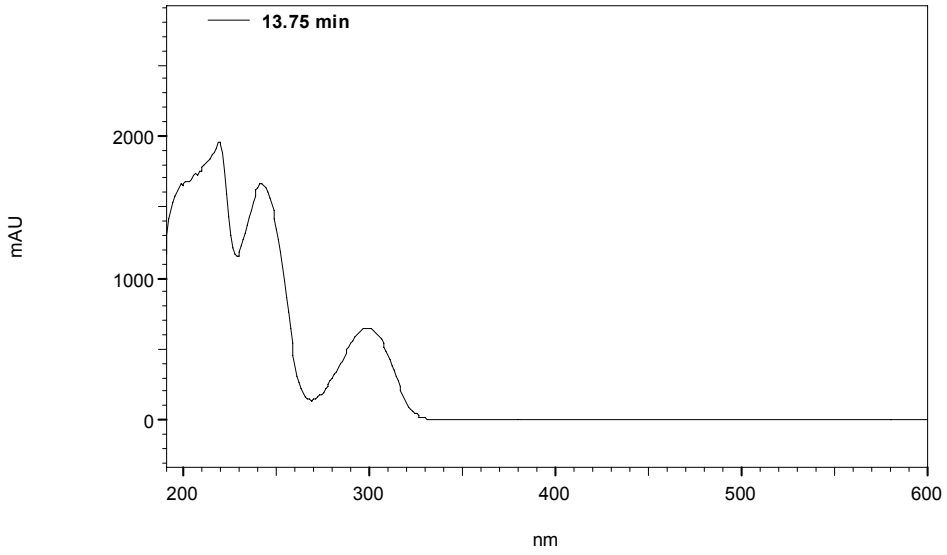


Fig. 3.49. UV spectrum of KT13/F4.5 (λ_{max}. 219, 241, 298 nm; in MeOH)

3.6.1.3. Structure Elucidation of Compounds KT13/1 and KT13/2

F4.4 and F4.5 were isolated in pure forms and named as KT13/1 and KT13/2, respectively. HR-MS analysis of compound KT13/1 indicates a $[M-H]^-$ ion at *m/z* 265.14096 suggesting a molecular weight of 266. In comparison of the MS spectra with Dictionary of Natural Products the compound was proposed as aspterric acid ($C_{15}H_{22}O_4$, Fig. 3.50).

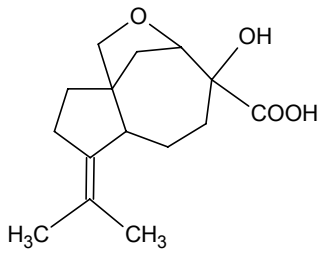


Fig. 3.50. Molecular structure of compound KT13/1 ($C_{15}H_{22}O_4$)

HR-MS analysis of compound KT13/2 shows a $[M-H]^-$ ion at *m/z* 265.13211 and a $[2M+Na]^+$ ion at *m/z* 555.29511 representing the same molecular weight of 266.

The molecular structure of compound KT13/2 indicated that this substance is a chromane derivative with dehydrobenzopyran as basic structure (Fig. 3.51). The presence of carbonyl group was confirmed by the IR spectrum with absorption band at 1693 cm^{-1}. The IR (KBr) spectrum also showed the presence of hydroxyl group (3430 cm^{-1}), aromatic group (2954 cm^{-1}) and olefinic bond (1384 cm^{-1}). Compared to the Chemical Abstracts of the American Chemical Society, the compound was determined as ruakuric acid with a molecular weight of 266 (Cutler *et al*., 1996). The absolute configuration however has to be determined.

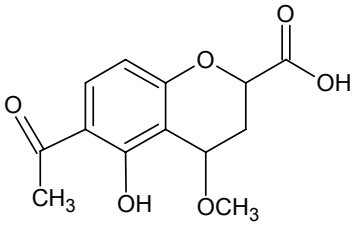


Fig. 3.51. Molecular structure of compound KT13/2 ($C_{13}H_{14}O_6$)

Interestingly, the compounds KT13/1 and KT13/2 contributed approximately 15% of the total crude extract. However, compound KT13/1 has to be further elucidated.

3.6.1.4. Biological Activity

The agar diffusion assay exhibited that KT13/1 was not active against *Bacillus subtilis,* but active against *Vibrio anguillarum*, on the contrary KT13/2 was active against both bacteria. MIC test showed that KT13/2 inhibited the growth of *B. subtilis* and *V. anguillarum* at concentrations of 0.28 mM and 70 µM, respectively. In the antifungal assay against phytopathogenic *Cladosporium cucumerinum*, compound KT13/1 inhibited the fungal growth with an inhibition area of 314 mm² at 200 µg/spot.

Cytotoxicity test of KT13/2 showed that the compound exhibited weak cytotoxicity with an IC_{50} value of 0.29 mM. As positive control etoposide possessed an IC_{50} value of 0.6 µM.

3.6.2. Isolated Compounds from KT19

3.6.2.1. Bioautographic Assay

Ethyl acetate extracts of isolate KT19 showed considerable antimicrobial activity. Fig. 3.52 shows that the extracts consist of complex mixtures. In order to determine the active components of the extracts, bioautographic assay towards *Bacillus subtilis* was performed. As can be seen in Fig. 3.53, two extracts obtained from different batch cultures showed similar activity against the test organism. Three inhibition zones were found which were caused by the components with R_f values of 0.64, 0.21 and 0.04. The most active components, however, appeared at R_f 0.64.

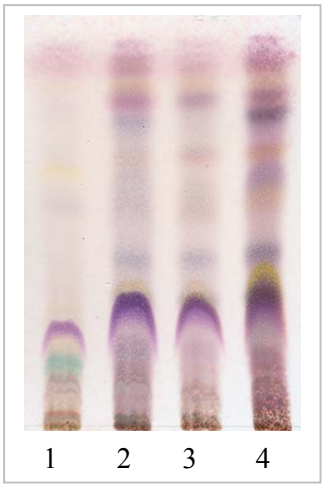


Fig. 3.52. TLC-profiles of KT19-EtOAc extracts from four batch cultures (spraying with AS reagent)

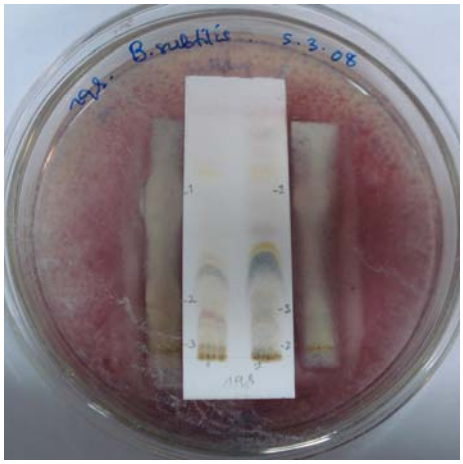


Fig. 3.53. Bioautograph of KT19-EtOAc extracts against *Bacillus subtilis*
(TLC plate as standard was sprayed with AS reagent)

3.6.2.2. Fractionation and Purification

Based on the bioautographic results, EtOAc extracts of KT19 (420 mg) were then subjected to column chromatography on silica gel and eluted with EtOAc:Acetone:*n*-Hexane (25:4:18, v/v), followed by pure EtOAc and MeOH to obtain 11 fractions (Fig. 3.54). Fractions 3, 5, 7-11 exhibited antibacterial activity against *Bacillus subtilis* and *Vibrio anguillarum*.

Fraction F7 was then chromatographed on Sephadex LH-20 and eluted with 90% MeOH. Subfractions F7.3 (compound KT19/1) and F7.5 (compound KT19/2) were obtained as brown crystals after grown on MeOH. The substances appeared at R_f 0.62 and 0.64, as brown and orange spots, respectively on TLC Si60 after spraying with AS reagent.

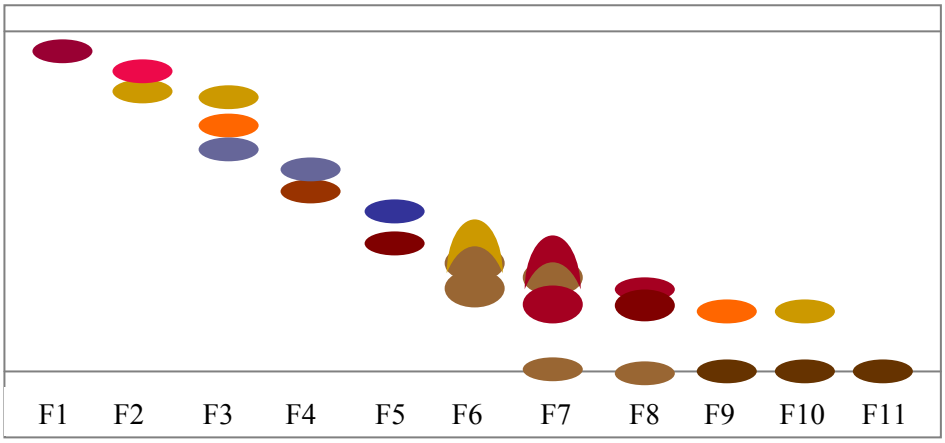


Fig. 3.54. TLC-chromatogram of collected fractions F1-11 of KT19 (detected under UV light at 254 nm and 366 nm, and spraying with AS reagent)

Second attempt of isolation bioactive compounds from the extract of KT19 was carried out. Ethyl acetate extract of strain KT19 was subjected to column chromatography using Sephadex LH-20 as stationary phase and eluted with 100% methanol. Based on TLC analyses, collected fractions were then combined into five fractions. Fig. 3.55 presents the TLC profiles of fractionated KT19 extracts.

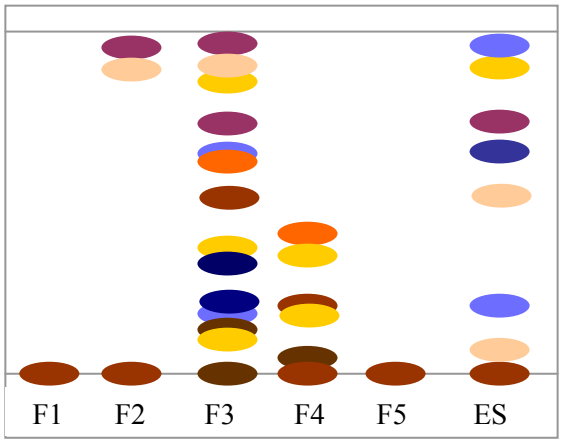


Fig. 3.55. TLC-chromatogram of collected fractions (F1-5) of KT19 (mobile phase: Toluene:Ethyl formiate:Formic acid (10:5:3, v/v); detected under UV light at 254 nm and 366 nm, and spraying with AS reagent; ES=Ergosterol stored at room temperature)

Based on TLC chromatograms of the fractions and in parallel comparing with chromatogram of ergosterol, fraction F4 was further separated using column chromatography with silica gel as stationary phase (0.040-0.063 mm) and varying solvent systems were used as follows.

- SS1 : EtOAc:*n*-Hexane (1:4, v/v)
- SS2 : EtOAc:*n*-Hexane (3:2, v/v)
- SS3 : EtOAc: *n*-Hexane (1:1, v/v)
- SS4 : EtOAc
- SS5 : EtOAc:MeOH (4:1, v/v)

TLC analyses revealed three main subfractions. Subfraction F4.1-3 was the major component and obtained as yellow powder. Fig. 3.56 demonstrates TLC profiles of separated fraction. Based on the visual performance of substance F4.1-3, its TLC chromatogram was compared to a well-known hydroxyanthraquinone emodin (Fig. 3.57).

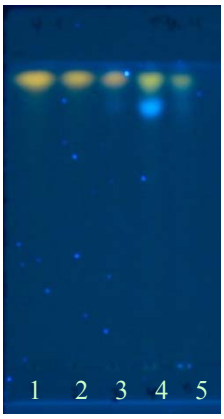


Fig. 3.56. TLC-chromatogram of collected fractions (F4.1-5) of KT19 (detected under UV light at 366 nm)

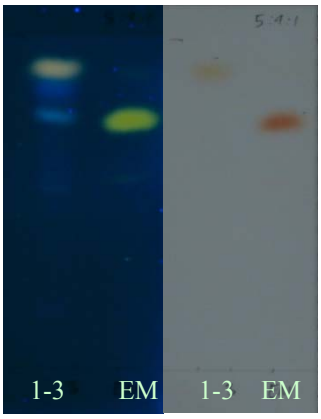


Fig. 3.57. TLC-chromatogram of F4.1-3 R_f 0.96 and emodin R_f 0.78 (detected under UV at 366 nm and day light after exposed with ammonia)

F4.1-3 was then purified by cartridge RP18 eluted with EtOAc and *n*-Hexane (1:4, v/v). For repeatedly used the cartridge was washed with pure methanol. The pure substance was then elucidated for its chemical structure (later noted as compound KT19/3).

3.6.2.3. Structure Elucidation of Compound KT19/1

The molecular formula of the compound was determined as $C_{10}H_{12}O_5$ by the molecular-ion peaks at *m/z* 235.05767 ($[M+Na]^+$) in positive-ion mode HR-FTICR-MS and at *m/z* 211.06126 ($[M-H]^-$) in negative-ion mode. The fragment ion peak was found at *m/z* 167.07163 ($[M-H-CO_2]^-$). This peak indicated the loss of carboxyl group. The molecular structure of the compound as presented in Fig. 3.58.

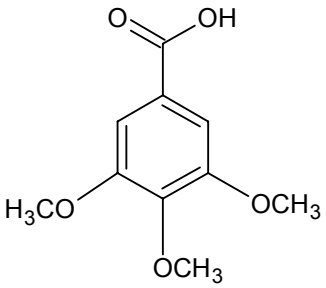


Fig. 3.58. Molecular structure of compound KT19/1 ($C_{10}H_{12}O_5$)

In comparison with Chemical Abstracts the compound was determined as trimethoxybenzoic acid. The compound was found abundantly as part of complex structures. Eriksson *et al.* (1984) reported the presence of trimethoxybenzoic acid as a result of syringic acid metabolism in white-rot fungus *Sporotrichum pulverulentum.*

3.6.2.4. Structure Elucidation of Compound KT19/2

Single-crystal X-ray diffraction analysis was conducted to determine the chemical structure of compound KT19/2. The molecular formula of the compound was determined to be $C_{10}H_{16}O_3$. Fig. 3.59 presents the relative configuration of the compound.

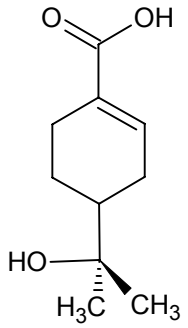


Fig. 3.59. Molecular structure of compound KT19/2 ($C_{10}H_{16}O_3$)

Based on its similarities to the known compound in the literature the isolated compound was determined as oleuropeic acid which was isolated from olive (*Olea europaea*) for the first time (Shasha & Leibowitz, 1959). In their report the characteristics of oleuropeic acid has been described, namely its solubility in ether, ethanol, and acetone; m.p. 160 ^oC, and strongly levorotatory ($[\alpha]_D$=-100^o in methanol).

3.6.2.5. Structure Elucidation of Compound KT19/3

The ESI-FTICR-MS of the compound showed a $[M\text{-}OH]^-$ ion at *m*/*z* 237.05540 (calc. for $C_{15}H_9O_3^-$ 237.05572) representing a molecular formula of $C_{15}H_{10}O_4$ (MW. 254).

Based on the colour of the compound it was subjected that it belongs to anthraquinones. Proton and ^{13}C-NMR data were in agreement with this suggestion. Comparison of the measured values with those obtained from the Chemical Abstracts for anthraquinone derivatives; the possible structure of the compound is as presented in Fig. 3.60.

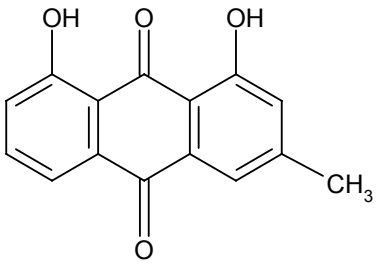


Fig. 3.60. Molecular structure of compound KT19/3 ($C_{15}H_{10}O_4$)

The substance was suggested as chrysophanol, an anthraquinone derivative which is abundantly found in nature. Related to this reason, no further elucidation was carried out.

3.7. Metabolites Isolated from Algicolous Fungi

Four of six marine algicolous isolates were further investigated for their active secondary metabolites. Bioassay-guided fractionation led to the isolation of the targeted biologically active compounds. The isolation process and structure elucidation of isolated compounds are described as follows.

3.7.1. Isolated Compounds from KT29

3.7.1.1. Bioautographic Assay

Ethyl acetate extracts of fungus KT29 showed considerable antibacterial activity against Gram-negative bacteria, both fish- and human pathogenic bacteria. The extracts also exhibited strong activity in fungicidal assay against phytopathogenic fungus *Cladosporium cucumerinum.* Therefore, further investigation in an attempt to obtain the compounds which were responsible for its bioactivity was conducted.

Fig. 3.61 presents the TLC profiles of EtOAc extracts of KT29 cultured in freshwater and seawater media. From agar diffusion assays was found that extracts isolated from seawater medium showed stronger activity. In order to determine the active components, bioautographic assay was carried out (Fig. 3.62).

Fig. 3.61. TLC profiles of EtOAc extracts of KT29 (a: Freshwater culture, b: Seawater culture; spraying with AS reagent)

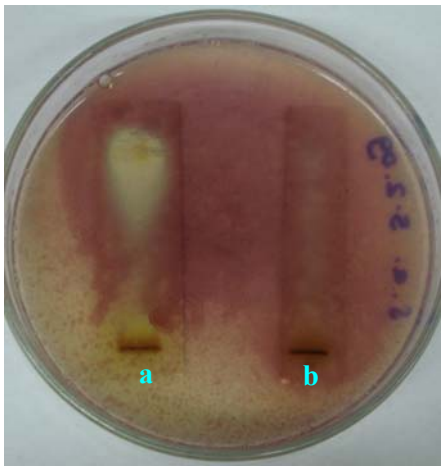


Fig. 3.62. Bioautograph of KT29 against *S. aureus* (a: Seawater culture, b: Freshwater culture; 0.4 mg/line; spraying with INT)

3.7.1.2. Fractionation and Purification

Ethyl acetate extract of KT29 (232.2 mg) was chromatographed on Sephadex LH-20 and eluted with 100% MeOH. Based on TLC analyses the fractions were combined into 10 fractions. Fraction F5 appeared as blue fluorescence both in short and long wavelength under UV detection (Fig. 3.63). Subsequently, F5 was fractionated by rechromatography on Sephadex LH-20 to afford two subfractions.

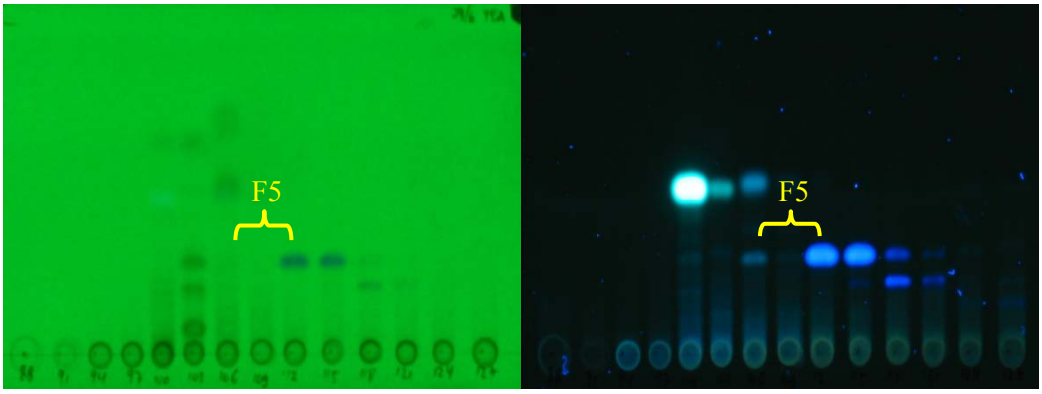


Fig. 3.63. TLC profiles of KT29 fractions (mobile phase: Toluene:Ethyl formiate: Formic acid (10:5:3 v/v); detected under UV light at 254 and 366 nm)

Subfraction F5.1 (KT29/1; 13.9 mg) was obtained as brown powder or brown crystals after grown on methanol. The substance appeared at R_f 0.45 as yellowish spot under day light and as blue fluorescence under UV detection (Si60 plate eluted with Toluene/Ethyl formate/Formic acid; 10:5:3, v/v) (Gill & Steglich, 1987).

3.7.1.3. Structure Elucidation of Compound KT29/1

Molecular formula of compound KT29/1 was determined to be $C_{12}H_{10}O_4$ (MW. 218) by the molecular ion peaks at *m/z* 241.04721 ($[M+Na]^+$) in positive-ion mode and 217.05042 ($[M-H]^-$) in negative-ion mode of HR-FTICR-MS. The UV spectrum of the substance exhibited maximum absorptions at 237, 355 and 343 nm.

In order to elucidate the compound, NMR measurement in MeOH-d_4 was carried out. The ^1H spectrum confirmed that KT29/1 was almost entirely aromatic, with all proton signals present at $\delta > 6.8$ ppm. More detail, five aromatic protons were observed – four as doublets at δ 7.889; 7.291; 7.126 and 6.855 and one as doublet of doublets at δ 7.366 ppm. A methyl signal was observed at δ 3.930 *s* ppm (H_3-12). The ^{13}C-NMR confirmed the presence of 12 carbon atoms.

The presence of hydroxyl groups was confirmed by the IR spectrum with the absorption bands at 3439 cm^{-1}. The IR (KBr) spectrum also exhibited the presence of

carbonyl group (1623 cm^{-1}), aromatic group (1584 cm^{-1}) and olefinic bond (1384 cm^{-1}). All signals were confirmed by 2D HMBC, HSQC and ROESY. Fig. 3.64 presents the 2D ROESY spectrum of the compound. In Table 3.6 the NMR data of the compound are presented.

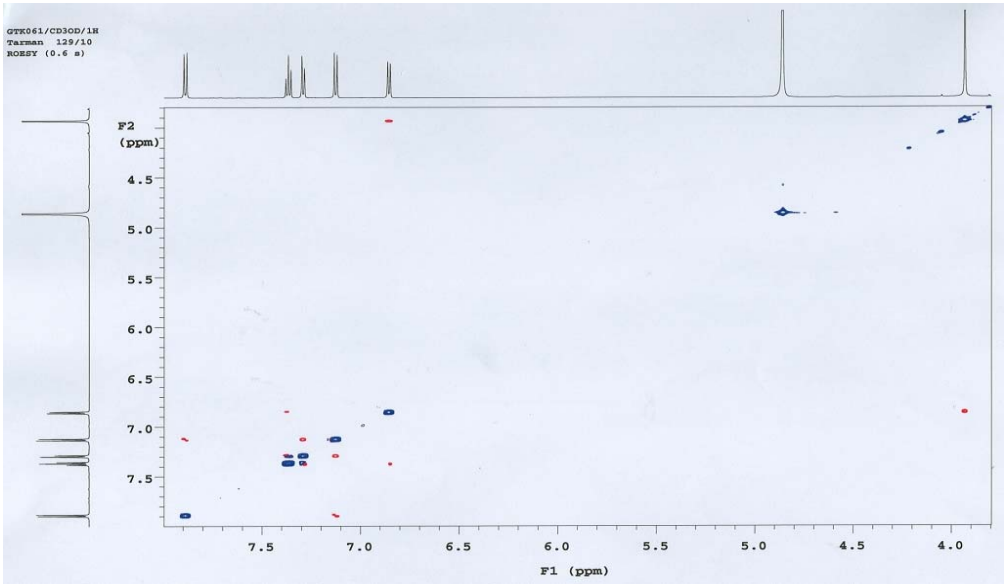


Fig. 3.64. 2D ROESY spectrum of compound KT29/1

Table 3.6. NMR data of compound KT29/1 in CD_3OD

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]
1	162.4	-
2	113.7	-
3	128.9	7.889 *d* (8.5)
4	117.9	7.126 *d* (8.5)
5	121.5	7.291 *d* (8.2)
6	129.0	7.366 *dd* (8.2(7.8)
7	106.4	6.855 *d* (7.8)
8	159.8	-
9	117.8	-
10	140.9	-
11	176.9	-
OMe	56.3	3.930

Fig. 3.65 presents the molecular structure of compound KT29/1. In comparison with the database of Chemical Abstracts the compound was determined to be 1-hydroxy-8-methoxy-2-naphthalene-carboxylic acid. The substance was optically inactive ($[\alpha]_D^{22}$ = +0.2° in methanol). Previously, the compound was obtained from chemical synthesis

(Eugster & Good, 1962; Bosshard *et al.,* 1964). According to this result, compound KT29/1 isolated from the fungus KT29 in this study was concluded as new natural product.

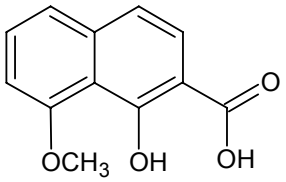


Fig. 3.65. Molecular structure of compound KT29/1 ($C_{12}H_{10}O_4$)

3.7.1.4. Biological Activity

In agar diffusion assays compound KT29/1 exhibited no antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* (at 100 µg/disc) and *Candida maltosa* (at 200 µg/disc). Compound KT29/1 was tested *in vitro* against human bladder carcinoma cell line 5637 with etoposide as the reference substance. The results showed that the compound exhibited weak cytotoxic activity with an IC_{50} value of 0.34 mM compared to etoposide with an IC_{50} value of 0.6 µM.

3.7.2. Isolated Compounds from *Xylaria psidii* KT30

3.7.2.1. Bioautographic Assay

In bioactivity assay, EtOAc extract of KT30 exhibited antimicrobial activity against bacteria and fungi. From bioautographic assay the active component of the mixture was subsequently found with an R_f value of 0.54. Unfortunately, the intensity of this active band was diverse from different cultures. A reason for this fluctuating metabolites production could not be found since the fungus was cultured in the same condition. In Figs. 3.66-67 TLC profile of the KT30-EtOAc extract as standard TLC chromatogram and the bioautographic assay against *Vibrio anguillarum* are presented.

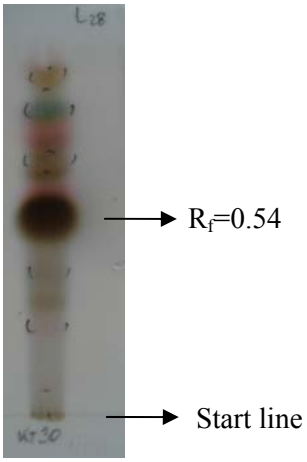


Fig. 3.66. TLC profiles of EtOAc extract of KT30 (detected under UV light at 254 and 366 nm, and spraying with AS reagent)

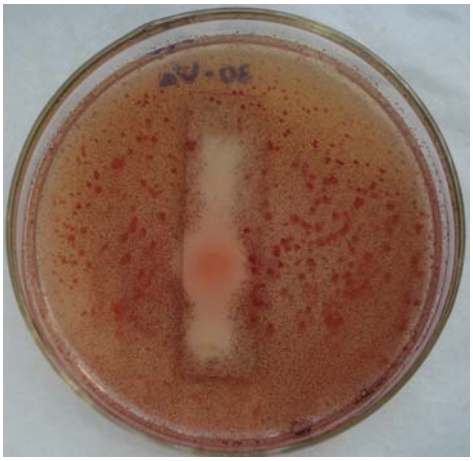


Fig. 3.67. Bioautograph of KT30 against *V. anguillarum* (0.4 mg/line; spraying with INT)

3.7.2.2. Fractionation and Purification of Compounds KT30/1 and KT30/2

EtOAc extract of *Xylaria psidii* KT30 (617 mg) was fractionated by column chromatography with silica gel (0.040-0.063) as stationary phase and mobile phase EtOAc:Acetone:*n*-Hexane (25:4:18, v/v) followed by EtOAc:*n*-Hexane (3:1, v/v) and EtOAc:*n*-Hexane:MeOH (3:1:4, v/v).

The fractions were combined according to their profiles on TLC plates detected under UV light at 254 nm and 366 nm, and by spraying with AS reagent. The chromatograms of the fractions are illustrated in Fig. 3.68.

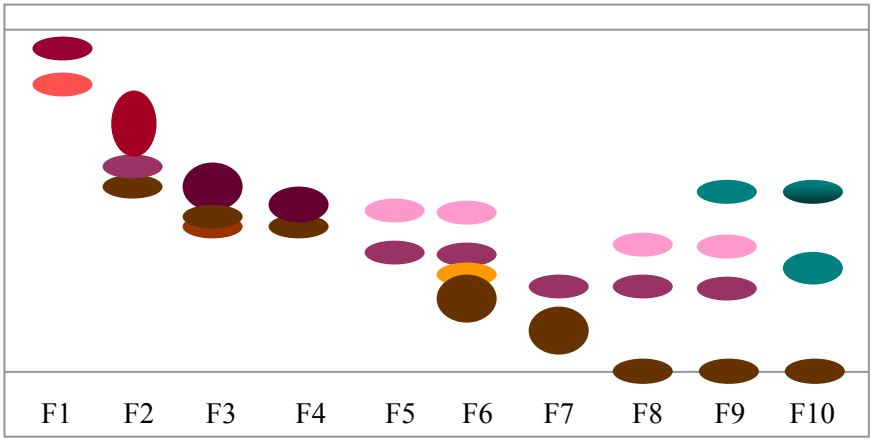


Fig. 3.68. TLC profile of collected fractions (F1-10) of *Xylaria psidii* KT30 (detected under UV light at 254 nm and 366 nm, and spraying with AS reagent)

Based on the bioassay of the collected fractions, F2, F3, F4 and F6 exhibited growth inhibition against *Bacillus subtilis* and/or *Vibrio anguillarum.* By considering the bioactivity and amount of the fractions, F2, F3 and F4 were further fractionated by preparative TLC using silica gel as stationary phase and DCM/EtOAc (65:35, v/v) as mobile phase.

Fraction F2 afforded four collected subfractions. Subfraction 2 (2.1 mg; R_f=0.70, mobile phase: DCM/EtOAc, 65:35, v/v) was crystallized from methanol and obtained as brown crystals (later written as compound KT30/1). The compound was then elucidated.

Fractions F3 and F4 were combined due to the similarity. From preparative TLC using the same method as described for F2, five components were obtained and then screened for their purity on TLC. The pure compound F3.3 was found as yellow band on TLC after spraying with AS reagent at R_f 0.44. Compound F3.3 (3.1 mg; later written as KT30/2) was obtained as light yellow crystals after grown on methanol.

3.7.2.3. Structure Elucidation of Compound KT30/1

Single-crystal X-ray diffraction analysis was undertaken to elucidate the molecular structure of compound KT30/1. This analysis revealed that the compound belongs to benzoic acid derivatives. The compound was proposed to be 4-(cyanomethoxy)-benzoic acid ($C_9H_7NO_3$, MW. 177; Fig. 3.69). Referring to Chemical Abstracts the compound was registered with CAS No. 792954-24-6. However, there was no reference reporting more about the compound. So that 4-(cyanomethoxy)-benzoic acid was suggested as new natural product.

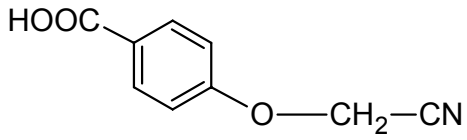


Fig. 3.69. Molecular structure of compound KT30/1 ($C_9H_7NO_3$)

3.7.2.4. Structure Elucidation of Compound KT30/2

The HR-FTICR-MS analysis of compound KT30/2 indicates a $[M+Na]^+$ ion at *m*/*z* 502.25609 (calc. for $C_{29}H_{37}NO_5Na^+$ 502.25639) suggesting a molecular formula of $C_{29}H_{37}NO_5$ (MW. 479).

HR-MS/MS fragmentation yielded 3 different groups of prominent ion peaks in the range of retention times of 19.94-21.54. The first peak was found at t_R 19.94 with positive-

ion mode at *m/z* 461.9 ($[M-H_2O]^+$), 443.9 ($[M-2H_2O]^+$) and 480 ($[M+H]^+$). The second peak appeared at t_R 20.67 with positive-ion mode at *m/z* 239.9 ($[1/2M+H]^+$). The third peak was found at t_R 21.54 with positive-ion mode at *m/z* 443.9 ($[M-2H_2O]^+$), 263.8, 461.9 ($[M-H_2O]^+$), 277.9 and 480 ($[M-H]^+$). Compared to the literature the compound was suggested to be the known compound cytochalasin B (Fig. 3.70).

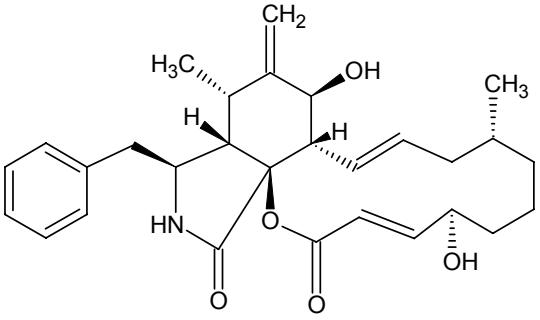


Fig. 3.70. Molecular structure of compound KT30/2 ($C_{29}H_{37}NO_5$)

Cytochalasin B is also known as phomin, an alkaloid which was isolated from a fungus *Phoma* sp. for the first time (Rothweiler & Tamm, 1966). Their study reported that phomin forms colourless needles with m.p. 218-220 ^oC and $[\alpha]_D^{25} = 83^o \pm 2^o$ (in methanol).

In TLC analyses cytochalasin B was detected at R_f values of 0.50 and 0.62 in solvent systems TEF (Toluene/EtOAc/90% Formic acid, 5:4:1, v/v) and CA (Chloroform/Acetone, 9:1, v/v), respectively. After spraying with 65% (v/v) sulphuric acid and heating at 100 ^oC, cytochalasin B was detected as blue fluorescent spot under UV (Scott *et al.*, 1975).

3.7.3. Isolated Compounds from KT31

3.7.3.1. Fractionation and Purification of Compounds KT31/1 and KT31/2

In an attempt to isolate the active compounds of KT31 as described in part **3.5.1**, ethyl acetate extract of the fungus (696 mg) was subjected to column chromatography on silica gel (0.015-0.040) and eluted with DCM/EtOAc (4:1, v/v). Based on TLC analyses, fractions were combined into 10 main fractions. Fig. 3.71 presents the TLC chromatogram of fractionated KT31-ethyl acetate extracts.

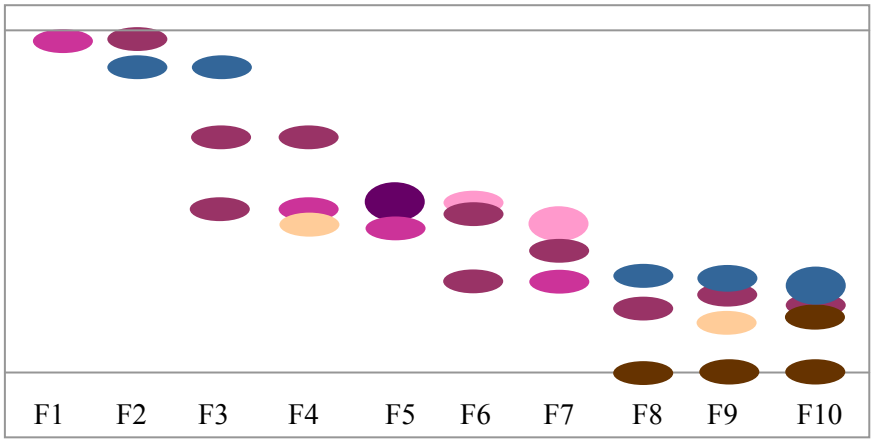


Fig. 3.71. TLC profiles of KT31-collected fractions (eluted with 90:10 to 63:35 DCM:EtOAc; detected at 254 nm and 366 nm, and spraying with AS reagent)

Antibacterial assays were conducted to trace the active fractions. *Bacillus subtilis* and *Vibrio anguillarum* were used as the test organisms. Fractions F5 and F10 showed antibacterial activity against *B. subtilis.* Unexpectedly, all fractions were active against *V. anguillarum.* However, the most active fraction was F5 followed by F8, F3 and F2 with corresponding IZ diameters of 23, 21, 17 and 15 mm.

By considering the available amount besides the bioactivity, fraction F2 was further purified. The fraction was subjected to semi-preparative HPLC with mobile phase 0-100% of methanol/water in 18 min followed by another 4 min eluted with 100-10% of methanol/water. The HPLC trace of the fraction is presented in Fig.3.72.

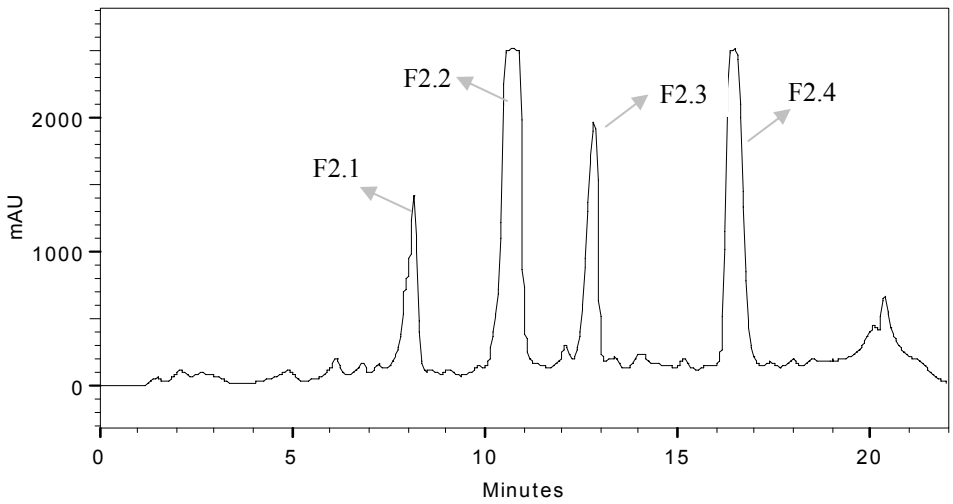


Fig. 3.72. HPLC trace of fraction KT31/F2

After concentration under a reduced pressure in rotary evaporator, all collected compounds were air dried at room temperature. Compounds F2.2 and F2.3 (later written as

KT31/1 and KT31/2) were obtained as colourless crystals after grown in methanol. Under UV analysis compound KT31/2 was detected at 232 and 292 nm (Fig. 3.73).

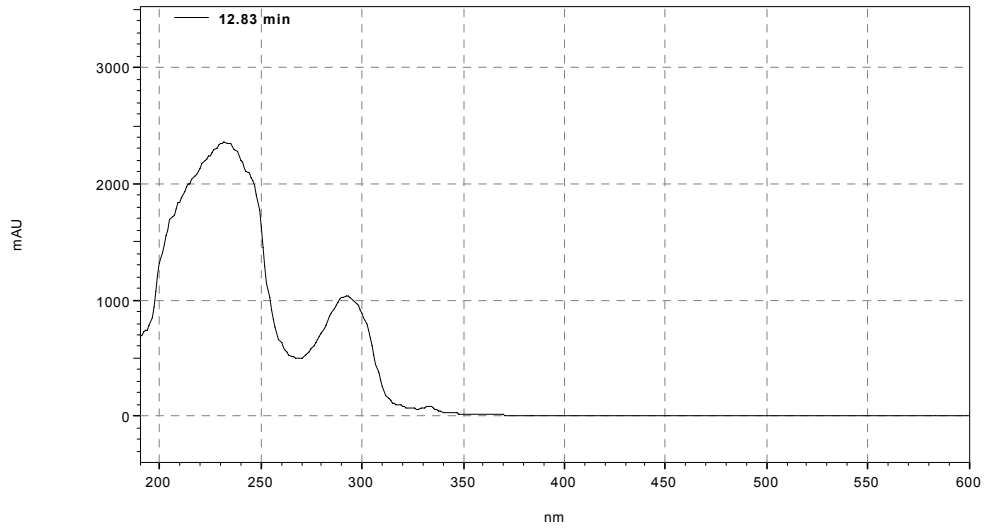


Fig. 3.73. UV spectrum of compound KT31/2 (λ_{max}. 232 and 292 nm in MeOH)

3.7.3.2. Fractionation and Purification of Compounds KT31/3 and KT31/4

Dichloromethane extract of KT31 (707 mg) was further investigated based on its antifungal activity against *Candida maltosa.* The single active band was found at R_f 0.9 which appeared as turkish band after spraying with AS reagent. The TLC profile of the DCM extracts of KT31 and their properties against *C. maltosa* on bioautographic assay are presented in Figs. 3.74-75.

Compared to the extract of seawater culture, the active band in the extract of freshwater culture appeared more intense. This band however was also present in the EtOAc extract of culture broth, but showed no activity against *Bacillus subtilis* and *Vibrio anguillarum* (as described in part **3.5.1**).

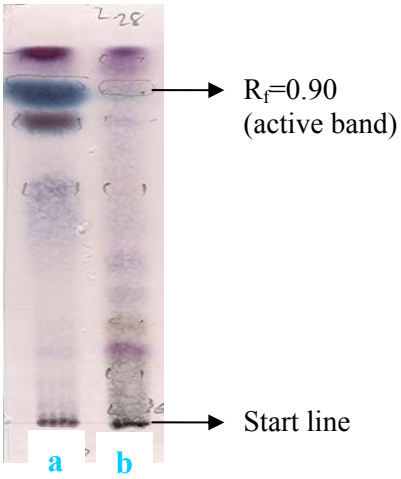


Fig. 3.74. TLC profiles of DCM extracts of KT31 (a. DCM extract of freshwater culture, b. from seawater culture)

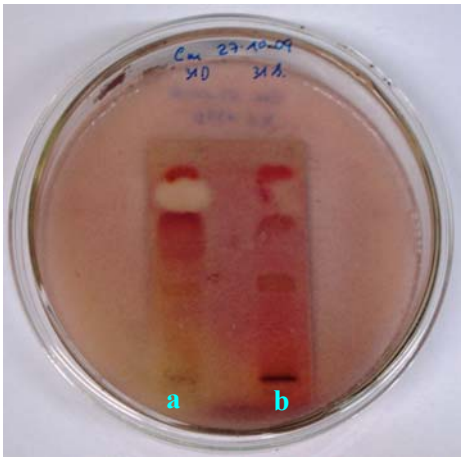


Fig. 3.75. Bioautograph of DCM extracts of KT31 against *C. maltosa* (a. DCM extract of freshwater culture, b. from seawater culture; 0.5 mg/line; spraying with INT)

The DCM extract was then chromatographed on silica gel using the same method for the ethyl acetate extract fractionation as described in **3.7.3.1.** Based on the bioautographic assay as presented in Fig. 3.75, fraction F2 was further purified. The fraction was subjected to semipreparative HPLC with mobile phase 0-80% of methanol/water in 25 min followed by another 5 min eluted with 80-30% of methanol/water. The HPLC trace of fraction F2 and UV spectrum of compound F2.3 (later written as KT31/3) are presented in Fig.3.76 and Fig. 3.77, respectively. Compound F2.4 was later noted as KT31/4.

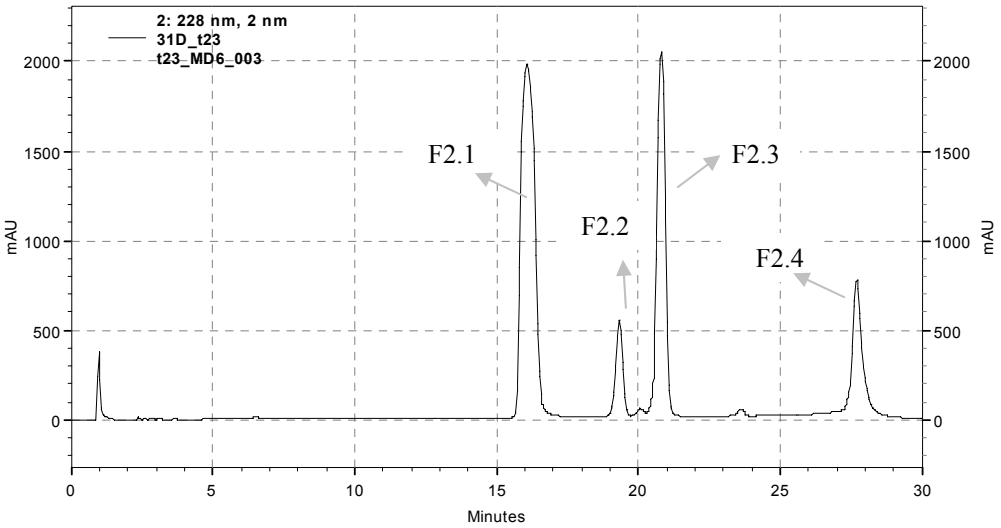


Fig. 3.76. HPLC trace of fraction KT31D/F2

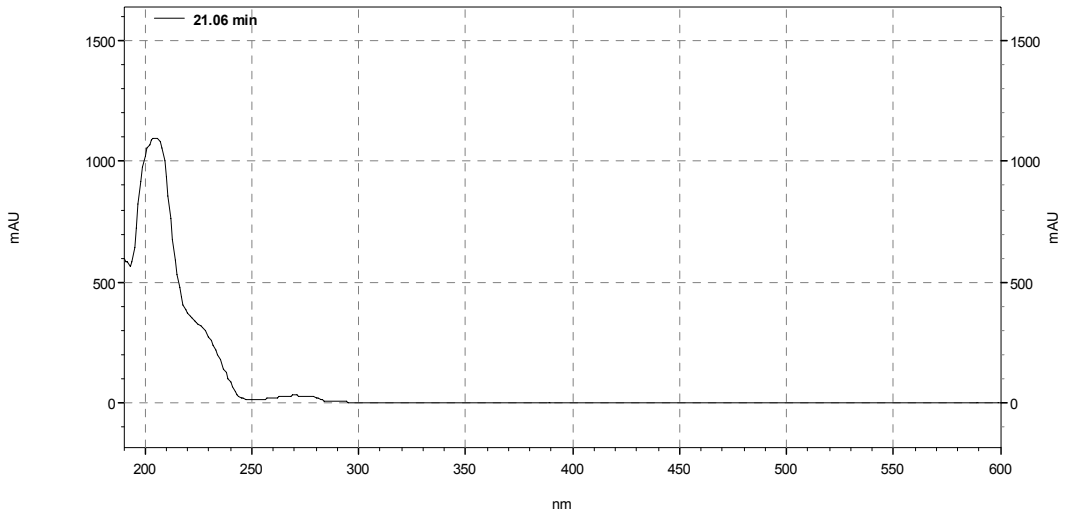


Fig. 3.77. UV spectrum of compound KT31/3 (λ_{max}. 206 nm in MeOH)

3.7.3.3. Structure Elucidation of Compounds KT31/1

Single-crystal X-ray diffraction analysis was undertaken to elucidate the molecular structure of compound KT31/1. This analysis revealed that the compound belongs to quinone derivatives. The compound was proposed to be $C_{18}H_{16}O_4$, MW. 296; Fig. 3.78). No similar compound could be found in Chemical Abstracts. Therefore, this quinone derivative was suggested as new natural product.

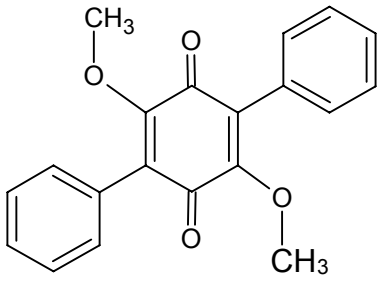


Fig. 3.78. Molecular structure of compound KT31/1 ($C_{18}H_{16}O_4$)

3.7.3.4. Structure Elucidation of Compounds KT31/2 and KT31/3

Compounds KT31/2 and KT31/3 were found to be prominent both intra- and extracellular. The compounds were obtained from EtOAc- and DCM-extracts as colourless needle-like crystals. The ESI-FTICR-MS of both compounds show an identical $[M+Na]^+$

ion at *m*/z 269.11466 (calc. for $C_{15}H_{18}O_3Na^+$ 269.11482) suggesting a molecular formula of $C_{15}H_{18}O_3$ (MW. 246).

MS/MS fragmentation yielded a positive-ion mode at *m/z* 166.9 $[M-79]^+$. This peak indicates the loss of aromatic group ($[C_6H_7]^+$) as resulting from a complex degradation. In Chemical Abstracts were found 4906 entries of substance with molecular formula of $C_{15}H_{18}O_3$. Therefore, further investigation has to be done to elucidate the active compounds produced by the fungus KT31.

3.7.3.4. Structure Elucidation and Biological Activity of Compound KT31/4

Compound KT31/4 was collected from DCM extract of KT31 after column chromatography (see part **3.7.3.2**). The substance was obtained as colourless crystals. The ESI-FTICR-MS of the compound shows a prominent $[2M+Na]^+$ ion at *m*/z 879.64740 (calc. for $C_{56}H_{88}O_6Na^+$ 879.64731) suggesting a molecular formula of $C_{28}H_{44}O_3$ (MW. 428).

Compound KT31/4 exhibited no antifungal activity against phytopathogenic fungus *Cladosporium cucumerinum* at 200 µg/spot. However, the compound showed considerable cytotoxicity against human bladder carcinoma 5637 cell line with an IC_{50} value of 8.5 µg/ml.

3.7.4. Isolated Compounds from KT32

Ethyl acetate extracts of KT32 showed medium activity in antimicrobial assays against Gram-positive and fish pathogenic bacteria. The extracts also possessed fungistatic activity toward *Cladosporium cucumerinum* at 200 µg/spot. Regarding to these results, bioautographic assay was carried out to determine the active components.

3.7.4.1. Bioautographic Assay

Fig. 3.79 presents TLC profile of complex mixtures found in ethyl acetate extract of KT32. After spraying with AS reagent, bands with R_f of 0.8 (yellow) and 0.89 (pink) appeared more intensely than others. However, on bioautographic assay against *Bacillus subtilis* both bands showed less activity compared to the component at R_f 0.72 as presented in Fig. 3.80.

Fig. 3.79. TLC profile of KT32-EtOAc extract (detected at 254 and 366 nm; spraying with AS reagent)

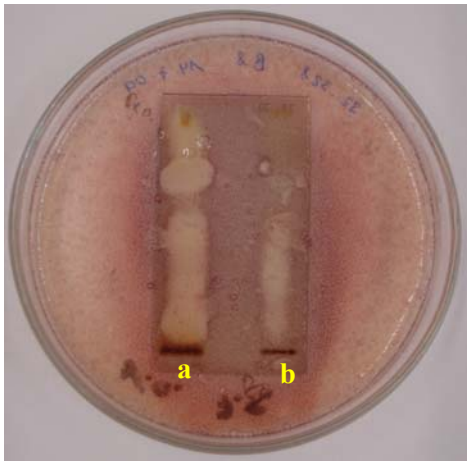


Fig. 3.80. Bioautograph of KT32 against *B. subtilis* (a: Freshwater culture, b: Seawater culture; 0.4 mg/line; spraying with INT)

3.7.4.2. Fractionation and Purification of Compounds KT32/1 and KT32/2

EtOAc extract of KT32 (524.6 mg) was chromatographed on silica-gel (0.015-0.040), stepwise and eluted with DCM/EtOAc (65:35, v/v to 100% EtOAc, lower phase) to give nine main fractions (F1-9) on the basis of TLC analysis. Fig. 3.81 shows the HPLC trace of fraction F3.

Fraction F3 (28.4 mg) was purified by semi-preparative RP-HPLC to afford compound F3.1 (later written as KT32/1; 8.6 mg). Semi-preparative HPLC was carried out with mobile phase 20-100% of methanol/water in 25 min followed by 50-10% methanol/water in 4 min. The HPLC chromatogram of the compound after purification is presented in Fig. 3.82.

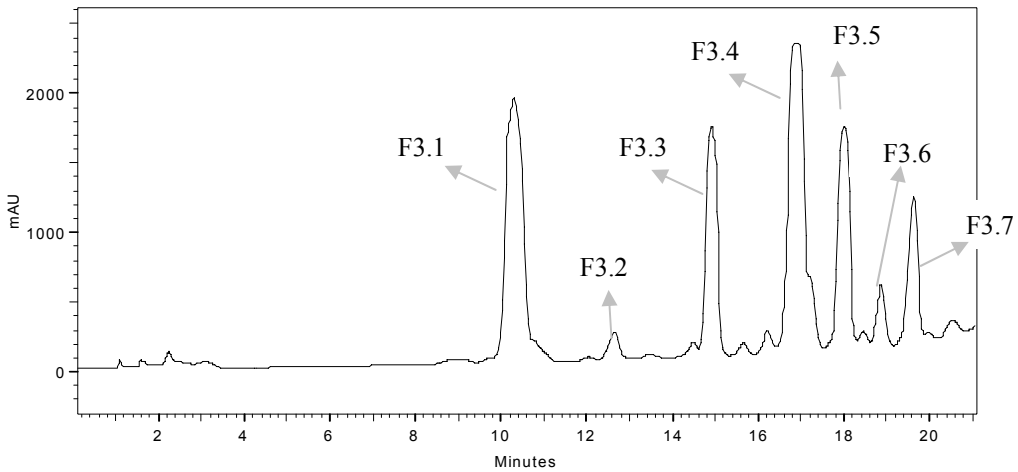


Fig. 3.81. HPLC chromatogram of KT32/F3 detected at 205 nm

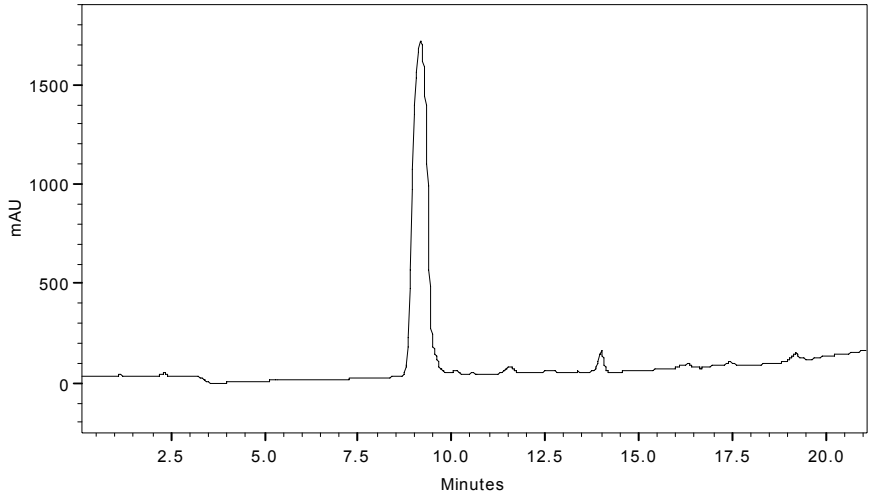


Fig. 3.82. HPLC chromatogram of compound KT32/1 detected at 212 nm

Substance KT32/1 was obtained as colourless crystals after grown on methanol. Under UV detection two maximum wavelengths were detected at 212 and 285 nm. Nevertheless, the most intense was detected at 212 nm. UV spectrum of the compound is illustrated in Fig. 3.83.

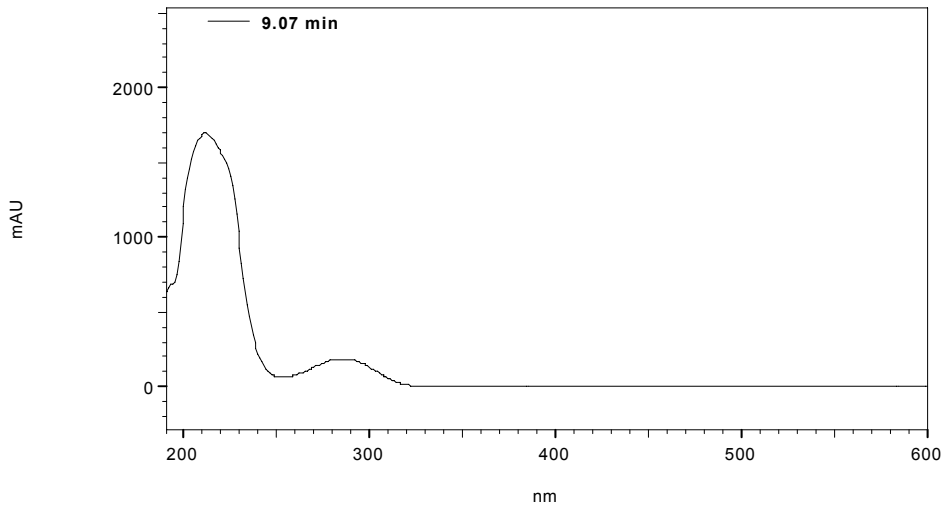


Fig. 3.83. UV spectrum of KT32/1 (λ_{max}. 212 and 285 nm; in MeOH)

In Fig. 3.84 chromatogram of F6 which contains five main components (F6.1-5) is presented. Fraction F6 (53.1 mg) yielded compound F6.4 (later written as KT32/2; 8.1 mg) after purification using semi-preparative RP-HPLC with mobile phase 20-90% of methanol/water in 20 min. The chromatogram of compound KT32/2 after purification is presented in Fig. 3.85.

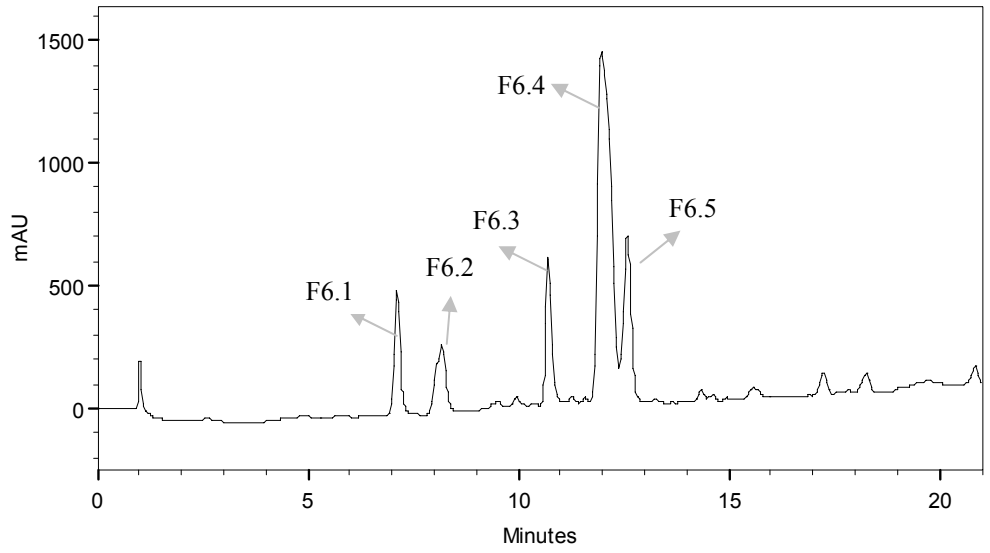


Fig. 3.84. HPLC chromatogram of KT32/F6 detected at 208 nm

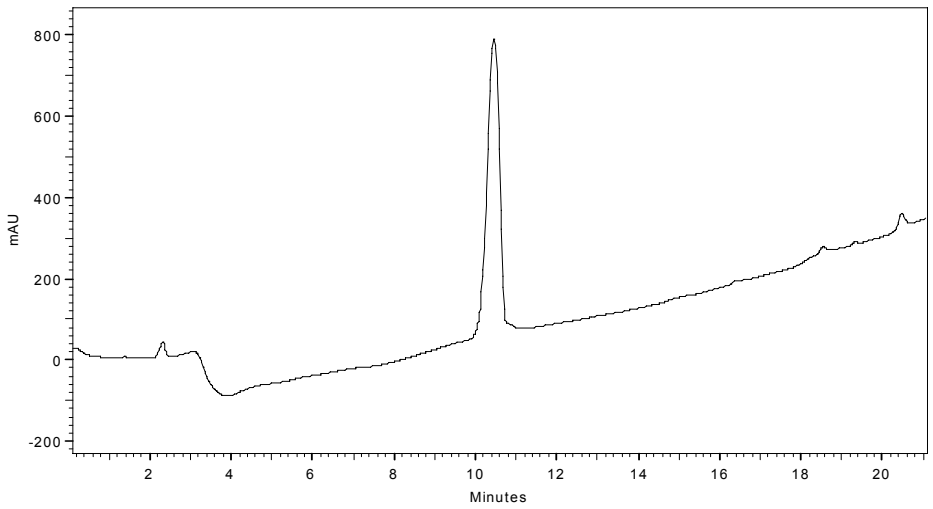


Fig. 3.85. HPLC chromatogram of compound KT32/2 detected at 203 nm

Compound KT32/2 was obtained as white powder after lyophilization. However, the compound was also obtained as colourless crystals after grown on methanol. The compound was detected at 203 nm under UV as presented in Fig. 3.86.

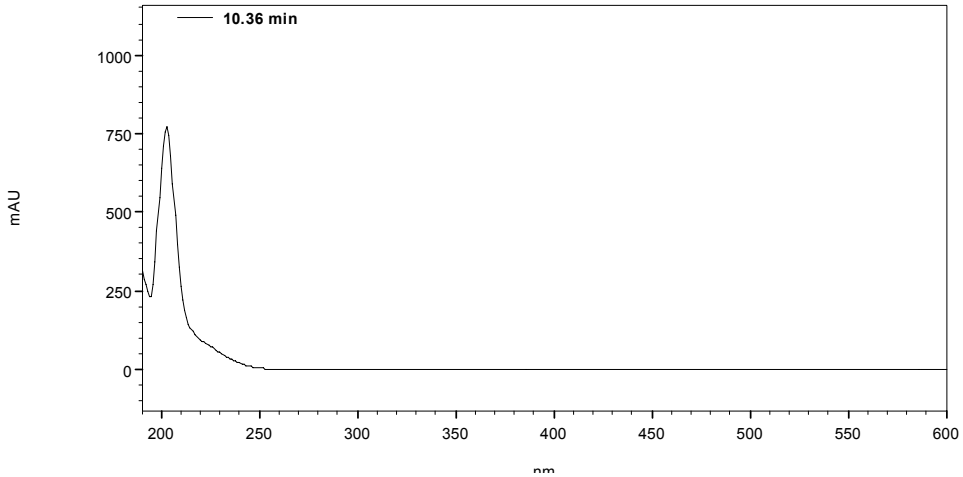


Fig. 3.86. UV spectrum of KT32/2 (λ_{max}. 203 nm; in MeOH)

3.7.4.3. Structure Elucidation of Compounds KT32/1 and KT32/2

Single-crystal X-ray diffraction analysis was performed to determine the molecular structure of compound KT32/1. One plate (600 x 150 x 5 µm) was cryocooled in a nitrogen stream at 100 K (Cryostream, Oxford Cryosystems, Oxford, UK) for X-ray diffraction data collection. The data were solved using SHELX (Sheldrick, 2008). According to this analysis the substance was determined as $C_{12}H_{18}O_3$. The ORTEP drawing and 3D model of the compound are presented in Figs. 3.87-88.

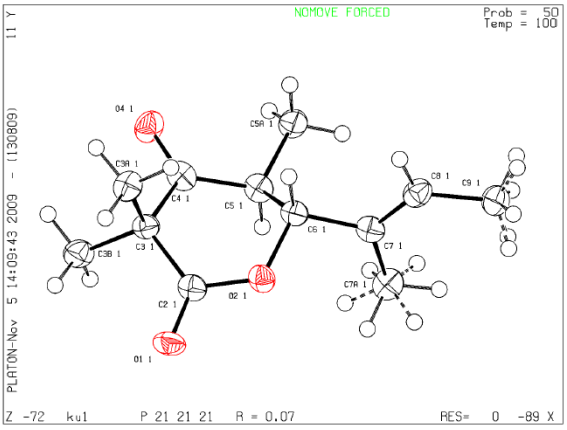


Fig. 3.87. ORTEP drawing of compound KT32/1 ($C_{12}H_{18}O_3$)

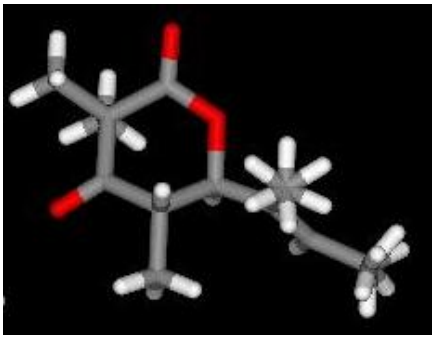


Fig. 3.88. 3D Model of the relative configuration of compound KT32/1 ($C_{12}H_{18}O_3$)

In comparison with Chemical Abstracts, it could be found two isomeric similar compounds, i.e. helicascolides A and B. The two lactones were isolated from marine fungus *Helicascus kanaloanus* (Poch & Gloer, 1989). However, the compound isolated from unidentified fungus KT32 in the present study was found for the first time. Therefore, crystallographic data for the structural analysis of the compound has been deposited with the Cambridge Crystallographic Data Centre (Deposition No. CCDC 793692).

Regarding to the previous related known compounds, the new compound found in this study was named helicascolide C. New lactone helicascolide C was isolated along with related structurally known compound helicascolide A (compound KT32/2). Both compounds were obtained as colourless crystals.

The molecular formula of helicascolide C was also established as $C_{12}H_{18}O_3$ by the molecular ion peaks at *m/z* 265.14082 ($[M+MeOH+Na]^+$) and 233.11464 ($[M+Na]^+$) in positive-ion mode ESI-FTICR-MS, and 179 ($[M-MeOH]^-$) in negative-ion mode ESI-MS. Interpretation of ^1H- and ^{13}C-NMR spectral data confirmed the presence of 12 carbon atoms, 18 protons, and two lactones C=O groups (Table 3.6). Five Me groups were observed, which by their chemical shifts were all bound to carbon atoms (δ(H) 1.704 (*br d*, Me(12)), 1.700 (*s*, Me(11)), 1.395 (*s*, Me(9)), 1.382 (*s*, Me(8)) and 0.945 (*d*, Me(10)). The positions of the Me groups (δ(H) 1.704 (*br d*, Me(12)) and 1.700 (*s*, Me(11)) were corroborated by the HMBCs Me(12)/C(6), Me(11)/C(6), Me(12)/C(7), and Me(11)/C(7), respectively. Fig. 3.89 presents the molecular structures of helicascolides A and C.

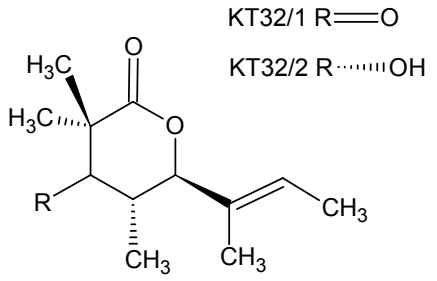


Fig. 3.89. Molecular structures of KT32/1 and KT32/2

The relative configurations at the stereogenic C-atoms were established by ROESY experiment. The vicinal coupling constant *J*(4,5) of 11.5 Hz indicated the *trans* position of the protons H – C(4) and H – C(5). This coupling was also found in helicascolide A along with the other vicinal coupling constant *J*(3,4) of 11.1 Hz showing *cis* position of the protons H – C(3) and H – C(4) which was not found in helicascolide C.

Essentially the NMR spectra of helicascolide A were very similar to those of helicascolide C as shown in Table 3.8. The main difference was revealed by a signal characteristic for ketone group in the ^{13}C-NMR at δ 209.9 ppm (213.8) (C(3)) of helicascolide C, while at the same position the signal of compound KT32/2 was replaced by hydroxyl group δ(C) 77.4. The interpretation was confirmed by the IR spectrum which exhibited the presence of carbonyl groups by the strong absorption at 1720-1712 cm^{-1} and there was lacking absorption of hydroxyl group found in region 3400-3200 cm^{-1}. Optical rotation of the compounds KT32/1 and KT32/2 were $[\alpha]_D^{22} = +21.2^o$ (0.34% in MeOH) and $[\alpha]_D^{22} = -35.3^o$ (0.14% in MeOH), respectively.

Table 3.7. NMR Data of Helicascolide C (KT32/1). Recorded in CD_3OD at 600 (^{1}H) and 150 MHz (^{13}C); δ in ppm, *J* in Hz

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm] m (*J* [Hz])	HMBC (to C)	Selected ROE
1	177.0	---		
2	52.6	---		
3	209.9	---		
4	44.5	2.927 *dq* (11.5; 6.7)	3, 5, 6, 10	8, 11
5	86.4	4.687 *d* (11.5)	3, 6, 7, 10, 11	7, 9 (?)
6	132.3	---		
7	129.1	5.710 *m*	5, 12	5, 10
8	23.8	1.382 *s*	1, 2, 3, 9	
9	23.9	1.395 *s*	1, 2, 3, 8	
10	10.2	0.945 *d* (6.7)	3, 4, 5	11
11	9.7	1.700 *s*	5, 6, 7	
12	13.3	1.704 *br d* (7.8)	6, 7	

Table 3.8. NMR Data of Helicascolide A (KT32/2). Recorded in CD_3OD at 600 (^1H) and 150 MHz (^{13}C); δ in ppm, *J* in Hz

Position	δ ^{13}C [ppm]	δ ^1H [ppm] m (*J* [Hz])	HMBC (to C)	Selected ROE
1	180.6	---		
2	45.6	---		
3	77.4	3.454 *d* (1.8)	1, 2, 4, 5, 8, 10	4, 10
4	32.4	2.333 *dqd* (11.1; 6.7; 1.8)	5, 6, 10	3, 8, 11
5	90.1	4.644 *d* (11.2)	4, 6, 7, 10, 11	7, 10
6	133.4	---		
7	127.2	5.575 *br qd* (6.7; 1.3)	5, 12	5, 10
8	26.9	1.280 *s*	1, 2, 3, 9	
9	23.3	1.285 *s*	1, 2, 3, 8	
10	14.2	0.895 *d* (6.8)	3, 4, 5	11
11	10.4	1.636 *dq* (1.3; 1.1)	5, 6, 7	
12	13.3	1.671 *dq* (6.7; 1.1)	6, 7	

In order to ensure that compound KT32/1 was not the result of oxidation process of the known compound helicascolide A, single crystal obtained from fraction F3 was directly elucidated by X-ray diffraction instead of further purified to reduce the isolation process. The single crystal analysis confirmed that the crystal was determined as the same compound, helicascolide C. The crystals of helicascolide C is presented in Fig. 3.90.

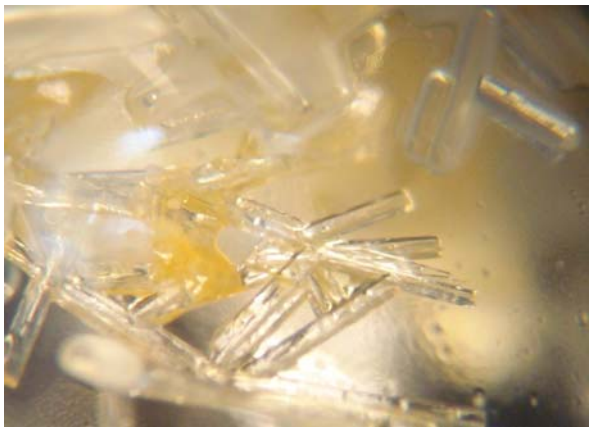


Fig. 3.90. Crystals form of KT32/F3

3.7.4.4. Biological Activity of Compounds KT32/1 and KT32/2

In biological activity assays, compound KT32/1 (helicascolide C) showed fungistatic activity at 200 µg/spot against phytopathogenic fungus *Cladosporium cucumerinum*. The positive control 80 ng of benomyl afforded inhibition area of 78.5 mm². However, both compounds showed no antibacterial activity at 300 µg/disc against *Staphylococcus aureus* and *Bacillus subtilis.* The 200 µg/disc of compounds KT32/1 and KT32/2 also showed no activity against *Escherichia coli* and *Pseudomonas aeruginosa* as well as exhibited no cytotoxic activity against human bladder carcinoma 5637 cell line at concentration of 1.18 and 1.19 mM, respectively. From the previously study the biological properties of helicascolides were not mentioned (Poch & Gloer, 1989).

3.7.4.5. Fractionation and Purification of Compound KT32/3

Fraction F8 isolated from EtOAc extracts of KT32 as described in **3.7.4.1** was obtained as brown needle-like crystals after grown on MeOH. The substance (later written as KT32/3) was detected as dark violet spot with R_f value of 0.2 on TLC Si60 eluted with DCM/EtOAc (65:35, v/v) after spraying with AS reagent.

3.7.4.6. Structure Elucidation of Compound KT32/3

Single-crystal X-ray diffraction analysis was conducted to determine the chemical structure of compound KT32/3. The molecular formula of the compound was determined to be $C_{10}H_{10}O_4$. Compared to the data of Chemical Abstracts the compound was known as 3-hydroxyisosclerone, a metabolite previously isolated from *Sclerotinia sclerotiorum.* The relative configuration of the compound is presented in Fig. 3.91.

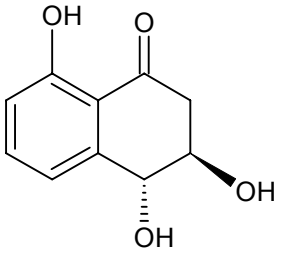


Fig. 3.91. Structure elucidation of compound KT32/3 ($C_{10}H_{10}O_4$)

HR-FTICR-MS of the compound shows a $[M-H]^-$ ion at *m/z* 193.05019 (calc. for $C_{10}H_9O_4^-$ 193.05063) suggesting a molecular formula of $C_{10}H_{10}O_4$ (MW. 194). Proton and ^{13}C-NMR data also confirmed the elucidated compound. Interpretation of ^{1}H- and ^{13}C-NMR spectral data corroborated the presence of 10 carbon atoms, 10 protons, and three hydroxyl groups (Table 3.9).

Table 3.9. NMR Data of compound KT32/3 recorded in CD_3OD at 600 (^1H) and 150 MHz (^{13}C); δ in ppm, *J* in Hz

Position	δ ^{13}C [ppm]	δ ^1H [ppm] m (*J* [Hz]) {pos.}
1	204.34	---
2	44.31	3.079 *dd* (17.2/4.0) {α} 2.701 *dd* (17.2/8.0) {β}
3	71.68	4.073 *ddd* (8.0/6.8/4.0) {α}
4	73.23	4.606 *d* (6.8) {β}
5	120.02	7.123 *ddd* (7.6/1.1/1.1)
6	138.00	7.532 *dd* (8.3/7.6)
7	117.78	6.862 *d* (8.3)
8	163.25	---
9	116.74	---
10	145.83	---

4. DISCUSSION

4.1. Isolation and Identification of Marine Fungi

Fungi, the second most diverse organism groups are still less explored. From about 1.5 Mio estimated fungal species only 5% have been described (Hawksworth, 1991; 2001). This percentage will be lower if used 9.9 Mio as the total number of fungi. There are various numbers of estimated fungal species in the world, varying from 0.5 to 9.9 Mio (Mueller & Schmit, 2007).

The proportion of the investigated fungi is probably higher, since plenty of literatures reported marine natural products isolated from unidentified fungi. However, fungi are still less explored and offer variety of novel natural products to be discovered. Cannon (1997) stated that the low level of knowledge in fungi is due to some factors as follows: most fungi are microscopic, many produce their fruit bodies ephemerally and unreliably, species concepts are archaic in most cases, and systematic mycology has historically been under-resourced compared with many other groups.

Identification of fungi in the context of marine natural products is necessary. Marine fungal identification leads to the isolation of certain groups of natural products, particularly novel compounds. In other words, identification of fungi is useful in dereplication purposes of isolated natural products. Thus, new compounds of the identified fungi can be determined easily based on the existing data of known natural products. These data are also helpful to trace the presence of new compounds resulted from metabolic engineering.

Fungal species is mostly determined according to the reproductive structure, namely spore because of its stability in form and size (Kohlmeyer & Kohlmeyer, 1979). This conventional method is currently complemented by molecular method. Nevertheless, fungal experts are still relying on spores to identify the fungi.

In this work 32 fungal strains have been successfully isolated from Indonesian marine area, which mostly belong to mould groups. Based on the fact that some moulds as *Penicillium* sp., *Aspergillus* sp. and *Mucor* sp. have been well investigated, thus the present study focused on 11 isolates of "higher" marine-derived fungi which seem not to belong to the mould groups except *Aspergillus* sp. KT13 which developed no conidiophores on basal medium used in the present study, i.e. Hagem medium.

In order to induce sporulation of the isolated fungi, different media have been used in our study. Media which are suitable for fungal sporulation consist of complex carbon

source, e.g. wood-mill, or poor nutrient content as corn-meal and carrot-potatoes agar. However, mostly the samples are still in vegetative status. Therefore, from 11 fungal isolates are only five strains which have been successfully identified, i.e. *Aspergillus* sp. KT13, *Lasiodiplodia theobromae* KT26 (identified by Dr. Peter Hoffmann), *Epicoccum nigrum* KT28 (identified by Dipl.-Biol. Beate Cuypers), *Xylaria psidii* KT30 (identified by PD. Dr. Marc Stadler) and *Coniothyrium* sp. KT33 (identified by Drs. Jan Kohlmeyer and Brigitte Volkmann-Kohlmeyer).

The difficulty in identification of marine fungi is mostly due to their microscopic size. It is different with the terrestrial fungi which mostly belong to the macroorganisms, therefore they can be distinguished more rapidly. Microfungi which can be determined simpler are of moulds. For example, *Aspergillus* sp. and *Penicillium* sp. are relatively easy to identify by their specific conidiophores form. However, more strains show similar spore forms.

Consequently, it is not a simple task to identify the marine fungi. In addition, fungi isolated from specific environment such as marine habitat do not easily sporulate outside of their own environment. Besides the unpredictably in spore production of the fungus, this work is also time-consuming. Ebel (2010) recently reviewed that it is difficult to obtain sexually reproducing forms and thus presently using molecular biology-based methods as sequencing rDNA should be preferred. The rapid taxonomic assessment of fungal strains would be useful for drug discovery because it could help to avoid replication of known substances. In future this task needs more attention.

In phylogenetic study of the fungi, molecular genetic study also played a significant role in finding the true ancestor of basidiomycetes. Since fungal structures did not fossilize well as other organisms, mycologist studied phylogeny of the fungi only based on morphology features of the extant fungi. Therefore, formerly it was supposed that the red algae are ancestor of the higher fungi basidiomycetes (Demoulin, 1974; 1985). However, the genetic study afterwards did not support this evolutionary relationship between red algae and fungi (Kwok *et al*., 1986). The study of Kwok *et al.* (1986) found that basidiomycetes are more closely related to other fungi than to red algae and protozoa; red algae are no more closely related to higher fungi than green algae, dictyostelids and other lower eukaryotes.

4.2. Nutrients and Fungal Growth

Fungi are heterotrophic eukaryotes that absorb their food (osmotrophic) (Kendrick, 2000). All fungi depend on organic carbon which is a major part of living plants, animals and microbes. Carbon in the form of structural materials such as cellulose, lignin, chitin and keratin are normally difficult to degrade. However, fungi are able to degrade all these molecules (Jennings & Lysek, 1999). Since fungi are non-diazotrophic, they need to be supplied with nitrogen-containing compounds, either in inorganic or organic form (Walker & White, 2005).

In the present study, we used Hagem medium as the basal medium. This medium was chosen according to the previous studies in our groups (Kusnick, 1997; Froben, 2000; Shushni *et al.* 2009). The results showed that Hagem medium was suitable for marine fungal growth as well as for bioactive metabolites production. Moreover, Kreisel and Schauer (1987) stated that complex medium such as Hagem medium which consists of glucose, malt-extract and ammonium succinate as carbon and nitrogen source is suitable for fungi to produce secondary metabolites.

Fungi cultivated in shake cultures produce more active compounds than those from still cultures (Helmholz *et al.,* 1999; Shushni *et al.,* 2009). However, a still culture may also induce bioactive metabolites production. Griffin (1994) stated that fungi cultivated in still culture undergo two different conditions. The surface layer is aerobic but has poor contact with the medium, whereas the underside of the mat is anaerobic. This situation may result in growth inhibition and excessive production of secondary metabolites, depending on the characteristics of the fungus. Therefore, as a comparison the still culture is necessary to observe in the future.

The pH of seawater is about 8 (Blomberg & Adler, 1993). However, the pH value of Hagem medium used in this study was set at 7.5, according to Helmholz *et al.* (1999). After 3 days of cultivation the pH value of most strains decreased to about 5. The pH depletion indicates that the ammonium salts are consumed (Kreisel & Schauer, 1987; Papagianni *et al.,* 2005) or H^+ ions are discharged by the fungal mycelia during exponential phase of growth (Kreisel & Schauer, 1987).

4.3. Interaction between Two Fungi

In an attempt to investigate the interaction between two fungi, isolate KT31 was cultured together with strains KT19, KT27 and *Xylaria psidii* KT30. The results showed that fungus KT31 grew well and were more aggressive than the other strains. However, no

inhibition zone was observed. This phenomenon indicates that the two fungi showed neutralism interaction. It was also possible that one strain produces antifungicides and the other detoxifies the compounds (Tsujiyama & Minami, 2005).

Performance of either or both components of two interacting mycelia or a mycelium interacting with another microorganism is modified through one of the three broad phenomena: antagonism, mutualism or neutralism (Cooke & Whipps, 1993). In this study, dual culture of strains KT31 and KT27 showed a salient feature of the mycelia due to interaction between the two fungi. Fungus KT27 developed chlamydospores and initiated fruiting bodies formation as responses to the fungus KT31. This result was in agreement with Cooke and Whipps (1993). They stated when mycelia of two species meet or intermingle; a frequent result is stimulation of sporulation in one or both fungi.

Pigmentation and browning of mycelia were observed in dual culture of KT19 and KT31 (Fig. 4.1). The pigmentation was found strongly in the confrontation region. This occurrence was suggested due to competitive action between the two fungi. Tsugiyama and Minami (2005) stated that pigmentation detected at confrontation region in dual culture was related to phenol-oxidizing enzymes which are useful in blocking invasion. Phenol-oxidizing enzymes such as laccase and peroxidase that are produced by white-rot fungi are confirmed to participate in detoxification of xenobiotic compounds, for example antifungicides (White & Boddy, 1992; Score *et al.,* 1997; Baldrian, 2004; Tsugiyama & Minami, 2005; Velásquez-Cedeño *et al.,* 2007; Hiscox *et al.,* 2010).

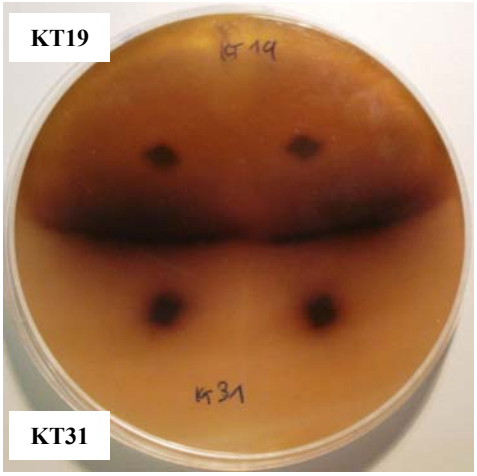


Fig. 4.1. Confrontation region in dual culture of strains KT19 and KT31

Laccases secreted by fungi have also been reported to possess an important role in the degradation of some toxic environmental pollutants, such as polychlorinated biphenyls (PCBs). Jonas *et al.* (1998) reported that laccases isolated from white-rot fungi *Trametes versicolor* and *Pycnoporus cinnabarinus* are capable to transform hydroxylated biarylic ethers to oligomerization products. Laccase produced by *P. cinnabarinus* was also reported to dechlorinate and detoxify chlorinated hydroxylbiphenyls (Schultz *et al.,* 2001; Keum & Li, 2004). The oligomerization process involved in the transformation process demonstrated the dehalogenization ability of laccase. Laccases from white-rot fungi *Pleurotus ostreatus* and *Myceliophthora thermophila* were also reported to be able to degrade chlorinated hydroxybiphenyls (Keum & Li, 2004; Kordon *et al.,* 2010). Regarding to the potential of laccase in the degradation of xenobiotic compounds, it would be interesting to investigate the capability of fungi KT19 and KT31 to degrade the toxic pollutants.

However, physiological complementarity between neighbouring individuals in some circumstances may provide mutualism benefit. For example, mixtures of cellulolytic species – each with its own spectrum of cellulases, may affect cellulolysis more efficiently than a single species acting alone (Cooke & Whipps, 1993).

4.4. Bioactive Compounds of Marine Fungi and Endophytic Fungi

Fungi living in marine environments are normally related to other organisms. The fact is obviously shown by their important roles in the environment. In general, existence of fungi in marine environment is classified into three main groups, which are saprophytic, parasitic and symbiotic fungi.

Production of secondary metabolites is often associated to their role in the environment. As an example, Schulz *et al.* (2002) reported that endophytic fungi which were isolated from healthy algae and plants produced natural products in higher proportion compared to the soil isolates. Based on this condition, it was assumed that the presence of the fungi in seaweeds or plants as their hosts affected the creativity of the fungi to produce bioactive metabolites.

From the present study were found that five fungal isolates, i.e. *Aspergillus* sp. KT13, *Mycelium sterilium* KT19, *Mycelium sterilium* KT29, *Xylaria psidii* KT30 and *Mycelium sterilium* KT31 exhibited considerable bioactivity against human- and fish pathogenic bacteria. Isolates *Aspergillus* sp. KT13 and KT31 were remarkably active against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, while strains

KT19, *X. psidii* KT30 and KT31 were predominantly active against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa.* All five isolates were active toward Gram-negative fish pathogenic bacteria *Vibrio anguillarum* and *Aeromonas salmonicida*. Three algicolous fungi KT29, *X. psidii* KT30 and KT31 showed significant antifungal activity against *Candida maltosa* and phytopathogenic *Cladosporium cucumerinum.*

Two strains *X. psidii* KT30 and KT31 which were isolated from the same algal specimen *Kappaphycus alvarezii* BRKA-1 showed strong cytotoxicity against cultivated human bladder carcinoma cell line 5637. Moreover, an unidentified fungus KT32 showed moderate activity in related cytotoxic assay. Besides ethyl acetate extracts isolated from culture broth, DCM extracts from mycelia of these fungi were found to be active in antifungal assay against *C. maltosa* as well as in cytotoxic assay.

Fungal strain KT15 was isolated from mollusc shell. Ebel (2010) mentioned that fungal strains isolated from marine invertebrates are so far less characterized; even they are among the most prolific secondary metabolites producers. Unfortunately, the fungus KT15 showed no significant bioactivity in our test systems.

There are several papers reporting the natural products of fungi isolated from marine algae, particularly from algal species found in temperate zones. Brown alga *Fucus serratus* as an example has been studied for a few years (Ebel, 2010). There are several active compounds isolated from fungi associated with *F. serratus* and other species as *Fucus vesiculosus* and *Fucus spiralis* (Schulz *et al.,* 2002). Abdel-Lateff *et al.* (2003) also reported some secondary metabolites from *Epicoccum* sp. which was isolated from *F. vesiculosus.* Marine fungi were also reported to be found in brown alga *Sargassum horneri* (Nguyen *et al.,* 2007), red alga *Acanthophora spicifera* (Greve *et al.,* 2008) and green algae *Ulva* sp. (Gamal-Eldeen *et al.,* 2009); *Ulva pertusa* (Cui *et al.,* 2010).

In the search for groups of microorganisms as good producers for biologically active metabolites, Schulz *et al*. (2002) concluded that endophytic fungi are a good source of novel secondary metabolites. From their study of thousands fungi isolated from Germany, Costa Rica and Mallorca they found that from a comparison of 135 isolated metabolites, endophytic fungi afforded more new structures with 51% proportion compared to soil isolates with 38%. This study also recommended concentrating in searching for novel metabolites on organisms that inhabit novel biotopes and/or hosts. Tan and Zou (2001) also reported that endophytes (either bacterial or fungal microorganisms) are a rich source of functional metabolites.

Endophytes moreover have been reported to make associations with higher life forms and may precede to biochemically mimic the host organism (Stierle *et al.,* 1993; Strobel *et al.,* 1996; Wang *et al.,* 2000; Zhou *et al.,* 2009). For example, *Taxomyces andreanae,* an endophytic fungus isolated from a yew tree (*Taxus* sp.) also demonstrated to produce taxol® (Strobel *et al.,* 1996; Strobel, 2003; Strobel & Daisy, 2003).

4.5. Screening Strategies for Bioactives Production

In the present study, varying of treatments and culture conditions were applied to induce the fungal cultures to produce bioactive secondary metabolites. However, the inductions were not always followed by production of more potent metabolites. Miller (1986) stated that the production of secondary metabolites of fungi in quantity in the laboratory is a difficult problem, since very limited understanding of secondary metabolites exists for filamentous fungi. Certain conditions are required for the fungi to produce secondary metabolites.

Bioactives production in fungi is influenced by such factors as the nature of the fungus, its substratum and stage of development. Primarily marine fungi are distributed on the basis of their response to salinity and temperature. In the case of lignicolous marine fungi, salinity, temperature and availability of substrate will determine a priori whether a settling fungal spore will grow (Miller, 1986).

Nutritional modifications such as carbon and nitrogen sources can affect productivity of marine fungi to produce active metabolites. As an example, Kansoh *et al.* (2010) reported that 2.0% starch and 0.24% potassium nitrate as a carbon and an inorganic nitrogen sources, respectively supported the highest level of antimicrobial production by *Penicillium viridicatum.*

Penicillium and *Aspergillus* are fungi that have been intensively investigated in searching for bioactive secondary metabolites, both terrestrial and marine origin. Thus, there has been philosophy to avoid common genera like *Penicillium* and *Aspergillus* due to the risk of rediscoveries. However, they are continuously showing to produce novel and biologically active compounds. According to Rømer-Rassing and Gürtler (2002), *Penicillium* and *Aspergillus* are among the genera with the highest index of creativity in the Kingdom of Fungi. It can be found from some recent literatures which reported new compounds isolated from both fungi (Ishikawa *et al.,* 2010; Lee *et al.,* 2010; Wei *et al.,* 2010; Zhao *et al.,* 2010).

4.5.1. Salinity

Culture condition affects the production of secondary metabolites in organisms. One of the environmental factors that distinguish between terrestrial and marine habitat is salinity. In addition, this parameter is relatively simple to modify. Thus, in the present study salinity was used as main factor to acquire the more active compounds, both quantitatively and qualitatively. The 3% of salinity applied here was determined according to the average of seawater salinity in Indonesia.

From 11 fungal strains, eight strains showed more activity when cultivated in the freshwater medium, in contrary three strains (KT19, KT28 and KT29) possessed stronger activity in the seawater medium. In general, the production of their secondary metabolites had negative correlation to their mycelial growth. Therefore, it could be roughly concluded that the most fungal strains in the present study preferred high salinity medium for their growth.

4.5.2. Fermentation Period

Production of secondary metabolites in organism is growth-depending. This phenomenon is obviously shown by the fluctuation of the secondary metabolites during cultivation. For example, concentrations of several antibiotics were shown to decrease in the course of prolonged cultivation. Demain (1986; 1998) furthermore stated that secondary metabolites often have unusual structures and their formation are regulated by nutrients, growth rate, feedback control, enzyme inactivation and enzyme induction.

There are some mechanisms to prevent suicide due to its own secondary metabolites which are normally called as self-defence mechanisms. Some proposed mechanisms in microorganisms are such as production of antibiotic-inactivating enzymes in producers; or not producing active drug molecules intracellularly. Another mechanism is metabolic shielding, whereby antibiotics are produced and secreted as inert prodrugs and subsequently will be activated during or following export (Cundliffe & Demain, 2010).

In the present study, the fungal strains mostly produced metabolites with the strongest bactericide properties in relatively short culture period, i.e. about one week of cultivation, whereas the pH value of this culture period was mostly low. In this period the growth phase of the fungi was mostly still in exponential growth phase. During the exponential phase, fungi are undergoing primary metabolism. In industrial fermentations requiring maximum cell biomass or primary metabolites production, such as baker's yeast

production, this growth phase is normally to be prolonged by fed-batch or continuous culture techniques (Walker & White, 2005).

Otherwise it was shown for the example KT31 that the chemical diversity of the extract is increasing with prolonged cultivation time. Therefore, in an attempt to obtain more variety of metabolites, the fungus was cultivated in relatively longer culture period and at the same time surely considered the metabolites yield.

However, secondary metabolites are mostly produced when fungi enter a stationary growth phase. This stationary phase is normally following the exponential phase due to nutrient deficiency. Besides nutrient deprivation, other physiological causes such as presence of toxic metabolites, low pH, high CO_2, variable O_2 and high temperature may promote fungal cells entering stationary phase (Walker & White, 2005).

4.5.3. Extreme Culture Condition

As mentioned before the production of secondary metabolites in marine microorganisms is due to their extreme environment. Therefore, in the present study salinity was used to set up the extreme condition to induce the marine fungus KT31 to produce more bioactive secondary metabolites.

The 10% of salinity was found to be suitable for fungal growth as can be seen from the double biomass yield compared to the biomass obtained from the culture in basal medium. This data was interesting since 10% salinity was much higher than the average salinity of seawater in Indonesia. As consequently, the extracts obtained from this culture showed less activity compared to the standard culture. This result was related to the study of Zalar *et al.* (2005) which reported that marine fungi, particularly those belonging to the indigenous halophilic microbial communities, can grow and adjust across the whole salinity range, from freshwater to almost saturated NaCl solutions. Blomberg and Adler (1993) previously stated that salt-tolerant fungi have developed very efficient mechanisms to solve salinity stress. However, 20% of salinity was found to be very extreme to the fungus KT31, so that it grew very slowly and mostly samples were dead.

Kunčič *et al.* (2010) reported that some marine fungi, e.g. *Wallemia muriae* and *Wallemia sebi,* demonstrated adaptive responses to the high salinity by increasing their size of mycelial pellets. The marine fungus *Wallemia ichthyophaga* showed adaptive response by increasing the cell wall thickness.

In our study, mycelia of the fungus KT31 cultured in freshwater and 10% salinity media were found to be similar. According to these results, it can be roughly assumed that

freshwater medium is an extreme condition to the marine fungus KT31. Furthermore, the fungus was found to be more adapted to the low salinity by producing more secondary metabolites than to the very high salinity.

Response of a fungus to salinity stress involves many diverse capacities of the organism (Blomberg & Adler, 1993). Fungi adapt to this situation primarily by excluding the ions from the cytoplasm and by employing alternative osmotica to restore the cell volume and turgor required for growth. Conway *et al.* (1954) reported that *Saccharomyces cerevisiae* was able to export Na^+ against the Na^+ concentration gradient across the membrane. It was also found that the rate of Na^+ efflux was a function of the intracellular Na^+ content (Rothstein, 1974).

Fungi require sodium ions for their growth (Jennings & Lysek, 1999). Moreover, zoosporic fungi like *Althornia* and *Thraustochytrium* demand Na^+ at high concentration that gives the element the status of macronutrient. Tresner and Hayes (1971) previously reported that the penicillia and the aspergilli could tolerate 20% NaCl or more. Therefore, it was not surprising that in this study fungus KT31 grew well in high salinity (~1.7 M NaCl).

Salt tolerance of the fungi is conditioned by quality of the carbon source (Bellinger & Larher, 1990). Their study on the influence of nutrient quality on the salt tolerance of *S. cerevisiae* showed that in acetate medium the yeast could grow in up to 0.45 M NaCl, whereas in glucose medium up to 1.5 M NaCl.

Salt tolerance of the fungi is also corresponding to the polyol accumulation (Adler *et al*., 1982; Davis *et al.,* 2000). Their study on *Aspergillus niger* and *Penicillium chrysogenum* showed that intracellular content of polyol was markedly enhanced by high medium salinity. Through out the entire growth cycle of the fungi at high salinity, only minor portions of the total polyols were excreted to the external medium. The polyol accumulation proceeded through the vegetative growth phase and reached a high and stable level during the late log and early stationary phase.

Furthermore, Adler *et al.* (1982) found that the increased polyol was due to the enhanced levels of glycerol and erythritol. Fermentation of *P. chrysogenum* in medium with 12% of NaCl concentration and using 1% glucose as carbon source amounted glycerol up to 20% of dry weight of the biomass. Cultivation of *A. niger* using the same medium yielded glycerol up to 21% of the biomass. The corresponding values of glycerol contents in mycelia grown in basal media were only 6 and 7%. According to Adler (1978), glycerol and other polyols are poor inhibitors of enzyme functions even in very high concentrations.

Therefore, glycerol may be a preferred compatible solute in fungi grown under salt stress (Adler *et al.,* 1982). Some reports stated that glycerol appears to be the primary osmotic adjuster in growing cells subjected to salinity stress (Adler & Gustafsson, 1980; Beever & Laracy, 1986; Wethered *et al*., 1985; Kelly & Budd, 1990).

4.5.4. Mixed Culture

Bioactive compounds in microorganisms which are mostly belonging to the secondary metabolites are produced as mechanism of defence or survive to their environmental condition. Culture competition or presence of strange substances in the culture thus can be used to stimulate the production of microbial secondary metabolites.

Cueto *et al.* (2001) reported that a marine deuteromycete *Pestalotia* sp. produced a benzophenone antibiotic (pestalone) when mixed fermented with an unidentified antibiotic-resistant marine bacterium. The pestalone was not detected when either strain was cultured individually. Ho *et al.* (2003) also found that culture filtrates obtained from mixed-culture of *Chloridium* sp. and *Sporochisma mirabile* showed antifungal activities against *Candida albicans*, while culture filtrates obtained from monoculture showed no activity.

In the present study we used autoclaved *Staphylococcus aureus* as the strange material. This treatment influenced the fungus to produce more active metabolites than the pure culture. Fungal culture which was added with the autoclaved *S. aureus* at the inoculation time exhibited stronger activity compared to the culture with the *S. aureus* death cells added on day 3 or standard culture. This study showed that addition of strange material in the fungal culture can be used to enhance bioactive metabolites production.

4.6. Bioactivity Screening

In the search for bioactive compounds, 55 extracts of 11 fungal isolates were tested in screen for antimicrobial, antifungal, antioxidant and cytotoxic activities. From each strain 5 extracts were obtained, i.e. ethyl acetate and ethanol extracts isolated from culture broth, while dichloromethane, methanol and water extracts were prepared from mycelia.

The results of biological activity screening presented that all ethyl acetate extracts were active at least against one of the test organisms. It is in sharp contrast to biological activity of mycelial extracts which was found very seldom. Only dichloromethane extracts of KT30 and KT31 showed medium antifungal activity against *Candida maltosa.* These results complied with mostly reported papers on bioactive compounds isolated from fungal

culture broth in several reviews (Debbab *et al.*, 2010; Ebel, 2010). Therefore, it can be assumed that marine-derived fungi mostly produce extracellular metabolites.

The presence of bioactive metabolites in the culture medium or known as extracellular metabolites is possibly relating to self-defence mechanisms of the fungi. The mechanisms are suggested to prevent suicide due to its own secondary metabolites. Various mechanisms in the fungi and other microorganisms have been presumed, as an example metabolic shielding mechanism. In this metabolic shielding mechanism, antibiotics are produced and secreted as inert prodrugs and subsequently will be activated during or following export (Cundliffe & Demain, 2010).

Since secondary metabolites in the fungi serve as competitive weapons against other microorganisms, it can be expected that bioactive compounds from fungi are normally secreted as extracellular metabolites. Taking into account that there is a great volume of water around the fungi this means that the metabolites should have a very strong activity or that they act only over short distances direct on neighbouring microorganisms. This could be also a mechanism of the fungi to minimize toxicity of the metabolites inside the cells. Vining (1990) furthermore mentioned that few secondary metabolites accumulate intracellularly.

From chemical structure point of view, Ebel (2010) stated that the spectrum of biological activity of new natural products isolated from marine-derived fungi was dominated by polyketides. These polyketides were then followed by other groups including meroterpenoids, nitrogen-containing polyketides, alkaloids, diketopiperazines, peptides, terpenes, lipids and phenylpropanoids.

4.7. Secondary Metabolite Profiling in Chemotaxonomy and Pigments in Marine Fungi

Marine fungi represent an ecological term instead of taxonomic classification. Morphologic as well as genetic characteristics are required to identify fungi taxonomically. In this work we define all fungi occurring in marine environment as “marine fungi”.

Reproductive spores are so far used particularly in morphological determination of fungi. Obviously spore is the most important morphological character of fungi, since this reproductive structure is relative constant in shape and size of each strain (Kohlmeyer & Kohlmeyer, 1979).

The production of reproductive spores is relatively different in fungi. Some fungal strains need obviously such special conditions for production of sexually reproductive

structures that these spores could not be found in our investigations. Therefore, it becomes a common problem in determination of fungal taxonomy. Besides the usual reproductive character, metabolites are used frequently to determine some fungal strains.

Chemotaxonomy is a term used for appliance of metabolite spectrum for classification purpose. Fungi are good producers of secondary metabolites; therefore secondary metabolites have been used frequently in fungal taxonomy. For example, fungal chemotaxonomic studies in *Alternaria, Aspergillus, Fusarium, Hypoxylon, Penicillium, Stachybotrys, Xylaria* and in few basidiomycete genera (Frisvad & Filtenborg, 1984; Frisvad *et al.,* 2007; Frisvad *et al.,* 2008) are mentioned. In our study, identification of *Aspergillus* sp. KT13 was successfully done according to this approach.

4.7.1. Chemotaxonomy in *Aspergillus* sp.

Aspergillus is well-known as producer of several toxins such as aflatoxins -potent human carcinogens, ochratoxins and patulin. There are plenty of publications reporting toxin of *Aspergillus* origin. Bräse *et al.* (2009) have been listed mycotoxins produced by *Aspergillus* and other prolific toxin sources, i.e. *Penicillium* and *Alternaria*. Moreover, some strains are categorized as human pathogenic strains which cause aspergillosis. Despite the "negative roles" of the fungus, *Aspergillus* produces also valuable metabolites particularly for drug discovery purposes.

In the present study a chromane derivative was effectively isolated from the fungal isolate KT13 which has not yet been identified due to the absence of its conidia (on Hagem agar as basal medium). The isolated compound was found very intense in the fungus which composed nearly 9% of the ethyl acetate extract. Comparison of the elucidation data of the compound with known compounds in Chemical Abstracts revealed that the compound belongs to the chromane derivatives.

In previously researches Fukami *et al*. (1991) and Cutler *et al*. (1996) isolated similar chromane derivatives from *Aspergillus niger* and *Aspergillus fumigatus* as metabolites of the polyketide pathway. A derivative isolated from *A. fumigatus* was also known as ruakuric acid.

Based on this result then the strain KT13 was cultured in two of several specific media for *Aspergillus*, i.e. Czapek-Dox agar (CZ) and malt-extract agar (MEA) media as described by Klich (1992; 2002). Definitely, after one week of cultivation the fungus produced conidiophores with orangish-white and greenish-white mycelia on CZ and MEA media, respectively. According to the specific form of their conidiophores, the isolate was

then determined as *Aspergillus* sp. Compared to the pictures and characteristics of *Aspergillus* described by Klich (2002), the fungus KT13 has similar characteristics to *Aspergillus versicolor, Aspergillus japonicus* and *Emericella nidulans.*

4.7.2. Pigments in Marine Fungi

In the liquid culture, fungal strains KT19, KT29 and *Xylaria psidii* KT30 appeared correspondingly as yellowish and reddish cultures. Based on the orangish-yellow pigment of the compound isolated from ethyl acetate extract of KT19, a compound was suggested belonging to anthraquinones. This suggestion was supported by the colour change of the compound on TLC plate from orange into red after exposed with ammonia (Gill & Steglich, 1987). Therefore, the compound was elucidated more rapidly.

A number of anthraquinones isolated from fungi, particularly macromycetes have been characterized. The presence of the compounds is closely related to the colour of the fungi. Pigments also have been used widely in taxonomy of macrofungi. For example, the red pigment mitorubrin has been used as the taxonomic pigment of *Hypoxylon* (Stadler & Fournier, 2006). Moreover, Melvyn Gill and his group have reported the pigments of macrofungi (Gill, 1999; 2003; 2004; Gill & Steglich, 1987; Gill *et al.* 1988; Gill & Kiefel, 1988; Gill & Gimenez, 1990; Gill & Qureshi, 1992; Gill & Morgan, 2001).

4.8. Isolated Compounds and their Biological Properties

In the present study, 14 isolated compounds were elucidated. The compounds were isolated from ethyl acetate- and dichloromethane extracts of six fungal strains and included 7 polyketides, 4 terpenes, 1 alkaloid and 2 till now undefined structures.

4.8.1. Metabolites from Marine-derived Fungi

Metabolites of *Aspergillus* sp. KT13

Ethyl acetate extracts of the fungus KT13 were active against majority of the test organisms, but the highest activity was against Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus.* In opposite to the better growth of the fungus in the salt medium, the extract from *Aspergillus* sp. KT13 which was cultivated without salt addition exhibited higher and broader antibacterial activity against both human- and fish pathogenic bacteria. This result was related to the previous study by Miao *et al.* (2006) which concluded that mycelial growth and bioactives production have negative correlation. The fungi produce bioactive substances as chemical weapons to survive in extreme condition. Therefore, an

effective screening process can be achieved through systematic manipulation of culture conditions.

Aspergillus strains from marine origin have been explored and produced diverse active metabolites such as unsaturated fatty acids, sesquiterpenes, steroids, alkaloids, chromane derivatives, difuranxanthone, asperxanthone, asperbiphenyl, cerebroside, asperiamide and anthraquinone derivatives (Rahbæk *et al.,* 1997, Lin *et al.,* 2001, Wahidulla *et al.,* 2002, Wu *et al.,* 2008; 2009). Different habitats or environmental factors might play a key role to influence the products. In addition, variation in species itself is possible to make them more diverse.

Based on the reported data on *Aspergillus* sp., therefore it is not surprising that *Aspergillus* sp. KT13 was found to possess considerable bioactivities. Bioactive compound isolated from *Aspergillus* sp. in the present report as described in part **4.7.1** was determined as a chromane derivative with a molecular weight of 266. However, further investigation has to be done to elucidate finally the molecular structure of compound KT13/1 which was proposed as aspterric acid. Moreover, it is necessary to observe other chemical and biological characteristics of the two compounds. Fukami *et al*. (1991) and Cutler *et al*. (1996) previously reported that the compounds which were isolated from *A. niger* and *A. fumigatus* as metabolites of the polyketide pathway showed biological activity as fungicides and fungistatics.

From biological activity investigation, it can be concluded that the chromane derivative possesses antibacterial and antifungal properties. The literatures stated that chromane derivatives have potential effect on nervous system disease such as Alzheimer's disease, Parkinson's disease and could be useful in treating depression or other mood disorders. In addition, these compounds have also potential for treating obesity relating to its effect to reduce appetite (Heffernan *et al.,* 2008). Considering these properties and also its abundance in *Aspergillus,* it is fascinating to study further on its characteristics and other valuable usage of the compound.

Aspterric acid was isolated for the first time from *Aspergillus terreus* (Tsuda *et al.,* 1978). Synthetic studies of the compound have been reported by Haramaya *et al.* (1983; 1987). Finally, alkaline hydrolysis of isopropylidene compound afforded 76% yield of aspterric acid (Haramaya *et al.,* 1987). Aspterric acid was also reported as inhibitor of pollen development in *Arabidopsis thaliana* at concentration of 38 µM. Furthermore, it was

reported that inhibition by aspterric acid occured at meiosis (Shimada *et al.,* 2002). However, it is still necessary to observe the biological activities of the compound.

Trimethoxybenzoic Acid Isolated from KT19

In the present study, trimethoxybenzoic acid was isolated from ethyl acetate extract of fungus KT19. The compound is naturally present in the syringic acid metabolism. As reported by Eriksson *et al.* (1984), trimethoxybenzoic acid was formed from syringic acid via methylation of the *p*-hydroxyl group. It is suggested that this reaction occurred regarding to detoxification of phenolics in wood lignin. Surprisingly, the fungus KT19 was originally not isolated from driftwood but from sandy habitat. To our knowledge there was no report relating to this result.

Syringic acid is naturally known as phenol in seeds, particularly in blueberry. The compound was also reported to exhibit antioxidant activity (Elzaawely *et al.,* 2007). However, syringic acid was also found in fungi (Eriksson *et al.,* 1984; Shin, 1995). Furthermore, Eriksson *et al.* (1984) proposed the metabolism of syringic acid based on their study on white-rot, soft-rot and brown-rot fungi (Fig. 4.2).

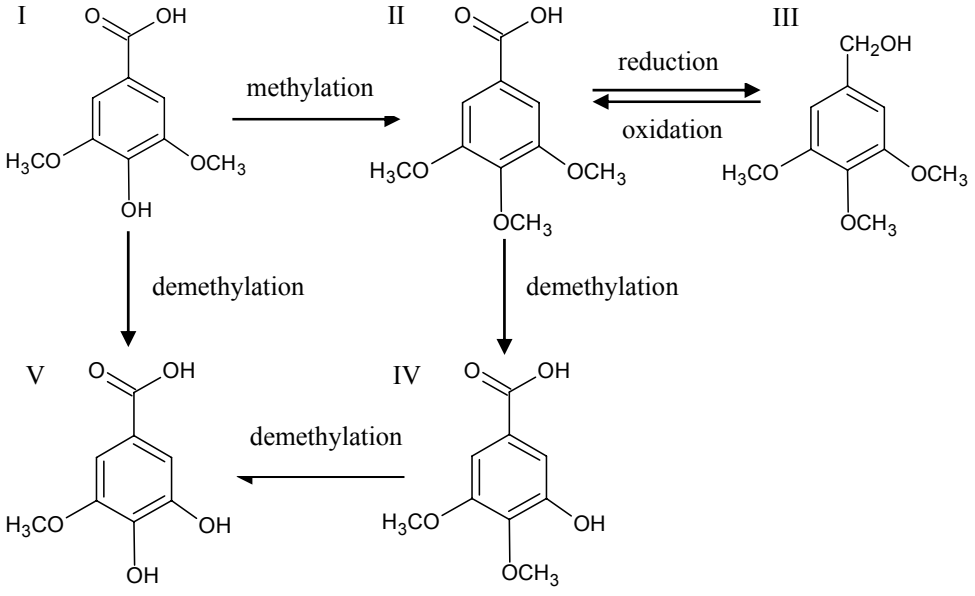


Fig. 4.2. Proposed reactions taking place during syringic acid metabolism by *S. pulverulentum* (I. Syringic acid, II. Trimethoxybenzoic acid, III. Trimethoxybenzyl alcohol, IV. Isomer syringic acid, V. Methylgallic acid; Eriksson *et al.,* 1984)

In our study, biological activity test of the substance has not been carried out due to the limited yield.

Oleuropeic Acid Isolated from Fungus KT19

Oleuropeic acid was obtained as light brown crystals from fungal strain KT19. The compound was isolated from the same fraction as trimethoxybenzoic acid.

On TLC analyses trimethoxybenzoic acid and oleuropeic acid appeared at very near R_f, namely of 0.62 and 0.64, and after spraying with AS reagent found as brown and orange spots, respectively. It is understandable regarding to their related molecular structures. Interestingly, both acids and their derivatives were known to possess antioxidant and cytotoxic activities (Elzaawely *et al.,* 2007; Hasegawa *et al.,* 2008; Tian *et al.,* 2009).

Oleuropeic acid was initially isolated from olive (*Olea europea*) (Shasha & Leibowitz, 1959). It was isolated using alkaline hydrolysis of oleuropein, a bitter principle present in the leaves, unripe fruit and root bark of the olive tree. Tian *et al.* (2009) recently reported five new oleuropeic acid derivatives, eucalmaidins A-E which were isolated from fresh leaves of *Eucalyptus maideni.* Oleuropeic acid was also reported as the result of biotransformation of some monoterpenes by *Pseudomonas incognita* as presented in Fig. 4.3 (Madyastha, 1984).

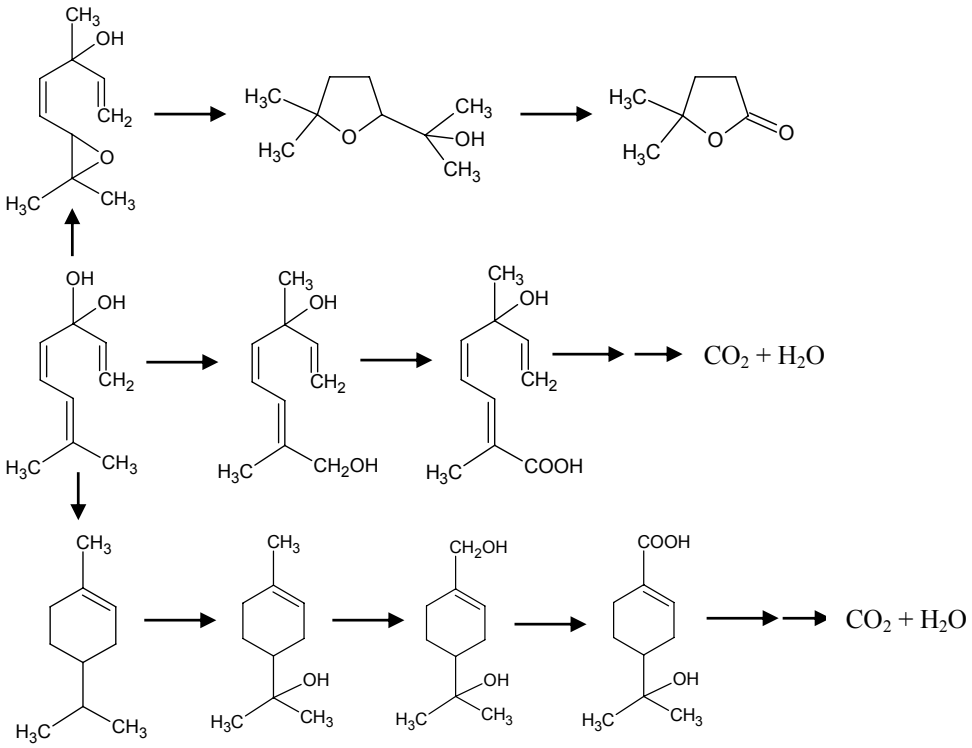


Fig. 4.3. Prosible pathways of degradation of linalool by *P. incognita* (Madyastha, 1984)

Oleuropeic acid which possesses antiallergic properties (Japan Kokai Tokyo Koho *in* Pellegata *et al.,* 1985) has attracted researchers to study its chemical synthesis.

Mechoulam *et al*. (1962) reported the chemical synthesis of oleuropeic acid for the first time. The racemic oleuropeic acid was obtained by condensed cyclohexanone-4-carboxylic acid with potassium acetylide in *t*-butanol as described in following Fig. 4.4. However, Pellegata *et al.* (1985) afterwards reported a complete synthesis of oleuropeic acid starting from β-pinene to afford 38% yield.

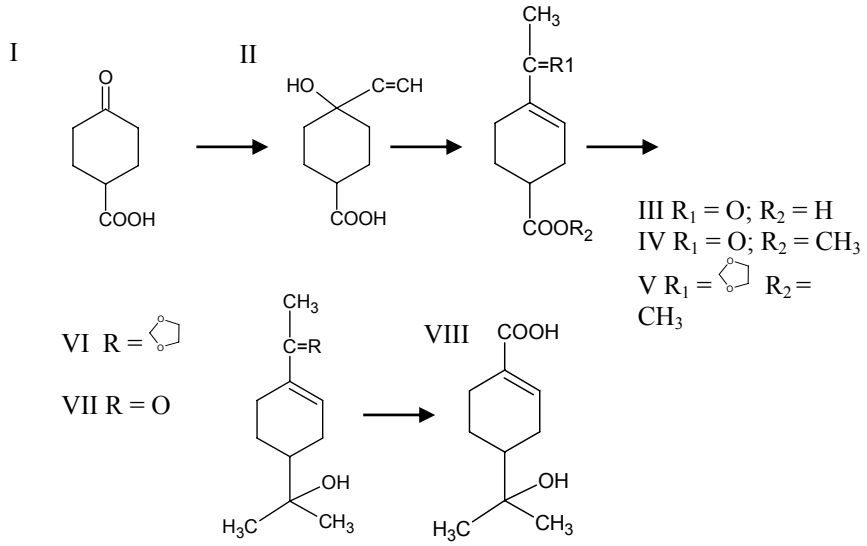


Fig. 4.4. Synthetic pathways of oleuropeic acid (Mechoulam *et al*., 1962)

Anthraquinone Derivative Isolated from Fungus KT19

Yellow pigment which was determined as anthraquinone derivative was isolated from an unidentified marine-derived fungus KT19. The compound was suggested to be chrysophanol, an anthraquinone derivative which has been found in many plants as well as fungi (Howard & Raistrick, 1950). Chrysophanol is highly active against powdery mildew, one of the most serious plant diseases causing large yield losses in crops (Choi *et al.,* 2004).

Chrysophanol together with physcion are natural anthraquinone derivatives found in three plant families: Fabaceae (*Cassia* spp.), Polygonaceae (*Rheum, Rumex* and *Polygonum* spp.) and Rhamnaceae (*Rhamnus* and *Ventilago* spp.). They can be extracted from the roots of the plants (Izhaki, 2002; Choi *et al.,* 2004; Yao *et al.,* 2006). These compounds are the major active ingredients in traditional herbal medicines widely used in clinics (Hsiang & Ho, 2008). Recent studies have reported that these compounds possess various biological activities, such as antitumor (Sun *et al.,* 2000), antioxidant (Yen *et al.,* 2000) and antivirus (Barnard *et al.,* 1992).

Chrysophanol, moreover, has been reported to act synergistically together with other anthraquinones such as physcion (Yang *et al.,* 2007). Choi *et al.* (2004) confidently

mentioned that chrysophanol may be used directly as fungicide or as lead molecule in the development of new fungicide with other chemical skeleton. Considering this potential therefore it could be interesting to observe its activity against some phytopathogenic fungi or powdery mildews.

4.8.2. Metabolites from Algicolous Fungi

Naphthalene Derivative Isolated from Fungus KT29

A naphthalene derivative isolated from fungus KT29 was determined as new natural product. The compound was found as brown crystals isolated from ethyl acetate extract of fungal broth. In comparison with Chemical Abstracts, the compound was known as an intermediate product in synthesis reaction of a naphthalene carboxylic acid ($C_{20}H_{18}O_8$) using a naphthalenediol ($C_{10}H_8O_2$) as starting compound as presented in Fig. 4.5 (Eugster & Good, 1962; Bosshard *et al.,* 1964).

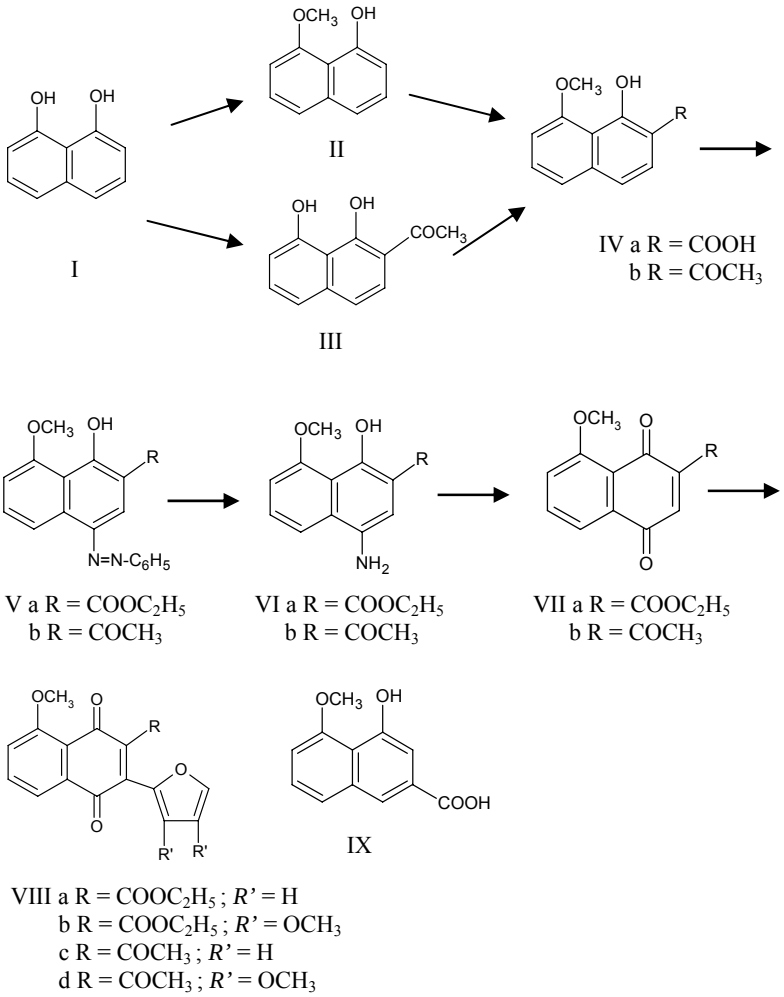


Fig. 4.5. Synthetic pathways of naphthalene carboxylic acid (Bosshard *et al.,* 1964)

Cytochalasin B Isolated from *Xylaria psidii* KT30

Fungi of the genus *Xylaria* are very diverse and well-known as one of most productive fungal genera. *Xylaria* sp. is one of rarely macrofungi that has been isolated from marine environment. This strain can be simply recognized through the presence of stromata under subculture. However, the exact identification is in general yet difficult since the presence of stromata is not always followed by reproductive spores. Fig. 4.6 presents the stromata form of the algicolous fungus *Xylaria psidii* KT30 and *Xylaria hypoxylon* from literature.

Fig. 4.6. Stromata form of *Xylaria* spp.
(a) 3 month-old culture of *Xylaria psidii* KT30, (b) *Xylaria hypoxylon*

Xylaria psidii is an inhabitant of *Psidium* (guava) seeds in Hawaii reported for the first time by Rogers *et al.* (1992). The fungus was found as pathogen to guava. To our knowledge, there was no publication reporting active compounds isolated from this fungus.

The diversity of the fungi with respect to their chemical constituents has been widely explored. However, new compounds are continuing to be found from the fungi belonging to genus *Xylaria.* The known compounds isolated from *Xylaria* were classified in various chemical groups, such as terpenoids, cyclopeptides, polyketides, cytochalasins, quinone, xanthones, benzofuran and unique unclassified xyloketals (Lin *et al*., 2001a; Lin *et al.,* 2001b; Boonphong *et al.,* 2001; Smith *et al.,* 2002; Jayasuriya *et al.,* 2004; Healy *et al.,* 2004; Davis & Pierens, 2006; Tansuwan *et al.* 2007; Hu *et al.,* 2008; Xu *et al.,* 2008; Rukachaisirikul *et al.,* 2009; Isaka *et al.,* 2010). Recently Li *et al.* (2010) reported new diterpenoid (xylarenolide) and three new sesquiterpenoids, xylaranol A and B, and xylaranic acid isolated from *Xylaria* sp. collected in Gaoligong Mountain, P.R. China.

Cytochalasins isolated from *Xylaria* sp. are quite diverse. Abate *et al.* (1997) found deacetyl 19-20-epoxy-cytochalasin Q; 19,20-epoxy-cytochalasin Q; 18-desoxy-19,20-epoxy-cytochalasin Q (xylobovatin); deacetyl 19,20-epoxy-cytochalasin C; and 19,20-epoxy-cytochalasin C isolated from *Xylaria obovata* collected in southern Ethiopia. Cytochalasin D, desacetylcytochalasin D, epoxy-cytochalasin N, cytochalasin O and cytochalasin Q and R were also reported to be found in *Xylaria* sp. (Espada *et al.,* 1997; Deyrup *et al.,* 2007; Pongcharoen *et al.,* 2007). Cytochalasins from *Xylaria* sp. were reported to possess cytotoxic activity (Abate *et al.,* 1997; Pongcharoen *et al.,* 2007; Zhang *et al.,* 2010).

Cytochalasins however are not only found in *Xylaria* sp. Previous studies reported that cytochalasins have been widely found in fungal strains such as *Phomopsis paspalli, Rhinocladiella* sp., *Eutypella scoparia, Endothia gyrosa*, *Spicaria elegans* and *Aspergillus terreus* (Patwardhan *et al.,* 1974; Wagenaar *et al.,* 2000; Pongcharoen *et al.,* 2006; Xu *et al.,* 2009; Lin *et al.,* 2009; Zhang *et al.,* 2010).

Cytochalasin B which is also known as phomin was for the first time isolated from *Phoma* sp. (Rothweiler & Tamm, 1966). Furthermore, their study reported its biological properties to prevent completely multiplication of HeLa cells *in vitro* at concentrations of 3-10 µg/ml and to inhibit 80% migration of chicken leucocytes at a concentration of 2.5 µg/ml.

Cytochalasin B (CB) affects a variety of cell functions (Painter *et al.* 1981). Some reports described in more detail that CB inhibits a wide variety of cellular movements such as cytokinesis, cell locomotion, cytoplasmic division (Carter, 1967; Bray, 1979). CB disrupts microfilament bundles by binding to high affinity sites on actin filaments and by blocking filament elongation (Brown & Spudich, 1979; Flanagan & Lin, 1980; Lin *et al.* 1980; Brown & Spudich, 1981). At the cellular level CB inhibits cytoplasmic division by disrupting actin-containing contractile proteins in the cleavage furrow (Schroeder, 1978 *in* Somers & Murphey, 1982). Another literature reported that CB shortens actin filaments by blocking monomer addition at the fast-growing end of polymers (Theodoropoulos *et al.,* 1994). In leukaemia HL-60 cells, CB blocks adenosine-induced apoptotic body formation without affecting activation of endogenous ADP-ribosylation (Tanaka *et al.,* 1994).

In respect to its potent bioactivity, there are several reports on optimization of cytochalasin B production through enriched media. Zabel *et al.* (1979) describe cytochalasin B production in *Helminthosporium dematoideum* in amount of 700 mg/l after

3 weeks of cultivation. This yield was much higher than those produced by *Phoma exigua* with 80-120 mg/l or from the same species *H. dematoideum* with 50 mg/l (Aldridge *et al.,* 1967; Rothweiler & Tamm, 1970 *in* Zabel *et al.,* 1979).

The present work is the first report of chytochalasin B isolated from *X. psidii*. The cytotoxicity assay of the fungal ethyl acetate extracts showed strong activity against human bladder carcinoma cell line with an IC_{50} value of 4 µg/ml. According to the well-known cytotoxic activity of the cytochalasins, it could be assumed that the cytochalasin B was responsible for the cytotoxic properties of the fungus *X. psidii* KT30.

Bioactive Compounds Isolated from Strain KT31

Fungal isolate KT31 exhibited considerable antimicrobial and cytotoxic activities. Interestingly, the fungus produced the most active metabolites in relative short fermentation period which was 6 days, while in longer culture period more diverse active metabolites were produced.

Extracts of the fungus, i.e. ethyl acetate extract isolated from culture broth and DCM extract from mycelia were found to be active in antifungal assay against *Candida maltosa* as well as in cytotoxic assay. Bioautographic assays of both extracts showed the presence of the active component at R_f 0.90. This component was found to be very intense in extracts obtained from freshwater cultures.

The ESI-FTICR-MS analyses of both compounds showed an identical $[M+Na]^+$ ion at *m*/*z* 269.11466 suggesting a molecular formula of $C_{15}H_{18}O_3$ (MW. 246). These results concluded that similar compounds were produced by the fungal strain KT31 intra- and extracellularly. Considering the biological activity and the abundance of the compounds both in culture broth and mycelia, further study dealing with chemical and biological characteristics of the compounds is needed to carry out.

In preliminary assays (not demonstrated in this work) it could be shown that the transformation of the mycelial biomass of KT31 into microparticles, so-called Maresome®, by a special homogenization process (Lukowski *et al.,* 2003) improves the antimicrobial activity. These experiments should be continued in the future and extended also for the mycelia of the other fungi.

Helicascolides A and C Isolated from Strain KT32

Ethyl acetate extract of fungus KT32 was found to contain highly diverse components as presented in its TLC profile (see **3.7.4.1**). Furthermore, the components of

the fungal extract were found frequently in crystals form after fractionation in column chromatography. Three compounds, i.e. helicascolides A and C, and isosclerone were also found in crystals form. These compounds have been successfully elucidated based on single-crystal X-ray diffraction and spectroscopic analyses.

Helicascolides isolated from fungus in the present study were found for the second time after Poch and Gloer (1989). Regarding to the previous compounds, then the new lactone isolated in our study was named as helicascolide C. Both compounds were found as colourless crystals.

The NMR spectra of helicascolide C were also very similar to those of helicascolide A (see **3.7.4.3**). The main difference was revealed by a signal characteristic for carbonyl group in the ^{13}C-NMR at δ 209.9 ppm (213.8) (C(3)) of compound helicascolide C, while at the same position the signal of helicascolide A was replaced by hydroxyl group δ(C) 77.4. The interpretation was corroborated by the IR spectra which exhibited the presence of carbonyl groups by the strong absorption at 1720-1712 cm^{-1} and there was lacking absorption of hydroxyl group found in region 3400-3200 cm^{-1}.

Based on the related data therefore helicascolide C was determined as new compound. In biological activity assays both compounds showed no antibacterial and antifungal activities, while helicascolide C exhibited fungistatic activity. In the previous study the biological properties of helicascolides were not mentioned (Poch & Gloer, 1989).

Isosclerone Isolated from Fungal Strain KT32

Isosclerone was isolated for the first time from the fungus *Sclerotinia sclerotiorum* (Morita & Aoki, 1974 *in* Kokubun *et al.,* 2003). Its absolute structure was determined as (4S)-3,4-dihydro-4,8-dihydroxy-1(2H)-naphthalenone. The substance was reported to possess activity in stimulation of root elongation of rice seedlings and at high concentrations in inhibiting the growth of shoots and roots. At the same time white spot (chlorosis) on leaves was caused.

Oxidation of isosclerone with manganese dioxide afforded juglone, a compound naturally produced by walnut trees (*Juglans regia*). Morita & Aoki (1974) revealed that the process afforded ca. 30% yield of juglone (Kokubun *et al.,* 2003) (Fig. 4.7).

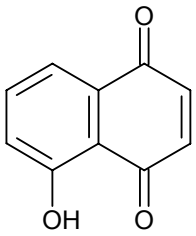


Fig. 4.7. Molecular structure of juglone ($C_{10}H_6O_3$)

Isosclerone was also found in *Tubakia dryina*, a fungus known as the casual agent of red oak (*Quercus rubra*) leaf spot (Ventkatasubbaiah & Chilton, 1992). Some fungal strains which were also reported to produce isosclerone are *Scytalidium* sp. (Findlay & Kwan, 1973), *Penicillium diversum* (Fujimoto *et al.,* 1986), *Pyricularia oryzae* (Iwasaki *et al.,* 1969 *in* Sviridov, 1991), *Discula* sp. (Ventkatasubbaiah & Chilton, 1991) and *Microsphaeropsis* sp. (Höller *et al*., 1999). Isosclerone isolated from marine-derived *Microsphaeropsis* sp. displayed antifungal activity against *Eurotium repens* at 50 µg level (Höller *et al*., 1999).

5. OUTLOOK

The present study showed an enormous potential of Indonesian marine-derived fungi in correlation with drug discovery from the sea. Five isolates, i.e. *Aspergillus* KT13, *Mycelium sterilium* KT19, *Mycelium sterilium* KT29, *Xylaria psidii* KT30 and *Mycelium sterilium* KT31 exhibited considerable bioactivity against human and fish pathogenic bacteria. An unidentified algicolous fungus KT32 was fascinating in respect to chemical diversity of its secondary metabolites. These six strains are noteworthy to investigate for further chemical and pharmacological characteristics.

Molecular biology study is needed to identify taxonomically the sterile fungal strains KT19, KT29, KT31 and KT32. Identification of these fungi leads to minimize replication in searching for bioactive natural products.

An algicolous *Xylaria psidii* KT30 showed markedly activity in the bioactivity assays. In order to enhance the bioactivity as well as to produce more diverse bioactives, a straightforward technique such as OSMAC approach (one strain many compounds) could be implemented in the future. Moreover, study of biosynthetic pathways of the fungus would be necessary to conduct. Subsequently, the use of gene cloning to manipulate bioactives production would be interesting to complete the conventional and proven approaches, in order to increase the yield and to produce useful novel bioactives. Besides it would be interesting to elucidate the structure of the red pigment of KT30. It is also necessary to investigate the strains KT19, KT29 and KT31 in respect to their enzymes production.

Four new natural compounds, i.e. a naphthalene derivative, a benzoic acid derivative, a quinone derivative and helicascolide C were correspondingly isolated from ethyl acetate extracts of fungal culture broths KT29, *X. psidii* KT30, KT31 and KT32. These substances should be produced in more amounts to accomplish comprehensive chemical characteristics and pharmacological properties of the compounds. Structure elucidation of undefined isolated compounds (see Appendix 3) should be carried out completely.

6. SUMMARY

Fungi are one of the most significant groups of organisms to be exploited for drug discovery purposes. Especially Fungi Imperfecti have provided mankind with numerous different bioactive secondary metabolites such as β-lactam antibiotics, griseofulvin, cyclosporine A or lovastatin. In agreement with their related terrestrial fungi, marine fungi are producers of a high variety of biologically active secondary metabolites. In recent years, marine fungi have been explored more intensely to obtain novel and biologically active compounds. However, compared to marine sponges and bacteria, marine fungi are still less explored.

In the search for biologically active natural products the present study deals with the isolation and identification of marine fungi, screening strategies for bioactives production, isolation and structure elucidation as well as pharmacological investigation of the isolated compounds.

Marine fungi represent an ecological term instead of taxonomic classification. In this work we define all fungi occurring in marine environment as “marine fungi”. Thirty two of Indonesian marine fungi have been isolated and cultivated. Thereof 11 strains have been investigated in respect to their broad spectrum of biological activities. From 11 fungal isolates, 5 strains could be identified, i.e. *Aspergillus* sp. KT13, *Lasiodiplodia theobromae* KT26, *Epicoccum nigrum* KT28, *Xylaria psidii* KT30 and *Coniothyrium* sp. KT33. *Aspergillus* sp. KT13 was identified according to the chemotaxonomic approach; the other ones according to the morphological approach.

Salinity of the medium in submerged fermentation can be used as preliminary screening to determine the suitable medium for fungal growth and bioactives production. Seven of 11 strains produced more active compounds in freshwater culture. Furthermore, yield and bioactivity of the secondary metabolites are related to the fermentation period. Mixed culture of fungus KT31 with autoclaved *Staphylococcus aureus* enhanced the production of bioactive secondary metabolites.

All ethyl acetate extracts obtained from the fungal fermentation broth showed antibacterial activity at least against one of the test organisms. Five isolates, i.e. *Aspergillus* KT13, *Mycelium sterilium* KT19, *Mycelium sterilium* KT29, *Xylaria psidii* KT30 and *Mycelium sterilium* KT31 exhibited considerable antimicrobial activity against human and fish pathogenic bacteria. Isolates *Aspergillus* sp. KT13 and strain KT31 were obviously

active against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, while strains KT19, *X. psidii* KT30, and KT31 were predominantly active against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa.*

All five isolates were active against Gram-negative fish pathogenic bacteria *Vibrio anguillarum* and *Aeromonas salmonicida*. Three algicolous fungi KT29, *X. psidii* KT30, and KT31 showed significant antifungal activity against *Candida maltosa* and phytopathogenic *Cladosporium cucumerinum.* Two strains, *X. psidii* KT30 and KT31 which were isolated from the same algal specimen *Kappaphycus alvarezii* BRKA-1 showed strong cytotoxicity against the human bladder carcinoma cell line 5637. Besides ethyl acetate extracts isolated from the culture broth, DCM extracts from mycelia of these fungi were found to be active in antifungal assay against *C. maltosa* as well as in cytotoxic assay. However, in the antagonistic study no inhibition zone in dual culture of the two fungi was observed.

Bioassay-guided fractionation led to the isolation of bioactive compounds. Altogether 14 compounds were isolated and further elucidated. The compounds were obtained from the ethyl acetate and dichloromethane extracts of six fungal strains. They included 9 polyketides, 2 terpenes, 1 alkaloid and 2 till now undefined structures.

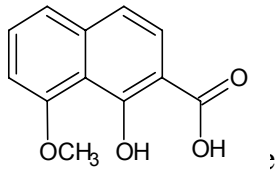
A chromane derivative has been isolated from *Aspergillus* sp. KT13. Together with the isolated aspterric acid the chromane derivative contributed approximately 15% of the total ethyl acetate extract. From an unidentified fungus KT19, two known compounds, trimethoxybenzoic acid and oleuropeic acid, were isolated from ethyl acetate extract of the fungal culture broth.

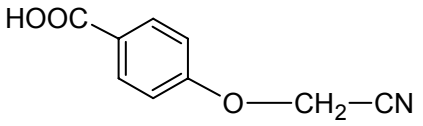
A new naphthalene derivative was isolated from an unidentified algicolous fungus KT29. In comparison with the database of Chemical Abstracts, the compound was known as an intermediate product in synthesis of a naphthalene carboxylic acid ($C_{20}H_{18}O_8$) using a naphthalenediol ($C_{10}H_8O_2$) as starting compound (Eugster & Good, 1962; Bosshard *et al.,* 1964). Therefore, isolated compound in this study was determined as new natural product.

The present work is the first report of chytochalasin B isolated from *X. psidii* (KT30) as well as the first report of a natural product isolated from this fungus. The cytotoxicity assay of the fungal ethyl acetate extracts showed strong activity against the human bladder carcinoma cell line 5637 with an IC_{50} value of 4 µg/ml. According to the well-known cytotoxic activity of the cytochalasins, it is presumably that the isolated cytochalasin B was responsible for the cytotoxic properties of the fungus *X. psidii* KT30. From the same extract the new natural product 4-(cyanomethoxy)-benzoic acid has been isolated. A new quinone

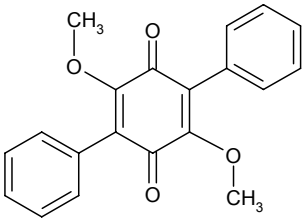
derivative together with two undefined compounds was isolated from the algicolous fungus KT31.

Single-crystal X-ray diffraction and spectroscopic analyses led to the identification of three compounds, i.e. helicascolides A and C, and isosclerone obtained from ethyl acetate extracts of an unidentified algicolous fungus KT32. New compound helicascolide C along with a related structurally known compound helicascolide A were found to be abundant in ethyl acetate extracts of culture broth of the fungus KT32.

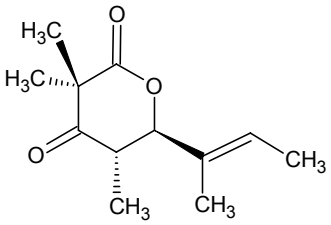




4-(Cyanomethoxy)-benzoic acid



Quinone derivative



Helicascolide C

In summary, the screening of marine-derived fungi accompanied by isolation, structure elucidation and pharmacological investigation of the isolated compounds provides a multitude of so far unexplored abundant resources available in Indonesian sea water in respect to drug discovery.

7. ZUSAMMENFASSUNG

Pilze sind eine für die Wirkstoffsuche sehr bedeutende Gruppe von Organismen. Besonders Fungi imperfecti sind Produzenten wichtiger Arzneistoffe, z.B. von β-Lactam-Antibiotika, Griseofulvin, Cyclosporin A oder Lovastatin. Ebenso wie terrestrische Pilze können auch marine Pilze eine hohe Vielfalt von biologisch aktiven Sekundärmetaboliten bilden. In den letzten Jahren wurden marine Pilze stärker in dieser Hinsicht untersucht. Im Vergleich zu marinen Schwämmen und Bakterien sind sie jedoch immer noch viel weniger erforscht.

Auf der Suche nach biologisch aktiven Naturstoffen befasst sich die vorliegende Arbeit mit der Isolierung, Identifizierung und Kultivierung von marinen Pilzen aus dem indonesischen Raum, mit geeigneten Screening-Strategien zur Auffindung bioaktiver Extrakte und Verbindungen sowie mit der Isolierung, Strukturaufklärung und pharmakologischen Untersuchung der isolierten Verbindungen.

Die Bezeichnung „Marine Pilze“ stellt einen ökologischen Begriff und keine taxonomische Einordnung dar. In dieser Arbeit werden alle aus dem marinen Milieu isolierten Pilzarten als "marine Pilze" bezeichnet. Insgesamt wurden 32 Marine Pilzstämme von Holz oder Algen aus indonesischen Küstengewässern isoliert und kultiviert. Davon konnten 11 Stämme in größerer Menge kultiviert und in Bezug auf ihre biologischen Aktivitäten untersucht werden. Von den 11 Isolaten konnten 5 Stämme identifiziert werden: es handelt sich um *Aspergillus* sp. KT13, *Lasiodiplodia theobromae* KT26, *Epicoccum nigrum* KT28, *Xylaria psidii* KT30 und *Coniothyrium* sp. KT33. Der Stamm *Aspergillus* sp. KT13 wurde nach dem chemotaxonomischen Ansatz identifiziert; die anderen nach dem morphologischen Ansatz.

Zur Vorauswahl eines geeigneten Kulturmediums wurde der Salzgehalt des Mediums variiert. Auswahlkriterien waren das Pilzwachstum und die antimikrobielle Aktivität gegen *Staphylococcus aureus.* Sieben von 11 Stämmen produzierten mehr Wirkstoffe in Süßwasser- als in Salzwasserkultur. Auch der Fermentationszeitraum und der Erntezeitpunkt haben einen großen Einfluss auf Ausbeute und Bioaktivität. Eine Mischkultur des Pilzes KT31 mit autoklavierten *Staphylococcus aureus* Zellen steigert die Produktion der bioaktiven Sekundärmetabolite.

Alle aus den Fermentationsbrühen gewonnenen Ethylacetatextrakte zeigten antibakterielle Aktivität gegen mindestens einen der Testorganismen. Fünf Isolate, d.h.

Aspergillus KT13, *Mycelium sterilium* KT19, *Mycelium sterilium* KT29, *Xylaria psidii* KT30 und *Mycelium sterilium* KT31 zeichneten sich durch eine besonders hohe Aktivität gegen human- und fischpathogene Bakterien aus. Während die Isolate *Aspergillus* sp. KT13 und KT31 deutlich aktiv gegen die Gram-positiven Bakterien *Staphylococcus aureus* und *Bacillus subtilis* waren, waren die Stämme KT19, *X. psidii* KT30 und KT31 überwiegend gegen die Gram-negativen Bakterien *Escherichia coli* und *Pseudomonas aeruginosa* aktiv. Alle fünf Isolate waren aktiv gegen die zu den Gram-negativen Keimen gehörenden fischpathogenen Bakterien *Vibrio anguillarum* und *Aeromonas salmonicida*. Die drei von Algen isolierten Pilzstämme KT29, *X. psidii* KT30 und KT31 zeigten antimykotische Aktivität gegen *Candida maltosa* und den phytopathogenen Pilz *Cladosporium cucumerinum*. Die zwei Stämme *X. psidii* KT30 und KT31, die beide von der Alge *Kappaphycus alvarezii* BRKA-1 isoliert worden waren, zeigten eine starke zytotoxische Wirkung gegen die menschliche Blasetumorzelllinie 5637. Aus den Myzelien dieser beiden Pilze gewonnene Dichlormethanextrakte waren im antimykotischen Test gegen *C. maltosa* sowie im zytotoxischen Assay mit 5637-Zellen ebenfalls aktiv. In einer Dual-Kultur der beiden Pilze waren jedoch keine antagonistischen Effekte zu finden.

Die biologisch geführte Fraktionierung führte zur Isolierung von bioaktiven Verbindungen. Insgesamt 14 Verbindungen wurden isoliert und weiter aufgeklärt. Die Verbindungen wurden aus den Ethylacetat- und Dichlormethan-Extrakten von sechs Pilzstämmen erhalten. Sie umfassen 9 Polyketide, 2 Terpene, 1 Alkaloid und 2 bis jetzt nicht zuordenbare Strukturen.

Aus *Aspergillus* sp. KT13 wurde ein Chromanderivat isoliert. Dieses macht zusammen mit der ebenfalls isolierten Aspterrin-Säure circa 15% des gesamten Ethylacetatextrakts aus.

Aus dem Ethylacetatextrakt des Kulturmediums des Pilzes KT19 wurden die Verbindungen Trimethoxybenzoesäure und Oleuropeinsäure isoliert.

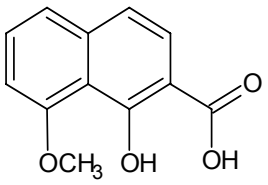
Ein als Naturstoff neues Naphthalinderivat wurde aus dem Pilz KT29 gewonnen. Laut Datenbank Chemical Abstracts wurde die Verbindung bisher nur als Zwischenprodukt bei der Reaktion einer Naphthalin- Kohlenstoffsäure ($C_{20}H_{18}O_8$) mit einem Naphthalindiol ($C_{10}H_8O_2$) beschrieben (Eugster & Good, 1962;. Bosshard *et al*, 1964).

In der vorliegenden Arbeit konnte erstmals Chytochalasin B in *X. psidii* (KT30) nachgewiesen werden. Die Substanz ist vermutlich für die hohe Zytotoxizität der Ethylacetatextrakte des Kulturmediums dieser Pilze gegen humane Blasentumorzellen (5637, IC_{50}-Wert 4 µg/ml) verantwortlich. Aus demselben Extrakt wurde als neuer

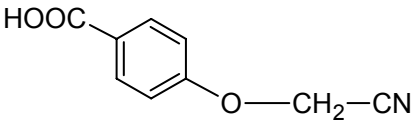
Naturstoff 4-(Cyanomethoxy)-benzosäure isoliert.

Ein neues Chinonderivat wurde aus dem Ethylacetatextrakt des Kulturmediums des Pilzes KT31 isoliert. Außerdem wurden zwei andere Verbindungen isoliert, die bis jetzt noch nicht aufgeklärt werden konnten.

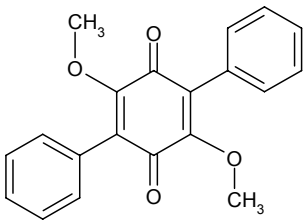
Einkristall-Röntgen- und spektroskopische Analysen führten zur Identifizierung von drei Verbindungen, den Helicascoliden A und C, sowie Isosclerone aus dem Ethylacetatextrakt des Mediums des Pilzes KT32. Bei Helicascolide C handelt es sich um einen bisher nicht beschriebenen Naturstoff.



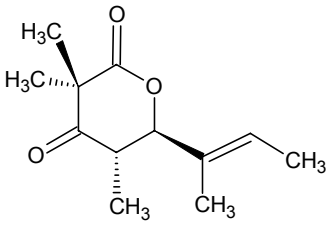
Naphthalinderivat



4-(Cyanomethoxy)-benzosäure



Chinonderivat



Helicascolide C

Zusammenfassend lässt sich feststellen, dass marine Pilze ein großes Potential zur Produktion bioaktiver Verbindungen besitzen. Dessen Erschließung kann einen wichtigen Beitrag zur Erforschung und Nutzbarmachung der marinen Ressourcen Indonesiens leisten.

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Appendix 1. Media for Sporulation

1. **Corn Meal Agar (BBL™)**, approx. formula (g/L):

Corn meal, infusion form (solids)	2.0 g
Agar	15.0 g
Final pH	6.0±0.2

2. **Rice Mill Agar**

Rice mill ("Vînh Thuân")	20.0 g
Agar (Roth)	10.0 g
Tap water or Seawater	1000 ml

3. **Rice Agar** (Modified from Kreisel & Schauer, 1987)

Rice ("Reismilch, Ja")	80.0 g
Agar (Roth)	10.0 g
Tap water or Seawater	1000 ml

4. **Wood Mill Agar**

Wood mill	50.0 g
Agar (Roth)	20.0 g
Tap water or Seawater	1000 ml

5. **Seaweed Agar**

Red seaweed (extraction waste)	20.0 g
Agar (Roth)	10.0 g
Tap water or Seawater	1000 ml

6. **Carrot-Potatoes Agar** (Kreisel & Schauer, 1987; Höller *et al.*, 2000)

Peeled carrot	20.0 g
Peeled potato	20.0 g
Agar (Roth)	20.0 g
Tap water	1000 ml

7. **Yeast Extract Agar (AW)** (Modified from Wright *et al.*, 2003)

Glucose	1.0 g
Yeast extract	0.5 g
Tryptone soy agar	0.5 g
Papain	10.0 g
Agar (Roth)	15.0 g
Seawater	1000 ml
pH before autoclaved	8

8. **Solid Malt Extract (SW)** (Modified from Wright *et al.*, 2003)

Malt extract	15.0 g
Tryptone soy agar	3.0 g
Papain	10.0 g
Agar (Roth)	7.6 g
Distilled water	200 ml
Seawater	800 ml
pH before autoclaved	5.8

Appendix 2. BLAST Analysis of *Xylaria psidii* KT30

BLAST

Basic Local Alignment Search Tool

Edit and Resubmit Save Search Strategies Formatting options Download

Nucleotide Sequence (489 letters)

Results for: lcl|31323 None(489bp)

Your BLAST job specified more than one input sequence. This box lets you choose which input sequence to show BLAST results for.

Query ID
lcl|31323
lcl|31323
Description
None
Molecule type
nucleic acid
Query Length
489

Database Name
nr
Description
All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences)
Program
BLASTN 2.2.24+ Citation

Reference
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.
Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

NCBI Blast:Nucleotide Sequence (489 letters)

```
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internal transcribed spacer 1, 5.8S ribosomal RNA gene,
and internal transcribed spacer 2, complete sequence; and
28S ribosomal RNA gene, partial sequence
Length=579

 Score =  887 bits (480),  Expect = 0.0
 Identities = 487/490 (99%), Gaps = 1/490 (0%)
 Strand=Plus/Plus

Query  1    TCATTAAAGAGTTATTACAACTCCCAACCCCATGTGAACTTACCGTACGTTGCCTCGGCA  60
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Sbjct  25   TCATTAAAGAGTTATTACAACTCCCAAACCCATGTGAACTTACCGTACGTTGCCTCGGCA  84

Query  61   GGTCGCGCTCACCCCGTAGCACCCTACCACGTA-GGGCCTACCCGGTGGCCGCGGACTAA  119
            |||||||||||||||||||||||||||||||| |||||||| ||||||||||||||||||
Sbjct  85   GGTCGCGCTCACCCCGTAGCACCCTACCACGTAGGGGCCTACTCGGTGGCCGCGGACTAA  144

Query  120  GCCTGTCGGTGGCCCAACCAAACTCTGTCAGTGATTGTGTCTTCTGAACTTGACAACGAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  GCCTGTCGGTGGCCCAACCAAACTCTGTCAGTGATTGTGTCTTCTGAACTTGACAACGAA  204

Query  180  ATACGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA  239
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Query  240  AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  299
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Query  300  TGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCC  359
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Query  360  CTGTTGCTTAGTGTTGGGAGCCTACAGCGATGTAGCTCCTCAAAGTTAGTGGCGGAGTCG  419
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Query  420  GTTACACACTCTAGGCGCAGTAAACTCTTTATCTCGTCTACGGTTGTGGCCGGTCCCTTG  479
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Sbjct  445  GTTACACACTCTAGGCGCAGTAAACTCTTTATCTCGTCTACGGTTGTGGCCGGTCCCTTG  504

Query  480  CCGTAAAACC  489
            ||||||||||
Sbjct  505  CCGTAAAACC  514

>gb|DQ322159.1|  Xylaria psidii isolate SUT125 internal transcribed spacer 1,
5.8S ribosomal RNA gene, and internal transcribed spacer 2,
complete sequence; and 28S ribosomal RNA gene, partial sequence
Length=516

 Score =  857 bits (464),  Expect = 0.0
 Identities = 478/484 (98%), Gaps = 3/484 (0%)
 Strand=Plus/Plus

Query  7    AAGAGTTATTACAACTCCCAACCCCATGTGAACTTACCGTACGTTGCCTCGGCAGGTCGC  66
            ||||| || |||||||||||| ||||||||||||||||||||||||||||||||||||||
```

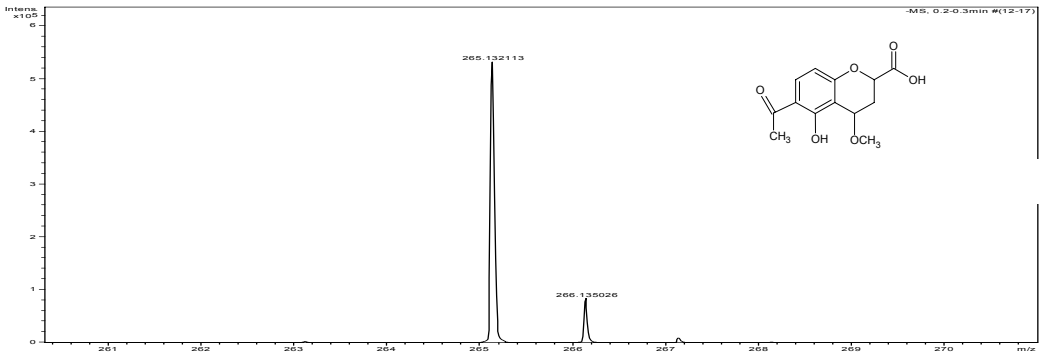
Appendix 3. MS Data of Unidentified Isolated Compounds

No.	Fungal Strain	Extract/ Fraction	Characteristic	*m/z*	Molecular Formula
1	KT03	EtOAc_F6	Yellow crytals	227; 205	$C_{12}H_{12}O_3$ (MW. 204)
2	KT19s	EtOAc_F2P1	Brown, solid	249; 227	$C_{12}H_{18}O_7$ (MW. 274)
3	KT19s	EtOAc_F9P7	Brown, solid		$C_{10}H_{12}O_5$ (MW. 212)
4	KT29s	EtOAc_F6P6	Yellow, solid	349	$C_{18}H_{30}O_5$ (MW. 326)
5	KT30	EtOAc_F1	Yellow, powder	305	$C_{15}H_{22}O_5$ (MW. 282)
6	KT30	EtOAc_F5P1	Light yellow	293	$C_{13}H_{18}O_6$ (MW. 270)
7	KT31	EtOAc_T90	Colorless crytals	585	$C_{33}H_{54}O_7$ (MW. 565)
8	KT31	EtOAc_F8	Brown crystals	277; 255	$C_{12}H_{10}N_2O_6$ (MW. 278)
9	KT31	DCM_F2P1	Pink crystals	305	$C_{19}H_{13}O_4$ (MW. 282)
10	KT31	DCM_F3P2	Orange, powder	369	$C_{20}H_{18}O_7$ (MW. 370)

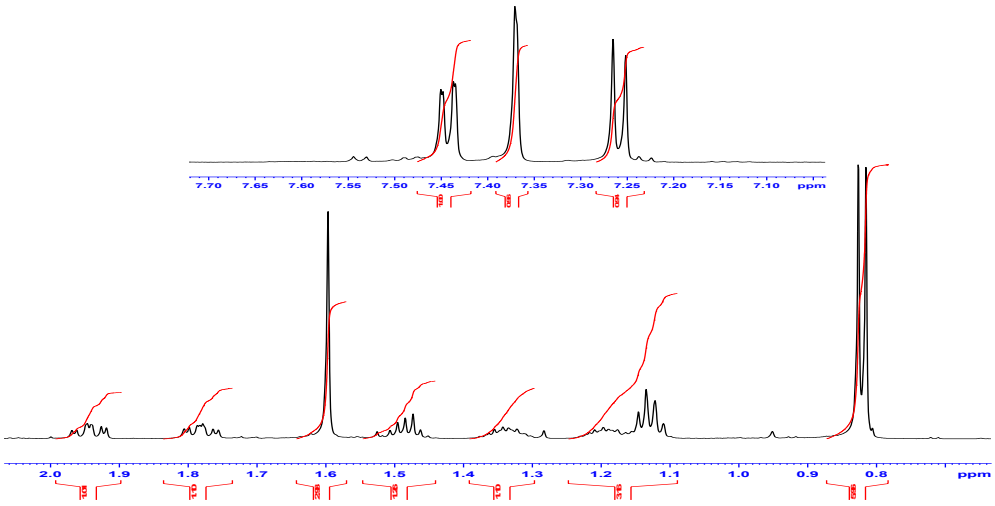
Appendix 4. IR, MS, NMR Data of Elucidated Compounds

1. Chromane derivative (compound KT13/2) from *Aspergillus* sp. KT13

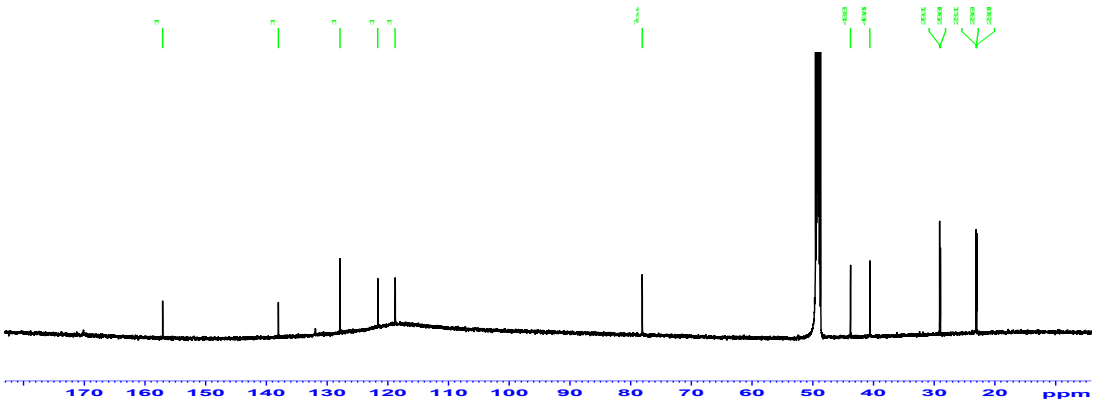
MS Spectrum of Compound KT13/2



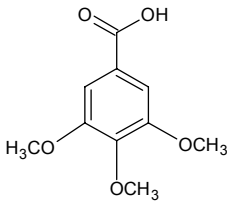
^{1}H-NMR Spectrum of Compound KT13/2



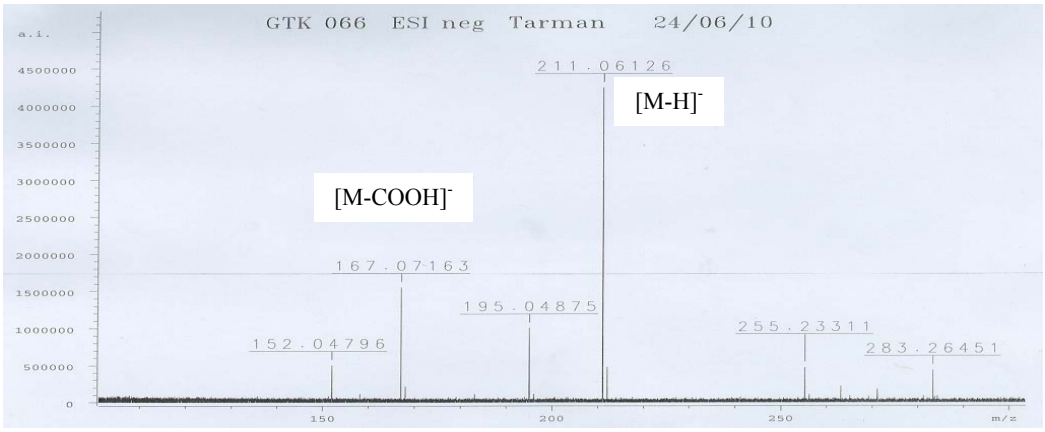
^{13}C-NMR Spectrum of Compound KT13/2



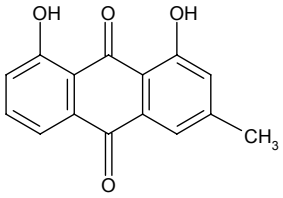
2. Trimethoxybenzoic acid (compound KT19/1) from strain KT19



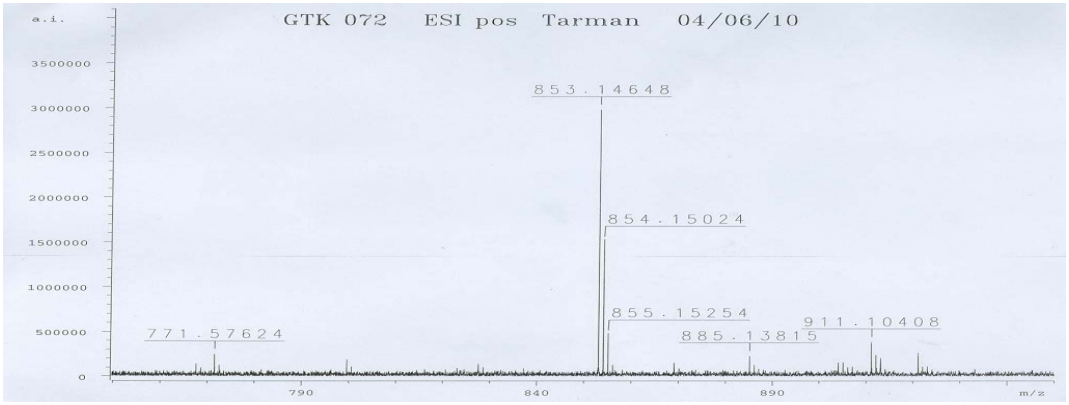
MS Spectrum of Compound KT19/1



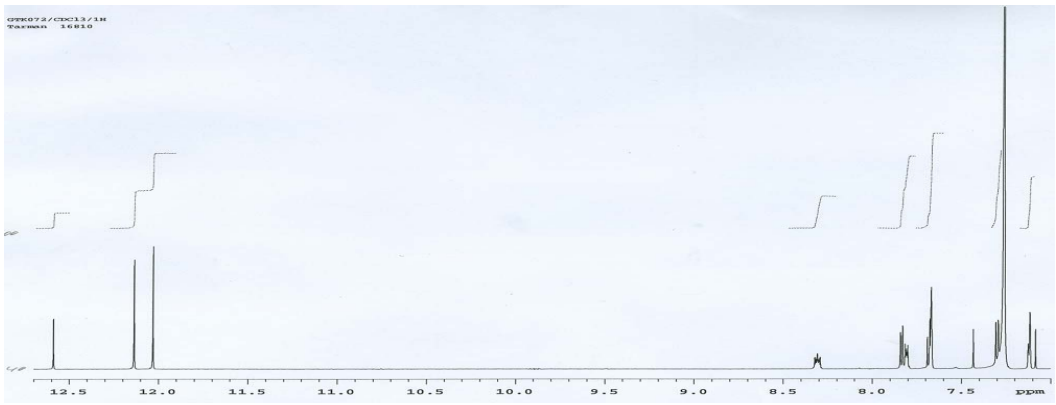
3. Anthraquinone derivative (compound KT19/3) from strain KT19



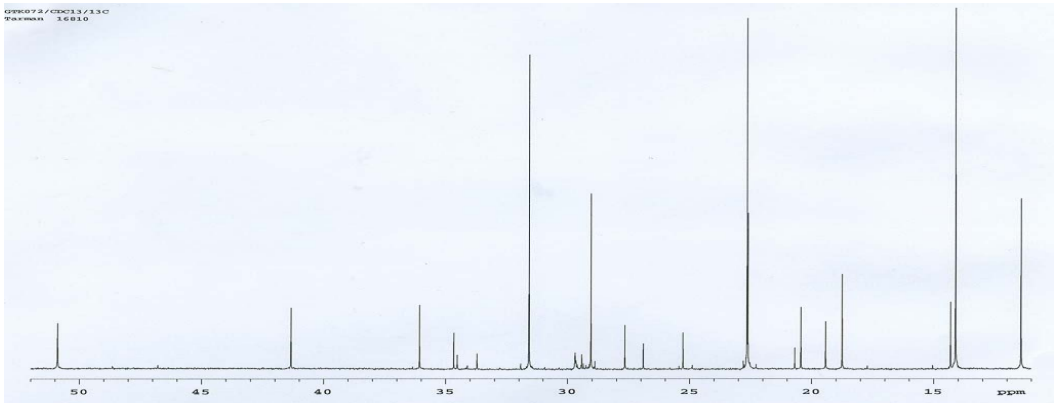
MS Spectrum of Compound KT19/3



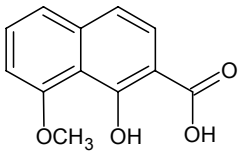
^{1}H-NMR Spectrum of Compound KT19/3



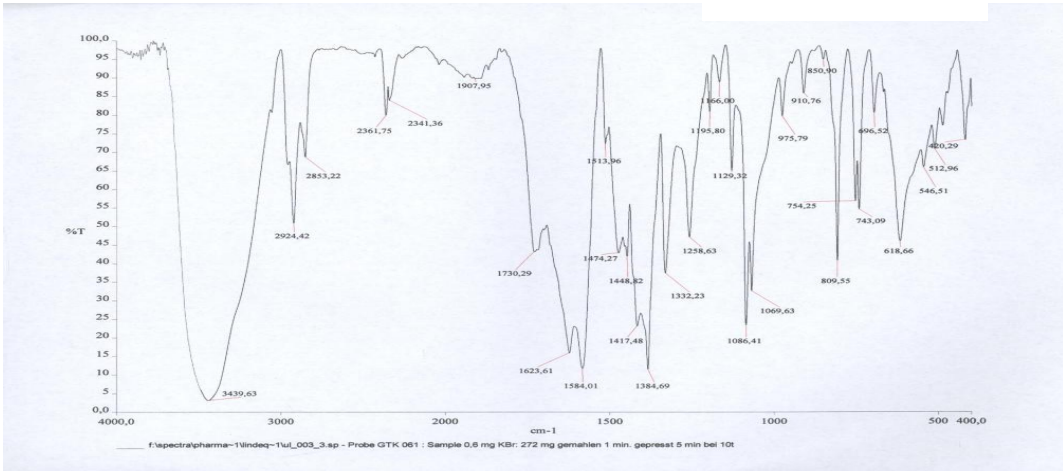
^{13}C-NMR Spectrum of Compound KT19/3



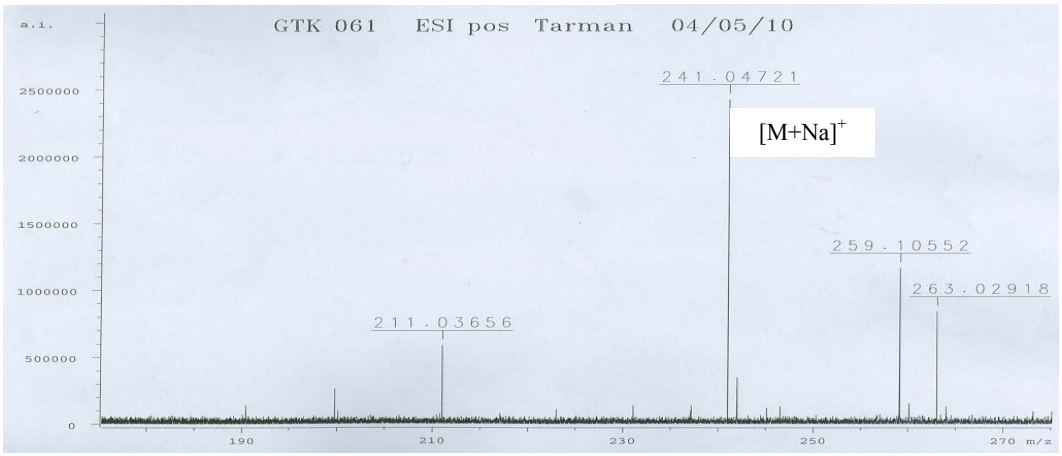
4. Naphthalene carboxylic acid (compound KT29/1) from strain KT29



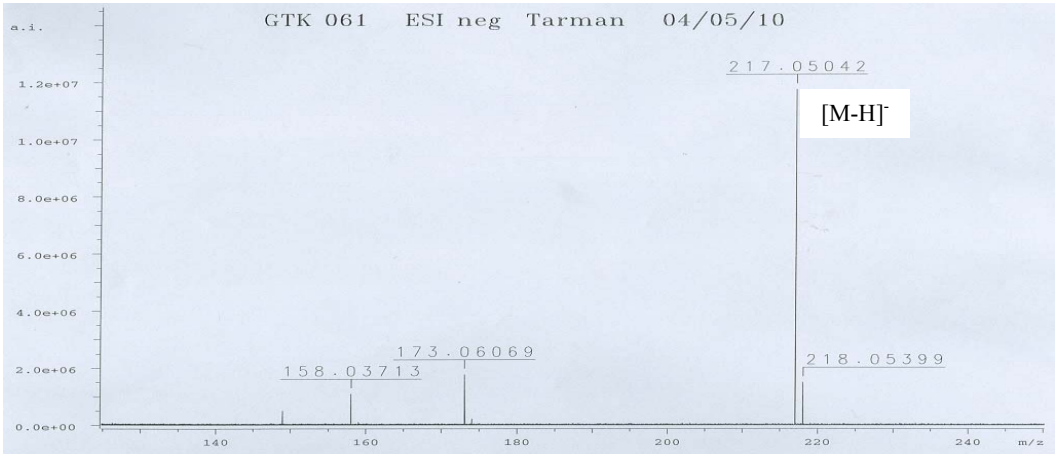
IR Spectrum of Compound KT29/1



MS Spectrum of Compound KT29/1

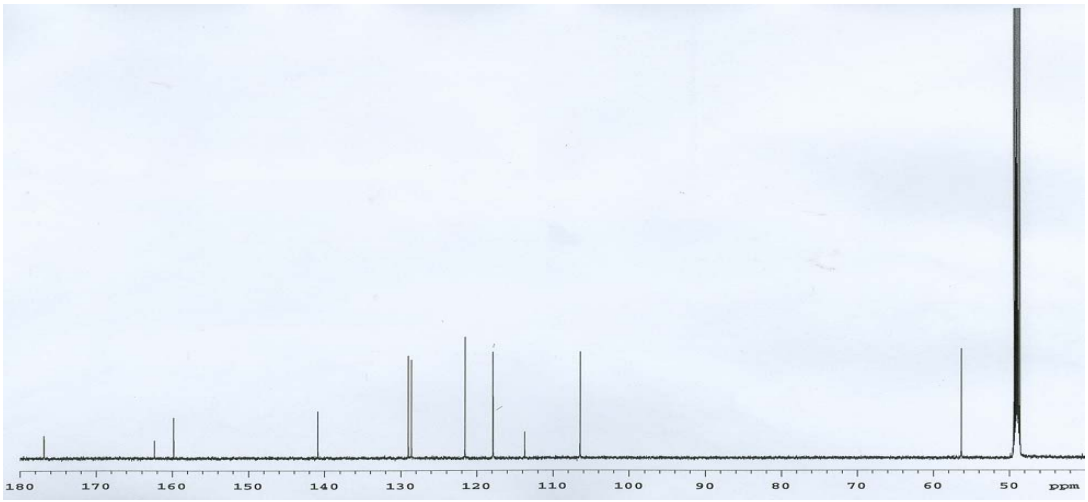


MS Spectrum of Compound KT29/1

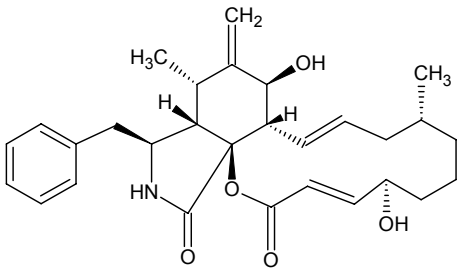


^{1}H-NMR Spectrum of Compound KT29/1

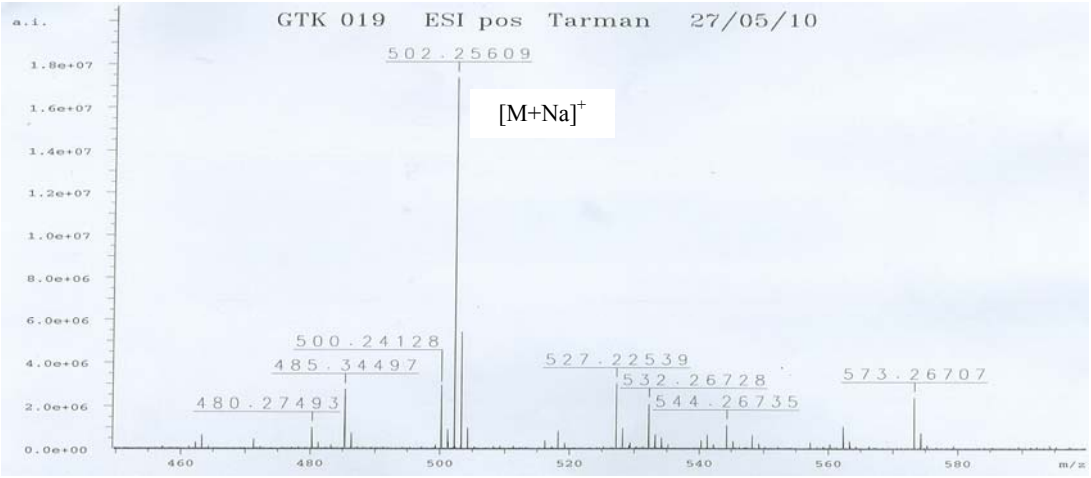
^{13}C-NMR Spectrum of Compound KT29/1



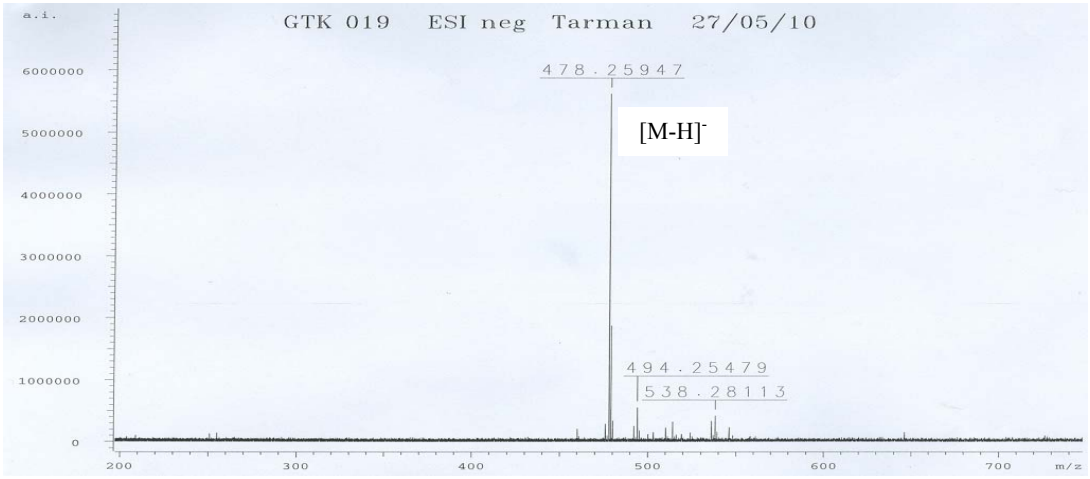
5. Cytochalasin B (compound KT30/2) from *Xylaria psidii* KT30



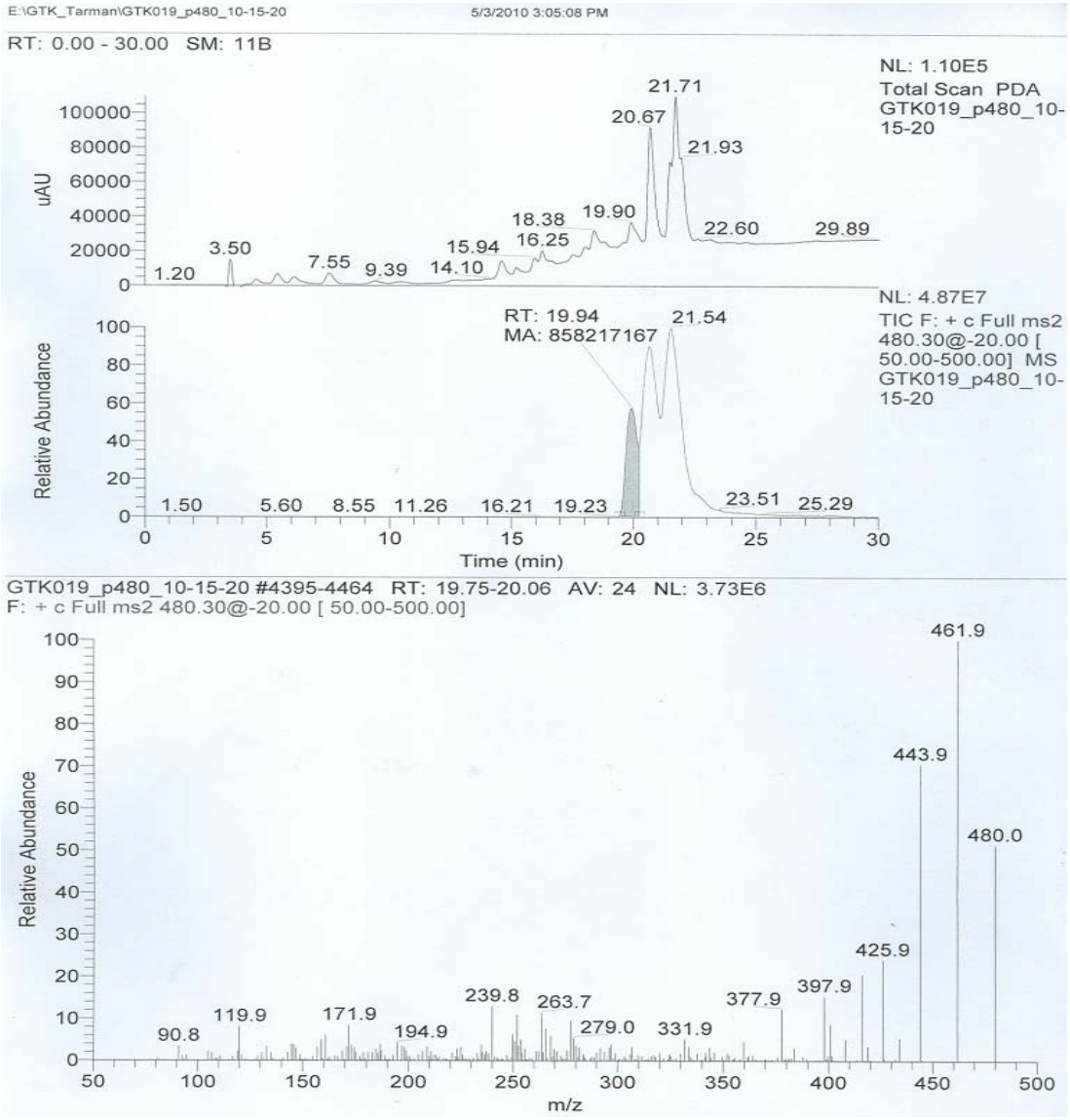
MS Spectrum of Compound KT30/2



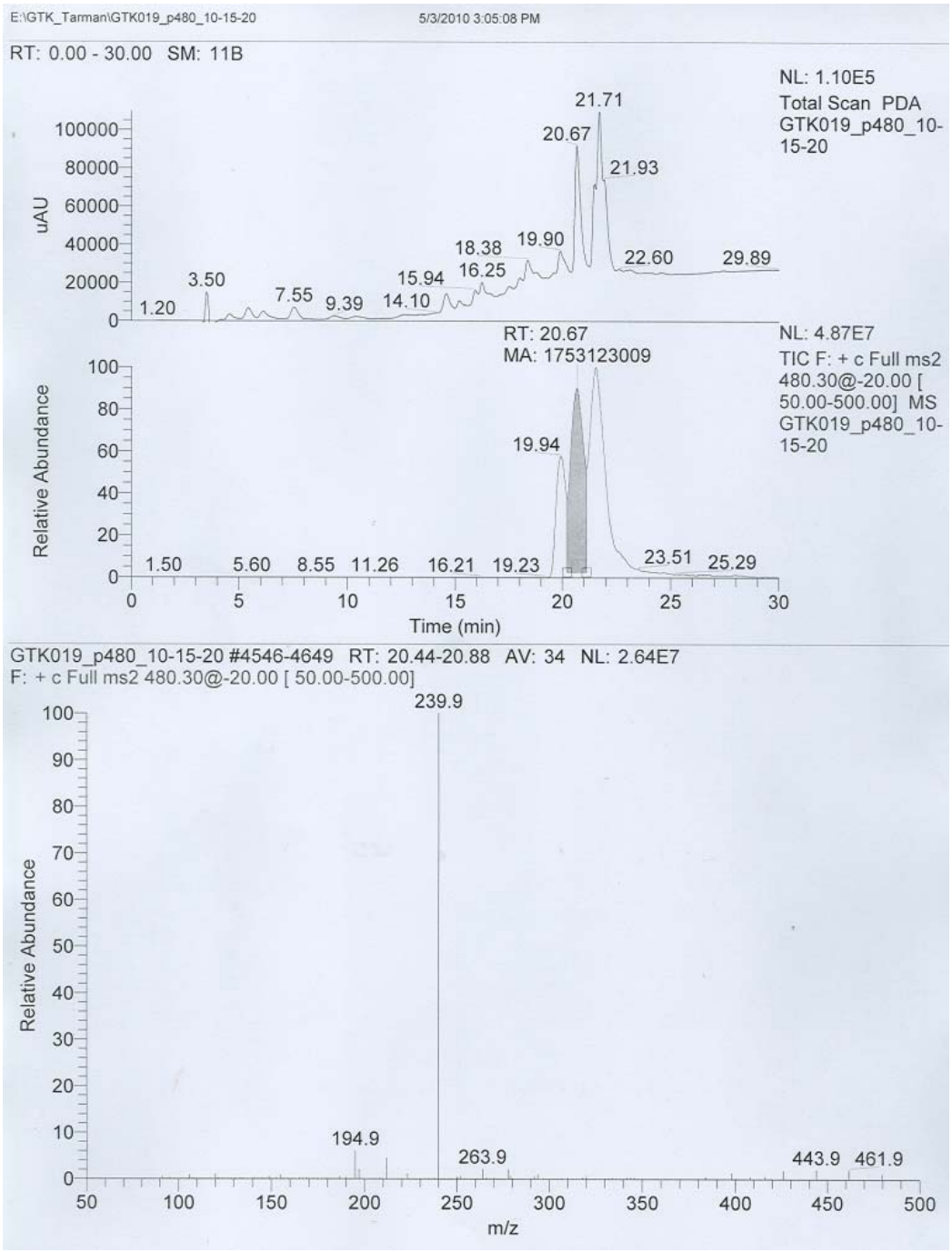
MS Spectrum of Compound KT30/2



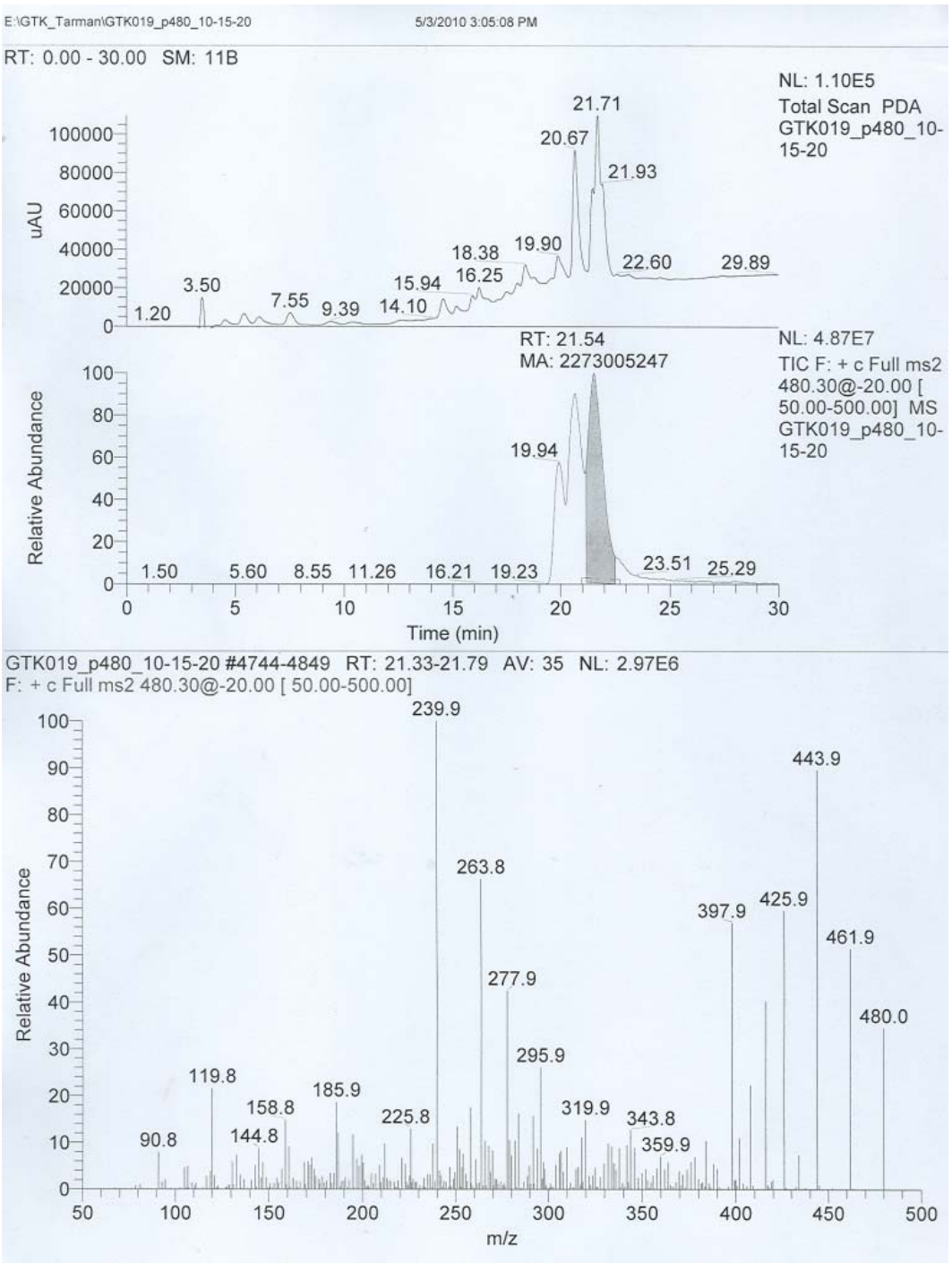
MS/MS Spectra of Compound KT30/2



MS/MS Spectra of Compound KT30/2

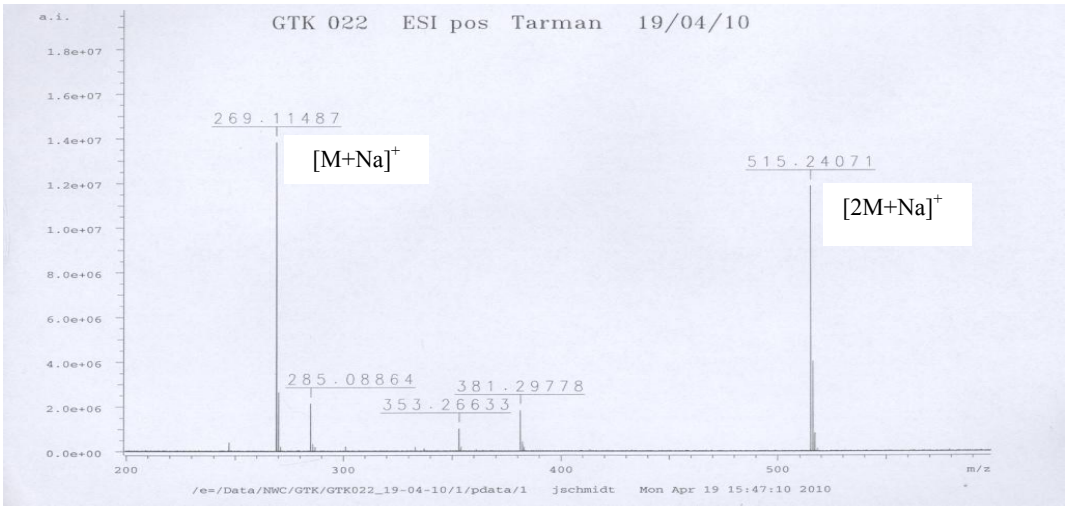


MS/MS Spectra of Compound KT30/2



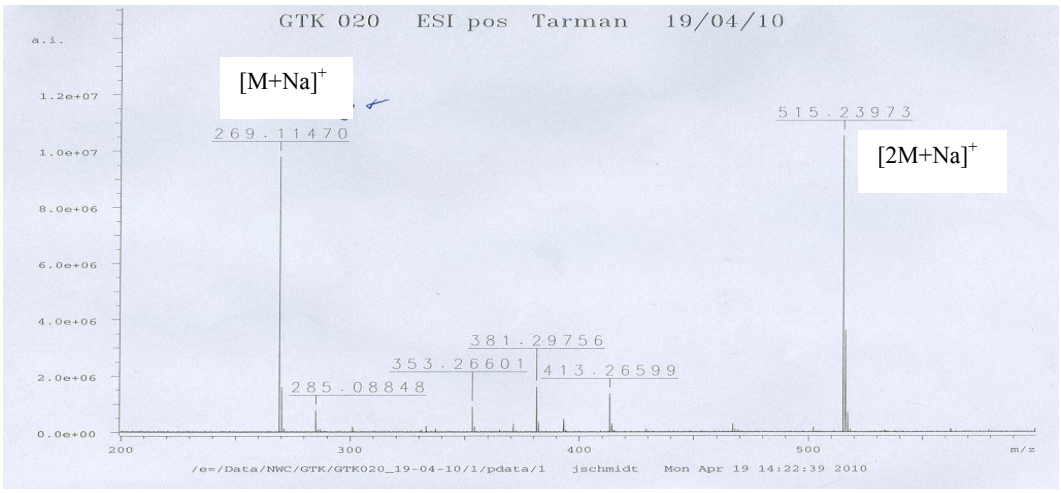
6. Compound KT31/2 from strain KT31

MS Spectrum of Compound KT31/2



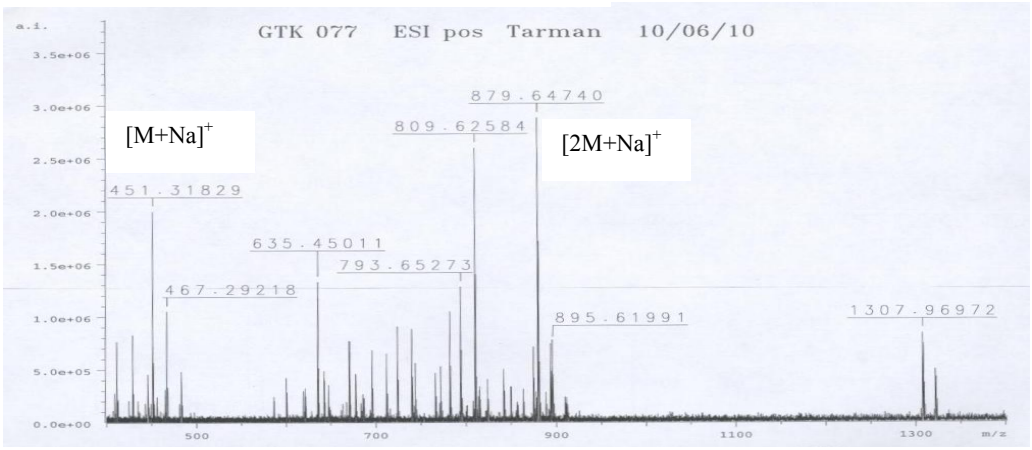
7. Compound KT31/3 from strain KT31

MS Spectrum of Compound KT31/3

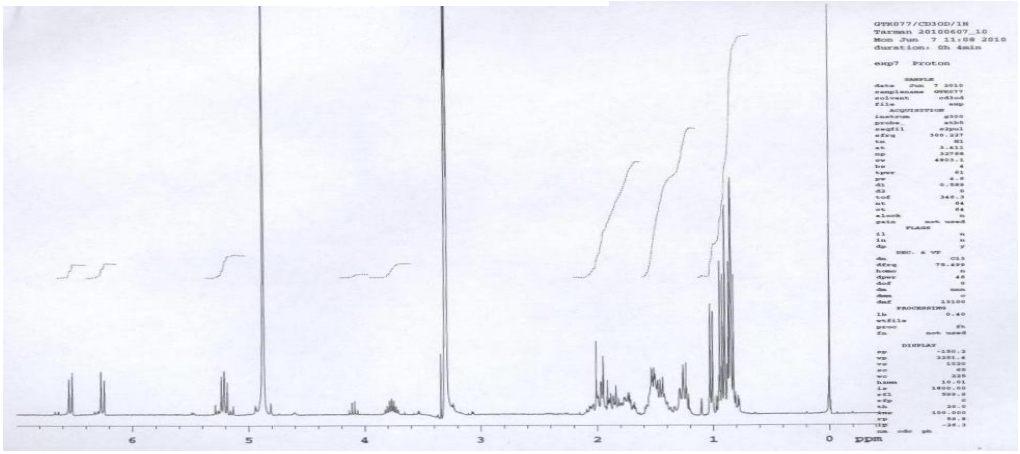


8. Compound KT31/4 from strain KT31

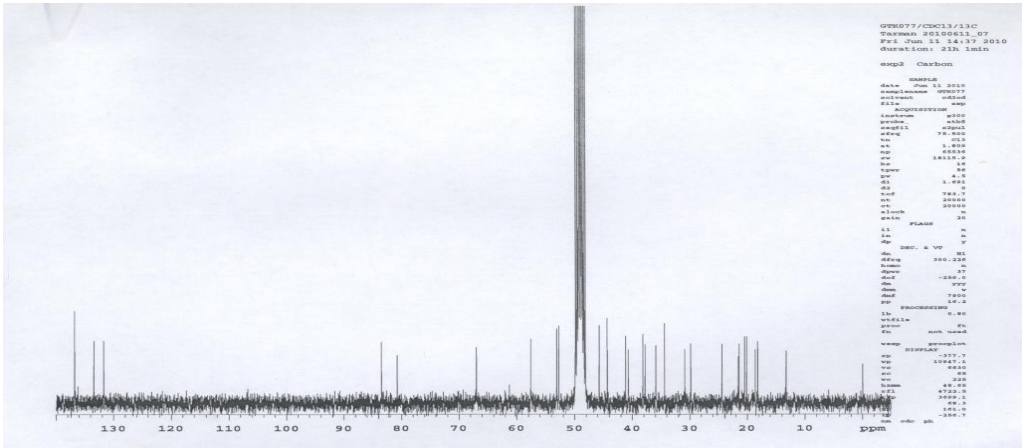
MS Spectrum of Compound KT31/4



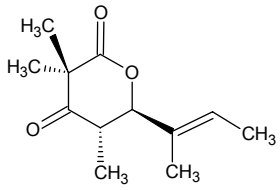
^{1}H-NMR Spectrum of Compound KT31/4



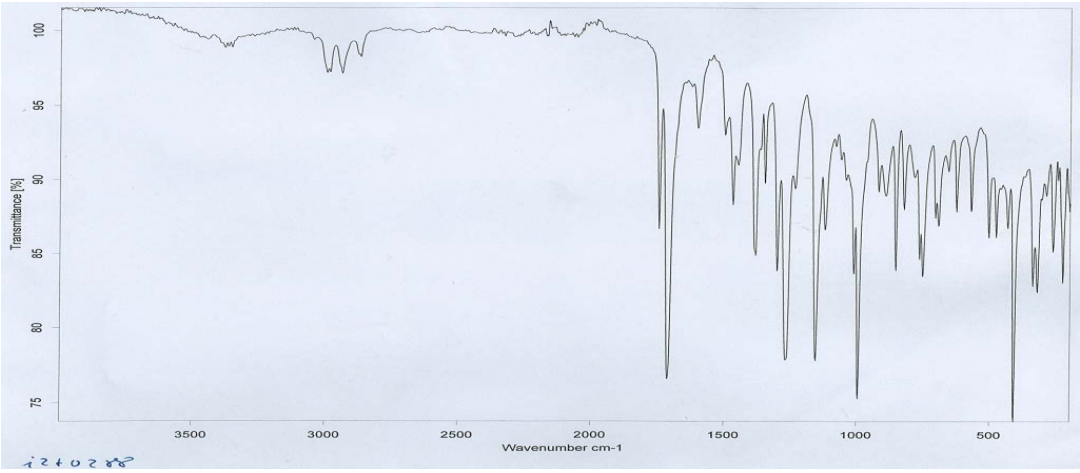
^{13}C-NMR Spectrum of Compound KT31/4



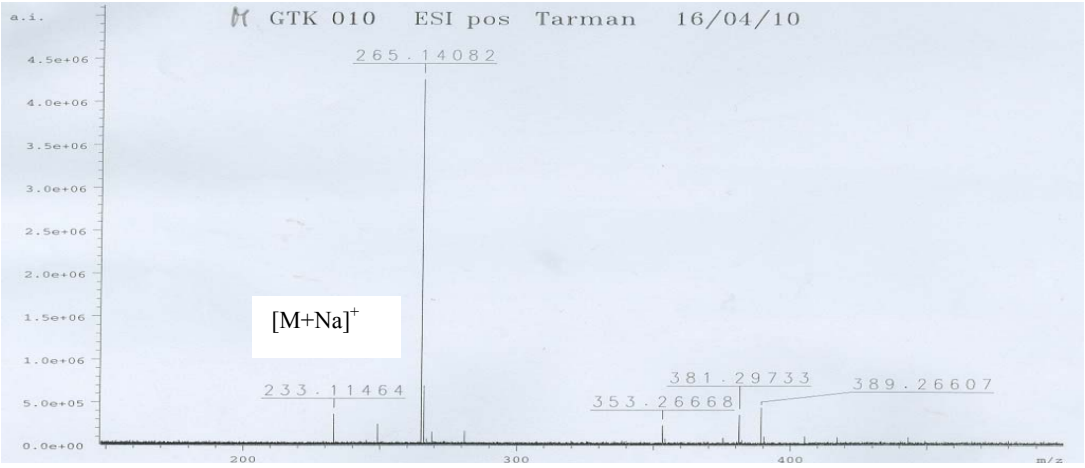
9. Helicascolide C (compound KT32/1) from strain KT32



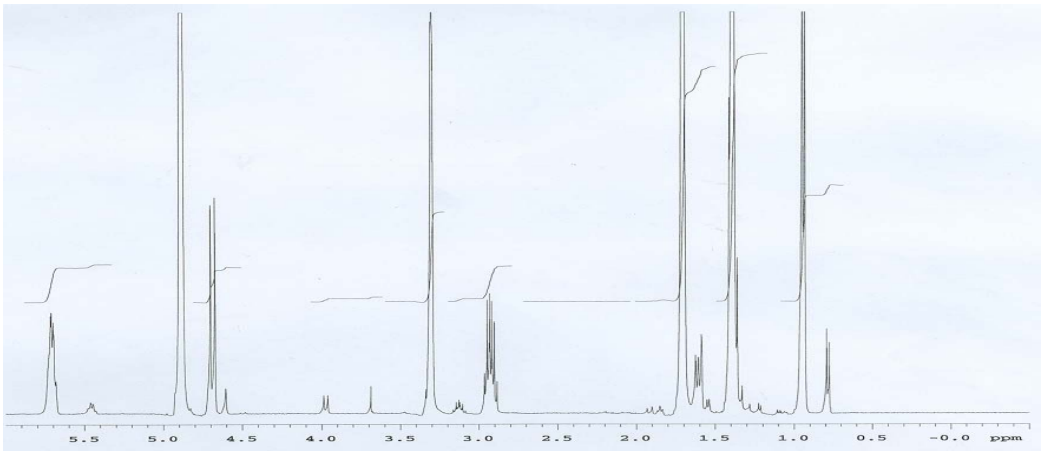
MS Spectrum of Compound KT32/1



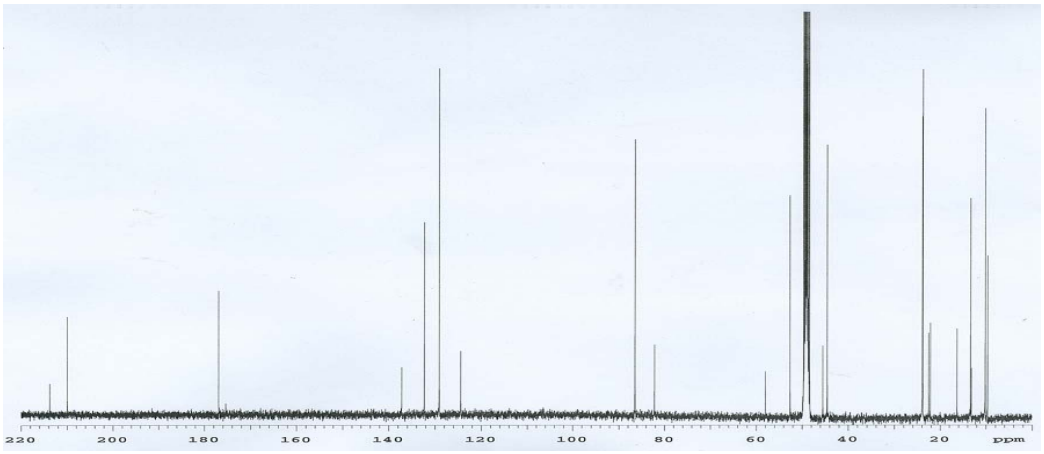
IR Spectrum of Compound KT32/1



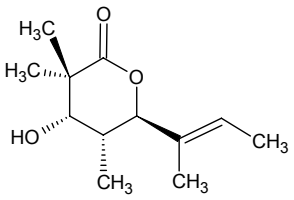
^{1}H-NMR Spectrum of Compound KT32/1



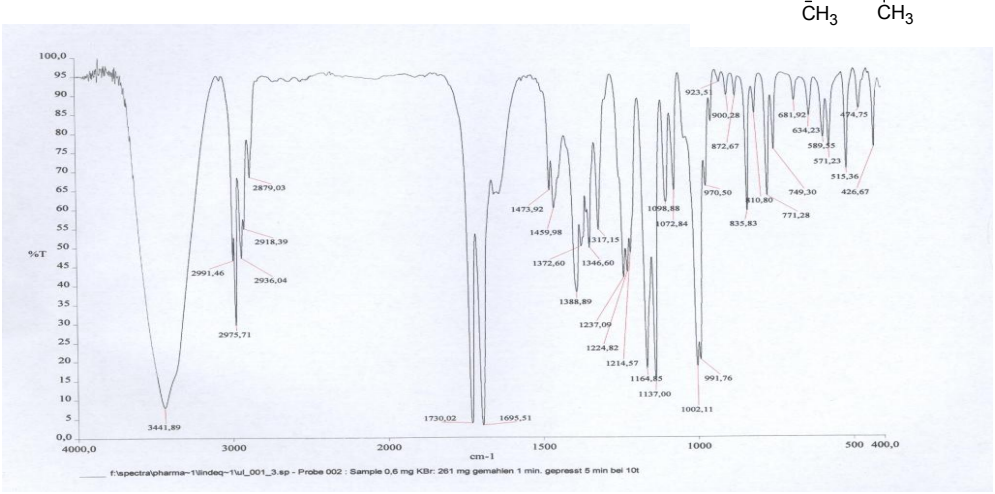
^{13}C-NMR Spectrum of Compound KT32/1



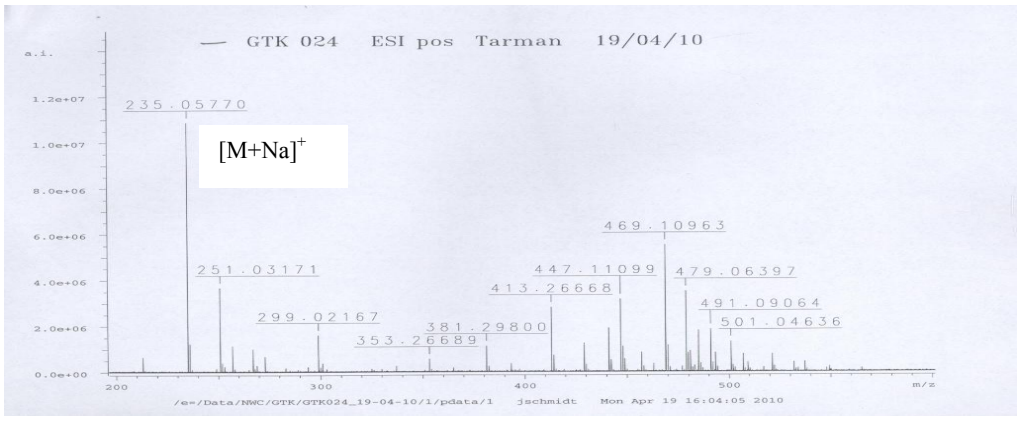
10. Helicascolide A (compound KT32/2) from strain KT32



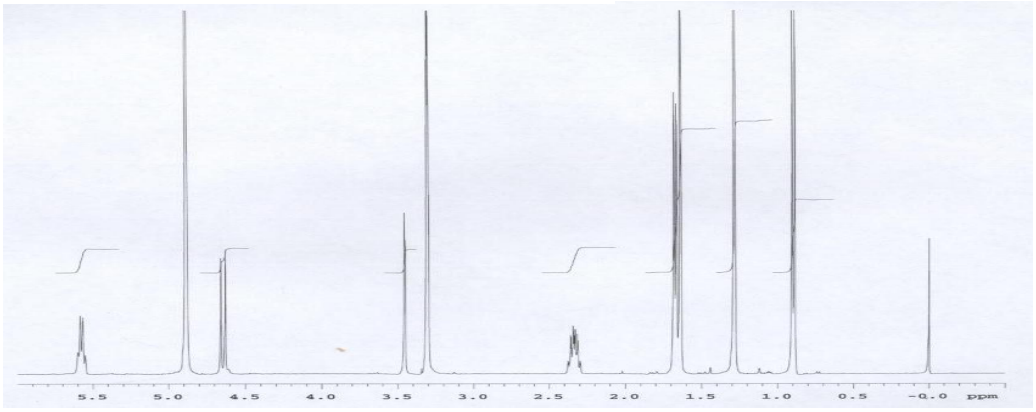
IR Spectrum of Compound KT32/2



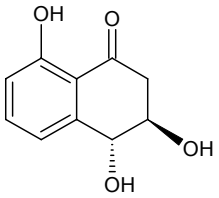
MS Spectrum of Compound KT32/2



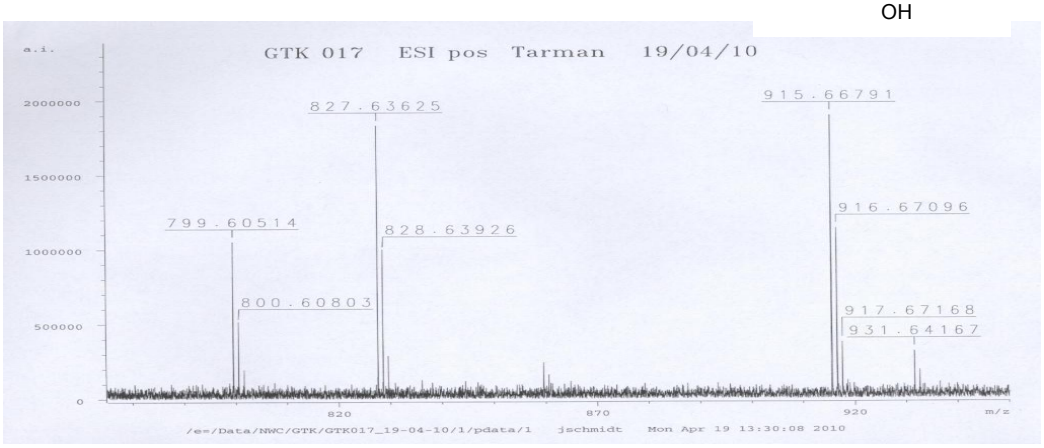
^{1}H-NMR Spectrum of Compound KT32/2



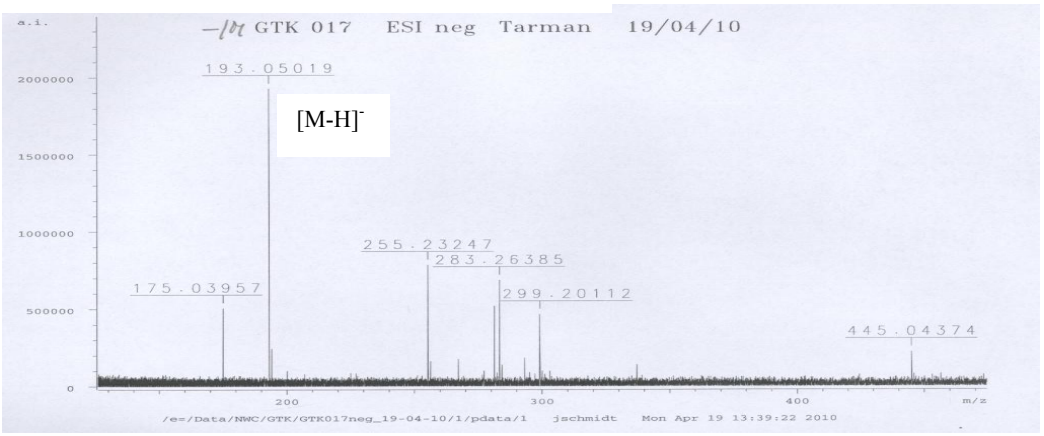
11. Isosclerone (compound KT32/3) from strain KT32



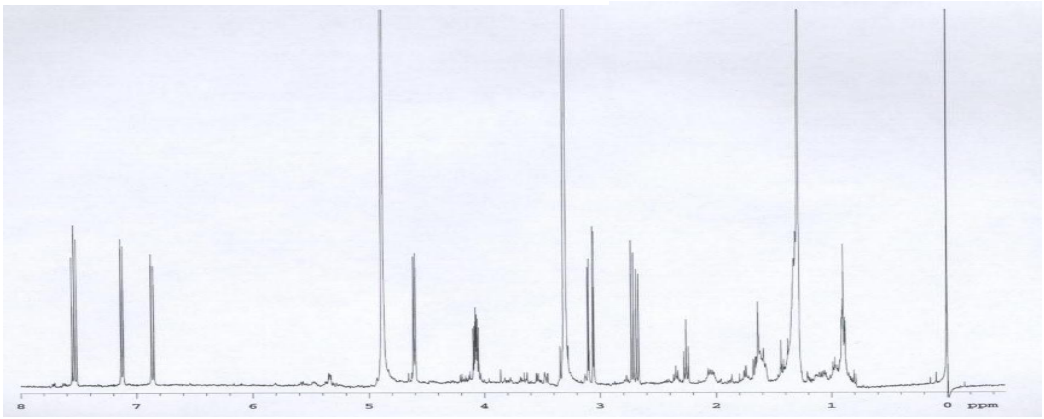
MS Spectrum of Compound KT32/3



MS Spectrum of Compound KT32/3



^{1}H-NMR Spectrum of Compound KT32/3



List of Publications and other Scientific Achievements

Parts of this thesis have been published or are in the publication process in the following conferences and journals.

Cytotoxic and antimicrobial activities of extracts and secondary metabolites from Indonesian marine-derived fungi
Kustiariyah Tarman, Ulrike Lindequist, Kristian Wende, Andrea Porzel, Norbert Arnold, Ludger A. Wessjohann
Marine Drugs 2011, 9 (3), pp. 294-306

Helicascolide C, a new lactone from an Indonesian marine algicolous fungus
Kustiariyah Tarman, Gottfried J. Palm, Andrea Porzel, Jürgen Schmidt, Ludger A. Wessjohann, Kurt Merzweiler, Norbert Arnold, Ulrike Lindequist
Phytochemistry Letters (Submitted)

Helicascolide C, a new lactone from an Indonesian marine algicolous fungus
Tarman K, Palm G, Lindequist U
Planta Medica 12, Vol. 76, August 2010, p. 1246 (Abstract Book)

Helicascolide C, a new lactone from an Indonesian marine algicolous fungus
Kustiariyah Tarman, Gottfried J. Palm, Andrea Porzel, Jürgen Schmidt, Ludger A. Wessjohann, Kurt Merzweiler, Norbert Arnold, Ulrike Lindequist
58th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 29 August-2 September 2010, Berlin, Germany
(Poster presentation)

Biological activity and compounds of marine-derived fungi isolated from tropical sea
Kustiariyah Tarman, Ulrike Lindequist, Kristian Wende, Michael Lalk, Martina Wurster, Beate Cuypers, Gudrun Mernitz
6th European Marine Natural Products Conference, 19-23 July 2009, Porto, Portugal
(Poster presentation)

Metabolic fingerprinting of a marine-derived *Aspergillus* sp. by HPLC and NMR
Kustiariyah Tarman, Michael Lalk, Ulrike Lindequist
Taibah International Chemistry Conference, 23-25 March 2009, Madinah, Saudi Arabia (Poster presentation)

Other publications:

Marine Natural Products: Prospects and Impacts on the Sustainable Development in Indonesia
Ariyanti S. Dewi, **Kustiariyah Tarman**, Agustinus R. Uria
Proceeding of Indonesian Students' Scientific Meeting (ISSM) 2008. ISSN: 0855-8692. Delft. The Netherlands.

Antibacterial Activity and Fatty Acid Components of *Skletonema costatum* Extract
Iriani Setyaningsih, **Kustiariyah**
Proceeding of National Seminar on Fisheries and Marine Sciences, 2007. ISBN: 978-979-99781-2-7. Yogyakarta, Indonesia.

Sea Slug (*Discodoris* sp.) as Functional Food
Nurjanah, **Kustiariyah**
Proceeding of National Seminar on Indonesian Fisheries, 2006. ISBN: 979-97119-9-1. Jakarta, Indonesia.

Separation Optimization of Sea Cucumber Antifungal on Thin Layer Chromatography using PRISMA Model
Kustiariyah, Endang Gumbira-Said, Khaswar Syamsu, Kaseno
Proceeding of International Seminar and Workshop on Marine Biodiversity and their Potential for Developing Biopharmaceutical Industry in Indonesia, 2006. ISBN: 978-979-25-0396-8. Jakarta, Indonesia.

Indonesian Marine Biotechnology has yet to be considered (In Bahasa: Bioteknologi Kelautan Indonesia Belum Dilirik)
SAMUDRA, Indonesian Fisheries Magazine, August 2006. Jakarta, Indonesia

Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Greifswald, den 22.11.2010

Kustiariyah Tarman

CURRICULUM VITAE

Kustiariyah Tarman

Born on the 18th August 1975 in Malang (East Java), Indonesia

Educational Background and Professional Experience

Oct 2007-present	Ph.D student at the Institute of Pharmacy, Ernst-Moritz-Arndt University of Greifswald, Germany.
15.04.-18.06.2010	Research work on Natural Products Isolation and Bioassay at Leibniz-Institute of Plant Biochemistry (IPB), Halle (Saale), Germany.
June-Sept. 2007	German course at Goethe Institute, Dresden, Germany; sponsored by DAAD, Germany.
2002-2006	MSc in Agro-Industrial Technology, Bogor Agricultural University, Indonesia
	Thesis under supervision of Prof. Dr. E. Gumbira-Said, Prof. Dr. Khaswar Syamsu and Dr. Kaseno, entitled: "Isolation, Characterization and Biological Activity Test of Sea Cucumber Steroid Compounds as Natural Aphrodisiac"
Jan.-Dec. 2005	Industrial Biotechnology Training Programme, GBF-Braunschweig (*now* the Helmholtz Centre for Infection Research-HZI) and Institute of Marine Biotechnology (IMaB), Greifswald University; conducted by InWEnt, Germany.
2005	German course at Carl Duisburg Centre (CDC) Saarbrücken and Dortmund, Germany; sponsored by InWEnt, Germany. January-April.
2005-present	Lecturer, Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Indonesia.
2000-present	Research Associate, Division of Marine Biotechnology, Centre for Coastal and Marine Resources Studies, Bogor Agricultural University, Indonesia.
1999-2001	Lecturer assistant for Marine Biotechnology, Department of Fisheries Product Technology (*now* Department of Aquatic Product Technology), Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Indonesia.
1994-1999	B.Sc in Technology of Fisheries Product, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Indonesia.

1991-1994 High school in Malang, East Java, Indonesia.

Greifswald, 22.11.2010

Kustiariyah Tarman

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