BIOACTIVE NATURAL PRODUCTS FROM THE SOFT CORAL, *SINULARIA* SP.

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BIOACTIVE NATURAL PRODUCTS FROM THE SOFT CORAL, *SINULARIA* SP.

BY

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ABSTRACT

BIOACTIVE NATURAL PRODUCTS FROM THE SOFT CORAL, *Sinularia* sp.

Lim Fei Shen

Soft coral is a rich source of bioactive secondary metabolites and one of the most prolific sources of new marine natural products. In this study, the soft coral, *Sinularia* sp. was chosen to study its photo-toxicity, antifungal and antibacterial activities. Lyophilized samples of *Sinularia* sp. were subjected to sequential extraction using organic solvents of increasing polarity, i.e. hexane, diethyl ether, chloroform, ethyl acetate, acetone, methanol and water at a ratio of 1 g: 10 mL. Microculture tetrazolium (MTT) test with light irradiation was used to study its photo-toxicity activity towards the leukemia cell line, HL 60. Chloroform extract with the highest photo-toxicity activity (2.25% of cell viability) was subjected to isolation using flash chromatography. However, the fractions were unstable and lost its activitiy after fractionation. In antimicrobial assay, a colorimetric broth microdilution method using *p*-iodonitrotetrazolium violet (INT) as growth indicator was employed to determine the minimum inhibitory concentrations (MICs) towards medically-important bacteria and fungi. Hexane extract which exhibited the strongest inhibitory effect towards *Cryptococcus neoformans* (MIC= 0.03 mg/mL, n=3) was selected for further *in vitro* bioassay-guided fractionation. It was purified using silica gel impregnated

with silver nitrate and semi-preparative thin layer chromatography (TLC). Fuscol ($C_{20}H_{32}O$, MW = 288) which was identified by gas chromatography mass spectrometry (GCMS) and nuclear magnetic resonance (NMR) analysis had a mean of MIC value 0.064 mg/mL towards *C. neoformans*. Fraction 1 from the hexane extract which demonstrated anti-fungal activity (MIC = 0.13 mg/mL) contains a mixture of hydrocarbons and eight other compounds identified using GCMS. Fraction 6, a white powder, was found to be a mixture of sterols (MIC = 0.06 mg/mL).

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FACULTY OF SCIENCE

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SUBMISSION OF THESIS

It is hereby certified that LIM FEI SHEN (ID No: 08UEM01996) has completed this thesis entitled "BIOACTIVE NATURAL PRODUCTS FROM THE SOFT CORAL, SINULARIA SP." under the supervision of Assoc. Prof. Dr. Khoo Kong Soo (Supervisor) from the Department of Chemical Science, Faculty of Science, and Assist. Prof. Dr. Sit Nam Weng (Co-supervisor) from Department of Biomedical Science, Faculty of Science.

I understood that the University will upload softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(LIM FEI SHEN)

APPROVAL SHEET

This thesis entitled "**BIOACTIVE NATURAL PRODUCTS FROM THE SOFT CORAL, *SINULARIA* SP."** was prepared by LIM FEI SHEN and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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DECLARATION

I _____ hereby declare that the thesis/dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____
(LIM FEI SHEN)

Date _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndromes
ATCC	American Type Culture Collection
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standard Institute
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulphoxide
FDA	Food and Drug Administration
GCMS	Gas Chromatography Mass Spectrametry
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
INT	*p*-Iodonitrotetrazolium violet
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
MOPS	3-(N-morpholino) propanesulfonic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance
PDA	Potato dextrose agar
PDT	Photodynamic therapy
Pha	Pheophorbide-A

RPMI	Roswell Park Memorial Institute
SARS	Severe acute respiratory syndrome
TIC	Total ion chromatogram
TLC	Thin layer chromatography
UV	Ultraviolet

CHAPTER 1.0

INTRODUCTION

1.1 Natural Products

Natural products are secondary metabolites from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and marine microorganisms (Chin et al., 2006). These compounds usually have molecular weights of less than 2000 amu. Examples of secondary metabolites are alkaloids, flavonoids, coumarins, glycosides, lignins, steroids, terpenoids and many more. They can be obtained after a number of processes accordingly which are extraction, fractionation and isolation (Sarker et al., 2006).

For thousands of years, natural products have played an important role in treating and preventing human diseases. The contribution of natural product is evidenced by approximately 75% of drugs for infectious diseases and 60% of anticancer compounds are either natural products or natural product derivatives. According to World Health Organization, roughly two-thirds to three quarters of the world's population relies upon medicinal plants for its primary pharmaceutical care (McChesney et al., 2007). Natural products provide a starting point for new synthetic compounds, with diverse structures and often with multiple stereo centers (Balunasand Kinghorn2005).

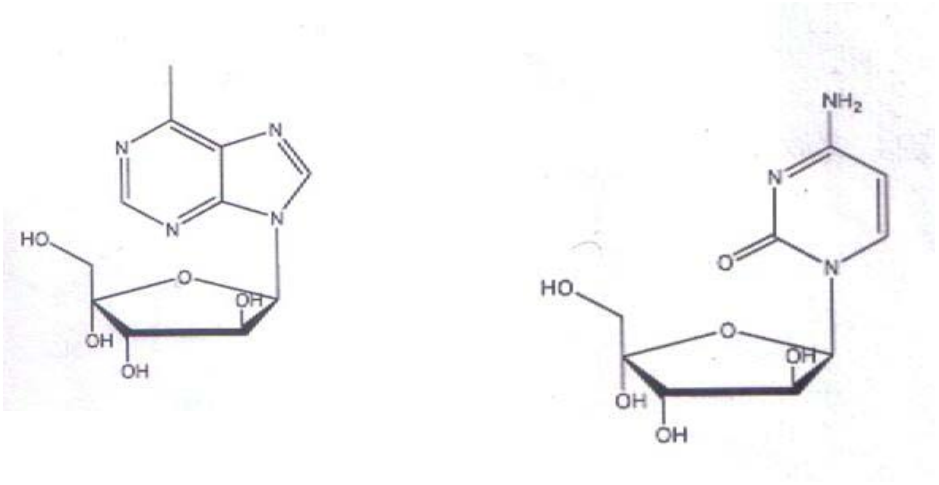
1.2 Marine Natural Products

Among the natural products, marine compounds provide numerous of sources of natural products since the oceans cover more than 70% of the earth's surface and represent greatest biodiversity in the world. There are thousands of marine life identified and millions more unknown (Murray et al., 1999). Among the known groups of organisms are such as coral, sponges, mollusks, tunicates, echinoderms, and algae.

These marine organisms produce valuable natural products that are useful to mankind. Ecological pressures, including predation, competition for space, the fouling of the surface and successfully reproducing have led to the evolution of secondary metabolites with various bioactivities (Donia and Hamann 2003). Among the natural sources, marine organisms such as sponges, sea cucumbers, mollusks and corals have a great potential for producing new drugs. They are potential sources of bioactive substances and have provided novel lead compounds for the pharmaceutical industry (Mayer et al., 2009).

Since early 1950s, the search for drugs in marine began when the first spongouridine and spongothymidine were isolated from the Caribbean sponge, *Cryptothecacrypta*. They were approved as an anticancer drug (cytosine arabinoside, Ara-C) and an antiviral drug (adenine arabinoside, Ara-A), which

received FDA approval in 1969 and 1976, respectively (Figure 1.1) (Jimeno et al., 2004; Chin et al., 2006; Glaser and Mayer 2009).



Adenine arabinoside, Ara-A **Cytosine arabinoside, Ara-C**

Figure 1.1: Adenine arabinoside, Ara-A and cytosine arabinoside, Ara-C.

1.3 *Sinularia* sp.

Coral reefs are a rich source of food and livelihood. They play a key role in stabilizing coastlines and also provide us with goods and services (Moberg and Folke, 1999). Coral reefs are made up of hard and soft coral. Corals acquire the majority of their energetic and nutrient requirements by two mechanisms either develop a symbiotic relationships with tiny yellow brown plants called zooxanthellae, or the direct ingestion of zooplankton and other organic particles in the water column by the cnidarian host (Lesser 2004). Hard corals have a very hardy-like appearance and made of rigid calcium carbonate (limestone) with hard

calcium skeletons. While the soft corals have a soft and leathery feel. Soft corals do have some rigid calcium carbonate but it is blended with protein, so it is less rigid compared to hard coral (Haider and Khan 2012).

Figure 1.2: *Sinularia* sp.

The soft coral *Sinularia* sp. (Figure 1.2) was used for this research. *Sinularia* is a soft coral in the kingdom of Animalia, phylum *Cnidaria*, class *Anthozoa*, order *Alcyonacea* and family *Alcyoniidae*. *Sinularia*sp. also referred as "thin finger leather coral", and it can be green, yellow or brown coloured. It is a slow growing coral. Although sessile, it is free from fouling organisms. Relatively high number of coral species in coral reef systems and the abundance of *Sinularia* sp. show that it has some mechanisms to compete for space and resist invasion by faster growing organisms. Although this soft coral is lacking in a physical defense system, it appears to be remarkably free of predators. Since the last two decades,

*Sinularia*sp. attracts considerably attention because it produces a wide range of secondary metabolites including sesquiterpenes, diterpenes, polyhydroxylated steroids, and polyamine compounds. These metabolites were shown to possess a range of biological activities such as antimicrobial (Kelman et al., 2006), anti-inflammatory (Williams and Faulkner 1996), antipredator (Alstyne et al., 1994) and cytotoxic activities (Sheuet al., 1999).

1.4 Antimicrobial

Over the years, antimicrobial resistance is a major health problem and challenges worldwide. New infectious diseases keep emerging and the use of certain antibiotics had caused undesirable side effects. Even though many antimicrobial compounds are marketed, the ability of microbes to develop resistance to antimicrobial compounds is amazingly rapid (Jayaraman et al., 2008). Besides antibiotic resistance, humankind is faced with the emergence of new epidemics threats such as severe acute respiratory syndrome (SARS), human immunodeficiency virus - human acquired immune deficiency syndrome (HIV-AIDS) and bird flu (Minh et al., 2005).

Fungal infections are a major cause of diseases and mortality for the past decade, in part as a consequence of the increase in AIDS, the greater use of immunosuppressive drugs in transplantation and chemotherapeutic agents in cancer, long term use of corticosteroids and even the indiscriminate use of

antibiotics. The common antifungal drugs available in the market are polyenes (such as amphotericin B and nystatin), echinocandins (such as caspofungin and micafungin), allylamines (such as niftifine and terbinafine), and azoles (such as fluconazole, ketoconazole, itraconazole, etc) (Aher et al., 2009). Besides, the development of vaccines against fungal pathogens is less advanced than bacterial pathogens due to the similar eukaryotic nature with humans and the target patients to be vaccinated are often immunocompromised (Segal and Elad 2006).

1.5 Photodynamic Therapy

Cancer is still a medical enigma that has yet to be solved.Surgery, radiotherapy and chemotherapy are the three major methods used by clinicians in the treatment of cancer. These methods are useful in some forms of cancer but there is no single method can be used against all forms of cancer.Photodynamic therapy (PDT) has been proposed as a promising new approach of medical treatment of cancer diseases and non-oncological disorders (Buytaert et al., 2007). The treatment is based upon the interaction of visible light with the photosensitizing molecules in target cells. Subsequent photochemical reactions result in the production of reactive oxygen species (ROS) inducing the cell death. PDT can induce both modes of cell death, apoptosis and necrosis in target cells (Plaetzer et al., 2003).

Photofrin is a photosensitizer approved by the European Union, the United States FDA and Asian countries use in PDT. Hypocrellin A, which is isolated from natural fungus sacs of *Hypocrellabambusae*, causes cell damage by photodynamic reactions (Chio-Srichan et al., 2008). Due to the cytotoxicity activities reported, many researchers have focused on the search for phototoxic compounds from marine sources.

1.6 Hypothesis

Among the marine invertebrates, soft corals continue to be one of the most prolific sources of new marine natural products. The secondary metabolites found in soft corals have diverse bioactivities, including anti-infective, anti-predatory, allelopathic, anti-fouling and cytotoxic properties (Wang, 2008). Since the soft coral, *Sinularia*sp. is a rich source of bioactive secondary metabolites (Wang, 2008) this research is focused on whether extracts from it could be used as anti-microbial drug.

1.7 Objectives

The objectives of this study were to extract antimicrobial compounds from *Sinularia* sp. Isolation and identification of the antifungal active extract were conducted by using flash chromatography impregnated with silver nitrate and gas chromatography mass spectrometer (GC-MS). This research is also focused on

whether extracts from it could be used as photosensitisers in photodynamic therapy (PDT) which exhibited minimally invasive therapeutic modality in the threatment of cancer diseases and non-oncological disorders (Buytaertet al., 2007). Besides, the toxicity of solvent on the microbes used was also carried out.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Products

Natural products are compounds that are obtained from living organisms which include plants, microorganisms and animals from land and marine (Table 2.1). The compounds can be obtained from the entire or part of the organism. For example, leaves, bark and stem of a plant or an individual organ of an animal. In chemistry, natural products are often referred as secondary metabolites or pure compounds that are extracted from the entire or part of the organism. The examples of secondary metabolites are alkaloids, coumarins, glycosides, lignins, flavonoids and terpenoids (Sarker et al., 2006).

Marine natural products are gaining more attention in the research field as many compounds are found to be useful from medical to bio-fuel industries. Many reviews and journals have published highlighting the promising future of marine natural products as the main source in drug development (Mayer et al., 2009; Nobili et al., 2009).

Table 2.1: Drugs developed from natural sources.

Drug	Medical use	Mechanism of action	Source
Aspirin	Analgesic, anti-inflammatory, antipyretic	Inhibition of cyclooxygenase	Plant
Atropine	Pupil dilator	Antagonistof acetylcholine at muscarinic receptors at post-ganglionic parasympathetic neuroeffector sites	Plant
Caffeine	Stimulant	Adenosine receptor antagonist	Plant
Codeine	Analgesic, antitussive	Opioid receptor agonist	Plant
Digoxin	For atrial fibrillation and congestive heart failure	Inhibition of the Na^+/K^+ ATPase membrane pump	Plant
Eugenol	Toothache	Reduces excitability of sensory nerves	Plant
Morphine	Analgesic	Opioid receptor agonist	Plant
Pilocarpine	Glaucoma	Muscarinic receptor agonist	Plant
Quinine	Malaria prophylaxis	Inhibition of protein synthesis in the malarial parasite	Plant
Taxol	Anticancer agent	Antimitotic (binds to and stabilizes microtubules)	Plant
Penicillin	Antibiotic	Inhibition of the synthesis of cell wall peptidoglycogen	Microbe
Tetracycline	Antibiotic	Inhibition of protein synthesis by binding to the ribosome 30S subunit	Microbe
Cyclosporin A	Immunosuppressant	Inhibition of clonal proliferation of T lymphocytes	Microbe
Aurantosides	Antifungal	Inhibition of tubulin polymerization	Marine organism
Spongistatin 1	Antifungal	Inhibition of tubulin polymerization	Marine organism
Manoalide	Analgesic, anti-inflammatory	Inhibition of phospholipase A2	Marine organism

(da Rocha et al.,2001)

2.2 Research on *Sinularia* sp.

Soft corals, or Alcyonacea, are included in the subclass Octacorallia because they have tentacles and internal body divisions in multiples of eight. They belong to the class of Anthozoa of phylum Cnidaria, the stinging-celled animals. They are found in parts of the reef with the greatest current, facing the open ocean. This is due to the fact that soft corals rely on currents to bring them food and oxygen, and carry away their wastes. Soft corals tend to produce smooth mucous coating and it is colonized by bacteria. Some bacteria can be pathogenic and cause disease but some bacteria could serve as beneficial symbionts. However, corals are able to get rid of the bacteria by self cleansing of mucus from their surface (Kelman et al., 2006).

The Genus *Sinularia* is one of the most widely distributed soft coral in the tropical reef. During the past decade, many bioactive metabolites have been reported and it hasyielded many new skeletal structures such as unusual sesquiterpenes, cembranediterpenes and sterols.

To date, not much researches that have been done on the soft coral S*inularia* sp. although a large variety of natural products is expected from the soft coral.Iguchi et al. (1995) isolated a norditerpenoid, named yonarolide (Figure 2.1) from *Sinularia* sp. Yonarolide has a novel tricycle tetradecane skeleton.

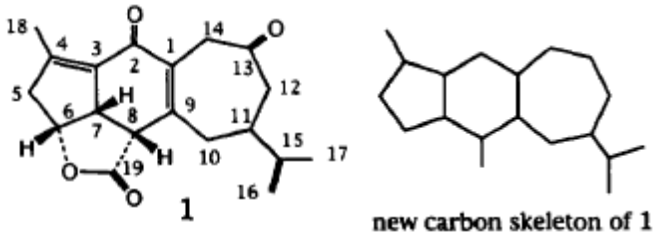
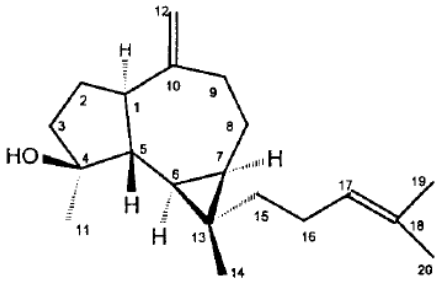
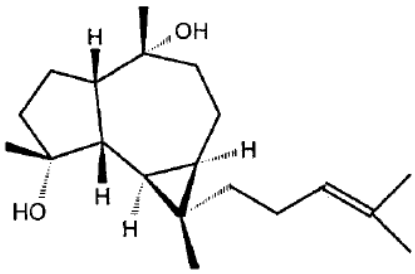


Figure 2.1: Yonarolide isolated from *Sinularia* sp. by Iguchi et al. (1995).

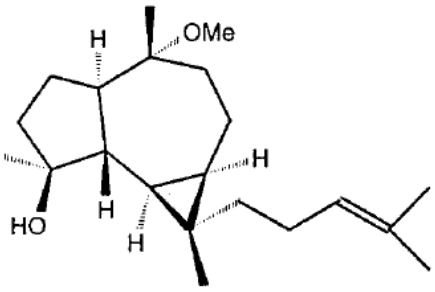
Anjaneyuluet al. (1997) successfully isolated nine compounds from S*inularia*sp. in which four of them (Figure 2.2) are novel diterpenoids having aromadendrane skeleton. This class of diterpenoids was only found once from terrestrial source, *Cneorumtricoccon* and *Neochemaeleapulverulenta*.



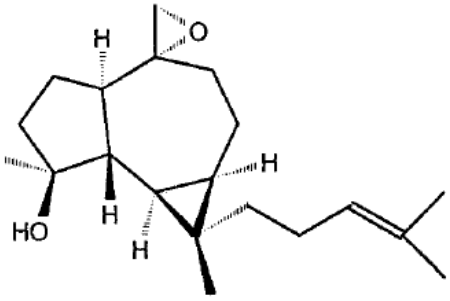
4β-hydroxyl-15–(3-methyl-2-butenyl)aromadendr-$\Delta_{10(12)}$-en



4α,10α-dihydroxyl-15–(3-methyl-2-butenyl)alloromadendrane



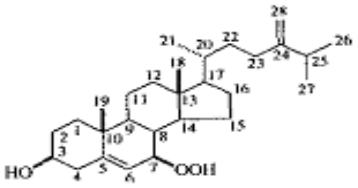
4β-hydroxyl-10α–methoxy-15–(3-methyl-2-butenyl)-aromadendrane



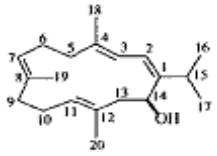
4β-hydroxyl-10,12-α–epoxy-15–(3-methyl-2-butenyl)-aromadendrane

Figure 2.2: Four novel diterpenoids isolated from *Sinularia* sp. by Anjaneyuluet al. (1997).

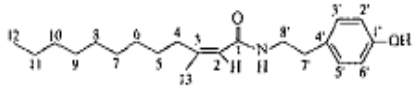
Sheuet al. (1999) found a new marine sterol, 7β-hydroperoxy-24-methylenecholesterol from *Sinularia* sp. Besides, five known compounds (Figure 2.3) were also isolated. All these six compounds showed significant cytotoxicity activities against P-388 (leukemic cell line), KB, A-549 (human lung adenocarcinoma) and HT-29 (human colon adenocarcinoma) cancerous cell lines.



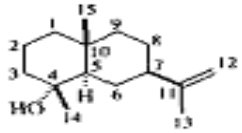
7β-hydroperoxy-24-methylenecholesterol



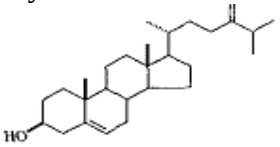
Sarchophytol A



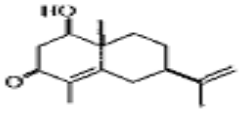
(Z)-N-[2-(4-hydroxyphenyl)ethyl]-3-methyldodec-2-enamide



5α,7αH-eduesm-11(13)en-4α-ol



24-methylenecholesterol

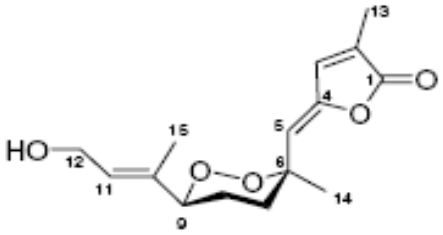


1β-hydroxy-α-cyperone

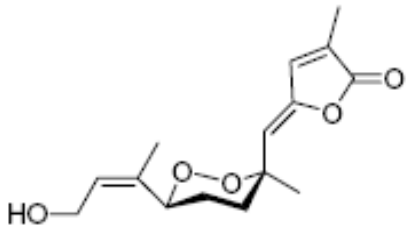
Figure 2.3: Six marine sterols isolated from *Sinularia* sp. by Sheuet al. (1999).

Sinularioperoxides A-D and sinularianins A (Figure 2.4) found by Chao et al. (2006(a)) and Chao et al. (2006(b)), respectively, are not active against a panel of cancer cell lines, including A549 (human lung adenocarcinoma), HepG2

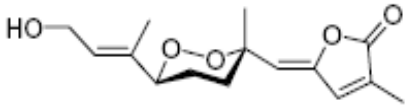
(human hepatocellular carcinoma), MCF7 and MAD-MB-231 (both human breast carcinoma) cells although cyclic peroxides often exhibit a wide spectrum of biological activities including cytotoxic against cancer cells and antiparasitic activities. Both sinularioperoxides and sinularianins A are sesquiterpenoids. Sinularioperoxides possess a cyclic peroxide and a γ-alkylidene-α-methyl-α,β-unsaturated γ-lactone moieties.While, sinularianins A, possesses a bicyclic skeleton,sinulariolane.SinularianinB found by Chao et al. (2006(b)) has a valerenane skeleton, with a spiro-butenolide moiety, which had been reported to occur in plants. Due to low yield of sinularianins B, the cytotoxicity of this metabolite toward the above cell lines has not been determined.



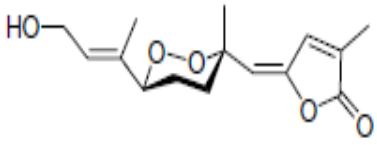
Sinularioperoxide A



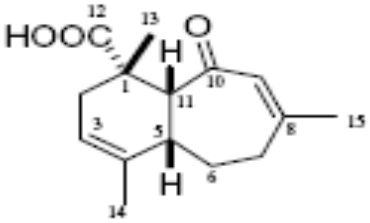
Sinularioperoxide B



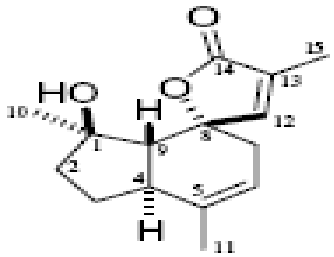
Sinularioperoxide C



Sinularioperoxide D



Sinularianins A



Sinularianins B

Figure 2.4: Six compounds isolated from *Sinularia* sp. by Chao et al. (2006).

Sataet al. (1999) reported *Sinularia*sp. containsacylatedspermidine derivatives. Bioassay-guided isolation done by Sataet al., yielded an active substance named sinulamide (Figure 2.5), which is an inhibitorof gastric H^+, K^+-ATPase.

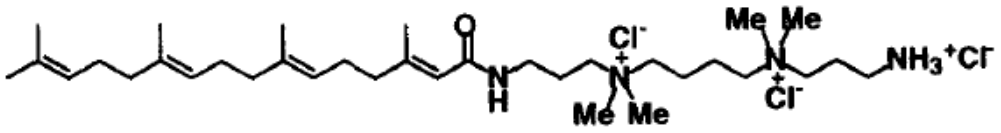


Figure 2.5: Sinulamide isolated from *Sinularia* sp. by Sataet al.(1999).

2.2.1 Research on*Sinulariadissecta*

*S. dissecta*has been found as a rich source of polyhydroxylated steroids by Jin et al. (2005). Of fifteen polyhydroxylated steroids (Figure 2.6), six are C-18 steroids. All the compounds possess 1, 3, 11-trioxygenated nucleus, except compound 6. The nature of side chains of the isolated steroids could be classified into gorgosterol-type (1-3, 7, 8), ergosterol-type (4, 5, 9), and 24-methylenecholesterol-type (10-15) derivatives. Compounds 2, 3, and 8 showed weak activity against HL-60 (leukemia cell line) and A-549, while the rest showed no inhibitory activities.

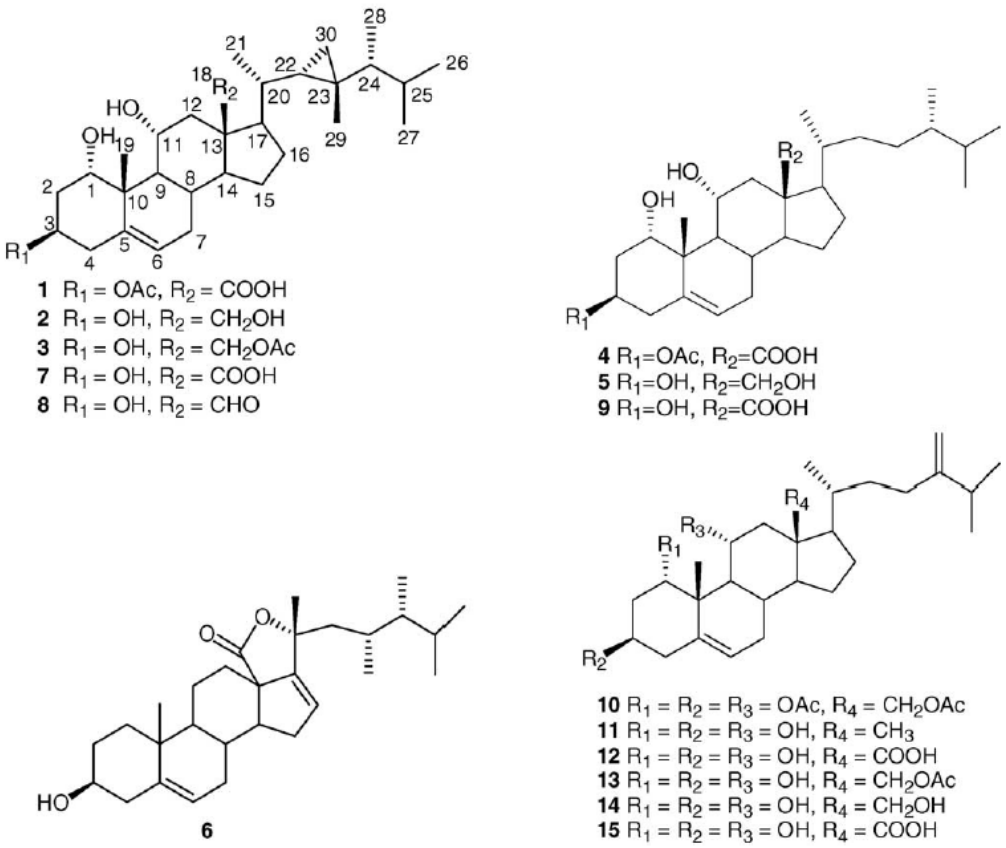
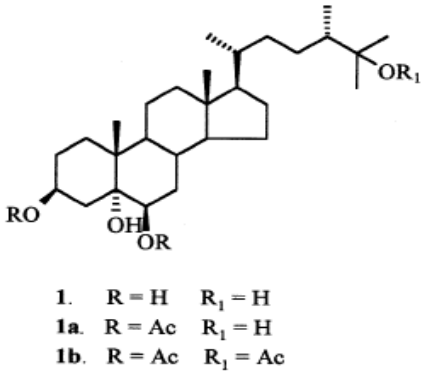


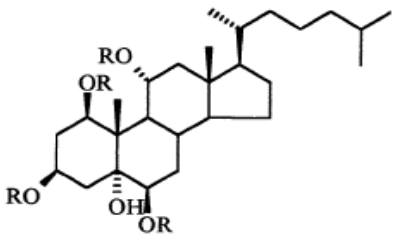
Figure 2.6: Fifteen steroids isolated from *Sinulariadissecta*by Jin et al.(2005).

Ramesh and Venkateswarlu (1999)isolated five steroids from this species, which include four new polyhydroxy steroids (compounds 1-4) and a novel polyoxygenated epoxy steroid (compound 5) (Figure 2.7). The steroid mixture was acetylated by using an acetic anhydride-pyridine mixture and then the acetylated mixture was separated on a silver nitrate-impregnated silica gel column to obtain those steroids.



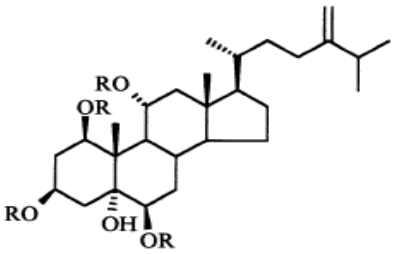
1. R = H R_1 = H
1a. R = Ac R_1 = H
1b. R = Ac R_1 = Ac

1a. 24(S)-methyl-3,6-diacetate-cholestan-3β,5α,6β,25-tetrol
1b. 24(S)-methyl-3,6,25-triacetate-cholestan-3β,5α,6β,25-tetrol



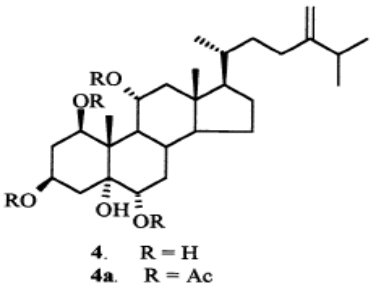
2. R = H
2a. R = Ac

2a. 1,3,6,11-tetraacetate-cholestan-1β,3β,5α,6β,11α-pentol



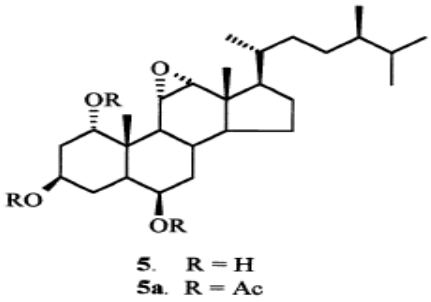
3. R = H
3a. R = Ac

3a. 24,methylene-1,3,6,11-tetraacetate-cholestan-1β,3β,5α,6β,11α-pentol



4. R = H
4a. R = Ac

4a. 24,methylene-1,3,6,11-tetraacetate-cholestan-1β,3β,5α,6α,11α-pentol

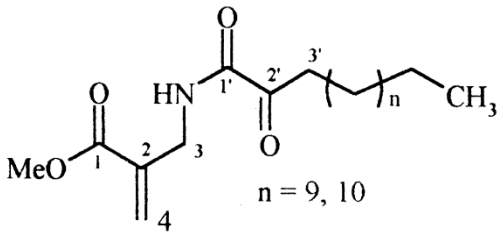


5. R = H
5a. R = Ac

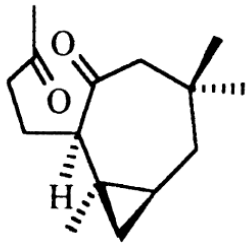
5a. 24(R)–methyl-11α,12α-epoxy-1,3,6 triacetate-cholestan-1α,3β,6β-triol

Figure 2.7: Five steroids isolated from *Sinulariadissecta* by Ramesh and Venkateswarlu (1999).

Ramesh et al. (1999) also isolated a homogeneous mixture of α-keto-2-methylene-β-alanine methyl esters and 4, 5-seco-african-4, 5-dione from *S. dissecta*. The mixture of α-keto-2-methylene-β-alanine methyl estersshowed mild activity against brine shrimp assay. Although 4, 5-seco-african-4, 5-dione was previously isolated from the plant *Lippiaintegrifolia*, but it wasisolated from marine source for the first time. The bioactivity of the compound was not reported.



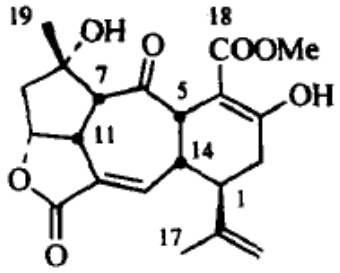
α-keto-2-methylene-β-alaninemethyl esters



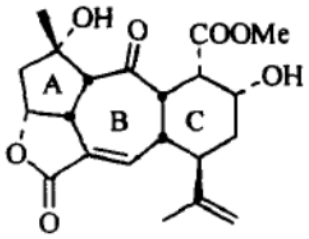
4,5-seco-african-4,5-dione

Figure 2.8: Two compounds isolated from *Sinulariadissecta*by Ramesh et al.(1999).

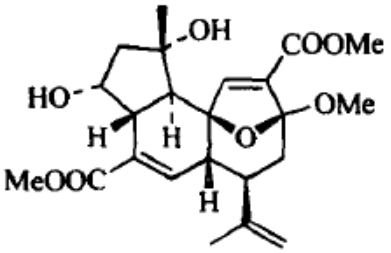
Rameswaralide, a novel diterpene possessed a 5-7-6 tricyclic skeleton was isolated from *S.dissecta* (Ramesh et al., 1998). A single isomer, dihydrorameswaralide was formed by selective reduction of enolic group with $NaBH_4$. Three new diterpenes, furanocembranediester, mandapamate and isomandapamate (Figure 2.9) were also isolated.



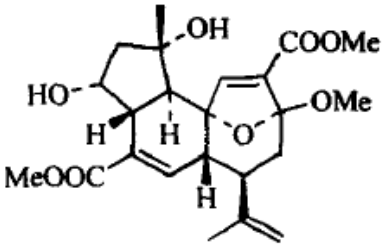
Rameswaralide



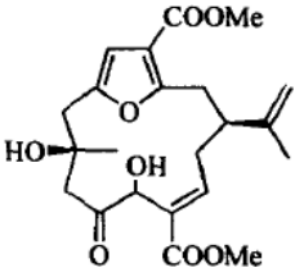
Dihydrorameswaralide



Mandapamate



Isomandapamate

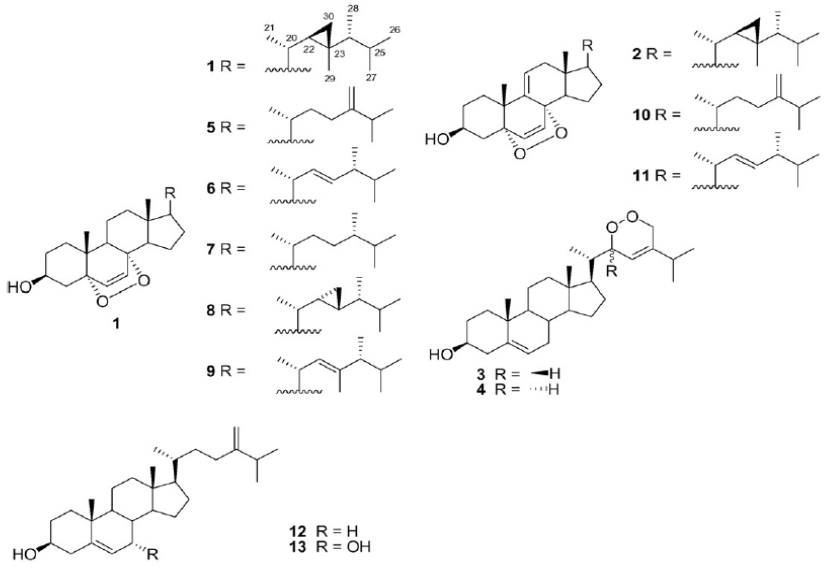


Furanocembranediester

Figure 2.9: Diterpenes isolated from *Sinulariadissecta*by Ramesh et al.(1998).

2.2.2 Research on *Sinulariaflexibilis*

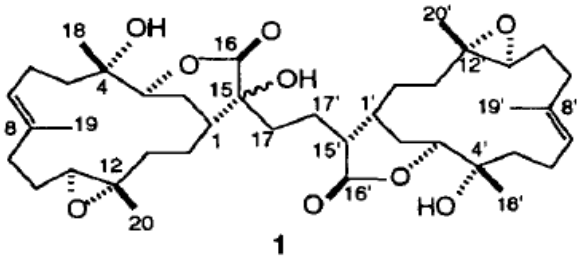
Yu et al. (2006) identified four new sterols 5α, 8α-epidioxygorgosta-6-en-3β-ol(**1**), 5α, 8α-epidioxygorgosta-6, 9(11)-dien-3β-ol(**2**), 22α, 28α-epidioxycholesta-5,23(*E*)-dien-3β-ol(**3**) and its C-22 epimer (**4**), along with nine known sterols (Figure 2.10) from S. *flexibilis*, however the bioactivities of these compounds are yet to be explored.



5. 5,8-epidioxy-24-methylcholesta-6-4(28)-dien-3β-ol
6. ergosterol peroxide
7. 5,8-epidioxy-24-methylcholesta-6-en-3β-ol
8. 5,8-epidioxy-23,24-methylene-24-methylcholesta-6-en-3β-ol
9. 5,8-epidioxy-23,24-dimethylcholesta-6,22-dien-3β-ol
10. 5,8-epidioxy-24-methylcholesta-6,9(11),24(28)-trien-3β-ol
11. 5,8-epidioxy-24-methylcholesta-6,9(11),22-trien-3β-ol
12. 24-methylcholesta-5,24(28)-dien-3β-ol
13. 24-methylcholesta-5,24(28)-diene-3,7-diol

Figure 2.10: Thirteen sterols isolated from *Sinulariaflexibilis*by Yu et al. (2006).

S. flexibilis is a rich source of cembranoidditerpene.Duh et al. (1998) isolated a novel cytotoxic biscembranoidditerpene, sinuflexin(Figure 2.11) from *S.flexibilis*, which exhibited cytotoxicity against P-388 (leukemic cell line) with $ED_{50}$of 1.32 µg/mL.



Sinuflexin

Figure 2.11: Biscembranoidditerpene isolatedfrom *Sinulariaflexibilis* by Duh et al. (1998).

2.3 Antimicrobial Susceptibility Testing Methods

Antimicrobial activity of a compound can be detected by observing the growth response of the microbes to samples that are placed in contact with them. Several *in vitro*antimicrobial susceptibility testing methods can be performed to detect the antimicrobial activity, the most common being disk diffusion, agar dilution, broth macrodilution and broth microdilution and bio-autography.

2.3.1 Disc Diffusion Method

The antimicrobial activity of different extracts and pure compounds can be measured using numerous *in vitro*assays, but discdiffusion methodremains the most commonly used method.It is based on the measurement of zones of growth inhibition from the effect of known concentrations of an antimicrobial agent diffuse from impregnated discs onto an agar plate, which has previously been inoculated with the test organism. The result obtained provides information whether the organism is sensitive, intermediate or resistant to the antimicrobial agent after suitable incubation period (McGrillet al., 2009). This method is technically easy to perform, relatively inexpensive, reproducible, and the drug combinations can be changed easily (Lestari et al., 2008).

2.3.2 Broth Dilution Method

Broth macrodilution andmicrodilution methods are recommended by Clinical and Laboratory Standards Institute (CLSI) for minimum inhibitory concentration (MIC) determination (NCCLS, 2003).Broth dilution is a method in which a predetermined concentration of suspension of bacterium is tested against varying concentrations of an antimicrobial agent in a liquid medium. The broth macrodilution method is performed in tubes containing a minimum volume of 2 mL while microdilutionis performed in smaller volumes using microtitration plates. It is usually performed as serial dilution. Broth dilution method is a more

complicated and expensive method compared with discdiffusion method but it is useful to quantify antimicrobial activity of various extracts for comparative purposes (Teffoet al., 2010). Besides, this method can avoid the problems associated with agar diffusion techniques, for example difficulties in diffusion of non-polar extracts through an aqueous agar matrix (Eloffet al., 2008). The use of microplates also allows various concentrations to be assessed in the same time. The MIC could be assessed either spectrophotometrically or with the aid of colorimetric growth indicator, the indicator commonly used are *p*-Iodonitrotetrazolium violet or resazurin (Eloff et al.,2007;Liu et al., 2007).

2.3.3 Bio-autography Method

Three approaches are used in bio-autographic method: (a) direct bio-autography in which the microorganism grow directly on the thin layer chromatographic (TLC) plate, (b) contact bio-autography in which the antimicrobial compounds are transferred from TLC plate onto an agar plate which contains the microorganism, (c) agar-overlay bio-autography in which the TLC plate places directly onto an agar plate which contains the microorganism (Cos et al., 2006). This method is useful in detecting the number of bioactive compounds present in an extract. However, this method is problematic with fungi as fungi grow more slowly than bacteria and thus it increases the contamination rate (Eloff et al., 2008).

2.4 Fungi

Fungi are eukaryotic microorganisms which include yeast, mould and dimorphism.Dimorphic fungi are fungi which can grow as a mould or as yeast (Gerard et al., 2012). Fungi are ubiquitous in the environment, and infection due to fungal pathogens has become more frequent. Fungal diseases can be divided clinically into superficial mycoses, subcutananeous mycoses, systemic mycoses and opportunistic mycoses. Clinically, candidiasis, aspergillosis and cryptococcosis are the three major infections in the immune-compromised patients.

2.4.1 *Cryptococcus neoformans*

Cryptococcus neoformans is a fungal pathogen and the causative agent of cryptococcosis. This organism produces a unique capsule which is a complex polymer, the major component of the capsule mass is glucuronoxylomannan (Heiss et al., 2009). Pigeon droppings and soil contaminated with fecal material are considered the reservoir of *C. neoformans* in nature. The primary disease caused by *C. neoformans* is chronic meningitis. The onset is slow, with a low-grade fever and headache progressing to altered mental state. Most patients are immune compromised, although some show no immune defect. This fungus is found throughout the world.The disease is transmitted through inhalation of the yeast cells. It is the most common fungal infection seen in acquired

immunodeficiency syndrome (AIDS) (Mader 2010). *C. neoformans* also causes infections in a wide range of domestic and wild animals, for example, cryptococcosis is the most common fungal disease in cats (Jacobs and Medleau, 1998).

2.4.2 *Candida*Species

*Candida albicans*exists in the yeast form as normal flora but becomes invasive in the filamentous form.Infection caused by*Candida* sp. is referred to as candidiasis and is the most common cause of opportunistic mycoses worldwide. *Candida* sp. is a normal inhabitant of human gastrointestinal and genital tract. *Candida* infection is a common disease of lower genital tract in women, young age, pre-menopausal status, use of oral contraceptives, and history of diabetes are associated with an increased risk (Parazziniet al., 2000). Besides, colonization of the mouth by *Candida* sp. has also been well known since the age of Hippocrates, isolated from various oral sites including tongue, cheek and palatal mucosa, and from dental plaque, dental caries, and the subgingival flora. Some have important roles in the development of oral and dental diseases (Senet al., 1997).

2.4.3*Aspergillusniger*

The genus of *Aspergillus* is moulds which arewidespread in the environment, being found in the soil, in the air, on plants and on decomposing

organic matter. *Aspergillus* sp. is also opportunistic pathogens that cause aspergillosis especially in immune-compromised patients. Besides, otomycosis is another fungal infection caused by *Aspergillus* sp. particularlyby *A. fumigatus*, *A. niger* and *A. flavus*. Otomycosis or fungal otitis external is described as fungal infection of the external auditory canal with infrequent complications involving the middle ear. Although otomycosisrarely life-threatening, but the disease requires long term treatment and follow-up, yet the recurrence rate remains high. This infection distributes worldwide but it is more common in tropical and subtropical regions and occurs in adults of both sexes but children are less commonly affected (Aktas and Yigit2009).

2.4.4 *Trichophytonmentagrophytes*

*Epidermophyton, Microsporum*and *Trichophyton* are the three genera of dermatophytes that affect keratinous tissue of humans and of other vertebrates, causing superficial infections (Karaca and Koc 2004). These fungi are highly adapted to the non-living, keratinized tissues of nail, hair and the stratum corneum of the skin. Production of keratinase allows dermatophytes to digest keratin in skin, nail and hairs. Dermatophytoses are slowly progressive eruptions of the skin and its appendages which are not painful or life-threatening, but typically involve induration, itching, erythema and scaling. Dermatophytoses are common skin infections in domestic animals, especially dogs and cats. The transmission of dermatophytes to humans from dogs and cats usually occurs through direct

contact or indirectly through fungus-bearing hair and scales from infected animals (Ateset al., 2008).

2.5 Bacteria

Bacteria are a large group of unicellular, prokaryotemicroorganisms. They are classified based on their cell wall characterization by Gram staining method. Gram staining methods are designed to make a staining agent binds to the bacteria's cell wall. The classification is largely based on the difference of cell wall structure. Gram-positive bacteria are classified as bacteria that retain a crystal violet dye during the Gram stain process and will appear blue or violet under microscope, whereas Gram-negative bacteria will appear red or pink (Mader 2010).

2.5.1 *Staphylococcusaureus*

Staphylococcus aureus is a Gram-positive, spherical bacterium. *S. aureus* frequently lives as commensal on human skin or in the nose of a healthy person. It also occurs in the throat and less commonly, may be found in the colon and urine. The most common infections are those of the skin and soft tissues, including cellulitis, impetigo and soft tissue abscesses. *S. aureus*is also the most common cause of bone and joint infections such as acute and vertebral osteomyelitis. The bacterial infection is also the cause of morbidity and mortality in renal patients

aleading cause ofhaemodialysis related bacteraemia (Casey et al., 2007; Rowe et al., 2002).

2.5.2*Bacillus cereus*

Bacillus cereus is a Gram-positive, aerobic, rod-shaped bacterium.It is commonly found in soil with low nutrient requirement. *B. cereus* is a common food poisoning organism; it can cause two types of food poisoning: a diarrheal type which is associated with consumption of contaminated milk product, meat and vegetables. While the emetic type is due to the consumption of contaminated rice and pasta.It is known to create heavy nausea, vomiting, and abdominal pain. These foodborne illnesses occur due to survival of the *B. cereus* spores. It is an opportunistic pathogen that caused variety of infections, such as endophthalmitis, bacteremia, septicemia, endocarditis,salpingitis, cutaenous infections, pneumonia and meningitis in immunocompromised patients (Kotirantaet al., 2000; Rasko et al.,2005).

2.5.3*Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped, and glucose-nonfermentativebacterium. It typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections. It is also one of the most prevalent pathogens in nosocomial pneumonia (Fujimura et

al.,2009).

2.5.4*Escherichia coli*

*Escherichia coli*is a Gram-negative, aerobic and facultative anaerobic, non-spore-forming, rod-shaped bacterium. It generally causes several intestinal and extra-intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and pneumonia(Madigan and Martinko 2006).

2.5.5*Klebsiellapneumoniae*

K. pneumoniae is a Gram-negative, lactosefermenting, facultative anaerobic, rod shaped bacterium distributed widely in the natural environment. It is also found in the normal flora of the skin, and intestines (Legakis et al., 1995). *K. pneumoniae* is an opportunistic pathogen that infects immune-compromised and immune-suppressed patients. Besides, these bacterial infections can also lead to human urinary or respiratory tractinfections (Huang et al., 2009).

2.6PhotodynamicTherapy

Cancer is a disease in which involves with changes or mutations in the genomes of cells. It causes a group of cells grow uncontrollably regardless the normal processes of cell division. There are two groups of factors result cancer:

external factors in the environment (chemical, tobacco, radiation and infectious microorganisms) and factors within the cell (hormones, mutations and immune condition) (Reddy et al., 2003).

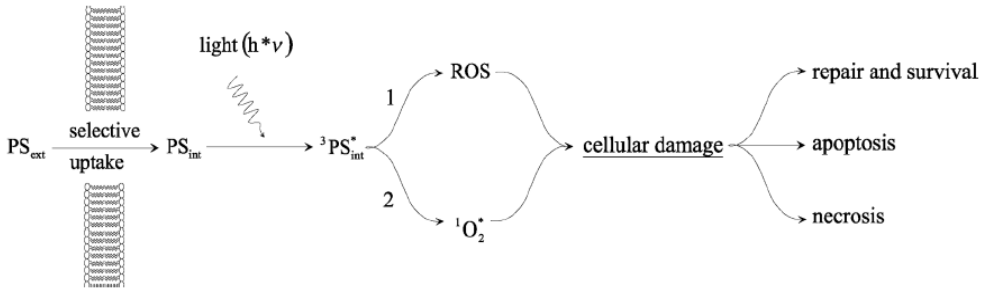


Figure 2.12: Principle of photodynamic treatment (Plaetzer et al., 2003).

Among the established treatments used by clinicians in the treatment of cancer, photodynamic therapy (PDT) is a promising new form of treatment against a variety of tumors and non-oncological disorders. In PDT treatment, photosensitizer, light of appropriate wavelength and molecular oxygen are the three main components. It involves the selective uptake of the photosensitizer into the cytoplasm of the cells and followed by the activation by irradiation of light with appropriate wavelength. The activated sensitizer then exchanges an electron or hydrogen atom with a nearby molecule to produce reactive oxygen species (ROS, type I photochemical reaction) or transfer energy to adjacent molecules yielding singlet-oxygen (type II photochemical reaction). These reactive species induce cellular damage or lead to cell death via apoptosis or necrosis (Buytaert et al., 2007;Chio-Srichan et al., 2008;Plaetzer et al., 2003).

CHAPTER 3.0

MATERIAL AND METHODS

3.1 Sample Collection and Extraction

The chemicals used were:

Hexane (Merck, AR grade), diethyl ether (Merck, AR grade), chloroform (Merck, AR grade), ethyl acetate (Merck, AR grade), acetone (Merck, AR grade), methanol (Merck, AR grade)

*Sinularia*sp. was collected from Tanjung Tuan Port Dickson, Negeri Sembilan on four different days, 13th May 2008, 28th April 2009, 12th December 2009 and 13th April 2012. The specimens were washed thoroughly with sea water to remove soil and debris and it was kept in the ice box while on the way to the laboratory. They were later frozen immediately and stored.

The soft coral was thawed before extraction was carried out, they were cut into small pieces and subjected to freeze drying (Model: Martin Christ, Alpha 1-4, LD plus). 1.58kg of lyophilized sample was blended into a fine powder.Lyophilized samples of *Sinularia* sp. were subjected to sequential extraction using organic solvents of increasing polarities, i.e. hexane (0), diethyl

ether (2.8), chloroform (4.1), ethyl acetate (4.4), acetone (5.1), methanol (5.1) and water (9) at a ratio of 1g: 10mL. The sample was immersed in hexane and left on an orbital shaker (Model: IKA) at 120 rpm overnight. The extract was filtered with Whatman 542 filter paper. The filtrates were kept while the sample was added with hexane again and shook for another night. The steps were repeated two to three times and continued with the next solvent. The steps were monitored by thin layer chromatography. The extracts collected were concentrated under vacuum by rotary evaporator (Model: BuchiRotavapor R-200) to remove the solvent and further dried using a vacuum concentrator. The seven different extracts were screened for their cytotoxicity, antibacterial and antifungal activities.

3.2 Microbial Susceptibility Testing

Principle:

The antimicrobial activity of compounds can be assessed by observing the growth response of the microbes that are in contact with them. Several *invitro* susceptibility testsmay be used for detecting antimicrobial activity, the most common being disk diffusion, agar dilution, broth macrodilution and broth microdilution and bio-autographic.

A colorimetric broth microdilution method was used in solvent toxicity testing, antibacterial and antifungal assays. Broth microdilution allows varied concentrations to be assessed at the same time on the same microplate(Liu *et al*.,

2007). This avoids the problems associated with agar diffusion techniques, for example difficulty in the diffusion of non-polar extracts through an aqueous agar matrix (Eloff*et al*., 2008). The MIC value could also be quantified easily either spectrophotometrically or with the aid of a colorimetric growth indicator (Liu *et al*., 2007).

Procedures:

The test was performed on sterile and untreated 96 U-bottom well plate. It was performed as serial two-fold dilutions, performed by removing 50µL of contents from wells in the second column and transferring these to the wells in the third column. Serial dilution was repeated until the eleventh column was reached. *p*-Iodonitrotetrazolium violet (INT) was used as the growth indicator in the assay. The MIC was defined as the lowest concentration of solvent/ extract/ fraction in which there was no microbial growth, indicated by the absence ofcolour change.The total activity (mL) of each fraction was calculated as the total mass (mg) of the extract divided by the MIC value (mg/mL). The total activity value indicates the volume to which an extract or a fraction derived from 1g of sample material can be diluted and still inhibits the growth of microbial cells (Eloff, 2004).

3.2 .1 Sample Extract Preparation

Methanol (Merck, HPLC grade).

Procedures:

20mg of each dried extract was dissolved in 1 mL of methanol and water mixture (2:1, v/v). The sample was filter-sterilized using a 0.2 μm nylon syringe filter.

3.2.2 Growth Indicator Preparation

The chemical used was *p*-iodonitrotetrazolium violet (Sigma Aldrich).

Procedures:

The *p*-iodonitrotetrazolium violet (INT) solution was prepared by dissolving 40 mg of INT in 100 mL of distilled water and further sterilized by using 0.2 μm nylon syringe filter and stored at 4°C until required.

3.2.3Antibacterial Testing

The antibacterial test wasperformed on 96 well plates. The layout of the plate is shown in Figure 3.1. 100 μL of broth medium was added to sterility control wells to ensure the sterility of the broth medium. 75 μL of broth medium and 25 μL of each extract were added to negative control wells to ascertain sterility of each extract. The viability of the microbes was assessed by adding 50 μL of the tested microbes to 50 μL of the broth medium in the growth control wells. For antibacterial testing and drug control, 50 μL of broth medium was

added from column 2 to column 11, then 50 µL of drug was added to well H2 while 50 µL of each extract tested was added to the respective wells of A2 to G2, after which a serial two fold dilution was performed from columns 2 to 11 followed by adding 50 µL of the bacterial inocula. The plates were incubated for 24 h, after which 20 µL of the *p*- iodonitrotetrazolium violet (INT) indicator was added to determine the MIC value of each extract towards the bacteria.

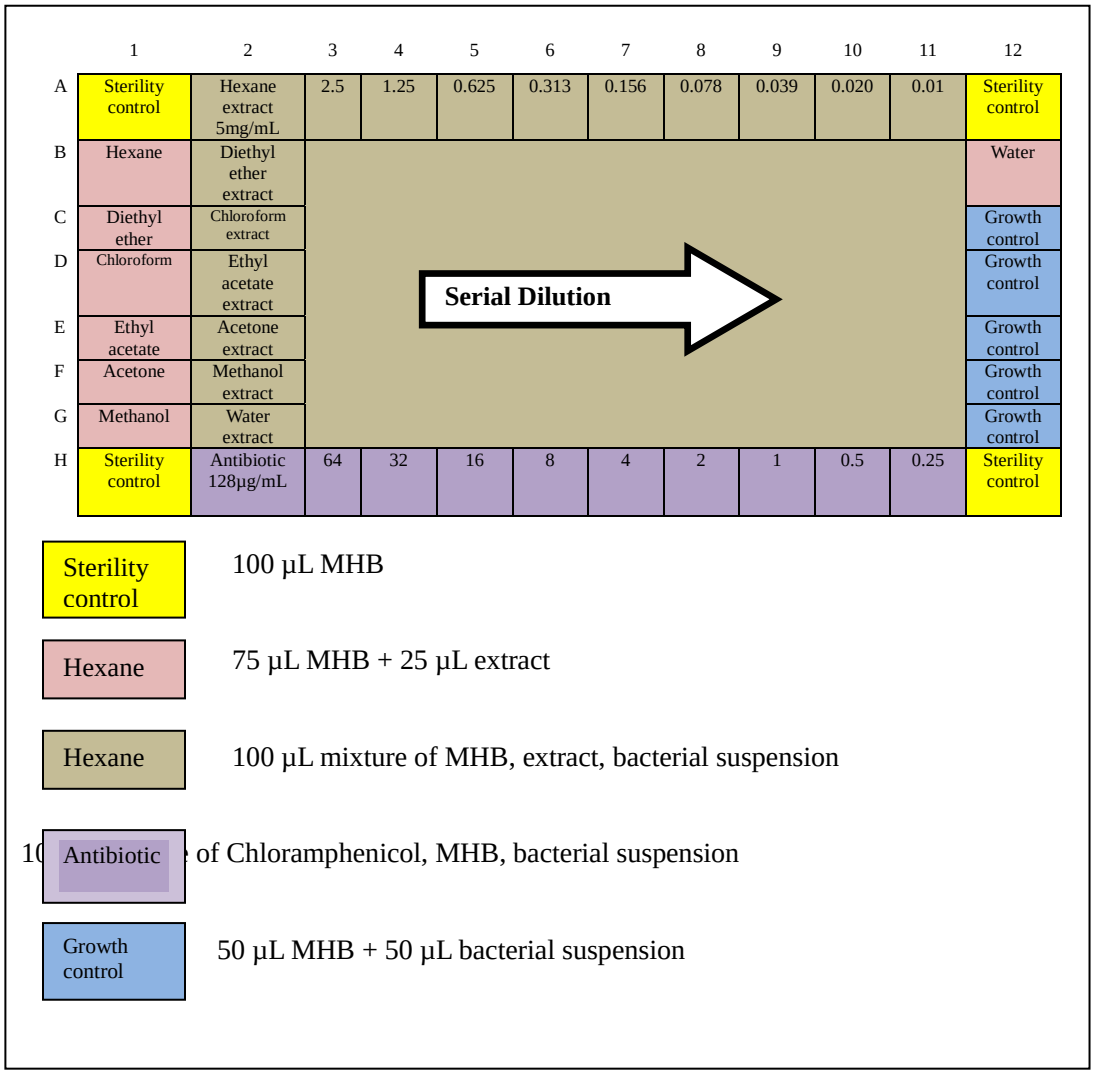


Figure 3.1: Design of the 96- well microplate for antibacterial testing.

3.2.3.1 Bacterial Strains

Both Gram-positive and Gram-negative bacteria were used in the study. The Gram-positive bacteria were *Staphylococcus aureus* (ATCC 29213) and *Bacillus cereus* (ATCC 11778). The Gram-negative bacteria were *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiellapneumoniae*(ATCC 13883), *Escherichia coli*- penicillin sensitive strain (ATCC 25922) and *E. coli*-penicillin resistant strain (ATCC 35218).

3.2.3.2 BacterialInocula Preparation

The chemicals used were:

Mueller-Hinton Agar(Oxoid), Mueller-Hinton Broth(Oxoid)

Procedures:

The bacteria were cultured using Mueller-Hinton agar at 37 ºC , incubation times were between 18 to 24 h. Mueller-Hinton Broth (MHB) was used as the broth medium and the concentrations of bacteria inocula were adjusted and measured using UV-Vis spectrophotometer set at 625 nm. A few colonies of bacteria were transferred to MHB and the optical density of the bacteria inoculum

was adjusted to fall between 0.08 to 0.10 absorbance units, where the concentration of bacteria is 1 x 10^8 CFU/mL. The dilution was carried out to obtain a final concentration of 1 x 10^6 CFU/mL.

3.2.3.3 Chloramphenicol Preparation

The chemical used was Chloramphenicol (Oxoid).

Procedures:

Chloramphenicol was dissolved in distilled water to concentration of 512 μg/mL and sterilized by filtration through a 0.2 μm syringe filter. The concentration rangeused as the positive control was between 0.25 to 128 μg/mL.

3.2.3.4 Broth Medium Preparation

The chemical used was MHB powder (Oxoid).

Procedures:

10.5 g of MHB powder was dissolved in 500 mL distilled water. The medium was sterilized by autoclaving at 121 ºC for 15 min.

3.2.4 Antifungal Testing

The antifungal test wasperformed using 96 well plates. The layout of the plate is illustrated in Figure 3.2. 100 µL of broth medium was added to sterility control wells to ensure the sterility of the broth medium. 75 µL of broth medium and 25 µL of each extract were added to negative control wells to check the sterility of each extract. Besides, the viability of the fungi was assessed by adding 50 µL of the tested fungi to 50 µL of the broth medium in the growth control wells. For the antifungal testing and drug control, 50 µL of broth medium was added from column 2 to column 11, after which 50 µL of drug was added to well H2 while 50 µL of each extract tested was added to the respective wells of A2 to G2, followed by a serial two-fold dilution was performed from column 2 to 11 followed by adding 50 µL of the fungal inocula. The plates were incubated accordingly to the incubation time of each fungus and lastly 20 µL of the *p*-iodonitrotetrazolium violet (INT) indicator was added to determine the MIC value of each extract.

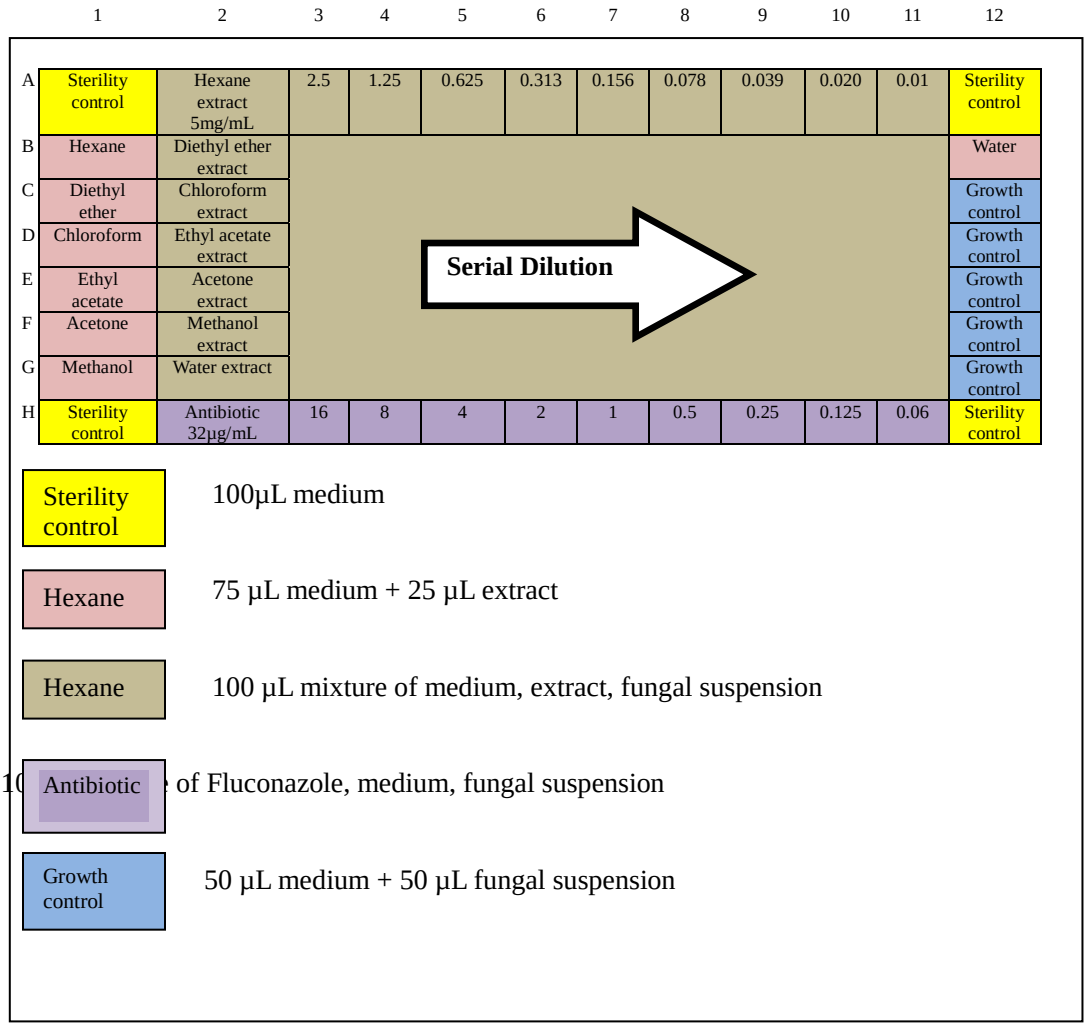


Figure 3.2: Design of the 96-well microplate for antifungal testing.

3.2.4.1 Fungal Strains

The yeast species used were *Candida albicans* (ATCC 90028), *Candida parapsilosis* (ATCC 22019) and *Cryptococcus neoformans*(ATCC 90112). The

mouldsspecies used were *Aspergillusniger* (ATCC 16404) and *Trichophytonmentagrophytes* (ATCC 9533).

3.2.4.2 Fungal Inocula Preparation

The chemicals used were:

Potato dextrose agar(Merck),RPMI-1640 with L-glutamine, without bicarbonate (Sigma Aldrich), 3-(N-morpholino) propanesulfonic acid (Merck)

Procedures:

The fungi were cultured using potato dextrose agar. Different incubation times were required for different species of fungus. *Candida* species was incubated for 24h while *C. neoformans* and *A. niger*were incubated for 48h. A 120h incubation period was used for *T. mentagrophytes*. RPMI-1640 with L-glutamine, without bicarbonate and 3-(N-morpholino) propanesulfonic acid at pH 7 was used as the broth medium. Similar to bacterial inocula, fungalinocula were prepared by transferring a few colonies of fungi to the medium broth. The concentrations of fungalinocula were adjusted by its optical density using a UV-Vis spectrophotometer set at 530 nm. The absorbance of inocula of *Candida* species and *C. neoformans*were adjusted within the range of 0.12 to 0.15, while the absorbance of inocula of *A. niger* and *T. mentagrophytes*were adjusted within the range of 0.09 to 0.11, whereby the concentration of fungi was 1-5 x 10^6 CFU/mL.Dilution was carried out to obtain a final concentration of fungal

inocula, *Candida* species at 0.5-2.5 x 10^3 CFU/mL, *C. neoformans*at 0.5-2.5 x 10^4 CFU/mL and *A. niger* and *T. mentagrophytes* at 0.5-2.5 x 10^5 CFU/mL.

3.2.4.3 Fluconazole Preparation

The chemicals used were:

Fluconazole (Merck), Dimethyl sulfoxide (Merck)

Procedures:

Fluconazole (Merck)was disssolved in dimethyl sulfoxide (DMSO) to a concentration of 6.4mg/mL. This was further diluted to a ratio of 1:50 in broth mediumand sterilized by filtration through a 0.2 μm filter.The concentrations tested were between 0.63 to 32 μg/mL.

3.2.4.4 Broth Medium Preparation

The chemicals used were:

RPMI-1640 medium supplemented with glutamine and phenol red (Sigma Aldrich), 3-(N-morpholino) propanesulfonic acid (MOPS) (Merck)

Procedures:

One liter of the broth medium was prepared as follows: 20.8 g of RPMI-1640 medium supplemented with glutamine and phenol red, without bicarbonate,

69.06 g of 3-(N-morpholino) propanesulfonic acid (MOPS) were dissolved in 800mL distilled water, adjusting the pH to 7.0 at 25°C with 1 mol/L sodium hydroxide solution. Water was added to make up the final volume to 1 L.The medium was filtered-sterilized and stored at 4°C.

3.3 Solvent Toxicity Testing

The solvents used were:

Hexane (Merck, HPLC grade), diethyl ether (Merck, HPLC grade), chloroform (Merck, HPLC grade), ethyl acetate (Merck, HPLC grade), methanol (Merck, HPLC grade), dimethyl sulfoxide (Merck, AR grade), ethanol (Merck, HPLC grade), butanol (Merck, HPLC grade), dichoromethane (Merck, HPLC grade), acetone (Merck, HPLC grade)

Procedures:

Solvent toxicity test was performed to investigate the toxicity of ten different organic solvents (hexane, diethyl ether, chloroform, ethyl acetate, methanol, dimethyl sulfoxide, ethanol, butanol, dichloromethane and acetone) towards fungi and bacteria. The concentrations of the solvent used varied from 0.05 to 25% (v/v). Chloramphenicol was used as positive control in antibacterial assay and fluconazole was used in antifungal assay.

The tests were performed on 96 well plates and the design of the plate was

arranged as in Figure 3.3. 100 µL of broth medium was added to the sterility control wells to check the sterility of the broth medium. 75 µL of broth medium and 25 µL of each selected solvent were added to negative control wells to check the sterility of each solvent. The viability of the microbes was assessed by adding 50 µL of the tested microbes to 50 µL of the broth medium to the growth control wells. For solvents testing and drug control wells, 50 µL of broth medium was added from column 2 to column 11, followed by 50 µL of drug was added to well H2 while 50 µL of each solvent tested was added to the respective wells of A2 to G2. Serial two fold dilution was then performed from column 2 to 11 followed by adding 50 µL of the microbial inocula. The plates for bacterial inocula were incubated at 37 ºC for 24 h. The plates for *Candida* species were incubated for 24h while *C. neoformans* and *A. niger*were incubated for 48h. A 120h incubation period was used for *T. mentagrophytes*. All the plates for fungal inocula were incubated at 37 ºC. Finally 20 µL of the *p*-iodonitrotetrazolium violet (INT) indicator was added and MIC value was determined.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterility control	Hexane 25%	12.5 %	6.25 %	3.13 %	1.56 %	0.78 %	0.39 %	0.2 %	0.10 %	0.05 %	Sterility control
B	Hexane	Diethyl ether										Ethanol
C	Diethyl ether	Chloroform										Growth control
D	Chloroform	Ethyl acetate										Growth control
E	Ethyl acetate	Methanol										Growth control
F	Methanol	Dimethyl sulfoxide										Growth control
G	Dimethyl sulfoxide	Ethanol										Growth control
H	Sterility control	Antibiotic										Sterility control

Serial Dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterility control	Buthanol										Sterility control
B	Buthanol	Dichloro-methane										Acetone
C	Dichloro-methane	Acetone										Growth control
D	Sterility control	Antibiotic										Sterility control
E												
F												
G												
H												

Serial Dilution

Sterility control — 100 µL medium

Hexane — 75 µL medium + 25 µL solvent

Hexane — 100 µL mixture of medium, solvent, microbial suspension

Antibiotic — 100 µL mixture of antibiotic, medium, microbial suspension

Growth control — 50 µL medium + 50µL microbial suspension

Figure 3.3: Design of the 96- well plate for solvent toxicity testing.

3.4 Photo-toxicity Assay

The chemicals used were:

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich), dimethyl sulfoxide (DMSO) (Sigma Aldrich, HPLC grade)

Principle:

A microculturetetrazolium assay using MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was developed by Mossman (1983) for rapid cytotoxic screening. The reduction of MTT into its formazan product is catalyzed by mitochondrial dehydrogenases which are active in living cells. The amount of formazan formed is directly proportional to the number of living cells and the amount is quantified spectrophotometrically at 550nm (Young et al., 2005).

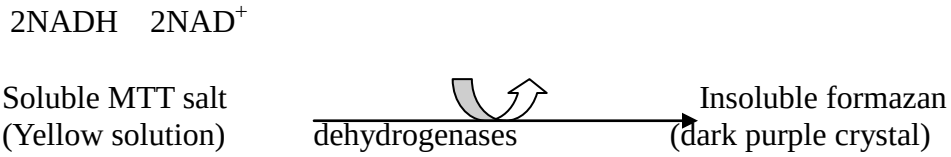


Figure 3.4: Principle of the MTT assay. The reduction of MTT into its formazan product is catalyzed by mitochondrial dehydrogenases.

Procedure:

The MTT test was performed in sterile, untreated 96 flat-bottom well plates. Sterility controls, positive controls, negative controls and growth controls were included. The seven extracts were dissolved inDMSO and the stock concentration was 100 mg/mL. The working stocks were vortexed and diluted to 40 mg/mL with RPMI medium without phenol red prior to addition to the cell suspension. 50 mL of the cells (approx. 15,000 cells/well) was added to each well, followed by 50 µL of the extracts. The plate was subjected to light irradiation for 10min at 9.6 J/cm after 2h of incubation. After 24h incubation, 20 µL of MTT solution at 5 mg/mL was added to each well and left for another 4 h for crystal formation. 75 µL of supernatant was removed and 100 µL of DMSO was added and mixed thoroughly to dissolve the purple coloured crystal. The absorbance of the wells was read immediately on a microplate reader at a wavelength of 570 nm. The average absorbance was calculated and used to determine the percentage of cell viability using the formula:

$$\text{Cell viability (\%)} = \frac{\text{S (average)} - \text{B (average)}}{\text{A (average)} - \text{B (average)}} \times 100$$

Where, S = Absorbance of the cells treated with extract

A = Absorbance of the cells without extract

B = Absorbance of the medium only

3.4.1 Preparation of Cell Line

The chemicals used were:

Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% (v/v) L-glutamine and 10% (v/v) Fetal Bovine Serum (Gibco®), HL 60 cell line (NCI).

Procedures:

A promylocytic leukemia cell line HL 60 was obtained from National Cancer Institute, Maryland, USA. The cells were maintained in the Roswell Park Memorial Institute (RPMI) 1640 supplemented with 1% (v/v) L-glutamine and 10% (v/v) Fetal Bovine Serum (Gibco) at 37 ºC and 5% CO_2atmosphere.

3.4.2 Photosensitizer Preparation

The chemicals used were:

Pheophorbide-A (Pha) (Frontier Scientific), dimethyl sulfoxide (DMSO) (Sigma Aldrich, HPLC grade)

Procedures:

Pheophorbide-A (Pha) (Frontier Scientific), a commercially available photo-cytotoxic compound was dissolved in DMSO to a concentration of 1mg/mL. The stock solution was kept at -20 °C in the dark. It was directly diluted in medium to obtain the desired initial working concentration at 20 µg/mL.

.

3.4.3 Isolation of Active Photo-cytotoxic Compounds

The chemicals used were:

Silica gel 230-400 mesh (Merck), hexane (Merck, HPLC grade), ethyl acetate (Merck, HPLC grade)

Procedures:

Activity-guided fractionation was performed on active chloroform extract. It was subjected to silica gel flash chromatography fitted with a 40 x 150 mm silica cartridge which was packed with silica gel 230-400 mesh. The column was eluted with five mobile phases (Table 3.1). Fractions of 50 mL each were collected and monitored by thin layer chromatography (TLC). Fractions with similar TLC patterns were combined and screened for photo-cytotoxicity activity. To avoid the active compounds being degraded by light, the procedure was performed under dim light.

Table 3.1: Mobile phases used in the isolation of active photo-toxicity chloroform extract.

Number of mobile phases	Solvents used in mobile phases	Volume of the mobile phases	Number of fraction collected
1	100% Hexane	50mL	1-2
2	90% Hexane: 10% Ethyl acetate	50mL	3-5
3	80% Hexane: 20% Ethyl acetate	50mL	6-9
4	70% Hexane: 30% Ethyl acetate	50mL	10-12
5	50% Hexane: 50% Ethyl acetate	100mL	13-15

3.5Isolation and Identification of Antifungal Compounds

Principle:

Ion exchange chromatography is a type of column chromatography performed by solid-liquid partition using the principle of ion charge. The charge of ionic resin was used to separate classes of components by differential adsorption between the stationary phase and the mobile phase.

In the study, flash chromatography was used to separate the compounds. It

is a very convenient and fast technique compared to conventional column chromatography. Briefly, a column is dry filled with silica gel, a sample is introduced, and then solvent is forced through the column under pressure. As the hexane extract is oily, a modified flash chromatography using silica gel impregnated with 1 % of silver nitrate as reported by Li et al. (2005) was used.

The chemicals used were:

Silica gel 230-400 mesh (Merck), preparative thin layer chromatography, silver nitrate, hexane (Merck, HPLC grade), ethyl acetate (Merck, HPLC grade)

Procedures:

The silica gel mixture was prepared by mixing 100 g silica gel (230-400mesh ASTM) with an aqueous solution of 11 g of silver nitrate dissolved in 60 mL of distilled water. The mixture was ground and then dried in oven for overnightat 70 °C.The dimension of the column used was 80mm x 150mm. The column was eluted first with nine mobile phases (Table 3.2). 200 mL fractions were collected and monitored by gas chromatography mass spectrometry (GCMS). Fractions with similar TIC patterns were combined and screened for antifungal activity.

Table 3.2: Mobile phases used in the isolation of active antifungal hexane extract.

Number of mobile phases	Solvents used in mobile phases	Volume of the mobile phases	Number of fraction collected
1	100% Hexane	200mL	1-5
2	98% Hexane: 2% Ethyl acetate	200mL	6-10
3	96% Hexane: 4% Ethyl acetate	200mL	11-15
4	94% Hexane: 6% Ethyl acetate	200mL	16-19
5	92% Hexane: 8% Ethyl acetate	200mL	20-24
6	90% Hexane: 10% Ethyl acetate	200mL	25-28
7	85% Hexane: 15% Ethyl acetate	200mL	29-34
8	80% Hexane: 20% Ethyl acetate	200mL	35-40
9	100% Ethyl acetate	200mL	41-42

The active fraction AgN27 (fraction 18-23) was further isolated using preparative thin layer chromatography to yield fraction AgN27-1. The fraction was developed with the solvent system hexane:ethyl acetate (80:20, v/v) to yield the isolated compound, fuscol.

3.5.1 Gas Chromatography Mass SpectrometryAnalysis

The chemical used was methanol (Merck, GC grade).

Procedures:

GC-MS is a chromatographic technique to identify the volatile compounds of a sample. The sample was dissolved in methanol, followed by filtration using 0.45 µm syringe filter prior to injection into GCMS. GCMS analysis was performed on an Agilent Technologies 6890N GC System equipped with Agilent Technologies 5975 inert Mass Selective Detector (70 eV direct inlet) on fused silica capillary column HP–5ms (30 m, ID 0.25 mm, 0.25 µm film thickness) with helium as carrier gas at a flow rate of 1 mL/min. The column temperature was programmed initially at 100°C, then increased 5°C min^{-1} to 300°C and was kept isothermally for 10min.The total ion chromatogram obtained was auto integrated by Chemstation and the constituents were identified by comparison with published mass spectra databases (Wiley 9N Mass Spectral Library).

3.5.2 ^{1}H Nuclear Magnetic Resonance and ^{13}C Nuclear Magnetic Resonance Spectroscopic Analysis on the Isolated Antifungal Compound

The chemical used was deuterated methanol D4.

Procedures:

The isolated antifungal compound was air dried and dissolved in deuterated methanol D4. The solution was then transferred to a NMR tube (Norell, 400 MHz) to a height of 4 cm. The compound was subjected to ^{1}H nuclearmagnetic resonance (NMR) and ^{13}C NMR (Model: Bruker, JOEL, Japan, 270 MHz) for structural elucidation.

CHAPTER 4.0

RESULTS AND DISCUSSION

4.1 Collection of *Sinularia*sp.

The soft coral, *Sinularia*sp., was collected from Tanjung Tuan PortDickson, Malaysia. The site offers a combination of high biological diversity and density of marine organisms including algae, sponges, mollusks, corals and fishes. After collection of sample, it was rinsed with sea water and kept in an ice box immediately. It is because the sample might die upon the exposure to air and rapidly decompose and cause the compounds present in the sample rapidly degraded by oxidative, enzymatic, or polymerization process. This will also lower the overall yield. After it was sent to the laboratory, immediate storage in the freezer was necessary to inactivate the enzymatic activity and reduce the possible degradation.

Freeze drying was chosen to prepare the sample instead of air drying. It eliminates water molecules and prevents the potential active constituents altered or lost. Many marine organisms including *Sinularia* sp. contain significant

quantities of water and often cause the extracts contain surface active agents which will initiate foaming and bubbling during the concentration process by rotary evaporator. The hardening of the sample simplified the crushing work of corals and allowed for easier breakage of the cell, which would then release the substances present. Pounding the samples into powder form allows a larger surface area exposed to a solvent for more efficient extraction. The dry weights of sample which were collected on four different days are shown in Table 4.1.

Table 4.1: Dry weights of *Sinularia* sp. collected on four different days.

Date of sample collection	Dry weight of sample
13th May 2008	430 g
28th April 2009	290 g
12th December 2009	465 g
13th April 2012	395 g

4.2 Sequential Extraction of *Sinularia* sp.

Compounds active in antimicrobial and cytotoxic tests, are expected to be of low solvent polarity. However, the sample was extracted sequentially using seven solvents, ranged from non-polar to polar, i.e. hexane, diethyl ether, chloroform, ethyl acetate, acetone, methanol and finally water. The use of solvents of ranging polarities permits better discrimination between extracts that show bioactivity (Cos et al., 2006). 1.58 kg of freeze dried sample was extracted in the

same solvent for 2-3 times and the extract was analysed by TLC to ensure complete extraction of the compounds with solvent of same polarity.

The yield of the extracts for each solvent and the colour of the extracts are summarized in Table 4.2.

Table 4.2: Yield of seven extracts from 1.58 kg of dried sample of *Sinularia* sp.

Solvent	Yield (g)	Colour of the extracts
Hexane	25.870	Dark brown
Diethyl ether	23.582	Dark brown
Chloroform	3.860	Brown
Ethyl acetate	0.0317	Green
Acetone	0.577	Light green
Methanol	43.436	Light green
Water	60.949	Green
Total	158.306	

4.3Solvent Toxicity

It is often necessary to dissolve natural compounds in organic solvents as some compounds have limited solubility in water. While it is desirable to use solvents that are non-toxic to the microorganisms used for bioassays, but there is no standard method for defining the most suitable solvent or the optimal solvent concentration to be used. Hence, a suitable method must be employed to determine the conditions that result in the lowest degree of toxicity on the microorganisms used for bioassays as some solvents may affect biological membranes of the microbes (Sikkemaet al., 1995).

For this purpose, a colorimetric broth microdilution assay was performed, using *p*-iodonitrotetrazolium violet (INT) as the growth indicator. The inhibition was indicated by the lack of colour change while microbial growth was indicated by a colour change to red. The concentrations of solvent tested were varied from 0.05 to 25%. Among the 10 solvents tested, only DMSO exhibited some degree of inhibitory activity (Table 4.3).*K. pneumoniae*was the strain that was least sensitive to DMSO, with an MIC value of 25% while *E. coli,* penicillin-resistant strain, was the most sensitive towards DMSO, with MIC value of 6.25%. The susceptibility of *S. aureus, B. cereus, P. aeruginosa* and *E. coli* (penicillin-sensitive strain) towards DMSO was approximately equal, with MIC values of 12.5%. Gram-positive bacteria appear to be more sensitive than Gram-negative bacteria towards DMSO. This could be due to the lack of the outer membrane which acts as a barrier against hydrophobic molecules (Denyer andMaillard, 2002).

The effects of organic solvents on fungi were also studied and the results are summarized in Table 4.4. The MIC was defined as the lowest concentration of solvent in which there was no microbial growth, indicated by the absence of colour change (Figure 4.1). The same MIC values (12.5%) were obtained for *C. albicans* and *C. parapsilosis*when DMSO was used.A lower MIC value of 6.5% was obtained for *C. neoformans.*The same degree of inhibition towards the growth of *A. niger* and *T. mentagrophytes*for which MIC values of 6.5% were recorded.

Inhibition of growth of the microorganisms might be due to the accumulation of solvents in the cytoplasmic membrane which would affect its structural properties thus preventing the cell from performing essential functions such as dissipation of pH and electrical potentials, disruption of the normal flow of ions, proteins, lipids and endogenous metabolites, and the effects on membrane protein (Sikkemaet al., 1994 & 1995). Some microorganisms have the ability to modify the physical properties of cellular membranes by increasing the rigidity of the cellular membrane thus making the cell less permeable to toxic compounds such as solvents, drugs and antibiotics (Fernandeset al., 2003).

Table 4.3: Percentage of the organic solvents that inhibited the growth of the tested bacteria.

	Percentage of solvent that inhibited the growth of bacteria (%, v/v)									
Solvents	Hexane	Diethyl Ether	Chloroform	Ethyl Acetate	Dimethyl sulfoxide	Ethanol	Methanol	Acetone	Butanol	Dicholomethane
Gram- positive bacteria										
S.aureus	-	-	-	-	12.5	-	-	-	-	-
B. cereus	-	-	-	-	12.5	-	-	-	-	-
Gram- negative bacteria										
P.aeruginosa	-	-	-	-	12.5	-	-	-	-	-
K.pneumoniae	-	-	-	-	25	-	-	-	-	-
*E.coli*penicillin sensitive strain	-	-	-	-	12.5	-	-	-	-	-
*E.coli*penicillinr esistant strain	-	-	-	-	6.25	-	-	-	-	-

All values are expressed as mean of three replicates
"- " : no inhibition

The solvent chosen for the bioassay must be miscible with the broth medium and the inoculum since these are water-based. DMSO, acetone, ethanol and methanol were water-miscible. The effect of organic solvent on the materials used in the assay should also be taken into account. Chloroform, diethyl ether, dichloromethane and acetone exhibit some degree ofcorrosiveness towardsthe polystyrene microplate, thus those solvents would not be a good choice for dissolving the dry extracts for further bioassay screening, unless compatible materials such as polypropylene microplatesare used.

A mixture of methanol and water at the ratio 2:1 was selected to dissolve the crude extracts prior to antimicrobial testing. The mixture was able to dissolve all the crude extracts completely. In addition, it may help to maintain the stated concentration of the stock solution by reducing the rapid evaporation of methanol.

Table 4.4: Percentage of the organic solvents that inhibited the growth of the tested fungi.

Solvents	Percentage of solvent that inhibited the growth of fungi (%, v/v)									
	Hexane	Diethyl Ether	Chloroform	Ethyl Acetate	Dimethyl sulfoxide	Ethanol	Methanol	Acetone	Butanol	Dicholoromethane
Yeast										
C. albicans	-	-	-	-	12.5	-	-	-	-	-
C. parapsilosis	-	-	-	-	12.5	-	-	-	-	-
C. neoformans	-	-	-	-	6.25	-	-	-	-	-
Mould										
A. niger	-	-	-	-	6.25	-	-	-	-	-
T. mentagrophytes	-	-	-	-	6.25	-	-	-	-	-

All values are expressed as mean of three replicates
"- " : no inhibition

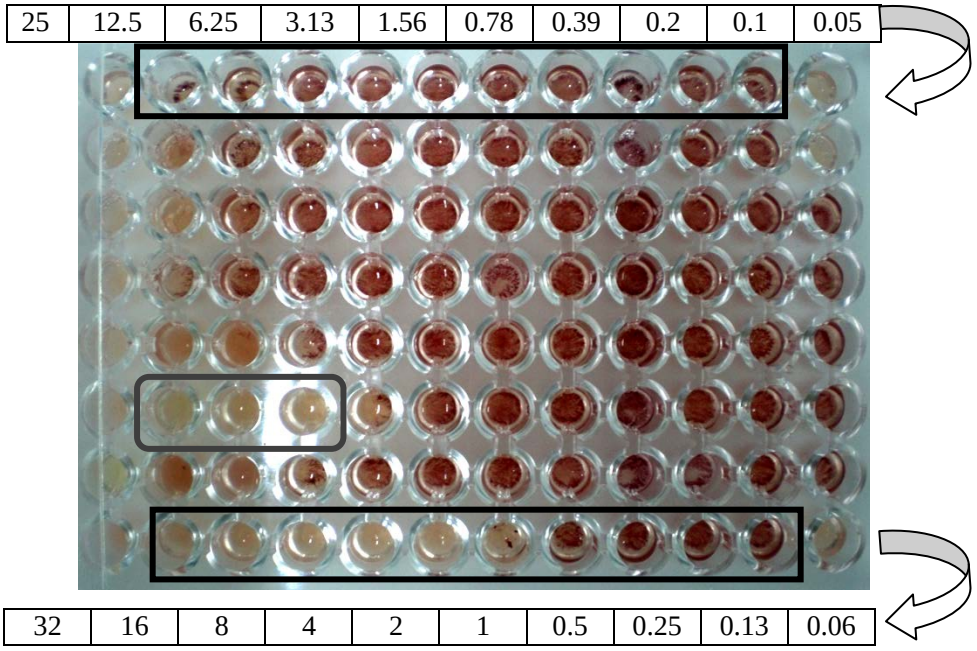


Figure 4.1: An example of microtitre plates showing the result of the organic solvents against *C. parapsilosis* (ATCC 22019).

4.4 Antimicrobial Activities

A colorimetric broth microdilution method was employed for quantifying the MIC value of each extract and fractions towards various microorganisms in the study since it has good reproducibility and well-defined end-points (Liu et al., 2007).This method overcomes the difficulties caused by the diffusion of non-polar extracts in disc diffusion method and thereby reducing the contamination rate encountered in the bio-autography method (Eloffet al., 2008). According to

Aligianniset al., (2001), the extracts are considered to exhibit strong inhibition if the MIC value is between 0.5 mg/mL and below; moderately inhibition if the MIC value is between 0.6 and 1.5 mg/mL and weak inhibition if the MIC value is more than 1.6 mg/mL.

4.4.1 Antibacterial Testing

In antibacterial testing, two Gram-positive bacteria, *S. aureus* and *B. cereus* and four Gram-negative bacteria, *K. pneumoniae*, *P. aeruginosa* and *E. coli* penicillin-resistant and sensitive-type were used. Chloramphenicol with concentrations ranging from 0.25 to 128 μg/mL was used as the positive control.

Table 4.5 summarizes the results of the extracts towards the six medically-important bacteria. All extracts except water demonstrated inhibition towards *S. aureus*, *B. cereus*, *K. pneumoniae* and *P. aeruginosa* with MIC values ranging from 0.08 to 5.00 mg/mL. The methanol extract only showed weak inhibition towards *S. aureus*, *K.pneumoniae* and *P. aeruginosa*with MIC values of 5 mg/mL, 2.5 mg/mL and 4.17 mg/mL. Among the extracts, only ethyl acetate extract showed inhibition towards both *E. coli* penicillin sensitive and penicillin resistant strains with MIC value of 5 mg/mL. The water extract showed no inhibition toward all the bacteria tested.

Table 4.6 shows the total activities of seven extracts towards tested bacteria. Diethyl ether extract showed highest total activity (186.57 mL/g) towards *K. pneumoniae*, this shows that 1 g of the extract can be dissolved in 186.57 mL of solvent and still have inhibitory activity towards *K. pneumoniae*.

Table 4.5: Mean MIC values (mg/mL) of the seven extracts of *Sinularia* sp. towards tested bacteria.

Extracts	MIC (mg/mL)							MIC (µg/mL)
	Hexane	Diethyl ether	Chloroform	Ethyl acetate	Acetone	Methanol	Water	Chloramphenicol
Gram-positive bacteria								
S.aureus	2.5	2.5	1.25-2.50	0.63-1.25	1.25-2.5	5	-	8
B.cereus	5	1.25	1.25	0.63	1.25	-	-	8
Gram- negative bacteria								
K.pneumoniae	0.16	0.08	0.31	0.16-0.31	0.63	2.5	-	4
P.aeruginosa	0.31	0.31	0.31	0.16-0.63	1.25	2.50-5.00	-	4
E.coli penicillin sensitive strain	-	-	-	5	-	-	-	4
*E.coli*penicillin resistant strain	-	-	-	5	-	-	-	4

All values are expressed as mean or rangeof three replicates
"- " : no inhibition

Table 4.6: Total activity (mL/g) of the seven extracts of *Sinularia* sp. towards tested bacteria.

Extracts	Total activity (mL/g)						
	Hexane	Diethyl ether	Chloroform	Ethyl acetate	Acetone	Methanol	Water
Gram positive bacteria							
S.aureus	6.5	5.97	0.98	0.02	0.15	5.50	-
B.cereus	3.27	11.94	1.95	0.03	0.29	-	-
Gram negative bacteria							
K.pneumoniae	102.33	186.57	7.88	0.06	0.58	11.00	-
P.aeruginosa	52.82	48.15	7.88	0.03	0.29	5.50	-
E.coli penicillin sensitive strain	-	-	-	0.004	-	-	-
E.coli penicillin resistant strain	-	-	-	0.004	-	-	-

All values are expressed as mean of three replicates
"- " : no inhibition

4.4.2 Antifungal Activities

The seven extracts were subjected to antifungal testing using colorimetric broth microdilution. Five medically-important fungi including yeasts and moulds were used in the testing. The yeasts used were *Calbicans, C. parapsilosis* and *C. neoformans* while the moulds used were *A. niger* and *T. mentagrophytes.*The antifungal drug, fluconazole with concentrations from 0.63 to 32 µg/mL was used as the positive control.

Table 4.7 summarizes the results of the extracts towards the five medically-important fungi. Hexane and diethyl ether extracts showed inhibitory activity towards yeasts and moulds, the MIC values towards *C.parapsilosis* and *C.neoformans* were ranged from 0.02 to 0.31 mg/mL. However, both extracts showed no inhibition towards *A. niger*. The MIC values of acetone and methanol extracts obtained were between 0.16 and 5 mg/mL. Acetone and ethyl acetate extracts showed good inhibition, but the yield from these two extracts were too little for further isolation (<0.05%). Water extract showed weak inhibition towards *Candida* species with MIC values ranged from 2.5 to 5 mg/mL.

The total activities of seven extracts towards fungi tested are summarised in Table 4.8. Hexane extract showed the highest total activity with a value of 409.34 mL/g towards *C. neoformans*, i.e. that 1 g of extract dissolved in 409.34 mL of solvent has inhibitory activity towards *C.neoformans*. Due to its strong inhibitory activity and sufficient quantity, hexane extract was selected for

bioassay-guided isolation to identify the bioactive compounds.

Table 4.7: Mean MIC values (mg/mL) of the seven extracts of *Sinularia* sp. towards tested fungi.

Extracts	MIC (mg/mL)							MIC (µg/mL)
	Hexane	Diethyl ether	Chloroform	Ethyl acetate	Acetone	Methanol	Water	Fluconazole
Yeast								
C.albicans	2.5-5	2.5-5	-	1.25	0.63	2.5-5	2.5	4
C.parapsilosis	0.04-0.16	0.16-0.31	2.5	0.08-0.16	0.16	0.31	2.5-5	8
C.neoformans	0.02-0.04	0.04-0.08	2.5-5	0.16-0.63	0.31-0.63	0.63-1.25	-	16
Mould								
A.niger	-	-	0.31-0.63	0.31-0.63	-	0.63-1.25	-	4
T.mentagrophytes	1.25	0.63-1.25	1.25-2.50	5	2.5-5	0.63-1.25	-	4

All values are expressed as mean or range of three replicates
"- " : no inhibition

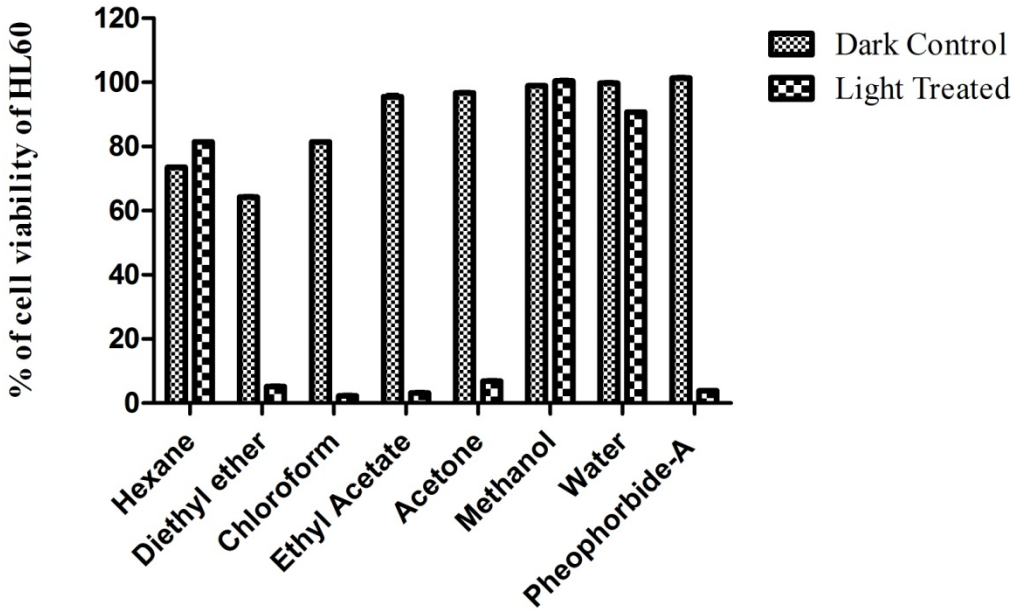
Table 4.8: Total activity (mL/g) of the seven extracts of *Sinularia* sp. towards tested fungi.

Extracts	Total activity (mL/g)						
	Hexane	Diethyl ether	Chloroform	Ethyl acetate	Acetone	Methanol	Water
Yeast							
C.albicans	3.27	2.99	-	0.02	0.58	5.50	15.43
C.parapsilosis	102.33	48.15	0.98	0.13	2.28	88.68	7.72
C.neoformans	409.34	186.57	0.49	0.03	0.60	21.99	-
Mould							
A.niger	-	-	3.88	0.03	-	21.99	-
T.mentagrophytes	13.10	11.94	0.98	0.004	0.07	21.99	-

All values are expressed as mean or range of three replicates
"- " : no inhibition

4.4.3 Photo- toxicity Activities

A MTT assay with light treatment was performed in order to assess the potential of *Sinularia* sp. as photosensitizer in photodynamic therapy. In the assay, the seven crude extracts were subjected to MTT assay with and without light treatment. The cytotoxicity activities of the crude extracts towards leukemia cell, HL 60 are shown in Figure 4.2. Diethyl ether, chloroform, ethyl acetate and acetone extracts showed good cytotoxicity activites with light treatment but showed no or low cytotoxicity activities without irridiation.

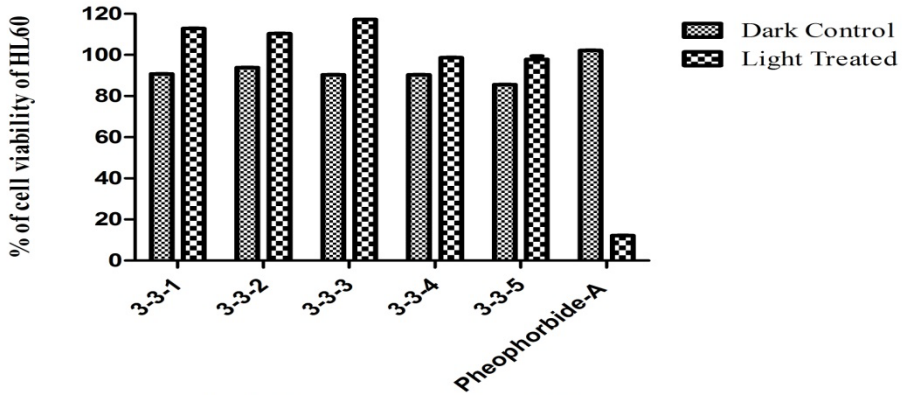


***Sinularia* sp. extracts 20µg/mL**

Figure 4.2: Percentage of cell viability of HL 60 versus *Sinularia*sp. extracts.

Diethyl ether, chloroform, ethyl acetate and acetone extracts showed high photo-toxicity activities towards HL 60 cell line among these extracts. However, only chloroform extract with highest photo-toxicity activities (2.25% of cell viability) was subjected to isolation using flash chromatography. The weight of the ethyl acetate and acetone extracts were not sufficient for isolation.

Three grams of the chloroform extract was further isolated to yield five fractions (3-3-1 to 3-3-5) and subjected to MTT test. However, all of the fractions lost its activities and the test was terminated. This phenomenon may have been due to synergistic effect where several fraction working together to show inhibition. A fraction by itself does not show cytotoxicity properties. The fraction might have lost its activity after exposure to air, or it was unstable.



***Sinularia* sp. fractions 20 µg/mL**

Figure 4.3: Percentage of cell viability of HL 60 versus *Sinularia*sp. fractions.

4.5 Isolation of Antifungal Components from Hexane Extract of *Sinularia*sp.

The hexane extract of *Sinularia*sp. was found to exhibit strong antifungal activity and thus was subjected to isolation techniques to obtain its active components.The hexane extract was subjected to modified column chromatography techniques described by Li et al., (2005). The hexane extract was fractionated into 30 fractions (AgN1 to AgN30) that were then subjected to another round of antifungal activity screening. All 30 fractions were subjected to GCMS analysis on an Agilent Technologies 6890N GC System. The total ion chromatogram obtained was auto integrated by Chemstation and the constituents were identified by comparison with published mass spectra databases (Wiley 9N Mass Spectral Library).

A few fractions were found to exhibit strong antifungal activities towards *C. neoformans* including AgN1 (MIC value 0.13 mg/mL), AgN6 (MIC value 0.06 mg/mL) and AgN27 (MIC value 0.016mg/mL). Fraction AgN1 is an oily mixture of hydrocarbons and eight other compounds in this fraction. These are waxy compounds common for protection and to prevent desiccation. The total ion chromatogram is shown in Figures 4.4 – 4.11 and Appendix A. This is the first report of waxy substances from the soft coral, *Sinularia*sp.

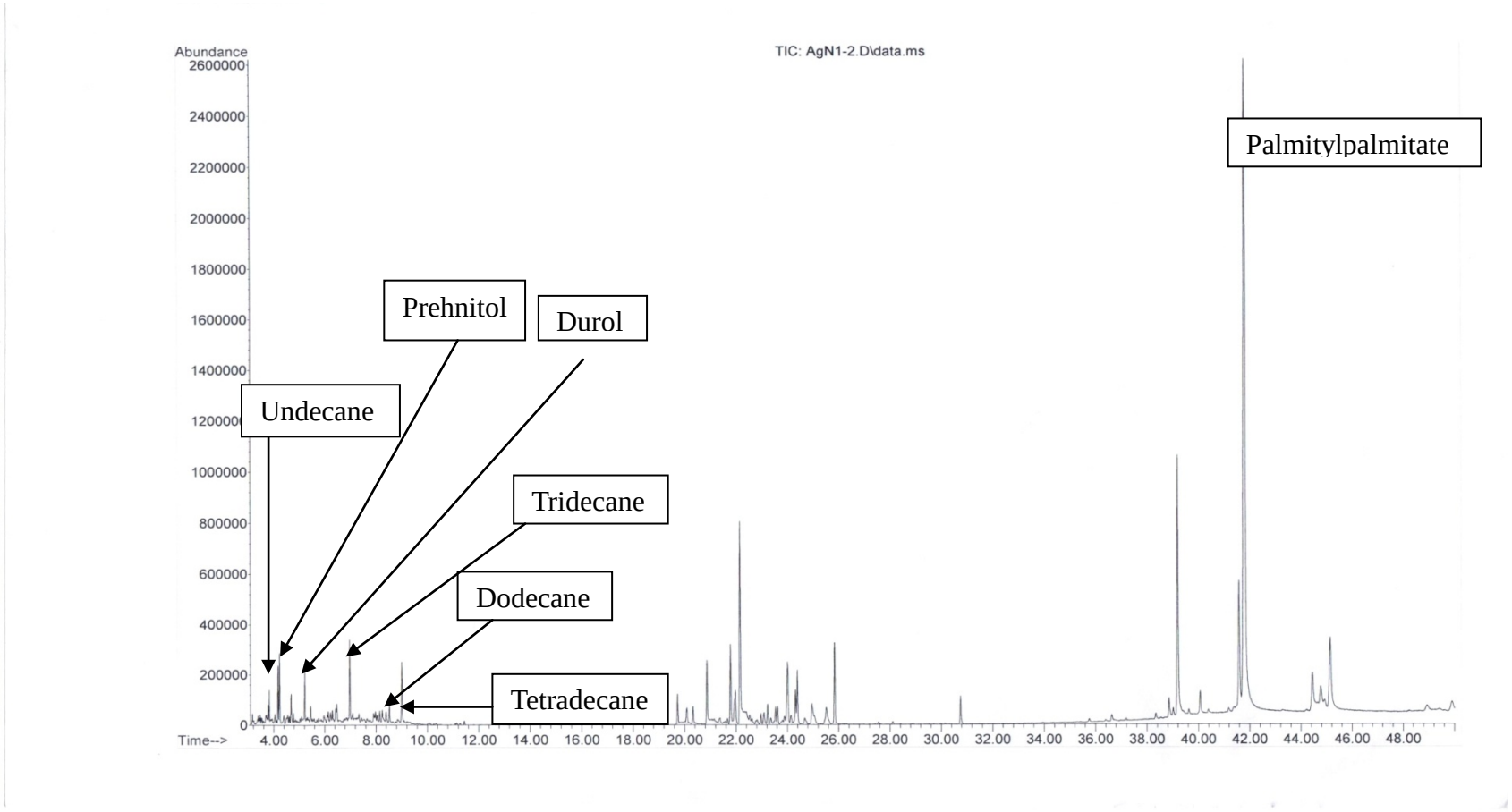


Figure 4.4: Total ion chromatogram of fraction AgN1.

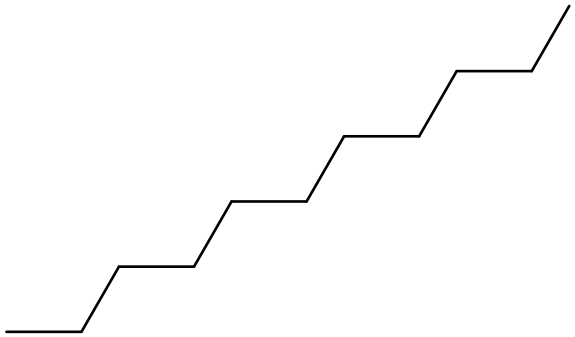


Figure 4.5: Chemical structure of undecane.

Undecane: m/z 156 [M^+], 127 [$M^+ – C_2H_5$], 98 [$M^+ – C_2H_5 – C_2H_5$], 85, 71, 57 [100%, base peak]

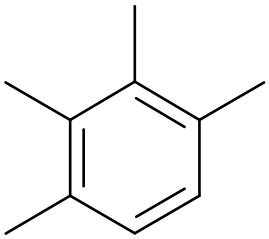


Figure 4.6: Chemical structure of prehnitol.

Prehnitol: m/z 134 [M^+], 119[$M^+ – CH_3$, 100%, base peak], 105, 95, 91, 77, 65

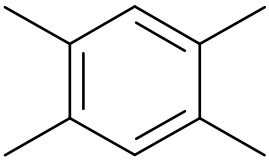


Figure 4.7: Chemical structure of durol.

Durol: m/z 134 [M^+], 119 [$M^+ – CH_3$, 100%, base peak], 105, 91, 77, 69

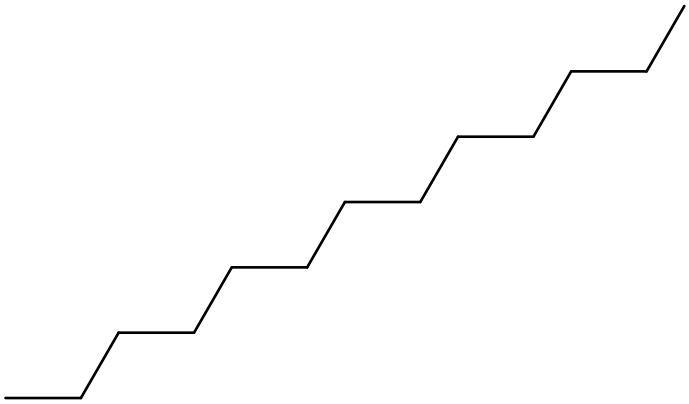


Figure 4.8: Chemical structure of tridecane.

Tridecane: m/z 184 [M^+], 155 [$M^+ – C_2H_5$], 141 [$M^+ – C_2H_5 – CH_2$], 126 [$M^+ – C_2H_5 – C_2H_5$], 99, 85, 71, 57 [100%, base peak]

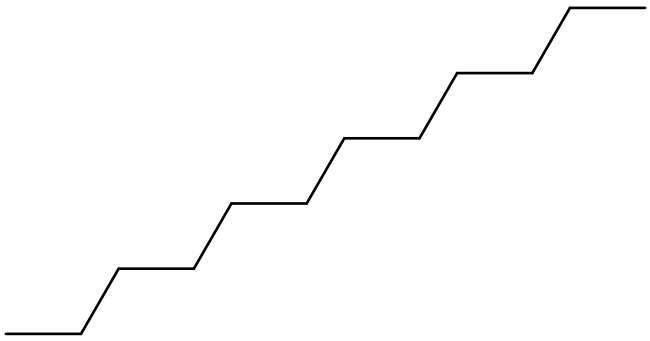


Figure 4.9: Chemical structure of dodecane.

Dodecane: m/z 170 [M^+], 141 [$M^+ – C_2H_5$], 128, 112 [$M^+ – C_2H_5 – C_2H_5$], 98, 85, 71, 57 [100%, base peak]

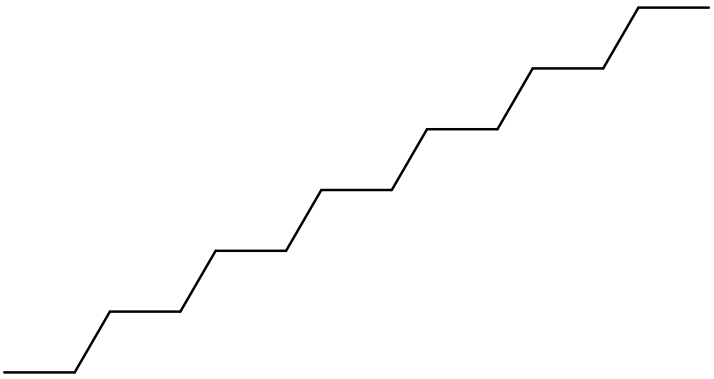


Figure 4.10: Chemical structure of tetradecane.

Tetradecane: m/z 198 [M^+], 169 [M^+– C_2H_5], 155 [M^+– C_2H_5– CH_2], 141 [M^+– C_2H_5–C_2H_4], 99, 85, 71, 57 [100%, base peak]

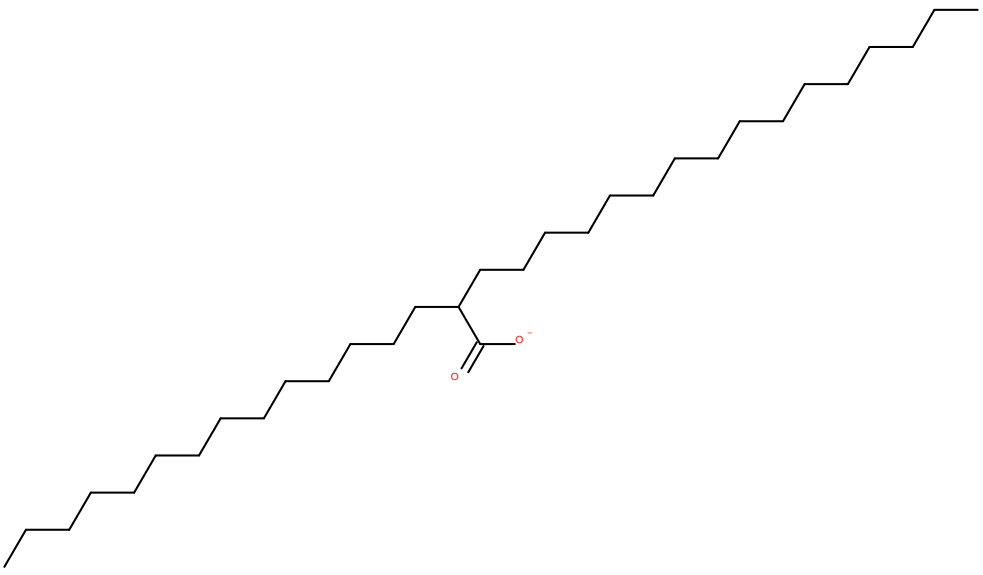


Figure 4.11: Chemical structure of Palmitylpalmitate.

Palmitylpalmitate: m/z 480 [M^+], 257, 239, 224, 196, 125, 111, 97, 83, 57 [100%, base peak]

Sterol, an abundant component was also found in the fraction because it is an essential component in the membranes of all eukaryotic organisms (Tang et al., 2012). Fraction AgN6, a whitish powder, was found to be a mixture of sterols by GC–MS analysis. It consisted of mainly lanol, ergost-5,8(14)-dien-3-ol, and campesterol. This is the first report of these sterols from the soft coral, *Sinularia*sp. The total ion chromatogram is shown in Figure 4.12 and Appendix B.

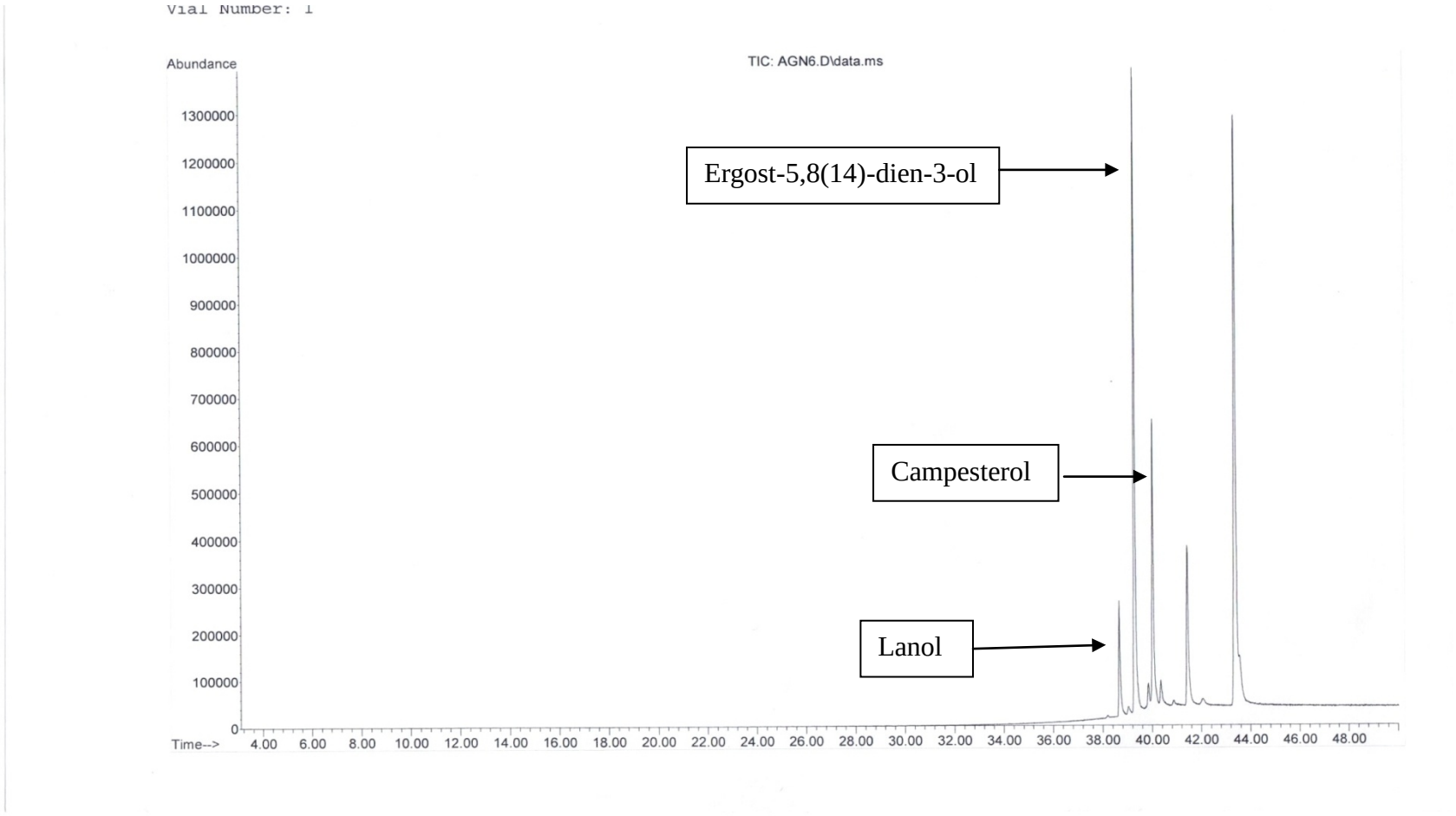


Figure 4.12: Total ion chromatogram offraction AgN6.

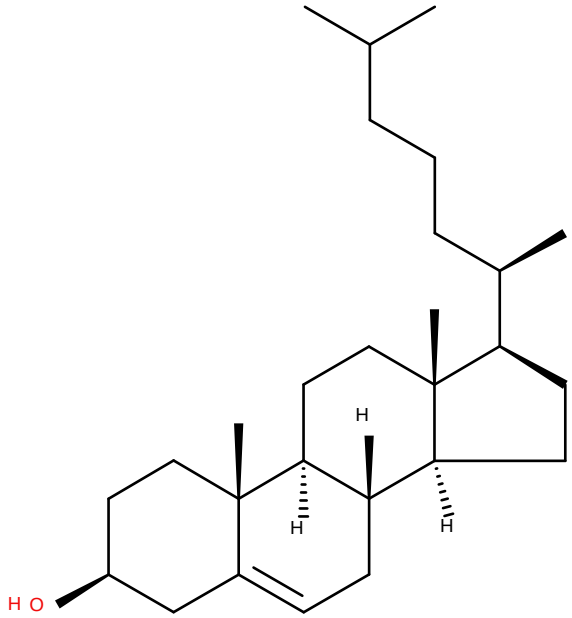


Figure 4.13: Chemical structure of lanol.

Lanol: m/z 386 [M^+], 368 [$M^+ – H_2O$], 353 [$M^+ – H_2O – CH_3$], 301, 275, 255, 207, 159, 145, 133, 105 [100%, base peak], 81, 67, 55.

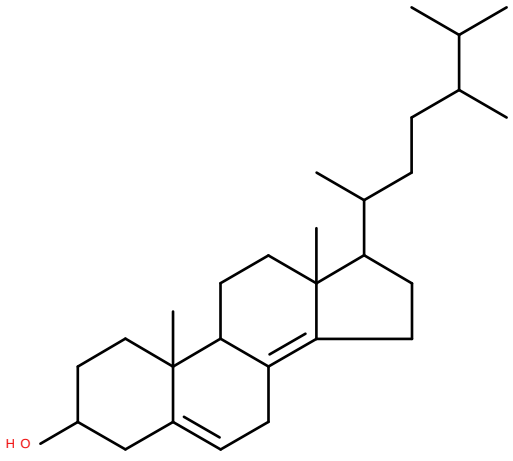


Figure 4.14:Chemical structure of ergost-5,8(14)-dien-3-ol.

Ergost-5,8(14)-dien-3-ol: m/z 398 [M^+], 380 [$M^+ – H_2O$], 365 [$M^+ – H_2O – CH_3$], 337, 300, 271, 255, 213, 159, 145, 133, 119, 105, 93, 81, 69, 55 [100%, base peak].

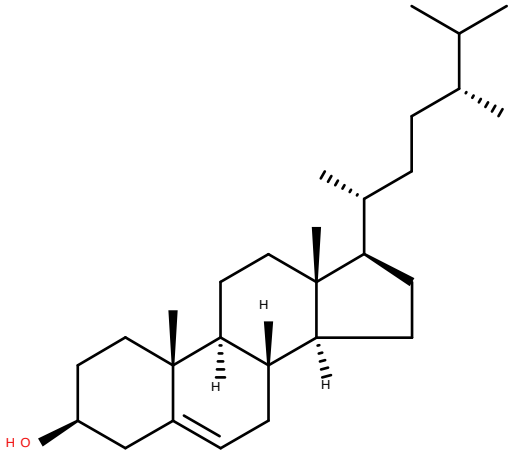


Figure 4.15: Chemical structure of campesterol.

Campesterol: m/z 400 [M^+], 382 [$M^+ – H_2O$], 367 [$M^+ – H_2O – CH_3$], 315, 289, 273, 255, 213, 159, 145, 133, 119, 105, 81, 67, 55 [100%, base peak].

4.6 Fuscol, Isolated Antifungal Compound from *Sinularia*sp.

AgN27-1, a colourless oil, was isolated from fraction AgN27 byflash chromatography and preparative thin layer chromatography. 42 fractions were collected from flash chromatography using hexane and ethyl acetate combination as mobile phase. Combined active fractions 18 to 23 were further purified on preparative TLC. The fraction was developed with the solvent system hexane:ethyl acetate (80:20) to yield 28.5 mg AgN27-1 (Figure 4.16).

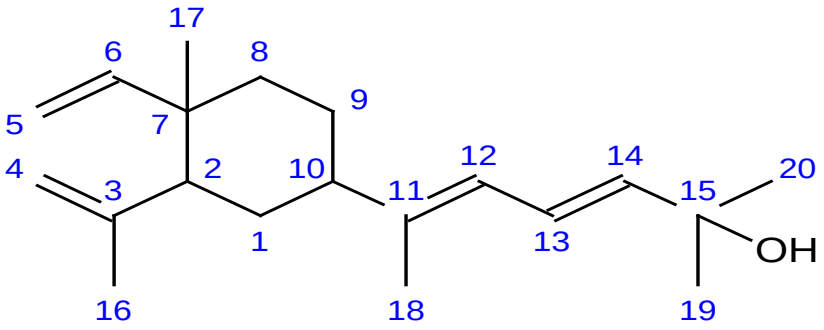


Figure 4.16: AgN27-1 ($C_{20}H_{32}O$, MW = 288).

The total ion chromatogram from GCMS analysis (Figure 4.17) showed AgN27-1 as a major peak at retention time of 23.780min and the mass spectrum indicated the presence of a molecular ion at m/z 288. The presence of molecular ion at m/z 288 of AgN27-1 is consistent with the molecular formula of fuscol, $C_{20}H_{32}O$ (Figure 4.18). AgN27-1 showed inhibition towards *C. neoformans* with MIC = 0.064 mg/mL.

Fuscol was first isolated by Gopichand and Schmitz in year 1978 (Gopichand and Schmitz, 1978). In the research, the compound was isolated from gorgonians, *Euniceafusca*. However, no research on the bioactivity of the compound was performed.

In 2012, Govindamet al. (2012) isolated fuscol from soft coral, *Lopophytumpauciflorum*.In their study, fuscol showed cytotoxic with IC_{50} of 0.52 µM towards human epidermoid carcinoma A431 cells.

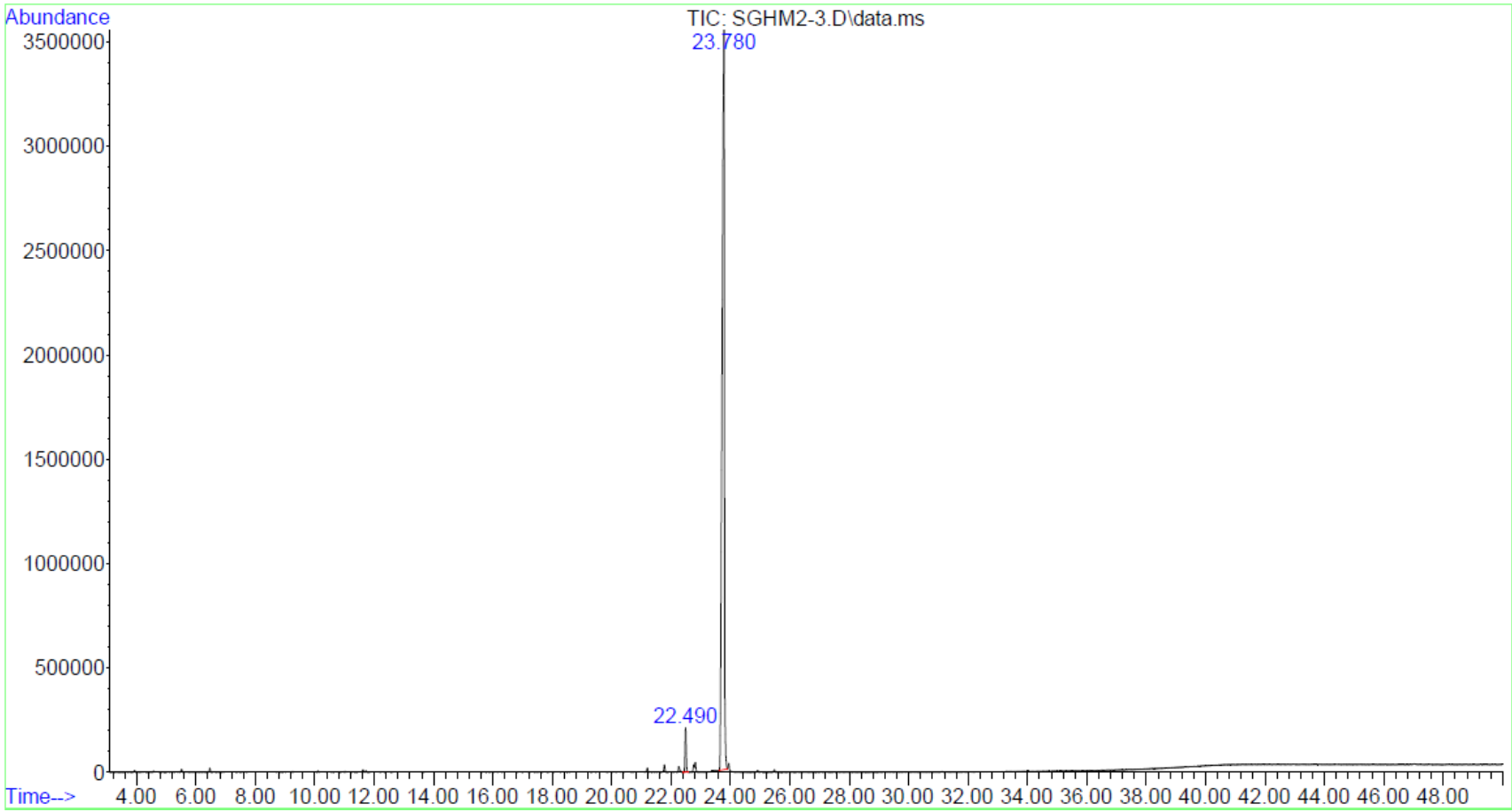


Figure 4.17: Total ion chromatogram of subfraction AgN27-1.

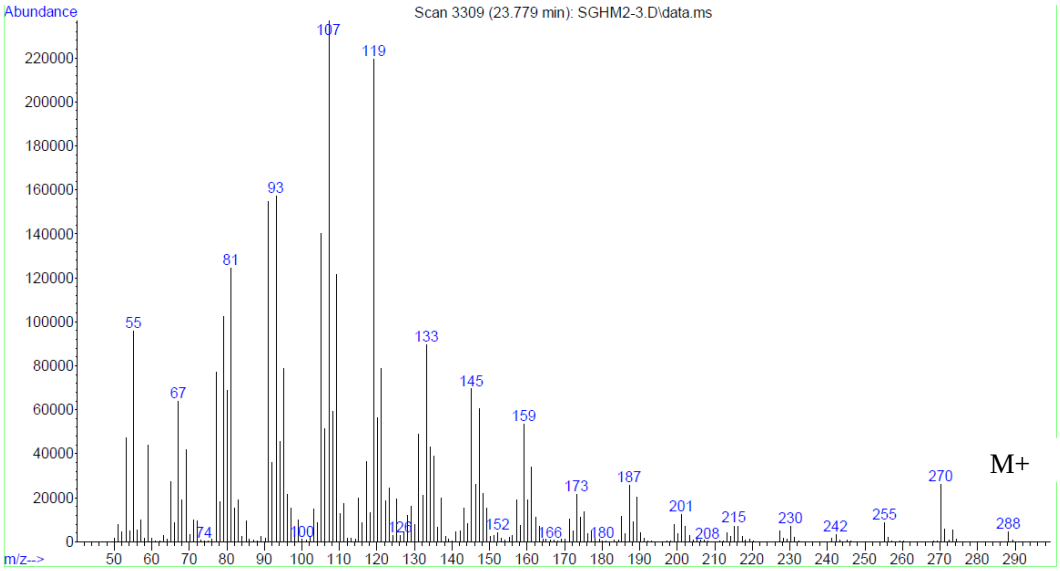


Figure 4.18: Mass spectrum of compound AgN27-1.

The ^{1}H NMR spectral data is consistent with those reported by the literature (Govindamet al., 2012) (Table 4.9).The ^{1}H NMR indicated the presence of one broad singlet peak at δ4.59 and one triplet peak at δ4.82 (*J* = 1.5 Hz) representing the *exo*-methylene protons for H4 while Govindam et al. (2012) reported two singlet peaks at δ4.58 and δ4.81 instead. The triplet peak with a small coupling constant value of 1.5 Hzobserved at δ 4.82may due to the long range coupling of proton H_b4 with the methyl protons H16. The multiplicity of the peak was different from those reported by Govindam et al. (2012), and the discrepancy could be due to the variation in experimental condition and sensitivity of NMR equipment used during the study. The ^{1}H NMR also showed the presence of two overlapped doubletpeaks at δ4.92 (*J* = 16.9 Hz) and at δ4.91 (*J* = 11.5 Hz) representing the *exo*-methylene protons for H5.Whilst Govindam et al. (2012) reported two doublet peaks at δ4.90 (*J* = 15.2 Hz) and δ4.91 (*J* = 11.6

Hz)representing the *exo*-methylene protons for H5.Govindam et al. (2012) also reported the presence of five singlet peaks at δ1.00, 1.35, 1.35, 1.70 and 1.79 representing the methyl protons of H16, H17, H18, H19 and H20, respectively, consistent with the ^{1}H NMR spectral data of this study which showed the presence of five singlet peaks at δ1.02, 1.36, 1.36, 1.71 and 1.80.

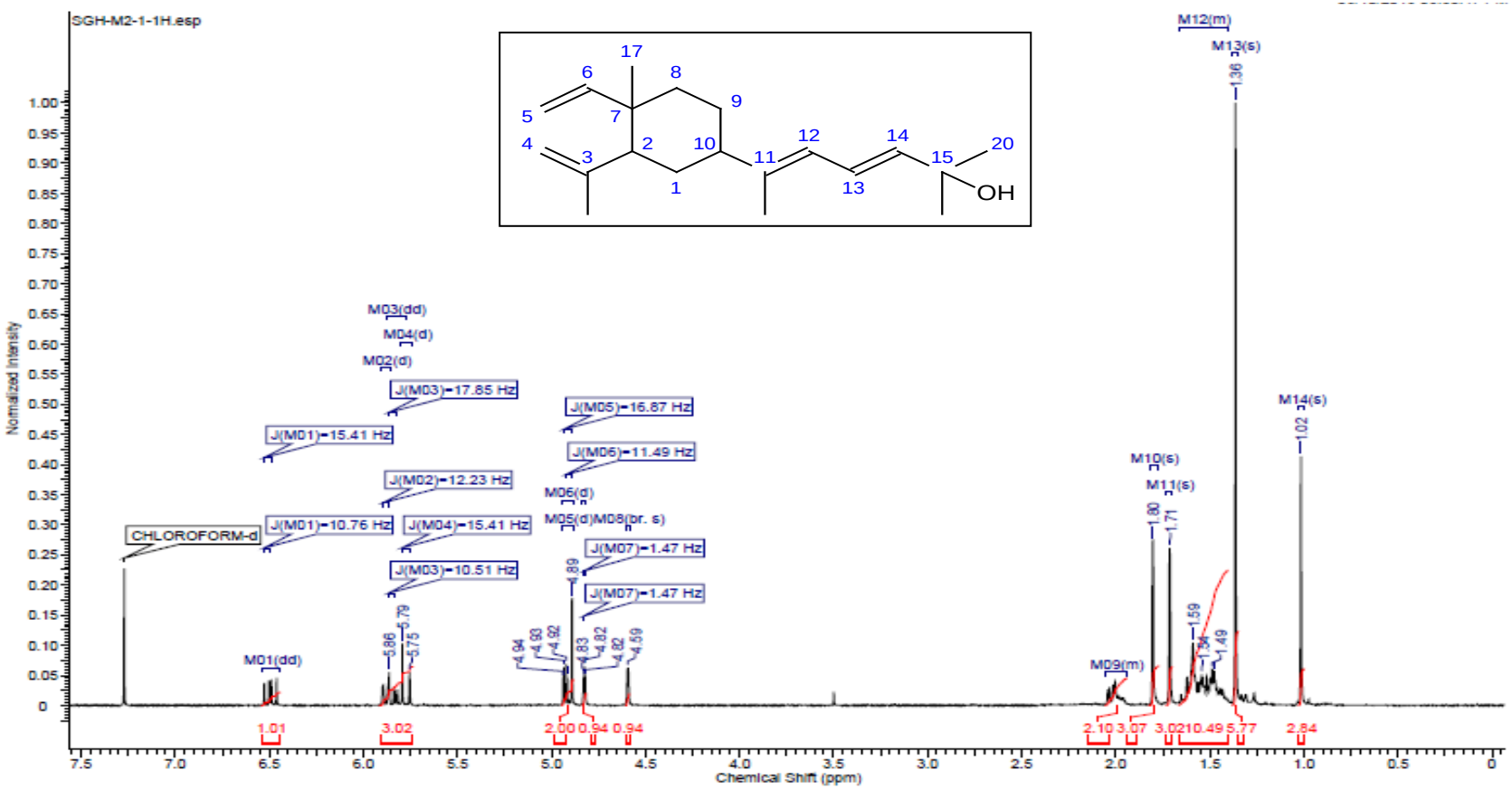


Figure 4.19: ^{1}HNMR spectrum of fuscol, isolated from *Sinularia* sp. (400 MHz, $CDCl_3$)

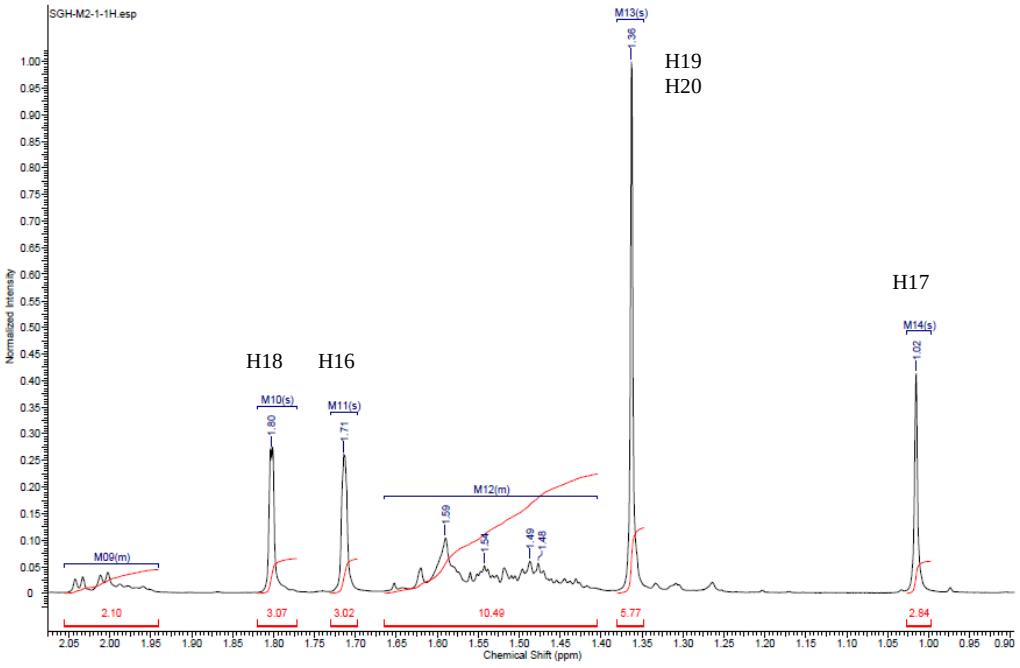


Figure 4.20: Expanded ^{1}H NMR spectrum of fuscol, isolated from *Sinularia* sp. (400 MHz, $CDCl_3$)

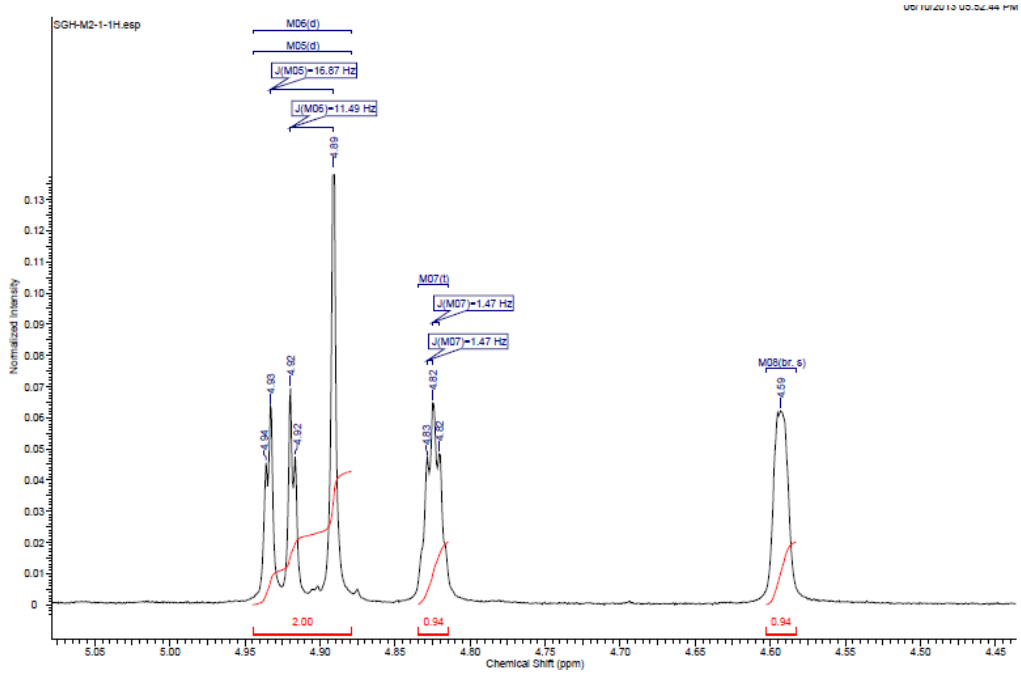


Figure 4.21: Expanded ^{1}H NMR spectrum of fuscol, isolated from *Sinularia* sp. (400 MHz, $CDCl_3$)

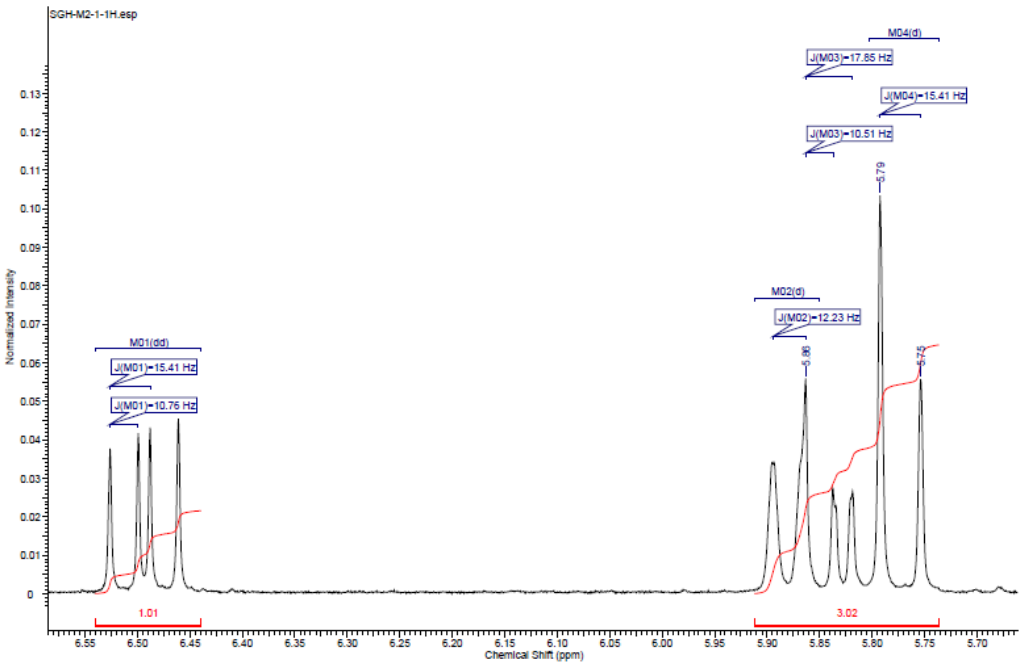


Figure 4.22: Expanded ^{1}H NMR spectrum of fuscol, isolated from *Sinularia* sp. (400 MHz, $CDCl_3$)

Table 4.9:^{1}H NMR data of isolated fuscol and those reported in the literature.

Position	Sample (δ, $CDCl_3$, 400 MHz)	Reference (δ, $CDCl_3$, 400 MHz)[a]
H1	1.60 – 1.20 (m, CH_2, 2H)	1.60 – 1.20 (m, CH_2, 2H)
H2	2.00 (m, CH, 1H)	2.00 (m, CH, 1H)
H4	4.59 (br s, CH_2, $1H_a$) 4.82 (t, *J* = 1.5 Hz, CH_2, $1H_b$)	4.58 (s, CH_2, $1H_a$) 4.81 (s, CH_2, $1H_b$)
H5	4.91 (d, *J* = 11.5 Hz, CH_2, $1H_a$) 4.92(d, *J* = 16.9 Hz, CH_2, $1H_b$)	4.90 (d, *J* = 11.6 Hz, CH_2, $1H_a$) 4.91 (d, *J* = 16.8 Hz, CH_2, $1H_b$)
H6	5.83 (dd, *J* = 17.9, 10.5 Hz, CH, 1H)	5.82 (dd, *J* = 16.8, 11.6 Hz, CH, 1H)
H8	1.60 – 1.20 (m, CH_2, 2H)	1.60 – 1.20 (m, CH_2, 2H)
H9	1.60 – 1.20 (m, CH_2, 2H)	1.60 – 1.20 (m, CH_2, 2H)
H10	2.00 (m, CH, 1H)	2.00 (m, CH, 1H)
H12	5.86 (d, *J* = 12.2 Hz, CH, 1H)	5.87(d, *J* = 11.0 Hz, CH, 1H)
H13	6.49 (dd, *J* = 15.4, 10.8 Hz, CH, 1H)	6.49 (dd, *J* = 15.2, 11.0 Hz, CH, 1H)
H14	5.77 (d, *J* = 15.4 Hz, CH, 1H)	5.76(d, *J* = 15.2 Hz, CH, 1H)
H16	1.71(s, CH_3, 3H)	1.70 (s, CH_3, 3H)
H17	1.02 (s, CH_3, 3H)	1.00 (s, CH_3, 3H)
H18	1.80 (s, CH_3, 3H)	1.79 (s, CH_3, 3H)
H19	1.36 (s, CH_3, 3H)	1.35 (s, CH_3, 3H)
H20	1.36 (s, CH_3, 3H)	1.35 (s, CH_3, 3H)

[a]Govindam et al., 2012

The ^{13}C NMR broadband spectrum (Figure 4.23) showed the presence of 20 carbons consisting of five methyl carbons at δ15.4, 16.7, 24.8, 29.9, 29.9, three methylene carbons at δ26.5, 32.6, 39.8, two *exo*–methylene carbons at δ 109.9, 112.1, six methine carbons at δ47.6, 52.7, 122.4, 123.0, 139.2, 150.2 and four quaternary carbons at δ39.3, 70.9, 143.4, 147.6. The assignment of the carbon types was confirmed through the DEPT analysis (Figure 4.24).

The structure of fuscol was further confirmed by the heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC) data as shown in Table 4.11. HSQC spectrum (Figure 4.25)showed the direct ^{1}H–^{13}C correlation in fuscol, while HMBC spectrum showed long range connectivity of proton to carbons separated by one or two atoms away and also correlate quaternary carbons with nearby protons in fuscol (Figure 4.26) (Silverstein and Webster, 1998).

Quaternary carbon C3 showed ^{2}J correlations with methine proton H2, *exo*-methylene protons H4 and methyl proton H16. There are ^{2}J correlations between quaternary carbon C7 and methyl proton H17 and methine protons H2 and H6, whilst it also showed ^{3}J correlation with *exo*-methylene protons H5. Quaternary carbon C11, on the other hand, showed ^{2}J correlation with methyl proton H18 and ^{3}J correlation with methine proton H13. As for quaternary carbon C15, it showed ^{3}J correlation with methine proton H13 and ^{2}J correlation with methine proton H14 and both methyl protons H19 and H20.

Exo-methylene carbonC4 showed ^{3}J correlations with methine proton H2 and methyl proton H16, whilst its protons exhibited^{3}J correlation with methine carbonC2 and methyl carbonC16. The *exo*-methylene protonH4 showed ^{2}J correlation with quaternary carbon C3.

Methyl carbon C16 has ^{3}J correlation with methine proton H2 and *exo*-methylene protons H4. Methyl proton H16 showed ^{2}J correlation with quaternary carbon C3, ^{3}J correlation with methine carbonC2 and *exo*-methylene carbon C4. As for methyl carbon C17, it showed ^{3}J correlation with methine protons H2 and H6 whilst its protons showed ^{2}J correlation with quaternary carbon C7, ^{3}J correlation with methine carbonsC2 and methylene carbon C8. There are ^{3}J correlations between methyl carbon C18 and methine protons H10 and H12 while methyl proton H18 has ^{2}J correlations with quaternary carbon C11 and ^{3}J correlations with methine carbons C10 and C12. Both methyl carbons C19 and C20 showed ^{3}J correlations with mehine proton H14 and each other protons. Both methyl protons of H19 and H20 exhibited ^{2}J correlation with quaternary carbon C15 and ^{3}J correlation with methine carbon C14. Both methyl protons of H19 and H20 also showed ^{3}J correlation with each other carbon.

The methine carbon C12 showed ^{2}J correlation with methine proton H13 and ^{3}J correlation with methyl protons H18. There are ^{3}J correlation between methine proton H12 with methine carbon C10 and methyl carbon C18. Methine proton H12also found to have^{2}J correlation with methine carbon C13.

Methinecarbon C13 has ^{2}J correlation with methine protons H12 and H14. As for its methine proton, H13 showed ^{2}J correlation with methine carbons C12 and C14, ^{3}J correlation with quaternary carbons C11 and C15. Methine proton H14 showed ^{2}J correlation with methine carbon C13 and quaternary carbon C15. It also showed ^{3}J correlation with methine carbon C13 and both methyl carbons C19 and C20. Whilst its methine carbon C14 showed ^{2}J correlation with methine proton H13 and ^{3}J correlations with methine proton H12 and both methyl protons H19 and H20.

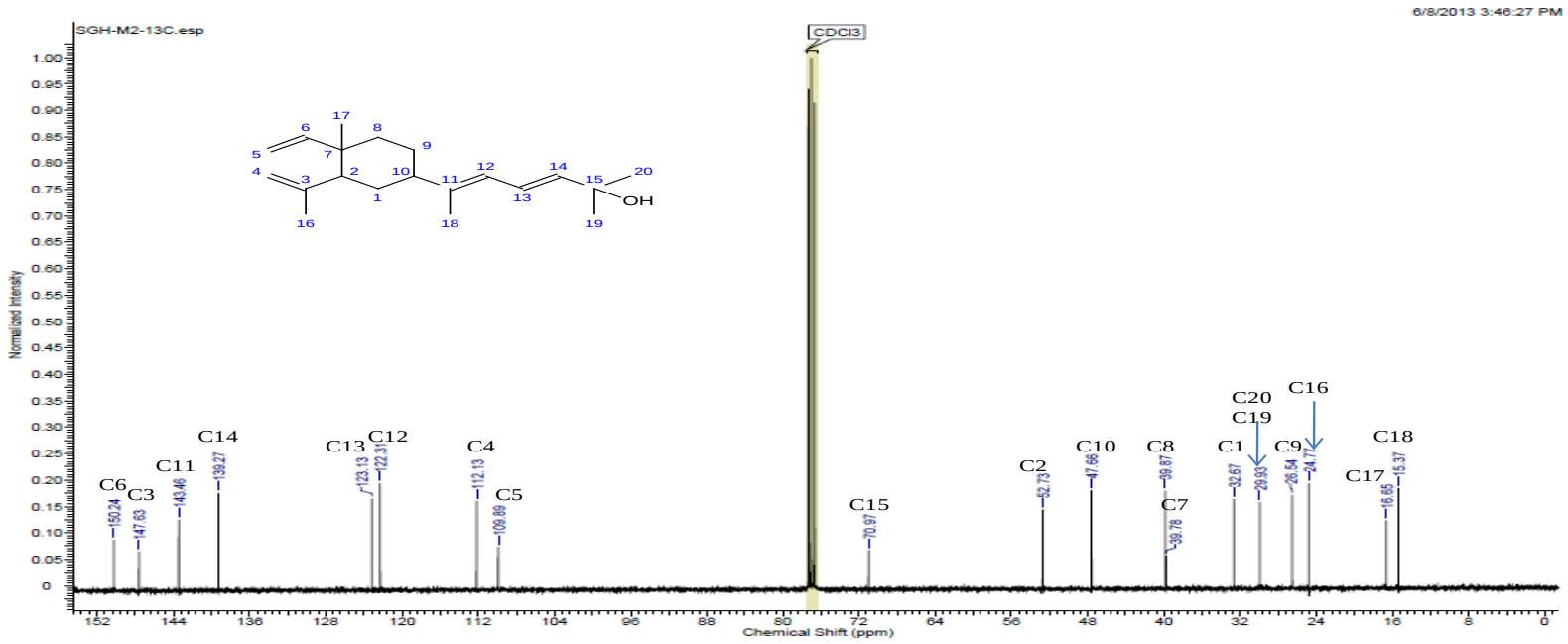


Figure 4.23: ^{13}CNMR broadband spectrum of fuscol.(400 MHz, $CDCl_3$)

Table 4.10: ^{13}C NMR data of isolated fuscol and those reported in the literature.

Position	Sample (δ, $CDCl_3$, 125 MHz)	Reference (δ, $CDCl_3$, 100 MHz)[a]
C1 (CH_2)	32.7	32.5
C2 (CH)	52.7	52.6
C3 (Cq)	147.6	147.5
C4 (CH_2)	112.1	112.0
C5 (CH_2)	109.9	109.6
C6 (CH)	150.2	150.1
C7 (Cq)	39.8	39.6
C8 (CH_2)	39.9	39.7
C9 (CH_2)	26.5	26.4
C10 (CH)	47.7	47.5
C11 (Cq)	143.5	143.2
C12 (CH)	122.3	122.9
C13 (CH)	123.1	123.0
C14 (CH)	139.3	139.1
C15 (C–OH, Cq)	71.0	70.9
C16 (CH_3)	24.8	24.7
C17 (CH_3)	16.7	16.4
C18 (CH_3)	15.4	15.3
C19 (CH_3)	29.9	29.8
C20 (CH_3)	29.9	29.8

[a]Shin and Fenical, 1991

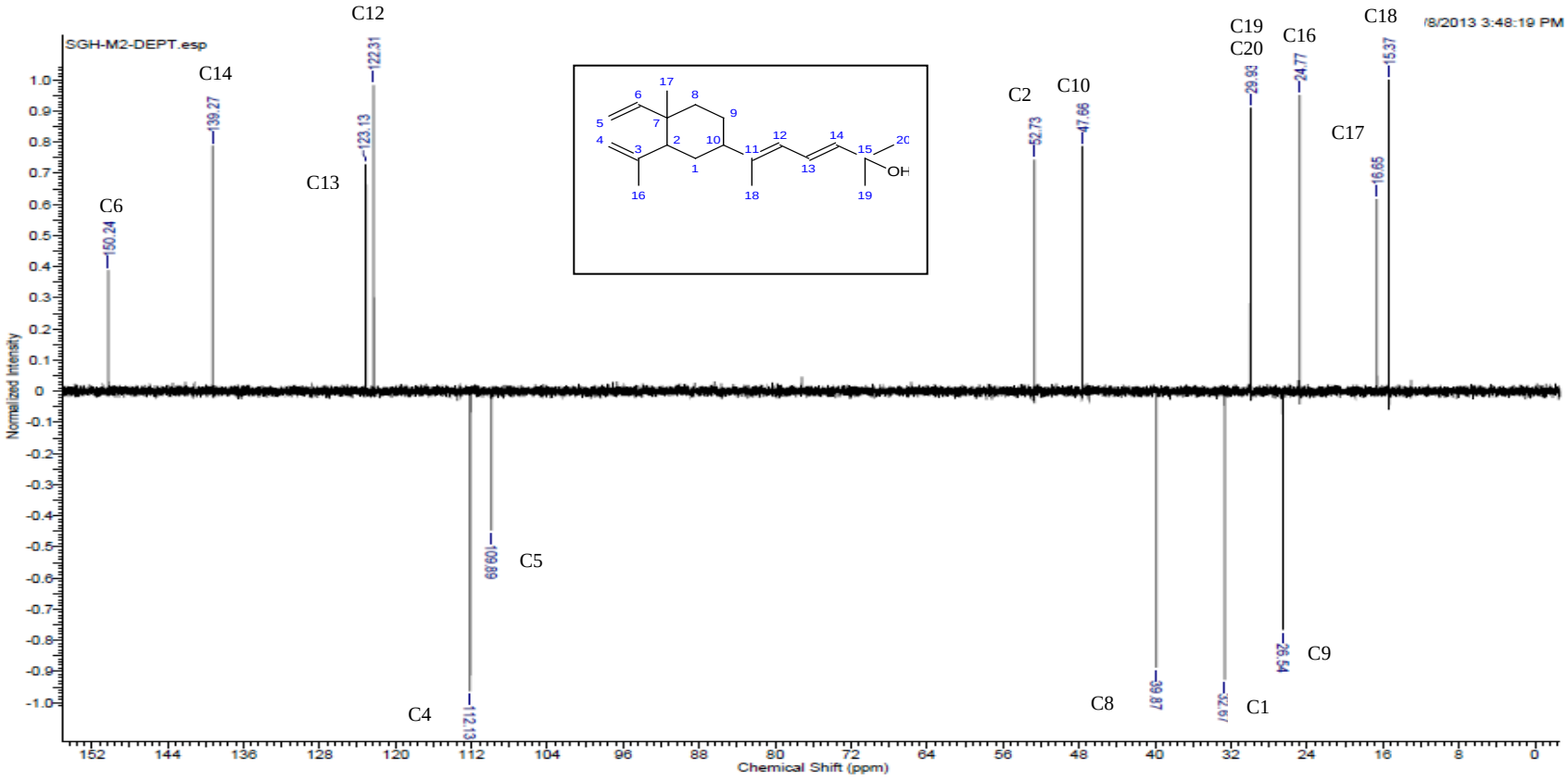


Figure 4.24:DEPT broadband spectrum of fuscol.(400 MHz, $CDCl_3$)

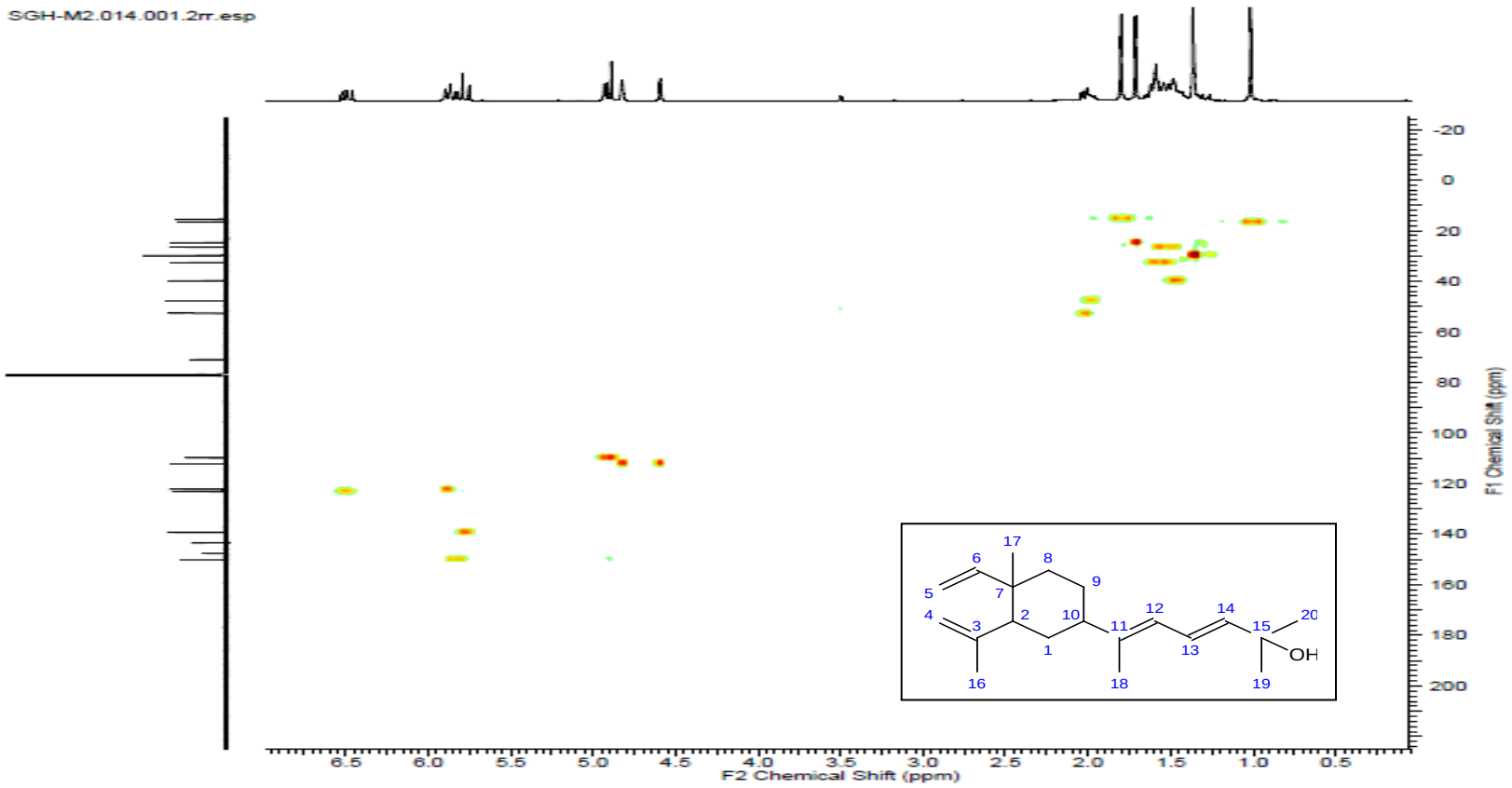


Figure 4.25: HSQC broadband spectrum of fuscol.(400 MHz, $CDCl_3$)

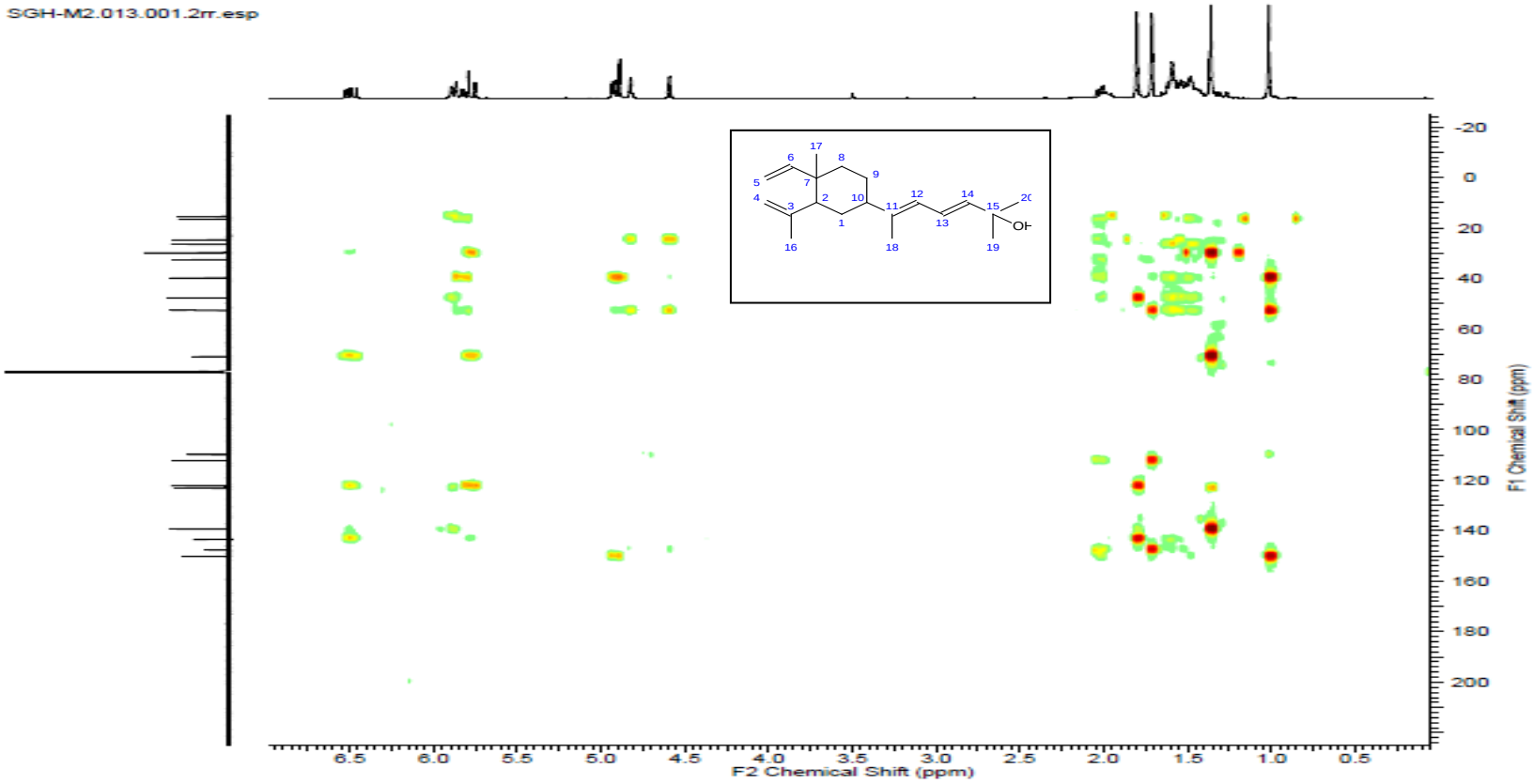


Figure 4.26:HMBC broadband spectrum of fuscol.(400 MHz, $CDCl_3$)

Table 4.11: HSQC and HMBC data of the isolated fuscol.

Position	HSQC		HMBC
	^{13}C (δ)	^{1}H (δ)	^{1}H – ^{13}C (δ)
2, CH	52.7	2.00	32.7 (C1), 147.6 (C3), 112.1 (C4), 150.2 (C6), 39.8 (C7), 39.9 (C8), 26.5 (C9), 47.7 (C10), 24.8 (C16), 16.7 (C17)
3, Cq	147.6	-	-
4, CH_2	112.1	4.59 4.82	52.7 (C2), 147.6 (C3), 39.8 (C7), 24.8 (C16)
5, CH_2	109.9	4.91 4.92	150.2 (C6), 39.8 (C7)
6, CH	150.2	5.83	52.7 (C2), 39.8 (C7), 39.9 (C8), 16.7 (C17)
7, Cq	39.8	-	-
10, CH	47.7	2.00	32.7 (C1), 39.9 (C8), 26.5 (C9), 15.4 (C18)
11, Cq	143.5	-	-
12, CH	122.3	5.86	47.7 (C10), 123.1 (C13), 139.3 (C14), 15.4 (C18)
13, CH	123.1	6.49	143.4 (C11), 122.3 (C12), 139.3 (C14), 71.0 (C15)

14, CH	139.3	5.77	122.3 (C12), 123.1 (C13), 71.0 (C15), 29.9 (C19), 29.9 (C20)
15, Cq	71.0	-	-
16, CH_3	24.8	1.71	52.7 (C2), 147.6 (C3), 112.1 (C4)
17, CH_3	16.7	1.02	52.7 (C2), 39.8 (C7), 39.9 (C8)
18, CH_3	15.4	1.80	47.7 (C10), 143.4 (C11), 122.3 (C12)
19, CH_3	29.9	1.36	139.3 (C14), 71.0 (C15), 29.9 (C20)
20, CH_3	29.9	1.36	139.3 (C14), 71.0 (C15), 29.9 (C19)

4.7 Summary

*Sinularia*sp. exhibited antifungal and antibacterial activities. Fuscol is the compound responsible for the antifungal activities. Fuscol was reported for the first time from the *Sinularia*sp. A mixture of sterols and hydrocarbons were also found from the active antifungal fractions. These compounds were also reported for the first time from *Sinularia*sp. The chloroform extract of *Sinularia*sp. showed photo-toxicity activity towards HL 60. However, the fractions of chloroform extract lost its activities after exposed to air or it was unstable.

4.8 Suggestions for Further Work

Fuscol, an isolated antifungal compound from *Sinularia*sp. is not as potent as standard antifungal drug. Chemical modification should be carried out in order to obtain a new compound with higher potency. Collection of sample at different locations should be performed to assess the effects of environment towards the antifungal activity of fuscol.

The isolation of antibacterial compound from diethyl ether extract should be carried out to elucidate the structure of antibacterial compounds. Characterization of the isolated antibacterial compounds should be also studied.

CHAPTER 5.0

CONCLUSION

The active antifungal hexane extract of *Sinularia* sp. was purified by bioassay-guided fractionation towards *C. neoformans*(ATCC 90112). Fuscol($C_{20}H_{32}O$, MW = 288), an antifungal compound with MIC value of 0.064 mg/mL was isolated from the extract. Besides, an oily mixture of hydrocarbons and seven other compounds (fraction AgN1) with MIC value of 0.13 mg/mL was also found. They are undecane, prehnitol, durol, tridecane, dodecane, tetradecane, and palmitylpalmitate. A whitish powder (fraction AgN6) consisted of cyclopropa [5,6]-33-norgorgostan-3-ol, lanol, ergost-5,8(14)-dien-3-ol, campesterol and stigmasterol were also found. The MIC value was 0.06 mg/mL. All these compounds were first time reported from the soft coral, *Sinularia*sp.

Diethyl ether extract exhibited strong inhibition towards *C. neoformans* and *K.pneumoniae.* However, bioassay-guided fractionation was not performed due to time constraint.

Chloroform extract showed positive result in photo-cytotoxicity test towards leukemia cell line HL 60. However, all of the fractions lost their activities after fractionation. It maybe due to synergistic effect where several fractions working together to show inhibition, or the fraction might have lost its activity after exposure to air, or it was unstable.

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Appendix A

Total ion chromatogram for fraction AgN1 integrated by Chemstation and the constituents identified by camparison published mass spectra databases (Wiley 9N Mass Spectral Library).

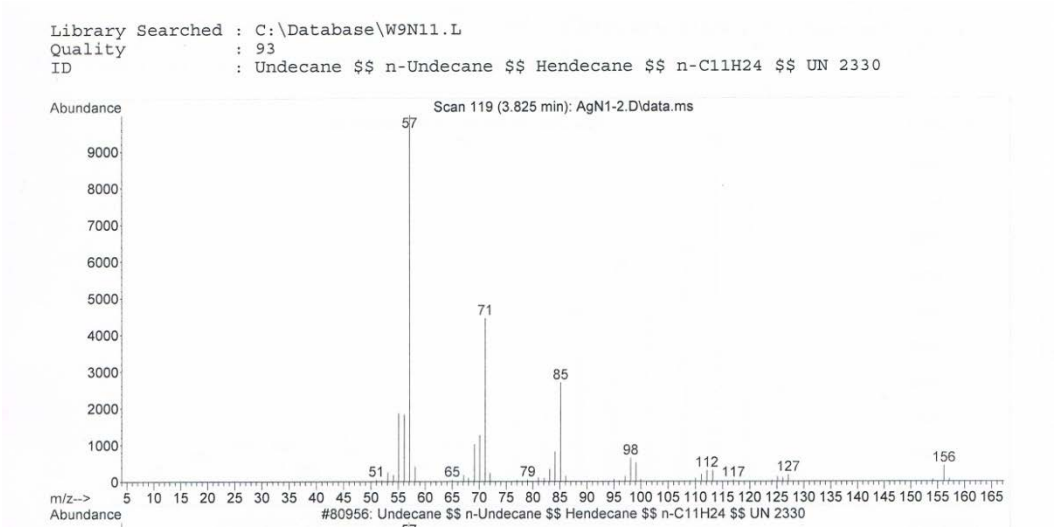


Figure 1: Undecane obtained fromfraction AgN1.

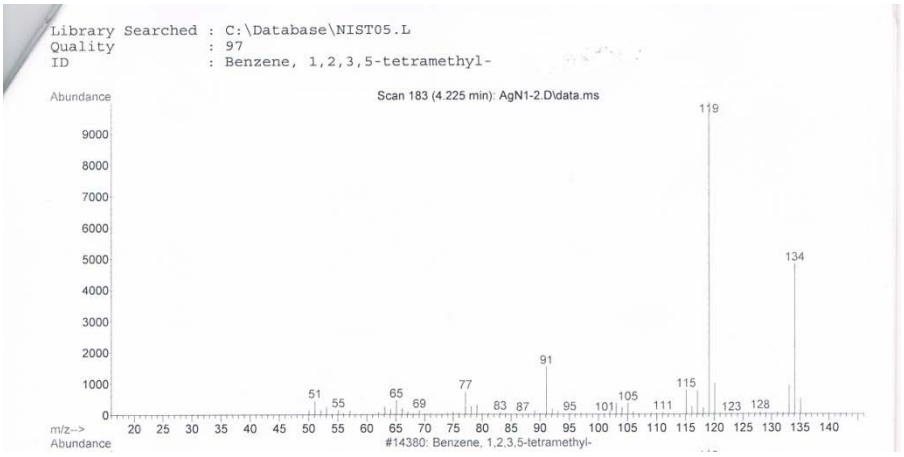


Figure 2: Prehnitolobtained fromfraction AgN1.

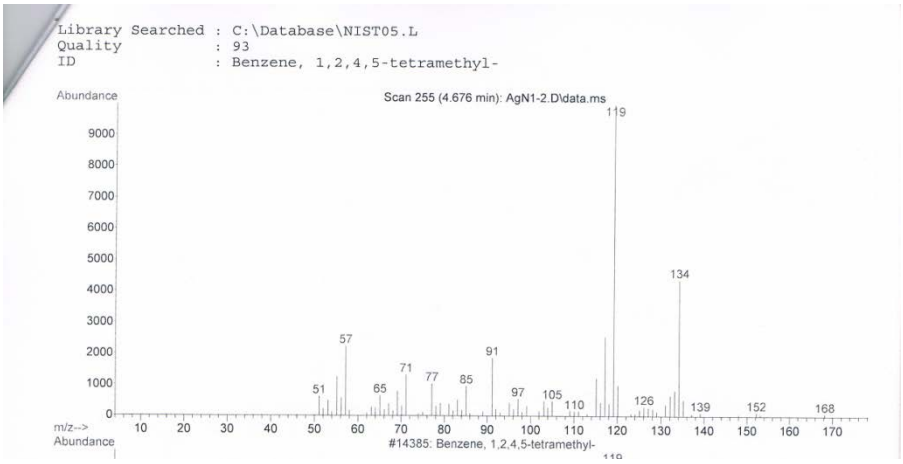


Figure 3: Durol obtained from fraction AgN1.

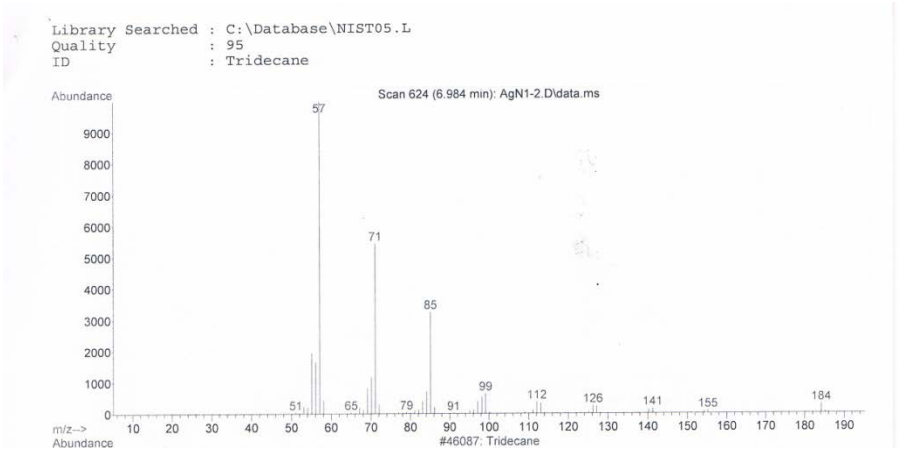


Figure 4: Tridecane obtained fromfraction AgN1.

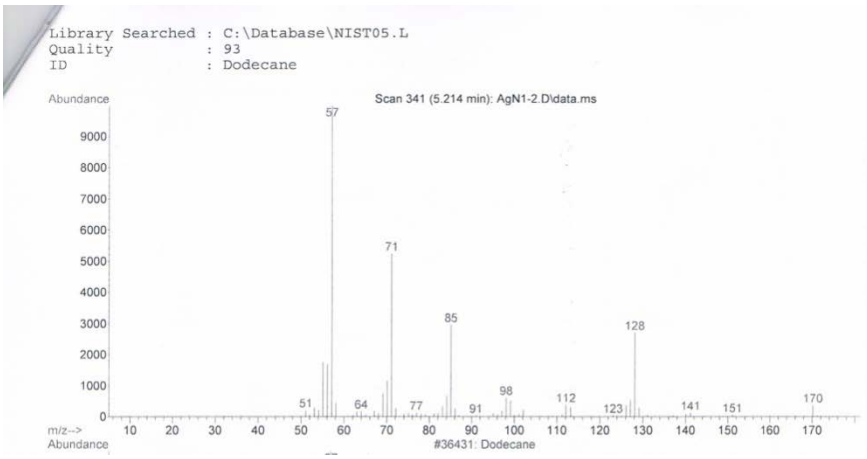


Figure 5: Dodecane obtained from fraction AgN1.

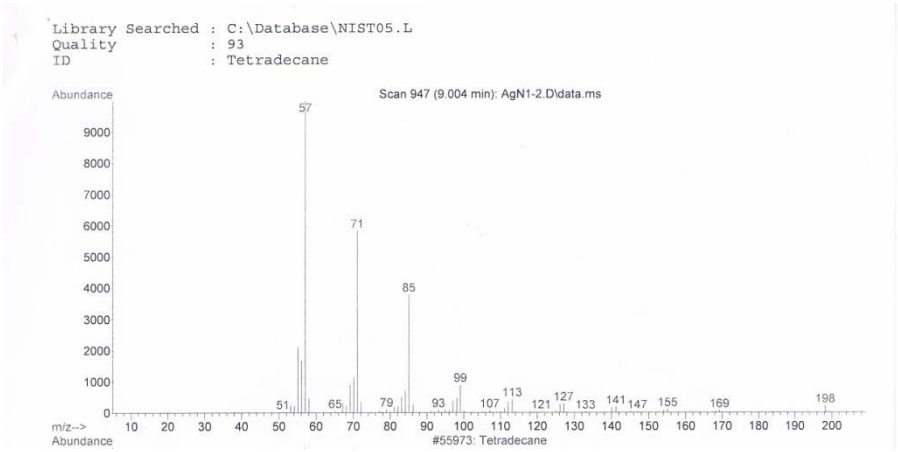


Figure 6: Tetradecane obtained from fraction AgN1.

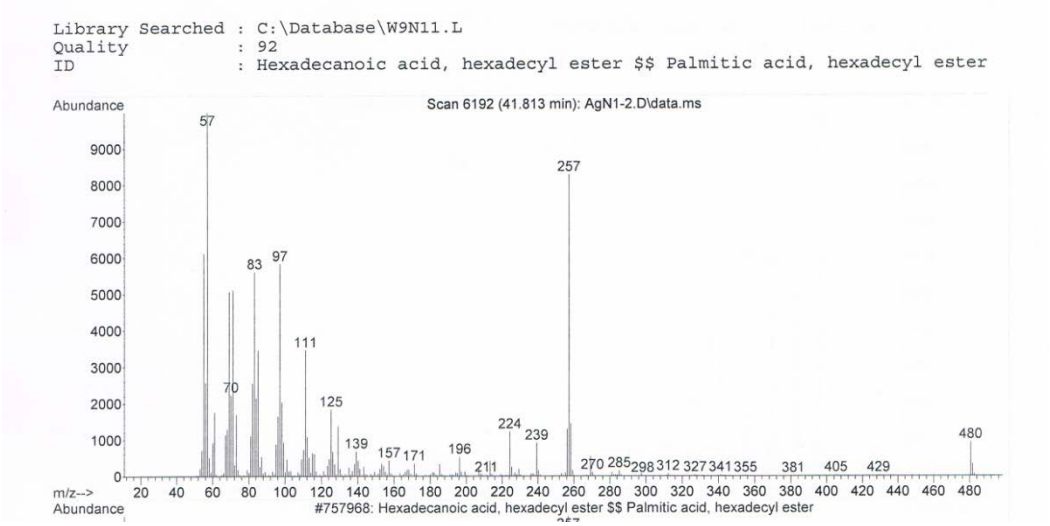


Figure 7: Palmitylpalmitate obtained from fraction AgN1.

Appendix B

Total ion chromatogram for fraction AgN6 integrated by Chemstation and the constituents identified by camparison published mass spectra databases (Wiley 9N Mass Spectral Library).

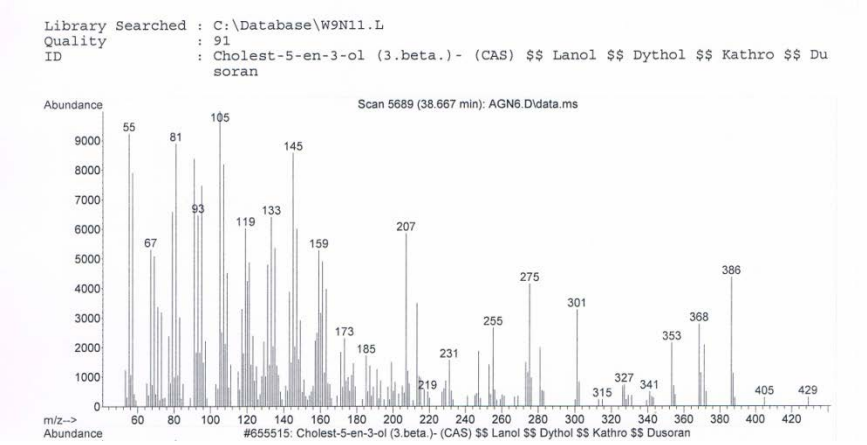


Figure 1: Lanol obtained from fraction AgN6.

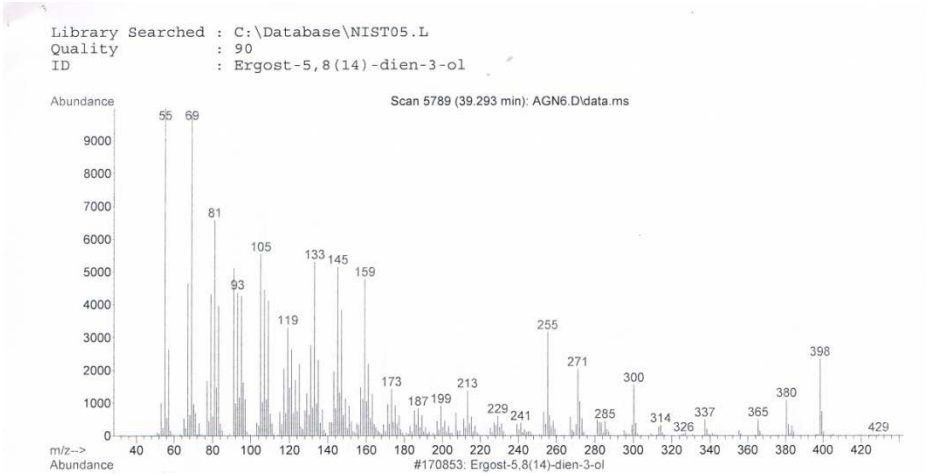


Figure 2: Ergost-5, 8(14)-dien-3-ol obtained from fraction AgN6.

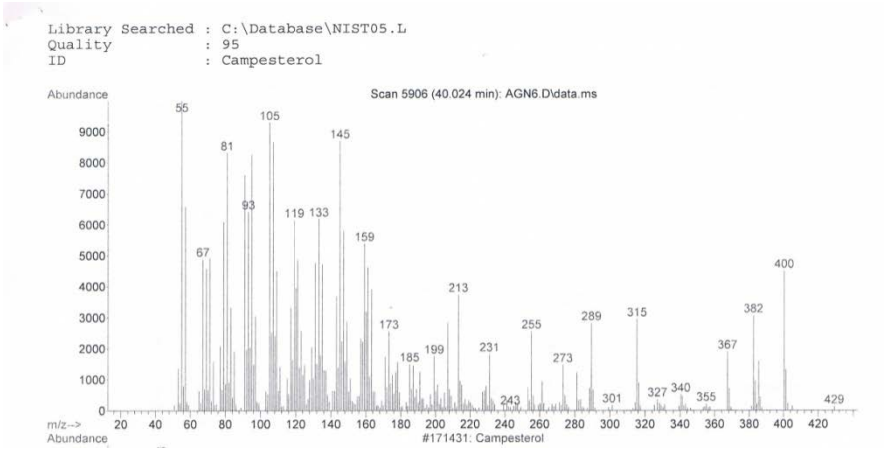
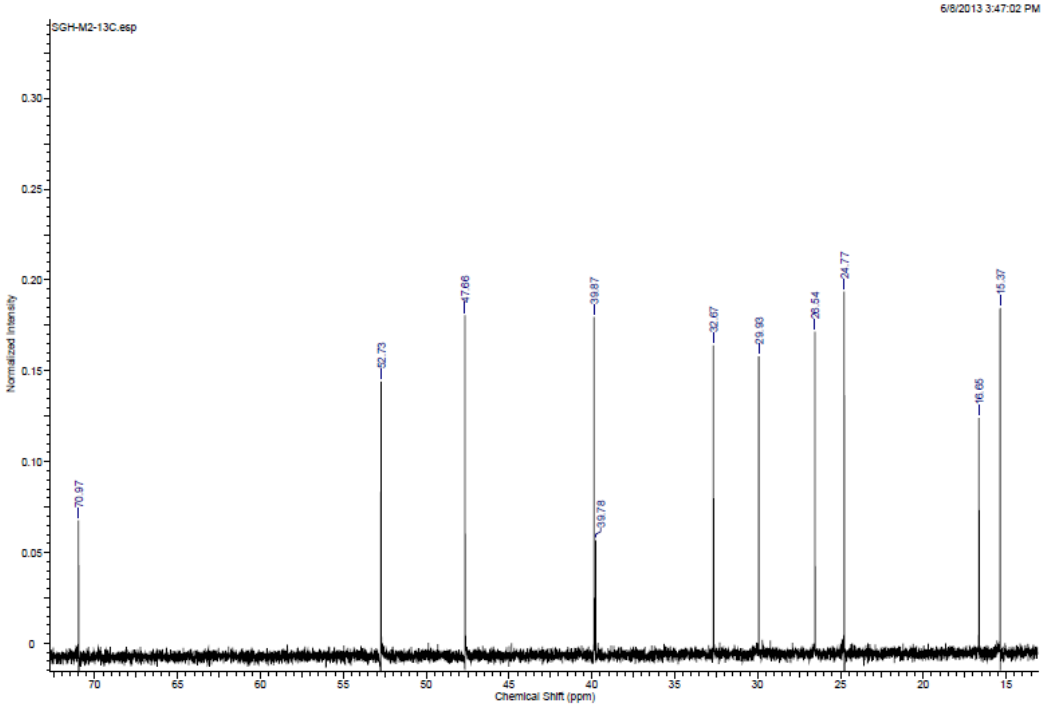
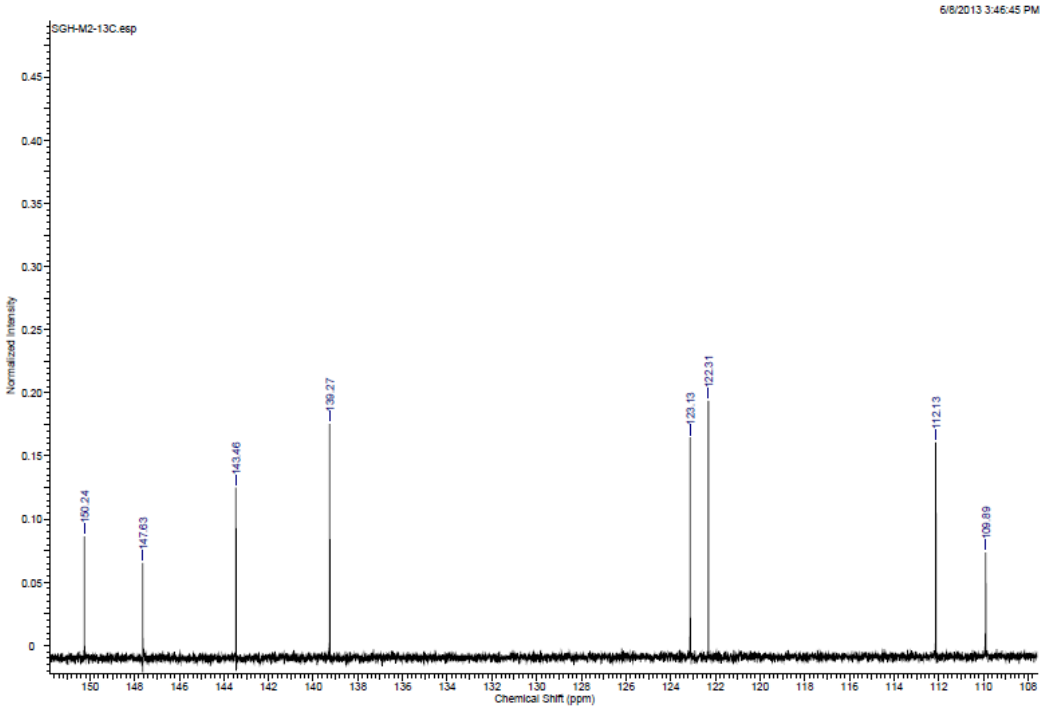


Figure 3: Campesterolobtained fromfraction AgN6.

Appendix C

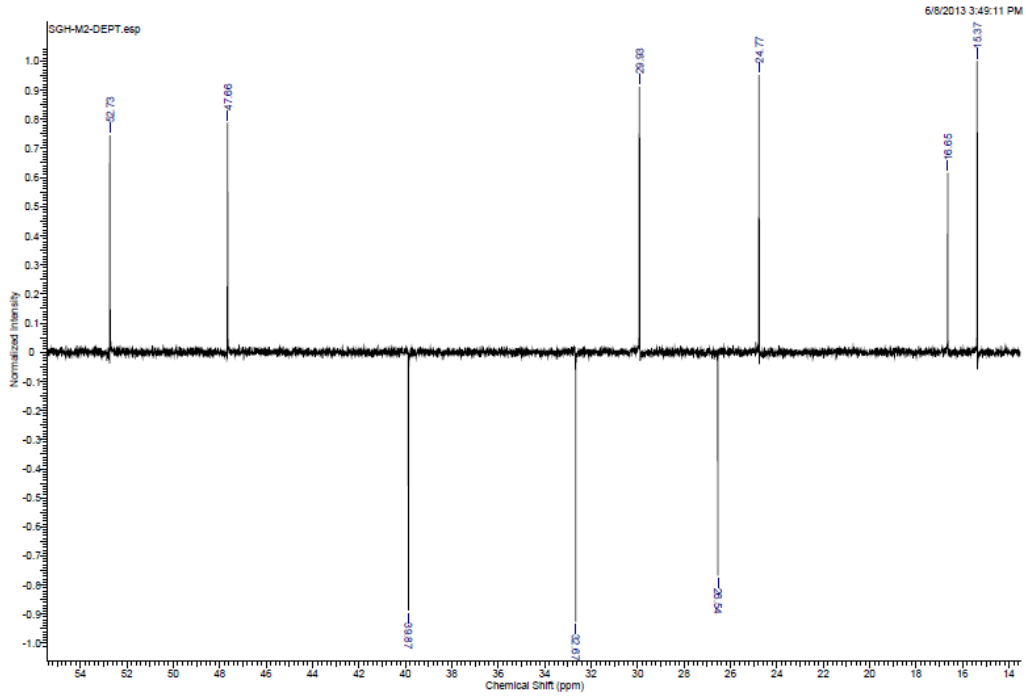
Expanded ^{13}C NMR spectrum of fuscol, isolated from *Sinularia* sp.

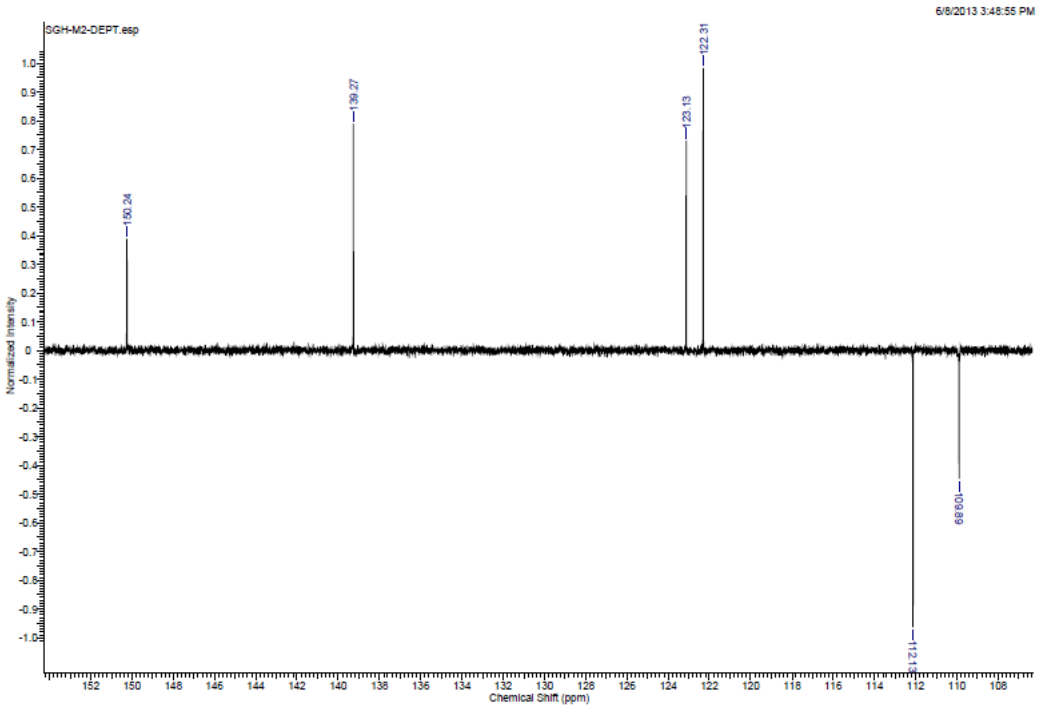




Appendix E

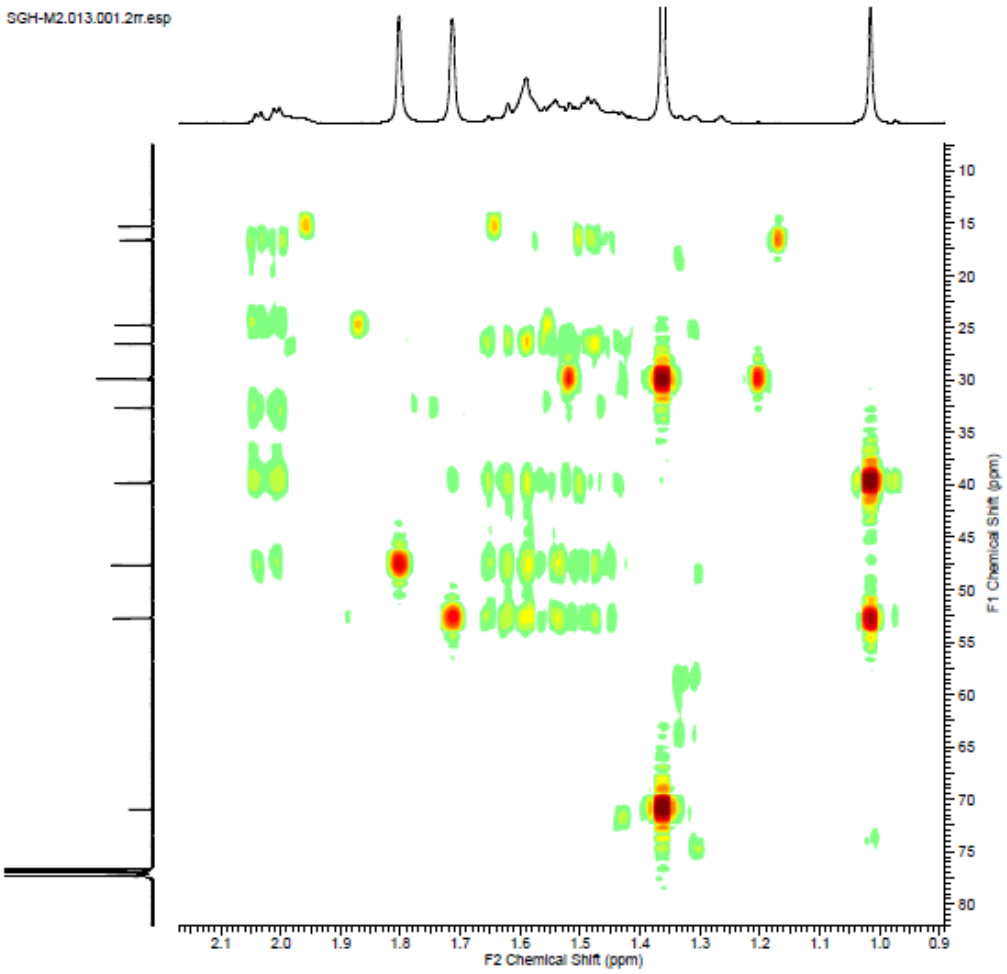
Expanded DEPT spectrum of fuscol, isolated from *Sinularia* sp.

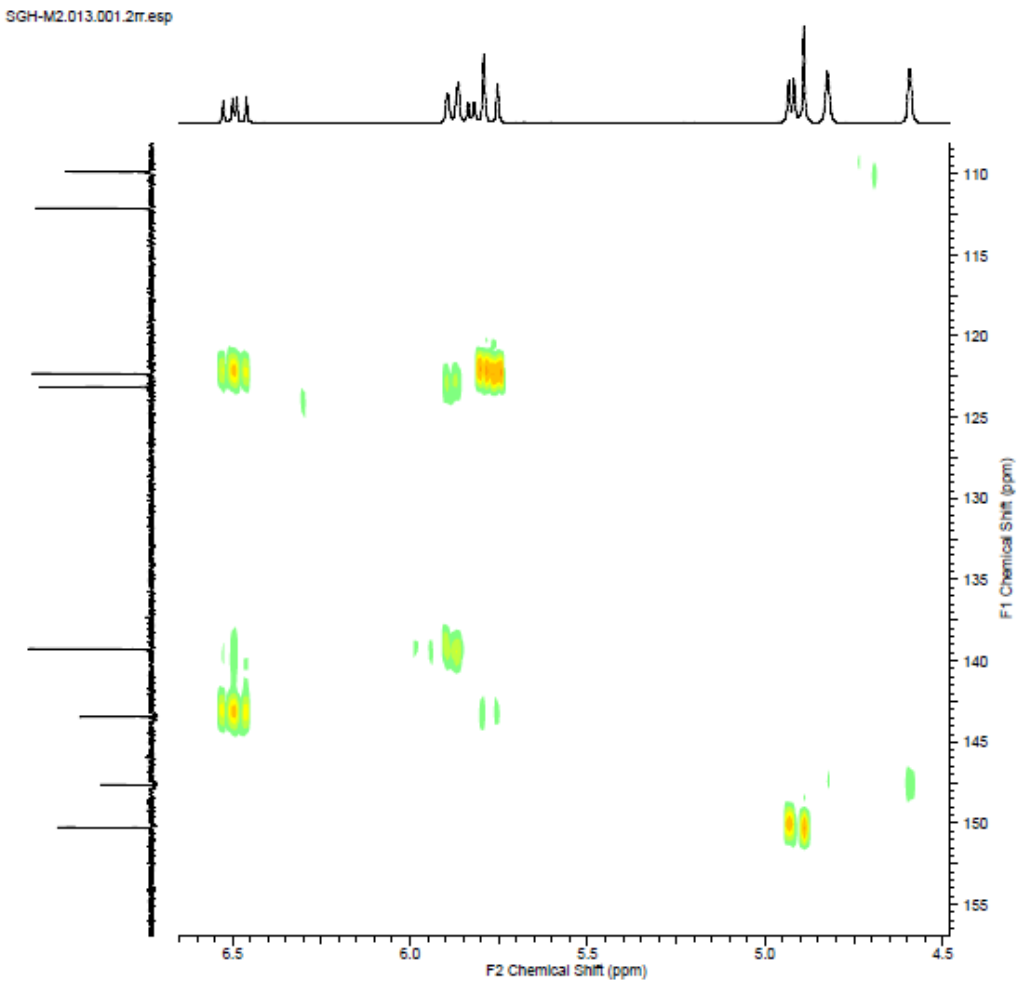


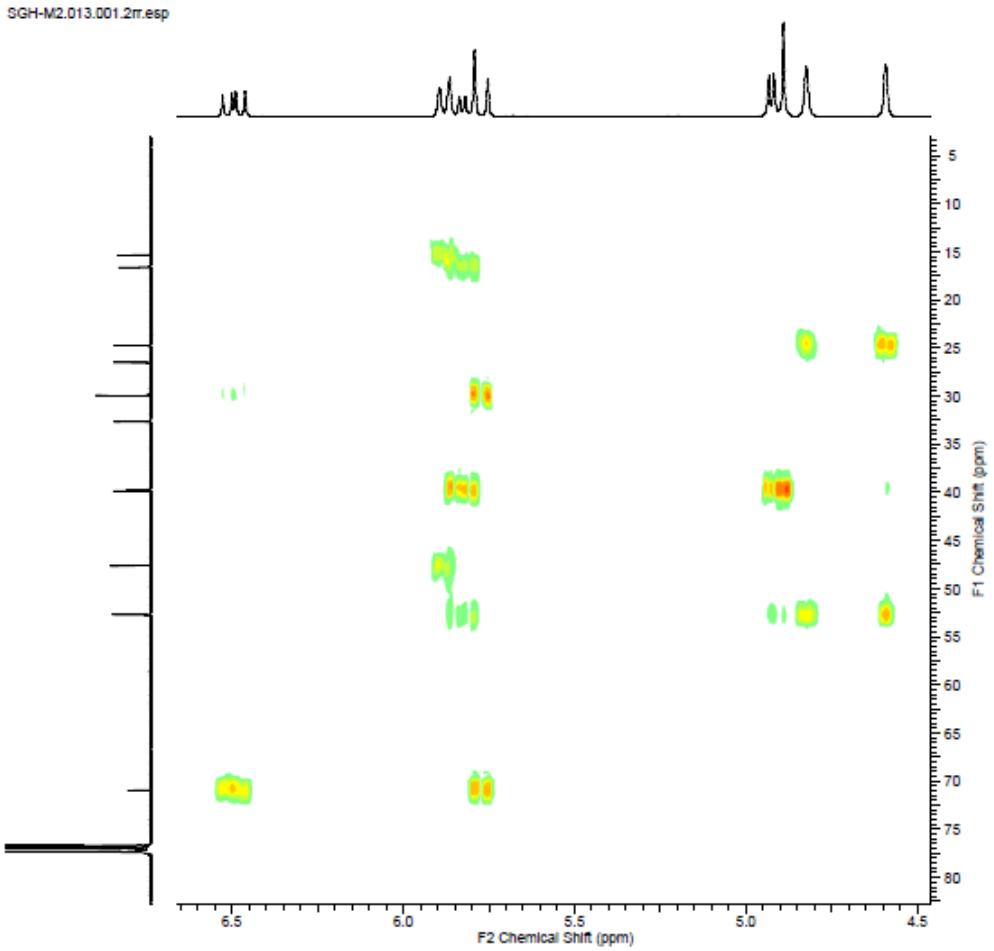


Appendix D

Expanded HMBCspectrum of fuscol, isolated from *Sinularia* sp.

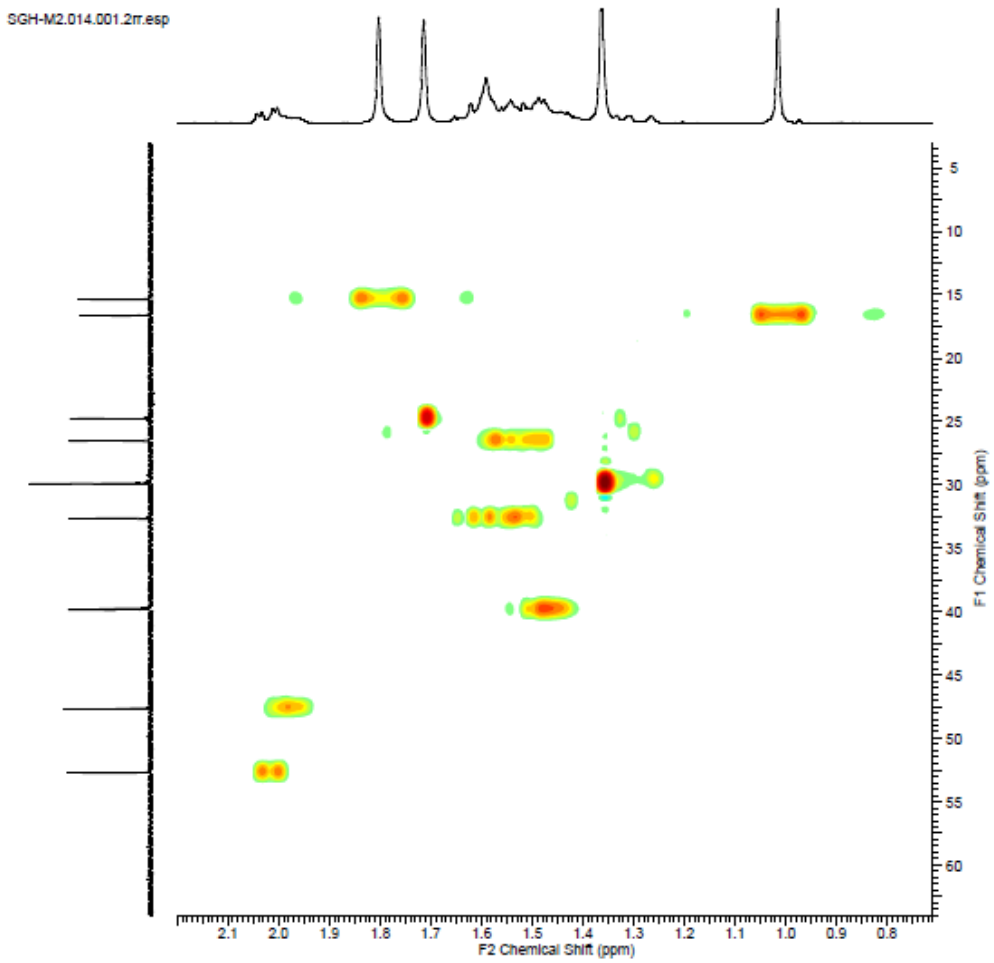


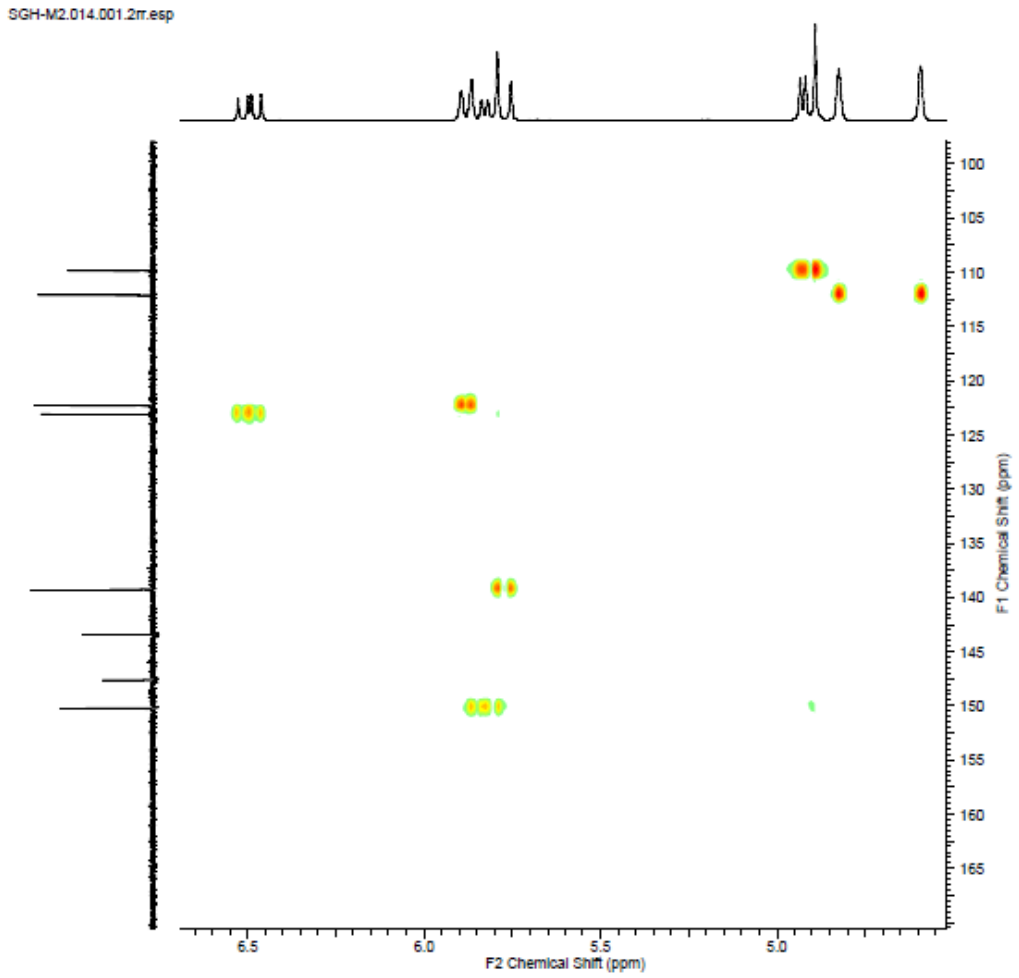

SGH-M2.013.001.2rr.esp
110
115
120
125
130
135
140
145
150
155
F1 Chemical Shift (ppm)
6.5
6.0
5.5
5.0
4.5
F2 Chemical Shift (ppm)


SGH-M2.013.001.2rr.esp
F1 Chemical Shift (ppm)
F2 Chemical Shift (ppm)

Appendix E

Expanded HSQC spectrum of fuscol, isolated from *Sinularia* sp.




SGH-M2.014.001.2rr.esp
100
105
110
115
120
125
130
135
140
145
150
155
160
165
F1 Chemical Shift (ppm)
6.5
6.0
5.5
5.0
F2 Chemical Shift (ppm)