Chemistry and bioactivity of Natural products from the gorgonian coral *Subergorgia reticulata*

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By

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Certificate

*This is to certify that the thesis entitled "**Chemistry and bioactivity of Natural products from the gorgonian coral Subergorgia reticulata.** " is an authentic record of the research work carried out by Mr. BYJU K, under my supervision and guidance at the Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, Kochi-682016, in partial fulfilment of the requirements for Ph.D degree of Cochin University of Science and Technology and no part of this has been presented before for any degree in any University. I further certify that all the relevant corrections and modification suggested by the audience during the pre-synopsis Seminar and recommended by the Doctoral Committee of Mr. BYJU K has been incorporated in the thesis.*

Dr. N. Chandramohanakumar
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Kochi - 682016
June, 2015

Declaration

*I hereby declare that the thesis entitled "**Chemistry and bioactivity of Natural products from the gorgonian coral Subergorgia reticulata."** is an authentic record of the research work carried out by me under the guidance and supervision of Dr. N. Chandramohanakumar, Professor, Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, and no part of this has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition from any University/Institution.*

Kochi-16 **BYJU K**
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Preface

Natural products have been produced as results of millions of years of evolution of terrestrial and marine organisms adapting to various abiotic and biotic stresses. They are there for encoded to be bioactive, for ages they have been used as a medicine and today they continue to be a reservoir of potential drugs. Almost all of the current natural product derived therapeutics have terrestrial origin, however finding novel sources such as marine environment will be a new hope for rare compounds which are not commonly available in the terrestrial environment. With this hope and knowledge this study focused to find such type of compounds responsible for specific biological activity from the marine sources. The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of unique chemical compounds with the potential for development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals. In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms. The focus of the present study is on the research topics dealing with the isolation and characterization of biologically active compounds derived from marine organism soft coral, coming under octacorallia gorgonian coral Subergorgia reticulata. These compounds are small molecules ideally suited to either as themselves or through chemical modifications as new pharmaceutical agents for the treatment of a variety of ailments.

The thesis is divided in to seven chapters including the overall summary and conclusion

Chapter 1, the introduction chapter of the thesis, describes the various application of Natural products derived from terrestrial as well as marine origin. The pharmaceutical applications of Marine natural products as well as the compounds which are in the current clinical trials are also discussed. The review of literature on Natural product concern with the genus of the gorgonian coral Subergorgia is also form part of the chapter. The objectives and scope of the thesis is also included in this chapter.

Chapter 2 describes the detailed procedure of sample collection, methods adapted for the morphological examination of the soft coral collected. The Morphology of Subergorgia reticulata is evaluated to state weather this organism having any special features.

Chapter 3, the biochemistry and significance of bioactive compounds were discussed briefly and this chapter is divided into two sections. Section 1 deals with the biochemical approach of the primary and secondary metabolites and also describes about the preliminary screening of active secondary metabolites, and quantitative analysis of protein, carbohydrates, etc. Analysis of volatile compounds, fatty acids, were done by GC-MS. Amino acid composition, Monosaccharide and disaccharide composition are also discussed in the section 1 of this chapter. Section 2 explains about the biological activity of crude extract as well as identified compounds by in vitro and in silico approach.

Chapter 4 describes the characterization of terpenoids and biological activity studies. In this chapter terpenoid compounds are isolated and characterized using GCMS, FTIR and NMR. The pharmacological applications of the identified and isolated compounds were studied via in vitro and in silico approach. The anticancer activities of the isolated compounds were studied for human breast cancer and Liver cancer. The in vitro approach of HIV1 RT assay of the isolated compound was also studied to know the activity towards AIDS.

Chapter 5 includes the characterization of sterols and biological activity studies. In this chapter sterol compounds are isolated and characterized using GCMS, FTIR and NMR. The pharmacological applications such as anticancer, apoptosis inducing activity of the identified and isolated compounds were studied via in vitro and in silico approach.

In the chapter 6, polysaccharides were isolated and characterized using FTIR, TGA, and CHNS. The applications were also studied. Chapter 7 is the overall summary and conclusion of the thesis.

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List of Abbreviations

AADS	Automated active site identification, Docking and Screening
AIDS	Acquired Immune Deficiency Syndrome
APHA	American Public Health Association
ARA	Arachidonic acid
ATR	Attenuated Total Reflectance
BSA	Bovine Serum Albumin
CMFRI	Central Marine Fisheries Research Institute
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DHA	Docosahexanoic acid
DLA	Dalton's lymphoma ascites
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDX	Electron Diffraction study
ELISA	Enzyme-linked immunosorbent assay
FAME	Fatty Acid Methyl Ester
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FRAP	Ferric Reducing Ability of Plasma
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometer
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Inhibitory Concentration at 50
ICP	Inductively Coupled Plasma
LCMS	Liquid Chromatography Mass spectroscopy

MDM2	Murine Double Minute2
MeOH	Methanol
MF	Molecular Formulae
MOSES	Molecular structure encoding system
MUFAs	Mono Unsaturated Fatty Acids
MW	Molecular weight
NIST	National Institute of Standard Technology
NMR	Nuclear Magnetic Resonance
OD	Optical Density
OES	Optical Emission Spectroscopy
PASS	Prediction of Activity Spectra of Substances
PBS	Phosphate Buffer saline
PITC	Phenyl Isothiocyanate
PUFAs	Poly Unsaturated Fatty Acids
RT	Reverse Transcriptase
SCUBA	Self Controlled Underwater Breathing Apparatus
SEM	Scanning Electron Microscopy
TPTZ	2,4,6-Tripyridyl-s-Triazine
TEA	Tri ethyl amine
TFA	Trifluro acetic acid
XRD	X-ray Diffraction Study
MTT	[3(4,5 Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium Bromide]

Chapter

1 INTRODUCTION

Contents

1.1 Natural Products Derived Drugs
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The majestic oceans, covering about 70% of the earth's surface, are unique in our solar system and contain roughly 97% of the earth's water resources. Life on earth originated in the oceans, and the oceans continue to be home to an incredibly diverse web of life. The first Census of Marine Life (www.coml.org) has just completed a decade inventory (2000–2010) that revealed an astounding level of biodiversity and amplified the estimate of known marine species from approximately 230,000 to nearly 250,000. Moreover, the project extrapolated their findings to at least a million of marine species and tens or even hundreds of millions of microbial species. The largely unexplored marine world that inevitably harbours the most biodiversity could be the vastest resource to discover novel 'validated' structures with novel modes of action that cover biologically relevant chemical space.

1.1 Natural Products Derived Drugs

Nature is an ancient pharmacy that used to be the solitary source of therapeutics for the early eras. A natural product is a chemical compound or substances produced by living organisms that are found in nature (Cutler and Cutler 2000). Over the centuries, people have been living in close association

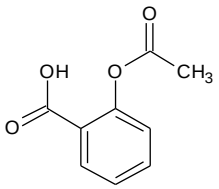
with the environment and relying on its flora and fauna as a source of food and medicine. Nearly 80% of the population still depends on the use of plant extract as a source of medicine (WHO 2002). Natural products, therefore encoded to be bioactive, for ages they have been used as medicines and today, they continue to be a reservoir of potential drugs (Newman and Cragg 2012).

Natural products have been the forefront of medicine to treat human disease (Dias et al 2012). There are written evidence for the usage of natural products for curing various diseases by ancient civilization of the Chinese, Indians and North Africans (Phillipson 2001). Sumerian's clay tablet, which used for the remedy of various illnesses, is the earliest known written document about natural product (Kong et al. 2003). Isolation of morphine Fig. 1.1(3) from *Papaver somniferum* in 1816 by Friedrich Serturner leads to the development of the analgesic, highly effective pain relievers (Benyhe 1994, Der Marderosian and Beutler 2002). A great impact on the investigation of nature as a source of new bioactive agents was made by the discovery of penicillin Fig. 1.1(6) from the filamentous fungus *Pencillium natatum* by Alexander Fleming in the year 1929. He won the Nobel Prize in physiology and medicine in 1945 for the discovery of Penicillin. A trend was observed after this discovery to isolate compounds from micro organisms (Buss and Waigh 1995, Williams 1999).

Historically apothecaries and then pharmaceutical companies utilized plant extracts to produce relatively crude therapeutic formulations. In the mid 20^{th} century, drug formulations of partially purified natural products became typical prior to single molecule medicines (Mishra and Tiwari 2011). Currently, 90% of the drugs which are directly from plant sources and 47% of the anticancer drugs in the market came from natural product mimics (Benowitz 1996, Newman and Cragg 2007). The most famous and well known

example of medicine derived from natural product is anti-inflammatory agent acetyl salicylic acid (aspirin) which is a synthetic product of salin Fig. 1.1(2), isolated from the bark of the willow tree *Salix alba L* (Der Marderosian and Beutler 2002). Another medicine Digitoxin Fig.1.1(4) and its analogues have been used in the congestive heart failure and used as a medicine in the treatment of heart deficiency (Der Marderosian and Beutler 2002). FDA in 2004 approved an oral formulation of Pilocarpine Fig 1.1(5), to treat dry mouth (Xerostomia), which is a side effect of radiation therapy for head and neck cancers (Aniszewski 2007).

Vancomycin Fig.1.1 (12), a glycopeptides antibiotic isolated from the cultures of *Amycolatopsis orientalis*, has known activity against bacteria. Another antibacterial drug Erythromycine Fig.1.1(13), was isolated from *Saccharopolyspora erythraea* (Butler 2004). Numbers of antiviral natural product or synthetically derived analogues were isolated from fungi (Newman and Cragg 2007). A triterpenoid, Betulinic acid Fig.1.1(7) isolated from *Betula pubescens* was well known for inhibitor of HIV replication (Kashiwada et al. 1996, Martin et al 2007). Betulinic acid is also in progression as a cancer preventive agent (Yogeeswari and Sriram 2005). Bevirimat isolated from Chinees herb *Syzygium claviflorum* is in pipeline to release as an anti HIV medicine (Heider et al. 2010). Ganoderic acid β, another anti HIV compound isolated from fruiting bodies and spores of *Ganoderma lucidum.* Amrubicin hydrochloride Fig. 1.1(10), similar to anthracycline and doxorubicin, was isolated from the fungus *Streptomyces peucetius*. The compound Doxorubicin Fig. 1.1(11) is used to treat acute leukemia, lung cancer, thyroid cancer and both Hodgkins and non-Hodgkins lymphomas (Dewick 2002, Butler 2004). Torreyanic acid from the natural plant *Torreya taxifolia* was shown to have grater potency of cytotoxicity.



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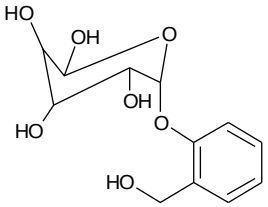

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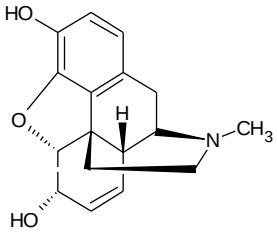

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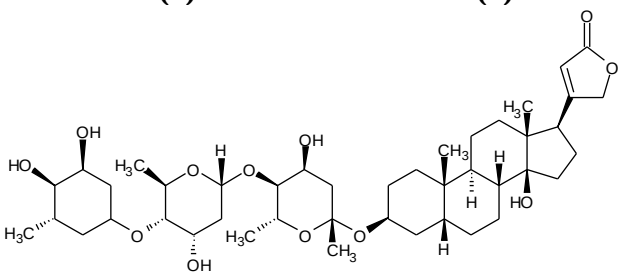

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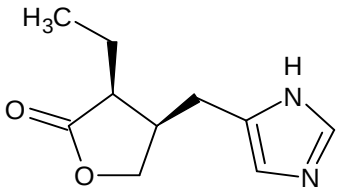

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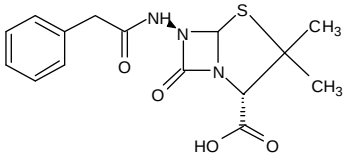

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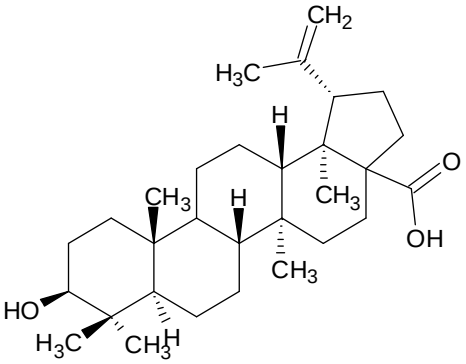

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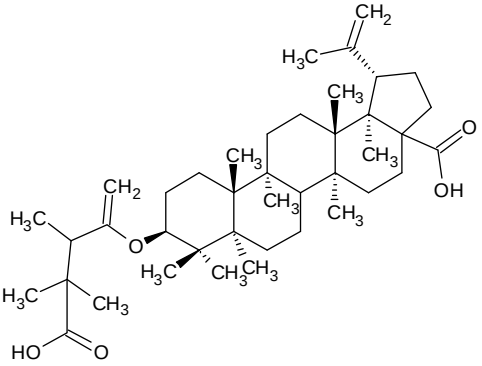

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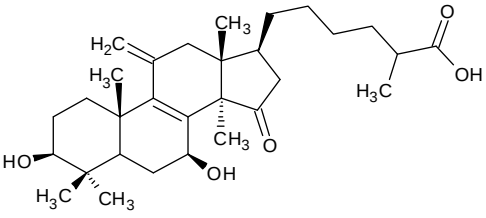

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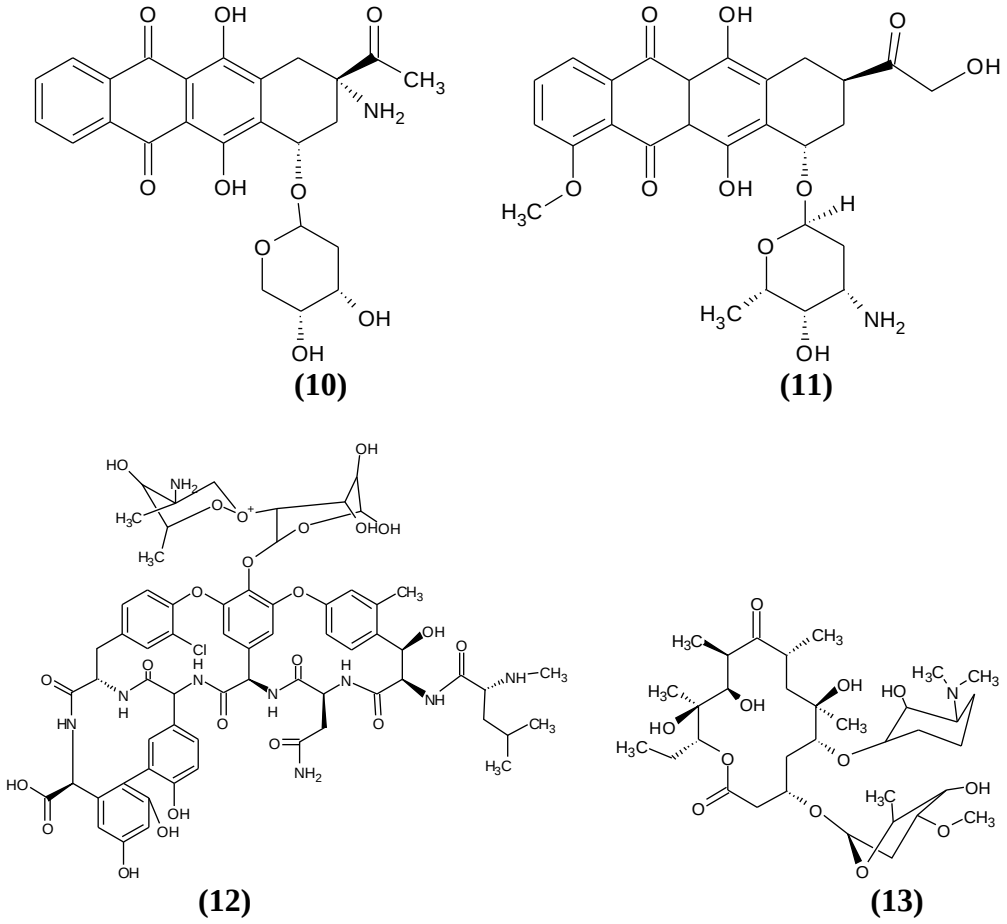


Figure 1.1 Structure of Natural products compounds derived drugs.

(1) Acetyl salicylic acid, (2) Salin, (3) Morphine, (4) Digitoxin,(5) Pilocarpine,(6) Penicilin G, (7)Betulinic acid, (8)Bevirimat, (9) Ganoderic acid β, (10) Amrubicin hydrochloride, (11) Doxorubicin, (12) Vancomycin and (13) Erythromycine

1.2 Marine Natural Products Derived Drugs

Although almost all of the current natural product-derived therapeutics has terrestrial origins, novel sources, such as the marine environment, will clear the way for chemical and biological novelties as well. A comparative analysis showed that marine natural products are superior to terrestrial natural products in terms of chemical novelty (Kong et al. 2010). In addition, marine organisms show higher incidence of significant bioactivity compared with

terrestrial organisms (Munro et al. 1999; Montaser and Luesch 2011). Although marine organisms have been used in medicine since ancient times, a systematic study of marine biodiversity for therapeutic purposes really started in the second half of the 20th century only. However the faith placed in marine pharmacology from the 1950's onwards soon came up against scientific and economic reality, and entering the third millennium, fewer than one tenth of the drug prepared using models inspired by the study of marine organisms are commercially available. The oldest known pharmacopeia, which describes about the use of marine algae for the pharmaceutical applications, was published round 2800 BC (Cragg and Newman 2005).

Even though great research in the field of marine pharmacology started from years before, only a very few compounds and their analogues have been approved and came in to market as drug or health food. Some of them are ω-3 polyunsaturated fatty acids (PUFA) Ara-C (cytarabine), Ara-A (antiviral), Zinconotide (analgesic), Trabectidin (anticancer) etc. Several more compounds are in pipeline such as bryostain, didehydrodidemnin, eribulin mesylate, dolastatins, kahalalide F, KRN 7000, Squalamin etc (Mayer et al. 2010). 3079 new compounds are added to literature during the period 2008-2011(Blunt et al 2010; 2011, 2012).

Food and Drug Administration (FDA) has so far approved 13 natural products in the phase of clinical trials from the marine sources (Alejandro et al. 2010).The first drug from the sea, Zinconotide (ω-conotoxin MV11A) a peptide originally from tropical marine cone snail was approved in the United States in 2004 for the treatment of chronic pain in spinal cord injury. Trabectedin is the second drug; the antitumor compound from a tropical sea-squirt was approved in October 2007(Newman and Cragg 2004; 2007, Newman 2008). The first marine derived anticancer drug to reach in the market is Ecteinascidin-743 Fig. 1.2(8).

The great first success of marine pharmacology occurred when a new generation of antibiotics was discovered during 1948 by an Italian professor Giuseppe Brotzu which is having very similar structure to pencilline (Nakajima 2003). Bergman and Feeney (1951) studied a Caribbean sponge *Cryptotethya cryota* and isolated similar structure close to purine and arabinose. With the help of this discovery, chemists found two synthetic analogus Ara-C and Ara-A as an antileukaemic drug in 1959.These compounds eventually lead to the development of derivatives Ara A (Vidarabine), and Ara C (Cytarabine) Fig 1.2(2), two nucleosides with significant anticancer properties which has been released in market for decades (Bergmann and Feeeney 1951, Bergmann and Stempien 1957, Mc Connell et al. 1994). In 1969, another compound (15)-epi-Prostaglandin A_2 was isolated from Caribbean gorgonian *Plexaura homomalla* (Weinheimer and Spraggins 1969).

It was found in 1968 that extracts of *Bugula nerittina* collected from the Gulf of Mexico exhibited activity against leukaemia cells (Pettit et al. 1982) and by 1982, the active compound bryostatin I Fig.1.2(5), was isolated (Pettit 1996).The promising anticancer activity of this bryostatin I were undergone numerous clinical trials for myeloid leukaemia, lymphocytic leukaemia, melanoma, non-Hodgkin's lymphoma and chronic lymphocytic leukaemia and other refractory malignancies (Varterasian et al 1996, 2000, 2001, Grant et al. 1998, Propper et al. 1998).

Aplidine also is well known for inhibition on angiogenesis in chick embryos and also active for metastatic melanoma, multiple myeloma, non Hodgkins lymphoma, acute lymphoblastic leukaemia, prostatic cancer and bladder cancer (Perez et al. 2003, Taraboletti et al. 2004). Dolastain 10 was another drug candidate compound isolated from the sea hare *Dolastain auricularia* from Indian Ocean. It was found to be effective for cancer cells such

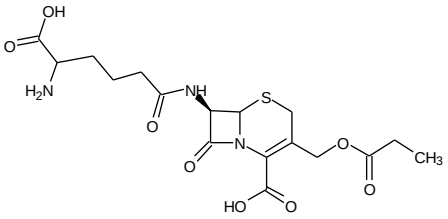
as melanoma, sarcoma and ovarian cancers (Pettit et al. 1987). Halichondrin B Fig. 1.2(6), norhalichondrin B and homohalichondrin B were isolated from the sponge *Homohalichondria okadai* is lead to the discovery and development of antitumor compounds such as Halichondrin B and its analogue Eribilin mesylate.(Uemura et al. 1985, Hirata and Uemura 1986). Kahalalide F from Sacoglossan *Elysia rufescens* was isolated in 1993 was found to be cytotoxic. Now this compound is undergoing phase I trial for therapeutic on advanced solid tumours (Hamann et al. 1993; Ciruelos et al. 2002).

Even though the antitumor properties of *Ecteinascidia turbinata* extracts were discovered in 1969 and the structure of the alkaloids named Ecteinascidins later in 1990 (Rinehart et al. 1990; Wright et al. 1990), it took many more years to come as drug candidate. Didemnin B Fig. 1.2(9) and aplidine are two closely related compounds isolated from different organisms such as *Tridemnum solidum* and *Aplidum albicans*. These compounds are cyclic peptides. Didemnin B was first isolated by Rinehart group in 1981 from the tunicates *T.solidum*. These compounds were found to be antiviral and have cytotoxic properties (Rinehart et al. 1981; Chun et al. 1986; Rinehart et al. 1990; Wright et al. 1990; Vera and Joullie 2002; Rinehart 2003; Jimeno et al. 2004). *In vitro* testing of Didemnin B against colorectal, lymphatic and prostate cells proved enormous activity (Scheithauer et al. 1988; Grubb et al. 1995; Geldof et al. 1999).

Chemical investigation of marine algae lead to isolation of so many classes of compounds such as heterocycles, phenazine derivatives, sterols, amino acids, amines and guanidine derivatives; mostly green, brown and red algae assessed for their antibacterial and antifungal activities (Baslow 1969, Faulkner 2002). In 1986 Japanese researchers discovered a specious *Patmaria palmate* with a high Kainic acid content, which is used as an anthelmintic led

to the preparation of a true drug against the parasitic nematode *Ascaris sp* Cephaloseporins Fig . 1.2(1).

Plitidepsin, a depsipeptide was isolated from the Mediterranean tunicate *Aplidium albians* was found effective in treating various cancers and acute lympho blastic leukemia(Alejandro et al. 2010). Although active anticancer compound spisulosine isolated from the marine clam *Spisula polynyma*, was under clinical trials, it was withdrawn in 2006 due to the toxic effect. Another example for the drug candidate withdrawn due to toxic side effects and lack of efficacy is Cryptophycin (Trimurtulu et al. 1994, Cuadros et al. 2000, Salcedo et al. 2003).



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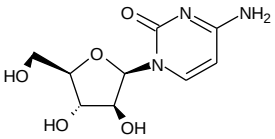

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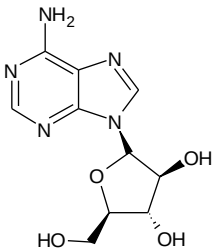

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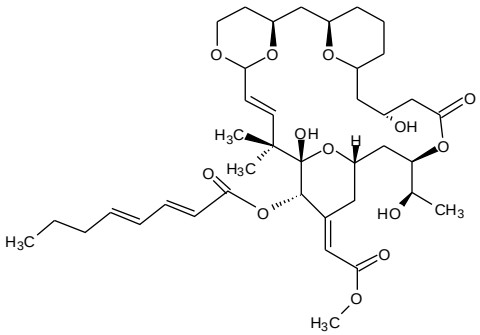

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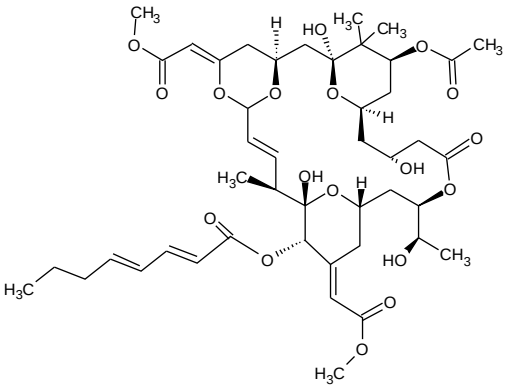

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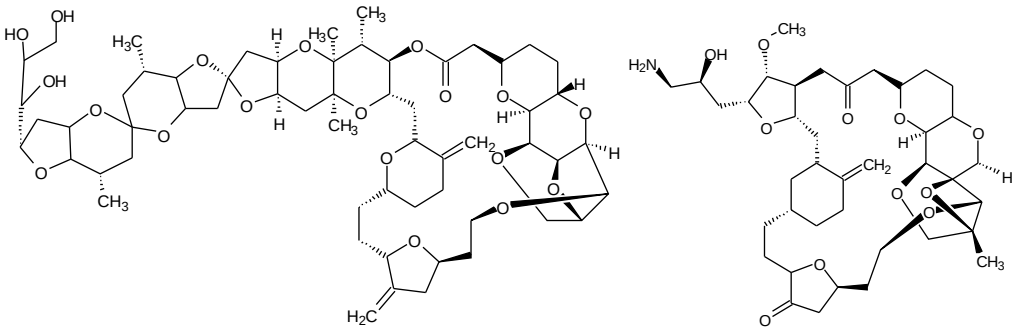

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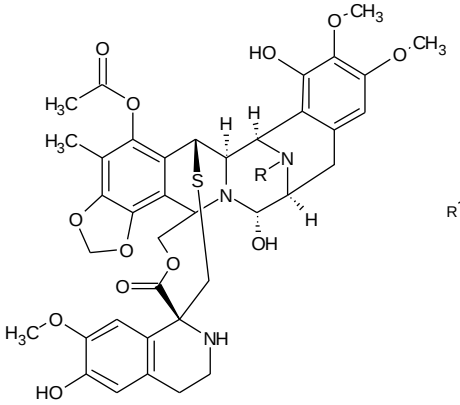

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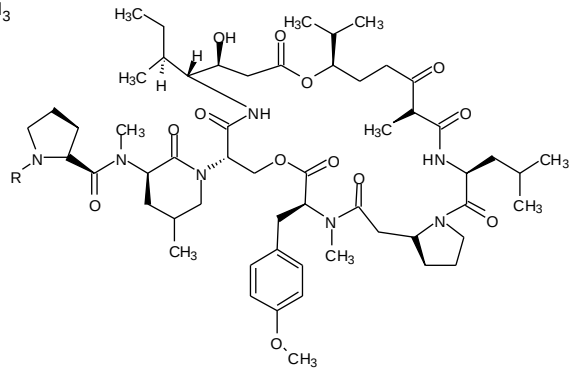

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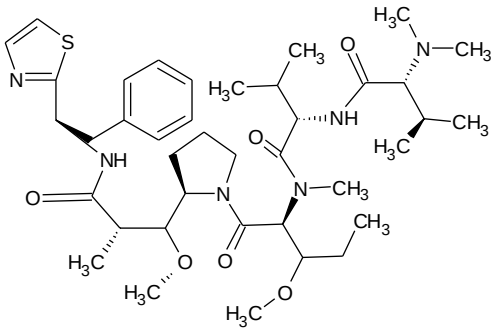

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Figure 1.2 Structure of marine natural product derived drug candidates

(1) Cephalosporin C, (2) Arabinosylcytosine (Ara-C), (3) Arabinosyladenine(Ara-A), (4) ,(5) bryostatin I, (6) Halichondrin B, (7)Eribulin mesylate,(8) Ecteinascidin-743, (9) Didemnin B, (10) Dolastain 10.

1.3 Bioactive Compounds from Corals

Corals, the major contributors to the formation of coral reef in tropical and subtropical waters, have an ancient geological history (Pratt et al. 2001). Corals are marine coelenterates belonging to the class Anthozoa. They are divided into two subclasses mainly based on the number of tentacles possessed by them. Those with eight tentacles are called octacorallia (Alcyonaria) Comprising soft corals, gorgonians, sea pens, and sea pansies. Those with more than eight tentacles in a multiple of six are called hexaacorallia (Zpantharia), and include reef building corals(Scleractinians), Sea anemones and zoanthids. Corals in general are also classified in to hard corals and soft corals based on their skeletal composition and texture (Daly et al. 2003). More than 3200 species of octacorals, have been discovered world over in which soft corals contibutes a big majority (Williams and Cairns 2013). The coral reef cover around 2×10^6 Km^2 in tropical oceans, the longest of them is the Great Barrier Reef which extends about 2000 Km along the east coast of Australia. In India coral reefs are mainly distributed in Gulf of Kutch, Lakshadweep, Gulf of mannar, Palk bay and Anadaman and Nicobar Islands.

Soft corals are rich sources of bioactive compounds such as prostanoid, sterols, cyclic diterpenoids and sesquiterpenoids. Many of the metabolites are also found to be pharmacologically important. The new cytotoxic prostanoids called claviridins A-D, isolated from Taiwanese soft coral *Clavularia viridis,* were active against HepG2 (Shen et al. 2010). Six new Withanolides isolated from a Formosan soft coral *Paraminabea acronocephala* in which the compounds such as puraminabeolides A-D showed mild anti-inflammatory properties and paraminabeolides A was also active against HepG2 cells. The diterpenoid isolated from soft coral *Sinularia gyrosa*, gyrosanols A&B showed activity against HCMV(human cytomegalo virus) and anti inflammatory

activity(Cheng et al. 2010). From an Indonesian octocoral *Cladiella sp*. isolated two new eunicellin type diterpenoids, cladielloids A and B having 2-hydroxybutyroxy group in their structures. Among these, compound cladielloid B was found to be active against human leukemia CCRF-CEM tumour cells (Cheng et al. 2010). From Indian soft coral *Cladiella krempfi* an important eunicellin derivatives, (-)6α-hydroxy polyanthellin A was isolated and it was exhibiting promising antifouling properties against barnacle larvae *Balanus Amphitrite* (Mol et al. 2011).

1.4 Gorgonide Subergorgiidae as Important Sources of Marine Natural Products

Gorgonians are major group of octocorallia. Their bodies are supported by skeletons mostly formed of flexible horny substances called gorgonin. There are about 500 different species of gorgonians in the world oceans, mostly distributed in the tropics and subtropics (www.oceans.udel.edu/kiosk/gorgonia.html).The colour of the gorgonians may vary often purple, red or bright colours. As like sponges gorgonians also does not possess physical means of defence and hence they are mainly depends on chemical means of defence mainly secondary metabolites. Octocorals are found in Indian waters and use more abundant in Gulf of Mannar, Gulf of Kutch, Lakshadweep Islands and Andaman Nicobar Islands. There are several novel compounds reported from this group having biomedical applications.

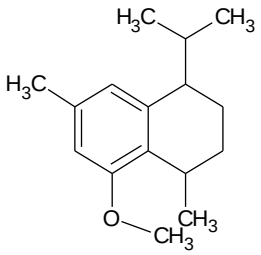
Subergorgia coming under gorgonian are soft corals under phylum Cnidaria. The continued and growing interest in the development of marine natural products as drugs candidates has resulted in numerous reviews (Coll 1992, Blunt et al. 2014). Specific classes of compounds are up to date reported for diterpenoids, sesquiterpenoids, sesquiterpene hydroquinones, heterolcycles,

indolocarbazole, astaxanthin, bioactive sulphur containing compounds, invertebrate pigments, imidazole, oxazole and thiazole alkaloids, polyether compounds, dysiherbaine, terpenoids, trisoxazole macrolides, bioactive hetero cyclic alkaloids and diterpenoids (Kashman 1979, Goh et al. 1996, Anjaneyulu et al. 1997, Parameswaran et al. 1998, Grassoff 2000, Grassoff and Bargibant 2001, Yang et al. 2005, Qi et al. 2007, 2008, Limna mol et al. 2011). The structure of compounds reported from *Subergorgia* genus is shown in Fig.1.3.

Two new sesquiterpenes, 8-methoxy-methoxy calamenene and 5-hydroxy-8-methoxy calamenene were isolated from the *Subergorgia hicksoni* coming under Subergorgiidae family. A new steroid 11α,15 α-diacetoxy-17 α-pregna-4,20-die-3-one along with a known steroid 17 α-pregna-4,20-dien-3one was isolated from *Subergorgia mollis* (Wu et al. 2004). Five known polyhydroxylated steroids anyaols A-E having cytotoxic activity were also isolated from the same species (Guo et al. 2004).Two new compounds reticulatic acid and reticulatin, along with a known compound 3,22,25-trihydroxy-16,24,20-24-bisepoxy-3β,16 β,20S,22R,24S-cholest5ene were also isolated from South China Sea gorgonian coral *S. reticulata* (Yang et al. 2005). Some other compounds isolated from *S. reticulata* were batyl alcohol, cholesterol,cholesta-7,22-diene 3β,5α,6β-triol, Ergosta-7,22-diene-3β,5α,6β-triol, 5, 8-epidioxycampesta-6,22-diene 3-ol, Guanine, Thrine, Thymine and Uracil (Yang et al. 2006). A new briarane type diterpenoid Reticulolide along with known compounds (-)-11α,20α-epoxy-4-deacetyljunceelolide D, Junceellin, Junceellolide A, Praelolide, Umbraculolide A and Umbraculolide C were isolated from the same species *S. reticulata*. Among these compounds Praelolide and Junceellin exhibited anti-settlement activity against the larva of *Bugula neritina* (Yang et al. 2007). *Subergorgia reticulata* were also found to be contain three new sesquiterpenes viz. (+)-(7R, 10S)-2, 5-dimethoxy

calamenene, (+)-(7R, 10S)-2 methoxy-5acetoxy calamenene and (+)-(7R, 10S)-2 methoxy calamenene. These calamenene compounds were found to be having potent inhibitory effects against Cyprids of *Balanus amphitrite and* also showed appreciable activity against *Artemia nauplii* (Limna mol et al. 2011 a&b). The calamenene compounds (+)-(7R, 10S)-2 methoxy calamenene, (+)-(7R, 10S)-2, 5-dimethoxy calamenene also showed antifouling activity against the Cyprids of *Balanus Amphitrite*.(Raveendran et al. 2011).

Subergorgic acid was isolated from the gorgonian coral *Subergorgia suberosa*, which showed cardiotoxic properties and significant activity for the toxicity in mice. Suberosenone a new cytotoxic sesquiterpenes, and known piscicidal sesquiterpenes buddledins C-D found to show significant cytotoxic activity against tumour cell lines (Groweiss et al. 1985, Tan et al. 1990, Bokesch et al. 1996). Isolation of a new steroid 3,9-dioxo-9,11-secocholesta-5,7-dien-11-al was reported for *Subergorgia suberosa* along with known compounds like avenasterol, cumpesterol, cholesterol, fucosterol, 3β-hydroxypregn-5ene20-one, 24-propylidene cholest-5-en-3β-ol, Subergorgic acid, lathosterol and zymosterol. *Subergorgia suberosa* collected from Indian ocean was reported to contain four new compounds Methyl 2β-hydroxysubergorgate, Methyl 2β acetoxy subergorgate, 2β hydroxyl subergorgic acid, Methyl subergorgate along with the known compound subergorgic acid (Parameswaran et al. 1998).



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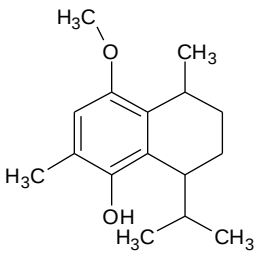

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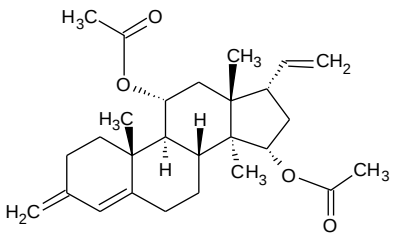

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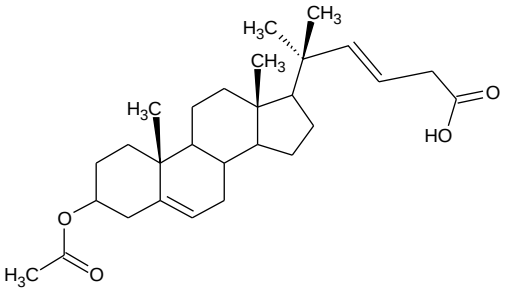

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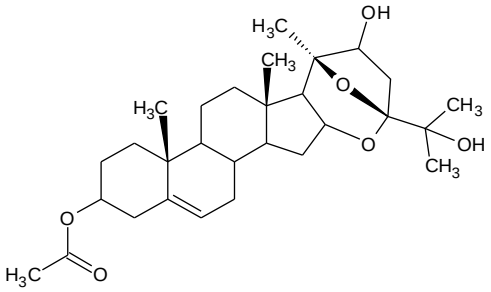

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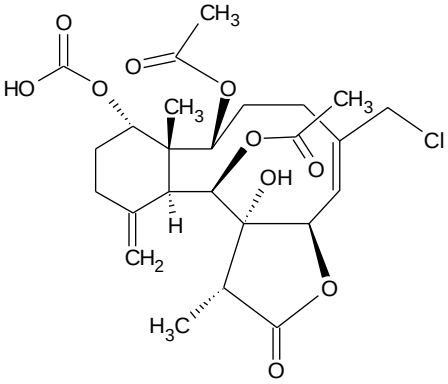

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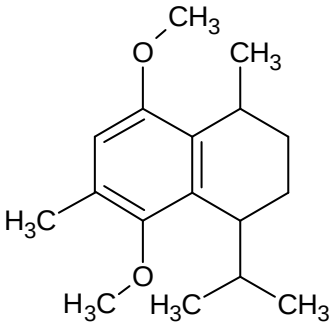

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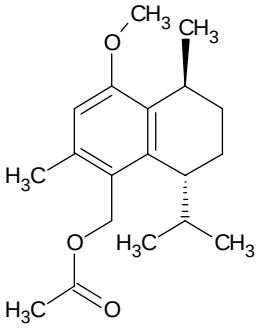

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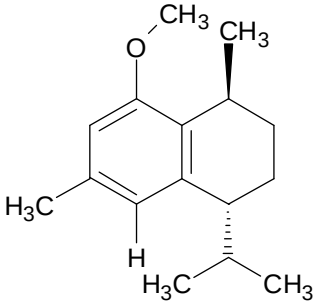

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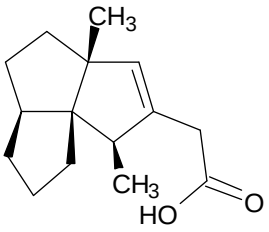

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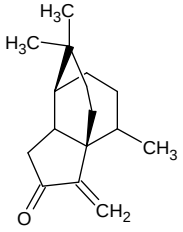

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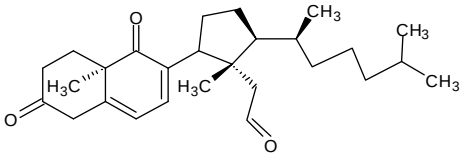

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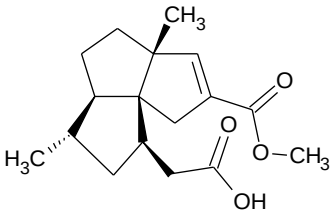

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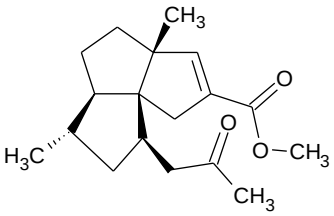

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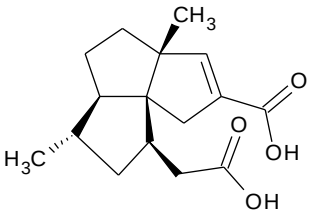

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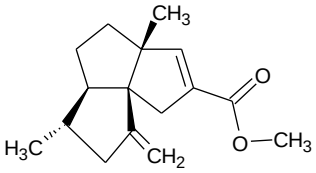

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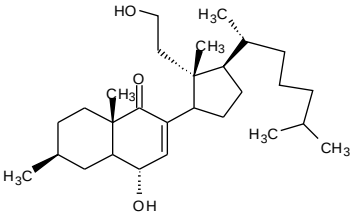

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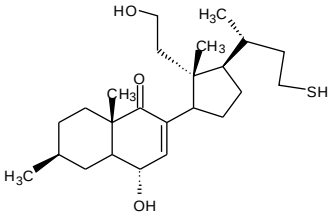

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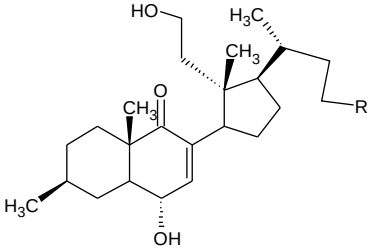

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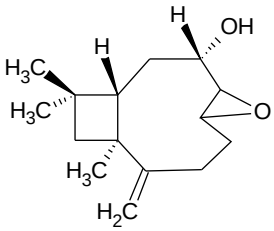

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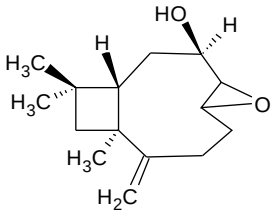

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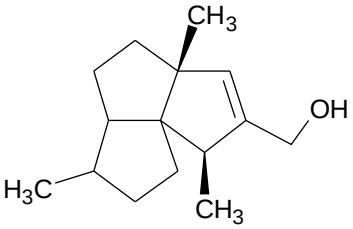

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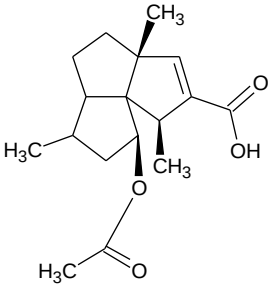

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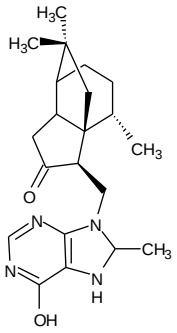
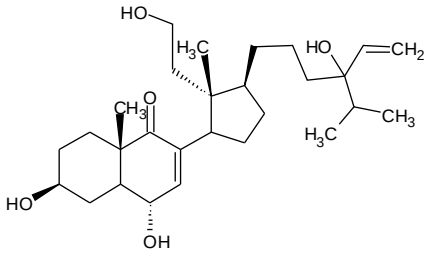
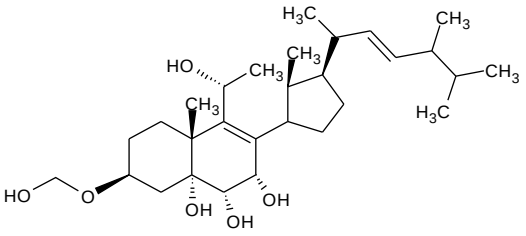


(24)



(25)



(26)

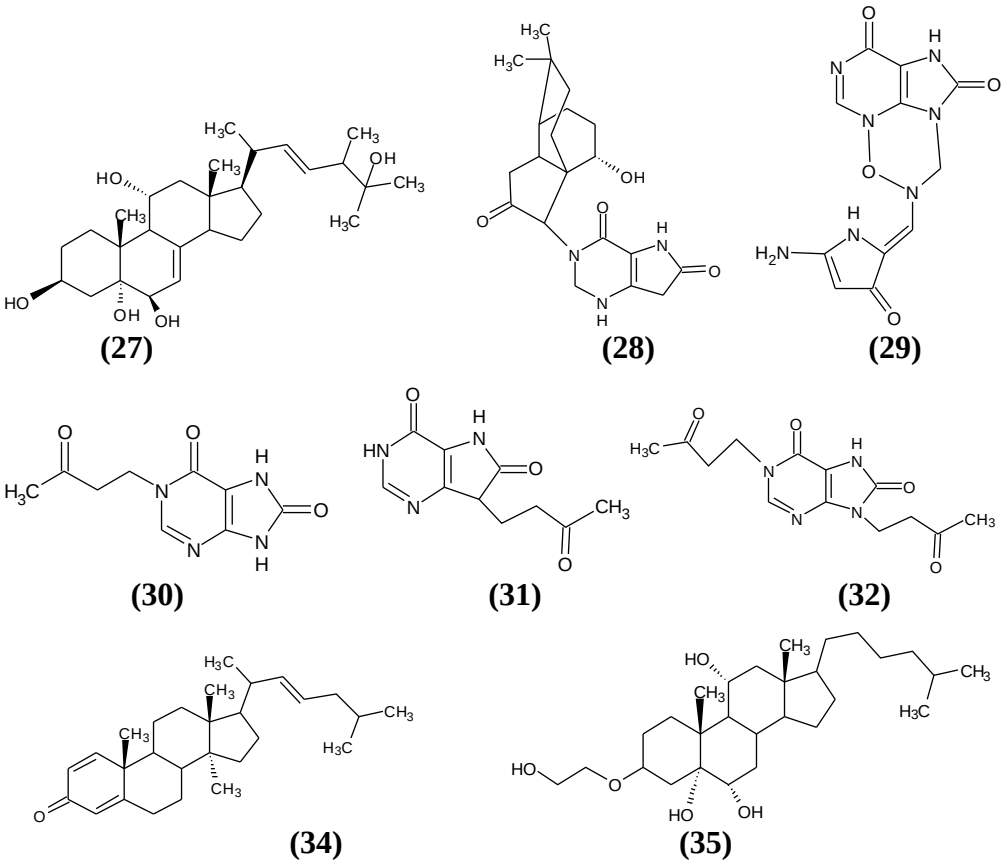


Figure 1.3 Structure of compounds isolated from *Subergorgia* genus

(1) 8-methoxy calamenene (2) 5-hydroxy-8-methoxy calamenene (3) 11α,15 α-diacetoxy-17 α-pregna-4,20-die-3-one (4) anyaols A (5) reticulatin (6) Reticulolide (7) (+)-(7R, 10S)-2, 5-dimethoxy calamenene (8) (+)-(7R, 10S)-2 methoxy-5acetoxy calamenene (9) (+)-(7R, 10S)-2 methoxy calamenene (10) Subergorgic acid (11) Suberosenone (12) 3,9-dioxo-9,11-secocholesta-5,7-dien-11-al (13) Methyl 2β-hydroxysubergorgate (14) Methyl 2β acetoxy subergorgate (15) 2β hydroxyl subergorgic acid (16) Methyl subergorgate (17) 3β,6α,11-trihydroxy-9,11-seco-5α-cholest-7-ene-9-one (18) 24S-methyl-3β,6α,11-trihydrxy-9,11-seco-5α-cholest-7,22E-diene-9-one (19) 24R-methyl-3β,6α,11-trihydrxy-9,11-seco-5α-cholest-7,22E-diene-9-one (20) Suberosols A (21) Suberosols D (22) subergorgiol (23) 2β-autoxy subergorgic acid (24) 6-(9'-purine-6,8-diolyl)-2β-suberosanone (25) 3β,6α,11,20β,24-pentahydroxy-9,11-seco-5α-24-ethylcholest-7,28-diene-9one (26) 3(1',2'-ethandiol)-24-methylcholest-8(9),22E diene-3β,5α,6α,7α,11α pentaol (27) 24-methyl cholest-7,22E diene-3β,5α,6β,25-tetraol (28) 6-(1'-purine-6,8-dionyl)suberosanone (29) 3,9-(2-imino-1-methyl-4-imidazoli-dinone-5-yl)isopropenylpurine-6',8'-dione (30) 1-(3-carbonyl butyl)purine-6,8-dione (31) 9-(3-carbonyl butyl)purine 6,8-dione (32) 4-carboxy-5,6-dihydro-4H, 8H-pyrimido(1,2,3-cd)purine-8,10(9H)-dione, (33) 7,9-dihydro-1-(3-oxobutyl)-1H-purine-6,8-dione (34) 7-hydro-9-(3-oxobutyl)-1H-purine-6,8-dione (35) (22E)-14α-hydroxy-cholesta-1,4,22-trien-3-one and 3-(1',2'-ethandiol)-cholest-3β,5α,6α,11α-tetraol.

Three new secosterols 3β,6α,11-trihydroxy-9,11-seco-5α-cholest-7-ene-9-one, 24S-methyl-3β,6α,11-trihydrxy-9,11-seco-5α-cholest-7,22E-diene-9-one and 24R-methyl-3β,6α,11-trihydrxy-9,11-seco-5α-cholest-7,22E-diene-9-one were isolated from *Subergorgia suberosa* (Aknin et al 1998). The compound isosubergorgic acid isolated from *Subergorgia suberosa* is claimed to revealed acetyl choline esterase inhibitory activity and it is suggested for the treatment of Alzheimer's disease (Al et al. 1999, Ye et al. 2001). Four new β-caryophyllene derived sesquiterpene alcohols, Suberosols A-D together with two known β caryophyllene derived sesquiterpene ketones, buddledinsC-D were isolated from Taiwanese gorgonian coral *Subergorgia suberosa* among them some exhibited cytotoxic activity(Ye et al. 2001,Wang et al. 2002).

From the ethyl acetate extracts of the Taiwanese gorgonian coral *Subergorgia suberosa,* two new subergane based sesquiterpenes, subergorgiol and 2β-autoxy subergorgic acid together with four known compounds such as subergorgic acid methyl ester, subergorgic acid 2β acetoxy methyl ester,2β-hydroxy subergorgic acid and subergorgic acid were isolated. Among these compounds, subergorgic acid methyl ester showed moderate cytotoxic activity against the growth of Hela cancer cells. Subrahmanyam et al. 2003 reported 3,3-dimethoxy-5α-pregnan-20-one from the same gorgonian coral *S.suberosa*.

A new sesquiterpene alkaloid 6-(9'-purine-6,8-diolyl)-2β-suberosanone was isolated from the South China Sea gorgonian *S. suberosa* along with three known compounds, suberosenol A, subergorgic acid and subergorgiol. The isolated sesquiterpene alkaloid exposed moderate cytotoxic activity against the human breast carcinoma cell lines (Subrahmanyam et al. 2005). *S. suberosa* collected from Mandapam coast were found to be present four compound, batyl alcohol, subergorgic acid N-hexadecanoyl-2-amino-1,3-dihyroxyoctadec-4-ene, and thymine. From these compounds a formulation was developed for the

isolation of a sesquiterpene ketone which has anticancer activity against tumour cells such as breast cancer, leukemia, oral cancer, liver cancer and lung cancer (Subrahmanyam et al. 2005, Qi et al. 2006). Three new polyhydroxylated sterols were isolated from the South China Sea gorgonian coral *S. suberosa* viz. 3β,6α,11,20β,24-pentahydroxy-9,11-seco-5α-24-ethylcholest-7,28-diene-9one, 3(1',2'-ethandiol)-24-methylcholest-8(9),22E diene-3β,5α,6α,7α,11α pentaol and 24-methyl cholest-7,22E diene-3β,5α,6β,25-tetraol, along with six known steroid compounds such as 24α-methyl cholest-7,22-diene-3β,5α,6β,9α-triol, 3β,6α,11-trihydroxy-9,11,seco 5α-choles-7-ene-9-one, 3β,6α,5,11-tetrahydroxy-9,11-seco-5α-cholest-7-ene-9-one, 3-*O*-β-D-glucopyranosyl-β-sitosterol and 25-O-acetyl-3-O-(β-D-arabinopyranosyl-oxy)-cholest-5-ene-3β,19,25-triol (Qi et al. 2008). An effective anticancer steroid compound 3β, 6α, 11, 20β, 24-pentahydroxy-9-11-seco-5α-24-ethylcholesta-7, 28-diene-9one isolated from *S. suberosa*. This compound was found to be active against gastric cancer, leukemia and liver cancer. An alkaloid 3,9-(2-imino-1-methyl-4-imidazolidionone-5-yl)-isopropenely-purine-6,8-dione, was also isolated from *S. suberosa*. This alkaloid was found to inhibit the growth of cancer cells of breast adenocarcinoma, liver cancer, gastric cancer and leukemia (Qi et al. 2008, Qi and Zhang 2008).

Four new purine alkaloids 6-(1'-purine-6,8-dionyl) suberosanone, 3,9-(2-imino-1-methyl-4-imidazoli-dinone-5-yl) isopropenylpurine-6',8'-dione, 1-(3-carbonyl butyl) purine-6,8-dione and 9-(3-carbonyl butyl) purine 6,8-dione along with three known compounds guanosine, thymidine and adenosine were extracted from *Subergorgia suberosa* (Qi et al. 2008). In these compounds 6-(1-purine-6,8-dionyl)suberosanone and 9-(3-carbonyl butyl)purine 6,8-dione exposed cytotoxic activity against human cancer cell line(Qi et al. 2008). Again from *S. suberosa* six known compounds were isolated such as subergorgic acid, pregn-4ene-3,20-dione(progesterone), 5β-pregn-3,20-dione,

3β-pregn-5-one-20-one-3ol, 3β,5β-pregn-20-one-3ol and stigma-7,22-diene3β,5α,6β-triol. These compounds were found to be antiviral against *B.amphitrite* and *B.neritina* and also antibacterial (Qi et al. 2010).

Chemical examination of *Subergorgia suberosa* led to the isolation of two new steroids such as 3β-O-palmitoyl-pregn-5-en-20-one-3ol and 3β-O-palmitoyl-5α-pregn-20-one-3-ol along with six other known steroids 5α-pregn-1-ene-3,20-dione, 3β,5α-pregn-20-on-3-ol, 3β-pregn-5-en-20-on-3ol 3β(Qi S et al. 2008). Three purine derivatives such as 4-carboxy-5,6-dihydro-4H, 8H-pyrimido(1,2,3-cd)purine-8,10(9H)-dione, 7,9-dihydro-1-(3-oxobutyl)-1H-purine-6,8-dione and 7-hydro-9-(3-oxobutyl)-1H-purine-6,8-dione were isolated from the South China gorgonian coral *S. suberosa* (Qi et al. 2008). Qi et al 2010 also isolated two new steroids (22E)-14α-hydroxy-cholesta-1,4,22-trien-3-one and 3-(1',2'-ethandiol)-cholest-3β,5α,6α,11α-tetraol from *S.suberosa*. These two compounds showed cytotoxic activity against human cancer cell lines A549, HONE1 and HeLa (Qi et al. 2010). Nine compounds were also isolated for the first time from *S. suberosa* such as cholesta-5ene-3β,7α-diol, cholestane-1β,3β,5α,6β-tetrol, cholesterol, (E)-N-2-(1,3-dihydroxy octadecan-4-en)-hexadecanide, batyl alcohol, thymidine, thymine, uracil and heptadecane (Jian et al. 2010). From s*ubergorgia* sp. astaxanthin was isolated and was found to be cytotoxic (Folmer et al. 2009). Thus a significant number of researches were done on the species *Subergorgia* and some of the compounds are pharmacologically important. It is very curious that the results of these compounds were not further studied for the clinical trials and some of the compounds are repeatedly isolated and hence more dedicated efforts is required for the compound to be come in the market as a drug candidate.

1.5 Scope of the Present Study

The survivals of many marine invertebrates that are sessile and soft bodies rely on chemical defences, which are natural products arose through their evolutionary history to deter predators, keep competitors away or to paralyse. Due to the predator deterrence corals are well known for the pharmacologically active toxic compounds. This knowledge is the main focus of this study as these types of natural products have immense potential to serve as drugs. Research into the pharmacological properties of marine natural products has led to the discovery of many potently active agents. Detailed review on the identification and isolation of natural product from the gorgonian coral *subergorgia* revealed a number of prospective chemical compounds, which possess biological activities. There are very few noted attempts to identify and isolate compounds from the gorgonian coral *subergorgia reticulata.* Since the compounds reported are very few compared to other species, the biological activity also was not studied in full.

The Lakshadweep archipelago located in the Arabian Sea comprise a group of 36 Islands in which 11 Islands are inhabited islands, 17 uninhabited islands, 6 submerged sand banks and 3 coral reef environments, situated between 8º-12º N latitude and 71º45'-73º45' E longitude. Lakshadweep Islands is unique marine ecosystem and known to possess very high degree of biodiversity and a number of endemic flora and fauna. The richest biodiversity of the coral reefs of Indian subcontinent is found in Lakshadweep Islands. The lagoons and atolls of Lakshadweep are home to a wide variety of organisms including crabs, lobsters, sponges, echinoderms, turtles, tuna and other fishes and birds.. About 103 species of corals, fewer than 37 genera, from Lakshadweep were identified (Pillai and Jasmine 1989). There were no dedicated works to understand the morphology and biochemistry of the

gorgonian coral *subergorgia reticulata* collected from the Lakshadweep islands. Attempts are made to study the biochemistry of the organism to record the nutritional uptake and the energy storage, transport etc.

The study is an attempt to isolate pharmacologically active compounds from *subergorgia reticulata* and to study about the chemistry and biological activity relationship. The compounds identified and isolated to study well for its action ie, biological action on various aspects. The *In silico* studies were mainly done as a starting and initial step prior to *in vitro* studies. The results of *in silico* studies were amazing that the identified compounds were shown to posses various biological activity. The biological activity is very difficult to access for chemists since it requires both practical as well as in depth knowledge. It is well established that the chemical compounds have its own role in the organism and on this back ground a detailed study of the entire chemistry of the organism is evaluated for the various applications of pharmacy as well as industry.

1.6 Objectives of the Study

The rich biodiversity of the marine environment are found to possess rich source of novel chemical compounds which have shown promising activities having ecological and biological significance. Noted among them are the potential value added products like drugs, pharmaceuticals and other organic compounds. With the advantage of modern chemical methods and sophisticated instruments, systematic exploration and investigation of the marine organisms will lead to the isolation and characterization of bioactive compounds having pharmacological properties which can be a great promise for the furtherance of current knowledge of marine natural products.

The present study focus on the isolation and characterisation of biologically active compounds derived from *Subergorgia reticulata*, a soft coral, coming under octocorallia gorgonian coral collected from Kavaratti Island of Lakshadweep archipelago. The objectives are

- To find out a natural compound responsible for a specific biological activity.
- To study the chemical dissection of the organism in order to identify and characterise its interesting metabolites.
- The characterisation of the structure of the isolated compounds.
- Screening of the biological activity& the drug potentiality of the isolated and identified bioactive compounds by *in vitro* and *in silico* studies.

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Chapter

2 MORPHOLOGY

Contents

2.1 Introduction
2.2 Materials and Methods
2.3 Results and Discussion
Conclusion
References

2.1 Introduction

The phylum Cnidaria includes about 10,000 species which are traditionally divided into three classes: Anthozoa, Hydrozoa and Scyphozoa. Within the single class Anthozoa, which is characterised by the complete absence of any medusoid stage, the best known representative of Anthozoa are the gorgonians (sea fan) and soft corals. They are exclusively marine, solitary or colonial and almost always fixed, and have a horny or calcareous skeleton. Gorgonians, sea feathers and soft corals are the major examples in this category. The horny skeleton is formed of 'gorgonin', a fibrous protein similar to the 'sponging' of sponges and containing limestone spicules called sclerites. The term sclerite refers to an element of a composite exoskeleton, one in which the elements are held together to form a protective coating like a coat of mail. Sclerites having characteristic shape and are functionally significant to organism, the shapes of these sclerites are often be used alone to identify the specimens to the generic level (Lewis and Von wallis 1991). In common usage, the term sclerite excludes vertebrate bones, echinoderm ossicles, and sponge spicules, which are all formed endoskeletally, and simple one- or two-part skeletons like gastropod or brachiopod shells (Bengtson and Hou 2001). Calcium carbonate is of animal origin and all of today's continental limestone

massif are the remnants of coral reefs from remote geological eras, which is evident from the many fossils they contain. The role played by the corals in the fixing of carbon dioxide in the form of limestone is considerable and similar to the role played by the terrestrial plants in fixing carbon as cellulose. Almost all corals are made up of aragonite and that the percentage of calcium in the coral corresponds to a concentration approximately 800 compared to the sea water (Jean Michel Kornprobst 2014).The mechanism by which biomineralization occurs in corals is poorly known**.**

It is reported that corals are composed of calcium carbonate in an organic matrix (Watanabe et al. 2003; Fukuda et al. 2003; Rahman et al 2005; Rahman and Isa 2005; Rahman et al. 2006; Rahman and Oomori 2008; Rahman et al. 2011). The organic matrix is formed prior to mineralization, and it has been suggested that some components of the matrix protein may serve as a template for mineral deposition (D'Souza et al. 1999; Weiner and Hood 1975).Since the advent of animal skeletons, calcium carbonates (mostly calcite, magnesian calcites and aragonite), phosphates (mostly apatite, particularly dahllite, $Ca_5(PO_4,CO_3)_3(OH)$), and opal (a hydrated gel of silica, SiO_2) have been the most common skeleton-forming minerals (Lowenstam and Weiner1989). It is categorically cited that in stony corals, calcium carbonate generally occurs as needle-like aragonite crystals, whereas in soft corals and gorgonians, it occurs as calcite forms (Milliman 1974; Kaczorowska et al. 2003; Rahman and Oomori 2009). Octocorals are sessile cnidarians, and they synthesize mineralized sclerites which is an intriguing aspect of interaction between biological and physicochemical factors (Towe 1972), and the sclerites act as a flexible exoskeleton to assist the body under varying seawater conditions (Chave 1954; Velimirov and Bohm 1976; Bayer 1981; Weinbauer and Velimirov 1995; Sethmann et al. 2007).

The shapes of gorgonian surface sclerites (spicules) can often be used alone to identify specimens to the generic level. In most gorgonians, surface sclerites differ from those of deeper layers, and these combinations are used for identification to the specific level (Bayer 1961).

2.2 Materials and Methods

2.2.1 Sampling location and collection

The organism for the present study was collected from Kavaratti Island of the Lakshadweep group of islands, the important coral reef system in the Indian waters. The Lakshadweep group of Island is composed of 36 Islands spread over 220-440Km off the Kerala coast of India, lying between 8^{o}-10^{o}N Latitude and 71^{o}-74^{o}E Longitude. Kavaratti, the capital Island of Lakshadweep is located between $10^{o}33^{1}$N Latitude and 72^{o} 38^{1}E Longitude (Fig. 2.1). A wide variety of marine life, especially soft corals and gorgonians are the general feature of the coral bed of Lakshadweep Island. The octocoral, that is the gorginan coral *Subergorgia reticulata* used for the present study were collected from this island by Self Controlled Underwater Breathing Apparatus (SCUBA) diving during the month December 2011. The collected organism were washed well with water and kept it in methanol for chemical studies.

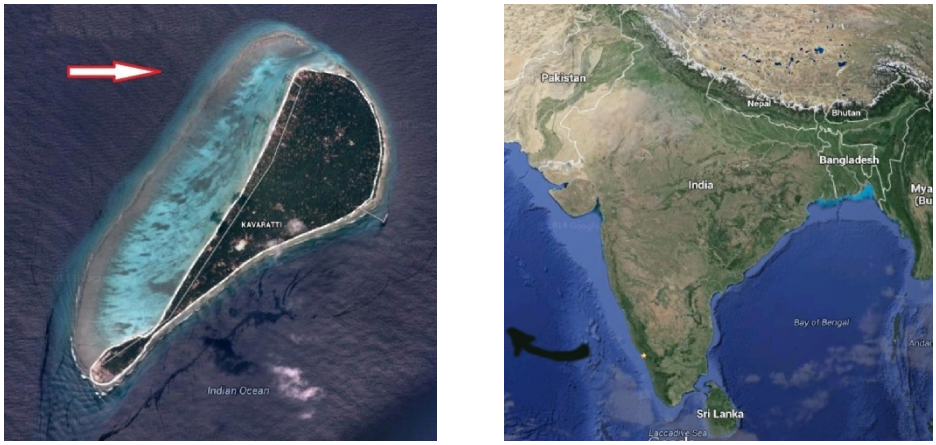

Fig. 2.1 Kavaratti Island (Sampling location)*

*https://www.google.co.in/maps/@11.9449124,75.2750377,1064385m/data=!3m1!1e3)

2.2.2 Identification

The soft corals were identified as *S. reticulata*, by Dr. P.A. Thomas, Emeritus Scientist, Central Marine Fisheries Research Institute (CMFRI), (ICAR),Cochin. The voucher specimen (IUCDMB.R.No.3) of the soft corals was preserved in the Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology. Taxonomical Details-Kingdom: Animalia; Phylum: Cnidaria; Class: Anthozoa; Subclass: Octocorallia; Order: Alcyonacea; Sub order: Scleraxonia; Family: Subergorgiidae; Genus: *Subergorgia*.

Fig.2.2 Gorgonian coral *Subergorgia reticulata*

2.2.3 Separation of Sclerites

Calcified endoskeletons (sclerites) were isolated from coral *subergorgia reticulata* colonies using a series of mechanical and chemical treatments. The coral colony was cut into small pieces using sharp scissors. The pieces were ground in a mixer machine and washed with 1 M NaOH for 2 hours, and then they were stirred vigorously in a 10% sodium hypochlorite (NaOCl) bleaching solution for 1 hour to remove fleshy tissues and debris. The treated samples were washed with water until the sclerites were completely cleaned. Finally, the samples were washed with MilliQ water till

the sclerites are free from contaminants. The sclerites were examined using a microscope (LEICA EZ4D) to ensure that the sclerites are tissue free. All steps in the isolation of sclerites from the colony were conducted at room temperature, and the materials obtained were stored at 4°C until further use.

2.2.4 SEM and Microscopic analysis

SEM EDX analysis was carried out to know the shape and material composition. The instrument (JEOL model JSM-6390LV coupled with EDX, JEOL model JED-2300) was used for analysis.

2.2.5 Biominaral characterisation of sclerite composition

FT-IR spectra of isolated sclerites were recorded by Perkin Elmer spectrum 100 FTIR Spectrophotometer in the range from 4000 to 650 cm^{-1}. The FTIR spectrophotometer was equipped with Universal Attenuated Total Reflectance accessory which permits solid and liquid sample analysis with much spectral reproducibility. SEM by using a Hitachi S4500 and JEOL JSM-6700F equipped with EDS at a 15-kV accelerating voltage. The mineralogy of crystals in the sclerites of the gorgonin coral *S. reticula* was determined by using a powder diffractometer, Bruker AXS D8 Advance (Cu Kα radiations (Ni filtered) of wavelength 1.5406 A° with Si(Li) PSD detector, 2θ range from 10 to 80° and step size of 0.02°.

2.2.6 Metals

Metal concentration were analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (Thermo scientific model:iCAP Duo).The standardisation was done with high pure standard (Sigma Aldrich). The reagent blank was used as ultrapure water and the concentrations of metals were read out from the standard graph.

The final concentration is obtained as given below.

$$C = \frac{a - b}{Wt} \times v$$

Where 'C' is Concentration in the test sample

'a'is the concentration in the test solution (mg/l)

'b' mean concentration of blank

'v' is the volume of the test solution made (ml)

Wt , weight of the test portion.

2.2.7 Organic matrix characterisation

Total protein content was determined using Lowry et al 1951 method and total carbohydrate content was determined by phenol sulphuric acid method by Dubois et al 1956.

2.2.8 Total Carbohydrates

Total carbohydrate content in the powdered sclerites of the gorgonian coral *S.reticulata* was determined colorimetrically by Phenol-Sulfuric acid method (Dubois et al. 1956). Exactly 0.5 mg of methanol extract of the sample is dissolved in 1ml of distilled water. To 0.1ml of the sample in a test tube, 1ml of 5% Phenol reagent followed by rapid addition of 96% sulphuric acid. After 10 minutes, the contents in the tube were shaken well and placed in water bath at 25-30˚C for 30 minutes. After cooling to room temperature, the absorbance was measured at 490 nm against a reagent blank in UV-Visible spectrophotometer (Analytikjena Specord 200). Glucose was used as standard and the results were expressed as percentage of dry weight of the sample.

$$\text{Conc.} = \frac{[X * V]}{1000} * \left[\frac{1}{W(mg)}\right] * 1000\,mg/g$$

- Conc – Concentration
- X - Spectrophotometric reading
- V – Volume of the solvent
- W – Weight of the sample

2.2.9 Total Protein

Total Protein content of powdered sclerites is determined colourimetrically by the method of Lowry et al. 1951. Exactly 0.5mg of the sample dissolved in 1ml of NaOH. To 0.1ml of the sample, 5ml of freshly prepared alakaline copper tartrate reagent was added followed by 0.5ml of 1:1 of Folin-Ciocalteu phenol reagent. The contents were mixed thoroughly and allowed to stand for 20minute for colour development. The absorbance was then read at 660 nm against a reagent blank using UV-Visible spectrophotometer (Analytikjene Specord 200). Bovine serum albumin was used as standard and the results are expressed as percentage of dry weight of the sample.

$$\text{Conc.} = \frac{[X * V]}{1000} * \left[\frac{1}{W(mg)}\right] * 1000\,mg/g$$

- Conc - Concentration
- X - Spectrophotometric reading
- V - Volume of the solvent
- W - Weight of the sample

2.2.10 Monosaccharide & Disaccharide Composition of sclerites using HPLC

20 mg of sclerite sample was dissolved in 2 ml of 2 M TFA in an ampoule (5 ml) (Fengel and Wegener 1979). The TFA method has been adapted with 2 major advantages: causing smaller losses and omitting the step of neutralization since the TFA can be removed by evaporation. The ampoule was sealed under a nitrogen atmosphere and kept in boiling water bath to hydrolyze the polysaccharide present in the sclerites into component monosaccharides for 10 hours. After being cooled to room temperature, the reaction mixture was centrifuged at 1000 rpm for 5 min. The supernatant was collected and dried under a reduced pressure. The hydrolyzed and dried sample solutions are added with 1 ml distilled water. 10µl of the sample was then injected into the HPLC system. A Shimadzu LCMS 2020 equipped with RID 10A detector and SUPELCOSIL LC-NH_2 column (purchased from SIGMA ALDRICH). The separation was conducted at 30ºC with the mobile phase Acetonitrile: water (8:2) at flow rate of 0.8ml per minute. The identification of monosaccharide, sugar units were done by comparing the retention time of sample with individual monosaccharide standards under same conditions. The calibration curve was plotted using varying concentrations of the standards starting from 5ppm to 100ppm. The concentration of the monosaccharide in the sample was determined from the peak area of the detected sugar.

2.2.11Total amino acid quantification

Calibration of amino acid using Standard solution

The standard containing seventeen amino acid were dissolved in 0.05 N HCl solutions. Glutamine converts to pyroglutamic acid after prolonged

storage in HCl. The pre-column derivatization method with PITC was used (Shang and Wang 1996). Dry samples were dissolved in 20 ml of ethanol: water:TEA (2:2:1) and dried again under vacuum. The derivatization reagent was made fresh and consisted of ethanol:water:TEA:PITC (7:1:1:1), where PITC was added to this solution under nitrogen. Derivatized amino acids were formed by adding 20 ml of the reagent to the dried samples under nitrogen atmosphere and sealing them for 30 min at room temperature. The reagents were then removed under vacuum at 45^{o}C, dissolved in buffer and injected. The samples were also done using the same derivatization procedure but in the hydrolysis step 6N HCl was used instead of 0.05N HCl.

Chromatographic conditions

The proposed solvent system consisted of two eluants. Solvent A, an aqueous buffer, was a solution of 50 mM sodium acetate containing TEA as modifier. The solution was degassed before the addition of TEA. The pH was adjusted to the desired value using glacial acetic acid, and the solution was filtered through a 0.2µm membrane filter. Solvent B consisted of water, acetonitrile, and methanol (4:3:3). Aqueous solutions containing acetonitrile and methanol are the most common solvents used in amino acid analysis in RP-HPLC. The column used was SUPELCO C_{18} having 25cm length, 4.6cm breadth and 5µm particle size. The pH and TEA content of solvent A, the composition of solvent B, the temperature, and the mobile phase flow rate are specified as required for the experimental design. Ultra violet detection was carried out at 254 nm. The column was equilibrated for 30 minutes with the mobile phase before the injection of the sample.

2.3 Results and Discussion

The yield of the sclerites were calculated after the removal of complex tissue from the organism and is given as;

Total weight: 50gm

Sclerite obtained: 15gm

Yield of sclerites: 30% by weight

2.3.1 SEM & Microscopic analysis

Three portions were notified from the gorgonian coral *S.reticulata* as axial, mid, and tip portion. When the three portions were analysed by microscopy three different types of sclerites were observed (Fig. 2.3). One is rod shaped, double head, and hexagon shaped. Bottom axial position was dominated by scale like and rod shaped sclerites and no dumbbell shaped sclerites were observed. Most of the gorgonians, sclerites are arranged in three distinct layers, in the colenchymal cortex. The layers are distinguished by sclerite types. Sclerites in this layers are usually short rods, spindles, radiates or spheroids (Lewis and Von Wallis 1991).The hexagonal shaped, double headed sclerites were the majority in the tip portion. The axial and surface sclerites differ and provide mechanical strength for the organism (Bayer 1961, Koehl 1982, Wainwright et al. 1982). The double head or dumb-bell is characteristic of the genera *Elliswlla* and *Lophogorgia* (Lewis and Von Wallis 1991).

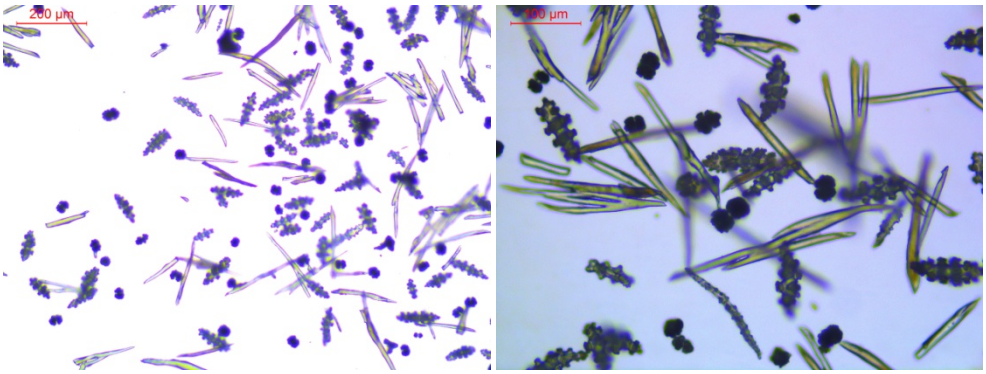

Fig. 2.3. Sclerites through microscope

SEM images of isolated Sclerites

Branches of *Subergorgia reticulata* gorgonian coral representing the common sclerite types were examined by scanning electron microscopy. To determine changes in position of sclerites associated with extension and compression, appropriate measures of sclerite density was taken. Surface sclerites (spicules), and axial bottom were taken for SEM. Sclerites from three different portions of the gorgonin soft coral *S.reticulata* were freeze-dried and polished. The polished surface was observed by SEM. A SEM image of (Fig.2.4) three different shapes of the colony were observed in the parts of the tip, axial and bottom sclerites of soft coral *S.reticulata.*Three types of sclerites, rod-like and dumbbell-shaped, were observed by SEM.

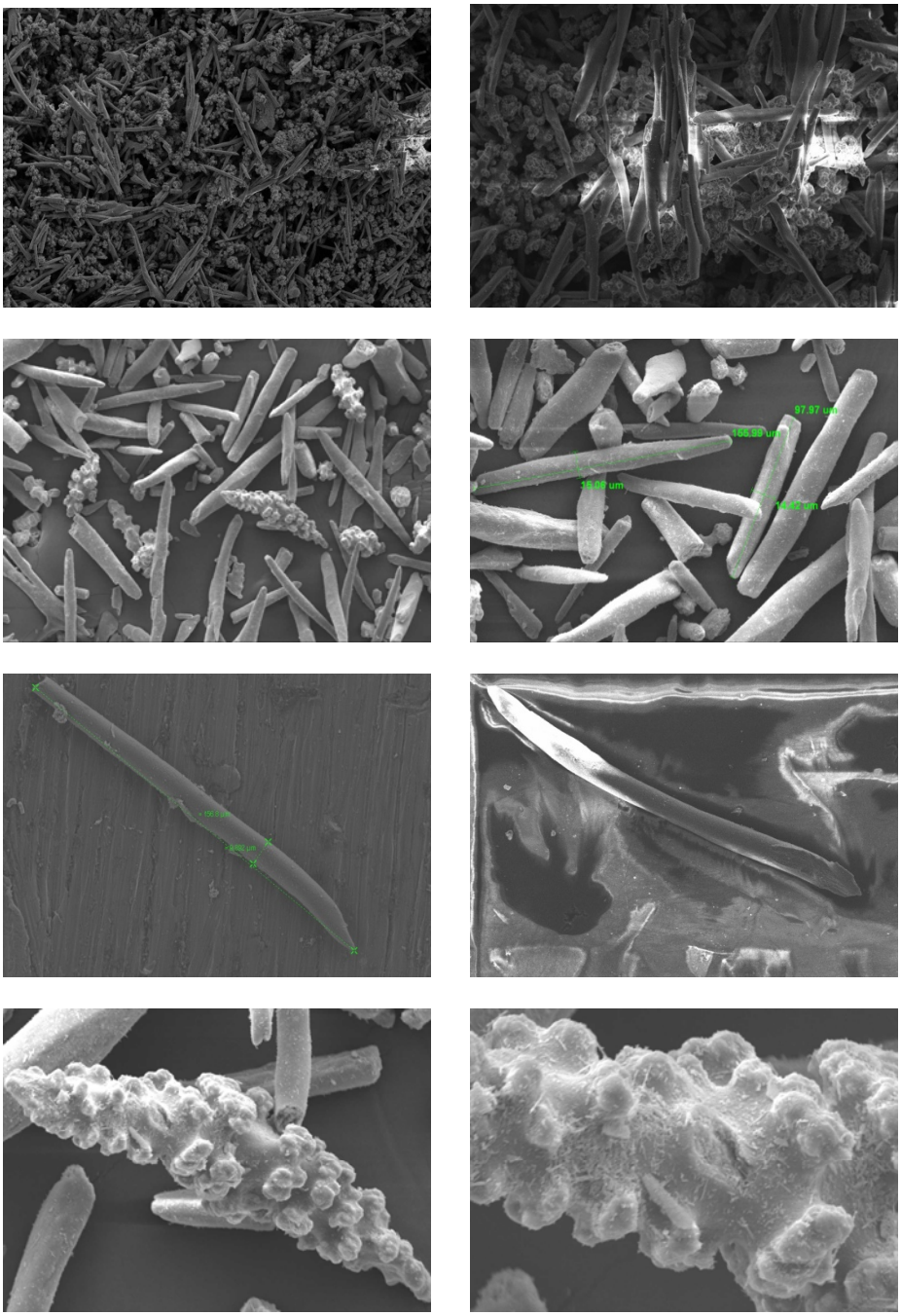

Fig.2.4. Scanning electron micrographs of *S.reticulata* sclerites in surface view (a) Magnification-209X,(b) Magnification-527X,(c) Magnification-250X,(d) Magnification-500X,(e) Magnification-1.74kX,(f) Magnification-1.28kX, (g) Magnification-1000X,(h) Magnification-2500X

2.3.2 Biominaral Composition of the Isolated Sclerites

FT-IR Analysis

The characteristic broadening of carbonate asymmetric stretching peak is observed at 1397.47 cm^{-1} (Fig.2.5). The absorption band of carbonate (in-plane bending), appearing at 716.65 cm^{-1} is typical for calcite (Table 2.1). The carbonate out-of-plane bending peak at 870.05 cm^{-1} is also characteristic of calcite. Thus, the three main calcite bands are visible from the FTIR spectrum of the sclerites of *S.reticulata*. Further, the peak intensities at 870.05 cm^{-1} and 716 cm^{-1} gives a peak height ratio gives higher than the value reported for pure synthetic calcite minerals (Politi et al. 2004; Long et al. 2011). Characteristic absorption peaks of neither vaterite (at 744, 876, and 1088 cm^{-1}) nor aragonite (at 700, 712, 852, and 1083 cm^{-1}) were observed.

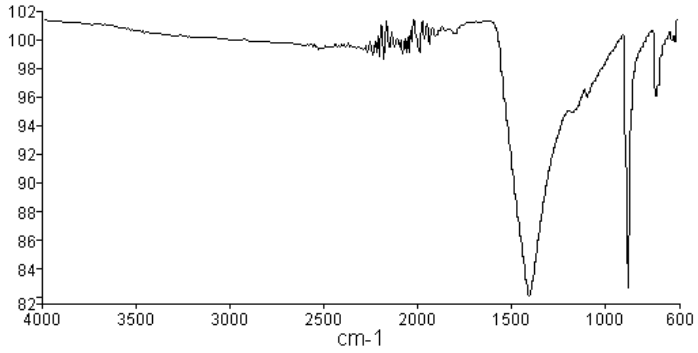

Fig. 2.5 FT IR spectrum of Sclerite separated from the soft coral *Subergorgia reticulata.*

Table 2.1 FTIR Major absorption wave length and intensity.

Transmittance in wave length (cm^{-1})	Intensity
716.65	96.08
870.05	82.59
1397.47	82.08
1987.24	99.26
2077.49	99.12
2185.92	98.69
2203.37	98.9

Electron Diffraction studies

EDX analysis was performed in the area defined by the box on the left panel. The EDX analysis reveals the presence of both Ca and Mg in the sclerites and identifies the minerals in the sclerites as low-Mg calcites. The pie chart displays the observed elemental weight fractions (inset). An SEM-combined EDX was used for spot analyses of the elements comprising selected sclerites and single crystals.

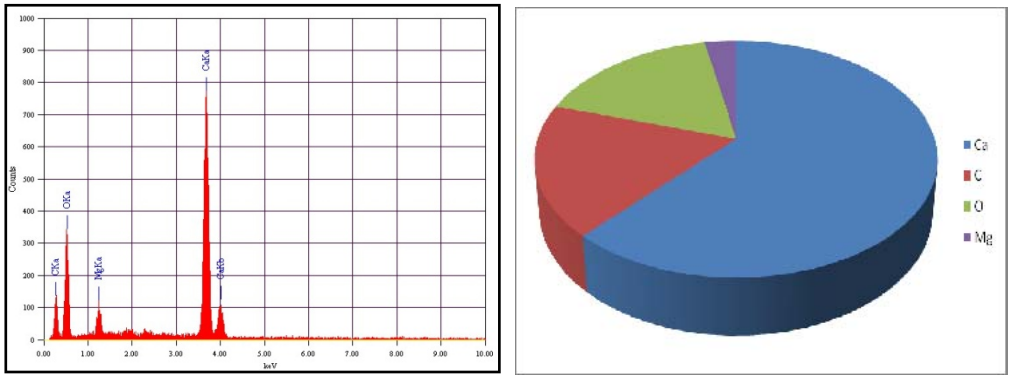

Fig. 2.6 (a). EDS of the sclerite separated from the soft coral *Subergorgia reticulata* (b) Pie diagram showing the elemental composition of sclerite from *Subergorgia reticulata*

The EDX analyses of the sclerites (Fig.2.6) substantiated that the magnesium-bearing calcite is the only calcium carbonate polymorph in *Subergorgia reticulata.* The EDX data of (mass and atom % of the elements) the sclerites are given in Table 2.2. The magnesium-coupled calcites in the sclerites of the soft corals reflect the high dissolved magnesium concentration of seawater (about 50 mmol/l) as compared with its calcium concentration (10mmol /1). But, in biomineralization, selection of $CaCO_3$ polymorph is a key prescript. It is the unique feature of biomineralizing pathways of $CaCO_3$ that organisms are able to shift the polymorphic direction at will, whereas the same biominerals are difficult to synthesize and to stabilize under any simulated conditions.

From the mineralogical analyses reports by Weinbauer et al. 2000; Rahman and Oomori 2008; Rahman et al. 2011 have shown an appreciable molar concentration (2-10 mole %) of Mg in the composition of sclerites of alcyonarians. In the present study the estimated Mg in the sclerites of *S.reticulata* is 2.9 mole%. Which is well below the threshold level reported for the destabilization of calcite crystals; significantly, at 12 mole% Mg content, calcite becomes less stable than aragonite (Chave et al. 1962; Katz 1973; Treves et al. 2003). Weinbauer et al. 2000 reported that the incorporation of Mg in calcite skeletons is facilitated by a hike in temperature and reported a value of 2.8-3.0% in Ceramium rubrum. Calcium concentrations generally did not vary with ecological factors, like water depth or colony region, whereas a high intraspecific variation of Mg/Ca ratios was accompanied in relation to water depth and colony region (Weinbauer and Velimirov 1995). Recently, this feature has been potentially exploited by using minor element ratios, like Mg/Ca in the calcite skeleton of corals, foraminifers, bivalves, and so on, as ecological indicators of water temperatures since they are independent of other seawater variables including salinity (Beck et al. 1992; Shen et al. 1996; Lea et al. 1999; Elderfield and Ganssen 2000; Quinn and Sampson 2002; Wanamaker et al. 2008). If so, temperature could also act as a cofactor in the enrichment of Mg in *S.reticulata* collected from the Lakshashweep archipelago, as it falls under a typical tropical climatic condition. Thus, the results suggest that the surrogate Mg calcite, rather than pure calcite is the only polymorph in the sclerites studied.

Table 2.2. Result of EDS analysis.

Element	(keV)	Mass%	Atom%
C	0.277	18	35.35
O	0.525	17.23	25.4
Mg	1.253	2.98	2.89
Ca	3.69	61.8	36.37
Total		100	100

From the SEM EDS analysis it was found that Calcium was the most deserted element present in the sclerites of the gorgonin coral *S.reticulata* followed by carbon, oxygen and magnesium which represents the existence of $CaCO_3$ structure.

X-Ray Powder Diffractometry (XRD)

X-Ray Powder Diffractometry is one of the most powerful and established techniques for the structural analysis of materials, and are capable of providing information about the structure of a material at the atomic level. X-ray diffraction studies were carried out to know the crystallinity of the sclerites separated from *S.reticulata.*

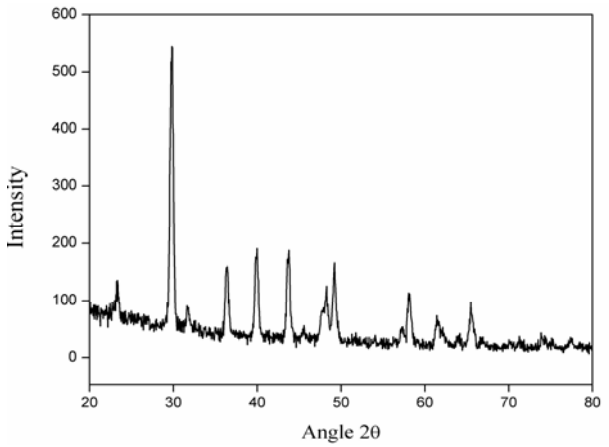

Fig. 2.7 The intensity of the absorption observed for different angle 2θ

The X-ray diffraction pattern (Fig.2.7) of sclerites of *Subergorgia reticulata* showed the strongest diffraction intensity of the calcite rhombohedrons (104) which appears at a *d* value 2.99 Å (2θ - 29.82°). The 2θ value at 23.34°,36.35°,47.67°,49.21° and 58.14° can be correlated to the hkl indices (012), (110),(024), (116) and (1010).Thus the X-ray diffraction analysis has shown that the crystalline form of calcium carbonate in the sclerites of *S.reticulata* is calcite with rombohydral shape. The shifting of

diffraction angle 2θ to 29.82° of the sclerites of *S.reticulta* has indicated the existence of calcite as Mg-calcite since the diffraction angle for pure calcite is 29.31° (Belchar et al 1996; Rahman and Oomori 2008).Further the presence of Mg in the sclerites of *S.reticulata* was also endorsed by EDX, ICP-OES discussed hereafter. The other studies in the skeletal parts of several marine species as compared with terrestrial ones (Falini et al. 1994; Raz et al. 2000; Gayathri et al. 2007; Lenders et al. 2012). Since Mg^{2+} being a smaller ion than Ca^{2+}, calcite crystals can incorporate Mg^{2+} ions inside the lattice, unlike that of aragonite (Treves et al. 2003; Brecevic and Kral 2007).

XRD examination of *S. reticulata* in this study did not indicate the presence of either the amorphous or the metastable crystalline forms of calcium carbonates (aragonite and vaterite) in the sclerites. Earlier reports have also shown that organic biopolymers like proteinous materials present in the matrix can direct a selective deposition of a specific polymorph of $CaCO_3$, specifically Mg-calcite, which leads to a particular crystal morphology in the sclerites of soft corals (Chave 1954; Rahman and Oomori 2008; Long et al. 2011; Lenders et al. 2012).

Metal composition of sclerites and comparison with tissue

Trace metals in tissue and skeleton have been investigated in various ways since the early seventies. ICP OES method was adopted for the detection of trace elements such as Ca, Mg, Sr, Ag, Al, B, Ba, Bi, Cd, Co, Cr, Fe, Ga, Mn, Ni, Pb, Tl and Zn. Corals are marine animals that widely regarded as pristine habitats which are not exposed to high heavy metal input(Mc Conchie and Harriot,1992; Anu et al. 2007) The study on metal accumulation in coral tissue and skeleton could be useful in examine environmental changes and pollution (Howard and Brown 1986; Shen and Boyle 1988; Esslemont 2000;

Hoffmann 2002). From the reported results of metals present in coral tissues and skeletons were able to assess metal accumulation, whether corals can uptake metals directly either from sea water or through feeding(Chan et al. 2012). There are some studies showing the uptake of elements by corals from sea water contains both $^{45}Ca^{++}$ and $H^{14}CO_3$, Calcium is deposited into the $CaCO_3$ skeleton and ^{14}C is fixed to the tissues as organic matter and into the skeleton as $^{14}CO_3$ (Goreau and Goreau1960; Ladd 1961). No toxic levels of metals are observed on analysis. Toxic metals such as Hg and As were not detected in the sclerites; whereas Pb and Cd were present at the permissible limit. Ca, Mg, K, Sr were the leading elements present in the sclerites. The concentration of metals obtained from sclerites is listed in table 2.3.

Table 2.3 Metal compostion in sclerites from *S.reticulata*

Metal (ppm)	Sclerites	Tissue
Calcium	179500	153400
Magnesium	21980	20220
Potassium	20160	3020
Aluminium	814	166
Strontium	1851	1594
Arsenic	ND	ND
Cadmium	0.136	2.77
Chromium	0.377	1.26
Copper	0.136	2.46
Iron	16.9	17.1
Mercury	ND	ND
Manganese	1.10	1.55
Lead	1.08	0.781
Tin	4.08	3.56
Zinc	14.9	17.8
Boron	9.973	13.37
Barium	56.11	2.637
Bismath	ND	0.4124
Cobalt	0.00435	0.07250
Gallium	0.073	0.1934
Lithium	3.942	3.562
Nickel	ND	ND
Indium	0.0674	ND

*ND- Not detected

2.3.3 Organic Matrix from Sclerites

Alcyonarian endoskeletons contain a protein-polysaccharide complex as their major component. It is also accepted that there is an organic matrix in the skeleton, which is believed to play an important role in the process of spicule formation. The organic matrix and inorganic calcium carbonate are also thought to be major components of the spicules. Organic matrices of bio-minerals generally consist of proteins, carbohydrates and lipids. The mature spicules contain 5.9% by weight of organic material, most of which is protein (Rahman and Oomori 2008). Proteins are always present in coral skeletons (Rahman and Oomori 2008) and they comprise about 0.3% by weight of the dry skeleton. In many mineralization processes, particularly those occurring in most skeletal hard parts, the space set up by cells and/or polymerized macromolecules is further subdivided prior to mineralization by an assemblage of so-called organic matrix macromolecules. The organic matrix is proposed to act as activators or inhibitors of crystal growth in corals by binding calcium.

Total protein and total carbohydrate

Protein, carbohydrates, and lipids are the major composition of organic matrix from spicule biominerals. A mature spicule contains organic material, most of which is protein (Kingsley and Watabe 1983).

Total protein content from sclerite: 56µg/mg

Total carbohydrate from sclerite:11µg/mg

Total amino acid content of the sclerite

Amino acids are fundamental components of all life forms, to some extent plants, fungi, bacteria, protista are able to synthesis all of the twenty protein amino acids. Amino acids which are essential must be obtained by animals from their environment. All vertebrates have the same nine essential amino acids are, threonine, valine, methionine, leucine, isoleucine, phenylalanine, histidine, lysine

and tryptophane. Glycine is elevated in the organic matrix of hard corals (Ingalls et al. 2003; Gupta et al. 2006). It was also reported that glycine is the most abundant amino acid in the organic matrix of gorgonian *Leptogorgia virgulata*. The amino acid concentration from the sclerite of *S.reticulata* was found to be rich of aspartic acid (Fig. 2.8). There are reports showing acidic amino acids are prominent as is of glycine and alanine (Kingsley and Watabe 1983). According to Weiner et al. 1983 aspartic acid have been found to be pre dominant in calcified structures as well. According to the amino acid concentration from sclerites of *S.reticulata,* the order of decreasing concentration of amino acid obtained were Aspartic acid> phenyl alanine> tyrosine> alanine> glutamic acid> leucine> serine> threonine> isoleucine> arginine> histidine (Table 2.4). Mitterer 1978, studied the amino acid composition scleractinians skeletal matrix and suggested that the aspartic acid and glutamic acid play a significant role in calcification and coral formation. However it is still mysterious that how the mucopolysacchrides and proteins in the matrix can provide binding sites for Ca^{2+} ions and contributes the $CaCO_3$ crystal formation.

Table 2.4. Total Amino acid concentration present in the sclerites of *S.reticulata.*

Amino acid	Concentration (µmol/ml)	Concentration (percentage)
Aspartic acid	11.918	72.4
Glutamic acid	0.558	3.39
Serine	0.235	1.43
Histidine	0.040	0.24
Glycine	0.033	0.182
Threonine	0.180	1.09
Arginine	0.084	0.510
Alanine	0.665	4.04
Tyrosine	0.833	5.06
Cysteine	ND	ND
Valine	ND	ND
Methionine	ND	ND
Phenylalanine	1.219	7.86
Isoleucine	0.122	0.74
Leucine	0.554	3.37
Lysine	ND	ND

*ND- Not detected

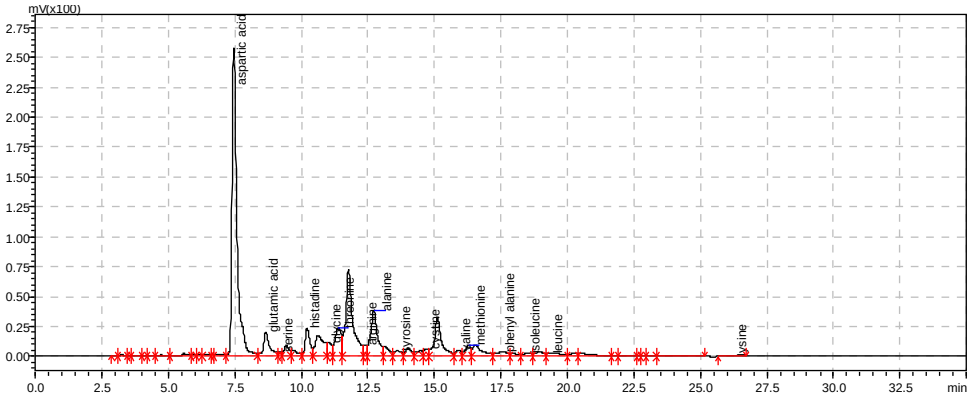

Fig.2.8. Chromatogram of amino acid compositions from *Subergorgia reticulata* sclerites

Monosaccharide & Disaccharide Composition of sclerites using HPLC

From the sugar unit analysis it was found that Fructose is the leading unit followed by arabinose, maltose, xylose, galactose, sucrose and lactose (Table 2.5). This results means to be very clear that some of the sugar unites were monosaccharide and some were disaccharides. Fructose is a sugar found widely in the diet as a free monosaccharide hexose, as a disaccharide, sucrose and in a polymerised form (fructans) (www.rayasahelian.com/fructose.html). Fructose is easily metabolized and changed in to fat. Studies in rodents, dogs and nonhuman primates eating diets high in fructose or sucrose consisting show elevated blood lipids. The metabolism of fructose in the liver drives the production of uric acid which utilizes nitric oxide, a key modulator of vascular function (George 2007). Arabinose is the monosaccharide containing five carbon atoms and was found to be second abundant sugar from the sclerites of *S.reticulata.*. The microbes are growing in association with coral by making utilization of the high concentration of protein, polysaccharides and lipids which produced at coral mucus (Ducklow and Mitchell 1979; Ferrier Pages et al. 2001). It was further studied about the mucus and found that significant amount of arabinose were present in the coral mucus. Arabinose is not a common constituent of animal cells and the possibility of involvement of zooxanthalle

cannot be omitted. The other sugar units present along with fructose and arabinose were maltose, galactose and lactose.

Table 2.5 The percentage composition of sugars units detected using HPLC

Sugar Unit	Concentration (ppm)
Ribose	ND
Xylose	2.210
Arabinose	4.875
Fructose	4.923
Mannose	ND
Glucose	ND
Galactose	2.169
Sucrose	1.569
Maltose	2.706
Lactose	1.364

*ND- Not detected

2.4 Conclusion

Investigation of sclerites from the gorgonian coral *S.reticulata* was found to be fascinating as it seems to be one of the wonderful creations in marine environment. Scientists are keenly involved in research to understand the relationship between the organic matrix formation in the sclerites to provide binding sites for Ca^{2+} ions and their contribution to $CaCO_3$ crystal formation. The clear SEM image of sclerite shows presence of three different types of sclerites. Three different parts of the organism were selected to study the difference in the arrangement of sclerites in the gorgonin matrix. The double headed and spindle shaped were found to be predominant in the top most part. Scale like sclerites are found to be rich at the bottom axial part, which may provide more mechanical support to the organism, with sessile habitat at the benthic region of sea. Mg- bound calcite crystalline rombohydral structure was proposed for the sclerites of *S.reticulata.* Apart from the pure calcite, the existence of calcite polymorphs with magnesium is observed from

XRD and EDX data, rather than a pure calcite. Instead of calcite, magnesium-bearing calcite has also been reported in many organisms from marine environments, such as sea urchin, sea star, corallina algae, octocorals and so on (Savassi 1999; Aizenberg et al. 2001; Aizenberg and Hendler 2004; Oaki and Imai 2006; Long et al.2011; Lenders et al. 2012). From the XRD analysis the crystalline nature was found to be $CaCO_3$ rhombohedral shape in which 2.9% is magnesium. Total protein and total carbohydrate analysis revealed that protein is the leading primary metabolite in the sclerite of *S.reticulata*. No toxic level of metals such as Hg and As were not detected in the sclerites; whereas Pb and Cd were present at the permissible limit. Ca, Mg, K, Sr were the leading elements present in the sclerites. Amino acid analysis indicated more information about the nature of the protein present in the organic matrix of sclerites. Several studies have been reported on the input role of aspartic acid rich proteins, in biomineralisation leading to the formation of calicium binding protein. The analysis of the amino acids from sclerites of *S.reticulata* has shown the richness of aspartic acid residues in sclerites. Hence the process of biomineralisation in the formation of sclerites in *S. reticulata* is also speculated to follow the procedure in other organisms. Analysis of sugars was also carried out to evaluate the contribution of sugar building unites in the biomineralisation process.

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Chapter

3 BIOCHEMISTRY AND SIGNIFICANCE OF BIOACTIVE COMPOUNDS

Contents

3.1 Introduction
3.2 Materials and methods
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Part I. Biochemistry

3.1 Introduction

Corals are symbiotic organisms formed by polyp animals. Soft corals stands along with hard corals in the phylum Cnidaria, having in common a very simple body plan and polyp structure. Most of the coral species build skeletons, which provide refuge againt predators and also help to provide support for attaching rocky substances (Rohwer et al. 2002). Gorgonian colony consists of individual polyps connected by common tissue, the coenenchyme, surrounding a long thin, axial endoskeleton. The colonies grow in fan, bush or tree like shapes. The body wall of individual polyps contains transport canals, calcareous spicules and endoskeleton is comprised of either calcareous or wood like collagenous matrix (Bayer 1961). Gorgonian corals undergoes both asexual and sexual reproduction. Asexual reproduction is mainly by fragmentation and sexual reproduction producing single sex colonies, and is called as gonochoric. Fragmentation is the processes by which parent colonies are break off and continue to be independent colonies (Walker and Bull 1983, Lasker 1984). In the sexual reproduction, all of the polyps in another colony

produce only eggs (Theodor 1967; Coma et al 1995; Zeevi Ben-Yosef and Benayuhu 1999; De Putron 2002).

Octocorals take nutritional food using a range of mechanisms and sources. Gorgonians posses a heterogeneous diet, they uses wide range of resources such as feeding on zooplankton, suspended particulate matter, dissolved organic matter, and micro plankton (Murdock 1978; Sterrer 1986; Coma et al 1994; Ribes et al 1999; Lasker 1981; Ribes et al. 1998). According to Lewis 1982, the mechanisms of octocoral feeding on particulate matter and zooplankton having a common strategy, individual polyp tentacles captured fine particulate matter; where as coordinated movement of tentacles enabled the uptake of larger particles. Most of the gorgonians, especially found in shallow, tropical waters have the ability to use photoautotrophic as well as heterotrophic food sources through a symbiotic relationship (Lasker et al. 1983).

Coming to the chemical entity, the primary constituent of the gorgonian axiel skeleton is of gorgonin which is a collagen protein complex. This was confirmed by Goldberg 1974 as collagen is the major structural element of gorgonin. According to Szmant-Froelich 1974 apart from collgen, saccharides, glucose and galactose were also present in the skeletal components. The other major compounds are coming under secondary metabolites such as acetogenins, sesquiterpenes, diterpenoids, and some steroids (Fenical 2006; Sung et al 2003, Reddy et al. 2005). It is also proven that gorgonians are sources of prostaglandins since Wein Heimer found PG-A_2 in *Plexaura* in 1969.Gorgosterols were used to be a biomarker since it is synthesized by this respective organism (Djerassi 1981; Whiters 1983). Like all animals, corals displays complex metabolism and these complex metabolic reaction results in the production of toxic substances such as steroids and terpenes (www.coralsciences.org; Iwagawa et al. 1995). Soft corals, like carnivorous

animals, find fuel for their metabolism in their prey; and in fact some fixed cnidarians need the assistance of photosynthetic organism to supplement their nutritional intake ie, carbohydrates, vitamins and hormones (Chevallier 1987).It has been established that about 143% of the daily energy requirement of corals are from symbiotic zooxanthellae (Davies1991).

The biochemical constituents are the major aspect for growth and development (Chu and Dupuy 1981).The variation of biochemical compositions may occurs within the species, season, age and sex of individual organism. The biochemical composition of the soft coral *Subergorgia reticulata* were analysed to understand the chemical composition and bioavailability. Biological significance of the organism play vital role in developing new pharmacologically active compounds. In this chapter antimicrobial properties of the extracts were widely studied and an approach to *in silico* biological activities of the compounds analysed in the biochemistry part were done. The detailed sample collection procedure is described in chapter 2.2.1.

3.2 Materials and methods

3.2.1 Preliminary qualitative tests

Methanolic extract of fresh *S.reticulata* was subjected to preliminary biochemical screening for the detection of various chemical constituents. The method of qualitative analysis is adapted from Jeffrey 1998.

Test for Alkaloids. Wagner's test: A fraction of extract was treated with Wagner's test reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) reddish brown colour precipitate indicate the presence of alkaloids.

Test for Flavonoids. NaOH test: A small amount of extract was treated with aqueous NaOH and HCl, yellow orange colour indicates the presence of flavonoids.

Test for Tannins. Braymer's test: Few ml of extract was treated with 10% alcoholic ferric chloride solution and formation of blue or greenish colour solution indicates the presence of tannin.

Test for Saponins. Foam test: A small amount of extract was shaken with water, formation of persistent foam indicate the presence of saponins.

Test for Carbohydrates. Molisch's test: Few drops of Molisch's reagent were added to each of the portion dissolved in distilled water; this was then followed by addition of 1 ml of conc. H_2SO_4 by the side of the test tube. The mixture was then allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet colour at the interphase of the two layers is a positive test.

Fehling's test: About 0.5 g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

Test for Quinones: A small amount of extract was treated with concentrated HCl the formation of yellow colour precipitate indicates the presence of Quinones.

Test for Terpenoids. Liebermann- Burchard test: Extract (1ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added, the formation of dark green colour indicates Terpenoids.

Test for Sterols. Liebermann- Burchard test: Extract (1ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added and observed for the formation of dark pink or red colour. H_2SO_4 test: The fraction of extract was treated with ethanol and H_2SO_4 and formation of violet blue or green colour indicate the presence of Sterols.

Test for Phenols. Ferric chloride test: The fraction of extract was treated with 5 % ferric chloride, the formation of deep blue or black colour indicates the presence of Phenols.

Test for Anthraquinones. Borntrager's test: About 50mg of powdered extract was heated with 10% ferric chloride solution and 1ml concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia; pink or deep red colourations of aqueous layer indicate the presence of anthraquinone.

Test for Anthocyanin. NaOH test: A small amount of extract was treated with 2M NaOH the formation of blue green colour indicate the presence of Anthocyanin.

Test for Proteins. Ninhydrin test (aqueous): The extract was treated with aqueous ninhydrin, purple colour indicates the presence of protein.

3.2.2 Quantitative estimation

Total carbohydrate content and total protein in the soft tissue of the gorgonian coral *S.reticulata* was determined colourimetrically by Phenol-Sulfuric acid method (Dubois et al. 1956) and Lowry et al. 1951. The detailed procedure is given in chapter 2.2.8 & 2.2.9.

3.2.3 Identification of compounds by GC MS

Gas chromatographic analysis of the purified methanol fraction was done on Perkin Elmer Clarus 680 GC-MS equipped with headspace auto sampler (Turbo matrix). Helium was employed as carrier gas and the ionizing voltage was kept at 70eV. Oven temperature was programmed at 10°C $min^{-1}$from 60°C to 290°C, when reached 290°C kept constant for 15 minutes. Injector and detector temperature were kept constant at 280°C and 290°C, respectively. The column used was Elite 5MS having 30m length and 250µm id. Mass spectrum of each peak in the total ion chromatogram was resolved and it was compared with National Institute of Standards and Technology (NIST) library spectra (Version: NIST MS Search 2.1) for the identification of known compounds.

3.2.4 Amino acid composition

The detailed procedure of hydrolysis of proteins using PITC method is described in Chapter 2.2.11 at according to the method (Shang and Wang 1996) .The whole dried tissue of the organism were weighed and hydrolysed, corresponding derivative were prepared according to the procedure and analysed by reverse phase HPLC using C_{18} column.

3.2.5 Monosaccharide and disaccharide composition from tissue

The detailed procedure of analysis is given in Chapter 2.2.10 as per the modified method by Fengel et al. 1979.

3.2.6 Identification of fatty acid methyl esters

The method described by Harvey 1994, was selected for the fatty acid analysis with some modifications. 10 mg of the crude residue obtained after methanol extraction were dissolved in mixture of 2:1 dichloromethane (DCM): methanol (MeOH). The extracts were combined and evaporated to dryness using rotary evaporation. The extracted residue was subjected to

mild alkaline hydrolysis by refluxing using 6 % methanolic KOH (70 ^{o}C for 6 hours). After the sample was cooled, the neutral lipids were removed using hexane from the alkaline solution. The aqueous layer containing the fatty acid salts was acidified to pH 2. Fatty acids in this polar-lipid fraction were recovered separately into dichloromethane. It is then converted to fatty acid methyl esters (FAMEs) by treating with 10 ml of 12% BF_3/MeOH (Sigma Aldrich) (70^{o}C for 30 minutes). The FAMEs were subsequently partitioned from the reaction solution into dichloromethane. The dichloromethane layer was evaporated to dryness, and the extract was then re-dissolved into 1ml n-hexane for Gas chromatographic analysis.

Analysis of FAMES was carried out by gas chromatography-mass spectrometry (GC-MS) using a Perkin Elmer Clarus GC 680, with MS detector equipped with a non-polar HP ultra-double-fused silica capillary column (30 m, 0.32 mm internal diameter, 0.25 mm film thickness). Operating conditions were as follows: ion source of 200°C electron voltage 70 eV. Spectra were scanned from 50 to 600 m/z with a scan time of 1.50 seconds. A two-step temperature program was used: from 50°C to 200°C at 2°C per min. It is then held for 5min. Then temperature again increases from 200°C to 280°C at 10°C per min. It is again held for 10 min. The detector was held at 290°C and helium was used as carrier gas. Data acquisition was obtained with the use of MS turbo mass version 5.3.2. The Fatty acid methyl esters were identified using the NIST (National Institute of Standard Technology version 2.1) library spectra by matching with those obtained for the compound from the fraction of fatty acid methyl esters.

Part II Significance of bioactive compounds

3.2.7 *In silico* biological activity of identified compounds

In silico biological activity of the identified compounds by GC-MS from *S. reticulata* was studied using PASS (Prediction Activity Spectra of Substances). PASS is a software product designed as a tool for evaluating the general biological potential of an organic drug-like molecule. This tool agree to estimate the probable profile of biological activity of a drug like organic compound whose molecular mass ranges from 50 to 1250Da. PASS provides simultaneous predictions of many types of biological activity based on the structure of organic compounds (Filimonov and Poroikov 2006, 2008). Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their biological testing (Lagunin et al. 2010). PASS has been well accepted by the community since 2000 and is now actively used in the field of medicinal chemistry for the prediction of biological activities. The average prediction accuracy calculated and is about 95 % (Filimonov et al. 2014). There are more than 200 publications from researchers using PASS (www.geneexplain.com/pass). The values obtained after running the software denoted by P_a and P_i, P_a (Probability to be active) estimates the chance that the studied compound is belonging to the sub class of active compound. P_i (Probability to be inactive) calculate approximately the chance that the studied compound is belonging to the subclass of inactive compounds.

3.2.8 Antimicrobial properties of extracts

The crude extracts, ethyl acetate and hexane fractions of the organism were screened for antimicrobial activity by disc diffusion method using the Kirby-Bauer technique (Bauer et al. 1966). The test was done on Mueller-Hinton agar for bacterial pathogens and Saboraud dextrose agar for yeast pathogen and expressed as diameter (mm) of the inhibition zone. The antimicrobial activity was observed after 24 hours incubation at 37 ^{o}C for

bacteria and 48 hours incubation at 28 ^{o}C for yeast. The antimicrobial activity of the extracts was tested against bacterial and yeast pathogens associated with human diseases viz. *Escherichia coli, Klebsiella* sp, *Proteus* sp, *Enterobacter* sp, *Bacillus* sp, *Planococcus* sp and *Candida* sp (yeast pathogen) and those associated with fish diseases viz. *Vibrio parahemolyticus* (MCCB 141), *V. harveyi* (MCCB 151), *V. alginolyticus* (MCCB142) and *Aeromonas* sp (MCCB 152). The pathogens were obtained from DDRC SRL Pvt Ltd, Panampaly Nagar, Cochin. The crude methanol extract was partitioned using various solvents like hexane, ethyl acetate, and water. All the solvent extracts were screened for antimicrobial activity by disc diffusion method using the Kirby-Bauer technique (Bauer et al. 1966) for the blank.

3.3 Results and Discussion

Part I. Biochemistry

3.3.1 Preliminary qualitative tests

Table 3.1 Preliminary test results of various extracts for biochemical compounds.

Compound tested	In methanol extract	In ethyl acetate extract	In hexane extract	In water extract
Alkaloids	+	+	+	+
Steroids	+	+	+	-
Flavanoids	+	+	_	+
Sesquiterpene	+	+	+	-
Saponins	+	_	_	+
Carbohydrate	+	_	_	+
Protein	+	_	_	+

+ indicates the presence of the compounds
-indicates the absence of the compounds.

3.3.2 Quantitative estimation

Protein, carbohydrates, lipids are molecules which serve for an organism for essential physiological functions. The main substances found in every cell are a combination of lipids, carbohydrates and Proteins. Fats are broken down and forms individual fatty acids and cholesterol molecules. Fatty acids and cholesterol are key components of the membranes that surround the cells. Carbohydrates are profound source of energy for most of the tissue in the living organisms. They are converted to glucose, which can be used as a source of energy stored in the form of glycogen. Fats provide more calories per gram than protein and carbohydrates. The quantitative estimation of these protein carbohydrates and lipids from the gorgonin coral *subergorgia reticulata* is found to be rich in lipids and proteins. Infact carbohydrates were found to be less compared to proteins and lipids. According to Willimer et al. 2000, the value of carbohydrates will change commonly to the conditions of temperature tropic stress and under food deprivation. The high lipid content 485.6±2 μg/mg (table 3.2) was found to a pointer that the organism is using longer term food constrains. According to Rossi and Tsounis 2006, lipids are considered to be good indicator to detect the longer term food. It has been reported that the gorgonian species *P.clavata* had found to have low lipid level in the Medes Islands during July 2003. So temperature here becomes the major factor in the depletion of biochemical factors (Coma et al. 2002). In fact it has been suggested that the organism *S.reticulata* was growing with high storage energy levels of lipids and proteins.

Table 3.2 Results of quantitative estimation of protein, lipids and carbohydrates

Protein (μg/mg)	Carbohydrate (μg/mg)	Lipids (μg/mg)
425.98±2	83.24± 1	485.6±2

3.3.3 Identification of compounds by GC-MS

GC MS analysis of the volatile compounds from *Subergorgia reticulata*

Most of the compounds from *S. reticulata* are found to be unknown and having very complex structure it cannot be possible to predict the structure with comparison with the NIST (National Institute Standard Technology) library. Those shown complete match with the spectrum obtained for the molecule and from the NIST library are shown below (Table 3.3). The compounds detected were a)1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl) ethyl) b) 2,6,6-Trimethyl-2-cyclohexene-1,4-dione c) Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl) d) Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl] e) cis,trans-1,7-Dimethylspiro[5.5]undecane f) trans-2-methyl-4-n-pentylthiane, S,S-dioxide g) Phenol 2,4,bis 1,1dimethylethyl and h) Vitamin E. There were no reports available for these compounds isolated from this gorgonian coral *S.reticulata*. Some compounds were previously found in nature and some were found to be toxic according to literature. (2R)-2,5,7,8-tetramethyl-2-(4R,8R)-4,8,12-trimethyl tridecyl)-6 chromanol is commonly known as Vitamin E, it is one of the most important lipophilic antioxidant compound found in plants and animals to prevent cells against lipid peroxidation. It was first isolated from salmon eggs and there has reports for the presence from the cold water marine organisms (Yorihiro Yamamoto et al. 2001).The compound 5 ethoxy 6(1-4 methoxyphenyl ethyl 1,3 benzodioxole is structurally similar to podophyllotoxin, which have the propery antimitotic activity (Batra et al. 1986). Podophyllotoxin is a non alkaloid toxin lignin found in the roots of Podophyllum species. It shows a range of activities such as purgative, antiviral, vesicant, and antihelminthic and its derivatives are existing leads for antitumor agent. This phodophyllotoxin is the pharmacological precursor for the important cancer drug etoposide (Gordaliza et al. 2004, Damayanthi and Lown, 1998). 2, 6, 6-trimethyl 2 cyclohexane 1, 4 dione is also

known as 4 keto isophorone and is the major component of saffron spice. It is a cyclic olefin and was reported as a product of the thermal degradation of β-carotene in aqueous suspension (Maggi et al. 2010).

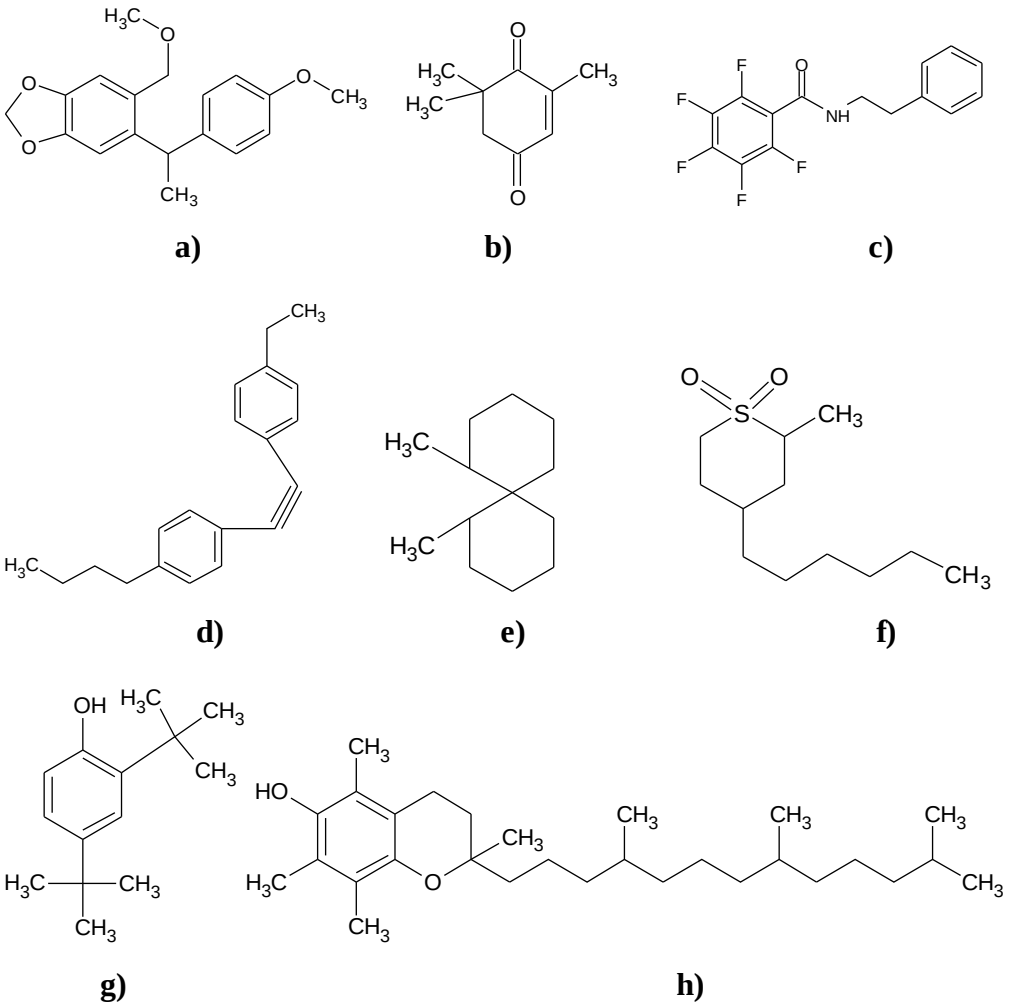

Fig 3.1 Structure of compounds obtained from *Subergorgia reticulta* methanolic fractions by GCMS analysis.

a) 1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl)ethyl) **b)** 2,6,6-Trimethyl-2-cyclohexene-1,4-dione **c)** Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl) **d)** Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl] **e)** cis,trans-1,7-Dimethylspiro[5.5]undecane **f)** trans-2-methyl-4-n-pentylthiane, S,S-dioxide **g)** Phenol 2,4,bis 1,1dimethylethyl **h)** (2R)-2,5,7,8-tetramethyl-2-(4R,8R)-4,8,12-trimethyl tridecyl)-6 chromanol

Table 3.3 Compounds from purified methanol extract of *Subergorgia reticulata* by GC-MS.

SL No	Retention Time	Compound	Molecular weight	Molecular Formula
1	14.32	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	152	$C_9H_{12}O_4$
2	16.939	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	218	$C_{11}H_{22}O_2S$
3	36.05	Phenol 2,4,bis 1,1dimethylethyl	206	$C_{14}H_{20}O$
4	50.74	Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl]	262	$C_{13}H_{18}O_4$
5	63.71	cis,trans-1,7-Dimethylspiro[5.5]undecane	180	$C_{13}H_{24}$
6	82.25	Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl)	315	$C_{13}H_{18}O_4$
7	88.22	1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl)ethyl)	300	$C_{18}H_{20}O_4$
8	92.44	(2R)-2,5,7,8-tetramethyl-2-(4R,8R)-4,8,12-trimethyl tridecyl)-6 chromanol	430	$C_{29}H_{50}O_2$

3.3.4 Amino acid composition

Amino acids, the building units of proteins are biologically important organic compounds containing both a carbonyl and an amino group. Amino acids play central roles both as building blocks of proteins and as intermediates in metabolism. The chemical properties of the amino acids of proteins determine the biological activity of the protein. Proteins not only catalyse the reactions in living cells, they control virtually all cellular processes (Wagner and Musso1983). Like other animals amino acids play important roles in the lives of corals for production of enzymes, tissue growth and skeleton formation (Goreau et al. 1971) and synthesis of organic matrix (Allemand et al. 2004). It is interesting that unlike other animals at least some of the corals can synthesis the essential amino acids. It was found in 1997 by Fitzgerald and Szmant that some different coral species such as *Montastraea favedata*, *Acropa cervicornis* are able to synthesis at least 15 amino acids, of which eight are essentials.

From the amino acid results of the tissue of the organism *S.reticulata*, it was found that threonine was the most abundant amino acid with a

concentration of 32.062µmol/ml. The concentration obtained for each amino acid is listed in the table 3.4. Argenin and Lysine was at bellow detection limit. Threonine is an essential amino acid which makes up elastin, collagen and enamel protein. Threonine is known for aiding the digestive and intestinal tracts to function more smoothly (www.aminoacidsguide.com). Tissue of gorgonian coral *S.reticulata* consists of collagenous matrix called gorgonin(Goldberg 1976; Barnes 1980).The presence of extreme concentration of threonine amino acid which may be due to the collagenous matrix gorgonin. Threonine is the important amino acid which needed to create glycine and serine that are necessary for the production of collagen, elastin and muscle tissue. It also helps connective tissue and muscles throughout the body strong and elastic (www.vitaminstuff.com/aminoacids-threonine.com). According to Fitzgerald and Szmant 1997, almost all amino acids can be synthesised by Cnidarians. Leucine acts as a fuel for the production of alanine and glutamine in skeletal muscle (Neil and Michael 1974). According to Anitra et al. 2003 total hydrolysable amino acid in unbleached samples ranged from 10-30 µmol/g or 29-86mg amino acid/100gm of coral samples. In fact, 79-120mg/100g were reported in coral for a Caribbean sample of *Montastraes faveolata* with 300 years old band (Nyberg et al. 2002). Aspartic acid is closely associated with the initiation and development of a coral skeleton, due to the strong interaction between aspartic acid and Calcium ions (Tong et al. 2004).Goldberg 1976 found that the aminoacid compostion of some corals contained mainly glycine, proline and hydroxyl proline. Collagen has a distinctive amino acid composition, nearly one third of its residue are glycine and another 15-30% are proline and hydroxyproline (Istin 1975; Voet and Voet 1990).

Table 3.4 Amino acid composition from tissue

Amino acid	Concentration (µmol/ml)	Concentration (%)
Aspartic acid	32.062	14.99
Glutamic acid	22.113	10.33
Serine	0.965	0.451
Histidine	1.009	0.471
Glycine	9.591	4.48
Threonine	36.816	17.215
Arginine	ND	ND
Alanine	21.882	11.634
Tyrosine	24.578	11.49
Cysteine	1.364	0.637
Valine	0.644	0.301
Methionine	6.300	2.94
Phenylalanine	20.901	9.77
Isoleucine	5.442	2.54
Leucine	30.192	14.11
Lysine	ND	ND

*ND Not Detected

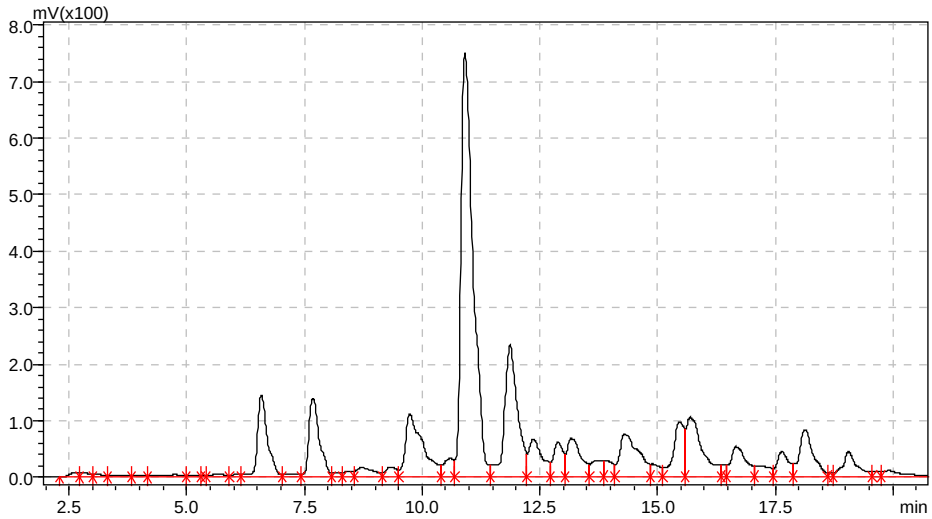

Fig.3.2 chromatogram of amino acid composition.

3.3.5 Monosaccharide and disaccharide composition from tissue

Gorgonin corals use their tentacles to capture zooplankton; they are small animals that live in water. Most corals only extent their polyps and tentacles at night when zooplankton is most abundant. Many corals have single celled algae called zooxanthallae. The algae use solar energy to convert CO_2 from seawater into energy rich sugar and fats. A major fraction of this food is given to the coral animals and helps the coral grow and produce its skeleton faster than a coral without the zooxanthallae; it is a mutuality symbiosis. (http://www.coexploration.org/bbsr/coral/html/body_basic_coral_biology.html).Carbohydrates are one of the main component of dissolved organic matter released by phytoplankton along with amino acids and fatty acids (Degens 1970; Handa 1970). It is well known that when nitrate has become drained the proportion of carbohydrates and thus C/N ratio increases as in the form of reserve carbohydrates (Mc Allister et al. 1961; Antia et al 1963; Myklestad 1974; Brockmann et al. 1977). The composition of extracellular monosaccharide varies, and also species specific (Allan et al. 1972). Sulphated glycoproteins and small low molecular weight compounds are the major component of coral mucus (Meikle et al. 1988). Glycosides, proteases and esterase are considered to be the growth substrate in corals (Vacelet and Thomassin 1991; Krediet et al. 2013). Arabinose was the sugar present in the maximum concentration by total percentage of 53.4 followed by ribose (20.68%), xylose (9.4%), glucose (8.41%), and galactose (8.27%) (Table 3.5).

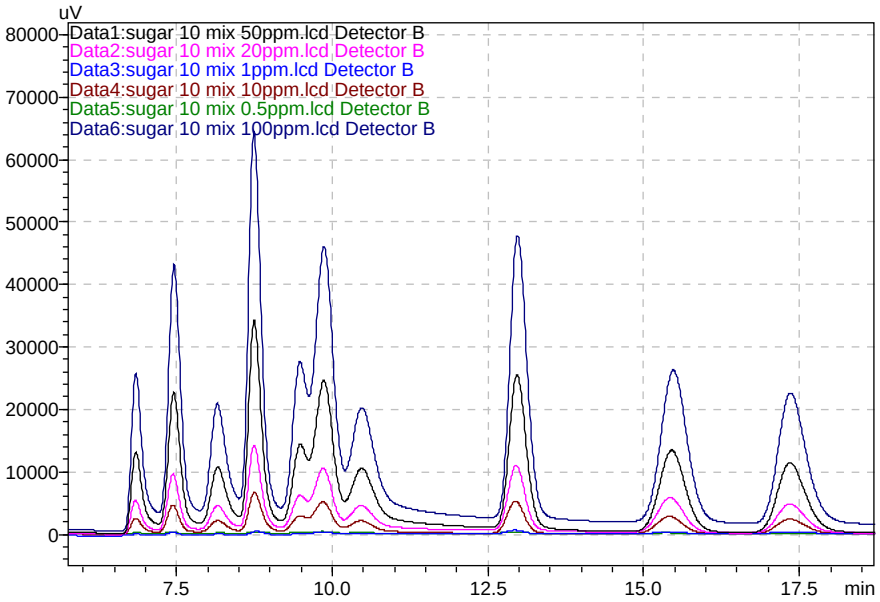

Fig 3.3 HPLC chromatogram of standard sugars with six different concentrations

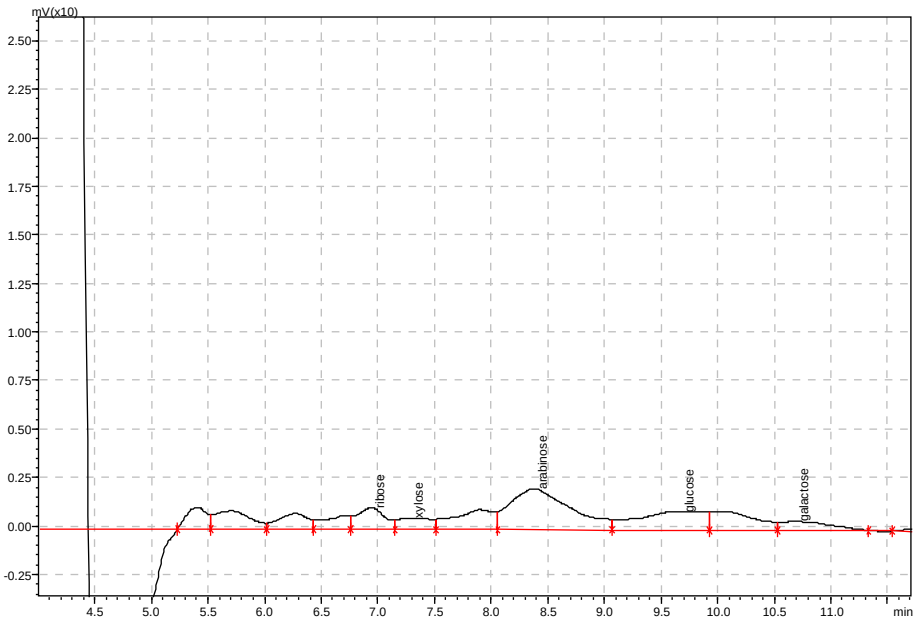

Fig 3.4 HPLC chromatogram of sugar units present in *S.reticulata* tissue

Table 3.5 Monosaccharide and disaccharides from *S.reticulata*

Name	Retention time	Monosaccharide Composition (%)
Ribose	6.771	20.68
Xylose	7.35	9.14
Arabinose	8.024	53.4
Glucose	9.312	8.41
Galactose	10.229	8.27

3.3.6 Identification of fatty acid methyl esters

Lipids are the main source of stored energy in the Coelentrates. Zooxanthallae associated with corals are important primary producers in the nutritional chain in tropical shallow water communities (Benson and Muscatine 1974; Patton and Burris 1983; Stimson 1987). It has been shown by many authors that photo synthetically fixed carbon are rapidly transformed into lipids. Which are then carried into 'host' tissues in the form of droplets consisting of triglycerides wax esters and free fatty acids (Patton and Burris 1983).These compounds lipid are the main sources of saturated fatty acids. Studies on fatty acids of reef building corals have not so far been systematic, although there are reports on the fatty acid composition of some lipid classes (Benson and Muscatine 1974; Patton and Burris 1983). Representatives of coelenterate phylum have remarkable peculiarities in their fatty acid composition. In general, compared with Octocorallia, saturated acids are significantly more abundant in Hexacorallia, in which they account for 40-50% of total lipids (on an average up to 75 % is possible) (Yamashiro et al. 1999). The content of polyunsaturated acids is about 35-50% with exception of low content 6%. The main fatty acids found in Anthozoa are C_{18}, C_{20}, C_{22}, and C_{24} acids, having two to six double bonds, and belongs to n-3, n-6 series.

Twenty four fatty acid methyl esters were detected using GC-MS analysis (Table 3.6). The lowest molecular weight fatty acid were found to be 2- methyl heptanoic acid (MW: 144 and molecule formulae $C_8H_{16}O_2$) and the highest molecuer weight fatty acids were found to be tetracosanoic acid methyl ester (MW: 382 and MF: $C_{25}H_{50}O_2$).The total ion chromatogram of the fatty acid methyl ester compounds were shown in the Fig. 3.5

According to Mark et al., 2011 the main source of fatty acids for corals are from the symbiotic zooxanthellae. Zooxanthellae contain an abundance of saturated fatty acids ARA, and DHA these compounds are transferred to the host corals. Zooxanthellae can provide up to more than 100% of daily requirement of the host healthy corals (Muscatine et al. 1981, Grottoli and Palardy 2006). In some cases heterotrophic feeding can be a significant source of fatty acid and can account more than 50% of fatty acids (Muscatine 1990). Heterotrophic food sources to sustaining coral health is more evident by the study from Muscatine et al. 1989; Grottoli and Wellington 1999; Houlbreque and Ferrier Pages 2008; Mark et al. 2011). However corals can acquire their essential biochemicals such as fatty acids from both autotrophic and heterotrophic sources.

Only very few attempts were done to study about the biosynthesis of lipid in marine invertebrates (Chu and Greaves 1991) and the pathway of polyunsaturated fatty acids biosynthesis in corals are virtually unexplored. To explain the observed peculiarities of fatty acid composition of corals it can be possible to apply the general principles of PUFA biosynthesis that have been established for plants and animals (Imbs 2013).

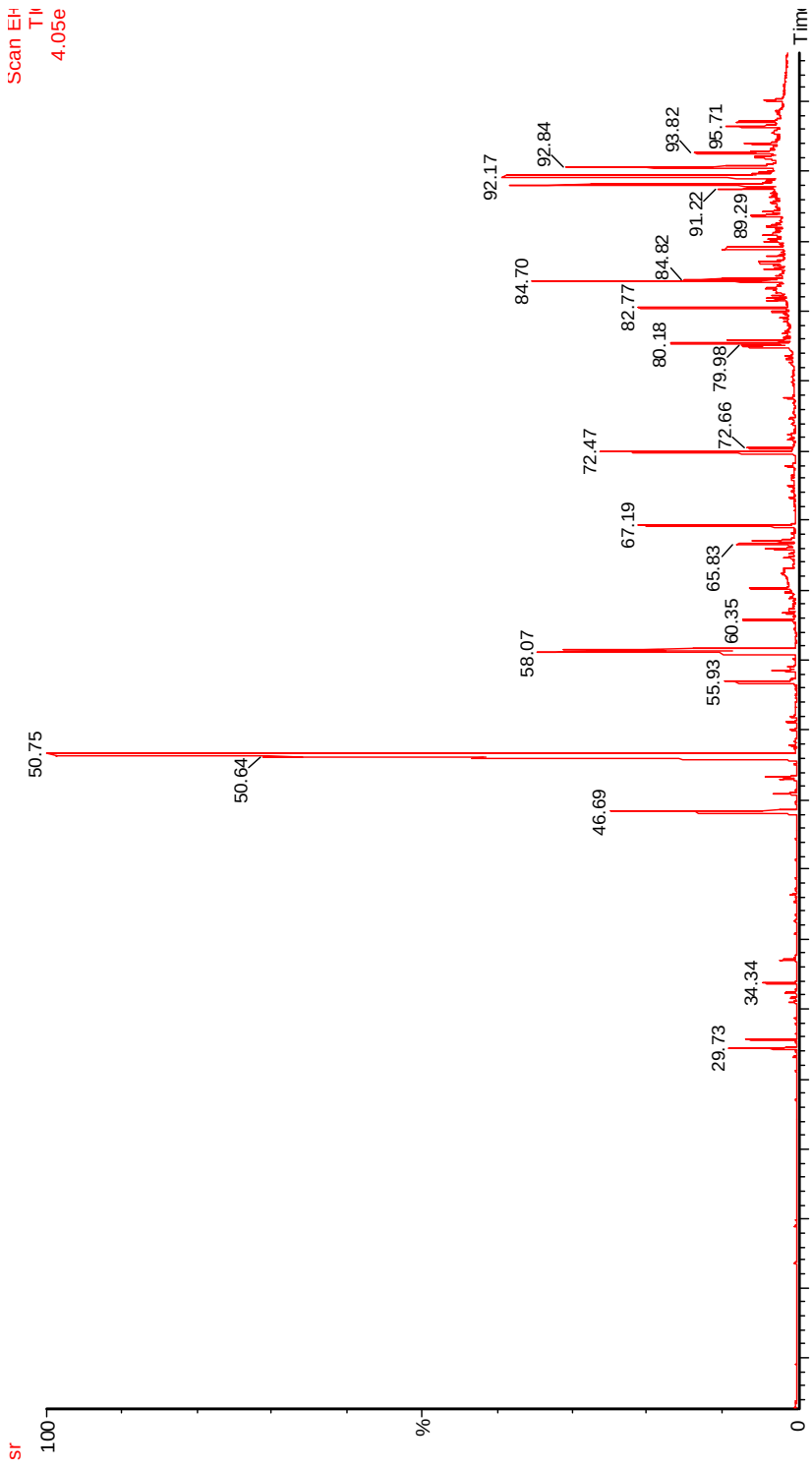

Fig. 3.5 Total ion chromatogram of fatty acid methyl ester compounds by GC-MS analyis.

Table 3.6 Total fatty acid methyl ester compounds from *S. reticulata*

SL No	Retention Time	Compound	Molecular weight	Molecular Formula
1.	40.70	Chlorocetic acid tera decyl ester	290	$C_{16}H_{31}O_2Cl$
2.	47.91	Methyl 12 methyl tridecanoate	242	$C_{15}H_{30}O_2$
3.	51.16	2-methylheptanoic acid	144	$C_8H_{16}O_2$
4.	53.03	Methyl 13 methyl tetradecanoate	256	$C_{16}H_{32}O_2$
5.	56.77	Methyl 11-hexadecanoate	268	$C_{17}H_{32}O_2$
6.	60.35	6-hexadeacanoic acid-7methyl methyl ester	282	$C_{18}H_{34}O_2$
7.	60.90	Methyl 15-methyl hexadecanoate	284	$C_{18}H_{36}O_2$
8.	64.85	Methyl 8,11,14,17,eicosatetraenoate	318	$C_{21}H_{34}O_2$
9.	66.08	Methyl 11-octadecenoate	296	$C_{19}H_{40}O$
10.	67.18	Methyl-16methyl heptadecanoate	298	$C_{19}H_{38}O_2$
11.	71.44	Tetradecanoic acid 10,13 Dimethyl methyl ester	270	$C_{17}H_{34}O_2$
12.	72.47	5,8,11,14 Eicosatetranoic acid methyl ester(all z)	318	$C_{21}H_{34}O_2$
13.	73.34	Methyl 8,11,14 Eicosatetraenoate	320	$C_{21}H_{36}O_2$
14.	73.62	Methyl 8,11,14,17 Eicosatetraenoate	318	$C_{21}H_{34}O_2$
15.	74.44	Methyl 12,15 octadecandienoate	294	$C_{19}H_{32}O_2$
16.	76.30	Eicosanoic acid,methyl ester	326	$C_{21}H_{42}O_2$
17.	79.04	4methyl -5pentyl furyl 2-undecanoic acid methyl ester	350	$C_{22}H_{38}O_3$
18.	80.18	4,7,10,13,16,19-Docosa hexadecanoic acid methyl ester(all z)	342	$C_{23}H_{34}O_2$
19.	80.44	Methyl 7,10,13,16 Docasatetraenoate	346	$C_{23}H_{38}O_2$
20.	80.59	n-propyl 5,8,11,14,17-Eicosapentaenoate	344	$C_{23}H_{36}O_2$
21.	81.77	13-Docasoenoic acid methyl ester	352	$C_{23}H_{44}O_2$
22.	82.45	Methyl 20-methyl heneicosanoate	354	$C_{23}H_{46}O_2$
23.	83.25	2,2 Dimethyl propanoic acid, heptadecyl ester	340	$C_{22}H_{44}O_2$
24.	86.46	Tetracosanoic acid methylester	382	$C_{25}H_{50}O_2$

Part II. Significance of Bioactive compounds

3.3.7 *In silico* biological activity study of identified compounds

The identified compounds by GC-MS were selected for the *In silico* prediction of biological activity by PASS (Prediction of Acivity Spectra of Substances). The eight compounds which were selected under the study are a)

1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl)ethyl) b) 2,6,6-Trimethyl-2-cyclohexene-1,4-dione c) Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl) d) Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl] e) cis,trans-1,7-Dimethylspiro[5.5]undecane f) trans-2-methyl-4-n-pentylthiane, S,S-dioxide g) Phenol 2,4,bis 1,1dimethylethyl h) (2R)-2,5,7,8-tetramethyl-2-(4R,8R)-4,8,12-trimethyl tridecyl)-6 chromanol. The different possible biological activity of the compounds were analysed by the Probability of activity (Pa) values. Those activity which were shown the probability of activity >.70 were taken into account since the possibility of these compounds to be active is high. Numbers of biological activity were shown by these compounds which have both pharmaceutical as well as economical importance. The important biological activity shown by most of the compounds were Antiezmatic, Carminative, anti-inflammatory, anti diabetic, phobic disorder treatment, Antiseborrheic and Antihypertensive. Vitamine E is previously known as the best antioxidant since it is used ever in the pharmacy industry as a strong antioxidant, the value shown by PASS prediction also tells its probability to be active is (Pa) .96. From the *in silico* prediction of biological activity it was very clear that the identified compounds have pharmacological importance. The compound cis, trans-1, 7-Dimethylspiro [5.5]undecane is found to be active for Antiobesity(Pa .976), anti hypertensive(Pa .624), antidiabetic (Pa .880), carminative (Pa .833), antiseborrheic (Pa .798) and antiviral (Pa .976). All of these activities except antiviral shown by cis, trans-1,7-Dimethylspiro[5.5]undecane having probability more than 70%. The compound Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl) is active for phobic disorder treatment with a probability factor of .874, anti neurotic(Pa

.710).antiezmatic activity (Pa .721). From the eight compounds detected three compounds were shown to posses antiezmatic activity such as Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl](Pa .946), 2,6,6-Trimethyl-2-cyclohexene-1,4-dione (Pa .681) and cis,trans-1,7-Dimethylspiro[5.5] undecane (Pa .790). Vitamin E which was already reported for its pharmacological importance and here from PASS prediction also showed activity such as antioxidant, acute neurological disorder treatment, apoptosis agonist, wound healing agent and anti-inflammatory. The major biological activity shown by the eight compounds were listed in the Table 3.7 below.

Table 3.7 The biological activity predicted by PASS for the identified compounds.

Compound	Biological activity predicted by PASS
1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl)ethyl)	Carminative, Acaricide, Fibrosis treatment.
2,6,6-Trimethyl-2-cyclohexene-1,4-dione	Carminative, Anti-inflammatory,anti eczmatic, cytoprotectant.
Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl)	Phobic disorder treatment, ant neurotic
Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl]	Anti-eczematic, Carminative, Kidney function stimulant
1,3,7-Trimethylpurine-2,6-dione	Growth hormone agonist
cis,trans-1,7-Dimethylspiro[5.5]undecane	Anti obesity, Anti diabetic
trans-2-methyl-4-n-pentylthiane, S,S-dioxide	Mucous membrane protector
Undecane	Sclerosant
(2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol	Antioxident, wound healing agent,acute neurological treatment, apoptosis agonist, anti inflammatory

3.3.8 Antimicrobial properties

Antimicrobial activity of some of the natural products has led to the discovery of new effective drugs (Choi et al. 1999). Due to the increase in resistance to antibiotics by several pathogens, marine extracts are of new interest as antimicrobial agents to combat infectious diseases. There are so many previous studies had done on screening of marine extracts for

antimicrobial activity particularly from Indian coasts (Vanisree and Subharajan 2002; Venkateshwar et al 2002; Ramasamy and Devadason 2003; Rodrigues et al. 2004; Tilvi et al. 2004). Corals are able to control microbe population with immune defences with the production of secrete chemicals that can inhibit on microbial growth (Slattery et al. 1995; Brown and Bythell 2005; Mydlarz and Jacob 2006; Mydlarz and Harvel. 2007). In the Caribbean corals, studies have reported that gorgonians possess compounds with antimicrobial activity against marine bacteria and also tested whether these compounds have significant role for the microbial defences (Kim 1994; Jensen et al 1996). The first report came for the antimicrobial activity of sponge extracts was published by Nigrelli et al. 1959. According to Jensen et al. 1996, the maintenance of broad spectrum antimicrobial activity is not a leading factor in the evolution of chemical defences in gorgonians; it is also necessary to address other mechanisms for microbial regulations in gorgonians. So many studies have reported antimicrobial activity of extracts from marine soft corals (Kelman et al. 2001; Harder et al. 2003). According to Laura et al. 2012 ethanol extracts of eight gorgonians showed significant and variable antimicrobial activity against Gram negative and Gram-positive bacterial stains and is relatively more against Gram- positive bacteria compared to Gram negative bacteria.

The results of the primary screening for antimicrobial activity which measures diameter of growth of inhibition is summarised in the Table 3.7 and Fig. 3.6. According to the results, the ethyl acetate fraction was active against *Escherichia coli* (12mm), *Klebsiella* sp (12mm), *Proteus* sp (10mm), *Planococcus* sp (15mm) against *Vibrio parahemolyticus* (9mm). The crude

fraction exhibited activity against *Escherichia coli, Klebsiella* sp, *Proteus* sp, *Planococcus* sp and *Vibrio parahemolyticus* (MCCB 141).The ethyl acetate fraction was active against *Escherichia coli* (12mm), *Klebsiella* sp (12mm), *Proteus* sp (10mm), *Planococcus* sp (15mm) against *Vibrio parahemolyticus* (9mm). From these results it seems to be posses different activity according to the polarity. The antimicrobial effects of body wall extracts of starfish *Pentaceraster affinis* have been studied earlier (Prabha Devi et al. 2001) and the activity was attributed primarily to saponins and saponin like compounds. According to Prabha Devi et al. 2011 invertebrates belongs to phylum coelenterates were exhibited mild activity against all pathogens except for moderate fungicidal activity against *Fusarium sp*, and *Nocardia sp*; the study also claims that the activity of soft corals of the genus *Sinularia* and *Labophytum sp*. may be due to soft coral derived terpenes. *Subergorgia reticulata* was found to contained terpene with unique structures which were detailed discussed in the upcoming Chapters. This indication leads to the confirmation of the observation of Prabha Devi et al. 2011,that the activity is due to the presence of terpenes. These studies indicates *S.reticulata* act as a significant source for new antimicrobial compound that as a future drug candidate, more specific studies required for the conformation of which compound is responsible for the antimicrobial activity.

Table 3.7 Zone inhibition of extracts on various pathogens from *S.reticulata*

Pathogens	Ethyl acetate fraction of the sample Zone of inhibition (mm)	Hexane fraction Zone of inhibition (mm)
Escherichia coli	12mm	-
Klebsiella sp	12mm	-
Proteus sp	10mm	-
Planococcus sp	15mm	-
Vibrio parahemolyticus (MCCB 141).	9mm	-

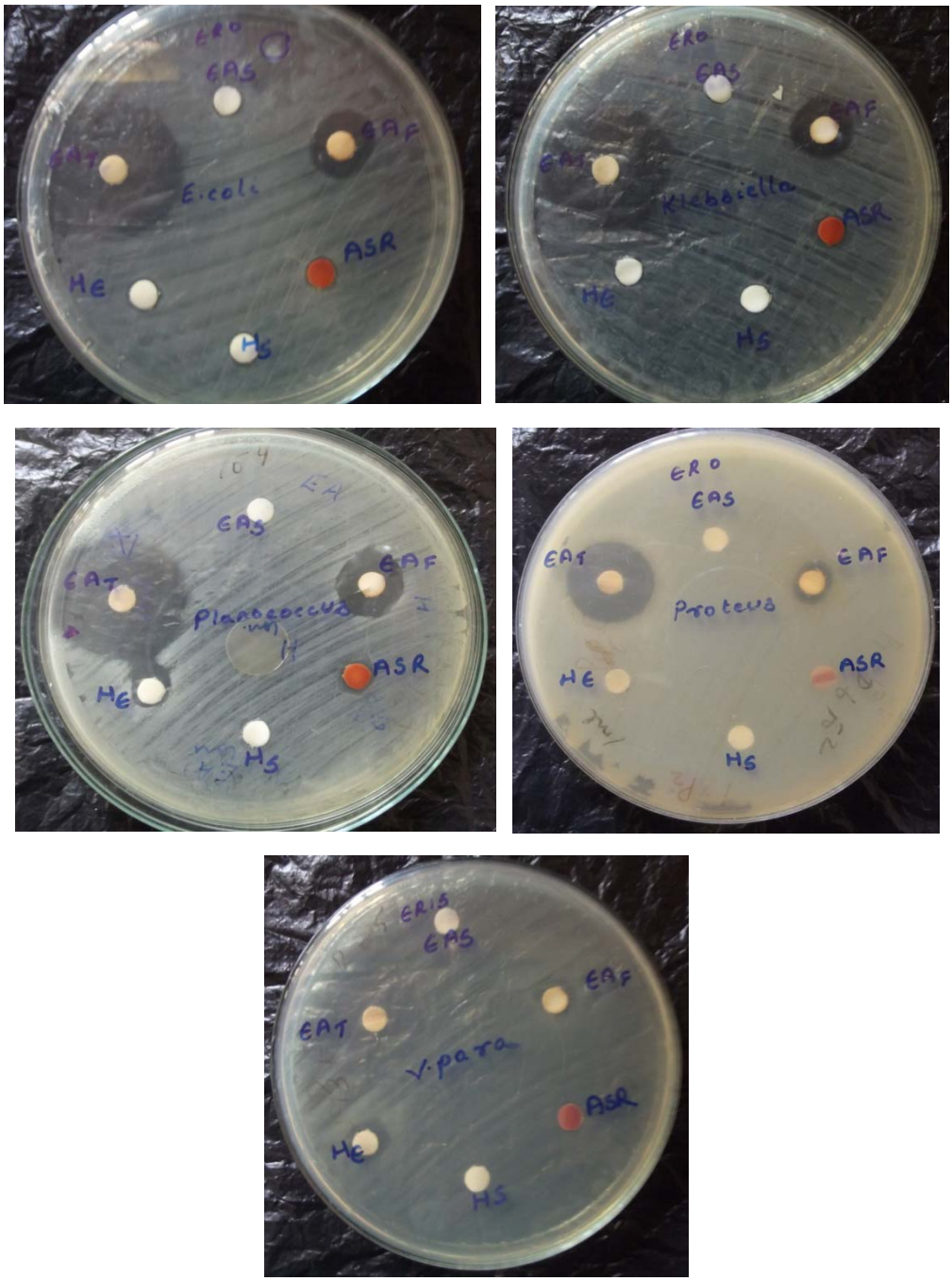

Fig. 3.6 Antimicrobial activity of extracts of *Subergorgia reticulata*

a) Escherichia coli b) Klebsiella sp *c) Proteus* sp *d) Planococcus* sp *e) Vibrio parahemolyticus* (MCCB 141).

3.4 Conclusion

The gorgonian coral *Subergorgia reticulata* were investigated for the biochemical composition, antimicrobial property and *in silico* biological activity studies. From the preliminary examination, it is very clear that almost all types of compounds present in the organism such as terpenes, alkaloids, steroids, carbohydrates, lipids, saponins, flavanoids and sesquterpenes. From the quantitative analysis, protein concentration was high compared to carbohydrates and lipids. It is understood that its tissue is made up collagenous matrix, which is a complex protein. From the preliminary analysis of the volatile compounds in the crude methanol fraction by GC MS analysis, it is confirmed the presence of eight compounds such as 1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl)ethyl) b) 2,6,6-Trimethyl-2-cyclohexene-1,4-dione c) Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl) d) Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl] e) cis,trans-1,7-Dimethylspiro[5.5]undecane f) trans-2-methyl-4-n-pentylthiane, S,S-dioxide g) Phenol 2,4,bis 1,1dimethylethyl h) (2R)-2,5,7,8-tetramethyl-2-(4R,8R)-4,8,12-trimethyl tridecyl)-6 chromanol. There is not much investigation about the chemical constituent of the organism, the identified compounds are seems to be new for this organism. From the sugar unites analysis results, it was found that arabinose is the leading monosaccharide present in the organism with a concentration of 53.4% compared to the other sugar units. Mucus membrane glycosides, which are found to be rich in arabinose and other sugar units were identified as ribose (20.68%), xylose (9.14%), glucose (8.41) galactose (8.27%). There are no previous reports available for the study of monosaccharides and sugar unites present in the organism. Even though studies had done to identify the organic matrix from the coral hard skeleton

and sclerites, still it is a mystery about which sugar units are present in the organic matrix.

Amino acid composition resulted in the presence of both essential and non essential amino acids in the organism. The high concentration of aspartic acid was found and it is very clear that Ca binding proteins which makes the sclerites to be hard, to give more support to the organism. The highest concentrated amino acid detected was threonine and which is an essential amino acid required for building the collaginous matrix, type of protein usually found in the outer tissue part of the gorgonian corals. This collagenous matrix makes the organism hard and flexible for the water column circulation. Other amino acids were also present in small amounts, compared to aspartic acid and threonine.

Twenty four fatty acids compounds were identified using GC-MS by NIST (National Institute Technology) library spectral search. Among them only one is found to be halogen containing fatty acid methyl ester, that is chloroacetic acid tetra decyl ester. Even though there are previous investigations to assess the fatty acid methyl ester composition of some corals, the preesnt study is the first report of fatty acid methyl esters from the gorgonian coral *Subergorgia reticulata*. The lowest molecular weight fatty acid were found to be 2- methyl heptanoic acid (MW: 144 and MF: $C_8H_{16}O_2$) and the highest molecuer weight fatty acids were found to be tetracosanoic acid methyl ester (MW: 382 and MF: $C_{25}H_{50}O_2$).

In silico biological activity studies by PAAS online prediction for the identified compounds, implied that all the eight compounds displayed particular biological activity. The important biological activity shown includes phobic disorder treatment, anti neurotic, anti obesity, Anti diabetic,

Antioxident, apoptosis agonist and anti inflammatory. These findings will be appended for the future drug candidate from *S.reticulata.* From the antimicrobial property of crude as well as ethyl acetate and hexane fractions it was found that ethyl acetate fraction was active against all pathogens including both Gram positive and Gram negative. The maximum zone inhibition shown was against *Planococcus sp.* and least for *Vibrio parahemolyticus.*

In this Chapter, the biochemistry and biological activity of the gorgonian coral was studied and in fact as like all other soft corals the biochemical composition was found to be similar. The important features here found to be the antimicrobial activity of the extracts and the eight identified compounds by GC-MS. The fatty acids compositions are found to be significant study from this organism to understand the relationship between the storage and intake of lipids as food and energy source.

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Chapter

4 CHARACTERISATION OF TERPENES AND BIOLOGICAL ACTIVITY STUDIES

Contents

4.1 Introduction
4.2 Materials and Methods
4.3 Results and Discussion
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Reference

4.1 Introduction

In the chemistry of Natural products, perhaps more than any other compounds, isoprenoids constitute the largest class of Natural products. The discovery of new terpenoids has been increased past ten years as s result of increase in sophistication of separation and analytical techniques (Fischer et al. 1991).The first observation of this distinct group of naturally occurring substances, may be formally derived from branched five carbon unit, was by Wallach 1887.The terpenoids as biosynthesised from branched five carbon modules was first stated by Ruzicka and Stoll (1922). Ruzicka's theory has inspired and guided an incredible amount of imaginative research in the structural determination and in organic synthesis. Terpenoids are found abundance in plants and also found in some insects and marine organisms. In the marine environment, many substituents of terpenes that are rarely found in terrestrial natural products occur. It is not possible to justify whether the distribution of various terpenoids in marine organisms parallel that found in terrestrial environment. Recent research into marine natural products has been shown that marine organisms are a prolific source of unusual terpenoids (Andersen 1990). Few ubiquitous monoterpenes such as geraniol α-pinene

have been reported from algal sources and further it was confirmed by Katayama 1962.Many substituent that are rarely found in natural product occurs in marine terpenoids. For example bromine and chlorine substituted terpenoids and isonitrile and isothiocyanate substitutes are studied and reported as a series of reviews (Hanson 1983, Banthorpe and Branch 1985, Beale and Macmillan 1988).

Monoterpenes are widely distributed in secretion tissues, such as oil glands or chambers and resin canals of higher plants, insects, fungi, and marine organisms. These are major constituent of volatile oils of plants. Monoterpenes occurs in more than 30 different known carbon skeleton, among them 20 are common and can be divided in to acyclic, monocyclic, and bicyclic types (Fig. 4.1) (Remigius 2013). Due to the economic importance, monoterpenoids have been studied with great interest over the past few decades (Charlwood and Charlwood 1991). Volatile monoterpenoids contribute to flavours and aromas in food and are important in perfume industry (Rozenbaum et al 2006).The importance of monoterpenoids and their glycosides in grapes contribute to the aromas of wine (Rapp and Mandery 1986). Limonene is the most abundant monocyclic monoterpene found in nature. It has wide range of flavours and medicinal properties in the same skeleton (Santos et al. 2010). The monoterpenes are found to posses a variety of biological activity including anti bacterial, sedative, antitumor, cytotoxic, anti inflammatory, insecticidal and molluscidal. Menthol is one of the examples used for antipruritic, analgesic preparations, antiseptic and flavouring agents. Linalool a monoterpene alcohol found to be inhibiting cell proliferation (Kamatou et al. 2013;Kehai et al.2012). Limonene is established for chemo preventive and therapeutic agent (Crowell 1999, Fabian 2001, Kris Etherton et al 2002). The mechanisms of action of monoterpene was studied

for the induction of apoptosis and was found to successful for various compounds coming under this category(Crowell 1999,, Fabian 2001, ,Crowell et al 2002).

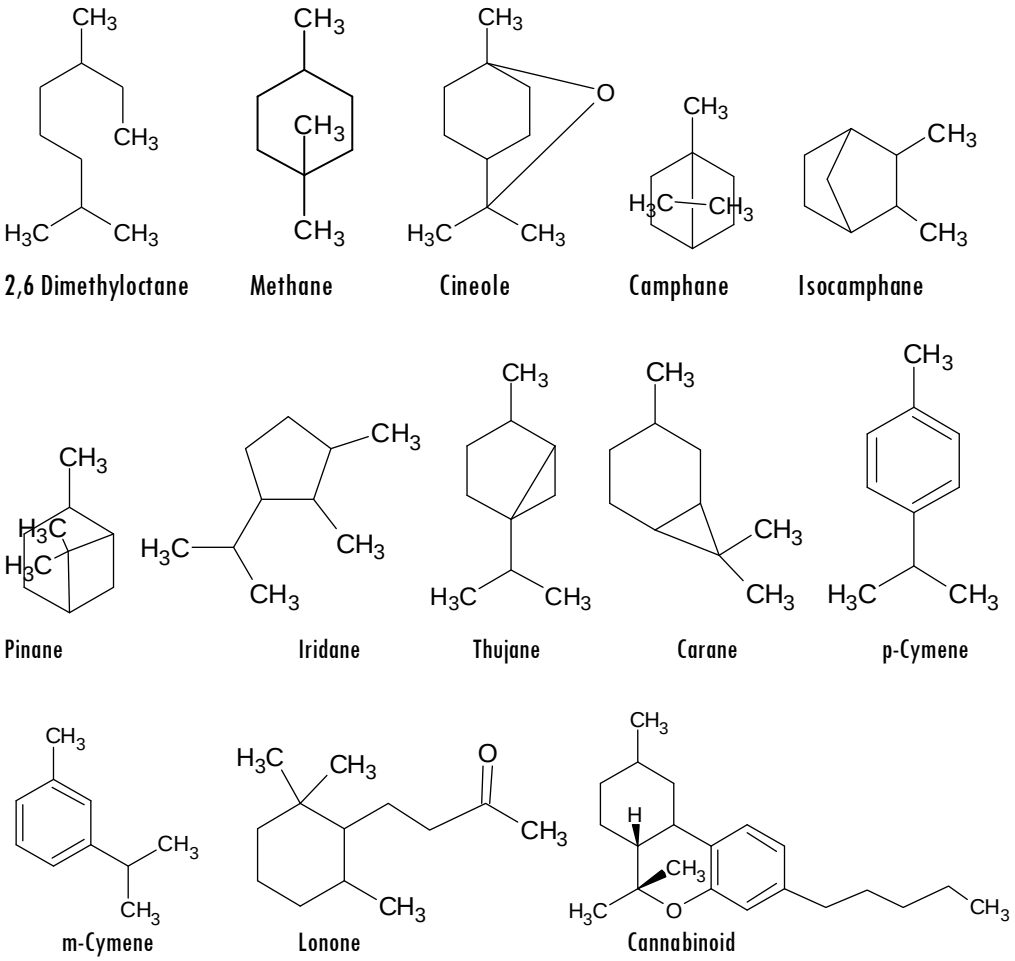
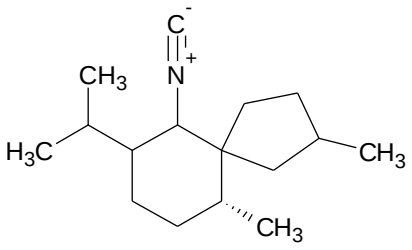


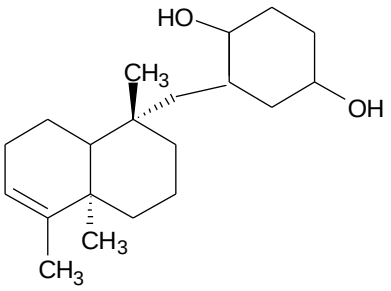
Fig.4.1 Monoterpene skeleton.

The largest; yet still very modest, number of terpenoids from marine sources have been studied are sesquiterpenoids. The sesquiterpenoids which are found in nature are similar properties to monoterpenoids and are generally less volatile than monoterpenes (Robbers et al. 1996; Heinrich et al. 2004, Dewick 2009). Sesquiterpene lactones are derivatives of sesquiterpenes widely

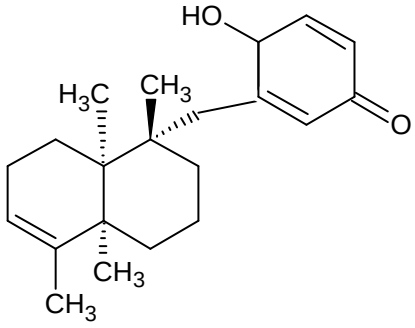
distributed in marine and terrestrial natural product and are well known for their biological activity (Asakawa et al 1986,Abraham 2001). Sesquiterpenes from marine environment were also found to be posses biological activity. Axisonitrile-3 (Fig.4.2) is an example showing biological activity against tuberculosis. Sesquiterpene quinines and hydroxyl quoinones and related compounds were also found to be pharmacologically important (Capon 1995). The important biological activity shown by sesquiterpene quinines and hydroxyl quoinones includes, anti human immunodeficiency virus (HIV) and protein kinase inhibition (Giannini et al 2001). Avarol, avarone (Fig.4.2) isolated from sponge were found to inhibit HIV reverse transcriptase and also found to be strong antibacterial and anticancer active compounds.



Axisonitrile-3



Avarol



Avarone

Fig.4.2 Sesquiterpenoids.

Non volatile terpenoids based on four isoprene units are known as diterpenoids. Abetic acids, Gibberellines, taxol are the well known diterpenoids (Robbers et al 1996, Hanson 2003). One of the simplest important acyclic diterpene is phytol (Fig. 4.3), which is used as biomarker found in aquatic environment. A wide range biological activity was observed for diterpenes from marine as well as terrestrial sources (Sung and Chen 2002, Bruno et al 2002, Hanson 2003). Eleutherobin isolated from marine soft coral *Eleutherobia* were shown to posses cytotoxic effect, an analogue of Eleutherobin (Fig. 4.3) ie, Eleuthesides were abundant in Caribbean gorgonian coral (Britton et al 2001, Cinel et al 2000). The extracts of the gorgonian coral *Pseudopterogorgia elisabethae* were found to be anti inflammatory and used as an ingredient in skin care products (Proksch et al 2002). Solenolide A, a diterpene lactone, isolated from gorgonian species of the genus *Solenopodium* were reported to inhibit rhinovirus, poliovirus, herpesvirus. According to Bryant 1969, more than 200 sesquiterpenoids, divisible in to 40 types, had been characterised and it is a justification for great versatility of these compounds, which are made by only fifteen carbon atoms. Up to 1971 the number of marine sesquiterpenoids have been characterised is fewer than 40 and is from only four marine phyla, the coelenterates(Cnidarians), the molluscs(Mollusca),the brown algae (Phaeophyta), and the red algae(Rhodophyta).

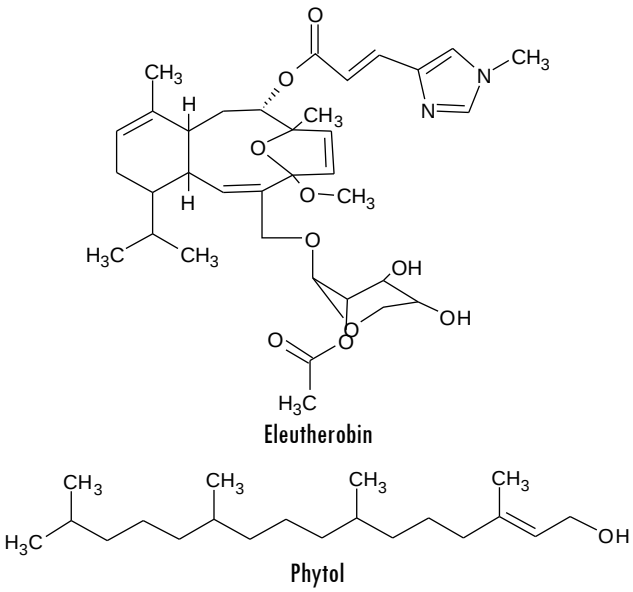


Fig. 4.3 Diterpenoids.

Sesterterpenoids are small group of natural products that occur mainly in marine organisms and some of them are found in terrestrial life. Even though they strictly contain $C_{25,}$ much compounds are there as nor and alkylated derivatives (Jaspers et al 2002). Triterpenoids having six isoprene units are widely distributed in plants, micro organisms, animals, and humans. Examples are steroids, which functions as sex hormones (Robbers et al 1996, Heinrich et al 2004). A few diterpenoid including C_{19}, C_{21} and a number of closely related triterpenoids of unique structure have been reported so far. Friedelin and taraxerol are the two widely distributed triterpenoid (Santos and Doty 1971).

The research to synthesis terpenoid compounds mainly for pharmaceutical applications are still a tedious task due to the similarity in carbon skeletons. Terpenoids from the terrestrial natural products are almost identified and still researches are continuing to come across any new mysterious compounds. Gas chromatography and GC MS technique are mainly used to analysis these volatile components.

4.2 Materials and Methods

4.2.1 Extraction, isolation and purification

The organisms were collected according to the procedure detailed in Chaper 2.2.1. The extraction procedure adopted here is the method given by Bhakuni and Rawat 2005 with some modifications. The extraction of the fragmented organism *S. reticulata* (850gm) was carried out at room temperature with methanol (Merk chem AR grade) for 4 days. The extraction was repeated until, it left negligible residue on removal of the solvent under reduced pressure. The residue was dissolved in minimum quantity of aqueous methanol (80%). In an effort to further characterise the chemical constituents in the original sample, the aqueous methanol sample was fractionated by partitioning with hexane and ethyl acetate**.** The ethyl acetate fraction concentrated under vacuum and weighed. The crude residue obtained after ethyl acetate partitioning was subjected to column chromatography by silica gel (Sigma Aldrich 60-120mesh) with hexane and ethyl acetate as eluent. Hexane: Ethyl acetate fraction were pooled according to the polarity range and thin layer chromatography examination.

4.2.2 Purification by flash chromatography.

From the thin layer chromatography similar fractions were combined and concentrated under vacuum. The residue again mixed with silica. The purification was done by Isolera 1 Flash chromatographic system equipped with UV VIS detector (Biotage). The wavelengths selected for the separation of terpenoid compounds were 210nm and 254nm. Silica fine cartridge, 15mm and 10cm were used for the fine purification. The active fractions were collected in test tube and tested by TLC and GC for the final purity of the compound.

4.2.3 Purification by HPLC

The fractions collected in test tube showed three major spot in TLC and further separated using preparative HPLC with C_{18} (octadecyl) reversed phase column (Supelco Sigma Aldrich) having 5µ particle size 10mm inner diameter and at two elengths 210nm and 254nm l. Injector with a 2.0ml loop and a differential UV VIS detector were used. The sample was dissolved in pure acetonitrile (Merck HPLC grade) and 1 ml injected. The total run time was at 60 minute using isocratic elution by mobile phase acetonitrile(Merck HPLC grade). The peaks were isolated and tested for the purity by Gas chromatography.

4.2.4 GC-MS analysis.

Gas chromatographic analysis of purified as well as fractions collected were done on Perkin Elmer Clarus 680 GC-MS equipped with headspace sampler (Turbomatrix 40 trap). Helium was employed as carrier gas and the ionizing voltage was 70eV. Oven temperature was programmed at 10°C min^{-1} from 60°C to 290°C and kept at 290° C for 15 minutes. Injector and detector temperature were kept constant at 280°C and 290°C, respectively. The column used was Elite 5MS having 30m length and 250µm id. Mass spectrum of each peak in the total ion chromatogram was resolved and it was compared with National Institute of Standards and Technology (NIST) library spectra for the identification of the compounds.

4.2.5 Structural Characterisation

FTIR analysis was recorded using Spectrum 100 ATR model from 4000cm^{-1} to 600cm^{-1}. ^{1}H, ^{13}C, DEPT, COSY, HMBC NMR were taken using Bruker Avance III, 400MHz instrument with 9.4 Tesla super-conducting Magnet using $CDCl_3$ solvent. Tetramethyl silane was used as the internal standard.

4.2.6 *In silico* Biological screening

In silico Biological screening of the identified compounds by GC-MS from *S. reticulata* was studied using PASS (Prediction Activity Spectra of Substances,). Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their biological testing (Lagunin et al 2010).The details about the software tool already mentioned at chapter 3.2.7.

4.2.7 *In vitro* Biological screening for anticancer activity

Anticancer activity of 2-methoxy 5-acetoxy calamenene by *In vitro* MTT Assay.

The MTT assay was performed to measure the metabolic activity of cells to reduce yellow coloured tetrazolium salt 3-4, 5-Dimethyl thiazol-2-yil)-2, 5- diphenyltetrazolium bromide to purple coloured formazan. Test material ie, the compound was a syrupy pale yellow liquid and dissolved in 1ml 5% DMSO in PBS and diluted with equal volume of MEM 2X to get stock concentration of 2mg/ml and filtered through 0.22µ filter. Different dilutions of test materials from stock solutions were prepared and transferred (300µl, 200 µl, 100 µl, and 50 µl) and the total concentration is 600µg/ml, 400µg/ml, 200µg/ml and 100µg/ml were used for test and further medium was added to each well to make 400µml. Cells cultured in normal medium was considered as well as control and 5%DMSO in PBS as reagent control were placed on sub confluent monolayer of MCF -7 cells in 24 well plate. After incubation of cells with various concentration of test sample and controls at 37±1^{o}C for 24 hours, extracts and control medium was replaced with 200µml MTT solution (1mg/ml in medium without supplements), wrapped with aluminium foil and were incubated at 37±2^{o}C for 2 hours. After discarding the MTT solution, 400µl of isopropanol was added to all wells and swayed the plates. The colour developed was quantified by measuring absorbance at 570nm using a

spectrophotometer. The data obtained for test sample and reagent control were compared with cell control (Mosmann 1983, Marshall 1995).

Anticancer activity of 2, 5 di methoxy calamenene by *In vitro* MTT Assay

Hep G2 cells (Human hepatocellular carcinoma) were seeded in to 96-well flat bottom titre plates (5000 cells/well) containing 200µl RPMI-1640 (Roswell Park Memorial Institute) with 10% FCS (Fetal Calf Serum) and incubated for 24 hrs at 37^{o}C with 5% CO_2 atmosphere for the attachment of cells. After incubation, various concentrations of the test compound were added to the wells and the incubation was continued for 48 hrs. 20µl of MTT (5mg/ml in PBS) was added to each well before 4 hours of the completion of incubation. After the incubation period, the plates were centrifuged, supernatant was removed and 100µl of DMSO was added to each well. The plate was then incubated at room temperature for 15 min and the optical density was measured at 540 nm. (Mosmann 1983, Marshall 1995)

The percentage of dead cells was determined using the formula:

% of dead cells= [1 – (OD of drug treated/OD of control)] x100

4.2.8 HIV1 RT assay of 2 methoxy calamenene

HIV 1 Reverse Transcriptase is colorimetric test designed for the quantification determination for the Reverse transcriptase activity in cell culture and other biological samples. This method was adapted by so many researchers for finding new anti HIV compounds (Ravindra et al. 2008, Utsab Debnath et al. 2015.).The assay is used to determine the propagation of retroviruses in retrovirus infected mammalian cells in culture. This assay is also used for *in vitro* screening for RT inhibitors in the case of acquired immunodeficiency syndrome (AIDS). Reverse transcriptase generally uses RAN, which is complexed with various primers, as a template for DNA

synthesis. Classically, for the detection or quantification of RT activity, the amount of incorporated radioactivity labelled nucleotides is measured. The reverse Transcriptase Assay, colorimetric takes advantage of the ability of reverse transcriptase to synthesis DNA using the hybrid poly (A)x oligo $(dT)_{15}$ as a template and primer .The detection and quantification of the synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. Biotin labelled DNA binds to the surface of streptavidin coated micro plate modules. In the next step, an antibody to digoxigenin, conjugated to peroxidise (anti-DIG-POD) is added and bound to the digoxigenin-labeled nucleotides. In the final step the peroxidise substrate ABTS is added. The peroxidise enzyme catalyzes the cleavage of the substrate to produce a coloured reaction product. The absorbance of the sample is determined using a micoplate (ELISA) reader and is directly correlated to the level of RT activity in the sample.

4.3 Results and Discussion

4.3.1 Extraction, Isolation and Purification

The crude extracts after partitioned with solvents like ethyl acetate and hexane were concentrated under vacuum and yield was noted. The ethyl acetate fraction was shown maximum yielded fraction and which is chosen for further purification process. This ethyl acetate fraction was purified under column chromatography using ethyl acetate and hexane up on varying polarity. Four fractions were found to be containing the similar types of compounds it was pooled and concentrated under vacuum and purified further by flash chromatography.

4.3.2 Purification by flash chromatography

The vials are kept in such a way to collect all peek point fractions which are coming in the wave length range 210 nm and 254nm. The most of the fractions were having similar types of compounds shown by gas

chromatographic analysis. Those fractions which contained three major compounds were again pooled and kept for final purification.

4.3.3 Purification by HPLC

The purest fraction which contained three major compounds by flash chromatography is undergone further purification into single compounds and collected in a vial and concentrated under vacuum, kept for further spectroscopic, GC-MS analysis. The chromatogram obtained is as shown in Fig.4.4.

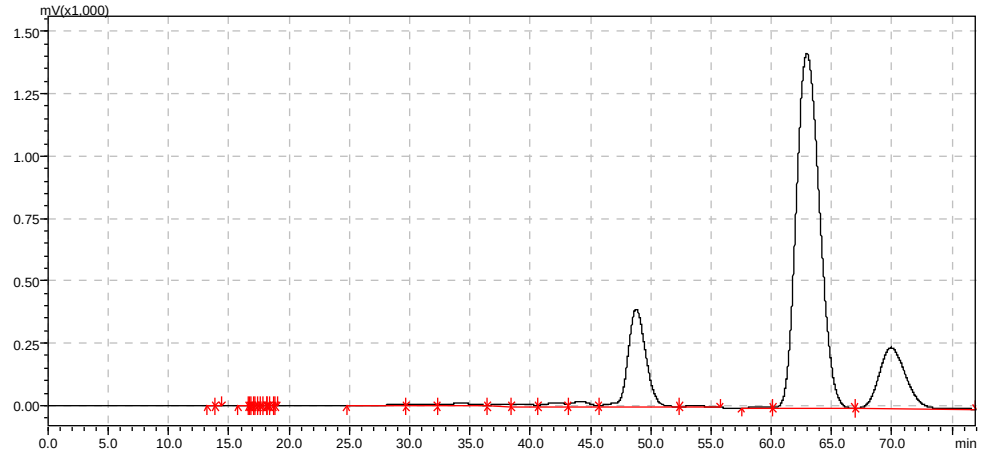


Fig. 4.4 Chromatogram of HPLC purification of three compounds.

4.3.4 GC-MS analysis

The GC-MS analysis of the three isolated compounds showed above 95% purity and is inferred it is ready for the NMR studies. The three compounds were shown molecular weight such as 290, 262, and 232 respectively. The mass spectra of the compound is resolved and checked whether it is matching with any of the spectra available in the NIST (National Institute of Standard Technology) library. It was found very curious that there no any matching spectra available in the NIST library. The mass spectra of each compound are shown as below in Fig.4.5,Fig.4.6 and Fig.4.7.

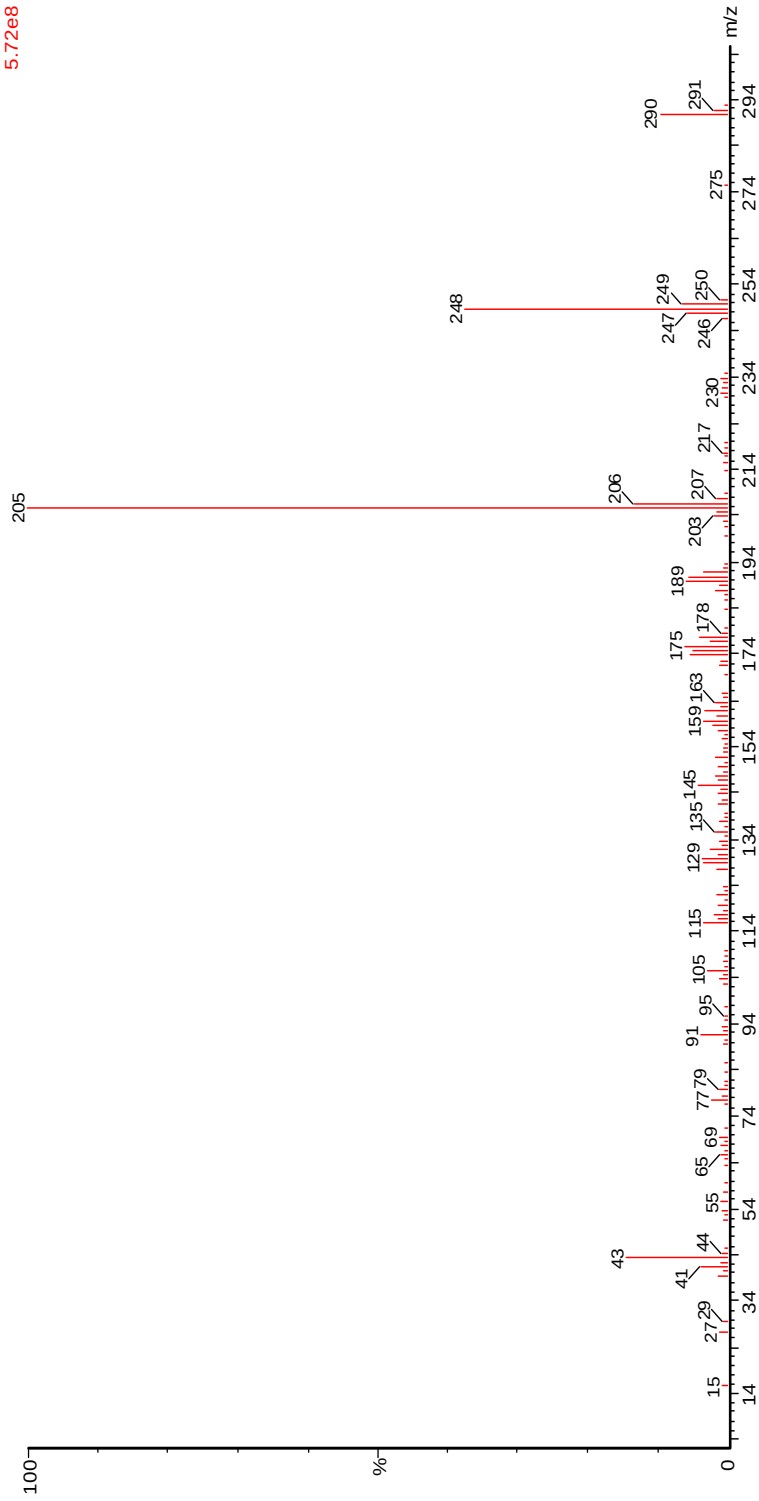


Fig.4.5 Mass spectra of isolated compound SR-1

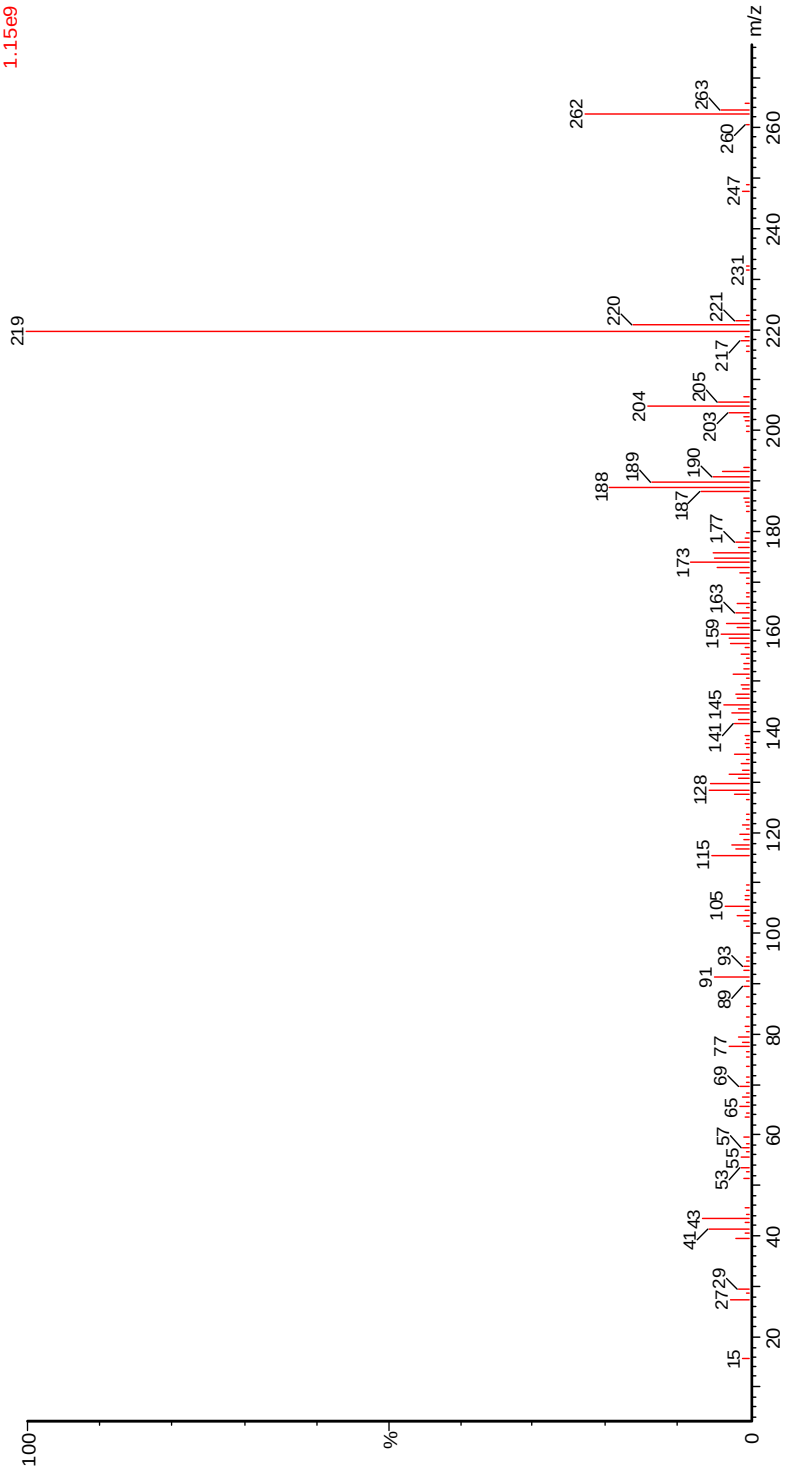


Fig.4.6 Mass spectra of isolated compound SR-2

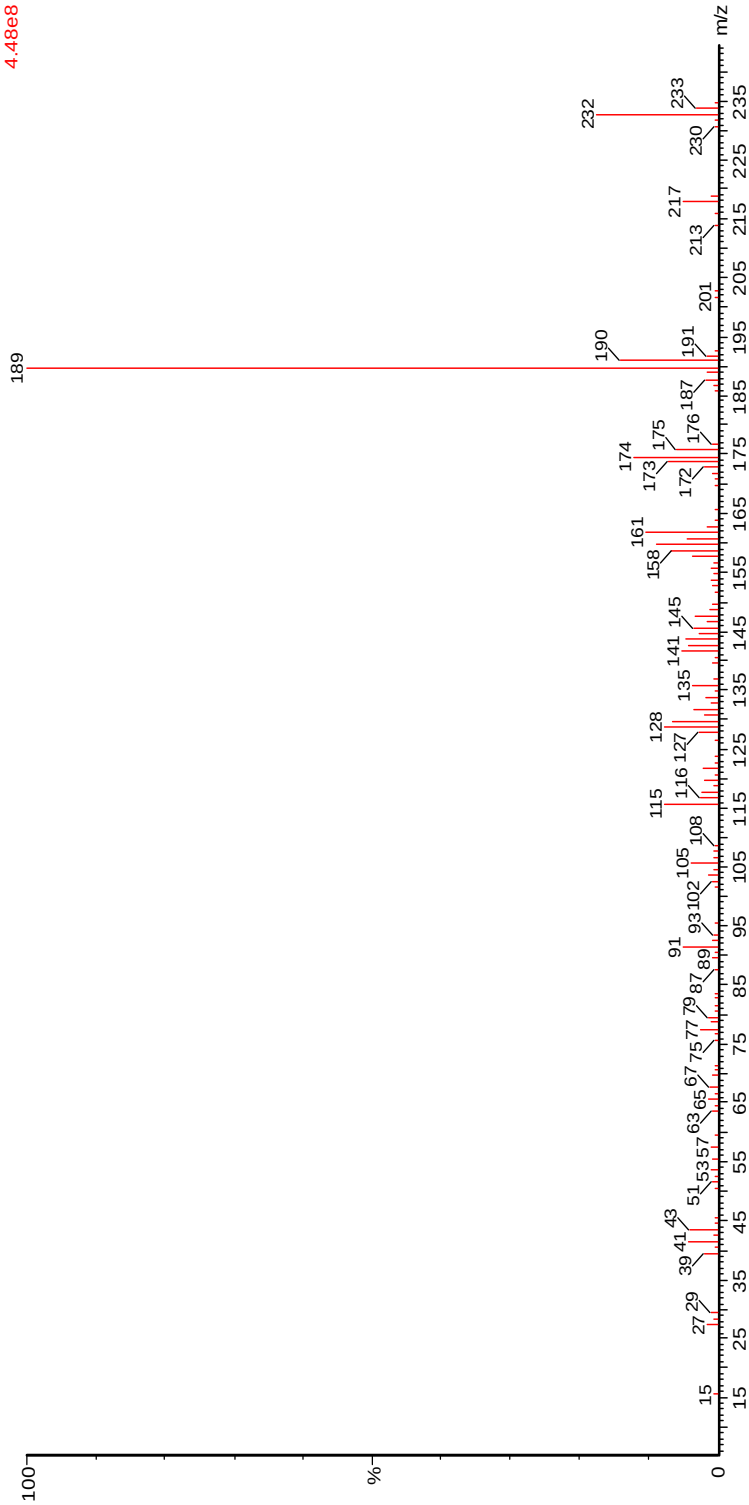


Fig.4.7 Mass spectra of isolated compound SR-3

The fractions collected after flash chomatography were undergone GC-MS analyis and it was found to be extremely intersting that plenty of compounds were present in the organism in very minute concentration.The mass spectra of each compounds were resolved and compared with NIST library spectra. It was found that most of the compounds were with unusual mass spectra and which is not having any similarity with the NIST library spetra. Infact some compounds showed 100% match with the spectra they are listed below in table 4.1. The structure of the compounds identified is given in fig. 4.10 & mass spectrum obtained is shown in Fig. 4.9.

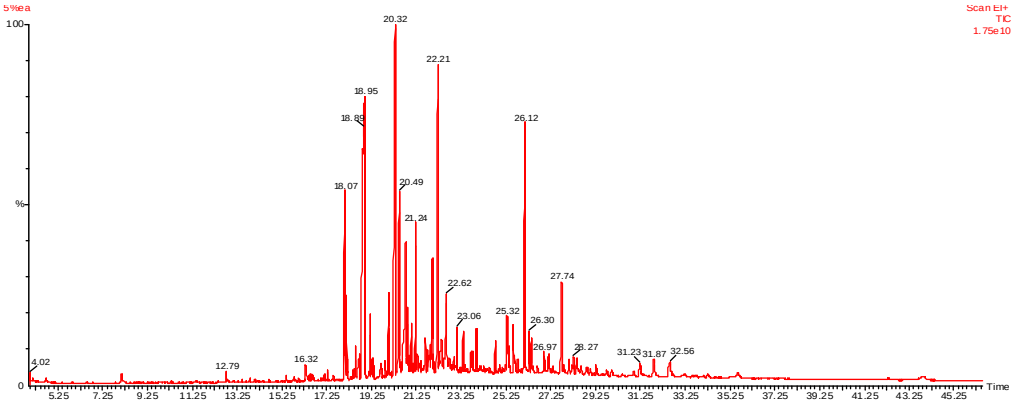
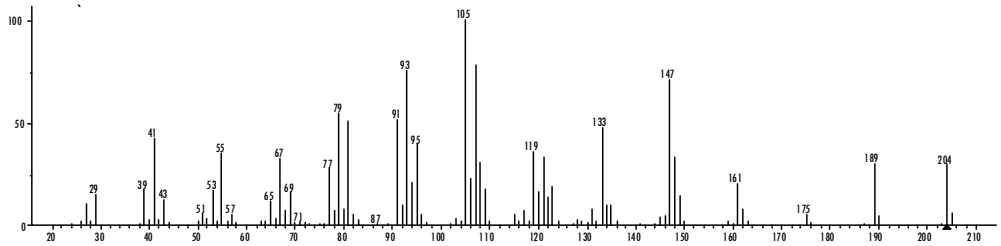
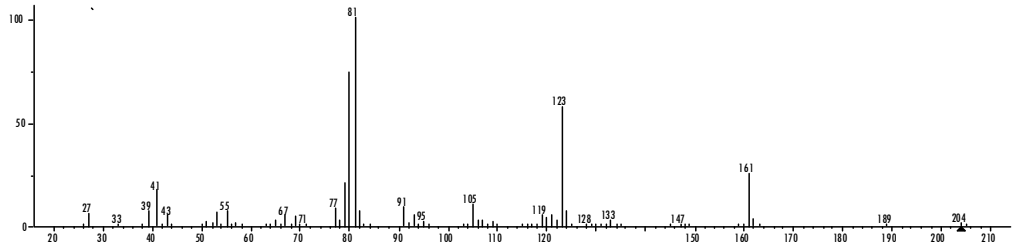


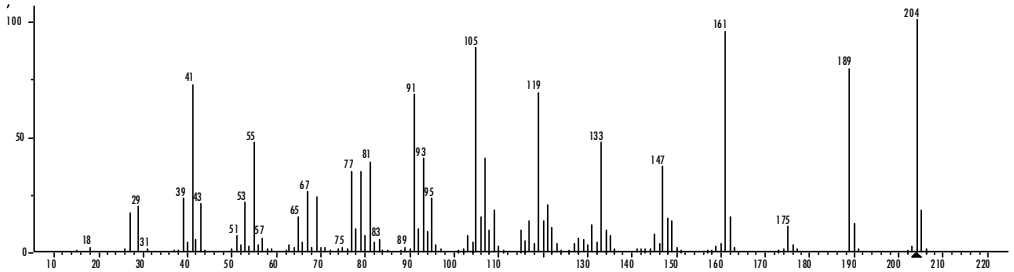
Fig. 4.8 Total ion chromatogram of the Ethyl acetate fraction of *S.reticulata*



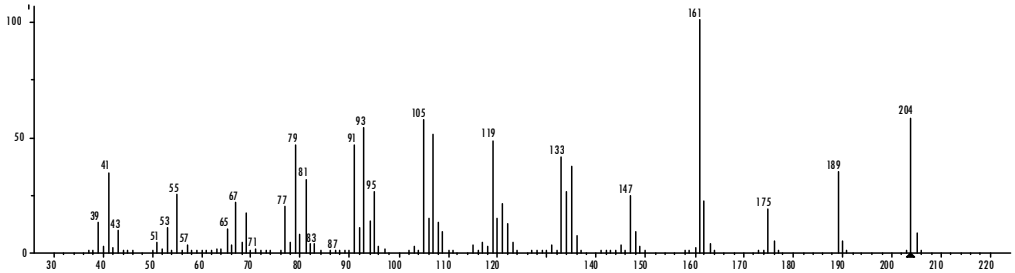
Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)



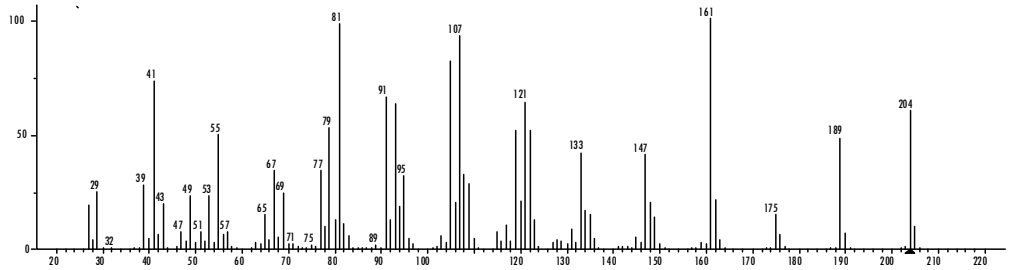
Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl)



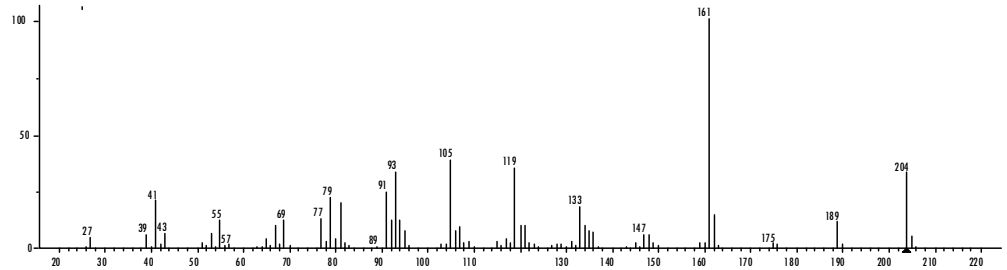
1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl



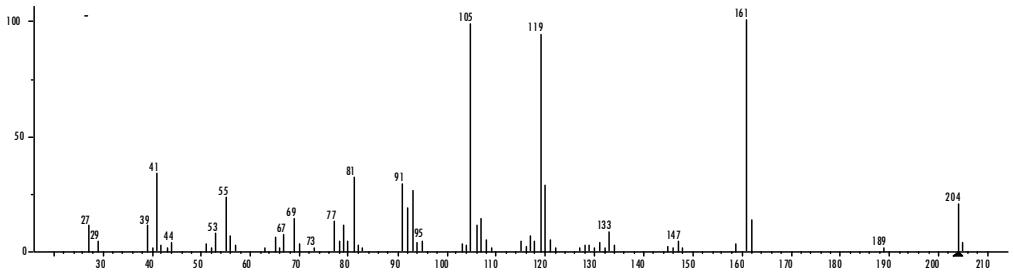
Naphthalene,1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)



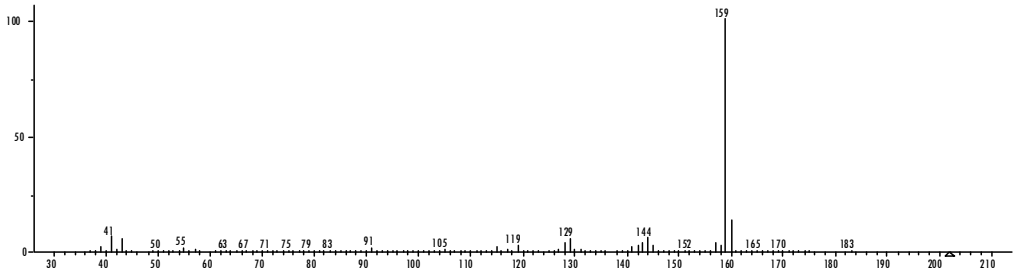
Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl).



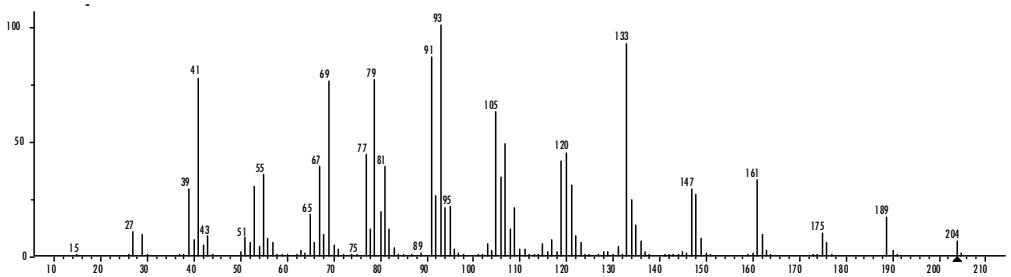
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl).



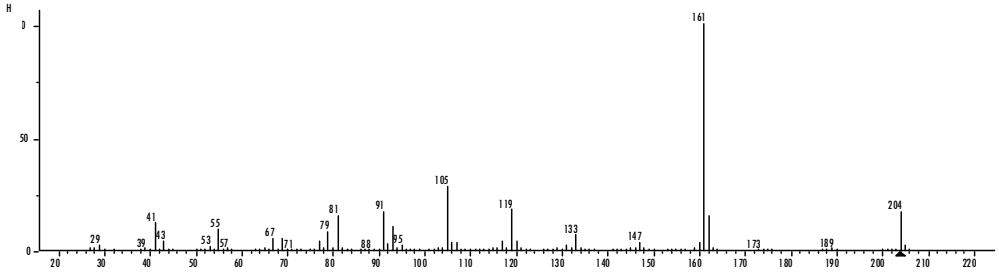
α-Cubebene



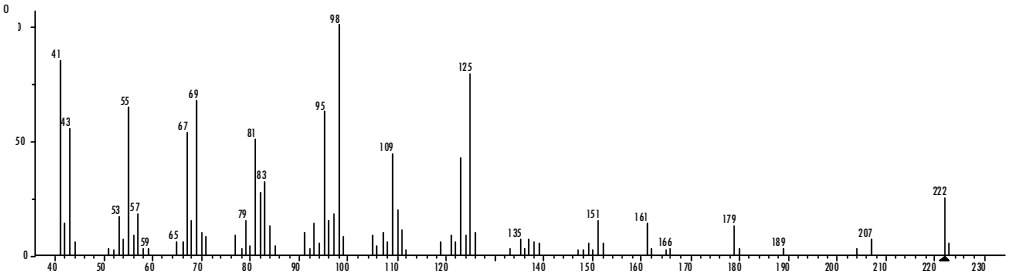
Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)



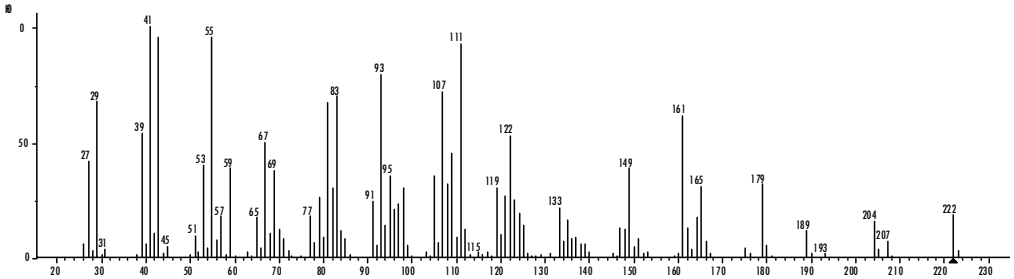
Caryophyllene



(+)-Epi-bicyclosesquiphellandrene



1(2H)-Naphthalenone, octahydro-4a,8a-dimethyl-7-(1-methylethyl)



4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl

Fig. 4.9 Mass spectrum of Identified terpene compounds

Table 4.1 Identified terpene compounds by GC-MS.

Sl No	Retention time	Compound	Molecular weight	Molecular formulae	m/z values
1	20.05	Azulene1,2,3,4,5,6,7,8 octahydro1,4dimethyl-7(1-methyl ethenyl)	204	$C_{15}H_{24}$	M+204, 105(b),41,55,67,77,79,93,91,95,119,133,147,161,175,189.
2	20.32	Cyclobutan(1,2,3,4)Dicyclopentene,Decahydro-3A-methyl-6-methyl	204	$C_{15}H_{24}$	M+204,81(b),41,55,67,91,105,123,161,189.
3	20.57	1H cyclopra(E) azulene1A,2,3,4,4A,5,6,7B octahydro1,1,4,7 tetram	204	$C_{15}H_{24}$	M+204(b),41,55,67,77,81,91,105,119,133,147,161,175,189
4	20.80	Naphthalen1,2,3,5,6,7,8,8A Octahydro-1,8A Dimethyl 7-(1-methylethyl)	204	$C_{15}H_{24}$	M+204,161(b),41,55,67,79,93,105,119,133,147,161,175,189
5	20.89	Azulene 1,2,3A,4,5,6,7 octahydro 1,4 Dimethyl-7methyl ethenyl	204	$C_{15}H_{24}$	M+204,161(b),41,55,81,91,107,121,133,147,175,189.
6	21.04	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1α,4aα,8aα)-	204	$C_{15}H_{24}$	M+204,161(b),41,55,79,93,105,119,133,147,189.
7	21.36	α- cubebene	204	$C_{15}H_{24}$	M+204,161(b),41,55,69,81,91,105,119,133,147,189
8	21.96	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	204	$C_{15}H_{22}$	M+204,159(b),41,55,77,91,105,119,129,144,159,170,183.
9	22.02	Caryophyllene	204	$C_{15}H_{24}$	M+204,93(b),41,55,69,79,91,93,105,120,133,147,161,175,189
10	22.07	(+)-Epibicyclosesquiphellandrene	204	$C_{15}H_{24}$	M+204,161(b),41,55,81,91,105,119,133,147,173,189.
11	22.22	1(2H)naphthalenone,octahydro-4A8Adimethyl7(1-methyl ethyl)	222	$C_{15}H_{26}O$	M+222,98(b),41,55,69,81,95,98,109,125,135,151,161,179,189,207
12	22.94	4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,4β,4aβ,7α,7aβ,7bα)]-	222	$C_{15}H_{26}O$	M+222,41(b),55,67,55,67,83,93,107,111,122,149,161,179,204,222

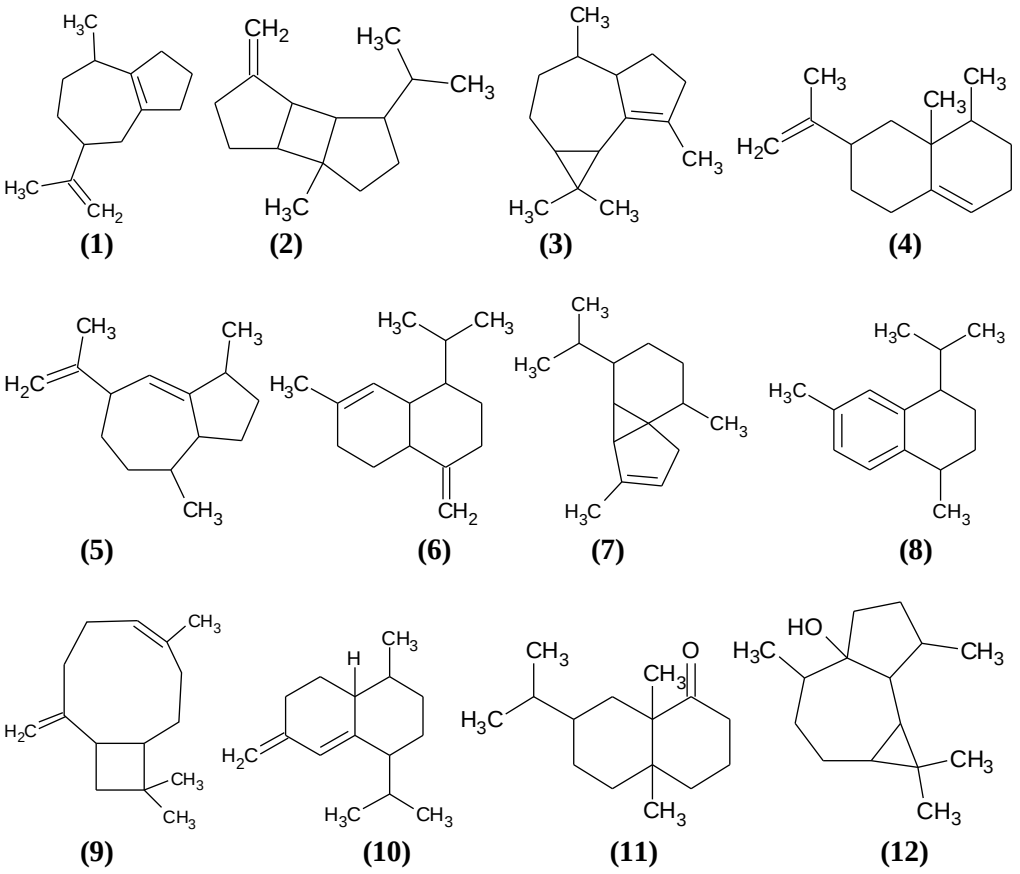


Fig.4.10 Structure of terpenoids from *Subergorgia reticulata.*

(1) Azulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl) (2) Cyclobuta [1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl) (3) 1H-Cycloprop[e]azulene,1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7 tetramethyl. (4) Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl). (5) Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl). (6) Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl). (7) α-Cubebene (8) Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl) (9) Caryophyllene (10) (+)-Epi-bicyclosesquiphellandrene (11)1(2H)-Naphthalenone, octahydro-4a, 8a-dimethyl-7-(1-methylethyl) (12)4aH-Cycloprop [e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl.

4.3.5 Structural Characterisation of isolated compounds

The three compounds isolated from the fraction by preparative HPLC were named as SR-1, SR-2 and SR-3. The complete charaterisation of these tree compounds were done using FTIR, ^{1}H, ^{13}C, DEPT, COSY, HMBC and HSQC.

Characterisation of Compound 1

Compound 1(SR-1) was obtained as a pale yellow syrupy liquid. The molecular formula of the compound 1 was deduced to be $C_{18}H_{26}O_3$ according to the GC MS and NMR results. Mass fragments obtained were m/z 290(M^+),205(base peak), 248, 189, 175, 159, 145, 135, 129, 115, 105, 91, 77, 65 and 43. The FTIR spectrum of the compound 1 has prominent peaks at 2869, 1759, 1612, 1464,1367,1203,1187 and 1098 cm^{-1}, indicative of alkyl – CH stretching, carbonyl stretching at 1759$cm^{-1}$and -CO stretching.

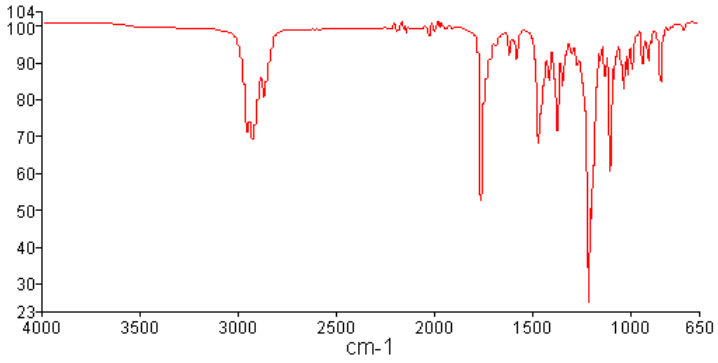


Fig.4.11 FT IR spetrum of compound 1

^{1}H NMR of Compound 1(SR-1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.55 (s, 1H), 3.80 (s, 3H), 3.16 (tt, J = 7.5, 6.1 Hz, 1H), 2.29 (s, 3H), 2.11 (s, 3H), 2.12 – 1.87 (m, 3H), 1.84 – 1.69 (m, 2H), 1.50 – 1.40 (m, 1H), 1.11 (d, J = 6.9 Hz, 3H), 0.89 (d, 7.0 Hz, 3H), 0.86 (d, J = 7.0 Hz, 3H).

^{13}C NMR of Compound 1(SR-1)

^{13}C NMR (400 MHz, $CDCl_3$) δ 169.24 , 154.63 , 141.49, 132.77 , 130.06 , 126.82 , 109.79 , 55.27 , 38.45 , 32.61 , 26.45 , 25.79 , 21.90 , 21.67 , 20.89 , 20.82 , 19.14 , 16.79 .

DEPT 135

^{13}C NMR (400 MHz, $CDCl_3$) δ 55.26, 32.61, 26.44, 25.78(CH_2), 21.89, 21.67, 20.82, 19.27(CH_2), 16.78

DEPT 90

^{13}C NMR (400 MHz, $CDCl_3$) δ 109.79, 38.42, 32.61, 26.44.

It has 18 carbon atoms of which six aromatic, six- CH_3, two- CH_2, and four-CHs.

The assigned structure of compound 1(SR-1) is 2 methoxy 5- acetoxy calamenene is given bellow in fig. 4.12.

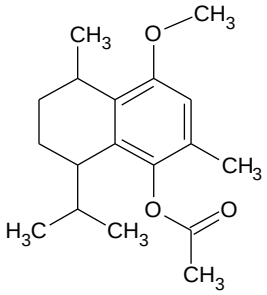


Fig 4.12. 2-methoxy 5- acetoxy calamenene

Characterisation of compound 2(SR-2)

Compound SR-2 was obtained as a pale yellow syrupy liquid. The molecular formula of the compound is $C_{17}H_{26}O_2$ and molecular weight 262.The other major mass fragments were **Mass**-262(m+), 219(b), 204, 188, 173, 159, 128, 115, 91. The FTIR spectrum showed prominent peaks at 2953,

2932, 2867, 1463, 1400, 1341, 1232, 1099 cm^{-1}. From FTIR spectra aromatic –CH stretching, phenyl ester group were clearly observed.

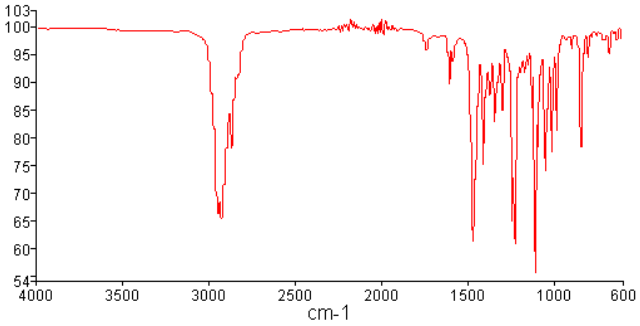


Fig.4.13. FT IR spetrum of compound 2

^{1}H NMR (400 MHz, $CDCI_3$): 6.502 (s, 1H); 3.781 (s, 3H); 3.633 (s, 3H); 3.138 (p, J=6.8,1H); 2.744 (td, J=5.6,2.1,1H); 2.266 (s, 3H); 2.013 (h, J=6.8,1H); 1.990 (qt, J=13.6,4,1H); 1.815 (t, J=6.5,1H); 1.785 (qq, J=6.7,2,1H); 1.438 (dt, J=13.3,3,1H); 1.116 (d, J=6.88,3H); 0.867 (d, J=9.6,3H); 0.849 (d, J=9.6,3H).

^{13}C NMR (100 MHz, $CDCI_3$): 153.1; 150.8; 134.0; 130.0; 127.2; 110.0, 60.1; 55.3; 38.1; 33.0; 26.6; 25.8; 22.2; 21.7, 20.9, 19.5; 16.5.

DEPT -135

Quaternary -153.1; 150.8; 134.0; 130.0; 127.2,

CH_3- 60.1; 55.3; 22.2; 21.7; 20.9; 16.5.

CH_2-25.8, 19.5

CH-110.0, 38.1; 33.0; 26.6.

From the ^{1}H and^{13} C it was very clear that compound 2 has six CH_3, two CH_2 and four CHs.

Finally the structure assigned as 2, 5 dimethoxy calamenene as given bellow in fig. 4.14.

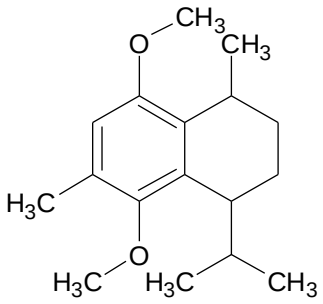


Fig 4.14. 2, 5 dimethoxy calamenene.

Characterisation of compound 3(SR-3)

From the FTIR results the absorption at 2917, 2849, 2159, 1706, 1464, 1242, 1025, 757cm^{-1} .Indicative of aliphatic –CH_2 stretching, aromatic –CH stretching and -OCH_3 stretches. The molecular mass of the compound is found to be m/z232 (M^+) and other fragments ions such as 189(b), 217, 174, 161, 158, 145, 141, 128, 115, 105, 91 and 77. The molecular formulae assigned as $C_{16}H_{24}O$ from GC-MS and NMR studies.

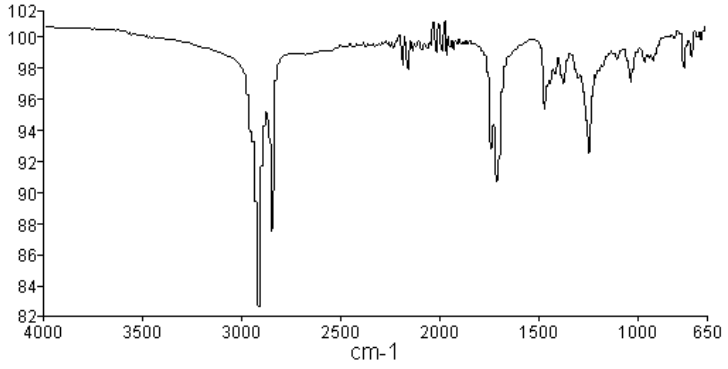


Fig. 4.15. FTIR spectrum of compound 2

^{1}H NMR of Compound 3 (SR-3)

^{1}H NMR (400 MHz $CDCl_3$); δ 6.60(d,J=1.4Hz,1H), 6.50(d,J=1.5Hz,1H), 3.80(s,3H), 3.13(pd,J=6.9,2.2 Hz,1H), 2.46(td,J=5.8,2.7Hz,1H), 2.30(s,3H), 2.09-1.67(m,4H), 1.47(ddt,J=11.1,3.3,1.9 Hz,1H), 1.332(d,J=7,3H), 0.982 (d,J=7,3H), 0.812(d,J=7,3H)

^{13}C NMR of compound 3

^{13}C NMR (400 MHz $CDCl_3$); 157.09, 140.66, 134.57, 128.50, 122.54, 108.53, 55.103, 43.04, 33.26, 27.19, 26.54, 22.17, 21.58, 21.47, 19.62, 19.11

DEPT 135

122.54, 108.55, 55.10, 43.05, 33.25, 27.28(CH_2), 26.60, 22.17, 21.58, 21.48, 19.62, 19.12(CH_2).

DEPT 90

122.54, 108.52, 43.03, 33.26, 26.54

From the ^{13}C, DEPT135 and DEPT 90 spectra it was found that 16 carbon atoms of which five CH_3, two CH_2, five CHs and four quaternary were present. Finally the structure of the molecule is assigned as 2-methoxy calamenene as given bellow in fig. 4.16.

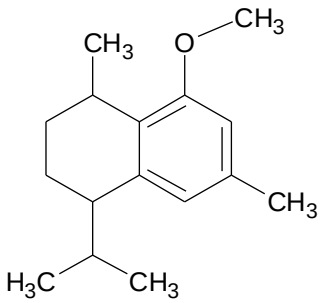


Fig. 4.16. 2-methoxy calamenene

The isolated compounds such as 2-methoxy 5-acetoxy calamenene, 2, 5 dimethoxy calamenene and 2-methoxy calamenene were reported previously by Limna mol et al 2011 from the same species, even though the biological activity of the compound is not studied well. The antifouling activity of the compounds were studied and found to be active. Similar compounds were also reported from different terrestrial sources (Nishizawa et al. 1983; Warmers and Konig 1999; Salmoun et al. 2007). Though it was reported earlier, the stereochemistry of the compound was not studied well accordingly it was given more importance to the biological activity of the compound. 2-methoxy 5-acetoxy calamenene was studied for anticancer activity against human breast cancer cell lines (MCF-7), 2,5 dimethoxy calamenene was studied for anticancer activity against human liver cancer cellline(HepG2) and the compound 2-methoxy calamenene were studied for anti HIV activity.

4.3.6 *In silico* Biological screening

The biological activities of identified terpene and terpenoid compounds were tested by *in silico* studies. PASS online prediction software tells about the incredible biological activity possibility with more accuracy. It was chosen because it gives number of biological activity with the probability of activity factor with in a very short time. The identified twelve compounds were shown various biological actions among them only the probability factor greater than seventy were considered because it is having more possibility than other values. The compound (+)-Epi-bicyclosesquiphellandrene was shown different biological activity by PASS prediction such as Ant infertility female (P_a .704), bone disease treatment (P_a .710), antieczmatic(P_a .904). The essential oil from *Helichrysum gymocephalaum* was studied by Afoulous et al in 2011 and was found that the essential oil is having biological activity such as cytotoxic, antimalarial and antioxidant. Interesting fact is that from this essential oil (+)-

Epi-bicyclosesquiphellandrene was found to be a major component. There is not much studies have done for the biological activities of pure (+)-Epi-bicyclosesquiphellandrene.The compound 1(2H)-Naphthalenone, octahydro-4a, 8a-dimethyl-7-(1-methylethyl) is not studied so far for its biological activities, but this compound was reported in natural essential oils. The major activities shown by this compound is cardiovascular analeptic (P_a 845), carminative (P_a .821), vascoprotector(P_a .822),antieczmatic(P_a .804), dermatologic (P_a .727), antipsoriatic(P_a .715)etc. The compound α-cubebene is shown to posses activities such as anti-inflammatory (P_a .888), antineoplastic (P_a .837), atherosclerosis treatment(P_a .776) and antieczmatic(P_a .737).according to Lee et al 2012 α iso cubebene is found to be active for certain cell lines, as toxic compound. In fact the PASS online prediction probability of apoptosis agonist is < .70. The pharmacological properties of α-cubebene is studied by Sung Kyan et al 2012 and it was suggested that wide range of activity was shown such as experimental sepsis, polymicrobial sepsis etc.

Caryophyllene is found to be active for number of biological activities such as antineoplastic(P_a .915), antieczmatic(P_a .897),apoptosis agonist(P_a .847),anti-inflammatory (P_a .745),ant psoriatic(P_a .734) and dermatologic(P_a .734).Even though it shown apoptosis agonist activity in PASS online prediction by a probability factor of 847 and it was also proved by Legault and Pichelte in 2007 that this Caryophyllene is a strong anticancer compound. The essential oil from *Cymbopogon Nardus(L Rendle)* was found to be possess antioxidant, antibacterial and anti mosquito activities and this essential oil associated with these compounds such as α-cubebebe and caryophyllene.

Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl) was found to be active for antineoplastic(P_a .930), antieczmatic(P_a .903), bone disease treatment(P_a .845),antiosteoprotic (P_a .829),

dermatologic(P_a .800),apoptosis agonist(P_a .734), antipsoriatic(P_a .722) and prostate disorder treatment(P_a .704).There is not much studies are available in the literature about the biological actions of this compound. 1H-Cycloprop[e]azulene,1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7 tetramethyl was active for transplant rejection treatment(P_a .920), Antieczmatic (P_a .810), Cardiovascular analeptic(P_a .735), and immunosuppressant(P_a .727). Rejection of transplanted organ is one of the main barrier of transplantation today. It occurs as a result of humoral and cell mediated responses by the recipient to specific antigens present in the donor tissue (www.biomed. brown.edu/course); here it was found that this compound 1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7 tetramethyl is active and useful for this particular biological activity.

Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl) was shown activities such as carminative(P_a .898), antieczematic(P_a .828) and anti-inflammatory(P_a .710).The similar compound Naphthalene,1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl) showed activities like carminative (P_a .888), antieczmatic (P_a .764) and dermatologic(P_a .738).

4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl was active for spasmolytic(P_a .982), which is used for suppress muscle spasms and analgesic(P_a .897). Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl) showed wide range of activities such as anti neoplastic(P_a .930), antieczematic(P_a .899), bone disease treatment(P_a .845), antiosteoprotic(P_a .829), dermatologic(P_a 800),apoptosis agonist(P_a .734), antipsoriatic(P_a .722) and prostate disorder treatment(P_a .704).Activities of Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl) was useful for carminative (P_a .888), antieczematic(P_a .785),dermatologic(P_a .748), antineoplastic(P_a .754). The activities shown by PASS software is 95% well agreement with *in vitro* studies which are tested and proved for the

trustworthiness of the particular *in silico* tool (Filimonov et al 2014). This seems to be the first study of such kind of biological activities of compounds from *S.reticulata.*

4.3.7 *In vitro* biological screening for anticancer activity

The movement of new anticancer medicines is a gruelling task that requires both chemical and biological effort. The main issue that it must be solved selectively because cancer cells are just like normal cells and without harming the similar healthy cells remains a challenging task. In recent years more researchers have come to the awareness that marine organisms seize immense potential as a source of novel molecules for cancer treatment. The marine environment offers drug hunters, attractive features not shared by terrestrial ecosystem. In the region of 16000 chemical compounds were isolated from marine sources (Bhakuni and Rawat 2005), albeit only very few compounds have subjected to comprehensive biological evaluation as chemotherapeutic agents. At present about 22 marine derived natural products are in clinical trials as a drug candidate for anticancer efficacy (Newman and Cragg 2006). Ecteinascidin 743 have shown activity against ovarian, breast and lung cancer cell lines and still it is under clinical trials(Cragg and Newman 2005, Newman and Cragg 2006). Auristain, a synthetic analogue of dolastain 10 isolated from Indian Ocean sea hare *Dolabella auricularia* (Cragg and Newman 2009). Preliminary *in vitro* screening against various cancer cell lines revealed remarkable cytotoxicity. Some other examples are Halichondrin B from the sponge *Halichondria okadai* and its synthetic derivative were exhibited anticancer activities (Cragg and Newman 2005), Bryostatin isolated from *Bugula neritina* (Shimek 2003) were also found promising anticancer effect. Eleutherobin was isolated from octocoral *Eleutherobia sp*. was found to be effective anticancer compounds. There are so many examples for the

inhibition of marine compounds and extracts on various cancerous cell lines but only very few are come across for human trials. Here from the isolated compounds only two were selected for the *in vitro* MTT assay because these two have showed high apoptosis inducing activity in the *in silico* prediction.

Anticancer activity of 2-methoxy 5-acetoxy calamenene by *In vitro* MTT Assay.

In this study the effect of the isolated compound 2-methoxy 5-acetoxy calamenene was tested on breast cancer cell line MCF-7. MCF-7 is a breast cancer cell line isolated in 1970 (Soule et al 1973). Breast cancer is the most danger and common type of cancer in women. At present the identification of new therapies is not satisfied by the scientist due to the negative toxic effect of the medicine, chemotherapeutical agents damage cell DNA effecting normal cells as well as the cancer cells. There are some studies which show the inhibition of compounds and extracts on MCF-7 and HepG2 cell lines (Baz et al 2014). MTT assay is well established *in vitro* model used to test cytotoxity of compounds against cancer cell lines and was used as one of the conventional method for screening of compounds with antitumor properties (Sieuwerts et al 1995). The test is based on the principle, viable cells convert the soluble yellow tetrazolium salt, the MTT compound (3-(4, 5-dimethyl thiazzol-2yl)-2, 5-diphenyl-tetrazolium bromide) to an insoluble purple coloured formazan precipitate by the mitochondrial enzyme succinate dehydrogenase where as dead cells not. There for untreated cells shows higher absorbance than the treated ones due to the formation of formazan this can be correlated with the number of viable cells in the culture.

Four doses such as 100µg/ml, 200µg/ml, 400µg/ml, 600µg/ml of the compound 2-methoxy 5-acetoxy calamenene were prepared and their effects

on the cells were analysed over time periods of 24 hours. A significant activity was observed for this compound 2-methoxy 5-acetoxy calamenene on MCF-7 cell lines (Fig.4.17, Fig.4.18 and 4.19). A decreasing trend was observed in cell viability (Table 4.2). The cells were almost arrested the growth above 100µg/ml concentration. The IC_{50} value of the cell viability is calculated as 59.36µg/ml. The activity of this compound is very high when compared to the normal drugs such as Xeloda. Xeloda is used to treat breast cancer and recommended dose of xelode is 1250mg/m^2. From the above results it can be suggested that the action of the compound is more than the normal breast cancer drugs. This result will be a centre of attention into the development of a new drug candidate from this thesis.

Table 4.2. The effects of the compound 2-methoxy 5-acetoxy calamenene and control on MCF-7 at varying concentration

	Cell control	600µg/ml	400µg/ml	200µg/ml	100µg/ml	Phenol	DMSO
	108.460	10.876	13.242	12.138	79.611	13.242	97.898
	95.375	9.615	8.828	13.242	79.453	11.981	114.766
	96.163	9.615	13.557	11.981	93.641	14.976	113.505
AVG	100	10.038	11.875	12.454	84.235	13.399	108.723
STDEV	7.337	0.725	2.644	0.687	8.146	1.5038	9.395

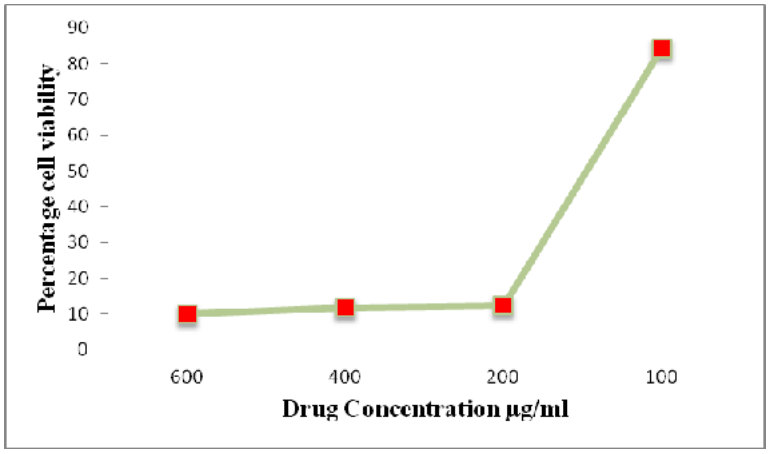
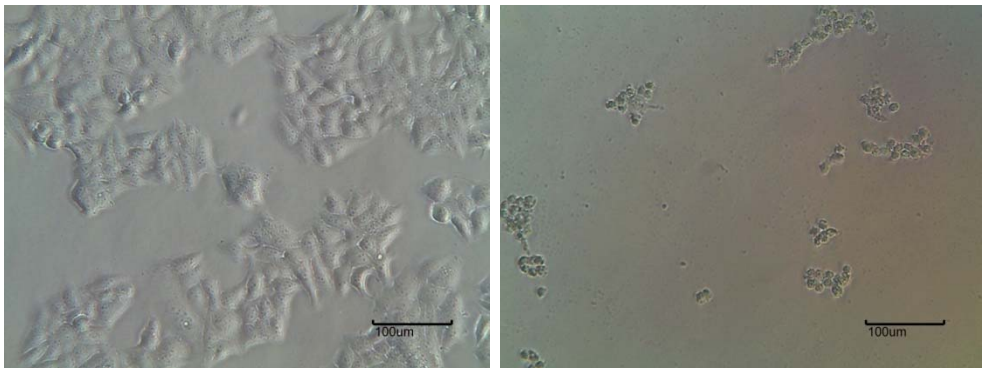
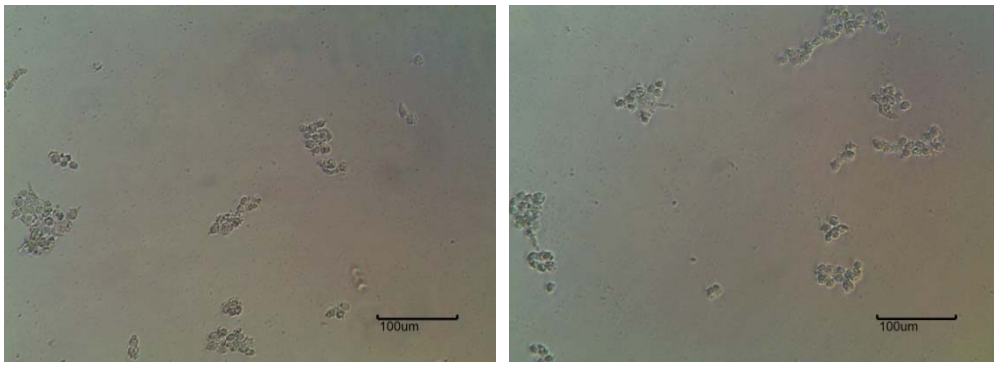


Fig. 4.17. The graph showing the cell viability for various concentration of the compound



a) MCF-7 cells after incubated with 50µl of sample b) MCF-7 cells after incubated with 100µl of sample



c) MCF-7 cells after incubated with 200µl of sample d) MCF-7 cells after incubated with 300 µl of sample

Fig No 4.18. MCF-7 cells after incubated with the compound 2 methoxy 5 acetoxy calamenene at different concentration.

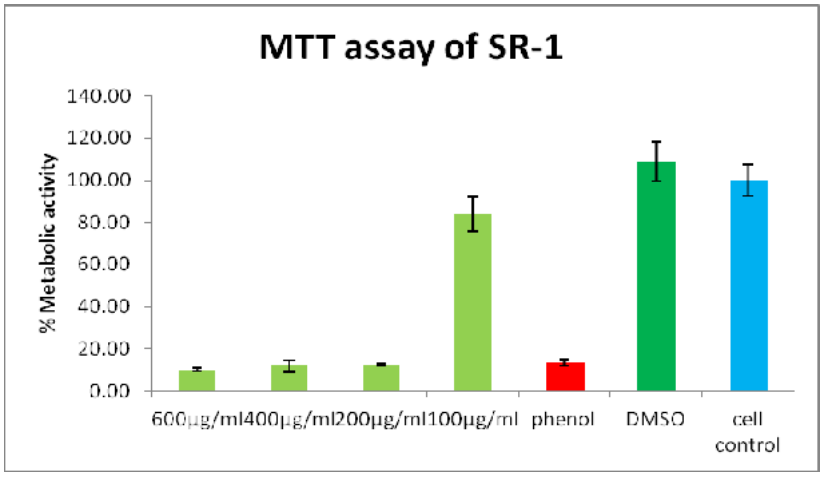


Fig 4.19. Metabolic activity of 2-methoxy 5-acetoxy calamenene, phenol, DMSO on MCF-7 cells.

Anticancer activity of 2,5 Dimethoxy calamenene by *In vitro* MTT Assay

Liver cancer is the fifth most common cancer worldwide and the third most deadly disease. HepG2 is a human liver cancerous tissue of a male. These are epithelial in morphology. Hepatocellular Carcinoma (HCC), a primary malignancy of the hepatocyte accounts for 85 to 90% all primary liver cancer (Kumar et al 2011). The isolated compound 2,5 Dimethoxy calamenene was studied for *in vitro* MTT assay on human Hep G2 cell lines in varying concentrations such as 0.1µg/ml,0.25 µg/ml, 0.5µg/ml, 1 µg/ml, 2.5 µg/ml, 5 µg/ml and 10 µg/ml. It was found that the compound is having significant cytotoxicity to HepG2 cells. The cell death or cell toxicity was found to be increased consecutively when the concentration is increased. The maximum toxicity 98.54% was observed for the concentration 10µg/ml and minimum cell death was observed at 0.1µg/ml by 2.24% cell death which is represented in table 4.3 and Fig. 4.20. This compound showed wonderful activity even in the very less concentration. The IC_{50} value calculated from the graph is 2.80µg/ml. It is also comparable to the normal cancer drug dosage such as Cisplatin and 5-fluourouracil.

Table 4.3 The effects of the compound 2,5 Dimethoxy calamenene and control on HepG2 cells at varying concentration

Drug Concentration (µg/ml)	OD 1	OD 2	OD 3	OD 4	OD 5	OD Average	% Toxicity
Control	0.896	0.894	0.889	0.885	0.890	0.891	-----
10	0.021	0.008	0.009	0.013	0.018	0.014	98.54 %
5	0.101	0.086	0.107	0.080	0.093	0.093	89.55 %
2.5	0.503	0.496	0.492	0.490	0.500	0.496	44.27 %
1	0.594	0.589	0.592	0.585	0.586	0.589	33.82 %
0.5	0.768	0.774	0.773	0.770	0.770	0.771	13.37 %
0.25	0.842	0.851	0.849	0.843	0.846	0.846	4.94 %
0.1	0.869	0.872	0.866	0.874	0.870	0.870	2.24 %

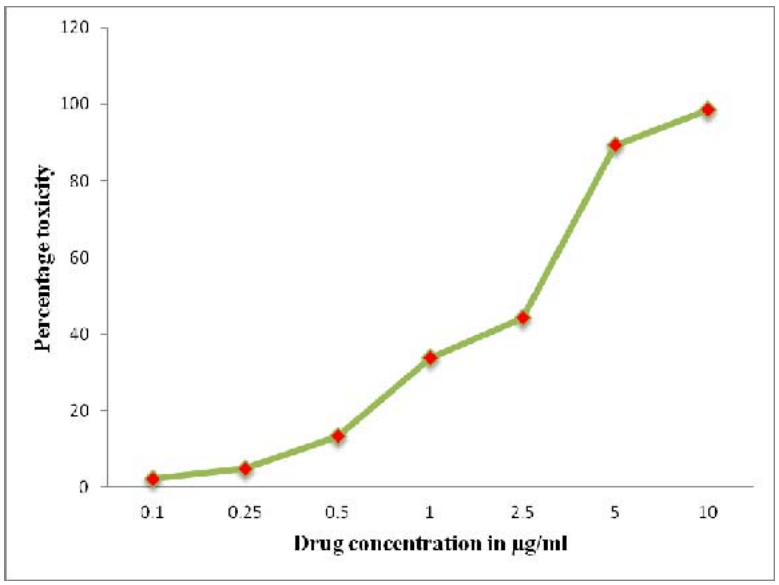


Fig.4.20. The Cell toxicity mesurement againt various concentration of isolated compound 2,5 Dimethoxy calamenene

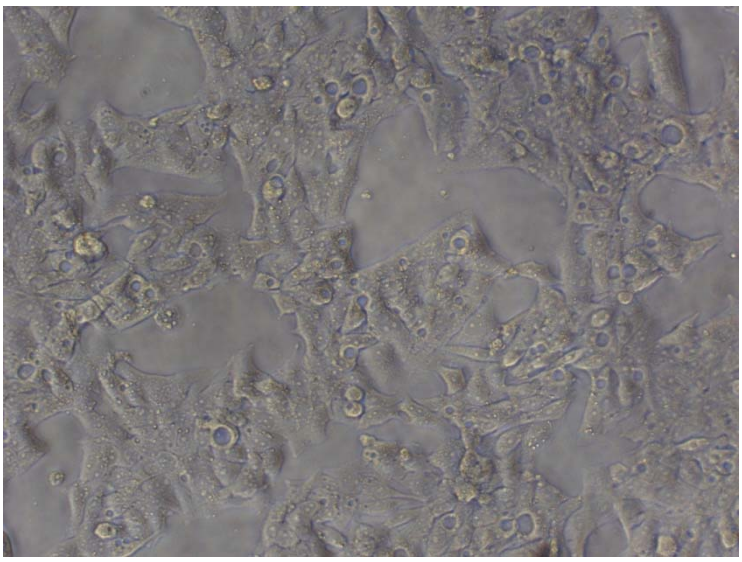


Fig no 4.21 Normal HEP G2 cells

4.3.8 HIV1 RT assay of compound 2-methoxy calamenene

From the HIV 1 RT assay it was found that the compound 2-methoxy calamenene is showing mild action compared to control. Two major concentrations were selected such as 10µg/ml and 100µg/ml for the assay due to less yield of the compound. In fact at lower concentration it shown minimum activity of 26.10% inhibition and it was not gradually increased when it was reached at 100µg/ml concentration (Table 4.4). But this result is a possibility for the anti HIV compound from the organism by any modification in side chain or adding function group via synthetic approach. At higher concentration the activity is found to be high. It is a hopeful result for the future drug candidate as an anti HIV compound.

Table 4.4 HIV1 RT assay of the isolated compound 2-methoxy calamenene

% Inhibition at 10µg/ml	26.13
% Inhibition at 100µg/ml	68.95
Control/(Nevirapine)	97.69

4.4 Conclusion

In this chapter characterisation of terpenes and their biological activity studies were done. Since corals are previously found to be rich of terpenes and terpenoid compounds, this gorgonian coral *S.reticulata* were also found to be rich of terepene compounds. Sesquiterpenes are mostly found in corals and they are chemical finger prints of soft corals. The compounds identified from GC-MS analysis were found to be very similar in structure and even they are very close retention time in GC. It was seems to be very difficult for the isolation of pure compound without the help of the instrument since they are having very similar structure. Three calamenenes sesquiterpenes such as 2-

methoxy 5-acetoxy calamenene, 2,5 Dimethoxy calamenene and 2-methoxy calamenene having cadinene skeleton have been isolated from this gorgonian coral *S.reticulata.* The compounds such as calamenenes were previously isolated from the same species by Limna mol et al but the biological activity of the compound is not studied well. It was also reported from different terrestrial sources. The compound 2-methoxy 5-acetoxy calamenene was found to be having anticancer activity against human breast cancer cell line (MCF-7). The activity of this compound 2-methoxy 5-acetoxy calamenene was found to be significant when compared to the normal drug dosage. IC_{50} value of the compound 2-methoxy 5-acetoxy calamenene was found to be 59.36µg/ml. The compound 2,5 Dimethoxy calamenene was found to be having anticancer activity against human liver cancer cell lines(HepG2). This activity is high when compared to the compound 2-methoxy 5-acetoxy calamenene, since the IC_{50} value seems to be very less 2.80µg/ml. This is the first time reporting such kind of activity for this compound. Anti HIV activity was tested for the compound 2-methoxy calamenene and it was found that the compound is having moderate activity. Since the anti HIV activity of the compound 2-methoxy calamenene is less than the positive drug control Nevirapine, it cannot be suggested for a good drug target. But it can be suggested for the future application by synthetic reactions for small variations in this compound 2-methoxy calamenene can lead to a drug target. The anticancer activity of the compounds such as 2-methoxy 5-acetoxy calamenene and 2,5 Dimethoxy calamenene can be considered to be a drug target, if more studies such as clinical trials are undergone.

From the GC-MS analysis twelve compounds were found to be exactly matched with the NIST library spectra they are **(**1) Azulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl), (2) Cyclobuta[1,2:3,4] dicyclopentene,

decahydro-3a-methyl-6-methylene-1-(1-methylethyl), (3)1H-Cycloprop[e] azulene,1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7 tetramethyl, (4) Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl), (5) Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl), (6) Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl), (7) α-Cubebene, (8) Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl), (9) Caryophyllene (10) (+)-Epi-bicyclosesquiphellandrene, (11)1(2H)-Naphthalenone, octahydro-4a, 8a-dimethyl-7-(1-methylethyl) and (12)4aH-Cycloprop[e] azulen-4a-ol, decahydro-1,1,4,7-tetramethyl.Even though most of the compounds showed similar molecular weight the structures were seems to be different. The identification of such terpenes were seems to be new to this organism since there no reports available for these compounds from this species. The *in silico* biological activity studies of these compounds by using PASS prediction software was found to be interesting due to the wide range of biological properties exhibited by these compounds. All compounds except 4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl was shown the activity antieczematic may be because of the structural similarities.

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Chapter

5 CHARACTERISATION OF STEROLS AND BIOLOGICAL ACTIVITY STUDIES

Contents

5.1 Introduction
5.2 Materials and Methods
5.3 Results and Discussion
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5.1 Introduction

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products (Williams and Carins 2013). There are more than 6100 coral species all over the world in which the class Anthozoa in the phylum of Coelenterata are the most dominant benthic invertebrates living mainly in tropical seas (Zhang et al. 2005). Cnidaria, formerly known as Coelenterata, is one of the largest among the phyla (Williams et al. 2013). The phylum Cnidaria possesses an array of secondary metabolites, mainly terpenes, and the soft coral group possesses more than 80% of all cnidarian compounds (Harper et al. 2001; Blunt et al. 2007). In the marine environment, the success of defence used by soft corals and gorgonians against consumers and competitors has been attributed to their production of secondary metabolites, many of which show predator deterrence and allelopathic activities (Changyun et al. 2008).

Steroids are highly diverse group of metabolically active compounds and there has been a continuing interest in the sterol and steroids of marine organisms ever since the earliest studies of Henze and Hoppe Seylor 1904 and Doree 1909. The resurgence of interest in marine sterols since 1960 stems in part from the

search for new marine natural products with useful pharmacological properties. Sterols are the major group of secondary metabolites characterizing corals next to sesquiterpenes and diterpenes (Coll 1992). Sterols advocate their dietary inclusion as an important strategy in prevention and treatment of cancer (Woyengo et al. 2009). The "usual" sterols have a 3β-hydroxy-Δ^5- (or Δ^0-) cholestane nucleus and a C_8-C_{10} side chain. There are over 200 such sterols, occurring in marine organisms as complex inseparable mixtures. It has been speculated that phytosterols inhibit the production of carcinogens, cancer-cell growth, invasion and metastasis, and promote apoptosis of cancerous cells (Ju et al. 2004). Mounting evidence suggests that phytosterols possess anti-cancer effects against cancer of the lung, stomach, ovary and estrogen-dependent human breast cancer (Mendilaharsu et al. 1998; De Stefani et al 2000; McCann et al. 2003; Ju et al. 2004; Choi et al. 2007) .

Cholesterol is usually the predominant sterol from Cnidaria with exception of some gorgonian species and member of Zoanthidea. The gorgoninans are distinguished by their content of gorgosterol and related sterol, with a cyclopropane group in the side chain (Schmitz 1977; Paul and Scheuer 1980). Among the genus *Subergorgia*, only very limited studies were reported on the chemical constituents of *Subergorgia reticulata*. Presence of some known polyhydroxylated steroids were reported from *S. reticulata* among which only few exhibited cytotoxic activity (Guo 2004). Yang et al. (2006) isolated nine compounds including three sterols and some alkaloids from *S. reticulata*. Apart from this, some diterpeniods and sesquiterpenoid compounds were also reported from *S. Reticulata* (Parameswaran et al. 1998; Yang et al. 2006; Limna Mol et al. 2011). The present study is an attempt to isolate and identify steroids from the *S. reticulata* collected from Lakshadweep

Island, Kavaratti for the tracing of the anticancer and apoptosis-inducing activities using *in vitro* and *in silico* methods.

5.2 Materials and Methods

5.2.1 Extraction, isolation and purification

The organisms were collected according to the procedure detailed in Chapter 2.2.1. The method of extraction procedure adopted here is from the book by Bhakuni and Rawat 2005 with some modifications. The extraction of the fragmented organism *S. reticulata* (400gm) was carried out at room temperature with methanol (Merk AR grade) for 4 days. The extraction was done under reduced pressure and repeated until, it left with negligible residue were seen on removal of the solvent. The crude residue collected after the was dissolved in minimum quantity of aqueous methanol (80%). In an effort to further characterise the chemical constituents in the original sample, the aqueous methanol sample was fractionated by partitioning with n-hexane (Merk AR grade)**.** The n-hexane extracts were combined, concentrated under vacuum and the n-hexane soluble portion was washed with water, dried over anhydrous $MgSO_4$ and the solvents were removed under reduced pressure. Column chromatography of the residue collected from hexane soluble fraction was done using silica gel with Hexane: Ethyl acetate on varying polarity from 0 to 100%. According to TLC 5% ethyl acetate fraction was selected for further analysis due to the presence of sterols. The fractions were collected and analysed by TLC and GC-MS.

5.2.2 GC-MS analysis.

Gas chromatographic analysis was done on GC-MS (Perkin Elmer Clarus 680) equipped with headspace sampler (Turbomatrix 40 trap). Helium was employed as carrier gas and the ionizing voltage was 70eV. Oven temperature was programmed at 10°C min^{-1} from 60°C to 290°C, where it remained constant for 15 minutes. Injector and detector temperature were kept constant at 280°C and 290, respectively. The column used was Elite 5MS having 30m length and 250µm

id. Mass spectrum of each peak in the total ion chromatogram was resolved and it was compared with National Institute of Standards and Technology (NIST) library spectra for the identification of the compounds.

5.2.3 Structural characterization

Structural characterization was done by FTIR (Perkin Elmer Spectrum 100 series), FTIR analysis was carried out using latest mode of analyzing samples such as solid, liquid, gel and powder with attenuated total reflectance mode (ATR).Gas chromatograph Mass spectrometer (Perkin Elmer clarus 680 equipped with head space) were used to find the molecular weight of the isolated compounds and NIST(National Institute of Standard Technology version 2.1) standard library were used for the spectral matching studies. ^{13}C NMR was done in Bruker NMR having 400MHz frequency.

5.2.4 Apoptosis inducing effect of identified sterols.

Apoptosis inducing effect of the identified sterols from *S. reticulata* was studied using PASS (Prediction Activity Spectra of Substances). The detailed description of the software tool PASS is given in chapter 3.2.7 (Lagunin et al. 2010). Apoptosis plays a crucial role in developing and maintaining the health of the body by eliminating old cells, unnecessary cells and unhealthy cells. The apoptosis agonist is the measure of the anticancer effect of the molecule (Russo et al 2006).

5.2.5 *In silico* docking studies

Docking of small molecule compounds in to the binding site of a receptor and estimating the binding affinity of the complex is an important part of the structure based drug design process. Here Auto dock version 4. (Daniel Seeliger and Bert L de Groot 2010) was selected to study the anticancer potential of the sterol molecule by inhibiting the murine double minute 2 protein (MDM2). The murine double minute 2 (MDM2) protein is the oncoprotein, primary cellular inhibitor of p53 (Chen et al. 1993; Picksley

and Lane 1994). The tumour suppressor protein p53 is the one which acts biochemically as well as biologically control cancer (Hollstein et al. 1991). Design and synthesis of different small molecule inhibitors that block the MDM2-p53 interaction has become an attractive strategy to activate p53 for the treatment of cancer and other diseases (Bottger et al. 1997). MDM2 oncoprotein was discovered by its over expression in a mouse cell line (Fakharzadeh et al. 1991; Momand et al. 1992) and it is the principal negative regulator of the p53 tumour suppressor protein. Loss of function of p53 (*TP53*) gene mutations occur in about half of human cancers (Feki and Irminger-Finger 2004). The inhibition of MDM2 function in cancer is therefore viewed as an attractive means of triggering or enhancing cancer cell death by promoting p53 induced cell cycle and apoptosis. So many compounds are under pre clinical trials through this *in silico* inhibition of MDM2 protein (Shaomeng et al. 2012). The inhibition of isolated sterol molecules from *S.reticulata* with MDM2 protein were studied for the activation of p53 protein. The 3D structure of the MDM2 protein was obtained from protein data bank (PDB ID: 4HFZ). The 3D structure of the sterol molecules were drawn by software Molecular Structure Encoding System (MOSES). Molecular structure encoding system is programming software for processing chemical structure, reactions, reaction generations, synthetic design, data analysis and database management (www.molecular-networks.com). The pdb file obtained was docked with MDM2 oncoprotein using Auto dock (version 4).

5.2.6 *In vitro* cytotoxicity study

In vitro cytotoxicity studies were carried out using Dalton's lymphoma ascites cells (DLA). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with phosphate buffered saline. Cell viability was determined by trypan blue exclusion method (Altman et al 1993; Mascotti et al 2000). Viable cell suspension (1×10^6 cells in 0.1 ml) in phosphate buffered saline (PBS) was used. Control tube contained only cell

suspension. These assay mixtures were incubated for three hours at 37^0C. Further cell suspension was mixed with 0.1ml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The numbers of stained and unstained cells were counted separately. Five different concentrations of the sterol fraction were prepared, 10 µg ml^{-1}, 20 µg ml^{-1}, 50 µg ml^{-1}, 100 µg ml^{-1}, and 200 µg ml^{-1} respectively.

$$\text{Percentage of deadcell} = \frac{\text{Number of dead cells}}{\text{Number of viablecells} + \text{Number of Dead cells}} \times 100$$

5.2.7 Purification of sterols

For the separation of sterol mixture for structural conformation preparative reverse phase HPLC with C_{18} (octadecyl) column (Supelco Sigma Aldrich) having 5µm particle size 10mm inner diameter and 250mm length were used. The elution was done with reverse phase isocratic mobile phase with HPLC grade Methanol (Merk). Injector with a 2.0ml loop and a differential UV VIS detector were used. The sample was dissolved in pure methanol (Merck HPLC grade) and 1 ml injected. The total run time was at 60 minute. The fractions were collected and analysed by GC-MS to know the molecular weight.

5.3 Results and discussion

5.3.1. Isolation purification and GCMS analysis.

From the 5% ethyl acetate fraction, six sterols were identified by comparison of their spectra with those in National Institute of Standard Technology (NIST) library (version 2.2). The total ion chromatogram obtained was shown in Fig. 5.1 and the respective mass spectra of the isolated sterols are shown in Fig. 5.2. The sterols obtained are 26,26-dimethyl-5,24(28)-ergostadien-3β-ol**,** β-sitosterol, cholesta-5,22-diene-3ol(3β), cholesterol, ergosta-5-22-dien 3βol (3 β,22E 24S) and fucosterol. The structures of the sterols were shown in Figure 5.3.

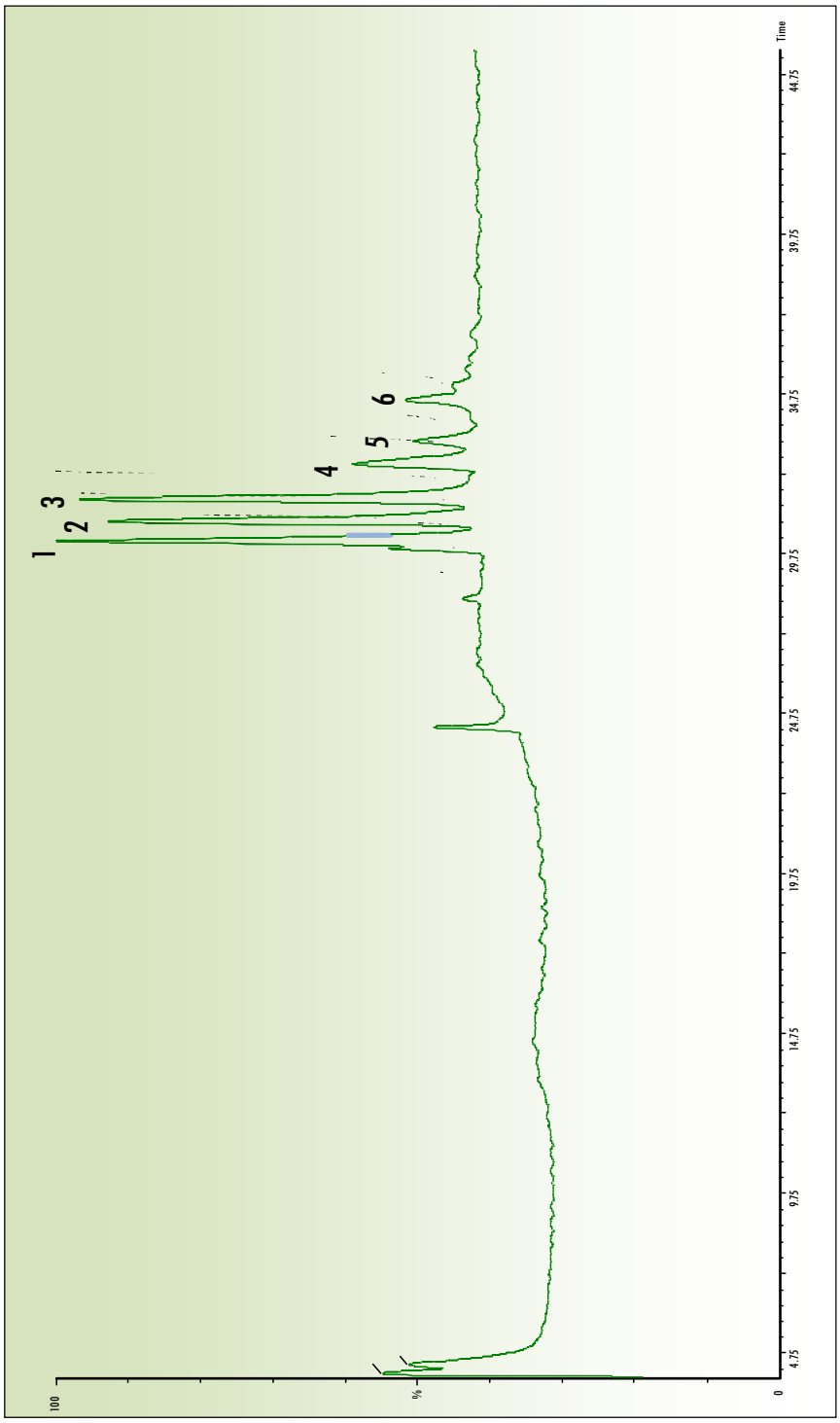
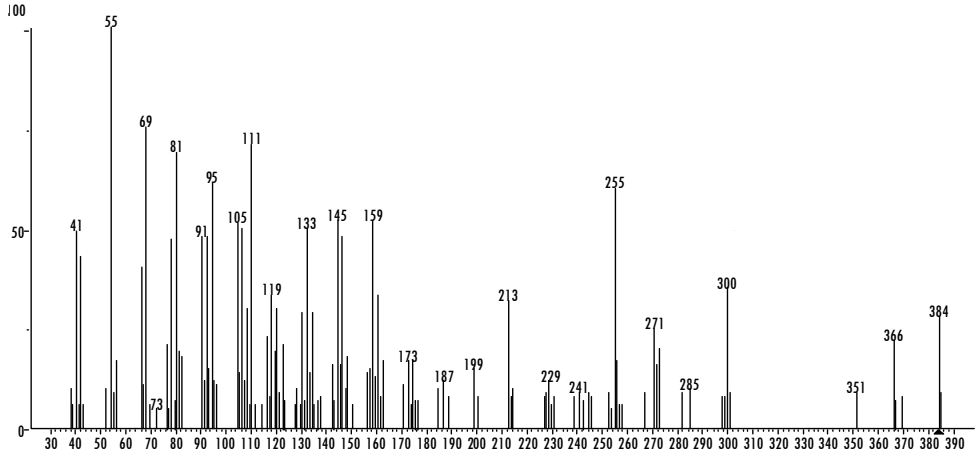
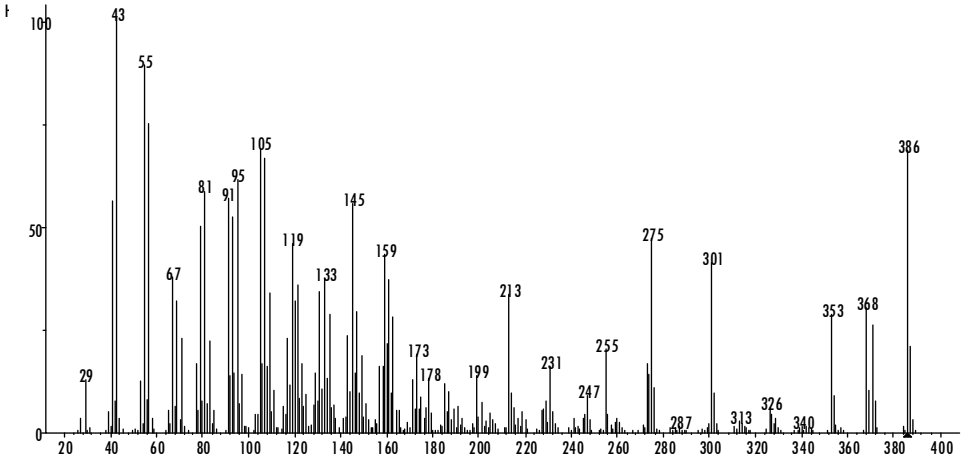


Fig. 5.1 Total ion chromatogram sterol fraction isolated from *S. reticulata.*

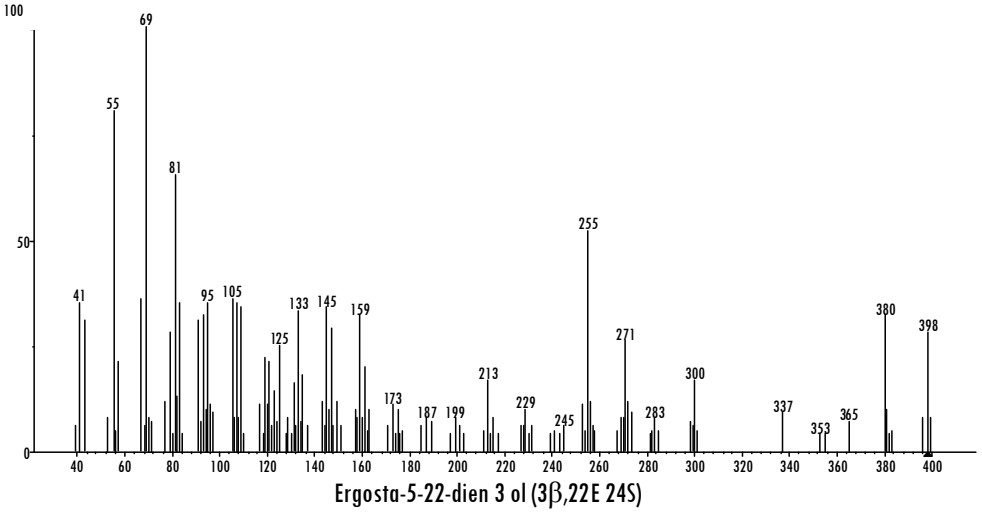
(1) Cholesta-5, 22-diene-3ol(3β) (2) Cholesterol (3) Ergosta-5-22-dien 3 ol (3β,22E 24S) (4) 26,26-Dimethyl-5,24(28)-ergostadien-3β-ol (5) β-sitosterol and (6) Fucosterol



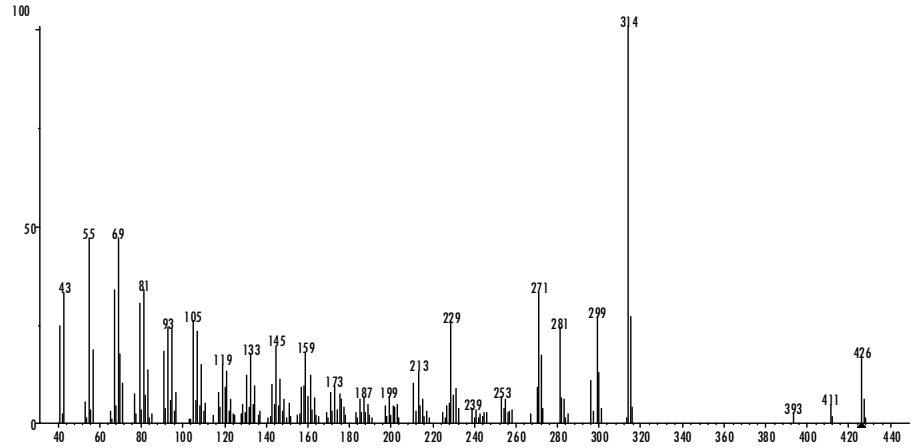
Cholesta-5, 22-diene-3ol(3β)



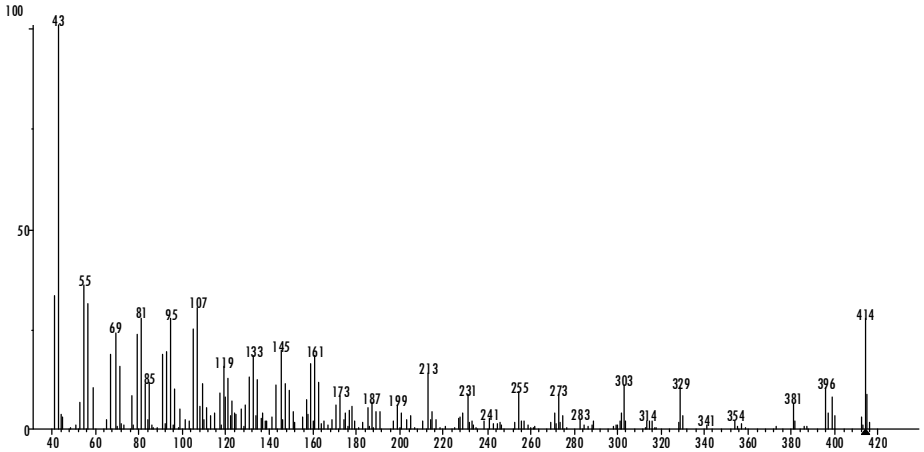
Cholesterol



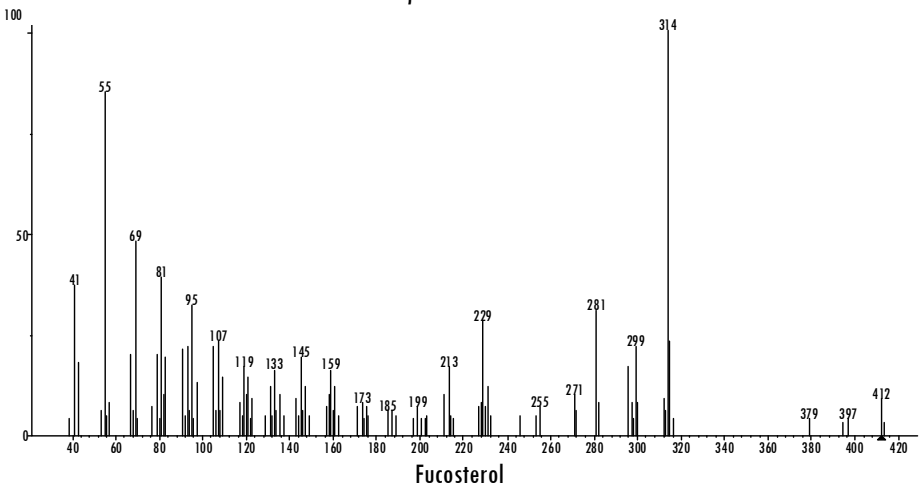
Ergosta-5-22-dien 3 ol (3β,22E 24S)



26,26-Dimethyl-5,24(28)-ergostadien-3β-ol



β-sitosterol



Fucosterol

Fig. No 5.2. Mass spectrum of compounds from the isolated sterol fraction.

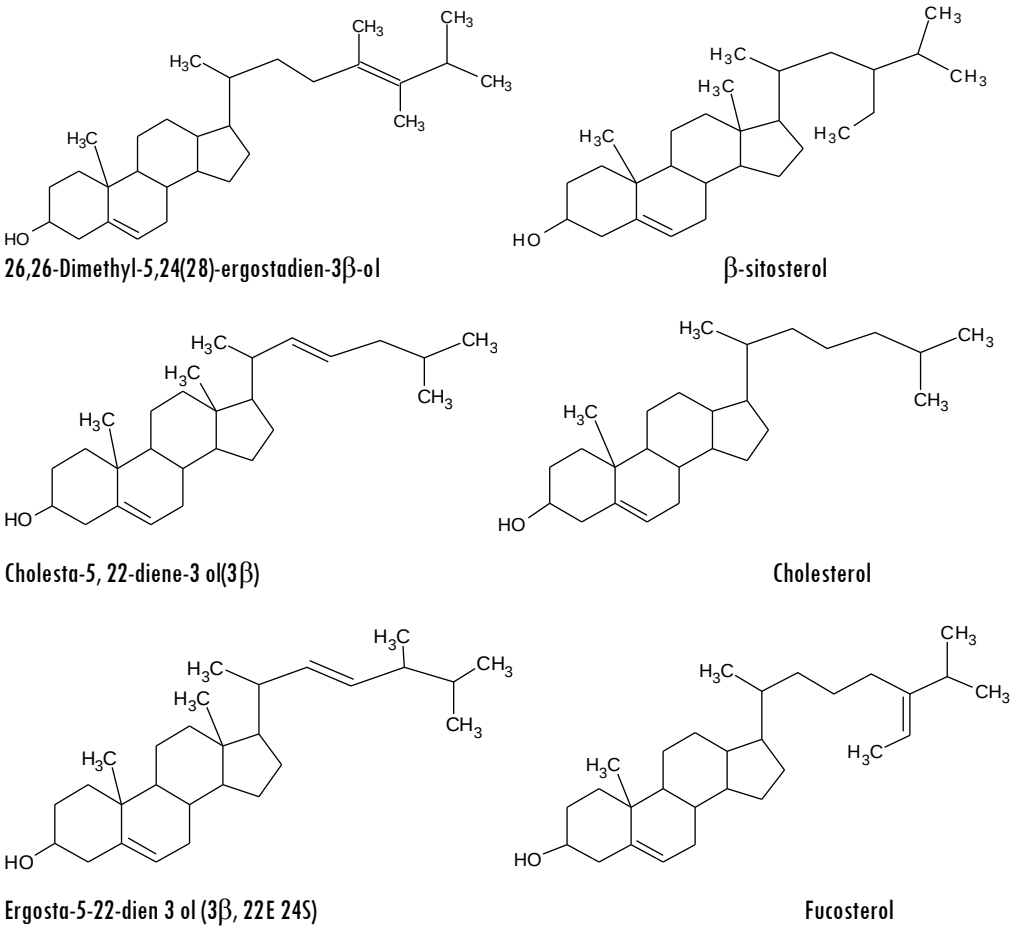


Fig. 5.3 The structure of identified sterols.

5.3.2 Characterisation of purified sterols

The 5% Ethyl acetate fractions obtained after column chromatography were subjected to purification by preparative HPLC and finally one compound was isolated, it was found to be single compound by TLC. The other compounds which were eluted were found to be mixture or not in a pure form. The molecular weight was obtained from GCMS analysis. Finally Cholesta-5, 22-diene-3-ol(3β) was isolated and confirmed by FITR, ^{13}C, and Mass spectrum.

FTIR Results

The IR absorption spectrum showed absorption peaks at 3370.15 indicates the presence of -OH stretching, the peak at 2938.23 is due to the stretching vibrations of aliphatic –C-H bond.

The double bond carbon stretching, -C=C- was observed at 1647.67 is a sharp peak, -C-H bending also observed at 1462.8 from -CH_2. These are the major observations from the FTIR spectrum of the isolated compound. These results are very similar to the sterol FTIR spectrum which obtained for Manish et al 2002 the results were comparable for free hydroxyl group, C-H stretching and -C=C- bond. The spectrum obtained and result are shown in Fig. 5.4 and Table 5.1.

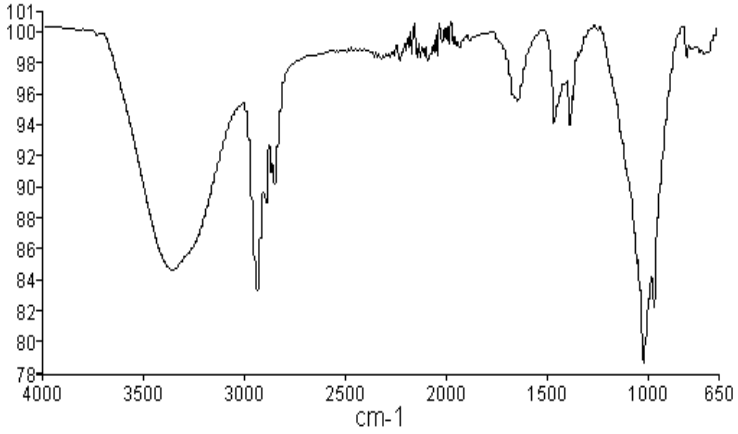


Fig 5.4 FTIR spectrum of purified compound Cholesta-5, 22-diene-3-ol(3β)

Table No.5.1 FTIR absorption peaks

Peak Name	X	Y
1	968.41	82.27
2	1011.74	78.48
3	1381.87	94.12
4	1462.8	94.19
5	1647.67	95.65
6	2091.41	98.24
7	2852.81	90.23
8	2938.23	83.3
9	3370.15	84.63

GC-MS analysis of the isolated compound

GCMS analysis was performed on Perkin Elmer Clarus 680 model equipped with head space. The method used for the analysis is described detail in this chapter 5.2.2 From the GCMS analysis the purity of the sample was found to be 100% (Fig. 5.5) and the MW was 384. It was found that the isolated compound was matching with the mass spectra of Cholesta 5, 22, diene 3-ol 3β from the NIST (National Institute of Standard Technology) library spectral match. Further it was confirmed by ^{13}C NMR studies Mass spectroscopic studies and FTIR.

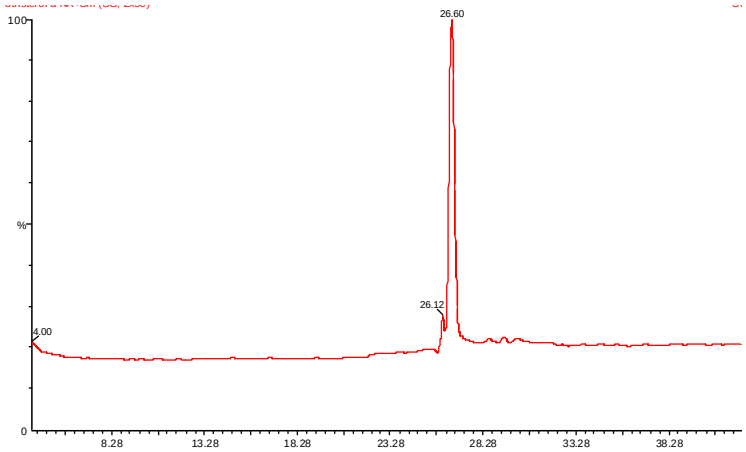


Fig. 5.5 Total ion chromatogram of GCMS analyisis of isolated compound.

^{13}C NMR of isolated compound

^{13}CNMR(CDCl3 400MHz) ^{13}C has given signals at C_1- 36.25, C_2-30.88,C_3-70.81,C_4-40.95,C_5-139.75,C_6-120.6,C_7-30.90,C_8-30.66,C_9-49.16,C_{10}-35.51,C_{11}-20.07,C_{12}-38.68,C_{13}-41.24,C_{14}-55.85,C_{15}-27.55,C_{16}-28.69,C_{17}-54.93,C_{18}-11.04,C_{19}-18.39,C_{20}-39.11,C_{21}-19.84,C_{22}-137.10,C_{23}-125.23,C_{24}-41.30,C_{25}-27.62,C_{26}-21.25,C_{27}-21.30.

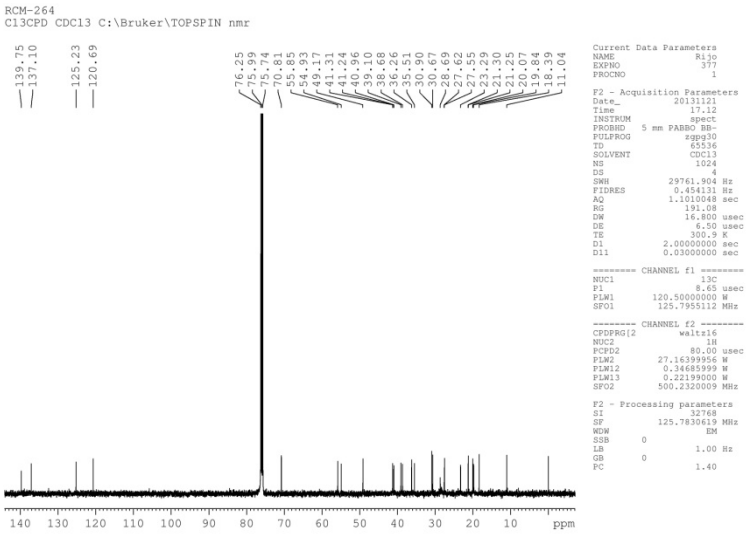


Fig 5.6 ^{13}C NMR spectrum of isolated compound.

5.3.3 Apoptosis inducing effect of identified sterols.

The probability of apoptosis inducing effect of identified sterols from *S. reticulata* was shown in Fig.5.7. The apoptosis agonist activity was found to be maximum for 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol(.803) followed by Cholesta-5, 22-diene-3-ol(3β), Cholesterol, Ergosta-5-22-dien 3-ol (3β,22E 24S), Fucosterol, and β-sitosterol. Cholesta-5, 22-diene-3ol(3β) is prominent among the six sterols identified, and the apoptosis agonist activity was found to be 0.773.

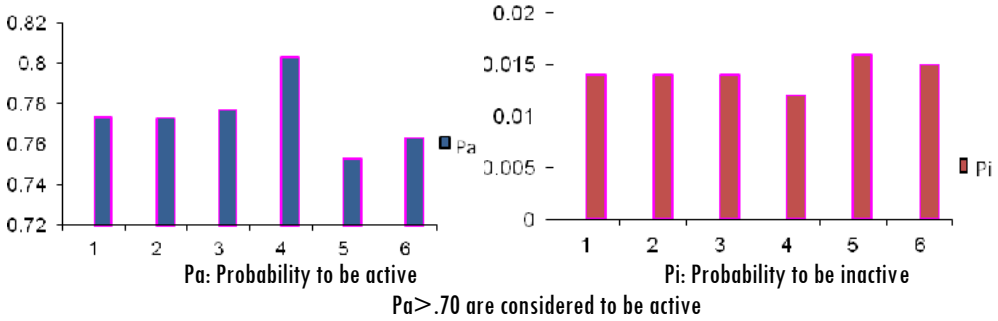


Fig 5.7 Apoptosis inducing effect of the six identified sterols.

(1) Cholesta-5, 22-diene-3-ol(3β) (2) Ergosta-5-22-dien 3-ol (3β,22E 24S) (3) Cholesterol
(4) 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol (5) β-sitosterol and (6) Fucosterol

5.3.4 *In silico* docking studies of sterols

Three sterols compounds, Cholesta-5, 22-diene-3-ol(3β), 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol and Ergosta-5-22-dien 3-ol (3β,22E 24S) were selected for docking studies according to the PASS(Prediction of Activity Spectra of Substances) software. These three compounds were selected because they exhibit the probability to be active maximum in the PASS (Prediction of Activity Spectra of Substances) online prediction. The binding energy calculated from Auto dock was well in agreement with the PASS online prediction. From the PASS online prediction it was found that 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol have maximum apoptosis agonist activity. The *In silico* docking results with MDM2 protein also having very less binding energy for 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol (-4.36 kcal/mol) and Binding Energy of Cholesta-5, 22-diene-3-ol(3β), Estimated Free Energy of Binding = -1.81 kcal/mol , Binding Energy of 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol, is -4.36 kcal/mol, Binding Energy of Ergosta-5-22-dien 3-ol (3β,22E 24S), is -0.06 kcal/mol. The active site of MDM2 protein was studied using AADS (Automated active site identification, Docking and Screening). According to AADS, three active sites were identified having depth of 12.89, 10.88 and 7.53. The ribbon structure of The Murine Double Minute2 (MDM2) protein is obtained from PBD (ID: 4HFZ) as shown in the Fig. 5.8.The active pocket was found by AADS (Automated active site identification, Docking and Screening.) is as shown in the Fig. 5.9 as indicated by the pink ball. The structure of MDM2 docked sterol molecule obtained by Auto dock is shown in the Fig. 5.10.

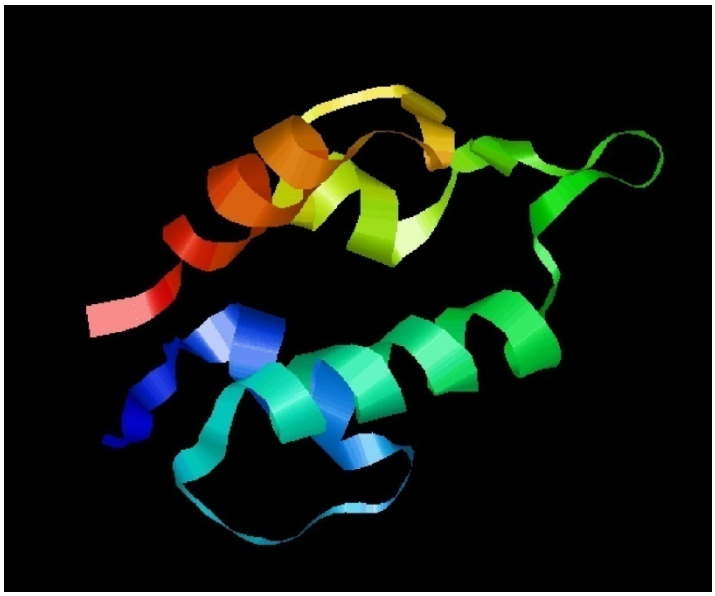


Fig 5.8 Ribbon Structure of MDM2 protein

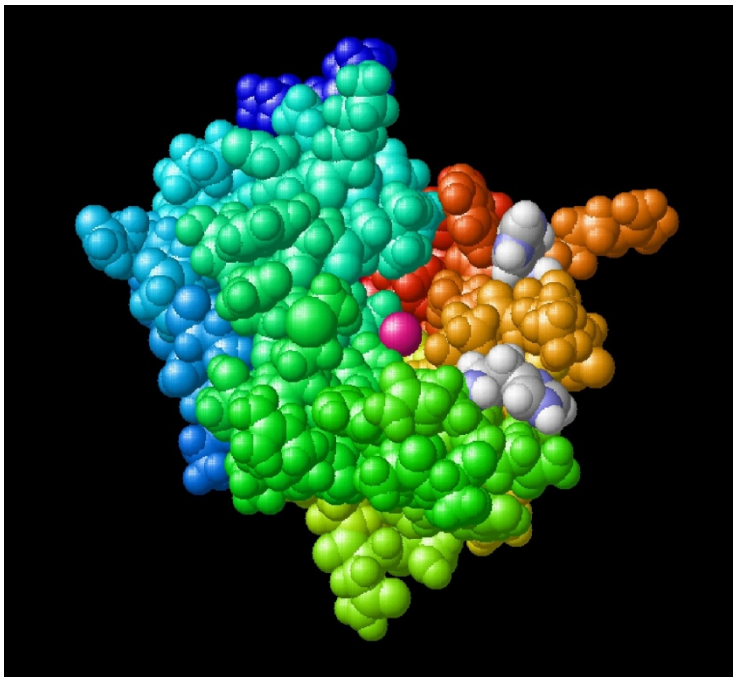


Fig 5.9 Available binding pocket of MDM2 protein (The pink dot indicate the available pocket)

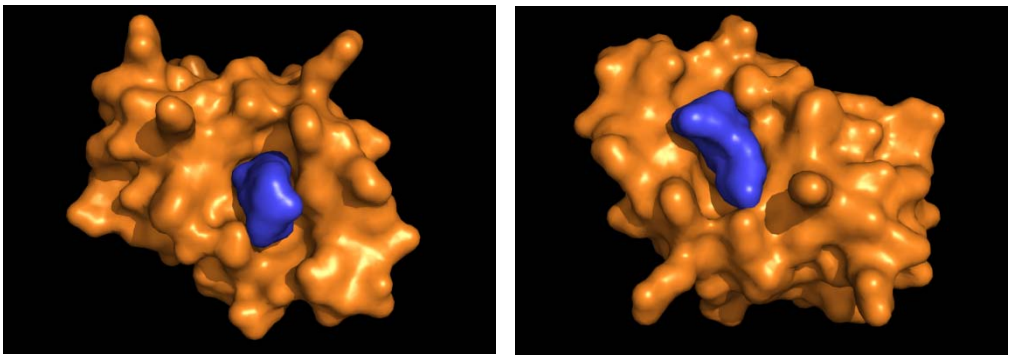


Fig 5.10 The structure of MDM2 protein and active site bind sterol molecule

(a) Cholesta-5, 22-diene-3-ol(3β) (b) 26, 26-Dimethyl-5, 24(28)-ergostadien-3β-ol

5.3.5. *In vitro* cytotoxicity study

The sterol mixture obtained after column chromatography ie 5% Ethyl acetate fraction was studied for short term *in vitro* cytotoxicity. The graph showing the variation of cell death of tumour cells on increase in concentrations of the sterol fraction is represented in Fig. 5.11 The study resulted in significant cell death, the number of dead cells was increased with increase in concentraction of the sterol fraction. 90% cell death was obseverd at 200 µg ml^{-1} concentration of the sterol fraction. The results are summarised in Table 5.2.

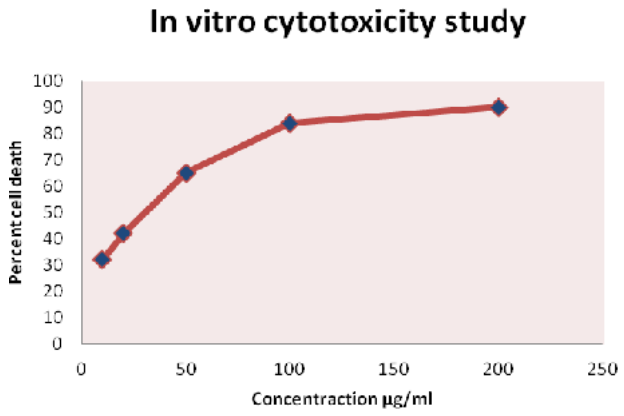


Fig 5.11 Effect of the application of the sterol fraction from *S.reticulata* on the tumour cells. Cell viability was determined by trypan blue exclusion method.

Table 5.2 Anticancer activity of sterol fraction from *S.reticulata*

Concentraction of sterol fraction in µg/ml	Percentage cell death (DLA)%
200	90
100	84
50	65
20	42
10	32

5.4 Conclusions

This study has succeeded in isolating the four sterols namely cholesta-5, 22-diene-3ol(3β), ergosta-5-22-dien 3ol (3β,22E 24S), 26,26-dimethyl-5,24(28)-ergostadien-3β-ol, and β-sitosterol for the first time from the soft coral *S. reticulata*. From the *in vitro* studies, it is evidenced that almost a saturation value (>85%) was attained at slightly above 100 µg ml^{-1} concentration of the sterol fraction. The anticancer activities of sterol fraction isolated from *S. reticulata* can be due to a natural mixture of its components, and a single constituent may not have an activity greater than that of the mixture. Individually cholesterol and β-sitostrol were reported for their potential for apoptosis agonist. β-Sitosterol is generally considered as a phytosterol with chemical structure similar to that of cholesterol and is the most common sterol in human diet. Studies have already indicated that β-sitosterol can inhibit the growth of various cultured cancer, and the simulation of apoptotic cell death (Raicht et al. 1980; Awad et al. 2003; Choi et al 2003). From the PASS prediction, the study found that although β-sitosterol exhibited apoptosis agonist activity as a natural component in cancer prevention as reported by Awad et al. 1996, it is the lowest among the sterols identified from *S. reticulata*.

The GC MS identification of sterol molecule Cholesta-5, 22-diene-3-ol(3β)was further confirmed by FTIR,NMR and Mass spectroscopy technique

found in well accordance with the results of the gas chromatographic separation and NIST library report. Eventhough these sterols have very similar structure the activity relationships are seemingly variable. The apoptosis agonist activity was maximum for 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol(Probability of activity is 80.3) and the probability of apoptosis inducing effect of all other sterols studies were found to be greater than 70%. There is no proven report for supporting 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol, ergosta-5-22-dien 3-ol (3β,22E 24S), fucosterol and Cholesta-5, 22-diene-3-ol(3β) that these are apoptosis agonist. It has been reported that intracellular cholesterol accumulation induces apoptosis of pancreatic cells(Lu et al. 2011), it also support the results with our findings that inducing effect of cholesterol is prominent with a probability of 0.77. Fucosterol is the most abundant phytosterol in brown algae and it was proved that fucosterol containing fraction of marine algae responsible for cytotoxic effect against breast and colon carcinoma cell lines (Khanavi et al. 2012). There is no evidence to prove fucosterol itself can induce apoptosis, but the PASS study conducted here have supported the findings of Khanavi et al. 2012 by predicting the probability of apoptosis inducing effect of fucosterol as 0.763.

From the *In silico* docking studies of sterols such as 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol, Cholesta-5, 22-diene-3-ol(3β) and ergosta-5-22-dien 3-ol (3β,22E 24S) suggest these sterol as a drug candidate in the cancer therapy. The compound 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol having very least amount of free energy of binding (-4.36 kcal/mol) is the best suited drug among the sterols identified form *S.reticulta* in the present study, because of the critical inhibitory role of MDM2 on p53, the sterol molecule 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol blocks the interaction of MDM2 and p53 by binding the active site of MDM2 protein.

Yang et al. (2006) reported the presence of cholesterol and isofucosterol from the South China Sea gorgonian coral *S. reticulata* with seven other compounds. Li et al. (2005) and Hsu et al. (2005) investigated that anticancer activity of some therapeutic substances is involved in the induction of apoptosis which can be used for cancer control. This is in accordance with our findings that apoptosis induction of these sterol molecules found to be anticancer agents. Induction of apoptosis in cancer cells is one useful strategy for anticancer drug development (Hu and Kavanagh 2003).

It has been realized well in case of octocorals that de novo biosynthesis is an important mechanism (Garson 1989). However, the alternative possibility must be considered that they are able to absorb sterols from food which may well comprise largely plankton and small crustacean species that are rich in sterols (Shimizu et al. 1976, Withers et al. 1978). Since soft corals are associated with plankton and micro organisms, the contribution of sterols from their part cannot be omitted. Biosynthesis of sterol from this soft coral *S. reticulata* is yet to be investigated (Dewick 1999) and it could be beneficial to know the sterol pathway to provide additional clues to understand its role in reproduction, chemical signalling or as defensive metabolite.

The apoptosis inducing effects and *in vitro* cytotoxicity studies of identified sterols from the soft coral *Subergorgia reticulata* were investigated. A significant apoptosis inducing effect was observed for each compound and it is the primary screening of pharmacological potential of sterols that we had identified. However, the determination of apoptosis by *in silico* may be considered as an indirect method, comparing to those performed in tissues. The cytotoxic results of this sterol compounds mixture confirm it as a natural potent chemopreventive and chemotherapeutic agent. In contrast, this apoptosis inducing effect was apparent when measured using PASS, online prediction assay give us an idea about the possibility for the development of an anticancer drug, and still more in vivo studies are required to prove the potentiality for an anticancer drug from the marine soft coral *Subergorgia reticulata*.

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Chapter

6 CHARACTERISATION OF POLYSACCHARIDES

Contents

6.1 Introduction
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6.3 Results and Discussion
6.4 Conclusion
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6.1 Introduction

Polysaccharides are complex carbohydrates and are most abundant of organic compounds; represent a structurally diverse class of macromolecules of relatively widespread occurrence in nature. They are macromolecules made up of many monosaccharide units joined together by glycosidic bonds and offer the highest capacity for carrying biological information due to the greatest potential for structural variability (Sharon and Lis 1993). Polysaccharides widely exist in the plants, microorganism (fungi and bacteria), algae, and animals. Together with proteins and polynucleotides, they are essential biomacromoleules in the life activities and play important roles in cell-cell communication, cell adhesion, and molecular recognition in the immune system (Dwek 1996). Since polysaccharides are major class of biomolecules, their bioactivity studies are limited (Stryer 1995). Wong et al. 1994 reported the biological property of polysaccharides such as glucans and proteoglucans from plants, and it is due to immunomodulatory action and would be useful in the treatment of cancer. Preclinical studies of several polysaccharides isolated from higher plants, mushrooms and seaweeds have demonstrated antitumour activity against transplantable tumours in mice (Sakagami and Minoru Takeda 1996; Yamada et

al. 1990). Biological properties of sulphated fucoidans extracted from marine invertibrates, seaweeds were found to be anticoagulant, antithrombotic and also active against cancer cells (Berteau and Mulloy 2003; Luyt et al. 2003; Durand et al. 2008). Antitumor, antiviral, anticoagulant, antioxidant, immuno-inflammatory and some other medicinal properties are unique for marine derived polysaccharides (Wei Wang et al. 2012).

Apart from the terrestrial plant derived polysaccharides, marine polysaccharides usually exhibit structure similar to mammalian glycosaminoglycans such as heparin and chondroitin suphate which coming under three category marine animal polysaccharides, marine plant polysaccharides and marine microbial polysaccharides (Jiao et al. 2012). Marine algal derived polysaccharides often have power over inhibitory effects on a variety of viruses (Jiao et al. 2011; Witvrouw and Clereq 1997).

The hunt for promising polysaccharides as antitumor agents probably stems from dissatisfaction and side effects from the existing cancer chemotherapy and radiotherapy. Various reports are available on chemical compounds which can act as anti cancer agents; but their cytotoxicity towards normal cells is unambiguous. Many of the potential anticancer drugs have considerable side effects and therefore have little clinical use. Hence, the discovery and identification of newer safe drugs which are active against tumours is an important motive of this work. This study is about bioactivities of marine polysaccharides isolated from *Subergogia reticulate*.

6.2 Materials and Methods

6.2.1 Collection of coral

The soft corals were collected from Lakshadweep Islands during December 2011. The sampling was carried out by SCUBA diving in the region at 25m depth. The organisms (Fig. 2.2) were brought to the laboratory for processing. The details of collection of sample and preservation is stated in Chapter 2.2.1 & 2.2.2

6.2.2 Extraction and isolation

The extraction of the fragmented organism, *S. reticulata* (100gm), was carried out at room temperature with methanol for 4 days. The process of extraction was repeated with hexane to remove all the lipophilic compounds present until, only negligible residue was left on removal of the solvent. The sample was further dried in freeze drier and 50gm of dried sample were extracted continuously with hot water at 60^0C in a water bath. The aqueous fraction was collected until the extraction was completed and lyophilised. The lyophilised water soluble fraction was further dissolved in minimum quantity of deionised water and ethanol was added until the complete precipitation of polysaccharides (Xue et al. 2012). The precipitated polysaccharides were washed with ethanol thrice and again dissolved in HPLC water, lyophilised, weighed and kept in -4^0C until analysis. 50 mgs of the sample were dissolved in minimum quantity of HPLC water and dialysed through dialysing membrane whose MW cut off 12000 Dalton (Purchased from SIGMA ALDRICH) with deionised water to remove all small molecules and impurities. After dialysing the sample was collected, lyophilised, weighed and kept in -4^oC.

6.2.3 Characterization of the polysaccharide extracted from the soft coral *S. reticulata*

Structure analyses of polysaccharides are very tedious due to their complex nature. Reported marine animal polysaccharides are found to be diverse structural features includes chitosan derived from crustaceans, chondroitin sulphates from cartilaginous fishes, sulphated polysaccharides from sponges and glycosamino glycans from scallops and abalone (Singh and Ray 2000; Zierer and Mourao 2000; Huang and Zheng 2004). Average structure of the repeating units of heparin and heparinoid polysaccharides is shown in Fig. 6.1.

Total carbohydrates and total Protein. Total carbohydrate content and total protein in the isolated polysaccharides of the gorgonian coral *S.reticulata* was determined colourimetrically by Phenol-Sulfuric acid method (Dubois et al. 1956) and Lowry et al. 1951 respectively. The detailed procedure is given in Chapter2.2.8 & 2.2.9

CHNS Analysis. Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) contents were analyzed using elemental analyzer, Elementar vario EL III, and using CHNS operation mode. The percentage of elements C, H, N, and S in an organic compound can be individually or simultaneously analyzed over a wide range of sample matrices and concentrations with the aid of elemental analyzer.

Sulphate estimation. The sulphate content was measured using modified $BaCl_2$- turbidimetric method of Tabatabai (1974). The reagent was prepared by dissolving 0.60g of Bacto gelatine in 200ml 60-70˚C of Milli Q water. This was allowing this to cool at 4ºC for 10 hrs. Then the temperature was increased to 20-25˚C and 2.0g of $BaCl_2$. $2H_2O$ were dissolved. The reagent

was stable for at least 2 months if stored at 2-4˚C. Reagent grade K_2SO_4 was used as standard in the range of 10-50µg/ ml. An accurately weighed sample (20mg) was hydrolysed for 2 hours at 100˚C in 0.5ml of 2N HCl in a sealed pyrex tube. The contents were then volumetrically transferred and made up to 10ml in a standard flask. 2ml of sample, 18.0ml of Milli Q water, and 2.0ml of 0.5N HCl were mixed in 50ml standard flask. 1ml of $BaCl_2$-Gelatin reagent was added and swirled. After 30min the contents of the flask were again mixed by swirling and turbidity was measured in a 2.0cm cell at 550nm against reagent blank.

FT-IR Analysis. FT-IR spectra of polysaccharide samples were recorded at $4cm^{-1}$ resolution with 4 scans on a FTIR Spectrophotometer (Perkin Elmer spectrum 100) in the range from 400 to 4000 cm^{-1}. The spectrophotometer was equipped with Universal Attenuated Total Reflectance accessory which permits solid and liquid sample analysis with much spectral reproducibility. The system was permanently maintained under nitrogen atmosphere to reduce atmospheric CO_2 and H_2O absorption. The background spectrum was measured for the atmosphere, and the sample spectra were automatically recorded against CO_2 and H_2O bands.

Thermal stability measurement. Thermal stability measurement of the polysaccharides was carried out using TGA/DTA (SIINT 6300-Japan) at a constant heating rate of 15^{o}Cmit in the temperature range from 30 to 900^{o}C. Alumina was used as the reference. The dried sample was weighed and analysed for the thermal stability measurement.

Monosaccharide Composition of Polysaccharide using HPLC. 20 mg of polysaccharide sample was dissolved in 2 ml of 2 M TFA and the subsequent

steps were followed according to the modified method of Fengel and Wegener 1979. The detailed procedure of analysis is given in Chapter 2.2.10.

6.2.4 Biological activity of the Polysaccharides

DPPH Radical Scavenging Assay. The DPPH assay was done according to the method of Brand-Williams et al. 1995 with some modifications. The stock solution was prepared by dissolving 24mg DPPH with 100ml methanol and then stored at -20℃ until needed. The working solution was obtained by mixing 10ml stock solution with 45ml methanol to obtain an absorbance of 1.170+/-02 units at 515nm using the spectrophotometer. Fruit extracts (150 µl) were allowed to react with 2850 ml of the DPPH solution for 24 h in the dark. Then the absorbance was taken at 515nm. The standard curve was linear between 25 and 800mM Trolox. Results are expressed in mM TE/g fresh mass. Additional dilution was needed if the DPPH value exceeds the linear range of the standard curve.

FRAP Reducing Power Assay. The FRAP assay was done according to Benzie and Strain (1996) with some modifications. The stock solutions included 300mM acetate buffer (3.1 gm $C_2H_3NaO_2.3H_2O$ and 16mL $C_2H_4O_2$), pH 3.6, 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40mM HCl, and 20mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ solution, and 2.5ml $FeCl_3.6H_2O$ solution and then warmed at 37℃ before using. Fruit extracts (150µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colour product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The standard curve was linear between 25 and 800 mM Trolox. Results are expressed in mM TE/g fresh mass. Additional dilution was needed if the FRAP value exceeds the linear range of the standard curve.

***In silico* prediction of Biological Activity.** *In silico* prediction of biological activity was done using the software Prediction of Activity Spectra for Substances (PASS) (Lagunin et al. 2010).The detailed description about the software which is already stated in Chapter 3.2.7. The *in silico* activity was analysed for monomer structures predicted in the three different forms of sulphated polysaccharides such as heparine, dextran sulphate, chitosan sulphate, and ulvanobiuronic acid (Fig.6.1).

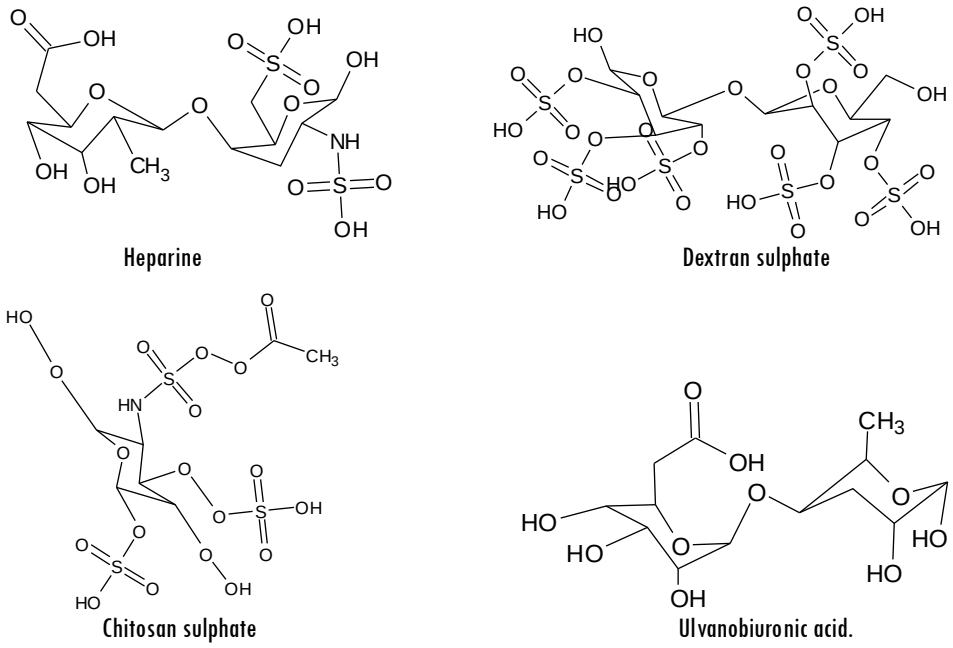

Fig 6.1. Average structure of the repeat unit of heparin and heparinoid polysaccharides
a. Heparine. b. Dextran sulphate. c. Chitosan sulphate. d. Ulvanobiuronic acid

***In vitro* cytotoxicity study.** *In vitro* cytotoxicity studies were carried out using Dalton's lymphoma ascites cells (DLA). The tumor cells aspirated from the peritoneal cavity of tumor bearing mice were washed thrice with phosphate buffered saline. Cell viability was determined by trypan blue exclusion method (Altman et al. 1993; Mascotti et al. 2000). Viable cell suspension (1×10^6 cells in 0.1 ml) in phosphate buffered saline (PBS) was used. Control tube

contained only cell suspension. These assay mixtures were incubated for 3 hours at 37°C. Further, cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 min and loaded on a hemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The numbers of stained and unstained cells were counted separately. Five different concentrations of the sterol fraction were prepared, respectively, as 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1}, 100 μg ml^{-1}, and 200 μg ml^{-1}.

$$\text{Percentage of dead cell} = \frac{\text{number of dead cells}}{\text{number of iable cells} + \text{number of dead cells}} \times 100$$

6.3 Results and discussion

6.3.1 Characterization of the polysaccharide extracted from the soft coral *S. reticulata*

CHNS analysis, total carbohygrate, and protein concentration were calculated for the preliminary information about the polysaccharides isolated from the gorgonian soft coral, *Subergorgia reticulata.* The major elements of the polysaccharides were found to be sulphur (14.9%) followed by carbon (8.86%) nitrogen (2.41%) and hydrogen (0.58%) The results are shown in (Table 6.1). The carbohydrate concentration of the polysaccharides was 12.06 mg/l and protein concentration was 67mg/l. From these observations the polysaccharide is found to contain both carbohydrate and protein concentration in which protein predominates than carbohydrate. These results furnish the design that polysaccharides may be a glycosaminoglycan that contains amino sugar along with sugar units.

Table 6.1. Result of CHNS analysis

Sample No	Sample Name	N%	C%	S%	H%
1	*S.reticulata* polysaccharide	2.41	8.86	14.90	0.58

Sulphate estimation

The observation for the standard and sample is shown in Table 6.2. The concentration of sulphate content in the sample was found to be 15mg/l from Fig. 6.2.

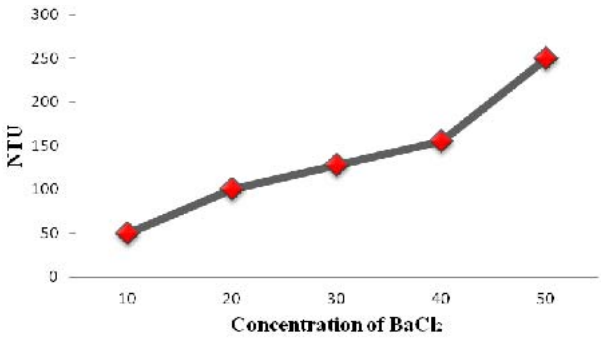

Fig. 6.2. Estimation of sulphate using $BaCl_2$ turbidimetric method.

Table 6.2. Reading obtained for the different concentration of $BaCl_2$ standard sample.

Turbidimetry Concentration (ppm)	Reading (NTU)
10	50
20	100
30	128
40	155
50	250
Sample	75

FTIR results

A broad band centred at 3271 cm^{-1} assigned to hydrogen bonded O-H stretching vibrations (Fig.6.3). Presence of sulphate ester group which is a characteristic component of sulphated polysaccharides (Ruperez et al 2002; Chandia and Matsuhiro 2008; Synytsya et al. 2010), were also observed at 1261cm^{-1} and a very sharp peak at 1083cm^{-1}.The peak at 1083 cm^{-1} is of presence

of glycosidic linkage containing sulphate ester bond. The study of carrageenans by FTIR showed the presence of very strong absorptions bands in 1261cm^{-1} regions (S=O of sulphate esters) and a sharp peak at 1083cm^{-1} is for glycosidic linkage (Robert and Quemener 1999, Leonel et al. 2003), and it was reported that 1210-1260 cm^{-1} region is assigned for sulphate ester O-SO^{3-} group (Knutsen et al. 1994). The wave number corresponding to 1632cm^{-1} indicates the presence of amide peaks, that is the isolated polysaccharide may be a glycoprotein or which contains heparin monomer. It was well established that the presence of amide peaks will be in the 1650cm^{-1}-1550cm^{-1} region (Mc cann et al. 1992).

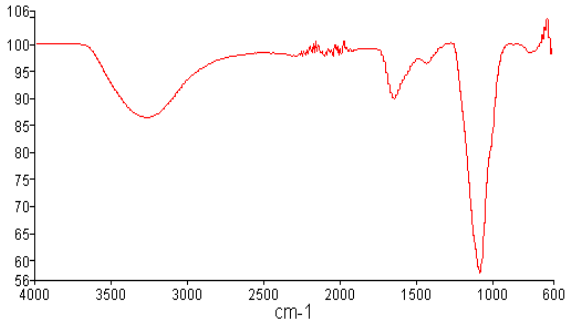

Fig.6.3. FTIR spectrum of the isolated polysaccharide from *Subergorgia reticulate*

Thermal analysis

First weight loss in the TGA is due to water content below 100^{o}C (Fig.6.4).The evidence of xylan and starch structure was obtained from the sharp peak in the DTA curve at 345^{o}C (Li et al. 2010). The second phase weight major weight loss is found to at 520^{o}C, 563^{o}C caused by the presence of disulfide bridges in the fibrinogen. The presence of 563^{o}C maxima in the DTA indicates higher cross linked structure of the macromolecular network probably due to the stronger association of the glycoprotein-proteoglycan-collagen complexes. Since the organism posses the Ca sclerites in the skeleton structure the association of this structure with calcium salts cannot be neglected (Bihari varga et al. 1988).

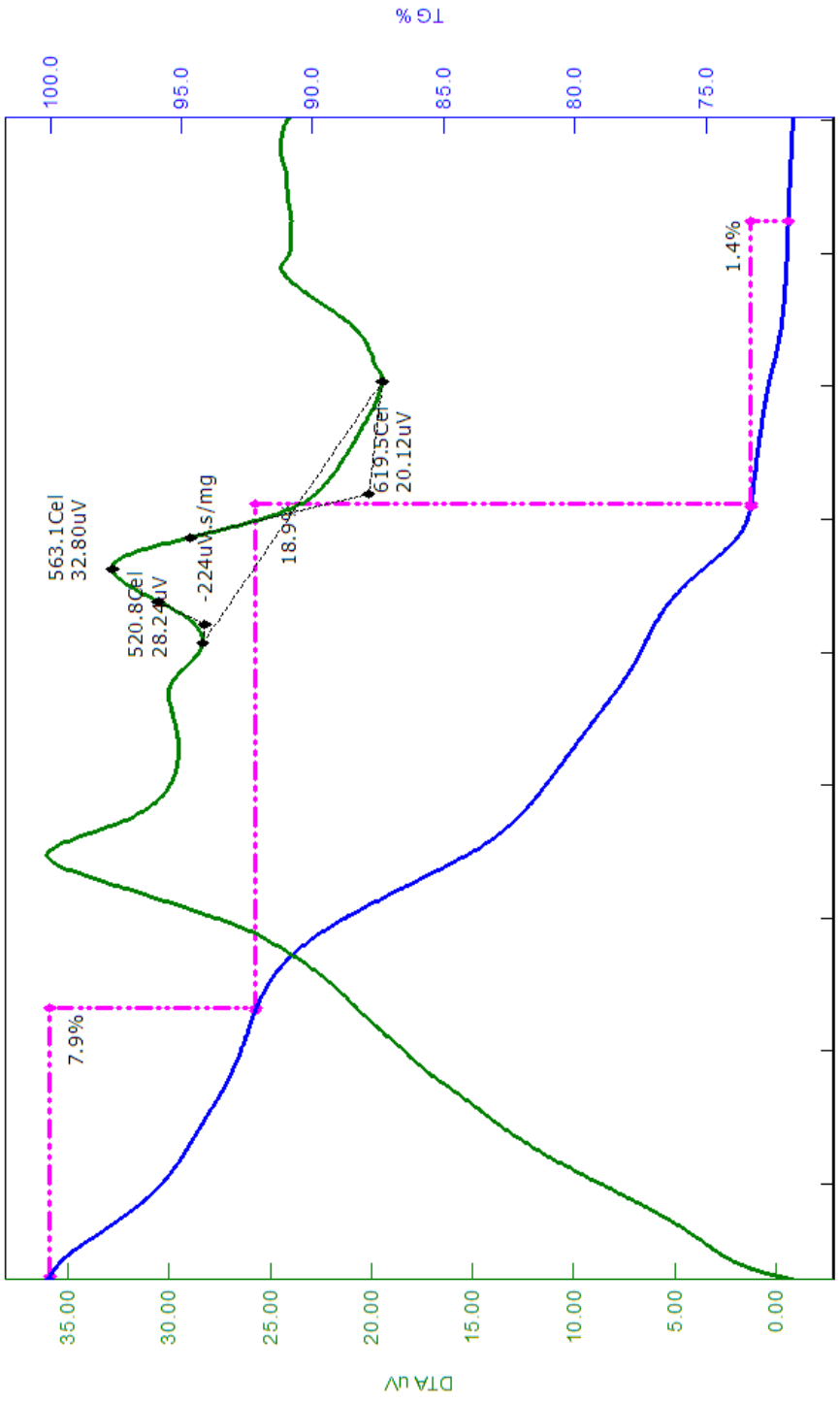

Fig.6.4. Thermo gravimetric (TGA) analysis of the polysaccharides isolated from the soft coral *Subergorgia reticulata*

Monosaccharide composition analysis

Ten known common sugars were used for the determination of monosaccharide and disaccharide concentrations. Arabinose found to be leading monomer unit with 26.64 mol% of total monosaccharide and disaccharide monomer followed by ribose(24%), Mannose (15.71%), galactose(13.81%), maltose(7.06%), sucrose(5.99%), glucose(3.75%) and lactose(2.4%) (Fig.6.5). Arabinose sugar unit has been reported to be higher in cell walls of plants susceptible to pathogens (Maxemiuc-Dietrich 1978; Aldington and Fry 1992). It was also reported that some plant species, which do not produce phytoalexins, exhibit higher amount of arabinose (Marcia et al. 1998). The results were shown in table 6.3.

Table 6.3. Results of monosaccharide and disaccharides composition.

Sugar unit	Retention time (minutes)	Monosaccharide& disaccharide composition (mol %)
Ribose	6.771	24
Arabinose	7.58	26.64
Mannose	8.024	15.71
Glucose	9.312	3.75
Galactose	10.229	13.81
Sucrose	12.864	5.99
Maltose	15.2	7.06
Lactose	16.562	2.4

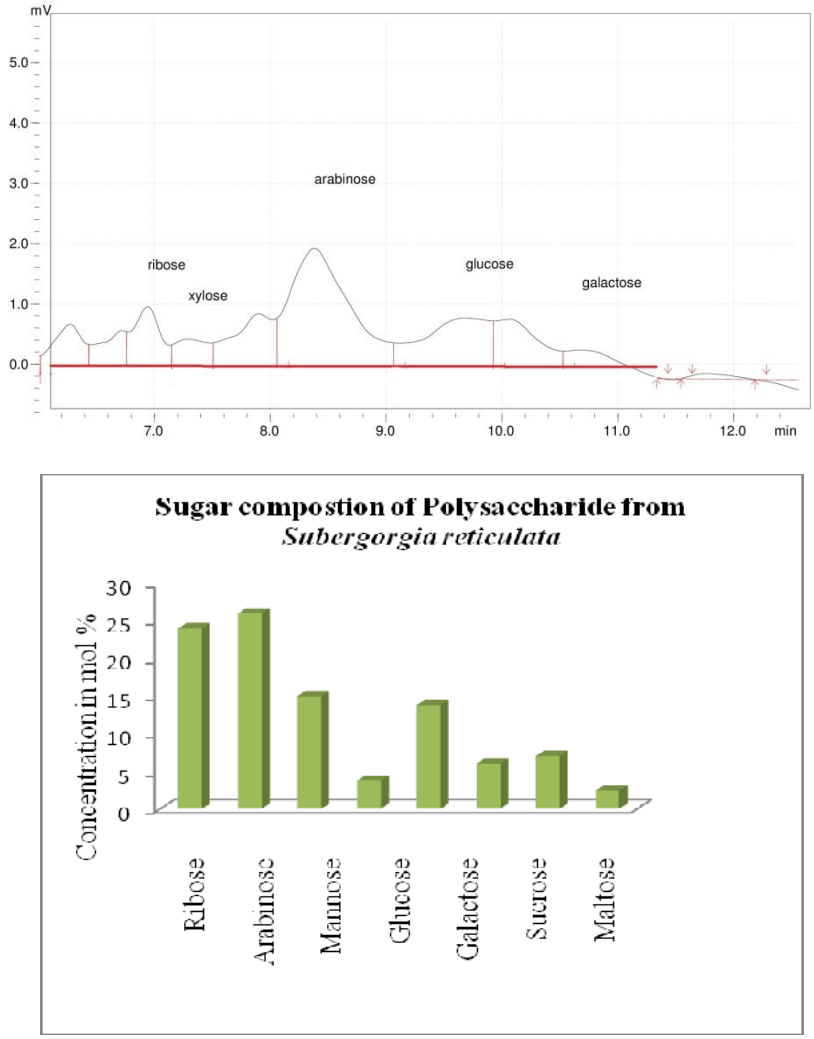

Fig 6.5 a. Chromatogram of sugar units present in the polysaccharides isolated from *S.reticulata* **b.** Composition of sugars in the isolated polysaccharide from *S.reticulata.*

6.3.2 Biological activity of the polysaccharide isolated from the soft coral *Subergorgia reticulata*

In silico prediction of biological activity

Using PASS (Prediction of Activity Spectra of Substances) prediction software it was found that the monomer unit having more sulfate group in the structure will possess more biological activity. The software was used to get

the information of biological activity of respective units prior to *in vitro* studies. The application of PASS prediction will help to understand and modify the molecule to get the results in a better way. There are ample of research are ongoing to make nontoxic drug candidate as polysaccharides, and still there is very few attempt was made to suggest why the biological activity varies among the structures. This attempt was made to confirm whether the sulfate group is incorporated in the biological activity, and it was found that the biological activity was found remarkable variation when the sulphate group incorporation was prominent. The major activities in which Pa value >7 only purposeful and accepted since the probability of action is more. The biological activity studies were fibroblast growth factor antagonist, cholesterol antagonist, hepatoprotectant, hepatic disorder treatment, antiinflomatory, sclerosant, antiviral and antibacterial. The comparison of these biological activities with the different monomer was given in Table.6.4

Table 6.4. List of biological activity predicted by PASS for the four selected monomer units of the sulphated polysaccharide reported in this investigation

Monomer units	Biological activity Pa >0.7							
	1	2	3	4	5	6	7	8
Heparine	+	-	-	-	-	-	-	+
Dextran sulphate	+	+	+	+	+	+	-	-
Chitosan sulphate	+	+	+	+	+	+	-	-
Ulvanobiuronic acid	-	-	+	-	-	-	-	+

1) Fibroblast growth factor antagonist
2) Cholesterol antagonist
3) Hepatoprotectant
4) Hepatic disorder treatment.
5) Antiinflomatory
6) Sclerosant
7) Antiviral
8) Antibacterial

In vitro cytotoxicity studies

In vitro cytotoxicity analysis were carried out by Tryphan blue exclusion method and it was found that it is cytotoxic at 200ug/ml causing 26% cell death (Fig. 6.6 a & b, Fig. 6.7) Marine polysaccharides containing suphate group enhances the biological activities such as antioxidant and anticancer activities (Qi et al. 2005;Yuan et al. 2005; Zou et al. 2008). From the structural studies sulphate concentration was found to be 14.9% which is indicative of the antioxidant as well as cytotoxic properties. From the *In silco* studies of the basic monomers of heparin, dextran of marine origin, such as heparin, dextran sulphate, chitosan sulphate are found to be fibroblast growth factor antagonist. Monosaccharide composition and sulphate content of isolated polysaccharides support the prevalence of dextran sulphate and heparin.

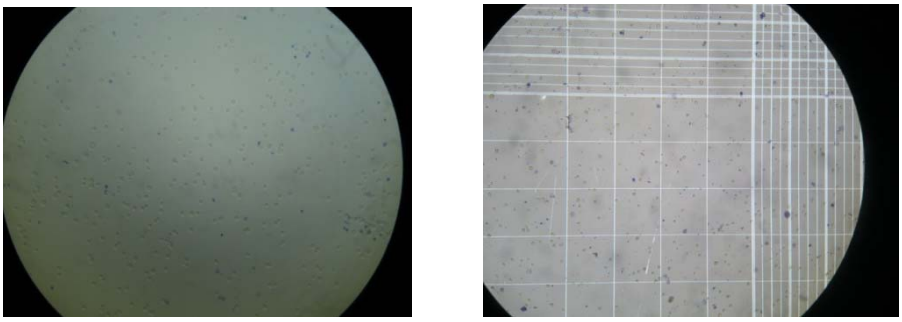

Fig.6.6 (a) *In vitro* anticancer activity of isolated polysaccharides from *subergorgia reticulata,* blue spot indicates the dead cells and white spot live cell lines at lower concentration of sample at 50µg/ml. **(b)**. At concentration of 200µg/ml.

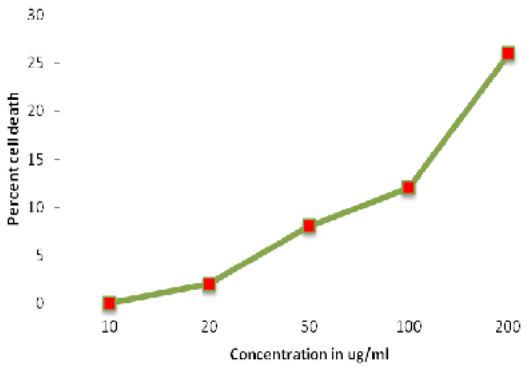

Fig. 6.7. Graph showing the effect of isolated polysaccharides in varying concentration on cancer cell lines.

Antioxidant properties of the polysaccharides isolated from *Subergorgia reticulata*

Antioxidant properties of the polysaccharides isolated were evaluated using DPPH and FRAP assay and it was found that it is a strong antioxidant as that of the standard used in the analysis. The effect of DPPH radical scavenging assay and FRAP assay is shown in Fig. 6.8. The scavenging property of the polysaccharide sample improved with increase in concentration. The inhibitory concentration at 50 (IC_{50}) from the graph was calculated and it was required 11.46mg/ml by weight. DPPH scavenging activity of the polysaccharides was strong free radical scavenger since the IC_{50} <1000µgml^{-1}which is near to the obtained value. However compared to the standard Trolox, showed obvious radical scavenging property which is comparable with the standard value. From the FRAP reducing assay the antioxidant property of the polysaccharides was high compared to the standard and which substantiate for the DPPH results.

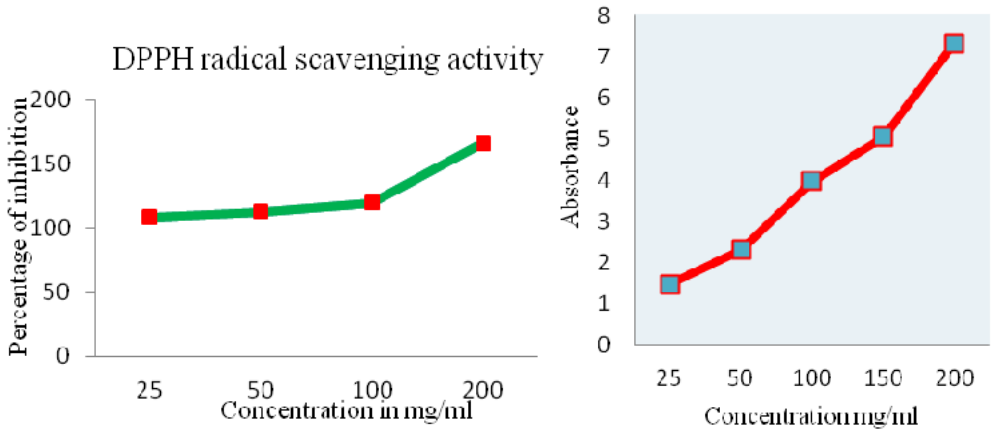

Fig. 6.8 (a) Graph showing the DPPH scavenging property of the polysaccharide isolated from gorgonin coral *subergorgia reticulata.* **(b).**Graph showing the FRAP scavenging property of the polysaccharide isolated from gorgonin coral *subergorgia reticulata.*

Conclusion

The polysaccharides isolated from the gorgonin coral *subergorgia reticulata* was found to be a strong antioxidant and having anticancer property. This study is the first report to the best of our knowledge on the carbohydrate characterisation of the gorgonin coral *subergorgia reticulata.* From the structural studies we substantiate that the isolated polysaccharide is sulphated in nature. From the monosaccharide composition studies, arabinose is found to be the leading sugar unit, which support the building monomer of the tissue of the organism as in the case of pectin polysaccharides in plants since it have more arabinose concentration. Presence of amide group and the presence nitrogen in the elemental composition focuses it should be a glycoprotein having glycosidic linkage with sugars. Thermal decomposition studies reveal the presence of high thermally stable polysaccharide consist of collagen-proteoglycan-glycoprotein complexes. The best suggested structure form the results obtained is the glycosaminoglycans, in view of the fact that it's a mucopolysaccharide is present in the organism. This is a preliminary study of the biological evaluation of the isolated polysaccharides.

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Chapter

7 SUMMARY

Natural products show a diversity of chemical structures that are not accessible even by synthetic concepts. Chemical investigations on natural products of marine origin can lead to the discovery of bioactive compounds of unique structure because natural products are good sources of drugs - those from terrestrial sources account for 47% of the drugs developed for human consumption. Even though 70% of the earth's surface is covered by water more than 70% of the earth's biodiversity is in the sea, rich sources of bioactive compounds from sea remains relatively untapped. Marine organisms have developed exquisitely complex biological mechanisms and evolved in the ability of synthesis chemical weapons to survive from predators. A huge number of natural products and novel chemical entities exist in the Oceans with biological activities that may be useful in the quest for finding drugs with grater efficacy and specificity for the treatment of human diseases.

Coral reefs, the special features of tropical and sub tropical oceans, are one of the Earth's most beautiful ancient and complex ecosystems. Soft corals are very common, especially in shallow waters, known to contain secondary metabolites such as terpenes, steroids and steroidal glycosides with unique structures and most of them exhibit various biological activities. The success of defense used by soft corals and gorgonians against consumers and competitors has been attributed to their production of secondary metabolites, many of which show predator deterrence and allelopathic activities. Although many bioactive compounds of soft corals were isolated, there are only very

few studies about its biological activity. This study focuses on isolation and identification of such types of compounds from the gorgonian coral *Subergorgia reticulata* collected from the Kavaratti Island of Lakshadweep archipelago. Lakshadweep archipelago is a coral island, rich in biodiversity with 89 species of marine algae, 7 species of seagrasses, 82 species of sponges, 45 species of crustaceans, 424 species of mollusca, 97 species of echinodermata and 603 species of fishes.

The main objective of this study was the examination of the chemistry and bioactivity of the compounds from the Indian gorgonian coral *Subergorgia reticulata.* To attain this objective, the study was focused in to the pharmaceutical applications of the identified and isolated compounds by *in silico* and *in vitro* approaches. The organism was extracted using solvents on polarity basis, according the method described in the text book Bioactive Marine Natural products by Bhakuni and Rawat 2005.Since the organism exhibited high yield in the Ethyl acetate fraction and also the significant antimicrobial properties of the fractions, this study focused in the isolation and identification of compounds from ethyl acetate fraction. The purification and identification were done using sophisticated instruments such as HPLC and GC-MS. The spectroscopic techniques such as FTIR and NMR were used to characterize the final structure of the isolated compound. To assess the biological activity of the isolated and identified compounds, *In silico* tools such as PASS and AutoDock and *In vitro* anticancer studies using MTT assay and HIV1 RT assay were used. The biological activities studied in this work such as investigations with the isolated compound on human breast cancer cellines MCF-7 and human liver cancer celllines HepG2 are much pertinent because till date the sciences have not found any ultimate antidote for these serious ailments. In this chapter the summary and conclusions of all the results

which were obtained in the analysis in both chemical as well as biological aspects were scrutinized and critically justified.

The morphology of the organism is studied to identify the special features of the studied organism and to examine the physical features. Investigation of sclerites from the gorgonian coral *S.reticulata* was done to understand the relationship between the organic matrix formation in the sclerites to provide binding sites for Ca^{2+} ions and their contribution to $CaCO_3$ crystal formation. The clear SEM image of sclerite shows presence of three different types of sclerites. The double headed and spindle shaped were found to be predominant in the top most part. Scale like sclerites are found to be rich at the bottom axial part, which may provide more mechanical support to the organism, with sessile habitat at the benthic region of sea. Mg- bound calcite crystalline rombohydral structure was proposed for the sclerites of *S.reticulata.* Apart from the pure calcite, the existence of calcite polymorphs with magnesium is observed from XRD and EDX data, rather than a pure calcite. From the XRD analysis the crystalline nature was found to be $CaCO_3$ rhombohydral shape in which 2.9% is magnesium. From the total protein and total carbohydrate analysis it was observed protein is the leading primary metabolite in the sclerite of *S.reticulata.* No toxic level of metals such as Hg and As were not detected in the sclerites. Whereas Pb and Cd were present at the permissible limit. Ca, Mg, K, Sr were the leading elements present in the sclerites. Amino acid analysis indicated more information about the nature of the protein present in the organic matrix of sclerites. The analysis of the amino acids from sclerites of *S.reticulata* has shown the richness of aspartic acid residues in sclerites. Hence the process of biomineralisation in the formation of sclerites in *S. reticulata* is also speculated to follow the procedure in other

organisms. Sugar analysis was also done to know the contribution of sugar building unites in the biomineralisation process.

From the preliminary examination, it is very clear that almost all types of compounds present in the organism such as terpenes, alkaloids, steroids, carbohydrates, lipids, saponins, flavanoids and sesquterpenes. From the quantitative analysis, protein concentration was high compared to carbohydrates and lipids. From the preliminary analysis of the volatile compounds in the crude methanol fraction by GC-MS analysis it is confirmed the presence of eight compounds such as 1,3-Benzodioxole, 5-ethoxy-6-(1-(4-methoxyphenyl)ethyl) b) 2,6,6-Trimethyl-2-cyclohexene-1,4-dione c) Benzamide, 2,3,4,5,6-pentafluoro-N-(2-phenylethyl) d) Benzene, 1-butyl-4-[(4-ethylphenyl)ethynyl] e) cis,trans-1,7-Dimethylspiro[5.5]undecane f) trans-2-methyl-4-n-pentylthiane, S,S-dioxide g) Phenol 2,4,bis 1,1dimethylethyl h) (2R)-2,5,7,8-tetramethyl-2-(4R,8R)-4,8,12-trimethyl tridecyl)-6 chromanol. Even though there is only very few investigations were done from the organism about the chemical constituent, the identified compounds are seems to be new from this organism. From the analysis of the sugar units , it was found that arabinose is the leading monosaccharide present in the organism with a percentage concentration of 53.4% compared to the other sugar units. The mucus membrane glycosides which are found to be rich of arabinose , and other sugar units were identified as ribose (20.68%), xylose (9.14%), glucose (8.41) galactose (8.27%). There are no previous reports available on the study of monosaccharides and sugar units present in the organism. It is a mystery about which sugar unit is present in the organic matrix. The presence of Xylose, glucose, galactose is may be due to the association of xylose derived glycoproteins. A high concentration of aspartic acid was found and it is very clear that Ca binding proteins, which makes the sclerites to be hard, give more support to the organism. The highest concentration of amino acid

detected was threonine, an essential amino acid required for building the collaginous matrix, the type of protein usually found in the outer tissue part of the gorgonian corals. This collagenous matrix makes the organism hard and flexible for the water column circulation. Other amino acids were also present in small amounts, compared to aspartic acid and threonine.

Twenty four fatty acids compounds were identified using GC-MS. Among them only one is found to be halogen containing fatty acid, which is chloroacetic acid tetra decyl ester. Even though there are some studies done on the fatty acid methyl ester composition in some of the corals, this study is the first report on the fatty acid methyl esters from the gorgonian coral *Subergorgia reticulata*. The lowest molecular weight fatty acid was found to be 2- methyl heptanoic acid (molecular weight 144 and molecule formulae $C_8H_{16}O_2$) and the highest molecular weight fatty acid was found to be tetracosanoic acid methyl ester(molecular weight 382 and molecular formulae $C_{25}H_{50}O_2$). *In silico* biological activity studies by PAAS online prediction for the identified compounds, it was found that all the eight compounds have one or other biological activity. The important biological activity shown includes phobic disorder treatment, anti neurotic, anti obesity, anti diabetic, antioxidant, apoptosis agonist and anti inflammatory. These findings will be appended for the future drug candidate from *S.reticulata*. From the antimicrobial property of crude as well as ethyl acetate and hexane fractions it was found that ethyl acetate fraction was active against all pathogens including both Gram positive and Gram negative. The important features are found to be the antimicrobial activity of the extracts and the eight identified compounds by GC-MS. The study of fatty acids compositions is found to be very significant.

Corals are reported to be rich of terpenes and terpenoid compounds and the present study also gave similar results. The compounds identified from

GC-MS analysis were found to be very similar in structure and even they have very close retention time. It seems to be very difficult for the isolation of pure compound without the help of the instrument since they are having very similar structure. Three calamenenes sesquiterpenes such as 2-methoxy 5-acetoxy calamenene, 2,5 Dimethoxy calamenene and 2-methoxy calamenene having cadinene skeleton have been isolated from this gorgonian coral *S.reticulata*. The compounds such as calamenenes were previously isolated from the same species and different terrestrial sources, but the biological activity of the compound was not systametically approached. The compound 2-methoxy 5-acetoxy calamenene was found to be having anticancer activity against human breast cancer cell line (MCF-7). The activity of this compound 2-methoxy 5-acetoxy calamenene was found to be significant when compared to the normal drug dosage. IC_{50} value of the compound 2-methoxy 5-acetoxy calamenene was found to be 59.36µg/ml. The compound 2,5 Dimethoxy calamenene was found to be having anticancer activity against human liver cancer cell lines(HepG2). This activity is high when compared to the compound 2-methoxy 5-acetoxy calamenene, since the IC_{50} value seems to be very low, 2.80µg/ml. Anti HIV activity was tested for the compound 2-methoxy calamenene and it was found that the compound is having moderate activity. Since the anti HIV activity of the compound 2-methoxy calamenene is less than the positive drug control Nevirapine, it cannot be suggested for a good drug target. But it can be suggested for the future application by synthetiic modifications. The anticancer activity of the compounds such as 2-methoxy 5-acetoxy calamenene and 2,5 Dimethoxy calamenene can be considered to be a drug target, if more studies such as clinical trials are undergone.

GC-MS analysis revealed the presence of twelve terpene compounds such as (1) Azulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl) (2)

Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl) (3)1H-Cycloprop[e]azulene,1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7 tetramethyl.(4) Naphthalene,1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl).(5) Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl). (6) Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl).(7) α-Cubebene (8) Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)(9) Caryophyllene (10) (+)-Epi-bicyclosesquiphellandrene (11)1(2H)-Naphthalenone, octahydro-4a, 8a-dimethyl-7-(1-methylethyl) and (12)4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl. However the compounds showed similar molecular weight the structures were seems to be different. The identification of such terpenes was found to be new to this organism since there no reports available for these compounds from this species. The *in silico* biological activity studies of these compounds by using PASS prediction software was found to be interesting due to the wide range of biological properties exhibited by these compounds. All compounds except 4aH-Cycloprop[e]azulen-4a-ol, decahydro-1,1,4,7-tetramethyl showed the activity antieczematic, may be because of the structural similarities.

This study has identified the four sterols namely cholesta-5, 22-diene-3ol(3β), ergosta-5-22-dien 3ol (3β,22E 24S), 26,26-dimethyl-5,24(28)-ergostadien-3β-ol, and β-sitosterol for the first time from the soft coral *S. reticulata.* From the *in vitro* cytotoxicity studies, it is evidenced that almost a saturation value (>85%) was attained at slightly above 100 µg ml^{-1} concentration of the sterol fraction. The anticancer activities of sterol fraction isolated from *S. reticulata* can be due to a natural mixture of its components, and a single constituent may not have an activity greater than that of the mixture. Cholesta-5, 22-diene-3-ol(3β)was further confirmed by FTIR,NMR and Mass spectroscopy technique and found to be in well accordance with the

NIST library report. Even though these sterols have very similar structure the activity relationships are seemingly variable. The apoptosis agonist activity was maximum for 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol and the probability of apoptosis inducing effect of all other sterols studies were found to be greater than 70%. There is no proven report for supporting 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol, ergosta-5-22-dien 3-ol (3β,22E 24S), fucosterol and Cholesta-5, 22-diene-3-ol(3β) that these are apoptosis agonist. There is no evidence to prove fucosterol itself can induce apoptosis, but the PASS study conducted here have supported the findings of other researchers by predicting the probability of apoptosis inducing effect of fucosterol as 0.763. The *In silico* docking studies of sterols such as 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol, Cholesta-5, 22-diene-3-ol(3β) and ergosta-5-22-dien 3-ol (3β,22E 24S) suggest these sterol as a drug candidate in the cancer therapy. The compound 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol having least amount of free energy of binding (-4.36 kcal/mol) is the best suited drug among the sterols identified form *S.reticulta*. In the present study, because of the critical inhibitory role of MDM2, the sterol molecule 26,26-Dimethyl-5,24(28)-ergostadien-3 β-ol blocks the interaction of MDM2 and p53 by binding the active site of MDM2 protein. This is in accordance with these findings that apoptosis induction of these sterol molecules found to be making it anticancer agents. Induction of apoptosis in cancer cells is one useful strategy for anticancer drug development. The apoptosis inducing effects and *in vitro* cytotoxicity studies of identified sterols from this soft coral *Subergorgia reticulata* were primary screening of anticancer potential of the sterols. However, the determination of apoptosis by *in silico* may be considered as an indirect method comparing to those performed in tissues, this study will be an expansion in the development of new pharmacologically

active sterols from this organism, still more *in vivo* studies are required to prove the potentiality for an anticancer drug.

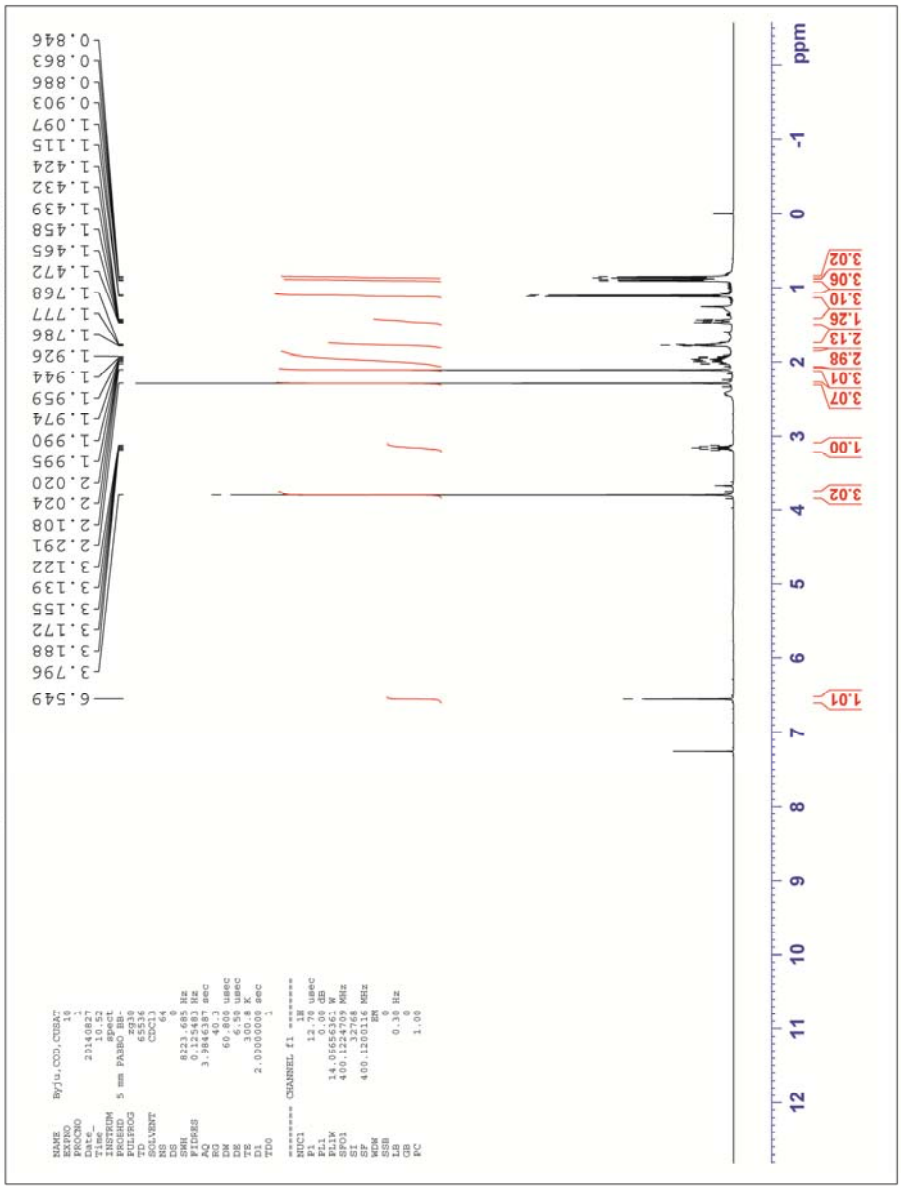
The polysaccharide isolated was found to be a strong antioxidant and having anticancer property. This is the first report on the carbohydrate characterization of this gorgonin coral *subergorgia reticulata*. From the structural studies it was found that the isolated polysaccharide is sulfated in nature. From the monosaccharide composition studies, arabinose is found to be the leading sugar unit, which supports the building monomer of the tissue of the organism as in the case of pectin polysaccharides in plants since it have more arabinose concentration. Presence of amide group and the presence nitrogen in the elemental composition focuses that it should be a glycoprotein having glycosidic linkage with sugars. Thermal decomposition studies reveal the presence of high thermally stable polysaccharide consisting of collagen-proteoglycan-glycoprotein complexes. The best suggested structure form the results obtained is the glycosaminoglycans, in view of the fact that it's a mucopolysaccharide present in the organism.

Natural products have been considered as the forefront of medicine to treat human diseases. Although almost all of the current natural product-derived therapeutics has terrestrial origins, marine natural products are believed to be superior to terrestrial natural products in terms of chemical novelty as the marine environment is a rich source of both biological and chemical diversity. The present study successfully isolated three terpene compounds and characterized. The biological activity of the compounds, the three aromatic sesquiterpene isolated, were found to be active against breast cancer, liver cancer and HIV virus. The identified compounds were also exhibited wide range of biological activity according to *in silico* studies. The sterols identified were found to be apoptosis agonist. The future prospect of these compounds needed further

clinical trials for clarification. The further development of these compounds as a drug candidate lies beyond the scope of this thesis, which requires more clinical trials and complex biological assays. The synthetic modifications of the isolated and identified compounds are good drug target since the biological activity of the compounds depends on the structural variability. This study recommend with a great hope for the future drug candidate by small changes in chemical structure of the identified compounds. It could be concluded that this gorgonian coral is a good source of biologically active compounds for the future development of drug candidate.

ANNEXURE

NMR Spectrum of Compound 1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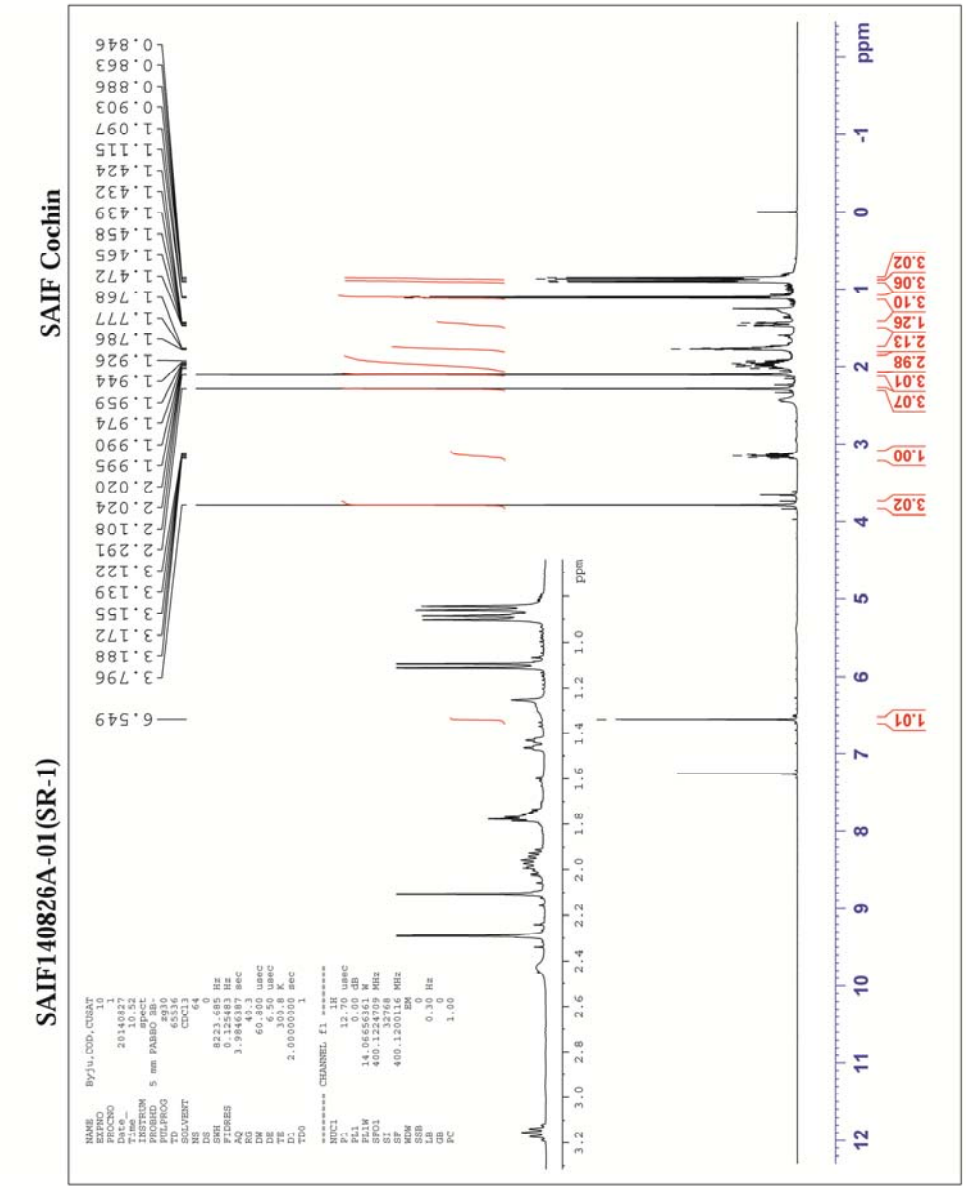
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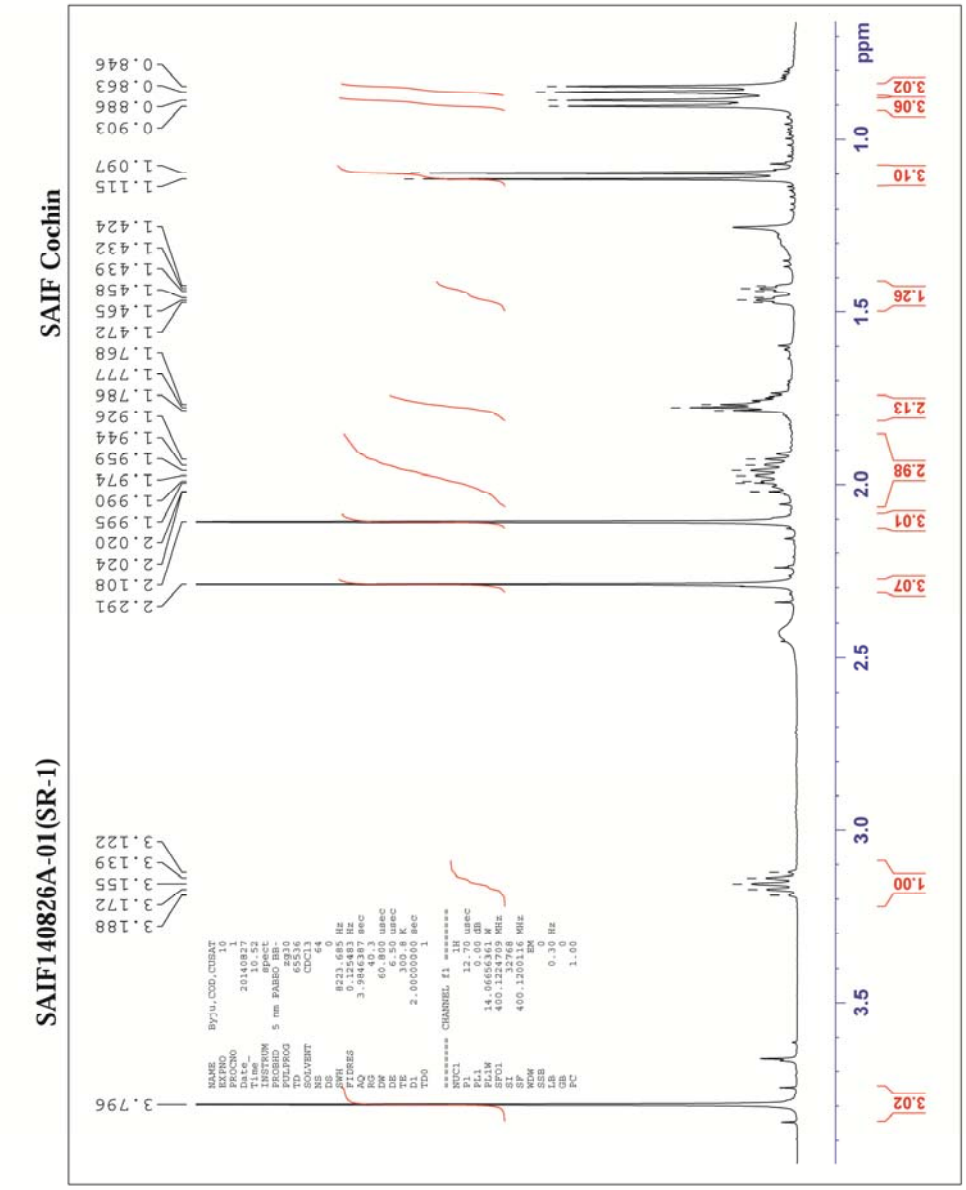
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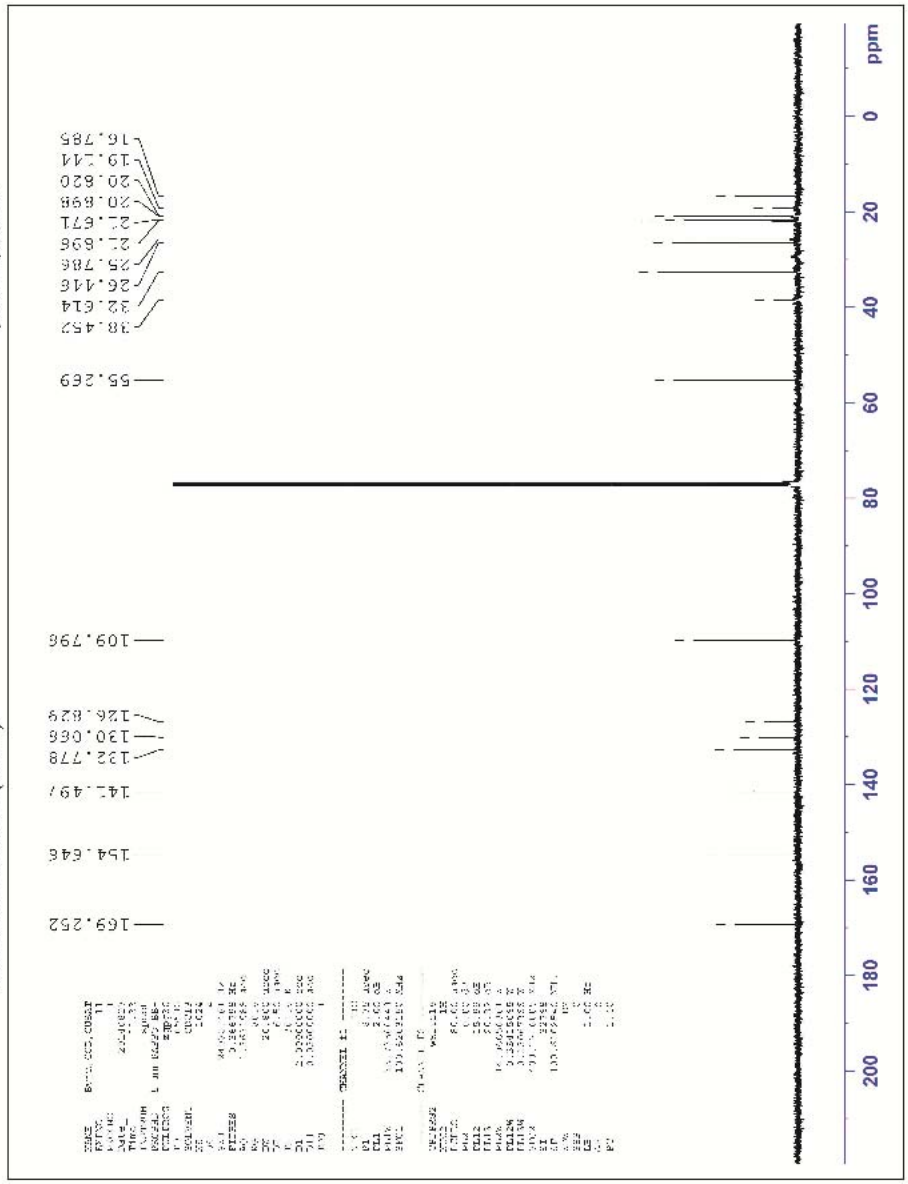
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20.820
20.898
21.671
21.898
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26.446
32.614
38.452
55.269
109.796
126.829
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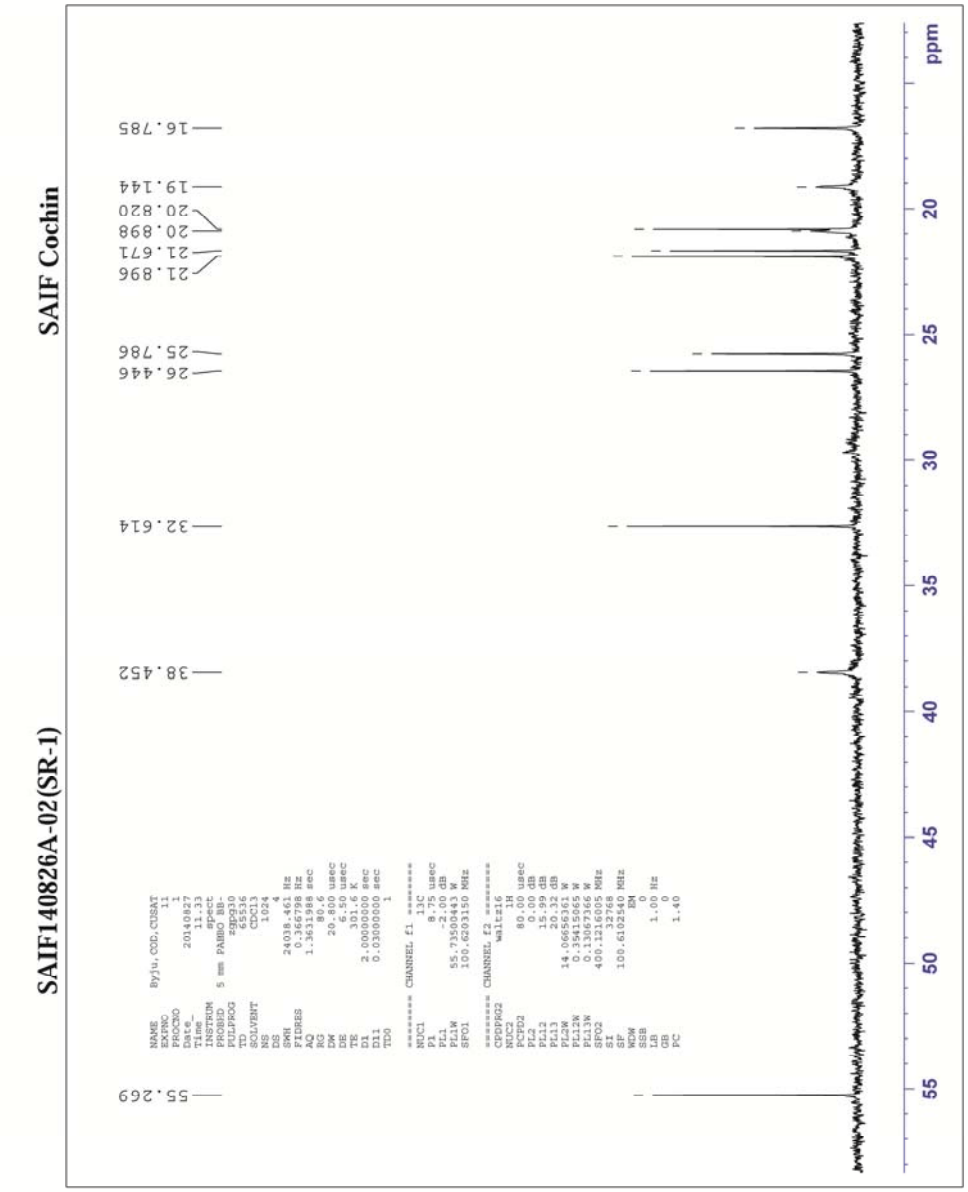
200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20.820
19.144
16.785
ppm
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FIDRES 0.366798 Hz
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DW 20.800 usec
DE 6.50 usec
TE 301.6 K
D1 2.00000000 sec
D11 0.03000000 sec
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PL1 -2.00 dB
PL1W 55.73500443 W
SFO1 100.6203150 MHz
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NUC2 1H
PCPD2 80.00 usec
PL2 0.00 dB
PL12 15.90 dB
PL13 20.32 dB
PL2W 14.06656361 W
PL12W 0.35415065 W
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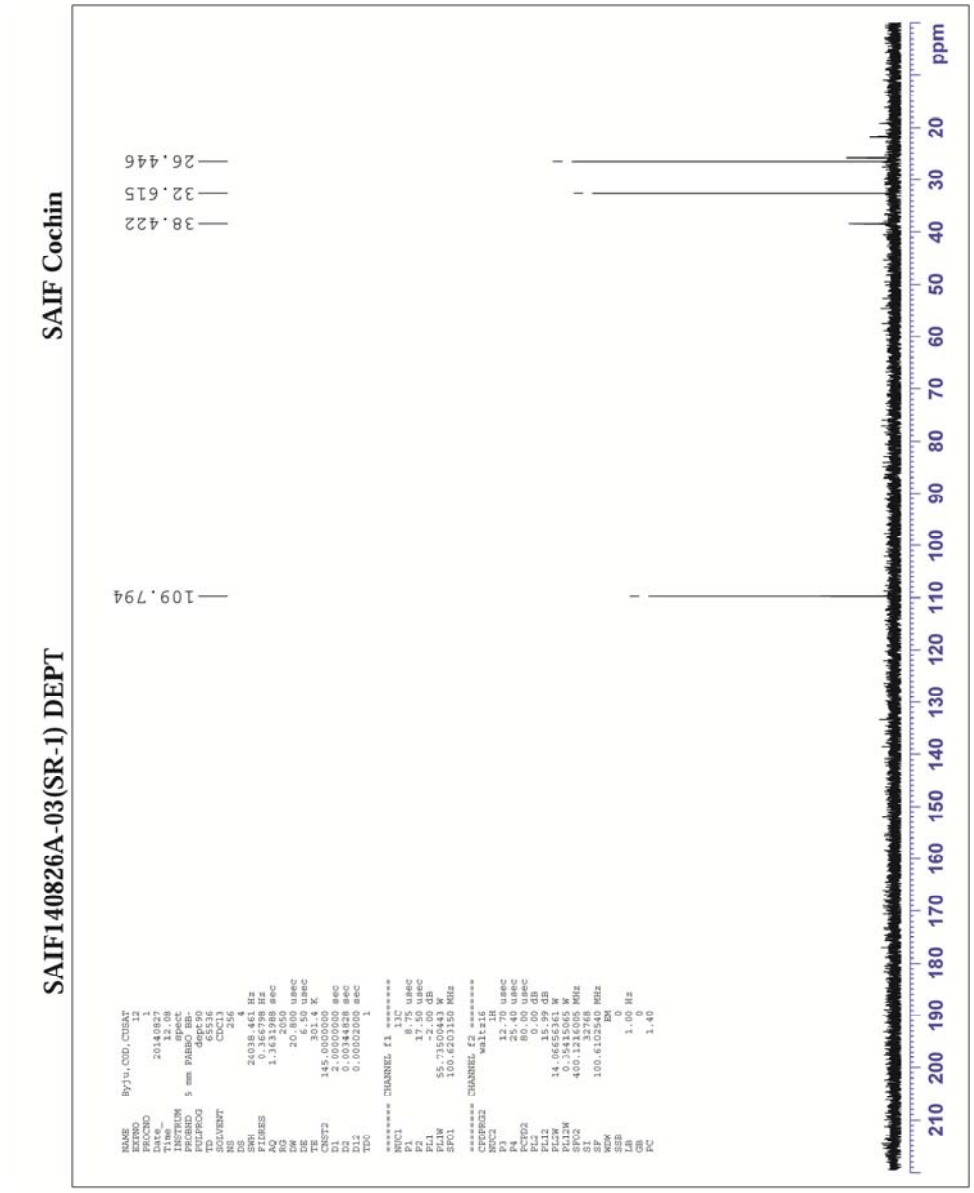
PC 1.40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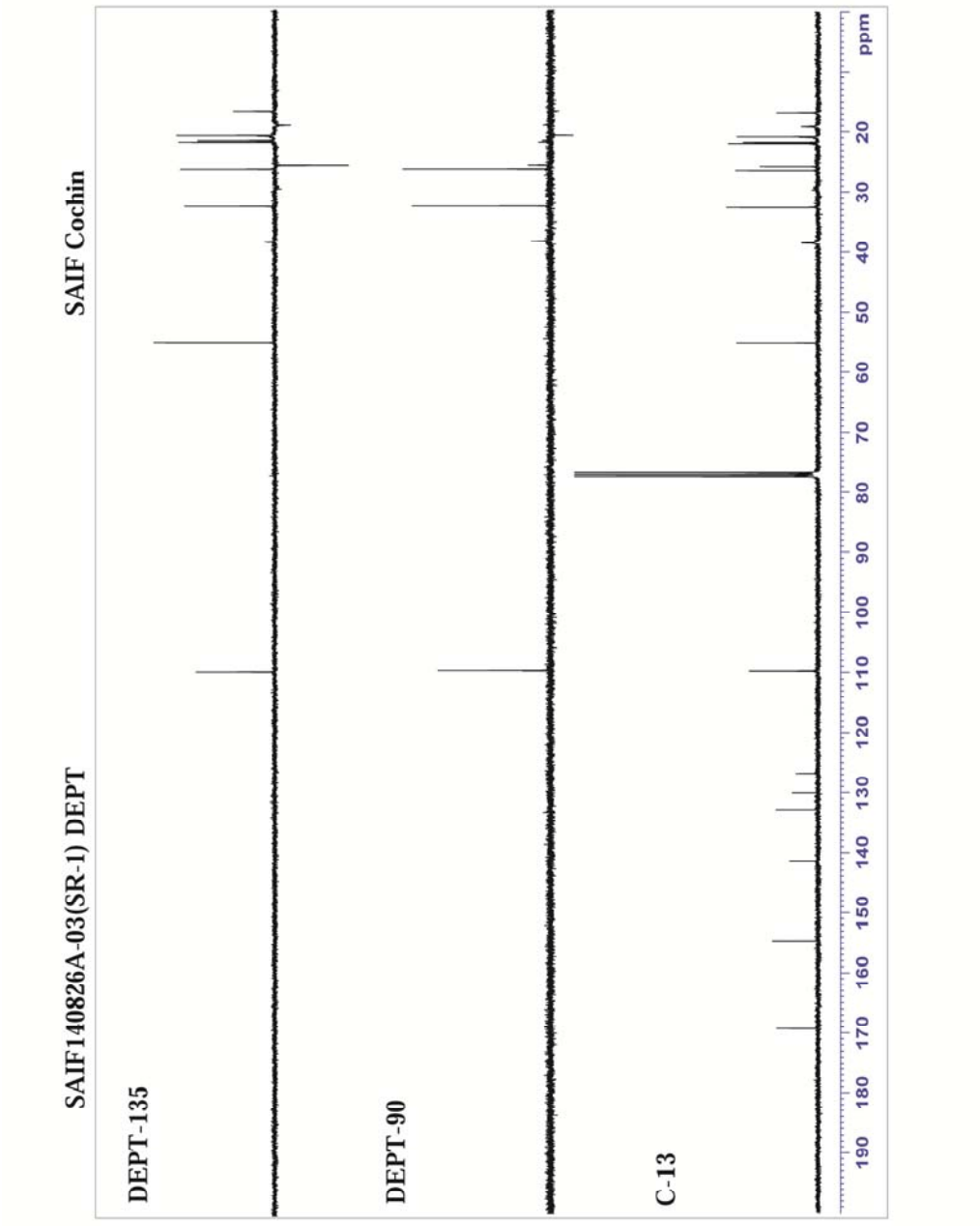
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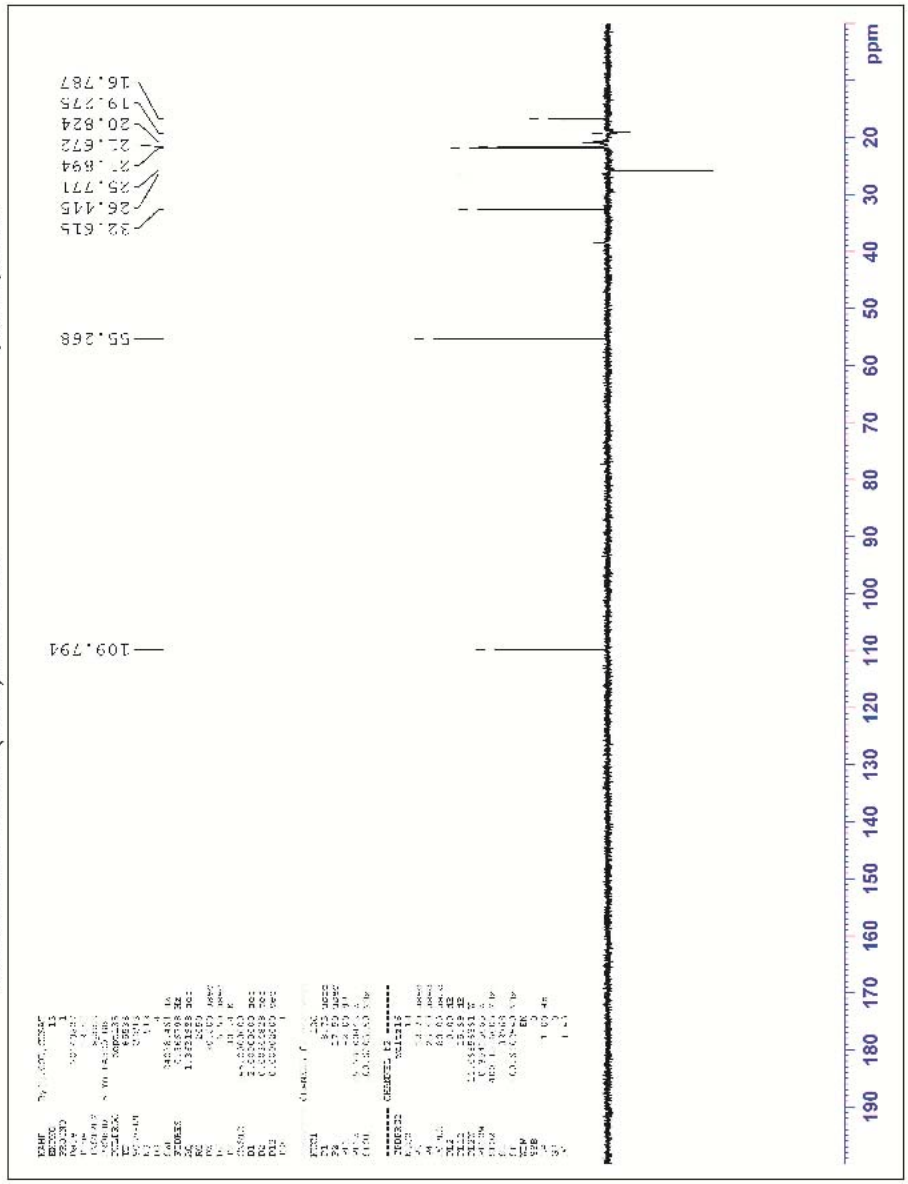
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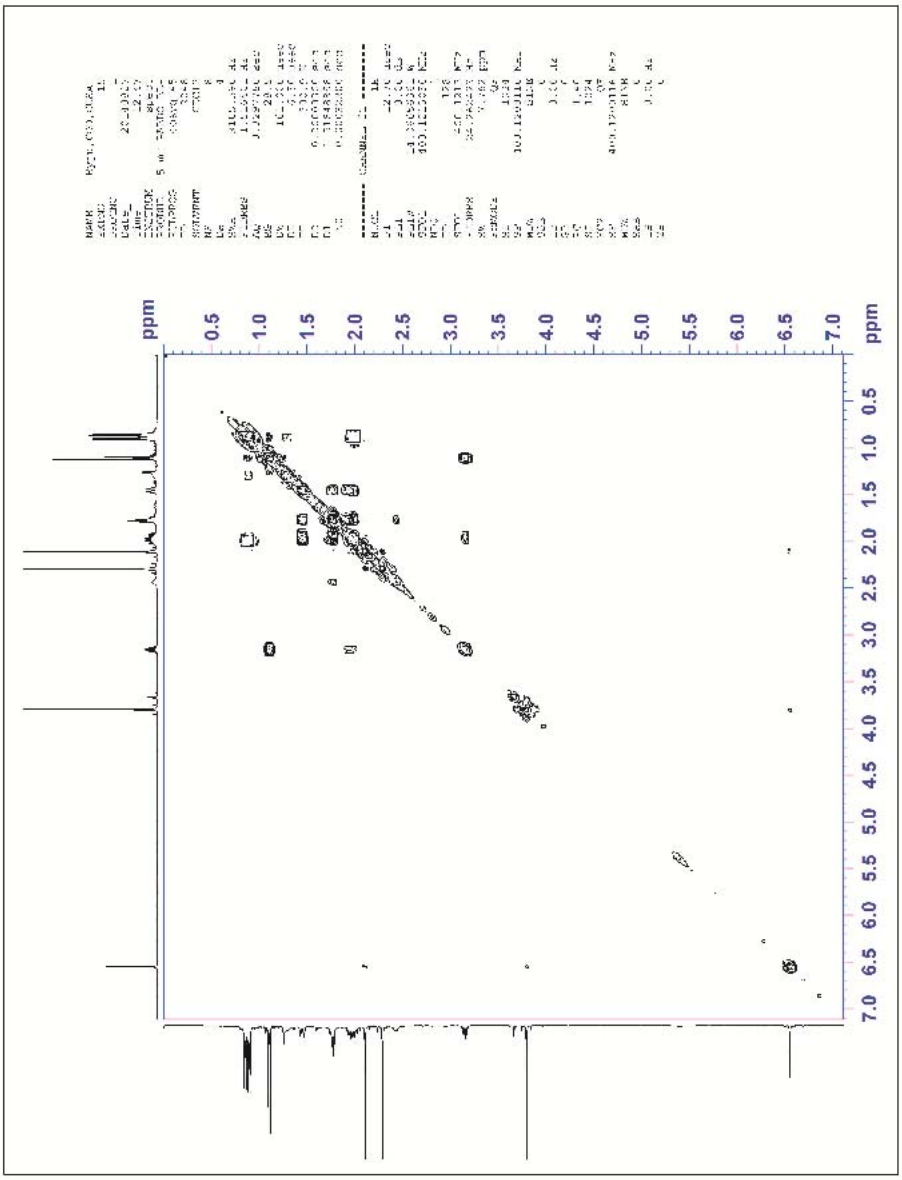
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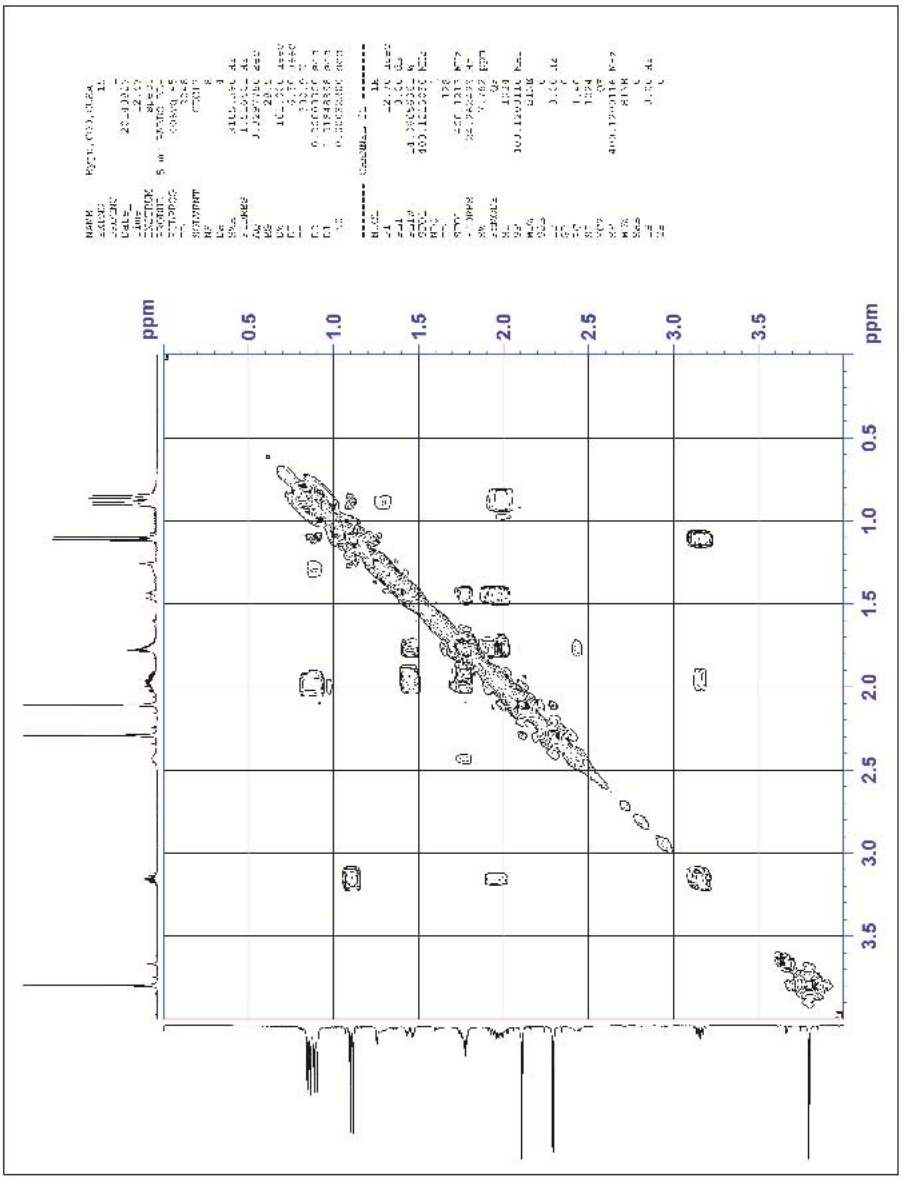
6.0
6.5
7.0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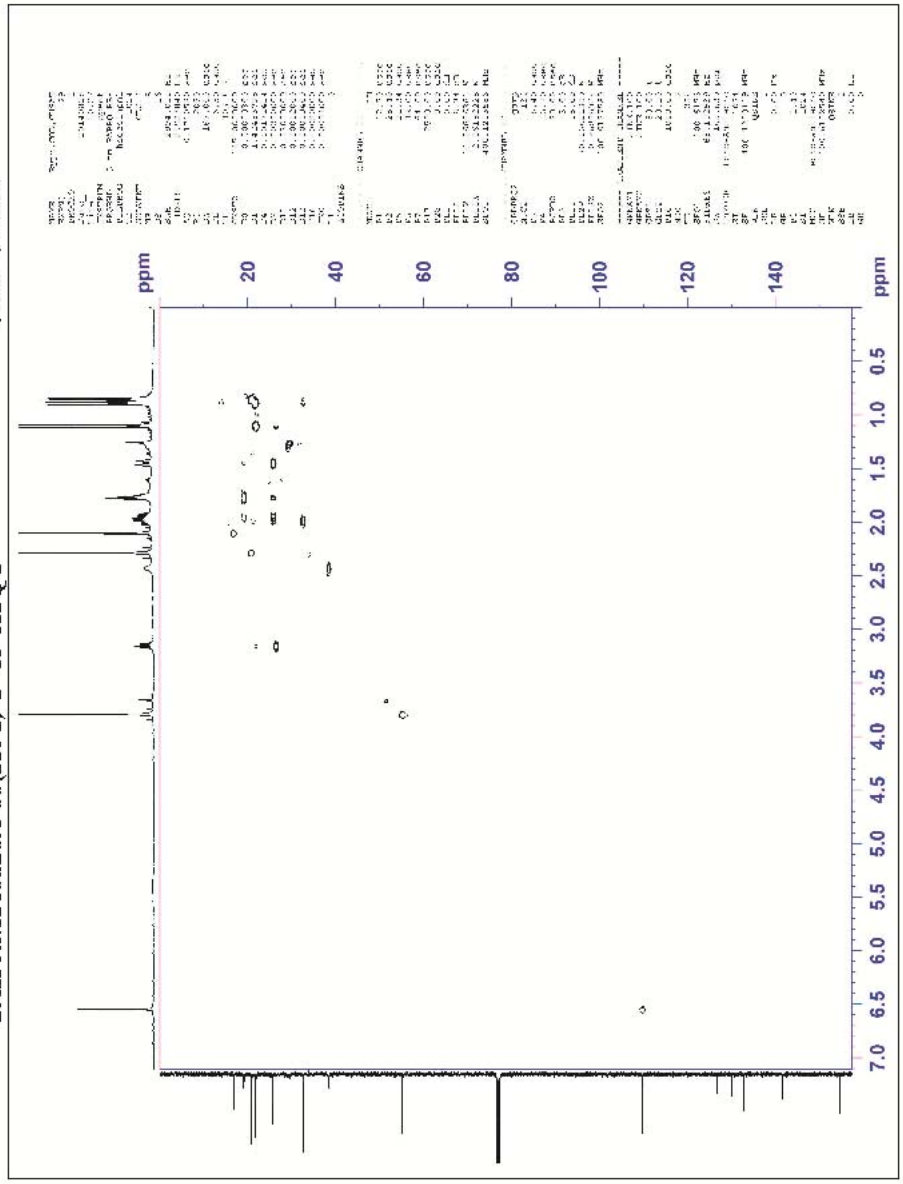
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120
140
ppm
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5.0
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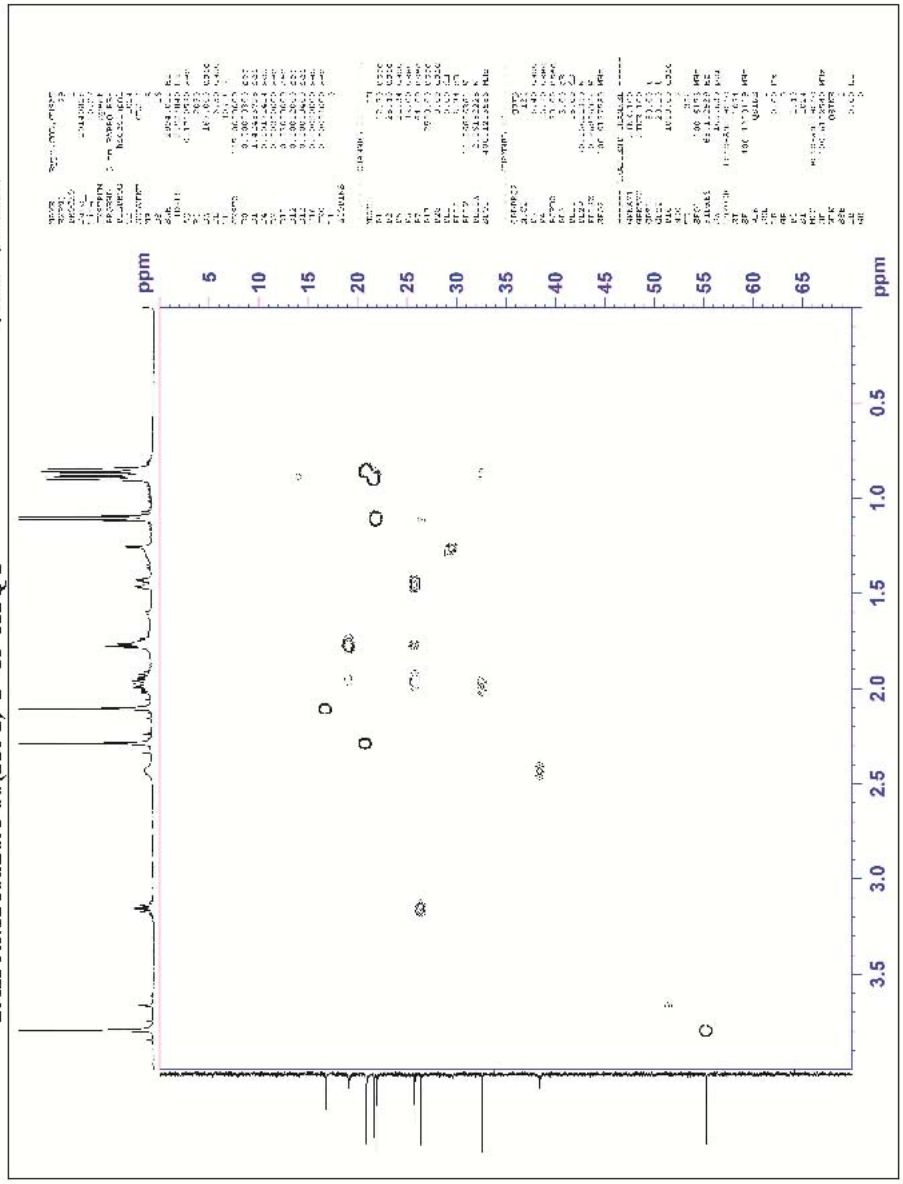
6.5
7.0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ppm
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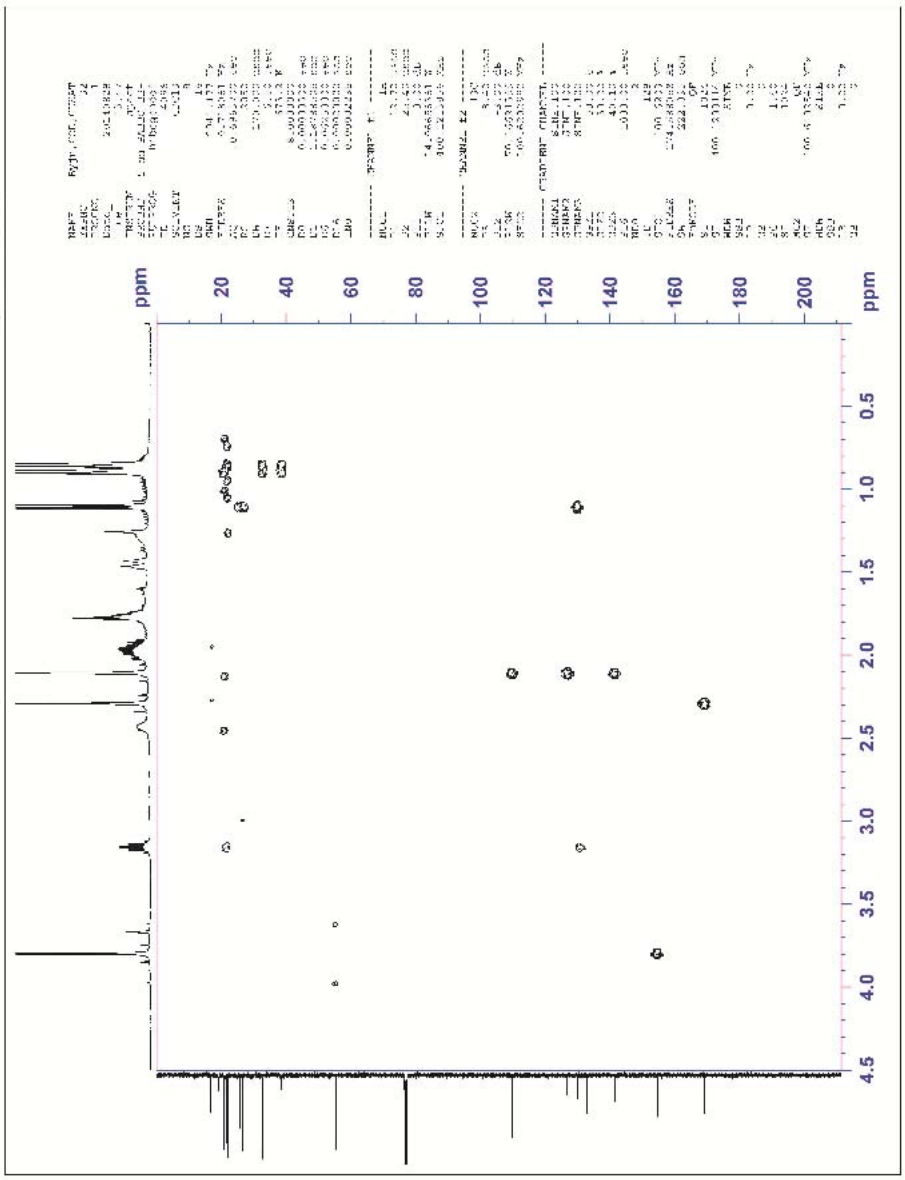
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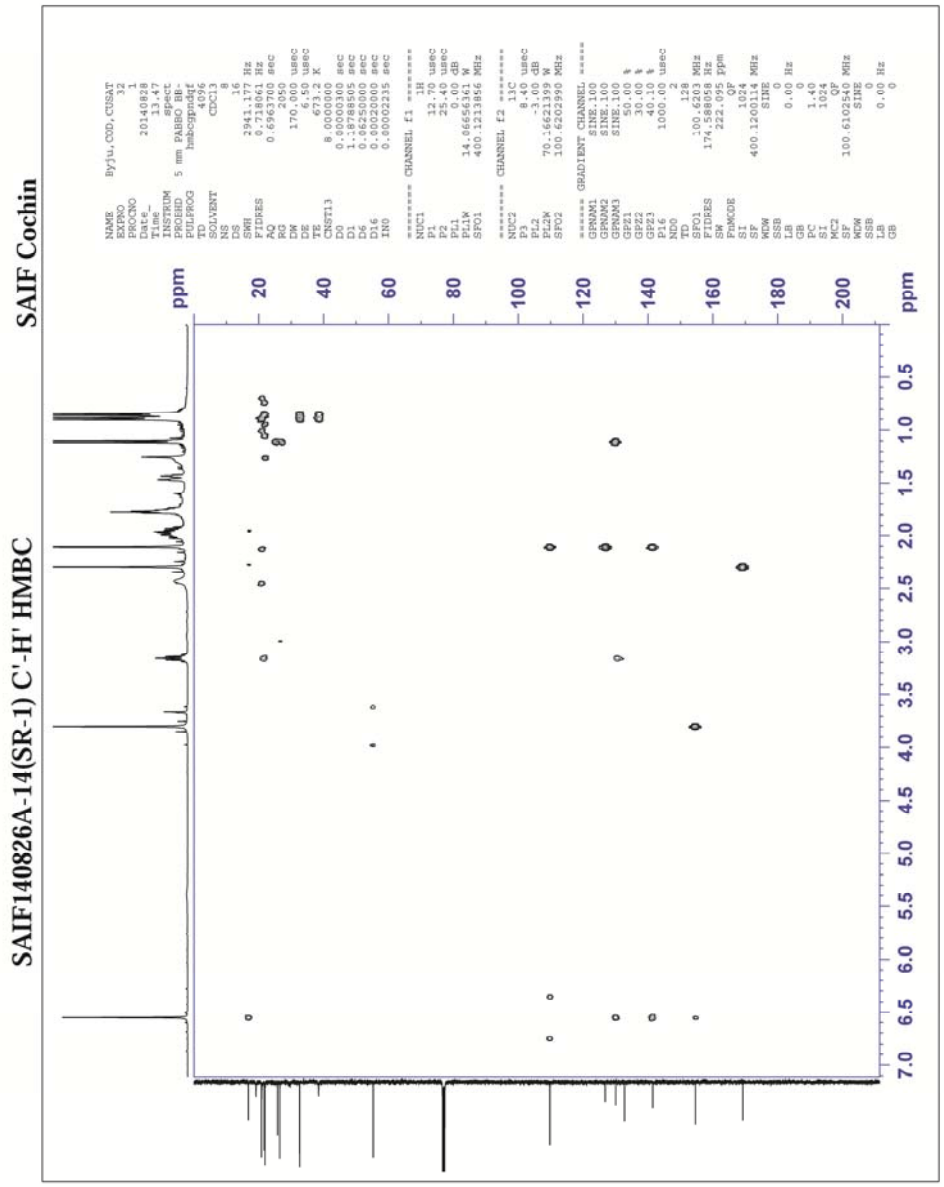
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1.5
1.0
0.5

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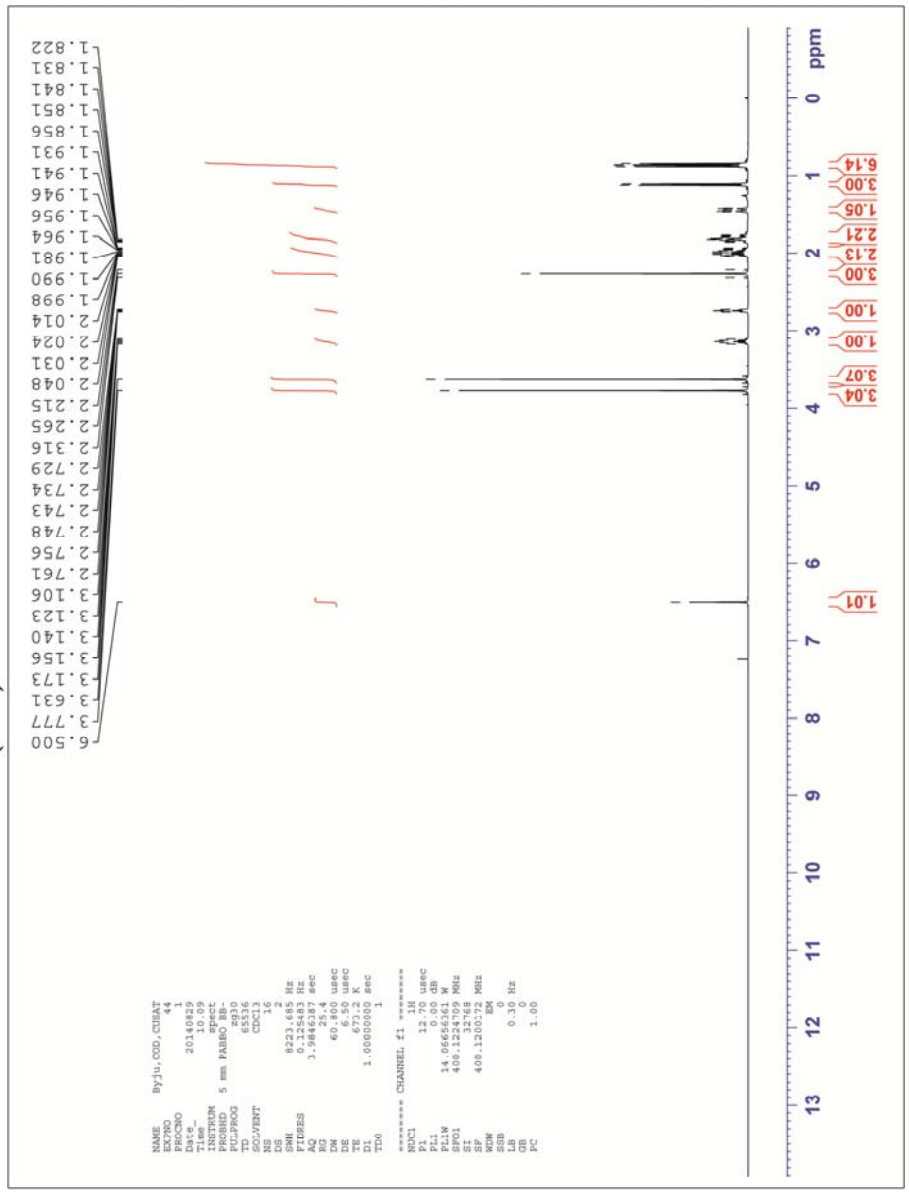
NMR Spectrum of Compound 2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2.729
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2.024
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SI 32768
SF 400.1200172 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
1.01
1.00
1.00
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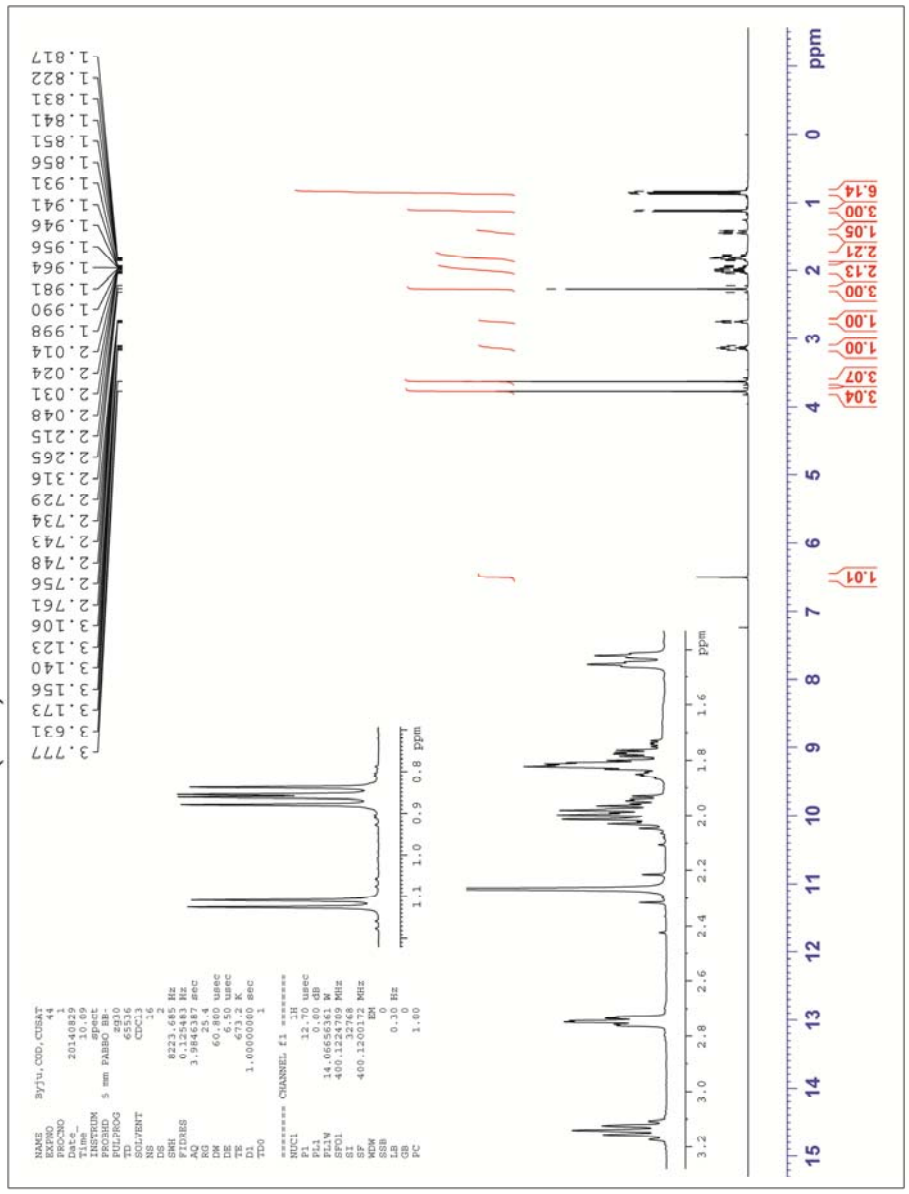
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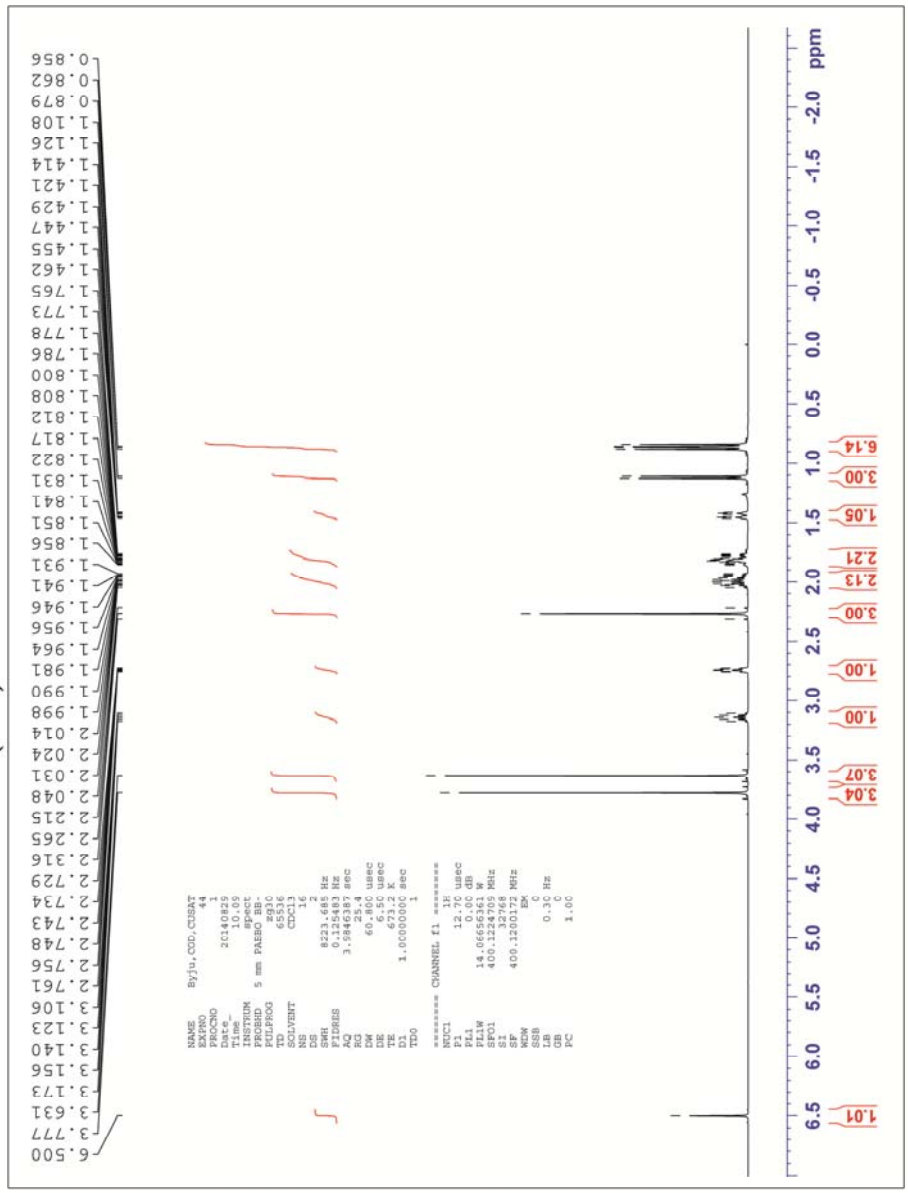
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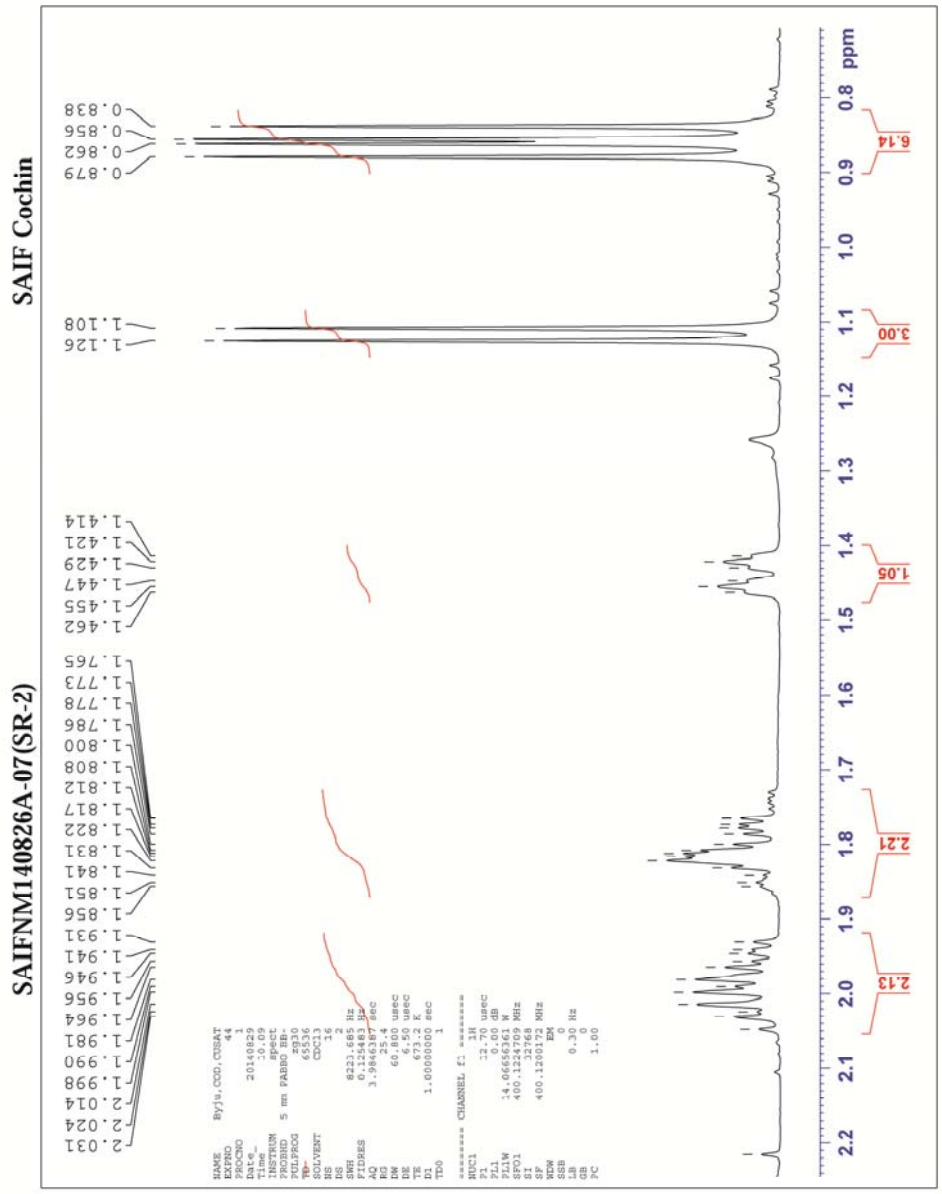
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1.421
1.414
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1.108
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0.862
0.856
0.838
2.13
2.21
1.05
3.00
6.14
2.2
2.1
2.0
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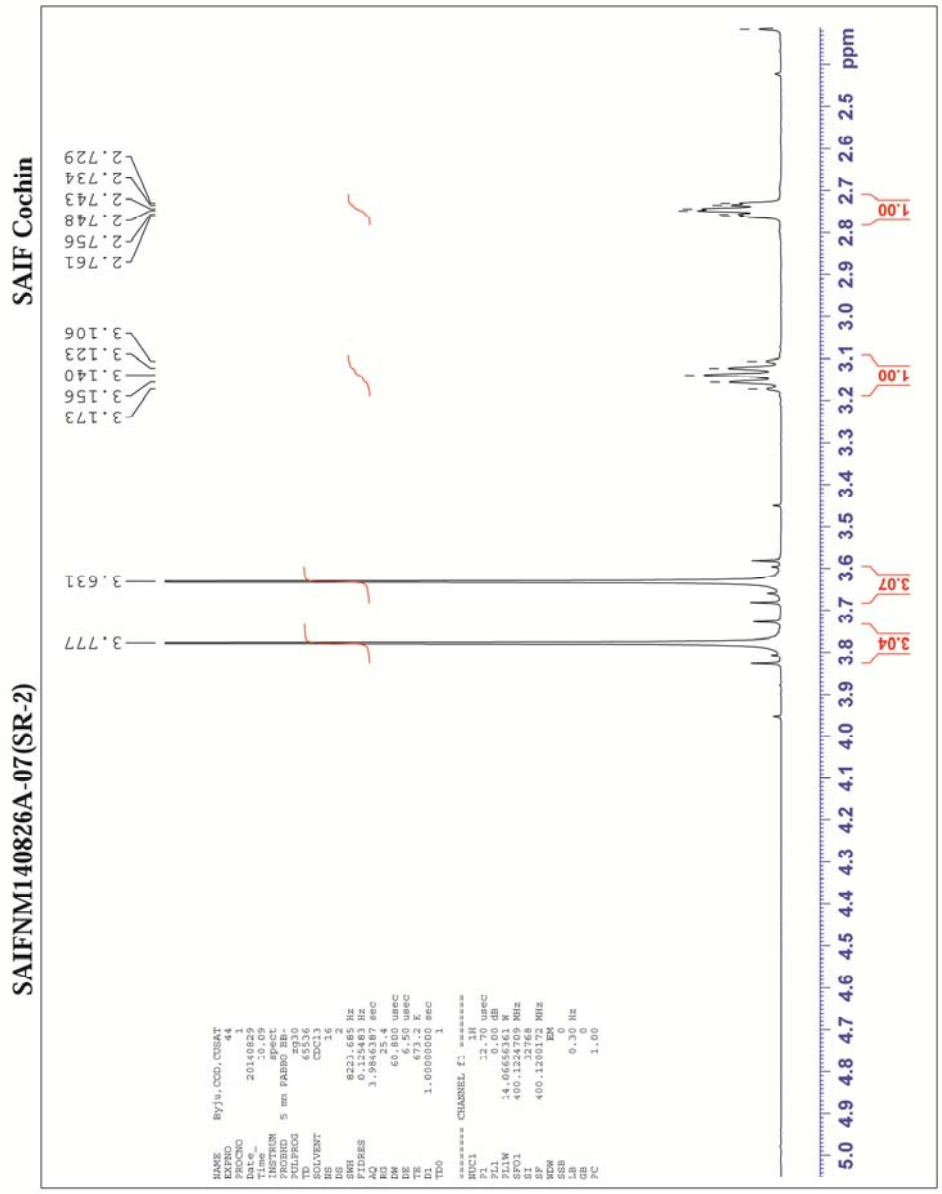
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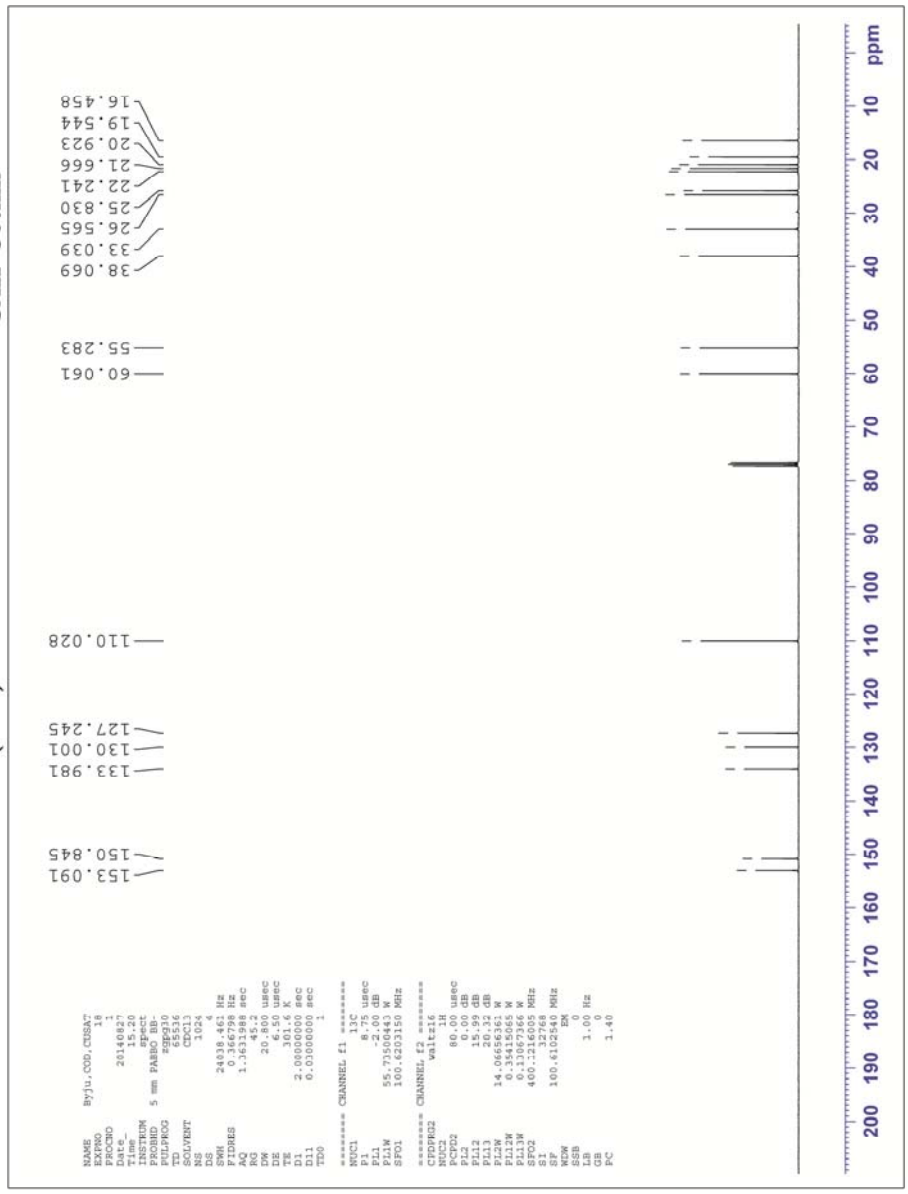
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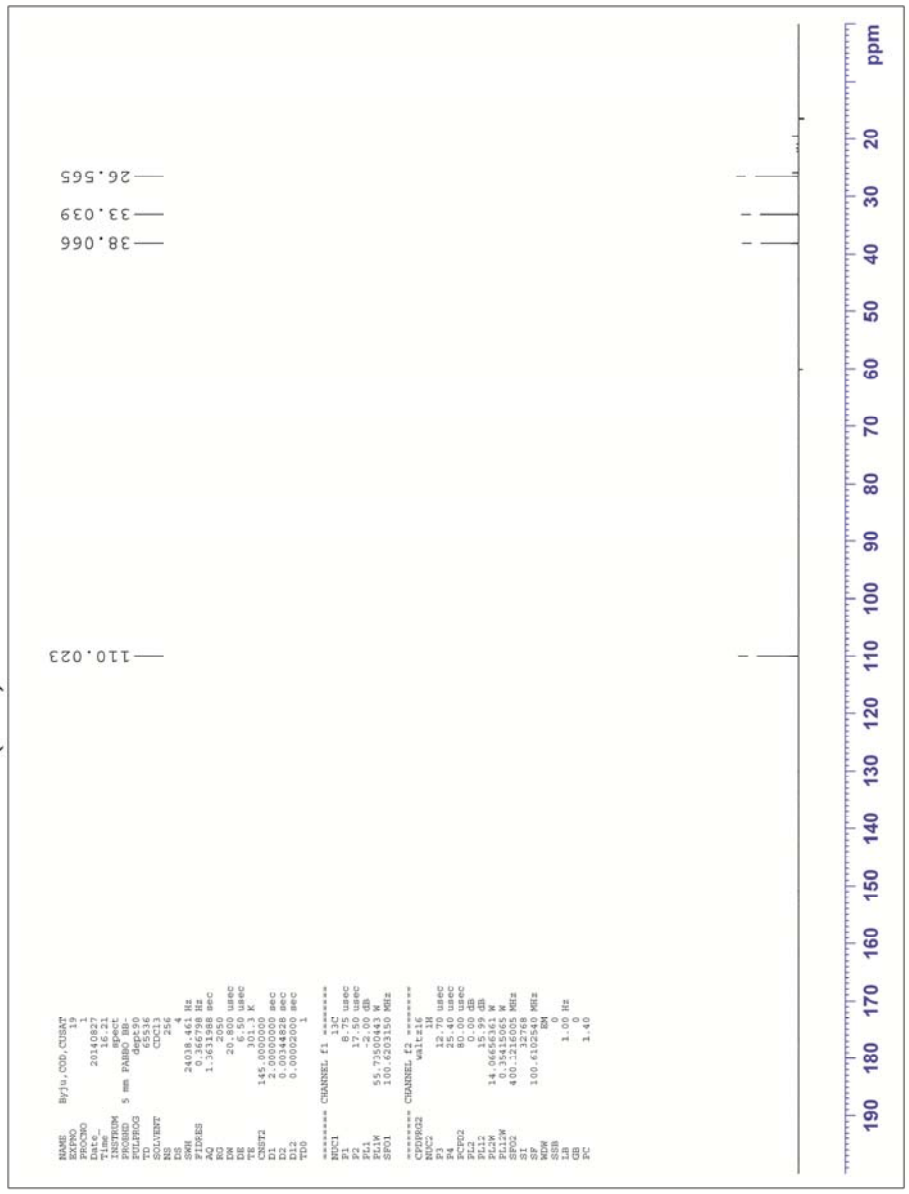
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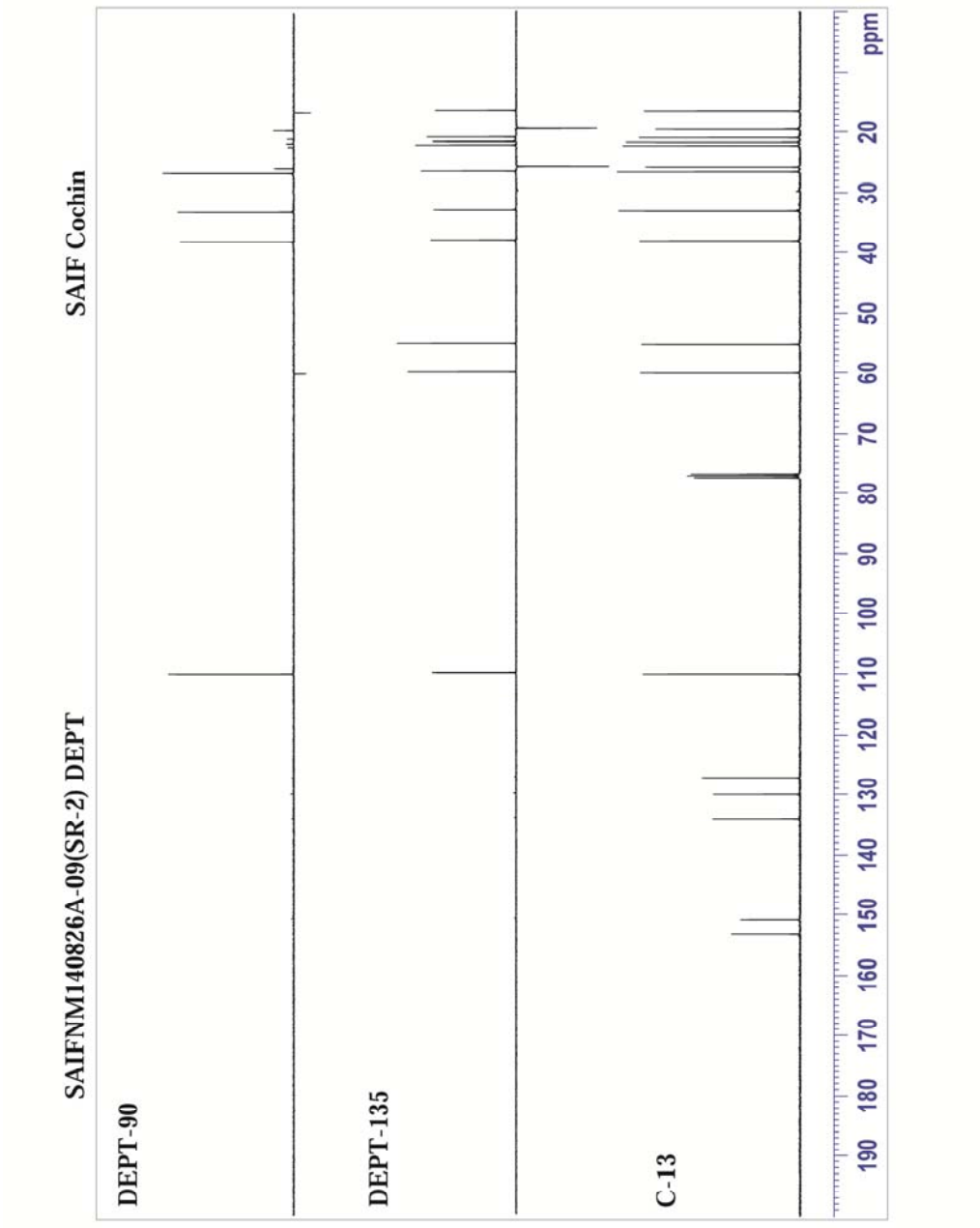
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DEPT-90
DEPT-135
C-13
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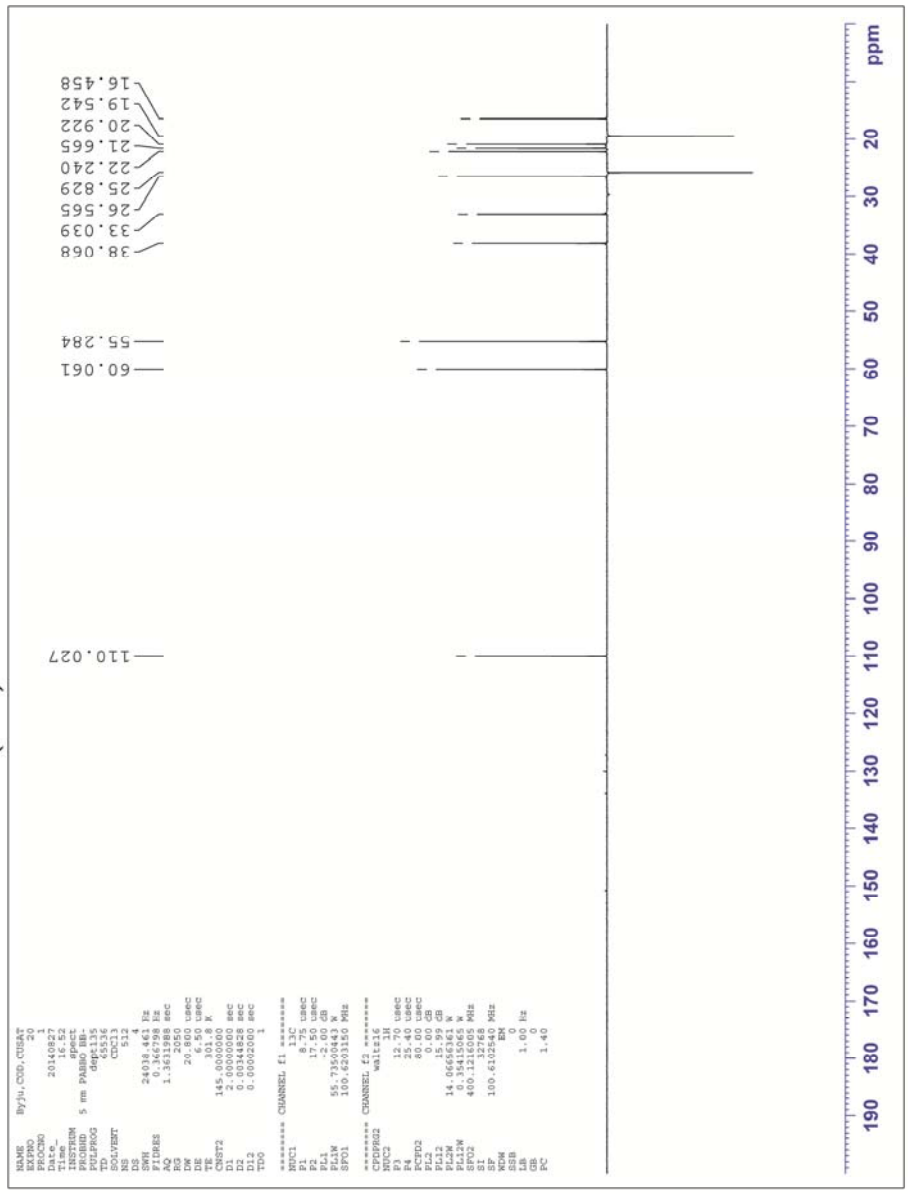
ppm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19.542
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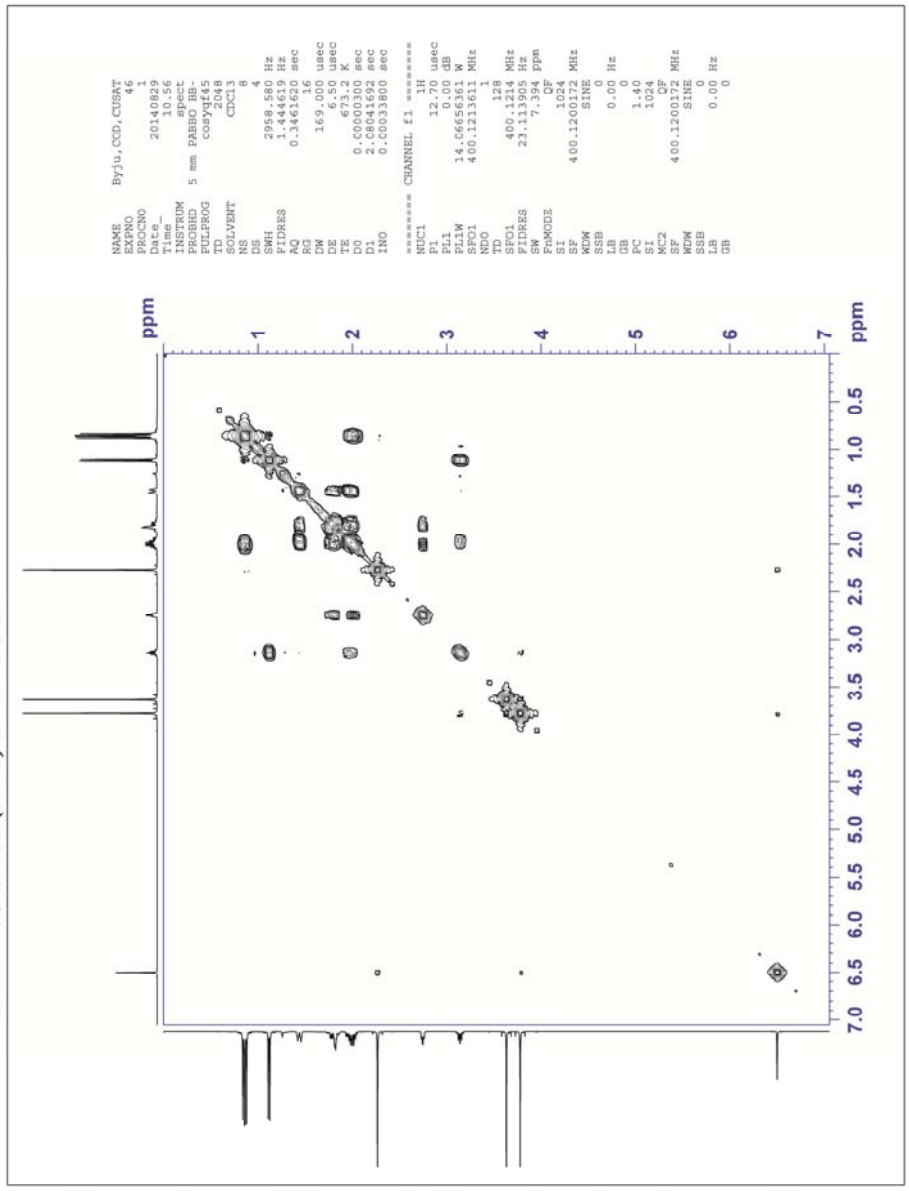
ppm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PULPROG cosygf4s
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SOLVENT CDCl3
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DS 4
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FIDRES 1.444619 Hz
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RG 16
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DE 6.50 usec
TE 673.2 K
D0 0.00001000 sec
D1 2.00001560 sec
D13 0.00033800 sec
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======== CHANNEL f1 ========
NUC1 1H
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PC 1.40
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MC2 QF
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WDW SINE
SSB 0
LB 0.00 Hz
GB 0
ppm
1
2
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4
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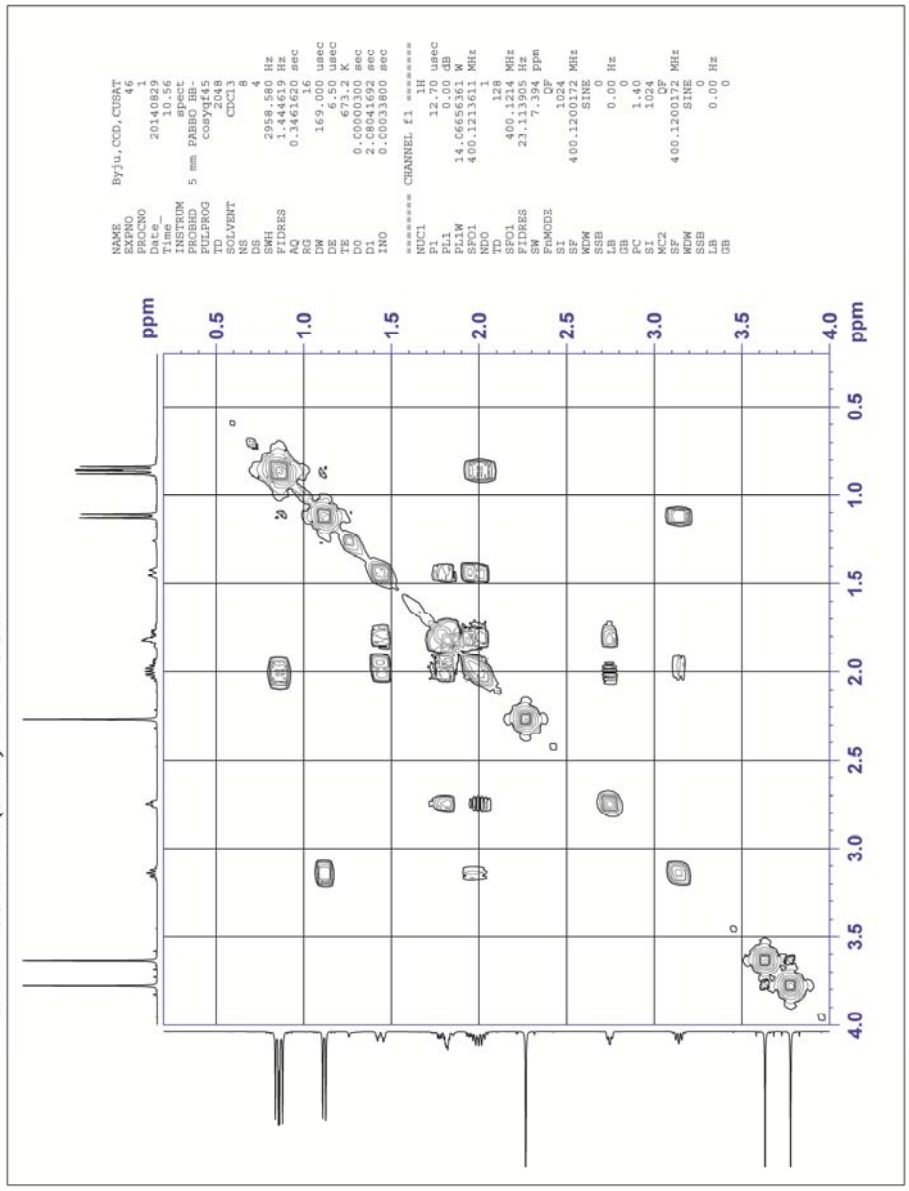
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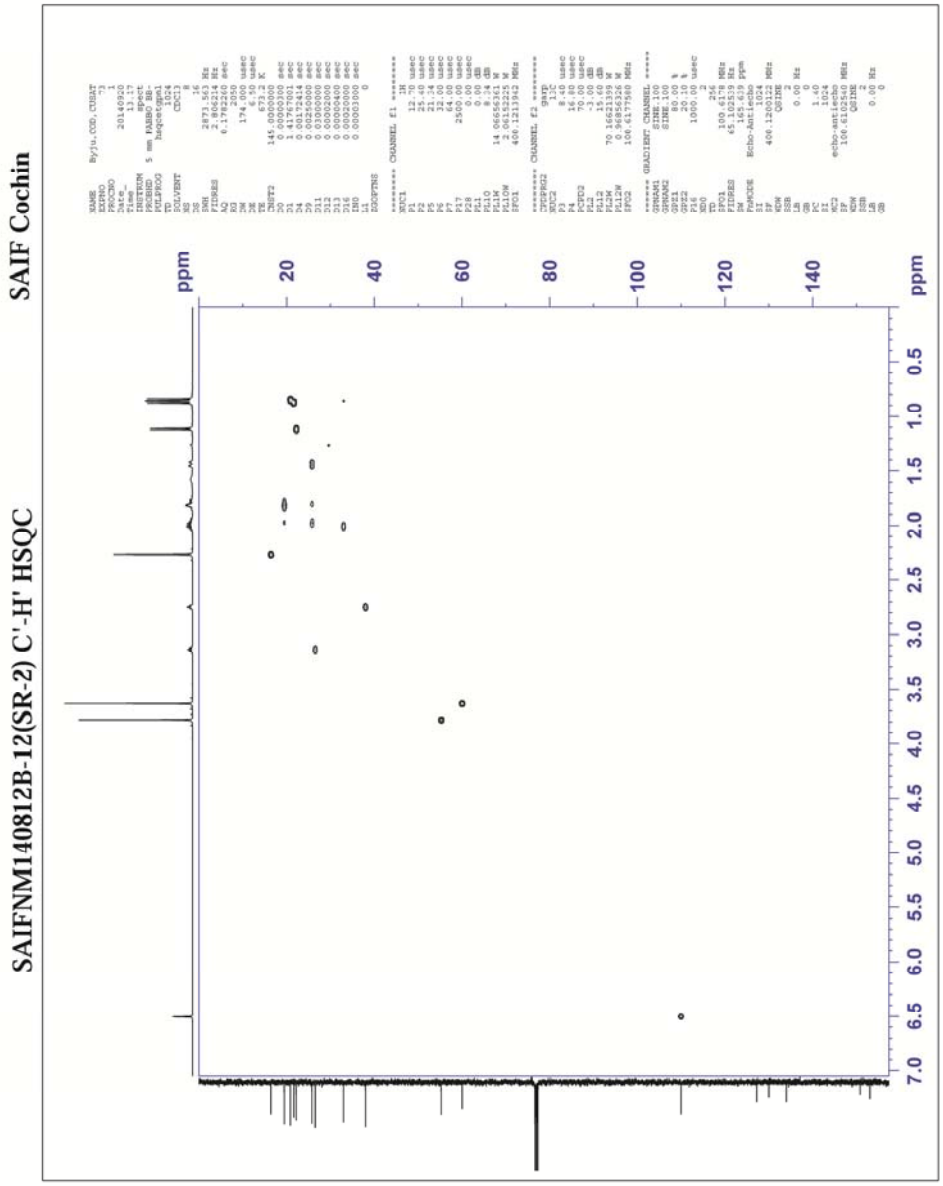
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60
80
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120
140
ppm
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1.0
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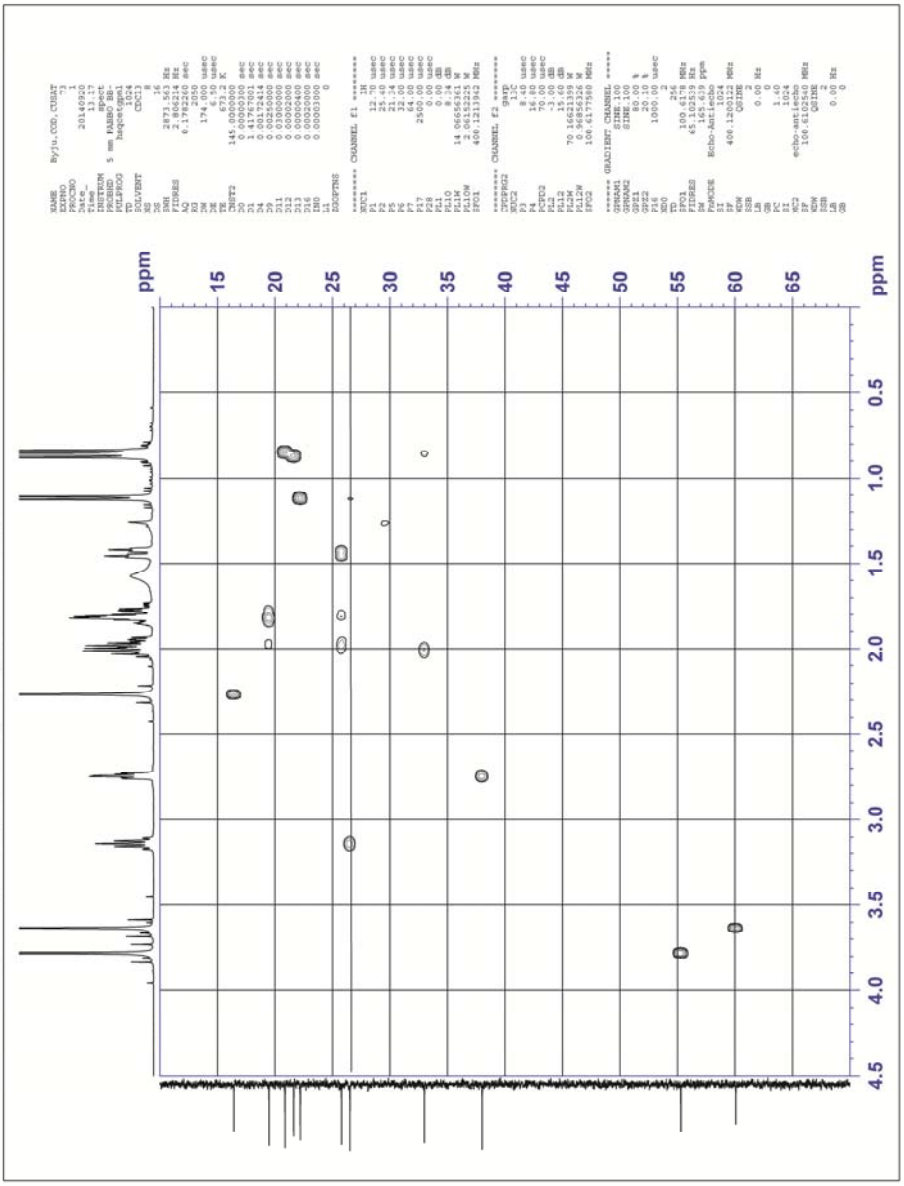
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2.0
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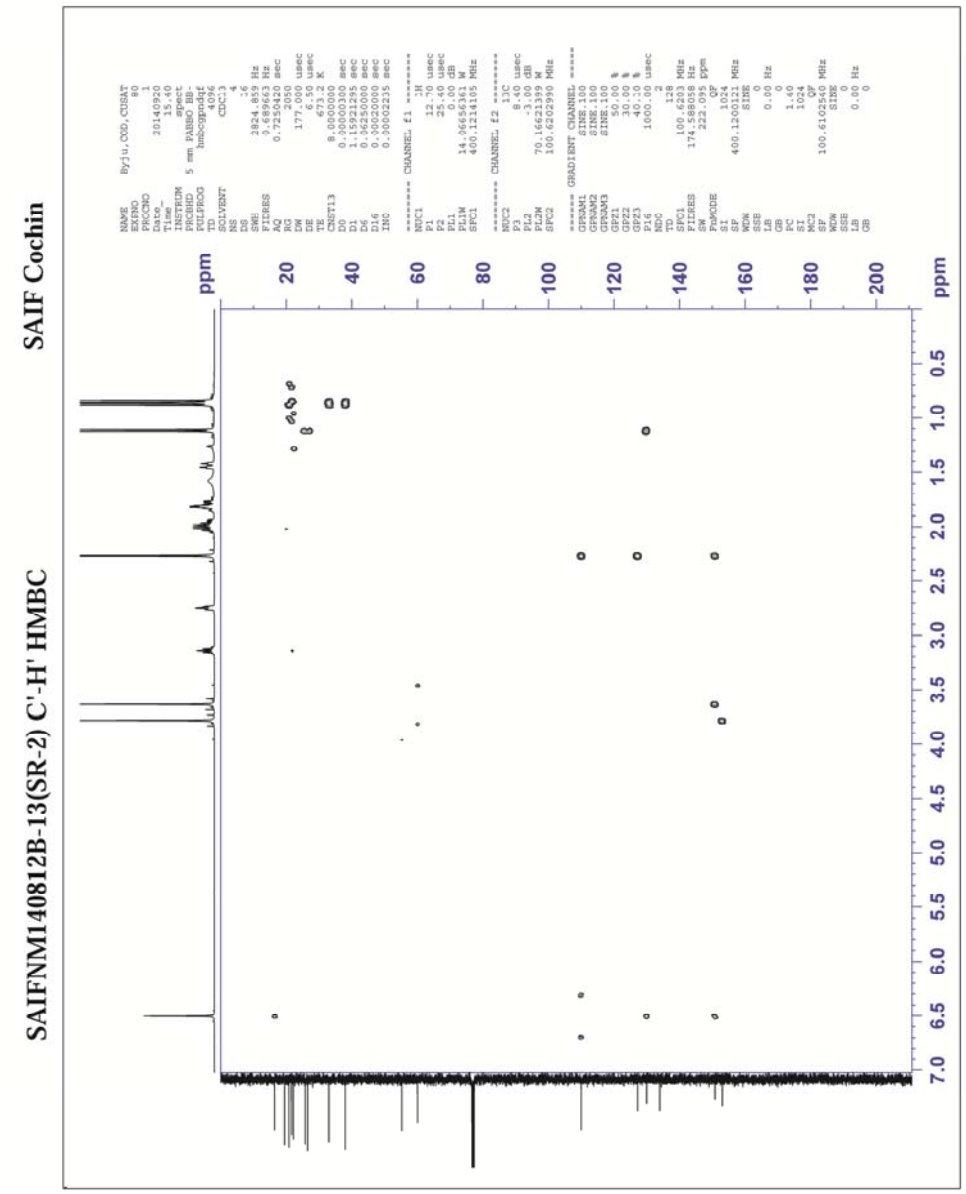
0.5
ppm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4.0
3.5
3.0
2.5
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ppm
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100
120
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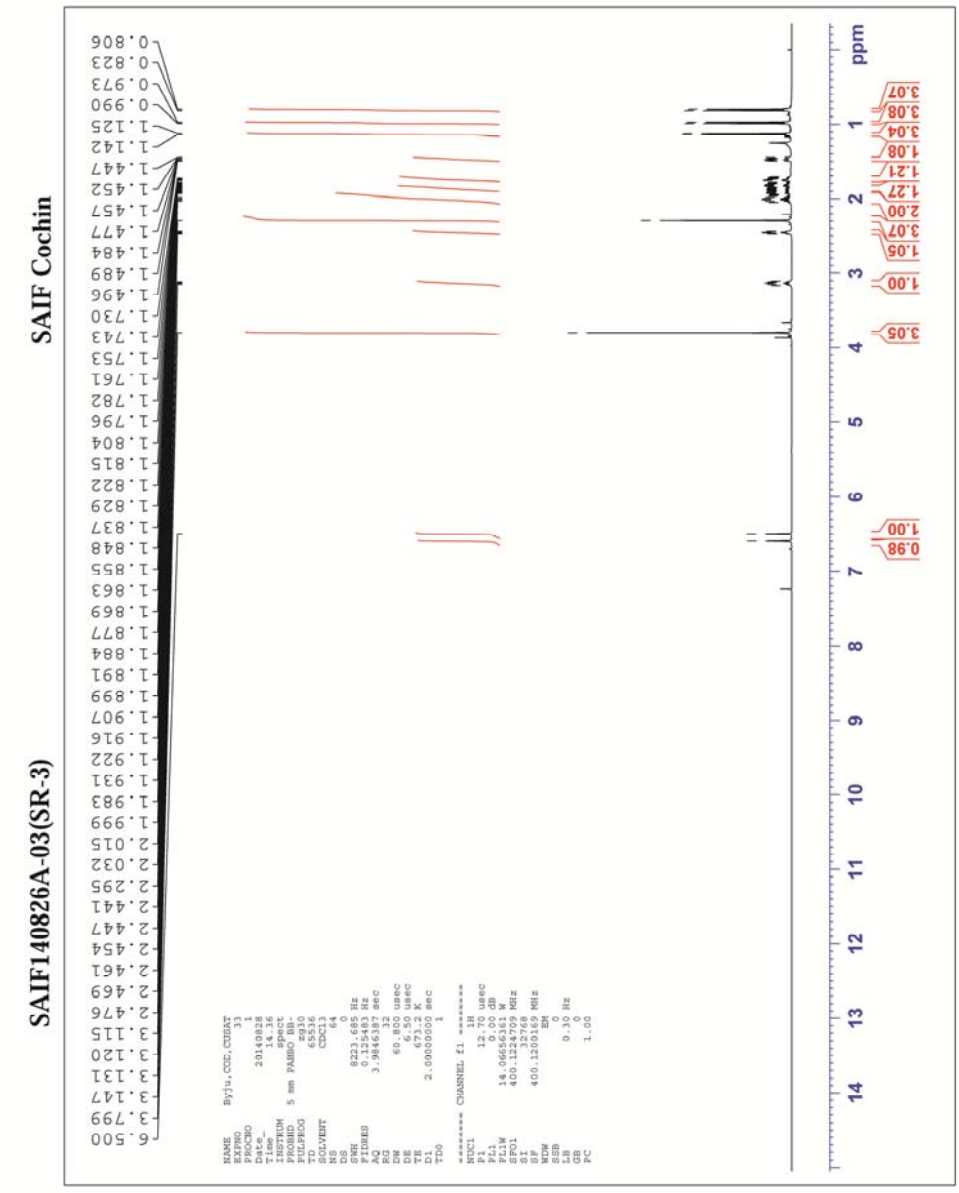
180
200

NMR Spectrum of Compound 3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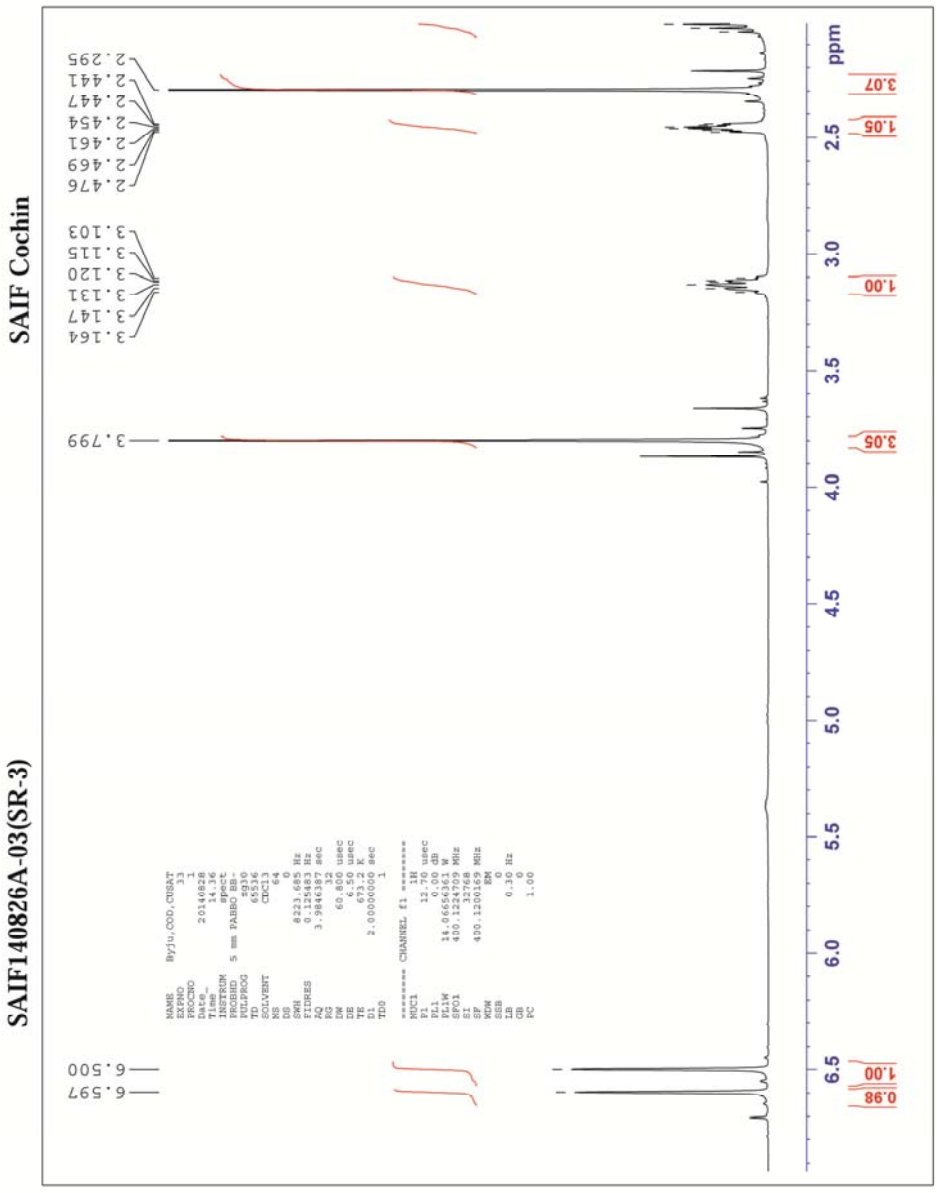
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2.447
2.441
2.295
ppm
6.5
6.0
5.5
5.0
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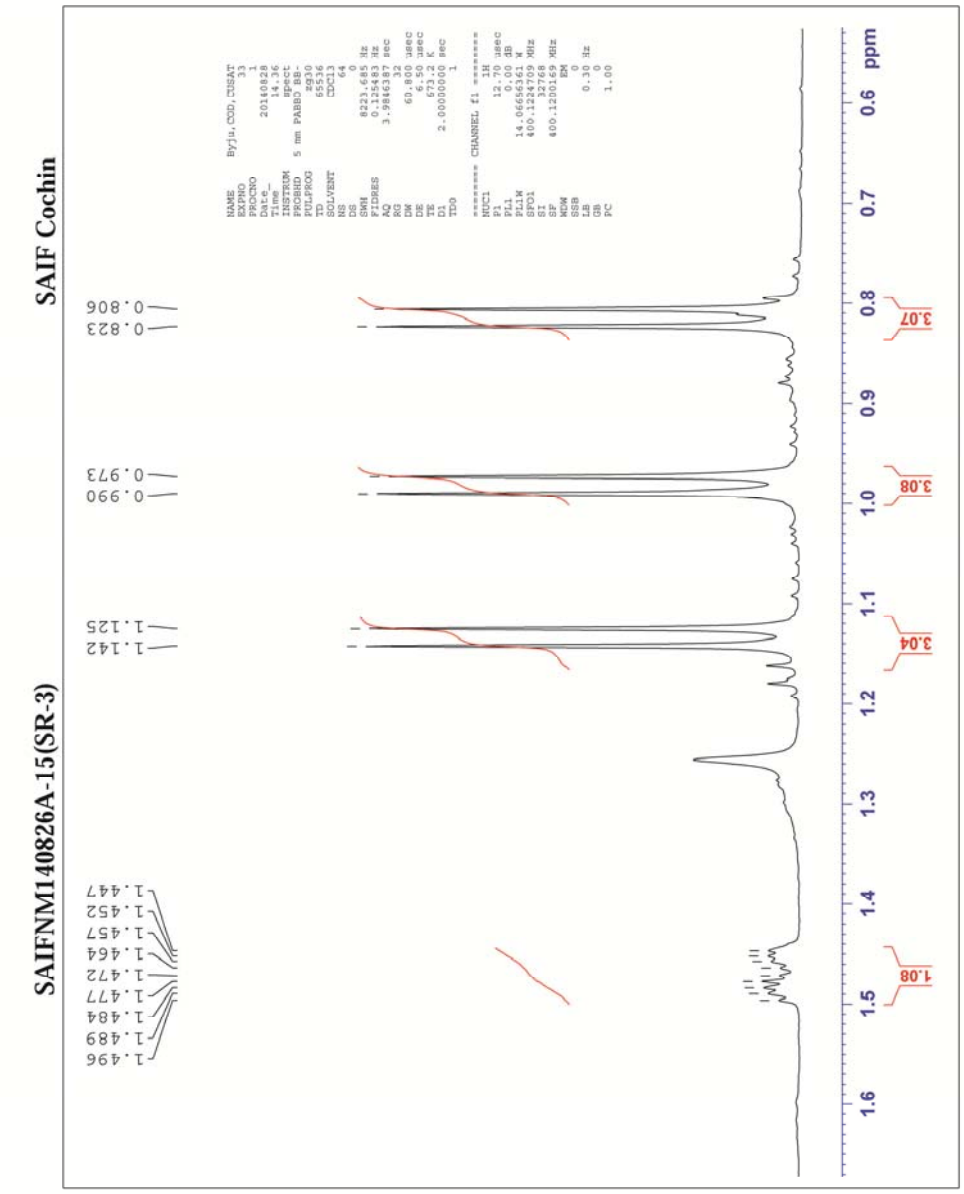
1.00
3.07
1.05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1.125
1.142
1.447
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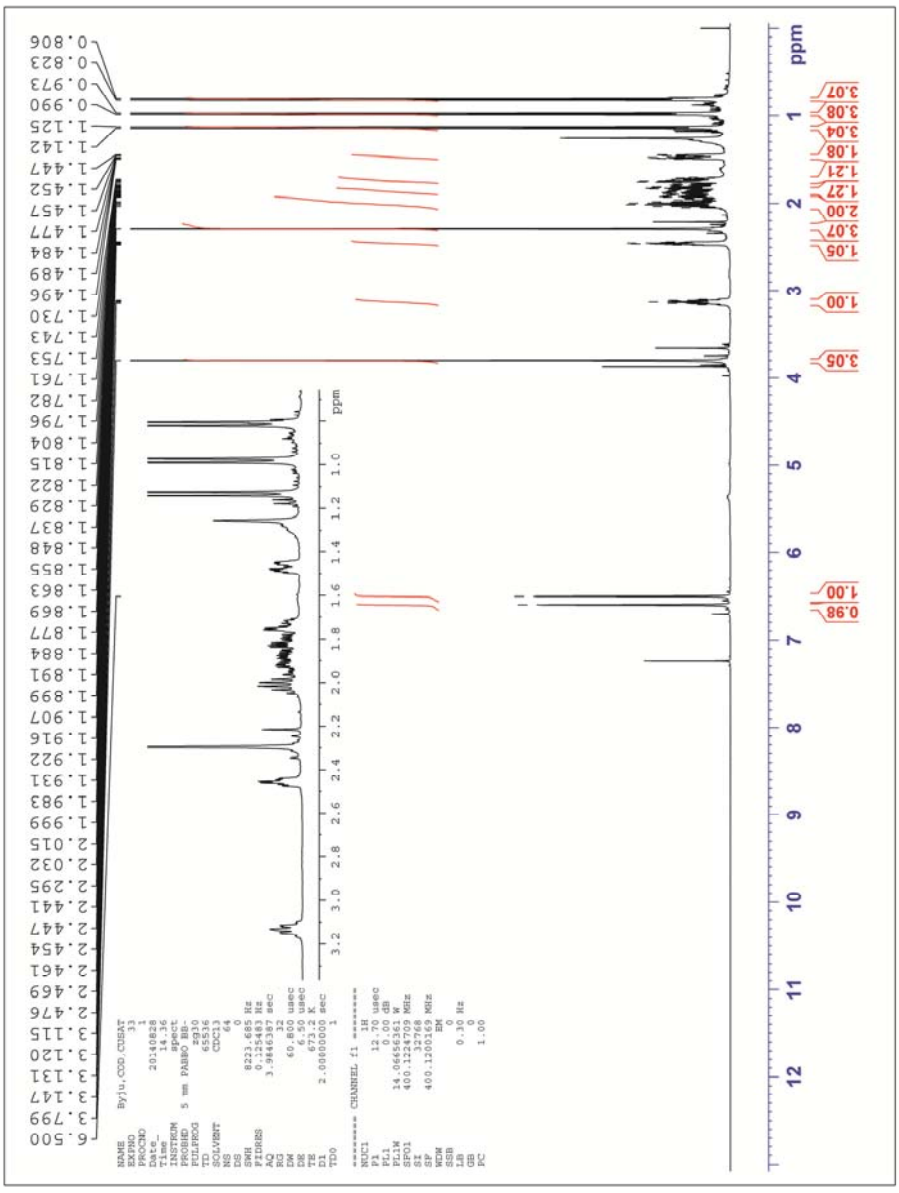
1.472
1.477
1.484
1.489
1.496
3.07
3.08
3.04
1.08
ppm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ppm
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1.05
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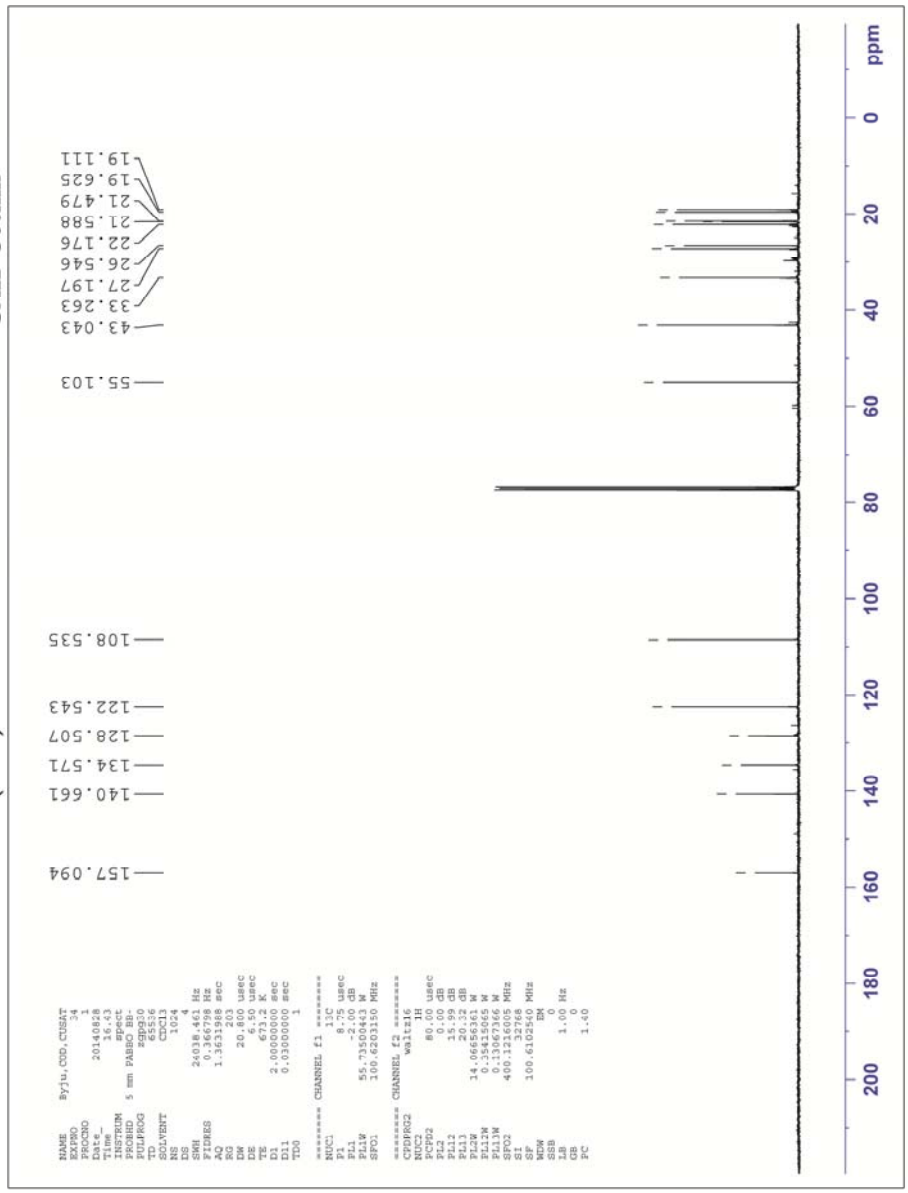
3.05
1.00
0.98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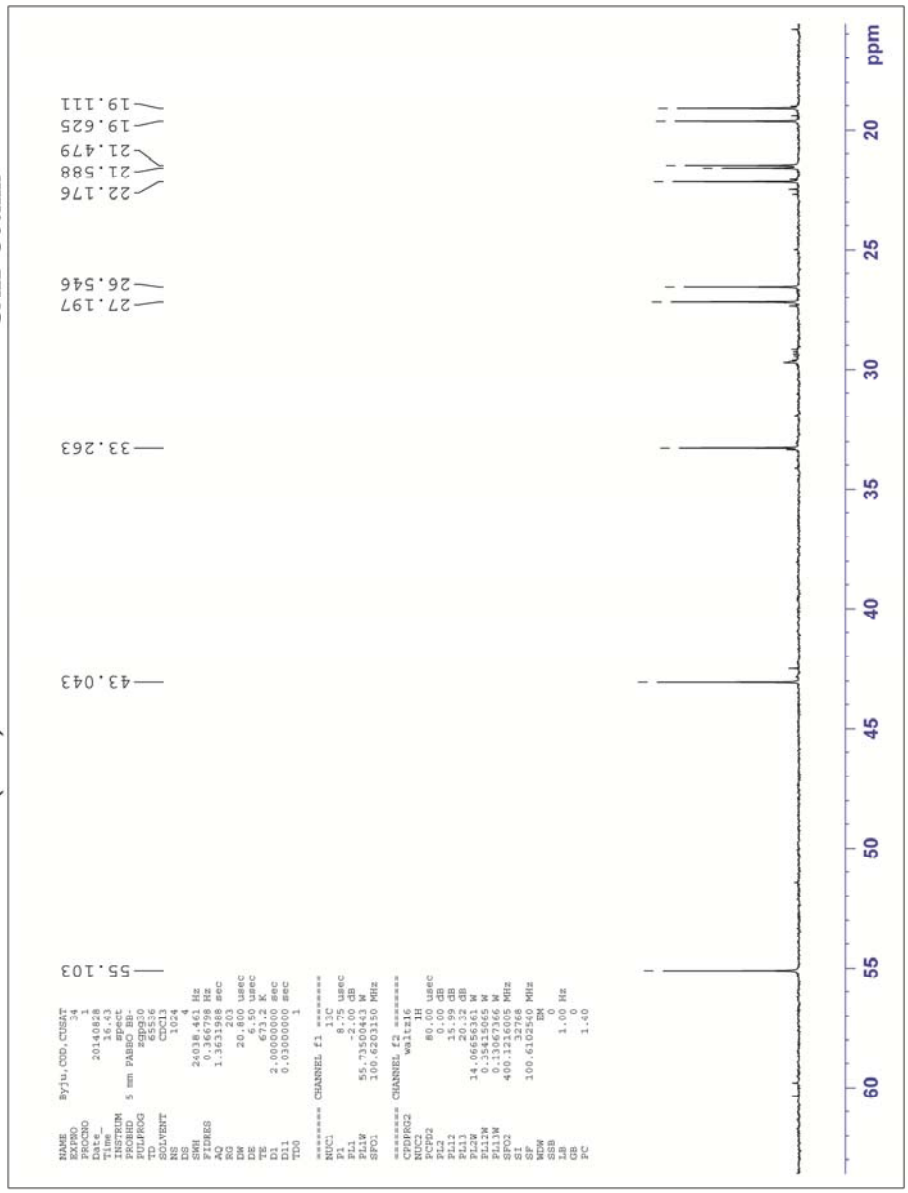
128.507
122.543
108.535
55.103
43.043
33.263
27.197
26.546
22.176
21.588
21.479
19.625
19.111
ppm
0
20
40
60
80
100
120
140
160
180
200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43.043
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27.197
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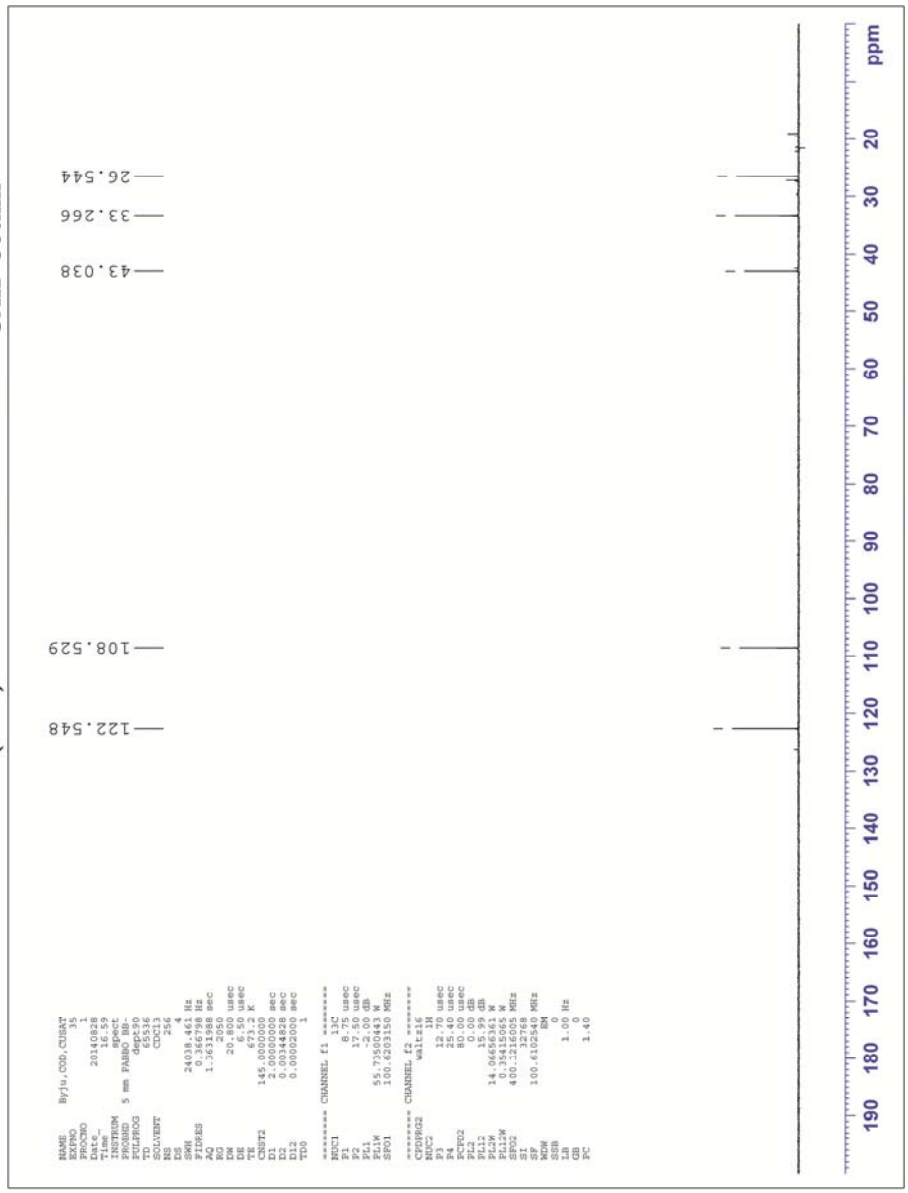
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43.038
33.266
26.544
ppm


SAIFNM140826A-17(SR-3) DEPT 90
SAIF Cochin
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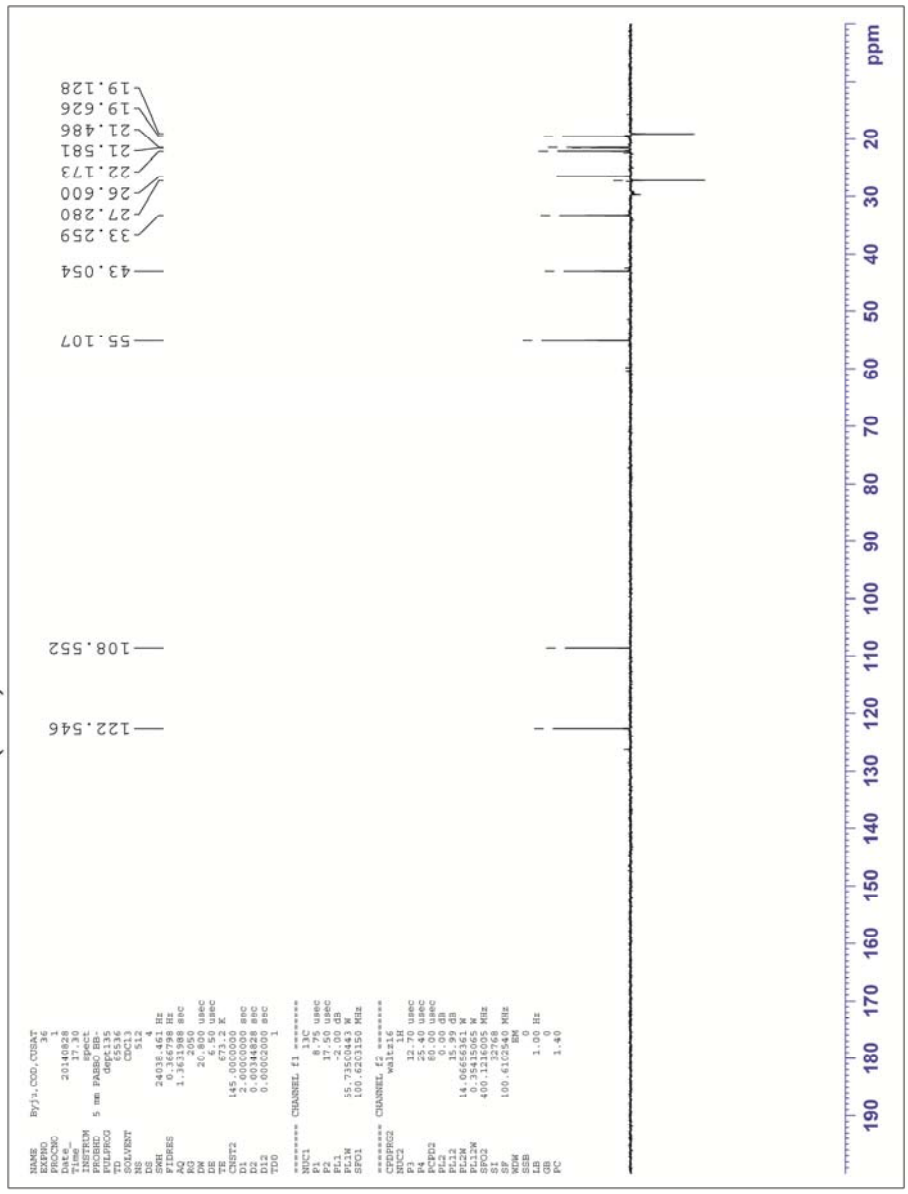
108.529
43.038
33.266
26.544
ppm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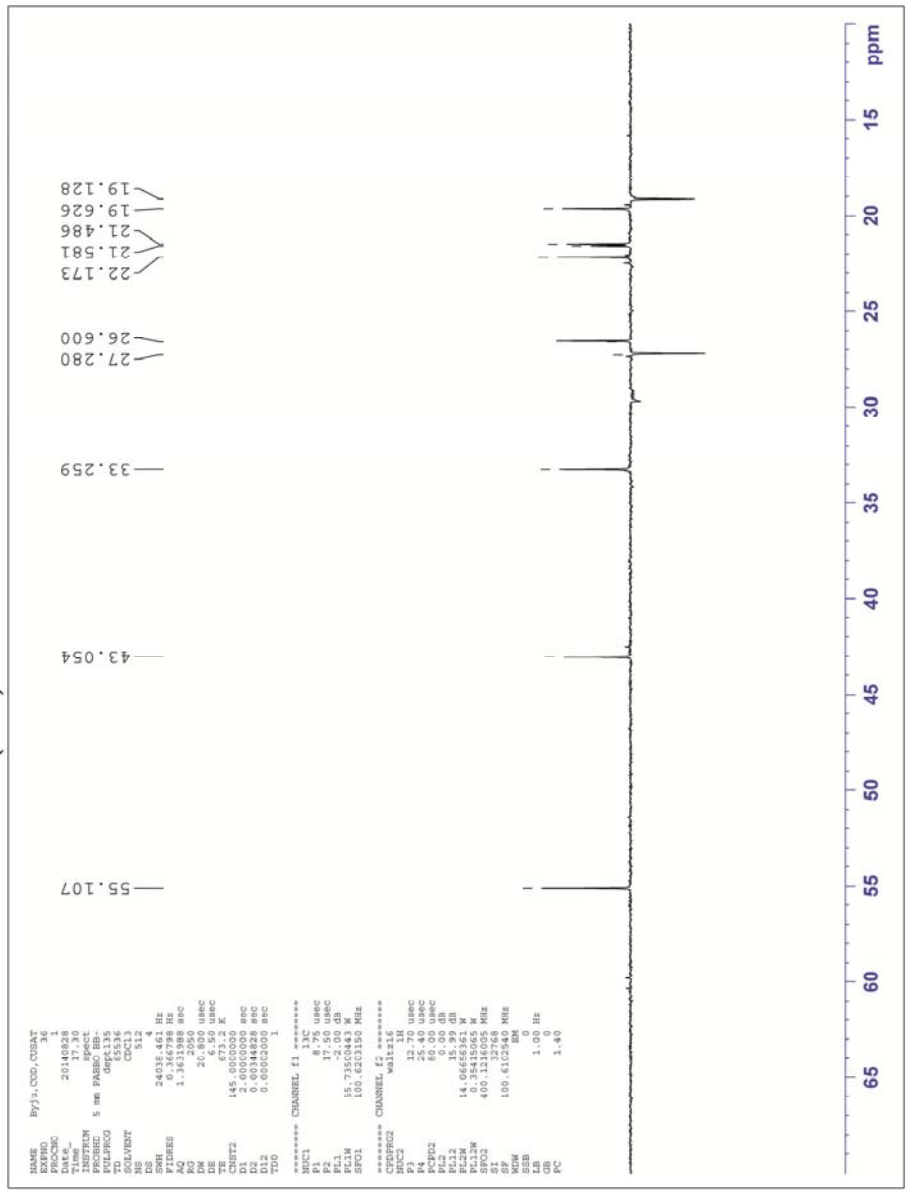
22.173
21.581
21.486
19.626
19.128
ppm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43.054
33.259
27.280
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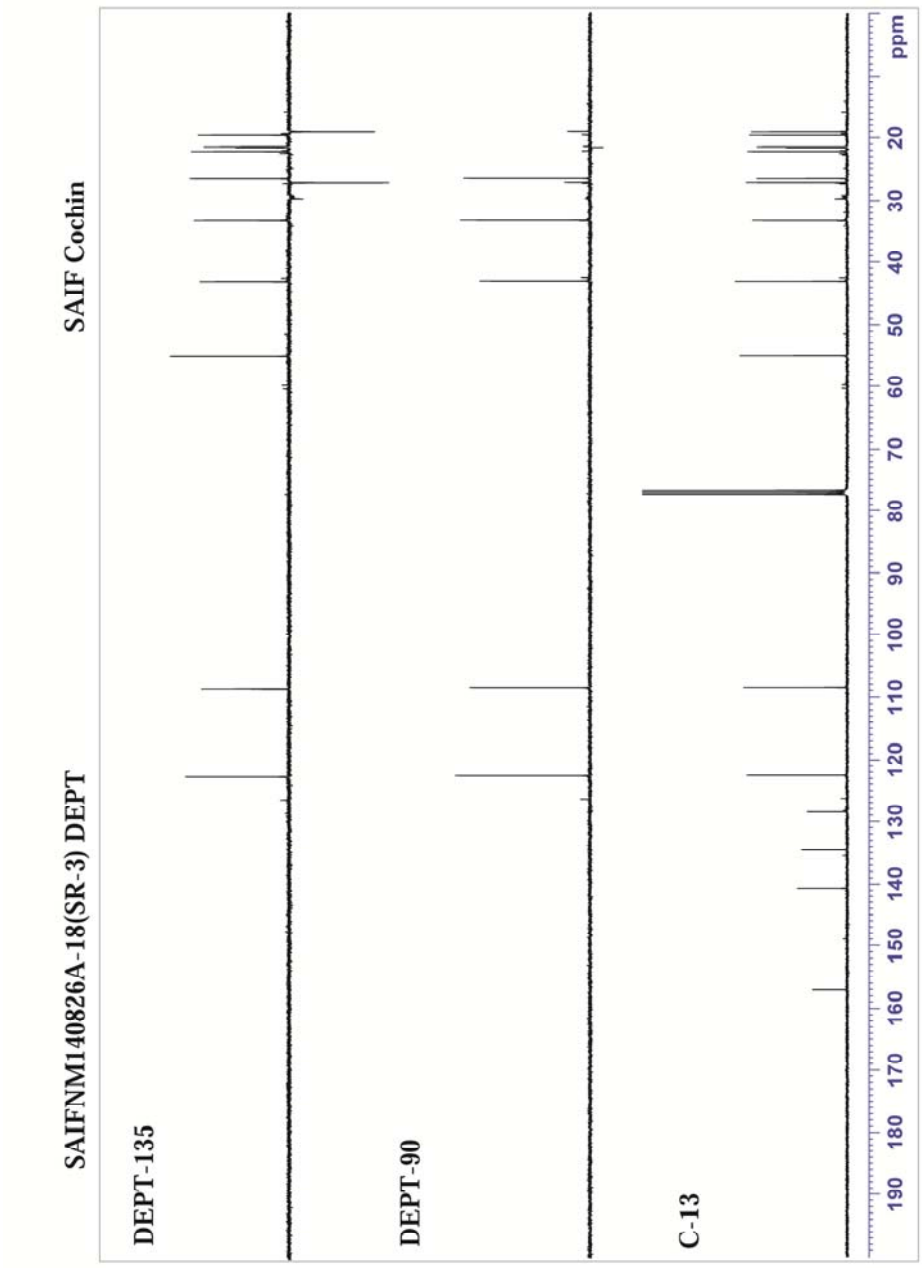
ppm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DEPT-135
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C-13
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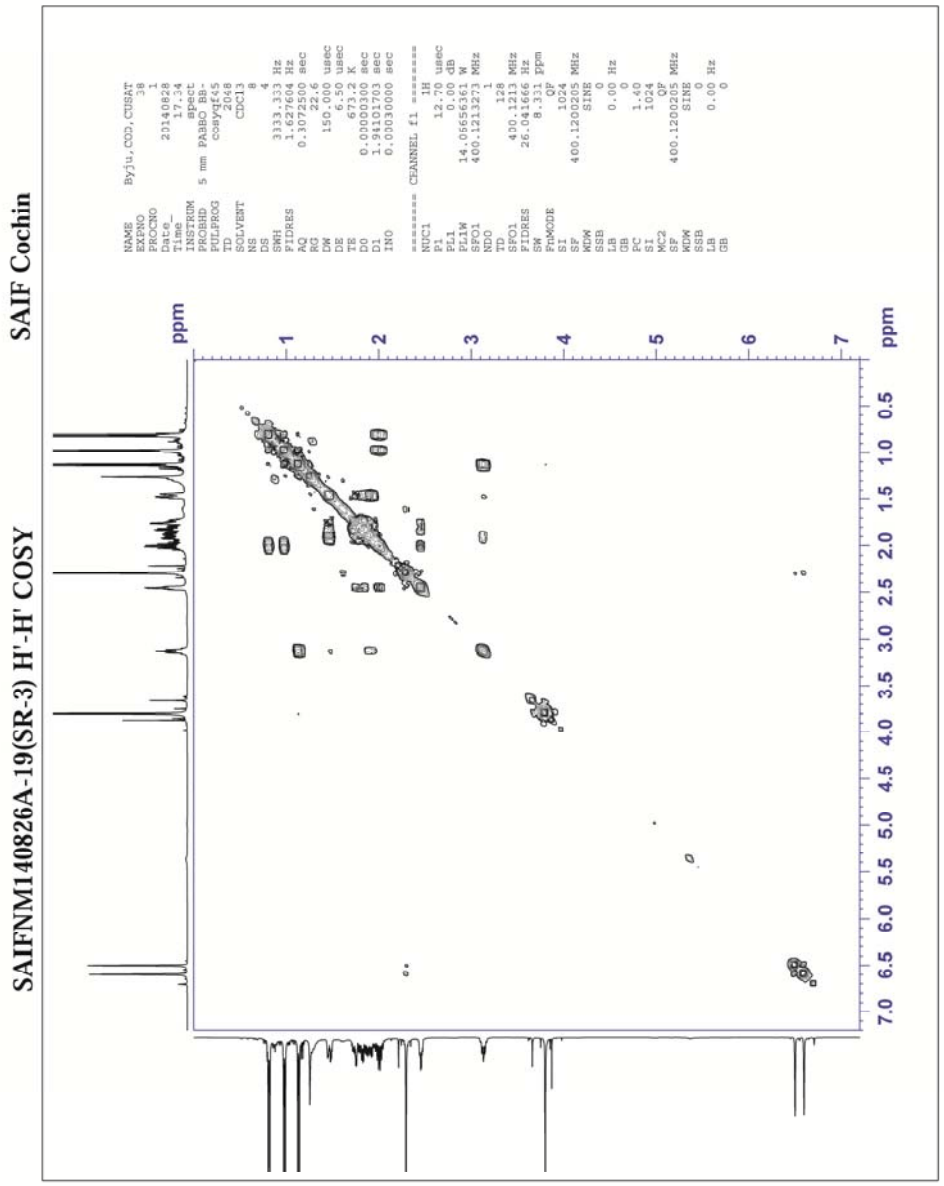
ppm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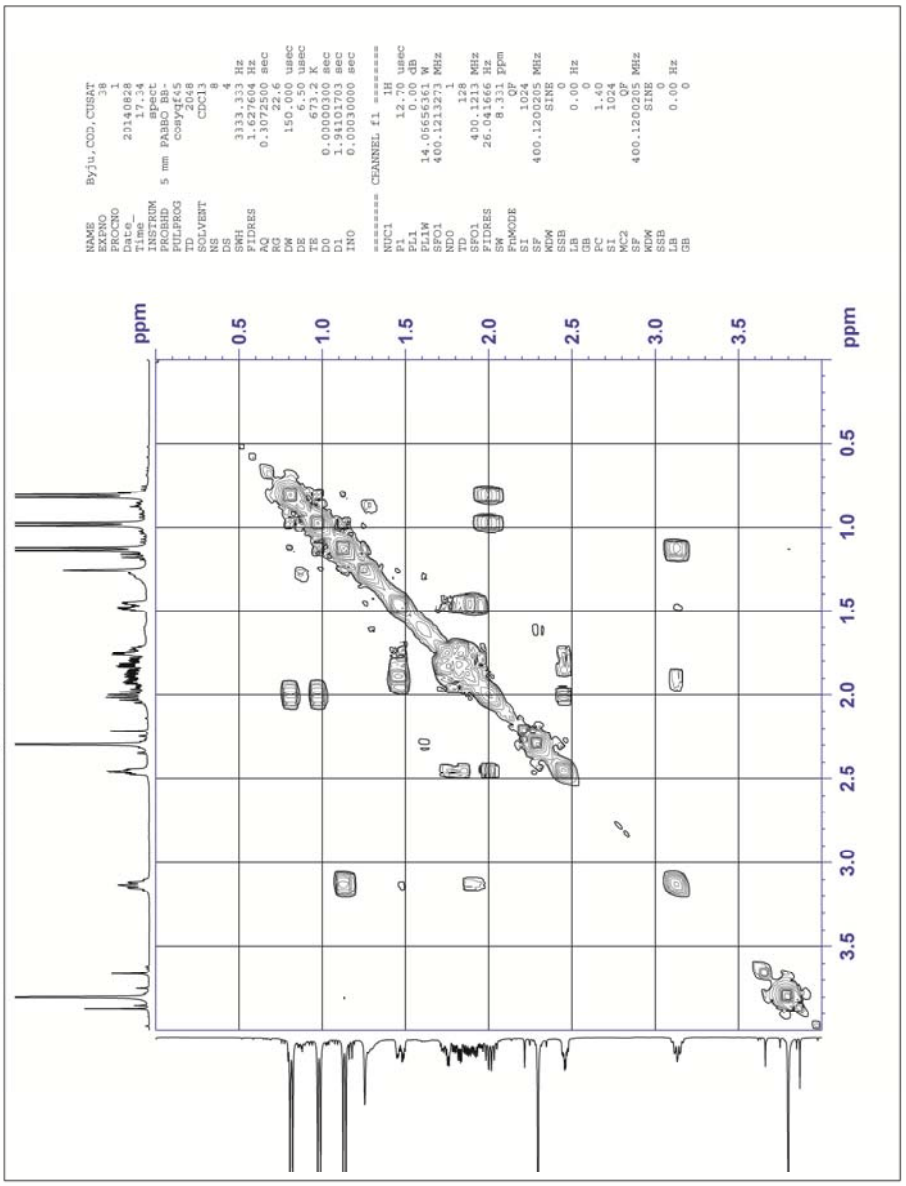
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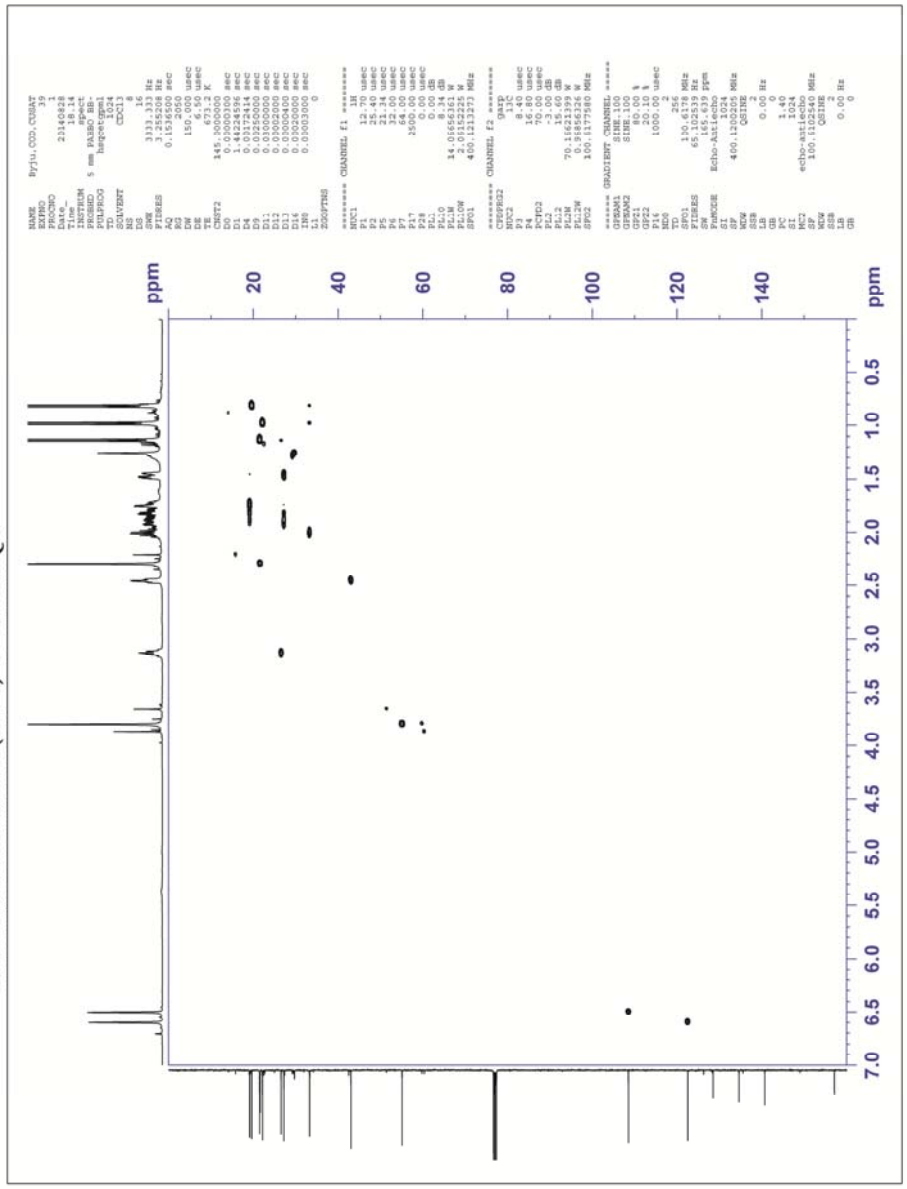
2.5
3.0
3.5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60
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100
120
140
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6.5
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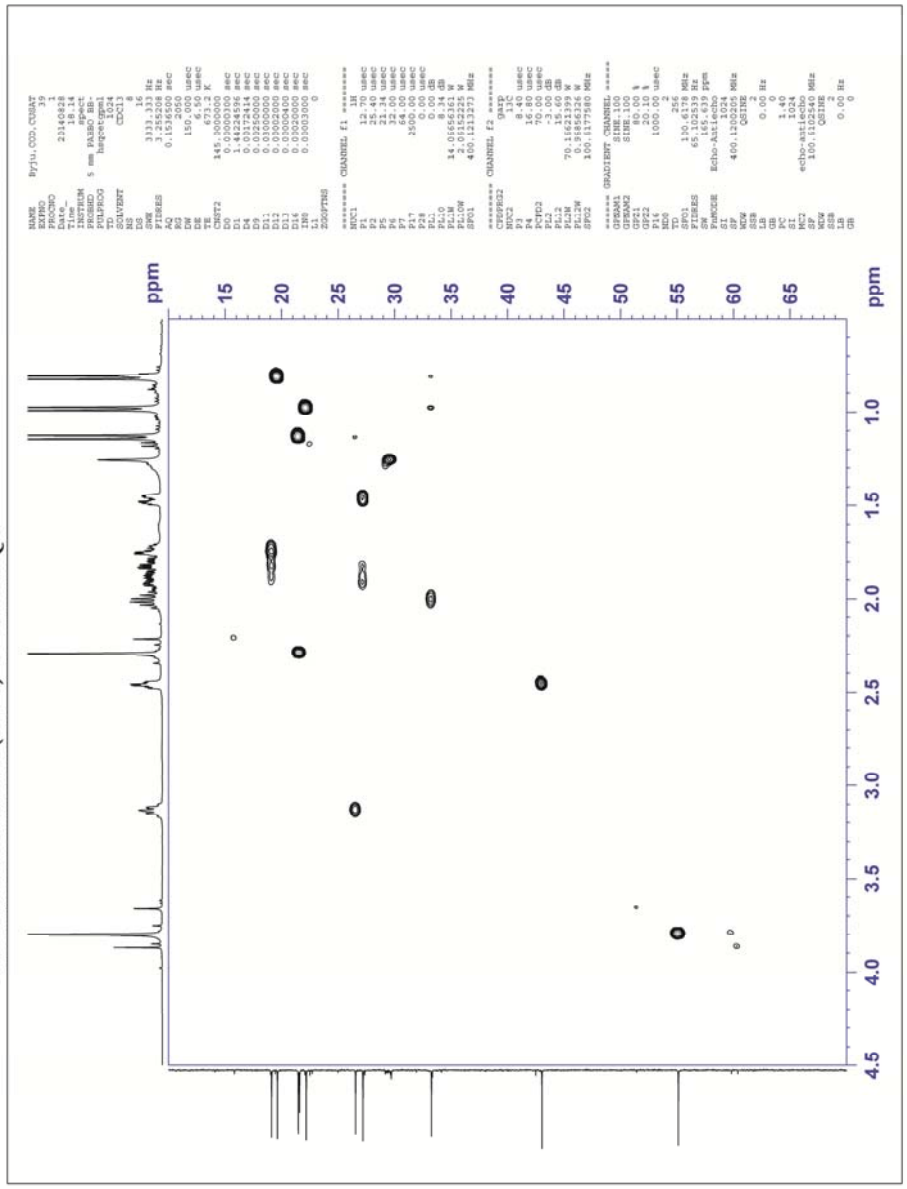
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm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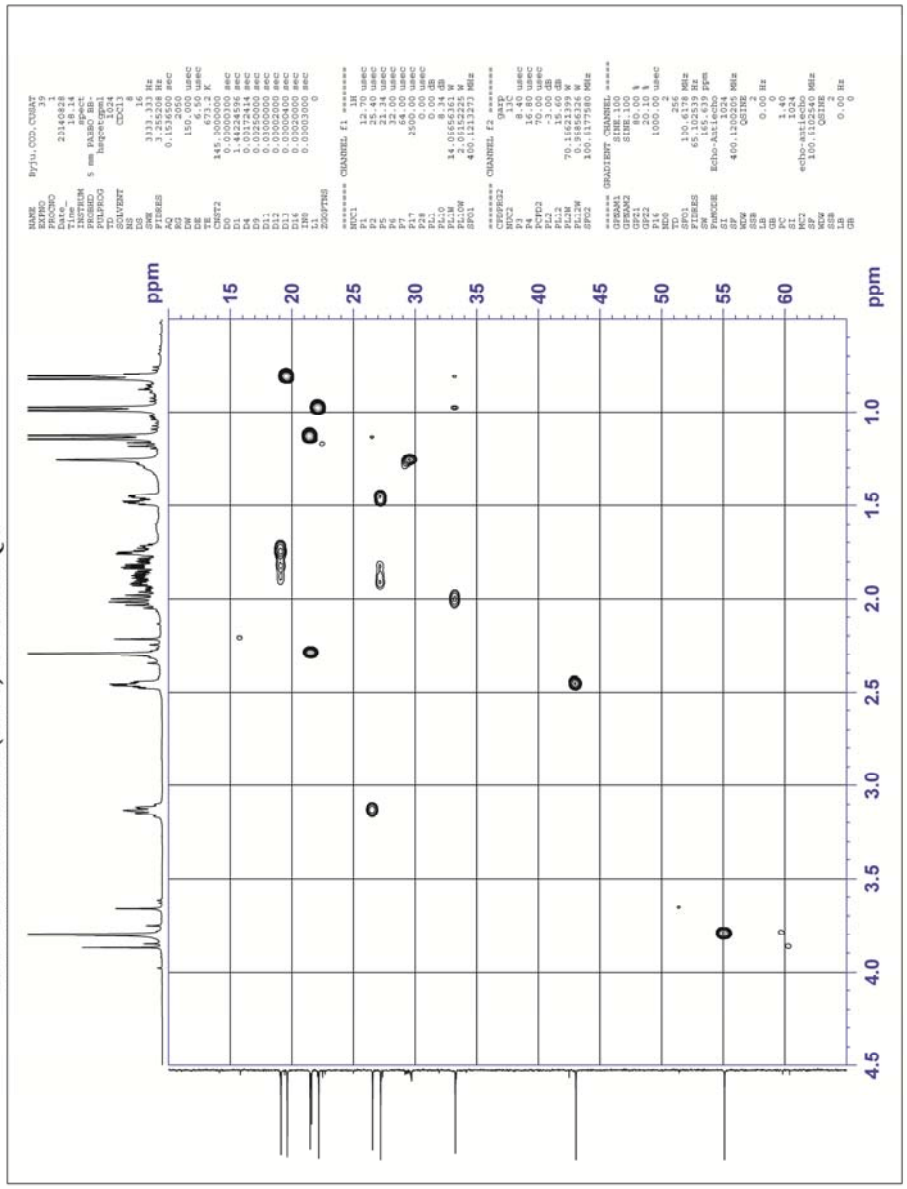
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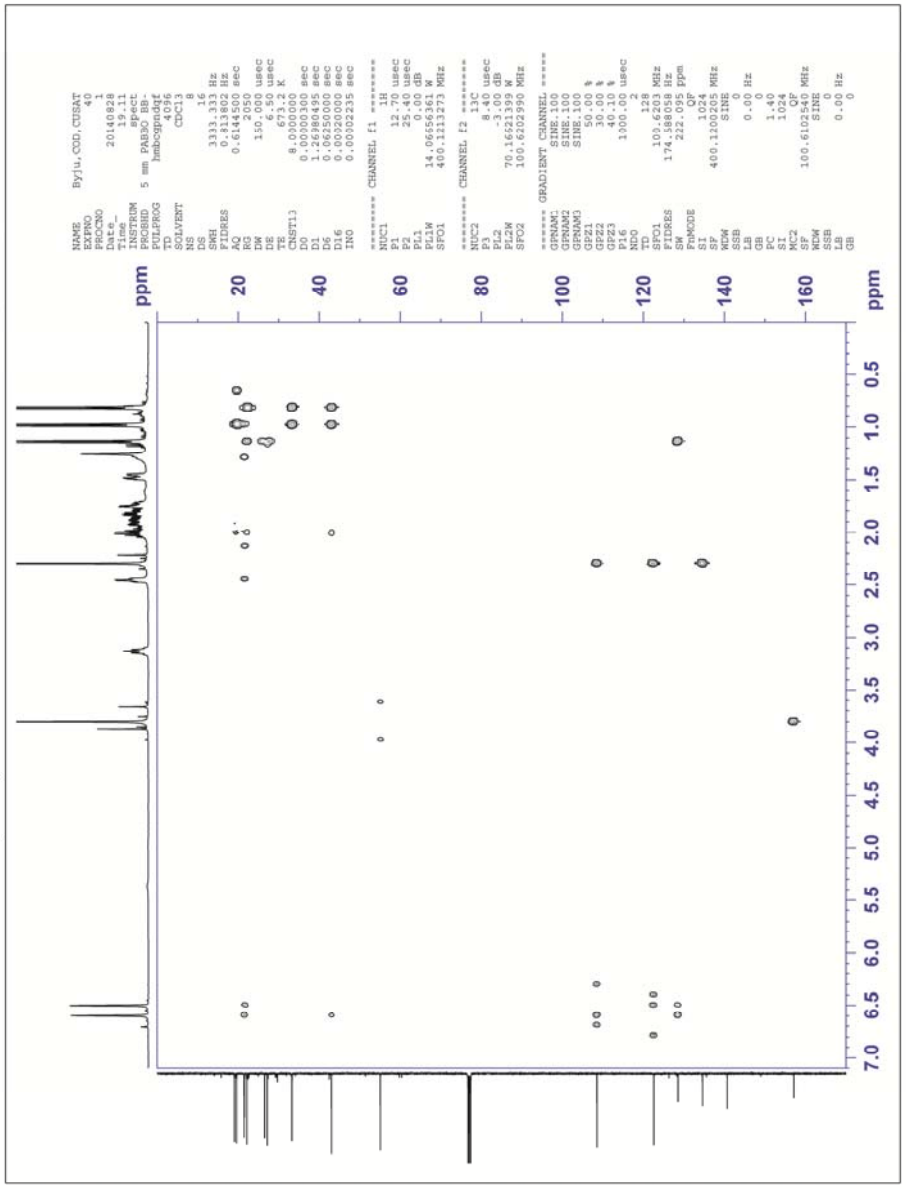
1.5
1.0
ppm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5.5
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4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
20
40
60
80
100
120
140
160

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LIST OF PUBLICATIONS

1. *In vitro* and *in silico* studies on the anticancer and apoptosis-inducing activities of the sterols identified from the soft coral, *subergorgia reticulata*, **Byju.K**, Anuradha.V, N.Chandramohanakumar, Vasundhara.G, S.MuraleedharanNair, **Pharmacognosy Magazine**, January-February 2014, Vol 10 , Issue 37, 65-71.
2. *In silico* biological activity of steroids from the marine sponge *Axinella carteri (*2013) Anuradha.V, **Byju.K**, Emilda Rosmine, AnuGopinath, S.MuraleedharanNair, N.Chandramohanakumar, Prashobpeter.K.J, Gireeshkumar,T.R, Vasundhara.G, **Medicinal Chemistry research**, 22: 1142-1146
3. Chemical characterization of the lipophilic extract *of Hydrilla verticillata*: a widely spread aquatic weed **Byju.K**, Anuradha.V, Emilda Rosmine, S.MuraleedharanNair, N.Chandramohanakumar, **Journal of plant biochemistry and biotechnology**, DOI 10.1007/s13562-012-0159-5
4. Evaluation of biochemical and nutritional potential of seaweeds from Lakshadweep archipelago. Anuradha V., **Byju K**., Kalajacob., Anu Gopinath., Prashobpeter K.J., Gireeshkumar T.R., Vasundhara G., Emilda Rosmine, Harishankar H.S., Kumar N.C. and Nair S.M (accepted for publication in "**Journal of Aquatic food product technology**")
5. Phytol – the precursor of vitamins in *Chaetomorpha antinnina* **Byju.K,** Vasundhara.G, Anuradha.V, Nair.S.M, Kumar.N.C- Accepted in **Mapana Journal of Sciences** 2013.
6. Sterol composition of the Indian green lipped mussel *Perna virdis*. Anuradha.V, Vineetha.P.V, Amrutha Manoj, Stephy.P.S, **Byju K,** Shameem.K, Vasundhara.G, N.C.Kumar, S.M. Nair. Accepted in **Mapana Journal of Sciences** 2013.
7. **K. Byju**, V. Anuradha, Emilda Rosmine, Hari Sankar, Anu Gopinath, K. J. Prashob Peter, T. R. Gireesh Kumar, G. Vasundhara, N. C. Kumar, Nair S.M .; DPPH scavenging property of active principles from the soft coral *sarcophyton flexuosum tixier-durivault*. **Pharmaceutical Chemistry Journal**- Article in press.

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PHCOG MAG. ORIGINAL ARTICLE

In vitro and *in silico* studies on the anticancer and apoptosis-inducing activities of the sterols identified from the soft coral, *subergorgia reticulata*

Kuniyil Byju, Vattoni Anuradha, Gopalakrishnapai Vasundhara, S. Muraleedharan Nair, N. Chandramohana Kumar

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Submitted: 13-04-2013 Revised: 18-04-2013 Published: 21-02-2014

ABSTRACT

Background: Gorgonians and other octocorals are known to possess a huge array of secondary metabolites in which sterols are the major group of secondary metabolites apart from sesquiterpenes and diterpenes, and the bioactive metabolites could show marked biomedical potential for future drug discovery. **Objective:** This study was intended for the isolation and identification of sterols from the octocoral *Subergorgia reticulata* and to evaluate the anticancer and apoptosis-inducing activities of the identified sterols through *in vitro* and *in silico* approach. **Materials and Methods:** The organism was collected from Lakshadweep Island. The isolated sterols were identified using Gas chromatography-mass spectrometry (GC-MS). The structure was confirmed by using comparison of their spectra those in National Institute of Standard Technology (NIST) library. The apoptosis inducing effect of identified sterols were determined by PASS online prediction. *In vitro* cytotoxity studies were carried out using Dalton's lymphoma ascites cells (DLA) and the cell viability was determined by trypan blue exclusion method. **Results:** Six sterols were identified from the soft coral *S. reticulata*. They are Cholesta-5,22-diene-3ol (3β), Ergosta-5-22-dien-3ol (3β,22E 24S), Cholesterol, 26,26-Dimethyl-5,24(28)-ergostadien-3β-ol. β-sitosterol, and Fucosterol. *In silico* predictions showed that the identified sterols exhibited remarkable apoptosis agonist activity. The probability of apoptosis agonist activity were found maximum for 26,26-Dimethyl-5,24 (28)-*S. reticulata* sterol fractions isolated were found to be having anticancer activity. **Conclusions:** These findings suggest that *S. reticulata* contained biologically active sterol compounds that may be useful in the treatment of cancer.

Key words: Apoptosis, cytotoxicity, Prediction of activity spectra for substances PASS, sterols, *Subergorgia reticulata*

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INTRODUCTION

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products.[1] There are more than 6,100 species all over the world, in which corals of the class Anthozoa in the phylum of Coelenterata are the most dominant benthic invertebrates living mainly in tropical seas.[2] Cnidaria, formerly known as Coelenterata, is one of the largest among the phyla[1] The phylum Cnidaria possesses an array of secondary metabolites, mainly terpenes, and the soft coral group possesses more than 80% of all cnidarian compounds.[3,4] In the marine environment, the success of defense used by soft corals and gorgonians against consumers and competitors has been attributed to their production of secondary metabolites, many of which show predator deterrence and allelopathic activities.[5]

Sterols are the major group of secondary metabolites characterizing corals next to sesquiterpenes and diterpenes.[6] Sterols advocate their dietary inclusion as an important strategy in prevention and treatment of cancer.[7] The "usual" sterols have a 3β-hydroxy-Δ^5- (or Δ^0-) cholestane nucleus and a C_8-C_{10} side chain. There are over 200 such sterols, occurring in marine organisms as complex inseparable mixtures, and their identification is usually done by Gas chromatography-mass spectrometry (GC-MS).

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E-mail: unnimurali@yahoo.com

Mounting evidence suggests that phytosterols possess anti-cancer effects[8] against cancer of the lung,[9] stomach,[10] ovary[11] and estrogen-dependent human breast cancer.[12] It has been speculated that phytosterols inhibit the production of carcinogens, cancer-cell growth, invasion and metastasis, and promote apoptosis of cancerous cells.[13]

Among the genus *Subergorgia*, very limited studies were reported on the chemical constituents of *S. reticulata*. Presence of few known polyhydroxylated steroids, among which some exhibited cytotoxic activity, were reported from *S. reticulata*[14] Yang *et al.*, (2006)[15] isolated nine compounds including three sterols and some alkaloids from *S. reticulata*. Apart from this, some diterpenoids and sesquiterpenoid compounds were also reported from *S. reticulata*.[15-17] The present study was aimed for the isolation and identification of steroids from the *S. reticulata* collected from Lakshadweep Island, Kavaratti and also for the tracing of the anticancer and apoptosis-inducing activities of the isolated steroids using *in vitro* and *in silico* methods.

MATERIALS AND METHODS

Collection of coral

The soft corals were collected from Lakshadweep Islands during December 2011. The collection was carried out by Scuba diving in the region at 25 m depth. The soft coral were collected in a glass bottle and brought to the shore, washed with fresh water free from detectable other adhering organisms like algae, cut into thin slices, and preserved in methanol in a glass bottle. A large specimen with clear morphological features was separately preserved in ethanol in small glass container for identification purpose. The organisms [Figure 1] were brought to the laboratory for processing.

Identification

The soft corals were identified as *S. reticulata*, by Dr. P.A. Thomas, Emeritus Scientist (ICAR). The voucher specimen (IUCDMB.R.No. 3) of the soft corals was preserved in the Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology. Taxonomical Details-Kingdom: Animalia; Phylum: Cnidaria; Class: Anthozoa; Subclass: Octacorallia; Order: Alcyonacea; Sub order: Scleraxonia; Family: Subergorgiidae; Genus: *Subergorgia*.

Extraction, isolation and purification

The extraction of the fragmented organism, *S. reticulata* (400 gm), was carried out at room temperature with methanol for 4 days. The process of extraction was repeated until, it left negligible residue on removal of the solvent. The solvent was removed under reduced pressure.

Figure 1: The soft coral *Subergorgia reticulata*

The residue was dissolved in minimum quantity of aqueous methanol. In an effort to further characterize the chemical constituents in the original sample, we fractionated the aqueous methanol sample by solvent partitioning with hexane. The hexane extracts were combined, concentrated under vacuum and the hexane soluble portion was washed with water, dried over anhydrous $MgSO_4$ and the solvents removed under reduced pressure. Column chromatography of the residue collected from hexane soluble fraction was done using silica gel with hexane: ethyl acetate on varying polarity. The fractions were collected and analyzed by Thin Layer Chromatography (TLC) and GC-MS. The fraction of 5% ethyl acetate was a mixture of six sterols.

GC-MS analysis

Gas chromatographic analysis was done on Perkin Elmer Clarus 680 GC-MS equipped with headspace sampler. Helium was employed as carrier gas and the ionizing voltage was 70 eV. Oven temperature was programmed from 60°C to 290°C at 10°C min^{-1}, where it remained constant for 15 min. Injector and detector temperature were kept constant at 280°C and 290°C, respectively. The column used was Elite 5MS having 30 m length and 250 µm id. Mass spectrum of each peak in the total ion chromatogram was resolved, and it was compared with National Institute of Standards and Technology (NIST) library spectra for the identification of the compounds.

Apoptosis inducing effect of identified sterols

Apoptosis inducing effect of the identified sterols from *S. reticulata* was studied using Prediction Activity Spectra of Substances (PASS). PASS is a software product designed as a tool for evaluating the general biological potential of an organic drug-like molecule. PASS provides simultaneous predictions of many types of biological activity based on the structure of organic compounds. Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their biological testing.[18] It was found

that the apoptosis inducing effect of identified sterols is prominent compared to other biological activity predicted by the PASS software.

In vitro cytotoxicity study

In vitro cytotoxicity studies were carried out using Dalton's lymphoma ascites cells (DLA). The tumor cells aspirated from the peritoneal cavity of tumor bearing mice were washed thrice with phosphate buffered saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension (1×10^6 cells in 0.1 ml) in phosphate buffered saline (PBS) was used. Control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37°C. Further, cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 min and loaded on a hemocytometer. Dead cells take up the blue color of trypan blue while live cells do not take up the dye. The numbers of stained and unstained cells were counted separately. Five different concentrations of the sterol fraction were prepared, respectively, as 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1}, 100 μg ml^{-1}, and 200 μg ml^{-1}.

$$\text{Percentage of dead cell} = \frac{\text{Number of dead cells}}{\text{Number of viable cells} + \text{Number of dead cells}} \times 100$$

RESULTS

Silica gel chromatography of the crude residue (4 g) obtained from hexane soluble fraction was done using hexane: ethyl acetate solvent composition. GC-MS analyses of the fractions collected were carried out. From the 5% ethyl acetate fraction, we identified six sterols by comparison of their spectra with those in National Institute of Standard Technology (NIST) library (version 2.2). The total ion chromatogram obtained was shown in Figure 2 and the respective mass spectra of the isolated sterols are shown in Figure 3. The sterols are 26,26-dimethyl-5,24 (28)-ergostadien-3β-ol, β-sitosterol, cholesta-5,22-diene-3ol (3β), cholesterol, ergosta-5-22-dien 3βol (3 β,22E 24S), and fucosterol. Cholesta-5,22-diene-3ol (3β) was the maximum yielded compound by area percentage followed by ergosta-5-22-dien 3βol (3 β,22E 24S), cholesterol, 26,26-dimethyl-5,24 (28)-ergostadien-3β-ol, β-sitosterol, and fucosterol. The structures of the sterols were shown in Figure 4.

The probability of apoptosis inducing effect of identified sterols from *S. reticulata* was shown in Figure 5. The apoptosis agonist activity was found to be maximum for 26,26-Dimethyl-5, 24 (28)-ergostadien-3β-ol (80.3) followed by Cholesta-5, 22-diene-3-ol (3β), Cholesterol, Ergosta-5-22-dien 3-ol (3β,22E 24S), Fucosterol, and β-sitosterol. Cholesta-5, 22-diene-3ol (3β) is prominent among the five sterols identified, and the apoptosis agonist activity was found to be 0.773.

The 5% ethyl acetate (steroid) fraction collected after the column chromatography of crude methanolic extract residue was concentracted under vacuum and the white residue obtained was weighed, five different concentrations were prepared, respectively, as 10 μg ml^{-1}, 20 μg ml^{-1}, 50 μg ml^{-1}, 100 μg ml^{-1}, and 200 μg ml^{-1} in Dimethyl sulfoxide (DMSO). The sterol mixture was studied for short term *in vitro* cytotoxicity. The graph showing the variation of cell death of tumor cells on increase in concentrations of the sterol fraction is represented in Figure 6. The study resulted in significant cell death, it was very clear that the number of dead cells was increased when the concentraction of the sterol fraction was increased. Ninety percent cell death was obseverd at 200 μg ml^{-1} concentration of the sterol fraction. The results are summarized in Table 1.

DISCUSSION

This is the first time reporting the four sterols cholesta-5, 22-diene-3ol (3β), ergosta-5-22-dien 3ol (3β,22E 24S), 26,26-dimethyl-5,24 (28)-ergostadien-3β-ol, and β-sitosterol

Table 1: Anticancer activity of sterol fraction from *Subergorgia reticulata*

Concentration of sterol fraction in μg ml^{-1}	Cell death (DLA) %
200	90
100	84
50	65
20	42
10	32

DLA: Dalton's lymphoma ascites

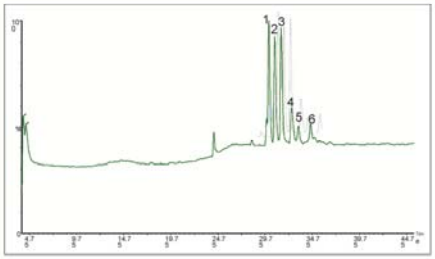

Figure 2: Total ion chromatogram of the sterol fraction isolated from *Subergorgia Reticulata* (1. Cholesta-5, 22-diene-3ol (3β), 2. Cholesterol, 3. Ergosta-5-22-dien-3-ol (3β,22E 24S), 4. 26,26-Dimethyl-5,24 (28)-ergostadien-3β-ol, 5. β-sitosterol, and 6. Fucosterol)

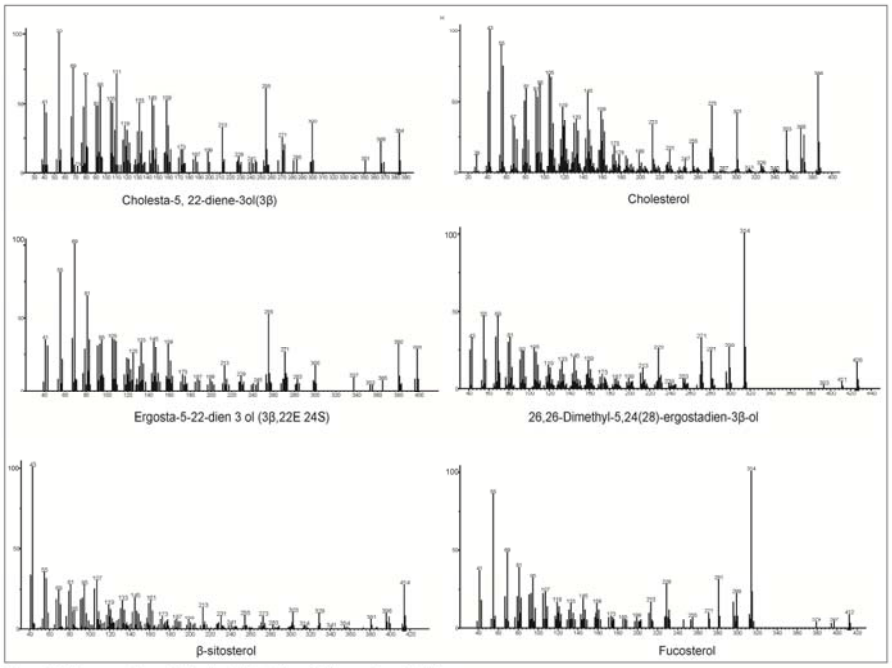

Figure 3: Mass spectrum of sterols isolated from *Subergorgia reticulata*

from the soft coral *S. reticulata*. From the *in vitro* studies, it is evidenced that almost a saturation value (>85%) was attained at slightly above 100 μg ml^{-1} concentration of the sterol fraction. The anticancer activities of sterol fraction isolated from *S. reticulata* can be due to a natural mixture of its components, and a single constituent may not have an activity greater than that of the mixture. Individually, cholesterol and β-sitostrol were reported for their potential for apoptosis agonist. β-sitosterol is generally considered as a phytosterol with chemical structure similar to that of cholesterol and is the most common sterol in human diet. Studies have indicated that β-sitosterol can inhibit the growth of various cultured cancer, and the simulation of apoptotic cell death.[19-21] From the PASS prediction, it is inferred that though β-sitosterol exhibited apoptosis agonist activity as a natural component in cancer prevention agreeing with findings of Award *et al.*, (1996)[22] it is found to be the lowest among the sterols studied from *S. reticulata*.

The apoptosis agonist activity was maximum for 26,26-Dimethyl-5,24 (28)-ergostadien-3 β-ol and the probability of apoptosis inducing effect of all other sterols studies were found to be greater than 70%. There is no proven report for supporting 26,26-Dimethyl-5,24 (28)-ergostadien-3 β-ol, ergosta-5-22-dien 3-ol (3β,22E 24S), fucosterol, and Cholesta-5, 22-diene-3-ol (3β) that these are apoptosis agonist. It has been reported that intracellular cholesterol accumulation induces apoptosis of pancreatic cells,[23] it also support the results with our findings that inducing effect of cholesterol is prominent with a probability of 0.77. Fucosterol is the most abundant phytosterol in brown algae and it was proved that fucosterol containing fraction of marine algae responsible for cytotoxic effect against breast and colon carcinoma cell lines.[24] There is no evidence to prove fucosterol itself can induce apoptosis, but the PASS study conducted here have supported the findings of Khanavi *et al.*,(2012)[24] by predicting the probability of apoptosis inducing effect of fucosterol as 0.763.

Yang *et al.*, (2006)[15] reported the presence of cholesterol and isofucosterol from the South China Sea gorgonian

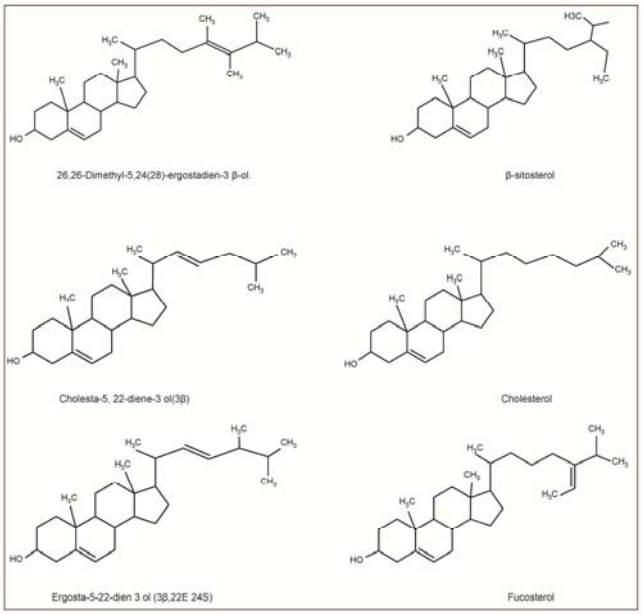

Figure 4: The structures of identified sterols from *Subergorgia reticulata*

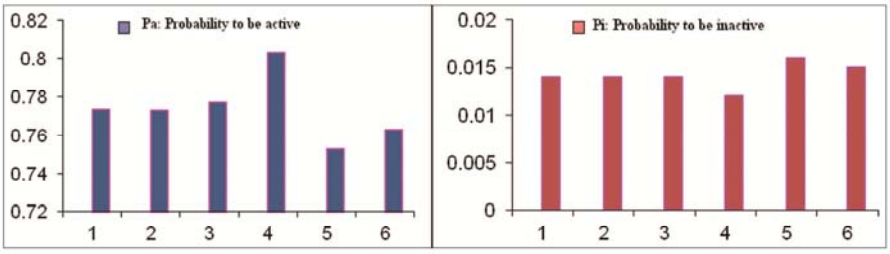

Figure 5: Apoptosis inducing effect of the six identified sterols (1. Cholesta-5, 22-diene-3-ol (3β). 2. Ergosta-5-22-dien 3-ol (3β,22E 24S). 3. Cholesterol, 4. 26, 26-Dimethyl-5, 24 (28)-ergostadien-3β-ol, 5. β-sitosterol, and 6. Fucosterol)

coral *S. reticulata* with seven other compounds. Li *et al.*,(2005)[25] and Hsu *et al.*,(2005)[26] investigated that anticancer activity of some therapeutic substances is involved in the induction of apoptosis which can be used for cancer control. This is in accordance with our findings that apoptosis induction of these sterol molecules found to be anticancer agents. Induction of apoptosis in cancer cells is one useful strategy for anticancer drug development.[27]

It has been realized well in case of octocorals that *de novo* biosynthesis is an important mechanism.[28] However, the alternative possibility must be considered that they are able to absorb sterols from food which may well comprise of largely plankton and small crustacean species that are rich in sterols.[29,30] Since soft corals are associated with plankton and micro organisms, the contribution of sterols from their part cannot be omitted. Biosynthesis of sterol from this soft coral, *S. reticulata* is yet to be investigated[31] and it

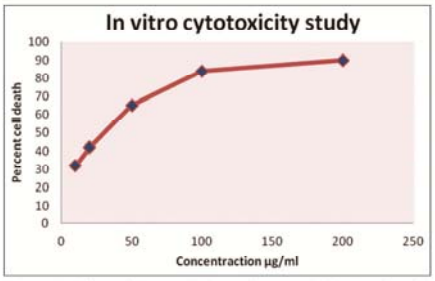

Figure 6: Effect of the application of the sterol fraction from S. reticulata on the tumor cells. Cell viability was determined by trypan blue exclusion method

could be beneficial to know the sterol pathway to provide additional clues to understand its role in reproduction, chemical signaling or as defensive metabolite.

CONCLUSIONS

The apoptosis inducing effects and *in vitro* cytotoxicity studies of identified sterols from the soft coral *S. reticulata* were investigated. A significant apoptosis inducing effect was observed for each compound and it is the primary screening of pharmacological potential of sterols that we had identified. However, the determination of apoptosis by *in silico* may be considered as an indirect method, comparing to those performed in tissues. The cytotoxic results of this sterol compounds mixture confirm it as a natural potent chemopreventive and chemotherapeutic agent. In contrast, this apoptosis inducing effect was apparent when measured using PASS, online prediction assay give us an idea about the possibility for the development of an anticancer drug, and still more *in vivo* studies are required to prove the potentiality for an anticancer drug from the marine soft coral *S. reticulata*.

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