Anticancer Potential of Sterols Isolated from *Turbinaria conoides* Against Lung and Liver Cancer

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To my loving family

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Certificate

This is to certify that the thesis entitled ***"Anticancer Potential of Sterols Isolated from Turbinaria conoides Against Lung and Liver Cancer"*** *is an authentic record of the research work carried out by Mrs. Kala K. Jacob., under my supervision and guidance at the Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, 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. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommendations by doctoral committee of the candidate have been incorporated in the thesis.*

Kochi – 16
March, 2016

Prof. (Dr.) N. Chandramohanakumar
(Supervising Guide)

Declaration

I hereby declare that the thesis entitled ***"Anticancer Potential of Sterols Isolated from Turbinaria conoides Against Lung and Liver Cancer"*** *is an authentic record of the research work carried out by me under the guidance and supervision of Dr. N. Chandramohanakumar, Emeritus 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.*

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Preface

Thesis entitled "Anticancer Potential of Sterols Isolated from Turbinaria conoides Against Lung and Liver Cancer", is exploring the cytotoxic potential of sterols isolated from a brown seaweed Turbinaria conoides. Various cytotoxic studies have identified the potential of sterols from Turbinaria conoides to suppress the proliferation of malignant cells effectively, however, their potential and mechanism of action to suppress lung and liver cancer are not studied so far in detail. Considering these facts, this brown sea weed, which are abundant along the shorelines of Arabian Sea and Bay of Bengal, is a promising source for developing safe drug combinations and hence needs immediate attention. The data generated in this study can be used for developing safe drug combinations to cure lung and liver cancer effectively without long and short term side effects in future..

First chapter collectively reflects the collaborative effort of researchers from various disciplines (natural product and medicinal chemistry) to comprehend natural products for developing safe therapeutically active products coast effectively. Particularly, role played natural products in containing the challenges raised by cancer effectively. Emphasis is given to bioactive molecules, cytotoxic, that are isolated from seaweeds. An assessment of their activity and details regarding the mechanism of action are also briefed, in order to emphasize their safety. In this chapter a section had exclusively reviewed various bioactivities shown by molecules isolated from Turbinaria conides. Finally, short description of the aim and scope of this work is also given.

In Chapter two, importance and scope of bioactivity based screening towards the drug development is highlighted in the introduction. Followed by material and method section which details the chromatographic methods used for the separation of complex lipid mixtures from Turbinaria conides, bioactive screening of these lipid fractions using SRB against A549 and Hep G2 cell line, and their composition analysis, using GCMS. Based on bioactive screening, some of the fractions were not just bioactive, they seem as a pool of secondary metabolites in which probability of finding potent cytotoxic agents are high on the

basis of their GCMS characterization. Based on the results and their analysis, it was presumed that prominent cytotoxic activity shown by NL3 fraction is an outcome of collective action of various sterol molecules identified in it. Composition of other active fractions, are also discussed in detail.

In chapter three, emphasis is given for the isolation, purification and spectroscopic characterization of sterols. Outlines of various spectroscopic methods used in the characterization of sterols are given the introduction section. In the materials and method section procedures followed for the isolation of sterols from Turbinaria conoides are briefed. This section also highlights the specification of instruments such as UV-Vis Spectroscopy, FTIR, NMR, GC-MS, FAB-MS used for the analysis. Methods adapted for the analyses of sterols using these instruments are also briefed. Observations of each analysis are tabulated and discussed in detailed to elucidate the structure of the two sterol molecules. Overall briefing of chemical features of sterols isolated from this sea weed is outlined in the conclusion.

Chapter four, core of this thesis highlights the potential of two sterol to suppress the progression of cancer cells, through apoptosis mechanism. This chapter highlights various methods adopted to study the cytotoxicity and the mechanism of action and why these methods were chosen for this particular study. Importance of this investigation is emphasized in the introduction section of this chapter. In the materials and method section, reasons for selecting Cell cultures (A549 and Hep G2), and adopting various assay protocols such as MTT assay, Neutral red assay, Lactate Dehydrogenase assay, double staining, flowcytometry and gene expression study for assessing the mechanism of cytotoxic action by sterols are highlighted. Results and observations of these investigations are further discussed.

Chapter five concludes with an overview of the major outcomes and importance of the two sterols for developing safe drug combinations.

Contents

Abbreviations

^{13}C-NMR	Carbon-13 nuclear magnetic resonance
7fl-HC	7fl-hydroxycholesterol
25 OHC	25-hydroxycholesterol
ABCG5	ATP-binding cassette sub-family G member 5 encoded by the *ABCG5* gene
ABCG8	ATP-binding cassette sub-family G member 8 encoded by the *ABCG8 gene*
AF1	Acid fraction 1
AF2	Acid fraction 2
AF3	Acid fraction 3
AF4	Acid fraction 4
AO	Acridine orange
ATP	Adenosine triphosphate
ATR-FTIR	Attenuated total reflectance fourier transform infrared spectroscopy
CDK1	Cyclin-dependent kinase 1
Bcl-2	B-cell lymphoma 2
C-Ab1	Chlorophyll A/B binding protein 1
cDNA	Complementary DNA
CDK2	Cyclin-dependent kinase
COSY	Correlation spectroscopy
Cox-2	Cyclooxygenase-2

DEPT	Distortionless enhancement by polarization transfer
DMEM/F2	Dulbeccos modified eagles media
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
EB	Ethidium bromide
ED_{50}	Median effective dose
EDTA	Ethylene diaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FABMS	Fast atom bombardment mass spectrometry
FACS	Fluorescence-activated cell sorting
FAO	Food and agriculture organization
FBS	Fetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GC-Ms	Gas chromatography–mass spectrometry
GFP	Green fluorescent protein
GPS	Global Positioning System
Ha-RAS	Harvey Rat Sarcoma Viral Oncogene
HDL	High density lipoprotein
H-H COSY	Proton proton correlation spectroscopy
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	High Performance Liquid Chromatography

HRFABMS	High resolution fast-atom bombardment mass spectroscopy
HSQC	Heteronuclear Single Quantum Coherence spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
IC_{50}	Half maximal inhibitory concentration
iNOS	Inducible nitric oxide synthase
IR	Infrared
KF	Kahalalide F
LPS	Lipopolysaccharide
LDH	Lactate dehydrogenase
LDL	Low-Density Lipoprotein
LXR	Liver X receptor
LXRβ	Crystal structure of human liver X receptor β
MAPK	Mitogen-activated protein kinase
MCT	Mercury cadmium telluride
mRNA	Messenger RNA
MTT	3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide
MHz	Megahertz
NADH	Nicotinamide adenine dinucleotide
NCCS	National Centre for Cell Science
NFB	Nuclear factor B
NK cells	Natural killer cells

NL1	Neutral lipid fraction 1
NL2	Neutral lipid fraction 2
NL3	Neutral lipid fraction 3
NMR	Nuclear magnetic resonance spectroscopy,
NPC1L1	Niemann-Pick C1-Like 1
NR dye	Neutral red dye
OD	Optical density
p53	Protein 53-kilo Daltons
p21	Protein 21- kilo Daltons
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
pRB	Retinoblastoma protein
QNP ^{1}H probe	Quattro Nucleus Probe
Rf	Retention factor
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SGOT	Serum glutamic oxaloacetic transaminas
SGPT	Serum glutamic-pyruvic transaminase
SOD	Superoxide dismutase
SRB	Sulforhodamine B
SR-BI	Scavenger receptor class B member 1
STAT1	Signal transducer and activator of transcription 1

TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TM	Primer melting temperature
TPA	Tetradecanoyl phorbol acetate
TUNEL	Terminal deoxynucleotidyl Transferase- mediated dUTP nick end labeling
US-FDA	United States Food and Drug Administration
UV-Vis	Ultraviolet-visible

Chapter 1

INTRODUCTION

Introduction

Lung and liver cancer survivors are facing serious rehabilitation issues such as physical, socio-economical and psychological mainly due to long term side effects of the treatment. Addressing this multidisciplinary issue is a major challenge not only for policy makers and institutions like World Health Organization, but also physicians and researchers working in this field (Gamble et al., 2011). One effective way is to develop treatment/medicine combinations which are devoid of any short term or long term side effects. Available statistics shows that due to lack of effective method to early diagnosis of lung and liver cancer, survival rate of patients suffering from these deadly diseases is very low. The treatment like radiation therapy is only partially successful in providing permanent cure (Gamble et al., 2011; Suda & Mitsudomi, 2014). In this regard many efforts are put in for identifying compounds with novel chemical structure and mode of action, which can be used with different medicinal combinations at various dose rates that can effectively control the proliferation of these malignant cells and

minimize the side effects of available remedies. To a great extent combinatorial chemistry and natural product research have contributed enormously to solve these issues (Messeguer & Cortés, 2007).

Even though progress in combinatorial chemistry has aided much towards drug discovery (Lahlou, 2013), most of the drugs and therapeutic strategies developed through these methods lack specificity and are often accompanied with a wide range of side effects and high cost (Galemmo et al., 1996; Salemme et al., 1997). Hence, researchers are primarily depending on natural products (phyco and phyto chemicals) for finding clues to solve, slow and/or reverse the cancer induction and its subsequent development and post treatment issues effectively (Hartwell, 1984; Newman et al., 2003). So far scientists have identified numerous natural products with unique structures, capable of suppressing not only tumors but also other human ailments effectively. About 62% of clinically proven cancer treating medicines has been derived from natural sources as per the survey data (1981-2002) published by United States Food and Drug Administration (US-FDA) (Newman et al., 2003).

Preference of natural products for treating various cancers and development of safer and more effective therapeutic agents (Mohan et al., 2011), are primarily due to their potential to specifically suppress the proliferation of malignant cells by inducing apoptosis (Sarkar & Li, 2009; Amin et al., 2011; Bajbouj et al., 2012). These mechanistic approaches are very useful in cancer chemoprevention, chemotherapy,

and to target multiple sites (Luo et al., 2014). Usually, this mode of malignant cell suppression occurs through their site-specific action on multiple cellular signaling pathways without inducing any undesired toxicity in normal cells. Hence, these natural agents are non-toxic and could be used in combination with conventional chemotherapeutic agents to achieve higher effectiveness towards the treatment of human malignancies with lower side effects (Demain & Vaishnav, 2011; Safarzadeh et al., 2014).

In this circumstances, successful development of first effective clinical anticancer agent cytarabine, was based on the clues provided by a natural product C-nucleosides isolated from the Caribbean sponge, *Cryptotheca crypta*. Presently, various malignant neoplasm such as leukaemia, lymphoma, pancreatic, breast, bladder, and non-small-cell lung cancers are treated using cytarabine and its fluorinated derivatives such as gemcitabine (Schwartsmann et al., 2000; Schwartsmann et al., 2001). A list of more than 3000 plant species used in the treatment of cancer has been documented in a review by Hartwell (1984). Vincristine, irinotecan, etoposide and paclitaxel derived from plant products are some routinely used anti-cancer agents (Da Rocha et al., 2001). Even though there are ample *in vitro* and *in vivo* evidence for the potential of drugs derived from primary and secondary plant metabolites, to suppress tumorigenesis (Awad & Fink, 2000; Woyengo et al., 2009), researchers are more focused on marine sources for discovering novel compounds and

mode of action to suppress malignant cells. This special interest in marine environment is due to unique set of biomolecules synthesized in marine plants, macro- and micro algae, microorganisms and sponges, owing to the presence of vast biodiversity and complex habitats (Lordan et al., 2011).

For example, a remarkable antitumour activity against tumour growing in athymic mice as well as *in vitro* was shown by didemnin B, a cyclic depsipeptide, commonly known as aplidine, isolated from the tunicate *Tridemnum solidum* (Geldof et al., 1999). Its presence has also been reported in Mediterranean tunicate, *Aplidium*. Preclinical results points out that rapidly proliferating tumour types were suppressed through interference of aplidine molecules with cell-cycle progression at G1, and accounts for their potentially high anticancer activity against various tumors (Urdiales et al., 1996). Selective and potent cytotoxic effects are exhibited by thiocoraline, a novel bioactive depsipeptide isolated from *Micromonospora marine*, against melanoma, lung and colon cancer cell lines (Amador et al., 2003). Independent investigations using this compound to identify the mechanism of action revealed a preferential antiproliferative effects in colon cancer cell lines with defective p53 systems (Erba et al., 1999). Another example for marine derived cytotoxic dessipeptide is Kahalalide F (KF), which blocks the cell cycle in G1 phase in a p53-independent manner (Garcia-Rocha et al., 1996) was first isolated from the mollusk *Elysia rubefescens* from Hawaii.

Sea hare, *Dolabella auricularia,* a mollusca found in the Indian Ocean are rich in cyclic and linear peptides known as dolastatins, are extremely potent *in vitro* cytotoxic compounds capable of inhibiting microtubule assembly, causing cells to accumulate in metaphase (Bai et al., 1990; Pathak et al., 1998). Another bicyclic peptide, depsipeptide isolated from a strain of *Chromobacterium violaceum* is capable of suppressing mRNA expression of the c-*MYC* oncogene. This mode of cytotoxic action is capable of causing cell-cycle arrest at G0–G1 in Ha-*RAS*-transformed NIH-3T3 cell line, and thereby inhibiting its growth (Ueda et al., 1994). Nakajima et al. (1998) identified its potential to acts as histone deacetylase inhibitor.

The ecteinascidins is a tetrahydroisoquinoline alkaloid, which acts by selective alkylation of guanine residues in the DNA minor groove (Zewail-Foote & Hurley, 1999), was derived from the Caribbean tunicate, *Ecteinascidia turbinate,* is effective in the suppression of both murine and human tumour cell lines (Garcia-Rocha et al., 1996). Granulatimide and isogranulatimide, two compounds capable of functioning as G2 checkpoint inhibitors, are aromatic alkaloids isolated from Brazilian tunicate, ascidian *Didemnum granulatum* (Berlinck et al., 1998). Topsentin and hamacanthin are two novel bisindole class of alkaloids, isolated from the Mediterranean sponge *Rhaphisia lacazei*, showcased significant *in vitro* antiproliferative activity against a series of human cancer cell lines (Casapullo et al., 2000).

Parahigginols and parahigginic acid, two novel sesquiterpenes isolated from a Taiwanese marine sponge *Parahigginsia* sp., demonstrated cytotoxic properties against murine P-388, human KB16, A549, and HT-29 tumour cells (Chen et al., 1999). Induction of antitumour activity in animal models through dose-dependent DNA cleavage via topoisomerase II was demonstrated by a group of amine compounds derived from marine sources (Matsumoto et al., 1999).

Sterols derived from marine resources are also promising bioactive source against lung (A549), colon (HT-29 and H-116), mice endothelial (MS-1) and human prostate carcinoma (PC-3) cell lines. An example for sterol showing these type of antiproliferative activity are 5α,6α-epoxy-24R-ethylcholest-8(14)-en-3β,7α-diol, 5α,6α-epoxy-24R-ethylcholest-8-en-3β,7α-diol and 3β-hydroxy-24R-ethylcholesta-5,8-dien-7-one isolated from marine sponge *Polymastia tenax* (Santafé et al., 2002). Proliferation in human leukemia (HL-60) cell line was effectively suppressed using orostanal, a class of sterol isolated from marine sponge *Stellatta hiwasaensis* (Miyamoto et al., 2001). Cytotoxic activity of sterols is attributed to chromatin condensation (Quang et al., 2011), modulating the expression of proteins (Huang et al., 2008), antioxidant activities (Van Minh et al., 2011), inhibition of superoxide anion generation and elastase release by human neutrophils (Liu et al., 2010).

Terpenoids, another class of secondary metabolites, also possess significant cytotoxicity against various malignant cells.

Brown algae *Sargassum tortile* is a rich source of meroterpenoids, such as sargol, sargol-I and sargol-II having significant cytoxic activity (Numata et al., 1992). Bifurcadiol, isolated from the brown algae *Bifurcaria bifurcate* by Di Guardia et al. (1999), is an example for a linear cytotoxic diterpene displaying significant activity against cultured human tumor cell lines such as A-549, SK-OV-3, SKL-2, XF 498 and HCT.

Even though various classes of molecules derived from marine sources such as cyclic depsipeptide, dolastatins, alkaloids, sesquiterpenes, sterols etc. have been found effective in the treatment of cancer, research on sterols is a promising area towards the development of cost effective cytotoxic drugs. As most of the sterols so far isolated from both marine and terrestrial sources has proved to be very effective as far as cancer treatments are concerned (Grattan, 2013).

1.1 Literature Review

In this review, cytotoxic potential of sterols from various sources and their mechanism of action are given in general, and particular emphasis is given for sterols isolated from Phaeophyceae (brown seaweeds). Seaweeds with an estimated gross production of 8.5 million metric tons (FAO, 2003) globally consist of numerous species, which have developed unique metabolic pathways and biosynthesized compounds as a part of their defense and survival. Seaweeds are capable of producing unique compounds in response to

even slight changes in the seasonal and climatic conditions (Chanda et al., 2010; Kendel et al., 2015), hence is a promising source for exploring novel sterol compounds. Pharmaceutical significance of sterols have been recorded in various investigations (Terry et al., 1995; Moreau et al., 2002; Ostlund et al., 2002; Jones & AbuMweis, 2009; Rasmussen et al., 2009; Rondanelli et al., 2013). Careri et al. (2006) reported the anti-inflammatory, anti-neoplastic, anti-pyretic and active immune modulating properties of β-sitosterol. Whereas ergosterolperoxide is an example for another class of sterols possessing inhibitory hemolytic activity along with anti-tumor, anti-viral, immune modulatory and anti-inflammatory activities. This sterol is also capable of mediating DNA topoisomerase I induced relaxation of supercoiled DNA (Bu et al., 2014). Moreover, both marine and phytosterols possess effective antibacterial and antifungal activities (Sánchez-Machado et al., 2004). However, in humans they showcase antidiabetic, antihypercholesterolemic (Moreau et al., 2002), anti-artherosclerotic and anti-ulcerative activities (Beveridge et al., 2002; Moreau et al., 2002; Lagarda et al., 2006). For the treatment of type-II diabetes and obesity, a potential drug was developed using the inhibitory potential of 24-ethylcholesta-4,24(28)-dien-3-one, stigmasta-5,28-dien-3β-ol, cholesta-5,14-dien-3β-ol and cholesta-5,23-dien-3β,25-diol isolated from the brown alga *Sargassum thunbergii* collected from East China Sea. These molecules have an IC_{50} of 2.24mg/mL (He et al., 2014). Ergostatrien-3β-ol, isolated from the

fungus *Antrodia camphorata*, has been used to alleviate the epidermis and sub-dermis damage due to their anti-inflammation capacity (Huang et al., 2010; Tung-Chou et al., 2015). Along with antiserum, β-sitosterol and stigmasterol are also used in snake venom neutralization (Gomes et al., 2007). These results along with the absence of reported toxic effect, side effects or biochemical anomalies on animal models has promoted their use in medicines and daily food supplements (Miettinen et al., 1995; Moreau et al., 2002).

Importance of incorporating sterols in food supplements particularly phytosterols are mentioned in various reports, as they are very effective in controlling the blood cholesterol levels (Dunford & King, 2000; Rondanelli et al., 2013). These lowering of cholesterol levels in serum (Jones et al., 1997), is very helpful in reducing the threat of cardiac arrests resulting from hypercholesterolemia (Moreau et al., 2002). This has been further supported by the findings of Jones & AbuMweis (2009), which highlighted the potential of phytosterols to specifically reduce the low density lipoproteins (LDL)-cholesterol levels in blood, whereas triglycerides and high density lipoproteins (HDL) levels are not affected. Even though exact mechanisms of cholesterol level reduction by sterols are not very well understood. Reduction in quantity of cholesterol available for absorption in gut through lowering their micellar solubility by sterols has been widely accepted as a major pathway for reducing blood cholesterol levels (Jones et al., 2000). On the basis of observations made by Park & Carr

(2010), the potential of sterols to function as signaling molecules to regulate the expression of cholesterol related genes are also well supported. This mode of increasing HDL-cholesterol through the selective suppression of LDL-cholesterol using phytosterols have vital role in reducing atherosclerosis (Moghadasian, 2006). Hedgehog protein, involved in embryonic development, are effectively modulated by oxysterols along with 25-Hydroxycholesterol (Lagace et al., 1999). Lagace et al. (1999) also investigated their role in the regulation of sphingomyelin biosynthesis, required for the formation of raft sub-domains in membranes. Further, using sterols expression of mRNA levels in hydroxy-3-methyl glutaryl CoA reductase (HMG-CoA reductase), NPC1L1, SR-BI and LDL receptor are suppressed effectively. Along with regulation of cholesterol homeostasis, neurodegenerative diseases can be effectively controlled by 24(*S*)-hydroxycholesterol and 24(*S*),25-epoxycholesterol, through specifically interacting with the nuclear receptors responsible for the expression and synthesis of proteins involved in sterol channeling. Associated with homeostasis, 24(*S*)-Hydroxycholesterol are also involved in and simultaneous down-regulation of amyloid precursor protein trafficking (Ridgway, 1995; Noguchi et al., 2014). These results highlight the importance of sterols from both phyco and phytosterols in various pharmaceutical applications.

Phytosterols, are very useful for treating patients suffering from breast cancer and other form of cancers such as the colon and prostate

(Awad & Fink, 2000; Awad et al., 2001, Awad et al., 2004). The healing effect of phytosterols, particularly β-sitosterol is attributed to their potential to stimulate antioxidant enzymes by an estrogen receptor/PI3-kinase-dependent pathway and thereby stimulating apoptosis in highly proliferative tumor cells (Moreno, 2003). Apoptosis-promoting potential of β-sitosterol against breast cancer is also well supported by the works of Awad et al. (2003) and Awad et al. (2007). These investigations have assessed the role of β-sitosterol and campesterol, two common dietary phytosterols, on the mevalonate and MAP Kinase (MAPK) pathways in MDA-MB-231 cells to understand the mechanistic basics for its antiproliferative effects on cancer cells. Further, a reduction in membrane sphingomyelin and an increase the ceramide levels in some tumor cells (Awad et al.,1997; von Holtz et al., 1998) induced by β-sitosterol also highlight the protective role played by these molecules against cancer (Platt et al., 2004).

Armartnol A and armartnol B are isolated and purified from soft coral *Nephthea armata* showcased significant cytotoxic activity against various malignant cells. Armartnol A had IC_{50} value of 7.6, 6.5 and 6.1mM, respectively against A549, HT-29, and P-388 (mouse lymphocytic leukemia) cell lines. However, cytotoxic activity of armartnol B against A549 cell lines were insignificant, whereas prominent activity was observed when studied against P-388 and HT-29 cells with IC_{50} values of 3.2 and 3.1mM, respectively (El-Gamal et al., 2004). Certonardosterol Q6 isolated and purified from starfish

Certonardoa s emiregularis is a potent cytotoxic agent against SK-OV-3 (human ovarian cancer), SK-MEL-2 (human skin cancer), A549, and HCT 15 cell line (Wang et al., 2004).

Eight Nebrosteroids (A-G) isolated from soft coral *Nephthea chabroli* Audouin (Nephtheidae) by Huang et al. (2008) are capable of effectively modulating the expression of proteins inducing the cell proliferation. Potential of Nebrosteroid D, E, and G sterols to significantly reduce the level of iNOS and COX-2 proteins expression at a concentration of 10mM was observed in LPS-stimulated RAW 264.7 cells. Whereas nebrosteroid A, B, C, and H were only effective in the suppression of iNOS protein expression (Huang et al., 2008). Another examples of sterols suppressing protein expression of proliferating cells, particularly COX-2 protein is stoloniferones T and (25S)-24-methylenecholestane-3,5,6-triol-26-acetate (Chang et al., 2008).

Considering the vast diversity of sterol structure in marine environment than in terrestrial environment, following discussion is focused towards sterols produced by seaweeds. In response environmental and seasonal factors seaweeds produce unique biochemical compounds, out of which more than 15000 primary and secondary metabolites have been reported (Faulkner, 2001; Blunt, et al., 2006; Cardozo et al., 2007; Blunt et al., 2011; Khairy & El-Shafay, 2013). Most of these molecular units identified were having potential medicinal value leading to intensification in chemical research on

algae products during the last years (Cardozo et al., 2007). Heilbron et al. (1934) isolated and purified a doubly unsaturated sterol from the brown algae *Pelvetia canicuhzta* and *Fucus vesiculosus* and named fucosterol, which was later found to be the abundant sterol of Phaeophyta along with biosynthetic precursors of fucosterol. From Phaeophyta, more than 500 novel biologically active metabolites were identified (Faulkner, 2001; Blunt et al., 2006). Even though the ecological differences, geographical origin and developmental stage of Pheophyta contribute enormously to the origin of unique phyco sterol combinations, distinctive biosynthetic pathway leads to a wide range side chain functnalization in these molecules (Kapetanović et al., 2005). Some of structurally exceptional cytotoxic sterols identified from seaweeds are ergosterol peroxide, 5,8-endoperoxides, 7-ketocholesterol, hydroxylated sterols, side chain oxy-sterols etc.

Ergosterol peroxides are potent anti tumor active compounds capable of suppressing multiple myeloma U266 cells, walker carcinosarcoma, human mammary adenocarcinoma cell lines, human gastric tumor cell line (SNU-1), human hepatoma cell line (SUN-354), human colorectal tumor cell line (SUN-C4), human prostate cancer cells, human leukaemia (HL60) cells, murine sarcoma-180 MDA-MB435, HCT-8 and SF-295 (Leon et al., 2008; Chen et al., 2009; Liu et al., 2009; Rhee et al., 2012; Wu et al., 2012). These results highlight their use in developing treatment methods to overcome drug-resistance of tumor cells (Wu et al., 2012). Potential of anti angiogenic activity

targeting JAK2/STAT3 signaling pathway mechanism of ergosterol peroxide is effectively used for multiple suppression of myeloma U266 cells (Rhee et al., 2012). Apoptosis induced cell death in human leukaemia (HL60) cells were reported by Liu et al. (2009) with IC_{50} at 25 μM. Their potential to induce suppression of inflammation induced by TPA and progression of tumor in mice along with mitogens induced suppression of proliferation of mouse and human lymphocytes were also observed (Liu et al., 2009). Both androgen-sensitive (LNCaP) and androgen-insensitive (DU-145) human prostate cancer cells were suppressed effectively by micro molar concentrations of ergosterol peroxide (Chen et al., 2009). These classes of sterol molecules can effectively alter the redox states in HT29 cells to suppress cell growth and STAT1 mediated inflammatory responses (Russo et al., 2010).

Sterol 5,8-endoperoxides is another molecule which has been subjected to various cytotoxic studies. Significant cytotoxic activity was displayed by mixture of the four steroids 5α,8α-epidioxy-24(*S*)-ethylcholest-6-en-3β-ol , 5α,8α- epidioxy-24(*R*)- ethylcholest-6-en-3β-ol, 5α,8α-epidioxy-24(*S*)-methylcholest-6-en-3β-ol and 5α,8α-epidioxy-24(*R*)-methylcholest-6-en-3β-ol against the human breast cancer cell line (MCF7WT) and human T-cell leukemia/lymphotropic virus type I (HTLV-I) (Gauvin et al., 2000; Bensemhoun et al., 2009). Sheu et al. (2000) reported the cytotoxic potential of (22*R*,23*R*,24*R*)- 5α,8α-epidioxy-22,23-methylene-24-methlycholest-6-en-3β-ol against P-388,

KB, A549, and HT-29 cells. Apoptosis-induced proliferation suppression was achieved in human leukemia (HL-60 cells) cells using 5α,8α-epidioxy-24(*S*)-methylcholest-6-en-3β-ol and 5α,8α-epidioxy-24(*R*)-methylcholest-6-en-3β-ol isolated from *Meretrix lusoria* (hard clam) (Pana et al., 2007). 5α,8α-endoperoxides sulfate sterol recorded prominent activity against P338 and HL-60 with IC_{50} 5.9 μM and 8.7 μM respectively. Whereas >100 μM concentration of 5α,8α-endoperoxides sulfate sterol was required to induce 50% cell death in A549 and BEL-7402 cell lines (Wang et al., 2008). However, Liu et al. (2011) reported mild cytotoxicity for 5α,8α-epidioxycholest-6-en-3β-ol against SGC-7901, HepG2 and HeLa cells. Proliferation of human breast adenocarcinoma MCF-7 cells were suppressed by inducing cell apoptosis with the aid of (22*E,24R*)-5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol isolated from fermentated mycelia of *Ganoderma lucidum*, and edible mushroom *Sarcodon aspratus* (Bensemhoun et al., 2009). From the gorgonian *Eunicella cavolini* and the ascidian *Trididemnum inarmatum*, potentially active growth inhibitory sterol, (22*E*,24*R*,25*R*)-5α,8α-epidioxy-24,26-cyclo-cholesta-6,22-dien-3β-ol, which suppressed the growth of human breast cancer cells (MCF-7), was isolated and studied by Ioannou et al. (2009).These sterols also exhibited very significant activity against HepG2, A549, HT29 and MDA-MB-231 cancer cell lines (Chang et al., 2013).

7-ketocholesterol, an oxidized derivative of cholesterol is another one of the most extensively studied keto-sterol, mainly due to

their potential to suppress various pathological conditions in humans (Hughes et al., 1994; Nelson & Alkon, 2005). These sterols induce apoptosis in cultured smooth muscle cells (Nishio et al., 1996), through the increased activation of Na^+/K^+ ATPase in fibroblasts (Sevanian et al., 1995). Meanwhile, reduced Ca^{2+} uptake in human erythrocytes (Neyses et al., 1985), effectively stimulate monocyte and lens epithelial cells differentiation (Hayden et al., 2002; Girao et al., 2003). Dushkin et al. (1998) reported their potential to impart oxidative stress through reactive oxygen intermediate generation in murine macrophages. Many vital cellular processes such as biosynthesis of cholesterol and sterol, proliferation, apoptosis and possibly inflammation are believed to be controlled effectively by the regulation and inhibition of HMGCoA-reductase by 7-ketocholesterol and other oxysterols (Kandutsch & Chen, 1973; Tomoyori et al., 2004; Ryan et al., 2005). These keto-sterols have been found to be effective in inducing apoptosis in cultured smooth muscle cells (Nishio & Watanabe, 1996) and regulate the cellular homeostasis (Mathieu et al., 2008). Kinase signaling pathways such as AKT-PKC-NFB, p38 MAPK, and ERK are also effectively controlled by keto-sterols and induce cytokines. Miyamoto et al. (2001) observed an inhibition of human leukemia (HL-60) cell line proliferation using orostanal, a sterol purified from marine sponge *Stellatta hiwasaensis*. Against this particular human cell line this sterol has an IC_{50} of 1.7mM. Sterols isolated from marine sponge *Lanthella* sp., petrosterol-3,6-dione,

5a,6a-epoxy-petrosterol and petrosterol also possessed significant cytotoxicity against two types of leukemia (HL-60 and U937) (Tung et al., 2009). These sterols were also active against A549, HT-29, breast (MCF-7) and human ovarian cancer (SKOV-3).

Cytotoxicity studies performed using lobophytosterol, isolated from soft coral *Lobophytum laevigatum*, against HL-60 provided sufficient data to support their potential to suppress leukemia through the induction of apoptosis. Chromatin condensation apoptotic bodies were observed after 24 hrs, when human HL60 cells treated with 5.6 µM of these sterols were assessed using Hoechst 33342 staining and fluorescent microscopy (Quang et al., 2011). Similar chromatin condensation apoptotic bodies were observed after 24 hrs, when human HCT116 cells treated with 3.2 µM and A549 cells with 4.5 µM lobophytosterol were assessed using Hoechst 33342 staining and fluorescent microscopy, highlighting apoptosis induced suppression of cell proliferation. These observations were further supported by MTT based viability assay (Quang et al., 2011).

Hydroxylated sterols are of special interest in cytotoxic, antiviral and anti-inflammatory studies, as they are capable of regulating immune functions by modulating biosynthetic pathway and cell membrane properties (Cyster et al., 2014). A specific example for this is oxysterols 25-hydroxycholesterol and 7α, 25-dihydroxycholesterol. These sterols accelerate the adaptive immune responses by engaging the G protein-coupled receptor EBI2,

and there by functioning as an immune cell guidance cue (Hannedouche et al., 2011). These oxy-sterols, due to their potential to alter the adaptive immune response, are of special interest in developing safe cytotoxic chemical combination against lymphocytes and murine transplanted tumors (Bischoff et al., 2000). There are also evidence for the down-regulation of Bcl-2 expression and activation of caspases and apoptosis in tumor cells by oxy-sterols (Li et al., 2001). Sterols 5β,6β-epoxyergost-24(28)-ene- 3β,7β-diol along with ergost-24(28)-ene-3α,5β,6β-triol are examples for this. Former has an ED_{50} 0.1mg/mL against HT-29 cell, whereas latter has ED_{50} 0.25mg/mL (Rueda et al., 2001). Likewise, prominent cytotoxic activity were observed against the A549 and HT-29 cell lines, when treated with poly hydroxy sterols, such as cholest-3β,5α,6β,7β-tetrol and cholest-3β,5α,6β-triol isolated from Caribbean gorgonian *Plexaurella grisea*. Effective dose ie., ED_{50} values of these sterols against these cells were 1mg/mL (Rueda et al., 2001).

Side chain oxy-sterols are another class of sterols which are subjected to rigorous investigations by researchers due to their wide range of cytoxic and other biological activities. Endogenous cholesterol synthesis has been significantly reduced in various cell lines through suppressing the cleavage of sterol regulatory element binding proteins, when treated with 25-hydroxy cholesterol (side-chain substituted oxysterol) and thereby influencing cell signaling (Adams et al., 2004). While, cell cultures treated with exogenous 25-hydroxy cholesterol

triggers apoptosis ie., programmed death in cells through growth inhibition and damage to cells, suppression of protein and DNA biosynthesis, altered triacylglycerols and phospholipids biosynthesis, suppression of reductase and LDL receptor mRNAs levels and cholesterol biosynthesis inhibitions (Kisseleva et al.,1999). However, concentration of these sterols in cell cultures is a decisive factor influencing the exogenous biological response of oxysterols. Significant activity displayed by oxysterols even at low concentrations have been attributed to the presence of functional groups present in the molecule such as additional oxygen group on the sterol nucleus or side chain along with C3-hydroxyl group, and an intact sterol ring structure with C17-hydrocarbon side chain (Larsson et al., 2006)

Nizamuddinia zanardinii, remarkable brown algae of Oman Sea, is a good source of hydroperoxy sterols with promising cytotoxic action on various cell lines particularly human colon adenocarcinoma (Moghadam et al., 2013). 24-saringosterol is another example for potent cholesterol lowering bio-molecule isolated from seaweeds. In a gene target study carried out in six different cell lines, selective transactivation of LXRβ-mediated pathway in cholesterol transport was shown by culture media treated with 24(S)-saringosterol. Whereas for 24(R)-saringosterol, selective transactivation of LXRβ-mediated pathway was comparatively low when compared to 24(S)-saringosterol. It has been presumed that the presence of oxygenated functional groups especially in the side chains of sterol molecule

resembling the native LXR ligands are more in seaweeds than in their terrestrial counterpart. This has been supported using sterols isolated from *Sargassum fusiforme* which showed significant cholesterol lowering in various cell lines such as Hep G2, THP-1 monocytes, HEK293T, THP-1-derived macrophages, RAW264.7 and intestinal Caco-2, due to selective LXRβ agonist activity (Chen et al., 2014).

Fucosterol an abundant sterol in brown algae, also possess hypocholesterolemic activity and can be used to increase the concentration level of plasma high-density lipoprotein effectively. This cholesterol lowering potential of fucosterol has been attributed to their potential to stimulate the transcriptional activity of both LXR-α and -β, critical transcription factors in reverse cholesterol transport. In a cell-free time-resolved fluorescence resonance energy transfer analysis, capacity of fucosterol to stimulate co-activator recruitment and there by inducing the transcriptional activation of ABCA1, ABCG1 and ApoE, key genes involved in the reverse cholesterol transport was observed. This significantly increased the efflux of cholesterol in THP-1-derived macrophages. Intestinal NPC1L1 and ABCA1 in Caco-2 cells are also effectively controlled by these sterols. Fucosterols are also capable of delaying a key hepatic lipogenic transcription factor (nuclear translocation of SREBP-1c) by modulating the upregulation of Insig-2a, thereby controlling the cellular triglyceride accumulation, particularly in HepG2 cells (Hoang et al., 2012). Hoang et al. (2012) concluded that fucosterol are dual-

LXR agonist which can effectively regulate the key genes expression involved in homeostasis of cholesterol without inducing hepatic triglyceride accumulation.

Oxygenated sterol isolated from seaweeds were capable of inducing apoptosis and there by effectively controlling the proliferation of cancer. 24(R)-hydroproxy-24-vinylcholesterol isolated from species of *Turbinaria* and *Padina* is an example for this (Ktari & Guyot, 1999; Caamal-Fuentes et al., 2014). Fucosterol also possess anti-oxidant and hepatoprotective properties. In an investigation carried out by Lee et al. (2003), significant anti-oxidant activity was observed for fucosterol isolated from *Pelvetia siliquosa*, marine algae, through decreasing the serum transaminase activities, resulting from CCl4-intoxication induced hepatic damage in rats. These sterols also inhibit the activities of SGOT and SGPT and anti-oxidant enzymes such as hepatic cytosolic superoxide dismutase (SOD), catalase and glutathione peroxidase. Human hepatocellular liver carcinoma cell line (Hep G2), skin carcinoma cell line (WI 38), vero and breast cancer cell lines (MCF-7) cultures treated with fucosterol isolated from *Sargassum* sp. showcased antioxidant and anticancer activities (Ayyad et al., 2011).

Hamdy et al. (2009), isolated and purified 3-keto-22-epi-28-nor-cathasterone, and cholest- 4-ene-3,6-dione from *Cystoseira myrica*, which were very active against Hep G-2(liver) and HCT116 (colon) human cancer cell lines. *Sargassum oligocystum*, yielded cytotoxic sterol compounds which were active against brine shrimp larvae models

(Permeh et al., 2012). Antiproliferative activity against osteosarcoma derived cell line cell MG 63 are shown by a mixture of fucosterol derivatives 24R, 28R- and 24S and 28 R-epoxy-24-ethylcholesterol isolated from *Hizikia fusiformis*. Significant apoptotic activity of 24(R)-hydroproxy-24-vinylcholesterol present in *Sargassum, Nizamuddinia zanardinii* and *Padina* species (Moghadam et al., 2013) were inferred from terminal deoxynucleotidyl transferase dUTP Nick End labeling assay results.

Inhibition of endogenous cholesterol synthesis and oxygenated sterol inclusion to membrane structure of cells are the some of the major mechanisms involved in antitumour effect. Experiments have revealed modulation of HMG-CoA reductase, a key enzyme involved in cholesterol and prenyl alcohols synthesis, by oxygenated sterol could be a major mechanism of deactivation of oncogenes (Schafer et al., 1989; Glomset et al., 1990). Involvement of immune related mechanism of controlling the proliferation of cancer cell by oxygenated sterols has been proposed by Moog et al. (1990), as macrophages and neutrophils exhibit significant *in vivo* affinity towards oxygenated sterols. Also there is ample evidence for this mode of mechanism as mitogens or alloantigens induced inhibitory effects on the early stages of lymphocyte division (Luu & Moog, 1991). Inhibiting proliferation and modulation of lymphocytes (blastogenesis), transformation of mixed lymphocyte response and activity of NK cells are other relevant mechanism proposed for cytotoxic potential of oxygenated sterols (Smith &

Johnson, 1989). Viability of EL4 lymphoma and K36 leukaemia cell lines (murine cells) in presence of protein inhibitors or RNA synthesis are effectively decreased by 7-hydroxycholesterols and 25-hydroxy sterols. Christ et al. (1993) confirmed these observations by carrying out controlled antiproliferative experiments on murine lymphoma cells (RDM4), highlighting the involvement of protein or RNA synthesis mediated mechanisms to induce toxicity to oxygenated sterol treated cells.

Cytosolic binding protein, a specific oxysterol binding protein related to the inhibition mechanism of HMG-CoA reductase were identified, and were found to be inducing cell death in different human leukaemic T-lymphocyte clones by Bakos et al. (1993). Ayala-Torres et al. (1994) identified the role of DNA-binding protein towards the regulation of oxygenated sterol-induced lymphoid cell viability and progression. Involvement of calcium dependent mechanism for 7-keto-cholesterol to induce cytotoxic effects on HL60 cells were suggested by Gregorio-King et al. (2004). On the basis of observations, this highlighted the involvement of an up-regulation of novel gene, RTKN2.

On the other hand, it was shown that cyclooxygenase-2 (Cox-2) and prostaglandin G-H synthase-2 expression in bovine mesenteric lymphatic, venous, and arterial endothelial cells were effectively induced by 25-hydroxycholestrol. Cellular proliferation was increased initially by the activation of Cox-2 expression in endothelial cells by 25-hydroxycholestrol. However, viability of endothelial cells was

significantly influenced when subjected to extended exposure (Nguyen et al., 2010)

Sterols isolated from the brown algae *Sargassum carpophyllum*, 3β,28xi-dihydroxy-24-ethylcholesta-5,23Z-dien, 2a-oxa-2-oxo-5alpha-hydroxy-3,4-dinor-24-ethylcholesta-24(28)-ene, fucosterol, 24-ethylcholesta-4,24(28)-dien-3,6-dione, 24xi-hydroperoxy -24-vinylcholesterol, 24-ketocholesterol, 24R,28R- and 24S, 28S-epoxy-24-ethylcholesterol possessed potent cytotoxicity against various cancer cell lines such as mouse lymphocytic leukemia (P-388), human promyelocytic leukemia (HL-60), human breast cancer (MCF-7), human ilececal cancer (HCT-8) human ovarian cancer (1A9) human bone tumor (HOS) and human prostate cancer (PC-3) (Tang et al., 2002). Mixture of 24-R and 24-S epimers of 24- hydroperoxy -24 Vinyl cholesterol retrived from Brazilian brown sea weeds *Dictyopteris justii* and *Spatoglossum schroederi* revealed apoptotic properties (Teixeira et al., 2006). Sheu et al. (1997) isolated 24-hydroperoxy-24-vinyl-cholesterol and 29-hydroperoxystigmasta-5,24(28)-dien-3beta-ol from the brown algae *Turbinaria ornata*, showed activity against various cell lines. Necrosis and acute inflammatory response in rats were effectively induced by subcutaneous implantation of cholestanetriol, 25-hydroxycholesterol and 26-hydroxycholesterol (Harland et al., 1973; Baranowski et al., 1982). *In vivo* antitumor activity of 7fl-hydroxycholesterol (7fl-HC) and its sodium dihemisuccinate against Krebs II tumours ila mice were even higher than methotrexate,

cyclophosphamide and 5-fluorouracil (antitumour drugs) (Rong et al., 1984). Nordmann et al. (1989) noted 7fl-HC is more toxic on hepatoma cells than on normal hepatocytes. On the other hand 7fl-HC was more cytotoxic on cultured mouse lymphoma cells than on normal lymphocytes (Hietter et al., 1986).

1.1a *Turbinaria* spp.

In tropical marine waters, brown algae, genus *Turbinaria* spp. are abundant and widespread on rocky substrates of costal ecosystem. Sterols and other secondary metabolites isolated from this phylum are very unique and invariable when compared to other phylum of seaweeds. These are sources of high quality carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals with unique bioactivity (Viron et al., 2000, Sanchez-Machado et al., 2004, Fayaz et al., 2005). About 30 number of *Turbinaria* species have been identified across the world (Guiry & Guiry, 2007). Out of which *Turbinaria conoides* and *Turbinaria ornata* are abundant in the coastal environments of Indian subcontinent (Chakraborty et al., 2013). As observed in other brown algae, fucosterol biosynthesized through the alkylation of 24-methylenecholesterol is exclusively present in *Turbinaria* spp. (Patterson, 1971; Kamenarska et al., 2003). Concentration of demosterol, a biosynthetic precursor of fucosterol is very low in these particular species. Traditionally extracts from *Turbinaria conoides* is used as remedy for antibacterial infection,

fever, and cancer and used as antioxidants and insect repellent (Erdmann & Bason, 2004).

Antimicrobial properties were shown by cyclohexane extract of *Turbinaria conoides*. Sterols isolated from these species found to poses anticholinergic and antihistaminic properties and proved to be effective in treating fungal infections. Kumar et al. (2010) has recorded the antimicrobial properties of 3,6,17-trihydroxy-stigmasta-4,7,24(28)-triene and 14,15,18,20-diepoxyturbinarin isolated from cyclohexane extract of *Turbinaria conoides*. Pathogens *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Aspergillus niger* were moderately suppressed by fucosterol (Kumar et al., 2009). Another compound 14,15,18,20-diepoxyturbinarin isolated from the extracts of these species was found to be very active against fungus, *Aspergillus niger* (Kumar et al., 2010). Further, studies using these compounds have also highlighted their role in developing effective antihistaminic treatment methods (Kumar et al., 2011). Potential of extracts from *Turbinaria conoides* to effectively control *Beauveria bassiana* infection in silkworm was identified by Kumari et al. (2011).

Fucoidan, a sulfated polysaccharide abundant in *Turbinaria conoides* is 2.36 times effective in inhibiting the A549 cell proliferation when compared to native fucoidan isolated from *Undaria pinnatifida* (Yang et al., 2008). In food processing, natural antioxidant potential of fucoidan is widely used for preservation by manufactures

(Chattopadhyay et al., 2010). Similarly cardio protective effects of fucoidan from *Turbinaria conoides* has been reported in rat by Krishnamurthy et al. (2012). Fucoidans with low molecular weight has proved to be potent inhibitor of human carcinoma cells, such as HT1080 fibrosarcoma, HeLa cervix adenocarcinoma, K562 leukemia, HL-60 leukemia cells, U937 lymphoma and A549 lung adenocarcinoma (Zhang et al., 2011; Marudhupandi et al., 2015). Invasion and angiogenesis of HT1080 fibrosarcoma cells are also effectively subdued by low molecular weight fucoidan. Results indicated that fucoidans, particularly low molecular weight fucoidan are capable of inducing apoptosis in MCF-7 cancer cells by means of mitochondria mediated pathway (Zhang et al., 2011).

Turbinaria conoides is also a source of cytotoxic hydroperoxysterols (Sheu et al., 1991). Oxygenated sterols such as 24ε-hydroperoxy-24-vinylcholesterol, 29-hydroperoxystigmasta-5,24(28)-dien-3β-ol, 24-ethylcholesta-4,24(28)-dien-3-one, 24ε-hydroperoxy-24-ethylcholesta-4,28(29)-dien-3-one, 24- ethylcholesta-4,24(28)-dien-3,6-dione, 24ε -hydroperoxy-24-ethylcholesta-4,28(29)-dien-3,6-dione, 6β-hydroxy-24-ethylcholesta-4,24(28)-dien-3-one , and 24 ε -hydroperoxy-6β-hydroxy-24-ethylcholesta-4,- 28(29)-dien-3-one isolated from *Turbinaria conoides* demonstrated cytotoxicity against cancer cell lines A-549, HT-29, KB and P-388. (Sheu et al., 1999). Infections caused by fungus *Curvularia lunata, Stachybotrys atra* and *Microsporum canis* can be treated by fucosterols (Choudhary et al., 1997). Fucosterols are

histamine and acetylcholine inhibitors and are also very effective antiviral agents (Yoon et al., 2008).

Numerous antibiotics, laxatives, anticoagulants, anti-ulcer products have been recovered from seaweeds (Rajasulochana et al., 2009). Based on available literature and data it can be presumed that *Turbinaria* spp. are also rich source of therapeutically valuable non toxic, unsaponifiable novel sterols (Orcutt & Richardson, 1970; Rajasulochana et al., 2009), which can effectively substitute the conventional expensive method of treatments. In this regard *Turbinaria conoides*, a promising source for discovering novel drugs are least explored.

1.2 Aim and Scope

Seaweeds are important source of new drug leads, and has played vital role in the progress of novel anticancer agents. Finding new bioactive molecules has been the primary focus of most of the natural product research. Detailed investigations of the pharmacological activities of these compounds are essential for the understanding of their mechanisms of action. Recent developments in molecular biology and analytical techniques have facilitated the development of new *in vitro* methods to examine the effect of compounds on intracellular pathways. In this work focus was to identify cytotoxic activity of fucosterol acetate against A549 and Hep

G2, and also effort was made to isolate new cytotoxic sterol with potent activity against A549 and Hep G2 from *Turbinaria conoides*.

The specific aims were

- Bioactivity based screening of lipids from *Turbinaria conoides* and characterization of active fractions in order to identify compounds of natural origin with cytotoxic activity against A549 and Hep G2.
- Extraction, isolation and structural elucidation of fucosterol acetate and novel sterol with potential cytotoxic activity against these cell lines.
- To elucidate the concentration-dependent cytotoxic effects of fucosterol acetate and novel sterol to determine their mechanisms of action on a molecular level.

By understanding the mechanism of cytotoxic action, in future these sterols could be in-cooperated in chemotherapeutic drugs for developing safer therapeutical methods of treating lung and liver cancer.

1.3 References

Adams, C. M., Reitz, J., De Brabander, J. K., Feramisco, J. D., Li, L., Brown, M. S., & Goldstein, J. L. (2004). Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. *Journal of Biological Chemistry*, *279*(50), 52772-52780.

Amador, M. L., Jimeno, J., Paz-Ares, L., Cortes-Funes, H., & Hidalgo, M. (2003). Progress in the development and acquisition of anticancer agents from marine sources. *Annals of Oncology*, *14*(11), 1607-1615.

Amin, A., Hamza, A. A., Bajbouj, K., Ashraf, S. S., & Daoud, S. (2011). Saffron: a potential candidate for a novel anticancer drug against hepatocellular carcinoma. *Hepatology*, *54*(3), 857-867.

Awad, A. B., & Fink, C. S. (2000). Phytosterols as anticancer dietary components: evidence and mechanism of action. *The Journal of Nutrition*, *130*(9), 2127-2130.

Awad, A. B., Chinnam, M., Fink, C. S., & Bradford, P. G. (2007). β-Sitosterol activates Fas signaling in human breast cancer cells. *Phytomedicine*, *14*(11), 747-754.

Awad, A. B., Williams, H., & Fink, C. S. (2003). Effect of phytosterols on cholesterol metabolism and MAP kinase in MDA-MB-231 human breast cancer cells. *The Journal of Nutritional Biochemistry*, *14*(2), 111-119.

Awad, A. B., Chinnam, M., Fink, C. S., & Bradford, P. G. (2004). Targeting ceramide by dietary means to stimulate apoptosis in tumor cells. *Current Topics in Nutraceutical Research*, *2*(2), 93-100.

Awad, A.B., Fink, C.S., Williams, H., & Kim, U. (2001). In vitro and in vivo (SCID mice) effects of phytosterols on the growth and dissemination of human prostate cancer PC-3 cells. *European Journal of Cancer Prevention*, *10*(6), 507–513.

Awad, A. B., Von Holtz, R. L., Cone, J. P., Fink, C. S., & Chen, Y. C. (1997). beta-Sitosterol inhibits growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle. *Anticancer Research*, *18*(1A), 471-473.

Ayala-Torres, S., Johnson, B. H., & Thompson, E. B. (1994). Oxysterol sensitive and resistant lymphoid cells: correlation with regulation of cellular nucleic acid binding protein mRNA. *The Journal of Steroid Biochemistry and Molecular Biology*, *48*(4), 307-315.

Ayyad, S. E. N., Ezmirly, S. T., Basaif, S. A., Alarif, W. M., Badria, A. F., & Badria, F. A. (2011). Antioxidant, cytotoxic, antitumor, and protective DNA damage metabolites from the red sea brown alga *Sargassum* sp. *Pharmacognosy Research*, *3*(3), 160-165.

Bai, R., Petit, G. R., & Hamel, E. (1990). Dolastatin 10, a powerful cytostatic peptide derived from a marine animal: inhibition of tubulin polymerization mediated through the vinca alkaloid binding domain. *Biochemical Pharmacology*, *39*(12), 1941-1949.

Bajbouj, K., Schulze-Luehrmann, J., Diermeier, S., Amin, A., & Schneider-Stock, R. (2012). The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines. *BMC Complementary and Alternative Medicine*, *12*(1), 69.

Bakos, J. T., Johnson, B. H., & Thompson, E. B. (1993). Oxysterol-induced cell death in human leukemic T-cells correlates with oxysterol binding protein occupancy and is independent of glucocorticoid-induced apoptosis. *The Journal of Steroid Biochemistry and Molecular Biology*, *46*(4), 415-426.

Baranowski, A., Adams, C. W. M., High, O. B., & Bowyer, D. B. (1982). Connective tissue responses to oxysterols. *Atherosclerosis*, *41*(2), 255-266.

Bensemhoun, J., Bombarda, I., Aknin, M., Faure, R., & Gaydou, E. M. (2009). 5a,8a epidioxysterols from the tunicate *Didemnum salary*. *Biochemical Systematics and Ecology*, *36*(12), 942-944.

Berlinck, R. G. S., Britton, R., Piers, E., Lim, L., Roberge, M., da Rocha, R. M., & Andersen, R. J. (1998). Granulatimide, isogranulatimide and didemnimide E, aromatic alkaloids isolated from the Brazilian ascidian *Didemnum granulatum*: structure elucidation, synthesis and G2 checkpoint inhibition activity. *The Journal of Organic Chemistry*, *63*, 9850-9856.

Beveridge, T. H., Li, T. S., & Drover, J. C. (2002). Phytosterol content in American ginseng seed oil. *Journal of Agricultural and Food Chemistry*, *50*(4), 744-750.

Bischoff, P. L., Holl, V., Coelho, D., Dufour, P., Luu, B., & Weltin, D. (2000). Apoptosis at the interface of immunosuppressive and anticancer activities the examples of two classes of chemical inducers, oxysterols and alkylating agents. *Current Medicinal Chemistry*, *7*(7), 693-713.

Blunt, J. W., Copp, B. R., Munro, M. H., Northcote, P. T., & Prinsep, M. R. (2006). Marine natural products. *Natural Product Reports*, *23*(1), 26-78.

Blunt, J. W., Copp, B. R., Munro, M. H., Northcote, P. T., & Prinsep, M. R. (2011). Marine natural products. *Natural Product Reports*, *28*(2), 196-268.

Bu, M., Yang, B. B., & Hu, L. (2014). Natural bioactive sterol 5α, 8α-endoperoxides as drug lead compounds. *Medicinal Chemistry*, *4*(10), 709-716.

Caamal-Fuentes, E., Moo-Puc, R., Freile-Pelegrín, Y., & Robledo, D. (2014). Cytotoxic and antiproliferative constituents from *Dictyota ciliolata, Padina sanctae-crucis and Turbinaria tricostata*. *Pharmaceutical Biology*, *52*(10), 1244-1248.

Cardozo, K. H., Guaratini, T., Barros, M. P., Falcão, V. R., Tonon, A. P., Lopes, N. P., Campos, S., Torres, M. A., Souza, A. O., Colepicolo, P., & Pinto, E. (2007). Metabolites from algae with economical impact. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, *146*(1), 60-78.

Careri, M., Elviri, L., & Mangia, A. (2006). Liquid chromatography-UV determination and liquid chromatography-atmospheric pressure chemical ionization mass spectrometric characterization of sitosterol and stigmasterol in soybean oil. *Journal of Chromatography A*, *935*(1), 249-257.

Casapullo, A., Bifulco, G., Bruno, I., & Riccio, R. (2000). New bisindole alkaloids of the topsentin and hamacanthin classes from the Mediterranean marine sponge *Rhaphisia lacazei*. *Journal of Natural Products*, *63*(4), 447-451.

Chakraborty, K., Praveen, N. K., Vijayan, K. K., & Rao, G. S. (2013). Evaluation of phenolic contents and antioxidant activities of brown seaweeds belonging to *Turbinaria* spp. (Phaeophyta, Sargassaceae) collected from Gulf of Mannar. *Asian Pacific Journal of Tropical Biomedicine*, *3*(1), 8-16.

Chanda, S., Dave, R., Kaneria, M., & Nagani, K. (2010). Seaweeds: A novel, untapped source of drugs from sea to combat infectious diseases. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex Research Center, Badajoz, Spain, 473-480.

Chang, F. R., Yen, C. T., El-Shazly, M., Yu, C. Y., Yen, M. H., Cheng, Y. B., Chang, F.R., Yen, C.T., El-Shazly, M., Yu, C.Y., Yen, M.H., Cheng, Y.B., Chen, S. L., & Wu, Y. C. (2013). Spirostanoids with, 4-dien-3-one or 3β,7α-diol-5,6-ene moieties from *Solanum violaceum*. *Bioorganic and Medicinal Chemistry Letters*, *23*(9), 2738-2742.

Chang, C. H., Wen, Z. H., Wang, S. K., & Duh, C. Y. (2008). New anti-inflammatory steroids from the Formosan soft coral *Clavularia viridis*. *Steroids*, 73 (5), 562–567.

Chattopadhyay, N., Ghosh, T., Sinha, S., Chattopadhyay, K., Karmakar, P., & Ray, B. (2010). Polysaccharides from *Turbinaria conoides*: Structural features and antioxidant capacity. *Food Chemistry*, *118*(3), 823-829.

Chen, Y. K., Kuo, Y. H., Chiang, B. H., Lo, J. M., & Sheen, L. Y. (2009). Cytotoxic activities of 9,11-dehydroergosterol peroxide and ergosterol peroxide from the fermentation mycelia of *Ganoderma lucidum* cultivated in the medium containing leguminous plant on Hep 3B cells. *Journal of Agricultural and Food Chemistry*, *57*(13), 5713-5719.

Chen, C. Y., Shen, Y. C., Chen, Y. J., Sheu, J. H., & Duh, C. Y. (1999). Bioactive sesquiterpenes from a Taiwanese marine sponge *Parahigginsia* sp. *Journal of Natural Products*, *62*(4), 573-576.

Chen, Z., Liu, J., Fu, Z., Ye, C., Zhang, R., Song, Y., Zhang, Y., Li, H., Ying, H., & Liu, H. (2014). 24 (S)- saringosterol from edible marine seaweed *Sargassum fusiforme* is a novel selective LXRβ agonist. *Journal of Agricultural and Food Chemistry*, *62*(26), 6130-6137.

Choudhary, M. I., Majeed, A., Shabbir, M., Ghani, U., & Shameel, M. (1997). A succinylanthranilic acid ester and other bioactive constituents of *Jolyna laminarioides*. *Phytochemistry*, *46*(7), 1215-1218.

Christ, M., Luu, B., Mejia, J. E., Moosbrugger, I., & Bischoff, P. (1993). Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes. *Immunology*, *78*(3), 455-460.

Cyster, J. G., Dang, E. V., Reboldi, A., & Yi, T. (2014). 25-Hydroxycholesterols in innate and adaptive immunity. *Nature Reviews Immunology*, *14*(11), 731-743.

Da Rocha, A. B., Lopes, R. M., & Schwartsmann, G. (2001). Natural products in anticancer therapy. *Current Opinion in Pharmacology*, *1*(4), 364-369.

Demain, A. L., & Vaishnav, P. (2011). Natural products for cancer chemotherapy. *Microbial Biotechnology*, *4*(6), 687-699.

Di Guardia, S., Valls, R., Mesguiche, V., Brunel, J. M., & Culioli, G. (1999). Enantioselective synthesis of (−)-Bifurcadiol: a natural antitumor marine product. *Tetrahedron Letters*, *40*(48), 8359-8360.

Dunford, N. T., & King, J. W. (2000). Phytosterol enrichment of rice bran oil by a supercritical carbon dioxide fractionation technique. *Journal of Food Science*, *65*(8), 1395-1399.

Dushkin, M., Schwartz, Y., Volsky, N., Musatov, M., Vereschagin, E., Ragino, J., Perminova, O., & Kozlov, V. (1998). Effects of oxysterols upon macrophage and lymphocyte functions in vitro. *Prostaglandins and Other Lipid Mediators*, *55*(4), 219-236.

El-Gamal, A. A., Wang, S. K., Dai, C. F., & Duh, C. Y. (2004). New nardosinanes and 19-oxygenated ergosterols from the soft coral *Nephthea armata* collected in Taiwan. *Journal of Natural Products*, *67*(9), 1455-1458.

Erba, E., Bergamaschi, D., Ronzoni, S., Faretta, M., Taverna, S., Bonfanti, M., Catapano, C.V., Faircloth, G., Jimeno, J., & D'incalci, M. (1999). Mode of action of thiocoraline, a natural marine compound with anti-tumour activity. *British Journal of Cancer*, *80*(7), 971-980.

Erdmann, A. M., & Bason, D. (2004). List of algal species in Komodo national park. (In) Erdmann A. M. A Natural history guide to Komodo National Park. Indonesia: *The Nature Conservancy*, 207-208.

FAO, (2003). http://www.fao.org/3/a-y4765e.pdf

Faulkner, D. J. (2001). Marine natural products. *Natural Product Reports*, *18*(1), 1R-49R.

Fayaz, M., Namitha, K. K., Murthy, K. C., Swamy, M. M., Sarada, R., Khanam, S., Subbarao, P.V. & Ravishankar, G. A. (2005). Chemical composition, iron bioavailability, and antioxidant activity of *Kappaphycus alvarezzi* (Doty). *Journal of Agricultural and Food Chemistry*, *53*(3), 792-797.

Galemmo, R. A., Fevig, J. M., Carini, D. J., Cacciola, J., Wells, B. L., Hillyer, G. L., G.L., Buriak, J., Rossi, K.A., Stouten, P.F., Alexander, R.S. & Hilmer, R. (1996). (N-acyl-N-alkyl) glycyl borolysine analogs: A new class of potent thrombin inhibitors. *Bioorganic and Medicinal Chemistry Letters*, *6*(24), 2913-2918.

Gamble, G. L., Gerber, L. H., Spill, G. R., & Paul, K. L. (2011). The future of cancer rehabilitation: emerging subspecialty. *American Journal of Physical Medicine and Rehabilitation*, *90*(5), S76-S87.

Garcia-Rocha, M., Bonay, P., & Avila, J. (1996). The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Letters*, *99*(1), 43-50.

Garcia-Rocha, M., Garcia-Gravalos, M. D., & Avila, J. (1996). Characterisation of antimitotic products from marine organisms that disorganise the microtubule network: ecteinascidin 743, isohomohalichondrin-B and LL-15. *British Journal of Cancer*, *73*(8), 875-883.

Gauvin, A., Smadja, J., Aknin, M., Faure, R., & Gaydou, E. M. (2000). Isolation of bioactive 5a,8a-epidioxy sterols from the marine sponge *Luffariella* cf. variabilis. *Canadian Journal of Chemistry* 78(7), 986-992.

Geldof, A. A., Mastbergen, S. C., Henrar, R. E., & Faircloth, G. T. (1999). Cytotoxicity and neurocytotoxicity of new marine anticancer agents evaluated using in vitro assays. *Cancer Chemotherapy and Pharmacology*, *44*(4), 312-318.

Girao, H., Shang, F., & Pereira, P. (2003). 7-ketocholesterol stimulates differentiation of lens epithelial cells. *Molecular Vision*, *9*, 497–501.

Glomset, J. A., Gelb, M. H., & Farnsworth, C. C. (1990). Prenyl proteins in eukaryotic cells: a new type of membrane anchor. *Trends in Biochemical Sciences*, *15*(4), 139-142.

Gomes, A., Saha, A., Chatterjee, I., & Chakravarty, A. K. (2007). Viper and cobra venom neutralization by β-sitosterol and stigmasterol isolated from the root extract of *Pluchea indica* Less. (Asteraceae). *Phytomedicine*, *14*(9), 637-643.

Grattan, B. J. (2013). Plant sterols as anticancer nutrients: Evidence for their role in breast cancer. *Nutrients*, *5*(2), 359-387.

Gregorio-King, C. C., Gough, T., Van Der Meer, G. J., Hosking, J. B., Waugh, C. M., McLeod, J. L., Mc Collier, F. & Kirkland, M. A. (2004). Mechanisms of resistance to the cytotoxic effects of oxysterols in human leukemic cells. *The Journal of Steroid Biochemistry and Molecular Biology*, *88*(3), 311-320.

Guiry, M. D., & Guiry, G. M. (2007). AlgaeBase version 4.2. world-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org/

Hamdy, A. H. A., Aboutabl, E. A., Sameer, S., Hussein, A. A., Díaz-Marrero, A. R., Darias, J., & Cueto, M. (2009). 3-Keto-22-epi-28-nor-cathasterone, a brassinosteroid-related metabolite from *Cystoseira myrica*. *Steroids*, *74*(12), 927-930.

Hannedouche, S., Zhang, J., Yi, T., Shen, W., Nguyen, D., Pereira, J. P., Guerini, D., Baumgarten, B.U., Roggo, S., Wen, B., & Knochenmuss, R. (2011). Oxysterols direct immune cell migration via EBI2. *Nature*, *475*(7357), 524-527.

Harland, W. A., Gilbert, J. D., & Brooks, C. J. (1973). Lipids of human atheroma: VIII. Oxidised derivatives of cholesteryl linoleate. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 316*(3), 378-385.

Hartwell, J. L. (1984). Plants used against cancer: a survey. Bioactive Plants. *2*, Quarterman Publications Inc.

Hayden, J. M., Brachova, L., Higgins, K., Obermiller, L., Sevanian, A., Khandrika, S., & Reaven, P. D. (2002). Induction of monocyte differentiation and foam cell formation in vitro by 7-ketocholesterol. *Journal of Lipid Research*, *43*(1), 26-35.

He, W. F., Li, G. Y., Hai, L. L., & Yue, W. G. (2014). Thunberol, a new sterol from the Chinese brown alga *Sargassum thunbergii*. *Journal of Asian Natural Products Research*, *16*(6), 685-689.

Heilbron, I., Phipers, R. F., & Wright, H. R. (1934). The chemistry of the algae. Part I. The algal sterol fucosterol. *Journal of the Chemical Society (Resumed)*, 1572-1576.

Hietter, H., Bischoff, P., Beck, J. P., Ourisson, G., & Luu, B. (1986). Comparative effects of 7 beta-hydroxycholesterol towards murine lymphomas, lymphoblasts and lymphocytes: selective cytotoxicity and blastogenesis inhibition. *Cancer Biochemistry Biophysics*, *9*(1), 75-83.

Hoang, M. H., Jia, Y., Jun, H. J., Lee, J. H., Lee, B. Y., & Lee, S. J. (2012). Fucosterol is a selective liver X receptor modulator that regulates the expression of key genes in cholesterol homeostasis in macrophages, hepatocytes, and intestinal cells. *Journal of Agricultural and Food Chemistry*, *60*(46), 11567-11575.

Huang, G. J., Huang, S. S., Lin, S. S., Shao, Y. Y., Chen, C. C., & Hou, W. C. (2010). Analgesic effects and the mechanisms of anti-inflammation of 456 ergostatrien- 3beta-ol from *Antrodia camphorata* submerged whole broth in mice. *Journal of Agricultural and Food Chemistry, 58*, 7445-7452.

Huang, Y. C., Wen, Z. H., Wang, S. K., Hsua, C. H., & Duh, C. Y. (2008). New anti-inflammatory 4-methylated steroids from the formosan soft coral *Nephthea chabroli*. *Steroids, 7*(11), 1181–1186.

Hughes, H., Mathews, B., Lenz, M. L., & Guyton, J. R. (1994). Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-ketocholesterol and 7-ydroxycholesterol. *Arteriosclerosis, Thrombosis, and Vascular Biology, 14*(7), 1177-1185.

Ioannou, E., Abdel-Razik, A. F., Zervou, M., Christofidis, D., Alexi, X., & Roussis, V. (2009). 5a,8a-Epidioxysterols from the gorgonian *Eunicella cavolini* and the ascidian *Trididemnuminarmatum*: Isolation and evaluation of their antiproliferative activity. *Steroids, 74*(1), 73-80.

Jones, P. J., & AbuMweis, S. S. (2009). Phytosterols as functional food ingredients: linkages to cardiovascular disease and cancer. *Current Opinion in Clinical Nutrition and Metabolic Care, 12*(2), 147-151.

Jones, P. J., Mac Dougall, D. E., Ntanios, F., & Vanstone, C. A. (1997). Dietary phytosterols as cholesterol-lowering agents in humans. *Canadian Journal of Physiology and Pharmacology, 75*(3), 217-227.

Jones, P. J., Raeini-Sarjaz, M., Ntanios, F. Y., Vanstone, C. A., Feng, J. Y., & Parsons, W. E. (2000). Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *Journal of Lipid Research, 41*(5), 697-705.

Kamenarska, Z. G., Dimitrova-Konaklieva, S. D., Stefanov, L. K., & Popov, S. S. (2003). A comparative study on the sterol composition of some brown algae from the Black Sea. *Journal of the Serbian Chemical Society*, *68*(4-5), 269-275.

Kandutsch, A. A., & Chen, H. W. (1973). Inhibition of sterol synthesis in cultured mouse cells by 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. *Journal of Biological Chemistry*, *248*(24), 8408 -8417.

Kapetanović, R., Sladić, D. M., Popov, S., Zlatović, M. V., Kljajić, Z., & Gašić, M. J. (2005). Sterol composition of the Adriatic Sea algae *Ulva lactuca, Codium dichotomum, Cystoseira adriatica* and *Fucus virsoides*. *Journal of the Serbian Chemical Society*, *70*(12), 1395-1400.

Kendel, M., Wielgosz-Collin, G., Bertrand, S., Roussakis, C., Bourgougnon, N., & Bedoux, G. (2015). Lipid composition, fatty acids and sterols in the seaweeds *Ulva armoricana,* and *Solieria chordalis* from Brittany (France): An analysis from nutritional, chemotaxonomic, and antiproliferative activity perspectives. *Marine Drugs*, *13*(9), 5606-5628.

Khairy, H. M., & El-Shafay, S. M. (2013). Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt. *Oceanologia*, *55*(2), 435-452.

Kisseleva, A. F., Goryunova, L. E., Medvedeva, N. V., Alquier, C., Morozkin, A. D., & Misharin, A. Y. (1999). Distribution of exogenous 25-hydroxycholesterol in Hep G2 cells between two different pools. *FEBS letters*, *446*(1), 163-168.

Krishnamurthy, M., Selvaraju, M., & Tamilarasan, M. (2012). *Turbinaria conoides* (J. Agardh) sulfated polysaccharide protects rat's heart against myocardial injury. *International Journal of Biological Macromolecules*, *50*(5), 1275-1279.

Ktari, L., & Guyot, M. (1999). A cytotoxic oxysterol from the marine alga *Padina pavonica* (L.) Thivy. *Journal of Applied Phycology*, *11*(6), 511-513.

Kumar, S. S., Kumar, Y., Khan, M. S. Y., & Gupta, V. (2010). New antifungal steroids from *Turbinaria conoides* (J. Agardh) Kutzing. *Natural Product Research*, *24*(15), 1481-1487.

Kumar, S. S., Kumar, Y., Khan, M. S. Y., Anbu, J., & De Clercq, E. (2009). Antihistaminic, anticholinergic and antiviral activities of fucosterol from *Turbinaria conoides* (J. Agardh) Kutzing. *Pharmacologyonline 1*, 1104-1112.

Kumar, S. S., Kumar, Y., Khan, M. S. Y., Anbu, J., & De Clercq, E. (2011). Antihistaminic and antiviral activities of steroids of *Turbinaria conoides*. *Natural Product Research*, *25*(7), 723-729.

Kumari, S. S., Rao, S. S., Misra, S., & Murty, U. S. (2011). Antifungal activity of *Turbinaria conoides* and evaluation for the effective concentration against the infection of *Beauveria bassiana* in silkworm larvae. *Journal of Microbiology*, *6*(2), 115-123.

Lagace, T. A., Byers, D. M., Cook, H. W., & Ridgway, N. D. (1999). Chinese hamster ovary cells over expressing the oxysterol binding protein (OSBP) display enhanced synthesis of sphingomyelin in response to 25-hydroxycholesterol. *Journal of Lipid Research*, *40*(1), 109-116.

Lagarda, M. J., Garcia-Llatas, G., & Farré, R. (2006). Analysis of phytosterols in foods. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(5), 1486-1496.

Lahlou, M. (2013). The success of natural products in drug discovery. *Pharmacology and Pharmacy, 4*(3A), 17-31.

Larsson, D. A., Baird, S., Nyhalah, J. D., Yuan, X. M., & Li, W. (2006). Oxysterol mixtures, in atheroma-relevant proportions, display synergistic and proapoptotic effects. *Free Radical Biology and Medicine*, *41*(6), 902-910.

Lee, S., Lee, Y. S., Jung, S. H., Kang, S. S., & Shin, K. H. (2003). Anti-oxidant activities of fucosterol from the marine algae *Pelvetia siliquosa*. *Archives of Pharmacal Research*, *26*(9), 719-722.

Leon, F., Brouard, I., Torres, F., Quintana, J., Rivera, A., Estevez, F., & Bermejo, J. (2008). A new ceramide from *Suillusluteus* and its cytotoxic activity against human melanoma cells. *Chemistry and Biodiversity*, *5*(1), 120-125.

Li, W., Dalen, H., Eaton, J. W., & Yuan, X. M. (2001). Apoptotic death of inflammatory cells in human atheroma. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *21*(7), 1124-1130.

Liu, X., Wang, C. Y., Shao, C. L., Wei, Y. X., Wang, B. G., Sun, L. L., Zheng, C. J., & Guan, H. S. (2009). Chemical constituents from *Sargassum pallidum* (Turn.) C. Agardh. *Biochemical Systematics and Ecology*, *37*(2), 127-129.

Liu, Y., Yan, H., Wen, K., Zhang, J., Xu, T., Wang, L., Zhou, X., & Yang, X. (2011). Identification of epidioxysterol from South China sea urchin

Tripneustes gratilla Linnaeus and its cytotoxic activity. *Journal of Food Biochemistry*, *35*(3), 932-938.

Liu, C. Y., Hwang, T. L., Lin, M. R., Chen, Y. H., Chang, Y. C., Fang, L. S., Wang, W.H., Wu, Y.C., & Sung, P. J. (2010). Carijoside A, a bioactive sterol glycoside from an octocoral *Carijoa* sp. (Clavulariidae). *Marine Drugs*, *8*(7), 2014-2020.

Liu, E. H., Qi, L. W., Wu, Q., Peng, Y. B., & Li, P. (2009). Anticancer agents derived from natural products. *Mini Reviews in Medicinal Chemistry*, *9*(13), 1547-1555.

Lordan, S., Ross, R. P., & Stanton, C. (2011). Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. *Marine Drugs*, *9*(6), 1056-1100.

Luo, F., Gu, J., Chen, L., & Xu, X. (2014). Systems pharmacology strategies for anticancer drug discovery based on natural products. *Molecular BioSystems*, *10*(7), 1912-1917.

Luu, B., & Moog, C. (1991). Oxysterols: biological activities and physicochemical studies. *Biochimie*, *73*(10), 1317-1320.

Marudhupandi, T., Kumar, T. T. A., Lakshmanasenthil, S., Suja, G., & Vinothkumar, T. (2015). In vitro anticancer activity of fucoidan from *Turbinaria conoides* against A549 cell lines. *International Journal of Biological Macromolecules*, *72*, 919-923.

Mathieu, J., Schloendorn, J., Rittmann, B. E., & Alvarez, P. J. (2008). Microbial degradation of 7-ketocholesterol. *Biodegradation*, *19*(6), 807-813.

Matsumoto, S. S., Haughey, H. M., Schmehl, D. M., Venables, D. A., Ireland, C. M., Holden, J. A., & Barrows, L. R. (1999). Makaluvamines vary in ability to induce dose dependent DNA cleavage via topoisomerase II interaction. *Anti-Cancer Drugs, 10*(1), 39-46.

Messeguer, A., & Cortés, N. (2007). Combinatorial chemistry in cancer research. *Clinical and Translational Oncology*, *9*(2), 83-92.

Miettinen, T. A., Puska, P., Gylling, H., Vanhanen, H., & Vartiainen, E. (1995). Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *New England Journal of Medicine*, *333*(20), 1308-1312.

Miyamoto, T., Kodama, K., Aramaki, Y., Higuchi, R., & Van Soest, R. W. (2001). Orostanal, a novel abeo-sterol inducing apoptosis in leukemia cell from a marine sponge, *Stelletta hiwasaensis*. *Tetrahedron Letters*, *42*(36), 6349-6351.

Moghadam, M. H., Firouzi, J., Saeidnia, S., Hajimehdipoor, H., Jamili, S., Rustaiyan, A., & Gohari, A. R. (2013). A cytotoxic hydroperoxy sterol from the brown alga, *Nizamuddinia zanardinii*. *DARU Journal of Pharmaceutical Sciences, 21*, 24.

Moghadasian, M. H. (2006). Dietary phytosterols reduce cyclosporine-induced hypercholesterolemia in apolipoprotein E-knockout mice. *Transplantation*, *81*(2), 207-213.

Mohan, S., Bustamam, A., Ibrahim, S., Al-Zubairi, A. S., Aspollah, M., Abdullah, R., & Elhassan, M. M. (2011). In vitro ultramorphological assessment of apoptosis on CEMss induced by linoleic acid-rich fraction from *Typhonium flagelliforme* tuber. *Evidence-Based Complementary and Alternative Medicine,* doi:10.1093/ecam/neq010.

Moog, C., Ji, Y. H., Waltzinger, C., Luu, B., & Bischoff, P. (1990). Studies on the immunological properties of oxysterols: in vivo actions of 7, 25-dihydroxycholesterol upon murine peritoneal cells. *Immunology*, *70*(3), 344-350.

Moreau, R. A., Whitaker, B. D., & Hicks, K. B. (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research*, *41*(6), 457-500.

Moreno, J. J. (2003). Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radical Biology and Medicine*, *35*(9), 1073-1081.

Nakajima, H., Kim, Y. B., Terano, H., Yoshida, M., & Horinouchi, S. (1998). FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Experimental Cell Research*, *241*(1), 126-133.

Nelson, T. J., & Alkon, D. L. (2005). Oxidation of cholesterol by amyloid precursor protein and β-amyloid peptide. *Journal of Biological Chemistry*, *280*(8), 7377-7387.

Newman, D. J., Cragg, G. M., & Snader, K. M. (2003). Natural products as sources of new drugs over the period 1981-2002. *Journal of Natural Products*, *66*(7), 1022-1037.

Neyses, L., Locher, R., Stimpel, M., Streuli, R., & Vetter, W. (1985). Stereospecific modulation of the calcium channel in human erythrocytes by cholesterol and its oxidized derivatives. *Biochemical Journal*, *227*(1), 105-112.

Nguyen, V. P., Chen, S. H., Pizzuto, K., Cantarutti, A., Terminesi, A., Mendonca, C., & Dumont, D. J. (2010). 25-Hydroxycholesterol exerts both a cox-2-dependent transient proliferative effect and cox-2-independent cytotoxic effect on bovine endothelial cells in a time-and cell-type-dependent manner. *Journal of Angiogenesis Research*, *2*(1), 1.

Nishio, E., & Watanabe, Y. (1996). Oxysterols induced apoptosis in cultured smooth muscle cells through CPP32 protease activation and bcl-2 protein down regulation. *Biochemical and Biophysical Research Communications*, *226*(3), 928-934.

Nishio, E., Arimura, S., & Watanabe, Y. (1996). Oxidized LDL induces apoptosis in cultured smooth muscle cells: a possible role for 7-ketocholesterol. *Biochemical and Biophysical Research Communications*, *223*(2), 413-418.

Noguchi, N., Saito, Y., & Urano, Y. (2014). Diverse functions of 24 (S)-hydroxycholesterol in the brain. *Biochemical and Biophysical Research Communications*, *446*(3), 692-696.

Nordmann, P., Diez-Ibanez, M., Chessebeuf-Padieu, M., Luu, B., Mack, G., & Mersel, M. (1989). Toxic effects of 70-hydroxycholesterol on rat liver primary cultures, epithelial lines and co-cultures. *Cell Biology and Toxicology*, *5*(3), 261-270.

Numata, A., Kanbara, S., Takahashi, C., Fujiki, R., Yoneda, M., Usami, Y., & Fujita, E. (1992). A cytotoxic principle of the brown alga *Sargassum tortile* and structures of chromenes. *Phytochemistry*, *31*(4), 1209-1213.

Orcutt, D. M., & Richardson, B. (1970). Sterols of *Oocystis polymorpma*, a green alga. *Steroids*, *16*(1), 429-446.

Ostlund, R. E., Racette, S. B., Okeke, A., & Stenson, W. F. (2002). Phytosterols that are naturally present in commercial corn oil significantly reduce cholesterol absorption in humans. *The American Journal of Clinical Nutrition*, *75*(6), 1000-1004.

Pana, M. H., Huang, Y. T., Chang, C. I., Ho, C. T., & Pan, B. S. (2007). Apoptotic-inducing epidioxysterols identified in hard clam (*Meretrix lusoria*). *Food Chemistry*, *102*(3), 788- 795.

Park, Y. K., & Carr, T. P. (2010). Regulation of sterol transporter expression by plant sterols in HepG2 cells. *The FASEB Journal*, *24*(1), 541.15.

Pathak, S., Multani, A. S., Ozen, M. U. S. T. A. F. A., Richardson, M. A., & Newman, R. A. (1998). Dolastatin-10 induces polyploidy, telomeric associations and apoptosis in a murine melanoma cell line. *Oncology Reports*, *5*(2), 373-379.

Patterson, G. W. (1971). The distribution of sterols in algae. *Lipids*, *6*(2), 120-127.

Permeh, P., Saeidnia, S., Mashinchian-Moradi, A., & Gohari, A. R. (2012). Sterols from *Sargassum oligocystum*, a brown algae from the Persian Gulf, and their bioactivity. *Natural Product Research*, *26*(8), 774-777.

Platt, D., Pelled, D., & Shulman, A. (2004). Oils enriched with diacylglycerols and phytosterolesters for use in the reduction of cholesterol and tryclycerides. *PCT Patent WO2004069150*, *23*.

Quang, T. H., Ha, T. T., Van Minh, C., Van Kiem, P., Huong, H. T., Ngan, N. T. T., Nhiem, N. X., Tung, N. H., Thao, N. P., Thuy, D. T. T., & Song, S. B. (2011). Cytotoxic and PPARs transcriptional activities of sterols from the Vietnamese soft coral *Lobophytum laevigatum*. *Bioorganic and Medicinal Chemistry Letters*, *21*(10), 2845-2849.

Rajasulochana, P., Dhamotharan, R., Krishnamoorthy, P., & Murugesan, S. (2009). Antibacterial activity of the extracts of marine red and brown algae. *The Journal of American Science*, *5*(3), 20-25.

Rasmussen, H. E., Blobaum, K. R., Jesch, E. D., Ku, C. S., Park, Y. K., Lu, F., Carr, T.P., & Lee, J. Y. (2009). Hypocholesterolemic effect of *Nostoc commune* var. *sphaeroides Kützing*, an edible blue-green alga. *European Journal of Nutrition*, *48*(7), 387-394.

Rhee, Y.H., Jeong, S.J., Lee, H.J., Lee, H.J., Koh, W., Jung, J.H., Kim, S.H., & Sung-Hoon, K. (2012). Inhibition of STAT3 signaling and induction of SHP1 mediate antiangiogenic and antitumor activities of ergosterol peroxide in U266 multiple myeloma cells. *BMC Cancer*, *12*(1), 28.

Ridgway, N. D. (1995). 25-Hydroxycholesterol stimulates sphingomyelin synthesis in Chinese hamster ovary cells. *Journal of Lipid Research*, *36*(6), 1345-1358.

Rondanelli, M., Monteferrario, F., Faliva, M. A., Perna, S., & Antoniello, N. (2013). Key points for maximum effectiveness and safety for cholesterol-lowering properties of plant sterols and use in the treatment of metabolic syndrome. *Journal of the Science of Food and Agriculture*, *93*(11), 2605-2610.

Rong, S., Bergmann, C., Luu, B., Beck, J. P., & Ourisson, G. (1984). In vivo antitumor activity of hydrosoluble derivatives of 7-hydroxycholesterols. *Comptes Rendus De l'Academie des Sciences. Serie III, Sciences de la Vie*, *300*(3), 89-94.

Rueda, A., Zubía, E., Ortega, M. J., & Salvá, J. (2001). Structure and cytotoxicity of new polyhydroxylated sterols from the Caribbean gorgonian *Plexaurella grisea*. *Steroids*, *66*(12), 897-904.

Russo, A., Cardile, V., Piovano, M., Caggia, S., Espinoza, C. L., & Garbarino, J. A. (2010). Pro- apoptotic activity of ergosterol peroxide and (22E)-ergosta-7, 22-dien-5α-hydroxy-3, 6-dione in human prostate cancer cells. *Chemico-Biological Interactions*, *184*(3), 352-358.

Ryan, E., Chopra, J., McCarthy, F., Maguire, A. R., & O'Brien, N. M. (2005). Qualitative and quantitative comparison of the cytotoxic and apoptotic potential of phytosterol oxidation products with their corresponding cholesterol oxidation products. *British Journal of Nutrition*, *94*(3), 443-451.

Safarzadeh, E., Shotorbani, S. S., & Baradaran, B. (2014). Herbal medicine as inducers of apoptosis in cancer treatment. *Advanced Pharmaceutical Bulletin*, *4*(1), 421-427.

Salemme, F. R., Spurlino, J., & Bone, R. (1997). Serendipity meets precision: the integration of structure-based drug design and combinatorial chemistry for efficient drug discovery. *Structure*, *5*(3), 319-324.

Sánchez-Machado, D. I., López-Hernández, J., Paseiro-Losada, P., & López-Cervantes, J. (2004). An HPLC method for the quantification of sterols in edible seaweeds. *Biomedical Chromatography*, *18*(3), 183-190.

Santafé, G., Paz, V., Rodríguez, J., & Jiménez, C. (2002). Novel cytotoxic oxygenated C29 sterols from the Colombian marine sponge *Polymastia tenax*. *Journal of Natural Products*, *65*(8), 1161-1164.

Sarkar, F. H., & Li, Y. (2009). Harnessing the fruits of nature for the development of multi-targeted cancer therapeutics. *Cancer Treatment Reviews*, *35*(7), 597-607.

Schafer, W. R., Kim, R., Sterne, R., Thorner, J., Kim, S. H., & Rine, J. (1989). Genetic and pharmacological suppression of oncogenic mutations in ras genes of yeast and humans. *Science*, *245*(4916), 379-385.

Schwartsmann, G., Brondani, A., Berlinck, R. G. S., & Jimeno, J. (2000). Marine organisms and other novel natural sources of new cancer drugs. *Annals of Oncology*, *11*(3), 235-243.

Schwartsmann, G., da Rocha, A. B., Berlinck, R. G., & Jimeno, J. (2001). Marine organisms as a source of new anticancer agents. *The Lancet Oncology*, *2*(4), 221-225.

Sevanian, A., Hodis, H. N., Hwang, J., McLeod, L. L., & Peterson, H. (1995). Characterization of endothelial cell injury by cholesterol oxidation products found in oxidized LDL. *Journal of Lipid Research*, *36*(9), 1971-1986.

Sheu, J. H., & Sung, P. J. (1991). Isolation of 24-hydroperoxy-24-vinylcholesterol and fucosterol from the brown alga *Turbinaria conoides*. *Journal of the Chinese Chemical Society*, *38*(5), 501-503.

Sheu, J. H., Chang, K. C., & Duh, C. Y. (2000). A cytotoxic 5α, 8α-epidioxysterol from a soft coral *Sinularia* species. *Journal of Natural Products*, *63*(1), 149-151.

Sheu, J. H., Wang, G. H., Sung, P. J., & Duh, C. Y. (1999). New cytotoxic oxygenated fucosterols from the brown alga *Turbinaria conoides*. *Journal of Natural Products, 62*(2), 224-227.

Sheu, J. H., Wang, G. H., Sung, P. J., Chiu, Y. H., & Duh, C. Y. (1997). Cytotoxic sterols from the formosan brown alga *Turbinaria ornata*. *Planta Medica, 63*(6), 571-572.

Smith, L. L., & Johnson, B. H. (1989). Biological activities of oxysterols. *Free Radical Biology and Medicine, 7*(3), 285-332.

Suda, K., & Mitsudomi, T. (2014). Successes and limitations of targeted cancer therapy in lung cancer. *Progress in Tumor Research, 41*, 62-77.

Tang, H. F., Yi, Y. H., Yao, X. S., Xu, Q. Z., Zhang, S. Y., & Lin, H. W. (2002). Bioactive steroids from the brown alga *Sargassum carpophyllum*. *Journal of Asian Natural Products Research, 4*(2), 95-101.

Teixeira, V. L., Barbosa, J. P., Rocha, F. D., Kaplan, M. A. C., Houghton, P. J., & Pereira, R. C. (2006). Hydroperoxysterols from the Brazilian brown seaweeds *Dictyopteris justii* and *Spatoglossum schroederi* (Dictyotales): a defensive strategy against herbivory. *Natural Product Communications, 1*(4), 293-297.

Terry, J. G., McGill, B. L., & Crouse, J. R. (1995). Evaluation of the use of beta-sitostanol as a nonabsorbable marker for quantifying cholesterol absorption. *Journal of Lipid Research, 36*(10), 2267-2271.

Tomoyori, H., Kawata, Y., Higuchi, T., Ichi, I., Sato, H., Sato, M., Ikeda, I., & Imaizumi, K. (2004). Phytosterol oxidation products are absorbed in the intestinal lymphatics in rats but do not accelerate atherosclerosis in apolipoprotein E–deficient mice. *The Journal of Nutrition, 134*(7), 1690-1696.

Tung, N. H., Van Minh, C., Ha, T. T., Van Kiem, P., Huong, H. T., Dat, N. T., Nhiem, N. X., Tai, B. H., Hyun, J. H., Kang, H. K., & Kim, Y. H. (2009). C29 sterols with a cyclopropane ring at C-25 and 26 from the Vietnamese marine sponge *Ianthella* sp. and their anticancer properties. *Bioorganic and Medicinal Chemistry Letters*, *19*(16), 4584-4588.

Tung-Chou, T., Yu-Tang, T., Jiunn-Wang, L., Hsin-Chung, T., Kowit-Yu, C., Hsiao-Ling, C., & Chuan-Mu, C. (2015). Anti-inflammatory effects of *Antrodia camphorata* and its active compound, ergostatrien-3β-ol, in a mouse skin ischemia model. *The FASEB Journal*, *29*(1), LB444.

Ueda, H., Nakajima, H., Hori, Y., Goto, T., & Okuhara, M. (1994). Action of FR901228, a novel antitumor bicyclic depsipeptide produced by chromo-bacterium violaceum No. 968, on Ha-ras transformed NIH3T3 Cells. *Bioscience, Biotechnology, and Biochemistry*, *58*(9), 1579-1583.

Urdiales, J., Morata, P., De Castro, I. N., & Sánchez-Jiménez, F. (1996). Antiproliferative effect of dehydrodidemnin B (DDB), a depsipeptide isolated from Mediterranean tunicates. *Cancer Letters*, *102*(1), 31-37.

Van Minh, C., Van Kiem, P., Nhiem, N. X., Cuong, N. X., Thao, N. P., Nam, N. H., Anh, H. L. T., Thung, D. C., Thuy, D. T. T., Kang, H. K., & Jang, H. D. (2011). Cytotoxic and antioxidant activities of diterpenes and sterols from the Vietnamese soft coral *Lobophytum compactum*. *Bioorganic and Medicinal Chemistry Letters*, *21*(7), 2155-2159.

Viron, C., Saunois, A., Andre, P., Perly, B., & Lafosse, M. (2000). Isolation and identification of unsaturated fatty acid methyl esters from marine micro-algae. *Analytica Chimica Acta*, *409*(1), 257-266.

von Holtz, R. L., Fink, C. S., & Awad, A. B.(1998). β-Sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. *Nutrition and Cancer*, *32*(1), 8–12.

Wang, F., Fang, Y., Zhang, M., Lin, A., Zhu, T., Gu, Q., & Zhu, W. (2008). Six new ergosterols from the marine-derived fungus *Rhizopus* sp. *Steroids*, *73*(1), 19-26.

Wang, W., Jang, H., Hong, J., Lee, C. O., Im, K. S., Bae, S. J., & Jung, J. H. (2004). Additional cytotoxic sterols and saponins from the starfish *Certonardoa s emiregularis*. *Journal of Natural Products*, *67*(10), 1654-1660.

Woyengo, T. A., Ramprasath, V. R., & Jones, P. J. H. (2009). Anticancer effects of phytosterols. *European Journal of Clinical Nutrition*, *63*(7), 813-820.

Wu, Q. P., Xie, Y. Z., Deng, Z., Li, X. M., Yang, W., Jiao, C.W., Fang, L., Li, S. Z., Pan, H. H., Yee, A. J., & Lee, D. Y. (2012). Ergosterol peroxide isolated from *Ganoderma lucidum* abolishes microRNA miR-378-mediated tumor cells on chemoresistance. *PLoS One*, *7*(8), e44579.

Yang, C., Chung, D., Shin, I. S., Lee, H., Kim, J., Lee, Y., & You, S. (2008). Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*. *International Journal of Biological Macromolecules*, *43*(5), 433-437.

Yoon, N. Y., Chung, H. Y., Kim, H. R., & Choi, J. S. (2008). Acetyl-and butyrylcholinesterase inhibitory activities of sterols and phlorotannins from *Ecklonia stolonifera*. *Fisheries Science*, *74*(1), 200-207.

Zewail-Foote, M., & Hurley, L. H. (1999). Ecteinascidin 743: a minor groove alkylator that bends DNA toward the major groove. *Journal of Medicinal Chemistry*, *42*(14), 2493-2497.

Zhang, Z., Teruya, K., Eto, H., & Shirahata, S. (2011). Fucoidan extract induces apoptosis in MCF-7 cells via a mechanism involving the ROS-dependent JNK activation and mitochondria-mediated pathways. *PLoS One*, *6*(11), e27441.

Chapter 2

BIOACTIVITY BASED SCREENING OF LIPID FRACTIONS EXTRACTED FROM *Turbinaria conoides*

2.1 Introduction

Bioactivity screening assays are usually fast and simple methods, opted by researchers all over the world to identify organic fractions, in which, the chances of finding natural products beneficial to mankind are high (Amador et al., 2003; McKay & Blumberg, 2006; Weller, 2012). Cell based assays have gained universal acceptance when compared to animal model as they are very economical and effective in identifying the compounds responsible for therapeutic/toxic effect from a pool of secondary metabolites ranging between hundreds and thousands (Li et al., 2000; Montalvão et al., 2014). As a result of extensive research, numerous bio-screening assays have been developed; which are even capable of disclosing the mechanism of action of compound/materials at the molecular level and deduce their side effects (Huang et al., 2004). However, the decisive factors for

selecting the type of assays to be performed are the objective of the investigation coupled with the extent of information we are looking for, screening strategies are continuing to evolve, probing new ideas and knowledge about cancer, introducing high throughput screening and new analytic methodologies (Riss et al., 2003). In part, the need for this continuing evolution has been stimulated by a desire to develop novel and less toxic therapies for cancer treatment (Weller, 2012). High throughput screening methods have enabled more sophisticated mechanism based screening, and subsequently required the move to pre-fractionation and peak library generation (Reetz et al., 1999). These ‘‘prior-to-screening’’ purifications have the consequence of reducing the complexity of screening materials, increasing the titer of low abundance components, segregating nuisance substances into discrete fractions, and generally speeding up the time line from detection of a primary screening hit to identification of a molecular structure for the active substance (Chen et al., 2005). It can generally be concluded that contemporary screening protocols in natural products chemistry are using chromatographic purification steps, sometimes producing pure compounds, before biological or enzymatic bioassay (Simmons et al., 2005).

Chromatographic methods are cheap and successful in analyzing a large set of samples rapidly. Moreover it provides evidence, necessary for the further investigations as they can be easily coupled with multiple spectroscopic tools such as mass spectroscopy,

nuclear magnetic resonance, infrared spectroscopy etc. to deduce the structural information of the isolated compound (Chen et al., 2005). Even though chromatographic methods offer the flexibility of simultaneous evaluation of bio-assay and structural features of the compound. However, authentic evaluations for the mechanism of action are difficult and are only useful in primary evaluations. Hence, these screening methods are usually coupled with other classical pharmacological models to evaluate the efficiency, side effect and toxicity of the medicine (Huang et al., 2004).

Limitations of chromatography based bioactivity screening can be easily overcome by performing in vitro assays using cellular and enzyme receptor models, capable of providing highly specific and sensitive data rapidly. After first reports of bioactive compound screening on the basis of receptor enzyme model, this field has shown an exponential growth (Moore & Rees, 2001; Wise et al., 2002; Peelman et al., 2006; Gaulton et al., 2012; Firempong et al., 2015). Molecular assays are target specific and are usually preferred, when details regarding mechanism of the action of test compound at the sub-cellular level, necessary for the development of drug are required. This specificity, a unique feature associated with molecular model based assay is also a major drawback of this method. Inhibition assays monitor only those classes of compounds with specific activity type, details of other bioactive compounds which lack these mechanism will be lost

(Moore & Rees, 2001). In this context, assays on cell models are useful for screening diverse bioactive compounds (Van Staveren et al., 2009).

Lu et al. (2002) identified the potential of polysaccharide isolated from *Acanthopanax giraldii Harms Var. Hispidus hoo* to selectively inhibit human gastric cancer cell by performing inhibition studies on various cell lines such as human embryonic tendocytes, human lymphocyte, and human gastric cancer cell. Using *in vitro* MTT assay on KBv200 cell line, traditionally used medicines in China were screened for multidrug resistance by Zhang et al. (2001). In an another study, potential of 29 out of 50 herbs collected from Guangxi province to suppress the proliferation of liver cancer was identified by Wei et al. (2003) with aid of MTT. Similarly, study by Peng et al. (2008) cell models were used to study the *in vitro* anti-tumor potential of steroidal glycoside extract from the root of *Cynanchum auriculatum.* All these results are direct outcome of novel innovations in the field of target selection using cellular format and advancement in the technology to monitor cytotoxicity, morphology, and cell cycle.

Even though chromatographic methods based bio-screening assays are widely used in the in situ monitoring of drug-protein binding, and to study the various protein-ligand interactions, such as, anti-cooperative, non-cooperative and cooperative interactions. In this chapter, chromatographic techniques were used for the fragmentation of crude extract from *Turbinaria conoides.* These neutral and acidic lipid

mixtures are then coupled with GC-MS and cell model to evaluate the structural features of the compounds present in each fraction which showcased considerable cytotoxicity. Sulforhodamine B (SRB) viability assay was carried out to evaluate the cytotoxic potential of each fraction. Test by means of SRB, which measures the reduction in total cellular protein content, useful for the effective quantification of clonogenicity, cell proliferation and cytotoxicity for large scale drug screening applications (Vichai & Kirtikara, 2006). Differential absorption of fluorescent dye binding proteins (eg. the SRB staining) is preferred due to its speed, easiness, stable end point, and accuracy. Cell binding, which can be effectively performed in the 96-well plates, is suitable for processing and storage to study the influence of drug dosage. Another advantage of SRB is its improved linearity and sensitivity in high density cells. Further, staining can easily differentiate between recently lyzed cells and cell debris, making it a highly sensitive cell viability assay (Vichai & Kirtikara, 2006; Houghton et al., 2007). Finally, the outcome of *in vitro* study and GC-MS evaluations used to identify the uniqueness of each fraction against lung and liver cancer, are processed and documented in this section.

2.2 Materials and Methods

2.2.1 Sampling and Processing

Turbinaria conoides (Fig. 2.1), a brown sea weed belonging to Sargassacea family, was collected from Mundapam coast, Gulf of

Mannar, south east coast of India during pre monsoon season. Coordinates of the sampling site, latitude 9° 45'N and longitude 79°E were recorded using global positioning system (Garmin GPS etrex 10). Samples were collected during low tide from rocky structures on the coastal area. Approximately 50 Kg. of wet *Turbinaria conoides* was collected, which were washed with sea water followed by distilled water and stored in deep freezer at -20°C until the extraction process.

Figure 2.1 Image of *Turbinaria conoides* collected from Mundapam coast, Gulf of Mannar.

2.2.2 Solvent Extraction of the Sea weed

Prior to analysis wet samples was air dried in shade, followed by grinded to fine powder for further analysis. All the chemicals used for the extraction of metabolites from *Turbinaria conoides* were

analytical grade, supplied by Merck. A solvent system prepared using methanol and chloroform in the ratio 4:1 were used for the total extraction. Algal powder, about 10 Kg dry weight, was treated with methanol:chloroform solvent system (Bligh & Dyer, 1959) followed by sonication using ultra sonicator (Sonics Vibra Cell) for effective extraction (Annegowda et al., 2012). An ice bath was used to dissipate the excess heat generated during sonication to reduce the vaporization of solvents and thermal degradation of compounds. Solvents saturated with organic compounds were collected and replaced by fresh solvent and sonicated, this process was repeated until the complete extraction was achieved. The extracts were concentrated using rotary evaporator (Heidolph 2 Germany) maintained at 40°C and 180 mbar vacuum conditions.

2.2.3 Saponification

In order to fractionate total extract into acidic and neutral fraction, they were saponified using 6% methanolic KOH in a water bath at 70°C for 3 hr. After refluxing, samples were left overnight at room temperature to complete the saponification (Heemken et al., 1997; Wang et al., 2000; Larsen & Christensen, 2005). An inert atmosphere was maintained while saponification through N_2 purging. Saponified samples were then treated with hexane to separate neutral fractions which consisted of alkanes, alkenes, sterols etc. Extraction of neutral lipids from aqueous layer using hexane was repeated several

times for maximum recovery. After the removal of lipid fraction, remaining aqueous fraction was treated with 6 N HCl solutions to recover acidic lipid. These solutions were solvent extracted using analytical grade hexane. Neutral and acidic fractions in hexane were passed through anhydrous sodium sulphate to remove moisture. Extracts were concentrated using rotary evaporator and preserved in a deep freezer at -20°C. Necessary arrangements were made using nitrogen to maintain inert atmosphere and molecular sieve to prevent moisture formation inside sample vials.

2.2.4 Separation of Acid and Neutral Fractions using Column Chromatography

Normal phase column chromatography was performed for the fractionation of neutral lipids, whereas to achieve effective separation of acid fraction, a silver nitrate impregnated column was used.

2.2.4 (a) *Preparation of silver nitrate impregnated column and separation of acid fraction*

Prior to impregnation of silica (60-120 mesh) with silver nitrate, it was activated by heating at 120°C for 3 hour. 100 gm of activated silica was made into paste using 200 mL of 10 % silver nitrate solution. These pastes were then oven dried at 45°C, followed by heating at 120°C for 30 minutes for activation. A white coloured coarse adsorbent thus obtained, were packed into 70×1.5 cm column. Throughout the fractionation, chances of column degradation from

photochemical decay were minimized by covering the surface of the column using dark paper (De Vries, 1963; Anderson & Hollenbach, 1965; Li et al., 1995).

Acid fractions were methylated using boron trifluoride methanol (1mL reagent /4-16 mg of lipid) prior to loading into the column. Esterification was carried out in a closed system using teflon tube with screw cap. Boiling water bath was set to attain thermal conditions necessary for the esterification. In order to recover the methyl ester, extraction using reagent grade hexane was repeated several times to attain maximum recovery. Excess solvents were removed and adsorbed onto silica gel (60-120 mesh) in rotary evaporator.

Methylated acid fractions adsorbed on silica gel were loaded to normal phase silica column and two fractions were separated using ethyl acetate (3%) - hexane (97%) solvent system, fraction I and fraction II. Excess solvents were removed from fraction I and adsorbed to silica gel, in rotary evaporator which was then applied to silver impregnated column. Polarity of the solvent system used to elute the acid fractions was prepared using ethyl acetate (5%) and hexane (95%). The three fractions (50 mL) eluted from the column using this solvent system were passed through NaCl to remove $AgNO_3$. Four acid fraction AF1 AF2, AF3 and AF4, were collected from fraction II and purified using silver impregnated column.

2.2.4 (b) Separation of neutral lipids using a normal phase silica column

Using a normal phase silica column, neutral lipids were separated into three factions. HPLC grade hexane was used in the collection of non polar fractions. Semipolar fractions were collected using 10% ethyl acetate–hexane solvent system. And 20% ethyl acetate-hexane solvent system for the elution of cyclic polar compounds. These three fractions were dried and preserved in inert atmosphere at $5^{\circ}C$.

2.2.5 Viability Assay using Sulforhodamine B (SRB) Staining

SRB, a cytotoxic viability assay is based on the ability of the protein dye sulforhodamine B to bind electrostatically and pH dependent manner on basic amino acid residues of trichloroacetic acid–fixed cells. Under mild acidic conditions it binds and under mild basic conditions it can be extracted from cells and solubilized for measurement. SRB is an anionic bright pink aminoxanthene protein dye ($C_{27}H_{30}N_2O_7S_2$) with two sulfonic groups (Vichai & Kirtikara, 2006). SRB assay was performed in triplicates with appropriated amount of sub fractions collected from acidic and neutral lipids on two cancer cell lines (A549 and Hep G2). Four fatty acid fractions which were collected in 10% ethyl acetate-hexane and all the neutral fractions (100% hexane, 5% ethyl acetate-hexane, 10% ethyl acetate-hexane, 15% ethyl acetate-hexane and 20% ethyl

acetate-hexane solvent system). SRB dissolved in 1% acetic acid store at 4°C protected from light (Rubinstein et al., 1990; Skehan et al., 1990; Griffon et al., 1995). Reagents used are shown in Table 2.1

Table 2.1 List of reagents used for SRB viability assay.

Reagents	**Concentration (w/v).**
TCA	10%
Acetic acid	1%
SRB	0.57%
Tris base (pH 10.5)	10 M

In the well plate without removing the cell culture supernatant, 100 µL of cold 10% TCA was added and incubated at 4°C for 1 hour. After which the plates were washed four times, with slow running tap water and excess water was removed using paper towels. The plate was dried using a blow dryer to dry them completely. Once the plates were dry, 100 µL of 0.057% SRB solution was added to each well. The stain was allowed to incubate 30 minutes followed by brief rinsing of the plates using 1% acetic acid four times to remove excess unbound dye. 200 µL of 10 mM Tris base solution (pH 10.5) was added to each well. The plates were placed in a shaker for 5 min to solubilize the protein-bound dye. The optical density (OD) was read in a microplate reader at 510 nm.

% viability = (OD of Test/ OD of Control) X 100

2.2.6 GC-MS Characterization

Characterization of bioactive acid and neutral fractions, which exhibited significant cytotoxicity against cancer cell lines A549 and Hep G2, were carried out using GC-MS (Perkin Elmer, Clarus 680-Clarus 600T). Analytical conditions used for the characterization of acid fractions are depicted in Table 2.2. GC-MS analytical conditions used for the attaining peak separation and identification of neutral lipids are detailed in Table 2.3.

Table 2.2 Analytical conditions used for the GC-MS characterization of acid fractions

Column	non polar HP ultra double fused silica capillary column
Injection quantity	1.0µL
Injection mode	Split (split ratio 1:20)
Vaporization chamber temperature :	280℃
Column oven temperature	60℃ (1 min) -> 200 (2℃/min) hold for four minute -> 280℃ (5℃/min) hold for 10 minutes
Detector	MS
[MS] Interface temperature	200℃
Ion source temperature	200℃
Inlet A	PSS1
Inlet B	CAP
Measurement mode	Scan
Sampling rate	12.50000 pts/s
Run time	101 minutes

Table 2.3 Analytical conditions of GC-MS for characterization of neutral fraction

Column	non polar HP ultra double fused silica capillary column
Injection quantity	1.0µL
Injection mode	Split (split ratio 1:20)
Vaporization chamber temperature :	280℃
Column oven temperature	150℃ (0 min) -> 200 (15℃/min) hold for one minute -> 250℃ (5℃/min) hold for 1 minute -> 280℃ (1℃/min) 45 minute hold.
Detector	MS
[MS] Interface temperature	200℃
Ion source temperature	200℃
Inlet A	PSS1
Inlet B	CAP
Data sampling time	16 to 80 min
Measurement mode	Scan
Mass range	m/z 45-330
Sampling rate	12.50000 pts/s
Run time	90 minutes

2.3 Results and Discussion

One acid fraction AF1 from normal phase and three acid fractions AF2, AF3, and AF4 were collected from silver-nitrate impregnate column. Normal phase chromatography was carried out to separate hydroxy-fatty acids from rest of the acid mixture. Otherwise hydroxy group will react with the silver ions (Metcalfe et al., 1966). Separation of three acid fractions in silver-nitrate impregnate column

is a direct consequence of hydrogen bond formation between Ag^+ and double bound. This principle has been used worldwide for the purification of fatty acids and the separation of geometrical isomers from crude (Kepler & Tove, 1967). Hydrogen bond formation results in the slight variations in the adsorption of fatty acids. During elution saturated fatty acids were easily eluted from the column, as they lack hydrogen bonding. Whereas for unsaturated fatty acids, order of elution was influenced by number of double bonds. (Nikolova-Damyanova et al., 1996; Nikolova-Damyanova & Momchilova, 2002; Dołowy & Pyka, 2015).

With the aid of normal phase chromatography three fractions NL1, NL2, and NL3 were collected from the neutral lipid extract. NL1 were collected using non polar solvent, hexane. Elution using hexane results in the mobilization of alkanes and alkenes. Semi polar chemical compounds (NL2) are mobilized using 10% ethyl acetate-hexane solvent system. Whereas 20% ethyl acetate-hexane are effective in the elution of polar compounds (NL3).

Results of SRB assay performed on A549 cell lines are shown in Fig. 2.2 and Hep G2 in Fig. 2.3. Three acid fractions (AF1, AF3, and AF4) displayed anticancer activity against both A549 and Hep G2 cell lines. AF2 recorded only moderate activity against above cell line. Whereas activity of NL1 and NL2 against these cancer cell lines were insignificant. Polar fraction (NL3) showed a prominent activity in Hep G2 and A549. While comparing the activity range of acidic fractions, AF1 fraction and AF3 fraction were having comparable activity in the group followed by AF4.

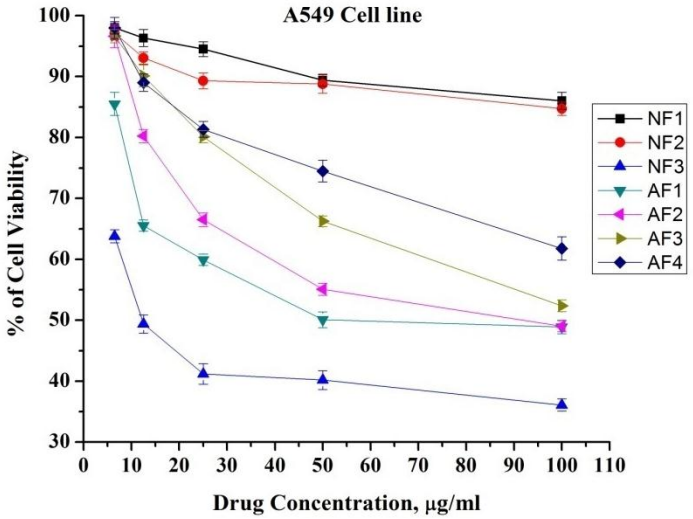


Figure 2.2 Graphical representation of percentage of cell viability in A549 cell lines exposed to different lipid extracts from *Turbinaria conoides*

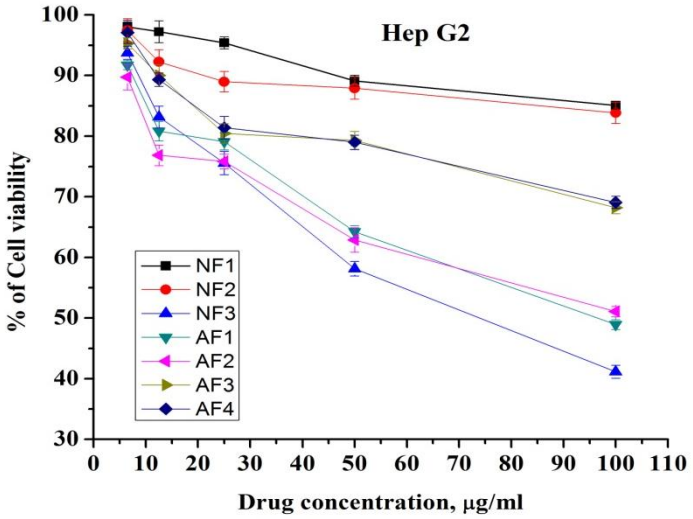


Figure 2.3 Graphical representation of percentage of cell viability in Hep G2 cell lines exposed to different lipid extracts from *Turbinaria conoides*

All the active fractions (AF1, AF2, AF3, AF4, and NL3) were characterized using GC-MS. Characteristic molecular ion fragmentation obtained with an ionization Voltage of 70 eV were compared with NIST library to deduce the chemical features of the individual compounds present in each mixture.

Total ion chromatogram of AF1 fraction is furnished in Fig. 2.4. On the basis of mass fragmentation pattern of this fraction, hydroxy fatty acids were predominant and eleven hydroxy fatty acids were identified (Table 2.4). Base peak m/z at 103 for almost all the compounds except one highlighted the presence of hydroxyl group in the alpha position of the fatty acids. This m/z at 103 (Fig. 2.5) has been attributed to $CH_3O(CO)CH_2CHOH$ (Ryhage & Stenhagen, 1960). Certain mass fragmentation pattern apart from m/z at 103 highlighted the presence of 3-OH fatty acids. For example, presence of m/z 166 and m/z 222 which are signature fragments of 3-OH–$C_{14:0}$ and 3-OH-$C_{18:0}$ respectively were also observed in this fraction. These fragments are the result of beta carbon cleavage and simultaneous removal of H_2O from the alkyl position (Ozen et al., 2004). The presence of hydroxy fatty acids in this seaweed highlight its importance, as only few Phaeophyceae are reported to produce this class of molecule (Proteau & Gerwick, 1993; Rorrer et al., 1997).

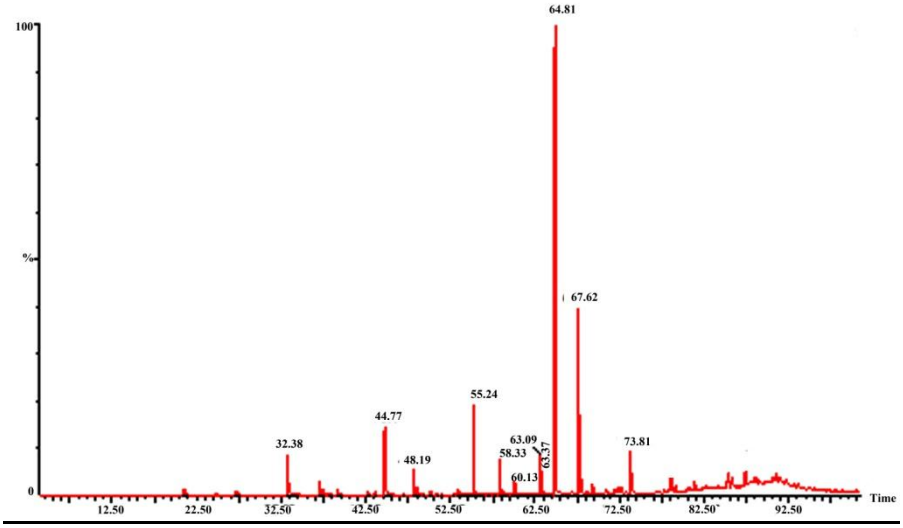


Figure 2.4 Total ion chromatogram of AF1 acid fraction conatining hydroxy fatty acids.

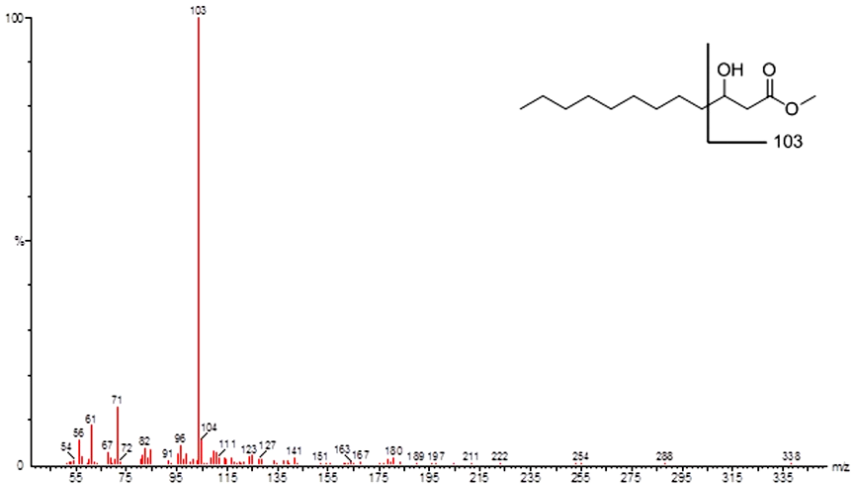
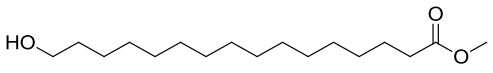
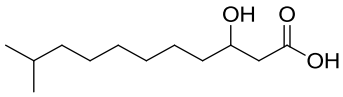
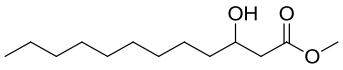
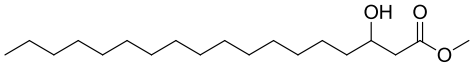
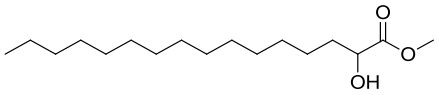
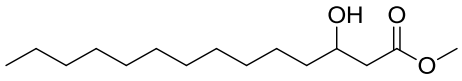
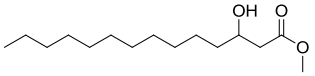


Figure 2.5 A representative mass fragmentation pattern of hydroxy fatty acid, methyl 3-hydroxydodecanoate, having base peak at *m/z* = 103. Produced by a characteristic cleavage *alpha* to the carbon with the hydroxy group and defines its position.

Table 2.4 List of hydroxy fatty acids present in fraction AF1.

SL. No	Fatty acids	Relative %	RT	Major m/z
1	Unknown	4.89	33.28	103, 74, 71, 69, 61
2	 methyl 16 hydroxyhexadecanoate	7.31	44.77	103, 71, 74, 75, 69, 61, 55, 96
3	Unknown	2.63	48.19	103,71, 74, 96, 194
4	 3-hydroxy-10-methylundecanoic acid	8.94	55.24	103, 71, 74, 97, 104, 215, 55, 61
5	 Methyl 3-hydroxy dodecanoate	3.36	58.33	103, 71, 104, 96, 61
6	 Octadecanoic acid ,3 hydroxy, Methyl ester	1.35	60.13	103, 74, 81, 96, 119
7	Unknown	3.61	63.09	55, 111, 227, 286
8	 Methyl-2-hydroxy hexadecanoate	1.08	63.372	103, 71, 74, 69, 96
9	 Methyl 3-hydroxytetradecanoate	44.68	64.81	103, 71, 74, 268, 256
10	 Methyl 3-hydroxyhexadecanoate	17.32	67.62	103, 74, 227, 250, 282, 286
11	Unknown	4.81	73.81	103, 74, 264, 292

Presence of eight dicarboxilic acids in AF2 was inferred from the occurrence of distinctive mass fragmentation pattern (Ryhage & Stenhagen, 1959; McCloskey, 1970). Total ion chromatogram for AF2 is depicted in Fig. 2.6 and the list of acids are shown in Table 2.5. Particularly those resulting from the loose of CH_3O $[M-31]^+$, 2 x CH_3O $[M-64]^+$, CH_3OCOCH_2, a MacLafferty ion $[M-73]^+$, $[M-92]^+$ resulting from the loss of neutral molecules CH_3OCO, CH_3O and 2H radicals, $[M-105]^+$ arising from the loss CH_3OCOCH_2, CH_3O, and H and finally, $[M-123]^+$ resulting from $CH_3OCOCH_2 + CH_3O + H_2O +$ H corresponding to series I (Fig. 2.7). Cyclic enol formed by the rearrangement leads to a m/z 84+14n, is a second series of abundant ions unique to these molecules. For both the series abundance of m/z is influenced by the chain length of the molecule for example m/z 74 resulting from the $[CH_3OOC(CH2)_n]^+$ is destabilized as the chain length increases (Ryhage & Stenhagen, 1959; McCloskey, 1970).

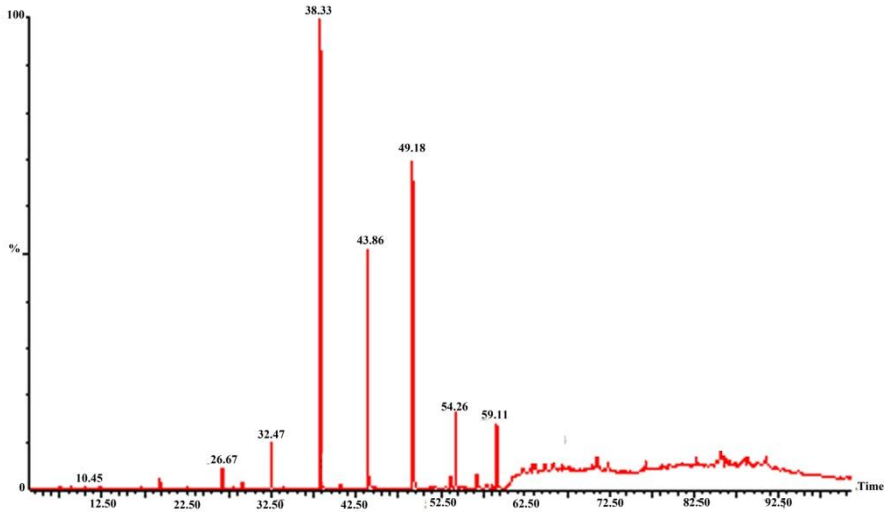


Figure 2.6 Total ion chromatogram of acid fraction AF2 containing dicarboxylic acid.

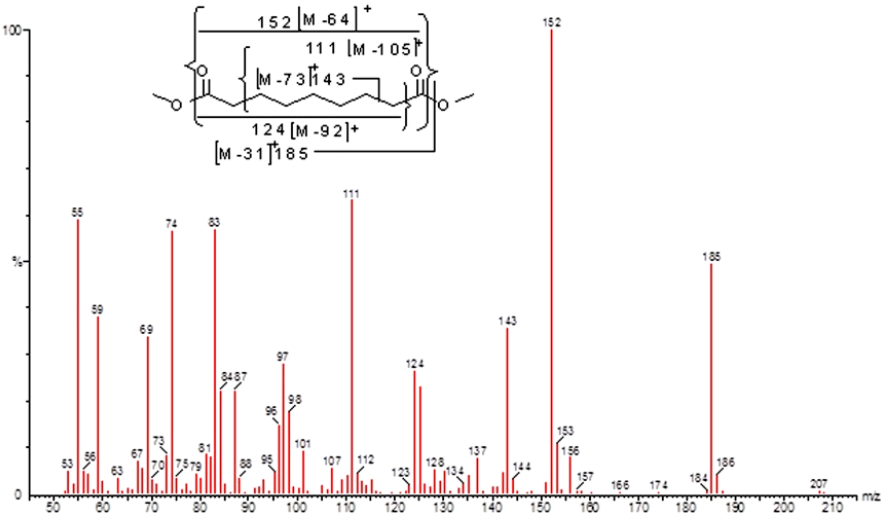
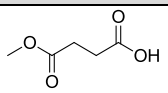
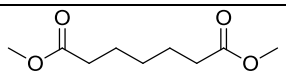
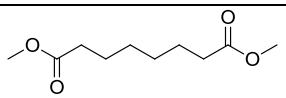
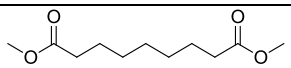
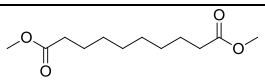
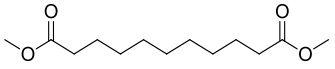
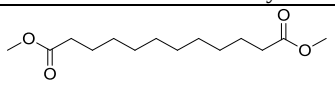
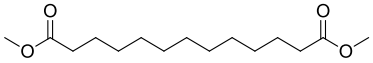


Figure 2.7 A representative mass fragmentation pattern of dicarboxilic acid, dimethyl nonanedioate, having base peak at *m/z* = 152.

Table 2.5 List of carboxylic fatty acids present in fraction AF2

Sl.No	Fatty acids	Relative %	RT	Major m/z
1	 Butanedioic acid, monomethyl ester	0.578	10.451	59, 128, 130, 131, 115, 101, 85, 75, 71
2	 Heptane dioic acid dimethyl ester	1.782	26.67	115, 111, 124, 128, 157, 74
3	 Octanedioic acid dimethyl ester	3.54	32.47	129, 111, 143, 138, 171, 74
4	 Nonanedioicacid dimethyl ester	36.854	38.33	152, 185, 143, 111, 124, 55, 69, 74
5	 Decanedioic acid dimethyl ester	19.47	43.86	55, 111, 125, 157, 199, 74
6	 Undecanedioic acid dimethyl ester	25.525	49.18	98, 111, 152, 185, 213, 74
7	 Dodecanedioic acid dimethyl ester	6.87	54.26	98, 153, 111, 124, 185, 227, 74
8	 Tridecanedioic acid dimethyl ester	5.374	59.11	74, 98, 111, 126, 184

Presences of poly unsaturated fatty acids (Table 2.6) particularly a series of methylene interrupted polyenes were observed in the total ion chromatogram (Fig. 2.8) of AF3 fraction. This inference was made on the basis of unique combination of m/z peaks such as m/z 79 (base peak) along with m/z 93, and 107 (Fig. 2.9) (Fellenberg et al., 1987).

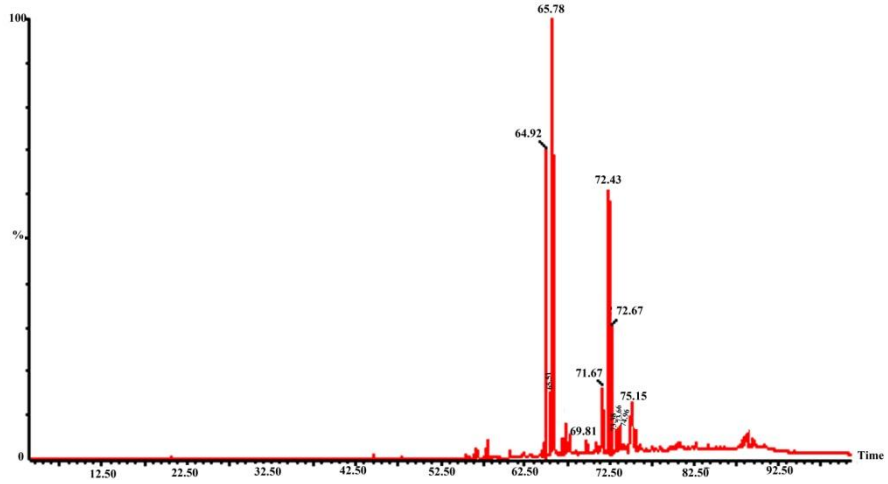


Figure 2.8 Total ion chromatogram of AF3 containing poly unsaturated fatty acid.

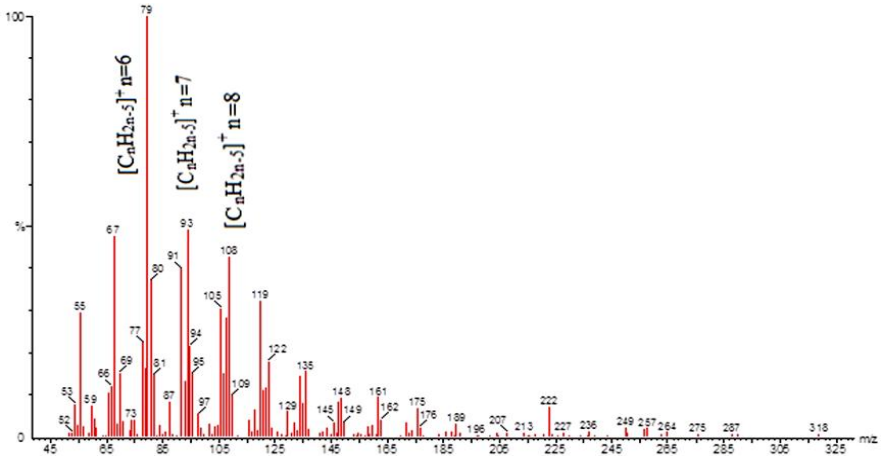
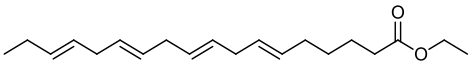
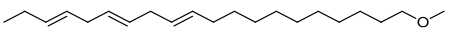
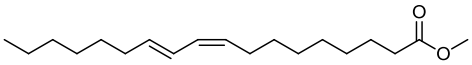
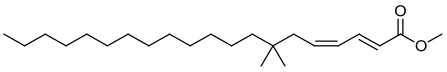
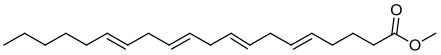
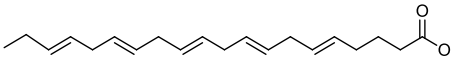
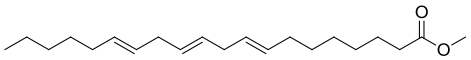
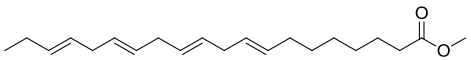
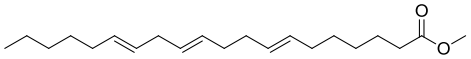
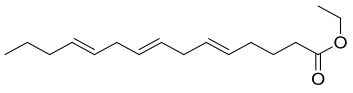


Figure 2.9 A representative mass fragmentation pattern of polyunsaturated fatty acid, methyl 18, 11, 14, 17, icosa tetraenoate, having base peak at *m/z* =79.

Table 2.6 List of poly unsaturated fatty acids present in the fraction AF3.

Sl. No	Fatty acid	Relative %	RT	Major m/z
1	 Ethyl 6, 9,12, 15-octadeca tetra enoate	21.615	64.92	79, 67, 91, 93, 108, 119, 55
2	 Methyl 12, 15-octadeca dienoate	4.146	65.51	67, 81, 95, 109, 294, 55
3	 Methyl 11, 14, 17- eicosatrienoate	31.4	65.78	95, 67, 81, 292, 294, 55
4	 Methyl 9-cis, 11-trans-octadecadienoate	1.266	69.81	67, 81, 95, 294, 55
5	 7,7, Dimethyl-(5Z,8Z)-Eicosadienoic acid Methyl Ester	5.44	71.67	71, 169, 230, 227, 294, 67, 55
6	 5,8,11,14-Eicosatetraenoic acid, methyl ester	19.0	72.43	79, 67, 81, 91, 95, 109, 55
7	 5,8,11,14,17-Eicosapentanenoic acid	8.472	72.67	79, 81, 95, 105, 119, 87, 143, 67
8	 Methyl 8,11,14-icostri enoate	1.678	73.38	67, 81, 95, 222, 320
9	 Methyl 8,11,14,17-icosa tetraenoate	1.607	73.66	79, 67, 81, 95, 106, 109, 55, 222
10	 Methyl 7, 11, 14 eicosa tri enate	1.298	74.96	67, 55, 57, 81, 95, 109, 294, 167
11	 Ethyl 6,9,12 Hexadecatrienoate	4.069	75.15	67, 81, 93, 95, 109, 135, 79, 55, 294, 318

Total ion chromatogram of AF4 is shown in Fig. 2.10. Mass fragmentation pattern of AF4 highlighted the presence of branched fatty acids (Fig. 2.11, Table 2.7), monoenes, and dienes. Allmost all mass spectra consisted of base peak at m/z 74 (Fig. 2.12), a stable ion resulting from the McLafferty rearrangement of the fatty acid molecule after fragmentation. Presence of fatty acids with carbon chain length ranging between C3-C15 observed in this fraction were inferred from the presence of peaks particularly those resulting from the removal of neutral aliphatic radicals such as m/z 87, 143, 199, and 255 (Table 2.7) (Odham & Stenhagen, 1972). Based on the m/z peak of 67, carbonyl carbon cleavage and subsequent lose of methoxy radicals from fatty acids is inferred (Christie, 1998). We can easily classify these fatty acids into branched particularly methyl branches. Presence of monoenes with varying double bond position in this fraction was also inferred from the mass fragmentation data. In this case, m/z 55 an unsaturated aliphatic radical, a unique fragmentation pattern of monoenes was dominated along with m/z at 69, 83, and 97 (Table 2.7). Most of these fragments are often stabilized through the formation of cyclic compounds (Brakstad, 1993).

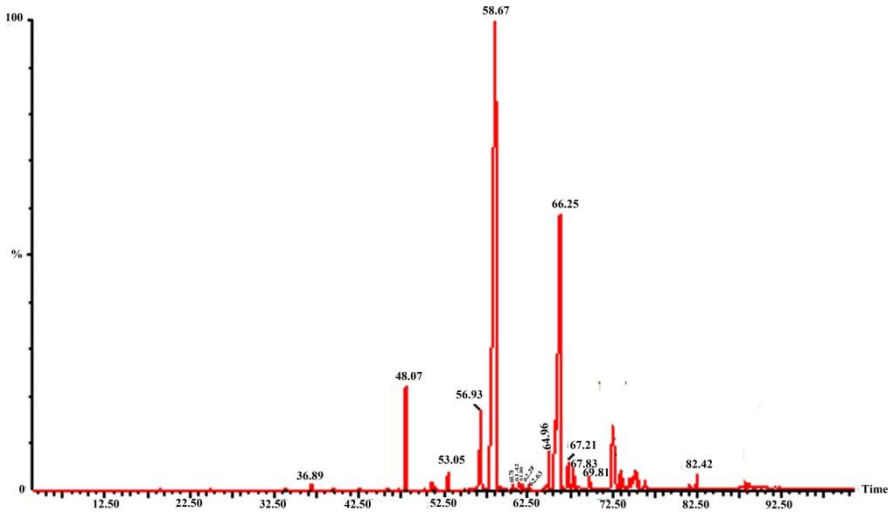


Figure 2.10 Total ion chromatogram of fraction AF4 containing branched acid.

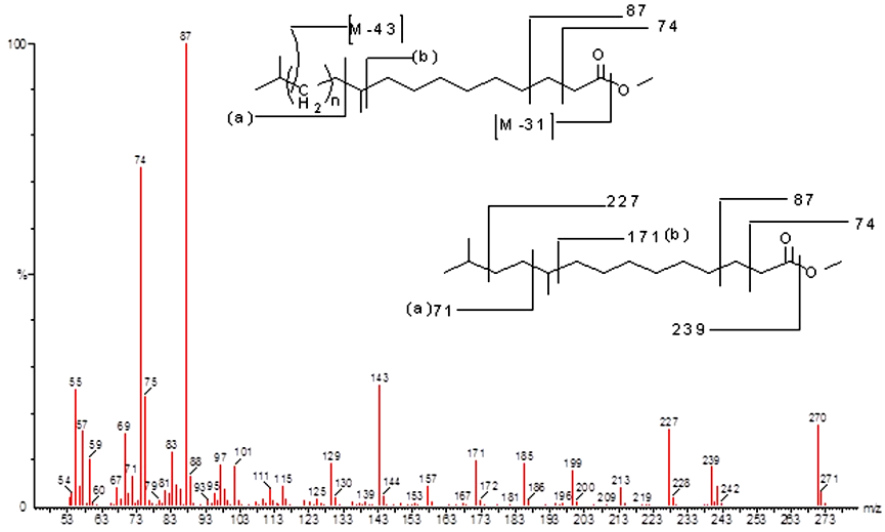


Figure 2.11 A representative mass fragmentation pattern of branched fatty acid, methyl 10, 13-dimethyltetradecanoate, having base peak at *m/z* =87.

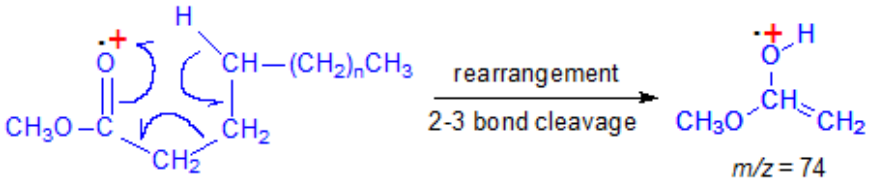


Figure 2.12 Base peak at m/z 74 resulting from the McLafferty rearrangement of the fatty acid molecule after fragmentation.

Presence of m/z 67, 81, 95, and 109 (Fig. 2.13) highlighted the presence of low molecular weight methylene interrupted dienes. In this case when comparing the abundance of m/z at 294 for the monoene and dienes of this class, this particular peak is more abundant than in monoenenes and also the presence of m/z 263 resulting from the removal of methoxyl radical is also observed (Christie, 1998).

Presence of base peak at m/z 55 (Fig. 2.14) for monoenes of low molecular weight compound, in this case result from the suppression of m/z 67, which is otherwise stabilized in methylene interrupted diene (Fellenberg et al., 1987). However, abundance of m/z 81 along with 55 is a unique feature of methylene interrupted fatty acids (Christie, 1998). In certain cases, however, discrepancies are observed in the mass spectra of these classes of molecules, particularly in the context of 2, 5 isomers due to the presence of double bond in the 2nd position (Christie, 1998).

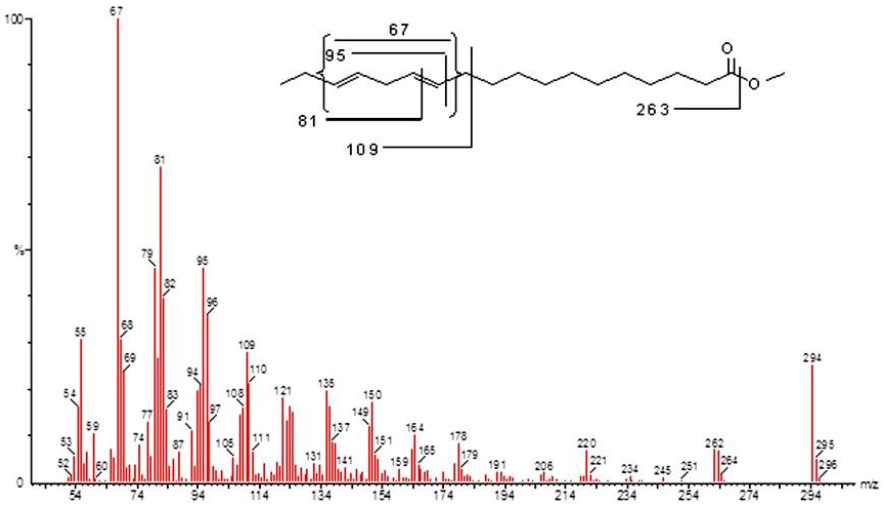


Figure 2.13 A representative mass fragmentation pattern of methylene interpreted dienes, methyl 12, 15 octa deca dienoate, having base peak at *m/z* =67.

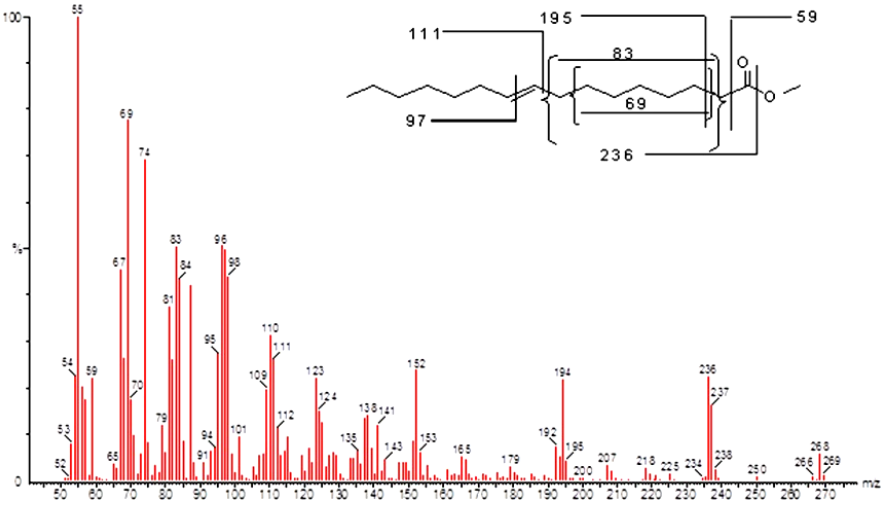
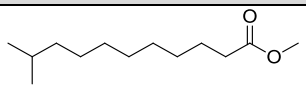
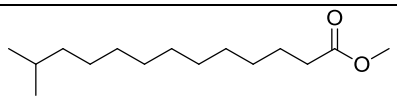
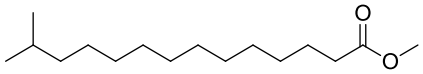
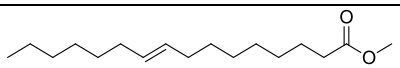
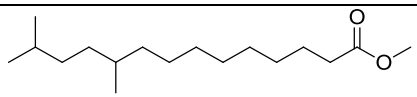
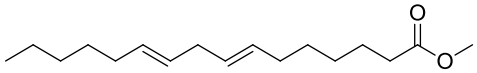
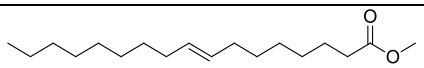
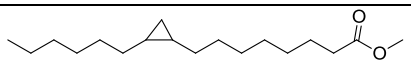
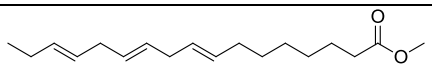
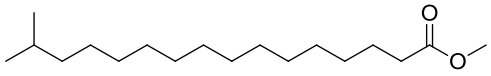
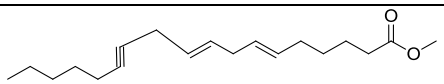
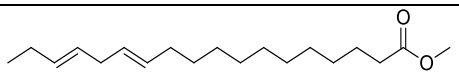
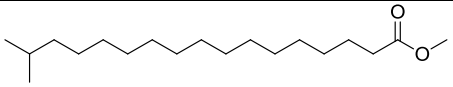
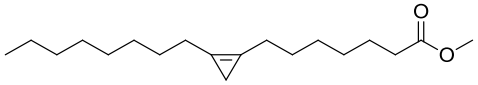
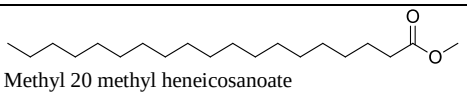


Figure 2.14 A representative mass fragmentation pattern of monoenes, methyl hexadec -9-enoate, having base peak at *m/z* =55.

Table 2.7 List of fatty acids present in the fraction AF4

Sl.No	Fatty acids	Relative %	RT	Major m/z
1	 Methyl 10 methyl undecanoate	0.213	36.89	74, 87, 199
2	 Methyl 12 methyl tridecanoate	5.677	48.07	74, 87, 143, 199, 242
3	 Methyl 13 methyl tetradecanoate	0.604	53.05	74, 87, 143, 199, 213, 256
4	 Methyl hexadec -9-enoate	4.299	56.93	55, 69, 83, 97, 74, 152, 194, 236
5	 Methyl 10,13-dimethyltetradecanoate	59.69	58.67	74, 87, 143, 171, 199, 227, 239, 270
6	 Methyl 7, 10-hexa deca dienoate	0216	60.786	55, 67, 81, 95, 234, 266
7	 Methyl 8-hepta decenoate	0.237	61.421	74, 55, 69, 81, 152
8	 Methyl 9, 10 methylene hexadecanoate	0.161	61.806	55, 69, 83, 110, 250
9	 Methyl 8, 11, 14 hepta deca trienoate	0.032	62.282	67, 79, 81, 108, 149,
10	 Methyl 15 methyl hexa decanoate	0.201	62.637	43, 55, 74, 143
11	 Methyl octadec-6-9diene-12-ynoate	1.703	64.96	79, 67, 91, 95, 105, 109, 119, 161, 290

12	 Methyl 15, 12-octadeca dienoate	24.212	66.25	55, 67, 69, 74, 81, 95, 97, 109, 264, 222, 296
13	 Methyl 16 methyl hepta decanoate	0.897	67.21	74, 87, 143, 199, 255, 298, 55, 57
14	 Methyl 8, 9-methylene hepta dec-8-enoate	0.905	67.83	95, 67, 81, 292, 81, 55
15	Un knowm	0.567	69.81	67, 81, 79, 95, 109, 294, 55
16	 Methyl 20 methyl heneicosanoate	0.436	82.42	55, 69, 87, 143, 255, 354

NL3 was the only active fraction in neutral lipid fraction collected from *Turbinaria conoides*. Activity of other two fractions NL1 and NL2 were negligible. Total ion chromatogram of NL3 is shown in Fig. 2.15. Mass spectral characterization of NL3 confirmed the dominance of phycosterols (Fig. 2.16, Fig. 2.17, Fig. 2.18, Table 2.8), in which an abundance of fucosterol along with other sterols with varying degrees of branching and unsaturation in the side chains were also identified. The mass fragmentation of the relatively abundant compound is having the $[M]^+$ at m/z 413, which is in agreement with the molecular formula $C_{29}H_{48}O$ with calculated molecular weight 412.69, indicating six degrees of unsaturation. The mass spectrum showed the base peak at m/z 314 is an outcome of "McLafferty" type of rearrangement (a → b), in this case results from the cleavage at 22-23 bond position together with a hydrogen

transfer from C-20 carbon atom. Presence of low intense m/z 271 peak corresponds to the loss of two hydrogen atoms from the steriod nucleus alongside chain (Wyllie & Djerassa, 1968). A feeble ion peak at m/z 255 corresponds to the loss of water molecule which supports the presence of hydroxyl group in the sterol ring of fragmented molecule. These mass fragmentations of compound isolated from *Turbinaria conoides* was similar to fucosterol skeleton, which was further confirmed by comparing the mass fragmentation data with NIIST library (Wyllie & Djerassa, 1968).

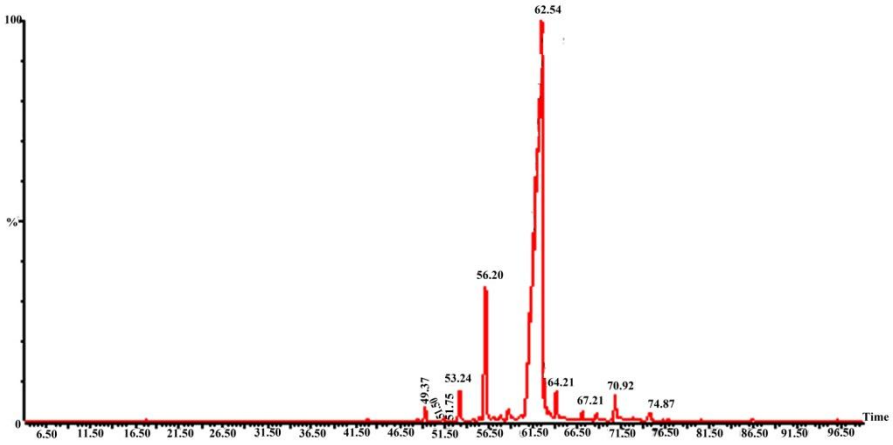


Figure 2.15 Total Ion Chromatogram of neutral fraction NL3.

Further, mass spectra of phyco sterols showcased m/z which corresponds to sterols possessing saturated and unsaturated side chains from the steroid nucleus. General mass fragmentation pattern of sterol molecule is shown in Fig. 2.16. Influence of the presence of unsaturation in the side chain in sterols can be easily deduced from the

presence of intense peak at m/z 257, which might be generated from the parent ion after the removal of hydrogen. This is analogues to the formation of m/z 271, ie., through the loss of side chain along with transfer of hydrogen atom (Fig. 2.17) (Wyllie & Djerassi, 1968). But in case of sterols with saturated side chains m/z 217 (Fig. 2.18) is usually abundant (Sklarz, 2013).

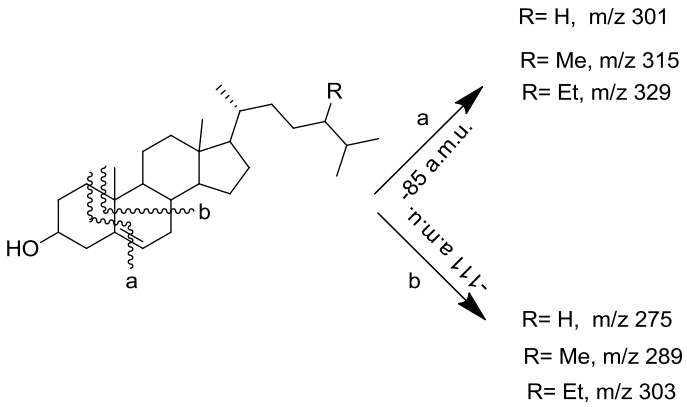


Figure 2.16 General mass fragmentation of sterol molecule ring cleavage.

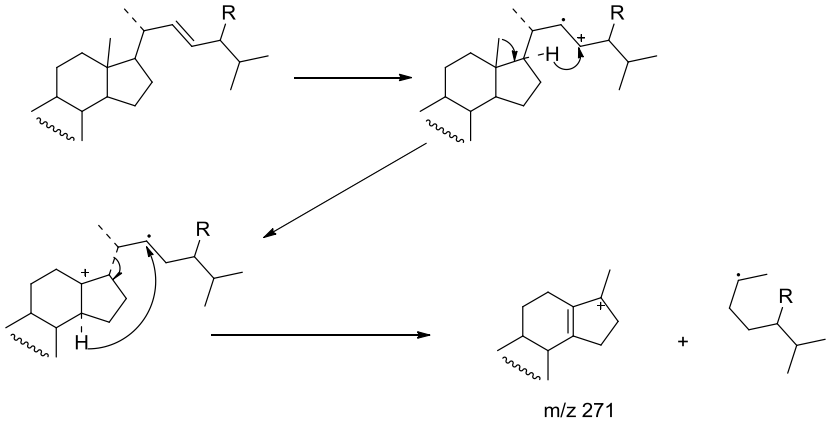


Figure 2.17 Mass fragmentation of unsaturated side chain of free sterol molecule producing m/z 271.

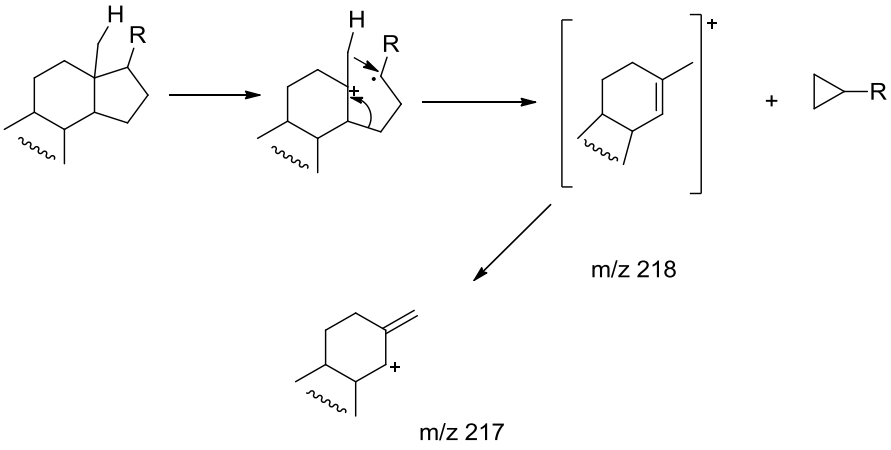
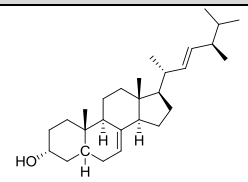
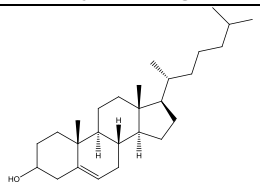
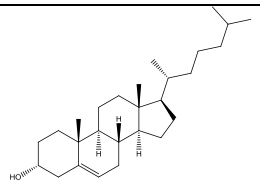
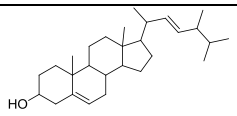
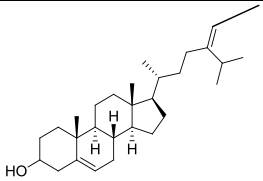
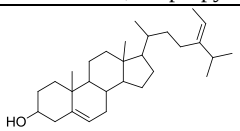
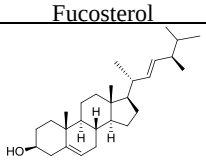
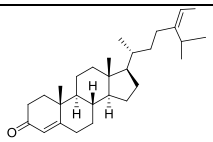
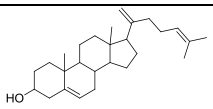
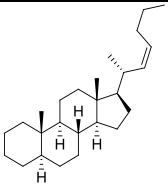


Figure 2.18 Mass fragmentation of saturated side chain of free sterol molecule producing m/z 217.

Table 2.8 List of sterols present in the neutral lipid fraction NL3.

SL No.	Sterols	Relative %	RT	Major m/z
1	 5, 6 Dihydro 3a ergosterol	0.18	49.37	43, 55, 271, 69, 255, 398
2	 Cholesterol	2.72	51.508	43, 105, 145, 213, 275, 301, 386
3	 (3a)-Cholest-5-en-3-ol	.02	51.758	215, 233, 108, 165, 389, 373

4	 Ergosta -5 -,22 –Dien -3-ol (3 Beta)	0.91	53.24	69, 55, 81, 107, 271, 300, 399
5	 Cholest-5-en-3-ol, 24-propylidene	1.26	56.20	55, 69, 41, 105, 271, 299, 314, 315
6	 Fucosterol	92.52	62.54	314, 271, 41, 315, 69, 81, 229
7	 Ergosta-5,22(E)-dien-3beta-ol	0.2	64.21	55, 69, 81, 95, 328, 271, 296, 395
8	 Stigmasta-4,24(28)-dien-3-one (24 (E))	1	67.21	312, 269, 69, 81, 297
9	 Cholesta -5,20,24-Trien-3 OL (3 Beta)	.09	70.92	43, 99, 271, 315, 95, 107, 367
10	 5 Alpha-Cholest-22-ENE (Z)	1.1	74.87	55, 69, 82, 98, 257, 259, 313, 271, 328

Considerable activity of fraction AF1against A549 and Hep G2 can be correlated to the dominance of hydroxy fatty acids in this fraction. Potential of hydroxy fatty acids and their derivatives to suppress the growth of cancer cells are very well established. 2-hydroxy oleic acid, the α-hydroxy derivative of oleic acid is well known for its ability to induce autophagy and endoplasmic reticulum stress (Marcilla-Etxenike et al., 2012). Even though mysteries of exact cellular mechanism for these are not completely revealed. Capacity of hydroxy fatty acids to trigger apoptosis can be linked to the down regulation of two potential proteins, E2F-1 and dihydrofolate reductase (Martínez et al., 2005; Lladó et al., 2009), mainly through altering the spatial organization of lipids. These initial stages of anticancer mechanism relate to the binding of 2-hydroxy oleic acid to the phosphatidylethanolamine membrane and associated alteration in the activity of proteins that are involved in the cell signaling during cancer cell proliferation. Another mechanism used by α-hydroxy fatty acid to inhibit cell cycle, cell proliferation, and autophagy is to specifically activate sphingomyelin synthase and there by suppressing RAS – MAPK pathway, a vital step crucial for the activation of CCND(Cyclin D)-CDK4-CDK6 and P13K-AKT1 pathway (Barceló-Coblijn et al., 2011; Terés et al., 2012).

Long chain hydroxy fatty acids possess potential to enhance anticancer defense, hence are used along with synthetic antitumor drug (PM02734). This enhancement of defense mechanism in cancer

cell lines have been linked to their potential to channelize through plasma membrane spontaneously after protonation or mediated by proteinaceous carriers (Pohl et al., 2008). These flip–flop of long chain hydroxy fatty acid across the plasma membrane influence their solubility and associated partitioning, consequently affecting the sensitivity of the cell line (Orbach & Finkelstein, 1980; Pohl et al., 2008).

The AF2 fraction is dominated by dicarboxylic acid. Total nine dicarboxylic acids were detected in this fraction. Cytotoxic potential of this fraction is comparatively very lower when compared to AF1. In a work carried out by Townsend et al. (1961) in AKR, leukemia cells, had observed that activity is closely related to the chain length and structure of the molecule. Any change in the chain length is reported to affect the activity (Carballeira, 2013). Hence, in the present case, reduction in the activity of the fraction (AF2) can be attributed to the dominance of short chain dicarboxylic acids (Townsend et al., 1961).

Suppressed lung and liver cancer cell lines viability by AF3 fraction can be attributed to the dominance of polyunsaturated fatty acids (Table 2.6). Cyclooxygenase independent pathway can be assumed for this activity. These modes of tumor cell suppression were observed when nude mice and culture cells were treated with polyunsaturated fatty acid (Jiang, 1998; Boudreau et al., 2001).

Potential of n-3 PUFA to inhibit vascular endothelial growth factor in human colon cancer cell was observed and reported by Calviello et al. (2004). Hardman (2004) had highlighted the role of gene expression, apoptosis induced by n-3 fatty acids and their derivatives to suppress the growth of tumor cells. Docosohexanoic acid manifested similar potential as n-3 fatty acids. Because of their angiogenesis and anti-inflammatory potential, their incorporation in the food supplements can reduce the risk of cancer (Healy et al., 2000).

Ample evidence for the potential of PUFA to suppress/alter tumorigenic signals and receptor activity along with lipid peroxidation and cyclooxygenase inhibition has been obtained by Lee et al. (2003). Another cytotoxic study carried out on HL-60 cell using eicosapentanoic acid gave evidence for down regulation of bcl-2 expression (Chiu & Wan, 1999), whereas Southgate et al. (1996), reported the potential of PUFA to directly stimulate apoptosis. Similar conclusion was arrived when arachidinoic acid was used to treat Hep G2–MV2E1-9 cells (Chen & Cederbaum, 1998).

Structural modification of plasma membrane is another mode of action through which saturated fatty acids suppress the activity of malignant cells. These modes of action are usually manifested by fatty acids having structural similarities to α- linolenic acid (Scheim, 2009).

AF4 fraction consists of branched fatty acids and unsaturated fatty acids. Moderate cytotoxicity exhibited by this fraction can be

attributed to branched fatty acid and unsaturated fatty acid with chain length ranging between 9 and 10 in this fraction. Potential of branched fatty acids to induce apoptosis through the regulation of AKT phosphorylation have been observed by Cai et al. (2013). This mode of vital pathways regulation by branched fatty acids triggers caspace activation.

Prominent activity of NL3 fraction can be attributed to the phycosterols present in them. List of compounds identified from this fraction are listed in the Table 2.8. This highlights the chances for multiple modes of induced cell death in lung and liver cancer cell lines, such as, through the inhibition of carcinogen production, cancer cell growth, angiogenisis, invasion and metastasis or through the promotion of programmed cell death (Woyengo et al., 2009). Presence of phycosterol may enhances the level of antioxidants, which simultaneously lead the suppression of oxidative stress and also capable of inducing apoptosis through cell membrane modification/alteration and also through lowering the level of blood cholesterol (da Silva et al., 2012).

2.4 Conclusion

SRB viability assay against A549 and Hep G2 cell lines using various lipid extract fractions collected from *Turbinaria conoides*, NF3 was the most active fraction identified. Characterization of this fraction using mass spectroscopy highlighted the dominance of fucosterol along

with other phycosterols with saturated and unsaturated side chain. Probability of finding sterols with potential cytotoxic activity against lung and liver cancer cell lines other than fucosterol is high in this fraction. Neutral fractions NF1 and NF2 did not exhibit any activity towards the cell lines under study. Silver nitrate impregnated column chromatography is effective in the separation of acid fractions collected from *Turbinaria conoides* to hydroxy, dicarboxylic, and polyunsaturated fatty acids. Presence of hydroxy fatty acids is the first report from this species. Both hydroxy and polyunsaturated fatty acids were the active acid fractions, followed by acid fraction AF4, consisting of monoenes, dienes and branched fatty acids. Dicarboxylic acid was the least active acid fraction as inferred from the viability assay carried out against cancer cell lines.

2.5 References

Amador, M. L., Jimeno, J., Paz-Ares, L., Cortes-Funes, H. & Hidalgo, M. (2003). Progress in the development and acquisition of anticancer agents from marine sources. *Annals of Oncology*, *14*(11), 1607-1615.

Anderson, R. L., & Hollenbach, E. J. (1965). Large-scale separation of fatty acid methyl esters by column chromatography on acid-washed florisil impregnated with silver nitrate. *Journal of Lipid Research*, *6*(4), 577-578.

Annegowda, H. V., Bhat, R., Min-Tze, L., Karim, A. A., & Mansor, S. M. (2012). Influence of sonication treatments and extraction solvents on

the phenolics and antioxidants in star fruits. *Journal of Food Science and Technology*, *49*(4), 510-514.

Barceló-Coblijn, G., Martin, M. L., de Almeida, R. F., Noguera-Salvà, M. A., Marcilla-Etxenike, A., Guardiola-Serrano, F., & Escribá, P. V. (2011). Sphingomyelin and sphingomyelin synthase (SMS) in the malignant transformation of glioma cells and in 2-hydroxyoleic acid therapy. *Proceedings of the National Academy of Sciences*, *108*(49), 19569-19574.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911-917.

Boudreau, M. D., Sohn, K. H., Rhee, S. H., Lee, S. W., Hunt, J. D., & Hwang, D. H. (2001). Suppression of tumor cell growth both in nude mice and in culture by n-3 polyunsaturated fatty acids: mediation through cyclooxygenase-independent pathways. *Cancer Research*, *61*(4), 1386-1391.

Brakstad, F. (1993). Accurate determination of double bond position in mono-unsaturated straight-chain fatty acid ethyl esters from conventional electron impact mass spectra by quantitative spectrum-structure modelling. *Chemometrics and Intelligent Laboratory Systems*, *19*(1), 87-100.

Cai, Q., Huang, H., Qian, D., Chen, K., Luo, J., Tian, Y., & Lin, T. (2013). 13-Methyltetradecanoic acid exhibits anti-tumor activity on T-cell lymphomas *in vitro* and *in vivo* by down-regulating p-AKT and activating caspase-3. *Plos One*, e65308

Calviello, G., Di Nicuolo, F., Gragnoli, S., Piccioni, E., Serini, S., Maggiano, N., & Palozza, P. (2004). n-3 PUFAs reduce VEGF expression in human colon cancer cells modulating the COX-2/PGE2 induced ERK-1 and-2 and HIF-1α induction pathway. *Carcinogenesis, 25*(12), 2303-2310.

Carballeira, N. M. (2013). Recent developments in the antiprotozoal and anticancer activities of the 2-alkynoic fatty acids. *Chemistry and Physics of Lipids*, *172*, 58-66.

Chen, H., Talaty, N. N., Takáts, Z., & Cooks, R. G. (2005). Desorption electrospray ionization mass spectrometry for high-throughput analysis of pharmaceutical samples in the ambient environment. *Analytical Chemistry*, *77*(21), 6915-6927.

Chen, Q. I., & Cederbaum, A. I. (1998). Cytotoxicity and apoptosis produced by cytochrome P450 2E1 in Hep G2 cells. *Molecular Pharmacology*, *53*(4), 638-648.

Chiu, L. C., & Wan, J. M. (1999). Induction of apoptosis in HL-60 cells by eicosapentaenoic acid (EPA) is associated with downregulation of bcl-2 expression. *Cancer Letters*, *145*(1), 17-27.

Christie, W.W. (1998). Gas chromatography-mass spectrometry methods for structural analysis of fatty acids. *Lipids*, *33*(4), 343-353

da Silva Marineli, R., Furlan, C. P. B., & Maróstica, M. R. (2012). Antioxidant effects of the combination of conjugated linoleic acid and phytosterol supplementation in Sprague–Dawley rats. *Food Research International*, *49*(1), 487-493.

De Vries, B. (1963). Quantitative separations of higher fatty acid methyl esters by adsorption chromatography on silica impregnated with silver nitrate. *Journal of the American Oil Chemists Society*, *40*(5), 184-186.

Dołowy, M., & Pyka, A. (2015). Chromatographic methods in the separation of long-chain mono-and polyunsaturated fatty acids. *Journal of Chemistry*. 1-20.

Fellenberg, A. J., Johnson, D. W., Poulos, A., & Sharp, P. (1987). Simple mass spectrometric differentiation of the n-3, n-6 and n-9 series of methylene interrupted polyenoic acids. *Biomedical and Environmental Mass Spectrometry*, *14*(3), 127-129.

Firempong, C. K., Cao, X., Tong, S., & Yu, J. (2015). Prospects for multitarget lipid-raft-coated silica beads: A remarkable online biomaterial for discovering multitarget antitumor lead compounds. *RSC Advances*. *5*, 49330-49342.

Gaulton, A., Bellis, L. J., Bento, A. P., Chambers, J., Davies, M., Hersey, A., Light, Y., McGlinchey, S., Michalovich, D., Al-Lazikani, B., & Overington, J. P. (2012). ChEMBL: A large-scale bioactivity database for drug discovery. *Nucleic Acids Research*, *40*(D1): D1100-D1107.

Griffon, G., Merlin, J. L., & Marchal, C. (1995). Comparison of sulforhodamine B, tetrazolium and clonogenic assays for *in vitro* radio sensitivity testing in human ovarian cell lines. *Anti-cancer Drugs*, *6*(1), 115-123.

Hardman, W. E. (2004). (n-3) fatty acids and cancer therapy. *The Journal of Nutrition*, *134*(12), 3427S-3430S.

Healy, D. A., Wallace, F. A., Miles, E. A., Calder, P. C., & Newsholme, P. (2000). Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids*, *35*(7), 763-768.

Heemken, O. P., Theobald, N., & Wenclawiak, B. W. (1997). Comparison of ASE and SFE with soxhlet, sonication, and methanolic saponification extractions for the determination of organic micropollutants in marine particulate matter. *Analytical Chemistry*, *69*(11), 2171-2180.

Houghton, P., Fang, R., Techatanawat, I., Steventon, G., Hylands, P. J., & Lee, C. C. (2007). The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods*, *42*(4), 377-387.

Huang, X., Kong, L., Li, X., Chen, X., Guo, M., & Zou, H. (2004). Strategy for analysis and screening of bioactive compounds in traditional Chinese medicines. *Journal of Chromatography B*, *812*(1), 71-84.

Jiang, W. G., Hiscox, S., Bryce, R. P., Horrobin, D. F., & Mansel, R. E. (1998). The effects of n6 polyunsaturated fatty acids on the expression of nm-23 in human cancer cells. *British Journal of Cancer*, *77*(5), 731-738.

Kepler, C. R., & Tove, S. B. (1967). Biohydrogenation of unsaturated fatty acids III. Purification and properties of a linoleate Δ12-cis, Δ11-trans-isomerase from *Butyrivibrio fibrisolvens*. *Journal of Biological Chemistry*, *242*(24), 5686-5692.

Larsen, E., & Christensen, L. P. (2005). Simple saponification method for the quantitative determination of carotenoids in green vegetables. *Journal of Agricultural and Food Chemistry*, *53*(17), 6598-6602.

Lee, J. Y., Plakidas, A., Lee, W. H., Heikkinen, A., Chanmugam, P., Bray, G., & Hwang, D. H. (2003). Differential modulation of Toll-like receptors by fatty acids preferential inhibition by n-3 polyunsaturated fatty acids. *Journal of Llipid Research*, *44*(3), 479-486.

Li, T. S., Li, J. T., & Li, H. Z. (1995). Modified and convenient preparation of silica impregnated with silver nitrate and its application to the separation of steroids and triterpenes. *Journal of Chromatography A*, *715*(2), 372-375.

Li, X. F., Zhang, S. Z., & Zheng, S. (2000). Apoptosis of K562 Cell and multidrug resistant cells (K562/vin, K562/dox) induced by matrine. *The Practical Journal of Cancer*, *6*, 002.

Lladó, V., Terés, S., Higuera, M., Álvarez, R., Noguera-Salva, M. A., Halver, J. E., & Busquets, X. (2009). Pivotal role of dihydrofolate reductase knockdown in the anticancer activity of 2-hydroxyoleic acid. *Proceedings of the National Academy of Sciences*, *106*(33), 13754-13758.

Lu, X., Su, M., Li, Y., Zeng, L., Liu, X., Li, J., & Wang, S. (2002). Effect of *Acanthopanax giraldii Harms Var. Hispidus Hoo* polysaccharides on the human gastric cancer cell line SGC-7901 and its possible mechanism. *Chinese Medical Journal*, *115*(5), 716-721.

Marcilla-Etxenike, A., Martín, M. L., Noguera-Salvà, M. A., García-Verdugo, J. M., Soriano-Navarro, M., Dey, I., Escribá, P. V., & Busquets, X. (2012). 2-Hydroxyoleic acid induces ER stress and autophagy in various human glioma cell lines. *PLoS ONE*, 7(10), e48235.

Martínez, J., Gutiérrez, A., Casas, J., Lladó, V., López-Bellan, A., Besalduch, J., & Escribá, P. V. (2005).The repression of E2F-1 is critical for the activity of minerval against cancer. *Journal of Pharmacology and Experimental Therapeutics*, *315*(1), 466-474.

McCloskey, J. A. (1970). Mass spectrometry of fatty acid derivatives. *Topics in Lipid Chemistry*, *1*, 369-440.

McKay, D. L., & Blumberg, J. B. (2006). A review of the bioactivity and potential health benefits of peppermint tea (*Mentha piperita* L.). *Phytotherapy Research*, 20(8), 619-633.

Metcalfe, L. D., Schmitz, A. A., & Pelka, J. R. (1966). Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Analytical Chemistry*, *38*(3), 514-515.

Montalvão, S. I. G., Singh, V., & Haque, S. (2014). Bioassays for bioactivity screening. *Comprehensive Analytical Chemistry*, *65*, 79-114.

Moore, K., & Rees, S. (2001). Cell-based versus isolated target screening: how lucky do you feel?. *Journal of Biomolecular Screening*, *6*(2), 69-74.

Nikolova-Damyanova, B., & Momchilova, S. (2002). Silver ion HPLC for the analysis of positionally isomeric fatty acids. *Journal of Liquid Chromatography and Related Technologies*, *25*(13-15), 1947-1965.

Nikolova-Damyanova, B., Christie, W. W., & Herslöf, B. (1996). Mechanistic aspects of fatty acid retention in silver ion chromatography. *Journal of Chromatography A*, *749*(1), 47-54.

Odham, G., & Stenhagen, E. I. N. A. R. (1972). Fatty acids. *Biochemical Applications of Mass Spectrometry*, 211-228.

Orbach, E., & Finkelstein, A. (1980). The nonelectrolyte permeability of planar lipid bilayer membranes. *The Journal of General Physiology*, *75*(4), 427-436.

Ozen, H. C., Bashan, M., Toker, Z., & Keskin, C. (2004). 3-Hydroxy fatty acids from the flowers of *Hypericum lysimachioides var. lysimachioides*. *Turkish Journal of Chemistry*, *28*(3), 223-226.

Peelman, F., Couturier, C., Dam, J., Zabeau, L., Tavernier, J., & Jockers, R. (2006). Techniques: new pharmacological perspectives for the leptin receptor. *Trends in Pharmacological Sciences*, *27*(4), 218-225.

Peng, Y. R., Li, Y. B., Liu, X. D., Zhang, J. F., & Duan, J. A. (2008). Antitumor activity of C-21 steroidal glycosides from *Cynanchum auriculatum Royle ex Wight*. *Phytomedicine*, *15*(11), 1016-1020.

Pohl, E. E., Voltchenko, A. M., & Rupprecht, A. (2008). Flip-flop of hydroxy fatty acids across the membrane as monitored by proton-sensitive microelectrodes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1778*(5), 1292-1297.

Proteau, P. J., & Gerwick, W. H. (1993). Divinyl ethers and hydroxy fatty acids from three species of Laminaria (brown algae). *Lipids*, *28*(9), 783-787.

Reetz, M. T., Becker, M. H., Klein, H. W., & Stöckigt, D. (1999). A method for high-throughput screening of enantioselective catalysts. *Angewandte Chemie International Edition*, *38*(12), 1758-1761.

Riss, T. E. R. R. Y., O'Brien, M. A. R. T. H. A., & Moravec, R. I. C. H. A. R. D. (2003). Choosing the right cell-based assay for your research. *Cell Notes*, *6*(1), 6-12.

Rorrer, G. L., Yoo, H. D., Huang, Y. M., Hayden, C., & Gerwick, W. H. (1997). Production of hydroxy fatty acids by cell suspension cultures of the marine brown alga Laminaria saccharina. *Phytochemistry*, *46*(5), 871-877.

Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., & Boyd, M. R. (1990). Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *Journal of the National Cancer Institute*, *82*(13), 1113-1117.

Ryhage, R., & Stenhagen, E. (1959). Mass spectrometric studies. 3. esters of saturated dibasic acids. *Arkiv for Kemi*, *14*(6), 497-509.

Ryhage, R., & Stenhagen, E. (1960). Mass spectrometry in lipid research. *Journal of Lipid Research*, *1*(5), 361-390.

Scheim, D. E. (2009). Cytotoxicity of unsaturated fatty acids in fresh human tumor explants: concentration thresholds and implications for clinical efficacy. *Lipids in Health and Disease*, *8*(1), 54.

Simmons, T. L., Andrianasolo, E., McPhail, K., Flatt, P., & Gerwick, W. H. (2005). Marine natural products as anticancer drugs. *Molecular Cancer Therapeutics*, *4*(2), 333-342.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., & Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute*, *82*(13), 1107-1112.

Sklarz, B. (Ed.). (2013). Mass spectrometry of natural products*: Plenary Lectures Presented at the International Mass Spectrometry Symposium on Natural Products, Rehovot, Israel.*

Southgate, J., Pitt, E., & Trejdosiewicz, L. K. (1996). The effects of dietary fatty acids on the proliferation of normal human urothelial cells *in vitro*. *British Journal of Cancer*, *74*(5), 728.

Terés, S., Lladó, V., Higuera, M., Barceló-Coblijn, G., Martin, M. L., Noguera-Salvà, M. A., & Escribá, P. V. (2012). Normalization of sphingomyelin levels by 2-hydroxyoleic acid induces autophagic cell death of SF767 cancer cells. *Autophagy*, *8*(10), 1542-1544.

Townsend, G. F., Brown, W. H., Felauer, E. E., & Hazlett, B. (1961). Studies on the *in vitro* antitumor activity of fatty acids: IV. The esters of acids closely related to 10-Hydroxy -2-Decenoic acid from royal jelly against transplantable mouse leukemia. *Canadian Journal of Biochemistry and Physiology*, *39*(11), 1765-1770.

Van Staveren, W. C. G., Solís, D. W., Hebrant, A., Detours, V., Dumont, J. E., & Maenhaut, C. (2009). Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells?. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1795*(2), 92-103.

Vichai, V., & Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols*, *1*(3), 1112-1116.

Wang, Y., Sunwoo, H., Cherian, G., & Sim, J. S. (2000). Fatty acid determination in chicken egg yolk: a comparison of different methods. *Poultry Science*, *79*(8), 1168-1171.

Wei, D. Z., Yang, J. Y., Liu, J. W., & Tong, W. Y. (2003). Inhibition of liver cancer cell proliferation and migration by a combination of (-)-epigallocatechin-3-gallate and ascorbic acid. *Journal of Chemotherapy*, *15*(6), 591-595.

Weller, M. G. (2012). A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. *Sensors*, *12*(7), 9181-9209.

Wise, A., Gearing, K., & Rees, S. (2002). Target validation of G-protein coupled receptors. *Drug Discovery Today*, *7*(4), 235-246.

Woyengo, T. A., Ramprasath, V. R., & Jones, P. J. H. (2009). Anticancer effects of phytosterols. *European Journal of Clinical Nutrition*, *63*(7), 813-820.

Wyllie, S. G., & Djerassi, C. (1968). Mass spectrometry in structural and stereochemical problems. CXLVI. Mass spectrometric fragmentations typical of sterols with unsaturated side chains. *The Journal of Organic Chemistry*, *33* (1), 305-313.

Zhang, H., Yang, L., Liu, S., & Ren, L. (2001). Study on active constituents of traditional Chinese medicine reversing multidrug resistance of tumor cells *in vitro*. *Journal of Chinese Medicinal Materials*, *24*(9), 655-657.

Chapter 3

CHEMICAL CHARACTERIZATION OF STEROLS ISOLATED FROM *Turbinaria conoides*

3.1 Introduction

Potential of marine seaweeds to bio-synthesize unusual steroid molecules have been exclusively verified by refined natural product isolation and purification techniques coupled with mass spectroscopy, NMR and FTIR data (Neudert et al., 1965; Tokes et al., 1968; Minale & Sodano, 1974a; Minale & Sodano, 1974b; Sheikh & Djerassi, 1974; Rubinstein et al., 1976; Schmitz et al., 1976; Blunt & Stothers, 1977; Itoh et al., 1983; Goad & Akihisa, 1997). With the aid of these advanced methodologies, stereo-chemical modifications occurring to the nucleus and the side-chain through oxygenation, alkylation, degradation, sulfate esterification, cis fused A/B ring junctions, aromatization or contraction in the A ring, unsaturation in the D ring, or secostructures with one of the rings oxidatively cleaved during biosynthesis can be easily presumed (Goad & Akihisa, 1997). In the

current investigation on the nonpolar sterol extract of *Turbinaria conoides*, which showed significant activity against lung and liver cancer during bio-screening assay (Chapter 2), aims to isolate fucosterol, the dominant sterol in *Turbinaria conoides*, as fucosterol acetate, and a novel sterol and structural elucidation of these sterols.

Sheu et al. (1999) separated nine cytotoxic oxygentated sterol compounds from *Turbinaria conoides* using a combination of adsorption and thin layer chromatography. Adsorption chromatography is a gravity driven liquid-solid chromatography, usually preferred in the primary separation and purification of polar compounds from crude mixtures weighing approximately 200 mg or more according to their polarity (Heupel, 1989; Goad & Akihisa, 1997). In this mode of separation organic mixture adsorbed on the solid stationary phase was eluted and partitioned under the influence of gravity using isocratic solvent systems as mobile phase. Partitioning of sterols during adsorption chromatography is closely related to their potential to form hydrogen bond and non-covalent interactions such as van der-waals forces and dipole-dipole interaction with vicinal and geminal silanol groups in the adsorbent (Gutmann, 1978; Nes, 1985; Kaliszan, 1987; Heupel, 1989; Forgacs & Cserhati, 1997; Kaliszan, 1997; Komsta et al., 2013). Sterols and other polar compound recovered from the adsorption chromatography are then purified using thin layer chromatography (TLC), in which capillary force plays a major role in the fine

purification and isolation process (Gruss, 1912; Fried & Sherma, 1999). As effective economical methods can be easily developed and manipulated during the course of running even for the simultaneous purification of multiple samples along with faster rate of sterol purification compared to column chromatography makes thin layer chromatography a form of solid-liquid chromatography, as one most preferred choices during phyto-chemistry work (Fried & Sherma, 1999; Spangenberg et al., 2011). Moreover, as the rate of mobility of each compound in a given solvent system is closely associated to hydrogen bonding strength, this method can be used as a first hand diagnostic tool to predict the position and number of methyl groups present in the molecule (Heupel, 1989). For example, mixture of 4-desmethyl, 4-monomethyl and 4,4-dimethyl sterols can be easily distinguished using the Retention factor (Rf) value. 4-desmethyl sterol form strong hydrogen bond with the adsorbent and have a relatively small Rf value compared to 4-monomethyl sterols. Whereas hydrogen bonding strength 4,4-dimethyl sterols is considerably masked by the presence and position of methyl groups and therefore displayed a high Rf value (Heupel, 1989).

Absorption data in the range of 200-400 nm, which are mainly associated to the changes in the energy of electrons involved in the formation of valence bonds, collected using UV-Vis Spectroscopy is another commonly used spectroscopic data to evaluate the sterols. As this range of absorption are associated with electron transitions,

structural information especially related to the nature of double bounds (conjugation) can be easily deduced, based on the position and intensity of UV absorption bands (Dorfman, 1953; Acuna-Johnson & Oehlschlager, 1989; Kerr et al., 1991). For example, 5,7 conjugated double bonded ergosterol have UV absorbance at 283 nm bands (Kerr et al., 1991), whereas 22,24(28) diene absorbs at 230 nm (Dusza, 1960). Even though bathochromic shift in the absorption values of diene system are useful in deducing the nature of alkyl substitution adjacent to them (Dorfman, 1953; Nes & McKean, 1977; Liljefors & Allinger, 1978; Acuna-Johnson & Oehlschlager, 1989), applicability of UV-Vis spectroscopy is limited as the same absorbance values are often obtained for different compounds with different conjugations (Acuna-Johnson & Oehlschlager, 1989). This limitation can be easily surpassed to some extent through the effective utilization of FTIR spectroscopy.

While evaluating the structure of sterol, IR spectroscopy is usually used to identify the nature and position of the double bonds (Nes & McKean, 1977; Thompson et al., 1980). Specific features of double bond, exo cyclic and endo cyclic present in the sterols can be deduced from the finger print region 650 and 1000 cm^{-1} (Neudert, 1965; Nes & McKean, 1977). Structural informations are usually presumed by comparing the obtained value against a reference value. Fucosterol with additional IR absorption at 820 cm^{-1} due to E-24(28) double bond is an apt example for the influence of substitution on

double bond absorption at 800-850 cm^{-1} (Nes & McKean, 1977; Gibbons et al., 1982), which otherwise exhibit a trisubstituted ethylene like absorption in this region as in sitosterol (Neudert, 1965). Presence of E-22 double bond in the sterol molecule can be easily predicted from the presence of 967 cm^{-1} peak (Neudert, 1965). Stigmasterol which differs from sitosterol by the presence of an additional double bound in E-22 is a fine example for this. Closely related sterols, such as spinasterol and stigmasterol, which differ by position of double bond also, have different absorption values in the finger print region (Neudert, 1965). However, to deduce the stereochemistry of side chain alkyl groups, mass fragmentation and NMR data are required (Goad & Akihisa, 1997).

Mass spectroscopy has been effectively used for determination of exact molecular weight of the sterol molecule and their fragmentation (Goad & Akihisa, 1997). Determination of exact molecular weight is very useful for developing an apt molecular formula, degree of unsaturation, and presence of elements such as halogens. Apart from molecular formula, mass fragmentations developed by these tools at 70 ev are very useful in providing conclusive evidence for structural elucidation of the molecule. As each fragmentation is a unique finger print of the molecule from which they are formed (Budzikiewicz et al., 1964; Eneroth et al., 1964; Djerassi et al., 1965; Eneroth et al., 1965).

Apart from giving vital informations on the nature of the functional groups present in the sterol nuclei, proton NMR spectra also gave crucial informations regarding the nature of the double bonds and side chain stereochemistry (Goad & Akihisa, 1997). For example, 24(28)-ethylidene stereochemistry of fucosterol, an abundant sterol in brown seaweed was finalized on the basis of number and nature of protons present on the C-28 and C-29 (Nes et al., 1966). A strong down field viz. between 5-6 ppm is observed for proton when it is attached to the double bond. Variations in C-18 and C-19 methyl resonance values is another data recovered from proton NMR, as it is very useful in predicting the structural information through comparison and calculations (Bhacca & Williams, 1964). Recent advancement in NMR instrumentation has improved their potential to distinguish the 24 alkyl isomers.

When compared to proton NMR, even slight modifications in sterols can be easily deduced using C-13 NMR spectroscopy due to high sensitivity of C-13 chemical shifts towards structural changes, making them a powerful tool in the elucidation of chemical features of sterols (Goad & Akihisa, 1997). Since stereo environment of carbon plays a vital role in the variation of chemical shifts at chiral centres, C-13 NMR is very effective in deducing the stereochemistry of the molecule. Based on the C-13 shift values ranging between 0-200 ppm, nature of the carbon can be easily assigned. For instance, saturated carbon in the cyclic structure of sterol molecules give

chemical shift values in the range of 10-60 ppm. Whereas for olifienic carbon this value lies in the range of 120-170 ppm. Hydroxylated carbon has characteristic chemical shift between 65-80ppm and carbonyl carbons at 170-220 ppm. However, the presence of ketones is usually detected at the low field, whereas esters are having a characteristic shift at high field. When compared to proton NMR, C-13 NMR shift data have established to be more valuable tool in the delineation and classification of C-24 epimers of 24-alkylsterols, (Blunt & Stothers, 1977; Akihisa et al., 1986; Akinhisa, 1989). For example, carbon signals at C-23, C-26 and C-29 are particularly useful in classifying C-24 diastereoisomers such as 24-ethylsterols. Similarly, as configuration of 24-methylsterols at C-24 can be easily assigned on the basis of signals at C-28 carbon and other side chains (Sright et al., 1978; Koizumi et al., 1979). Further carbon shift signals are particularly useful while studying the 24-methyl-A22-sterols, as each epimers of these sterols provide distinctive data for C-28 and C-16 with regard to shielding/ deshielding effect (Goad & Akihisa, 1997).

Even though one dimensional NMR (proton and C-13) are very effective in providing useful leads towards the structure of sterol under investigation, this data are often complicated by overlapping signals (Goad & Akihisa, 1997). Most of these short comes of one dimensional NMR can be easily overcome with aid of multidimensional homonuclear and heteronuclear NMR data

acquisition (Benn & Gunther, 1983; Croasmun et al., 1987; Derome, 1987; Sashida et al., 1991; Croasmun & Carlson, 1994). With the aid of NMR instruments equipped with advanced data processing units capable of achieving high sensitivity. 2D experiments such as scalar/spatial homotropic (^{1}H-^{1}H/^{13}C-^{13}C) measurements, essentially in combination with heteronuclear experiments, measuring both scalar and spatial coupling retrieved using these facilities gave conclusive evidence for deducing the unique features of sterols/unknown compounds. Hence, these pulsed methods are indispensible tool for a phyto-chemist to achieve the major vision of the project (Sashida et al., 1991; Croasmun & Carlson, 1994).

This chapter report the methodologies used for the isolation of a novel sterol and conversion of fucosterol to fucosterol acetate. Structure of fucosterol acetate were verified using spectroscopic tools such as UV-Vis, FTIR, proton NMR, C-13 NMR, H-H COSY, and HSQC. Whereas HMBC was used to confirm the structure of novel sterol molecule isolated.

3.2 Materials and Methods

3.2.1 Separation and Purification

Prior to purification of most bioactive lipid fraction (NF3) using TLC, these fractions were subjected to column chromatography.

3.2.1a Column chromatography

Initial separation and purification of the lipid fraction consisting of sterols were performed by Silica-gel column chromatography. Silica gel 60-120 mesh for column chromatography was purchased from Merck. In order to activate the silica gel, they were kept in oven over night, at 120°C. Activated silica was loaded homogeneously up to 1.5m in to a 2.0 m high borosil column with 0.5 cm internal diameter. Bioactive fraction adsorbed on silica was loaded to this column. Fractions were eluted using a solvent system (15% Ethylacetate and 85% Hexane). The flow rate was controlled at 1mL/minute, and the eluate from the column was collected in fractions of 25 mL. These subfractions were screened using TLC for further analysis.

3.2.1b Acetylation of fucosterol to fucosterol acetate

Subfractions consisting of dominant sterol, fucosterol (data shown in appendix) was pooled and concentrated. Concentrated fractions of fucosterol was used to synthesize fucosterol acetate through acetylation. Acetylation of fucosterol was carried out using acetic anhydride and pyridine (Kawagishi et al., 1988). Acetylated samples were recovered using hexane which was then dried in inert environment and purified using TLC.

3.2.1c Thin Layer Chromatography

Fine purification of fucosterol acetate (compound I) and novel sterol (compound II) isolated was done using TLC Silica gel 60 F_{254},

Merck Germany. 20X20 cm aluminium sheets. To this TLC plate, sterols dissolved in HPLC grade ethyl acetate were loaded and eluted using a hexane ethyl actetate solvent system. Prior to elution, TLC chamber were equilibrated with the solvent vapour. After equilibration, TLC plates were immersed in the solvent system for developing the plate. A reference was collected from the developed plate and monitored using sterol specific stain (Phosphoric acid 50 mL: Water 50 mL) (Neher & Wettstein, 1952). After the application of stain these reference plates were then kept in an oven maintained at 120°C for 20 minutes to complete the chemical reaction. Developed spots were monitored in both visible and UV light in a UV chamber. Comparing with the reference, sterols were purified and recovered.

3.2.2 UV-Vis Spectroscopy

Absorbance maxima of the isolated compounds were measured using UV-Visible spectrophotometer (Genesys 10UV). 1 mg of the isolated compounds was dissolved in chloroform and absorbances were measured from 200 nm to 750 nm. Chloroform was used as reagent blank.

3.2.3 FTIR Spectroscopic Characterization

ATR-FTIR spectra of sterols were collected using single bounce ATR-FTIR spectrometer (Perkin Elmer, Spectrum 100) with a diamond internal reflection element at ambient temperature. The samples were dried in inert nitrogen environment to remove excess moisture. IR

spectra were collected using a diamond crystal against the air background. Each scan took 1 second and 12 scans were collected and averaged for each sample. The resolution was set at 4 cm^{-1}. Prior to FTIR analysis, N_2 gas was purged on the surface of the instrument to remove moisture. High resolution (4 cm^{-1}) ATR transmission mode spectra for sterols were acquired over the mid infrared region (4000-600 cm^{-1}). The instrument is equipped with a mercury cadmium telluride (MCT) detector and was operated at frequency of 40 Hz. FTIR transmission spectrum was obtained after the background subtraction. To determine individual peak frequencies, peak centroid frequency was collected. However, the shoulder peaks were picked slightly away from the overlapping peaks.

3.2.4 Mass Spectroscopic Characterization

3.2.4a High Resolution Mass Spectroscopy analyisis

High resolution mass of sterols were acquired with the aid of FAB mass spectra (Joel SX 102/DA-6000 mass spectrometer) operated at 6 kV, 10 mA, is a type of mass spectrometry in which sample mixed with a liquid matrix is bombarded with a stream of atoms generated by noble gas and detect the secondary ions ejected as result of this impact. This is a widely used technique used to confirm the exact mass of thermally labile and non volatile organic compounds (Silverstein et al., 2014). Xenon was used as the carrier gas and m-nitrobenzyl alcohol as the matrix. Sterol sample (0.1 mg) in 100 µL was used for the analysis.

The quasimolecular ions $[M+H]^+$ spectrum was recorded at room temperature. The spectrum is obtained at resolution of 1000 amu.

3.2.4b GC-MS

Mass fragmentation of sterols isolated and purified was carried out using GC-MS (Perkin Elmer, Clarus 680-Clarus 600T). Analytical conditions used for characterization of sterols are shown in Table 3.1.

Table 3.1 Analytical conditions of GC-MS for characterization of sterols

Column	non polar HP ultra double fused silica capillary column
Injection quantity	1.0µL
Injection mode	Split (split ratio 1:20)
Vaporization chamber temperature :	280℃
Column oven temperature	150℃ (0 min) -> 200 (15℃/min) hold for one minute -> 250℃ (5℃/min) hold for 1 minute -> 280℃ (1℃/min) 45 minute hold.
Detector	MS
[MS] Interface temperature	200℃
Ion source temperature	200℃
Inlet A	PSS1
Inlet B	CAP
Data sampling time	16 to 80 min
Measurement mode	Scan
Mass range	m/z 45-330
Sampling rate	12.50000 pts/s
Run time	90 minutes

3.2.5 NMR

Prior to the ^{1}H and ^{13}C solution state NMR spectroscopic experiments, aliquots of sterols were dissolved in $CDCl_3$.

3.2.5a Proton NMR

NMR data were acquired using the instrument Bruker Avance III, 400MHz, 9.4 Tesla super-conducting Magnet, with Z-gradient, 2H lock NMR spectrometer fitted with a QNP ^{1}H probe, operating at a ^{1}H resonating frequency. All experiments were carried out at very low concentrations (1 mg).

3.2.5b C-13 NMR

^{13}C NMR spectra of sterols isolated from *Turbinaria conoides* were acquired with a Bruker Avance III, 400MHz, 9.4 Tesla super-conducting Magnet, with Z-gradient, 2H lock; for observation ^{13}C operating at a ^{13}C resonating frequency of 75.475 MHz. Depending on the density of the sample and the amount of sample available, approximately 10 mg dry weight per sample dissolved in $CDCl_3$, 500 µL was added in to 5mm NMR tube with polyethylene cap. Scans (10 to 30) were averaged to obtain adequate signal-to-noise on the solutions. ^{13}C NMR spectra were measured at 22°C in $CDCl_3$ solution (5 to 150 mM) and referenced to $CDCl_3$ at 77.0 ppm. Sweep rates of 2 or 4 Hz/sec at 50 or 100 Hz sweep width were used but slower sweep rates were employed when necessary to resolve closely spaced lines.

3.2.5c DEPT

Standard Bruker software was used to acquire DEPT (distortionless enhancement by polarization transfer)

3.2.5d Homonuclear Two-Dimensional Spectra

Data acquisition program necessary to plot two-dimensional H-H COSY following the double Fourier transform, which is required for developing phase-sensitive cross peak and diagonal peak multiplets with anti-phase fine structure were performed using Bruker software.

3.2.5e Heteronuclear Single-Quantum Coherence (HSQC) Spectroscopy

In HSQC analysis, an INEPT pulse transfer polarization from 1H to X via 1J(X) initially. Followed by an antiphase ^{13}C magnetization evolver during the variable t1 evolution period under the influence of X chemical shift. There after by applying a 180^{o} 1H pulse, heteronuclear (1H-X) coupling are refocused. X magnetization is converted to inphase 1H magnetization by aretro-INEPT pulse train. Further, X decoupling is performed for the acquisition of proton in HSQC analysis.

3.2.5f Heteronuclear Multiple Bond Correlation (HMBC) Spectroscopy

In HMBC analysis, to minimize direct responses, an optimal low pass J-filter is used after the initial 90^{o} 1H pulse. Followed by

optimization of defocusing period to ½*^{n}J(CH) (5-10 Hz). In this case refocusing period is omitted. Without decoupling, inter phase d_2 delay was optimized to ½*^{n}J(CH) (50-70 ms) to collect the HMBC spectra.

3.2.6 Characterization of Physical properties (Melting point and Specific Rotation)

Purified samples were used to estimate the melting point and specific rotation of the sample. Conventional methods were used to identify the melting point. In order to characterize the specific rotation, 0.1% sample solution in HPLC grade chloroform at 25°C (Fieser & Fieser, 1949; Fieser & Fieser, 1959) using a 10 cm cylindrical cell and the sodium D line with an optics manual polarimeter.

3.3 Results and Discussion

3.3.1 Compound I

Compound I (17.33 mg) was obtained as white amorphous solid with melting point at 119-120°C, specific rotation $[\alpha]_D$ (25°C) -43 and UV maximum at 282 nm. The molecular formula $C_{31}H_{50}O_2$ of the compound I was resolved from the molecular $[M+H]^+$ m/z ion peak at 455.538 from HRFABMS mass data. From the molecular formula, index of hydrogen deficiency was calculated to be 7 which highlight seven degrees of unsaturation in this compound.

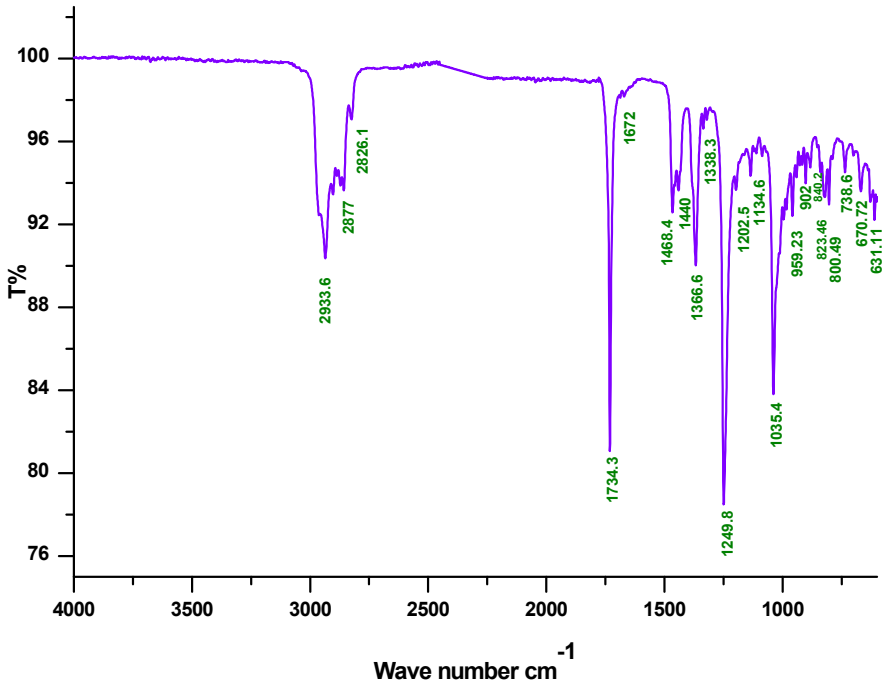


Figure 3.1 FTIR spectra of compound I.

In this molecule IR transmittance at 3482 cm^{-1} was absent in IR spectrum (Fig. 3.1) indicated the absence of hydroxyl groups. However, presence of intense band at 1734 cm^{-1} indicates the presence of C=O stretch in the molecule. Further, the presence of isolated double bonds in the molecule was deduced from the IR peaks at 1672 cm^{-1}. Peak at 1672 cm^{-1} along with C-H bending at 806 cm^{-1} validate C_5=C_6 double bond in the molecule. In this molecule CH_2 adjacent to olifienic carbon can be corroborated from 1468 and 1440 cm^{-1}. Peak at 1366 cm^{-1} corresponds to CH in plane bend, methyl groups.

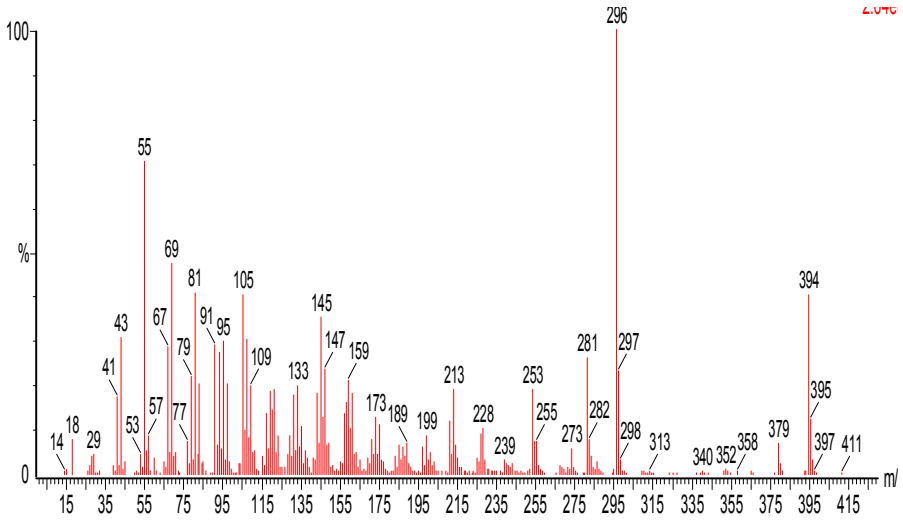


Figure 3.2 Mass spectra of compound I

Electron impact mass spectra of compound I with molecular weight 454 showed a moderately strong peak at m/z 394 (Fig. 3.2). Formation of an intense m/z peak at 394 is consistent with deacetylation of the compound [M-60]. This loss of [M-60] is a diagnostic tool for the ▲5 steryl acetates (Budzikiewicz et al., 1964; Galli & Maroni, 1967; Knights, 1967). This compound also consisted of m/z resulting from the fragmentation of neutral molecule elimination, specific hydrogen migration along with the loss of ring structures and alkyl side chain characteristic of an acetylated compound was observed. Absence of base peak or m/z at 217 is a direct indication for the lack of saturated side chain structure, which indirectly implies the presence of unsaturated side chain attached to the steroid nuclei. Formation of a very very feeble peak at m/z 411

indicate loss of m/z 43 ie., CH_3-C=O from the parent ion, leading to the formation of keto steroid ion. Meanwhile, m/z 122 results suggest the loss of C and D rings from▲^{1,4} -3 keto steroid fragment. Further, the presence of m/z at 213 and 255 indicate two unsaturation in the keto-steroid fragment. Again, a minor peak in the region of m/z 229 and 271 imply the presence of two unsaturation along with one oxygen functionality. Here, the deacylation proceeds through McLafferty rearrangement. Cleavage of C_{22}-C_{23} of deacteylated steroid nucleus result in the base peak at m/z 296 (Brooks et al., 1972).

Based on HRFABMS, FTIR and GC-MS data structure of the compound I can be tentatively assigned as shown in Fig. 3.3

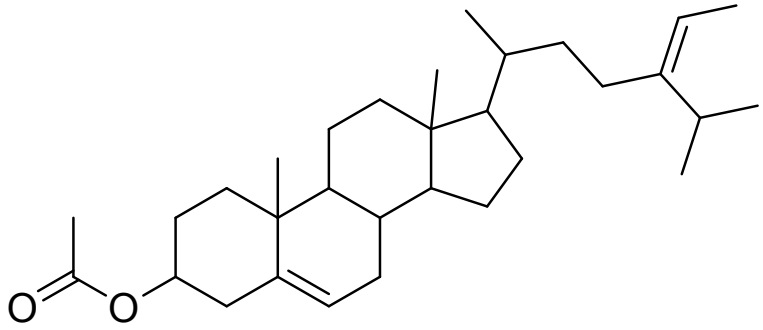


Figure 3.3 Structure of compound I

The proton NMR spectrum (Table 3.2) contained signals for seven methyl groups at δ_H 0.623, δ_H 0.68, δ_H 0.811, δ_H 0.98, δ_H 1.01, δ_H 1.57, and δ_H 2.02. Protons attached to C-3 position bearing a acetoxy group appears in the δ 4.4-4.8 region due to deshielding. Hence, the presence of 4.61 (dddt, *J* = 11.1, 9.0, 7.1, 4.8 Hz,) in

proton NMR spectra indicated the presence of a characteristic acetoxy group at C-3. J value of 11.1 Hz indicate an angle of 0^o or 180^o between the vicinal protons as the coupling constant (J value) is dependent on the angle between axial and equatorial orientation of proton in this position along with number and nature of the adjacent proton (Goad & Akisha, 1997). As substitution like methy group or unsaturation slightly shift the δ_H values. δ_H values of methyl group attached to acetoxy group 2.02 suggest the presence of unsaturation at Δ^4/ $\Delta^{5,7}$/ $\Delta^{5,7\ (9,11)}$. Presence of two olefinic protons were deduced from the presence of δ 5.37 (dq, *J* = 5.1, 1.5 Hz, 0H), and 5.18 ppm.

Table 3.2 Showing major proton splitting pattern and J values.

Sl No:	Proton shift	Splitting	J vaues
1	5.37	dq,	*J* = 5.1, 1.5 Hz
2	4.61	dddt	*J* = 11.1, 9.0, 7.1, 4.8 Hz
3	2.37 – 2.27	m	
4	2.03	s	
5	2.13 – 1.79	m	
6	1.67 – 1.36	m	
7	1.40 – 1.20	m	
8	1.23 – 1.01	m	
9	1.02	s	
10	1.04 – 0.78	m	
11	0.69	s	

The methyl, methylene and methine carbons present in this compound were assigned using the DEPT NMR measurements taken at 135°, 90°, and 45°. The results are shown in table 3.3. These carbons obtained were assigned to their corresponding directly attached protons using the HSQC experiments. Presence of 31 carbon atoms were corroborated from C-13 spectra (Table. 3.3). By comparing the C-13 and DEPT NMR presence of five quaternary carbons were inferred from δ_c values at 170, 146, 139.67, 42.36 and 36.61, nine methine carbon were observed in δ_c 122.621, 115, 74, 56.69, 55.63, 50.06, 36.430, 34.775 and 31.902, twelve methylene (δ_c 39.729, 38.140, 37.016, 35.224, 31.920, 28.234, 27.79, 25.733, 24.318 and 21.040 and seven methyl carbons at δ_c 22.24, 22.13, 21.41, 19.30, 18.76, 13.16 and 11.85. A partial molecular formula $C_{31}H_{50}$ can be accounted from these C-13 resonances. The presence of three double bounds in the compound accounts for three degree of unsaturation. This gives an indirect evidence for the presence of four cyclic ring structures.

Table 3.3 DEPT assignments and proton values based on HSQC data of compound I.

Sl No:	DEPT	C-13	H
1	C	170	
2	C	146	
3	C	139.67	
4	CH	122.621	5.37
5	CH	115	5.18
6	CH	74.15	4.60

7	CH	56.69	1.03
8	CH	55.63	1.27
9	CH	50.06	0.98
10	C	42.36	
11	CH2	39.729	2
12	CH2	38.14	2.32
13	CH2	37.016	1.87
14	C	36.61	
15	CH	36.430	1.88
16	CH2	35.224	0.98
17	CH	34.775	2.171
18	CH2	31.92	1.95
19	CH	31.902	1.46
20	CH2	28.234	1.08
21	CH2	27.79	1.87
22	CH2	25.733	2.034
23	CH2	24.318	1.58
24	CH3	22.24	0.98
25	CH3	22.13	0.98
26	CH3	21.41	2.02
27	CH2	21.04	1.55
28	CH3	19.3	1.01
29	CH3	18.76	1.01
30	CH3	13.16	1.57
31	CH3	11.85	0.68

In the case of compound I, C-13 and protons assignments were exclusively based on COSY correlations.

In the COSY spectrum, methine hydrogen atom attached to acetoxy group have proton signal at δ_H 4.60 (δ_c 74.15 as shown in HSQC spectrum) exhibited correlations with δ_H 2.32 and 1.87 of methylene carbons δ_c 38.14 and δ_c 27.79 respectively. The methylene carbon at δ_c 27.79 had ^1H chemical shift at δ_H 1.87. This ^1H signal at δ_H 1.87 is correlated to at δ_H 1.87 attached to δ_C 37.016. However, this ^1H signal is not correlated further to any other signal implying the presence of an adjacent quaternary carbon which might be connected to a methyl group. Absence of further connectivity between proton attached to δ_c 38.14 suggest, this signal is in between methine and a quaternary carbon. On the basis of these information first ring for this compound can be deduced as shown below. This accounts for the first unsaturation in the compound (second degree, I^{St} ring, Fig 3.4).

The chemical shift of quaternary carbon at δ_c 139.67 and δ_c 122.621 together with absence of adjacent sp^2 C suggests δ_c 139.67 and 121.621 are connected to each other. From HSQC spectral data δ_c 122.621 is attached to methine hydrogen atom at δ_H 5.37. The methine hydrogen at δ_H 5.37 (δ_c 122.621) displayed COSY coupling with methylene hydrogen at δ_H 1.95. In proton NMR spectra a doublet of quartet is having coupling constant (J) of 5.1 and 1.5 Hz. These small J values indicate, coupling methylene hydrogens are having small dihedral angle (Goad & Akisha, 1997). On the basis of HSQC δ_H 1.95 is having attachment to δ_c 31.92 and having

connection to δ_H 1.46 of δ_c 31.902. Further, this signal is connected to methine hydrogen δ_H 1.03 and δ_H 0.98. From HSQC, this proton signal at δ_H 1.03 is attached to 56.69. And δ_H 0.98 to δ_c 50.08. δ_c is showing only two connections one as already assigned to δ_c 31.902 and other to δ_c 21.04 suggest this methine carbon is connected to quarternary carbon of the first ring as shown below. This accounts for the next two unsaturation in the compound (Fig 3.4).

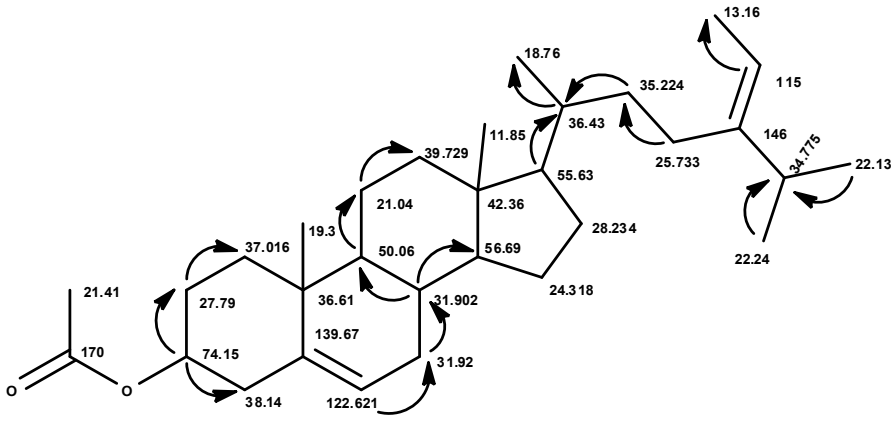


Figure 3.4 Image showing COSY correlations of compound I.

Proton Signal δ_c 21.10 is having connections to methylene δ_H 2.00 (δ_c 39.73). However, this methylene proton signal lacks further connections implying the presence of quaternary carbon having a methyl group attached to it. Similarly, only one COSY connection for δ_c 56.78 to δ_H 1.57 (δ_c 24.32) also points out to the presence of adjacent quaternary carbon atom as shown below. Which, thereby accounting for the forth unsaturation in the molecule (Fig 3.4).

Methyl hydrogen at δ_H 1.57 (δ_c 24.32) is having COSY connections towards methyl δ_H 1.08 which is attached to δ_c 28.234 as per HSQC data. This methyl ^1H signal at δ_H 1.08 is having connection to methine δ_H 1.27 (δ_C 55.63). Presence of only one connection, ie., to δ_H 1.88 (δ_c 36.43) further suggest, it might be connected to quaternary carbon, resulting in the forth ring and there by accounting for the fifth unsaturation in the molecule as shown Fig. 3.4 .

In the side chain δ_H 1.88 attached to δ_c 36.43 is correlated to δ_H 1.01 of δ_c 18.76 and δ_H 1.88 of δ_c 35.224. Proton shift at δ_H 1.88 is also correlated δ_H 2.034 of δ_c 25.733. However, there was no other correlation observed for δ_H 2.034 suggesting its attachment to a quarternary carbon. Other COSY correlations observed are between δ_H 5.18 of δ_c 115 and δ_H 1.57 of δ_c 13.6 along with δ_H 0.98 of δ_c 22.24 and δ_H 0.98 of δ_c 22.13 to δ_H 2.18 (δc 34.77). Only one correlation shown by δ_H 5.18 of δ_c 115 suggest it is bonded to quaternary carbon δ_c 146 which account for the sixth unsaturation. Seventh unsaturation in the molecule is accounted to carbonyl double bond in this molecule (Fig 3.4).

Based on melting point, specific optical rotation , FTIR, UV-Vis, GC-MS, proton NMR , C-NMR, DEPT, H-H COSY and HSQC, compound I is Stigmasta-5,24(28)-dien-3β-ol, acetate (IUPAC-IUBJCBN, 1989) commonly known as fucosterol acetate. This structure is further supported by the work of Itoh et al. (1980) and Akihisa et al. (1991) reported in journal Lipids.

3.3.2 Compound II

Compound II (8.35 mg) was isolated as white amorphous solid with melting point at 156-157, specific rotation $[\alpha]_D$ (25^0C) -28 and UV maximum at 243 nm. The molecular formula $C_{32}H_{52}O$ of the compound II was resolved from the molecular m/z $[M+H]^+$ ion peak at 453.63 from HRFABMS mass data. From the molecular formula, index of hydrogen deficiency was calculated to be 7 which highlight seven degrees of unsaturation in this compound.

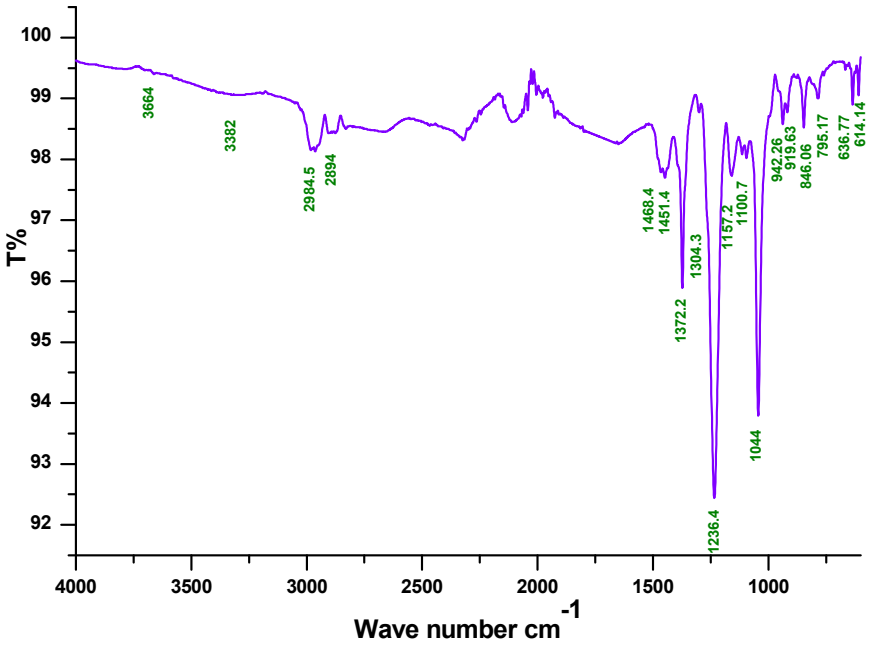


Figure 3.5 FTIR spectra of compound II.

Presence of an IR transmittance at 3382 cm^{-1} in IR spectrum (Fig. 3.5) indicated the presence of hydroxyl groups in the molecule.

Presence of double bonds in the molecule was deduced from the IR peaks at 1672 cm^{-1}. Occurrence of IR peak at ~1044 cm^{-1} corresponds to the presence of highly polarized OH group vibration at C3 along with the existence of C_5=C_6 double bond in the molecule. Further, formation of this intense vibration at 1044 cm^{-1} results from the increase in π electron density along with the formation of intra-molecular hydrogen bond between C_3-OH and C_5=C_6 double bond. Stretching of C=C at 1672 cm^{-1} along with C-H bending observed at 846 cm^{-1} substantiate the presence of double bond at C_5=C_6 position. Presence of 1451 cm^{-1} suggests the existence of CH_2 attached to olifienic carbon in this compound.

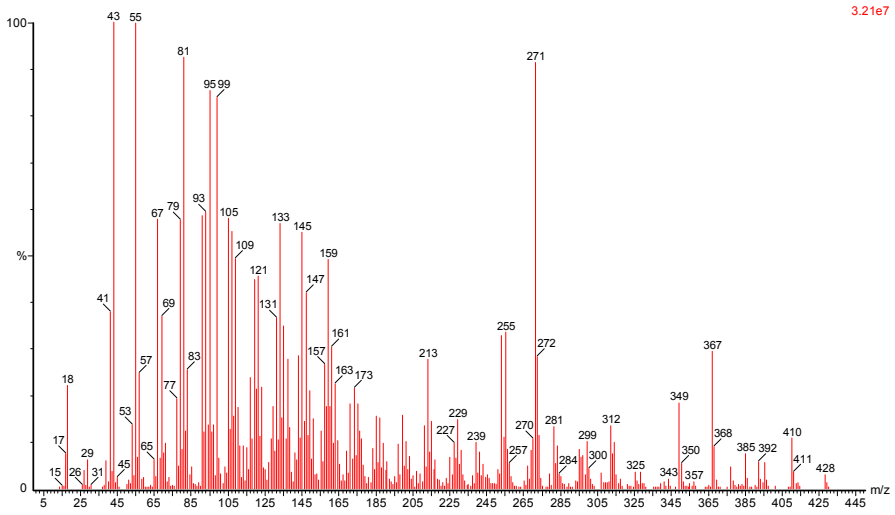


Figure 3.6 Mass spectra of compound II.

Electron impact mass spectra of this compound is shown in the Fig. 3.6 Base peak of this compound was observed at m/z 271. This

intense peak at m/z 271 result from the fragmentation of the compound in which side chain along with two hydrogen atoms are lost from the nucleus of the compound. This loss from the nucleus of the molecule is inferred from the presence of peak at 191.5, a metastable peak. Further, in the mass spectra, compared to m/z 271 a low intensity peak at m/z 314 is observed. This low intensity peak at m/z 314 corresponds to the cleavage of 22-23 bond along with the transfer of a hydrogen atom from the charged parent ion. In natural sterols, this mode fragmentation resulting in the formation of m/z 314 have been substantiated by "McLafferty" type rearrangement. In this case hydrogen atom which participate in the transfer originate from the C-20. Presence of peak at m/z 257 might be generated from the parent ion after the removal of hydrogen. Here the fragmentation is analogous to the formation of m/z 271 ie., through loss of side chain along with transfer of hydrogen atom influenced by the presence of unsaturation in the side chain. In this case, C-17 provides one of the hydrogen atom among the two that are participating in the rearrangement of hydrogen. Skeletal rearrangement is inferred as transfer of hydrogen from C-17 through two bond cleavage is not possible while mass fragmentation. In this skeletal rearrangement, a migration of methyl group from C-18 to C-17, which is deficient in electron due to the loss of hydrogen atom. Homolytic cleavage of C17- C20 bond after the abstraction of hydrogen atom leads to the formation of allylic stabilized m/z 257. Cleavage of 24-25 carbon bond along with transfer of two hydrogen atom from the charged

molecular ion result in the formation of m/z 349. A small m/z at 410 is analogous to M-43, corresponds to the loss of isopropylidiene group together with two hydrogen atom from the M^+.

Based on HRFABMS, FTIR and GC-MS data structure of the compound II can be tentatively assigned as shown in Fig. 3.7

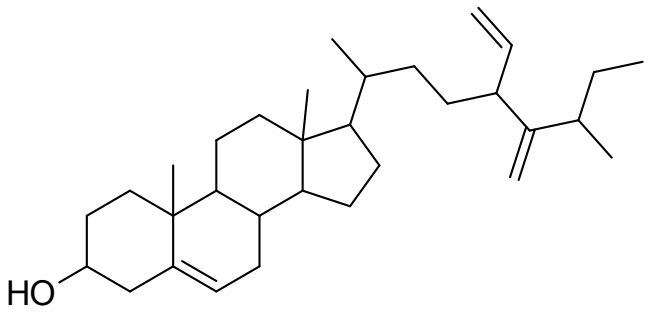


Figure 3.7 Structure of compound II

The proton NMR spectrum contained signals (Table 3.4) for five methyl groups at δ_H 0.68, δ_H 0.88, δ_H 0.89, δ_H 0.92 and δ_H 1.01. Presence of 3.52 in proton NMR spectra indicated the presence of a characteristic hydroxyl group at C-3. Protons attached to C-3 position bearing a hydroxyl group appears in the δ_H 3.5-4.5 region due to dishielding. Axial and equatorial orientation of proton in this position along with number and nature of the adjacent proton plays a vital role in the shape and position of this proton resonance. J value of 11.0 Hz indicates an angle of 0° or 180° between the vicinal protons as the coupling constant (J value) is dependent on the angle between these

protons (Goad & Akisha, 1997). Usually the signals are observed as septet; however, geometry of the adjacent double bond has profound influence on the value of shift. Presence of three olefinic protons were deduced from the presence of δ 5.80 (ddd, *J* = 17.5, 10.9, 5.5 Hz, 2H), 5.35 (dt, *J* = 5.3, 1.9 Hz, 2H), 5.24 – 5.09 (m, 2H).

Table 3.4 Major proton splitting pattern and J values.

Sl No:	Proton shift	Splitting	J vaues
1	5.80	ddd	*J* = 17.5, 10.9, 5.5 Hz
2	5.35	dt,	*J* = 5.3, 1.9 Hz
3	5.24 – 5.09	m	
4	3.52	tdd	*J* = 11.0, 5.3, 4.1 Hz,
5	2.37 – 2.17	m	
6	2.05 – 1.92	m	
7	1.89 – 1.78	m	
8	1.83 – 1.68	m	
9	1.73 – 1.50	m	
10	1.54 – 1.38	m	
11	1.42 – 1.25	m	
12	1.26	d	*J* = 2.6 Hz
13	1.27 – 1.03	m	
14	1.01	s	
15	1.02-0.79	m	
16	0.67	s	

The methyl, methylene and methine carbons present in this compound were assigned using the DEPT NMR measurements taken at 135°, 90°, and 45°. The results are shown in table 3.5. These carbons obtained were assigned to their corresponding directly attached protons using the HSQC experiments. Presence of 32 carbon atoms were corroborated from C-13 and DEPT NMR spectra (Table 3.5). Presence of four quarternary carbons were inferred from δ_c values at 142.50, 140.78, 36.52 and 36.52. ten methine carbon were observed in δ_c 142.579, 121.689, 71.816, 56.779, 55.855, 50.149, 36.146, 36.087, 35.928, and 31.923, thirteen methylene (δ_c 112.89, 112.854, 42.38, 39.78, 37.273, 34.801, 31.73, 31.94, 29.113, 28.219, 28.171, 24.284, and 21.091, and five methyl carbons at δ_c 11.857, 16.462, 17.559, 18.790, and 19.935. A partial molecular formula $C_{32}H_{51}$ can be accounted from these C-13 resonances, which, suggest the presence of hydroxyl group, supported by the FTIR data. Presence of three double bounds in the compound accounts for three degree of unsaturation. This gives an indirect evidence for the presence of four cyclic ring structures.

Table 3.5 DEPT assignments and proton values based on HSQC data of compound II.

Sl No:		C-13	H
1	CH	142.579	5.81
2	C	142.50	
3	C	140.78	
4	CH	121.689	5.36
5	CH2	112.89	5.16
6	CH2	112.854	5.21
7	CH	71.816	3.53
8	CH	56.779	1.07
9	CH	55.855	1.09
10	CH	50.149	0.92
11	CH2	42.38	2.28, 2.23
12	CH2	39.78	1.15, 2.00
13	CH2	37.273	1.08, 1.83
14	C	36.52	
15	C	36.52	
16	CH	36.146	1.72
17	CH	36.087	1.73
18	CH	35.928	1.38
19	CH2	34.801	1.57, 1.60
20	CH	31.923	1.96
21	CH2	31.94	1.49, 1.95
22	CH2	31.73	1.83
23	CH2	29.113	1.02
24	CH2	28.219	1.41
25	CH2	28.171	1.83
26	CH2	24.284	1.57
27	CH2	21.091	1.49
28	CH3	19.935	1.01
29	CH3	18.790	0.93
30	CH3	17.559	0.88
31	CH3	16.462	0.91
32	CH3	11.857	0.68

Structural determination of the compound II was exclusively based on COSY and HMBC correlations. In the COSY spectrum, methine hydrogen atom attached to –OH having proton signal at δ_H 3.53 (δ_c 71.816 as shown in HSQC spectrum) showed correlations with methylene carbons at δ_c 42.38 and δ_c 31.73. The methylene carbon at δ_c 31.73 had ^1H chemical shift at δ_H 1.83. This ^1H signal at δ_H 1.83 is correlated to at δ_H 1.08 attached to δ_C 37.273. However, this ^1H signal is not correlated further to any other signal implying the presence of an adjacent quaternary carbon connected to a methyl group. Absence of further connectivity between proton attached to δ_c 42.38 (δ_H 2.28) suggest this signal is in between methine and a quaternary carbon. This information accounts for first ring of this compound and also for the first unsaturation in the compound (second degree, I St ring, Fig. 3.8).

The chemical shifts of quarternary carbon at δ_c 140.78 and δ_c 121.689 together with absence of adjacent sp^2 C suggest δ_c 140.78 and 121.689 are connected to each other. From HSQC spectral data δ_c 121.689 is attached to methine hydrogen atom at δ_H 5.36. The methine hydrogen at δ_H 5.36 (δ_c 121.68) showed COSY coupling with methylene hydrogen at δ_H 1.95. In proton NMR spectra a doublet of triplet having coupling constant (J) of 5.3, 1.9 Hz. These small J values indicate, methylene hydrogens with small dihedral angle. On the basis of HSQC, δ_H 1.95 is having attachment to δ_c 31.94 and having connection to δ_H 1.96 (δ_c 31.923). Further more, this signal is connected to methine

hydrogen $\delta_{H.}$ 0.92 and δ_H 1.07 From HSQC, this proton signal at δ_H 0.92 is attached to 50.149. And δ_H 1.07 to δ_c 56.779. δ_c 50.149 is exhibited only two connections one as already assigned to δ_c 31.923 and other to δ_c 21.091 suggest this methine carbon is connected to quarternary carbon of the first ring as shown in Fig. 3.8, accounts for the next two unsaturation in the compound.

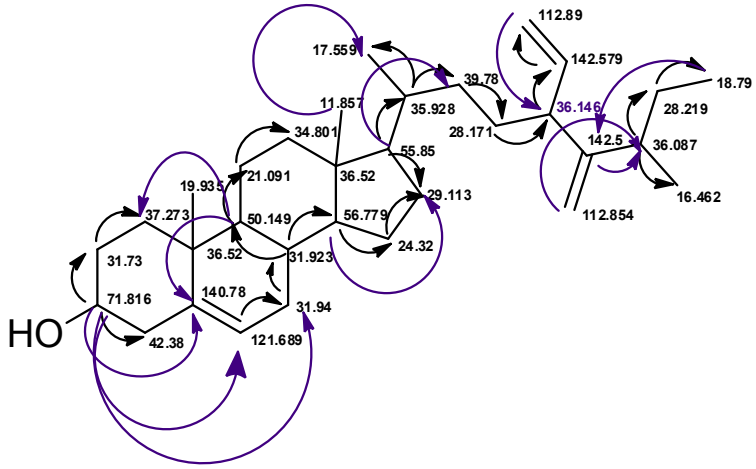


Figure 3.8 COSY correlations of compound II are shown in black arrows, HMBC correlations are shown in violet arrow.

Proton Signal δ_c 21.091 is having connections to methylene δ_H 1.57 (δ_c 34.801). However, this methylene proton signal lacks further connections implying the presence of qurternery carbon having a methyl group attached to it. Similarly, only one COSY connection for δ_c 56.779 to δ_H 1.62 (δ_c 24.32) also points to the presence of adjacent quarternary carbon atom as shown below. This confirms the forth unsaturation in the molecule (Fig. 3.8).

Methyl hydrogen at δ_H 1.62 (δ_c 24.32) is having COSY connections towards methyl δ_H 1.02 which is attached to δ_c 29.113 as per HSQC data. This methyl ^1H signal at δ_H 1.02 is having connection to methine δ_H 1.09 (δ_C 55.855). Presence of only one connection, ie. to δ_H 1.38 (δ_c 35.928) further suggest that it might be connected to quarternary carbon, resulting in the forth ring and there by accounting for the fifth unsaturation in the molecule (Fig. 3.8).

Methine δ_H 1.38 (δ_c 35.928) is connected to proton correlated δ_c 17.559 (δ_H 0.88) and δ_c 39.78 (δ_H 1.15). δ_H 1.15 is correlated to δ_H 1.83 (δ_C 28.171). This methyl proton attached to δ_C 28.219 is correlated to δ_H 1.72 attached to δ_C 36.146. This proton attached to methine carbon is connected to methine carbon δ_C 142.579 with δ_H value 5.81; however, further connections were not observed suggesting the presence of adjacent quaternary carbon δ_C 142.5. Whereas δ_H 5.81 is connected to δ_H 5.16 which is attached to δ_C 112.89 accounting for the sixth unsaturation in the molecule. From the absence of correlations by δ_H 5.21 which is attached to δ_C 112.854, it can be presumed that it formed a bond with quaternary carbon δ_C 142.5, accounting for the seventh unsaturation. Other correlations observed are between δ_H 1.73 to δ_H 1.41 and δ_H 0.91 of δ_C 28.219 (methylene) and δ_C 16.462 (methyl carbon) respectively. Proton attached to methylene is further connected to δ_H 0.93 of methyl carbon (δ_C 18.79) (Fig. 3.8).

Side chain assignments of compound II were further confirmed by HMBC correlations (Fig. 3.8). Carbon at δ_C 11.857 is correlated to δ_C 11.857, δ_C 55.855 to δ_C 39.78, δ_C 112.89 to δ_C 36.146, δ_C 112.85 to δ_C 36.087 and δ_C 142.5 to 18.79. Finally, some of the prominent ring correlations was observed between δ_C 140.78 and δ_C 42.38, and week correlations of δ_C 31.94 towards δ_C 71.816 . These correlations along with δ_C 50.149 to δ_C 37.273 and δ_C 56.779 to δ_C 29.113 establish the structure of compound II.

Based on melting point, specific optical rotation, FTIR, UV-Vis, GC-MS, proton NMR, C-NMR, DEPT, H-H COSY, HSQC, and HMBC, compound II is 27ethyl, 27methyl-stigmasta-5, $24^1(24^2)$, 25-triene-3β-ol (IUPAC-IUBJCBN, 1989). The compound has assigned the trivial name as "Turbiconol".

3.4 Conclusion

Compound I

IUPAC name: Stigmasta-5, 24(28)-dien-3β-ol, acetate

OR

(E)-17-(5-isopropylhept-5-en-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradechydro-1H-cyclopenta[a]phenanthren-3-yl acetate

Trivial name: Fucosterol acetate

Molecular formula: $C_{31}H_{50}O_2$

Melting point M.p. ($^{\circ}$C) = 143-144

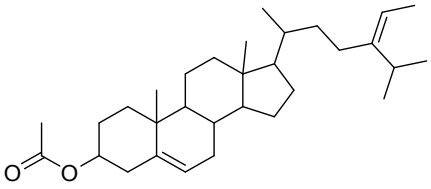
Specific rotation $[\alpha]_D$ (25°C) = -33

UV maximum λ_{max} at 282 nm.

IR: 1734 cm^{-1} corresponds to C=O stretch, 1672 cm^{-1} corresponds to double bond in the sterol molecule, 1468 cm^{-1} and 1440 cm^{-1} corresponds to olefinic carbon.

MS m/z: 296, 294, 281, 353

Based on proton NMR, C-13 NMR, DEPT, H-H COSY and HSQC data, structure of the compound can represented as given below.



Compound II

IUPAC name: 27 ethyl, 27 methyl-stigmasta-5,

$24^1(24^2)$, 25-triene-3β-ol

OR

10,13-dimethyl-17-(7-methyl-6-methylene-5-vinylnonan-2-yl 2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-3-ol

Trivial name: Turbiconol

Molecular formula: $C_{32}H_{52}O$

Melting point M.p. ($^{\circ}C$) = 156-157

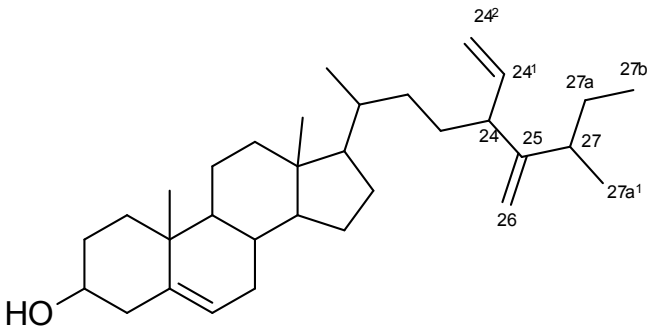
Specific rotation $[\alpha]_D$ ($25^{\circ}C$) = -28

UV maximum λ_{max} at 243 nm.

IR: 3382 cm^{-1} corresponds to OH, 1044 cm^{-1} corresponds to highly polarized –OH adjacent to C_5=C_6 conjugated and 1672 cm^{-1} corresponds to double bond in the sterol molecule.

MS m/z: 271, 257, 314, 367, 410

Based on proton NMR, C-13 NMR, DEPT, H-H COSY, HSQC and HMBC data, structure of the compound can represented as.



3.5 References

Acuna-Johnson, P., & Oehlschlager, A. C. (1989). Identification of sterols and biologically significant steroids by ultraviolet and infrared spectroscopy. *Analysis of Sterols and Other Biologically Significant Steroids*, 267-84.

Akihisa, T., Kokke, W. C. M. C., Tamura, T., & Matsumoto, T. (1991). Sterols of Kalanchoe pinnata: First report of the isolation of both C-24 epimers of 24-alkyl-Δ25-sterols from a higher plant. *Lipids*, *26*(8), 660-665.

Akihisa, T., Nes, W. D., & Parish, E. J. (1989). Analysis of sterols and other biologically significant steroids. *San Diego: Academic Press*, 251-284.

Akihisa, T., Thakur, S., Rosenstein, F. U., & Matsumoto, T. (1986). Sterols of cucurbitaceae: The configurations at C-24 of 24-alkyl-Δ5-, Δ7- and Δ8-sterols. *Lipids*, *21*(1), 39-47.

Benn, R., & Günther, H. (1983). Modern pulse methods in high-resolution NMR spectroscopy. *Angewandte Chemie International Edition in English*, *22*(5), 350-380.

Bhacca, N. S., & Williams, D. H. (1964). Applications of NMR spectroscopy in organic chemistry; illustrations from the steriod field . "Holden-Day series in physical techniques in chemistry, Holden-Day,San Francisco.

Blunt, J. W., & Stothers, J. B. (1977). 13C NMR spectra of steroids-a survey and commentary. *Organic Magnetic Resonance*, *9*(8), 439-464.

Brooks, C. J. W., Knights, B. A., Sucrow, W., & Radüchel, B. (1972). The characterisation of 24-ethylidene-sterols. *Steroids*, *20*(4), 487-497.

Budzikiewicz, H., Djerassi, E., & Williams, D. H. (1964). Structure elucidation of natural products by mass spectrometry, vol. 2. Holden Day, San Francisco

Croasmun, W. R., Carlson, R. M. K., Croasmun, W. R., & Carlson, R. M. K. (1987). Methods in stereochemical analysis. VCH publishers, New York.

Croasmun, W. R., & Carlson, R. M. (1994). Two-dimensional NMR spectroscopy: applications for chemists and biochemists (Vol. 15). John Wiley & Sons, Hoboken , New jersey.

Derome, A. E. (1987). Modern NMR techniques for chemical research. *Tetrahedron Organic Chemistry Series*, *6*.

Djerassi, C., Karliner, J., & Aplin, R. T. (1965). Mass spectrometry in structural and stereochemical problems LXXVIII. Steroidal Δ 4-3, 6-diketones. *Steroids*, *6*(1), 1-8.

Dorfman, L. (1953). Ultraviolet absorption of steroids. *Chemical Reviews*, *53*(1), 47-144.

Dusza, J. P. (1960). Contributions to the study of marine products. XLIX. Synthesis of 29- Isofucosterol1, 2. *The Journal of Organic Chemistry*, 25(1), 93-96.

Eneroth, P., Hellström, K., & Ryhage, R. (1964). Identification and quantification of neutral fecal steroids by gas–liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens. *Journal of Lipid Research*, *5*(2), 245-262.

Eneroth, P., Hellström, K., & Ryhage, R. (1965). Identification of two neutral metabolites of stigmasterol found in human feces: Bile acids and sterols 162. *Steroids*, *6*(6), 707-720.

Fieser, L. F., & Fieser, M. (1949). Natural Products Related To Phenanthrene. 3rd. Reinhold. New York.

Fieser, L. F., & Fieser, M. (1959). Steroids, Reinhold. New York, 271-274.

Fried, B., & Sherma, J. (1999). Thin-layer chromatography, revised and expanded (Vol. 81). CRC Press

Forgacs, E., & Cserhati, T. (1997). Molecular basis of chromatographic separation. (In) Adsorption phenomena and molecular interactions in chromatography, pp 10-13, New York ; CRC Press Inc.

Galli, G., & Maroni, S. (1967). Mass spectrometric investigations of some unsaturated sterols biosynthetically related to cholesterol. *Steroids*, *10*(3), 189-197.

Gibbons, G. F., Mitropoulos, K. A., & Myant, N. B. (1982). Biochemistry of cholesterol. Elsevier Biomedical Press,-Amsterdam.

Goad, J., & Akihisa, T. (1997). Analysis of sterols. Springer Science and Business Media, Berlin Germany.

Gruss, W. J., & Demarest, D. C. (1912). U.S. Patent No. 1,025,185. Washington, DC: U.S. Patent and Trademark Office.

Gutmann, V. (1978). The Donor-acceptor approach to molecular interactions. Plenum Press, New York, USA.

Heupel, R. C. (1989). Isolation and primary characterization of sterols . Academic Press, Inc.: San Diego, CA, USA.

IUPAC-IUBJCBN. (1989). The nomenclature of steroids. Recommendations 1989. *European Journal of Biochemistry, 186*, *429*-458.

Itoh, T., Sakurai, S., Tamura, T., & Matsumoto, T. (1980). Occurrence of 24 (E)-ethylidene sterols in two solanaceae seed oils and rice bran oil. *Lipids*, *15*(1), 22-25.

Itoh, T., Sica, D., & Djerassi, C. (1983). Minor and trace sterols in marine invertebrates. Part 35. Isolation and structure elucidation of seventy-four sterols from the sponge *Axinella cannabina*. *Journal of the Chemical Society, Perkin Transactions*, *1*, 147-153.

Kaliszan, R. (1987). Quantitative structure-chromatographic retention relationships. United States: John Wiley and Sons, New York.

Kaliszan, R. (1997). Structure and retention in chromatography: A chemometric approach. Harwood Academic Publishers, Amsterdam, the Netherlands.

Kawagishi, H., Katsumi, R., Sazawa, T., Mizuno, T., Hagiwara, T., & Nakamura, T. (1988). Cytotoxic steroids from the mushroom Agaricus blazei.*Phytochemistry*, *27*(9), 2777-2779.

Kerr, R. G., Kerr, S. L., Pettit, G. R., Herald, D. L., Groy, T. L., & Djerassi, C. (1991). Sterols of marine invertebrates. 63. Isolation and structure elucidation of sutinasterol, the major sterol of the marine sponge *Xestospongia sp*. *The Journal of Organic Chemistry*, 56(1), 58-62.

Knights, B. A. (1967). Identification of plant sterols using combined GLC/mass spectrometry. *Journal of Chromatographic Science*, *5*(6), 273-282.

Koizumi, N., Fufunoto, Y., Takeshita, T., & Ikekawa, N. (1979). Carbon-13 nuclear magnetic resonance of 24-substituted steroids. *Chemical and Pharmaceutical Bulletin ,* 27(1), 38-42.

Komsta, L., Waksmundzka-Hajnos, M., & Sherma, J. (Eds.). (2013). Thin layer chromatography in drug analysis. CRC Press.

Liljefors, T., & Allinger, N. L. (1978). Conformational analysis. 128. The Woodward-Fieser rules and. Alpha, beta, unsaturated ketones. *Journal of the American Chemical Society*, *100*(4), 1068-1073.

Minale, L., & Sodano, G. (1974a). Marine sterols: 19-nor-stanols from the sponge *Axinella polypoides*. *Journal of the Chemical Society, Perkin Transactions*, *1*, 1888-1892.

Minale, L., & Sodano, G. (1974b). Marine sterols: unique 3β-hydroxymethyl-A-nor-5α-steranes from the sponge *Axinella verrucosa*. *Journal of the Chemical Society, Perkin Transactions*, *1*, 2380-2384.

Neher, R., & Wettstein, A. (1952). About steroid. 109. Communication. Paper chromatography of weakly polar compounds. *Helvetica Chimica Acta*, *35*(1), 276-283.

Nes, W. R., & McKean M. L. (1977). Biochemistry of steroids and other isopentanoids, University park Press, Baltimore, MD, USA.

Nes, W. R. (1985). [1] A comparison of methods for the identification of sterols. *Methods in Enzymology*, *111*, 3-37.

Nes, W. R., Castle, M., McClanahan, J. L., & Settine, J. M. (1966). Confirmation of the structure of fucosterol by nuclear magnetic resonance spectroscopy. *Steroids*, *8*(5), 655-657.

Neudert, W., Ropke, H., & Leane, J. B. (1965). Atlas of steroid spectra. Springer-Verlag, Berlin.

Rubinstein, I., Goad, L. J., Clague, A. D. H., & Mulheirn, L. J. (1976). The 220 MHz NMR spectra of phytosterols. *Phytochemistry*, *15*(1), 195-200.

Sashida, Y., Kawashima, K., & Mimaki, Y. (1991). Novel polyhydroxylated steroidal saponins from *Allium giganteum*. *Chemical and Pharmaceutical Bulletin*, *39*, 698-703.

Schmitz, F. J., Campbell, D. C., & Kubo, I. (1976). Marine natural products: 5α-cholestane-3β, 5, 6β, 9-tetrol: a polyhydroxylated steroid from the gorgonian *Pseudopterogorgia elisabethae*. *Steroids*, *28*(2), 211-221.

Sheikh, Y. M., & Djerassi, C. (1974). Steroids from sponges. *Tetrahedron*, *30*(23), 4095-4103.

Sheu, J. H., Wang, G. H., Sung, P. J., & Duh, C. Y. (1999). New cytotoxic oxygenated fucosterols from the brown alga *Turbinaria conoides*. *Journal of Natural Products*, *62*(2), 224-227.

Silverstein, R. M., Webster, F. X., Kiemle, D. J., & Bryce, D. L. (2014).Spectrometric identification of organic compounds. John Wiley and Sons.

Spangenberg, B., Poole, C. F., & Weins, C. (2011). Quantitative thin-layer chromatography: a practical survey. Springer-Verlag, Berlin Heidelberg.

Sright, J. L. C., McInnes, A. G., Shimizu, S., Smith, D. G., Walter, J. A., Idler, D., & Khalil, W. (1978). Identification of C-24 alkyl epimers of marine sterols by 13C nuclear magnetic resonance spectroscopy. *Canadian Journal of Chemistry*, *56*(14), 1898-1903.

Thompson, M. J., Patterson, G. W., Dutky, S. R., Svoboda, J. A., & Kaplanis, J. N. (1980). Techniques for the isolation and identification of steroids in insects and algae. *Lipids*, *15*(9), 719-733.

Tokes, L., Jones, G., & Djerassi, C. (1968). Mass spectrometry in structural and stereochemical problems. CLXI. Elucidation of the course of the characteristic ring D fragmentation of steroids. *Journal of the American Chemical Society*, *90*(20), 5465-5477.

Chapter 4

CYTOTOXICITY OF STEROLS ISOLATED FROM *Turbinaria conoides* ON A549 AND Hep G2 CELL LINES

4.1 Introduction

Drug screening assays, a primary assessment for the pharmaceutical potential of a compound, are generally based on various cell functions such as enzyme activity, cell permeability, cell adherence, ATP production, co-enzyme production and nucleotide uptake activity. However, several models of screening studies for drug testing are ethically and practically difficult to perform in animals. Hence, cell lines are extensively preferred in drug screening, as it is possible to handle and execute molecular (genetic and/or epigenetic) characterization easily (Van Staveren et al., 2009). Further, these cell models can be both exclusively and actively used to deduce the key cell signaling pathways and in the disclosure of genes involved (Jordan & Wilson, 2004; Garnett, 2012). Additionally, drug testing in tumor cell lines provides access to a

large number of potential drugs before they are subjected to expensive *in vivo* clinical studies. When compared to other models used for studying the therapeutical potential of anti-cancer agents, data generated using cell lines not just evaluate the cytotoxicity but also give detailed information regarding the effect of drugs, mode of action, identification of resistance/sensitivity etc. (Tsuruo et al., 2003; Lacroix & Leclercq, 2004; Van Staveren et al., 2009).

Informations, regarding the mechanisms of action and resistance/sensitivity to chemotherapeutic agents in cancer treatment and simultaneous detection of specific markers for genetic and epigenetic changes with the aid of cell lines are vital for the development of new cancer drugs with specificity (Lacroix & Leclercq, 2004; Nakatsu et al., 2005; Louzada et al., 2012). It can be effectively used in the design of drug therapy and to identify new opportunities to specifically target cancer cells (Finlay & Baguley, 1984; Copeland et al., 2007). Finally, cell lines are cells with activated oncogene, which retain transcription of the genomic liberalization of the primary tumors and are independent of extracellular regulators, making them one of the best models for testing anticancer drugs (Neve et al., 2006).

To study the pharmaceutical potential of the compounds fucosterol acetate and turbiconol isolated from *Turbinaria conoides*, different assays have been collectively performed in these cell lines

for generating reliable data. Choosing of applicable and apt test conditions are important to provide relevant results. To determine whether apoptosis or secondary necrosis is the main mechanism of cell death, close examination of metabolism, membrane integrity and cytoplasmic content in the culture medium, prolonged incubation periods are essential. These experiments are so effective that they not only give the mechanism of the cell death but also give informations on the kinetics of cell death in a model system critical for drug development. Basic understanding of the changes that occurs in the different mechanisms of cell death helps us to decide the end point of the cytotoxicity tests (Finlay & Baguley, 1984; Copeland et al., 2007).

Even though assays can measure multiple markers indicating the number of dead cells (cytotoxicity test), the number of living cells (viability assay), and cell death mechanism (for example, apoptosis), reagents used for this metabolic markers should be stable at room temperature for a sufficient period of time; so as to generate stable and reproducible signal output (Farfan et al., 2004; Riss et al., 2005). Furthermore, using one of the cell lines in suspension instead of using cell lines that tend to grow in clumps for optimum incubation period is also adapted to reduce the edge effect, maintain the reproducibility and the stability of rehearsals signal. This offers the convenience and flexibility in recording data and minimizes differences in the treatment of large number of replicates. Finally, to

generate high-quality data with greater accuracy, speed and efficiency homogenous assay along with multiplexing is preferred. As it provide the benefit of performing multiple assays simultaneously and a holistic picture of what's going right in the cell viability when dosed with different drug concentration in a stretch (Riss et al., 2005).

There are only few effective ways to control/treat cancer, hence there is a great deal of research going on to control this menace. Researchers are therefore, developing novel drugs and identifying new pathways and mechanism to target specific part of cancer cells/their surroundings to suppress their activity. In this chapter, a conscious effort is made to delineate the cytotoxic effect of isolated compounds and mechanism by which sterols inhibits cancer cell growth, for that we examined the effect of two sterols isolated from *Turbinaria conoides* to induce antiproliferative and apoptotic events in on A549 (lung carcinoma) and Hep G2 (hepatic carcinoma) cells cultures with appropriate control. MTT, Neutral red assay, Lactate dehydrogenase assay and double staining are opted in the present investigation. These examinations explored basic anti-cancer activity of isolated sterols and mode of action. Further, these studies also investigated the cell cycle arrest by FAC Scan flow cytometry. Finally, appropriate gates were also defined for the evaluation of DNA content in G1, S and G2 phase. These results were further

supported by examining the expression of p21 gene in sterol treated cancer cell lines.

4.2 Materials and Methods

4.2.1 Cell Culture

For providing reliable and reproducible cytotoxic results in this study, apt selection of different assay sets and biological representative cell lines are crucial. In this regard, cell line A549 cells were selected to investigate anticancer potential of fucosterol acetate and turbiconol, against lung cancer and Hep G2 cells for liver cancer.

Cell line A549 cells are human epithelial basal alveolar adenocarcinoma, which can synthesize lecithin and contains high level of desaturated fatty acids, useful for the maintenance of cell membrane phospholipids (Speirs et al., 1991; McCormick & Freshney, 2000). Specifically these cell lines give fundamental informations regarding the drug metabolism in type II pulmonary (lung) epithelial cells and function as an *in vitro* model for its transfection host. In biological systems, these cells are responsible for the propagation of squamous substances such as water and electrolytes into the alveoli of the lungs (Speirs et al., 1991; McCormick & Freshney, 2000). Moreover, Hep G2 cell lines, the human liver cancer, used in this study is a convenient model system for the *in vitro* study of polarized human hepatocytes in the

laboratory. Due to their high degree of morphological and functional differentiation *in vitro*, HepG2 cell model is appropriate for studying the dynamics of intracellular transport and bile ducts and the sinusoidal membrane proteins and lipids in human hepatocytes (Ihrke et al., 1998; Herrema et al., 2006; Abreu et al., 2011).

Human alveolar epithelial carcinoma (A549) and Hepatic carcinoma cell lines used in the present study were purchased from NCCS Pune. Each cell lines maintained in Dulbeccos modified eagles media (DMEM/F2) supplemented with 10% FBS and antibiotics such as 100 µg/mL pencilin G, 100 mg/mL streptomycin and 250 ng/mL amphotericin (Invitrogen) in an incubator maintained at humidified 5% CO_2 atmosphere and an optimum temperature of 37°C. When the cells reached confluence after the four days of incubation they were treated with 0.5% (W/V) trypsin (0.25% , Invitrogen, USA) EDTA for 3 minutes at 37°C. All the chemicals used for culturing and cell preservation were purchased from Invitrogen and Gibco. These cell suspensions (2 X 10^5 cell/mL) are then transferred to culture plates and 10 cm culture dishes to study anti cancer effects. Assays such as MTT, LDH, neutral red, double staining, flow cytometry and gene expression studies were carried out. Initially the culture medium was replaced at an interval of 24 hours and there after it was replaced at every three days.

4.2.2 Anti-Proliferative Assays

4.2.2a Investigation of morphological changes of cell lines

Morphological changes of A549 and Hep G2 human cell lines were studied using Olympus CKX41. Olympus CKX41 is a compact inverted microscope with tilting and trinocular head options along with modern facilities to perform GFP and other fluorescence applications. High quality images of cells with optimum contrast and resolution were produced using this facility.

4.2.2b MTT Assay

The feasibility assessment using (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium (MTT) reduction, which offers the benefit of enhanced sensitivity of absorption measurement of the test medium, marker for viable cells, is a widely adapted homogenous cell viability estimation using 96-well format. Evaluation of cell viability by means of MTT is ideal for both cell viability and cytotoxicity test as it is easy to use, safe, has a high efficiency and reproducibility in screening (Loveland et al., 1992; Sylvester, 2011). Examination of conducting enzyme activity using MTT give fundamental information regarding the effect of test molecules on cell proliferation, cytotoxic effects and associated genetic expressions which inhibits the cell multiplication/ their obliteration.

Confluent cells were treated with different concentrations of fucosterol acetate and turbiconol for a period of 24 hours and

viability was measured by MTT assay. All the chemicals used in this analysis were purchased from Merck Inc. Later, these incubated cell suspensions were washed with phosphate buffer saline (PBS) followed by addition of 30 µL MTT solution (5mg/mL) dissolved in PBS. These were then incubated for 3 hours at 37°C to complete the reaction. After this incubation step, excess MTT is removed by washing with PBS. These cell lines were then treated with 200 µL DMSO and incubated at room temperature to dissolve the formazan product. Cell free suspensions for measuring optical density were obtained by removing the cell debris by centrifuging at top speed for 2 minutes. Optical density of solubalised formazan product was measured at max 540 nm keeping DMSO as blank using microplate reader Elisascan (Erba).

4.2.2c Neutral Red Assay

Even though MTT can be effectively used as marker for viable cell metabolism, it has its own limitations. As this assay depends only on the mitochondrial activity of the cell, they cannot be used for deducing the vital cell proliferation mechanism. MTT assay is effective only for low cell density solutions, linearity is loosed at high cell density. Further, possibility of fomazan crystal formation in the assay medium and toxicity of the MTT compound will adversely influence the cell membranes during excocytosis and affect the signal output, limiting this as a endpoint assay (Mosmann, 1983; Van de

Loosdrecht et al., 1994; Sumantran, 2011). Hence, neutral red assay a sensitive indicator of both the inhibition of cellular integrity and growth inhibition was performed to investigate the cellular viability. This test provides a quantitative estimate of viable cells in the medium. In comparison to other cytotoxicity tests, which estimates the release of the enzymes/proteins contents, procedure adopted for neutral red uptake assay is cheaper and more sensitive. It will not take more than three hours to complete the analysis. However, the test cannot distinguish between cytotoxic and cytostatic drugs or compounds (Elliott & Auersperg, 1993; Repetto et al., 2008).

Borenfreund & Puerner (1985) developed a cell viability assay using neutral red, a positively charged dye. Ability of living cells to uptake the diffused neutral red in the cytoplasm and stores them in the acidic environment of lysosomes was the basic concept used by the Borenfreund & Puerner (1985) to develop the neutral red cytotoxicity assay. To perform this viability test, cell lines pretreated with test compounds were incubated at 37°C for 3 hours along with neutral red dye. While incubation pH of the reagent was maintained at 6.35 using KH_3PO_4. After incubation, cells were washed with PBS to remove the excess reagent. Neutral red dye stored in the acidic environment of lysosomes was eluted using ethanol/acetic acid solvent system. Gentle shaking for 10 minutes was carried out to achieve complete dissolution prior to elution. To measure the optical

density at 540 nm, aliquots of the resulting solutions were analyzed using spectrophotometer.

4.2.2d Lactate Dehydrogenase Assay

Assay for lactate dehydrogenase (LDH) activity was performed to estimate cell death, because it is more reliable and the activity remains intact than other enzyme based evaluation of cell death. LDH assay directly detect the enzymes that leak out of the dead cells, useful for assessing membrane integrity and quantify dead cells. Further, investigation using LDH assay provides vital information on the ability of cellular glycolysis. However, reduced sensitivity and half-life are the major drawbacks of this cell viability test. Even though this test provides an accurate measure of cytotoxicity induced by the substance it fails to provide basic information concerning the primary mechanism of observed cytotoxicity (Fotakis & Timbrell, 2006; Smith et al., 2011).

In lactate dehydrogenase assay developed by Kuznetsov & Gnaiger (2010), cell free supernatant collected from tissue culture plates incubated with test compounds were used to investigate LDH leakage assay.

Prior to spectrophotometric reading, 100 µL of cell free supernatant was mixed with 2.7 mL potassium phosphate buffer, 0.1 mL 6 mM NADH solution, 0.1 mL sodium pyruvate solution in a cuvette. Optical density reading at 340 nm was measured at 0^{th} and 5^{th} minutes of incubation.

4.2.2e Calculation of ED50

To calculate ED50 values from MTT assay and neutral red assay using ED50plus V1.0.

4.2.3 Determination of Anti-proliferative Mechanism

4.2.3a Estimation of apoptosis using double staining

Optical fluorescence microscopy which utilizes the differential absorption of fluorescent DNA-binding dyes (such as EB / AO staining) is the method of choice for the sake of simplicity, speed and accuracy to study chromatin condensation and fragmentation of nuclei along with cell death mechanism (Ribble et al., 2005). In this assay as there is no further cell binding step, apoptosis index and the integrity of cell membrane can be evaluated at the same time without articrafts. This method allows a better evaluation to overcome the disadvantages of existing cytotoxicity assays. As compared to other classical staining techniques, multiple assays can be performed using very small quantity of materials to revel apoptosis morphological condition. Further, it can be combined with other experiments such as cell viability assay (MTT), cell death analysis (LDH), or some of the caspase activity assays. Thus, several end points of cell death and apoptosis can be calculated in a single experiment. Thus apoptotic and necrotic cells can be quantified simultaneously in real time making it a format suitable for high-throughput screening of drugs (Renvoize et al., 1998; Raju et al., 2004).

DNA-binding dyes Acridine orange (AO) and Ethidium bromide (EB), used for morphological differentiation of apoptotic

and nectrotic cells, were purchased from Sigma Aldrich, USA. Both viable and nonviable cells assimilate AO and upon intercalation with DNA emit green fluorescence (Gherghi et al., 2003). However, EB is taken up by non-viable cells only and emit red fluorescence when intercalated with DNA. This differential absorption of dyes by viable and non -viable cells along with variation in the emitted fluorescence was effectively used to determine exact mechanism of cell death. Cell lines after incubation with test samples, ie., ED_{50} of fucosterol acetate and ED_{50} of turbiconol, were stained with AO (100 µg/mL) and EB (100 µg/mL) at room temperature for ten minutes, after washing with cold PBS, before their visualization in a blue filter of fluorescence microscope (Olympus CKX41 with Optika Pro5 camera). The cells were divided into four category based on the observation. They are living cells with normal green nucleus, early apoptotic cells are having bright green nucleus with condensed or fragmented chromatin, late apoptotic are observed as orange stained nuclei with chromatin condensation and finally, necrotic cells are having uniformly orange stained cell nuclei.

4.2.4 Determination of Sterols Effect on Cell Cycle Pathway

4.2.4a Flow cytometry

Since reduction in the number of viable cells / total cellular protein may be associated with a decrease in cell proliferation, assays used for measuring the loss of cell viability is insufficient to study

the cytotoxic potential of the compound. Hence, evaluating the ability of cells to proliferate is critical to assess the health of the cells during cytotoxicity studies. Direct measurement of DNA synthesis using flow cytometry, a powerful tool for the detection of the distribution of cells in the three major phases of the cell cycle (G1, S, G2/M), is an accurate method of doing this. This assay provides single cell level cytotoxicity evaluation and cytolytic effect on cells. They have proven to be reliable, sensitive, specific and very effective. These investigations also give information on the possible DNA errors or DNA damage, which will eventually lead to apoptosis or disruption of cell cycle (Rabinovitch, 1994; Gupta et al., 2011).

For performing fluorescence activated sorting analysis, cell lines treated with samples were incubated at 37°C for 24 hours. After incubation the cells were collected by trypsinization and washed twice with 1X PBS. These concentrated cells were thoroughly washed with PBS. After washing by PBS, these cells were fixed using 1 mL ice cold ethanol (Merck) and incubated overnight at -20°C. Again an additional washing of ethanol fixed cells were carried out using PBS. These cells were then uniformly mixed with muse cell cycle reagent (Millipore, USA), and incubated for 30 minutes in dark. After incubation these cells were analyzed using muse cell flow cytometry. A set of untreated cells were used as control (Pozarowski et al., 2004; Darzynkiewicz & Zhao, 2014).

4.2.4b Determination p21 cancer suppressor path way

Tumor suppressor gene p21 is a potent cyclin-dependent kinase inhibitor (CKI). This protein inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes through bonding with these complexes and thereby regulate the progression of cell cycle at G_1 and S phase. Identifying the links between gene expression and chemoactivity potential of test compounds, such as its effectiveness, and toxicity to cells are vital prior to their development into anticancer drugs. Hence, the expression of the p21 gene is studied to deduce precious information in screening chemoactivity of fucosterol acetate and turbiconol (Roninson, 2002; Sassano & Platanias, 2008; Abbas & Dutta, 2009).

Briefly in order to study the p21 gene expression in A549 and Hep G2 cell lines treated with test samples were subjected to total RNA isolation kit followed by corresponding cDNA synthesis. RT-PCR was performed to study the p21 gene expression. The specific primers for p21 gene were as follows: Forward 5'-GCAGATCCACAGCGATATCC-3' with Tm 64.5 and Reverse 5'-CAACTGCTCACTGTCCACGG-3' with Tm 67.1.

4.2.4b(i) Total RNA isolation

In order to isolate pure, intact and high quality total RNA from cell lines, total RNA isolation kit manufactured by Invitrogen USA was used. Further, processing of the sample was carried out according to the guidelines given by the manufacturer. As per the instructions, A549 and

Hep G2 cell lines in 6 multiwell plates were incubated overnight with isolated sterols at 37°C, followed by addition of 1 mL trizol reagent to initiate the cell lysis. These cell lines were then homogenized to fine paste and transferred into an eppendorf tube. Homogenised cell lines were then mixed with chloroform and shaked vigorously for 15 sec and incubated again at room temperature for 2-3 minutes prior to separation of aqueous layer containing total RNA. These cell lines after incubation were subjected to centrifugation at 14000 rpm for fifteen minutes at 4°C to separate the aqueous layer. Total RNA was precipitated from the aqueous layer using 5µL of isopropanol (100%). For the complete precipitation of total RNA, the isopropanol treated aqueous layer was incubated at room temperature for 10 minutes. RNA precipitates recovered as white pellets were washed with 1 mL 75% ethanol for further purification. RNA pellets after purification were then centrifuged at 10000 rpm for 5 minutes at 4°C to remove the excess solvents. Finally, pure RNA pellets recovered were dried and dissolved in TE buffer for further analysis **(**Chomczynski & Sacchi, 1987; Chomczynski, 1993).

4.2.4b(ii) Reverse Transcriptase PCR (RT-PCR)

For synthesizing cDNA (Verso cDNA synthesis kit, Thermoscientific USA) and amplification, PCR master mix was used, 5 µL of total RNA, 2 µL forward primer and reverse primer were added to RNAse free tube and mixed with 25 µL primer RT-PCR premix, 1

µL enzyme mix and 1.5 µL RT enhancer and made upto 50 µL using sterile distilled water. Using micropipette, final solution was homogenized by gently pipetting up and down. Further, reaction was carried out in a thermal cycler, programmed to perform cyclic cDNA synthesis and amplification. Each cycle consisted of following steps. Initially, synthesis of cDNA was carried out at 42°C. This process which lasted for 30 minutes was immediately followed by denaturation at 94°C for ten minutes. After denaturation, one minute annealing at 58°C and extension at 72°C for one minute was performed. These steps were repeated for next 35 cycles and final extention lasted for 5 minutes at 72°C. Finally, amplified PCR product was separated and visualized using gel electrophoresis (Glazer et al., 2010).

4.2.4b(iii) Agarose gel electrophoresis

For agarose gel preparation, eletrophoretic grade agarose powder was purchased from Lonza and all other chemicals used were molecular biology grade. For all preparative mini gel system, apparatus from biorad OWL™ EasyCast™ B1 mini gel eletctrophoresis system was used. Using a constant power source, steady current was maintained. The temperature of the system was controlled using a cooling circulator. Following the procedures developed by Peacock & Dingman (1968), Tris-EDTA-borate buffer having pH 8.3 was prepared and used throughout the experiment. Agarose gel (1.5 %) was synthesized in 1X TE buffer. After loading the gel with samples, analysis were performed

at 50V for 30 minutes. PCR product separated was stained and visualized and imaged in a gel documentation system (E gel imager, Invitrogen).

4.3 Results and Discussion

Based on multiple viability assays, results of anti-proliferative potential of phycosterols isolated from *Turbinaria conoides* against Hep G2 and A549 cancer cell lines are graphically represented and discussed in detail. Observations and findings revealed that sterols induced early and late apoptotic events on cancer cells under investigation. Meanwhile, evaluation of cell cycle distribution and gene expression strongly support apoptosis induced anti-proliferative potential of sterols.

4.3.1 Antiproliferative Effect of Sterols on A549 and Hep G2 Cells

To delineate the antiproliferative effect of sterols, phase contrast microscopic imaging, MTT, Neutral red assay, and LDH were performed. On the basis of phase contrast microscopic images (Fig. 4.1[a-c] and 4.2[a-c]), alteration in the morphology along with reduction in cell adhesion capacity were found in concentration dependent manner. Results of MTT, Neutral red assay and LDH (Fig. 4.3, 4.4, and 4.5) in the range of 6.5 - 100 µg/mL, highlighted a strong anti-proliferative activity of fucosterol acetate and turbiconol. These results shows the cytotoxic nature of both fucosterol acetate and turbiconol.

4.3.1a Morphological change on cancerous cell line

Qualitative morphological peculiarities of A549 and Hep G2, before and after sterol treatment were displayed in Fig. 4.1 [a-c] and 4.2 [a-c] respectively. Cyto-morphological changes were found to be strictly concentration dependent in both the cell lines. Untreated cells were intact and retained their polygonal shape. Whereas those treated with test sterols at various concentrations ranging from 6.5 to 100 µg/mL showcased cytoplasmic shrinkage and detached from each other. Rounding up of cells were observed in both treated cell lines, even at lower concentrations (6.5 and 12.5 µg/mL). Typical morphology in most of the cells was lost in higher concentrations (25-100 µg/mL). Destroyed cell structures were visualized as blurry and floating under phase contrast microscope.

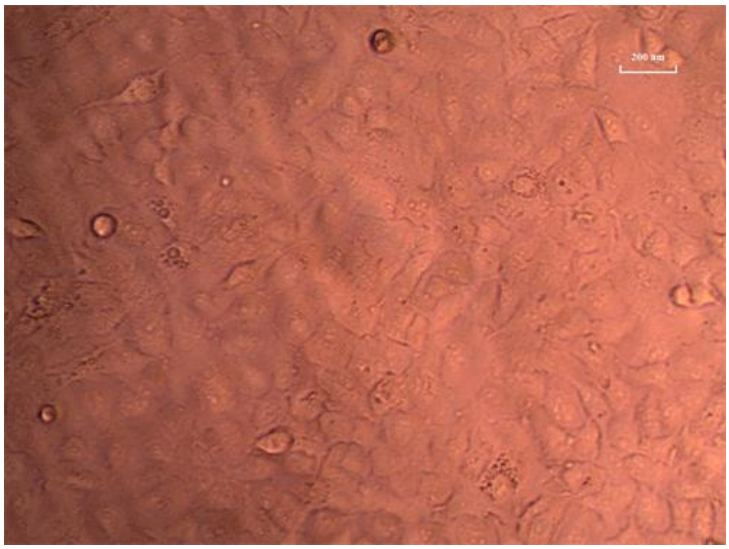


Figure 4.1a Phase contract image of A549 cell line control morphology.

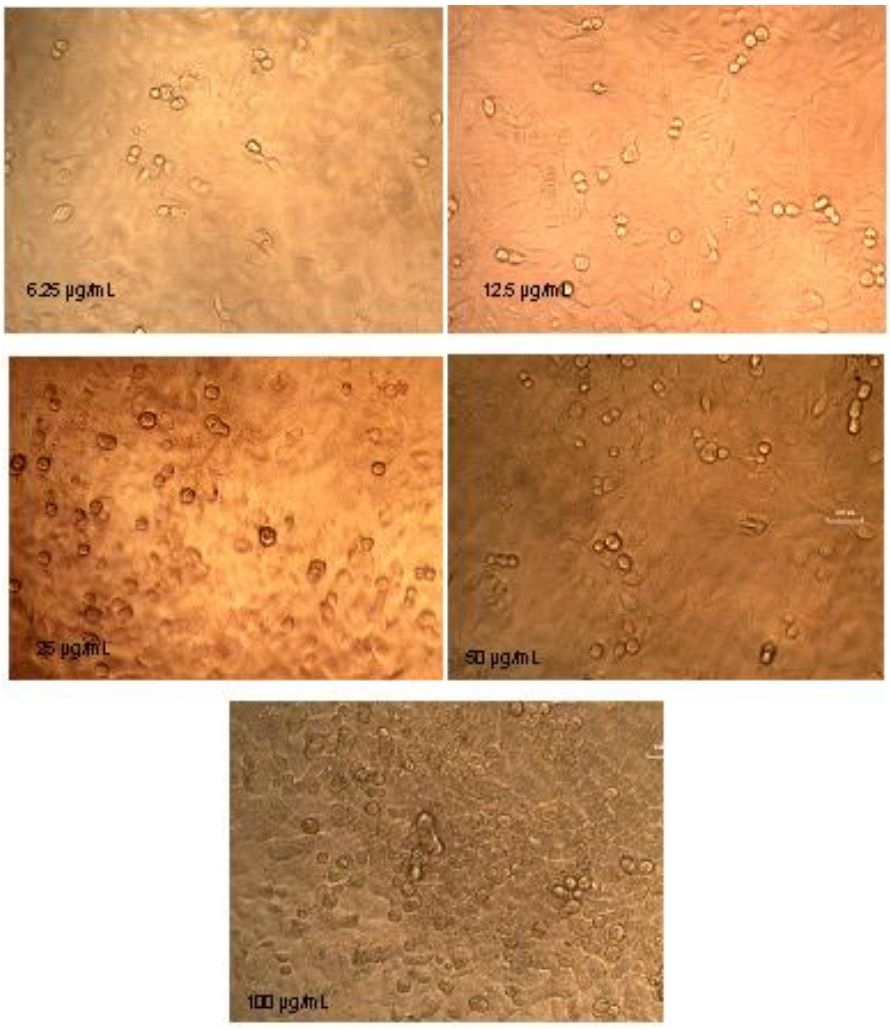


Figure 4.1b Morphological alteration of A549 cell line exposed to fucosterol acetate.

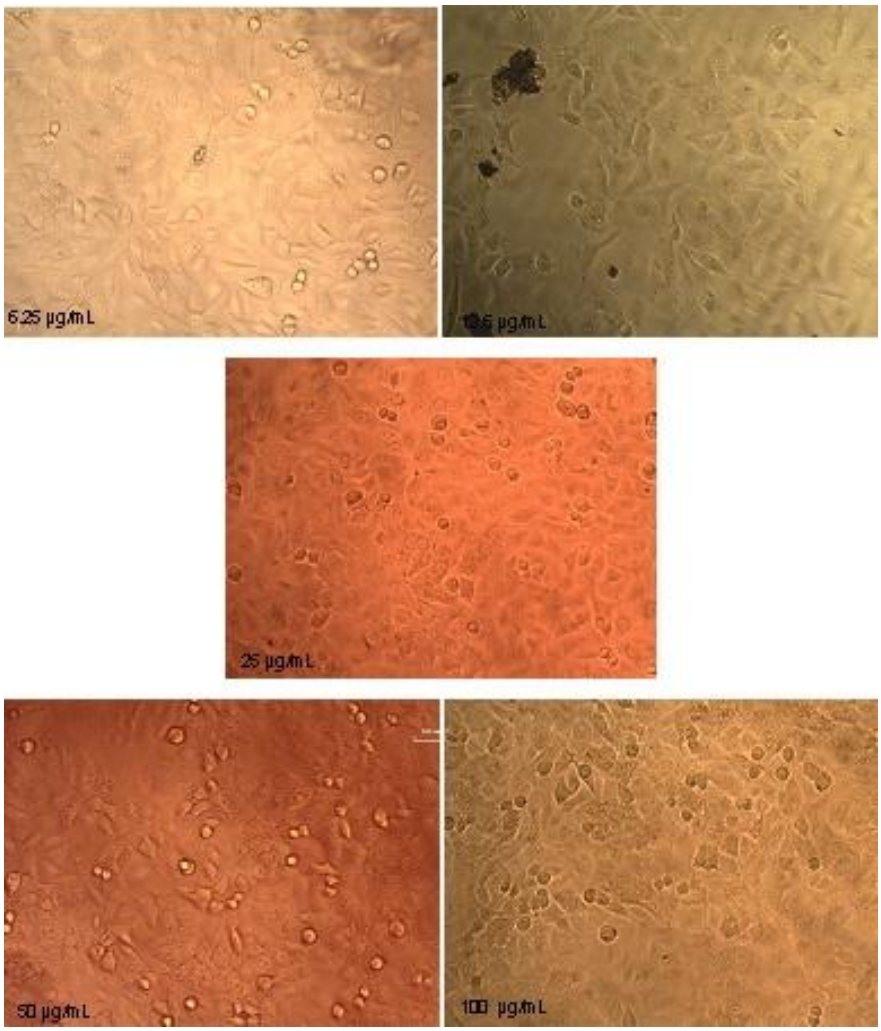


Figure 4.1c Morphological alteration of A549 cell line exposed to turbiconol.

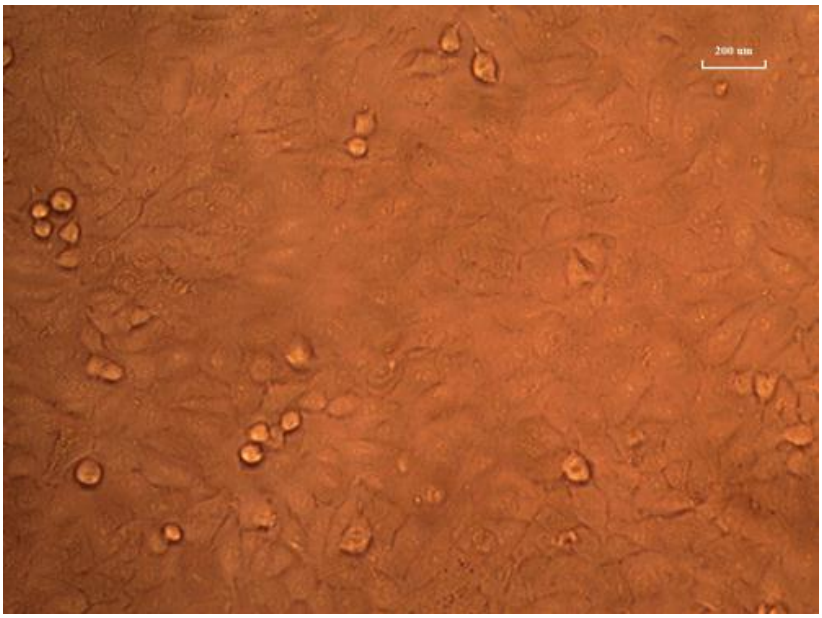


Figure 4.2a Phase contract image of Hep G2 cell line control morphology.

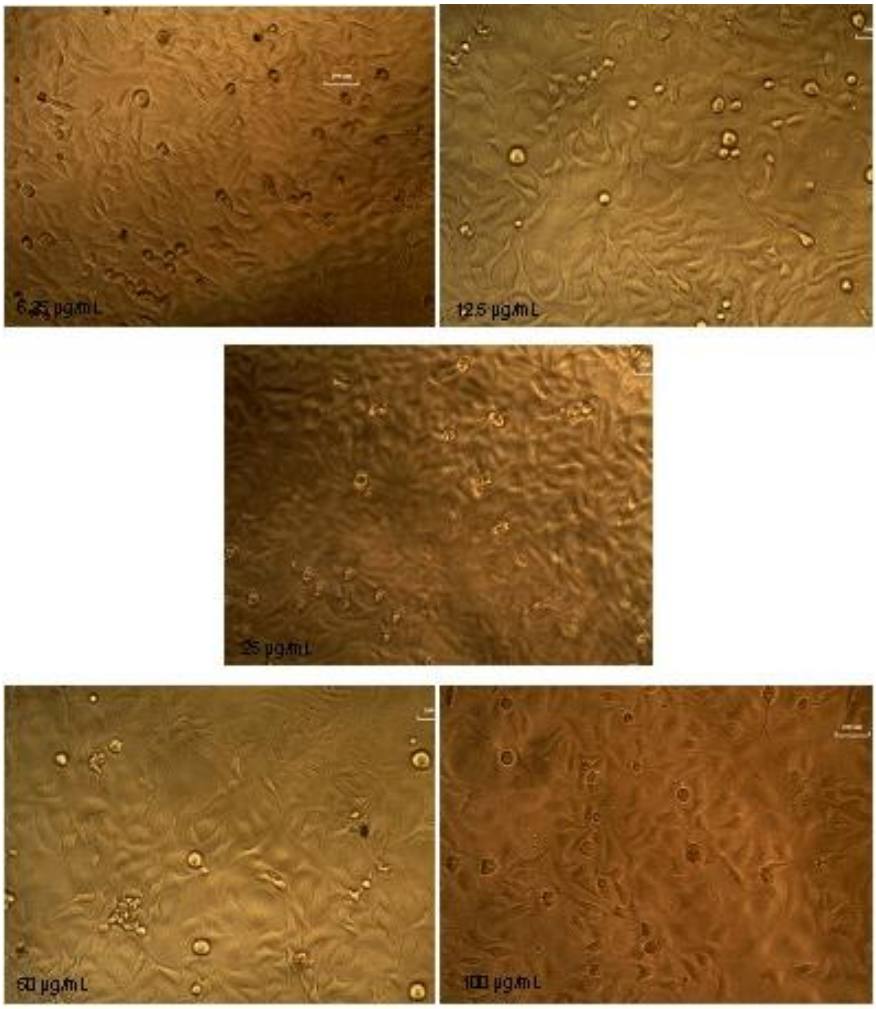


Figure 4.2b Morphological alterations of Hep G2 cell line exposed to fucosterol acetate.

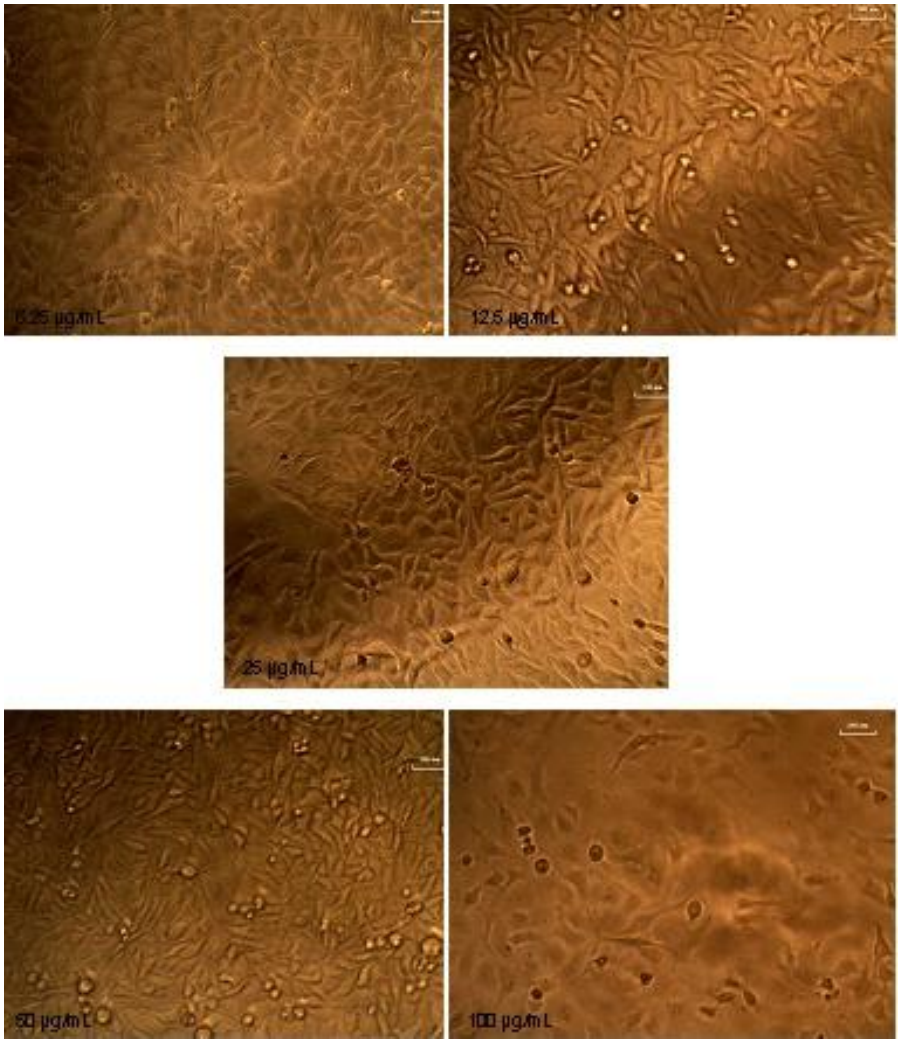


Figure 4.2c Morphological alterations of Hep G2 cell line exposed to turbiconol.

Most of the cytotoxicity studies represent cyto morphological changes as first hand indication of drug dosage response (Sgonc & Wick, 1994; Chakravarthy et al., 2006). Cytoplasmic shrinkage, rounding up of cells, loss of cell adhesion and nuclear condensation are marker for apoptotic cell death (Earnshaw et al., 1999; Los et al., 1999; Rashedi et al., 2007; Vijayarathna & Sasidharan, 2012). Ghavami and coworkers in 2009 reported morphological changes as a result of the expression of caspase-9 by activated receptors on the cell surface.

4.3.1b MTT assay

Cell viability was found to exhibit a dose dependent pattern with compound treatment. ED_{50} values for fucosterol acetate against A549 is 48.518 µg/mL. Whereas for turbiconol against A549 ED_{50} is 66.353 µg/mL. The results of fucosterol acetate showed better anti-proliferative activity towards both the cell lines. Activities of sterols against A549 are shown in Fig. 4.3a. At lowest concentration of 6.5µg/mL fucosterol acetate induced a cell death of 26.63% in lung cancer cell line, whereas at this dosage, activity of turbiconol induced 13.59% of cell death. This observation is comparable to the effect in Hep G2 Fig. 4. 3b. In this case for fucosterol acetate and turbiconol, gradual increase in cell death percentage was observed as the concentration increased. Significant cell death was observed with 67.74% and 65.02% for sterol fucosterol acetate and turbiconol, respectively at the higher concentration range (100 µg).

ED_{50} values for fucosterol acetate against Hep G2 is 46.316 µg/mL. Whereas for turbiconol, against Hep G2 ED_{50} is 60.98 µg/mL. Fucosterol acetate exhibited 30.87% cell death in Hep G2 human cell lines at 6.5 µg/mL concentration. Whereas turbiconol, at this concentration induced only 18.37% cell death. However, activity of both sterols increased by almost 10 % when concentration was increased to 12.5 from 6.5 µg/mL. At 50 µg/mL concentration of fucosterol acetate, only 44.89% cell was viable, whereas in the case of turbiconol, 50.21% cells were viable. Corresponding to increase in concentration, there was significant increment in the cell death.

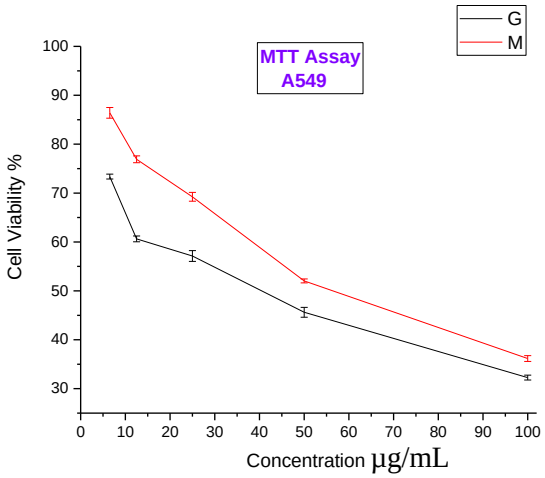


Figure 4.3a Graphical representation of cytotoxicity activity of fucosterol acetate (G) & turbiconol (M) against A549 cell line by MTT assay.

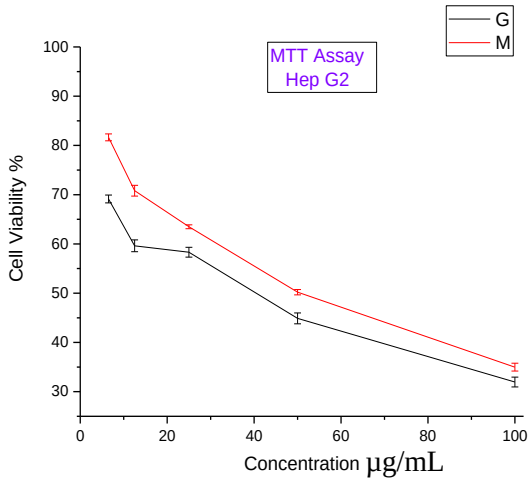


Figure 4.3b Graphical representation of cytotoxicity activity of fucosterol acetate (G) & turbiconol (M) against Hep G2 cell line by MTT assay.

Numerous reports have utilized the versatility of MTT assay to evaluate the cell viability for various drug screening. Since the rate of energy metabolism regulates the formazan formation, even metabolically activated cells can be quantified. In the absence of cell proliferation further formazan production is proportional to the quantity in incubation media even though MTT is limited by loss of its efficiency at higher concentration cell density. Chances of error to occur are reduced as there are minimum washing steps in the protocol. Variation on formazan absorption associated with change in pH could be overcome by using buffer solution (Plumb et al., 1989).

4.3.1c Neutral Red assay

The results of neutral red assay performed on A549 and Hep G2 are shown in Fig. 4.4 [a-b]. Significant observations of this assay can be summarized as follows. ED_{50} values for Fucosterol acetate against A549 is 50.34 µg/mL. Whereas for turbiconol, against A549 ED_{50} is 66.79 µg/mL. A cell death of approximately 37.08% was observed in A549 cells when treated with 12 µg/mL fucosterol acetate, whereas 22.06% cell death was observed when treated with same concentration of turbiconol. Further, when treated with 50µg/mL fucosterol acetate and turbiconol, cell death reached a maximum of 53.57% and 48.73% respectively.

Significant reduction in the density of viable cells was observed when Hep G2 cell lines were dosed with 6.5µg/mL fucosterol acetate and turbiconol. ED_{50} values for fucosterol acetate against Hep G2 is 49.47 µg/mL. Whereas as for turbiconol, against Hep G2 ED_{50} is 60.914 µg/mL. When treated with 100 µg/mL of fucosterol acetate and turbiconol, viability of these cells decreased to 32.19% and 35.06% respectively. The results clearly indicate a significant loss of cell organelle integrity following compound treatment which can be correlated to cell death (Borenfreund & Puerner, 1985).

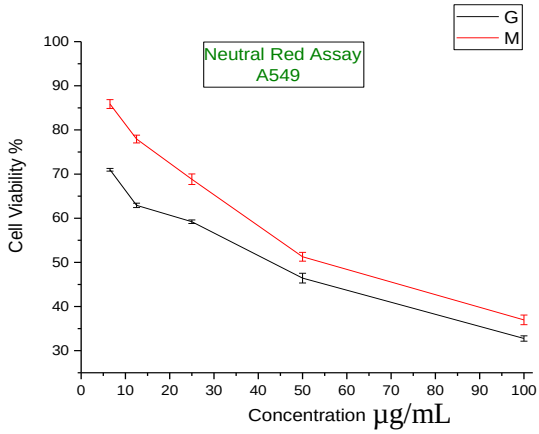


Figure 4.4a Graphical representation of cytotoxicity activity of fucosterol acetate (G) & turbiconol (M) against A549 cell line by Neutral red assay.

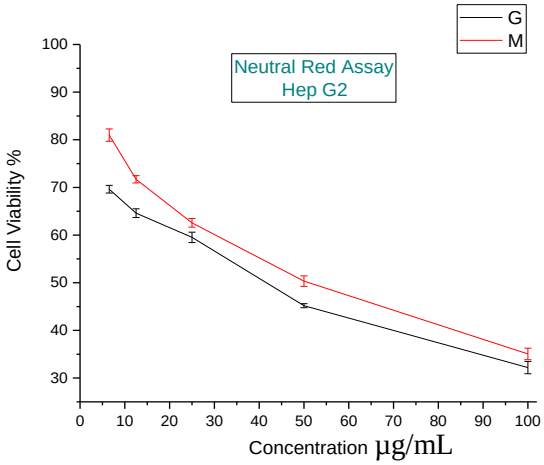


Figure 4.4b Graphical representation of activity of fucosterol acetate (G) & turbiconol (M) against Hep G2 cell line by Neutral red assay.

4.3.1d LDH Assay

LDH leakage directly measures the extent of membrane integrity following compound treatment. From the results of the present investigations it can be observed that addition of compound significantly induced membrane damage resulting in increase of LDH leakage. As membrane damage and loss of cytosolic enzymes to media can be considered as a measure of apoptotic effects of compounds (Kuznetsov & Gnaiger, 2010).

There was a gradual increase in the release of enzyme volumes when treated with fucosterol acetate and turbiconol against A549 and Hep G2 cell lines (Fig. 4.5[a-b]). When A549 cell lines were treated with 100 µg/mL of fucosterol acetate volume of LDH reach up to 1.29 U/mL from initial control value of 0.0048 U/mL. Whereas when dosed with turbiconol at this concentration, volume of LDH released was 1.01 U/mL. Similarly, Hep G2 cell lines when treated with fucosterol acetate and turbiconol, volume of enzyme released at 100 µg/mL reached 1.42 U/mL and 1.31 U/mL respectively from initial control value (0.0039 U/mL). LDH leakage levels highlighted the potential of fucosterol acetate and turbiconol to regulate the cellular glycolytic activity of the A549 and Hep G2. In cancer cells, LDH play a vital role in the initiation promotion and acceleration of cell growth and replication through fermentative glycolysis (Kuznetsov & Gnaiger, 2010). Variations in the LDH activity suggest the potential of fucosterol acetate and turbiconol, to inhibit the anaerobic respiration pathway. However, further investigations need to be carried out in detail.

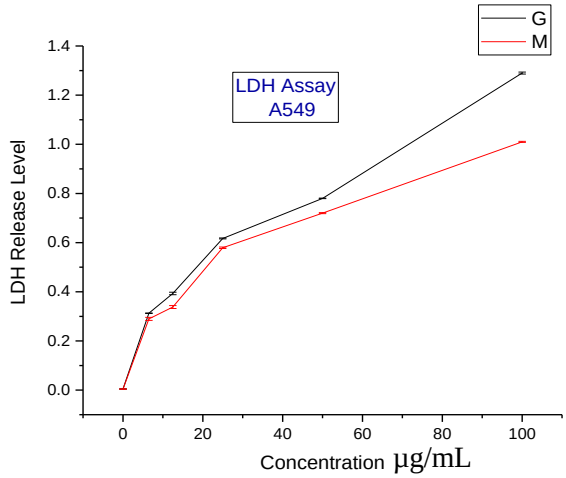


Figure 4.5a Graphical representation of cytotoxicity activity of fucosterol acetate (G) & turbiconol (M) against A549 cell line by LDH assay.

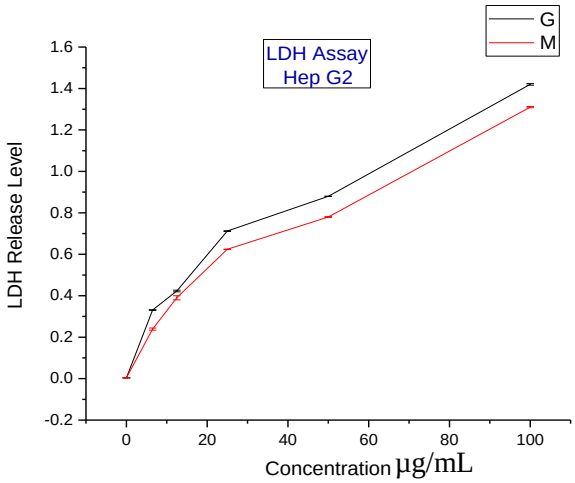


Figure 4.5b Graphical representation of cytotoxicity activity of fucosterol acetate (G) & turbiconol (M) against Hep G2 cell line by LDH assay.

Based on MTT, neutral red assay and LDH cytotoxicity assay anticancer potential of fucosterol acetate and turbiconol against A549 and Hep G2 were inferred. Both fucosterol acetate and newly isolated turbiconol are cytotoxic towards these two cancer cell lines. Even though assay results of sterols supports their potential to suppress both Hep G2, and A549, both sterols were more sensitive towards Hep G2.

4.3.2 Determination of Anti-proliferative Mechanism

4.3.2a Double staining

Normal cells were observed with bright green nucleus (Fig. 4.6a). Nucleus of A549 cell after sterol treatment consisted of condensed/fragment chromatin and nucleus appeared bright green in colour. These observations predict early apoptotic pathway initiated by fucosterol acetate & turbiconol. Hep G2 cells on the other hand when dosed with the same sterols, nuclei were observed as orange (Fig. 4.6b). Moreover, these cells suffered extensive chromatin condensation or fragmentation. These features are consistent towards late apoptotic pathway of cell death in Hep G2 (Gherghi et al., 2003; Liu et al., 2015).

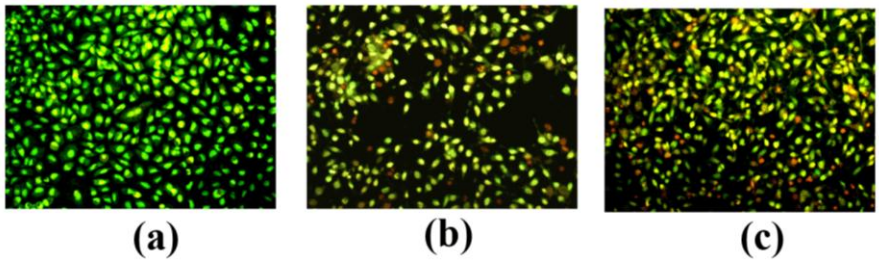


Figure 4.6a Detection of anti-proliferative mechanism (apoptosis /necrosis) treated with acridine orange and ethidium bromide double staining (a) Untreated A549 Control cells showing intact cells with green fluorescent nuclei (b) A549 cells treated with fucosterol acetate (c) A549 cells treated with turbiconol.

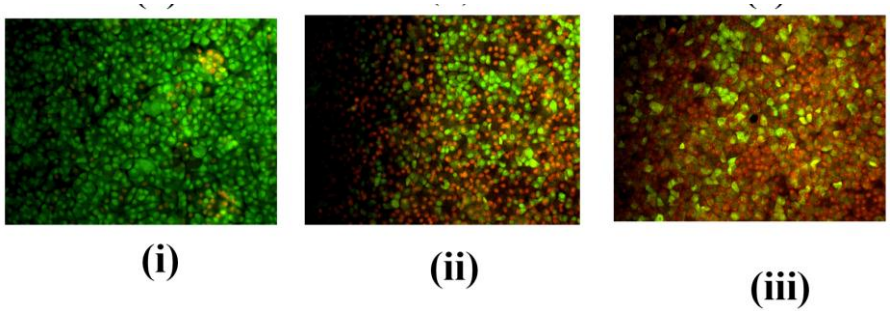


Figure 4.6b Detection of anti-proliferative mechanism (apoptosis/ necrosis) treated with acridine orange and ethidium bromide double staining (a) Untreated Hep G2 control cell lines showing intact cells with green fluorescent nuclei (b) Hep G2 Cells treated with fucosterol acetate (c) Hep G2 cells treated with turbiconol.

Detection of drug action mechanism is necessary, as cell viability assays for cytotoxiciy studies cannot distinguish among mechanisms of cell death and its impact on normal cells (Evan & Vousden, 2001). Drugs with limited clinical application due to their toxic/poisoning effect on cells can be easily screened. Hence, assessment of tumor cell mechanism is more informative than common cell viability evaluation. In this regard, fluorescent acridine orange (AO) /ethidium bromide (EB) dual staining dyes, a relatively new mode of approach are very effective to differentiate and quantify the basic morphological changes between normal cells, early and late apoptotic cells, and necrotic cells and even mild DNA injuries can be diagonised (Gherghi et al., 2003; Liu et al., 2015). Normal and early apoptotic cells with intact membranes are stained only by AO dye, and emit when fluorescing green bound to DNA. Whereas EB dye can bound only to concentrated DNA fragments or apoptotic bodies and emit orange-red fluorescence, resulting from late apoptotic or along with dead cells (Ribble et al., 2005). In order to identify the antiproliferative mechanism of fucosterol acetate and turbiconol, AO/EB double staining assay method was used. From Fig. 4 6[a-b], intercalation of AO stain with DNA of viable A549 and Hep G2 control cells were inferred from the emission of green fluorescence. In both the cases, all the control cell lines were viable and observed as bright green nucleus, with very well organized structure.

Nucleus of A549 cells after sterol treatment consisted of condensed/fragment chromatin and nucleus appeared yellow green in colour. These observations predict early apoptotic pathway initiated

by fucosterol acetate and turbiconol. Cells still with intact membranes but have started to undergo DNA cleavage interacts with AO stain and emits yellow green fluorescence predominantly, a direct consequence of early apoptosis. Presence of bright green patches or fragments observed in this case clearly highlights the perinuclear chromatin condensation. However, initiation of late apoptosis by sterol molecules is also inferred from the scattered emission of orange-red florescence (Ribble et al., 2005).

Hep G2 cells on the other hand when dosed with the same sterols, nuclei were observed as orange. Further, these Hep G2 cells suffered extensive chromatin condensation or fragmentation. These features are consistent towards a late apoptotic pathway of cell death in Hep G2, as necrotic cells have uniformly orange to red nuclei with a condensed structure (Ribble et al., 2005).

The rates of cell proliferation and apoptosis are major controlling factors influencing the tumor growth (Elmore, 2007). Both phyto and phycosterols are effective apoptosis (programmed cell death) stimulants. Major mechanism involved in the activation of apoptosis by sterols are membrane structure alteration, membrane fluidity, modulation of membrane bound enzyme activity, signal transduction pathways and membrane integrity (Awad et al., 2007). However, exact mechanisms are still elusive.

Sterols are easily incorporated into the membranes due to their closely related structural similarities to cholesterol (an integral lipid

component of biological membranes). But assimilation of sterols by tumor cells alters the concentration of two phospholipids, ie., sphingomyelin and phosphatidylcholine, which are involved in signal transduction pathways, there by inducing apoptosis in malignant cells (Pörn & Slotte, 1995; Slotte, 1999; Awad & Fink, 2000).

Proper fluidity balance is vital for the membrane functioning. Sterols are effective in altering the composition of lipid membranes, and thereby influencing their fluidity and suppressing the progression of tumor growth (Spector & Yorek, 1985). For example, liver membrane fluidity of rats is altered by feeding them with supplements incorporated with 5% sterols for 21 days (Leikin & Brenner, 1989). This was inferred from the elevation of hepatic fatty acid desaturases (Δ9, Δ6 and Δ5) activity when sterol levels were increased, a compensatory mechanism for the decreased fluidity. However, to understand their exact role in tumor progression, further investigations are required. The activities of membrane-bound enzymes hepatic and prostatic 5 α-reductase in prostatic aromatase in rats, involved in the metabolism of testosterone, when provided with a diet mixed with 2% sterols was decreased by about 33–44% and 55% respectively (Awad et al., 1998). This have been shown to considerably reduce the development of prostate hyperplasia and prostate cancer (Howie & Shultz, 1985; Schweikert et al., 1993), linked to the levels of both androgens and estrogens resulting from testosterone metabolism, by hepatic and prostatic 5 α-reductase (Mettlin, 1997).

4.3.3 Sterols Effect on Cell Cycle Pathway

In this section, cell cycle arrest and gene expression levels were analyzed and discussed to understand the effect of sterols on cell cycle pathway in cancer cell lines.

4.3.3a Flow cytometry

Various studies have shown that cell proliferation and regulation of cell cycle progression are interrelated to each other (Alenzi, 2004). According to the preliminary flow cytometry assays results (Fig. 4.7[a-c] and 4.8[a-c]), for A549 when treated with fucosterol acetate, cell cycle arrest at G0/G1 phase was not prominent (3.8%). In this case, only slight increase was observed in cells arrested at G0/G1 phase when compared to Hep G2, whereas A549 treated with turbiconol exhibited a mild cell arrest (2.6%) at G2/M phase (Fig. 4.7[a-c]). In the present investigation, growth inhibition effect of fucosterol acetate and turbiconol on Hep G2 cells are shown in Fig. 4.8[a-c]. After 24 hours there was an elevation in the number of cells in the G0/G1 phase of the cell cycle treated with fucosterol acetate and turbiconol. From the results of flow cytometric analysis, 34.2% Hep G2 cells were arrested at G0/G1 phase when treated with sterol (fucosterol acetate) compared with untreated control, whereas the S phase cells decreased proportionally. Similarly Hep G2 cell lines treated with sterol (turbiconol) were also arrested at G0/G1 phase. In the present study cell cycle arrest at G0/G1 phase of Hep G2 was evident with concomitant decrease in the G2/M

phases (13.3 %), indicates that cell cycle distribution was blocked significantly in the G0/G1 phase in cells treated with sterols from *Turbinaria conoides.*

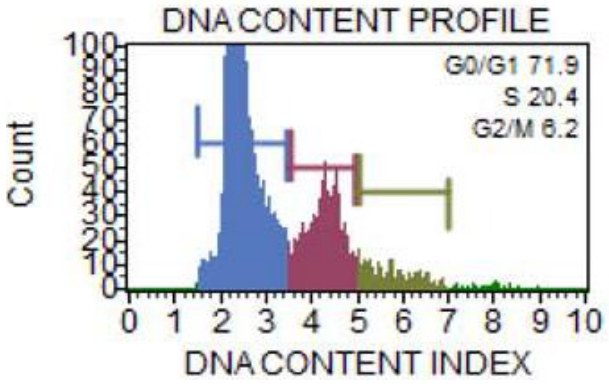


Figure 4.7a Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of control cells of A549 cell lines.

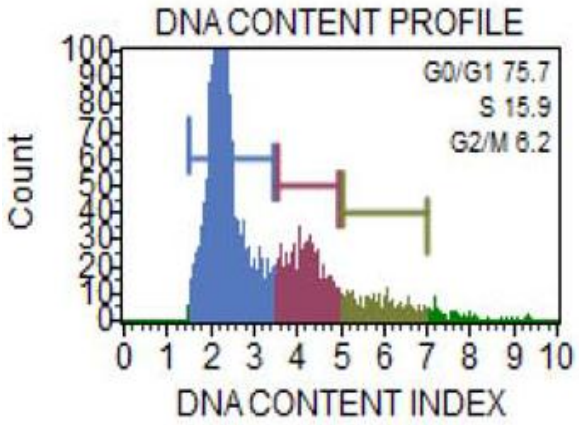


Figure 4.7b Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of A549 cell lines treated with fucosterol acetate.

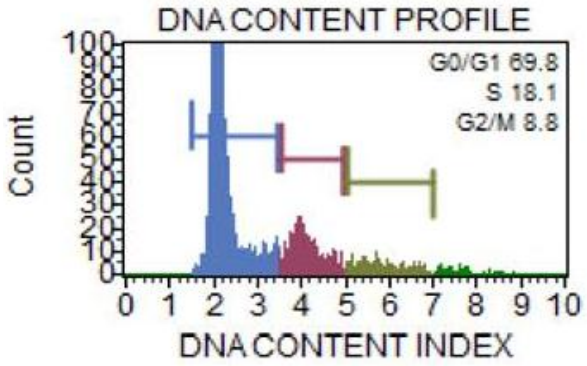


Figure 4.7c Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of A549 cell line treated with sterol (turbiconol)

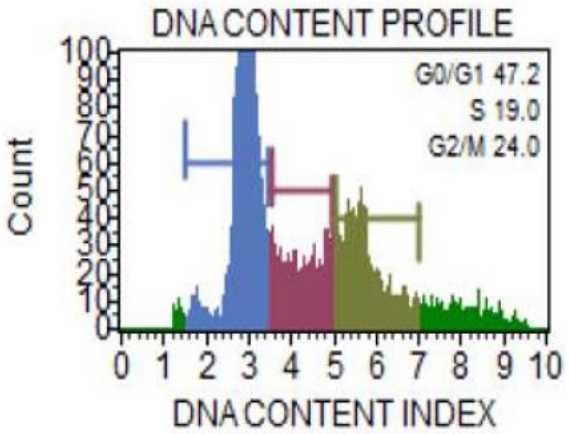


Figure 4.8a Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of control cells of Hep G2 cell line.

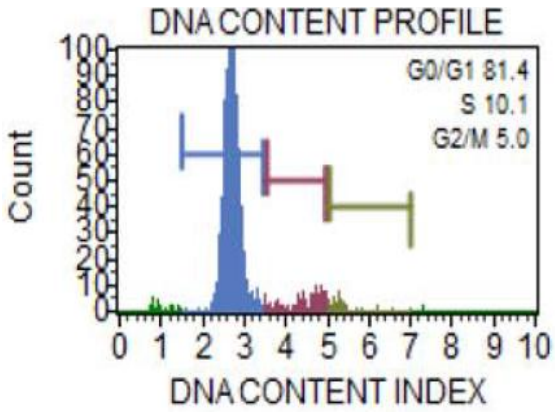


Figure 4.8b Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of Hep G2 cell line treated with fucosterol acetate.

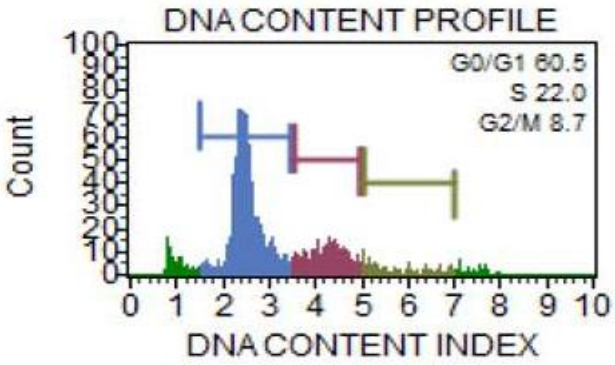


Figure 4.8c Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of Hep G2 cell line treated with turbiconol.

4.3.3b Gene expression

Suppression of G1-S transition is correlated to the activation of protein kinase-C regulated p53 gene transcription, in mock cells this process usually initiate a cellular repair mechanism (Bullock &

Fersht, 2001; Whal & Car, 2001). An explanation for this is related to the expression of p53, a tumor suppressor, which controls the functioning of p21 gene (Li et al., 2012). Hence, p21 gene expression studies are useful to delineate the mode of sterol action in the suppression of cancer. Therefore, p21 gene expression study was performed to support the cell cycle arrest observed in both cell lines when treated with fucosterol acetate and turbiconol.

p21 gene expression results are shown in Fig. 4.9. Expression level of p21 in A549 cell lines were not clearly affected. This observation is consistent with the magnitude of cell arrest for A549 cell cycle. When Hep G2 were treated with fucosterol acetate and turbiconol, expression of p21 was modified as inferred from variation in the band width of the gene. In this case intensity of band width was increased when compared to blank. These results confirm G0/G1 cell phase arrest in Hep G2 cell cycle.

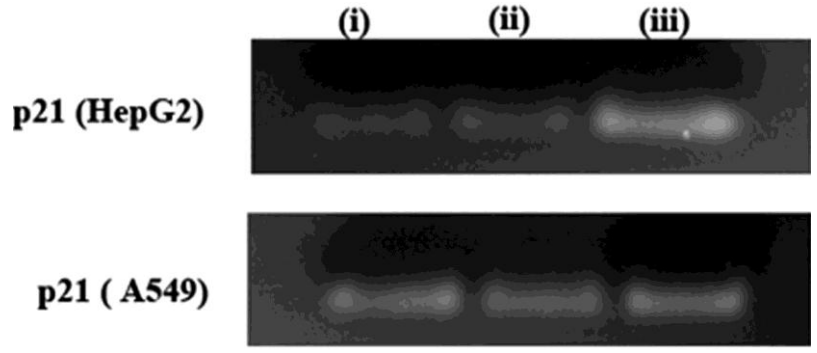


Figure 4.9 Expression of p21 in A549 and HepG2 cell lines. (I) corresponds to control, (II) corresponds to fucosterol acetate and (III) corresponds to turbiconol.

On the basis of observation made by Lukas and coworkers in 1994, G0/G1 phase cell arrest can also be linked to inhibition of phosphorylation at multiple sites and reducing the pace of C-Ab1 functions, resulting from malfunctioning of cyclin D1 expression. Researchers have identified numerous cyclin/CDK complexes and their role in cell cycle progression. However, D1/CDK4/6 and cyclin B1/CDK1 are of great significance as the former controls G1/S transition and the later regulate G2/M transition (Lew et al., 1991, Galaktionov & Beach, 1991).

Evidence for the promotion of programmed cell death (apoptosis) in cancer cells are available in various literature (Hassan et al., 2014). Basic mechanism of apoptosis induction by sterols is shown in Fig. 4.10. These are reports which mainly highlight the potential of anticancer compounds to participate in cell cycle to induce G1, S, or G2/M phase arrest with relation to their selectivity (Gallagher, 2007; Boesze-Battaglia et al., 2009; Zhao et al., 2011). Many researchers have observed the down regulation of CDK2 found with cyclin E and CDK inhibitors, when treated with cancer suppressing compounds in normal progression of G1 to S phase (Guadagno & Newport, 1996). Cell arrest at G2/M phase is also suggested by various researchers as major pathway for the initiation of apoptosis by phytosterols. G2/M cell cycle arrest results mainly from inappropriate cdc2 activation and hence, is used in combination with chemotherapy and radiation treatment methods (Awad et al., 2005).

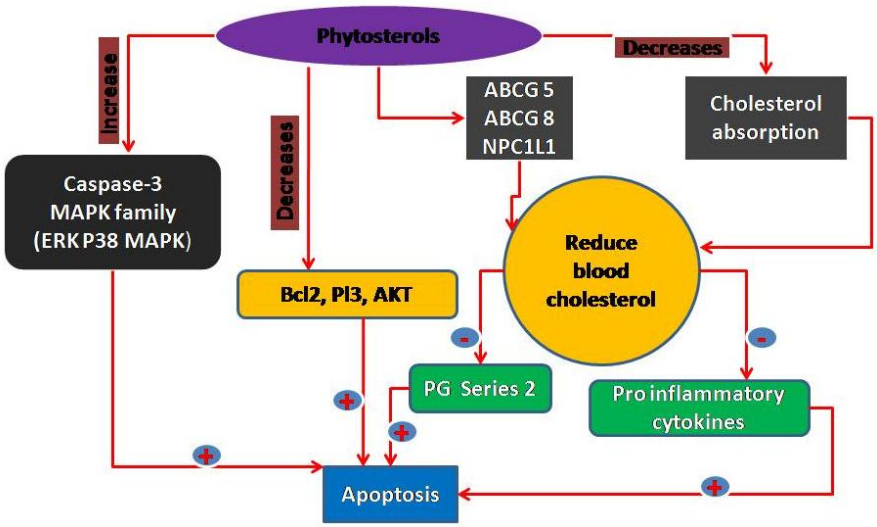


Figure 4.10 Proposed mechanism of anti-cancer activity shown by phytosterols.

Phytosterols mainly work through the activation of caspase enzyme by inactivating the Bcl-2 pathway in the cell cycle. Park et al. (2007) reported the activation of pro-apoptosis enzymes MAPK, through reducing the expression of Bcl -2 protein. Moon et al. (2007) proposed a mechanism for the activation of caspase using phytosterols which develops extracellular signals complimentary to the pathways of mitochondria. Potential to initiate endoreduplication and apoptosis by phytosterols through modifying phosphatidylino sito1-3 Kinase /Akt and ERk independent pathway is an important strategy used to suppress the cell proliferation (Woyengo et at., 2009).

During the progression of cell cycle from G1 to S phase, following cyclins D-CDK,D-CDK6 and E-CDK2 are activated along

with pRB phosphorylation (Li et al., 2012). Deactivation of pRB, through hyperphosphorylation disable their normal functioning and initiate the dissociation of E2F transcription factor from PRB, leading to the inactivation of vital pathways during different stages of cell cycle, from G1 to S. CDK inhibitors consisting of a group of proteins are found to be negatively regulating the CDK activity. A protein identified as P21 WAF1/C p1 is found to be a major down regulation of CDK activity through blocking the active site of cyclin E-CDK 2 complexes. This blocking of active cycles results in the hypophosporylation of pRB, consequently leading the halting of cell cycle transition from G1-S phase.

4.4 Conclusions

On the basis of examinations carried out in Hep G2 and A549 cell lines, a positive feedback regarding the potential of fucosterol acetate and turbiconol for the treatment of lung and liver cancer were obtained. Potential of fucosterol acetate and turbiconol to selectively induce apoptosis and G0/G1 cell cycle arrest in Hep G2 were identified. However, only fucosterol acetate induced G0/G1 cell cycle arrest in A549 human cancer cell lines. On the other hand G2/M phase cell cycle arrest was observed in A549 cell lines when treated with turbiconol. G2/M arrest facilitate the cells, particularly p53 deficient cells, to sensitize cells towards chemotherapy and radiation. Hence, turbiconol seems particularly useful in this case as

it induces G2/M arrest in A549. These results strongly supports the chemotherapeutic prospective of fucosterol acetate and turbiconol in developing safe treatment methods.

4.5 References

Abbas, T., & Dutta, A. (2009). p21 in cancer: intricate networks and multiple activities. *Nature Reviews Cancer*, *9*(6), 400-414.

Abreu, R. M., Ferreira, I. C., Calhelha, R. C., Lima, R. T., Vasconcelos, M. H., Adega, F., Chaves, R., & Queiroz, M. J. R. (2011). Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno [3, 2-b] pyridine-2-carboxylate derivatives: *in* vitro evaluation, cell cycle analysis and QSAR studies. *European Journal of Medicinal Chemistry*, *46*(12), 5800-5806.

Alenzi, F. Q. (2004). Links between apoptosis, proliferation and the cell cycle. *British Journal of Biomedical Science*, *61*(2), 99-102.

Awad, A. B., Chinnam, M., Fink, C. S., & Bradford, P. G. (2007). β-Sitosterol activates Fas signaling in human breast cancer cells. *Phytomedicine*, *14*(11), 747-754.

Awad, A. B., Burr, A. T., & Fink, C. S. (2005). Effect of resveratrol and β-sitosterol in combination on reactive oxygen species and prostaglandin release by PC-3 cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *72*(3), 219-226.

Awad, A. B., & Fink, C. S. (2000). Phytosterols as anticancer dietary components: evidence and mechanism of action. *The Journal of nutrition*, *130*(9), 2127-2130.

Awad, A. B., Hartati, M. S., & Fink, C. S. (1998). Phytosterol feeding induces alteration in testosterone metabolism in rat tissues. *The Journal of Nutritional Biochemistry*, *9*(12), 712-717.

Borenfreund, E., & Puerner, J. A. (1985). Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters*, *24*(2), 119-124.

Boesze-Battaglia, K., Brown, A., Walker, L., Besack, D., Zekavat, A., Wrenn, S., Krummenacher, C. & Shenker, B. J. (2009). Cytolethal distending toxin-induced cell cycle arrest of lymphocytes is dependent upon recognition and binding to cholesterol. *Journal of Biological Chemistry*, *284*(16), 10650-10658.

Bullock, A. N., & Fersht, A. R. (2001). Rescuing the function of mutant p53. *Nature Reviews Cancer*, *1*(1), 68-76.

Chakravarthy, U., Soubrane, G., Bandello, F., Chong, V., Creuzot-Garcher, C., Dimitrakos, S. A., Korobelnik, J.F., Larsen, M., Monés, J., Pauleikhoff, D., & Pournaras, C. J. (2006). Evolving European guidance on the medical management of neovascular age related macular degeneration. *British Journal of Ophthalmology*, *90*(9), 1188-1196.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*, *15*(3), 532-4.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, *162*(1), 156-159

Copeland, R. L., Das, J. R., Bakare, O., Enwerem, N. M., Berhe, S., Hillaire, K., White, D., Beyene, D., Kassim, O. O. & Kanaan, Y. M. (2007). Cytotoxicity of 2, 3-dichloro-5, 8-dimethoxy-1,4-naphthoquinone in androgen-dependent and-independent prostate cancer cell lines. *Anticancer Research*, *27*(3B), 1537-1546.

Darzynkiewicz, Z., & Zhao, H. (2014). Cell cycle analysis by flow cytometry. *Els*.

Earnshaw, W. C., Martins, L. M., & Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annual Review of Biochemistry*, *68*(1), 383-424.

Elliott, W. M., & Auersperg, N. (1993). Comparison of the neutral red and methylene blue assays to study cell growth in culture. *Biotechnic and Histochemistry*, *68*(1), 29-35.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology*, *35*(4), 495-516.

Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature*, *411*(6835), 342-348.

Farfan, A., Yeager, T., Moravec, R., & Niles, A. (2004). Multiplexing homogeneous cell-based assays. *Cell Notes*, *10*, 2-5.

Finlay, G. J., & Baguley, B. C. (1984). The use of human cancer cell lines as a primary screening system for antineoplastic compounds. *European Journal of Cancer and Clinical Oncology*, *20*(7), 947-954.

Fotakis, G., & Timbrell, J. A. (2006). In *vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, *160*(2), 171-177.

Galaktionov, K., & Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B- type cyclins: evidence for multiple roles of mitotic cyclins. *Cell*, *67*(6), 1181-1194.

Gallagher, J., & Brian, M. (2007). Microtubule-stabilizing natural products as promising cancer therapeutics. *Current Medicinal Chemistry*, *14*(28), 2959-2967.

Garnett, M. J., Edelman, E. J., Heidorn, S. J., Greenman, C. D., Dastur, A., Lau, K. W., Greninger, P., Thompson, I. R., Luo, X., Soares, J., & Liu, Q. (2012). Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*, *483*(7391), 570-575.

Ghavami, S., Hashemi, M., Ande, S. R., Yeganeh, B., Xiao, W., Eshraghi, M., Bus, C. J., Kadkhoda, K., Wiechec, E., Halayko, A. J., & Los, M. (2009). Apoptosis and cancer: mutations within caspase genes. *Journal of Medical Genetics*, *46*(8), 497-510.

Gherghi, I. C., Girousi, S. T., Voulgaropoulos, A. N., & Tzimou-Tsitouridou, R. (2003). Study of interactions between DNA-ethidium bromide (EB) and DNA-acridine orange (AO), in solution, using hanging mercury drop electrode (HMDE). *Talanta*, *61*(2), 103-112.

Glazer, L., Shechter, A., Tom, M., Yudkovski, Y., Weil, S., Aflalo, E. D., Pamuru, R. R., Khalaila, I., Bentov, S., Berman, A., & Sagi, A. (2010). A protein involved in the assembly of an extracellular calcium storage matrix. *Journal of Biological Chemistry*, *285*(17), 12831-12839.

Guadagno, T. M., & Newport, J. W. (1996). Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2–cyclin B kinase activity. *Cell*, *84*(1), 73-82.

Gupta, V., Zhang, Q. J., & Liu, Y. Y. (2011). Evaluation of anticancer agents using flow cytometry analysis of cancer stem cells. (In) *Drug Design and Discovery* (pp.179-191). Humana Press.

Hassan, M., Watari, H., Abualmaaty, A., Ohba, Y., & Sakuragi, N. (2014). Apoptosis and molecular targeting therapy in cancer. *BioMed Research International*, *ID 150845*, 1-23.

Herrema, H., Czajkowska, D., Théard, D., van der Wouden, J. M., Kalicharan, D., Zolghadr, B., Hoekstra, D., & van Ijzendoorn, S.C. (2006). Rho kinase, myosin-II, and p42/44 MAPK control extracellular matrix-mediated apical bile canalicular lumen morphogenesis in HepG2 cells. *Molecular Biology of the Cell*, *17*(7), 3291-3303.

Howie, B. J., & Shultz, T. D. (1985). Dietary and hormonal interrelationships among vegetarian seventh-day adventists and nonvegetarian men. *The American Journal of Clinical Nutrition*, *42*(1), 127-134.

Ihrke, G., Martin, G. V., Shanks, M. R., Schrader, M., Schroer, T. A., & Hubbard, A. L. (1998). Apical plasma membrane proteins and endolyn-78 travel through a subapical compartment in polarized WIF-B hepatocytes. *The Journal of Cell Biology*, *141*(1), 115-133.

Jordan, M. A., & Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, *4*(4), 253-265

Kuznetsov, A. V., & Gnaiger, E. (2010). Laboratory protocol lactate dehydrogenase cytosolic marker enzyme. *Mito Physiol Net*, *8*, 1-8.

Lacroix, M., & Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Research and Treatment*, *83*(3), 249-289

Leikin, A. I., & Brenner, R. R. (1989). Fatty acid desaturase activities are modulated by phytosterol incorporation in microsomes. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1005*(2), 187-191.

Lew, D. J., Dulić, V., & Reed, S. I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell*, *66*(6), 1197-1206.

Li, L., Dai, H. J., Ye, M., Wang, S. L., Xiao, X. J., Zheng, J., Chen, H. Y., Luo, Y. H., & Liu, J. (2012). Lycorine induces cell-cycle arrest in the G0/G1 phase in K562 cells via HDAC inhibition. *Cancer Cell International*, *12*(1), 49.

Liu, K., Liu, P. C., Liu, R., & Wu, X. (2015). Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Medical Science Monitor Basic Research*, *21*, 15.

Los, M., Wesselborg, S., & Schulze-Osthoff, K. (1999). The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. *Immunity*, *10*(6), 629-639.

Louzada, S., Adega, F., & Chaves, R. (2012). Defining the sister rat mammary tumor cell lines HH-16 cl. 2/1 and HH-16. cl. 4 as an *in vitro* cell model for Erbb2. *PloS One*, *7*(1), e29923.

Loveland, B. E., Johns, T. G., Mackay, I. R., Vaillant, F., Wang, Z. X., & Hertzog, P. J. (1992). Validation of the MTT dye assay for

enumeration of cells in proliferative and antiproliferative assays. *Biochemistry International*, *27*(3), 501-510.

Lukas, J., Pagano, M., Staskova, Z., Draetta, G., & Bartek, J. (1994). Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumour cell lines. *Oncogene*, *9*(3), 707-718.

Mettlin, C. (1997). Recent developments in the epidemiology of prostate cancer. *European Journal of Cancer*, *33*(3), 340-347.

Moon, D. O., Lee, K. J., Choi, Y. H., & Kim, G. Y. (2007). β-Sitosterol-induced-apoptosis is mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells. *International Immunopharmacology*, *7*(8), 1044-1053.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1), 55-63.

McCormick, C., & Freshney, R. I. (2000). Activity of growth factors in the IL-6 group in the differentiation of human lung adenocarcinoma. *British Journal of Cancer*, *82*(4), 881.

Nakatsu, N., Yoshida, Y., Yamazaki, K., Nakamura, T., Dan, S., Fukui, Y., & Yamori, T. (2005). Chemosensitivity profile of cancer cell lines and identification of genes determining chemosensitivity by an integrated bioinformatical approach using cDNA arrays. *Molecular Cancer Therapeutics*, *4*(3), 399-412.

Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., Clark, L., Bayani, N., Coppe, J. P., Tong, F., & Speed, T. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, *10*(6), 515-527.

Park, C., Moon, D. O., Rhu, C. H., Choi, B. T., Lee, W. H., Kim, G. Y., & Choi, Y. H. (2007). Beta-sitosterol induces anti-proliferation and apoptosis in human leukemic U937 cells through activation of caspase-3 and induction of Bax/Bcl-2 ratio. *Biological and Pharmaceutical Bulletin*, *30*(7), 1317-1323.

Peacock, A. C., & Dingman, C. W. (1968). Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry*, *7*(2), 668-674.

Plumb, J. A., Milroy, R., & Kaye, S. B. (1989). Effects of the pH dependence of 3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Research*, *49*(16), 4435-4440.

Pörn, M. I., & Slotte, J. P. (1995). Localization of cholesterol in sphingomyelinase-treated fibroblasts. *Biochemical Journal*, *308*(1), 269-274.

Pozarowski, P., Grabarek, J., & Darzynkiewicz, Z. (2004). Flow cytometry of apoptosis. *Current Protocols in Cell Biology*, 18-8.

Rabinovitch, P. S. (1994). DNA content histogram and cell-cycle analysis. *Methods Cell Biology*, *41*, 263-296.

Raju, J., Patlolla, J. M., Swamy, M. V., & Rao, C. V. (2004). Diosgenin, a steroid saponin of Trigonella foenum graecum (Fenugreek), inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon cancer cells. *Cancer Epidemiology Biomarkers and Prevention*, *13*(8), 1392-1398.

Rashedi, I., Panigrahi, S., Ezzati, P., Ghavami, S., & Los, M. (2007). Autoimmunity and apoptosis-therapeutic implications. *Current Medicinal Chemistry*, *14*(29), 3139-3151.

Renvoize, C., Biola, A., Pallardy, M., & Breard, J. (1998). Apoptosis: identification of dying cells. *Cell Biology and Toxicology*, *14*(2), 111-120.

Repetto, G., del Peso, A., & Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*, *3*(7), 1125-1131.

Ribble, D., Goldstein, N. B., Norris, D. A., & Shellman, Y. G. (2005). A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnology*, *5*(1), 12.

Riss, T. E. R. R. Y., Moravec, R., & Niles, A. (2005). Selecting cell-based assays for drug discovery screening. *Cell Notes*, *13*, 16-21.

Roninson, I. B. (2002). Oncogenic functions of tumour suppressor p21 Waf1/Cip1/Sdi1: association with cell senescence and tumour-promoting activities of stromal fibroblasts. *Cancer Letters*, *179*(1), 1-14.

Sassano, A., & Platanias, L. C. (2008). Statins in tumor suppression. *Cancer Letters*, *260*(1), 11-19.

Schweikert, H. U., Tunn, U. W., Habenicht, U. F., Arnold, J., Senge, T., Schulze, H., Schröder, F. H., Blom, J. H. M., Ennemoser, O., Horniger, W., & Bartsch, G. (1993). Effects of estrogen deprivation on human benign prostatic hyperplasia. *The Journal of Steroid Biochemistry and Molecular Biology*, *44*(4), 573-576.

Sgonc, R., & Wick, G. (1994). Methods for the detection of apoptosis. *International Archives of Allergy and Immunology*, *105*(4), 327-332.

Slotte, J. P. (1999). Sphingomyelin–cholesterol interactions in biological and model membranes. *Chemistry and Physics of Lipids*, *102*(1), 13-27.

Smith, S. M., Wunder, M. B., Norris, D. A., & Shellman, Y. G. (2011). A simple protocol for using a LDH-based cytotoxicity assay to assess the effects of death and growth inhibition at the same time. *PloS One*, *6*(11), e26908.

Spector, A. A., & Yorek, M. A. (1985). Membrane lipid composition and cellular function. *Journal of Lipid Research*, *26*(9), 1015-1035.

Speirs, V., Ray, K. P., & Freshney, R. I. (1991). Paracrine control of differentiation in the alveolar carcinoma, A549, by human foetal lung fibroblasts. *British Journal of Cancer*, *64*(4), 693.

Sumantran, V. N. (2011). Cellular chemosensitivity assays: an overview. (In) *Cancer Cell Culture* (pp. 219-236). Humana Press.

Sylvester, P. W. (2011). Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. (In) *Drug Design and Discovery* (pp. 157-168). Humana Press.

Tsuruo, T., Naito, M., Tomida, A., Fujita, N., Mashima, T., Sakamoto, H., & Haga, N. (2003). Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Science*, *94*(1), 15-21.

Van Staveren, W. C. G., Solís, D. W., Hebrant, A., Detours, V., Dumont, J. E., & Maenhaut, C. (2009). Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells?. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1795*(2), 92-103.

Van de Loosdrecht, A. A., Beelen, R. H. J., Ossenkoppele, G. J., Broekhoven, M. G., & Langenhuijsen, M. M. A. C. (1994). A

tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *Journal of Immunological Methods*, *174*(1), 311-320.

Vijayarathna, S., & Sasidharan, S. (2012). Cytotoxicity of methanol extracts of Elaeis guineensis on MCF-7 and Vero cell lines. *Asian Pacific Journal of Tropical Biomedicine*, *2*(10), 826-829.

Wahl, G. M., & Carr, A. M. (2001). The evolution of diverse biological responses to DNA damage: insights from yeast and p53. *Nature Cell Biology*, *3*(12), E277-E286.

Woyengo, T. A., Ramprasath, V. R., & Jones, P. J. H. (2009). Anticancer effects of phytosterols. *European Journal of Clinical Nutrition*, *63*(7), 813-820.

Zhao, Y. Y., Shen, X., Chao, X., Ho, C. C., Cheng, X. L., Zhang, Y., Lin, R.C., Du, K.J., Luo, W.J., Chen, J.Y., & Sun, W. J. (2011). Ergosta-4, 6, 8 (14), 22-tetraen-3-one induces G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells.*Biochimica et Biophysica Acta (BBA)-General Subjects*, *1810*(4), 384-390.

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Chapter 5

SUMMARY

Thesis entitled "Anticancer Potential of Sterols Isolated from *Turbinaria conoides* Against Lung and Liver Cancer", is exploring the cytotoxic potential of sterols isolated from a brown seaweed *Turbinaria conoides.* Various cytotoxic studies have identified the potential of sterols from *Turbinaria conoides* to suppress the proliferation of malignant cells effectively. However, their potential and mechanism of action to suppress lung and liver cancer are not studied so far in detail. Considering these facts, this brown sea weed, which are abundant along the shorelines of Arabian Sea and Bay of Bengal, is a promising source for developing safe drug combinations and hence needs immediate attention. The data generated in this study can be used for developing safe drug combinations to cure lung and liver cancer effectively without long and short term side effects.

Globally the cancer cases are increasing at an alarming rate, mainly due to changing life style and food habits. Even though significant amount of research are going on to contain this dread full disease, treatment methods developed are only partially successful in preventing their advancement and lead to serious side effects in some instances to lack of specificity. In this perspective, chemicals derived

from various natural sources due to their potential to destroy malignant cells by specifically targeting the affected region have gained significance in the treatment of cancer. As far as anticancer-lead compounds are concerned, unique chemical moieties in marine floras are least explored for their cytotoxic potential and are under-represented in current pharmacopoeia.

Collaborative efforts of researchers from various disciplines (natural product and medicinal chemistry) have made foundation for screening of therapeutically active products coast effectively from complex matrix and simultaneous mixture ingredient analysis. And in this case, SRB viability assay of lipid fractions collected from *Turbinaria conoides* against A549 and HEP G2 cell line, and their composition analysis using GCMS, revealed that fractions were not just bioactive, they seem as a pool of secondary metabolites in which probability of finding potent cytotoxic agents are high. Based on the SRB assay, third neutral fraction was the most active fraction and on the basis of GC-MS characterization, it was presumed that prominent cytotoxic activity shown by this fraction is an outcome of collective action of various sterol molecules identified in it. Further, selective extraction and chromatographic technique can be applied effectively to isolate and characterize bioactive molecule from the lipid fraction dominated with sterol.

In this study two sterols have been identified from *Turbinaria conoides*. One of the sterol have been identified as stigmasta-5, 24(28)-dien-3β-ol, acetate (fucosterol acetate) and other as 27 ethyl 27methyl-stigmasta-5, $24^1(24^2)$, 25-triene-3β-ol (Turbiconol). The structure of these compound were determined using high resolution mass spectroscopy (FABMS), GC-MS, 1D (proton NMR, C-13 NMR, DEPT) and 2D NMR (H-H COSY, HSQC, and HMBC) melting point, UV-Vis spectroscopy, specific rotation and FTIR. Oxygenated sterols such as 24*ε*-hydroperoxy-24-vinylcholesterol, 29-hydroperoxystigmasta-5,24(28)-dien-3*β*-ol, 24-ethylcholesta-4,24(28)-dien-3-one, 24*ε*-hydroperoxy-24-ethylcholesta-4,28(29)-dien-3-one, 24- ethylcholesta-4,24(28)-dien-3,6-dione, 24*ε* -hydroperoxy-24-ethylcholesta-4,28(29)-dien-3,6-dione, 6*β*- hydroxy-24-ethylcholesta-4,24(28)-dien-3-one , and 24 *ε* -hydroperoxy-6*β*-hydroxy-24-ethylcholesta-4,- 28(29)-dien-3-one along with fucosterol, 14,15,18,20-diepoxyturbinarin and 3,6,17-trihydroxy-stigmasta-4,7,24(28)-triene have been previously reported *Turbinaria conoides*. In this context turbiconol reported in this work have not been isolated from *Turbinaria conoides*, *Turbinaria sp.* or any other natural sources. Therefore this compound is a latest compound to the list of sterols identified in *Turbinaria conoides* and is found to be a novel compound.

Anti-proliferative potential of phycosterols isolated from *Turbinaria conoides* against Hep G2 and A549 cancer cell lines are

reveled using multiple cytotoxicity assays (MTT, Neutal Red, LDH). Potential of sterol molecule to induce early and late apoptotic events on cancer cells under investigation were confirmed using double staining. The impact of test compound on cell cycle is vital while evaluating the theraputical activity of compounds as uncontrolled cell division is the root cause of any cell acquiring malignancy and provide a better insight into the interactions occurring at molecular level. Particularly, chromosomal translocations or other genetic alterations that directly affect the function of critical cell cycle proteins such as cyclins as well as tumor suppressors, eg., p53. Here, evaluation of cell cycle distribution by flowcytometry and p21gene expression study strongly support apoptosis induced anti-proliferative potential of sterols. Against Hep G2, both fucosterol acetate and turbiconol selectively induced apoptosis and G0/G1 cell cycle arrest. Whereas G2/M phase cell cycle arrest was observed in A549 cell lines when treated with turbiconol and fucosterol acetate induced G0/G1 cell cycle arrest in A549 human cancer cell lines.

Hence, these results advocates sterols in dietary inclusion and in drug combination for developing cell cycle and cell division targets for limiting cancer cell proliferation, as an important strategy, to modulate signaling pathways leading to cell cycle regulation or directly alter cell cycle regulatory molecules in prevention and treatment of cancer. Overall, aim of the study was achieved. However, there are still more sterol compounds in this fraction of

extract that need to be identified. Isolation of these sterols and its structural characterization and bio-assays might provide a useful solution to problems faced by humans in the treatment of cancer and other ailments.

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APPENDIX

Appendix I

Notes

- Bcl2 (B-cell lymphoma 2) is a regulator proteins that regulate cell death.
- cDNA (complementary DNA) is a double-stranded DNA synthesized from a messenger RNA (mRNA).
- E-CDK2 is a protein which modulates multiple cellular processes by phosphorylating numerous downstream proteins.
- HMG-CoA reductase controls the enzyme activity in mevalonate pathway, which controls the metabolism of cholesterol and other isoprenoids.
- Myc (c-Myc) is a gene that codes for the regulation of transcription factor.
- NPC1L1 (Niemann-Pick C1-Like 1) is a gene associated with Nieman Pick disease.
- p38 MAPK (P38 mitogen-activated protein kinases) are a group of mitogen-activated protein kinases. These classes of proteins respond quickly to stress stimuli, particularly

ultravioletirradiation, heat shock, and osmotic shock, and are associated with cell differentiation, apoptosis and autophagy.

- p21 is a potent cyclin-dependent kinase inhibitor (CKI). This protein inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes through bonding with these complexes and thereby regulate the progression of cell cycle at G_1 and S phase.
- G0 is the resting phase in cell cycle where the cell devision has stopped.
- G1 (Gap 1), this phase in cell cycle make sure that every thing is in order for synthesis of DNA. And size of the cells increases in Gap 1.
- S (Synthesis), in this phase of cell cycle replication of DNA occurs.
- G2 (Gap 2) is the check point which make sure that everything is prepared to enter the M(mitosis) phase and divide. Growth of cell will progress through the gap between DNA synthesis and mitosis.
- M (Mitosis), is a stage in cell cycle were the growth of cell stops and cellular energy is mainly used for the orderly division into two daughter cells.

Appendix II

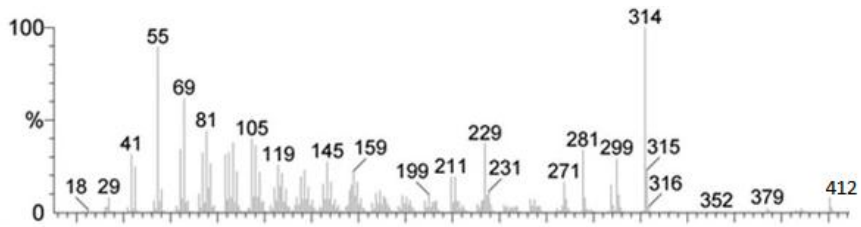

Figure I Mass spectra of fucosterol.

Figure II Developed TLC plate visualized in UV chamber after phosphoric acid staining, and bright spot corresponds to fucosterol acetate.

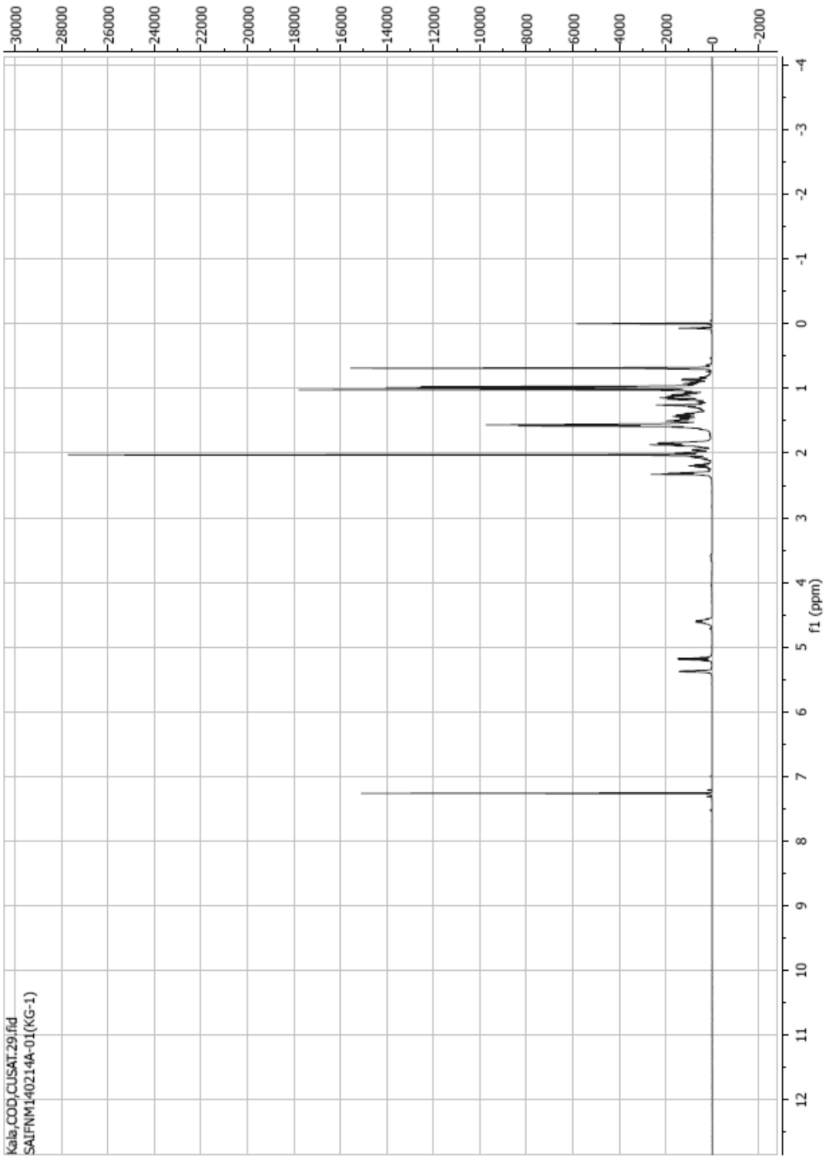

Figure III (1) Proton NMR spectra of fucosterol acetate in $CDCl_3$.

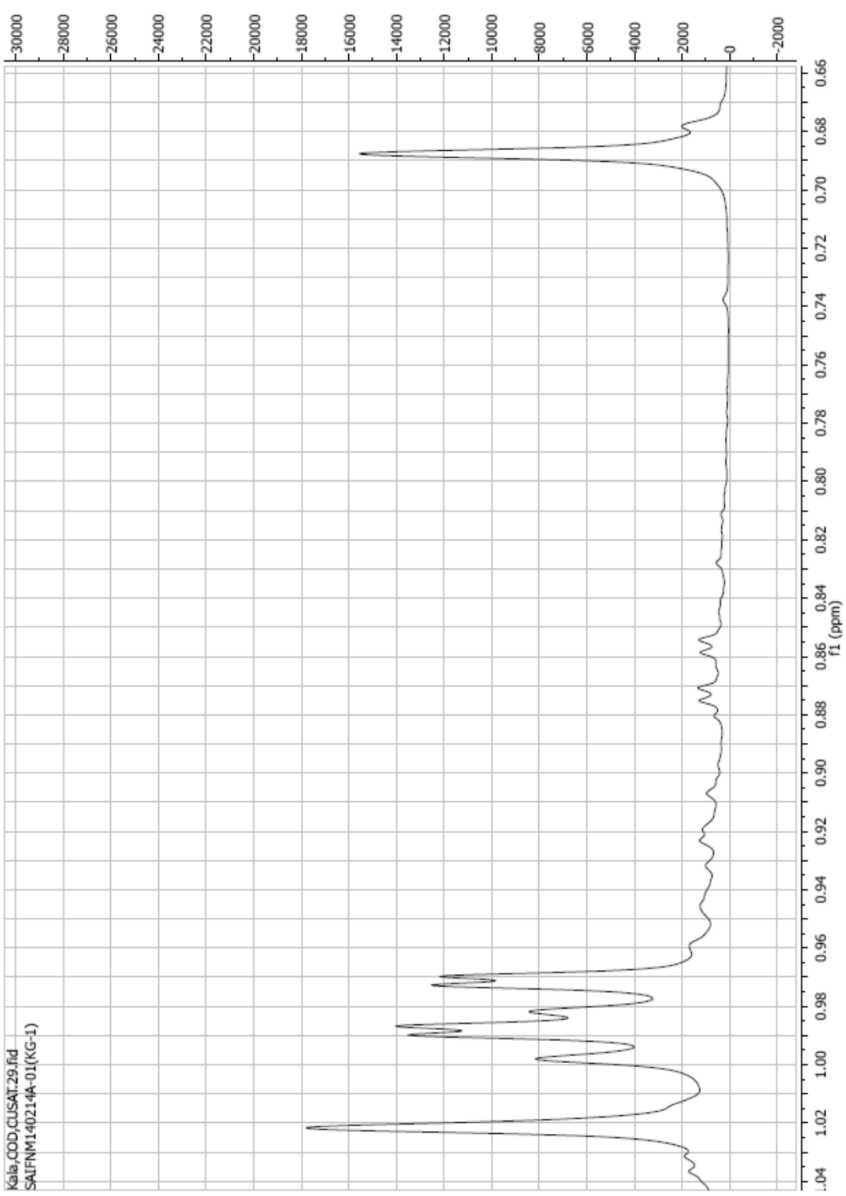

Figure III (a) An enlarged portion (0.66-1.04 ppm) of proton NMR spectra of fucosterol acetate in $CDCl_3$.

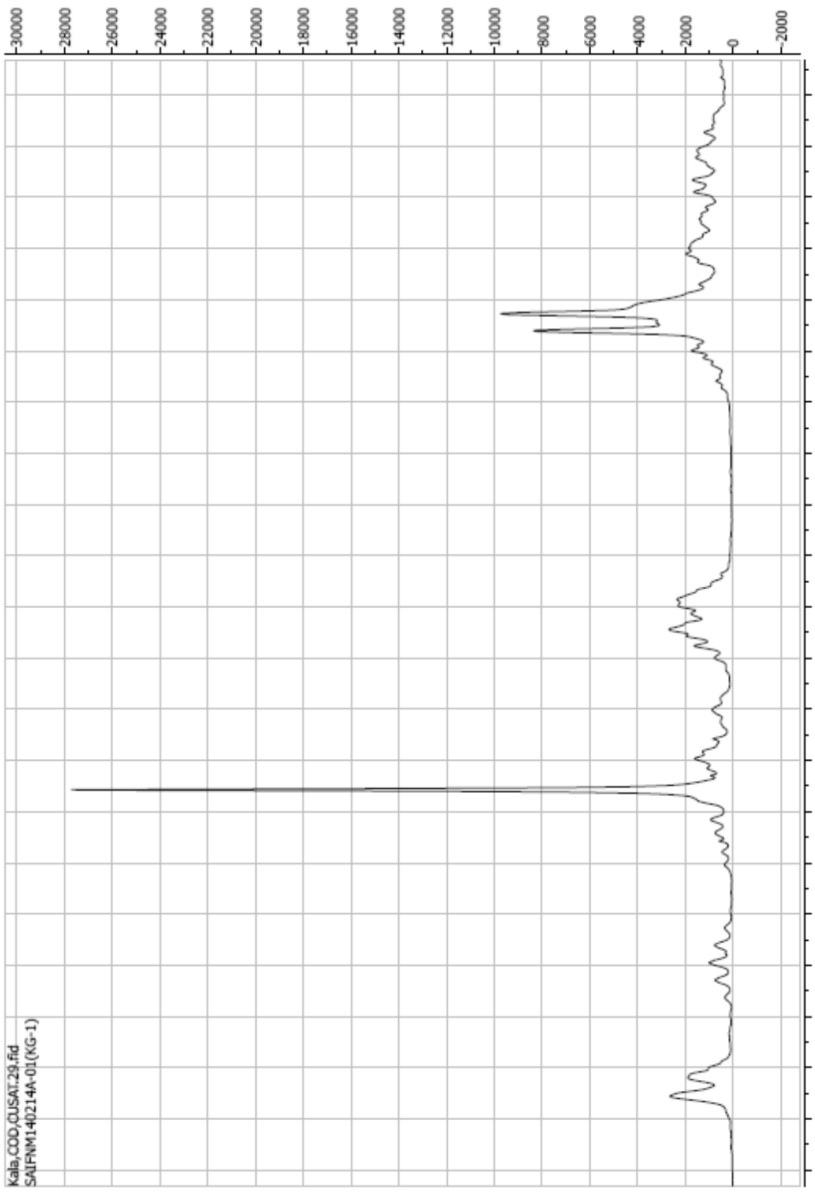

Figure III (1b) An enlarged portion (1.04-2.40 ppm) of proton NMR spectra of fucosterol acetate in $CDCl_3$.

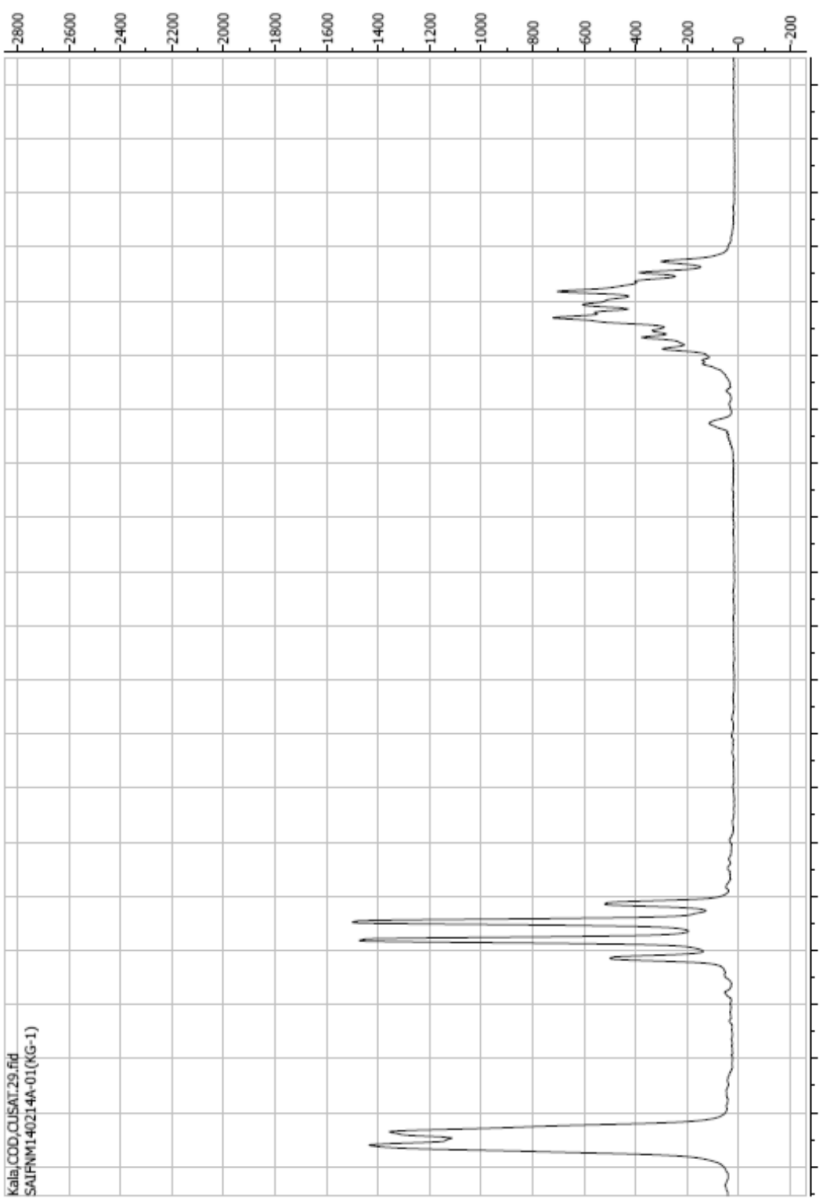

Figure III (1c) An enlarged portion (4.40-5.40 ppm) of proton NMR spectra of fucosterol acetate in $CDCl_3$.

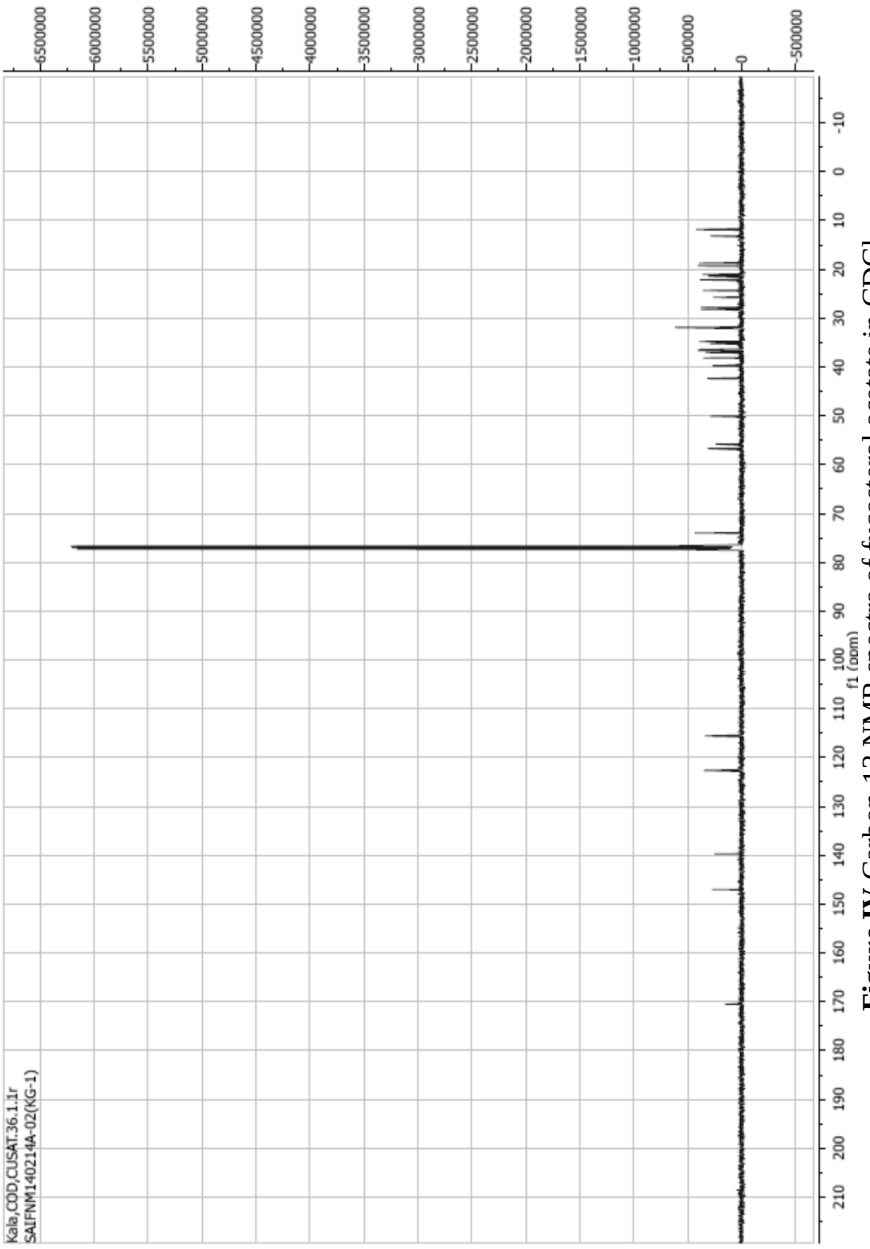

Figure IV Carbon-13 NMR spectra of fucosterol acetate in $CDCl_3$.

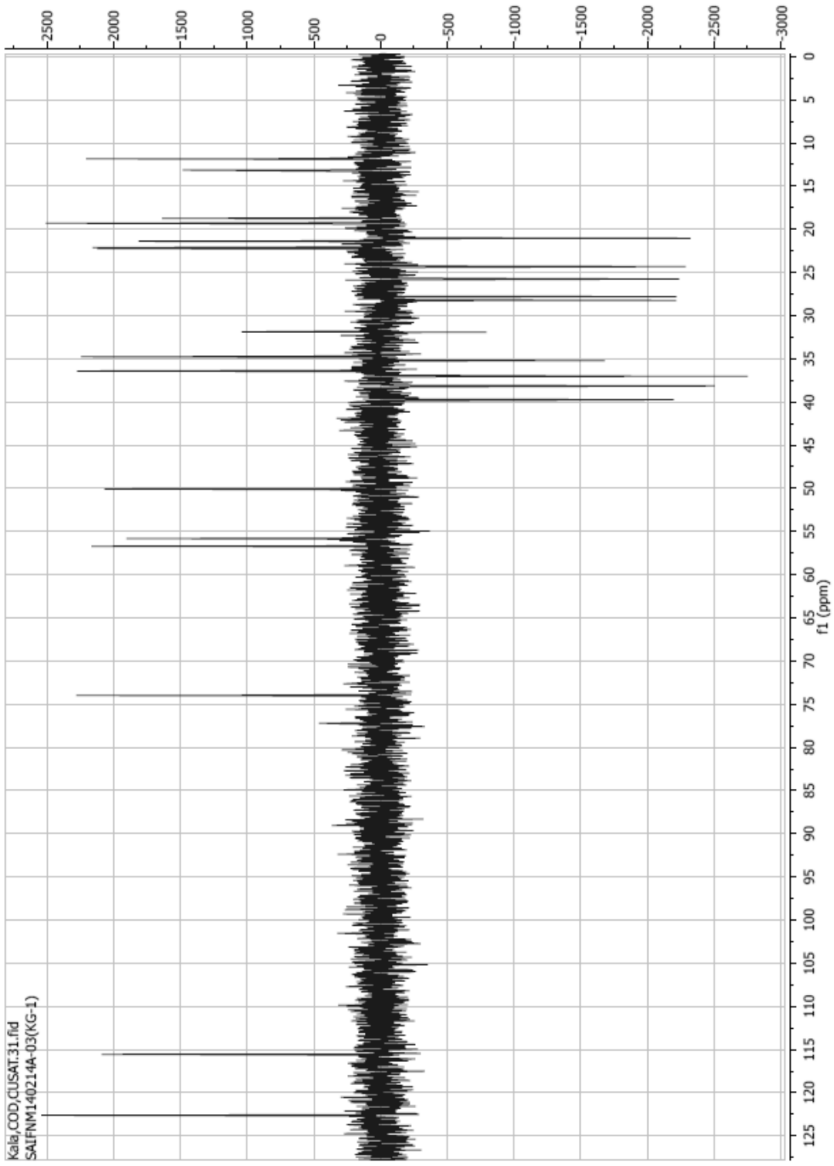

Figure V DEPT-135 spectra of fucosterol acetate in $CDCl_3$.

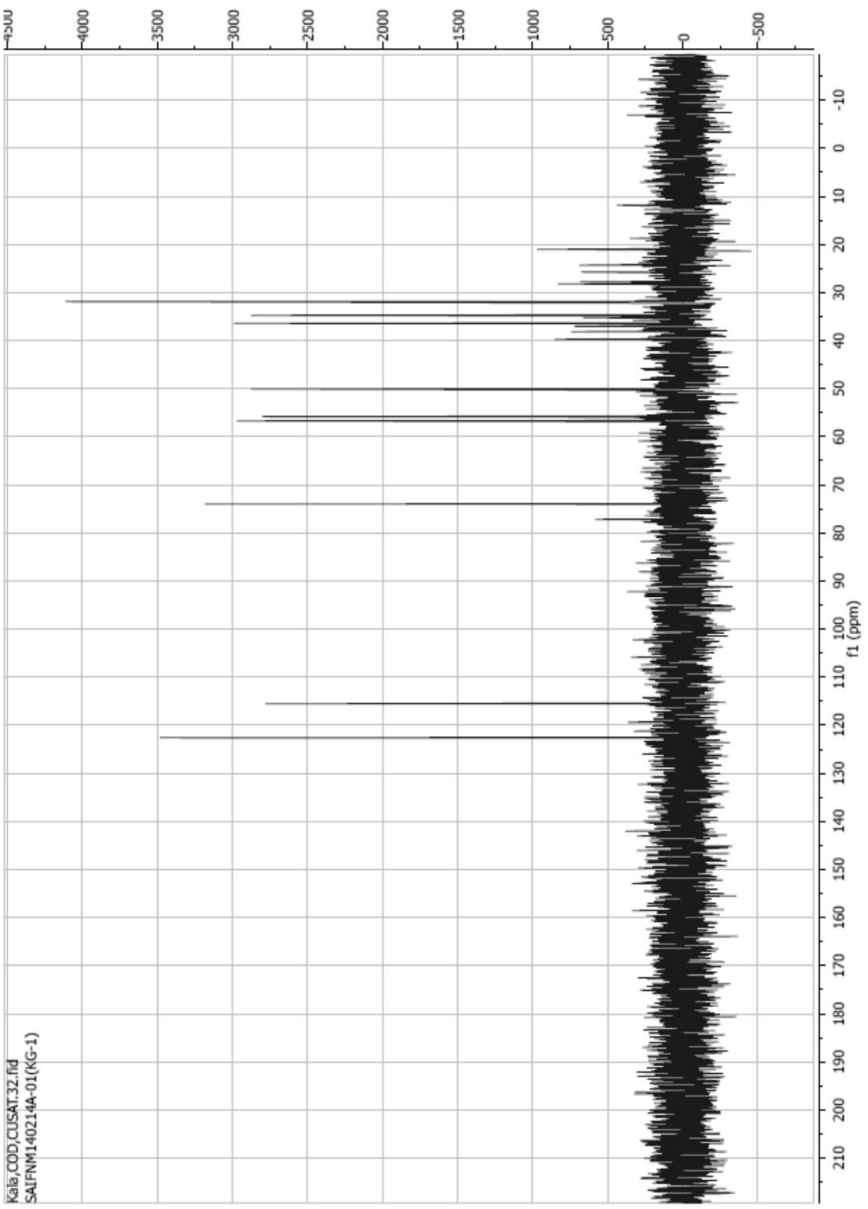

Figure VI DEPT-90 spectra of fucosterol acetate in $CDCl_3$.

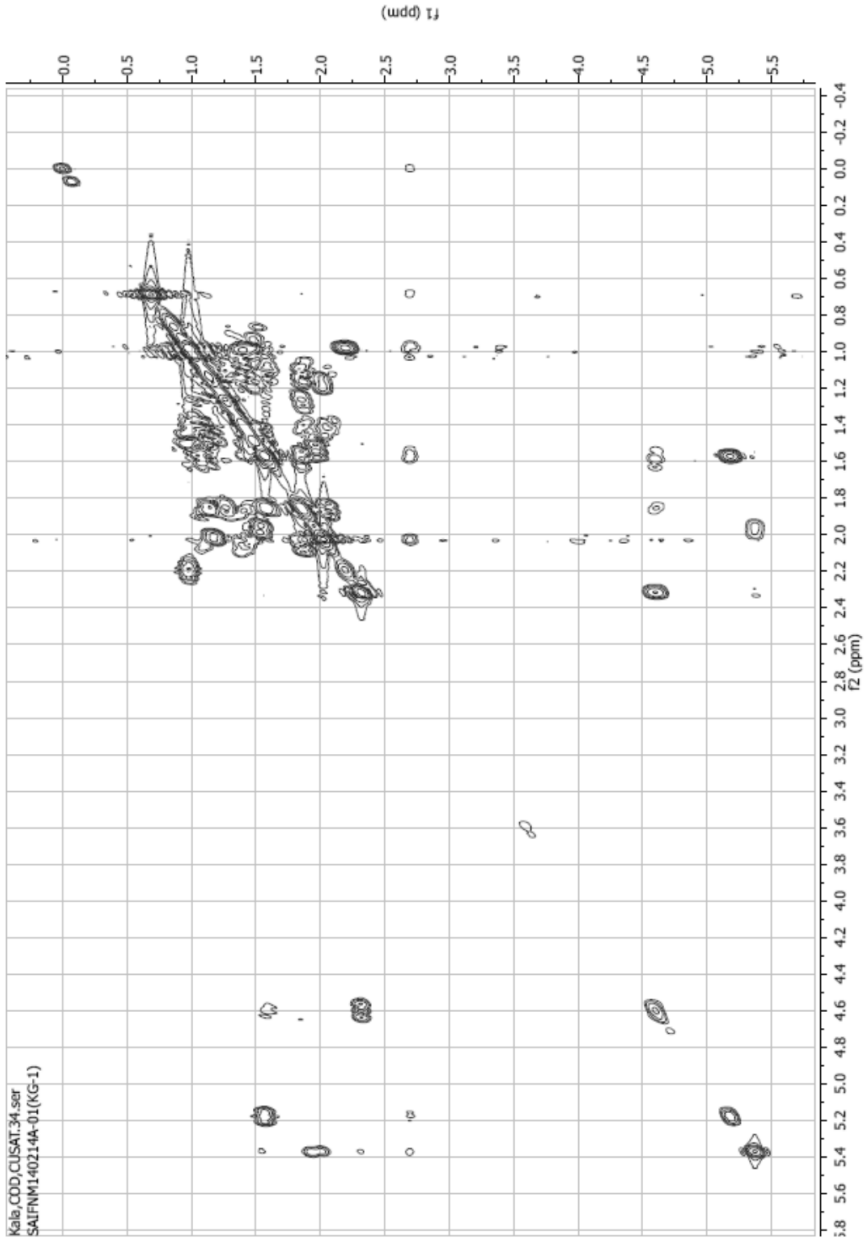

Figure VII COSY NMR spectra of fucosterol acetate in $CDCl_3$.

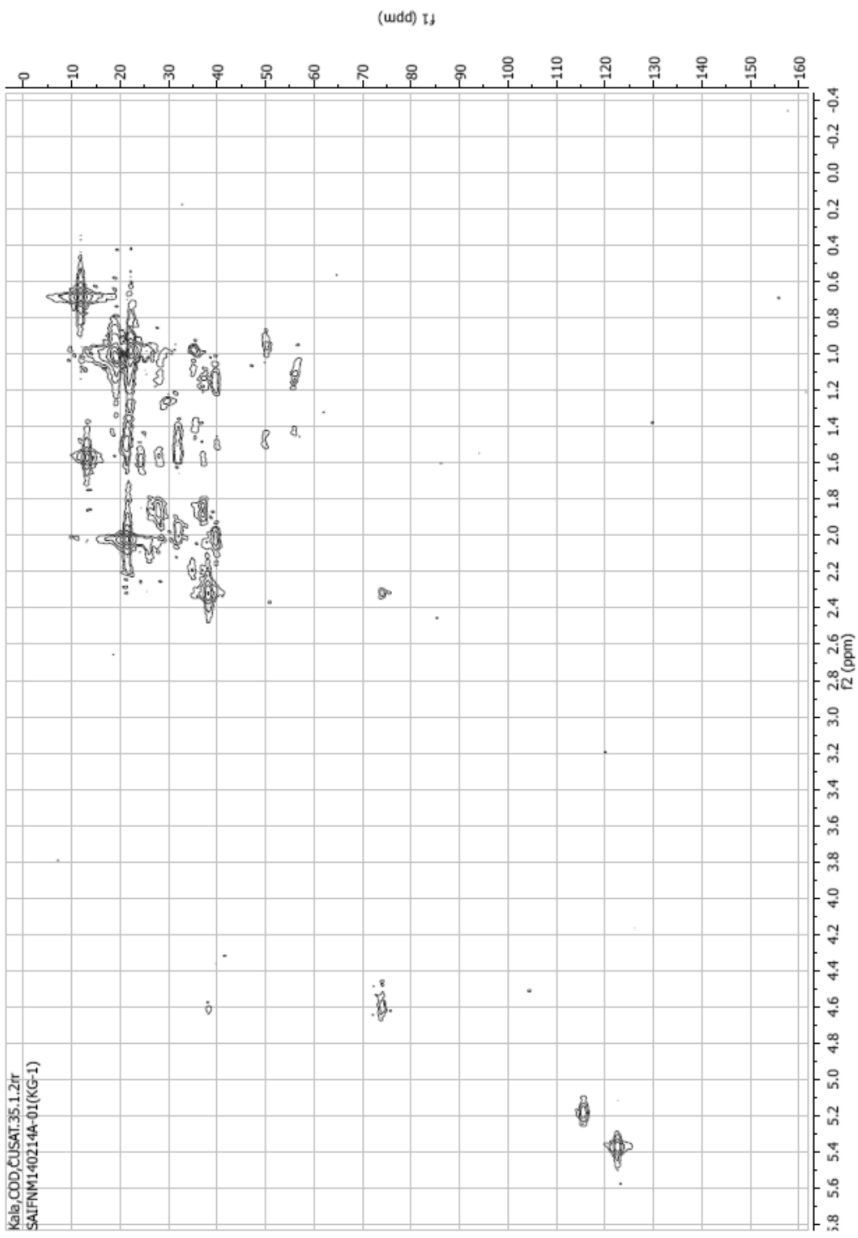

Figure VIII HSQC NMR spectra of fucosterol acetate in $CDCl_3$.

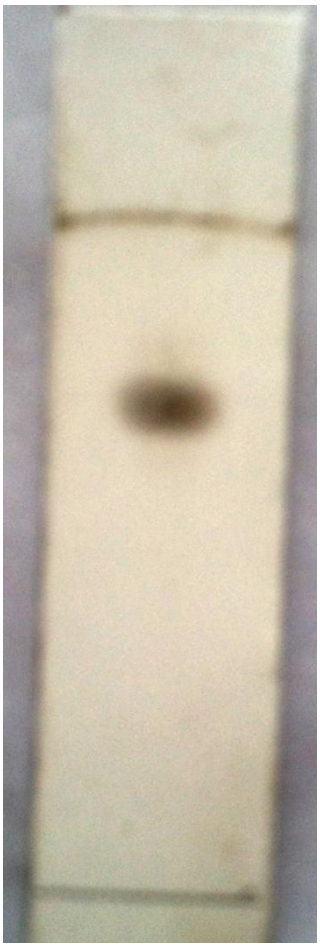

Figure IX Developed TLC plate after phosphoric acid staining, and spot corresponds to turbiconol.

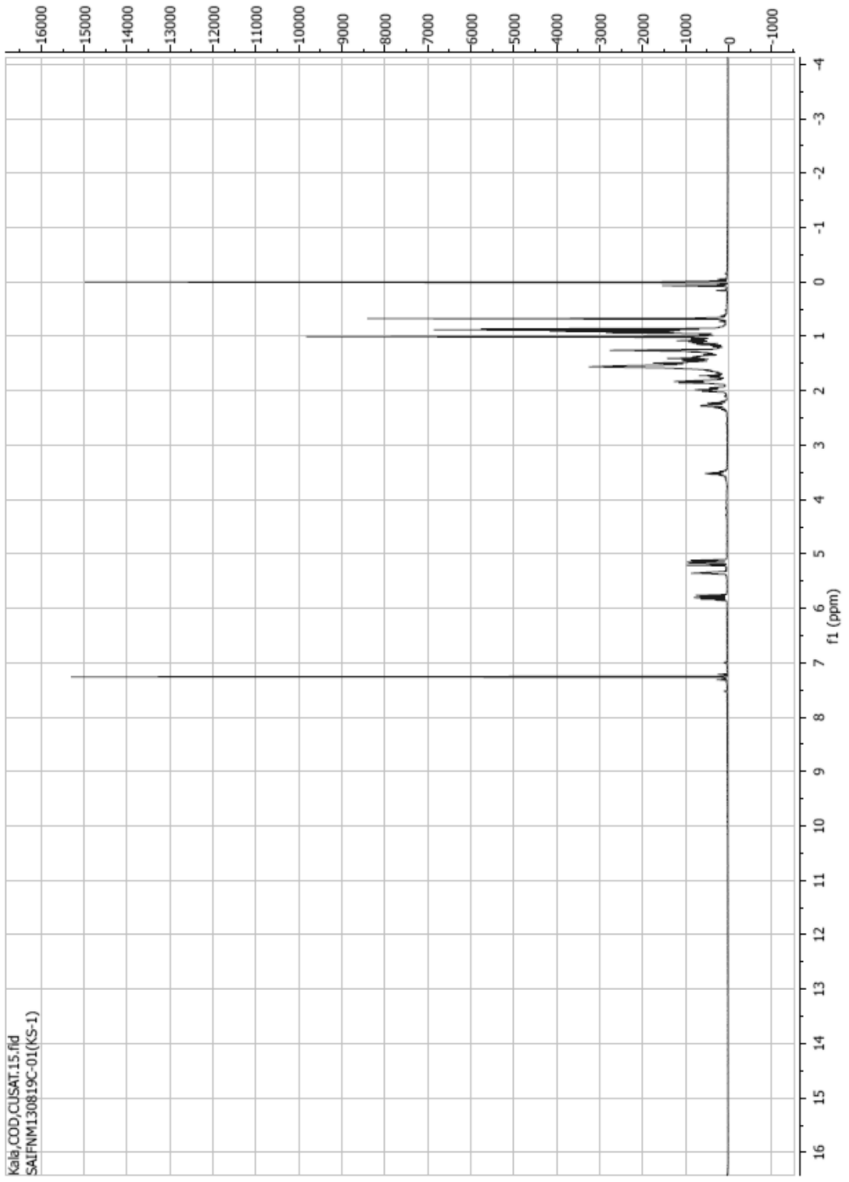

Figure X (1) Proton NMR spectra of turbiconol in $CDCl_3$.

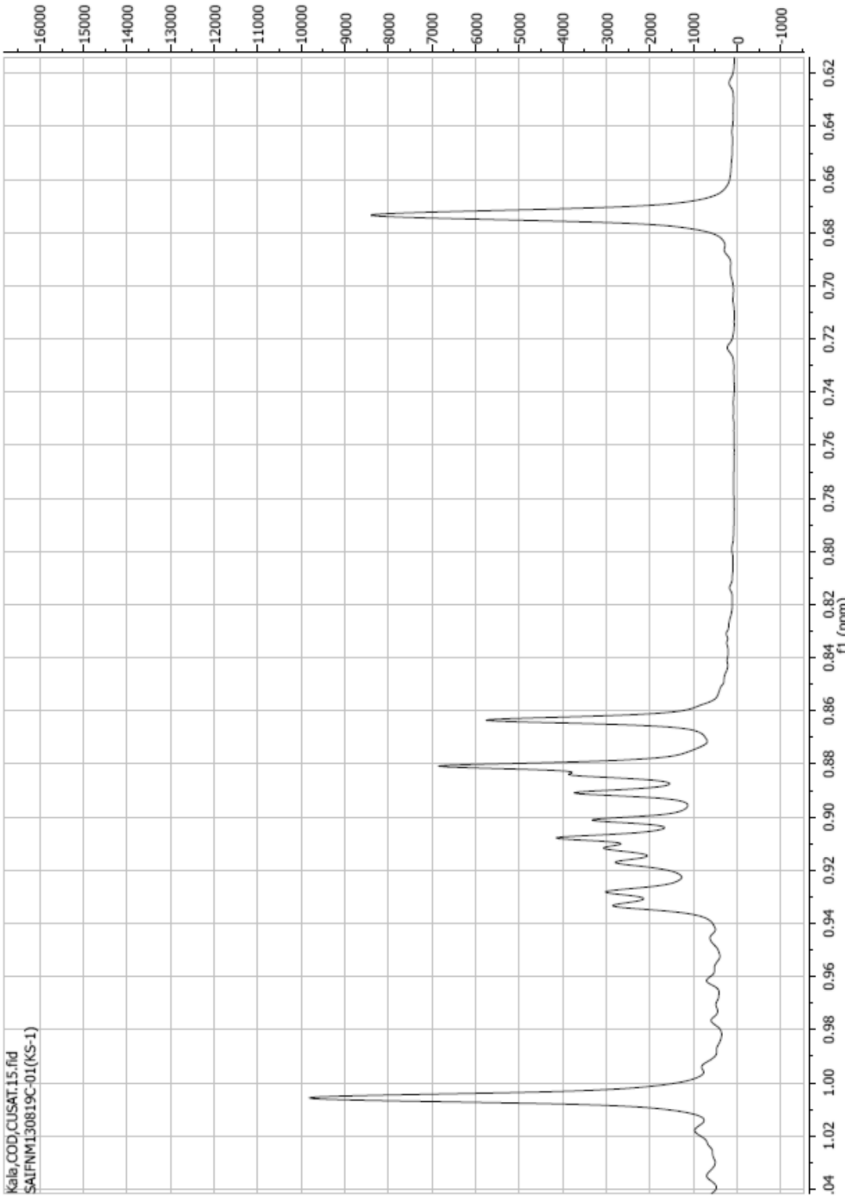

Figure X (1a) An enlarged portion (0.62-1.04 ppm) of proton NMR spectra of turbiconol in $CDCl_3$.

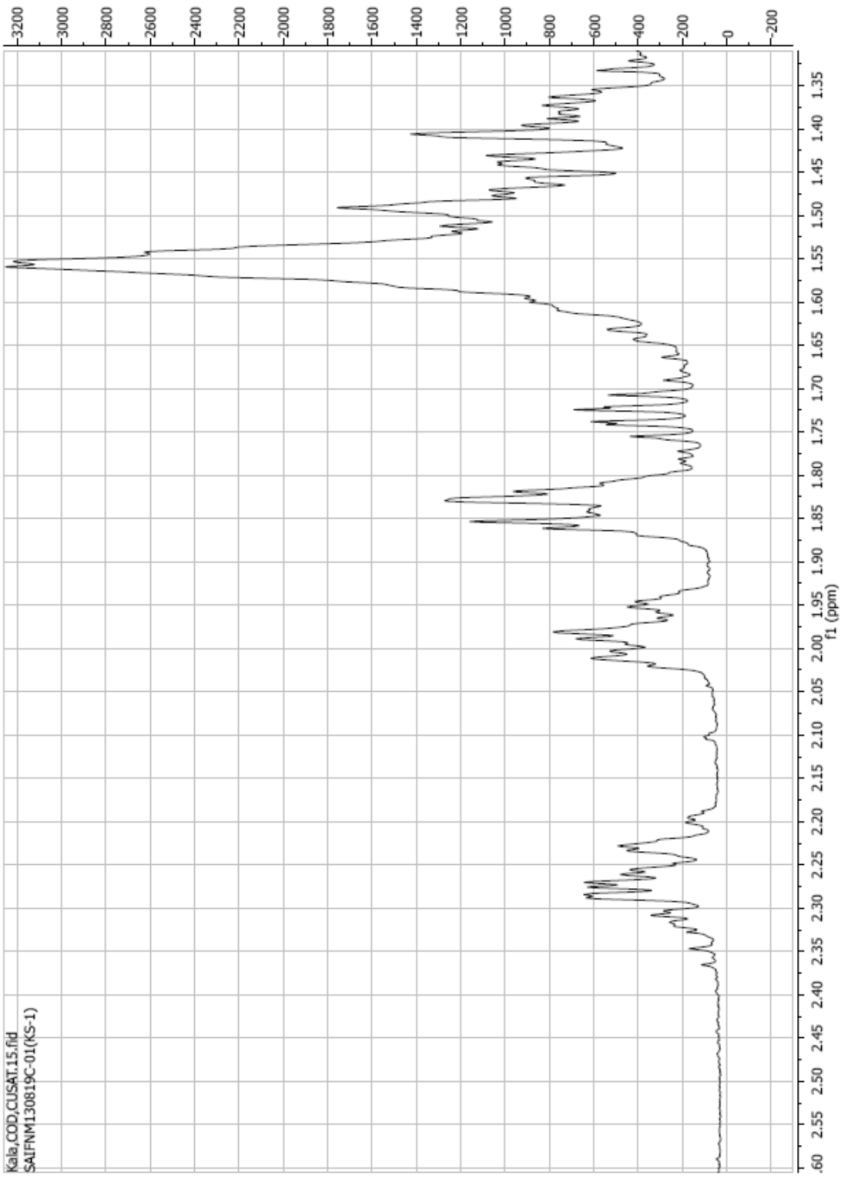

Figure X (1b) An enlarged portion (1.35-2.60 ppm) of proton NMR spectra of turbiconol in $CDCl_3$.

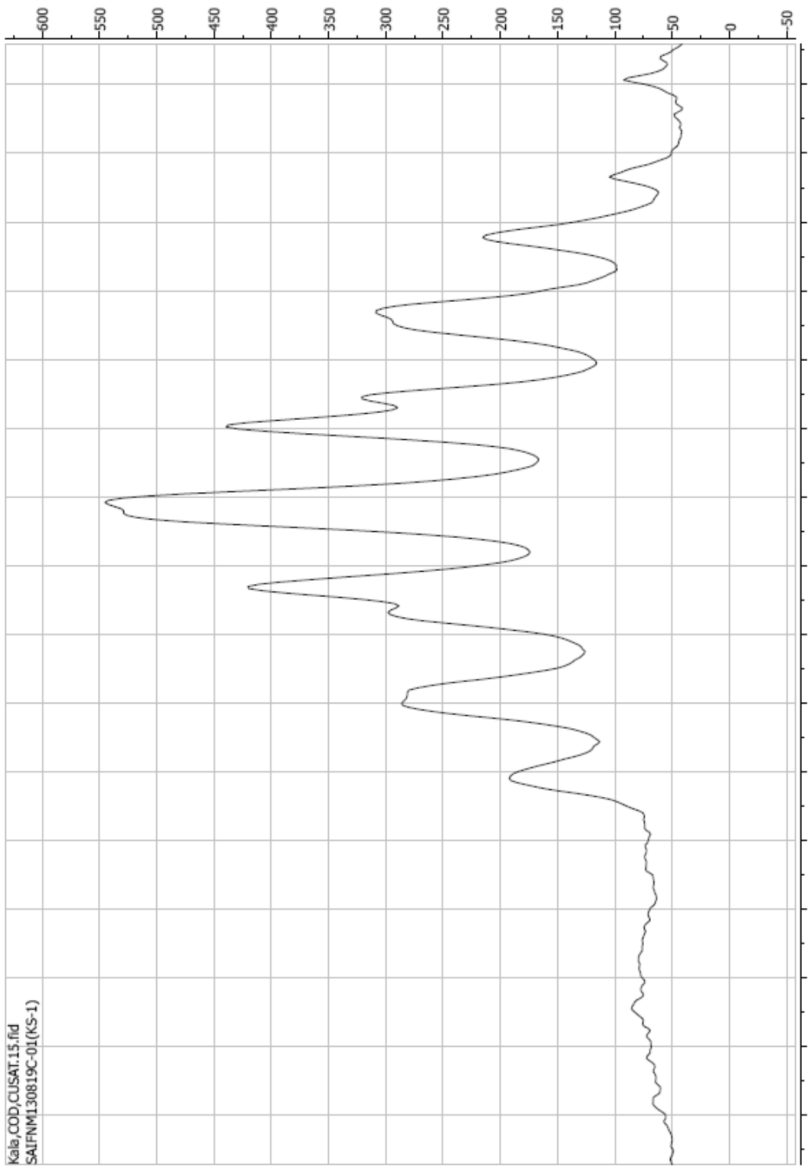

Figure X (1c) An enlarged portion (3.45-3.61 ppm) of proton NMR spectra of turbiconol in $CDCl_3$.

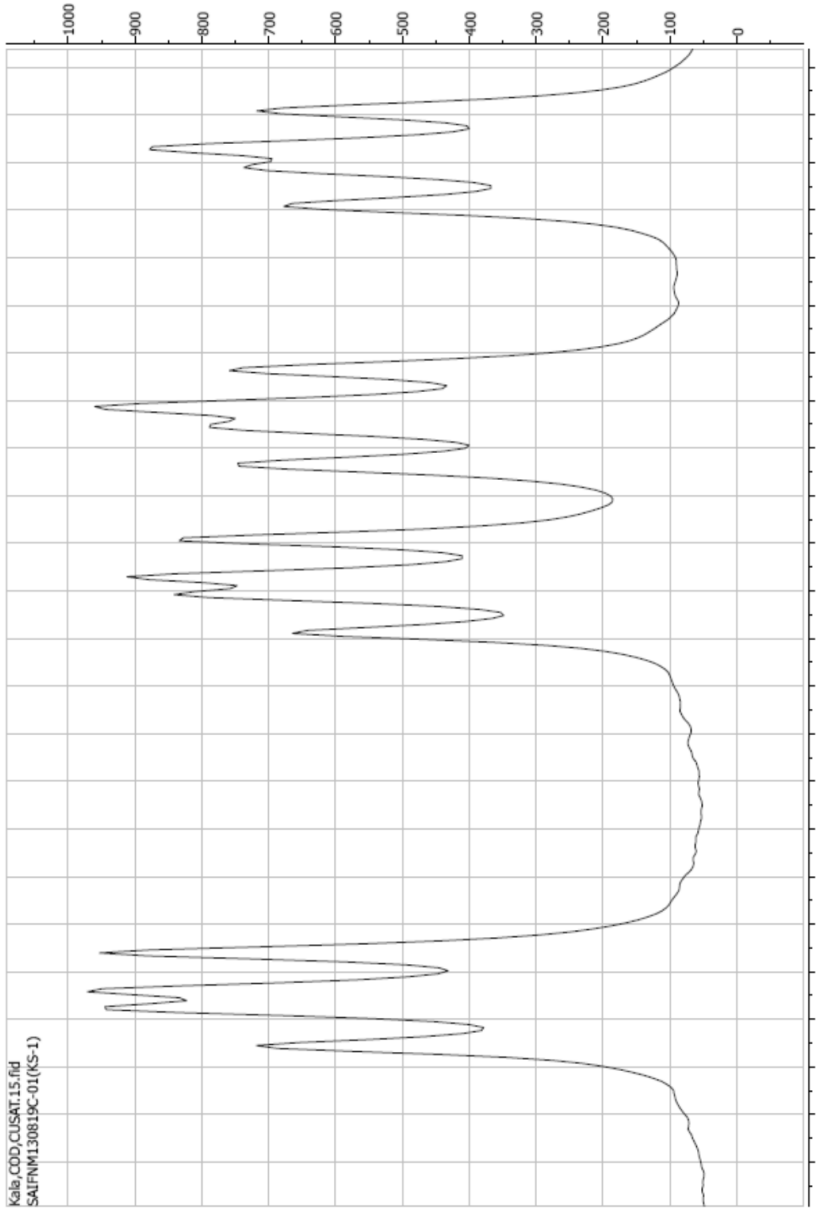

Figure X (1d) An enlarged portion (5.110-5.225 ppm) of proton NMR spectra of turbiconol in $CDCl_3$.

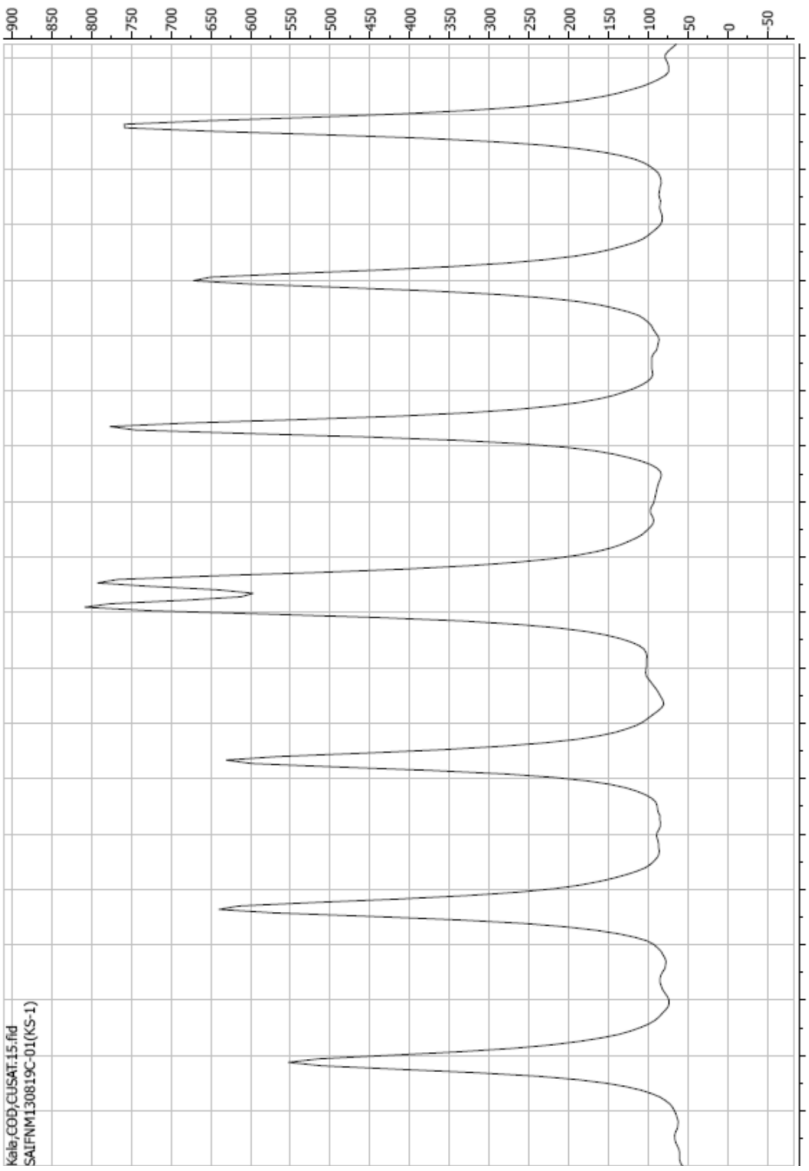

Figure X (1e) An enlarged portion (5.775-5.850 ppm) of proton NMR spectra of turbiconol in $CDCl_3$.

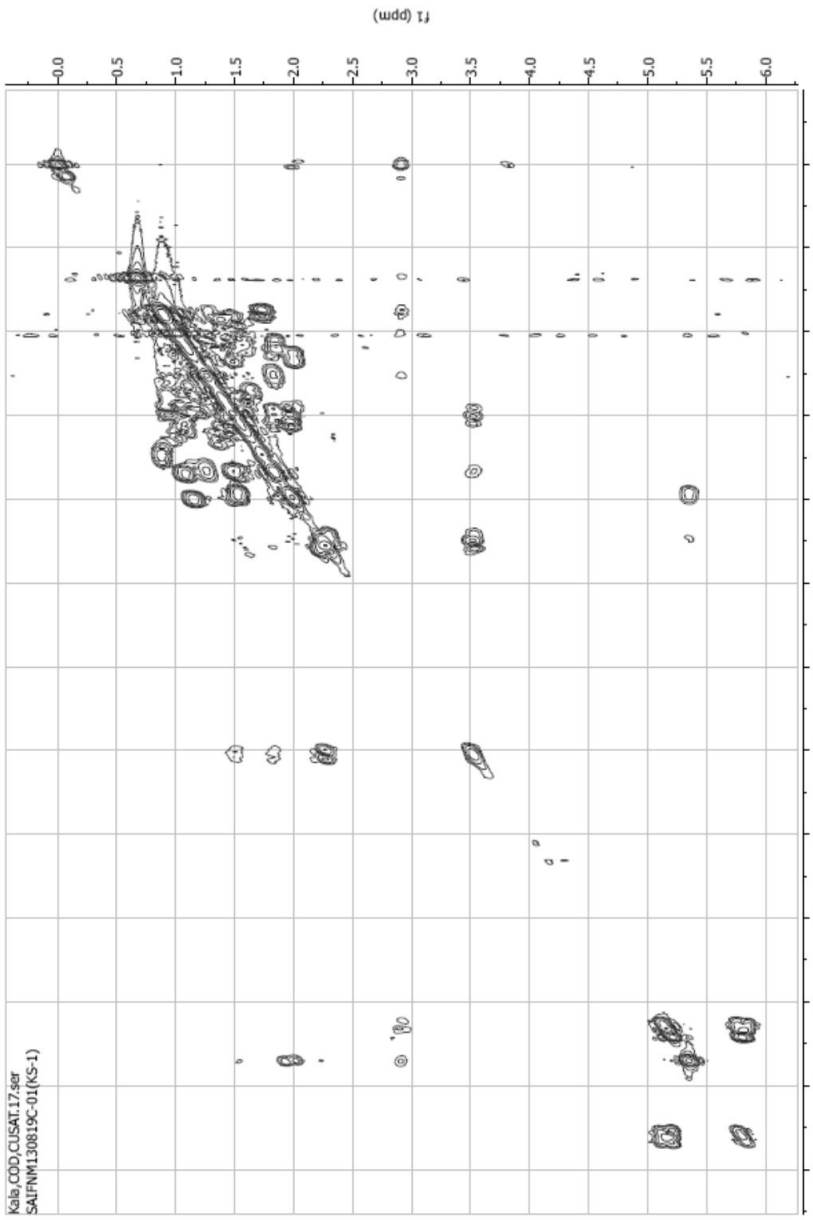

Figure XI Proton NMR spectra of turbiconol in $CDCl_3$.

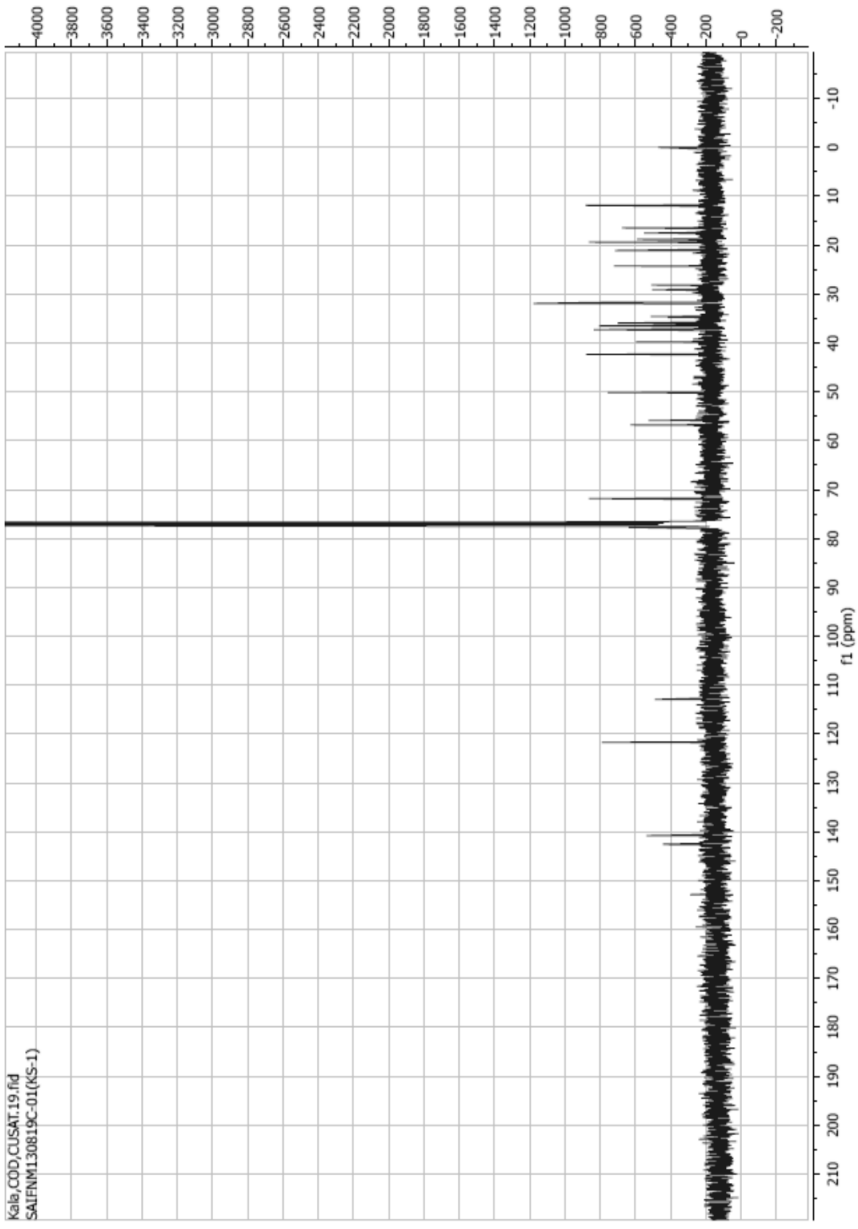

Figure XII Carbon NMR spectra of turbiconol in $CDCl_3$.

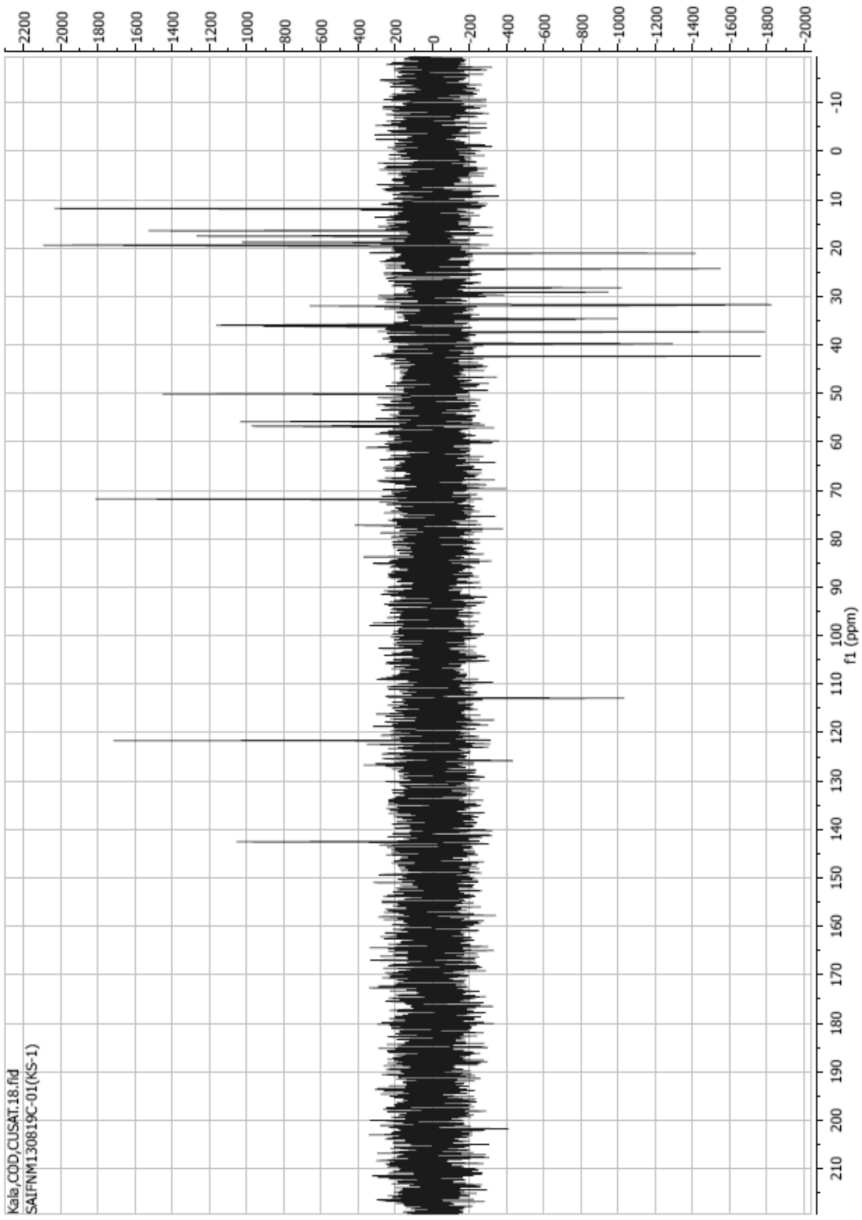

Figure XIII DEPT-135 spectra of turbiconol in $CDCl_3$.

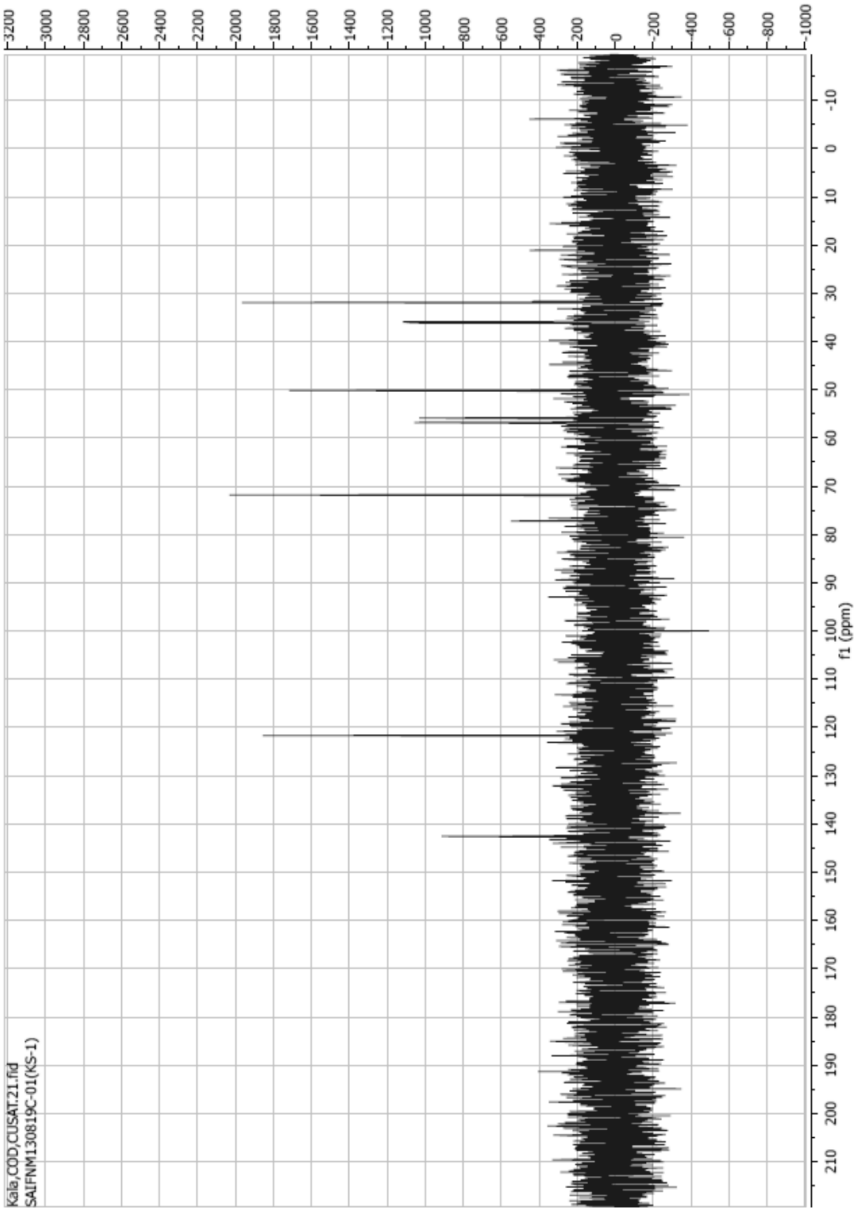

Figure XIV DEPT-90 spectra of turbiconol in $CDCl_3$.

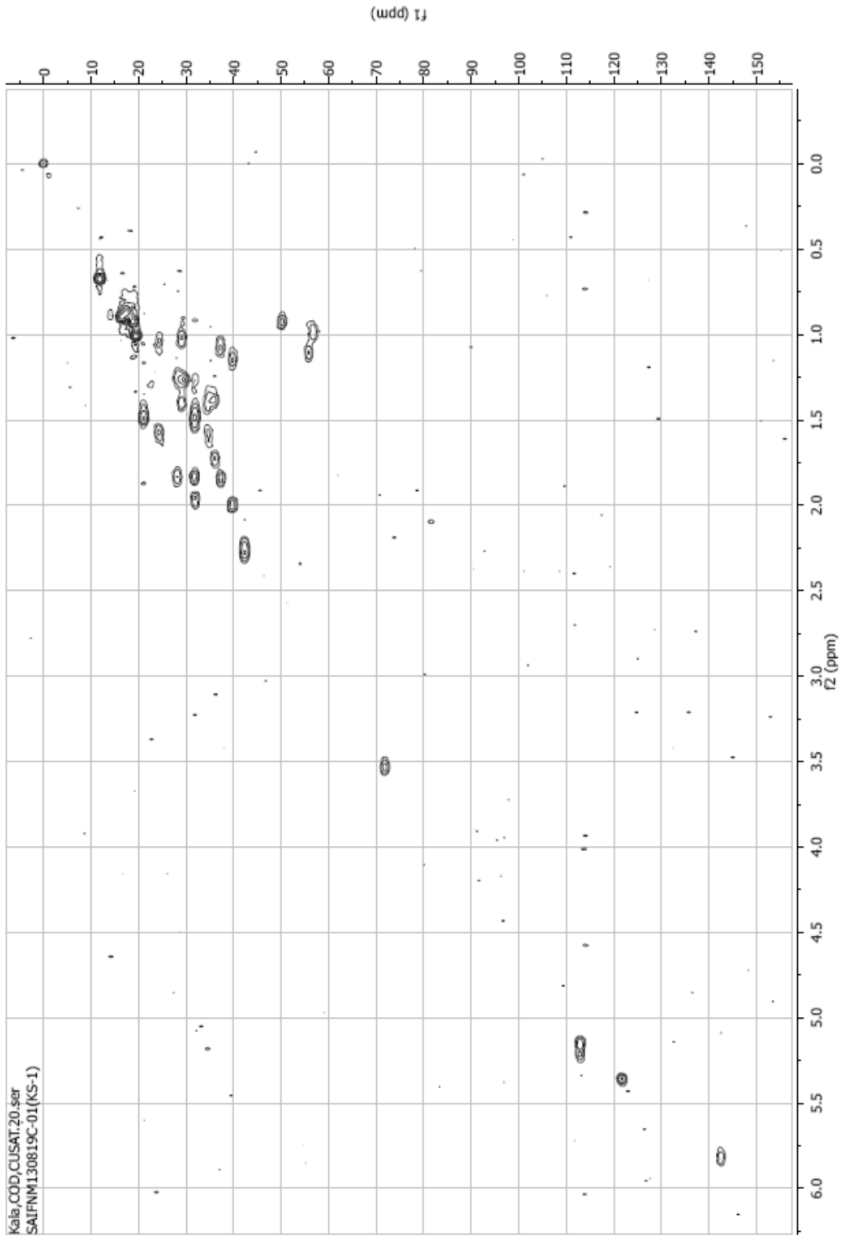

Figure XV HSQC spectra of turbiconol in $CDCl_3$.

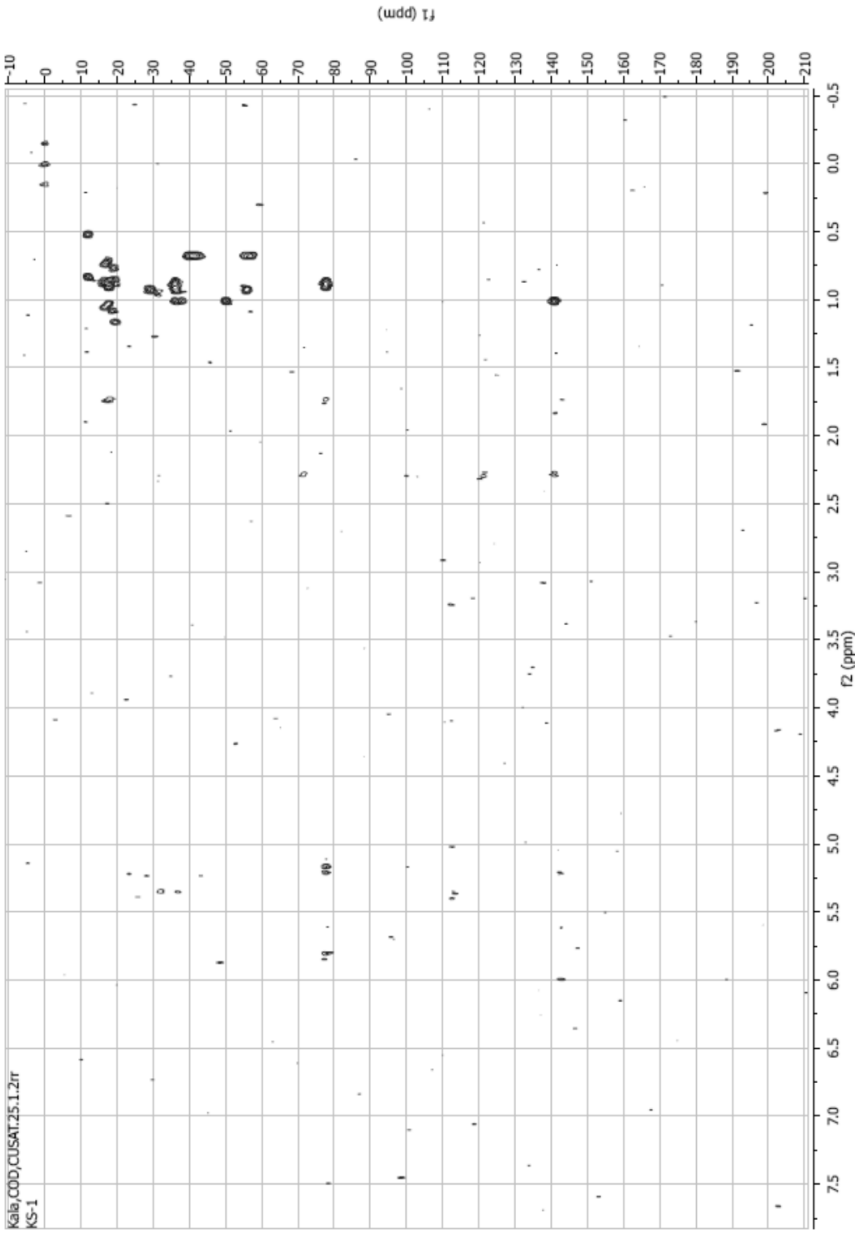

Figure XVI HMBC spectra of turbiconol in $CDCl_3$.

Appendix III

LIST OF PUBLICATIONS

Kala, K. J., Prashob Peter, K. J, & Chandramohanakumar, N. (2015) Cyto-toxic potential of fucosterol isolated from *Turbinaria conoides* against Dalton's Lymphoma Ascites. *International Journal of Pharmacognosy and Phytochemical Research*, 7(6); 1217-1221.

Anuradha, V., Byju, K., **Kala K. J.,** Gopinath, A., Prashob Peter, K. J., Gireesh Kumar, T. R., Vasundhara, G., Rosamine, E., Harishankar, H.S., Kumar, N.C. & Nair, S. M. (2015). Evaluation of biochemical and nutritional potential of seaweeds from Lakshadweep Archipelago. *Journal of Aquatic Food Product Technology*, *24*(7), 639-647.