Isolation and characterization of glycolipids from *Synechocystis* sp. and its cytotoxic potential against colon cancer

Thesis submitted to

Cochin University of Science and Technology

in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

in

Natural Product Chemistry
Under the Faculty of Marine Sciences

by

Saritha S.
Reg. No. 4007

Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi – 682016

October **2017**

Isolation and characterization of glycolipids from *Synechocystis* sp. and its cytotoxic potential against colon cancer

Ph.D. Thesis under the Faculty of Marine Sciences

Author

Saritha S.
Research Scholar
Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016
Email: s.sarithababu@gmail.com

Supervising Guide

Dr. S. Muraleedharan Nair
Professor
Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016
Email: muralis@cusat.ac.in

Department of Chemical Oceanography
School of Marine Sciences
Cochin University of Science and Technology
Kochi - 682016

October, 2017

DEDICATION

To my respected teachers, beloved family and friends for always supporting, helping and standing by me.

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
DEPARTMENT OF CHEMICAL OCEANOGRAPHY
Fine Arts Avenue
Kochi - 682016, India

Dr. S. Muraleedharan Nair
Professor

Tel: 91-484-2383131(O)
91-484-2863402(O)
Fax: 91-848-2374164
E-mail: muralis@cusat.ac.in

Certificate

This is to certify that the thesis entitled "**Isolation and characterization of glycolipids from *Synechocystis* sp. and its cytotoxic potential against colon cancer**" is an authentic record of the research work carried out by Ms. Saritha S. under my supervision and guidance at the Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, Kochi-16, in partial fulfilment of the requirements for PhD degree of Cochin University of Science and Technology and no part of this has been presented before for any degree in any university. I further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.

Kochi - 682016
October, 2017

Dr. S. Muraleedharan Nair
(Supervising Guide)

Declaration

I hereby declare that the thesis entitled "**Isolation and characterization of glycolipids from *Synechocystis* sp. and its cytotoxic potential against colon cancer**" is an authentic record of the research work carried out by me under the guidance and supervision of **Dr. S. Muraleedharan Nair**, 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.

Kochi-16 *Saritha S.*
October, 2017

Acknowledgements

This thesis has been concluded with the support of many generous and knowledgeable people. It has been a wonderful trip in my life that is now completed due to their financial and field assistance, scientific knowledge and emotional encouragement.

I am extremely grateful to my research guide and supervisor Dr. S. Muraleedharan Nair, Professor and Head, Department of Chemical Oceanography, Cochin University of Science and Technology (CUSAT) for giving me an opportunity to work under him. I am thankful to him for his constant support, encouragement and constructive advice throughout the research work. His magnanimity in making pertinent observation and rendering timely corrections enabled me to complete my research and bring out this thesis in the present form.

I am immensely thankful and obliged to Professor (Dr.) N. Chandramohanakumar, Hon Director, Inter University Centre for Development of Marine Biotechnology, CUSAT for his invaluable suggestions and encouragement in improving my research work. I am highly indebted to Dr. Jacob Chacko, Emeritus Professor, Department of Chemical Oceanography, CUSAT for his inspirational talks and suggestions during the course of my research work. I thankfully acknowledge Professor (Dr.) C.H. Sujatha, Department of Chemical Oceanography, CUSAT for her valuable suggestions, motivation and affection during the work. I am thankful to Dr. Habeeb Rahman, Assistant Professor, Department of Chemical Oceanography, CUSAT for his friendly and inspiring discussions.

I am grateful to Dr. K. Sajan, Director, School of Marine Sciences, CUSAT, Dr. A.N. Balachand, Dean, Faculty of Marine Sciences, CUSAT, Dr. K. Mohan Kumar, Former Director, School of Marine Sciences, Dr. H.S. Ram Mohan, Former Dean and Director School of Marine Sciences, for providing facilities for the research work.

I would like to acknowledge Council for Scientific and Industrial Research (CSIR) for providing financial support. I would like to place my sincere gratitude towards Inter University Centre for Development of Marine Biotechnology (IUCDMB) for providing Research facilities particularly GC-MS, LC-MS and ATR-FTIR.

I am thankful to Dr. A. V. Saramma, Professor, Department of Marine Biology, Microbiology and Biochemistry, and reseach scholars Ms. Lekshmi S., Mrs. Vijayaleksmi, for the isolated cyanobacteria culture.

I am grateful to Dr. K.J. Joseph, Professor (Retd.), Department of Marine Biology, Microbiology and Biochemistry, for his kind help in the morphological identication of cyanobacteria. I also extend my sincere thanks to Dr. Valsamma Joseph, Director, National Centre for Aquatic Animal Health (NCAAH), CUSAT and Mrs.Jisha Kumaran, PhD student, for their invaluable help in the molecular identification of cyanobacteria. I am also thankful to Dr. Rosamma Philip, Professor, Department of Marine Biology, Microbiology and Biochemistry, and Mrs. Aiswarya, PhD student, and Mr. Dibu Divakaran, PhD student, Dept. of Chemical Oceanography for their valuable suggestions in identifying the organism.

I express my deep sense of gratitude to Dr. P. R. Rajamohanan, Scientist, Central NMR Facility, National Chemical Laboratory (NCL), Pune, for the help extended by him in carrying out NMR analyses of my samples. I also express my sincere thanks to Dr. K. Ashok Kumar, Principal Scientist, Central Institute of Fisheries Technology (CIFT) for helping me in LC MS/MS analyses of the samples.

I gratefully acknowledge Dr. Luxmi Varma R., Associate Principal Sientist, NIIST Thiruvananthapuram and technical staff Mrs. S. Viji for providing facility for HRMS Analyses. I am thankful to Dr. K. P. Prathish (Scientist, NIIST), Dr. T. Lakshmikanth and Ms. Reethu Haridas (NIIST) for their help in the mass spectral studies. I am grateful to Mr. Saji, STIC - CUSAT for conducting 400 MHz NMR analyses. I am indebted to Dr K.K.C. Nair, former Scientist-in-Charge, Regional Centre, NIO, Kochi, for his invaluable advice during research progress assessments. I would like to thank Dr. J. Sundaresan Pillai, Scientist, NISCAIR (CSIR), for his generous help during my research work.

My sincere thanks also goes to Biogenix Research Center for Molecular Biology and Applied Science, for providing access to the laboratory and research facilities to successfully carry out cytotoxic studies. I greatly appreciate the valuable suggestions of Ms. Dhaneesha M., PhD student, NCAAH, CUSAT, regarding the cytotoxicity studies.

I owe special thanks to my former class mate and friend, Dr. Prashob Peter K. J. (Post-Doctoral Fellow, Department of Chemical Oceanography, CUSAT) for his

unstinted support all through the course of this research work. The numerous, laborious and tedious lab works became an enjoyable experience and beautiful memory because of his generous help and encouragement.

I am grateful to Dr. Ratheeshkumar C. S. and Dr. Byju K., for their consistent encouragement and help during my research period. I am also thankful to Dr. Gireeshkumar T. R. and Dr. Deepulal P. M. for their valuable advice and support during my study.

I profoundly thank my friends Mr. Shameem K, Dr. Salas P. M., Ms. Ragi A. S., Mr. Eldhose Cheriyan and Dr. Resmi P., and Dr. Martin G. D. for giving me such a good friendly company. Special thanks to you for listening, offering me advice, and supporting me in the compilation of thesis.

I am thankful to Dr. Shaiju P., Technical assistant, Department of Chemical Oceanography for his support and encouragement during my work.

I extend my special gratitude towards Dr. Manju Mary Joseph, Dr. Renjith K. R., Ms. Movitha M, Dr. Kala K. Jacob, Dr. Manju M. N. (late), Dr. Sanil Kumar K. S, Mr. Sudheesh V., Dr. Akhil P. S., Mrs. Anuradha V., Mr. Renjith P. K.,Mr. Libin baby., Mr. Rahul R., Ms. Gaby, for their invaluable help during my research work.

The help and support extended by my friends, Mr. Manu Mohan, Mrs. Leena P. P., Mrs. Ramzi A., Mrs. Nisha K, Mrs. Anju E. R, Mr. Udayakrishnan P. B., Dr. Soumen Ghosh, Dr. Siby Mathew, Dr. Ejin K. T., Mr. Anoop C. Shekhar, Dr. Nayeem Muhammed, Dr. Sruthi T. R. S., Dr. Harishankar, Dr. Nebula M., Dr. Bindhu K. R., Mrs. Dayala V. T., Dr. Renjitha Raveendran, Mrs. Asha C. V., Dr. Abhilash P., Mrs. Shibini mol, Dr. Radhika R., and Ms. Divya K. R, is highly appreciated.

I acknowledge the help and encouragement rendered by Mrs. Souda., Ms. Anu Susan cheriyan, Ms. Moushmi K. S., Mr. Ajeesh Kumar S., Akhil Dileep, Dr. Manju P. Nair, Neetu V., Krishnaveni K. S., Ms. Thulasi, Mrs. Arsha Krishnan, Mrs. Nisa, Mr. Midhun T. R., Mrs. Aneeta Thomas, Mr. Joshua, Mrs. Navya, Mrs. Jyothi, Mrs. Nisari, Ms. Haritha J. R, Mrs. Raihanath, Mr. Eldose P. Jacob and all other research scholars, M.Sc/M. Phil students in the Department of Chemical Oceanography, CUSAT. I

would like to thank for whoever being with me in all times of need and helping me at keeping my spirits up.

I thank office staff of Department of Chemical Oceanography, Cochin University of Science and Technology for helping the administrative work of my research.

I record my thanks to Mr. Binoop Kumar (Indu Photos) for formatting my thesis.

This PhD journey would not have been possible without the support of my family. I am forever indebted to my father Mr. B. Babu, my mother Mrs. S. Sarala, my sister Dr. S. Sandhya and my brother in law Mr. Dileep G. for their inspiration, endless patience and unconditional love. I am thankful to my nephew sweet little Dhyan Dileep for filling my mind with lot of happiness even during the stressful period of thesis writing.

Above all, I thank God, the almighty for blessing me with this opportunity and granting me the capability to proceed successfully.

Saritha S.

Preface

Glycolipids are amphiphilic compounds that regulate several key biological processes in prokaryotic and eukaryotic organisms. They serve as important cell surface components and involved in processes such as cell recognition, adhesion, signalling etc. Glycolipids composed of a hydrophilic polar sugar head group and a hydrophobic apolar lipid moiety anchoring the molecule in the membrane. By virtue of their sugar residues and location on the cell surface, glycolipids can interact with a wide variety of small molecules and proteins that either resides in the aqueous phase or on the surface of neighbouring cells. Among glycolipids, glycoglycerolipids constitute 70–80% of membrane lipids in photosynthetic cells and comprise the most abundant lipid class in the biosphere. They are predominant in chloroplasts of plants, eukaryotic algae and cyanobacteria. A number of glycolipids were isolated from cyanobacteria, which are known to possess specific biological activities.

Mono and di-galactosyl derivatives are found to be the major contributors of glycolglycerolipids and found to play an important role in establishing membrane characteristics. Glycolipids with only one sugar in the head group exhibit non-bilayer forming properties, whereas diglycosyldiacylglycerols are always bilayer forming. Certain heterocystous cyanobacteria are found to produce glycolipids containing pentose moieties. A number of unconventional glycolipids were isolated in which glycosyl moiety linked to the hydroxyl group of a fatty alcohol or a hydroxy fatty acid, or to the carboxyl group of a fatty acid, other than acetylated glycerol moiety. Depending on the structure, glycolipids found to possess various immunogenic properties including antiviral, anti-tumor and anti-inflammatory activities and therefore structural characterization of glycolipids is particularly important in medicinal chemistry.

Both natural and synthetic analogs of glycoglycerolipids are widely studied in cytotoxic chemotherapeutics due to their ability to selectively target proliferating cells, which are enriched in tumors. With this

background, this study focused to find new glycolipid drug candidates having cytotoxic potential against colon cancer. Colon cancer is a world-wide health problem and the second-most dangerous type of cancer, affecting both men and women. The modern diet and lifestyles has led to an increasing mortality rate for colon cancer. As a result, there is a need to develop novel and environmentally benign drug therapies for colon cancer. Several chemical anticancer drugs with prolonged survival have been developed, but they often possess adverse effects and off target actions. Based on this, nutraceuticals and phytochemicals have been investigated for colon cancer therapeutics.

The thesis entitled "**Isolation and characterization of glycolipids from *Synechocystis* sp. and its cytotoxic potential against colon cancer**" gives insight to the structure of major glycolipids isolated from the cyanobacteria *Synechocystis* sp. and also focused on its cytotoxic effects on colon cancer cells. The main objectives of the thesis are:

1) Isolation and structural characterization of major glycolipids from *Synechocystis* sp.
2) Screening cytotoxic effect of the isolated glycolipids against colon cancer
3) Prediction of mechanism of cell cycle arrest and induction of apoptosis

The thesis is divided into five chapters,

Chapter 1, gives a general introduction on the structure and function of glycolipids in various organisms. Pharmaceutical applications of important glycolipids isolated, especially from cyanobacteria, are also discussed. A review on colon cancer epidemiology along with importance of phytochemicals in colon cancer therapeutics is also described briefly. The objectives and scope of the thesis is included in this chapter.

Chapter 2, describes culture conditions and, morphological and molecular level identification of *Synechocystis* sp. Fatty acid composition through GC-MS analyses is discussed in a chemotaxonomic perspective. Mass culturing of *Synechocysis* sp. for glycolipid isolation is also described. Solvent extraction of lyophilized biomass and isolation of glycolipids using different chromatographic techniques such as column chromatography, TLC, HPLC along with LC-MS analyses for detecting the mass and purity of the isolates are also outlined in this chapter.

Chapter 3, emphasizes on the structure elucidation of isolated glycolipids. Sugar composition of each fraction using LC-RID is discussed in the beginning of this chapter. The spectral data consisting FTIR, NMR (^{1}H, ^{13}C, DEPT, HSQC, COSY, HMBC), HRMS, LC-MS^{2} are tabulated and discussed in detail to elucidate the structure of major glycolipids. Overall inferences on the chemical features of isolated glycolipids are outlined at the end.

Chapter 4, highlights the potential of glycolipids to suppress proliferation of colon cancer cells. Cytotoxic potential of glycolipids observed from MTT assay screening is briefly discussed. Induction of apoptosis and cell cycle arrest of the highest active glycolipid, through stimulation of reactive oxygen species, DNA damage and mitochondrial-dependant pathways, are discussed in detail. This is achieved based on the different analyses such as fluorescence, flow cytometry, comet assay and gene expression studies. An overall mechanism for the cytotoxic action of glycolipids against colon cancer is suggested.

Chapter 5, provides a brief summary and conclusion on the achievements of the study and indicates the scope of future work.

All the chapters have a brief introduction, materials and methods, discussions and conclusion sections. Chapters end with the respective references.

Contents

List of Abbreviations

$[\alpha]_D$ (25 °C)	Specific rotation measured in sodium D line at 25 °C
5-FU	5-fluorouracil
7-AAD	7-aminoactinomycin D
AIFs	Mitochondrial apoptosis initiation factors
AO	Acridine orange
APCI	Atmospheric pressure chemical ionization
APT	Attached proton test
ATR	Total reflectance accessory
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
COSY	Correlation spectroscopy
CTLs	Cytotoxic T lymphocytes
DCF	2',7' Dichlorofluorescein
DCFH-DA	2'-7'-Dichlorodihydrofluorescein diacetate
DEPT	Distortionless enhancement by polarization transfer
DGDG	Digalactosyldiacylglycerols
DHA	Docosahexaenoic acid
DMEM	Dulbecos modified Eagles medium
DMH	1,2-Dimethyl hydrazine
DNA	Deoxyribonucleic acid
EB	Ethidium bromide
EGCG	Epigallocatechin-3 gallate
EI	Electron impact
EPA	Eicosapentanoic acid
ESI	Electrospray ionization
FAB	Fast atom bombardment
FAME	Fatty acid methyl esters
FBS	Fetal bovine serum
FPG	Formamidopyrimidine-DNA glycosylases
FT-IR	Fourier transform infrared spectroscopy

FT-MS	Fourier transform mass spectrometry
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography–mass spectrometry
GFP	Green fluorescent protein
HMBC	Heteronuclear multiple-bond coherence
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum coherence
IC_{50}	Half maximal inhibitory concentration
INEPT	Insensitive nuclei enhanced by polarization transfer
LC-MS	Liquid chromatography–mass spectrometry
MGDG	Monogalactosyldiacylglycerols
MMP	Mitochondrial membrane potential
MPLC	Medium-pressure liquid chromatography
MTT	3-(4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acid
NCBI	National center for biotechnology information
NCCS	National centre for cell sciences
NIST	National institute of standards and technology
NMA	Normal melting point agarose
NMR	Nuclear Magnetic Resonance
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRAP	Proline rich acidic protein
PUFA	Polyunsaturated fatty acids
R_F	Retention factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCGE	Single cell gel electrophoresis

SFA	Saturated fatty acid
SQDG	Sulfoquinovosylacylglycerols
SQMG	Sulfo-quinovosylmonoacylglyceride
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
TOF	Time of flight
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
UV/Vis	Ultraviolet-visible

…..ꕥ…..

Chapter 1

INTRODUCTION

Contents

Glycolipids are important components of cell membranes and can be found in plants, bacteria as well as higher organisms. They are found to play crucial roles in various cellular events. Glycolipids can interact with a wide variety of small molecules and proteins that either resides in cytoplasm or on surface of neighbouring cells, depending on the nature of sugar residues and location on the cell surface (Bush et al., 1999). They also take part in lateral sorting and clustering of membrane embedded proteins (Lingwood & Simons, 2010).

Glycolipids are generally divided into three main groups, glycoglycerolipids, glycosphingolipids, and isoprenoid glycosides, depending on their lipid moiety, which can be an acylated glycerol, an acylated sphingosine (ceramide) or a terpene alcohol respectively. However, a number of glycolipids exist, that cannot be classified in any

of these groups (Leão et al., 2015; Peddie et al., 2015; Ngwoke et al., 2017). These compounds may contain a glycosyl moiety (one or several saccharide units) bound to carboxyl group of a fatty acid through ester linkage or hydroxyl group of a fatty alcohol or a hydroxy fatty acid through ether linkage. They are typically found in bacteria and yeasts and frequently possess interesting biological properties (Costantino et al., 2006). Both natural and synthetic glycolipids have been identified as potential drug candidates with antiviral (Morales-Serna et al., 2010; Papp et al., 2011; Stanley et al., 2012), anticancer (Song et al., 2010; Li et al., 2011; Zhang et al., 2011) as well as antimicrobial activities (Azim et al., 2006; Ding et al., 2011; Poláková et al., 2011). Biological properties of glycolipids may depend on various factors such as nature of sugar moiety, glycosidic linkage position, length and location of the acyl chain and anomeric configuration of the sugar (Mattos et al., 2011; Cortes-Sanchez et al., 2013; Surovtseva et al., 2016; Szeja et al., 2017).

1.1 Structure and function of glycolipids

Glycoglycerolipids are important constituents in surface membranes of photosynthetic organisms, where it is found to involve in diverse cellular functions. Major glycoglycerolipids observed are mono and digalactosyldiacylglycerols (MGDG and DGDG) and anionic sulfoquinovosylacylglycerols (SQAG) (Figure 1.1). General structure of glycoglycerolipids consists of a carbohydrate moiety which is beta or alpha-linked to sn-3 position of glycerol, that is acylated at the residual hydroxyls by fatty acids of different lengths and degrees of unsaturation (Colombo et al., 2013).

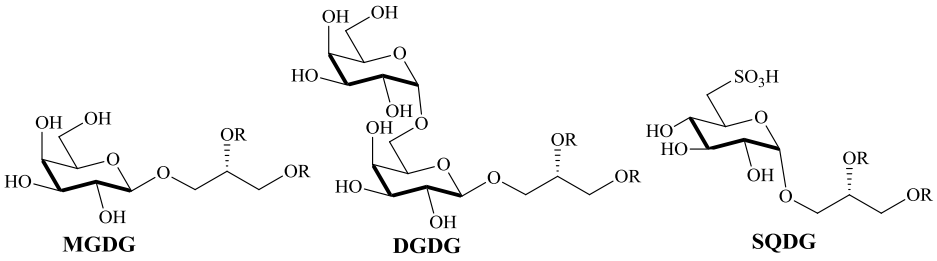


Figure 1.1 General structures of three main glycoglycerolipids (**R**-acyl chain)

Naturally occurring glycoglycerolipids generally possess a Y-shaped structure (Figure 1.2 a) with a polar carbohydrate head group linked to a 1,2- or 1,3-di-*O*-acyl or alkyl-*sn*-glycerol (Mannock et al., 2001; von Minden et al., 2002 a,b). But only a few compounds with an inverse structure (Figure 1.2 b) were reported (Fischer et al., 1994; von Minden et al., 2002a). Most bacterial and plant glycolipids are glycerol-containing compounds (glycoglycerolipids), whereas in animals and man, glycosphingolipids predominate.

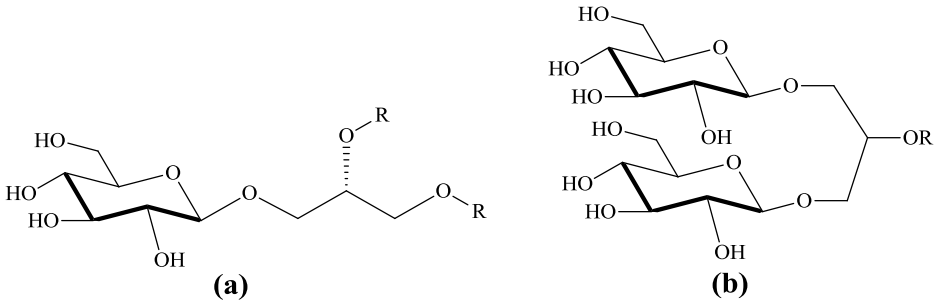


Figure 1.2 (a) Normal Y-shaped glycolglycerolipid and (b) inverted structure

a) Bacterial glycolipids

Structures of bacterial glycolipids are immensely varied and only a few species have been examined in detail (Kondakov & Lindner,

2005; Lebeer et al., 2010; Javed et al., 2017). Mycobacterial plasma membranes are found to contain numerous highly immunogenic glycolipids and have been studied extensively, due to their pathological significance. Major glycolipids identified in mycobacteria are classified as lipooligosaccharides, glycopeptidolipids and phenolic glycolipids (McNeil & Brennan, 1991). Bacterial membranes are shown to possess mono- to penta-glycosylated diacylglycerol with a wide variety of sugars, including glucose, galactose, mannose, rhamnose, trehalose, glucuronic acid and glucosamine, with numerous modifications (e.g. methylation, alkylation or acetylation) (Shimamura, 2008; Abdel-Mawgoud et al., 2010; Elshahawi et al., 2015). Archaebacterial membrane lipids are found to be unique in structure consisting of derivatives of a C_{20}-C_{20} diacylglycerol ether and its dimer. These unusual glycolipids stabilise bacterial membranes in harsh habitats due to their exceptional physical properties and hence, they are gaining special research interests. Structure of those macrocyclic tetraether lipids is as shown in Figure 1.3 (Hanford & Peeples, 2002). Gram-negative bacteria possess an extremely complex class of glycolipids, which are neither diacylglycerol nor sphingoid based. The structure consists of a glycosyl head group having approximately 40 sugar residues linked to lipid unit. The basic lipid component is diglucosamine phosphate with amide linked and ester-bound p-hydroxy myristic acid (Huguet et al., 2006; Becker et al., 2013).

Functions of glycolipids in bacterial membranes are generally based on their ability to form inter lipid hydrogen bonding via glycosyl head groups. In the case of species without cell walls, such as mycoplasma, plasma membrane is necessitated to provide resistance to

osmotic stress, and thus needs extra stabilization (Curatolo, 1987a). Also archaebacteria, in contrast to eubacteria, generally do not possess peptidoglycan based cell walls. Plasma membranes of these organisms, which are found stable to lysis by stress factors in the cell environment, comprise highly glycosylated proteins and unique membrane lipids. Hence, macrocyclic tetraether based glycolipid found in all archaebacterial membranes, suggested that those lipids have functions common to all archaebacteria. A bipolar monolayer of two headed lipids along the entire archaebacterial membrane was evidenced from the unique structure of the glycolipids. Alkyl ether bonds in glycolipid are found to stabilize the membrane at high temperatures and also over a wide range of pH (Kates, 1992; Chong, 2010). Along with the membrane stabilizing effects, these glycolipids can also contribute to specific cellular functions by influencing ion transport, proton transport and conductance, energy transduction and nutrient permeability (Wu et al., 2005). Detailed understanding of these highly unusual glycolipids will possibly help to reveal functions of similar lipids in eukaryotes.

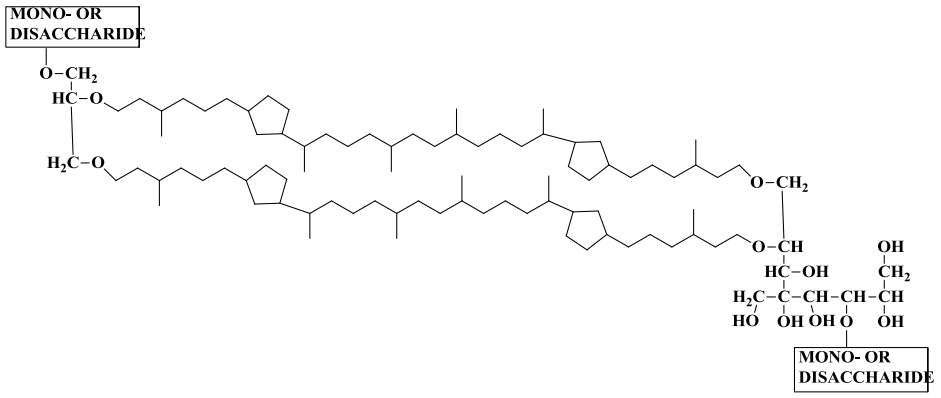


Figure 1.3 Basic structures of archaebacterial macrocyclic tetraether lipids

b) Plant glycolipids

Glycolipids based on diacylglycerol, particularly mono and digalactosyldiacylglycerol are major components of stacked thylakoid membranes of chloroplasts in plants (Kelly & Dörmann, 2004). Sulfoquinovosyldiacylglycerol is charged glycolipid of thylakoids, possessing a glycosyl headgroup which is sulfated at position-6 (Nishihara et al., 1980). Galactose residue in monogalactosyldiacyl glycerol (MGDG) is normally bound to sn-3 position of glycerol backbone through β-anomeric linkage (β-D-gal) (Awai et al., 2001). Digalactosyldiacylglycerol (DGDG) head group is characterized by a terminal α-galactose moiety (1=>6) linked to inner β-galactose residue (α-D-Gal(1=>6)β-D-Gal) (Froehlich et al., 2001; Kelly et al., 2003). Certain plants, for example Adzuki bean was found to contain DGDG forms with the two galactose residues in β-anomeric configuration (β-D-Gal(1=>6)β-D-Gal) and also, oligogalactolipids with three or more galactose residues in different anomeric configurations (Kojima et al., 1990). These uncommon lipids are produced only in specific tissues and under certain growth conditions. Apart from the conserved head group structures of MGDG and DGDG, fatty acyl chains of galactolipids display a high variability in chain lengths, degree of unsaturation and distribution to the sn-1 and sn-2 position of the glycerol backbone (Hölzl & Dörmann, 2007).

Monogalactosyldiacylglycerol does not form bilayers spontaneously when dispersed in purified form, but rather adopts hexagonal configurations (inverted micelles or tubes). This character was evidenced by the formation

of highly curved edges in thylakoid membranes (Williams, 1998). MGDGs and DGDGs showed specific physiological functions such as maintenance of ion permeability and electrical properties of thylakoid membrane, and insertion and stabilization of the large photosystem protein complexes in the thylakoid membranes. Plant sulfolipid, sulfoquinovosyldiacylglycerol plays a vital role in electron transport of photosynthesis (Dörmann & Benning, 2002; Shimojima, 2011; Boudière et al., 2014). Investigations to find specific function of glycolipid in plants remains as a challenging field owing to complexity in structure and function of thylakoid membranes.

c) Animal glycolipids

Most of animal glycolipids consist of long chain aminodiol sphingosine (4-sphingenine) as the lipid backbone and are termed as glycosphingolipids. Fatty acids of varying lengths are attached to the amino group of sphingosine via amide bonds, forming the glycolipid's hydrophobic tail, called ceramide. Carbohydrate units are bound to the primary hydroxyl of ceramide via glycosidic linkages. Glycosphingolipids are found primarily in the plasma membrane of all vertebrate tissues, and they are predominant in the nervous system. Major glycolipid of mammalian brain is galactosylceramide (galactocerebroside), which constitutes about 16% of total lipid in adult brain (Agranoff et al., 1994; Robert et al., 2009) (Figure 1.4). Lactosyl ceramide, and neutral tri- and tetraglycosylceramides are abundant in all tissues (Macher & Sweeley, 1978).

Glycosphingolipids with complex branched oligosaccharide structures have gained special interest as they serve as blood group

antigens, and their modifications act as human cancer antigens (Hakomori, 2000; Reis et al., 2010). Sometimes, sulfation of the saccharide part turns neutral glycosphingolipids into charged sulfatides. The major mammalian sulfatide consists of a monogalactosylceramide in which position 3 of the galactosyl headgroup is sulfate. Gangliosides are another important group of acidic glycosphingolipids characterized by the presence of one or more sialic acids (Kolter et al., 2002; Schnaar et al., 2014). Glycosphingolipids in invertebrates, particularly insects, were found to be quite similar to structures in vertebrates; however, two main differences were observed. Mannose replaced galactose in oligosaccharide chain, and glucuronic acid took the place of sialic acids, which insects are unable to synthesize. Such glucuronic acid containing insect glycosphingolipids have been named as arthrosides in synonymy to the gangliosides (Wiegandt, 1992).

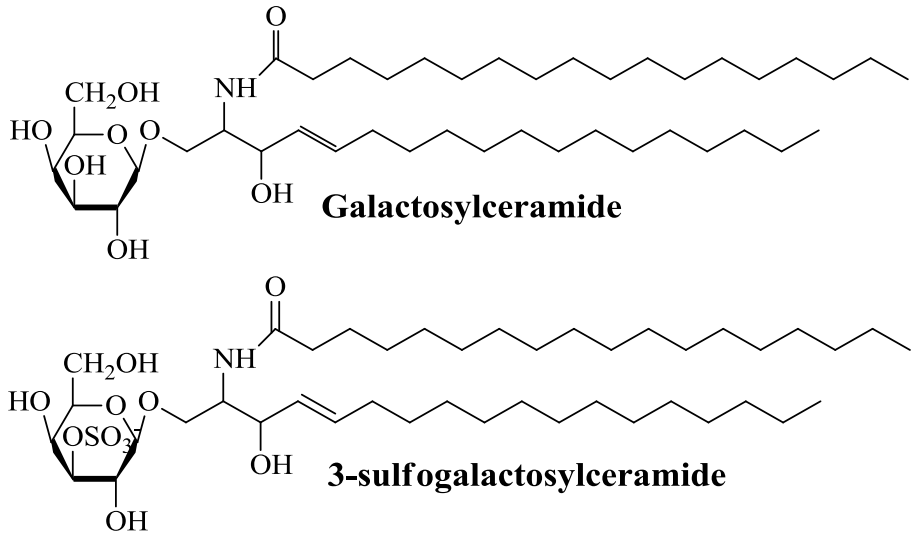


Figure 1.4 Structural components of glycosphingolipids

Glycosphingolipids contribute to the structural rigidity of surface membrane which is evidenced from physicochemical properties and localization of glycosphingolipids in the outer half of the lipid bilayer (Curatolo, 1987b). Involvement of glycosphingolipids in important cellular functions such as cell adhesion, cell-cell interaction and recognition has also been established (Lopez & Schnaar, 2009). Glycolipid patterns were found to change dramatically during hematopoiesis, and gangliosides act as differentiation inducers for hematopoietic cells (Monti et al., 2010; Yang et al., 2011). Leucocyte differentiation antigens based on glycosphingolipids mediate cellular interactions via cell adhesion molecules (Zhang & Kiechle, 2004; Wang et al., 2017) and also they interact with the T-cell receptor, thereby influencing T-cell activation (Morrison et al., 1993; Fleurence et al., 2017). Gangliosides can influence Ca^{2+} influx from extracellular sources (Liu et al., 2008). Sphingolipid breakdown products act in second messenger mechanisms (Ballou, 1992; Venkataraman & Futerman, 2000). Besides their action as antigenic determinants, glycosphingolipids are involved in mechanisms leading to proliferation, differentiation and activation of lymphocytes.

d) Cyanobacterial glycolipids

Glycolipid composition of cyanobacteria was found to be more or less similar to chloroplasts. In most of cyanobacterial glycoglycerolipids, anomeric head group configuration is found to be similar to plant lipids. Conservation of composition and structure of glycerolipid between cyanobacteria and chloroplasts can be explained according to the endosymbiont hypothesis. According to this, the first plant cell was

supposed to be originated by consumption of cyanobacterial cell by a eukaryotic progenitor. Some physiological processes were assumed to be transferred into the plant cell during this process and thus, gave explanation to common physiological aspects shown by cyanobacteria and plants (Wada & Murata, 1989; Hölzl & Dörmann, 2007).

In cyanobacteria, galactolipids play an important role in establishing membrane characteristics owing to their high abundance. Galactolipid content and composition affect the ratio of bilayer to non-bilayer forming lipids which is crucial for maintaining membrane stability and functional activity of membrane proteins (Williams, 1998). Bilayer characteristics of membranes depend on degree of unsaturation and chain length of the fatty acids in the glycolipids, and plants and cyanobacteria show high variability in this respect (Murata & Wada, 1995). Studies on enzymatic pathways for the generation of glycolipid indicated that prevalent fraction of glycolipids produced in cyanobacteria displays a C18 or C16 fatty acid distribution (Sato, 1982). Galactolipids, MGDG, DGDG and SQDG, were recognized as the major glycolipid compounds in cyanobacteria (Hölzl & Dörmann, 2007). Some structural variations were also observed in cyanobacterial glycolipids depending on type of organism and growth conditions. Some marine endosymbiotic cyanobacteria were identified to produce glycoglycerolipids containing pentose (C_5) moieties (Bale et al., 2015). Glycolipids consisting of sugar moieties glycosidically bound to long-chain diols, triols, keto-ols and keto-diols have been identified in Nostocaceae and Rivulariaceae families of cyanobacteria (Bauersachs et al., 2009).

Synechocystis represents one of the widely studied genus of cyanobacteria because of their ability to adapt to different environmental stress conditions viz. osmotic and salt stresses (Yu et al., 2013). This cyanobacterial genus is being developed as potent producers of valuable products such as alcohols, alkanes, bioplastics, fatty acids, etc. through certain genetic modifications (Savakis & Hellingwerf, 2015). *Synechocystis* is considered as model cyanobacteria for several metabolic and genomic studies (Li et al., 2011; Mo et al., 2015). *Synechocystis* sp. PCC 6803 has recently been engineered metabolically to produce a plant derived diterpenoid manoyl oxide possessing broad range of pharmaceutical applications (Englund et al., 2015). The species was also used to study regulatory changes to facilitate nitrogen fixation in photosynthetic organisms (Mueller et al., 2016)

Synechocystis cells are found to accumulate compatible solutes such as glycosylglycerol to acclimate to the varying salt conditions (Hagemann, 2010; Iijima et al., 2015). They are known as important producers of glycolipids. Galactoglycerolipids were found to contribute more than 90% of the total glycerolipids in the intact cells of *Synechocystis* sp. PCC 6803 (Wada & Murata, 1998; Awai et al., 2007). Lysoglycerolipids corresponding to their monoacylglycerol types have been isolated from the total lipid extract of cyanobacterium *Synechocystis* sp. PCC 6803 (Kim et al., 1999). Aromatic glycolipids, known as bartolosides, containing xylose and rhamnose units were also isolated from two different cyanobacteria *Synechosystis salina* and *Nodosilinea* sp. (Figure 1.5) (Leão et al., 2015).

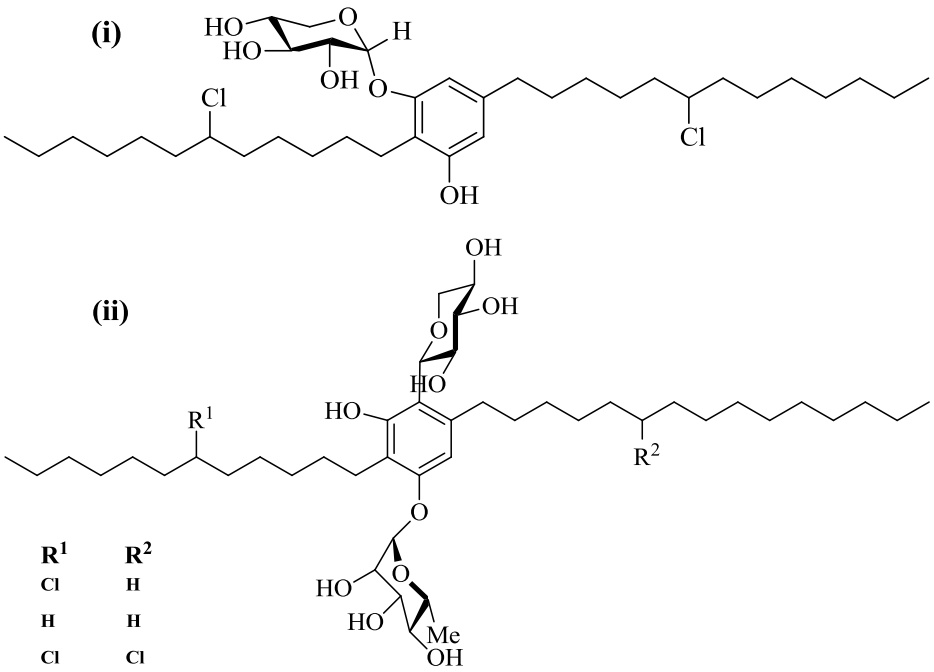


Figure 1.5 Bartolosides from *Nodosilinea* sp. (i) and *Synechosystis salina* (ii)

1.2 Bioactive glycolipids from natural sources

Numerous bioactive glycolipids have been identified from natural sources and are used for developing new drugs for a variety of diseases (Mayer et al., 2011; Hamed et al., 2015). Some of the important bioactive glycolipids isolated from different aquatic flaura and fauna are briefly discussed in this section. Pharmaceutical significances of glycoglycerolipids have been recorded in various investigations particularly in inhibiting *in vitro* and *in vivo* tumor-promoting activity (Murakami et al., 1995; Colombo et al., 2013). Thylakoid membranes of cyanobacteria, algae and plants were identified as important source for glycoglycerolipids (Hölzl & Dörmann, 2007; Larsen & Christensen, 2007).

a) Anti-inflammatory and antimicrobial effects

Glycolipids, especially from cyanobacteria and algae, are identified to have anti-inflammatory and antimicrobial properties. Glycolipid fraction from *Scytonema julianum*, a filamentous cyanobacterium isolated from Greek cave, was found to have ability to inhibit platelet-activating factor (PAF) and thrombin-induced washed rabbit platelet aggregation, *in vitro*. Among the three glycolipids separated, two were identified as phosphoglyco-analog of acyl-acetylated sphingosine and the third one as glyco-analog of phosphatidylglycerol (Antonopoulou et al., 2005a). Similar biological activity was also reported for glycolipid fractions of a very primitive photosynthetic and versatile cyanobacterium *Chroococcidiopsis* sp. (Antonopoulou et al., 2005b). Significant anti-inflammatory activity was observed for two sulfoglycolipids, (2S)-1-O-eicosapentaenoyl-2-O-myristoyl-3-O-(6-sulfo-a-D-qu- inovopyranosyl)-glycerol and (2S)-1-O-eicosapentaenoyl-2-O-palmitoyl-3-O-(6-sulfo-α-D-quinovopyranosyl)-glycerol, isolated from the macro algae *Palmaria palmata* (Banskota et al., 2014). Those glycolipids demonstrated nitric oxide inhibitory activity with IC_{50} values of 36.5 and 11.0 μM, respectively.

Glycolipid extracts of microalgae, *Tetraselmis*, *Nannochloropsis* spp. and *Porphyridium cruentum* were found to show anti-inflammatory effects especially for the treatment of NO-mediated disorders (Banskota et al., 2013a). Bioactive extract of *Tetraselmis* contained mainly two glycolipids, MGDG (18:3/16:4) and MGDG (18:4/16:4) (da Costa et al., 2016) and that of *Nannochloropsis* contained four molecular species of MGDG, namely MGDG (20:5/14:0), MGDG (20:5/16:0), MGDG

(20:5/16:1) and MGDG (20:5/20:5) (Banskota et al., 2013b). Fraction of *Porphyridium cruentum*, inhibiting NO accumulation, was found to contain five sulfolipids with large amounts of palmitic (16:0), arachidonic (20:4) and eicosapentaenoic (20:5) acids (Bergé et al., 2002). Two galactolipids, MGDG (18:4/16:0) and DGDG (18:4/16:0) from thermophilic blue-green alga ETS-05, were found to have significant anti-inflammatory effects on croton oil-induced ear oedema and carrageenan-induced paw oedema, in specific in vivo mouse models. MGDG with a high saturated fatty acid content and SQDG showed less anti-inflammatory activity (Bruno et al., 2005).

Studies on MGDGs isolated from the extract of *Sargassum muticum* (coast of Britanny, France), by Plouguerné et al. (2010) revealed that it is having inhibitory activity against some microorganisms involved in marine microfouling such as bacteria *Shewanella putrefaciens* and *Polaribacter irgensii*, and fungi *Halosphaeriopsis mediosetigera*, *Asteromyces cruciatus*, *Lulworthia uniseptata* and *Monodictys pelagica*. The inhibitory activity was reported for concentration 0.75 μg/mL. Two MGDGs, (2S)-1-O-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-2-O-(9Z,12Z, 15Z-octadecatrienoyl)-3-O-β-galactopyranosyl-sn-glycerol and (2S)-1-O-(9Z,12Z,15Z-octadecatrienoyl)-2-O-(6Z,9Z,12Z,15Z-octadecatetraenoyl)-3-O-β-D-galactopyranosyl-sn-glycerol, isolated from *Sargassum thunbergii* were found to have antifungal effects on *Candida albicans* (Kim et al., 2007). Three SQDGs, 1-O-palmitoyl-2-O-myristoyl-3-O-(6-sulfo-α-D-quinovopyranosyl)-glycerol, 1,2-di-O-palmitoyl-3-O-(6-sulfo-α-D-quinovopyranosyl)-glycerol and 1-O-palmitoyl-2-O-oleoyl-3-O-(6-sulfo-α-D-quinovopyranosyl)-glycerol isolated from the algae *Lobophora*

variegate from the Yucatan coast (Mexico) demonstrated activity against the protozoa *Trichomonas vaginalis*, *Giardia intestinalis* and *Entamoeba histolytica*, with good selectivity (Cantillo-Ciau et al., 2010).

Monogalactosyl diglyceride (MGDG) separated from ethyl acetate extract of *Fucus evanescens*, collected from the Arctic coast of Ungava Bay, Canada, showed strong antibacterial activity against *Hemophilus influenza*, *Legionella pneumophila*, *Propionibacterium acnes* and *Streptococcus pyogenes* (Amiguet et al., 2011), and the compound was identified as 2',3'-propyldilinolenate-β-D-galactopyranoside. The monogalactolipid, 1-eicosapentanoyl-2-palmitoyl-3-O-galactopyranosyl-glycerol, separated from Chondria armata was shown to have significant inhibition on growth of bacteria *Klebsiella* sp., yeast *Candida albicans* and fungus *Cryptococcus neoformans* (Al-Fadhli et al., 2006). Antibacterial and antiviral activities from sulfolipids extracts of Mediterranean macroalga *Ulva fasciata* was highlighted by El Baz et al. (2013). Xylolipids produced by probiotic bacteria such as *Lactococcus lactis* showed great anti-bacterial potential against multiple drug resistant pathogens *Escherichia coli* and *Staphylococcus aureus* (Saravanakumari & Mani 2010). Mannosyl erythritol lipids and sophorolipids produced by microorganisms were also found to show significant antibacterial activity (Deml et al., 1980; Baek et al., 2003).

b) Antiviral properties

Several glycolipid compounds have demonstrated potent antiviral properties. A group of five diacylated sulfoglycolipids isolated from cyanobacterium *Scytonema* sp. was found to show inhibitory effect on

HIV-1 RT enzymatic activity. Structure of a prominant sulfoglycolipid was identified as (2*S*)-1-linoleoyl 2,2”’,3”’-tripalmitoyl-3-*O*-α-D-6”’-sulfoquinovopyranosylglycerol. Other glycolipids also showed similar structure with variations in the fatty acyl chain. Four acylated diglycolipids isolated from cyanobacterium *Oscillatoria raoi* were also found to inhibit HIV-1 RT enzymatic activity. One of the major diglycolipids from *Oscillatoria raoi* was identified as 1,2,3””-triacyl-3-*O*-[α-galactopyranosyl-(1””-6”’)-*O*-*β*-galactopyranosyl]glycerol (Reshef et al., 1997; Tziveleka et al., 2003).

Sulfolipid (SQDG) isolated from microalgae, *Porphyridium purpureum* and *Spirulina platensis*, has exhibited antiviral effects on herpes simplex virus (HSV-1) (Naumann et al., 2007; Chirasuwan et al., 2009). Investigations on the structure and biological activities of sulfolipids from Mediterranean algae *Dilophus fasciola* and *Taonia atomaria* gave inference to antibacterial and antiviral activities of sulfolipid extracts and the major components were recognized as SQDG and SQMG (sulfo-quinovosylmonoacylglyceride) (El Baz et al., 2013).
SQDGs obtained from organic extract of the Brazilian macroalga *Sargassum vulgare* were found to have inhibitory effect on Herpes simplex virus, HSV1 and HSV2, and the main compound responsible for activity was identified as 1, 2-di-*O*-palmitoyl-3-*O*-(6-sulfo-α-D-quinovopyranosyl)-glycerol (Plouguerné et al., 2013). Sulfolipid isolated from the algae *Gigartina tenella* (Sagami Bay, Japan) showed inhibition of DNA polymerase α, DNA polymerase β and HIV-reverse transcriptase type 1, with respective IC_{50} values of 0.25, 3.6, and 11.2 μM. Structure of the SQDG was identified as 1-(1’-O-α-D-sulfoquinovosyl)-2-palmitoyl-3-

[5"(E),8"(E),11"(E),14"(E),17"(E)-eicosapentaenyl]-syn-glycerol (Ohta et al., 1998). Sulfolipid, 1,2-di-O-acyl-3-O-(6-deoxy- 6-sulfo-α-D-glucopyranosyl)-sn-glycerol, isolated from the extract of *Osmundaria obtusiloba*, has also showed anti-HSV (herpes simplex virus) activity (de Souza et al., 2012). Sulfolipids from *Laurencia papillosa* and *Galaxaura cylindrica*, collected from the Red Sea, were found to have significant antibacterial and antiviral activities (El Baz et al., 2013). SQDG, (2S)-1,2-di-O-palmitoyl-3-O-(61-sulfo-α-D-quinovopyranosyl)glycerol isolated from n-butanol fraction of *Caulerpa racemosa* (South China Sea) was found to be active against HSV-2 with IC_{50} of 15.6 mg/mL (Wang et al., 2007).

c) Anticancer activities

Several glycolipids were recognized for their anticancer potential. Lipid fraction rich in SQDG isolated from microalgae, *Porphyridium cruentum* showed inhibitory effects on human colon (DLD-1), breast (MCF-7), prostate adenocarcinoma (PC-3) and malignant melanoma (M4-Beu) cell lines and the bioactive fraction contained SQDGs with 16:0, 16:1, 20:4 and 20:5 fatty acids (Bergé et al., 2002). Galactolipid fractions, especially MGDG (16:2/16:2) and MGDG (16:3/16:2), isolated from the green microalga *Chlorella vulgaris*, have demonstrated antitumor activities in human lymphoblastoid cells (Morimoto et al., 1995). Digalactosyldiacylglycerides (DGDG) and sulfoquinovosyldiacylglycerides (SQDG), isolated from Japanese macroalgae *Sargassum horneri*, were found to induce apoptosis in human colon carcinoma, Calco-2 cells (Hossain et al., 2005). Monogalactosyldiacylglycerides (MGDG) isolated from the algae *Petalonia binghamiae* was found to act as specific

inhibitor on activities of mammalian DNA polymerase α (Mizushina et al., 2001). A highly unsaturated monogalactosyldiacylglycerol (MGDG) (1-*O*-(5Z,8Z,11Z,14Z,17Z-eicosapentanoyl)-2-*O*-(6Z,9Z,12Z, 15Z-octadecatetraenoyl)-3-*O*-β-D-galactopyranosyl-sn-glycerol), isolated from *Fucus evanescens*, collected from the Sea of Okhotsk, Russia, demonstrated inhibition to growth of human melanoma cells (Imbs et al., 2013). Glycolipid,1-O-(palmitoyl)-2-O-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-3-O-β-D-galactopyranosylglycerol also known as lithonoside isolated from cytotoxic hexane soluble extract of Fijian coralline macroalgae *Hydrolithon reinboldii* displayed antineoplastic activity with IC_{50} value of 19.8 μM (Jiang et al., 2008). SQDGs, from macroalgae *Porphyra crispate*, were found to have cytotoxic potential against human hepatocellular carcinoma cell line (HepG2), with IC_{50} 126 μg/mL (Tsai & Pan, 2012).

Rhamnolipids produced by microorganism, *Pseudomonas aeruginosa*, have showed antiproliferative effects against human breast cancer cells, at a concentration of 6.25 μg/mL (Thanomsub et al. 2007; Cortes-Sanchez et al., 2013). Succinoyl trehalose glycolipids from *Rhodococcus* sp. showed growth inhibition of HL60 cells of promyelocytic human leukemia (Sudo et al., 2000; White et al., 2013). In addition, trehalose lipids were found to cause hemolysis of human erythrocytes by a colloid osmotic mechanism resulting in the formation of areas with improved permeability or pores in the erythrocyte membrane (Zaragoza et al., 2010). Diacetylated lactone derivatives of sophorolipids, synthesized by yeast, *Wickerhamiella domercqiae*, have demonstrated cytotoxic effects in several cancerous cell lines. These compounds were found to induce apoptosis in liver cell line H7402 (Chen et al. 2006).

Sophorolipids from *Candida bombicola* were found to show biological activity against cancerous cells in pancreas (Fu et al., 2008). Mono and diacetylated lactone species of sophorolipids with a hydrophobic portion of oleic acid synthesized by *Wickerhamiella domercqiae* showed high cytotoxic activity against esophagus cancer cells at concentrations over 60 μg/mL and 30 μg/mL, respectively (Shao et al., 2012). Lactonic sophorolipids from *Starmerella bombicola* was recently identified to have anticancer activity against human cervical cancer cells (Li et al., 2017). Mannosyl erythritol lipids, produced by microorganism *Ustilago maydis*, have demonstrated growth inhibition, morphological changes, activities of induction and differentiation against lipids of human myelogenous (K562), promyelocytic (HL60) and basophilic (KU812) leukemia (Kitamoto et al., 2002).

Two monogalactosyl diacylglycerols having ability to induce apoptosis in mammalian cell lines were isolated from the marine diatom *Phaeodactylum tricornutum* (Andrianasolo et al., 2008). The compounds were identified as (2S)-1-O-5,8,11,14,17-eicosapentaenoyl-2-O-6,9,12-hexadecatrienoyl-3-O-[β-D-galactopyranosyl]glycerol and (2S)-1-O-3,6,9,12,15-octadecapentaenoyl-2-O-6,9,12,15-octadecatetraenoyl-3-O-β-D-galactopyranosyl-sn-glycerol (Figure 1.6). Two glucopyranosyl diacylglycerols isolated from *Sargassum fulvellum* were found to exhibit fibrinolytic activity in the reaction system of single chain urokinase-type plasminogen activator and plasminogen (Wu et al., 2009). MGDGs capsofulvesin A and B, along with MGMG capsofulvesin C, isolated from macro alga *Capsosiphon fulvescens* (southern coastal area of Wando, Korea), exhibited cholinesterase inhibitory activity (Fang et al., 2012).

Structure of the compounds were identified as (2S)-1-O-(6Z,9Z, 12Z, 15Z-octadecatetraenoyl)-2-O-(4Z,7Z,10Z,13Z-hexadecatetraenoyl) -3-O-β-D-galactopyranosyl glycerol (capsofulvesin A), (2S)-l-O-(9Z, 12Z,15Z-octadecatrienoyl)-2-O-(10Z,13Z-hexadecadienoyl)-3-O-β-D galactopyranosyl glycerol (capsofulvesin B) and (2S)-1-O-(6Z,9Z,12Z,15Z-octadecatetraenoyl)-3-O-β-D-galacatopyranosyl glycerol (capsofulvesin C).

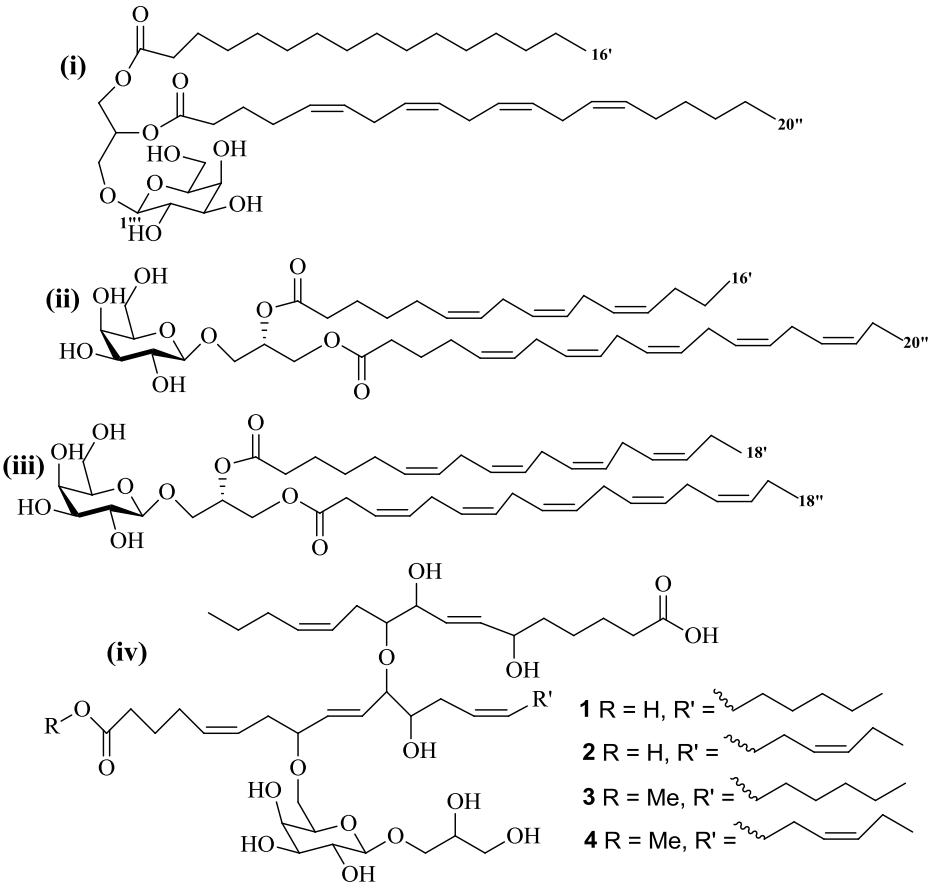


Figure 1.6 Structures of some important glycolipids showing cytotoxic potential; lithonoside from *Hydrolithon reinboldii* (i), two monogalactosyl diacylglycerols from *Phaeodactylum tricornutum* (ii) & (iii), nigricanosides from *Avrain*V*illea nigricans* (iv).

Aldose reductase inhibitory activities of those compounds were later revealed by Islam et al. (2014). Such observations pointed out potential health benefits of *Capsosiphon fulvescens* in improving neurotransmission as well as in preventing diabetic complications. A class of ether-linked glycoglycerolipids called nigricanosides, isolated from the green alga *Avrainvillea nigricans* (Dominica) are found to show potent antimitotic activity and ability to promote tubulin polymerization (Williams et al., 2007).

Certain nitrogenous glycolipids having biological significances were also identified. An example is acetylated nitrogenous glycolipid, isolated from a marine sponge *Plakinastrella clathrata* (Gneerings Reef, Queensland, Australia), having moderate anti-inflammatory activity by inhibition of prostaglandin E2 (PGE2) (Katavic et al., 2013). Peddie et al. (2015) isolated a class of glycolipids known as stellettosides possessing three arabinopyranose units (Figure 1.7) from a marine sponge, *Stelletta* sp.

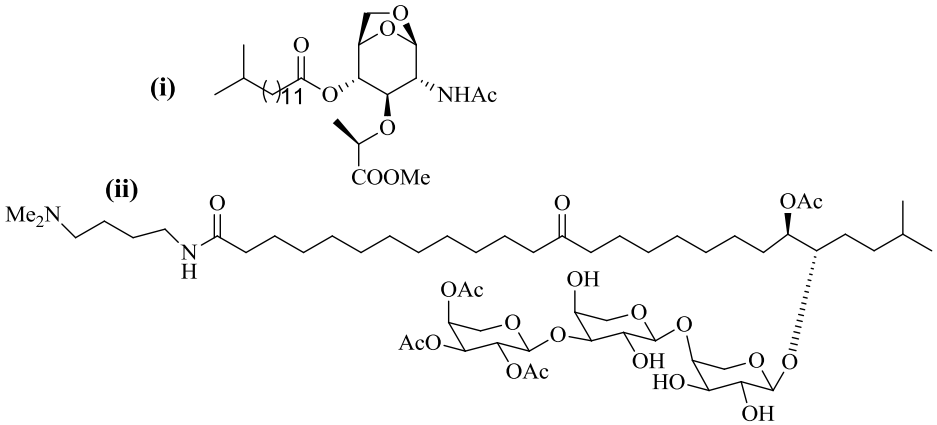


Figure 1.7 Structures of two cytotoxic nitrogenous glycolipids; nitrogenous glycolipid from *Plakinastrella clathrata* (i), stellettoside from *Stelletta* sp. (ii).

Those glycosylated fatty acid amides were found to have cytotoxic activity against human cervical cancer cells HeLa cells with an IC_{50} value of 9 μM. Structural variations and cytotoxic potentials shown by glycolipids from different natural sources project it as a potential target for new natural product discoveries.

1.3 Colon cancer: Pathology and risk factors

Colon cancer is the second most common cause of cancer related death in Western countries. Incidence of colon cancer is found to be increasing in the Asian Pacific region (Goh et al., 2005; Siegel et al., 2017). Of the 3.5 million cases of cancer reported in India, 35,000 are found to be due to colon cancer (Shrikhande et al., 2007; Gowri, 2013; Marley & Nan, 2016). Rate of its occurrence is seems to be increasing at an alarming rate with changing life style. Colon cancer can be any malignant neoplasm developing from the inner lining of the colonic epithelium. One of the major threats related to colon cancer is its ability to spread to other body parts such as liver, lung, ovaries and other gastrointestinal organs (Cappell, 2007). Epidemiological studies have suggested a large geographic difference in the global distribution of colon cancer (Boyle & Langman, 2000; Janout & Kollárová, 2001). Risk factors associated with colon cancer can be grouped into two categories. The first group includes unmodifiable factors such as age and hereditary factor. Environmental and lifestyle factors are included in the second group. Studies on colon cancer incidence have suggested that it is having strong correlation with age, viz., about 90% of cases were reported in people at the age of fifty or above. Until age of fifty years,

both men and women have equal possibility for colon cancer incidence, whereas in later life males predominate with the risk of this malignancy (Fairley et al., 2006). Epidemiological studies have proposed that colon cancer is manifestation of a number of inherited cancer predisposition syndromes, including familial adenomatous polyposis, hereditary non-polyposis colorectal cancer and personal or family history of colorectal cancer and/or polyps, and inflammatory bowel disease (Rowley, 2005; Boardman et al., 2007; Grande et al., 2008).

Environmental causes such as cultural, social and lifestyle factors also affect the rate of colon cancer incidence (Papapolychroniadis, 2004). Theoretically, those modifiable causes can be readily identified and a large proportion of colon cancer cases can be prevented (Johnson & Lund, 2007). Risk of colon cancer incidence is found to be higher in urban areas, which is evidenced from statistical studies on migrants of different countries (Parkin et al., 2005). Nutritional practices were found to strongly influence incidence of colon cancer (Willett, 2005; Kim & Milner, 2007). Diets rich in fat, especially animal fat, are found to be major risk factor for colon cancer. Consequence of high fat intake is that it favours development of bacterial flora capable of degrading bile salts to potentially carcinogenic N-nitroso compounds (Larsson & Wolk, 2006; Santarelli et al., 2008). Fundamental mechanism for positive association of red meat consumption with colorectal cancer includes the presence of heme iron in red meat (Kabat et al., 2007). In addition, over cooking of some meats at high temperatures are resulted in the production of heterocyclic amines and polycyclic aromatic hydrocarbons, both of which are believed to have carcinogenic properties (Sinha, 2002).

Certain studies also suggested that people following diet low in fruits and vegetables may have a higher risk of colorectal cancer. High dietary fibre intake may reduce colorectal cancer incidence rates (Haggar & Boushey, 2009).

Two major lifestyle related factors linked to colon cancer are physical inactivity and excess body weight. Several reports suggested that higher overall levels of physical activity are associated with lower risk of colon cancer (Lee et al., 2007). Regular moderate physical activity increases the metabolic rate and raises maximal oxygen uptake. Such sustained physical activity is found to reduce blood pressure and insulin resistance, and increase gut motility (Bazensky et al., 2007). Lack of physical activity in daily routines also can be attributed to the increased incidence of obesity in men and women, another important factor associated with colorectal cancer (Campbell et al., 2007). Cigarette smoking and heavy alcohol consumption is also reported as causes for colon cancer. Studies showed that 12% of colorectal cancer deaths are attributed to smoking (Zisman et al., 2006). Carcinogens in tobacco cause formation and growth of adenomatous polyps, the precursor lesions of colorectal cancer (Tsong et al., 2007; Botteri et al., 2008). Reactive metabolites of alcohol such as acetaldehyde can act as causative agents for colon cancer. Alcohol may also function as solvent, enhancing penetration of other carcinogenic molecules into mucosal cells (Pöschl & Seitz, 2004).

Colon cancer survival is highly dependent upon stage of disease at diagnosis (Jemal et al., 2004). Various treatment options such as

chemotherapy, surgery, radiation and phytotherapy are employed at different stages of colon cancer. Best known drug used for colon cancer chemotherapy is 5-fluorouracil (5-FU), acting as an inhibitor of DNA synthesis. Even though, synthetic chemical anticancer drugs provide prolong survival, they often have adverse effects and off-target actions. Therefore, natural products have widely been investigated for colon cancer therapeutics (Rajamanickam & Agarwal, 2008). Neutraceuticals have been identified as beneficial for reducing colon cancer risks (Donaldson, 2004; Wargovich et al., 2010). Selenium is associated with up to a 50% decrease in incidence of colon cancer and is found as an important dietary mineral in broccoli extract, dietary fiber, red wine, soya, pepper, fenugreek, cloves, ginger, apple and other vegetables (Finley et al., 2000). Yellow mustard oil containing complex mixture of long-chain polysaccharides, derived from brassica family of plants, was found to play a protective role against colon cancer formation (Nobili et al., 2009). Consumption of fish and fish products, rich in eicosapentanoic acid (EPA), docosahexaenoic acid (DHA) and other omega-3 fatty acids, is found to reduce the risk of colon cancer progression (Pandey et al., 2010).

1.4 Natural products in colon cancer therapy

Several nutraceuticals and phytochemicals were identified to have cytotoxic activity against various colon cancer cell lines (Figure 1.8). Antioxidant fruits such as raspberry, strawberry and grape seeds were proven to reduce occurrence of intestinal tumours (Seeram et al., 2006; Kuppusamy et al., 2014). Garlic, possessing diverse nutritional roles, is found to contain organo sulfur and S-allyl cysteine compounds with the

ability to inhibit proliferation of colon cancer cells. Experimental studies on mice by oral administration of garlic extract exhibited decreased multiplication of cancer cells in the initiation stage, but the extract was not effective during late stages. There is no clear scientific evidence for cancer preventive efficacy of garlic extract, and it is also found to have some toxic effects such as increased hemolysis and anaemic condition of the patient (Delshad et al., 2010). Fenugreek has high content of diosgenin, belongs to the steroidal group of saponins. It also showed anti-proliferative activity against colon cancer cell lines, but the mechanism of action is not completely studied (Raju & Bird, 2007). Omega 3-fatty acids (Larsson et al., 2004; Cockbain et al., 2012) and vitamins, especially vitamin C, B complex and vitamin D, were found to play important roles in colon cancer chemoprevention (Tangpricha et al., 2001; Lee et al., 2003).

Carotenoids are identified as important phytochemicals for the prevention of colon and gastrointestinal cancer. Halocynthiaxanthin, a carotenoid isolated from sea squirt *Halocynthia roretzi*, is found to induce apoptosis in DLD-1colon cancer cells by combining with tumor necrosis factor related apoptosis inducing ligand (TRAIL). Mechanism of action involves important anticancer effects such as poly (ADP-ribose) polymerase cleavage, induction of caspase inhibitors and nuclear condensation, and hence halocynthiaxanthin was identified to have the potential to regulate programmed cell death in colon cancer cells (Yoshida et al., 2007). Carolenoids such as xanthophyll, cryoptoxanthin and zeaxanthin metabolites have also been used for the treatment of colon cancer (Slattery et al., 2000).

Astaxanthin is a kind of carotenoid abundantly found in a single celled green algae, *Haematococcus pluvialis* and also in some crabs and marine animals, and chemically named as 3,3'-dihydroxy-β,β-carotene-4,4'-dione. It is shown to have significant cytotoxicity against colon cancer cells (Yang et al., 2013). Astaxanthin was found to regulate expression of several tumour necrosis factors and inhibit proliferation and induce apoptosis of cancer cells. Astaxanthin has specific functional groups to regulate the NF-κB proteins, which is a transcription factor participating in a wide range of cellular roles and cancer pathways (Pashkow et al., 2008; Nagendraprabhu & Sudhandiran, 2011; Kavitha et al., 2013). Astaxanthin is also used as food supplement to maintain optimal health for humans. Dietary astaxanthin significantly suppressed the formation of colonic mucosal ulcers and dysplastic crypts in an animal model. Additionally, astaxanthin was found to markedly reduce 1,2-dimethyl hydrazine (DMH), a potent DNA methylating agent, induced colon carcinogenesis (Prabhu et al., 2009). Siphonaxanthin, isolated from the marine green alga, *Codium fragile* was also suppressed cell viability and induces apoptosis in human colon cancer cells (Ganesan et al., 2010; Ganesan et al., 2011).

Luteolin, a flavonoid, found in several edible plants and vegetables such as green pepper, celery and perilla, was found to induce apoptosis in HT-29 colon carcinoma cells (Lim et al., 2007). Luteolin suppresses over expression of some antiapoptotic proteins in the cancer cells and regulate expression and activity of specific proteins that induce G2/M cell cycle arrest (Demidenko & Blagosklonny, 2004). Fisetin, another naturally occurring flavonoid was found to induce apoptosis in colon cancer cells through mitochondrial pathway by caspase 3 activation

(Khan et al., 2008; Suh et al., 2008). Epigallocatechin-3 gallate (EGCG), a polyphenol normally found in tea extracts was found to have anti colon cancer activity (Yang et al., 2011; Alshehri, 2012). Curcumin, one of the important secondary metabolite, induces apoptosis in colon cancer cells, HCT 116, by affecting molecular level protein expressions and is used clinically as a chemopreventive agent at initiation and progression stages of digestive tract cancer (Huang et al., 1994; Wang et al., 2006). Quercetin, a plant derived flavonoid was found to be effective for inducing apoptosis in SW480 colon cancer cells (Shan et al., 2009). Polysaccharide, lentinan, a β-1,3-glucan with β-1,6 branching, isolated from edible mushroom *Lentinus edodes*, is one of the important drugs used clinically for colon cancer treatment (Ng & Yap, 2002).

Acetylapoaranotin, diketopiperazine disulphide, isolated from marine *Aspergillus* sp., was found to induce apoptosis in human colon cancer cells (HCT116). Disulfide bridges in the compound were identified to be responsible for the cytotoxicity. Acetylapoaranotin showed potential to regulate several pro-apoptotic proteins. Treatment of the cancer cell line HCT116 with different concentrations of acetylapoaranotin was shown to initiate the caspase8 activation in intrinsic and extrinsic pathways (Choi et al., 2011). Eugenol, a natural compound derived from honey and from plant extracts including clove oil, cinnamon, citrus and balm, was found to promote apoptosis in colon cancer cells. It stimulates cell division in sub-G1 phase inducing apoptosis in regular time-dependent manner and it also showed increased p53 activation and proline rich acidic protein (PRAP) cleavage in treated cell lines (Jaganathan et al., 2011).

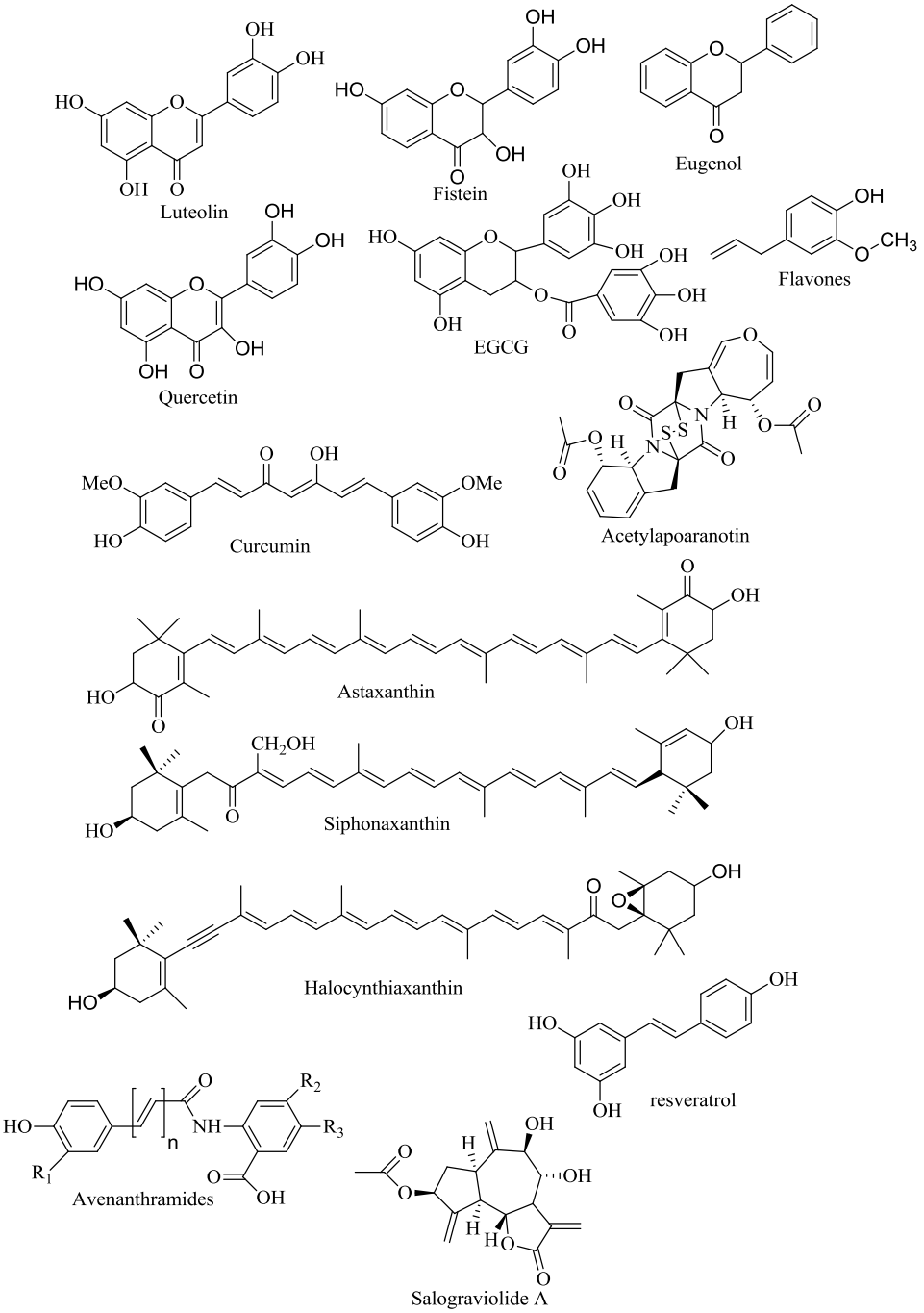


Figure 1.8 Structures of some clinically important nutraceuticals and phytochemicals used in colon cancer treatment.

Avenanthramides, unique polyphenols found in oats and oatmeal, have found to inhibit proliferation of human colon cancer cells (Guo et al., 2010). Another polyphenol, resveratrol present in wine and grapes, demonstrated apoptosis induction in colon cancer cells in a p53 independent pathway (Mahyar-Roemer et al., 2001). Salograviolide A, a sesquiterpene lactone, isolated from the extracts of *Centaurea ainetensis*, a Lebanon plant, was found to suppress proliferation of human colon carcinoma cells HCT-116 (El-Najjar et al., 2008).

Sunassee et al. (2014) have isolated two steroidal alkaloids, plakinamines N and plakinamines O from a Philippines collection of the marine sponge *Corticium niger*, with significant cytotoxicity towards colon carcinoma cell lines, COLO205 and KM12. Five bisindole alkaloids of vobasinyl-iboga type, isolated from an African medicinal plant *Tabernaemontana elegans* was found to have ability to induce apoptosis and arrest cell cycle in human HCT-116 colon and HepG2 liver carcinoma cells (Paterna et al., 2016). A group of cytotoxic glycosides, known as cardiac glycosides found in limited number of plant families, exhibited significant cytotoxicity towards colon cancer cells. Among those glycosides, convallatoxin, oleandrin and proscillaridin A, were identified as potent compound with submicromolar IC_{50} values against HT29 colon cancer cell line (Felth et al., 2009; Surovtseva et al., 2016) (Figure 1.9). Though efficiency of natural compounds for colon cancer treatment is well established, their mechanism of action needs further development and better molecular identification.

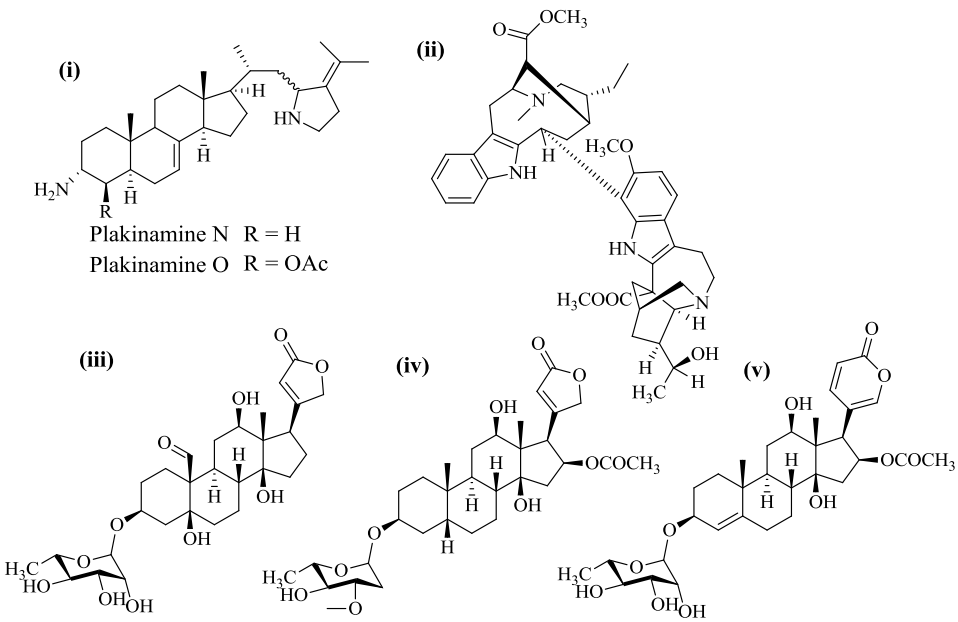


Figure 1.9 Structures of some natural products showing cytotoxicity against colon cancer, steroidal alkaloids, plakinamines from *Corticium niger* (i), bisindole alkaloid from *Tabernaemontana elegans* (ii), cardiac glycosides convallatoxin (iii), oleandrin (iv) and proscillaridin A (v).

1.5 Scope and objectives

Cyanobacteria are known as rich source of bioactive natural products. Their metabolites are found to be varying depending on nature of species and growth conditions. Both marine and freshwater cyanobacteria have been actively pursued for their unique and bioactive components. *Synechocystis* is the most widely studied genus among cyanobacteria, owing to their abilities of natural transformation and fast growth. They can accumulate compatible solutes from the environment to adapt with varying salt conditions. They are also identified as target for several metabolic and genomic studies. *Synechocystis* is recognized as an important source for

glycolipids. Some *Synechocystis* strains were found to produce glycolipids with certain structural variations along with common glycoglycerolipids. Therefore, it is important to study structural features of individual glycolipid compounds in *Synechocystis* strains.

Glycolipids have been identified as important candidates for new drug development and its therapeutic potential is found to depend on the chemical structure. It is being established as effective drug source for varieties of human cell carcinomas. Inhibitory activity of glycolipids against colon cancer, one of the emerging threats across the world, is less studied in comparison with ant-proliferative properties on other carcinomas. The modern diet and lifestyles have led to a tremendous increase in colon cancer mortality rate. Hence, there is a need for developing novel drug therapies with fewer side effects to prevent colon cancer. This work focuses mainly on isolation and structural studies of different glycolipid components of a freshwater *Synechocystis* species and also on anti-proliferative potential of the major glycolipids against colon cancer. Identification of the cyanobacteria is achieved through molecular analyses and fatty acid chemotaxonomy. Isolation and structural characterization of individual glycolipids from the cyanobacteria, using different spectroscopic methods, serve as vital element of the study. The study also highlights anti-proliferative potential of major glycolipids against colon cancer cells. Findings on induction of apoptosis and cell cycle arrest by the highest active glycolipid compound envisage new drug development strategies to suppress colon cancer proliferation.

Specific objectives of the study are

- Molecular level and chemotaxonomic identification of *Synechocystis sp.* Mass culturing of *Synechocystis* sp. for isolation of glycolipids.
- Extraction and isolation of glycolipid fractions using different chromatographic methods.
- Structure elucidation of major glycolipids using various spectroscopic techniques such as NMR, FTIR and Mass spectroscopy.
- Screening of anti-proliferative activities of major glycolipids on SW480 colon cancer cells using MTT assay.
- Analysis of cell cycle arrest and apoptosis induction by highest active glycolipid in SW480 cells using different molecular methods.
- Investigations on involvement of certain specific proteins in the inhibitory activity of glycolipids against colon cancer cells.

Better understanding on mechanism of cell cycle arrest and apoptosis, induced by major glycolipids from *Synechocystis* sp., may enable to assess therapeutic potential of the glycolipid against colon cancer and also to introduce new natural drug source for further *in vivo* and clinical trials.

References

Abdel-Mawgoud, A. M., Lépine, F., & Déziel, E. (2010). Rhamnolipids: diversity of structures, microbial origins and roles. *Applied Microbiology and Biotechnology*, 86(5), 1323-1336.

Agranoff, B. W., Benjamins, J. A., & Hajra, A. K. (1994). Properties of Brain Lipids. *Basic Neurochemistry, edited by Siegel GJ, Agranoff BW, Albers RW, Molinoff PB. New York: Raven*, 117-143.

Al-Fadhli, A., Wahidulla, S., & D'souza, L. (2006). Glycolipids from the red alga *Chondria armata* (Kütz.) Okamura. *Glycobiology*, 16(10), 902-915.

Alshehri, A. (2012). Molecular and biochemical evaluation of anti-proliferative effect of (*Cichorium endivia* L.) phenolic extracts on cancer cell line HCT-116. *Academic Journal of Cancer Research*, 5(2), 53-60.

Amiguet, T. V., Jewell, L. E., Mao, H., Sharma, M., Hudson, J. B., Durst, T., & Arnason, J. T. (2011). Antibacterial properties of a glycolipid-rich extract and active principle from Nunavik collections of the macroalgae *Fucus evanescens C. Agardh* (*Fucaceae*). *Canadian Journal of Microbiology*, 57(9), 745-749.

Andrianasolo, E. H., Haramaty, L., Vardi, A., White, E., Lutz, R., & Falkowski, P. (2008). Apoptosis-inducing galactolipids from a cultured marine diatom, *Phaeodactylum tricornutum*. *Journal of Natural Products*, 71(7), 1197-1201.

Antonopoulou, S., Nomikos, T., Oikonomou, A., Kyriacou, A., Andriotis, M., Fragopoulou, E., & Pantazidou, A. (2005a). Characterization of bioactive glycolipids from *Scytonema julianum* (cyanobacteria). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 140(2), 219-231.

Antonopoulou, S., Karantonis, H. C., Nomikos, T., Oikonomou, A., Fragopoulou, E., & Pantazidou, A. (2005b). Bioactive polar lipids from *Chroococcidiopsis* sp.(Cyanobacteria). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 142(3), 269-282.

Awai, K., Maréchal, E., Block, M. A., Brun, D., Masuda, T., Shimada, H., & Joyard, J. (2001). Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, 98(19), 10960-10965.

Awai, K., Watanabe, H., Benning, C., & Nishida, I. (2007). Digalactosyldiacylglycerol is required for better photosynthetic growth of *Synechocystis* sp. PCC 6803 under phosphate limitation. *Plant and Cell Physiology*, 48(11), 1517-1523.

Azim, A., Shah, V., Doncel, G. F., Peterson, N., Gao, W., & Gross, R. (2006). Amino acid conjugated sophorolipids: a new family of biologically active functionalized glycolipids. *Bioconjugate Chemistry*, 17(6), 1523-1529.

Baek, S. H., Sun, X. X., Lee, Y. J., Wang, S. Y., Han, K. N., Choi, J. K., & Kim, E. K. (2003). Mitigation of harmful algal blooms by sophorolipid. *Journal of Microbiology and Biotechnology*, 13(5), 651-659.

Bale, N. J., Hopmans, E. C., Zell, C., Sobrinho, R. L., Kim, J. H., Damsté, J. S. S., & Schouten, S. (2015). Long chain glycolipids with pentose head groups as biomarkers for marine endosymbiotic heterocystous cyanobacteria. *Organic Geochemistry*, 81(4), 1-7.

Ballou, L. R. (1992). Sphingolipids and cell function. *Immunology Today*, 13(9), 339-341.

Banskota, A. H., Gallant, P., Stefanova, R., Melanson, R., & O'Leary, S. J. (2013a). Monogalactosyldiacylglycerols, potent nitric oxide inhibitors from the marine microalga *Tetraselmis chui*. *Natural Product Research*, 27(12), 1084-1090.

Banskota, A. H., Stefanova, R., Gallant, P., & McGinn, P. J. (2013b). Mono-and digalactosyldiacylglycerols: potent nitric oxide inhibitors from the marine microalga *Nannochloropsis granulata*. *Journal of Applied Phycology*, 25(2), 349-357.

Banskota, A. H., Stefanova, R., Sperker, S., Lall, S. P., Craigie, J. S., Hafting, J. T., & Critchley, A. T. (2014). Polar lipids from the marine macroalga *Palmaria palmata* inhibit lipopolysaccharide-induced nitric oxide production in RAW264. 7 macrophage cells. *Phytochemistry*, 101(5), 101-108.

Bauersachs, T., Compaoré, J., Hopmans, E. C., Stal, L. J., Schouten, S., & Damsté, J. S. S. (2009). Distribution of heterocyst glycolipids in cyanobacteria. *Phytochemistry*, 70(17), 2034-2039.

Bazensky, I., Shoobridge-Moran, C., & Yoder, L. H. (2007). Colorectal cancer: an overview of the epidemiology, risk factors, symptoms, and screening guidelines. *Medsurg Nursing*, 16(1), 46.

Becker, K. W., Lipp, J. S., Zhu, C., Liu, X. L., & Hinrichs, K. U. (2013). An improved method for the analysis of archaeal and bacterial ether core lipids. *Organic Geochemistry*, 61(2), 34-44.

Bergé, J. P., Debiton, E., Dumay, J., Durand, P., & Barthomeuf, C. (2002). *In vitro* anti-inflammatory and anti-proliferative activity of sulfolipids from the red alga *Porphyridium cruentum*. *Journal of Agricultural and Food Chemistry*, 50(21), 6227-6232.

Boardman, L. A., Morlan, B. W., Rabe, K. G., Petersen, G. M., Lindor, N. M., Nigon, S. K., & Gallinger, S. (2007). Colorectal cancer risks in relatives of young-onset cases: is risk the same across all first-degree relatives?. *Clinical Gastroenterology and Hepatology*, 5(10), 1195-1198.

Botteri, E., Iodice, S., Raimondi, S., Maisonneuve, P., & Lowenfels, A. B. (2008). Cigarette smoking and adenomatous polyps: a meta-analysis. *Gastroenterology*, 134(2), 388-395.

Boudière, L., Michaud, M., Petroutsos, D., Rébeillé, F., Falconet, D., Bastien, O., & Block, M. A. (2014). Glycerolipids in photosynthesis: composition, synthesis and trafficking. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1837(4), 470-480.

Boyle, P., & Langman, J. S. (2000). ABC of colorectal cancer: Epidemiology. *BMJ: British Medical Journal*, 321(1), 805.

Bruno, A., Rossi, C., Marcolongo, G., Di Lena, A., Venzo, A., Berrie, C. P., & Corda, D. (2005). Selective *in vivo* anti-inflammatory action of the galactolipid monogalactosyldiacylglycerol. *European Journal of Pharmacology*, 524(1), 159-168.

Bush, C. A., Martin-Pastor, M., & Imbery, A. (1999). Structure and conformation of complex carbohydrates of glycoproteins, glycolipids, and bacterial polysaccharides. *Annual Review of Biophysics and Biomolecular Structure*, 28(1), 269-293.

Campbell, P. T., Cotterchio, M., Dicks, E., Parfrey, P., Gallinger, S., & McLaughlin, J. R. (2007). Excess body weight and colorectal cancer risk in Canada: associations in subgroups of clinically defined familial risk of cancer. *Cancer Epidemiology and Prevention Biomarkers*, 16(9), 1735-1744.

Cantillo-Ciau, Z., Moo-Puc, R., Quijano, L., & Freile-Pelegrín, Y. (2010). The tropical brown alga *Lobophora variegata*: A source of antiprotozoal compounds. *Marine Drugs*, 8(4), 1292-1304.

Cappell, M. S. (2007). From colonic polyps to colon cancer: pathophysiology, clinical presentation, screening and colonoscopic therapy. *Minerva Gastroenterologica e Dietologica*, 53(4), 351-373.

Chen, J., Song, X., Zhang, H., Qu, Y. B., & Miao, J. Y. (2006). Sophorolipid produced from the new yeast strain *Wickerhamiella domercqiae* induces apoptosis in H7402 human liver cancer cells. *Applied Microbiology and Biotechnology*, 72(1), 52-59.

Chirasuwan, N., Chaiklahan, R., Kittakoop, P., Chanasattru, W., Ruengjitchatchawalya, M., Tanticharoen, M., & Bunnag, B. (2009). Anti HSV-1 activity of sulphoquinovosyl diacylglycerol isolated from *Spirulina Platensis*. *Science Asia*, 35(1), 137-141.

Choi, E. J., Park, J. S., Kim, Y. J., Jung, J. H., Lee, J. K., Kwon, H. C., & Yang, H. O. (2011). Apoptosis-inducing effect of diketopiperazine disulfides produced by *Aspergillus* sp. KMD 901 isolated from marine sediment on HCT116 colon cancer cell lines. *Journal of Applied Microbiology*, 110(1), 304-313.

Chong, P. L. G. (2010). Archaebacterial bipolar tetraether lipids: physico-chemical and membrane properties. *Chemistry and Physics of Lipids*, 163(3), 253-265.

Cockbain, A. J., Toogood, G. J., & Hull, M. A. (2012). Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer. *Gut*, 61(1), 135-149.

Colombo, D., Gagliardi, C., Vetro, M., Ronchetti, F., Takasaki, M., Konoshima, T., & Tokuda, H. (2013). New 6-amino-6-deoxy-glycoglycerolipids derived from 2-O-β-d-glucopyranosylglycerol: insights into the structure–activity relationship of glycoglycerolipids as anti-tumor promoters. *Carbohydrate Research*, 373(4), 64-74.

Cortes-Sanchez, A. J., Sánchez, H. H., & Jaramillo-Flores, M. E. (2013). Biological activity of glycolipids produced by microorganisms: new trends and possible therapeutic alternatives. *Microbiological Research*, 168(1), 22-32.

Costantino, V., Fattorusso, E., Imperatore, C., & Mangoni, A. (2006). Glycolipids from sponges. part 17. clathrosides and isoclathrosides, unique glycolipids from the Caribbean sponge *Agelas c lathrodes*. *Journal of Natural Products*, 69(1), 73-78.

Curatolo, W. (1987a). Glycolipid function. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*, 906(2), 137-160.

Curatolo, W. (1987b). The physical properties of glycolipids. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*, 906(2), 111-136.

da Costa, E., Silva, J., Mendonça, S. H., Abreu, M. H., & Domingues, M. R. (2016). Lipidomic approaches towards deciphering glycolipids from microalgae as a reservoir of bioactive lipids. *Marine Drugs*, 14(5), 101-128.

de Souza, L. M., Sassaki, G. L., Romanos, M. T. V., & Barreto-Bergter, E. (2012). Structural characterization and anti-HSV-1 and HSV-2 activity of glycolipids from the marine algae *Osmundaria obtusiloba* isolated from Southeastern Brazilian coast. *Marine Drugs*, 10(4), 918-931.

Delshad, A. A., Heshmati, M., & Ghaini, M. H. (2010). Garlic extract can induce apoptotic cell death in the human colon adenocarcinoma HT29 cell line. *Iranian Journal of Pathology*, 5(3), 126-131.

Demidenko, Z. N., & Blagosklonny, M. V. (2004). Flavopiridol induces p53 via initial inhibition of Mdm2 and p21 and, independently of p53, sensitizes apoptosis-reluctant cells to tumor necrosis factor. *Cancer Research*, 64(10), 3653-3660.

Deml, G., Anke, T., Oberwinkler, F., Giannetti, B. M., & Steglich, W. (1980). Schizonellin A and B, new glycolipids from *Schizonella melanogramma*. *Phytochemistry*, 19(1), 83-87.

Ding, N., Zhang, Z., Zhang, W., Chun, Y., Wang, P., Qi, H., & Li, Y. (2011). Synthesis and antibacterial evaluation of a series of oligorhamnoside derivatives. *Carbohydrate Research*, 346(14), 2126-2135.

Donaldson, M. S. (2004). Nutrition and cancer: a review of the evidence for an anti-cancer diet. *Nutrition Journal*, 3(1), 19.

Dörmann, P., & Benning, C. (2002). Galactolipids rule in seed plants. *Trends in Plant Science*, 7(3), 112-118.

El Baz, F. K., El Baroty, G. S., El Baky, H. A., El-Salam, O. A., & Ibrahim, E. A. (2013). Structural characterization and biological activity of sulfolipids from selected marine algae. *Grasas y Aceites*, 64(5), 561-571.

El-Najjar, N., Dakdouki, S., Darwiche, N., El-Sabban, M., Saliba, N. A., & Gali-Muhtasib, H. (2008). Anti-colon cancer effects of Salograviolide A isolated from *Centaurea ainetensis*. *Oncology Reports*, 19(4), 897-904.

Elshahawi, S. I., Shaaban, K. A., Kharel, M. K., & Thorson, J. S. (2015). A comprehensive review of glycosylated bacterial natural products. *Chemical Society Reviews*, 44(21), 7591-7697.

Englund, E., Andersen-Ranberg, J., Miao, R., Hamberger, B., & Lindberg, P. (2015). Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide. *ACS Synthetic Biology*, 4(12), 1270-1278.

Fairley, T. L., Cardinez, C. J., Martin, J., Alley, L., Friedman, C., Edwards, B., & Jamison, P. (2006). Colorectal cancer in US adults younger than 50 years of age, 1998–2001. *Cancer*, 107(S5), 1153-1161.

Fang, Z., Jeong, S. Y., Jung, H. A., Choi, J. S., Min, B. S., & Woo, M. H. (2012). Capsofulvesins A–C, cholinesterase inhibitors from *Capsosiphon fulvescens*. *Chemical and Pharmaceutical Bulletin*, 60(11), 1351-1358.

Felth, J., Rickardson, L., Rosén, J., Wickström, M., Fryknäs, M., Lindskog, M., & Gullbo, J. (2009). Cytotoxic effects of cardiac glycosides in colon cancer cells, alone and in combination with standard chemotherapeutic drugs. *Journal of Natural Products*, 72(11), 1969-1974.

Finley, J. W., Davis, C. D., & Feng, Y. (2000). Selenium from high selenium broccoli protects rats from colon cancer. *The Journal of Nutrition*, 130(9), 2384-2389.

Fischer, S., Fischer, H., Diele, S., Pelzl, G., Jankowski, K., Schmidt, R. R., & Vill, V. (1994). On the structure of the thermotropic cubic mesophases. *Liquid Crystals*, 17(6), 855-861.

Fleurence, J., Fougeray, S., Bahri, M., Cochonneau, D., Clémenceau, B., Paris, F., & Birklé, S. (2017). Targeting O-Acetyl-GD2 Ganglioside for Cancer Immunotherapy. *Journal of Immunology Research*, 2017(1), 1-16.

Froehlich, J. E., Benning, C., & Dörmann, P. (2001). The digalactosyldiacylglycerol (DGDG) synthase DGD1 is inserted into the outer envelope membrane of chloroplasts in a manner independent of the general import pathway. *Journal of Biological Chemistry*, 276(34), 31806-31812.

Fu, S. L., Wallner, S. R., Bowne, W. B., Hagler, M. D., Zenilman, M. E., Gross, R., & Bluth, M. H. (2008). Sophorolipids and their derivatives are lethal against human pancreatic cancer cells. *Journal of Surgical Research*, 148(1), 77-82.

Ganesan, P., Matsubara, K., Ohkubo, T., Tanaka, Y., Noda, K., Sugawara, T., & Hirata, T. (2010). Anti-angiogenic effect of siphonaxanthin from green alga, *Codium fragile*. *Phytomedicine*, 17(14), 1140-1144.

Ganesan, P., Noda, K., Manabe, Y., Ohkubo, T., Tanaka, Y., Maoka, T., & Hirata, T. (2011). Siphonaxanthin, a marine carotenoid from green algae, effectively induces apoptosis in human leukemia (HL-60) cells. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1810(5), 497-503.

Goh, K. L., Quek, K. F., Yeo, G. T. S., Hilmi, I. N., Lee, C. K., Hasnida, N., & Ong, K. T. (2005). Colorectal cancer in Asians: a demographic and anatomic survey in Malaysian patients undergoing colonoscopy. *Alimentary Pharmacology & Therapeutics*, 22(9), 859-864.

Gowri, S. (2013). Chemopreventive potential of Caralluma adscendens against 1,2 dimethyl hydrazine induced colon cancer. *PhD Thesis. Bharathiar University, India*, 10-41.

Grande, M., Milito, G., Attinà, G. M., Cadeddu, F., Muzi, M. G., Nigro, C., & Farinon, A. M. (2008). Evaluation of clinical, laboratory and morphologic prognostic factors in colon cancer. *World Journal of Surgical Oncology*, 6(1), 98.

Guo, W., Nie, L., Wu, D., Wise, M. L., Collins, F. W., Meydani, S. N., & Meydani, M. (2010). Avenanthramides inhibit proliferation of human colon cancer cell lines in vitro. *Nutrition and Cancer*, 62(8), 1007-1016.

Hagemann, M. (2010). Molecular biology of cyanobacterial salt acclimation. *FEMS Microbiology Reviews*, 35(1), 87-123.

Haggar, F. A., & Boushey, R. P. (2009). Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinics in Colon and Rectal surgery*, 22(4), 191-197.

Hakomori, S. I. (2000). Traveling for the glycosphingolipid path. *Glycoconjugate Journal*, 17(7-9), 627-647.

Hamed, I., Özogul, F., Özogul, Y., & Regenstein, J. M. (2015). Marine bioactive compounds and their health benefits: a review. *Comprehensive Reviews in Food Science and Food Safety*, 14(4), 446-465.

Hanford, M. J., & Peeples, T. L. (2002). Archaeal tetraether lipids. *Applied Biochemistry and Biotechnology*, 97(1), 45-62.

Hölzl, G., & Dörmann, P. (2007). Structure and function of glycoglycerolipids in plants and bacteria. *Progress in Lipid Research*, 46(5), 225-243.

Hossain, Z., Kurihara, H., Hosokawa, M., & Takahashi, K. (2005). Growth inhibition and induction of differentiation and apoptosis mediated by sodium butyrate in Caco-2 cells with algal glycolipids. *In Vitro Cellular & Developmental Biology-Animal*, 41(5), 154-159.

Huang, M. T., Lou, Y. R., Ma, W., Newmark, H. L., Reuhl, K. R., & Conney, A. H. (1994). Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. *Cancer Research*, 54(22), 5841-5847.

Huguet, C., Hopmans, E. C., Febo-Ayala, W., Thompson, D. H., Damsté, J. S., & Schouten, S. (2006). An improved method to determine the absolute abundance of glycerol dibiphytanyl glycerol tetraether lipids. *Organic Geochemistry*, 37(9), 1036-1041.

Iijima, H., Nakaya, Y., Kuwahara, A., Hirai, M. Y., & Osanai, T. (2015). Seawater cultivation of freshwater cyanobacterium *Synechocystis sp*. PCC 6803 drastically alters amino acid composition and glycogen metabolism. *Frontiers in Microbiology*, 326(6), 1-10.

Imbs, T. I., Ermakova, S. P., Fedoreyev, S. A., Anastyuk, S. D., & Zvyagintseva, T. N. (2013). Isolation of fucoxanthin and highly unsaturated monogalactosyldiacylglycerol from brown alga *Fucus evanescens C Agardh* and in vitro investigation of their antitumor activity. *Marine Biotechnology*, 15(5), 606-612.

Islam, M. N., Choi, S. H., Moon, H. E., Park, J. J., Jung, H. A., Woo, M. H., & Choi, J. S. (2014). The inhibitory activities of the edible green alga *Capsosiphon fulvescens* on rat lens aldose reductase and advanced glycation end products formation. *European Journal of Nutrition*, 53(1), 233-242.

Jaganathan, S. K., Mazumdar, A., Mondhe, D., & Mandal, M. (2011). Apoptotic effect of eugenol in human colon cancer cell lines. *Cell Biology International*, 35(6), 607-615.

Janout, V., & Kollárová, H. (2001). Epidemiology of colorectal cancer. *Acta-Universitatis Palackianae Olomucensis Facultatis Medicae*, 13(2), 5-10.

Javed, S., Azeem, F., Hussain, S., Rasul, I., Siddique, M. H., Riaz, M., & Nadeem, H. (2017). Bacterial lipases: A review on purification and characterization. *Progress in Biophysics and Molecular Biology*. 130(2), 1-12.

Jemal, A., Clegg, L. X., Ward, E., Ries, L. A., Wu, X., Jamison, P. M., & Edwards, B. K. (2004). Annual report to the nation on the status of cancer, 1975–2001, with a special feature regarding survival. *Cancer*, 101(1), 3-27.

Jiang, R. W., Hay, M. E., Fairchild, C. R., Prudhomme, J., Le Roch, K., Aalbersberg, W., & Kubanek, J. (2008). Antineoplastic unsaturated fatty acids from Fijian macroalgae. *Phytochemistry*, 69(13), 2495-2500.

Johnson, I. T., & Lund, E. K. (2007). Nutrition, obesity and colorectal cancer. *Alimentary Pharmacology & Therapeutics*, 26(2), 161-181.

Kabat, G. C., Miller, A. B., Jain, M., & Rohan, T. E. (2007). A cohort study of dietary iron and heme iron intake and risk of colorectal cancer in women. *British Journal of Cancer*, 97(1), 118.

Katavic, P. L., Yong, K. W., Herring, J. N., Deseo, M. A., Blanchfield, J. T., Ferro, V., & Garson, M. J. (2013). Structure and stereochemistry of an anti-inflammatory anhydrosugar from the Australian marine sponge *Plakinastrella clathrata* and the synthesis of two analogues. *Tetrahedron*, 69(37), 8074-8079.

Kates, M. (1992). Archaebacterial lipids: structure, biosynthesis and function. *Biochemical Society Symposium,* 58(2), 51-72.

Kavitha, K., Kowshik, J., Kishore, T. K. K., Baba, A. B., & Nagini, S. (2013). Astaxanthin inhibits NF-κB and Wnt/β-catenin signaling pathways via inactivation of Erk/MAPK and PI3K/Akt to induce intrinsic apoptosis in a hamster model of oral cancer. *Biochimica et Biophysica Acta*, 1830(10), 4433-4444.

Kelly, A. A., & Dörmann, P. (2004). Green light for galactolipid trafficking. *Current Opinion in Plant Biology*, 7(3), 262-269.

Kelly, A. A., Froehlich, J. E., & Dörmann, P. (2003). Disruption of the two digalactosyldiacylglycerol synthase genes DGD1 and DGD2 in *Arabidopsis* reveals the existence of an additional enzyme of galactolipid synthesis. *The Plant Cell*, 15(11), 2694-2706

Khan, N., Afaq, F., Syed, D. N., & Mukhtar, H. (2008). Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. *Carcinogenesis*, 29(5), 1049-1056.

Kim, Y. H., Choi, J. S., Yoo, J. S., Park, Y. M., & Kim, M. S. (1999). Structural Identification of glycerolipid molecular species isolated from cyanobacterium *Synechocystis* sp. PCC 6803 using fast atom bombardment tandem mass spectrometry. *Analytical Biochemistry*, 267(2), 260-270

Kim, Y. H., Kim, E. H., Lee, C., Kim, M. H., & Rho, J. R. (2007). Two new monogalactosyl diacylglycerols from brown alga *Sargassum thunbergii*. *Lipids*, 42(4), 395-399.

Kim, Y. S., & Milner, J. A. (2007). Dietary modulation of colon cancer risk. *The Journal of Nutrition*, 137(11), 2576S-2579S.

Kitamoto, D., Isoda, H., & Nakahara, T. (2002). Functions and potential applications of glycolipid biosurfactants from energy-saving materials to gene delivery carriers. *Journal of Bioscience and Bioengineering*, 94(3), 187-201.

Kojima, M., Seki, K., Ohnishi, M., Ito, S., & Fujino, Y. (1990). Structure of novel glyceroglycolipids in Adzuki bean (*Vigna angularis*) seeds. *Biochemistry and Cell Biology*, 68(1), 59-64.

Kolter, T., Proia, R. L., & Sandhoff, K. (2002). Combinatorial ganglioside biosynthesis. *Journal of Biological Chemistry*, 277(29), 25859-25862.

Kondakov, A., & Lindner, B. (2005). Structural characterization of complex bacterial glycolipids by Fourier transform mass spectrometry. *European Journal of Mass Spectrometry*, 11(5), 535-546.

Kuppusamy, P., Yusoff, M. M., Maniam, G. P., Ichwan, S. J. A., Soundharrajan, I., & Govindan, N. (2014). Nutraceuticals as potential therapeutic agents for colon cancer: a review. *Acta Pharmaceutica Sinica B*, 4(3), 173-181.

Larsen, E., & Christensen, L. P. (2007). Common vegetables and fruits as a source of 1, 2-di-o-α-linolenoyl-3-o-β-d-galactopyranosyl-sn-glycerol, a potential anti-inflammatory and antitumor agent. *Journal of Food Lipids*, 14(3), 272-279.

Larsson, S. C., Kumlin, M., Ingelman-Sundberg, M., & Wolk, A. (2004). Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *The American Journal of Clinical Nutrition*, 79(6), 935-945.

Larsson, S. C., & Wolk, A. (2006). Meat consumption and risk of colorectal cancer: a meta-analysis of prospective studies. *International Journal of Cancer*, 119(11), 2657-2664.

Leão, P. N., Nakamura, H., Costa, M., Pereira, A. R., Martins, R., Vasconcelos, V., & Balskus, E. P. (2015). Biosynthesis-assisted structural elucidation of the bartolosides, chlorinated aromatic glycolipids from cyanobacteria. *Angewandte Chemie*, 127(38), 11215-11219.

Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nature Reviews. Microbiology*, 8(3), 171.

Lee, K. J., Inoue, M., Otani, T., Iwasaki, M., Sasazuki, S., & Tsugane, S. (2007). Physical activity and risk of colorectal cancer in Japanese men and women: the Japan Public Health Center-based prospective study. *Cancer Causes & Control*, 18(2), 199-209.

Lee, K. W., Lee, H. J., Surh, Y. J., & Lee, C. Y. (2003). Vitamin C and cancer chemoprevention: reappraisal. *The American Journal of Clinical Nutrition*, 78(6), 1074-1078.

Li, C., He, X. P., Zhang, Y. J., Li, Z., Gao, L. X., Shi, X. X., & Tang, Y. (2011). Click to a focused library of benzyl 6-triazolo (hydroxy) benzoic glucosides: Novel construction of PTP1B inhibitors on a sugar scaffold. *European Journal of Medicinal Chemistry*, *46*(9), 4212-4218.

Li, H., Guo, W., Ma, X. J., Li, J. S., & Song, X. (2017). *In vitro* and *in vivo* anticancer activity of sophorolipids to human cervical cancer. *Applied Biochemistry and Biotechnology*, 181(4), 1372-1387.

Li, T., Yang, H. M., Cui, S. X., Suzuki, I., Zhang, L. F., Li, L., & Huang, F. (2011). Proteomic study of the impact of Hik33 mutation in *Synechocystis* sp. PCC 6803 under normal and salt stress conditions. *Journal of Proteome Research*, 11(1), 502-514.

Lim, D. Y., Jeong, Y., Tyner, A. L., & Park, J. H. (2007). Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 292(1), G66-G75.

Lingwood, D., & Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *Science*, 327(5961), 46-50.

Liu, Z., Fei, X. W., Fang, Y. J., Shi, W. J., Zhang, Y. Q., & Mei, Y. A. (2008). PLC-dependent intracellular Ca^{2+} release was associated with C_6-ceramide-induced inhibition of Na^+ current in rat granule cells. *Journal of Neurochemistry*, 106(6), 2463-2475.

Lopez, P. H., & Schnaar, R. L. (2009). Gangliosides in cell recognition and membrane protein regulation. *Current Opinion in Structural Biology*, 19(5), 549-557.

Macher, B. A., & Sweeley, C. C. (1978). [24] Glycosphingolipids: Structure, biological source, and properties. *Methods in Enzymology*, 50(1), 236-251.

Mahyar-Roemer, M., Katsen, A., Mestres, P., & Roemer, K. (2001). Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. *International Journal of Cancer*, 94(5), 615-622.

Mannock, D. A., Harper, P. E., Gruner, S. M., & McElhaney, R. N. (2001). The physical properties of glycosyl diacylglycerols. Calorimetric, X-ray diffraction and Fourier transform spectroscopic studies of a homologous series of 1, 2-di-O-acyl-3-O-(β-d-galactopyranosyl)-sn-glycerols. *Chemistry and Physics of Lipids*, 111(2), 139-161.

Marley, A. R., & Nan, H. (2016). Epidemiology of colorectal cancer. *International Journal of Molecular Epidemiology and Genetics*, 7(3), 105.

Mattos, B. B., Romanos, M. T. V., Souza, L. M. D., Sassaki, G., & Barreto-Bergter, E. (2011). Glycolipids from macroalgae: potential biomolecules for marine biotechnology?. *Revista Brasileira de Farmacognosia*, 21(2), 244-247.

Mayer, A. M., Rodríguez, A. D., Berlinck, R. G., & Fusetani, N. (2011). Marine pharmacology in 2007–8: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 153(2), 191-222.

McNeil, M. R., & Brennan, P. J. (1991). Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. *Research in Microbiology*, 142(4), 451-463.

Mizushina, Y., Sugiyama, Y., Yoshida, H., Hanashima, S., Yamazaki, T., Kamisuki, S., & Yoshida, S. (2001). Galactosyldiacylglycerol, a mammalian DNA polymerase α-specific inhibitor from a sea alga, *Petalonia bingbamiae*. *Biological and Pharmaceutical Bulletin*, 24(9), 982-987.

Mo, R., Yang, M., Chen, Z., Cheng, Z., Yi, X., Li, C., & Ge, F. (2015). Acetylome analysis reveals the involvement of lysine acetylation in photosynthesis and carbon metabolism in the model cyanobacterium *Synechocystis* sp. PCC 6803. *Journal of Proteome Research*, 14(2), 1275-1286.

Monti, E., Bonten, E., D'Azzo, A., Bresciani, R., Venerando, B., Borsani, G., & Tettamanti, G. (2010). Sialidases in vertebrates: a family of enzymes tailored for several cell functions. *Advances in Carbohydrate Chemistry and Biochemistry*, 64(2), 403-479.

Morales-Serna, J. A., Boutureira, O., Serra, A., Matheu, M. I., Díaz, Y., & Castillón, S. (2010). Synthesis of hyper branched β-galceramide containing dendritic polymers that bind HIV-1 rgp120. *European Journal of Organic Chemistry*, 2010(14), 2657-2660.

Morimoto, T., Nagatsu, A., Murakami, N., Sakakibara, J., Tokuda, H., Nishino, H., & Iwashima, A. (1995). Anti-tumour-promoting glyceroglycolipids from the green alga, *Chlorella vulgaris*. *Phytochemistry*, 40(5), 1433-1437.

Morrison, W. J., Young, K., Offner, H., & Vandenbark, A. A. (1993). Ganglioside (GM1) distinguishes the effects of CD4 on signal transduction through the TCR/CD3 complex in human lymphocytes. *Cellular and Molecular Biology Research*, 39(2), 159-165.

Mueller, T. J., Welsh, E. A., Pakrasi, H. B., & Maranas, C. D. (2016). Identifying regulatory changes to facilitate nitrogen fixation in the nondiazotroph *Synechocystis* sp. PCC 6803. *ACS Synthetic Biology*, 5(3), 250-258.

Murakami, A., Nakamura, Y., Koshimizu, K., & Ohigashi, H. (1995). Glyceroglycolipids from *Citrus hystrix*, a traditional herb in Thailand, potently inhibit the tumor-promoting activity of 12-O-tetradecanoylphorbol 13-acetate in mouse skin. *Journal of Agricultural and Food Chemistry*, 43(10), 2779-2783.

Murata, N., & Wada, H. (1995). Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *Biochemical Journal*, 308(1), 1-10.

Nagendraprabhu, P., & Sudhandiran, G. (2011). Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFkB and COX-2. *Investigational New Drugs*, 29(2), 207-224.

Naumann, I., Darsow, K. H., Walter, C., Lange, H. A., & Buchholz, R. (2007). Identification of sulfoglycolipids from the alga *Porphyridium purpureum* by matrix-assisted laser desorption/ionisation quadrupole ion trap time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 21(19), 3185-3192.

Ng, M. L., & Yap, A. T. (2002). Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes*). *The Journal of Alternative & Complementary Medicine*, 8(5), 581-589.

Ngwoke, K. G., Orame, N., Liu, S., Okoye, F. B., Daletos, G., & Proksch, P. (2017). A new benzophenone glycoside from the leaves of *Mitracarpus villosus*. *Natural Product Research*, 31(20), 2354-2360.

Nishihara, M., Yokota, K., & Kito, M. (1980). Lipid molecular species composition of thylakoid membranes. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 617(1), 12-19.

Nobili, S., Lippi, D., Witort, E., Donnini, M., Bausi, L., Mini, E., & Capaccioli, S. (2009). Natural compounds for cancer treatment and prevention. *Pharmacological Research*, 59(6), 365-378.

Ohta, K., Mizushima, Y., Hirata, N., Takemura, M., Sugawara, F., Matsukage, A., & Sakaguchi, K. (1998). Sulfoquinovosyldiacylglycerol, KM043, a new potent inhibitor of eukaryotic DNA polymerases and HIV-reverse transcriptase type 1 from a marine red alga, *Gigartina tenella*. *Chemical and Pharmaceutical Bulletin*, 46(4), 684-686.

Pandey, M., Verma, R. K., & Saraf, S. A. (2010). Nutraceuticals: new era of medicine and health. *Asian Journal of Pharmaceutical and Clinical Research*, 3(1), 11-15.

Papapolychroniadis, C. (2004). Environmental and other risk factors for colorectal carcinogenesis. *Techniques in Coloproctology*, 8(3), 7-9.

Papp, I., Sieben, C., Sisson, A. L., Kostka, J., Böttcher, C., Ludwig, K., & Haag, R. (2011). Inhibition of influenza virus activity by multivalent glyco-architectures with matched sizes. *ChemBioChem*, 12(6), 887-895.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *Cancer Journal for Clinicians*, 55(2), 74-108.

Pashkow, F. J., Watumull, D. G., & Campbell, C. L. (2008). Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *The American Journal of Cardiology*, 101(10), 58-68.

Paterna, A., Gomes, S. E., Borralho, P. M., Mulhovo, S., Rodrigues, C. M., & Ferreira, M. J. U. (2016). Vobasinyl–iboga alkaloids from *Tabernaemontana elegans*: cell cycle arrest and apoptosis-inducing activity in HCT116 colon cancer cells. *Journal of Natural Products*, 79(10), 2624-2634.

Peddie, V., Takada, K., Okuda, S., Ise, Y., Morii, Y., Yamawaki, N., & Matsunaga, S. (2015). Cytotoxic glycosylated fatty acid amides from a *Stelletta* sp. Marine Sponge. *Journal of Natural Products*, 78(11), 2808-2813.

Plouguerné, E., de Souza, L. M., Sassaki, G. L., Cavalcanti, J. F., Villela Romanos, M. T., da Gama, B. A., & Barreto-Bergter, E. (2013). Antiviral Sulfoquinovosyldiacylglycerols (SQDGs) from the Brazilian brown seaweed *Sargassum vulgare*. *Marine Drugs*, 11(11), 4628-4640.

Plouguerné, E., Ioannou, E., Georgantea, P., Vagias, C., Roussis, V., Hellio, C., & Stiger-Pouvreau, V. (2010). Anti-microfouling activity of lipidic metabolites from the invasive brown alga *Sargassum muticum (Yendo) Fensholt*. *Marine Biotechnology*, 12(1), 52-61.

Poláková, M., Beláňová, M., Mikušová, K., Lattová, E., & Perreault, H. (2011). Synthesis of 1, 2, 3-triazolo-linked octyl (1→ 6)-α-D-oligomannosides and their evaluation in mycobacterial mannosyl transferase assay. *Bioconjugate Chemistry*, 22(2), 289-298.

Pöschl, G., & Seitz, H. K. (2004). Alcohol and cancer. *Alcohol and Alcoholism*, 39(3), 155-165.

Prabhu, P. N., Ashokkumar, P., & Sudhandiran, G. (2009). Antioxidative and antiproliferative effects of astaxanthin during the initiation stages of 1, 2-dimethyl hydrazine-induced experimental colon carcinogenesis. *Fundamental & Clinical Pharmacology*, 23(2), 225-234.

Rajamanickam, S., & Agarwal, R. (2008). Natural products and colon cancer: current status and future prospects. *Drug Development Research*, 69(7), 460-471.

Raju, J., & Bird, R. P. (2007). Diosgenin, a naturally occurring furostanol saponin suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression and induces apoptosis in HCT-116 human colon carcinoma cells. *Cancer Letters*, 255(2), 194-204.

Reis, C. A., Osorio, H., Silva, L., Gomes, C., & David, L. (2010). Alterations in glycosylation as biomarkers for cancer detection. *Journal of Clinical Pathology*, 63(4), 322-329.

Reshef, V., Mizrachi, E., Maretzki, T., Silberstein, C., Loya, S., Hizi, A., & Carmeli, S. (1997). New acylated sulfoglycolipids and digalactolipids and related known glycolipids from cyanobacteria with a potential to inhibit the reverse transcriptase of HIV-1. *Journal of Natural Products*, 60(12), 1251-1260.

Robert, K. Y., Nakatani, Y., & Yanagisawa, M. (2009). The role of glycosphingolipid metabolism in the developing brain. *Journal of Lipid Research*, 50(Supplement), 440-445.

Rowley, P. T. (2005). Inherited susceptibility to colorectal cancer. *Annual Review of Medicine*, 56(2), 539-554.

Santarelli, R. L., Pierre, F., & Corpet, D. E. (2008). Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. *Nutrition and Cancer*, 60(2), 131-144.

Saravanakumari, P., & Mani, K. (2010). Structural characterization of a novel xylolipid biosurfactant from *Lactococcus lactis* and analysis of antibacterial activity against multi-drug resistant pathogens. *Bioresource Technology*, 101(22), 8851-8854.

Sato, N. (1982). Lipid biosynthesis in the blue green alga, *Anabaena variabilis*. II. Fatty acids and lipid molecular species. *Biochimica et Biophysica Acta*, 710(1), 279-289.

Savakis, P., & Hellingwerf, K. J. (2015). Engineering cyanobacteria for direct biofuel production from CO_2. *Current Opinion in Biotechnology*, 33(1), 8-14.

Schnaar, R. L., Gerardy-Schahn, R., & Hildebrandt, H. (2014). Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiological Reviews*, 94(2), 461-518.

Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., & Heber, D. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *Journal of Agricultural and Food Chemistry*, 54(25), 9329-9339.

Shan, B. E., Wang, M. X., & Li, R. Q. (2009). Quercetin inhibit human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through Wnt/β-catenin signaling pathway. *Cancer Investigation*, 27(6), 604-612.

Shao, L., Song, X., Ma, X., Li, H., & Qu, Y. (2012). Bioactivities of sophorolipid with different structures against human esophageal cancer cells. *Journal of Surgical Research*, 173(2), 286-291.

Shimamura, M. (2008). Non-reducing end α-mannosylated glycolipids as potent activators for invariant Vα19 TCR-bearing natural killer T cells. *Carbohydrate Research*, 343(12), 2010-2017.

Shimojima, M. (2011). Biosynthesis and functions of the plant sulfolipid. *Progress in Lipid Research*, 50(3), 234-239.

Shrikhande, S. V., Saoji, R. R., Barreto, S. G., Kakade, A. C., Waterford, S. D., Ahire, S. B., & Shukla, P. J. (2007). Outcomes of resection for rectal cancer in India: The impact of the double stapling technique. *World Journal of Surgical Oncology*, 5(1), 35.

Siegel, R. L., Miller, K. D., Fedewa, S. A., Ahnen, D. J., Meester, R. G., Barzi, A., & Jemal, A. (2017). Colorectal cancer statistics, 2017. *A Cancer Journal for Clinicians*, 67(3), 177-193.

Sinha, R. (2002). An epidemiologic approach to studying heterocyclic amines. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*, 506(1-2), 197-204.

Slattery, M. L., Benson, J., Curtin, K., Ma, K. N., Schaeffer, D., & Potter, J. D. (2000). Carotenoids and colon cancer. *The American Journal of Clinical Nutrition*, 71(2), 575-582.

Song, S. X., Zhang, H. L., Kim, C. G., Sheng, L., He, X. P., Long, Y. T., & Chen, G. R. (2010). Expeditious preparation of triazole-linked glycolipids via microwave accelerated click chemistry and their electrochemical and biological assessments. *Tetrahedron*, 66(52), 9974-9980.

Stanley, M., Cattle, N., McCauley, J., Martin, S. R., Rashid, A., Field, R. A., & Streicher, H. (2012). 'TamiGold': phospha-oseltamivir-stabilised gold nanoparticles as the basis for influenza therapeutics and diagnostics targeting the neuraminidase (instead of the hemagglutinin). *MedChemComm*, 3(11), 1373-1376.

Sudo, T., Zhao, X., Wakamatsu, Y., Shibahara, M., Nomura, N., Nakahara, T., & Yokoyama, K. K. (2000). Induction of the differentiation of human HL-60 promyelocytic leukemia cell line by succinoyl trehalose lipids. *Cytotechnology*, 33(1-3), 259-264.

Suh, Y., Afaq, F., Johnson, J. J., & Mukhtar, H. (2008). A plant flavonoid fisetin induces apoptosis in colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-κB-signaling pathways. *Carcinogenesis*, 30(2), 300-307.

Sunassee, S. N., Ransom, T., Henrich, C. J., Beutler, J. A., Covell, D. G., McMahon, J. B., & Gustafson, K. R. (2014). Steroidal alkaloids from the marine sponge *Corticium niger* that inhibit growth of human colon carcinoma cells. *Journal of Natural Products*, 77(11), 2475-2480.

Surovtseva, Y. V., Jairam, V., Salem, A. F., Sundaram, R. K., Bindra, R. S., & Herzon, S. B. (2016). Characterization of cardiac glycoside natural products as potent inhibitors of DNA double-strand break repair by a whole-cell double immunofluorescence assay. *Journal of the American Chemical Society*, 138(11), 3844-3855.

Szeja, W., Grynkiewicz, G., & Rusin, A. (2017). Isoflavones, their glycosides and glycoconjugates, synthesis and biological activity. *Current Organic Chemistry*, 21(3), 218-235.

Tangpricha, V., Flanagan, J. N., Whitlatch, L. W., Tseng, C. C., Chen, T. C., Holt, P. R., & Holick, M. F. (2001). 25-hydroxyvitamin D-1α-hydroxylase in normal and malignant colon tissue. *The Lancet*, 357(1), 1673-1674.

Thanomsub, B., Pumeechockchai, W., Limtrakul, A., Arunrattiyakorn, P., Petchleelaha, W., Nitoda, T., & Kanzaki, H. (2007). Chemical structures and biological activities of rhamnolipids produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste. *Bioresource Technology*, 98(5), 1149-1153.

Tsai, C. J., & Pan, S. B. (2012). Identification of sulfoglycolipid bioactivities and characteristic fatty acids of marine macroalgae. *Journal of Agricultural and Food Chemistry*, 60(34), 8404-8410.

Tsong, W. H., Koh, W. P., Yuan, J. M., Wang, R., Sun, C. L., & Yu, M. C. (2007). Cigarettes and alcohol in relation to colorectal cancer: the Singapore Chinese Health Study. *British Journal of Cancer*, 96(5), 821-830.

Tziveleka, L. A., Vagias, C., & Roussis, V. (2003). Natural products with anti-HIV activity from marine organisms. *Current Topics in Medicinal Chemistry*, 3(13), 1512-1535.

Venkataraman, K., & Futerman, A. H. (2000). Ceramide as a second messenger: sticky solutions to sticky problems. *Trends in Cell Biology*, 10(10), 408-412.

von Minden, H. M., Morr, M., Milkereit, G., Heinz, E., & Vill, V. (2002a). Synthesis and mesogenic properties of glycosyl diacylglycerols. *Chemistry and Physics of Lipids*, 114(1), 55-80.

von Minden, H. M., Milkereit, G., & Vill, V. (2002b). Effects of carbohydrate headgroups on the stability of induced cubic phases in binary mixtures of glycolipids. *Chemistry and Physics of Lipids*, 120(1), 45-56.

Wada, H., & Murata, N. (1989). *Synechocystis* PCC6803 mutants defective in desaturation of fatty acids. *Plant and Cell Physiology*, 30(7), 971-978.

Wada, H., & Murata, N. (1998). Membrane lipids in cyanobacteria. *Lipids in Photosynthesis: Structure, Function and Genetics, edited by Paul-André S, Norio M.* Netherlands: Springer, 65-81.

Wang, G., Spassieva, S. D., & Bieberich, E. (2017). Ceramide and S1P signaling in embryonic stem cell differentiation. *Methods in Molecular Biology, edited by Turksen K. New Jersey: Humana Press,* 1-19.

Wang, H., Li, Y. L., Shen, W. Z., Rui, W., Ma, X. J., & Cen, Y. Z. (2007). Antiviral activity of a sulfoquinovosyldiacylglycerol (SQDG) compound isolated from the green alga *Caulerpa racemosa*. *Botanica Marina*, 50(3), 185-190.

Wang, X., Wang, Q., Ives, K. L., & Evers, B. M. (2006). Curcumin inhibits neurotensin-mediated interleukin-8 production and migration of HCT116 human colon cancer cells. *Clinical Cancer Research*, 12(18), 5346-5355.

Wargovich, M. J., Morris, J., Brown, V., Ellis, J., Logothetis, B., & Weber, R. (2010). Nutraceutical use in late-stage cancer. *Cancer and Metastasis Reviews*, 29(3), 503-510.

White, D. A., Hird, L. C., & Ali, S. T. (2013). Production and characterization of a trehalolipid biosurfactant produced by the novel marine bacterium *Rhodococcus* sp., strain PML026. *Journal of Applied Microbiology*, 115(3), 744-755.

Wiegandt, H. (1992). Insect glycolipids. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1123(2), 117-126.

Willett, W. C. (2005). Diet and cancer: an evolving picture. *Jama*, 293(2), 233-234.

Williams, D. E., Sturgeon, C. M., Roberge, M., & Andersen, R. J. (2007). Nigricanosides A and B, antimitotic glycolipids isolated from the green alga *Avrainvillea nigricans* collected in Dominica. *Journal of the American Chemical Society*, 129(18), 5822-5823.

Williams, W. P. (1998). The physical properties of thylakoid membrane lipids and their relation to photosynthesis. *Lipids in Photosynthesis: Structure, function and Genetics, edited by Paul-André S, Norio M. Netherlands: Springer,* 103-118.

Wu, D., Xing, G. W., Poles, M. A., Horowitz, A., Kinjo, Y., Sullivan, B., & Ho, D. D. (2005). Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proceedings of the National Academy of Sciences of the United States of America*, 102(5), 1351-1356.

Wu, W., Hasumi, K., Peng, H., Hu, X., Wang, X., & Bao, B. (2009). Fibrinolytic compounds isolated from a brown alga, *Sargassum fulvellum*. *Marine Drugs*, 7(2), 85-94.

Yang, G., Zheng, W., Xiang, Y. B., Gao, J., Li, H. L., Zhang, X., & Shu, X. O. (2011). Green tea consumption and colorectal cancer risk: a report from the Shanghai Men's Health Study. *Carcinogenesis*, 32(11), 1684-1688.

Yang, H. J., Jung, K. Y., Kwak, D. H., Lee, S. H., Ryu, J. S., Kim, J. S., & Choo, Y. K. (2011). Inhibition of ganglioside GD1a synthesis suppresses the differentiation of human mesenchymal stem cells into osteoblasts. *Development, Growth & Differentiation*, 53(3), 323-332.

Yang, Y., Kim, B., & Lee, J. Y. (2013). Astaxanthin structure, metabolism, and health benefits. *Journal of Human Nutrition & Food Science*, 1(3), 1-1003.

Yoshida, T., Maoka, T., Das, S. K., Kanazawa, K., Horinaka, M., Wakada, M., & Sakai, T. (2007). Halocynthiaxanthin and peridinin sensitize colon cancer cell lines to tumor necrosis factor–related apoptosis-inducing ligand. *Molecular Cancer Research*, 5(6), 615-625.

Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y. J., & Zhang, F. (2013). Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory. *Marine Drugs*, 11(8), 2894-2916.

Zaragoza, A., Aranda, F. J., Espuny, M. J., Teruel, J. A., Marqués, A., Manresa, A., & Ortiz, A. (2010). Hemolytic activity of a bacterial trehalose lipid biosurfactant produced by Rhodococcus sp.: evidence for a colloid-osmotic mechanism. *Langmuir*, 26(11), 8567-8572.

Zhang, H. L., He, X. P., Sheng, L., Yao, Y., Zhang, W., Shi, X. X., & Chen, G. R. (2011). Synthesis of novel 6-triazologlycolipids via click chemistry and their preliminary cytotoxicity assessments. *Molecular Diversity*, 15(4), 889-900.

Zhang, X., & Kiechle, F. L. (2004). Glycosphingolipids in health and disease. *Annals of Clinical & Laboratory Science*, 34(1), 3-13.

Zisman, A. L., Nickolov, A., Brand, R. E., Gorchow, A., & Roy, H. K. (2006). Associations between the age at diagnosis and location of colorectal cancer and the use of alcohol and tobacco: implications for screening. *Archives of Internal Medicine*, 166(6), 629-634.

Chapter 2

CULTURING OF *Synechocystis* sp. AND ISOLATION OF GLYCOLIPIDS

Contents

2.1 Introduction

Cyanobacteria, also known as blue green algae embody a large group within the prokaryotic kingdom. They are recognized for their ability to perform oxygenic photosynthesis and biological nitrogen fixation. Cyanobacteria can adapt to extreme environmental conditions and are distinguished by showing typical anabiosis and rapid restoration of activity under favourable conditions (Pankratova et al., 1987). They are believed to be the oldest photosynthetic organisms on earth, and photosynthetic organelle in eukaryotes is thought to have originated by the process of endosymbiosis between a phagotrophic host and a cyanobacterium (Löffelhardt & Bohnert, 2004; Lau et al., 2015). Cyanobacteria are morphologically diverse and widely distributed throughout the world. They exist in different forms including unicellular,

filamentous, planktonic or benthic, and colonial ones (Burja et al., 2001; Whitton & Potts, 2012). They can thrive in a wide range of ecological habitats, extending from marine, freshwater to terrestrial environments. Because of their simple growth needs, cyanobacteria are favourite model organisms for deeper understanding of several metabolic processes and for the production of recombinant compounds of medicinal and commercial value (Gupta et al., 2013).

According to the botanical system of classification, cyanobacterial diversity has been traditionally grouped into five orders, *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales* and *Stigonematales*, which generally correspond to the five subsections proposed in the Bergey's Manual of Systematic Bacteriology (Knoll, 2008; Whitton, 2008). The variety of phenotypes of cyanobacteria is accompanied by morphological plasticity changing accordingly to different environmental or culture conditions. This can result in misidentifications when using morphological analysis alone (Lyra et al., 2001). Thus, the inclusion of other characteristics, such as genetic information, is an important complement for the accurate identification and classification of cyanobacteria. The analysis of genes encoding the small subunit ribosomal RNA, the 16S rRNA, is currently the most used approach for phylogenetic classification of cyanobacteria, because their sequences are independent from culture or growth conditions. Furthermore, the universality and conservation of this gene makes it suitable for broad phylogenetic studies (Nübel et al., 1997). Nevertheless, phylogenetic studies have revealed contradictory results with the morphological classification of several cyanobacterial taxa. That is the case of

Oscillatoria and *Microcoleus* that are described as morphologically different genera, but 16S rRNA analyses grouped them within the same genera (Wilmotte et al., 1992). Primary metabolite patterns addressing cellular fatty acid composition (Gugger et al., 2002), have also been used, together with the traditional morphological taxonomy (Thacker & Paul, 2004) for the classification of cyanobacteria. Chain length and number and position of double bonds of fatty acids are genetically determined. It is reasonable to suggest that a taxon has a specific maximal length of the fatty acid chain and maximal number of double bonds. Caudales & Wells (1992) showed that the filamentous heterocystous *Nostoc* and *Anabaena* genera could be distinguished on the basis of their cellular fatty acids. Fatty acid compositions of *Chroococcidiopsis* and the other baeocytes-forming strains of the order *Pleurocapsales* showed much difference which is in agreement with the 16S rRNA analysis. The overwhelming available knowledge on the diversity and physiology of cyanobacteria serves as an excellent base for exploring their applications in biotechnology.

Cyanobacteria are known as potent sources of bioactive compounds, biofertilizers, bioplastics, energy, food and have currently been used in drug discovery, medical diagnostics and bioremediation (Abed et al., 2009; Rishi & Awasthi, 2015; Senhorinho et al., 2015; Kumar et al., 2017). In recent years, cyanobacteria have gained much attention as a promising source of bioactive compounds with diverse biological activities including antiviral, antibacterial, antifungal, antimalarial, antitumoral and anti-inflammatory properties (Gerwick et al., 1994a; Patterson et al., 1994; Kajiyama et al., 1998; Jaki et al., 2000a, Jaki

et al., 2000b), having therapeutic, industrial and agricultural significance. Major isolated compounds from cyanobacteria belong to groups of polyketides, glycolipids, amides, alkaloids, fatty acids, indoles and lipopeptides. Some compounds isolated from cyanobacteria having significant biological activities are listed in Table 2.1. Few studies on glycolipids from cyanobacteria reported them as having specific biological activities. Some of the compounds are showing interesting results and have successfully reached to phase II and phase III of clinical trials. These compounds also serve as lead compounds for the development of synthetic analogues with improved bioactivity. Glycolipids from cyanobacteria *Scytonema julianum* are found to have ability to inhibit platelet-activating factor (PAF) (Antonopoulou et al., 2005). Cyanobacteria have a glycolipid composition very similar to that of the chloroplasts of leaves (Awai et al., 2006; Hölzl & Dörmann, 2007). Monogalactosyl diglycerides, digalactosyl diglycerides and sulfoquinovosyl diglycerides contribute majority of glycolipids in cyanobacteria (Bishop et al., 1986; Kim et al., 1999; Marcolongo et al., 2006). In addition, several unconventional glycolipis have been isolated from cyanobacteria. A type of chlorinated aromatic glycolipids known as bartolosides possessing xylose or rhamnose was isolated recently from cyanobacteria, *Synechocystis salina* and *Nodosilinea* sp. (Leão et al., 2015).

Glycolipids from cyanobacteria are thus compounds with high biotechnological potential for food and health applications. Despite of the interesting bioactive properties of glycolipids, their structure and diversity are far from being entirely recognized and therefore identification

of particular glycolipid signature of distinct cyanobacteria lineage is gaining added significance. Hence, suitable methods have to be employed for isolation of individual glycolipids. In general, lipidomics applications require sample preparation methodology that is fast, reproducible and able to extract a wide range of analytes with different polarities compatible with the instrumental technique (da Costa et al., 2016). Several factors need to be taken into account while extracting the compounds of interest from non-soluble matrix in which they are embedded. These include the polarity and stability of the extractives and the toxicity, volatility, viscosity and purity of the extraction solvent, the probability of artefact formation during the extraction process, and the amount of bulk material to be extracted (Bucar et al., 2013). Majority of isolation procedures still utilize simple extraction methods with organic solvents of different polarity, water and their mixtures (Ghisalberti, 1993; Sticher, 2008; Seidel, 2012). Grinding of the raw material and breaking tissue to lose cell integrity is important to increase the extraction yield of metabolites. The methods include maceration, percolation, soxhlet extraction, ultrasound-assisted extraction and turbo-extraction. All the reported applications have shown that ultrasound-assisted extraction is a green and economically viable alternative to conventional techniques for food and natural products (Boonkird et al., 2008; Adjé et al., 2010). Classical techniques such as maceration or thermal extraction may require long processing time and may be less efficient which sometimes cause thermal degradation of the compound. Ultrasonic extraction is important among modern extraction techniques with improved yield and quality of extracted products.

A wide range of liquid chromatographic methods with solid stationary phases either as planar or column chromatography is available for fractionation and final purification of natural products. The choice largely depends on the level of purity of the extract or fraction, and purpose of the isolated natural products. Column chromatography is a popular method for fractionation of crude extracts due to its ease of use and high sample capacity. Eluted fractions are usually analysed by thin layer chromatography (TLC) for their composition. Due to its simplicity in use and relatively low costs for isolation of natural products, TLC is still a frequently used technique although the number of applications is lower than those of column chromatography. An attractive feature of TLC is the wide range of chemical detection methods characteristic for compound classes which can be carried out on a narrow section of the plate leaving most of the compound unchanged and available for isolation (Marston, 2011; Zheng et al., 2011). However, in many cases medium-pressure liquid chromatography (MPLC) or semipreparative and preparative HPLC with higher peak resolution power need to be applied for final purification. Nowadays hyphenated techniques like LC-MS are used to identify a large number of molecular species from the polar lipid classes in the total lipid extract (He et al., 2011; Yao et al., 2015). Hence, combinations of several chromatographic and spectroscopic methods are required for isolation and purification of a natural product.

This chapter mainly focuses on the molecular level and chemotaxonomic identification of *Synechocystis* sp. along with various methods employed for isolation and purification of major glycolipids

Table 2.1 Bioacive molecules produced by various cyanobacteria

Bioactive molecules	**Cyanobacteria**	**Bioactivity**	**References**
Borophycin	*Nostoc linckia, Nostoc spongiaeforme*	Anticancer	Hemscheidt et al. (1994)
Curacin A	*Lyngbya majuscule*	Anticancer	Gerwick et al. (1994b)
Diacylglycerols; C-phycocyanin	*Aphanizomenon flos-aquae*	Anticancer	Tokuda et al. (1996)
Cryptophycins	*Nostoc* sp.	Anticancer	Subbaraju et al. (1997)
Calothrixins A, B	*Calothrix* sp.	Anticancer	Rickards et al. (1999)
Lyngbyabellin B	*Lyngbya majuscule*	Anticancer	Luesch et al. (2000)
Pitipeptolides A, B	*Lyngbya majuscule*	Anticancer	Luesch et al. (2001a)
Symplostatin	*Symploca* sp.	Anticancer	Luesch et al. (2001b)
Apratoxins	*Lyngbya majuscula*, *L. sordid*, *L. bouilloni*	Anticancer	Luesch et al. (2002), Gutiérrez et al. (2008a), Tidgewell et al.(2010)
Malevamide	*Symploca hydnoides*	Anticancer	Horgen et al. (2002)
Obyanamide	*Lyngbya confervoides*	Anticancer	Williams et al. (2002)
Homodolastatin 16	*Lyngbya majuscule*	Anticancer	Davies-Coleman et al. (2003)

Table 2.1 Continued…

Tasiamide	*Symploca* sp.	Anticancer	Williams et al. (2003a)
Tasipeptins	*Symploca* sp.	Anticancer	Williams et al. (2003b)
Ulongapeptin	*Lyngbya* sp.	Anticancer	Williams et al. (2003c)
Jamaicamides	*Lyngbya majuscule*	Anticancer	Edwards et al. (2004)
Kalkitoxin	*Lyngbya majuscule*	Anticancer	White et al. (2004)
Ankaraholide A	*Geitlerinema* sp.	Anticancer	Andrianasolo et al. (2005)
Nostoflan	*Nostoc flagelliforme*	Antiviral	Kanekiyo et al. (2005)
Palauamide	*Lyngbya* sp.	Anticancer	Zou et al. (2005)
Wewakpeptins	*Lyngbya semiplena*	Anticancer	Han et al. (2005)
Aurilide	*Lyngbya majuscule*	Anticancer	Han et al. (2006)
Carmabin A, B	*Lyngbya majuscule*	Antimalarial	McPhail et al. (2007)
Symplocamide A	*Symploca* sp.	Anticancer	Linington et al. (2007)
Coibamide	*Leptolyngbya* sp.	Anticancer	Medina et al. (2008)
Dragonamide C, D	*Lyngbya polychroa*	Anticancer	Gunasekera et al. (2008)
Grassypeptolide	*Lyngbya majuscule*	Anti-proliferative	Kwan et al. (2008)
Largazole	*Symploca* sp.	Anticancer	Taori et al. (2008)
Majusculamide	*Lyngbya majuscule*	Anticancer	Pettit et al. (2008)

Table 2.1 Continued…

Symplocamide A	*Symploca* sp.	Anticancer	Linington et al. (2008)
Biselyngbyaside	*Lyngbya* sp.	Anticancer	Teruya et al. (2009a)
Bisebromoamide	*Lyngbya* sp.	Anticancer	Teruya et al. (2009b)
Palmyramide	*Lyngbya majuscule*	Anticancer	Taniguchi et al. (2009)
Aplysiatoxin	*Lyngbya majuscule*	Anticancer	Chlipala et al. (2010)
Caylobolide	*Phormidium* sp.	Anticancer	Salvador et al. (2010)
Hoiamide	*Association of Lyngbya majuscula and Phormidium gracile*	Anticancer	Choi et al. (2010)
Cocosamides B	*Lyngbya majuscule*	Anticancer	Gunasekera et al. (2011)
Isomalyngamide	*Lyngbya majuscule*	Anticancer	Chang et al. (2011)
Lagunamide	*Lyngbya majuscule*	Anticancer	Tripathi et al. (2011)
Malyngamide 3	*Lyngbya majuscule*	Anticancer	Gunasekera et al. (2011)
Veraguamides	*Symploca cf. hydnoides*	Anticancer	Salvador et al. (2011)
Sulfolipids	*Lyngbya lagerhimii, Phormidium tenue*	Anti-HIV	Gustafson et al. (1989)
Bauerines A–C	*Dichotrix baueriana*	Anti-HSV-2	Larsen et al. (1994)
Calcium spirulan	*Spirulina platensis*	Anti-HIV	Hayashi et al. (1996)

Table 2.1 Continued…

Sulfoglycolipid	*Scytonema* sp.	Anti-HIV	Loya et al. (1998)
Cyanovirin	*Nostoc ellipsosporum*	Anti-HIV	Dey et al. (2000)
Scytovirin	*Scytonema varium*	Anti-HIV	Xiong et al. (2006)
Ambiguines	*Fischerella* sp.	Antibacterial	Raveh & Carmeli (2007)
Ichthyopeptins	*Microcystis ichthyoblabe*	Antiviral	Zainuddin et al. (2007)
Didehydromirabazole	*Scytonema mirabile*	Antibacterial	Stewart et al. (1988)
Tjipanazoles	*Tolypothrix tjipanasensis*	Antifungal	Bonjouklian et al. (1991)
Tolyporphin	*Tolypothrix nodosa*	Antibacterial	Prinsep et al. (1992)
Muscoride	*Nostoc muscorum*	Antibacterial	Nagatsu et al. (1995)
Bis-(v-butyrolactones)	*Anabena variabilis*	Antibacterial	Ma & Led (2000)
Noscomin	*Nostoc commune*	Antibacterial	Jaki et al. (1999)
Dolastatins	*Lyngbya* sp., *Symploca* sp.	Antimalarial	Fennell et al. (2003)
Nostocine A	*Nostoc spongiaeforme*	Antibacterial	Hirata et al. (2003)
Ambiguine	*Fischerella ambigua*	Antibacterial	Raveh & Carmeli (2007)
Abietane diterpenes	*Microcoleous lacustris*	Antibacterial	Gutiérrez et al. (2008b)
Hapalindole	*Nostoc CCC537*, *Fischerella* sp.	Antibacterial	Asthana et al. (2009)

2.2 Materials and methods

2.2.1 Cyanobacteria, medium and culture conditions

The unicellular cyanobacteria used in the study were isolated from the water column of Cochin Estuary, southwest coast of India. The water sample (2 L) was filtered sequentially through 200 micron nylon mesh, 70 and 30 micron cell strainers (Genetix Biotech Asia, India) and 0.2 µ nitrocellulose filter. Subsequently, the particles collected on 0.2 micron nitrocellulose filter was inoculated into SN nutrient media (Stanier et al., 1971) and incubated at 25 ± 2 °C with a light: dark cycle of 12:12 h. The growth of cyanobacteria was regularly monitored under the 4x objective of an inverted microscope and purified by serial dilution. Hence, purified cyanobacterial strain was cultured in 2L of sterilized water of zero salinity enriched with SN media at the same conditions of inoculation. The cyanobacteria were harvested after 25 days in the stationary phase.

2.2.2 Molecular identification

The genomic DNA was isolated from cyanobacterial culture following a modified protocol of Cheng & Jiang (2006). Briefly, 10 mL cell suspension of cyanobacteria was centrifuged at 12000 rpm for 5 min, washed with 400 µL of STE buffer twice by centrifugation at 12000 rpm for 5 min. The cells were re-suspended in 200 µL of TE buffer, vortexed for 2 min with 20 mg glass beads and 100 µL of tris saturated phenol, centrifuged for 5 min at 12000 rpm and 4 °C to separate the aqueous phase from the organic phase. Upper aqueous phase (~ 160 µL) was transferred to 1.5 mL micro centrifuge tube and 40 µL TE buffer was

added to make 200 µL, and mixed with 100 µL chloroform. The mixture was centrifuged twice at 12000 rpm and 4 °C for 10 min and 160 µL of upper aqueous phase was transferred to a clear 1.5 mL microcentrifuge tube. Further, 40 µL TE buffer and 5 µL RNase (10 µg/mL) buffer were added. After incubation of the tubes for 10 min at 37 °C, 100 µL of chloroform was added and centrifuged for 5 min at 12000 rpm and 4 °C, and then transferred the aqueous phase (~ 150 µL) containing genomic DNA to a clean 1.5 mL tube. The integrity of DNA was checked by 1% agarose gel electrophoresis and the gel images were analyzed using a gel documentation system (Gel Doc™ XR+, Bio Rad, USA). PCR amplification of 16S rRNA gene was carried out using primer "16S rRNA gene universal forward primer (5'- AGA GTT TGA TCC TGG CTC AG-3')". The PCR products were confirmed on 1% agarose gel to determine size of the products using standard molecular weight marker (1kbp, New England Biolabs). PCR product was sequenced by Sanger sequencing on an ABI 3730xl DNA analyzer (SciGenom Labs Pvt. Ltd, India). The sequences were then analyzed by using the basic local alignment search tool (BLAST) of NCBI (National center for biotechnology information**)** (Altschul et al., 1990). The gene sequences were aligned using the Clustal X program. A phylogenetic tree was generated based on Neighbor-Joining method with Tamura 3-parameter model using the software Mega 6.0. A bootstrap analysis was performed in 1000 trial replications in order to provide confidence estimates for the topology of phylogenetic tree (Tamura et al., 2013).

2.2.3 Extraction of fatty acids

Fatty acid composition of dried algal biomass was determined and expressed as fatty acid methyl esters (FAME) (Bligh & Dyer, 1959; Harvey, 1994). Lyophilized samples were ultrasonicated with DCM: methanol (2:1) and the solvent was removed under reduced pressure. The lipid extract thus obtained was hydrolysed using 6% methanolic KOH to convert the fatty acids into their potassium salts. Neutral lipids were separated by sovent extraction. Polar fraction was acidified with 6 N HCl and the resulting free fatty acids were extracted with CH_2Cl_2.

2.2.4 Gas chromatographic–mass spectrometric analysis

Dried fatty acid samples were converted into fatty acid methyl esters (FAME) using BF_3/methanol and used for GC-MS analysis. GC-MS analysis was performed using gas chromaotography (Perikin Elmymer Clarus 680) coupled with mass spectrometer (Perikin Elmymer Clarus 600). 1 μL of sample was injected to GC equipped with non-polar HP ultra-double fused silica capillary column. Initially, the temperature was increased from 50 °C to 200 °C at a rate of 2 °C per min and held at 200 °C for 5 min. Then, the temperature was again increased from 200 °C to 280 °C at a rate of 10 °C per min and held at 280 °C for 10 min. MS operating parameters were as follows: ionization energy - 70 eV; ion source temperature - 200 °C, solvent delay - 4 min and scan range 40–600 u. Identification of the components was based on direct comparison of mass spectral data with NIST library version 2.1.

2.2.5 TLC separation of hydroxy fatty acids

The fatty acid extract showing traces of hydroxyl fatty acids, was fractionated using TLC silica gel plates 60 F254 (Merck) with hexane/ ethyl acetate (50:50 v/v). The polar fatty acid fraction thus obtained was subjected to GC-MS analysis at the same conditions of FAME analysis.

2.2.6 Mass culturing of cyanobacteria

Synechocystis sp. was mass cultured in 150 L sterilized water with sufficient supply of nutrients in optimum light:dark ratio (12:12 hrs). Continuous aeration was given and the temperature was maintained at 25 °C. The biomass was separated through refrigerated ultracentrifugation and freeze dried to obtain around 6.4 g dry weight. Figure 2.1 shows the mass culture of *Synechocystis* sp.

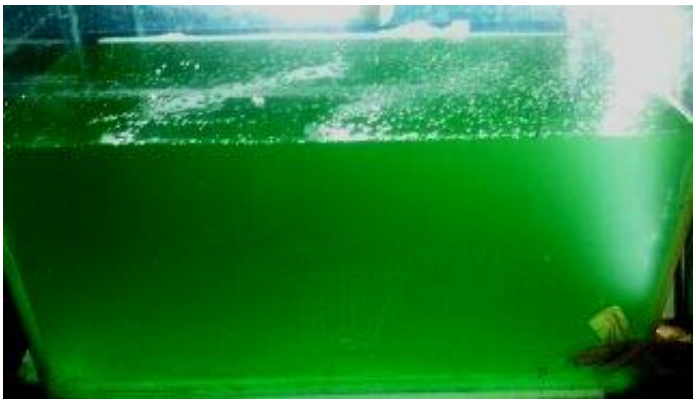


Figure 2.1: Mass culture of *Synechocystis* sp.

2.2.7 Solvent extraction of *Synechocystis* sp.

Lyophilized biomass was subjected to solvent extraction for isolating the metabolites. All the chemicals used for extraction were analytical

grade, supplied by Merck. A solvent system prepared using chloroform, methanol and water in the ratio 10:5:1 v/v/v was used for the total extraction. Algal powder, about 6 g dry weight, was treated with chloroform: methanol:water 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.

2.2.8 Separation and purification of glycolipids

The crude cyanobacterial extract was subjected to column chromatography, TLC, size exclusion chromatography and semipreparative HPLC purification for obtaining individual glycolipids.

(a) Column chromatography

Initial separation and purification of the lipid fraction consisting of glycolipids was performed by column chromatography using silica gel 60-120 mesh (0.015-0.040 mm Merck KGaA). Silica gel was activated by keeping it overnight in oven, at 120 °C. Activated silica was loaded homogeneously up to 50 cm in a borosil column with 3 cm internal diameter. Extract adsorbed on silica was loaded to this column. Solvent systems of increasing polarity from 90% EtOAc in hexanes to 100%

EtOAc to 100% MeOH, was applied to yield seven fractions (A-G). These subfractions were screened using TLC and LC-MS for further separation.

(b) Thin layer chromatography

Fine purification of major glycolipids was achieved using TLC silica gel 60 F_{254}, Merck Germany, 20 x 20 cm aluminium sheets. To this TLC plate, glycolipid fractions dissolved in HPLC grade methanol were loaded and developed using a chloroform/methanol solvent system. TLC chamber was equilibrated with solvent vapour before immersing the plate. A reference was collected from the developed plate and monitored using glycolipid specific stain (mixture of 20 mL 10% ethanolic diphenylamine solution, 100 mL 37% hydrochloric acid and 80 mL glacial acetic acid) (Jatzkewitz & Mehl, 1960). After application of stain, these reference plates were then heated at 100 °C for 5-10 min to obtain blue-grey spots. Comparing with the reference, glycolipids were purified and recovered.

(c) Purification by semipreparative HPLC

Glycolipids were further purified using preparative HPLC with C18 (octadecyl) reversed phase column (Supelco Sigma Aldrich) having 5 micron particle size 10 mm inner diameter. Refractive index detecor (RID) was used to identify peaks. Injector loop was having sample capacity of 2 mL. Samples dissolved in 1.0 mL pure acetonitrile (Merck HPLC grade) were loaded on to the column. The total run time was at 60 min using isocratic elution by mobile phase 85% acetonitrile in water (Merck HPLC grade). The peaks were isolated and tested for purity by LC-MS analysis.

(d) LC-MS analysis of glycolipid fraction

The fractions G-1 to G-4 separated were analysed using LC-MS (Shimadzu LC-MS 2020) in order to identify the mass and purity of isolates. Fractions having peaks with close retention time but different molecular weights were further separated using size-exclusion chromatography on a Sephadex LH-20 column eluting with DCM/MeOH (1:1, v/v). Six glycolipid fractions G-1 to G-6 were obtained and analysed using LC-MS.

(e) FT-IR analysis

FT-IR spectra of isolated glycolipids were recorded by FT-IR spectrophotometer (Perkin Elmer spectrum 100) in the range from 4000 to 650 cm^{-1}. The instrument was equipped with universal attenuated total reflectance accessory (ATR) which permits solid and liquid sample analysis with much spectral reproducibility.

2.3 Results and discussion

2.3.1 Phylogenic analysis of *Synechocystis* sp.

Microscopic image of the cyanobacterial strain is as shown in Figure 2.2. The typical round shape characteristic of *Synechocystis* sp. was observed. The BLAST search of sequenced data obtained from 16S rRNA analysis, on NCBI–nucleotide database showed 99% similarity to *Synechocystis* sp. (GenBank Accession No. MF444861). Cladding of the cyanobacterial strain is highlighted in the phylogenic tree (Figure 2.3). Taxonomic details of the strain are as follows; Kingdom: Bacteria; Phylum: Cyanobacteria; Order: *Chroococcales*; Genus: *Synechocystis*.

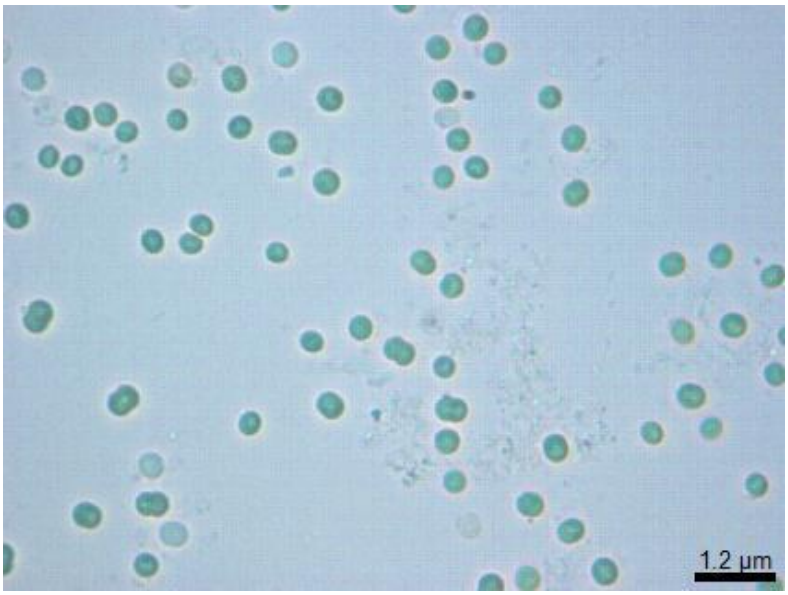


Figure 2.2 Microscopic image of *Synechocystis* sp.

2.3.2 Chemotaxonomy based on fatty acids

Fatty acid composition of *Synechocystis* sp. culture was obtained from the GC-MS total ion chromatogram of FAME (Figure 2.4). Relative abundance of different fatty acids listed in Table 2.2, depicts the presence of palmitic (C16:0) acid as the major fatty acid with a relative percentage of 36.14 ± 0.034. Oleic (18:1 n-9), stearic (18:0), palmitoleic (16:1 n-7) followed palmitic acid with a percentage abundance of 16.04 ± 0.016, 15.37 ± 0.010 and 13.09 ± 0.012 respectively. It showed higher content of saturated and monounsaturated fatty acids (SFA and MUFA) compared with polyunsaturated fatty acids (PUFA).

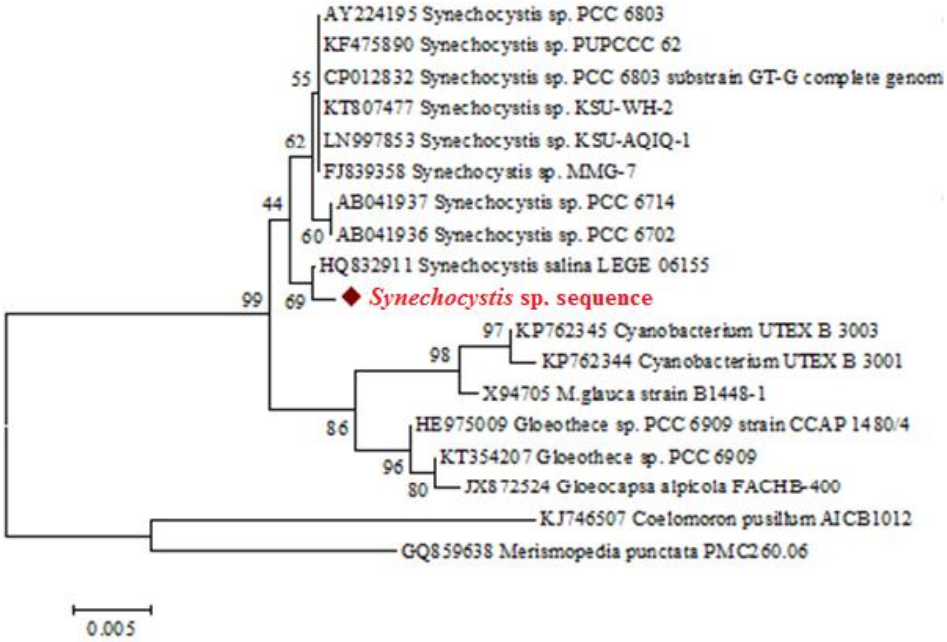


Figure 2.3 Phylogenetic tree of 16srRNA gene sequence of *Synechocystis* sp. (Gen Bank Accession No. MF444861) constructed based on Neighbor-Joining method with Tamura 3-parameter model using the software Mega 6.0.

Earlier reports on the fatty acid composition of cyanobacterial taxa also indicated higher palmitic acid abundance in *Lyngbya birgei* (59.6%), *Phormidium valderianum* (34%), *Synechocystis pevalekii* (34%) and *Phormidium tenue* (32%) (Burman et al., 2012). Cyanobacteria comprise higher saturated fatty acid (SFA) content which mainly contributed by C16:0 and 18:0 (~60%) (Patil et al., 2007). The fatty acid profile obtained in the present study also showed higher abundance of SFA (52.97 ± 0.057%). Higher amount of monounsaturated fatty acids (MUFAS) along with saturated fatty acids (SFAs) in cyanobacteria make it desirable for biodiesel production (Knothe, 2006; Meher et al., 2006). Poor oxidative

stability of polyunsaturated fatty acids (PUFA) is the most important challenge in biodiesel production from other microalgae classes compared with cyanobacteria (Stansell et al., 2012).

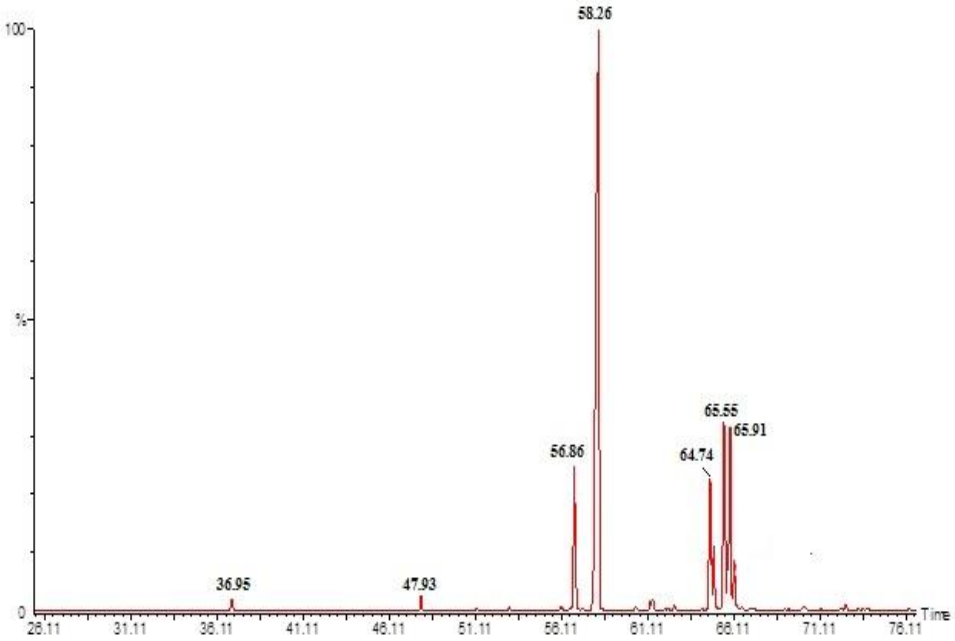


Figure 2.4 Total ion chromatogram of whole cell fatty acids from *Synechocystis* sp.

Synechocystis sp. showed a higher MUFA content of 29.81 ± 0.033% along with higher SFA and these observations emphasize on the occurrence of this species in cyanobacterial taxa and its potential application as biofuel. Fatty acid profile of *Synechocystis* sp. also showed presence of linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3) with a relative percentage of 11.25 ± 0.005 and 4.63 ± 0.003. Lipids of some filamentous cyanobacterial taxa are rich in essential fatty acids such as

C_{18} linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids and their C_{20} derivatives - eicosapentaenoic acids (20:5 n-3) and arachidonic acid (20:4 n-6) and are used as essential components of important feed additives in aquaculture (Borowitzka, 1988). But most of the recent studies on fatty acid profile of cyanobacteria suggest that maximum chain length of fatty acid in cyanobacteria is up to 18 carbon atoms (Li & Watanabe, 2001; Iliev et al., 2011) and sometimes with small quantities of C_{20} fatty acids (Singh et al., 2002). In the present study, there are very small quantities of 20:5 n-3 (0.27 ± 0.003%) and 20:4 n-6 (0.13 ± 0.002%). Very small amount of hydroxy fatty acids was also observed in the fatty acid fraction (0.47 ± 0.001%). In most of the studies on the fatty acid profile of cyanobacteria, the composition of hydroxy fatty acids was neglected because of their minor contents. Generation of hydroxy fatty acids can be attributed more to the bacterial characteristics of cyanobacteria than algal features. Hydroxy fatty acids are known to exhibit potent medicinal properties including anti-bacterial, anti-fungal, and anti-diabetic activities (Bajpai *et al*., 2009; Hou, 2009; Paul *et al*., 2010). They are widely used as surfactants, lubricants, cosmetics and pharmaceutical intermediates (Sandoval et al., 2005; Arias et al., 2008; Cao & Zhang, 2013). Thus, the polar hydroxyl fatty acid fraction was separated from the total fatty acid fraction using TLC.

Table 2.2 List of fatty acids in whole cell fatty acid fraction of *Synechocystis* sp.

SL No	Retention Time	Compound	Percentage abundance	Molecular Weight
1	36.95	Methyl dodecanoate (12:0)	0.65 ± 0.009	214
2	45.23	Methyl tetradec-7-enoate (14:1 n-7)	0.05 ± 0.001	240
3	47.93	Methyl tetradecanoate (14:0)	0.81 ± 0.004	242
4	56.86	(Z)-methyl hexadec-9-enoate (16:1 n-7)	13.09 ± 0.012	268
5	58.26	Methyl hexadecanoate (16:0)	36.14 ± 0.034	270
6	64.74	(9Z,12Z,15Z)-methyl octadecatrienoate (18:3 n-3)	4.63 ± 0.003	292
7	64.96	(9Z, 12Z)-methyl octadecadienoate (18:2 n-6)	11.25 ± 0.005	294
8	65.55	(Z)-methyl octadeca-9-enoate (18:1 n-9)	16.04 ± 0.016	296
9	65.61	(Z)-methyl octadeca-7-enoate (18:1 n-11)	0.63 ± 0.004	296
10	65.91	Methyl octadecanoate (18:0)	15.37 ± 0.010	298
11	72.34	(5Z,8Z,11Z,14Z)-methyl eicosatetraenoate (20:4 n-6)	0.13 ± 0.002	318
12	72.59	(5Z,8Z,11Z,14Z,17Z)-methyl eicosapentaenoate (20:5 n-3)	0.27 ± 0.003	316
13	73.88	Hydroxy fatty acids	0.47 ± 0.001	
		ΣSFA	52.97 ± 0.057	
		ΣMUFA	29.81 ± 0.033	
		ΣPUFA	16.71 ± 0.013	

The total ion chromatogram of the hydroxyl fatty acid fraction is as shown in Figure 2.5. Polar hydroxyl fatty acids were separated with nearly 73% abundance (Table 2.3). C14:0 3-OH (29.6 ± 0.25%), C16:0 3-OH (25.8 ± 0.33%) and C16:1 3-OH (11.6 ± 0.17%) were the major fatty acids found in the fraction. Base peak at m/z 103 in the mass fragmentation pattern of individual peaks in GC-MS chromatogram is indicating the presence of only 3-hydroxy fatty acids (Ryhage & Stenhagen, 1960) as shown in Figure 2.6. The presence of 2-hydroxy fatty acid was not detected and is in agreement with earlier findings. Certain cyanobacterial strains like *Anabaena* and *Nostoc* are differentiated by their 3-hydroxy and non-polar fatty acid content (Vargas *et al*., 1998). Eventhough there are small variations in the relative abundance, the fatty acid profile of *Synechocystis* sp. is in accordance with various cyanobacterial taxa and is in congruent with the morphologic and genetic data.

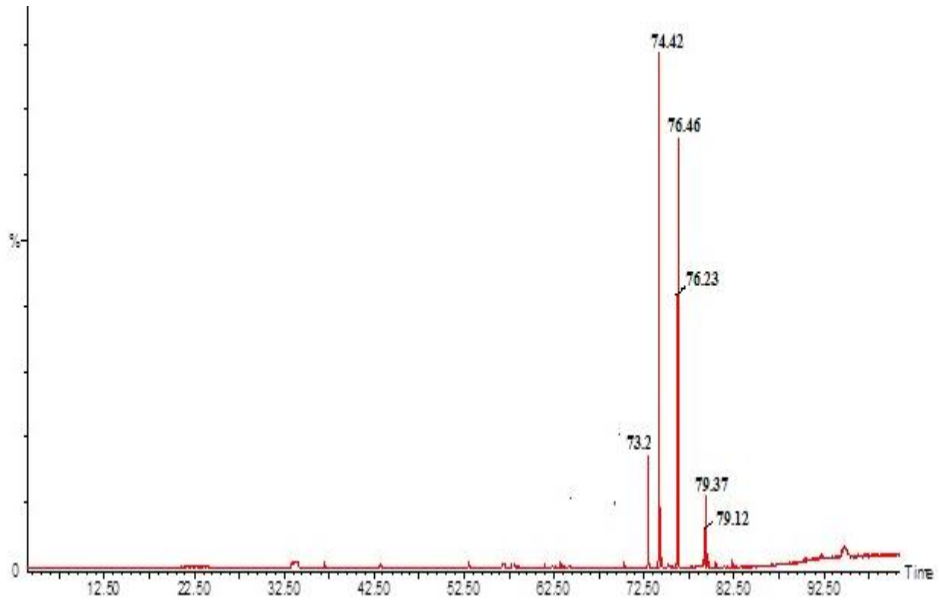


Figure 2.5 Total ion chromatogram of hydroxyl fatty acid fraction from *Synechocystis* sp.

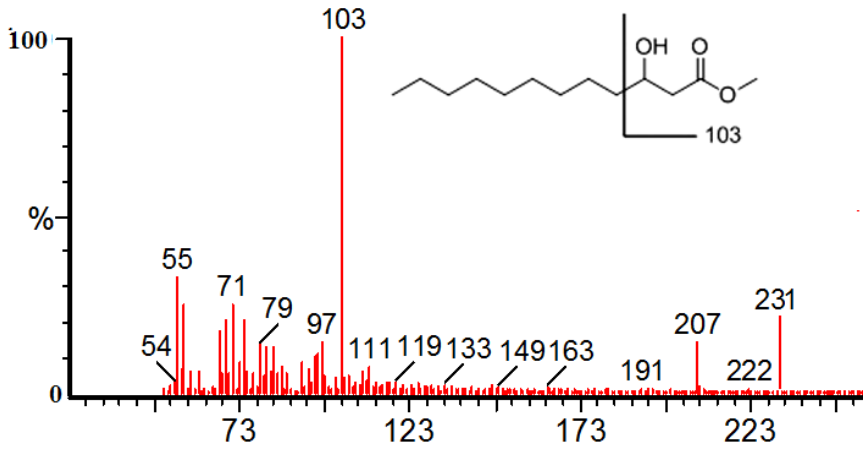
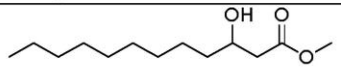
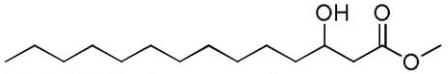
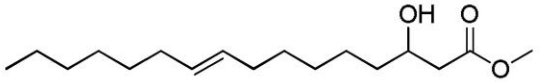
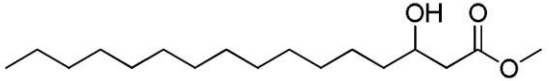
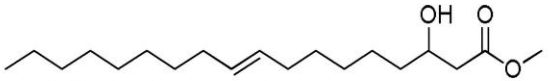
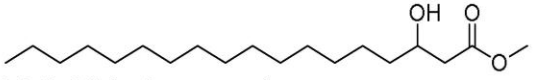


Figure 2.6 A representative mass fragmentation pattern of hydroxy fatty acid, methyl 3-hydroxydodecanoate, having base peak at *m/z* = 103., produced by a characteristic cleavage *alpha* to the carbon with the hydroxy group and defines its position.

Table 2.3 List of hydroxy fatty acids in the polar fraction separated from *Synechocystis* sp.

Sl No	Retention Time	Compound	Relative %	Molecular weight
1	73.21	 Methyl 3-hydroxy dodecanoate	2.84 ± 0.012	230
2	74.42	 Methyl 3-hydroxy tetradecanoate	29.6 ± 0.25	258
3	76.23	 Methyl 3-hydroxy hexadec-9-enoate	11.6 ± 0.17	284
4	76.46	 Methyl 3-hydroxy hexadecanoate	25.8 ± 0.33	286
5	79.12	 Methyl 3-hydroxy octadec-9-enoate	1.82 ± 0.007	312
6	79.37	 Methyl 3-hydroxy octadecanoate	1.62 ± 0.003	314

2.3.3 Analysis of purity of glycolipids through LC-MS analysis

The total lipid extract can be analyzed by LC-MS allowing identification of the glycolipids (Yan et al., 2010; Yan et al., 2011; Kim et al., 2013). However, the presence of other polar lipids may suppress some of the signals of glycolipid molecular species in the mass spectra. Therefore, several studies utilize a previous fractionation step of total lipid extracts in order to obtain isolated fractions (da Costa et al., 2016). TLC analyses of the seven fractions obtained after column chromatographic separation have shown that only three fractions viz., A, B, C have significant amount of glycolipids. LC-MS analysis of the fraction A eluted using ethyl acetate: methanol (4:1 v/v) indicated the presence of two prominent peaks at 759.54 and 780.39 along with several other mass peaks of low intensity (Figure 2.7). Further, TLC screening of this fraction using different combinations of chloroform:methanol:acetic acid solvent system resulted in observation of two spots. With the solvent system chloroform:methanol:acetic acid (75:15:8), it was possible to resolve the fraction into two well separated zones of R_F values 0.45 and 0.62, leaving other impurities in the origin. The R_F values of the two glycolipids varied at different times, but the separation of the two zones was always complete and the relative distance between the two spots was constant. The two compounds G-1 and G-2 thus obtained were further purified using semi preparative HPLC with methanol/ water solvent system to yield 2.34 and 8.55 mg of compounds respectively (Figure 2.8). It is observed from TLC that fraction A represents the least polar fractions among the glycolipid fractions. Monoglycosyl diglycerides are the commonly known

glycolipid with least polarity (Wood et al., 1965). There can be small variations in the R_F value depending on the side chains in the glyceride moiety. The two compounds G-1 and G-2 showed similar nature and movement as that of monogalactosyl diglyceride in TLC.

The fraction B eluted using ethyl acetate:methanol (4:2 v/v) was also subjected to LC-MS analysis. Two peaks of higher intensity were observed at m/z 836.21 and 918.76 (Figure 2.9). Further, TLC screening of this fraction using chloroform:methanol:acetic acid (65:25:8) solvent system resulted in observation of two spots but the R_F of those spots were very close such that separation may not be effective and cause reduction in the yield. Hence, size exclusion chromatography was also employed for separation of these two compounds. Further purification using semi preparative HPLC resulted in two glycolipids G-3 and G-4 of yield 1.67 and 2.51 mg respectively (Figure 2.8). These two compounds showed similar R_F range as that of diglycosyl diglycerides isolated from certain photosynthetic bacteria (Constantopoulos & Bloch 1967).

The fraction C eluted using ethyl acetate:methanol (3:2 v/v) was also analysed using LC-MS. Two intense peaks are observed at m/z 804.32 and 832.69 (Figure 2.10). This fraction showed the presence of two highly polar lipid fractions whose R_F in TLC does not match with that of conventional galactosyl glycolipids. Two spots were observed with the sovent combination chloroform:methanol:acetic acid (55:40:5) in TLC with slight R_F difference. Hence separation of these two compounds was achieved through semi preparative HPLC. Compound with m/z 804.32 (6.45 mg)

obtained in major quantity and other with m/z 832.69 (0.82 mg) in minor amounds (Figure 2.8).

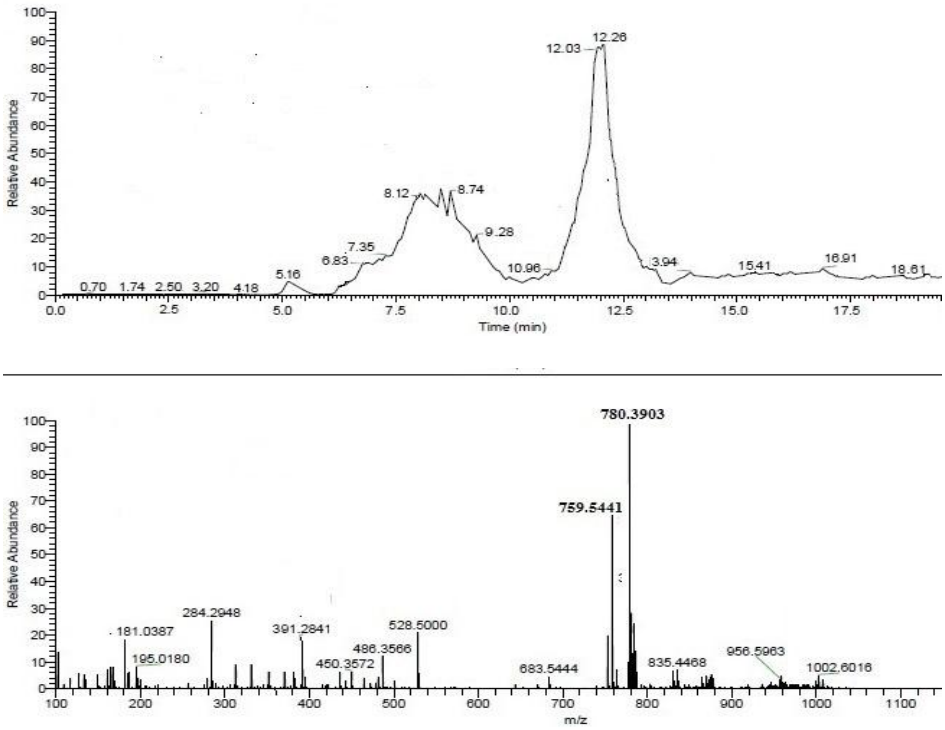


Figure 2.7 LC-MS chromatogram of fraction A

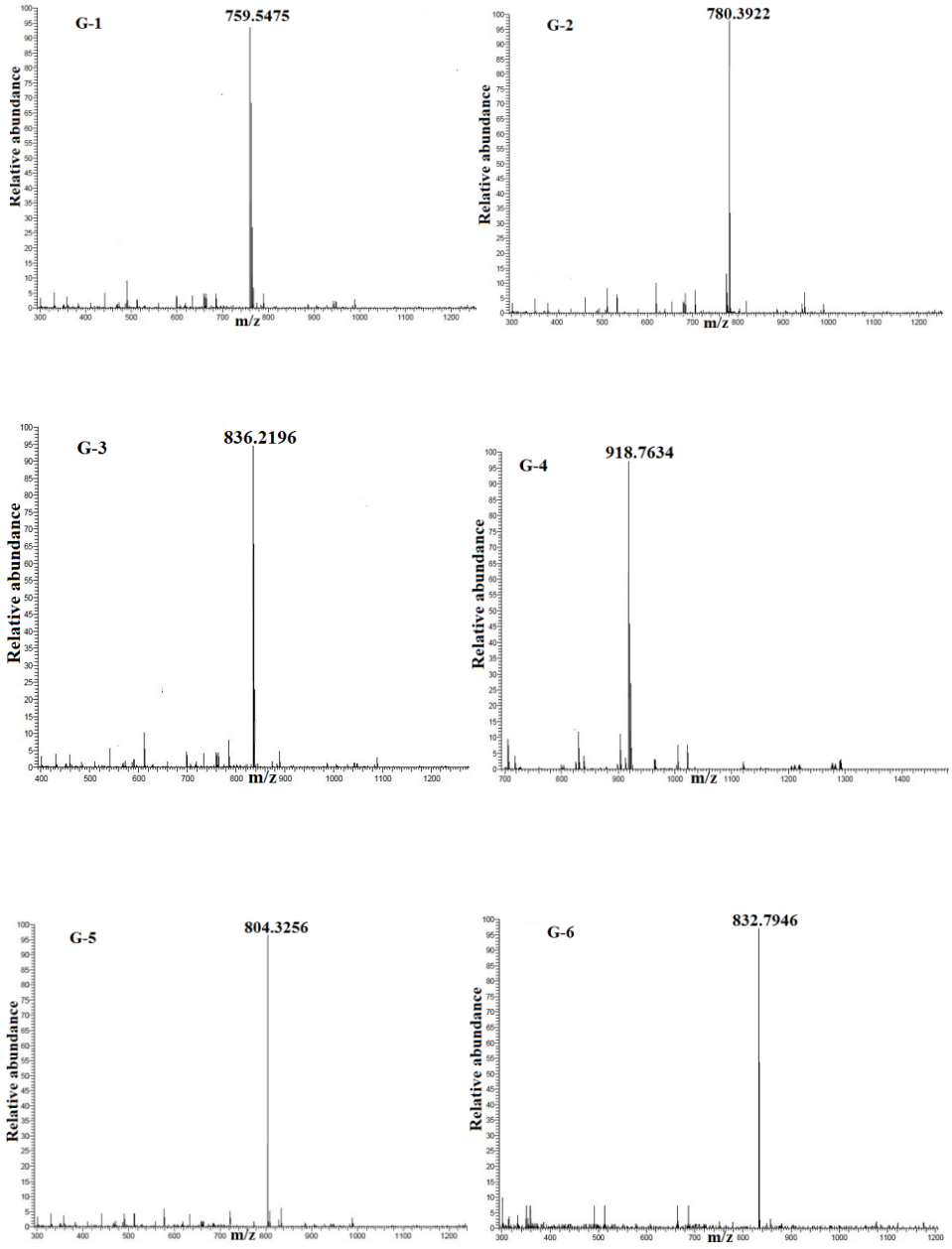


Figure 2.8 LC-MS analysis of isolated glycolipid fractions G-1 to G-6

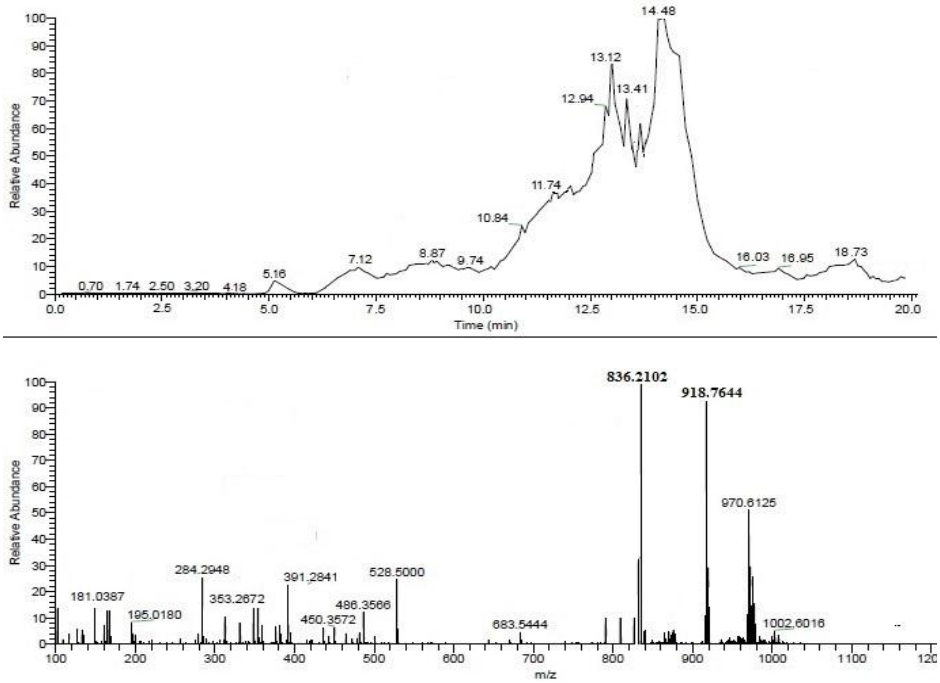


Figure 2.9 LC-MS chromatogram of fraction B

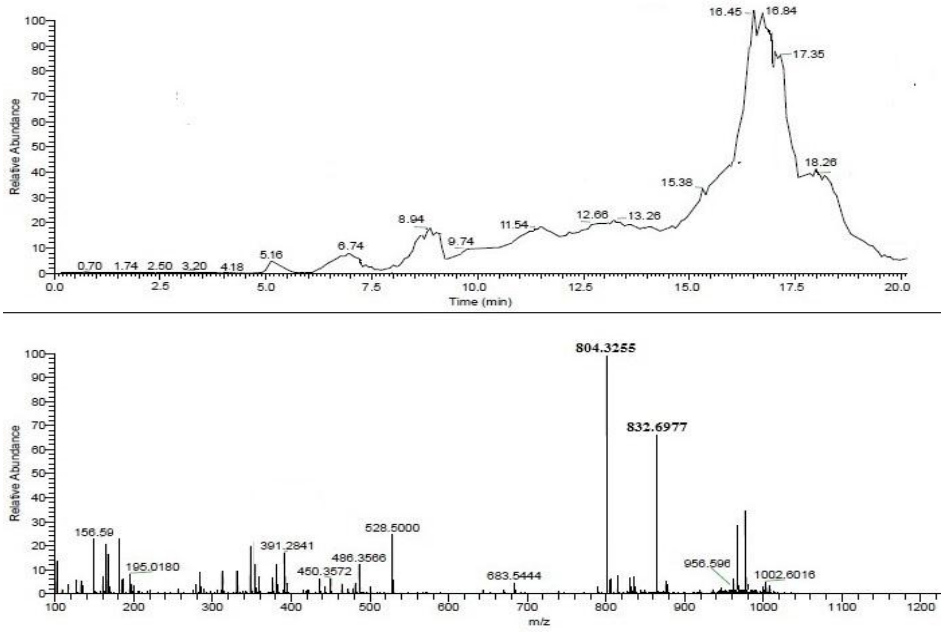
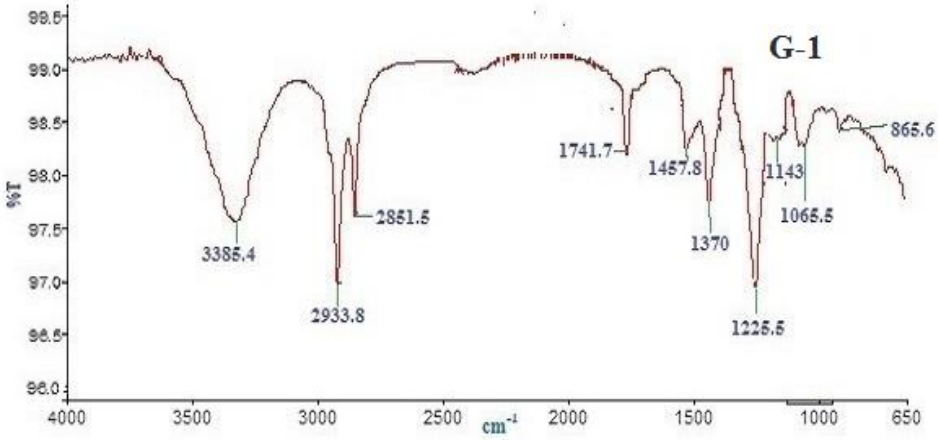
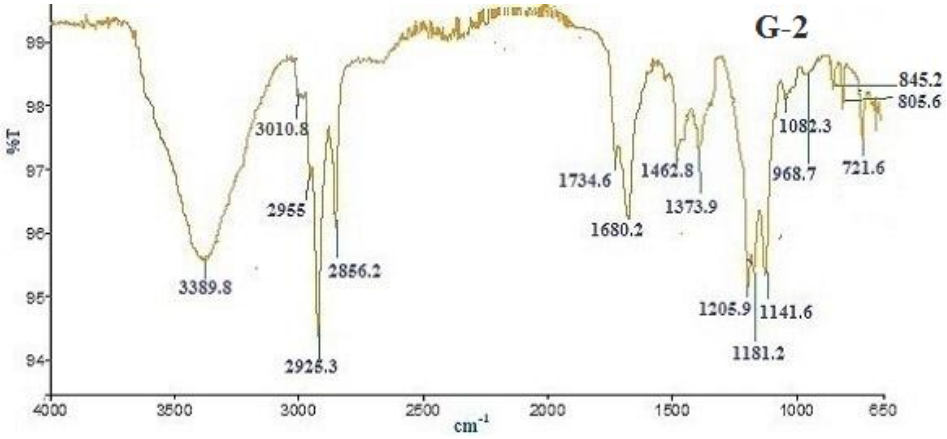
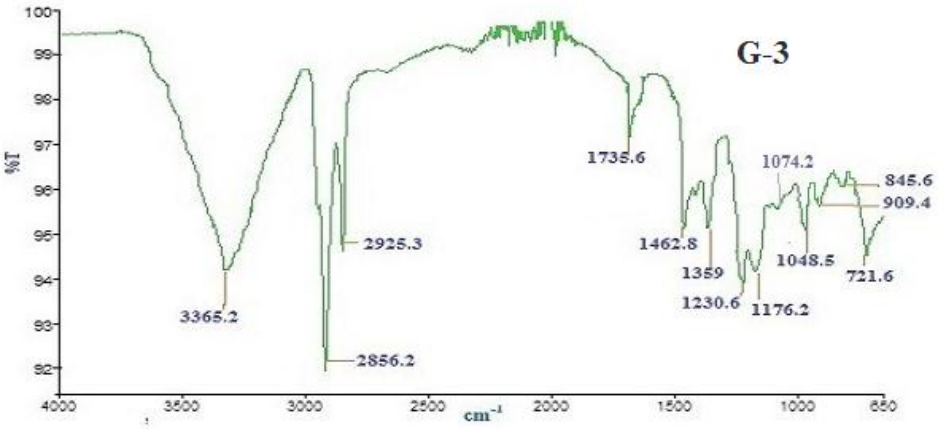


Figure 2.10 LC-MS chromatogram of fraction C


G-1
%T
cm-1
3385.4
2933.8
2851.5
1741.7
1457.8
1370
1225.5
1143
1065.5
865.6


G-2
%T
cm-1
3389.8
3010.8
2955
2925.3
2856.2
1734.6
1680.2
1462.8
1373.9
1205.9
1181.2
1141.6
1082.3
968.7
845.2
805.6
721.6


G-3
%T
cm-1
3365.2
2925.3
2856.2
1735.6
1462.8
1359
1230.6
1176.2
1074.2
1048.5
845.6
909.4
721.6

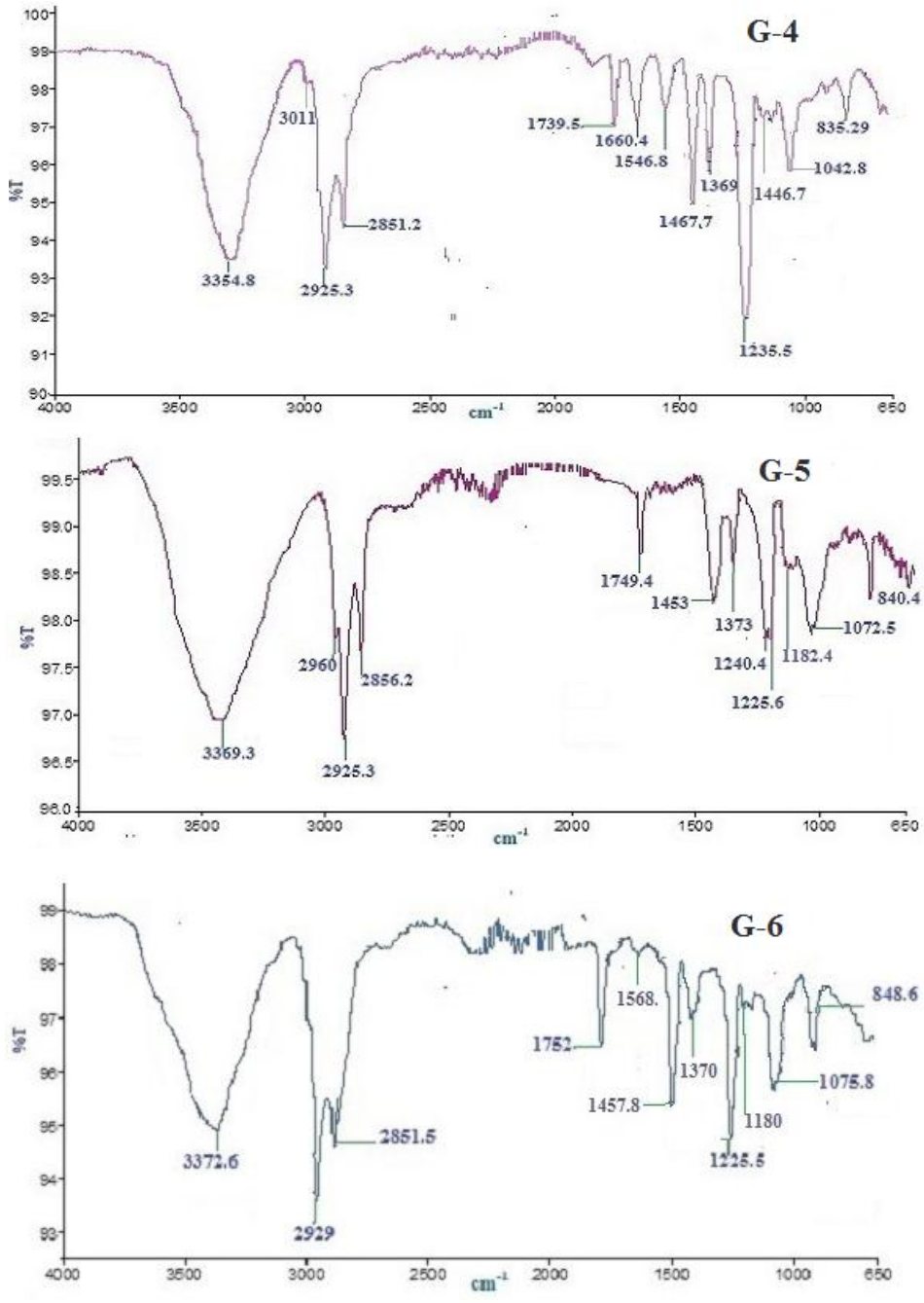


Figure 2.11 FT-IR spectra of glycolipid fractions (G-1 to G-6)

2.3.4 FT-IR charecterization of glycolipids

FT-IR analysis of all the six glycolipid fractions showed similarity in the spectra but with slight variations in intensity (Figure 2.11). Observation of FT-IR spectra revealed the presence of aliphatic hydrocarbon chains (lipid) along with polysaccharide moiety which confirmed the glycolipid nature (Singh & Tiwary, 2016). The absorption bands at 3500-3300 cm^{-1} indicated the presence of free –OH groups due to H-bonding of polysaccharides. The absorption peak in the region 2940-2920 cm^{-1} suggests the stretching vibration of –CH bond of respective sugar moiety. The absorption peaks between 1740 and 1708 cm^{-1} indicated the C = O stretching in ester groups of lipids and fatty acids while the deformation vibration of alkyl groups was confirmed by the presence of peaks between 1384 and 1361 cm^{-1}. The absorption peaks at around 1458 and 1180 cm^{-1} indicated the stretching bands of carbon atoms with hydroxyl groups in the structure of sugar moiety and bands in the region 1040-1080 and 820-850 cm^{-1} associated with the stretching vibrations of glycosidic linkage which confirmed the glycolipid nature. The FT-IR spectra of glycolipids were nearly the same as those reported for other glycolipid produced by microorganisms (Sharma et al. 2015; Hemlata et al. 2015).

FT-IR spectra of G-2 showed a shoulder peak at 3010 cm^{-1} and a peak at 1680 cm^{-1} characteristic of alkenyl C-H and C=C stretch respectively. This is an indication of double bond in the G-2 fraction, probably in the acyl fatty acid chain. G-4 fraction also showed similar observation with less intense peaks at 3011 and 1620 cm^{-1} and thus may contain double bond in the structure. Relative intensity of the bands in the

region 1080 and 848 are slightly higher in G-3 and G-4 compared to those of G-1 and G-2. This can be attributed to extra sugar units present in the former fractions through glycosidic linkages. Fractions G-5 and G-6 showed peaks of highest intensity in this region and may contain more glycosidic linkages compared with other fractions. This observation gives emphasis to the highly polar nature of G-5 and G-6 found through chromatographic analysis. Thus, G-5 and G-6 may possess maximum number of sugar units among the glycolipid fractions.

2.4 Conclusion

Taxonomic characters of cyanobacterial taxa are hardly distinguishable morphologically. Thus, molecular genetics and chemotaxonomic tools are useful and convenient for identification. 16S rRNA gene sequences phylogenetic analysis of the species under study showed 99% similarity to *Synechocystis* sp. (GenBank Accession No. MF444861). Chemotaxonomic analysis based on fatty acids showed that relative abundances of saturated and monounsaturated fatty acids (SFA and MUFA) are higher compared with polyunsaturated fatty acids. This is in agreement with the fatty acid profile of most of the cyanobacterial community. Palmitic (C16:0) acid was found to be the most prominent fatty acid followed by oleic (18:1 n-9), stearic (18:0) and palmitoleic (16:1 n-7) acids. Relative abundance of saturated and monounsaturated fatty acid was found to be 52.97 ± 0.05% and 29.81 ± 0.03 % respectively of the total fatty acid content. The characteristic presence of 3-hydroxy fatty acids in the polar fatty acid fraction also supports taxonomic features of cyanobacteria.

Mass culturing of *Synechocystis* sp. was done under suitable conditions to isolate major glycolipids. TLC staining and LC-MS guided fractionation of cyanobacterial extract yielded six glycolipid fractions (G-1 to G-6). The functional group characterization based on FT-IR analysis affirms the glycolipid nature of all the six fractions. There is a small variation in the FT-IR spectra of different fractions which may be due to differences in the number of hydroxyl groups, glycosidic linkages, double bonds, etc. FT-IR spectra of G-2 and G-4 fractions have shown bands indicating presence of double bond in the structure. More intense bands at glycosidic linkage positions inferred from FT-IR spectra of G-5 and G-6, can be attributed to the more sugar units in those fractions and also to their higher polar character compared to other fractions. Further spectroscopic studies to elucidate the structural features of the isolated glycolipids are discussed in chapter 3.

References

Abed, R. M., Dobretsov, S., & Sudesh, K. (2009). Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology*, 106(1), 1-12.

Adjé, F., Lozano, Y. F., Lozano, P., Adima, A., Chemat, F., & Gaydou, E. M. (2010). Optimization of anthocyanin, flavonol and phenolic acid extractions from *Delonix regia* tree flowers using ultrasound-assisted water extraction. *Industrial Crops and Products*, 32(3), 439-444.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410.

Andrianasolo, E. H., Gross, H., Goeger, D., Musafija-Girt, M., McPhail, K., Leal, R. M., & Gerwick, W. H. (2005). Isolation of swinholide A and related glycosylated derivatives from two field collections of marine cyanobacteria. *Organic Letters*, 7(7), 1375-1378.

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.

Antonopoulou, S., Nomikos, T., Oikonomou, A., Kyriacou, A., Andriotis, M., Fragopoulou, E., & Pantazidou, A. (2005). Characterization of bioactive glycolipids from *Scytonema julianum* (cyanobacteria). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *140*(2), 219-231.

Arias, S., Sandoval, A., Arcos, M., Cañedo, L. M., Maestro, B., Sanz, J. M., & Luengo, J. M. (2008). Poly-3-hydroxyalkanoate synthases from *Pseudomonas putida*: substrate specificity and ultrastructural studies. *Microbial Biotechnology*, 1(2), 170-176.

Asthana, R. K., Tripathi, M. K., Srivastava, A., Singh, A. P., Singh, S. P., Nath, G., & Srivastava, B. S. (2009). Isolation and identification of a new antibacterial entity from the Antarctic cyanobacterium *Nostoc* CCC 537. *Journal of Applied Phycology*, 21(1), 81.

Awai, K., Kakimoto, T., Awai, C., Kaneko, T., Nakamura, Y., Takamiya, K. I., & Ohta, H. (2006). Comparative genomic analysis revealed a gene for monoglucosyldiacylglycerol synthase, an enzyme for photosynthetic membrane lipid synthesis in cyanobacteria. *Plant Physiology*, 141(3), 1120-1127.

Bajpai, V. K., Kim, H. R., Hou, C. T., & Kang, S. C. (2009). Bioconverted products of essential fatty acids as potential antimicrobial agents. *New Biotechnology*, 26(3), 122-130.

Bishop, D. G., Kenrick, J. R., Kondo, T., & Murata, N. (1986). Thermal properties of membrane lipids from two cyanobacteria, *Anacystis nidulans* and *Synechococcus* sp. *Plant and Cell Physiology*, 27(8), 1593-1598.

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.

Bonjouklian, R., Smitka, T. A., Doolin, L. E., Molloy, R. M., Debono, M., Shaffer, S. A., & Patterson, G. M. (1991). Tjipanazoles, new antifungal agents from the blue-green alga *Tolypothrix tjipanasensis*. *Tetrahedron*, 47(37), 7739-7750.

Boonkird, S., Phisalaphong, C., & Phisalaphong, M. (2008). Ultrasound-assisted extraction of capsaicinoids from *Capsicum frutescens* on a lab-and pilot-plant scale. *Ultrasonics Sonochemistry*, 15(6), 1075-1079.

Borowitzka, M. A (1988). Fats, oils and hydrocarbons. *Micro-algal Biotechnology, edited by Borowitzka, MA, Borowitzka, LJ. Cambridge: Cambridge University Press,* 257-287.

Bucar, F., Wube, A., & Schmid, M. (2013). Natural product isolation–how to get from biological material to pure compounds. *Natural Product Reports*, 30(4), 525-545.

Burja, A. M., Banaigs, B., Abou-Mansour, E., Burgess, J. G., & Wright, P. C. (2001). Marine cyanobacteria—a prolific source of natural products. *Tetrahedron*, 57(46), 9347-9377.

Burman, N., Satpati, G. G., SenRoy, S., Khatoon, N., Sen, R., Kanjilal, S. & Pal, R. (2012). Mapping algae of Sundarban origin as lipid feedstock for potential biodiesel application. *Journal of Algal Biomass Utilization*, 3(2), 42- 49.

Cao, Y., & Zhang, X. (2013). Production of long-chain hydroxy fatty acids by microbial conversion. *Applied Microbiology and Biotechnology*, 97(8), 3323-3331.

Caudales, R., & Wells, J. M. (1992). Differentiation of free-living *Anabaena* and *Nostoc* cyanobacteria on the basis of fatty acid composition. *International Journal of Systematic and Evolutionary Microbiology*, 42(2), 246-251.

Chang, T. T., More, S. V., Lu, I. H., Hsu, J. C., Chen, T. J., Jen, Y. C., & Li, W. S. (2011). Isomalyngamide A, A-1 and their analogs suppress cancer cell migration in vitro. *European Journal of Medicinal Chemistry*, 46(9), 3810-3819.

Cheng, H. R., & Jiang, N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnology Letters*, 28(1), 55-59.

Chlipala, G. E., Tri, P. H., Hung, N. V., Krunic, A., Shim, S. H., Soejarto, D. D., & Orjala, J. (2010). Nhatrangins A and B, aplysiatoxin-related metabolites from the marine cyanobacterium *Lyngbya majuscula* from Vietnam. *Journal of Natural Products*, 73(4), 784-787.

Choi, H., Pereira, A. R., Cao, Z., Shuman, C. F., Engene, N., Byrum, T., & Gerwick, W. H. (2010). The hoiamides, structurally intriguing neurotoxic lipopeptides from Papua New Guinea marine cyanobacteria. *Journal of Natural Products*, 73(8), 1411-1421.

Constantopoulos, G., & Bloch, K. (1967). Isolation and characterization of glycolipids from some photosynthetic bacteria. *Journal of Bacteriology*, 93(6), 1788-1793.

da Costa, E., Silva, J., Mendonça, S. H., Abreu, M. H., & Domingues, M. R. (2016). Lipidomic approaches towards deciphering glycolipids from microalgae as a reservoir of bioactive lipids. *Marine Drugs*, 14(5),101-128.

Davies-Coleman, M. T., Dzeha, T. M., Gray, C. A., Hess, S., Pannell, L. K., Hendricks, D. T., & Arendse, C. E. (2003). Isolation of Homodolastatin 16, a new cyclic depsipeptide from a Kenyan collection of *Lyngbya majuscula*. *Journal of Natural Products*, 66(5), 712-715.

Dey, B., Lerner, D. L., Lusso, P., Boyd, M. R., Elder, J. H., & Berger, E. A. (2000). Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. *Journal of Virology*, 74(10), 4562-4569.

Edwards, D. J., Marquez, B. L., Nogle, L. M., McPhail, K., Goeger, D. E., Roberts, M. A., & Gerwick, W. H. (2004). Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. *Chemistry & Biology*, 11(6), 817-833.

Fennell, B. J., Carolan, S., Pettit, G. R., & Bell, A. (2003). Effects of the antimitotic natural product dolastatin 10, and related peptides, on the human malarial parasite *Plasmodium falciparum*. *Journal of Antimicrobial Chemotherapy*, 51(4), 833-841.

Gerwick, W. H., Roberts, M. A., Proteau, P. J., & Chen, J. L. (1994a). Screening cultured marine microalgae for anticancer-type activity. *Journal of Applied Phycology*, 6(2), 143-149.

Gerwick, W. H., Proteau, P. J., Nagle, D. G., Hamel, E., Blokhin, A., & Slate, D. L. (1994b). Structure of curacin A, a novel antimitotic, antiproliferative and brine shrimp toxic natural product from the marine cyanobacterium *Lyngbya majuscula*. *The Journal of Organic Chemistry*, 59(6), 1243-1245.

Ghisalberti, E. L. (1993). Detection and isolation of bioactive natural products. *Bioactive Natural Products: Detection, Isolation, and Structural Determination, edited by Colegate SM, Molyneux RJ. Boca Raton: CRC Press*, 9-57.

Gugger, M., Lyra, C., Suominen, I., Tsitko, I., Humbert, J. F., Salkinoja-Salonen, M. S., & Sivonen, K. (2002). Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena*, *Aphanizomenon*, *Microcystis*, *Nostoc* and *Planktothrix* (cyanobacteria). *International Journal of Systematic and Evolutionary Microbiology*, 52(3), 1007-1015.

Gunasekera, S. P., Owle, C. S., Montaser, R., Luesch, H., & Paul, V. J. (2011). Malyngamide 3 and cocosamides A and B from the marine cyanobacterium *Lyngbya majuscula* from Cocos Lagoon, Guam. *Journal of Natural Products*, 74(4), 871-876.

Gunasekera, S. P., Ross, C., Paul, V. J., Matthew, S., & Luesch, H. (2008). Dragonamides C and D, linear lipopeptides from the marine cyanobacterium brown *Lyngbya polychroa*. *Journal of Natural Products*, 71(5), 887-890.

Gupta, V., Ratha, S. K., Sood, A., Chaudhary, V., & Prasanna, R. (2013). New insights into the biodiversity and applications of cyanobacteria (blue-green algae)—prospects and challenges. *Algal Research*, 2(2), 79-97.

Gustafson, K. R., Cardellina, J. H., Fuller, R. W., Weislow, O. S., Kiser, R. F., Snader, K. M., & Boyd, M. R. (1989). AIDS-antiviral sulfolipids from cyanobacteria (blue-green algae). *Journal of The National Cancer Institute*, 81(16), 1254-1258.

Gutiérrez, O. M., Mannstadt, M., Isakova, T., Rauh-Hain, J. A., Tamez, H., Shah, A., & Wolf, M. (2008a). Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. *New England Journal of Medicine*, 359(6), 584-592.

Gutiérrez, R. M. P., Flores, A. M., Solís, R. V., & Jimenez, J. C. (2008b). Two new antibacterial norabietane diterpenoids from cyanobacteria, *Microcoleous lacustris*. *Journal of Natural Medicines*, 62(3), 328-331.

Han, B., Goeger, D., Maier, C. S., & Gerwick, W. H. (2005). The wewakpeptins, cyclic depsipeptides from a Papua New Guinea collection of the marine cyanobacterium *Lyngbya semiplena*. *The Journal of Organic Chemistry*, 70(8), 3133-3139.

Han, B., Gross, H., Goeger, D. E., Mooberry, S. L., & Gerwick, W. H. (2006). Aurilides B and C, cancer cell toxins from a Papua New Guinea collection of the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*, 69(4), 572-575.

Harvey, H. R. (1994). Fatty acids and sterols as source markers of organic matter in sediments of the North Carolina continental slope. *Deep Sea Research Part II: Topical Studies in Oceanography*, 41(4-6), 783-796.

Hayashi, K., Hayashi, T., & Kojima, I. (1996). A natural sulfated polysaccharide, calcium spirulan, isolated from *Spirulina platensis*: *in vitro* and *in vivo* evaluation of anti-herpes simplex virus and anti-human immunodeficiency virus activities. *AIDS Research and Human Retroviruses*, 12(15), 1463-1471.

He, H., Rodgers, R. P., Marshall, A. G., & Hsu, C. S. (2011). Algae polar lipids characterized by online liquid chromatography coupled with hybrid linear quadrupole ion trap/Fourier transform ion cyclotron resonance mass spectrometry. *Energy & Fuels*, 25(10), 4770-4775.

Hemlata, B., Selvin, J., & Tukaram, K. (2015). Optimization of iron chelating biosurfactant production by *Stenotrophomonas maltophilia* NBS-11. *Biocatalysis and Agricultural Biotechnology*, 4(2), 135-143.

Hemscheidt, T., Puglisi, M. P., Larsen, L. K., Patterson, G. M., Moore, R. E., Rios, J. L., & Clardy, J. (1994). Structure and biosynthesis of borophycin, a new boeseken complex of boric acid from a marine strain of the blue-green alga *Nostoc linckia*. *The Journal of Organic Chemistry*, 59(12), 3467-3471.

Hirata, K., Yoshitomi, S., Dwi, S., Iwabe, O., Mahakhant, A., Polchai, J., & Miyamoto, K. (2003). Bioactivities of nostocine A produced by a freshwater cyanobacterium *Nostoc spongiaeforme* TISTR 8169. *Journal of Bioscience and Bioengineering*, 95(5), 512-517.

Hölzl, G., & Dörmann, P. (2007). Structure and function of glycoglycerolipids in plants and bacteria. *Progress in Lipid Research*, 46(5), 225-243.

Horgen, F. D., Kazmierski, E. B., Westenburg, H. E., Yoshida, W. Y., & Scheuer, P. J. (2002). Malevamide D: Isolation and structure determination of an Isodolastatin H analogue from the marine cyanobacterium *Symploca hydnoides*. *Journal of Natural Products*, 65(4), 487-491.

Hou, C. T. (2009). Biotechnology for fats and oils: new oxygenated fatty acids. *New Biotechnology*, 26(1), 2-10.

Iliev, I., Petkov, G., Lukavsky, J., Furnadzhieva, S., & Andreeva, R. (2011). Do cyanobacterial lipids contain fatty acids longer than 18 carbon atoms?. *Zeitschrift Für Naturforschung C*, 66(5-6), 267-276.

Jaki, B., Orjala, J., & Sticher, O. (1999). A novel extracellular diterpenoid with antibacterial activity from the cyanobacterium Nostoc commune. *Journal of Natural Products*, 62(3), 502-503.

Jaki, B., Heilmann, J., & Sticher, O. (2000a). New antibacterial metabolites from the cyanobacterium *Nostoc commune* (EAWAG 122b). *Journal of Natural Products*, 63(9), 1283-1285.

Jaki, B., Orjala, J., Heilmann, J., Linden, A., Vogler, B., & Sticher, O. (2000b). Novel extracellular diterpenoids with biological activity from the cyanobacterium *Nostoc c ommune*. *Journal of Natural Products*, 63(3), 339-343.

Jatzkewitz, H., & Mehl, E. (1960). On thin layer chromatography of cerebral lipids, their conversion and decomposition products. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie*, 320(1), 251-257.

Kajiyama, S. I., Kanzaki, H., Kawazu, K., & Kobayashi, A. (1998). Nostofungicidine, an antifungal lipopeptide from the field-grown terrestrial blue-green alga *Nostoc commune*. *Tetrahedron Letters*, 39(22), 3737-3740.

Kanekiyo, K., Lee, J. B., Hayashi, K., Takenaka, H., Hayakawa, Y., Endo, S., & Hayashi, T. (2005). Isolation of an antiviral polysaccharide, Nostoflan, from a terrestrial cyanobacterium, *Nostoc flagelliforme*. *Journal of Natural Products*, 68(7), 1037-1041.

Kim, S. H., Liu, K. H., Lee, S. Y., Hong, S. J., Cho, B. K., Lee, H. & Choi, H. K. (2013). Effects of light intensity and nitrogen starvation on glycerolipid, glycerophospholipid, and carotenoid composition in *Dunaliella tertiolecta* culture. *PLoS One*, 8(9), 1-12.

Kim, Y. H., Choi, J. S., Hong, J., Yoo, J. S., & Kim, M. S. (1999). Identification of acylated glycoglycerolipids from a cyanobacterium, *Synechocystis* sp., by tandem mass spectrometry. *Lipids*, *34*(8), 847-853.

Knoll, A. H., 2008. Cyanobacteria and earth history. *The Cyanobacteria: Molecular Biology, Genomics and Evolution, edited by Herrero A, Flores E. Norfolk : Caister Academic Press*, 1–19.

Knothe, G. (2006). Analyzing biodiesel: standards and other methods. *Journal of the American Oil Chemists' Society*, 83(10), 823-833.

Kumar, A., Kaushal, S., Saraf, S. A., & Singh, J. S. (2017). Cyanobacterial biotechnology: an opportunity for sustainable industrial production. *Climate Change and Environmental Sustainability*, 5(1), 97-110.

Kwan, J. C., Rocca, J. R., Abboud, K. A., Paul, V. J., & Luesch, H. (2008). Total structure determination of grassypeptolide, a new marine cyanobacterial cytotoxin. *Organic Letters*, 10(5), 789-792.

Larsen, L. K., Moore, R. E., & Patterson, G. M. (1994). β-Carbolines from the blue-green alga *Dichothrix baueriana*. *Journal of Natural Products*, 57(3), 419-421.

Lau, N. S., Matsui, M., & Abdullah, A. A. (2015). Cyanobacteria: photoautotrophic microbial factories for the sustainable synthesis of industrial products. *Bio-Medical Research International*, 2015(1), 1-9.

Leão, P. N., Nakamura, H., Costa, M., Pereira, A. R., Martins, R., Vasconcelos, V., & Balskus, E. P. (2015). Biosynthesis-asssisted structural elucidation of the Bartolosides, chlorinated aromatic glycolipids from cyanobacteria. *Angewandte Chemie*, 127(38), 11215-11219.

Li, R., Watanabe, M. M. (2001). Fatty acid profiles and their chemotaxonomy in planktonic species of *Anabaena* (Cyanobacteria) with straight trichomes. *Phytochemistry*, 57(5), 727-731.

Linington, R. G., Clark, B. R., Trimble, E. E., Almanza, A., Ureña, L. D., Kyle, D. E., & Gerwick, W. H. (2008). Antimalarial peptides from marine cyanobacteria: isolation and structural elucidation of gallinamide A. *Journal of Natural Products*, 72(1), 14-17.

Linington, R. G., Edwards, D. J., Shuman, C. F., McPhail, K. L., Matainaho, T., & Gerwick, W. H. (2007). Symplocamide A, a potent cytotoxin and chymotrypsin inhibitor from the marine cyanobacterium *Symploca* sp. *Journal of Natural Products*, 71(1), 22-27.

Löffelhardt, W., & Bohnert, H. J. (1994). Molecular biology of cyanelles. *The Molecular Biology of Cyanobacteria, edited by Bryant DA, Netherlands: Springer*, 65-89.

Loya, S., Reshef, V., Mizrachi, E., Silberstein, C., Rachamim, Y., Carmeli, S., & Hizi, A. (1998). The inhibition of the reverse transcriptase of HIV-1 by the natural sulfoglycolipids from cyanobacteria: contribution of different moieties to their high potency. *Journal of Natural Products*, 61(7), 891-895.

Luesch, H., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2000). Isolation and structure of the cytotoxin Lyngbyabellin B and absolute configuration of lyngbyapeptin A from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*, 63(10), 1437-1439.

Luesch, H., Pangilinan, R., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2001a). Pitipeptolides A and B, new cyclodepsipeptides from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*, 64(3), 304-307.

Luesch, H., Moore, R. E., Paul, V. J., Mooberry, S. L., & Corbett, T. H. (2001b). Isolation of dolastatin 10 from the marine cyanobacterium *Symploca* species VP642 and total stereochemistry and biological evaluation of its analogue symplostatin. *Journal of Natural Products*, 64(7), 907-910.

Luesch, H., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2002). New apratoxins of marine cyanobacterial origin from Guam and Palau. *Bioorganic & Medicinal Chemistry*, 10(6), 1973-1978.

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L., Sivonen, K. (2001). Molecular characterization of planktic cyanobacteria of Anabaena, Aphanizomenon, Microcystis and Planktothrix genera. *International Journal of Systematic and Evolutionary Microbiology*, 51(2), 513-526.

Ma, L., & Led, J. J. (2000). Determination by high field NMR spectroscopy of the longitudinal electron relaxation rate in Cu (II) plastocyanin from *Anabaena variabilis*. *Journal of the American Chemical Society*, 122(32), 7823-7824.

Marcolongo, G., De Appolonia, F., Venzo, A., Berrie, C. P., Carofiglio, T., & Ceschi Berrini, C. (2006). Diacylglycerolipids isolated from a thermophile cyanobacterium from the Euganean hot springs. *Natural Product Research*, 20(8), 766-774.

Marston, A. (2011). Thin-layer chromatography with biological detection in phytochemistry. *Journal of Chromatography A*, 1218(19), 2676-2683.

McPhail, K. L., Correa, J., Linington, R. G., González, J., Ortega-Barría, E., Capson, T. L., & Gerwick, W. H. (2007). Antimalarial linear lipopeptides from a Panamanian strain of the marine cyanobacterium Lyngbya majuscula. *Journal of Natural Products*, 70(6), 984-988.

Medina, R. A., Goeger, D. E., Hills, P., Mooberry, S. L., Huang, N., Romero, L. I., & McPhail, K. L. (2008). Coibamide A, a potent antiproliferative cyclic depsipeptide from the Panamanian marine cyanobacterium *Leptolyngbya* sp. *Journal of American Chemical Society*, 130(20), 6324-6325.

Meher, L. C., Sagar, D. V., & Naik, S. N. (2006). Technical aspects of biodiesel production by transesterification-a review. *Renewable and Sustainable Energy Reviews*, 10(3), 248-268.

Nagatsu, A., Kajitani, H., & Sakakibara, J. (1995). Muscoride A: a new oxazole peptide alkaloid from freshwater cyanobacterium *Nostoc muscorum*. *Tetrahedron Letters*, 36(23), 4097-4100.

Nübel, U., Garcia-Pichel, F., & Muyzer, G. (1997). PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology*, 63(8), 3327-3332.

Pankratova, E. M. (1987). Participation of Cyanobacteria in the soil nitrogen cycle and formation of soil fertility. *Advances in Microbiology*, 21(1), 212-242.

Patil, V., Källqvist, T., Olsen, E., Vogt, G., & Gislerød, H. R. (2007). Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquaculture International*, 15(1), 1-9.

Patterson, G. M., Larsen, L. K., & Moore, R. E. (1994). Bioactive natural products from blue-green algae. *Journal of Applied Phycology*, 6(2), 151-157.

Paul, S., Hou, C. T., & Kang, S. C. (2010). α-Glucosidase inhibitory activities of 10-hydroxy-8 (E)-octadecenoic acid: an intermediate of bioconversion of oleic acid to 7,10-dihydroxy-8 (E)-octadecenoic acid. *New Biotechnology*, 27(4), 419-423.

Pettit, G. R., Hogan, F., Xu, J. P., Tan, R., Nogawa, T., Cichacz, Z. & Herald, C. L. (2008). Antineoplastic Agents. 536. New sources of naturally occurring cancer cell growth inhibitors from marine organisms, terrestrial plants, and microorganisms. *Journal of Natural Products*, 71(3), 438-444.

Prinsep, M. R., Caplan, F. R., Moore, R. E., Patterson, G. M., & Smith, C. D. (1992). Tolyporphin, a novel multidrug resistance reversing agent from the blue-green alga *Tolypothrix nodosa*. *Journal of American Chemical Society*, 114(1), 385-387.

Raveh, A., & Carmeli, S. (2007). Antimicrobial ambiguines from the cyanobacterium *Fischerella* sp. collected in Israel. *Journal of Natural Products*, 70(2), 196-201.

Rickards, R. W., Rothschild, J. M., Willis, A. C., de Chazal, N. M., Kirk, J., Kirk, K., & Smith, G. D. (1999). Calothrixins A and B, novel pentacyclic metabolites from *Calothrix* cyanobacteria with potent activity against malaria parasites and human cancer cells. *Tetrahedron*, 55(47), 13513-13520.

Rishi, V., & Awasthi, A. K. (2015). A brief review on potential applications of cyanobacteria. *Indian Journal of Biotechnology & Biochemistry*, 2(1),1-38.

Ryhage, R., & Stenhagen, B. (1960). Mass spectrometry in lipid research. *Journal of Llipid Research*, 1(2), 361-390.

Salvador, L. A., Paul, V. J., & Luesch, H. (2010). Caylobolide B, a macrolactone from symplostatin 1-producing marine cyanobacteria *Phormidium* spp. from Florida. *Journal of Natural Products*, 73(9), 1606-1609.

Salvador, L. A., Biggs, J. S., Paul, V. J., & Luesch, H. (2011). Veraguamides A− G, Cyclic Hexadepsipeptides from a Dolastatin 16-Producing Cyanobacterium Symploca cf. hydnoides from Guam. *Journal of Natural Products*, 74(5), 917-927.

Sandoval, Á., Arias-Barrau, E., Bermejo, F., Cañedo, L., Naharro, G., Olivera, E. R., & Luengo, J. M. (2005). Production of 3-hydroxy-n-phenylalkanoic acids by a genetically engineered strain of *Pseudomonas putida*. *Applied Microbiology and Biotechnology*, 67(1), 97-105.

Seidel, V. (2012). Initial and bulk extraction of natural products isolation. *Natural Products Isolation*. *Methods in Molecular Biology (Methods and Protocols), edited by Sarker S, Nahar L. Netherlands: Springer,* 27-41.

Senhorinho, G. N., Ross, G. M., & Scott, J. A. (2015). Cyanobacteria and eukaryotic microalgae as potential sources of antibiotics. *Phycologia*, 54(3), 271-282.

Sharma, D., Saharan, B. S., Chauhan, N., Procha, S., & Lal, S. (2015). Isolation and functional characterization of novel biosurfactant produced by *Enterococcus faecium*. *SpringerPlus*, 4(1), 1-14.

Singh, P., & Tiwary, B. N. (2016). Isolation and characterization of glycolipid biosurfactant produced by a *Pseudomonas otitidis* strain isolated from Chirimiri coal mines, India. *Bioresources and Bioprocessing*, 3(1), 42.

Singh, S. C., Sinha, R. P., & Hader, D. P. (2002). Role of lipids and fatty acids in stress tolerance in cyanobacteria. *Acta Protozoologica*, 41(4), 297-308.

Stanier, R. Y., Kunisawa, R., Mandel, M., & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews*, 35(2), 171-205.

Stansell, G. R., Gray, V. M., & Sym, S. D. (2012). Microalgal fatty acid composition: implications for biodiesel quality. *Journal of Applied Phycology*, 24(4), 791-801.

Stewart, J. B., Bornemann, V., JIAN, L., Moore, R. E., Caplan, F. R., Karuso, H., & Patterson, G. M. (1988). Cytotoxic, fungicidal nucleosides from blue green algae belonging to the *Scytonemataceae*. *The Journal of Antibiotics*, 41(8), 1048-1056.

Sticher, O. (2008). Natural product isolation. *Natural Product Reports*, 25(3), 517-554.

Subbaraju, G. V., Golakoti, T., Patterson, G. M., & Moore, R. E. (1997). Three new cryptophycins from Nostoc sp. GSV 224. *Journal of Natural Products*, 60(3), 302-305.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725-2729.

Taniguchi, M., Nunnery, J. K., Engene, N., Esquenazi, E., Byrum, T., Dorrestein, P. C., & Gerwick, W. H. (2009). Palmyramide A, a cyclic depsipeptide from a Palmyra Atoll collection of the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*, 73(3), 393-398.

Taori, K., Paul, V. J., & Luesch, H. (2008). Structure and activity of largazole, a potent antiproliferative agent from the Floridian marine cyanobacterium *Symploca* sp. *Journal of American Chemical Society*, 130(6), 1806-1807.

Teruya, T., Sasaki, H., Kitamura, K., Nakayama, T., & Suenaga, K. (2009a). Biselyngbyaside, a macrolide glycoside from the marine cyanobacterium *Lyngbya* sp. *Organic letters*, 11(11), 2421-2424.

Teruya, T., Sasaki, H., Fukazawa, H., & Suenaga, K. (2009b). Bisebromoamide, a potent cytotoxic peptide from the marine cyanobacterium *Lyngbya* sp.: isolation, stereostructure, and biological activity. *Organic Letters*, 11(21), 5062-5065.

Thacker, R. W., & Paul, V. J. (2004). Morphological, chemical, and genetic diversity of tropical marine cyanobacteria *Lyngbya* spp. and *Symploca* spp.(Oscillatoriales). *Applied and Environmental Microbiology*, 70(6), 3305-3312.

Tidgewell, K., Engene, N., Byrum, T., Media, J., Valeriote, F. A., & Gerwick, W. H. (2010). Evolved diversification of a modular natural product pathway: apratoxins F and G, two cytotoxic cyclic depsipeptides from a Palmyra collection of *Lyngbya bouillonii*. *ChemBioChem*, 11(10), 1458-1466.

Tokuda, H., Nishino, H., Shirahashi, H., Murakami, N., Nagatsu, A., & Sakakibara, J. (1996). Inhibition of 12-O-tetradecanoylphorbol-13-acetate promoted mouse skin papilloma by digalactosyl diacylglycerols from the fresh water cyanobacterium *Phormidium tenue*. *Cancer Letters*, 104(1), 91-95.

Tripathi, A., Puddick, J., Prinsep, M. R., Rottmann, M., Chan, K. P., Chen, D. Y. K., & Tan, L. T. (2011). Lagunamide C, a cytotoxic cyclodepsipeptide from the marine cyanobacterium *Lyngbya majuscula*. *Phytochemistry*, 72(18), 2369-2375.

Vargas, M. A., Rodriguez, H., Moreno, J., Olivares, H., Del Campo, J. A., Rivas, J., & Guerrero, M. G. (1998). Biochemical composition and fatty acid content of filamentous nitrogen-fixing cyanobacteria. *Journal of Phycology*, 34(5), 812-817.

White, J. D., Xu, Q., Lee, C. S., & Valeriote, F. A. (2004). Total synthesis and biological evaluation of (+)-kalkitoxin, a cytotoxic metabolite of the cyanobacterium *Lyngbya Majuscula*. *Organic & Biomolecular Chemistry*, 2(14), 2092-2102.

Whitton, B. A. (2008). Cyanobacterial diversity in relation to the environment. *Algal Toxins: Nature, Occurrence, Effect and Detection, edited by Evangelista V, Barsanti L, Frassanito AM, Passarelli V, Gualtieri P. Netherlands: Springer,* 17-43.

Whitton, B. A., & Potts, M. (2012). Introduction to the cyanobacteria. *Ecology of Cyanobacteria II, edited by Whitton B. Netherlands: Springer,*1-13.

Williams, P. G., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2002). Isolation and structure determination of obyanamide, a novel cytotoxic cyclic depsipeptide from the marine cyanobacterium *Lyngbya confervoides*. *Journal of Natural Products*, 65(1), 29-31.

Williams, P. G., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2003a). The isolation and structure elucidation of Tasiamide B, a 4-amino-3-hydroxy-5-phenylpentanoic acid containing peptide from the marine cyanobacterium *Symploca* sp. *Journal of Natural Products*, 66(7), 1006-1009.

Williams, P. G., Yoshida, W. Y., Moore, R. E., & Paul, V. J. (2003b). Tasipeptins A and B: new cytotoxic depsipeptides from the marine cyanobacterium *Symploca* sp. *Journal of Natural Products*, 66(5), 620-624.

Williams, P. G., Yoshida, W. Y., Quon, M. K., Moore, R. E., & Paul, V. J. (2003c). Ulongapeptin, a cytotoxic cyclic depsipeptide from a Palauan marine cyanobacterium *Lyngbya* sp. *Journal of Natural Products*, 66(5), 651-654.

Wilmotte, A., Turner, S., Peer, Y., & Pace, N. R. (1992). Taxonomic study of marine Oscillatoriacean strains (cyanobacteria) with narrow trichomes. II. Nucleotide sequence analysis of the 16S ribosomal RNA1. *Journal of Phycology*, 28(6), 828-838.

Wood, B. J. B., Nichols, B. W., & James, A. T. (1965). The lipids and fatty acid metabolism of photosynthetic bacteria. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 106(2), 261-273.

Xiong, C., O'keefe, B. R., Byrd, R. A., & McMahon, J. B. (2006). Potent anti-HIV activity of scytovirin domain 1 peptide. *Peptides*, 27(7), 1668-1675.

Yan, X., Chen, D., Xu, J., & Zhou, C. (2011). Profiles of photosynthetic glycerolipids in three strains of *Skeletonema* determined by UPLC-Q-TOF-MS. *Journal of Applied Phycology*, 23(2), 271-282.

Yan, X., Li, H., Xu, J., & Zhou, C. (2010). Analysis of phospholipids in microalga *Nitzschia closterium* by UPLC-Q-TOF-MS. *Chinese Journal of Oceanology and Limnology*, 28(1), 106-112.

Yao, L., Gerde, J. A., Lee, S. L., Wang, T., & Harrata, K. A. (2015). Microalgae lipid characterization. *Journal of Agricultural and Food Chemistry*, 63(6), 1773-1787.

Zainuddin, E. N., Mentel, R., Wray, V., Jansen, R., Nimtz, M., Lalk, M., & Mundt, S. (2007). Cyclic depsipeptides, ichthyopeptins A and B, from *Microcystis ichthyoblabe*. *Journal of Natural Products*, 70(7), 1084-1088.

Zheng, X. Y., Zhang, L., Cheng, X. M., Zhang, Z. J., Wang, C. H., & Wang, Z. T. (2011). Identification of acetylcholinesterase inhibitors from seeds of plants of genus Peganum by thin-layer chromatography-bioautography. *JPC-Journal of Planar Chromatography-Modern TLC*, 24(6), 470-474.

Zou, B., Long, K., & Ma, D. (2005). Total synthesis and cytotoxicity studies of a cyclic depsipeptide with proposed structure of palau'amide. *Organic Letters*, 7(19), 4237-4240.

.....ꕥ.....

Chapter 3

CHEMICAL CHARACTERIZATION OF GLYCOLIPIDS FROM *Synechocystis* sp.

Contents

3.1 Introduction

Natural products are compounds possessing broad structural diversity, produced by all classes of living organisms. Many natural product structures have found successful applications in medicine and industry. About half of the drugs currently in clinical use are natural products or synthetic molecules based on natural product scaffolds (Koehn & Carter, 2005; Patridge et al., 2016). Natural product structure elucidation was considered as an extremely difficult and lengthy task consisting numerous synthetic and degradation to provide needed clues for eventual determination of the structure. However, increasing availability and improvement of commercial spectroscopic instrumentation has revolutionized natural product structure elucidation (Colegate & Molyneux, 1993; Havlicek & Spizek, 2014). Fourier transform infrared (FT-IR) and UV/Vis spectrometry are routinely used in natural product chemistry for

functional group characterization (Braude & Nachod, 2013). FT-IR spectrometry is widely used to study structural details of glycolipids and polysaccharides, such as type of monosaccharide present, glycosidic linkages and other functional groups (Mathlouthi & Koenig, 1987; Brandenburg & Seydel, 1998; Yang & Zhang, 2009). However, structure analysis of natural products is dominated by mass spectrometry and NMR spectroscopy.

Mass spectrometry (MS) is primarily used to obtain molecular weights of compounds. MS is based on production of gaseous, positively or negatively charged ions that are subsequently separated according to their mass-to-charge (m/z) ratio and detected. In traditional MS, purified samples were subjected to high-energy ionization under ultra-high vacuum conditions (De Hoffmann & Stroobant, 2007). Development of electrospray ionization MS (ESI-MS) has made remarkable change in the study of natural products. In contrast to earlier ionization techniques such as EI (electron impact), which were applicable only to thermally stable, low molecular weight volatile compounds, ESI-MS can be used to analyze any ions ranging from inorganic salts to large macromolecules such as proteins and it rarely generates fragments (Shipovskov & Reimann, 2007; Ntai, 2008). Another advantage of ESI-MS is that it can be directly coupled to high performance liquid chromatography (HPLC). As a result, the interface of HPLC with ESI-MS has provided an excellent method in identification and isolation of new metabolites from complex extracts (Stecher et al., 2001; Zhang et al., 2004).

In mass spectroscopy, molecules are ionized by protonation, cationization or deprotonation to form pseudomolecular ions. During protonation and cationization, positively charged ions are formed such as $[M+H]^+$ and $[M+Na]^+$, respectively. During deprotonation, a proton is removed from the molecule resulting in negatively charged ions $[M-H]^-$ (Liao & Allison, 1995; Fabre et al., 2001). Performing both positive ion and negative ion ESI-MS experiments can give reliable evidence for the molecular weight of a compound. In addition, depending on the mass analyzer used, high accuracy in the determination of molecular weight can be achieved. From this accurately determined molecular mass (± 0.001 mass units), chemical formula and number of double bonds, rings or heteroatoms can be inferred (Vestal et al., 1995; El-Aneed et al., 2009; Dawson, 2013). Mass spectrometry can be used to easily determine number and type of monosaccharide unit components of glycosides. Most of the glycosides isolated nowadays are sequenced by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) in the negative or positive ion modes. Examples are glycosides of flavonoids (Grayer et al., 2000; Crublet et al., 2003; Carbone et al., 2004; Cuyckens & Claeys, 2004; Mihara et al., 2004), pregnanes (Plaza et al., 2005), triterpenes (Schöpke et al., 1995; Voutquenne et al., 2003; Liu et al., 2004), aminocyclitol antibiotics (Hu et al., 2000), naphtopyranones (Piacente et al., 2001), cardenolides (Guan et al., 1999; Grosa et al., 2005), xanthones (Řezanka & Dembitsky, 2003) and polar natural oligosaccharides (Tolstikov & Fiehn, 2002). Different strategies to sequence depend on the accessible instruments. Ion traps, which have ability to perform MS^n, are the most suitable for this purpose (Huhman & Sumner, 2002).

Mass spectrometry is a highly sensitive technique that is amenable to dereplication of natural products (Shin & van Breemen, 2001). Especially, LC-MS is a widely used dereplication tool as it can analyze complex mixtures. Molecular mass is one of the highly desired information for dereplication, as it is available in most databases of natural products and is not dependent on source, sample preparation and conditions of measurement. High-resolution mass spectroscopic techniques, such as fast atom bombardment (FAB) and quadrupole time of flight (TOF), determine molecular mass at low ppm accuracy (Liptáak & Heerma, 1993). FT-MS (Fourier transform mass spectrometry) is even more precise at sub-ppm accuracy. However, database search of an observed accurate molecular mass typically results in a list of natural products of which molecular masses are close to the observed one (Potterat et al., 2000). The intrinsic danger of this approach is that compounds in the list are often false positives. The risk of false positive identification can be reduced by MS/MS or LC-MS/MS as it provides some information about substructures of natural products (Stefanowicz et al., 2001; Fredenhagen et al., 2005; Konishi et al., 2007). The fragmentation patterns obtained in electrospray MS or LC-MS/MS are dependent on the type of mass spectrometer, mode of ionization, collision energy, etc.

While mass spectrometry may be much more sensitive, it can also be extremely selective. Therefore complete structural determination of new natural product requires complete ^{1}H and ^{13}C NMR data assignment. Technology and application potential of NMR spectroscopy have been enormous in the field of natural products. The higher the frequency of an

NMR spectrometer, the higher will be its sensitivity. In the early stages, utility of NMR spectroscopy was limited by low sensitivity of continuous wave NMR spectrometers which especially restricted ability to obtain ^{13}C spectra (Yates, 1967; Stothers, 2012). However, this problem was minimized by development of pulsed Fourier transform NMR spectrometers (Ernst & Anderson, 1966) and the use of ^{13}C NMR for structure elucidation became much more common (Levy & Nelson, 1972). This was followed relatively quickly by the introduction of higher field FT-NMR spectrometers using superconducting solenoids. This allowed development of multiple pulse sequences which greatly increased usefulness of NMR for natural product investigations. ^{1}H and ^{13}C NMR spectra carry information about the qualitative and quantitative composition of an unknown compound and they are used first for determination of the molecular formula. Introduction of two-dimensional (2D) NMR by Aue et al. (1976) was a critical step, followed by development of pulse sequences for editing of ^{13}C spectra based on numbers of attached proton. Some of the basic 2D pulse sequences for structure elucidation includes correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond coherence (HMBC), etc. (Reynolds & Enríquez, 2002; Berger & Braun, 2004).

The ability to edit ^{13}C spectra in terms of number of attached protons for each carbon peak can be very helpful, particularly for identifying known compounds at the early stages of investigation of natural products. The simplest of spectral editing sequences is the attached proton test (APT) sequence (Patt & Shoolery, 1982). This

sequence provides partial spectral editing with methylene and quaternary carbon signals upright, and methane and methyl carbons inverted. However, it is less sensitive than the basic ^{13}C sequence and is very sensitive to variations in one bond ^{13}C-^{1}H coupling constants, $^{1}J_{CH}$. As a result, it can give unreliable editing, particularly in cases where the coupling constants are unusually large (Breton & Reynolds, 2013). Two alternative choices, based on polarization transfer from ^{1}H to ^{13}C, are the INEPT (insensitive nuclei enhanced by polarization transfer) (Morris, 1980) and DEPT (distortionless enhancement by polarization transfer) sequences (Doddrell et al., 1982). Both sequences only give peaks for protonated carbons. However, due to polarization transfer, these peaks are significantly more intense than those in a standard ^{13}C spectrum obtained in the same scan time. Since DEPT is much less sensitive than INEPT to variations in $^{1}J_{CH}$ (Torres & McClung, 1991), it is the preferred choice. The DEPT-135 sequence gives methine and methyl carbons upright, and methylene carbons inverted. Alternatively, one can acquire three separate spectra with final pulses of 45, 90 and 135 degrees. These can then be combined in various ways to generate separate CH, CH_2 and CH_3 spectra. While this involves a small sensitivity loss, relative to a single DEPT-135 spectrum obtained in the same total time, which gives a much clearer display. In addition, overlapping peaks of opposite phase can be clearly observed in the fully edited DEPT-135 spectrum (Reynolds & Enríquez, 2002).

The HSQC spectrum shows resonances (heteronuclear correlations) which arise as a result of $^{1}J_{CH}$ couplings between ^{13}C nuclei and protons attached to the corresponding atoms. Edited version of the gradient-

selected HSQC sequence not only provides similar carbon editing information to DEPT-135 but also simultaneous assignment of chemical shifts of the attached protons (Willker et al., 1993). This often takes less time than acquiring a DEPT spectrum. Edited HSQC spectrum, in connection with a standard ^{13}C spectrum, appears as an effective approach for structure elucidation (Elyashberg et al., 2002). The ^{1}H-^{1}H correlation (COSY) spectrum usually reveals homonuclear correlations (spin couplings) between vicinal hydrogens separated by three bonds ($^{3}J_{H-H}$). This makes it possible to identify neighbour carbon atoms connected by a chemical bond. COSY spectra serve as a key tool for organic structure elucidation with off-diagonal correlation peaks indicating pairs of coupled protons (Bax & Freeman, 1981). However, original COSY sequence, which relied on phase-cycling for coherence pathway selection, has now almost entirely been replaced by the gradient-selected version of COSY which often allows obtaining a COSY spectrum with only one scan per time-increment spectrum. This generally gives better artefact suppression (Hurd, 1990; Marion, 2012). Heteronuclear multiple-bond coherence (HMBC) spectrum reveals heteronuclear correlation between ^{1}H and ^{13}C nuclei separated by two or three chemical bonds, allowing users to detect unclear fragments around a given carbon atom. However, depending on the spatial configuration of a molecule, correlations longer than standard correlations can also be observed (Bax & Summers, 1986; Furrer, 2011; Schoefberger et al., 2011). There are only a few approaches that would allow determining number of bonds between intervening ^{1}H-^{13}C pairs (Krishnamurthy et al., 2000; Nyberg et al., 2005). Therefore, information carried by HMBC is at

times uncertain by nature. While COSY and HMBC spectra used together could determine complete molecular structures.

NMR is a very powerful tool for studies of molecular order and structure of lipid bilayer components such as glycolipids and phospholipids (Castro et al., 2007). Biological functions of glycolipids are closely connected to the structure of sugar moiety. NMR serves as an efficient and non-destructive method for structure determination of glycolipids (Dabrowski et al., 1980; Koerner et al., 1983). Complete structural assignment of many naturally occurring lipopolysaccharides, including ring structure of sugar unit and linkage positions, were achieved by employing different two dimensional NMR techniques (Vinogradov & Bock, 1999; Duus et al., 2000). An example is the spin system assignment in furanose vs. pyranose units of carbohydrates. The rigid chair-like conformation in the latter in combination with large couplings for diaxial H-atoms leads to COSY spectra with good cross-peak intensities. In contrast, five membered furanose rings would give less intense COSY peaks. Also linkage points in carbohydrates are defined by using H-C long range interactions especially by HMBC technique (Bross-Walch et al., 2005).

This chapter describes structural characterization of glycolipids isolated from the cyanobacteria *Synechocystis* sp. using various spectroscopic methods. Structure elucidations of two major glycolipids using different NMR methods (^{1}H, ^{13}C and 2D NMR), LC-MS/MS and FT-IR spectroscopy are discussed. Structure analyses of other glycolipids based on mass fragmentation pattern are also included in this chapter.

3.2 Materials and methods

3.2.1 Sugar analysis of glycolipids

Each of the six isolated glycolipid samples (G-1 to G-6) were dissolved in 2 ml of 2 M trifluoroacetic acid (TFA) in an ampoule (5 mL) (Fengel &Wegener, 1979). The ampoule was sealed under nitrogen atmosphere and was hydrolysed at 100 °C for 10 h to obtain individual monosaccharide units present. All the reaction mixtures were cooled to room temperature and were centrifuged at 1000 rpm for 5 min. Supernatants were collected and dried under reduced pressure and dry hydrolysates were dissolved in 1 mL distilled water. 10 µL each of the samples was then injected to the HPLC system. Shimadzu LC-MS 2020 equipped with RID 10A detector and SUPELCOSIL LC-NH_2 column (purchased from SIGMA ALDRICH) was used for analysis. Separation was conducted at 30 °C with the mobile phase acetonitrile:water (8:2) at flow rate of 0.8 mL/min. Identification of monosaccharide sugar units was done by comparing the retention time of sample with individual monosaccharide standards under same conditions.

3.2.2 Acetylation of glycolipids

Portions of each glycolipid were acetylated by treatment with 1 mL acetic anhydride in pyridine at 50 °C for 6 h (Ferrier et al., 1973). Completion of reaction was checked using TLC. The reaction mixture was dried under reduced pressure and acetylated glycolipids were separated in hexane. Acetylation was done to prevent rapid degradation of compounds while performing various spectroscopic characterizations

in different solvents. The acetylated samples were further purified using TLC.

Molecular weights of glycolipid samples were obtained though high resolution mass spectral (HRMS) analysis on a JEOL JM AX 505 HA instrument. Electrospray ionization (ESI) in positive mode was employed and hence masses of glycolipids were recorded as $[M+H]^+$. Using HRMS, it is able to obtain molecular masses accurate up to four decimal points. Glycolipids obtained in higher yields (G-2 & G-5) were taken for further spectroscopic characterization.

3.2.3 FT-IR analysis

Complete acetylation of hydroxy groups of glycolipids can be confirmed through FT-IR analysis. FT-IR spectra of acetylated glycolipids were recorded by FT-IR spectrophotometer (Perkin Elmer spectrum 100) in the range from 4000 to 650 cm^{-1}. The instrument was equipped with universal attenuated total reflectance accessory (ATR) which permits solid and liquid sample analysis with much spectral reproducibility.

3.2.4 NMR analysis

a) Proton and ^{13}C NMR

Major glycolipids G-2 and G-5 were taken for NMR characterization. Prior to the ^{1}H and ^{13}C solution state NMR spectroscopic experiments, aliquots of acetylated glycolipids were dissolved in acetone-d6. ^{1}H, ^{13}C and all 2D NMR measurements of G-2 were done using Bruker Avance III, 400MHz NMR spectrometer equipped with single-axis Z gradients

and 9.4 Tesla super-conducting magnet. ^{13}C experiments were conducted for 2 h (2500 scans) in order to get resolved spectra as the sample quantity is less. ^{1}H and 2D NMR measurements of acetylated glycolipid G-5 were conducted on Bruker Avance HD 700 MHz spectrometer with single-axis Z gradients and a standard-bore UltraShield Plus 16.44 Tesla magnet. ^{13}C and DEPT measurements were done on Bruker AV 500 MHz spectrometer with single-axis gradients and a narrow bore Bruker Ultrashield 11.75 Tesla magnet. ^{13}C experiments of G-5 were conducted overnight (>10000 scans) for getting well resolved spectra due to high molecular weight and less quantity of the sample.

b) DEPT NMR

Carbon multiplicity is determined from DEPT (distortionless enhancement by polarization transfer) NMR experiments obtained using standard Bruker software. Intensity of the signal in DEPT depends on magnitude of the flip angle and the number of proton attached to a carbon based on the formula, I = n sinθ cos^(n-1)θ. Therefore, 135° angle gives all CH and CH_3 in a phase opposite to CH_2 (Hore, 2015).

c) Heteronuclear single-quantum coherence (HSQC) spectroscopy

Heteronuclear single-quantum coherence (HSQC) analysis was performed using standard Bruker software to obtain a 2D heteronuclear chemical shift correlation map between directly-bonded ^{1}H and ^{13}C nuclei. 2D HSQC pulse sequence consists of four independent parts. An initial INEPT (Insensitive nuclei enhanced polarization transfer**)** pulse train transfers polarization from ^{1}H to ^{13}C via $^{1}J(C-H)$, followed by an

antiphase ^{13}C magnetization evolved during variable t1 evolution period, under the influence of ^{13}C chemical shift. Heteronuclear (^{1}H-^{13}C) couplings are refocused by applying a 180º 1H pulse at the middle of this period. Thereafter, ^{13}C magnetization is converted to in-phase ^{1}H magnetization by a retro-INEPT pulse train. Further, proton acquisition is performed with ^{13}C decoupling (Hore, 2015).

d) Correlation spectroscopy (2D COSY)

Diagonal and off-diagonal cross peaks in phase-sensitive ^{1}H-^{1}H correlation spectroscopy (COSY) spectra were generated by performing suitable data acquisition program followed by double Fourier transform in Bruker NMR software. Diagonal peaks shows in-phase negative pure dispersion pattern, whereas off-diagonal peaks shows anti-phase pure absorption pattern with respect to active J-coupling, and in-phase with all passive J-couplings in phase-sensitive spectra. Hence off-diagonal peaks reveal J-coupling connectivity (Hore, 2015).

e) Heteronuclear multiple bond correlation (HMBC) spectroscopy

Two dimensional heteronuclear multiple bond correlation (HMBC) experiment was performed to obtain a 2D heteronuclear chemical shift correlation map between long-range coupled ^{1}H and ^{13}C. In HMBC analysis, an optimal low pass J-filter is used after the initial 90º 1H pulse to minimize direct responses. Defocusing period is optimized to ½ $^{n}J(CH)$ (5-10 Hz) and refocusing period is omitted. Proton acquisition is performed without ^{13}C decoupling. The interpulse d2 delay is usually optimized to ½ ^{n}JCH (50-70 ms) in HMBC experiments (Hore, 2015).

3.2.5 Mass spectroscopic analysis

LC-MS/MS analysis of glycolipids (G-1 to G-6) was performed on a 4000 QTRAP LC-MS/MS system (AB Sciex, Foster City, CA, USA) equipped with a Turbo Ion Spray (electrospray ionization; ESI). LC-MS/MS method for glycolipid analysis was generated according to the literature reported by Auray-Blais et al. (2011). A multiple reaction monitoring (MRM) experiment giving a precursor molecular ion (Q1) and a respective fragment ion (Q3) was normally applied. The instrument was operated in a positive-ion $[M + H]^+$ mode. The quadrupoles, Q1 and Q3, were tuned with unit resolution, and the MS conditions were optimized for maximum signal intensity.

3.2.6 GC-MS analysis of fatty acid fragments of glycolipids

Individual glycolipid fractions were hydrolysed using 6% methanolic KOH for 3h to convert fragmented fatty acids into their potassium salts and partitioned between CH_2Cl_2 and water. Polar fraction was acidified with 6 N HCl and the resulting free fatty acids were extracted with CH_2Cl_2. Dried fatty acid samples were converted into fatty acid methyl esters (FAME) using BF_3/methanol and used for GC-MS analysis (Harvey, 1994). GC-MS analysis was performed using gas chromaotography (Perikin Elmymer Clarus 680) coupled with mass spectrometer (Perikin Elmymer Clarus 600). 1µL of sample was injected to GC equipped with non-polar HP ultra-double fused silica capillary column. Initially, the temperature was increased from 50 °C to 200 °C at a rate of 2 °C per min and held at 200 °C for 5 min. Then, the temperature was again increased from 200 °C to 280 °C at a rate of 10 °C per min and

held at 280 °C for 10 min. MS operating parameters were as follows: ionization energy - 70 eV; ion source temperature - 200 °C, solvent delay - 4 min and scan range 40–600 u. Identification of the components was based on direct comparison of mass spectral data with NIST library version 2.1.

3.2.7 Determination of specific rotation

Purified samples were used to estimate specific rotation. Specific rotation of 0.1% glycolipid samples in acetone were measured at 25 °C ($[\alpha]_D$ (25 °C) using a 10 cm cylindrical cell and the sodium D line with an optics manual polarimeter.

3.3 Results and discussion

3.3.1 Analysis of sugars

Sugar analysis results of glycolipids (G-1 to G-6) obtained from HPLC are as shown in Table 3.1. Sugar residues were confirmed in comparison with retention times of monosaccharide standards. Sugar molecules obtained from glycolipids G-1 to G-4 were found to show similar retention times as that of galactose (~ 10.6 min), whereas, sugars from G-5 and G-6 were found at retention time ~ 7.7 min similar to xylose units (Figure 3.1). Molecular weights of the acetylated glycolipids obtained through HRMS analyses along with yield of each compound are also given in Table 3.1. It is observed that glycolipids G-2 and G-5 only possess significant quantities for complete structural characterisation.

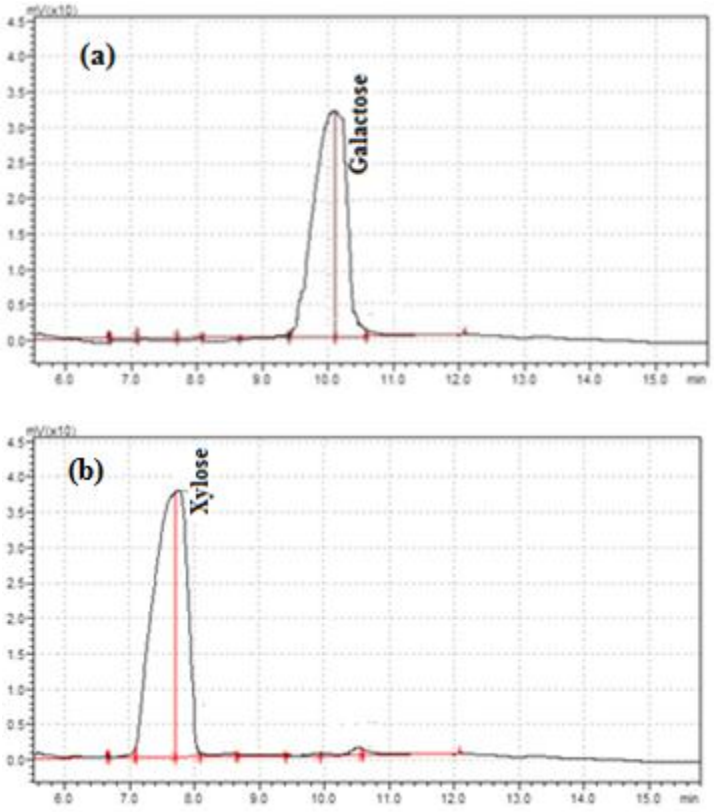


Figure 3.1 Representative chromatographs of sugar analysis of glycolipids, galactose units of glycolipid G-2 (a); xylose units of glycolipid G-5 (b)

Table 3.1 Sugar composition and molecular weights of glycolipids

Compound	Yield	Sugar	Mass (HRMS) of acetylated glycolipids $[M+H]^+$ (m/z)
G-1	2.8 mg	Galactose	928.6344
G-2	12.7 mg	Galactose	949.5289
G-3	1.7 mg	Galactose	1131.4764
G-4	3.2 mg	Galactose	1213.5478
G-5	10.6 mg	Xylose	1223.4576
G-6	0.5 mg	Xylose	1251.6785

3.3.2 Structural characterization of acetylated glycolipid G-2

Molecular mass of acetylated glycolipid G-2 was recorded as m/z 949.5289 $[M+H]^+$. According to earlier TLC analysis (Section 2.3.3, Chapter 2), G-2 is expected as monoglycosyl diglyceride. Complete acetylation of G-2 was confirmed by FT-IR analysis (Figure 3.2). The broad absorption band at 3500-3300 cm^{-1} representing free –OH groups are fully absent in the spectra which ensured protection of hydroxyl groups through acetylation. FT-IR spectra of G-2 displayed characteristic peaks of glycolipids along with peaks showing presence of unsaturation (Singh & Tiwary, 2016) (3010 cm^{-1} and 1680 cm^{-1}), as already discussed in Chapter 2. Further with the use of NMR, LC/MS/MS and fragmentation studies, chemical structure of G-2 is established as shown in Figure 3.3. Assignments of carbon and hydrogen atoms along with multiplicities are given in Table 3.2.

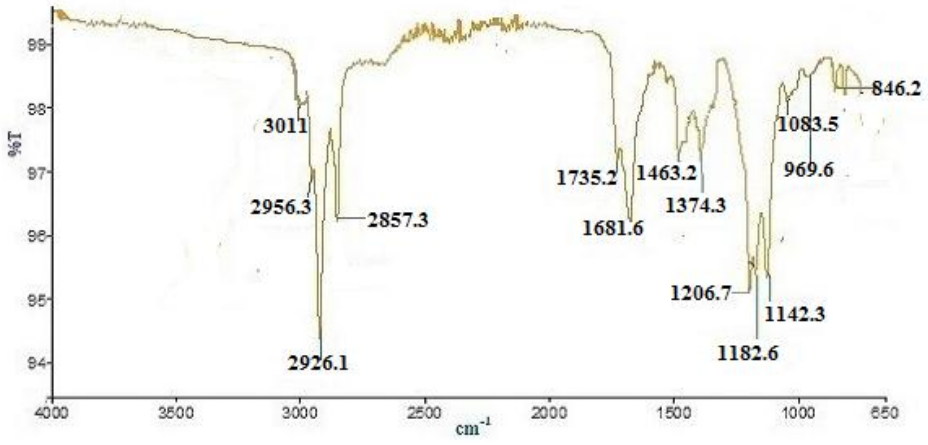


Figure 3.2 FT-IR spectra of glycolipid G-2

Based on sugar analysis, it is observed that G-2 contain galactose as the sugar unit. ^{1}H NMR spectra of G-2 showed four singlets of equal intensity from δ 1.79 to δ 2.00, which may be due to methyl protons of four acetylated hydroxyl groups of galactose residue (Figure 3.4). There are multiplets at δ 0.75 which may also represent other methyl protons (18" and 18"') in glycolipid G-2. Another peak at δ 1.16 having high intensity may be due to methylene protons present in fatty acid side chain of glycolipid. There is no peak beyond 5.23 in the ^{1}H NMR spectra which confirmed aliphatic nature of glycolipid G-2. Further assignment of peaks in proton NMR was made in comparison with ^{13}C NMR spectra.

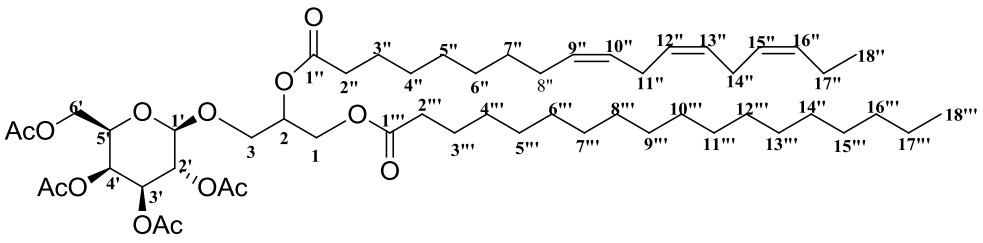


Figure 3.3 Chemical structure of glycolipid G-2

Table 3.2 NMR spectroscopic data of glycolipid G-2 (400 MHz, acetone-d^6)

Position	**δ_C**		**δ_H/ mult., J (Hz)**
1a, 1b	62.12	CH_2	4.02 dd (5.8, 11.6)/4.05 dd (3.2,11.6)
2	71.57	CH	4.98 m
3a, 3b	68.45	CH_2	3.85 dd (5.6, 11.2)/ 3.68 dd (5.6, 11.2)
1’	102.22	CH	4.64 d (6.9)
2’	71.57	CH	5.09 m
3’	68.28	CH	5.25 m
4’	70.72	CH	4.99 m
5’	69.64	CH	5.08 m
6’a, 6’b	63.00	CH_2	4.04 dd (6.8, 11.8)/4.22 dd (4.7, 11.8)
1’’	173.12	qC	
1’’’	173.35	qC	
2’’	34.69	CH_2	2.21 m
2’’’	34.36	CH_2	2.21 m
3’’	32.64	CH_2	1.16 m
3’’’	32.24	CH_2	1.16 m
4’’	30.41	CH_2	1.16 m
4’’’	30.41	CH_2	1.16 m
5’’	30.26	CH_2	1.16 m
5’’’	30.41	CH_2	1.16 m
6’’	30.07	CH_2	1.16 m
6’’’	30.413	CH_2	1.16 m
7’’	29.78	CH_2	1.19 m
7’’’	30.413	CH_2	1.16 m
8’’	27.82	CH_2	1.20 m
8’’’	30.26	CH_2	1.16 m
9’’	128.56	CH	5.24 m

Table 3.2 Continued…

9’’’	30.26	CH_2	1.16 m
10’’	128.88	CH	5.25 m
10’’’	30.26	CH_2	1.16 m
11’’	25.27	CH_2	2.73 m
11’’’	30.37	CH_2	1.16 m
12’’	128.97	CH	5.23 m
12’’’	30.07	CH_2	1.16 m
13’’	129.06	CH	5.25 m
13’’’	29.78	CH_2	1.16 m
14’’	25.66	CH_2	2.73 m
14’’’	27.82	CH_2	1.19 m
15’’	130.43	CH	5.23 m
15’’’	27.53	CH_2	1.19 m
16’’	130.93	CH	5.25 m
16’’’	26.24	CH_2	1.20 m
17’’	27.53	CH_2	1.20 m
17’’’	23.33	CH_2	1.20 m
18’’	14.34	CH_3	0.75 m
18’’’	14.34	CH_3	0.75 m
2’-OAc	20.50, 169.77	CH_3, CO	1.79 s
3’-OAc	20.55, 170.22	CH_3, CO	1.86 s
4’-OAc	20.59, 170.56	CH_3, CO	1.90 s
6’-OAc	20.73, 170.75	CH_3, CO	2.00 s

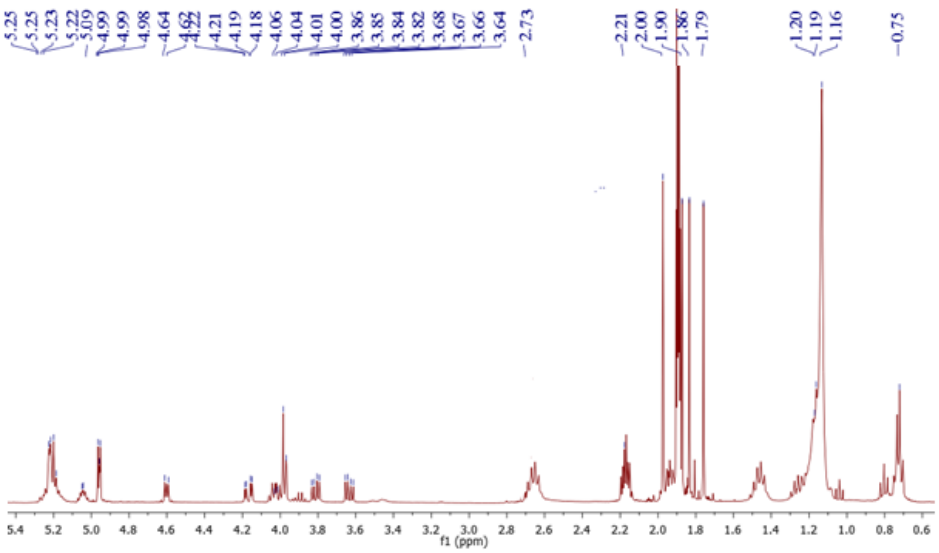


Figure 3.4 ^{1}H NMR spectra of glycolipid G-2

^{13}C NMR spectra also showed four peaks of similar intensity at δ_C 20.50, 20.55, 20.59 and 20.73. This can be assigned to four methyl groups in acetylated galactose (Figure 3.5). Single peak at δ_C 102.22 indicates presence of glycosidic linkage. There are six peaks from δ_C 128.56 to δ_C 130.93 which most probably indicate unsaturation (9", 10", 12", 13", 15", 16") in glycolipid fatty acid chain. Four distinct peaks from δ_C 169.77 to δ_C 170.75 can be indicative of the four carbonyl groups (2'-OAc, 3'-OAc, 4'-OAc, 6'-OAc) in acetyl moieties of galactose. Two peaks at δ_C 173.12 and δ_C 173.35 suggest that it is obtained from ester linkage (1"and 1"') of the fatty acid chains.

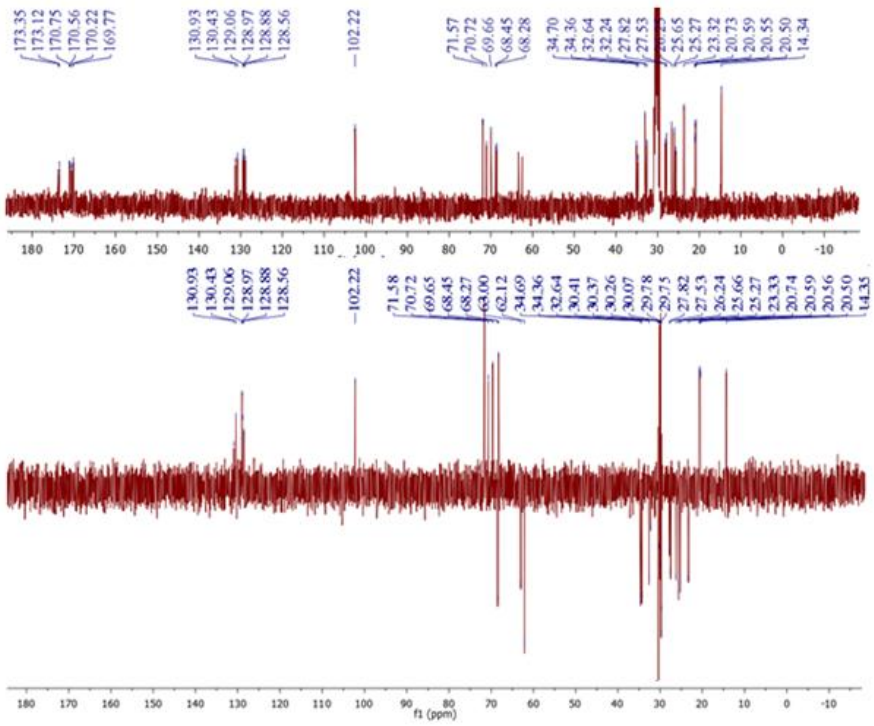


Figure 3.5 ^{13}C NMR (upper) and DEPT 135 (lower) NMR spectra of glycolipid G-2

DEPT 135 NMR measurements were used to differentiate methine (CH) and methyl (CH_3) carbon atoms from methylene (CH_2) carbon atoms. It is observed that all ^{13}C peaks found between δ_C 23.33 and δ_C 34.69 represent methylene groups, most probably found in the fatty acid side chain. There are two peaks of similar intensity at 34.69 and 34.36 which may be due to methylene groups (2" and 2"') attached to carbonyl groups in two distinct fatty acid chains. There are three CH_2 peaks at δ_C 62.12, 63.00 and 68.45. This may be due to two CH_2 groups in glyceride moiety (1 and 3) and one in galactopyranose ring (6'). Four methine peaks from δ_C 68.28 to 71.57 indicated four acetylated CH groups (2', 3', 4', 5')

in galactose ring. Six peaks from δ_C 128.56 to 130.93 were found to represent methine (CH) carbon atoms (9", 10", 12", 13", 15", 16") and therefore it indicated presence of three double bonds probably within fatty acid chain. All peaks from δ_C 169.77 to δ_C 170.75 were found to be absent in DEPT spectra which gave confirmation to the presence of quaternary carbonyl groups (2'-OAc, 3'-OAc, 4'-OAc, 6'-OAc) in that part of ^{13}C NMR spectra.

HSQC spectra were used to confirm the number and nature of carbon atoms and particularly to assign proton shifts for each carbon atom in comparison with ^{13}C and DEPT NMR measurements. Methine (CH) and methyl (CH_3) carbon atoms were observed as blue contours and methylene carbon atoms (CH_2) were observed as red contours in HSQC spectra of G-2 (Figure 3.6). It is inferred from the spectra that peak at δ_C 14.34 in the ^{13}C spectra have correlation with muliplet found at δ_H 0.76. This may represent the two terminal methyl goups (CH_3) (18" & 18"') of two distinct fatty acid chains. Methine carbon atom at δ_C 102.22 is found to have correlation with δ_H 4.64 and therefore doublet at δ_H 4.64 may correspond to 1' CH proton of galactose ring at the glycosidic linkage position. Methylene carbon atom (CH_2) at 68.45 is found to have correlation with two different protons at δ_H 3.85 (dd) and 3.68 (dd). This may represent CH_2 group at position 3 of glyceride moiety which is linked to sugar unit through glycosidic bond. Similarly methylene carbon atom at δ_C 63.00 is found to have correlation with δ_H 4.22 (dd) and 4.04 (dd) and this may represent 6' CH_2 protons of galactose residue. Another CH_2 carbon atom at δ_C 62.12 is correlated with δ_H 4.02 (dd) and 4.05 (dd). This may denote methylene protons in

the carbon number 1 of glyceride moiety, which is ester linked to fatty acid chain. Methine carbon atom at δ_C 71.57 from ^{13}C spectra is found to have correlation with δ_H 4.94 and 5.00.

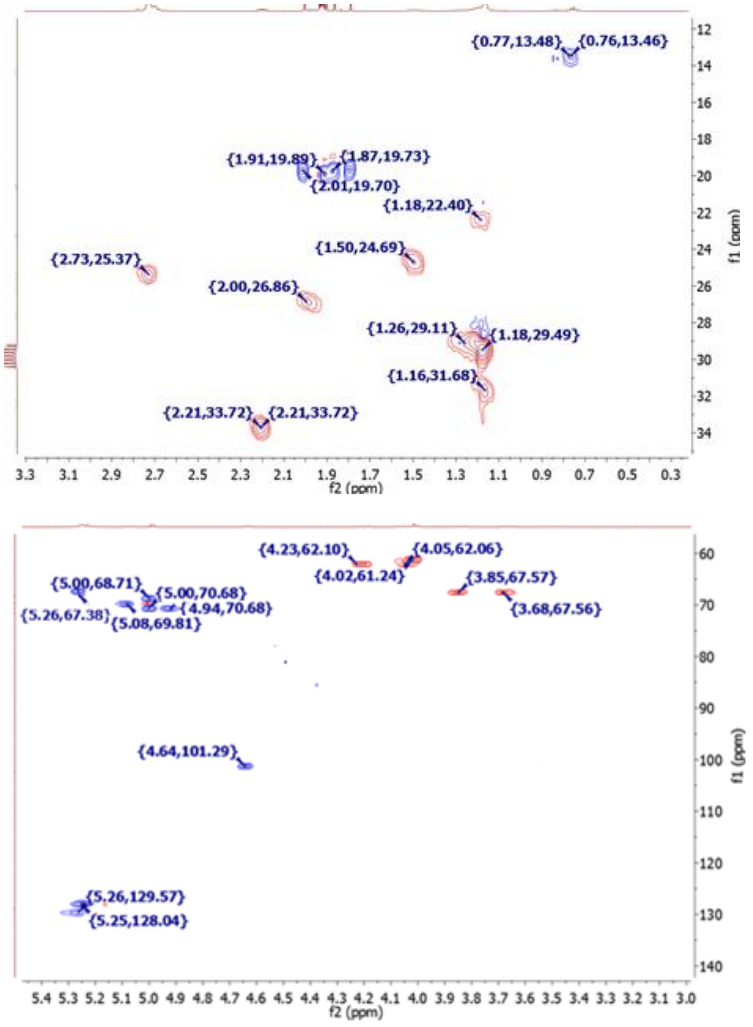


Figure 3.6 HSQC spectra of glycolipid G-2

Hence, they found to represent two different carbon atoms. δ_H at 4.94 may correspond to CH group in position 2 of glyceryl moety. δ_H at

5.00 may correspond to CH group at 2' position of galactose ring. Four peaks at δ_C 68.28, 69.66, 70.72 (from ^{13}C spectra) (Table 3.2) corresponding to multiplets from δ_H 5.00 to 5.24 can be assigned to 3', 4' and 5' positions respectively of the galactose ring. Six unsaturated CH groups ((9", 10", 12", 13", 15", 16") observed between 128.56 – 130.93 are found to have correlation with the multiplets observed in δ_H 5.25-5.26. One group signal at δ_C 102.22 and δ_H 4.64 (d, J=6.9 Hz, H-1') along with other CH signals from δ_C 68.28-71.57 suggested a β-D galactopyranose unit (Andrianasolo et al., 2008). D-configuration of the sugar is confirmed by comparing its optical rotation ($[\alpha]_D$ (25 °C) +51.9) with that reported in literature for an authentic sample i.e., optical rotation $[\alpha]_D$ (25 °C) +52.8 in H_2O (Takahashi & Ono, 1973; Larsen et al., 2003).

Spatial correlations were revealed from ^1H-^1H COSY and ^1H-^{13}C HMBC spectra. COSY NMR correlation of δ_H 3.85 and 3.68 with δ_H 5.08 indicated interaction between CH and CH_2 protons at 2 and 3 positions respectively of glyceride moiety (Figure 3.7). Correlation between 1' and 2' positions (δ_H 4.64, 5.00) of galactose residue is also visible from COSY spectra. Similar interaction is also observed between 6'CH_2 and 5'CH protons of galactose. Correlation among hydrogen atoms on adjacent carbon atoms of sugar moiety and also of the glyceride unit can be clearly understood from the COSY spectra. Olefinic protons at δ_H 5.23 were found to have correlation with δ_H 2.73 and 2.04. The strong signal at δ_H 2.73 corresponding to δ_C 25.37 obtained from HSQC, gave inference to the presence of two bis-allylic methylene carbons in fatty acid chain. Since bis-allylic carbon signals of *Z* and *E*-isomers are normally observed at *δ*C ~26 and ~32, respectively

(Scribe et al., 1988; Ishii et al., 2006), the 25.37ppm shift suggests the double bonds to have a cis geometry (*Z*).

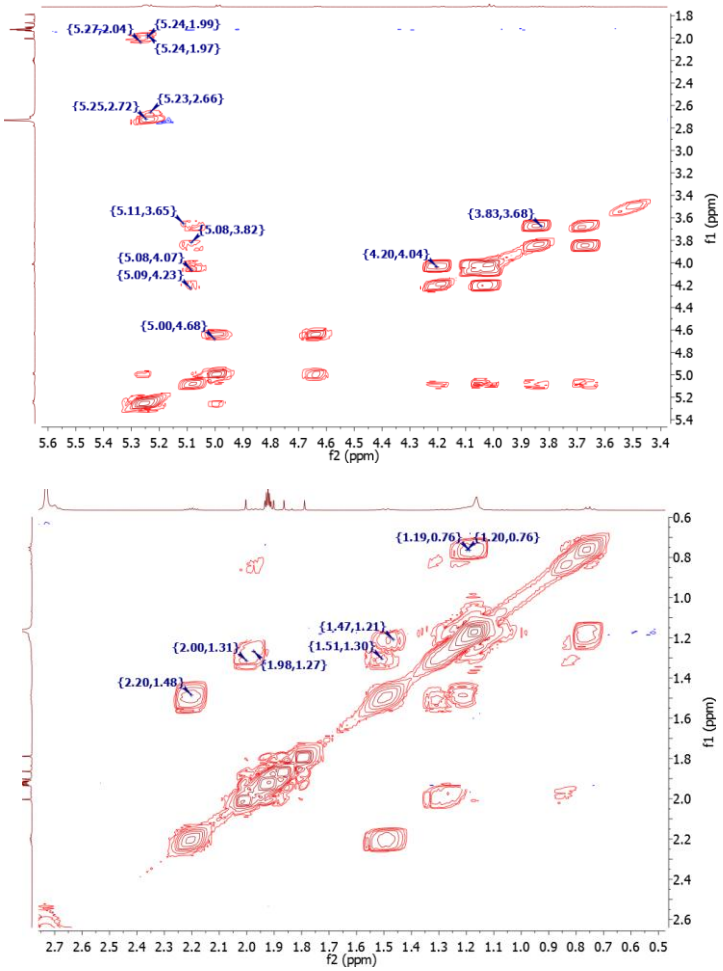


Figure 3.7 COSY spectra of glycolipid G-2

The glyceride structure is further confirmed from HMBC correlation between 1 and 3 positions (δ_H 3.83, δ_C 61.17; δ_H 4.02, δ_C 67.44) (Figure 3.8). Glycosidic bond connecting sugar and glyceride moiety was also inferred from spatial relation between 1'carbon atom (δc 101.56) of

galactose residue, and CH_2 proton (δ_H 3.70) at position 3 of glyceride moiety, found in the HMBC spectra. Two –CO- groups found at δ_C 172.24 and δ_C 172.10 are found to be correlated to methylene protons at δ_H 2.21 and 2.18 respectively. This gave indication on the presence of two fatty acyl chains connected to two non-glycosylated hydroxy groups of glyceride through ester linkage. Major COSY and HMBC correlations of glycolipid G-2 are represented in Figure 3.9.

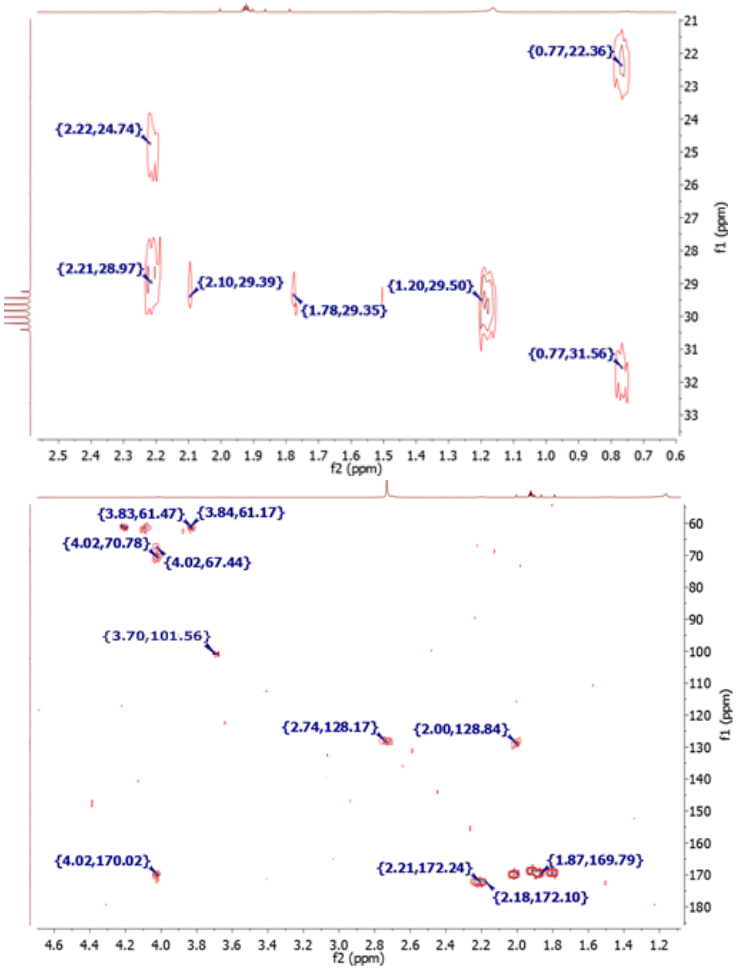


Figure 3.8 HMBC spectra of glycolipid G-2

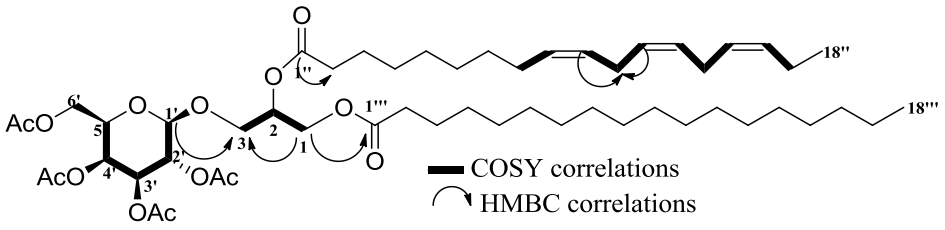


Figure 3.9 Key COSY and HMBC correlations of glycolipid G-2

To confirm the assignment of G-2, ESI-MS/MS was used on protonated molecular ion (HRMS m/z = 949.5289 for $[C_{53}H_{88}O_{14}+H]^+$) (Table 3.1) in positive ion mode. Two peaks at m/z 672.7 and m/z 666.1 observed in mass fragmentation spectrum of G-2 (Figure 3.10) were attributed to loss of α-linolenic acid (18:3 n-3) and stearic acid (18:0) respectively. As the loss of fatty acid linked to sn-1 position of glycosyldiglycerides produces a more intense peak, larger intensity of m/z = 672.75 fragment indicates that α-linolenic acid is esterified at this position in the glyceride (Guella et al., 2003). Signal for the loss of sugar unit (m/z 618.34) was also identified, as were acetylated galactose itself (m/z 332.76). Other m/z peaks observed may be due to multiple fragmentations and deacetylations. Peak at m/z 163.23 attributed to non-acetylated galactose fragment give further evidence for presence of hexose ring in the structure (Soulé et al., 2000). Fragmentation of the compound is predicted based on the MS/MS spectra (Figure 3.11). GC-MS analysis of fatty acids had given further confirmation to the exact nature of fatty acids. Two fatty acid peaks were observed at m/z 293 and 299 corresponding to methyl esters of α-linolenic acid (18:3 n-3) and stearic acid (18:0) respectively (Figure 3.12). Specific rotation $[\alpha]_D$ (25 °C) of the compound was found to be -8°.

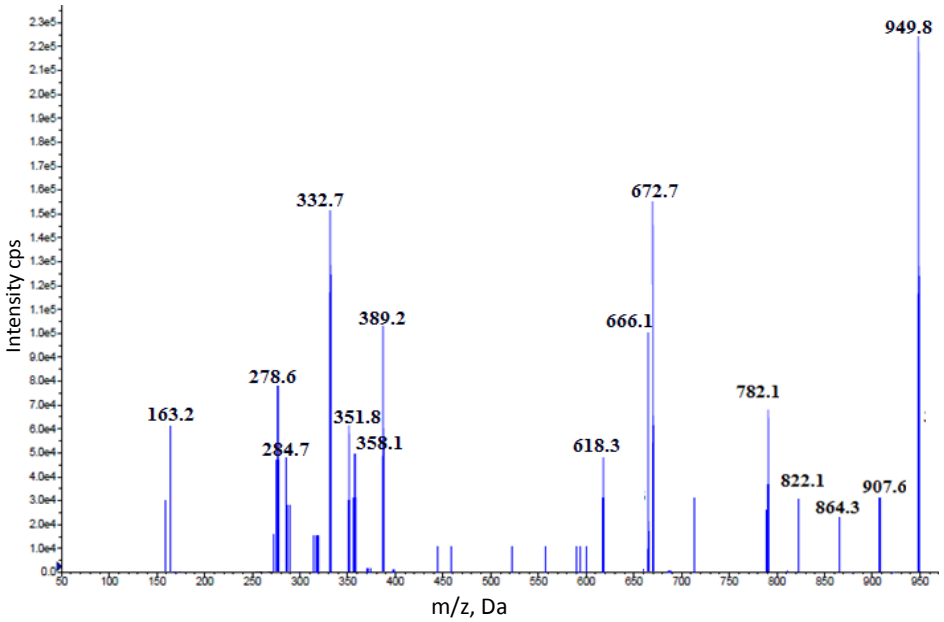


Figure 3.10 LC-MS/MS spectra of Galactosyl glycolipid G-2

The configuration at C-2 in the glyceride moiety of G-2 is presumed to be *S* on the basis of comparison of specific rotation with literature values. Structure of G-2 was found to be similar to monogalactosyldiacylglycerols isolated from several marine diatoms and cyanobacteria (Tokuda et al., 1996; d'Ippolito et al., 2004; Cutignano et al., 2006; Andrianasolo et al., 2008; Shan et al., 2016). Monogalactosyl diacylglycerols are known for the specific roles played by them in light-initiated reactions of photosynthesis and for considerable bioactivities, which include antiviral (Bergsson et al., 2001), antimitotic (Williams et al., 2007), tumor suppressor (Morimoto et al., 1995; Mizushina et al., 2012), antistress (Gupta et al., 2007) and anti-inflammatory properties (Larsen et al., 2003; Lopes et al., 2014). β-D-galactosyl glycolipid containing polyunsaturated fatty acid in the side chain, isolated from marine diatom

Phaeodactylum tricornutum was found to possess apoptosis inducing properties (Andrianasolo et al., 2008). This gave evidence for suitability of monogalactosyl glycolipid G-2 for anticancer studies. From all the spectral data, structure of G-2 is finalized as (2S)-2-O-9,12,15-octadecatrienoyl-1-O-octadecanoyl-3-O-[β-D-galactopyranosyl]-glycerol.

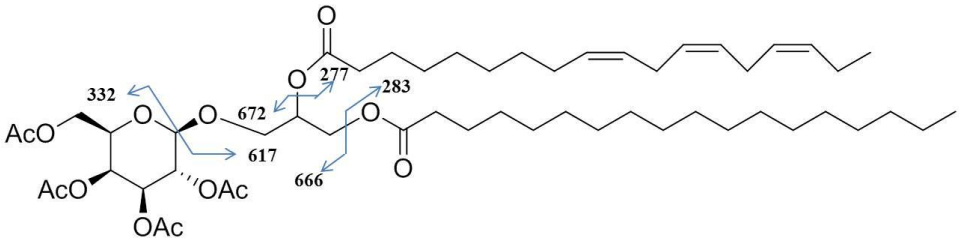


Figure 3.11 Fragmentation of glycolipid G-2 based on LC/MS analysis

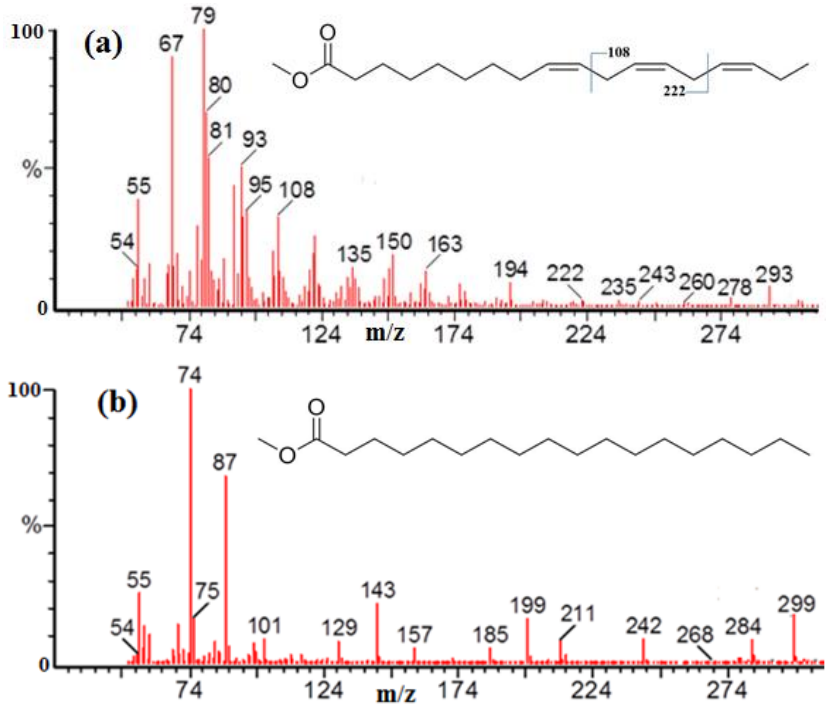


Figure 3.12 GC-MS mass spectra of α-linolenic acid (18:3 n-3) (a) and stearic acid (18:0) (b) obtained after fragmentation of G-2.

3.3.3 Structural characterization of acetylated glycolipid G-5

Molecular mass of acetylated glycolipid G-5 was recorded as m/z 1223.4576 $[M+H]^+$ through HRMS analysis. Sugar analysis of G-5 indicated that it contain xylose as the sugar unit (Table 3.1). Complete acetylation of hydroxyl groups of G-5 was verified by FT-IR analysis (Figure 3.13). Broad absorption band at 3500-3300 cm^{-1} was absent in the spectra which ensured protection of hydroxyl groups through acetylation. Bands in the region 1080 and 848 were found to be more intense in G-5 compared with G-2 which indicated more number of glycosidic linkages, as already discussed in chapter 2. TLC analysis of G-5 had shown that it is more polar than the galactosyl glycolipids (Section 2.3.3, Chapter 2). Therefore, it may contain more number of hydroxyl groups. Further with the use NMR, LC/MS/MS analysis and fragmentation studies chemical structure of G-5 is predicted as shown in Figure 3.14. Assignments of carbon and hydrogen atoms along with multiplicities are given in Table 3.3.

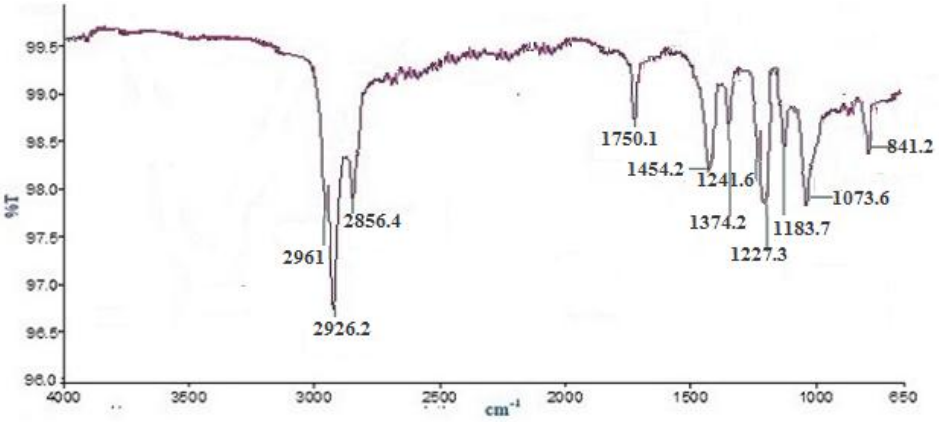


Figure 3.13 FT-IR spectra of xylosyl glycolipid G-5

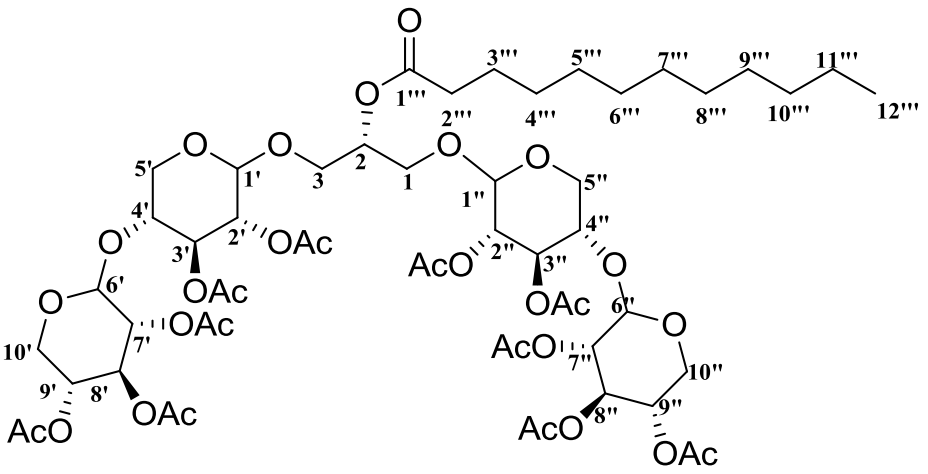


Figure 3.14 Chemical structure of xylosyl glycolipid G-5

Table 3.3 NMR spectroscopic data of glycolipid G-5 {700 MHz (^{1}H); (500 MHz (^{13}C), acetone-d^{6}}

Position	**δC**		**δ_H/ mult., J (Hz)**
1a/1b; 3a, 3b	67.42	CH_2	3.80, dd (11.2, 5.6)/ 3.97, dd (11.2, 5.6)
2	69.95	CH	5.18, m
1’	101.24	CH	4.78 d (8.2)
1”	101.24		4.78 d (8.2)
2’	68.72	CH	5.46, m
2”	68.72	CH	5.46, m
3’	67.36	CH	5.31, m
3”	67.36	CH	5.31, m
4’	70.64	CH	4.21, m
4”	70.64	CH	4.21, m
5’a/5’b	61.22	CH_2	4.15, dd (11.2, 4.9)/ 4.32, dd (11.9, 4.2)
5”a/5”b	61.22	CH_2	4.15, dd (11.2, 4.9)/ 4.32, dd (11.9, 4.2)
6’	101.28	CH	4.78 d (8.2)
6”	101.28	CH	4.78 d (8.2)

Table 3.3 Continued…

7’	68.72	CH	5.39 m
7”	68.72	CH	5.39 m
8’	70.61	CH	5.18 m
8”	70.61	CH	5.18 m
9’	70.64	CH	5.13 m
9”	70.64	CH	5.13 m
10’a/10’b	61.94	CH_2	4.15, dd (11.2, 4.9)/ 4.31, dd (11.9, 4.2)
10”a/10”b	61.94	CH_2	4.15, dd (11.2, 4.9)/ 4.31, dd (11.9, 4.2)
1’’’	172.63	CO	
2’’’	33.51	CH_2	2.32 m
3’’’	31.73	CH_2	1.61 m
4’’’	29.49	CH_2	1.30 m
5’’’	29.47	CH_2	1.30 m
6’’’	29.44	CH_2	1.30 m
7’’’	29.16	CH_2	1.30 m
8’’’	29.13	CH_2	1.30 m
9’’’	28.85	CH_2	1.30 m
10’’’	24.71	CH_2	1.30 m
11’’’	22.42	CH_2	1.30 m
12’’’	13.45	CH_3	0.89 m
2’, 2”-OAc	19.61, 168.95	CH_3, CO	1.93 s
3’, 3”-OAc	19.66, 169.36	CH_3, CO	2.01 s
7’,7”-OAc	19.69, 169.55	CH_3, CO	2.04 s
8’, 8”-OAc	19.81, 169.71	CH_3, CO	2.04 s
9’, 9”-OAc	20.00, 169.89	CH_3, CO	2.15 s

^{1}H NMR spectra of G-5 showed four singlets from δ1.93-δ2.15 (Figure 3.15). The singlet at δ2.04 was having doubled intensity compared to other three singlets. This may be due to five equivalent methyl protons of acetylated hydroxy groups (2', 2"; 3', 3"; 7', 7", 8', 8"; 9', 9"). The triplet observed at δ 0.89 may corresponds to other methyl protons in the glycolipid (12"'). High intense peak observed at δ1.30 can be due to CH_2 protons of similar environment in fatty acid side chain of glycolipid. There was no peak beyond 5.46 in the ^{1}H NMR spectra which confirmed aliphatic nature of glycolipid G-5. Further assignment of peaks in proton NMR was made in comparison with ^{13}C NMR spectra.

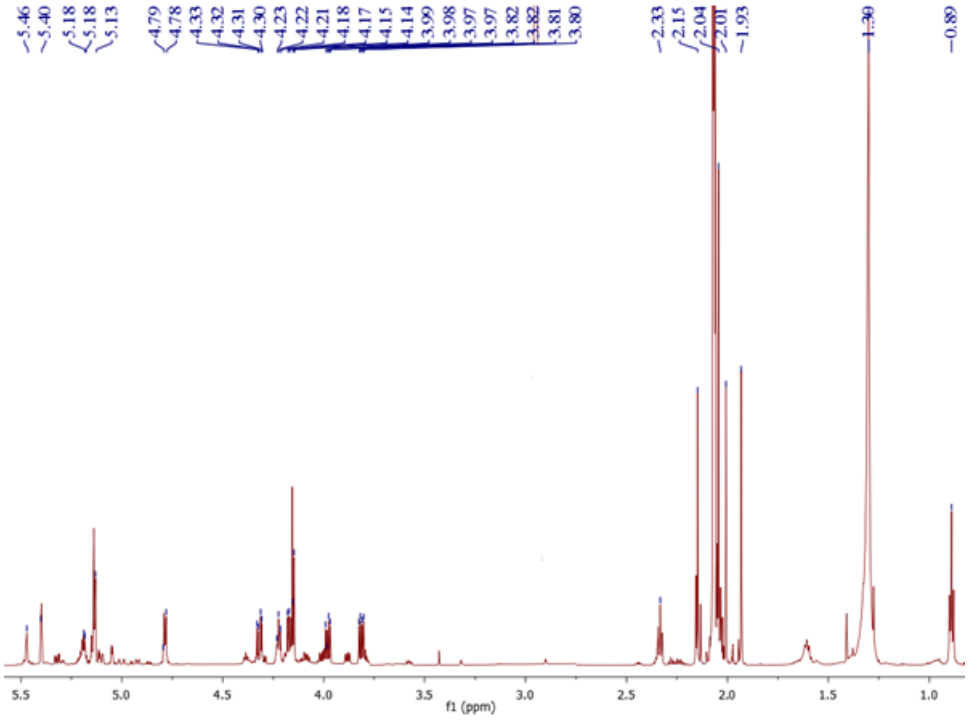


Figure 3.15 ^{1}H NMR spectra of xylosyl glycolipid G-5

^{13}C NMR spectra of G-5 showed five singlets from δ19.61 - δ 20.00 (Figure 3.16). This can be assigned to five methyl carbon atoms, as indicated from ^{1}H NMR spectra. Two peaks were found at δ 101.24 and δ 101.28 which indicate existence of two glycoside linkage positions. Five distinct peaks from δ 168.95 – δ 169.89 gave indication to five carbonyl groups in the acetylated sugar residue. As the sugar unit present is found to be xylose (Table 3.1), five acetylated hydroxyl groups along with two glycosidic linkage positions have given inference to a dixylosyl moiety in the structure. One peak at δ_C 172.65 suggests that it may come from ester linkage of fatty acid chain.

Methine (CH) and methyl (CH_3) carbon atoms were differentiated from methylene (CH_2) carbon atoms using DEPT 135 NMR measurements (Figure 3.16). It was observed that all ^{13}C peaks found between δ_C 22.42 and δ_C 33.51 correspond to CH_2 groups, probably arise from fatty acid side chain. Peak at δ_C 33.51 may represent carbon atom adjacent to carbonyl group of fatty acid chain. Two CH_2 peaks observed at δ_C 61.22, 61.94 can be assigned to methylene carbons at 5', 5" and 10', 10" positions respectively of the sugar chain. Another CH_2 peak observed at δ_C 67.42 may correspond to equivalent methylene carbon at 1, 3 position of glyceride moiety. Five methine peaks from δ_C 67.36 to 70.65 indicated five acetylated CH groups in the dixylopyranosyl moiety (2', 2"; 3', 3"; 7', 7", 8', 8"; 9', 9"). Peaks from δ_C 168.95 to δ_C 172.65 were not detected in DEPT spectra which give confirmation for quaternary carbonyl groups in that part of ^{13}C spectra.

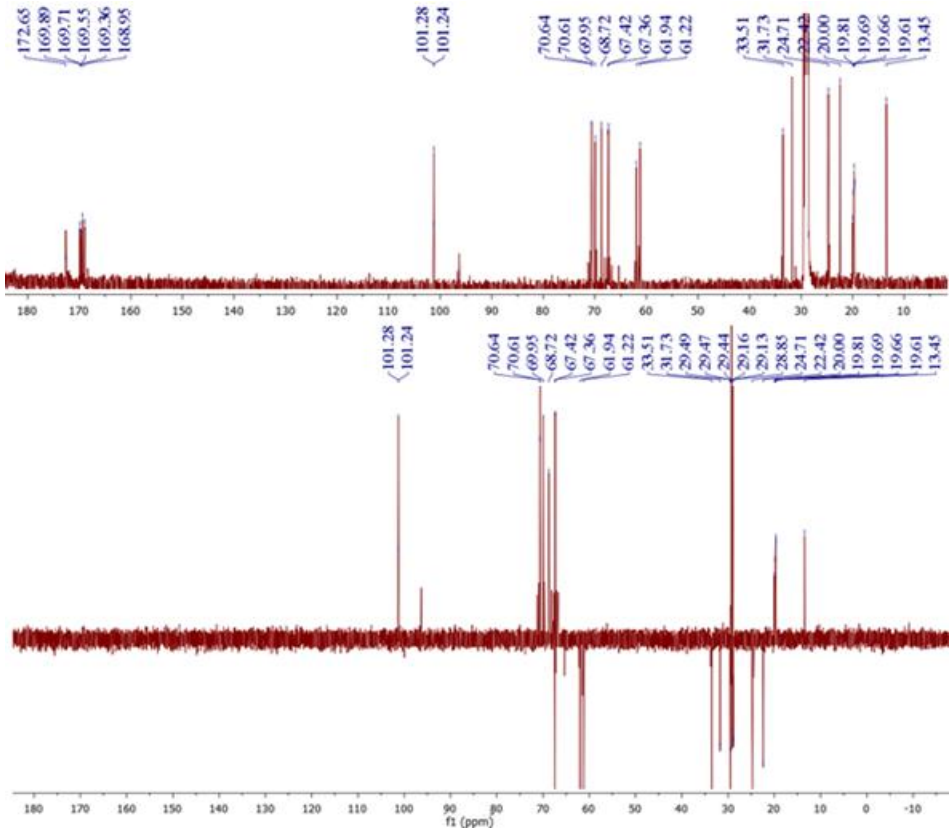


Figure 3.16 ^{13}C NMR (upper) and DEPT 135 (lower) NMR spectra of glycolipid G-5

HSQC spectra are also used to determine number and nature of carbon atoms and at the same time to get proton chemical shifts corresponds to each carbon atom (Figure 3.17). Methine (CH) and methyl (CH_3) carbon atoms were observed as red contours and methylene carbon atoms (CH_2) were observed as blue contours. Peak at δ_C 13.48 was found to be a methyl carbon atom and have correlation with a triplet at δ_H 0.89. This may represent terminal methyl goup (CH_3) in the fatty acid chain (12'''). Methine carbon atoms at δ_C 101.24 and δ_C

101.28 are found to have correlation with multiplets observed at δ_H 4.776-4.787. These two equivalent sets of CH groups may correspond to 1', 1" and 6', 6" positions of the dixylose. Methylene carbon atom (CH_2) at 67.42 is found to have correlation with two distinct δ_H 3.80 (dd) and 3.97 (dd). This may be due to the different chemical environments of protons in the same CH_2 groups. Hence, it represents two equivalent sets of CH_2 groups at 1, 3 positions of glyceride moiety which are connected to the sugar chain through glycosidic linkage. Methylene carbon atom at δ_C 61.22 is found to have correlation with δ_H 4.15 (dd) and 4.32 (dd) and this may correspond to CH_2 groups at 5', 5" positions of xylosyl residue. Similarly, another CH_2 carbon atom at δ_C 61.94 is found to have correlation with δ_H 4.15 (dd) and δ_H 4.31 (dd). This may denote methylene protons at 10', 10" positions of dixylosyl moiety. CH peak at δ_C 70.64 corresponds to two δ_H shifts at 4.22 and 5.13. Peak showing correlation between δ_C 70.64 and δ_H 5.314 may represent CH group at position 2 of glyceride moiety which is attached to the fatty acid chain. δ_C 70.64 and δ_H 4.217 may correspond to 4', 4" positon of xylopyranose ring which is O-linked to the other xylose residue. Methine peaks observed between δ_C 68.72 and δ_C 70.61 correlated to multiplets at δ_H 5.134 - δ_H 5.397 may represent other CH groups in the dixylose residue. Signal observed at δ_C 101.24 and δ_H 4.787 (d, J=8.2 Hz) suggest presence of β D-xylose residue (Chaturvedula & Prakash, 2011). A D-xylose form is proposed, due to its natural abundance (Yemm & Willis, 1954) and is confirmed by comparing with standards (Table 3.1).

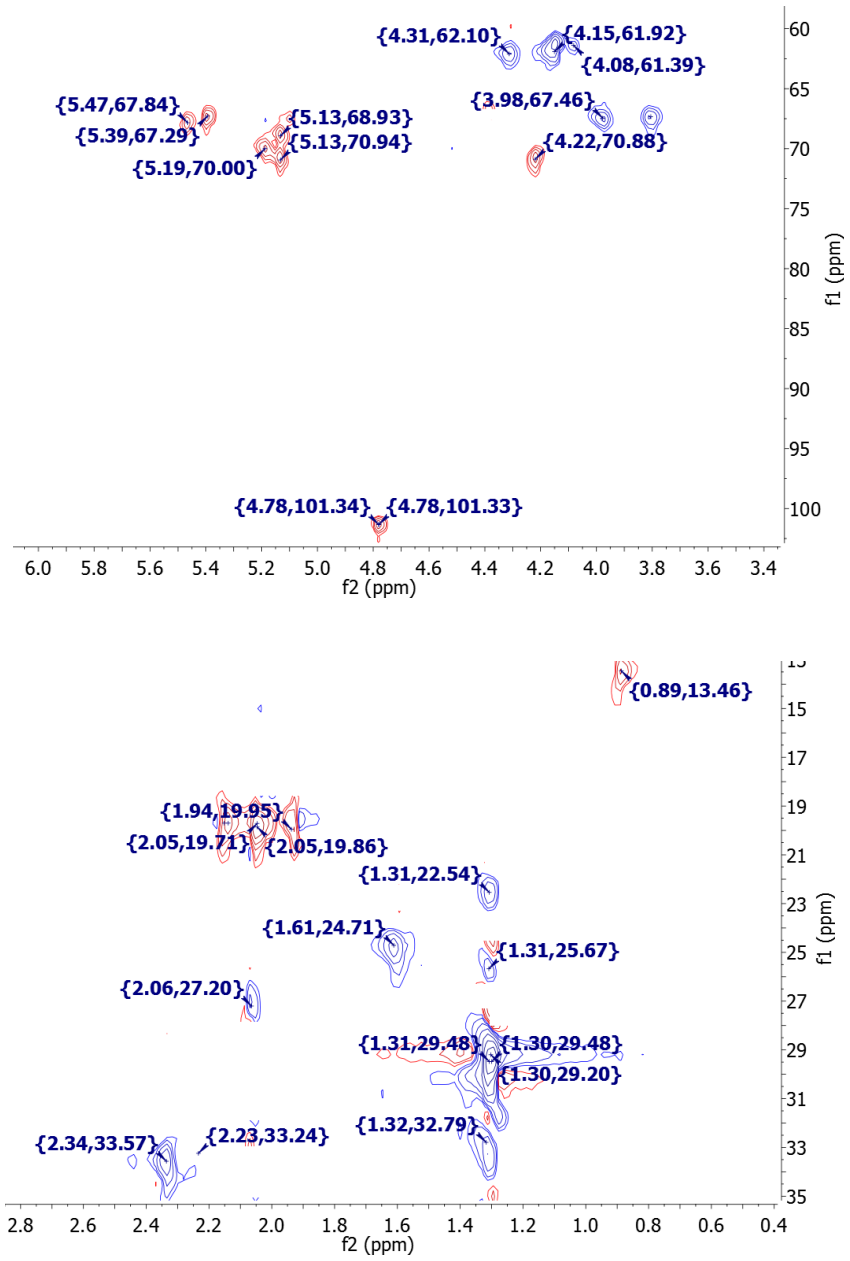


Figure 3.17 HSQC spectra of xylosyl glycolipid G-5

Spatial correlations can be revealed from ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC. COSY NMR correlation of δ_H 3.81 and 3.98 with δ_H 5.18 indicated interaction between CH_2 protons at 1, 3 positions with CH proton at position 2 of the glyceride moiety (Figure 3.18). Correlation between 10', 10" and 9', 9" positions (δ_H 4.17, 4.31 with δ_H 5.18) of the dixylosyl residue is also visible from COSY spectra. Correlation among hydrogen atoms on adjacent carbon atoms of sugar moiety and also of the glyceride unit can be clearly understood from the COSY spectra. Correlation between 2 and 1, 3 positions of glyceride moiety is further confirmed by HMBC correlations (δ_C 69.92 with δ_H 3.81) (Figure 3.19). Correlation between δ_H 3.81 at 1, 3 position of glyceride and δ_C 101.28 at 1', 1" position of xylose give more evidence for glycoside linkage between those positions. HMBC correlation between δ_H 4.22 and δ_C 101.25 indicate the glycosidic linkage between 4' and 6' (4" and 6") positions of xylose residue. Interaction among other CH and CH_2 groups of dixylose moiety is confirmed by HMBC spectra. The –CO- group at δ_C 172.55 is found to be correlated to methylenic protons at δ_H 2.34. This give indication on the presence of fatty acyl chain attached to position 2 of glyceride moiety. Prominent COSY and HMBC correlations of glycolipid G-5 are as shown in Figure 3.20.

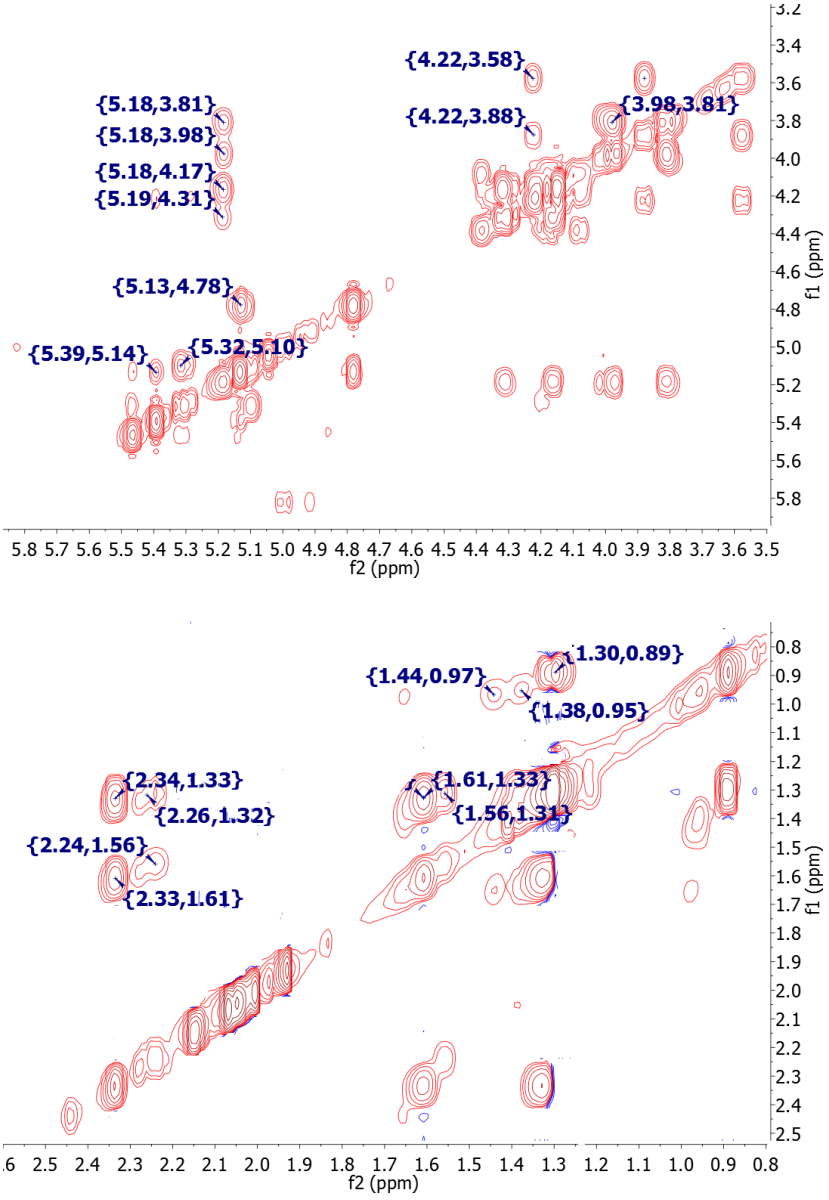


Figure 3.18 COSY spectra of xylosyl glycolipid G-5

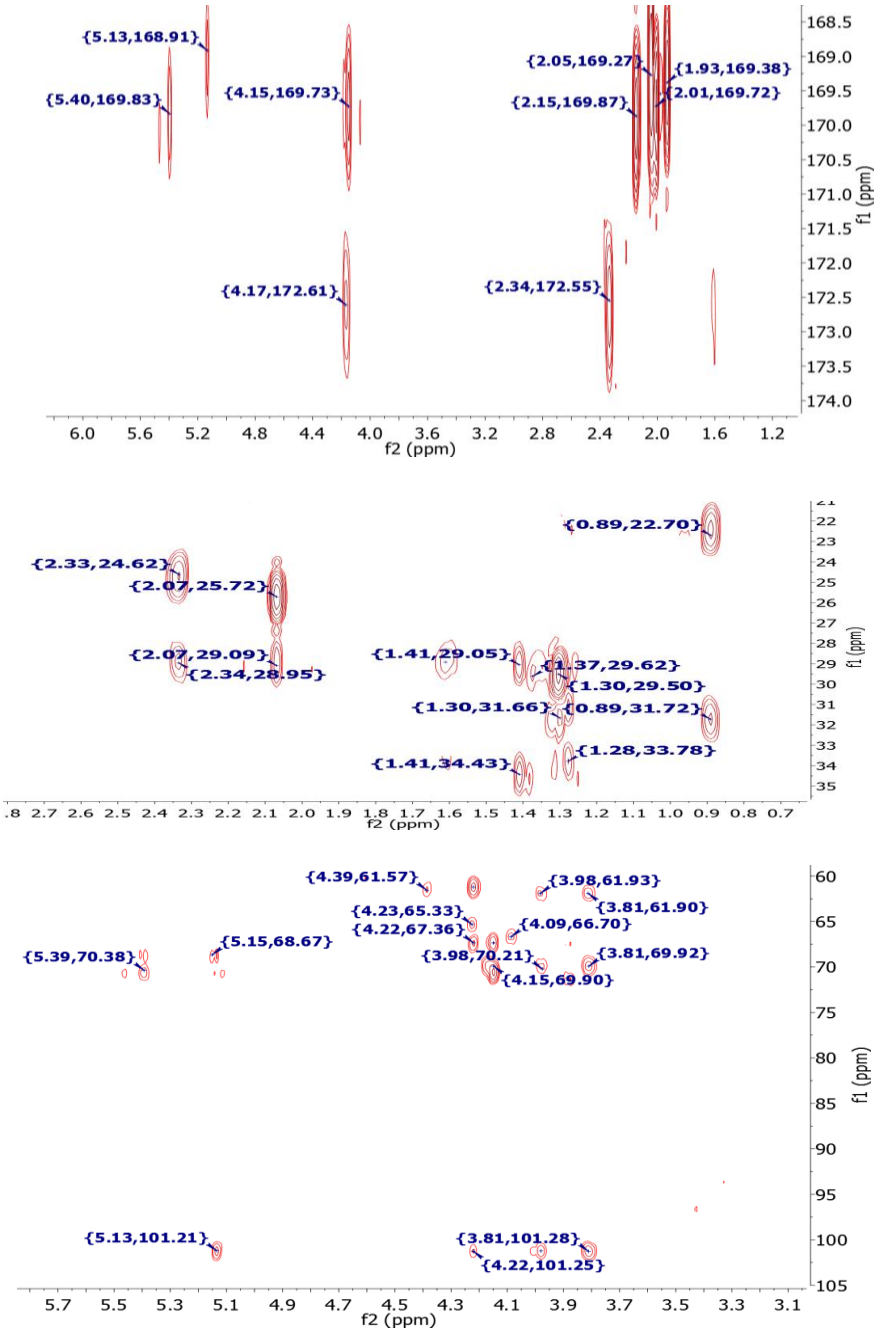


Figure 3.19 HMBC spectra of xylosyl glycolipid G-5

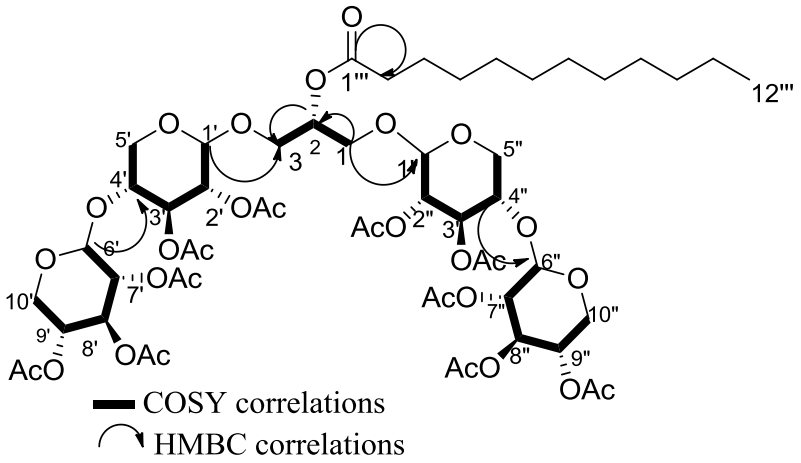


Figure 3.20 Key COSY and HMBC correlations of glycolipid G-5

To confirm the assigments of atoms in G-5 according to NMR predictions, ESI MS/MS analysis was performed on the protonated molecular ion (HRMS m/z 1223.4576 for $[C_{55}H_{82}O_{30}+H]^+$) (Table 3.1) in positive ion mode. Intense peak observed at m/z 1024 in mass spectrum of G-5 is attributed to the loss of dodecanoic acid (12:0). Peaks indicating loss of xylose (m/z 964) was also observed (Figure 3.21). A pair of signals observed at m/z 476 and 748 can be assigned to the fragmentation of dixylose unit. Peak observed at m/z 764 can be due to multiple fragmentations by loss of one pentose and fatty acid (Sauvageau et al., 2012). Other m/z peaks observed may be due to the multiple fragmentations and deacetylations. Peak at m/z 133 corresponding to non-acetylated xylose fragment further give confirmation to presence of pentose ring in the structure (Voutquenne et al., 2003). Fragmentation of G-5 is represented in Figure 3.22. GC-MS analysis of fragmented fatty acids showed single peak at m/z 215 corresponding to mass spectra of dodecanoic acid methyl ester (12:0) and had given further confirmation to the structure of G-5 (Figure 3.23).

Specific rotation $[\alpha]_D$ (25 °C) of the compound G-5 was found to be -22° and hence C-2 position in the glycolipid G-5 is assumed to have an S configuration. After combining all spectral data, structure of glycolipid G-5 is concluded as (2S)-2-O-dodecanoyl-1,3-di-O-[β-D-xylopyranose(1=>4)β-D-xylopyranosyl]-glycerol.

Natural glycolipids containing pentose sugar unit have been identified in less numbers. Chlorinated aromatic glycolipids containing xylose units have been isolated from two cyanobacteria *Nodosilinea* sp. LEGE 06102 and *Synechocystis salina* LEGE 06155 (Leão et al., 2015). Cytotoxic glycolipids containing tri-arabinose units have been isolated from a marine sponge *Stelletta* sp. (Peddie et al., 2015). Biological activities of glycolipids are known to be influenced by nature of the sugar moiety (Colombo et al., 1996) and hence it is important to study glycolipids containing different sugar units.

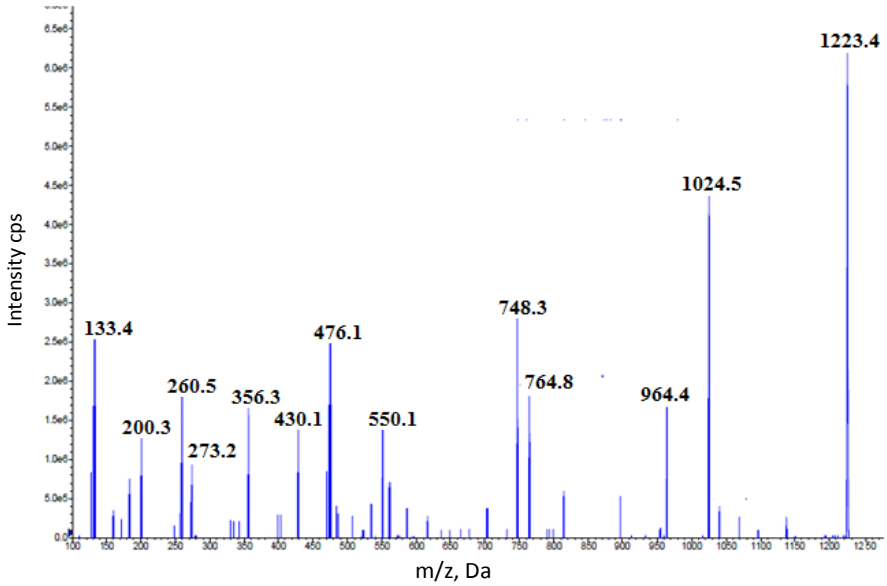


Figure 3.21 LC-MS/MS spectra of xylosyl glycolipid G-5

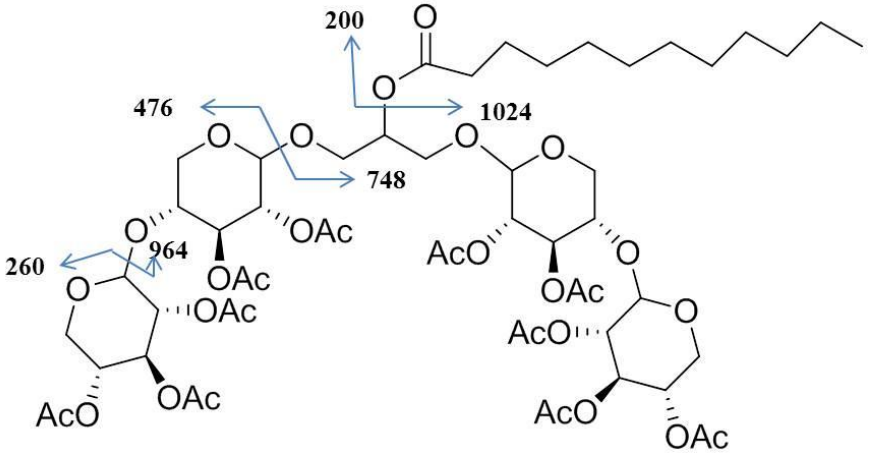


Figure 3.22 Fragmentation of xylosyl glycolipid G-5 based on MS/MS spectra.

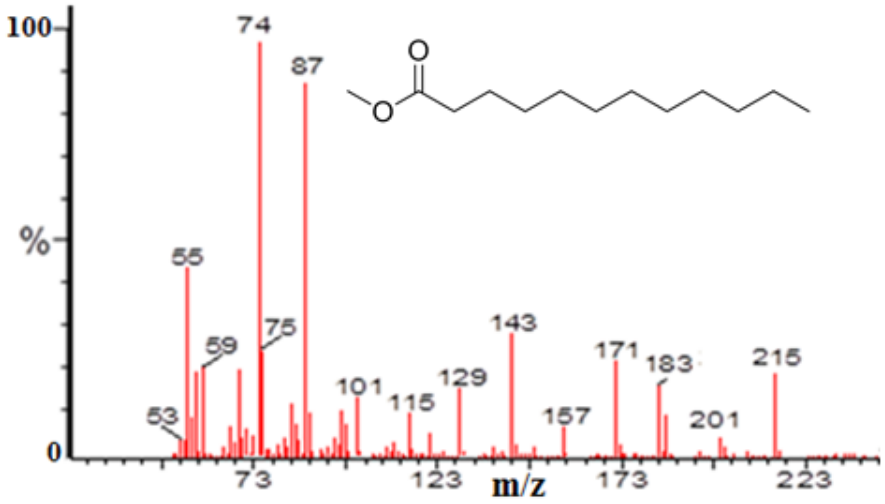


Figure 3.23 GC-MS mass spectra of dodecanoic acid (12:0) obtained after fragmentation of G-5.

3.3.4 Mass spectroscopic characterization of glycolipids G-1, G-3, G-4 and G-6

Structure of glycolipids G-1, G-3, G-4 and G-6 were predicted using LC-MS/MS analysis and fragmentation studies. Sugar analyses revealed that G-1, G-3 and G-4 contain galactose as the sugar unit whereas G-6 contain xylose unit (Table 3.1). Fatty acid fragments obtained from each fraction are listed in Table 3.4.

Table 3.4 Fatty acid composition of Glycolipids

Glycolipid	Fatty acid	
G-1	Palmitic acid (16:0)	Stearic acid (18:0)
G-3	Dodecanoic acid (12:0)	Palmitic acid (16:0)
G-4	Palmitic acid (16:0)	Oleic acid (18:1)
G-6	Tetradecanoic acid (14:0)	

The protonated molecular ion (HRMS m/z = 928.6344 for $[C_{51}H_{90}O_{14}+H]^+$) (Table 3.1) of galactosyl glycolipid G-1 was fragmented by ESI-MS/MS in positive ion mode (Figure 3.24). Fragments obtained at m/z 671 and 643 are attributed to loss of palmitic acid (16:0) and stearic acid (18:0) respectively. Among the two peaks, m/z 671 showed higher instensity and hence palmitic acid may be linked to sn-1 position of the galactosyl diglyceride (Guella et al., 2003). Peaks indicating loss of galactose unit (m/z 597) along with acetylated galactose itself (m/z 332) were also identified. Other peaks due to multiple fragmentations and deacetylations were also detected. Peak at m/z 162 attributed to non-acetylated galactose fragment further give confirmation to presence of hexose ring in the structure (Soulé et al., 2000). Fragmentation of the gactosyl glycolipid G-1 is represented in Figure 3.25. GC-MS analysis

of fatty acid fragments showed two peaks corresponding to palmitic acid (16:0) (m/z 271) and stearic acid (18:0) (m/z 299) (Figure 3.26). Hence G-1 is identified as a monogalactosyl diacylglyceride.

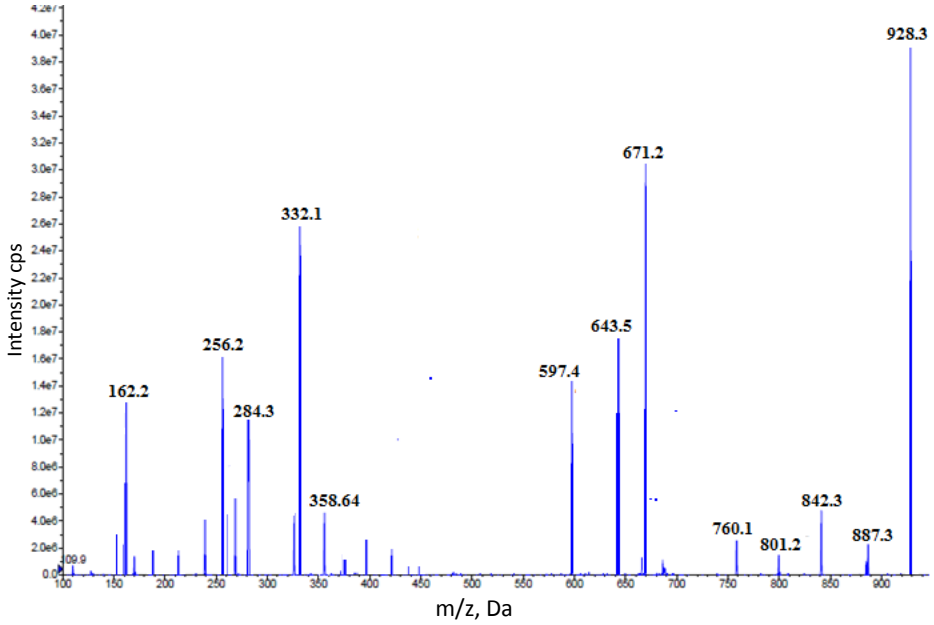


Figure 3.24 LC-MS/MS spectra of galactosyl glycolipid G-1

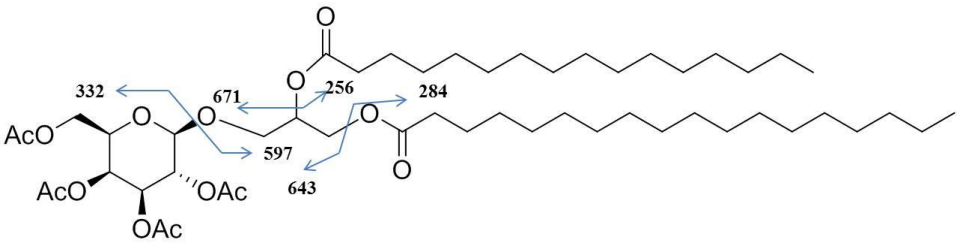


Figure 3.25 Fragmentation of galactosyl glycolipid G-1 based on MS/MS analysis

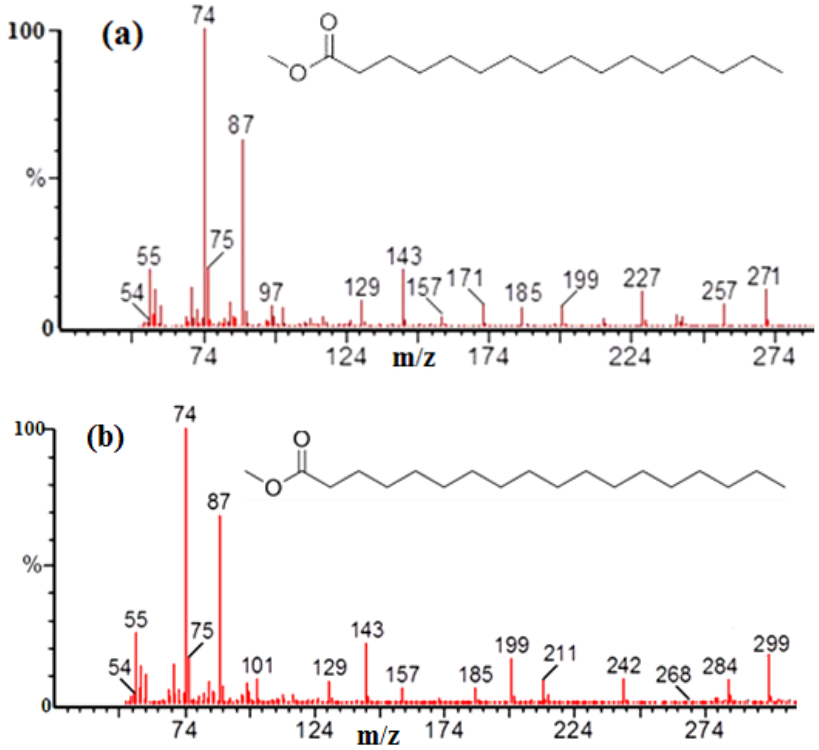


Figure 3.26 GC-MS mass spectra of palmitic acid (16:0) (a) and stearic acid (18:0) (b) obtained after fragmentation of G-1.

ESI MS/MS fragmentation pattern of galactosyl glycolipid G-3 (HRMS m/z = 1131.4764 for $[C_{57}H_{94}O_{22}+H]^+$) (Table 3.1) showed signals attributed to the loss of dodecanoic acid (12:0) and palmitic acid (16:0) at m/z 932 and m/z 876 respectively (Figure 3.27). Peak at m/z 932 has higher intensity indicating presence of dodecanoic acid (12:0) at sn-1 position of glyceride. Peaks at m/z 800 and 332 represent fragmentation at glycosidic linkage position. Peak at m/z 620 denoted the separated acetylated digalactose residue and the remaining fragment was obtained at m/z 512. Multiple fragmentation signals, such as formed by the loss of both fatty acids (m/z 677) and by the loss of galactose and either of two fatty acids

(m/z 601 and m/z 545) were also identified. Hence, G-3 is recognized as digalactosyl diacylglyceride. Mass fragmentation of G-3 is represented in Figure 3.28. GC-MS analysis of fatty acid fragments showed two peaks corresponding to methyl esters of dodecanoic acid (12:0) (m/z 215) and palmitic acid (16:0) (m/z) (Figure 3.29).

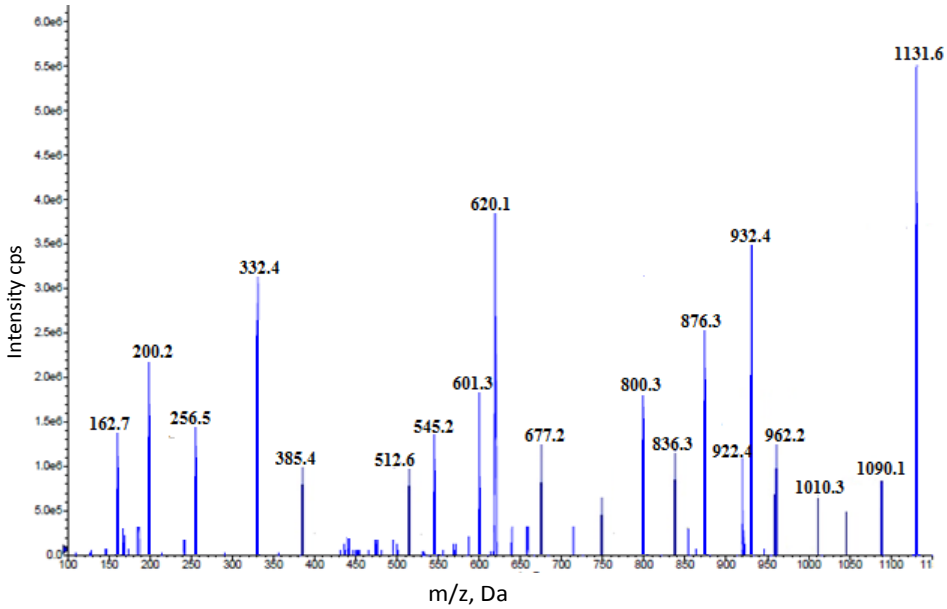


Figure 3.27 LC-MS/MS spectra of galactosyl glycolipid G-3

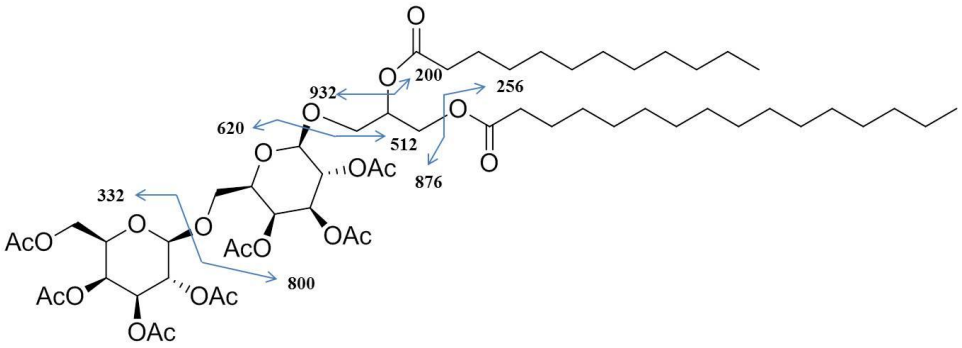


Figure 3.28 Mass fragmentation of galactosyl glycolipid G-3 based on MS/MS spectra

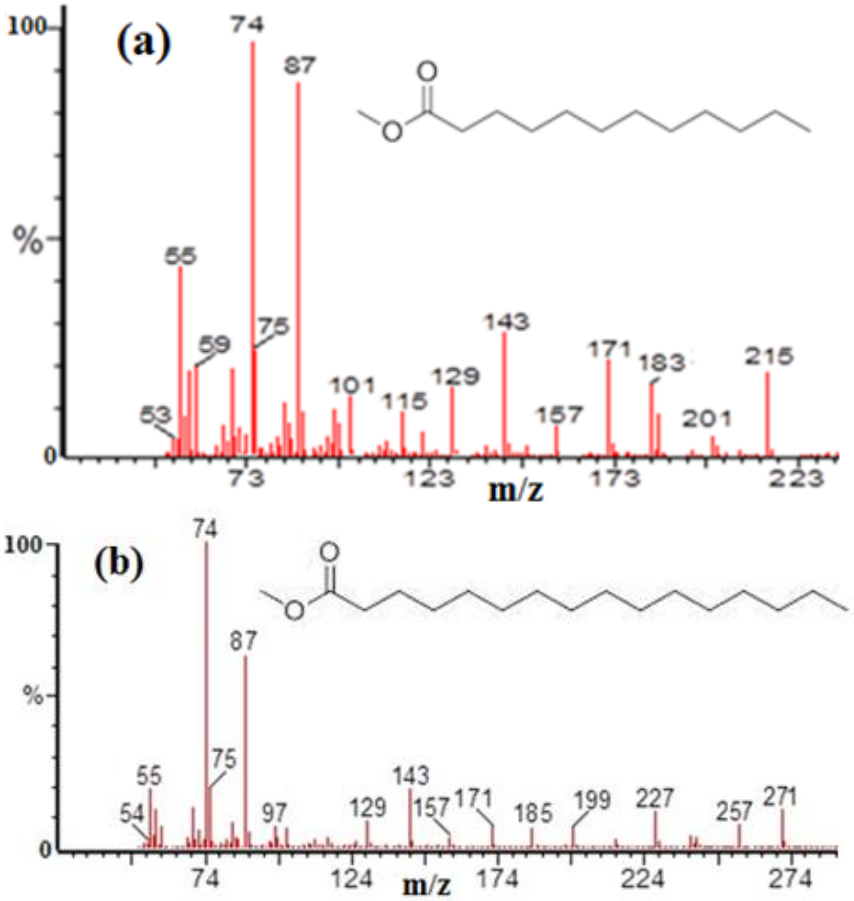


Figure 3.29 GC-MS mass spectra of dodecanoic acid (12:0) (a) and palmitic acid (16:0) (b) obtained after fragmentation of G-3.

Mass fragmentation pattern of galactosyl glycolipid G-4 (HRMS m/z = 1213.5478 for $[C_{63}H_{104}O_{22}+H]^+$) (Table 3.1) showed m/z at 620 similar to G-3 which also give inference to the loss of a digalactose residue (Figure 3.30). Observations on other peaks also support the structure of glycolipid G-4 to be a digalactosyl diacylglyceride. Prominent peaks at m/z 957 and 931 correspond to palmitic acid (16:0) and oleic acid (18:1) respectively, palmitic acid possibly occupy sn-1

position of glyceride due to comparatively higher intensity of m/z 957. Multiple fragmentation peaks due to loss of both fatty acids (m/z 677) and one sugar unit and either of the two fatty acids (m/z 600 and 627) were also observed in the spectra. Mass fragmentation of G-4 is reperesented in Figure 3.31. Fatty acid fragmentation analysis using GC-MS also give evidence for palmitic and oleic acids side chain in the glycolipid (Figure 3.32). Digalactosyl glycolipids were recognized to have significant cytotoxic potential (de Souza et al., 2012; Singh et al., 2017) and cyanobacteria are known as important source for them (Burja et al., 2001; Pandey et al., 2007). A group of digalactolipids isolated from the lipophilic extracts of cyanobacteria *Scytonema* sp. and *Oscillatoria raoi* were identified with a potential to inhibit the reverse transcriptase of HIV-1 (Reshef et al., 1997).

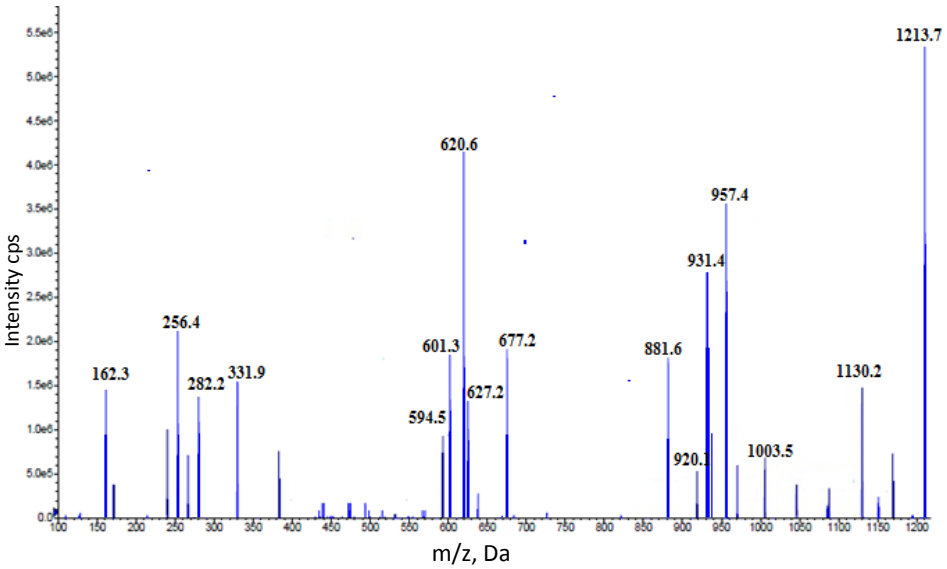


Figure 3.30 LC-MS/MS spectra of galactosyl glycolipid G-4

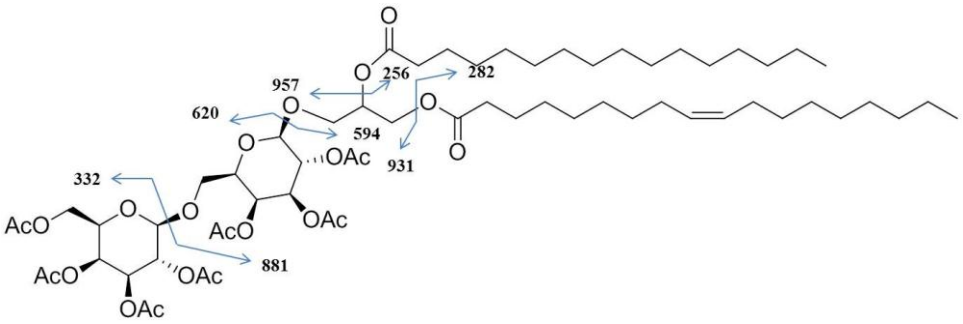


Figure 3.31 Mass fragmentation of galactosyl glycolipid G-4 based on MS/MS analysis

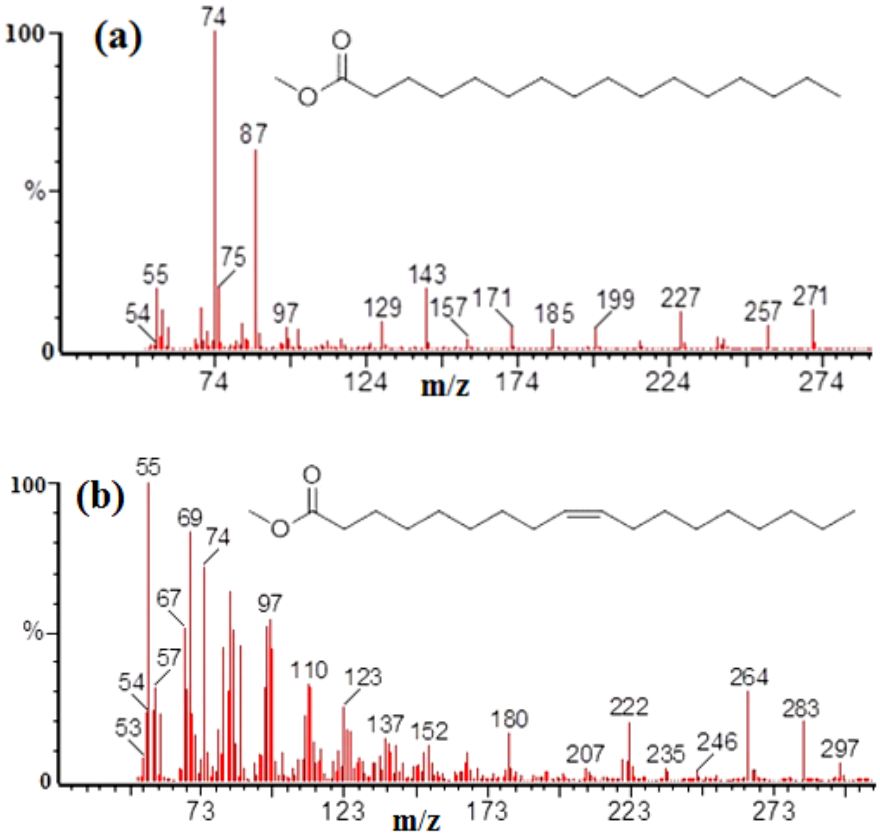


Figure 3.32 GC-MS mass spectra of palmitic acid (16:0) (a) and oleic acid (18:1) (b) obtained after fragmentation of G-4.

Mass fragmentation analysis of xylosyl glycolipid G-6 (HRMS m/z = 1251.6785 for $[C_{57}H_{86}O_{30}+H]^+$) (Table 3.1) using ESI MS/MS has given inference on a structure related to glycolipid G-5 due to intense peak at 1024 in the mass fragmentation pattern of G-6 similar to glycolipid G-5 (Figure 3.33). Fragmentation pattern indicated the attached fatty acid to be tetradecanoic acid (14:0). Predicted structure of glycolipid G-6 is as shown in Figure 3.34. Signals observed at m/z 476 and 776 can be correspond to fragmentation of dixylose. Peaks due to multiple fragmentations (m/z 764) and deacetylations were also detected. GC-MS analysis of fragmented fatty acid has also given peak corresponding to methyl ester of tetradecanoic acid (14:0) (Figure 3.35). Due to less sample quantity, further spectroscopic characterization to determine exact structure of glycolipid G-6 is limited.

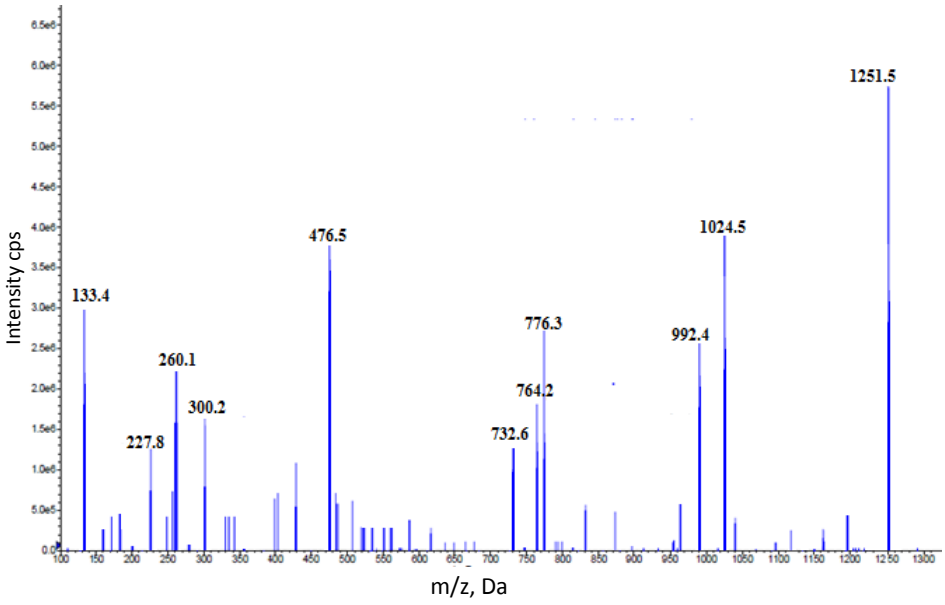


Figure 3.33 LC-MS/MS spectra of xylosyl glycolipid G-6

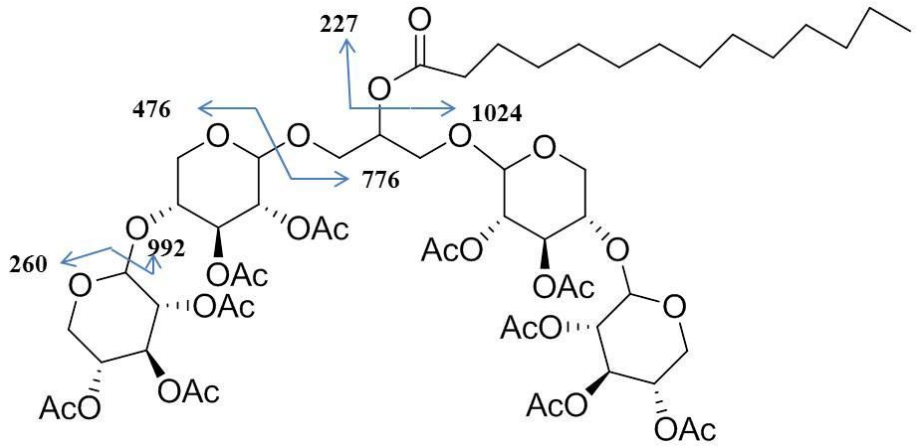


Figure 3.34 Mass fragmentation of xylosyl glycolipid G-6 based on MS/MS analysis

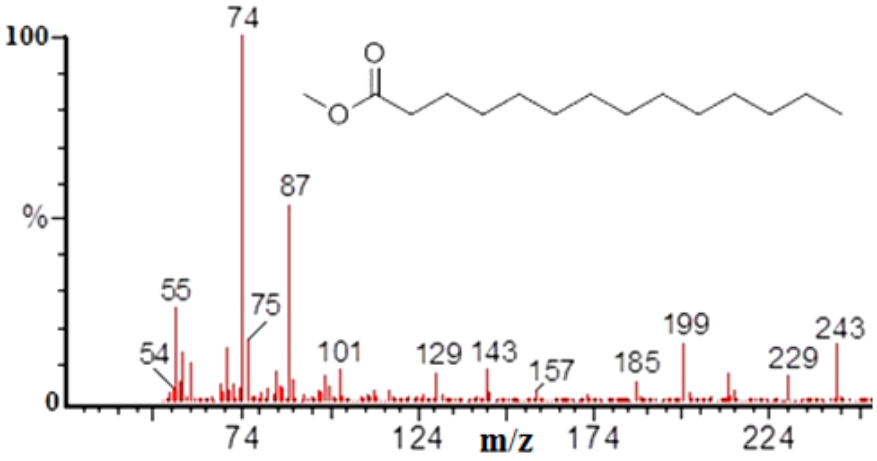


Figure 3.35 GC-MS mass spectra of tetradecanoic acid (14:0) obtained after fragmentation of G-6.

3.4 Conclusion

Sugar analysis of six glycolipids isolated from *Synechocystis* sp. indicated that four of them (G-1 to G-4) possess galactose as the sugar unit. Two glycolipids found to contain xylose units (G-5 and G-6). Masses of acetylated glycolipids were recorded on HRMS. Structural

features of glycolipids G-2 and G-5 obtained in higher yields were identified using NMR and mass spectroscopic studies. Galactosyl glycolipid G-2 is found to be similar in structure with monogalactosyl glycolipids found in photosynthetic organisms. Molecular mass of G-2 was recorded as m/z 949.5289 on HRMS and deduced molecular formula as $C_{53}H_{88}O_{14}$. Through NMR and ESI MS/MS analysis structure of glycolipid G-2 is established as (2S)-2-O-9,12,15-octadecatrienoyl-1-O-octadecanoyl-3-O-[β-D-galactopyranosyl]-glycerol. Optical rotation of the compound was found to be -8°.

Molecular weight of xylosyl glycolipid G-5 was obtained as m/z 1223.4576 through HRMS and the inferred molecular formula was $C_{55}H_{82}O_{30}$. Only a few xylosyl glycolipids of natural origin have been identified. Based on NMR and MS/MS analysis, structure of G-5 is concluded as (2S)-2-O-dodecanoyl-1,3-di-O-[β-D-xylopyranose(1=>4) β-D-xylopyranosyl]-glycerol. Optical rotation of the compound was found to be -22°. MS/MS analysis along with GC-MS studies of fatty acid fragments have given information on the structure of other isolated glycolipids. Glycolipid G-1 (m/z 928.6344) is assumed to be monogalactosyl diglyceride containing palmitic acid (16:0) and stearic acid (18:0) in the fatty acyl chain. G-3 (m/z 1131.4764) and G-4 (m/z 1213.5478) are identified as digalactosyl diglycerides. G-3 was shown to possess dodecanoic acid (12:0) and palmitic acid (16:0) in the fatty acyl chains of diglyceride. Fatty acid analysis of G-4 showed presence of two fatty acids, palmitic acid (16:0) and oleic acid (18:1). Glycolipid G-6 (m/z 1251.6785), which is a xylosyl glycolipid showed comparable mass spectra as that of glycolipid G-5. Hence, it is assumed to have similar

structure with that of G-5 and found to contain tetradecanoc acid (14:0) in the fatty acyl chain. Further, spectroscopic characterization is needed to find exact structure of G-6.

References

Andrianasolo, E. H., Haramaty, L., Vardi, A., White, E., Lutz, R., & Falkowski, P. (2008). Apoptosis-inducing galactolipids from a cultured marine diatom, *Phaeodactylum tricornutum*. *Journal of Natural Products*, 71(7), 1197-1201.

Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976). Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *The Journal of Chemical Physics*, 64(5), 2229-2246.

Auray-Blais, C., Bhérer, P., Gagnon, R., Young, S. P., Zhang, H. H., An, Y., & Millington, D. S. (2011). Efficient analysis of urinary glycosaminoglycans by LC-MS/MS in mucopolysaccharidoses type I, II and VI. *Molecular Genetics and Metabolism*, 102(1), 49-56.

Bax, A., & Freeman, R. (1981). Investigation of complex networks of spin-spin coupling by two-dimensional NMR. *Journal of Magnetic Resonance*, 44(3), 542-561.

Bax, A., & Summers, M. F. (1986). Proton and carbon-13 assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *Journal of the American Chemical Society*, 108(8), 2093-2094.

Berger, S., & Braun, S. (2004). The Second Dimension. *200 and more NMR experiments: A practical course, edited by Berger S, Braun S. Weinheim: Wiley-Vch*, 362-453.

Bergsson, G., Arnfinnsson, J., Steingrímsson, Ó., & Thormar, H. (2001). *In vitro* killing of *Candida albicans* by fatty acids and monoglycerides. *Antimicrobial Agents and Chemotherapy*, 45(11), 3209-3212.

Brandenburg, K., & Seydel, U. (1998). Infrared spectroscopy of glycolipids. *Chemistry and Physics of Lipids*, 96(1), 23-40.

Braude, E. A., & Nachod, F. C. (2013). *Determination of Organic Structures by Physical Methods, edited by Braude EA, Nachod FC. New York: Elsevier*, 131-219.

Breton, R. C., & Reynolds, W. F. (2013). Using NMR to identify and characterize natural products. *Natural Product Reports*, 30(4), 501-524.

Bross-Walch, N., Kühn, T., Moskau, D., & Zerbe, O. (2005). Strategies and tools for structure determination of natural products using modern methods of NMR spectroscopy. *Chemistry & Biodiversity*, 2(2), 147-177.

Burja, A. M., Banaigs, B., Abou-Mansour, E., Burgess, J. G., & Wright, P. C. (2001). Marine cyanobacteria-a prolific source of natural products. *Tetrahedron*, 57(46), 9347-9377.

Carbone, V., Montoro, P., de Tommasi, N., & Pizza, C. (2004). Analysis of flavonoids from *Cyclanthera pedata* fruits by liquid chromatography/ electrospray mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 34(2), 295-304.

Castro, V., Dvinskikh, S. V., Widmalm, G., Sandström, D., & Maliniak, A. (2007). NMR studies of membranes composed of glycolipids and phospholipids. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1768(10), 2432-2437.

Chaturvedula, V. S., & Prakash, I. (2011). Additional minor diterpene glycosides from *Stevia rebaudiana*. *Natural Product Communications*, 6(8), 1059-1062.

Colegate, S. M., & Molyneux, R. J. (1993). *Bioactive Natural Products Detection, Isolation, and Structural Determination, edited by Colegate SM, Molyneux RJ. CRC press: Boca Raton*, 1-267.

Colombo, D., Scala, A., Taino, I. M., Toma, L., Ronchetti, F., Tokuda, H., & Sakakibara, J. (1996). 1-O-, 2-O-and 3-O-β-glycosyl-sn-glycerols: Structure-anti-tumor-promoting activity relationship. *Bioorganic & Medicinal Chemistry Letters*, 6(10), 1187-1190.

Crublet, M. L., Long, C., Sévenet, T., Hadi, H. A., & Lavaud, C. (2003). Acylated flavonol glycosides from leaves of *Planchonia grandis*. *Phytochemistry*, 64(2), 589-594.

Cutignano, A., d'Ippolito, G., Romano, G., Lamari, N., Cimino, G., Febbraio, F., & Fontana, A. (2006). Chloroplastic glycolipids fuel aldehyde biosynthesis in the marine diatom *Thalassiosira rotula*. *ChemBioChem*, 7(3), 450-456.

Cuyckens, F., & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass spectrometry*, 39(1), 1-15.

Dabrowski, J., Hanfland, P., & Egge, H. (1980). Structural analysis of glycosphingolipids by high-resolution proton nuclear magnetic resonance spectroscopy. *Biochemistry*, 19(24), 5652-5658.

Dawson, P. H. (2013). *Quadrupole Mass Spectrometry and Its Applications, edited by Dawson PH New York: Elsevier*, 121-284.

De Hoffmann, E., & Stroobant, V. (2007). *Mass Spectrometry: Principles and Applications, edited by De Hoffmann E, Stroobant V: John Wiley & Sons*, 15-79.

de Souza, L. M., Sassaki, G. L., Romanos, M. T. V., & Barreto-Bergter, E. (2012). Structural characterization and anti-HSV-1 and HSV-2 activity of glycolipids from the marine algae *Osmundaria obtusiloba* isolated from southeastern Brazilian coast. *Marine Drugs*, 10(4), 918-931.

d'Ippolito, G., Tucci, S., Cutignano, A., Romano, G., Cimino, G., Miralto, A., & Fontana, A. (2004). The role of complex lipids in the synthesis of bioactive aldehydes of the marine diatom *Skeletonema costatum*. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1686(1), 100-107.

Doddrell, D. M., Pegg, D. T., & Bendall, M. R. (1982). Distortionless enhancement of NMR signals by polarization transfer. *Journal of Magnetic Resonance*, 48(2), 323-327.

Duus, J. Ø., Gotfredsen, C. H., & Bock, K. (2000). Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. *Chemical Reviews*, 100(12), 4589-4614.

El-Aneed, A., Cohen, A., & Banoub, J. (2009). Mass spectrometry, review of the basics: electrospray, MALDI, and commonly used mass analyzers. *Applied Spectroscopy Reviews*, 44(3), 210-230.

Elyashberg, M. E., Blinov, K. A., Williams, A. J., Martirosian, E. R., & Molodtsov, S. G. (2002). Application of a new expert system for the structure elucidation of natural products from their 1D and 2D NMR data. *Journal of Natural Products*, 65(5), 693-703.

Ernst, R. R., & Anderson, W. A. (1966). Application of Fourier transform spectroscopy to magnetic resonance. *Review of Scientific Instruments*, 37(1), 93-102.

Fabre, N., Rustan, I., de Hoffmann, E., & Quetin-Leclercq, J. (2001). Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *Journal of the American Society for Mass Spectrometry*, 12(6), 707-715.

Fengel, D., & Wegener, G. (1979). Hydrolysis of polysaccharides with trifluoroacetic acid and its application to rapid wood and pulp analysis. *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis, edited by Brown DR, Jurasek L. Germany: ACS, 145-158.*

Ferrier, R. J., Hay, R. W., & Vethaviyasar, N. (1973). A potentially versatile synthesis of glycosides. *Carbohydrate Research*, 27(1), 55-61.

Fredenhagen, A., Derrien, C., & Gassmann, E. (2005). An MS/MS library on an ion-trap instrument for efficient dereplication of natural products. Different fragmentation patterns for $[M+ H]^+$ and $[M+ Na]^+$ ions. *Journal of Natural Products*, 68(3), 385-391.

Furrer, J. (2011). Recent developments in HMBC studies. *Annual Reports on NMR Spectroscopy, edited by Webb GA, London: Elsevier*, 293-310.

Grayer, R. J., Kite, G. C., Abou-Zaid, M., & Archer, L. J. (2000). The application of atmospheric pressure chemical ionisation liquid chromatography-mass spectrometry in the chemotaxonomic study of flavonoids: characterisation of flavonoids from *Ocimum gratissimum var. gratissimum*. *Phytochemical Analysis*, 11(4), 257-267.

Grosa, G., Allegrone, G., & Del Grosso, E. (2005). LC–ESI-MS/MS characterization of strophanthin-K. *Journal of Pharmaceutical and Biomedical Analysis*, 38(1), 79-86.

Guan, F., Ishii, A., Seno, H., Watanabe-Suzuki, K., Kumazawa, T., & Suzuki, O. (1999). Identification and quantification of cardiac glycosides in blood and urine samples by HPLC/MS/MS. *Analytical Chemistry*, 71(18), 4034-4043.

Guella, G., Frassanito, R., & Mancini, I. (2003). A new solution for an old problem: the regiochemical distribution of the acyl chains in galactolipids can be established by electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 17(17), 1982-1994.

Gupta, P., Yadav, D. K., Siripurapu, K. B., Palit, G., & Maurya, R. (2007). Constituents of *Ocimum sanctum* with antistress activity. *Journal of Natural Products*, 70(9), 1410-1416.

Harvey, H. R. (1994). Fatty acids and sterols as source markers of organic matter in sediments of the north Carolina continental slope. *Deep Sea Research Part II: Topical Studies in Oceanography*, 41(4-6), 783-796.

Havlicek, V., & Spizek, J. (2014). *Natural products Analysis: Instrumentation, Methods, and Applications, edited by Havlicek V, Spizek J. John Wiley & Sons: New Jersy*, 1-571.

Hore, P. J. (2015). *Nuclear Magnetic Resonance*, e*dited by Hore PJ, USA: Oxford University Press*, 80-99.

Hu, P., Chess, E. K., Brynjelsen, S., Jakubowski, G., Melchert, J., Hammond, R. B., & Wilson, T. D. (2000). Collisionally activated dissociations of aminocyclitol-aminoglycoside antibiotics and their application in the identification of a new compound in tobramycin samples. *Journal of the American Society for Mass Spectrometry*, 11(3), 200-209.

Huhman, D. V., & Sumner, L. W. (2002). Metabolic profiling of saponins in *Medicago sativa* and *Medicago truncatula* using HPLC coupled to an electrospray ion-trap mass spectrometer. *Phytochemistry*, 59(3), 347-360.

Hurd, R. E. (1990). Gradient-enhanced spectroscopy. *Journal of Magnetic Resonance*, 87(2), 422-428.

Ishii, T., Okino, T., & Mino, Y. (2006). A ceramide and cerebroside from the starfish *Asterias amurensis* Lütken and their plant-growth promotion activities. *Journal of Natural Products*, 69(7), 1080-1082.

Koehn, F. E., & Carter, G. T. (2005). The evolving role of natural products in drug discovery. *Nature Reviews Drug discovery*, 4(3), 206.

Koerner, T. A., Prestegard, J. H., Demou, P. C., & Yu, R. K. (1983). High-resolution proton NMR studies of gangliosides - use of two-dimensional nuclear Overhauser effect spectroscopy and sialylation shifts for determination of oligosaccharide sequence and linkage sites. *Biochemistry*, 22(11), 2687-2690.

Konishi, Y., Kiyota, T., Draghici, C., Gao, J. M., Yeboah, F., Acoca, S., & Purisima, E. (2007). Molecular formula analysis by an MS/MS/MS technique to expedite dereplication of natural products. *Analytical Chemistry*, 79(3), 1187-1197.

Krishnamurthy, V. V., Russell, D. J., Hadden, C. E., & Martin, G. E. (2000). ^{2}J, ^{3}J-HMBC: a new long-range heteronuclear shift correlation technique capable of differentiating ^{2}J-CH from ^{3}J-CH correlations to protonated carbons. *Journal of Magnetic Resonance*, 146(1), 232-239.

Larsen, E., Kharazmi, A., Christensen, L. P., & Christensen, S. B. (2003). An antiinflammatory galactolipid from rose hip (*Rosa canina*) that inhibits chemotaxis of human peripheral blood neutrophils *in vitro*. *Journal of Natural Products*, 66(7), 994-995.

Leão, P. N., Nakamura, H., Costa, M., Pereira, A. R., Martins, R., Vasconcelos, V., & Balskus, E. P. (2015). Biosynthesis-assisted structural elucidation of the bartolosides, chlorinated aromatic glycolipids from cyanobacteria. *Angewandte Chemie*, 127(38), 11215-11219.

Levy, G. C., & Nelson, G. L. (1972). *Carbon-13 Nuclear Magnetic Resonance for Organic Chemists, edited by Levy GC, Nelson, GL, New York: Wiley-Interscience*. 1-222.

Liao, P. C., & Allison, J. (1995). Ionization processes in matrix-assisted laser desorption/ionization mass spectrometry: Matrix-dependent formation of $[M+ H]^{+}$ vs $[M+ Na]^{+}$ ions of small peptides and some mechanistic comments. *Journal of Mass Spectrometry*, 30(3), 408-423.

Liptáak, M., & Heerma, W. (1993). Fast-atom bombardment mass spectrometric study of some N-and S-glycosides of acetylated hexose isomers. *Rapid Communications in Mass Spectrometry*, 7(7), 676-679.

Liu, S., Cui, M., Liu, Z., Song, F., & Mo, W. (2004). Structural analysis of saponins from medicinal herbs using electrospray ionization tandem mass spectrometry. *Journal of the American Society for Mass Spectrometry*, 15(2), 133-141.

Lopes, G., Daletos, G., Proksch, P., Andrade, P. B., & Valentão, P. (2014). Anti-inflammatory potential of monogalactosyl diacylglycerols and a monoacylglycerol from the edible brown seaweed *Fucus Spiralis Linnaeus*. *Marine Drugs*, 12(3), 1406-1418.

Marion, D. (2012). Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of $^{1}H–^{1}H$ spin–spin coupling constants in proteins. *Biochemical and Biophysical Research Communications*, 425(3), 519-526.

Mathlouthi, M., & Koenig, J. L. (1987). Vibrational spectra of carbohydrates. *Advances in Carbohydrate Chemistry and Biochemistry*, 44(1), 7-89.

Mihara, R., Mitsunaga, T., Fukui, Y., Nakai, M., Yamaji, N., & Shibata, H. (2004). A novel acylated quercetin tetraglycoside from oolong tea (*Camelia sinensis*) extracts. *Tetrahedron Letters*, 45(26), 5077-5080.

Mizushina, Y., Hada, T., & Yoshida, H. (2012). *In vivo* antitumor effect of liposomes with sialyl Lewis X including monogalactosyl diacylglycerol, a replicative DNA polymerase inhibitor, from spinach. *Oncology Reports*, 28(3), 821-828.

Morimoto, T., Nagatsu, A., Murakami, N., Sakakibara, J., Tokuda, H., Nishino, H., & Iwashima, A. (1995). Anti-tumour-promoting glyceroglycolipids from the green alga, *Chlorella vulgaris*. *Phytochemistry*, 40(5), 1433-1437.

Morris, G. A. (1980). Sensitivity enhancement in nitrogen-15 NMR: polarization transfer using the INEPT pulse sequence. *Journal of the American Chemical Society*, 102(1), 428-429.

Ntai, I. (2008). Biosynthetic studies of secondary metabolites by mass spectrometry. *PhD Thesis. Vanderbilt University, USA*, 4-20.

Nyberg, N. T., Duus, J. Ø., & Sørensen, O. W. (2005). Heteronuclear two-bond correlation: suppressing heteronuclear three-bond or higher NMR correlations while enhancing two-bond correlations even for vanishing ^{2}J CH. *Journal of the American Chemical Society*, 127(17), 6154-6155.

Pandey, V. D., Gupta, R. K., & Singh, S. K. (2007). Cyanobacteria as a source of pharmaceutical compounds. *Advances in Applied Phycology, edited by Gupta RK, Pandey VD, New Delhi: Daya Publishing House*, 250-260.

Patridge, E., Gareiss, P., Kinch, M. S., & Hoyer, D. (2016). An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Discovery Today*, 21(2), 204-207.

Patt, S. L., & Shoolery, J. N. (1982). Attached proton test for carbon-13 NMR. *Journal of Magnetic Resonance*, 46(3), 535-539.

Peddie, V., Takada, K., Okuda, S., Ise, Y., Morii, Y., Yamawaki, N., & Matsunaga, S. (2015). Cytotoxic glycosylated fatty acid amides from a *Stelletta* sp. marine sponge. *Journal of Natural Products*, 78(11), 2808-2813.

Piacente, S., Campaner dos Santos, L., Mahmood, N., Zampelli, A., Pizza, C., & Vilegas, W. (2001). Naphthopyranone glycosides from *Paepalanthus microphyllus*. *Journal of Natural Products*, 64(5), 680-682.

Plaza, A., Perrone, A., Balestrieri, M. L., Felice, F., Balestrieri, C., Hamed, A. I., & Piacente, S. (2005). New unusual pregnane glycosides with antiproliferative activity from *Solenostemma argel*. *Steroids*, 70(9), 594-603.

Potterat, O., Wagner, K., & Haag, H. (2000). Liquid chromatography–electrospray time-of-flight mass spectrometry for on-line accurate mass determination and identification of cyclodepsipeptides in a crude extract of the fungus *Metarrhizium anisopliae*. *Journal of Chromatography A*, 872(1), 85-90.

Reshef, V., Mizrachi, E., Maretzki, T., Silberstein, C., Loya, S., Hizi, A., & Carmeli, S. (1997). New acylated sulfoglycolipids and digalactolipids and related known glycolipids from cyanobacteria with a potential to inhibit the reverse transcriptase of HIV-1. *Journal of Natural Products*, 60(12), 1251-1260.

Reynolds, W. F., & Enríquez, R. G. (2002). Choosing the best pulse sequences, acquisition parameters, postacquisition processing strategies, and probes for natural product structure elucidation by NMR spectroscopy. *Journal of Natural Products*, 65(2), 221-244.

Řezanka, T., & Dembitsky, V. M. (2003). Identification of acylated xanthone glycosides by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry in positive and negative modes from the lichen *Umbilicaria proboscidea*. *Journal of Chromatography A*, 995(1), 109-118.

Sauvageau, J., Ryan, J., Lagutin, K., Sims, I. M., Stocker, B. L., & Timmer, M. S. (2012). Isolation and structural characterisation of the major glycolipids from *Lactobacillus plantarum*. *Carbohydrate Research*, 357(2), 151-156.

Schoefberger, W., Schlagnitweit, J., & Müller, N. (2011). Recent developments in heteronuclear multiple-bond correlation experiments. *Annual Reports on NMR Spectroscopy, edited by Webb GA, London: Elsevier*, 1-32.

Schöpke, T., Thiele, H., Wray, V., Nimtz, M., & Hiller, K. (1995). Structure elucidation of a glycoside of 2β, 3β, 23-Trihydroxy-16-oxoolean-12-en-28-oic acid from *Bellis bernardii* using mass spectrometry for the sugar sequence determination. *Journal of Natural Products*, 58(1), 152-155.

Scribe, P., Guezennec, J., Dagaut, J., Pepe, C., & Saliot, A. (1988). Identification of the position and the stereochemistry of the double bond in monounsaturated fatty acid methyl esters by gas chromatography/mass spectrometry of dimethyl disulfide derivatives. *Analytical Chemistry*, 60(9), 928-931.

Shan, Y., Liu, Y., Yang, L., Nie, H., Shen, S., Dong, C., & Liu, H. (2016). Lipid profiling of cyanobacteria *Synechococcus* sp. PCC 7002 using two-dimensional liquid chromatography with quadrupole time-of-flight mass spectrometry. *Journal of Separation Science*, 39(19), 374.

Shin, Y. G., & van Breemen, R. B. (2001). Analysis and screening of combinatorial libraries using mass spectrometry. *Biopharmaceutics & Drug Disposition*, 22(7-8), 353-372.

Shipovskov, S., & Reimann, C. T. (2007). Electrospray ionization mass spectrometry in enzymology: uncovering the mechanisms of two-substrate reactions. *Analyst*, 132(5), 397-402.

Singh, P., & Tiwary, B. N. (2016). Isolation and characterization of glycolipid biosurfactant produced by a *Pseudomonas otitidis* strain isolated from Chirimiri coal mines, India. *Bioresources and Bioprocessing*, 42(3), 1-16.

Singh, R., Parihar, P., Singh, M., Bajguz, A., Kumar, J., Singh, S., & Prasad, S. M. (2017). Uncovering potential applications of cyanobacteria and algal metabolites in biology, agriculture and medicine: current status and future prospects. *Frontiers in Microbiology*, 8(515), 1-37.

Soulé, S., Güntner, C., Vázquez, A., Argandona, V., Moyna, P., & Ferreira, F. (2000). An aphid repellent glycoside from *Solanum laxum*. *Phytochemistry*, 55(3), 217-222.

Stecher, G., Huck, C. W., Popp, M., & Bonn, G. K. (2001). Determination of flavonoids and stilbenes in red wine and related biological products by HPLC and HPLC–ESI–MS–MS. *Fresenius' Journal of Analytical Chemistry*, 371(1), 73-80.

Stefanowicz, P., Prasain, J. K., Yeboah, K. F., & Konishi, Y. (2001). Detection and partial structure elucidation of basic taxoids from *Taxus wallichiana* by electrospray ionization tandem mass spectrometry. *Analytical Chemistry*, 73(15), 3583-3589.

Stothers, J. B. (2012). *Carbon-13 NMR Spectroscopy: Organic Chemistry, A Series of Monographs, edited by Stothers JB, New York: Elsevier*, 12-126.

Takahashi, K., & Ono, S. (1973). Calorimetric studies on the mutarotation of D-galactose and D-mannose. *The Journal of Biochemistry*, 73(4), 763-770.

Tokuda, H., Nishino, H., Shirahashi, H., Murakami, N., Nagatsu, A., & Sakakibara, J. (1996). Inhibition of 12-O-tetradecanoylphorbol-13-acetate promoted mouse skin papilloma by digalactosyl diacylglycerols from the fresh water cyanobacterium *Phormidium tenue*. *Cancer Letters*, 104(1), 91-95. 5-3753.

Tolstikov, V. V., & Fiehn, O. (2002). Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. *Analytical Biochemistry*, 301(2), 298-307.

Torres, A. M., & McClung, R. E. D. (1991). J-compensated DEPT sequences. *Journal of Magnetic Resonance*, 92(1), 45-63.

Vestal, M. L., Juhasz, P., & Martin, S. A. (1995). Delayed extraction matrix-assisted laser desorption time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 9(11), 1044-1050.

Vinogradov, E., & Bock, K. (1999). The structure of the core part of *Proteus mirabilis* O27 lipopolysaccharide with a new type of glycosidic linkage. *Carbohydrate Research*, 319(1), 92-101.

Voutquenne, L., Guinot, P., Thoison, O., Sevenet, T., & Lavaud, C. (2003). Oleanolic glycosides from *Pometia ridleyi*. *Phytochemistry*, 64(3), 781-789.

Williams, D. E., Sturgeon, C. M., Roberge, M., & Andersen, R. J. (2007). Nigricanosides A and B, antimitotic glycolipids isolated from the green alga *Avrainvillea nigricans* collected in Dominica. *Journal of the American Chemical Society*, 129(18), 5822-5823.

Willker, W., Leibfritz, D., Kerssebaum, R., & Bermel, W. (1993). Gradient selection in inverse heteronuclear correlation spectroscopy. *Magnetic Resonance in Chemistry*, 31(3), 287-292.

Yang, L., & Zhang, L. M. (2009). Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. *Carbohydrate Polymers*, 76(3), 349-361.

Yates, P. (1967). *Structure Determination: Commentaries on the Determination of the Structures of Some Natural products, edited by Yates P. New York: WA Benjamin*, 1-175.

Yemm, E. W., & Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal*, 57(3), 508.

Zhang, H., Shen, P., & Cheng, Y. (2004). Identification and determination of the major constituents in traditional Chinese medicine Si-Wu-Tang by HPLC coupled with DAD and ESI–MS. *Journal of Pharmaceutical and Biomedical analysis*, 34(3), 705-713.

Chapter 4

CYTOTOXIC POTENTIAL OF GLYCOLIPIDS FROM *Synechocystis* sp. AGAINST COLON CANCER

Contents

4.1 Introduction

Growth of multicellular organisms is governed by a balance between cell division and cell death. Disorders of these processes have pathologic consequences and sometimes lead to the development of cancer. Cancer cells generally exhibit highly proliferative, migrative and matrix-invasive characteristics by modulating expression of signalling molecules (Sherr, 2004). Promising anticancer agents with strong inhibitory effects on survival-related proteins or the ability to activate apoptosis-associated proteins have been proposed, especially those found in natural sources. Many studies have focused on selectively killing tumor cells through programmed cell death processes (Ferreira et al., 2002).

One of the emerging schemes in drug discovery is to develop agents that target cell-cycle checkpoints which are responsible for control of cell-cycle phase progression. Cells transverse the cell-cycle in several well-controlled phases (Sherr, 2000; Gabrielli et al., 2012) (Figure 4.1). In G1 phase, cells commit to enter the cell-cycle and prepare to duplicate their DNA in S phase. After S phase, cells enter the G2 phase, where repair might occur along with preparation for mitosis in M phase. In the M phase, chromatids and daughter cells separate. After M phase, the cells can enter G1 or G0, a quiescent phase. Entry into each phase of the cell-cycle is carefully regulated by receptor collectives, termed cell-cycle checkpoints. Hence, they serve as important control mechanisms that ensure proper execution of cell cycle events.

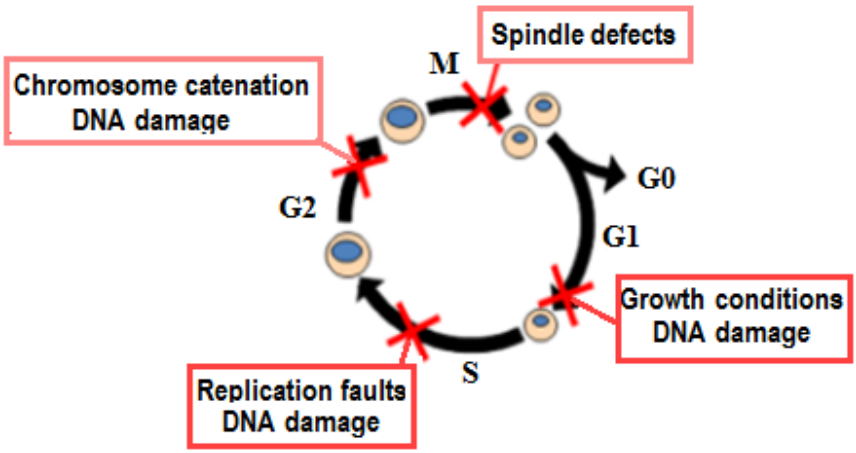


Figure 4.1 Cell cycle check points (Gabrielli et al., 2012)

The major currently used anticancer therapeutics may directly damage DNA or target and upset basic cell division mechanisms like DNA

replication and chromosome segregation. These changes elicit activation of elaborate checkpoint pathways to arrest cell cycle progression and promote repair or, in case of unrepairable damage, stimulate cell death (Medema & Macurek, 2012; Visconti et al., 2016). The cell-cycle DNA damage checkpoints occur late in G1, which prevents entry to S phase, and late in G2, which prevents entry to mitosis. The checkpoint control system is regulated by a family of protein kinases, the Cdks, which are in turn controlled by a complex array of proteins, including cyclins (Senderowicz & Sausville, 2000). At the G1 checkpoint in late G1 phase, the cell either exits to G0 or commits to the cell-cycle and enter into S phase. Analogous to G1 checkpoint, the G2 checkpoint allows the cell to repair DNA damage before entering mitosis. In fact, DNA damage that occurs in a cancer cell with a defective G1 checkpoint may result in more profound G2-M arrest. Defects in the G1 arrest checkpoint may lead cancer cell to enhanced proliferation, and efforts to correct these problems may slow growth and induce cell death. Defects in the G2-M arrest checkpoint may allow a damaged cell to enter mitosis and undergo apoptosis, and efforts to enhance this effect may increase the cytotoxicity of DNA damaging agents (Jackson et al., 2000; Hirose et al., 2001). Alternatively, efforts to increase G2-M arrest have also been associated with enhanced apoptosis.

Apoptosis is a kind of programmed cell death that upon receiving specific signals instructing the cells and causes specific morphological changes such as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process (Jacobson et al., 1997;

Fadeel et al., 1999). Apoptosis occurs on a continuous basis in the body in order to maintain homeostasis of 'good' verses 'bad' cells. This plays an essential role as a protective mechanism against tumor cells which are damaged or excess cells that have been improperly produced (Ellis et al., 1991; Cotter, 2009). Decreased apoptotic rate favours carcinogenesis by allowing cell survival and thus it is considered as central mechanism of tumorigenesis (Thompson, 1995). Thus, cancer treatment strategies have focused on targeting apoptotic inhibitors to stimulate apoptotic signals. The mechanisms of apoptosis are highly complex, involving an energy dependent cascade of molecular events. To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that both pathways are linked and that molecules in one pathway can influence the other (Igney & Krammer, 2002). Both mechanisms activate caspases, a family of cysteine aspartic proteases, functionally divided in two groups viz. initiators and executors, that induce proteolysis of cellular proteins and determine apoptotic cell death (Martin & Green, 1995; Alnemri et al., 1996; Kumar & Lavin, 1996; Singh, 2013).

In intrinsic pathway of apoptosis, permeabilization of the outer mitochondrial membrane by various cytotoxic stimuli and proapoptotic proteins leads to caspase activation (Decaudin et al., 1998; Green & Kroemer, 2004; Negi, 2013). Mitochondria play crucial role in the apoptotic process. Control of apoptosis by mitochondria occurs at several levels, by maintaining production of ATP and also by regulating mitochondrial membrane potential and mitochondrial membrane permeability for the release of certain apoptogenic factors from the inter

membrane space into the cytosol (Green & Reed, 1998). Mitochondrial dysfunction has been suggested to be central to the apoptotic pathway which participates in the induction of apoptosis. Mitochondrial membrane potential (MMP) is the electrochemical gradient which is maintained by the pumping of electrons across the inner mitochondrial membrane. Reduction in MMP stimulate opening of the mitochondrial permeability transition pores leading to the release of mitochondrial apoptosis initiation factors (AIFs) (Marchetti et al., 1996; Susin et al., 1996; Zamzami et al., 1996). Mitochondrial permeabilization is monitored by Bcl-2 family proteins, mitochondrial lipids and the permeability transition pore components (Certo et al., 2006). Set of proteins such as cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G which are present in the space between the inner and outer mitochondrial membranes are released on the disruption of outer mitochondrial membrane. On reaching cytosol, these apoptogenic proteins activate the cell death by triggering caspase activation (Saelens et al., 2004).

The extrinsic signaling pathways of apoptosis involve transmembrane death receptor-mediated interactions. Death receptors are members of the tumor necrosis factor (TNF) receptor gene super family that consists of more than 20 proteins. They regulate cell death, cell survival, differentiation or immune response (Walczak & Krammer, 2000; Liu & Chang, 2011). Members of the TNF receptor family share similar, cysteine-rich extracellular domains. They consist of cytoplasmic death domain of about 80 amino acids which transmits the death signal from the cell surface to intracellular signaling pathways (Ashkenazi & Dixit, 1998). Extrinsic pathway is activated by the ligation of cell surface death

receptors. The best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-a/TNFRl, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Chicheportiche et al., 1997; Peter & Kramer, 1998; Suliman et al., 2001). On binding of respective ligands (FasL and TNF-a) to their receptor, both Fas and TNF1 forms a complex with adapter proteins and procaspase 8. This complex prompts cleavage of procaspase 8 into active caspase 8, which further activates caspase 3 and results in the induction of apoptosis. Fas signaling plays vital role in immune surveillance and in the removal of self-reactive lymphocytes. It has been reported that defects in this pathway leads to many malignancies and autoimmune diseases. Therefore, various therapeutic strategies have been developed, targeting death receptors to enhance cancer cell apoptosis (Ashkenazi, 2008; Negi, 2013).

The relationship between cell cycle arrest and apoptosis is complex. Signaling pathways following apoptosis-inducing stimuli either arrest the cell cycle to allow time for DNA repair or switch to initiation of apoptosis after apoptogenic agents. An important cell cycle regulator plays a central role in the regulation of cell cycle arrest and cell death is the tumor suppressor, p53 (Vogelstein et al., 2000). It has recently been discovered that the p53 activates apoptosis through parallel pathways that may depend on transcription events or not (Fuster et al., 2007). Under many types of stress, p53 induces cell cycle arrest and apoptosis to maintain genomic integrity and prevent damaged DNA being passed on to daughter cells. Tumor cells have inactivated p53. About 50% of this inactivation is achieved through mutations in its sequence and the rest by disabling key components that lie upstream or downstream of

p53 in a common signaling pathway (Boyer et al., 2004). Thus, reactivation of these signals is important in cancer therapy.

Chemoprevention is the most promising and novel strategy for the prevention, suppression and reversal of carcinogenesis by the use of natural, synthetic or biologic chemical agents (Tsao et al., 2004; Demain & Vaishnav, 2011; Meiyanto et al., 2012; Bishayee & Sethi, 2016). Chemopreventive agents inhibit the development of invasive cancer by inhibiting one, or more events involved in the tumor promotion process (Sporn, 1976; Hong & Spom, 1997). The molecular mechanisms associated with chemoprevention are modulation of signaling cascades, regulation of cell proliferation, apoptosis and the suppression of chronic inflammation, metastasis and angiogenesis. An ideal chemopreventive agent should be nontoxic to normal cells, highly effective against multiple sites, economical to use, and should possess a known mechanism of action (Wattenberg, 1985; Greenlee, 2012; Yarla et al., 2016). Therefore, the concept of chemoprevention is gaining attention now a days because it serves as a cost-effective method of cancer treatment.

One of the common strategies for chemoprevention is the use of nonsteroidal anti-inflammatory drugs (NSAIDs). Several studies have shown that NSAIDs increase the production of reactive oxygen species (ROS) and create an oxidative stress (Giardina & Inan, 1998; Minami et al., 2005; Negi, 2013). ROS have been widely reported to play a significant role in induction of apoptosis by chemotherapeutic agents (Matés & Sánchez-Jiménez, 2000; Huang et al., 2003; Park et al., 2004). Aerobic metabolism generates ROS such as hydrogen peroxides, hydroxyl radicals

and superoxide anion, and controls many biochemical and pathological processes (Inoue et al., 2003). ROS is associated with initiation, progression, and metastasis of cancer (Pelicano et al., 2004). Low levels of ROS may contribute to the oncogenic phenotype by inducing cell proliferation and genetic instability. However, high concentration of ROS can act as anti-tumorigenic species by inducing DNA damage- and oxidative stress-mediated apoptosis (Valko et al., 2006; Trachootham et al., 2009). These studies suggest that ROS-induced DNA damage and oxidative stress represent a major anti-cancer mechanism of chemopreventive agents (Rigas & Sun, 2008).

Various types of tumours were known to have different histopathologies, genetic and epigenetic variations, and clinical outcomes (Vargo-Gogola & Rosen, 2007). Therefore, it is important to understand mechanisms of action of chemotherapeutics in developing novel therapies. Diverse experimental models have been evolved to study cancer pathobiology, because certain types of manipulations for genetic and DNA methylation analysis and drug testing are ethically, and in practice, difficult to perform in animals (Engel et al., 1978; Van Staveren et al., 2009). Cell lines emerge as a feasible alternative to overcome the issues in animal models, being easy to manipulate and molecularly characterize (Ruhe et al., 2007). This cell model is exceptional for the fundamental study of cellular pathways and for disclosing critical genes involved in cancer. Also, characterization of cancer cell lines is essential for understanding the action mechanisms and resistance/sensitivity patterns of chemotherapeutics already in use and for the development of more targeted anticancer drugs (Ferreira et al., 2013).

In the present study, mono galactosyl glycolipid G-2 and xylosyl glycolipid G-5 obtained in good yield from *Synechocystis* sp. were screened for their antiproliferative activity against SW480 colon cancer cell line. Cytotoxic potential of the highest active glycolipid was further studied using different methods. Apoptosis induction, DNA damage, ROS production, cell cycle checkpoint arrest and mitochontrial potential changes were determined using specific assay techniques. Involvement of selected regulatory proteins in apoptosis induction and cell cycle arrest was also assessed.

4.2 Materials and methods

4.2.1 Cell culture

SW480 (Colon carcinoma) cell line, purchased from National Centre for Cell Sciences (NCCS), Pune, India, was maintained in Dulbecos modified Eagles medium (DMEM) (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS (Fetal bovine serum), L-glutamine, sodium bicarbonate and antibiotic solution containing, penicillin (100 µg/mL), streptomycin (100 µg/mL) and amphoteracin B (2.5 µg/mL) (Wang, 2003). Cultured cell lines were kept at 37 °C in humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Two days old confluent monolayer of cells was trypsinized using 500 µL of 0.025% trypsin in PBS (phosphate buffered saline**)**/0.5 mM EDTA solution (Himedia) and then cells were suspended in 10% growth media. 100 µL of cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated for 24 h. 1 mg each of the glycolipids G-2 and G-5 was added to 1mL of DMEM and dissolved

completely by cyclomixer. Further, solution was filtered through 0.2 µm millipore syringe filter to ensure the sterility. Freshly prepared samples were five times serially diluted by two fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg in 100 µL of 5% DMEM) and 100 µL each of the diluted samples was added in triplicates to the respective wells after removing the growth media, and incubated. Viability of cells was evaluated by direct observation of cells through inverted phase contrast microscope and also by MTT (3-(4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide) assay method.

4.2.2 Cytotoxicity assay by direct microscopic observation

Morphological alterations of SW480 cell lines after exposure to major glycolipid fractions G-2 and G-5 were observed at an interval of each 24 h; up to 72 h in an inverted phase contrast tissue culture microscope, Olympus CKX41 with Optika Pro5 CCD camera (Pattillo & Gey, 1968). Olympus CKX41 inverted microscope with tilting and trinocular head options and fluorescence upgrade capability is suitable for advanced technques such as imaging of GFP (green fluorescent protein) and other fluorescence applications. High quality images of cells with optimum contrast and resolution were produced using this facility.

4.2.3 Cytotoxicity assay by MTT method

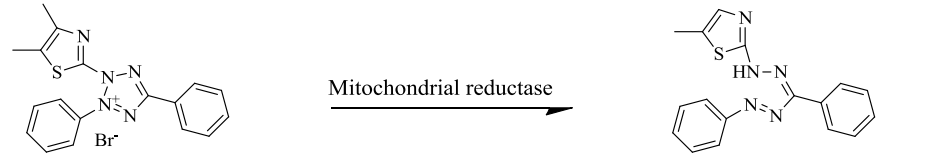
MTT (3-(4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide) assay is a method used to measure cell viability and hence to evaluate cytotoxicity of compounds (Mosmann, 1983). In this method, metabolic activities of the cells are measured spectrophotometrically through the

reduction of yellow-coloured MTT salt to purple formazan (E,Z-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan) crystals (Figure 4.2). The reaction is catalysed by mitochondrial succinate dehydrogenase which occur only in viable cells (Gerlier & Thomasset, 1986; Loveland et al., 1992). The greater the purple colour of reaction, the lesser the extent at which a particular compound induces cell death. Samples for cytotoxicity measurements are mostly dissolved in DMSO because of its high solubilizing capacity and low viscosity, and also there is no reports on the cytotoxicity of DMSO.

Viability of SW480 cells, treated with different concentrations of glycolipids G-2 and G-5, was measured using MTT assay. All the chemicals used in this analysis were purchased from Merck India. Incubated cell suspensions were washed with phosphate buffer saline (PBS) followed by addition of 30 µL MTT solution (5 mg/mL) dissolved in PBS and incubated for 3 h at 37 °C to complete the reaction. After incubation, excess MTT was removed by washing with PBS. Cell lines were further treated with 200 µL DMSO and incubated at room temperature to dissolve the formazan product. The solution was centrifuged at top speed for 2 min to precipitate cell debris and to obtain cell free suspensions. Optical density (OD) of solubalised formazan product was measured at 540 nm keeping DMSO as blank in a microplate reader (ELISASCAN, ERBA).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability } = \frac{Mean\ OD\ samples}{Mean\ OD\ of\ control} \times 100$$



3-(4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide **(MTT)**

E,Z-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl formazan **(Formazan)**

Figure 4.2 Reduction of MTT to formazan crystals

4.2.4 Comet assay

Oxidative DNA damage was detected by means of an endonuclease (formamidopyrimidine-DNA glycosylases (FPG) modified comet assay (Collins et al., 1993; Siddique et al., 2005). The cells were cultured in 6 well plates and treated with IC_{50} (17.88 µg/mL) of the highest active xylosyl glycolipid, G-5 and incubated overnight. The cells were trypsinzed, washed with fresh media and used for comet assay. Control was also maintained by using cell suspensions without adding the glycolipid, in the same reaction conditions. Fully frosted microscope slides were precoated with 1 mL of 0.75% normal melting point agarose (NMA Invitrogen, USA) and stored at 4 °C. This layer was removed before use and 120 µL of 0.75% NMA was pipetted on to the slides. Cell suspensions were mixed with 10 µL of low melting point agarose (Invitrogen, USA) and pipetted over the first layer of agarose. NMA (80 µL) was used as a final protective layer and the slides were covered with cover slips. After each step, the slides were incubated at 4 °C for

10 mins to allow for agarose polymerization. The cover slip was removed and the slide was immersed in freshly prepared, chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1.0% Triton X-100, pH 10) for 2 h. For the enzyme treatment, the slides were removed from the lysis buffer and incubated with enzyme reaction buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/mL BSA, adjusted to pH 8 with KOH) for 5 min. Thereafter, 50 µL of formamidopyrimidine-DNA glycosylases (FPG) was added to the slide and the reaction was covered with a cover slip. The slides were incubated at 37 °C for 30 min. After the enzyme treatment, the cover slips were removed and the slides were placed in a horizontal electrophoresis platform (Life Technologies, Gaithersburg, MD, USA) containing fresh chilled electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13). The slides were incubated in the platform for 10 min to allow the DNA to unwind. The samples were subsequently electrophoresed at 0.7 V/cm (300 mA/25 V) at 4 °C for 15 min using a power supply from Techno Source Pvt. Ltd. (Mumbai, India). The slides were then washed three times with 0.4 M tris buffer (pH 7.5) at 4 °C to neutralize the reaction. The slides were stained with 50 µL ethidium bromide (20 µg/mL) for 10 min in the dark. After staining, the slides were dipped once in chilled distilled water to remove the excess stain and cover slips were placed over the slides. All steps after the single gel preparation were performed under dimmed light to avoid any light-induced DNA damage. The slides, both control and samples, were photographed using inverted epifluroscent microscope Olympus CKX41 attached with Opitka Pro5 CCD camera. Comets were scored using Tritek comet scoring software and correlated statistically.

4.2.5 Flow cytometric analysis of the cell cycle distribution

Monitoring cell's ability to proliferate is critical for assessing cell health during toxicity studies. The most accurate method of doing this is by directly measuring DNA synthesis using flow cytometry (Nicoletti et al., 1991). DNA histogram gives a static picture of the proportion of cells in different phases of cell cycle. The Muse™ cell cycle kit allows for facile and rapid quantitative measurements of percentage of cells in each phase of cell cycle on the Muse™ cell analyzer. The basic principle of this assay is the standard ethanol fixation and detergent permiabilization which is sufficient to gain access to DNA during active cell cycle. The kit utilizes a premixed reagent which includes nuclear DNA intercalating stain, propidium iodide (PI), which discriminates cells at different stages of cell cycle based on differential DNA content in presence of RNAase and hence increases specificity of DNA staining in each phase (G0/G1, S and G2/M) (Fried et al., 1976). SW480 colon cancer cells were cultured as per standard procedures described earlier and treated with IC_{50} value of glycolipid G-5 for 24 h. The cell sample was transferred to a 12×75-mm polystyrene tube. The minimum recommended number of cells for fixation in a tube is 1×106 cells. The samples were centrifuged at 3000 rpm for 5 min and supernatant was removed without disturbing the pellet. After centrifugation, the cell pellet forms a white film on the bottom of the tube. Appropriate volume of PBS was added to each tube (i.e., 1 mL of PBS per 1×106 cells) and the contents were mixed by vortexing gently followed by centrifugation at 3000 rpm for 5 min. The supernatant was discarded without disturbing the cell pellet, leaving approximately 50 µL of PBS per 1×106 cells. The cell pellet was resuspended in the residual

PBS by vortexing. The resuspended cells were added drop wise into a tube containing 1 mL of ice cold 70% ethanol while vortexing at medium speed, and the tube was capped and frozen at -20 °C. After the overnight incubation, samples were centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was removed and 250 µL PBS was added to the pellet, and centrifuged again at the same rpm and time. The pellet was taken after discarding the supernatant and 250 µL of cell cycle reagent (propidium iodide) was added. It was incubated at dark for 30 min followed by analysis using a flow cytometer. Gating was performed with reference to untreated control cells and cell count was measured.

4.2.6 Apoptosis assessment using AO/EB double staining

DNA-binding dyes acridine orange (AO) and ethidium bromide (EB) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells (Zhang et al, 2002). Differential absorption of dyes by viable and non-viable cells along with variation in the emitted fluorescence was effectively used to detect apoptosis in tumor cells. The cells grown to 60-70% confluency was treated with IC_{50} of xylosyl glycolipid G-5 for 24 hours. Cells were washed by cold PBS and then stained with a mixture of AO (100 µg/mL) and EB (100 µg/mL) at room temperature for 10 min. The stained cells were washed twice with PBS and visualized in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green yellow nucleus with condensed or fragmented chromatin), late

apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).

4.2.7 Reactive oxygen species (ROS) detection

Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses. Most of the chemotherapeutic agents destroy cancer cells by augmenting ROS stress. Common techniques for measuring ROS utilize cell permeable fluorescent and chemiluminescent probes. One of the most widely used techniques employ 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell (Eruslanov & Kusmartsev, 2010). It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time. DCHF-DA can be deacetylated by intracellular esterase to non-fluorescent DCFH, which can be oxidized by ROS to the fluorescent compound 2',7' dichlorofluorescein (DCF) (Figure 4.3). The fluorescent intensity of DCF is proportional to the amount of ROS produced by the cells (Karlsson et al., 2010).

SW480 cells were seeded onto 96-well plates at a density of 5000–10000 cells per well. After 24 h seeding (at 40–70% density), cells were washed with PBS and treated with 100 μM DCFH-DA (diluted in DMEM+1% FBS) for 1 h (at 37 °C, 5% CO_2). Cells were then washed with PBS, and treated with IC_{50} of xylosyl glycolipid followed by incubation for 24 h. Untreated wells were considered as control. DCF fluorescence was imaged in a fluorescent microscope (Olympus CKX41 with optika pro5 CCD camera). Excess dye was washed with PBS and

the fluorescence was measured using a fluorimeter at 470 nm excitation and emission at 635 nm (Qubit 3.0 , Life technologies, USA) and expressed in arbitrary units.

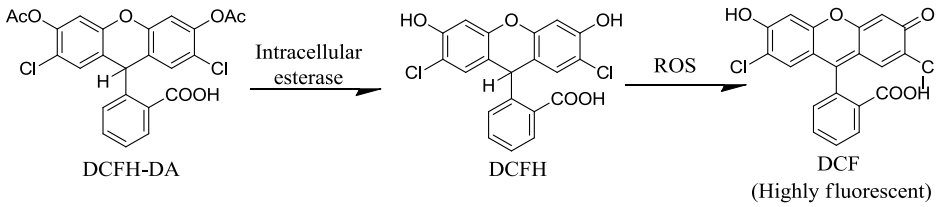


Figure 4.3 Generation of green fluorescent DCF by reactive oxygen species (ROS)

4.2.8 Mitochondrial membrane potential detection

Measurement of changes in mitochondria membrane potential ($\Delta\psi_m$) was performed with mitopotential assay kit (Muse™, EMD Millipore Bioscience). The assay utilizes a cationic, lipophilic mitopotential dye to detect changes in the mitochondrial membrane potential and 7-AAD (7-aminoactinomycin D) as an indicator of cell death (King, 2000). High membrane potential drives accumulation of mitopotential dye within inner membrane of intact mitochondria resulting in high fluorescence. Cells with depolarized mitochondria demonstrate a decrease in fluorescence and a downward shift. This parameter is displayed in the mitopotential axis. Dead cell marker (7-AAD) is used as an indicator of cell membrane structural integrity and cell death. It is excluded from live, healthy cells, as well as early apoptotic cells. Dead cells thus show increased fluorescence in the viability axis. Therefore, this flow-cytomety-based assay differentiates four

populations of cells; live cells with depolarized mitochondrial membrane: mitopotential$^-$/7-AAD$^-$; live cells with intact mitochondrial membrane: mitopotential$^+$/7-AAD$^-$; dead cells with depolarized mitochondrial membrane: mitopotential$^-$/7-AAD$^+$ and dead cells with intact mitochondrial membrane: mitopotential$^+$/7-AAD$^+$.

SW480 cells seeded on T25 flasks were grown to 70% confluency and added with IC_{50} concentrations of the glycolipid. Untreated cells were kept as control and the samples were incubated for 24 h. Cells were trypsinized and subjected to flow cytometery as per the following procedures. A mitopotential working solution was prepared by diluting the Muse™ mitopotential dye (1:1000) in 1X assay buffer. The cells after centrifugation were resuspended in 1X assay buffer and added with 95 µL of mitopotential working solution. Solution was mixed thoroughly by pipetting and incubated for 20 min in CO_2 incubator at 37 °C. After incubation, 5 µL of 7-AAD was added to each well and mixed thoroughly by vortexing for 3 to 5 s and incubated for 5 min at room temperature. The samples were loaded on to a flow cytometer (Millipore, USA) and events were acquired after gating and correlated with controls.

4.2.9 Regulatory protein expression

a) Isolation of total RNA (trizol method)

Total RNA was isolated using total RNA isolation kit according to the manufacturer instructions (Invitroge, USA). 70% confluent SW480 cells in 6 well plate (approximately 4 X 10^5 cells) were treated with IC_{50} of the glycolipid and incubated at 37 °C for 24 h in a CO_2 incubator. A

set of untreated control cells was also incubated at the same conditions. After removing incubation media aseptically, 200 µL of trizol reagent was added to initiate cell lysis and incubated for 5 min. The contents were then transferred to a fresh sterile eppendorf tube. 200 µL of chloroform was added and shaken vigorously for 15 s and incubated for 2-3 min at room temperature, followed by centrifugation at 14000 rpm for 15 min at 4 °C. The aqueous layer was collected and 500 µL of 100% isopropanol was added to precipitate total RNA. It was incubated for 10 min at room temperature and then centrifuged at 14000 rpm for 15 min at 4 °C. Supernatant was discarded and pellet thus obtained was washed with 200 µL of 75% of ethanol (Merck). It was then centrifuged at 14000 rpm for 5 min at 4 °C in a cooling centrifuge (Remi CM12). The RNA pellet was dried and suspended in TE buffer for further analysis **(**Chomczynski & Sacchi, 1987; Chomczynski, 1993).

b) Reverse transcriptase PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction commonly used in molecular biology to analyse gene expression in plant and animal models. In RT-PCR, an RNA strand is first reverse transcribed into its DNA complement (cDNA) catalyzed by the enzyme reverse transcriptase (Shiao, 2003). The cDNA thus obtained can be amplified using PCR to obtain multiple copies. RT-PCR technique was performed in the present study to analyze p53, FasL and Mapk p38 expressions using specific primers (Table 4.1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal/housekeeping control, which is essential to normalize cancer

related gene expression. The most important characteristic of such genes is that their presence and expression levels at all cells remain relatively constant under different experimental conditions. GAPDH has been identified as an optimal choice of housekeeping gene to determine the expression of oxidative stress induced genes in different tumors such as human hepatocellular carcinoma, colon carcinoma, lung adenocarcinoma, etc (Said et al., 2009).

The cDNA synthesis was performed using cDNA synthesis kit (iScript™, BIO-RAD). About 4 µL of 5x iScript reaction mix , 1 µL of reverse transcriptase, 5 µL of RNA template were added to an RNAse free tube and made up to 20 µL with sterile distilled water. The solution was mixed by pipetting gently up and down. cDNA synthesis was performed in the previously programmed thermal cycler (Eppendorf Master Cycler). Cycling conditions employed were 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C, and held at 4 °C. The amplification was done using Thermoscientific amplification kit. 25 µL of PCR master mix, 2 µL of forward primer (0.1-1.0 µM), 2 µL of reverse primer(0.1-1.0 µM), 5 µL of template DNA (10 pg - 1 µg) were mixed and made upto 50 µL with sterile distilled water (nuclease-free). Denaturation was performed at 95 °C for 30 s followed by annealing at Tm (basic melting temperature) for 30 s, extension at 72 °C for 1 min which was repeated for 35 cycles and the final extension at 72 °C for 5 min. After amplification, the PCR product was separated by agarose gel electrophoresis (Glazer et al., 2010).

c) Agarose gel electrophoresis

Agarose gel electrophoresis is a method for separating and visualizing DNA fragments. The fragments are separated based on charge and size, and move through agarose gel matrix when subjected to an electric field. 1.5% agarose gel was prepared in 1X TE buffer and melted in hot water bath at 90 °C. Melted agarose was cooled down to 45 °C and 6 µL of ethidium bromide (10 mg/mL) was added and mixed. The warm agarose solution was poured into gel casting apparatus with gel comb. Gel was allowed to set completely and the comb was removed carefully. Electrophoresis buffer was poured in the electrophoresis tank and gel platform was placed in it so as to immerse the gel. Samples of DNA in buffer were loaded on to the gel and were run at 50 V for 30 min. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen) (Peacock & Dingman, 1968). `

Table 4.1 Primer sequences used in RT-PCR

Primer	Forward	Reverse
p53	5'-CACCATGAGCGCTGCTCA-3'	5'-GCAGGGGAGGGAGAGATG-3'
FasL	5'-GAGCACCAGACCTACTGCCAG-3'	5'-AATTTCTGGAGCCCTGTACCA-3'
Mapk p38	5'-CAGCTTCAGCAGATTATGCGT-3'	5'-GTACTGAGCAAAGTAGGCATGT-3'
GAPDH	5'ACTCAGAAGACTGTGGATGG-3'	5'-GTCATCATACTTGGCAGGTT-3'

4.3 Results and discussion

4.3.1 Morphological changes on SW480 cell lines

Morphological alterations of SW480 cell lines on treatment with different concentrations of mono galactosyl glycolipid G-2 and xylosyl glycolipid G-5 in comparison with control, are displayed in Figures 4.4 [a-f] and 4.5 [a-f] respectively. Cyto-morphological changes were found to be strictly concentration dependent for both the fractions. Treatment of glycolipids at various concentrations ranging from 6.5 to 100 µg/mL, found to cause cytoplasmic shrinkage, rounding up of cells, loss of cell adhesion, etc., in the cancer cells. Rounding up of cells were observed even at lower concentrations (6.5 and 12.5 µg/mL) and is more prominent in the xylosyl glycolipid treated cell line. At higher concentrations (25-100 µg/mL) the cells became shrunken and showed signs of detachment from the surface of the wells, which denoted cell death. Detectable changes in the morphology of cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm, and pyknotic nuclei were considered as indicators of cytotoxicity (Borenfreund & Puerner, 1985; Wyllie & Duval, 1992; Bozzuto et al., 2010; Brandhagen et al., 2013; Moses et al., 2016).

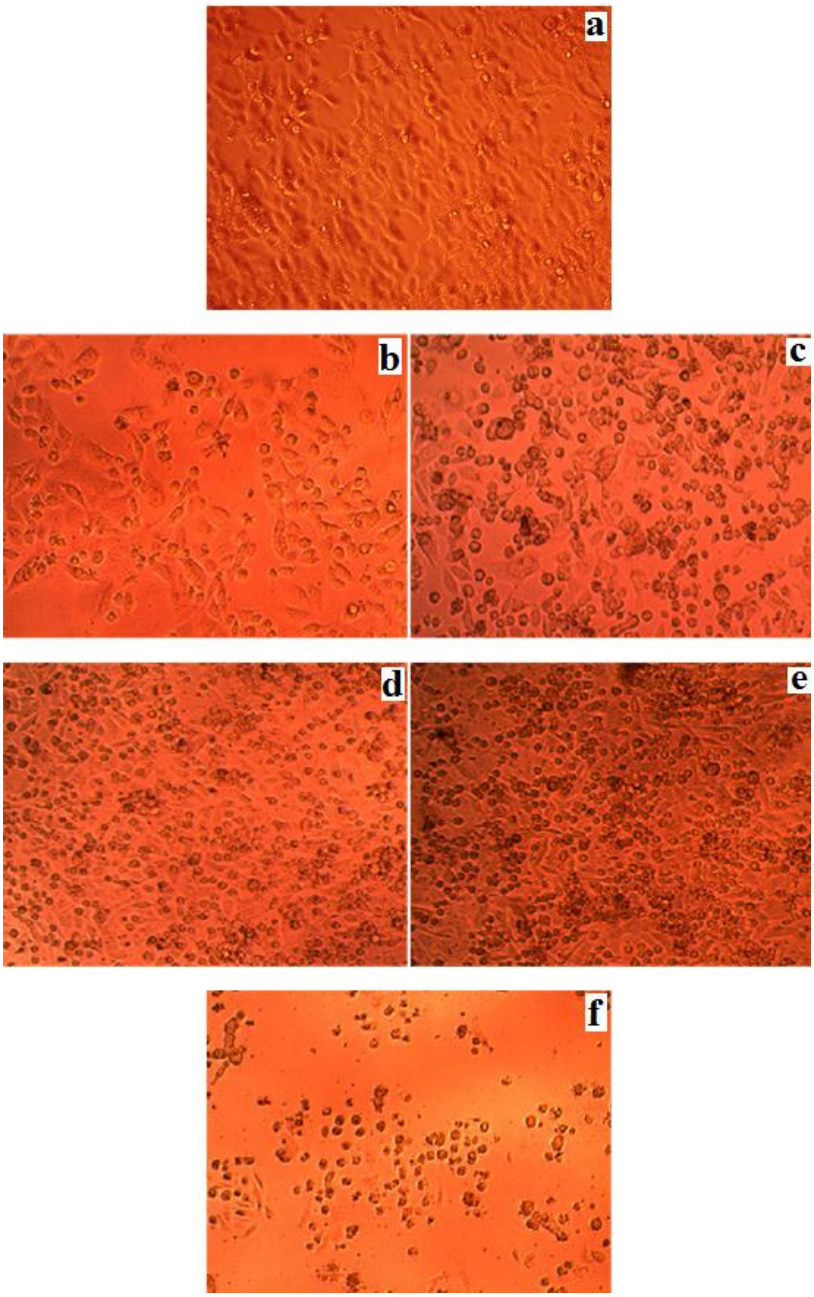


Figure 4.4 Morphological changes of SW480 cell line with different concentrations of mono galactosyl glycolipid G-2.;
a) Control; b) 6.25 µg/mL G-2; c) 12.5 µg/mL G-2;
d) 25 µg/mL G-2; e) 50 µg/mL G-2; f) 100 µg/mL G-2

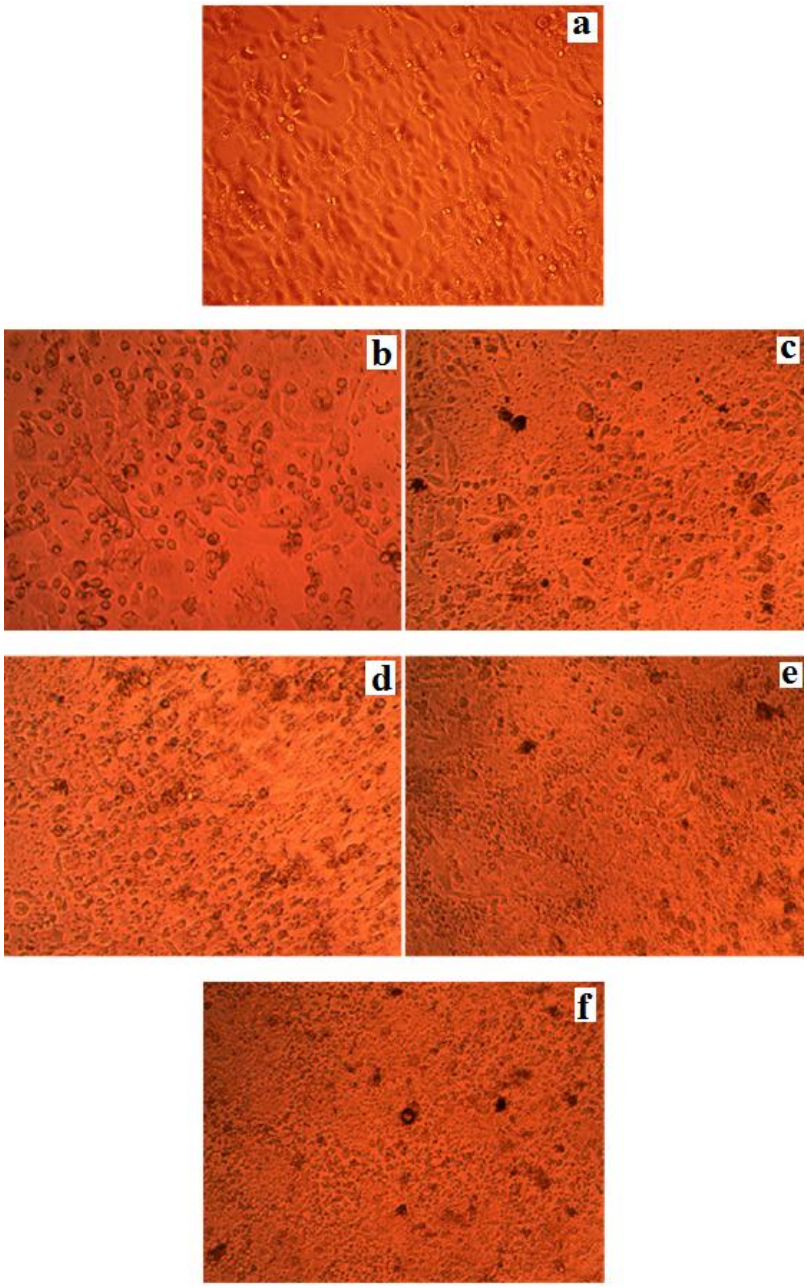


Figure 4.5 Morphological changes of SW480 cell line with different concentrations of xylosyl glycolipid G-5.;
a) Control; b) 6.25 µg/mL G-5; c) 12.5 µg/mL G-5;
d) 25 µg/mL G-5; e) 50 µg/mL G-5; f) 100 µg/mL G-5.

4.3.2 MTT assay

MTT assay based screening of antiproliferative activity indicated that both galactosyl and xylosyl glycolipids have significant effect on cell viability and is found to show dose dependent pattern with compound treatment. IC_{50} value of xylosyl glycolipiod G-5 against SW480 cell line was found to be 17.88 µg/mL and thus it possess higher activity against SW480 colon cancer cells compared with galactosyl glycolipid G-2 having IC_{50} 52.26 µg/mL. This indicated that G-5 has higher antiproliferative effect towards the colon cancer cell line. Activities of the glycolipids against SW480 are shown in Figure 4.6. MTT cytotoxicity assay is considered as the most reliable method of measuring cell death (Mosmann, 1983; Sylvester, 2011), and it was thus employed to measure the cytotoxicity of the glycolipid fractions. 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. The assay depends mainly on the activity of mitochondrial dehydrogenases. Thus, it measures metabolic activity, not necessarily viability (Fotakis & Timbrell, 2006). Some cells can be perfectly viable but not necessarily with a lot of metabolic activity. So it is always important to validate the results using something that directly measures cell apoptosis or necrosis. Hence, apoptosis induction by xylosyl glycolipid G-5 possessing higher antiproliferative potential was further analysed using suitable assays.

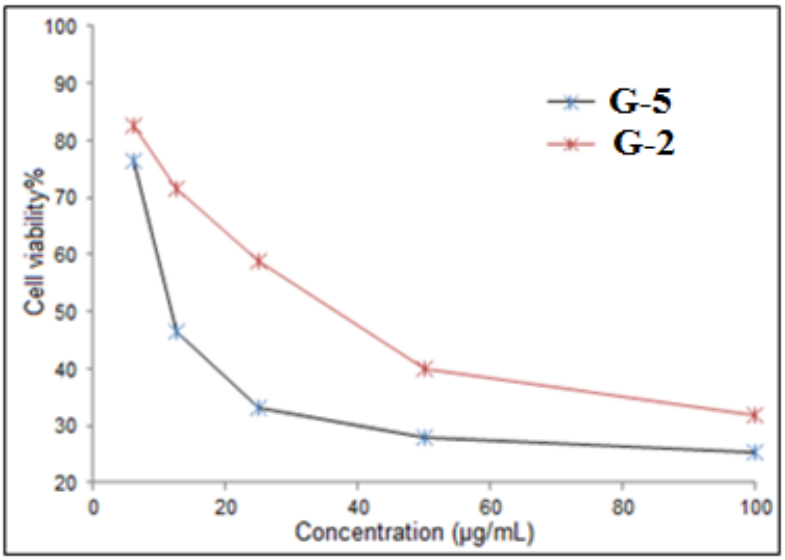


Figure 4.6 Graphical representation of cytotoxicity activity of mono galactosyl glycolipid (G-2) and xylosyl glycolipid (G-5) against SW480 cell line by MTT assay, concentration ranging between 6.25-100 µg/mL.

4.3.3 DNA damage induced by the compounds

Traditionally, single cell gel electrophoresis (SCGE) also known as comet assay has been considered as an easy, economical and speedy method to semi-quantify DNA damage such as single- and double-stranded breaks (Collins et al., 2008; Wasson et al., 2008). Because of its simplicity, sensitivity and the small number of cells/nuclei required to obtain robust results, it has been extensively applied to evaluate genotoxicity (Chen et al., 2011; Rodriguez et al., 2011). Tail length, tail moment and tail DNA percentage of comets were used to analyse DNA damage in the cancer cells. As the frequency of DNA breaks increases, so does the fraction of DNA extending towards the anode, forming the comet tail. Comets with DNA fragments in the tail represent early stages

of apoptosis (Lorenzo et al., 2013). In this study, formamidopyrimidine DNA glycosylase (FPG), that specifically recognizes the number of oxidized purine bases (Smith et al., 2006), was used to determine whether the xylosyl glycolipid G-5 could induce cellular DNA damage in SW480 cells. Comet images from control and the G-5 treated SW480 cells were analyzed. As shown in Figure 4.7, the nuclei (the stained circles) from the control essentially remained intact with almost no visible comet trail. After treating SW480 cells with the glycolipid, the nuclei exhibited not only a shrunken morphology but also a prominent comet tail. Tail moment and tail DNA percentage were found to be significantly high in glycolipid treated cells compared with control cells (Table 4.2). This implied that xylosyl glycolipd G-5 can elicit DNA damaging effects. It is well known that DNA fragmentation is a hallmark of apoptosis and mitotic catastrophe (Castedo et al., 2004). In the current study, the comet assay demonstrated DNA damage, which provided another experimental endpoint to validate existence of the apoptotic process in SW480 cells.

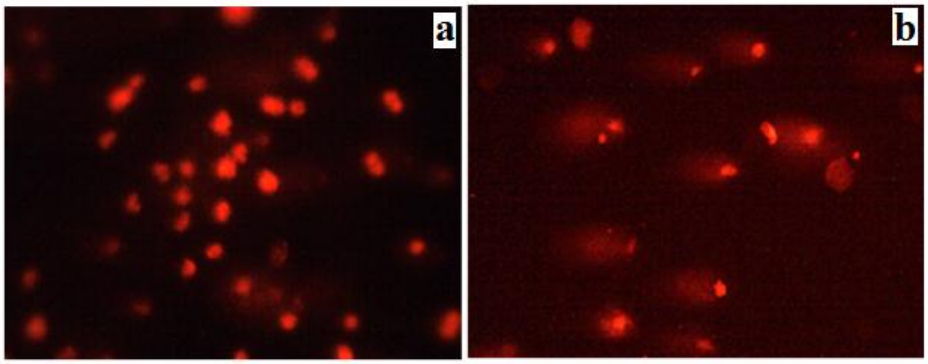


Figure 4.7 Comet assay image of EB-stained SW480 (a) control (b) after exposure to IC_{50} of xylosyl glycolipid G-5 for 24 h.

Table 4.2 Comet parameters of control and glycolipid treated SW480 cells

Comet Parameters	SW480 control		Treated with IC_{50} of G-5	
	Min.	Max.	Min.	Max.
Tail Length	0.000	2.000	3.000	15.000
% DNA in tail	0.007	1.952	7.167	83.334
Tail moment	0.085	1.155	1.314	24.246

4.3.4 Cell cycle arrest by flow cytometry

DNA distribution histograms of SW480 cells obtained from cell cycle flow cytometry analysis of control and glycolipid treated sample are as shown in Figure 4.8 a & b respectively. The percentage of cells in G2/M checkpoint in the SW480 sample treated with IC_{50} of glycolipid G-5 for 24 h was found to be 22.8 ± 1.2, which is significantly high compared with the G2/M cell count of control (8.8 ± 1.3). This was accompanied by a corresponding reduction in the percentage of cells in the G0/G1 and S phase. The results indicate that the antiproliferative mechanism induced by the glycolipid on SW480 cells was G2/M phase arrest. G2/M phase checkpoints play a key role in providing time for DNA repair, and may cause apoptosis to remove irreparably damaged cells (King et al., 1994). Several anticancer drugs lead to DNA damage, resulting in cell cycle modulations (Wang & Cho, 2004; Sakaue-Sawano et al., 2011). In general, cells with DNA damage cannot complete cell cycle while this DNA is not repaired, leaving to cellcycle arrest and cytotoxicity. G2/M checkpoint prevents cells from initiating mitosis after DNA injuries during G2 phase (Levine, 1997; Suganuma et al., 1999; Kastan & Bartek, 2004). It was reported that enhanced garlic

consumption is closely related with reduced colon cancer incidence (Haenszel et al. 1972; Buiatti et al., 1989).

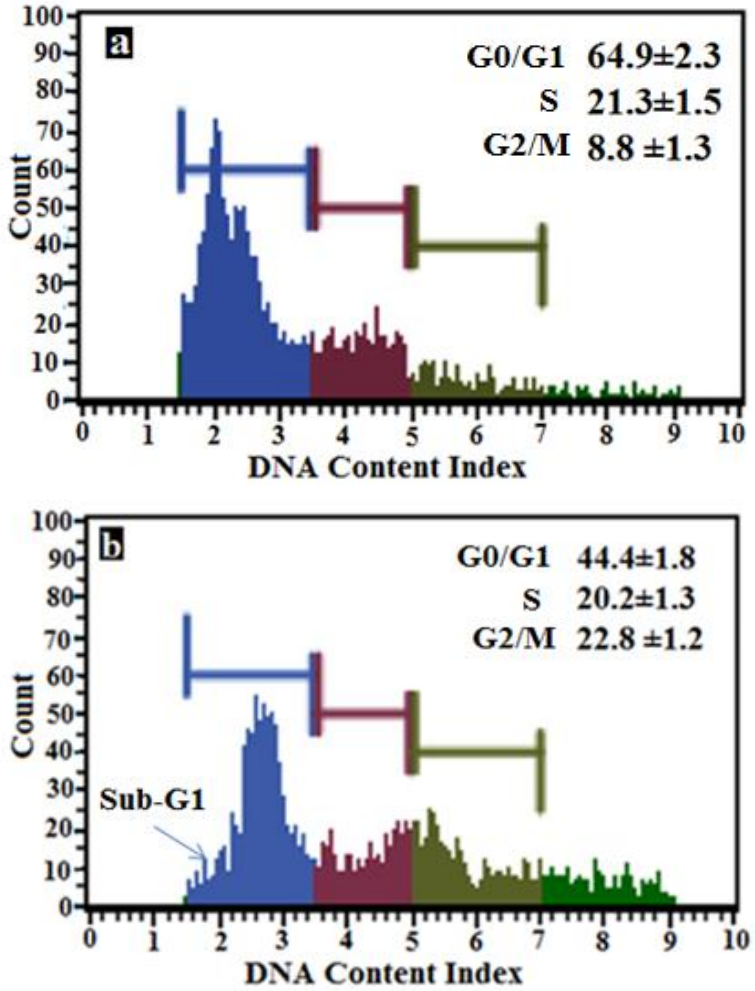


Figure 4.8 Flow cytometry cell cycle analysis using propidium iodide DNA staining. DNA content index of SW480 (a) control (b) after exposure to IC_{50} of glycolipid G-5.

Diallyl disulfide (DADS), an important oil-soluble organosulfur component of garlic (*Allium sativum*) has been reported to posess IC_{50} of 22.47 μM on COLO 205 colon cancer cells in vitro which induced

significant G2/M cell cycle arrest and apoptosis (Yang et al., 2009). Results from the mechanism of action of glycolipid G-5 on SW480 cell is found to be compatable with those findings. Sub-G1 accumulation was also observed in cell cycle analysis after glycolipid exposure. Glycolipid G-5 induced a G2/M cell cycle arrest suggesting that DNA repair process is activated and repairs the DNA damage by arresting the cell cycle in the G2/M phase. However, extensive DNA damage may eventually cause the cells to undergo a transition from the G2/M phase to the apoptotic phase, as evidenced by the appearance of the sub-G1 peak (Yeh et al., 2014).

4.3.5 Apoptosis studies

Apoptosis is a physiological pattern of cell death characterized by morphological features and extensive DNA fragmentation, the frequency and time of appearance of which depend on the cell line and apoptosis inducing signal. AO/EB double staining is a relatively new approach and is found to be more economical and convenient method to detect cell apoptosis. In comparison to AO staining, AO/EB method improves detection of apoptosis and can distinguish between late apoptotic and dead cells. AO can penetrate to normal and early apoptotic cells with intact membranes and emit green fluorescence when bound to DNA. EB can only enter to cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies (Liu et al., 2015). SW480 cells treated with IC_{50} of xylosyl glycolipid G-5 was analysed after AO/EB staining. In the control, the living SW480 cells were

stained bright green in spots with intact nuclei (Figure 4.9 a). After treating with compound for 24 h, AO/EB staining clearly shows condensed and fragmented nuclei. Bright green cells with nuclear margination and chromatin coagulation indicating early apoptotis, and orange cells with apoptotic bodies and chromatin fragmentation representing late apoptotis, were observed in the sample (Figure 4.9 b). The results suggested that the xylosyl glycolipid G-5 can induce apoptosis in SW480 colon cancer cells. Glycoglycerolipid analogues have gained importance in cancer chemoprevention due to their promising inhibitory effect on tumor growth. The nature of the sugar moiety, fatty acyl chain length and its position may influence the apoptosis inducing activity (Colombo et al., 1999; Colombo et al., 2000). Galactolipids isolated from marine diatom *Phaeodactylum tricornutum* were reported to reactivate proapoptotic genes disabled in the cancer cells and hence result in the induction of apoptosis (Andrianasolo et al., 2008).

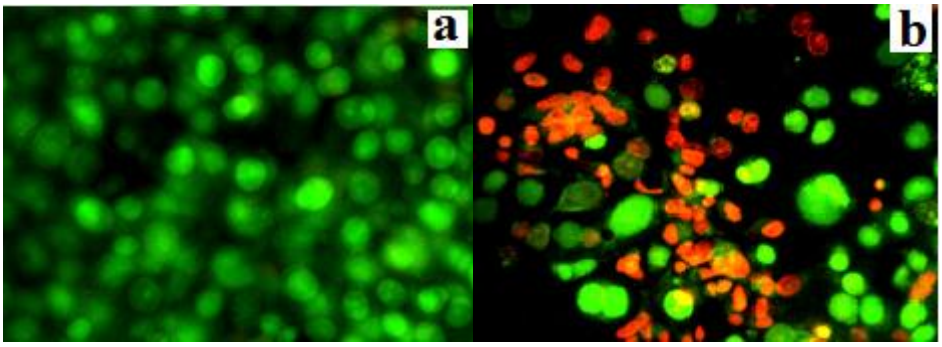


Figure 4.9 Detection of anti-proliferative mechanism (apoptosis /necrosis) treated with acridine orange and ethidium bromide double staining (a) Untreated SW480 control cells showing intact cells with green fluorescent nuclei (b) SW480 cells treated with IC_{50} of xylosyl glycolipid G-5

4.3.6 Imaging of intracellular ROS generation by fluorescent microscopy

Because ROS (reactive oxygen species) generation plays an important role in the proapoptotic activities of various agents (Genestra, 2007; Fauser et al., 2013), it was also investigated whether ROS generation was required in xylosyl glycolipid induced apoptosis. Intracellular ROS generation in the control and compound treated SW480 cells were assessed using fluoresecence measurements following staining with 2', 7' dichloro-dihydrofluorescei diacetate (DCHF-DA). Increase in intensity of the green fluorescent compound, DCF (2', 7' dichlorofluorescein) formed by the oxidation of non-fluorescent DCFH, which in turn produced by deacetylation of DCHF-DA by intracellular esterase, could be indicative of the extent of ROS generation induced by the compound (Karlsson et al., 2010). ROS generation in the present study was confirmed by measuring average green emission at 470 nm. The emission intensity was found to increase from 2462.29 in control to 3280.50 in sample and this was also visible from the fluorescence microscopic image of control and sample (Figure 4.10 a & b). Increased generation of ROS is presumed to be a final consequence of imbalance of the redox system and a shift from the reductive to the oxidative state in a cell (Aw, 1999). Findings on the dependence of cell viability to ROS production suggest that increasing in ROS levels exceeds the capacity of the cellular antioxidant defense system, and causes cells to enter a state of oxidative stress which results in damage of cellular components such as lipids, protein and DNA (Lovrić et al., 2005). The persistent oxidative stress that characterizes cancer cells has been proposed as the basis for a

strategy to develop new therapeutic approaches based on ROS. The increase in intracellular ROS generation induced by the xylosyl glycolipid G-5 can be an event upstream of changes in the mitochondrial membrane potential and caspase activation.

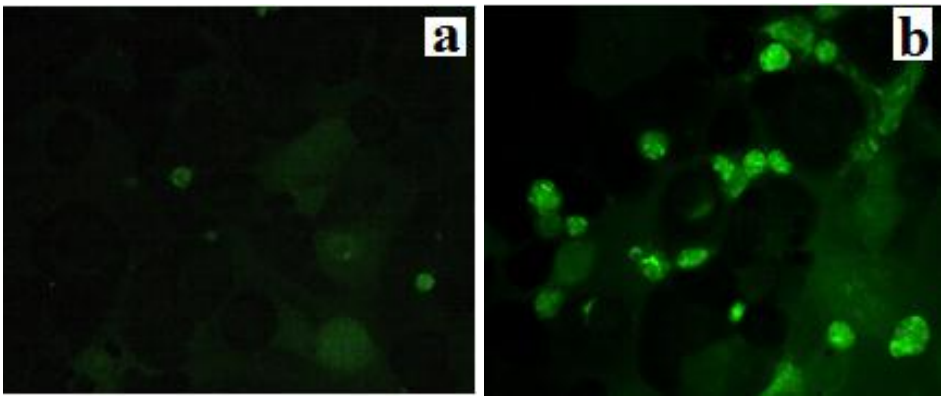


Figure 4.10 Measurement of ROS generation in SW480 cells using DCFH-DA (a) Untreated Control cells (b) Cells treated with IC_{50} of xylosyl glycolipid G-5.

4.3.7 Determination of mitochondrial membrane potential by flow cytometery

Since mitochondria are the main source of ROS as a byproduct of energy metabolism (Brown, 1992), the effect of xylosyl glycolipid G-5 on mitochondria potential ($\Delta\psi_m$) was analyzed. Change in inner mitochondrial membrane potential ($\Delta\psi_m$) of SW480 cells before and after treatment with IC_{50} of xylosyl glycolipid G-5 was examined quantitatively by flow cytometry analysis (Figure 4.11). The cells were distinguished by treatment with cationic, lipophilic mitopotential dye and a death cell marker 7-AAD (7-aminoactinomycin D). Total depolarized cell count in the sample was found to be 60.20% which is much higher than that in the control (1.04%), which envisaged the decrease in $\Delta\psi_m$ and hence mitochondrial dysfunction.

Major portion of the total depolarized cell percentage in the sample was contributed by depolarized live cells (56.55%), which indicate the mitochondria mediated apoptosis caused by xylosyl glycolipid G-5. It is well documented that loss of mitochondrial membrane potential gives rise to the release of different apoptogenic factors from the mitochondria into the cytoplasm (Gupta et al., 2009). Several studies have demonstrated the role of ROS in mitochondria toxicity by activating different mechanisms, including the increase of mitochondrial permeability, which opens transition pores (Mitochondria Outer Membrane Permeabilization-MOMP), or promoting the release of pro-apoptotic factors, such as cytochrome c. Thus, ROS can mediate activation of apoptosis regulators in the cytoplasm, which insert on outer mitochondria membrane, and onset MOMP (Malik et al., 2007; de Sá Júnior et al., 2016) culminating in apoptosis. In this scenario, the results obtained pointed out that xylosyl glycolipid G-5 exhibits a pronounced mitochondrial toxicity as evidenced by the loss of $\Delta\psi_m$ concomitant to ros production.

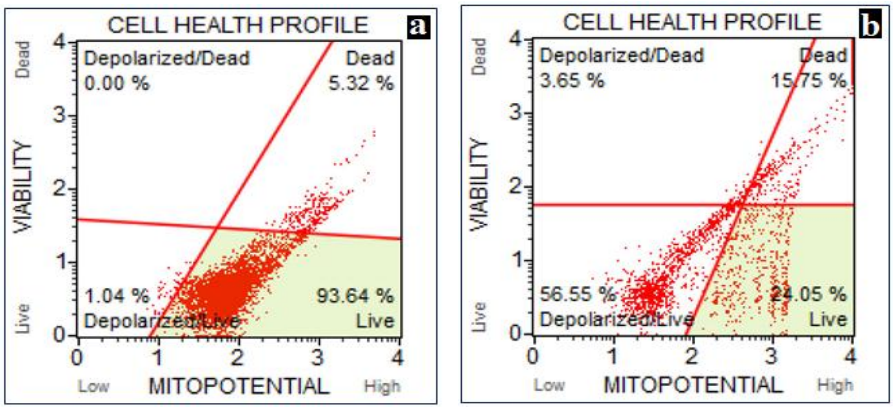


Figure 4.11 Flow cytometric analysis of the changes in mitochondrial potential ($\Delta\psi_m$) (a) Untreated control SW480 cells (b) Cells treated with IC_{50} of xylosyl glycolipid G-5.

4.3.8 Expression of proteins regulating apoptosis

Several tumour suppressor genes are invoved in the pathways operating to inhibit cancer cell proliferation. Cells undergoing apoptosis are killed by activation of a family of cysteine proteases called caspases (cysteine aspartate-specific proteinase), which cleave specific cellular proteins causing the typical morphology of apoptotic cell death (Salvesen & Dixit, 1997). In chemotherapy induced apoptosis, a number of pathways to caspase activation have been identified. The best known involves the nuclear phosphoprotein p53, which is stabilized and activated by DNA damage causing either cell cycle arrest through transactivation of cyclin-dependent kinase inhibitor or death of the cell by apoptosis (El-Deiry et al., 1994). p53 can increase the expression of redox-related genes leading to the formation of reactive oxygen species that damage mitochondria causing release of substances, including cytochrome C, that activate caspases (Polyak et al., 1997; Fridman & Lowe, 2003).

p53 expression study was carried in the SW480 cells and it was found to be over expressed in test sample treated with xylosyl glycolipid G-5 compared to the control (Figure 4.12 (i)) and the upregulation of p53 gene is represented by the bar diagram Figure 4.12 (ii). It was reported that multiple overlapping p53-dependent and p53-independent pathways regulate the G2/M transition in response to genotoxic stress (Agarwal et al., 1995; Taylor & Stark, 2001). The effect of p53 on the G2/M transition in response to DNA damage depends on the cell type. A role for p53 in the G2/M checkpoint arrest was reported ealier by using a

derivative of the human colorectal tumor cell line HCT116 in which p53 was inactivated by homologous recombination, which made G2/M cell cycle arrest unstable compared to those possessing active p53 (Bunz et al., 1998). Upregulation of p53 in the xylosyl glycolipid teated SW480 cells was also indicated involvement of p53-dependant pathways in G2/M cell cycle arrest and mitochondrial dependant apoptotic phenomenon.

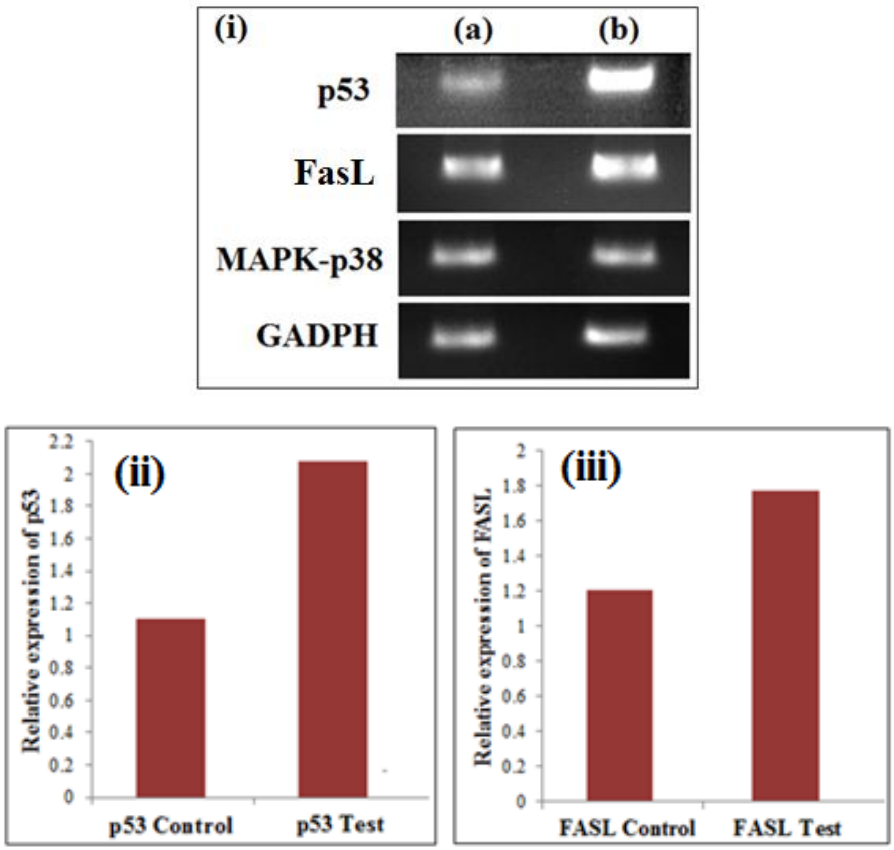


Figure 4.12 Relative gene expression on SW80 cells (i) Gel picture showing expression of selected genes (a) Untreated control cells (b) Cells treated with IC_{50} of xylosyl glycolipid G-5; (ii) Bar diagram representing upregulation of p53, (iii) Bar diagram representing upregulation of FasL.

There can be another pathway involving the Fas receptor (CD95/APO-1) and its ligand FasL. This pathway does not always require p53 and can activate caspases directly without mitochondria necessarily being involved (O'connell et al., 1997; Ogawa et al., 2004). FasL belongs to the tumor necrosis factor family (Nagata 1997). It is a type-II membrane protein that exists as a trimer in the plasma membrane with its N-terminus in the cytoplasm of the cell. Crosslinking of Fas to Fas ligand induces apoptosis through activation of caspases (Watson, 1999). FasL expression was also studied in the SW480 cells and it was found to be upregulated in the test sample treated with xylosyl glycolipid G-5 compared to the control SW480 cells (Figure 4.12 (i) & (iii)).

Fas is known to be highly expressed in normal human colon epithelial cells. It was reported that Fas protein level is down-regulated in primary human colon carcinoma and complete loss of Fas expression often occurs in metastatic human colon carcinoma (Möller et al., 1994). It is known that FasL on cytotoxic T lymphocytes (CTLs) plays an essential role in suppression of spontaneous tumor development (Caldwell et al., 2003; Liu et al., 2006; Fingleton et al., 2007; Afshar-Sterle et al., 2014). Therefore, human colon carcinoma may use down-regulation of Fas expression as a mechanism to escape host cancer immune surveillance. Therapeutic means to upregulate FasL expression and resultant caspase activation represent effective approach to suppress human colon carcinoma immune escape (Coe et al., 2016). The present study is therefore having much significance. FasL can either operate through an extrinsic pathway independent of mitochondria or through a pathway dependent of mitochondria or both. Upregulation of both p53

and FasL along with mitochondrial dysfunction found in the present study give inference that there can be a chance of occuring both mechanisms together to induce apoptosis. It was also repoted that death receptor ligand FasL, could induce apoptosis in various cancer cells through the activation of the p38 MAPK signalling pathway (Kim et al., 2015). But no change in the expression of p38 MAPK was observed after treatment with xylosyl glycolipid (Figure 4.12 (i). Hence p38 MAPK signalling pathway may not be involved in the apoptotic process induced by the glycolipid.

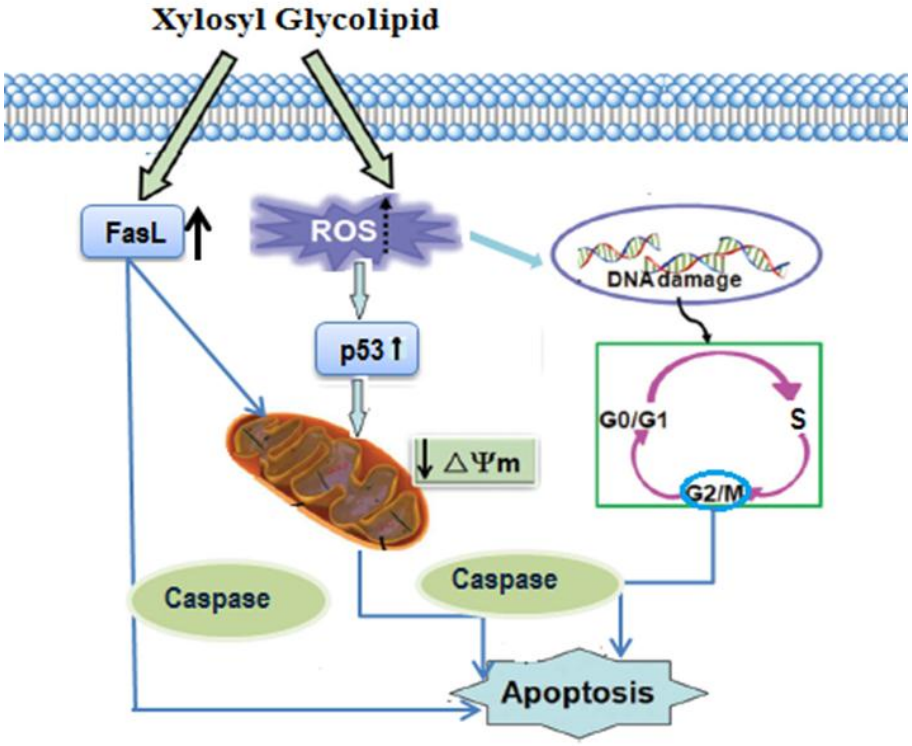


Figure 4.13 Outline of overall processes observed in the cytotoxic activity of xylosyl glycolipid G-5

4.4 Conclusion

Screening of anticancer activity of mono galactosyl glycolipid G-2 and xylosyl glycolipid G-5 isolated from *Synechocystis* sp. using MTT assay depicted that G-5 possess the highest antiproliferative property against SW480 colon carcinoma cells with an IC_{50} of 17.88 µg/mL. Higher cytotoxic potential of G-5 may be due to more number of hydroxyl group present compared with G-2 and therefore further cytotoxicity analysis was performed on xylosyl glycolipid G-5. DNA damage in SW480 cells induced by the compound was evidenced through comet assay analysis. The study demonstrated that glycolipid G-5 induces abrogation of G2/M phase associated to intense ROS production and mitochondrial toxicity triggering apoptosis in cancer cells. Decrease in mitrochondrial membrane potential indicative of mitochondrial dysfunction is inferred from increase in depolarized cell count observed through flowcytometry.

The tumour suppressor protein p53 was found to be upregulated by the action of the glycolipid compound G-5 on cancer cells and, this shows potential of the compound to induce apoptosis through mitochondrial dysfunction and also occurance of p53 dependant G2/M arrest. FasL, a death receptor ligand which can direct apoptosis through either mitochondrial dependant or independent pathway also found to be upregulated by the action of G-5. This data suggest that there can be a coexistence of intrinsic and extrinsic pathways of apoptosis. The overall inferences obtained from cytotoxicity analysis of xylosyl glycolipid G-5 are outlined in Figure 4.13. It is therefore necessary to futher investigate the involvement of various regulatory proteins and caspases, for understanding different signalling and execution pathways of apoptosis completely.

References

Afshar-Sterle, S., Zotos, D., Bernard, N. J., Scherger, A. K., Rödling, L., Alsop, A. E., & O'reilly, L. A. (2014). Fas ligand-mediated immune surveillance by T cells is essential for the control of spontaneous B cell lymphomas. *Nature Medicine*, 20(3), 283-290.

Agarwal, M. L., Agarwal, A., Taylor, W. R., & Stark, G. R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proceedings of the National Academy of Sciences*, 92(18), 8493-8497.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., & Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell*, 87(2), 171.

Andrianasolo, E. H., Haramaty, L., Vardi, A., White, E., Lutz, R., & Falkowski, P. (2008). Apoptosis-inducing galactolipids from a cultured marine diatom, *Phaeodactylum tricornutum*. *Journal of Natural Products*, 71(7), 1197-1201.

Ashkenazi, A. (2008). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine & Growth Factor Reviews*, 19(3), 325-331.

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science*, 281(5381), 1305-1308.

Aw, T. Y. (1999). Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. *The American Journal of Clinical Nutrition*, 70(4), 557-565.

Bishayee, A., & Sethi, G. (2016). Bioactive natural products in cancer prevention and therapy: Progress and promise. *Seminars in Cancer Biology*. 40(1), 1-3.

Borenfreund, E., & Puerner, J. A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicology Letters*, 24(2-3), 119-124.

Boyer, J., McLean, E. G., Aroori, S., Wilson, P., McCulla, A., Carey, P. D., & Johnston, P. G. (2004). Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. *Clinical Cancer Research*, 10(6), 2158-2167.

Bozzuto, G., Ruggieri, P., & Molinari, A. (2010). Molecular aspects of tumor cell migration and invasion. *Annali dell'Istituto Superiore Di Sanita*, 46(1), 66-80.

Brandhagen, B. N., Tieszen, C. R., Ulmer, T. M., Tracy, M. S., Goyeneche, A. A., & Telleria, C. M. (2013). Cytostasis and morphological changes induced by mifepristone in human metastatic cancer cells involve cytoskeletal filamentous actin reorganization and impairment of cell adhesion dynamics. *BMC Cancer*, 13(1), 35.

Brown, G. C. (1992). Control of respiration and ATP synthesis in mammalian mitochondria and cells. *Biochemical Journal*, 284(1), 1-13.

Buiatti, E., Palli, D., Decarli, A., Amadori, D., Avellini, C., Bianchi, S., & Marubini, E. (1989). A case-control study of gastric cancer and diet in Italy. *International Journal of Cancer*, 44(4), 611-616.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., & Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, 282(3), 1497-1501.

Caldwell, S. A., Ryan, M. H., McDuffie, E., & Abrams, S. I. (2003). The Fas/Fas ligand pathway is important for optimal tumor regression in a mouse model of CTL adoptive immunotherapy of experimental CMS4 lung metastases. *The Journal of Immunology*, 171(5), 2402-2412.

Castedo, M., Perfettini, J. L., Roumier, T., Valent, A., Raslova, H., Yakushijin, K., & Vainchenker, W. (2004). Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene*, 23(25), 4362-4370.

Certo, M., Moore, V. D. G., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S. A., & Letai, A. (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*, 9(5), 351-365.

Chen, B. H., Chang, H. W., Huang, H. M., Chong, I. W., Chen, J. S., Chen, C. Y., & Wang, H. M. (2011). (−)Anonaine induces DNA damage and inhibits growth and migration of human lung carcinoma h1299 cells. *Journal of Agricultural and Food Chemistry*, 59(6), 2284-2290.

Chicheportiche, Y., Bourdon, P. R., Xu, H., Hsu, Y. M., Scott, H., Hession, C., & Browning, J. L. (1997). TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *Journal of Biological Chemistry*, 272(51), 32401-32410.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*, 15(3), 532-534.

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

Coe, G. L., Redd, P. S., Paschall, A. V., Lu, C., Gu, L., Cai, H., & Liu, K. (2016). Ceramide mediates FasL-induced caspase 8 activation in colon carcinoma cells to enhance FasL-induced cytotoxicity by tumor-specific cytotoxic T lymphocytes. *Scientific Reports*, 6(3), 1-15.

Collins, A. R., Duthie, S. J., & Dobson, V. L. (1993). Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis*, 14(9), 1733-1735

Collins, A. R., Oscoz, A. A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., & Stetina, R. (2008). The comet assay: topical issues. *Mutagenesis*, 23(3), 143-151.

Colombo, D., Compostella, F., Ronchetti, F., Scala, A., Toma, L., Mukainaka, T., & Nishino, H. (1999). Inhibitory effects of monoacylated 2-O-β-galactosylglycerols on Epstein–Barr virus activation: the significant role of the hexanoyl chain. *Cancer Letters*, 143(1), 1-4.

Colombo, D., Compostella, F., Ronchetti, F., Scala, A., Toma, L., Tokuda, H., & Nishino, H. (2000). Glycoglycerolipid analogues active as anti-tumor-promoters: the influence of the anomeric configuration. *European Journal of Medicinal Chemistry*, 35(12), 1109-1113.

Cotter, T. G. (2009). Apoptosis and cancer: the genesis of a research field. *Nature Reviews. Cancer*, 9(7), 501.

de Sá Júnior, P. L., Câmara, D. A. D., Costa, A. S., Ruiz, J. L. M., Levy, D., Azevedo, R. A., & Fonseca, P. M. M. (2016). Apoptotic effect of eugenol envolves G2/M phase abrogation accompanied by mitochondrial damage and clastogenic effect on cancer cell *in vitro*. *Phytomedicine*, 23(7), 725-735.

Decaudin, D. I., Marzo, I. S., Brenner, C. A., & Kroemer, G. U. (1998). Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy. *International Journal of Oncology*, 12(1), 141-193.

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

El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., & Wiman, K. G. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Research*, 54(5), 1169-1174.

Ellis, R. E., Yuan, J., & Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annual Review of Cell Biology*, 7(1), 663-698.

Engel, L. W., Young, N. A., Tralka, T. S., Lippman, M. E., O'Brien, S. J., & Joyce, M. J. (1978). Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Research*, 38(10), 3352-3364.

Eruslanov, E., & Kusmartsev, S. (2010). Identification of ROS using oxidized DCFDA and flow-cytometry. *Advanced Protocols in Oxidative Stress II*, 57-72.

Fadeel, B., Orrenius, S., & Zhivotovsky, B. (1999). Apoptosis in human disease: a new skin for the old ceremony?. *Biochemical and Biophysical Research Communications*, 266(3), 699-717.

Fauser, J. K., Matthews, G. M., Cummins, A. G., & Howarth, G. S. (2013). Induction of apoptosis by the medium-chain length fatty acid lauric acid in colon cancer cells due to induction of oxidative stress. *Chemotherapy*, 59(3), 214-224.

Ferreira, C. G., Epping, M., Kruyt, F. A., & Giaccone, G. (2002). Apoptosis. *Clinical Cancer Research*, 8(7), 2024-2034.

Ferreira, D., Adega, F., & Chaves, R. (2013). The importance of cancer cell lines as *in vitro* models in cancer methylome analysis and anticancer drugs testing. *Oncogenomics and Cancer Proteomics-Novel Approaches in Biomarkers Discovery and Therapeutic Targets in Cancer, edited by César LC, Elena AO. Portugal: In Tech*, 139-141.

Fingleton, B., Carter, K. J., & Matrisian, L. M. (2007). Loss of functional Fas ligand enhances intestinal tumorigenesis in the Min mouse model. *Cancer Research*, 67(10), 4800-4806.

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

Fridman, J. S., & Lowe, S. W. (2003). Control of apoptosis by p53. *Oncogene*, 22(56), 9030.

Fried, J., Perez, A. G., & Clarkson, B. D. (1976). Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. Properties of the method and mathematical analysis of the data. *The Journal of Cell Biology*, 71(1), 172-181.

Fuster, J. J., Sanz-González, S. M., Moll, U. M., & Andrés, V. (2007). Classic and novel roles of p53: prospects for anticancer therapy. *Trends in Molecular Medicine*, 13(5), 192-199.

Gabrielli, B., Brooks, K., & Pavey, S. (2012). Defective cell cycle checkpoints as targets for anti-cancer therapies. *Frontiers in Pharmacology*, 3(9), 1-6.

Genestra, M. (2007). Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular Signalling*, 19(9), 1807-1819.

Gerlier, D., & Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods*, 94(1-2), 57-63.

Giardina, C., & Inan, M. S. (1998). Nonsteroidal anti-inflammatory drugs, short-chain fatty acids, and reactive oxygen metabolism in human colorectal cancer cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1401(3), 277-288.

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

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science*, 305(1), 626-629.

Green, D. R., & Reed, J. C. (1998). Mitochondria and apoptosis. *Science-AAAS-Weekly Paper Edition*, 281(1), 1309-1311.

Greenlee, H. (2012). Natural products for cancer prevention. *Seminars in Oncology Nursing*, 28(1), 29-44.

Gupta, S., Kass, G. E., Szegezdi, E., & Joseph, B. (2009). The mitochondrial death pathway: a promising therapeutic target in diseases. *Journal of Cellular and Molecular Medicine*, 13(6), 1004-1033.

Haenszel, W., Kurihara, M., Segi, M., & Lee, R. K. (1972). Stomach cancer among Japanese in Hawaii 2. *Journal of the National Cancer Institute*, 49(4), 969-988.

Hirose, Y., Berger, M. S., & Pieper, R. O. (2001). Abrogation of the Chk1-mediated G2 checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Research*, 61(15), 5843-5849.

Hong, W. K., & Sporn, M. B. (1997). Recent advances in chemoprevention of cancer. *Science*, 278(2), 1073-1077.

Huang, H. L., Fang, L. W., Lu, S. P., Chou, C. K., Luh, T. Y., & Lai, M. Z. (2003). DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation. *Oncogene*, 22(50), 8168-8177.

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. *Nature Reviews Cancer*, 2(4), 277-288.

Inoue, M., Sato, E. F., Nishikawa, M., Park, A. M., Kira, Y., Imada, I., & Utsumi, K. (2003). Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Current Medicinal Chemistry*, 10(23), 2495-2505.

Jackson, J. R., Gilmartin, A., Imburgia, C., Winkler, J. D., Marshall, L. A., & Roshak, A. (2000). An indolocarbazole inhibitor of human checkpoint kinase (Chk1) abrogates cell cycle arrest caused by DNA damage. *Cancer Research*, 60(3), 566-572.

Jacobson, M. D., Weil, M., & Raff, M. C. (1997). Programmed cell death in animal development. *Cell*, 88(3), 347-354.

Karlsson, M., Kurz, T., Brunk, U. T., Nilsson, S. E., & Frennesson, C. I. (2010). What does the commonly used DCF test for oxidative stress really show?. *Biochemical Journal*, 428(2), 183-190.

Kastan, M. B., & Bartek, J. (2004). Cell-cycle checkpoints and cancer. *Nature*, 432(7015), 316-323.

Kim, J. S., Oh, D., Yim, M. J., Park, J. J., Kang, K. R., Cho, I. A., & Kim, D. K. (2015). Berberine induces FasL-related apoptosis through p38 activation in KB human oral cancer cells. *Oncology Reports*, 33(4), 1775-1782.

King, M. A. (2000). Detection of dead cells and measurement of cell killing by flow cytometry. *Journal of Immunological Methods*, 243(1), 155-166.

King, R. W., Jackson, P. K., & Kirschner, M. W. (1994). Mitosis in transition. *Cell*, 79(4), 563-571.

Kumar, S., & Lavin, M. F. (1996). The ICE family of cysteine proteases as effectors of cell death. *Cell Death and Differentiation*, 3(3), 255-267.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell*, 88(3), 323-331.

Liu, K., Caldwell, S. A., Greeneltch, K. M., Yang, D., & Abrams, S. I. (2006). CTL adoptive immunotherapy concurrently mediates tumor regression and tumor escape. *The Journal of Immunology*, 176(6), 3374-3382.

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(1), 15-20.

Liu, W. H., & Chang, L. S. (2011). Fas/FasL-dependent and-independent activation of caspase-8 in doxorubicin-treated human breast cancer MCF-7 cells: ADAM10 down-regulation activates Fas/FasL signaling pathway. *The International Journal of Biochemistry & Cell Biology*, 43(12), 1708-1719.

Lorenzo, Y., Costa, S., Collins, A. R., & Azqueta, A. (2013). The comet assay, DNA damage, DNA repair and cytotoxicity: hedgehogs are not always dead. *Mutagenesis*, 28(4), 427-432.

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.

Lovrić, J., Cho, S. J., Winnik, F. M., & Maysinger, D. (2005). Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. *Chemistry & Biology*, 12(11), 1227-1234.

Malik, F., Kumar, A., Bhushan, S., Khan, S., Bhatia, A., Suri, K. A., & Singh, J. (2007). Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by N-acetyl cysteine. *Apoptosis*, 12(11), 2115-2133.

Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N., Hirsch, T., Macho, A., & Kroemer, G. (1996). Mitochondrial permeability transition is a central coordinating event of apoptosis. *Journal of Experimental Medicine*, 184(3), 1155-1160.

Martin, S. J., & Green, D. R. (1995). Protease activation during apoptosis: death by a thousand cuts?. *Cell*, 82(3), 349-352.

Matés, J. M., & Sánchez-Jiménez, F. M. (2000). Role of reactive oxygen species in apoptosis: implications for cancer therapy. *The International Journal of Biochemistry & Cell Biology*, 32(2), 157-170.

Medema, R. H., & Macůrek, L. (2012). Checkpoint control and cancer. *Oncogene*, 31(21), 2601-2613.

Meiyanto, E., Hermawan, A., & Anindyajati, A. (2012). Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. *Asian Pacific Journal of Cancer Prevention*, 13(2), 427-436.

Minami, T., Adachi, M., Kawamura, R., Zhang, Y., Shinomura, Y., & Imai, K. (2005). Sulindac enhances the proteasome inhibitor bortezomib-mediated oxidative stress and anticancer activity. *Clinical Cancer Research*, *11*(14), 5248-5256.

Möller, P., Koretz, K., Leithäuser, F., Brüderlein, S., Henne, C., Quentmeier, A., & Krammer, P. H. (1994). Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. *International Journal of Cancer*, 57(3), 371-377.

Moses, S. L., Edwards, V. M., & Brantley, E. (2016). Cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cells, without harming MCF-10A healthy cells. *Journal of Nanomedicine & Nanotechnology*, *7*(369), 2.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55-63.

Nagata, S. (1997). Apoptosis by death factor. *Cell*, 88(3), 355-365.

Negi, A. K.(2013). Role of apoptotic mechanism in chemoprevention of experimental mammary carcinogenesis by celecoxib and fish oil. *PhD Thesis. Panjab University, India*, 6-47.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., & Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *Journal of Immunological Methods*, 139(2), 271-279.

O'connell, J., Bennett, M. W., O'sullivan, G. C., Collins, J. K., & Shanahan, F. (1997). The Fas counterattack: a molecular mechanism of tumor immune privilege. *Molecular Medicine*, 3(5), 294.

Ogawa, S., Nagao, M., Kanehiro, H., Hisanaga, M., Ko, S., Ikeda, N., & Nakajima, Y. (2004). The breakdown of apoptotic mechanism in the development and progression of colorectal carcinoma. *Anticancer Research*, 24(3A), 1569-1580.

Park, S. J., Wu, C. H., Gordon, J. D., Zhong, X., Emami, A., & Safa, A. R. (2004). Taxol induces caspase-10-dependent apoptosis. *Journal of Biological Chemistry*, 279(49), 51057-51067.

Pattillo, R. A., & Gey, G. O. (1968). The establishment of a cell line of human hormone-synthesizing trophoblastic cells *in vitro*. *Cancer Research*, 28(7), 1231-1236.

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.

Pelicano, H., Carney, D., & Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates*, 7(2), 97-110.

Peter, M. E., & Krammer, P. H. (1998). Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Current Opinion in Immunology*, 10(5), 545-551.

Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., & Vogelstein, B. (1997). A model for p53-induced apoptosis. *Nature*, 389(6648), 300-305.

Rigas, B., & Sun, Y. (2008). Induction of oxidative stress as a mechanism of action of chemopreventive agents against cancer. *British Journal of Cancer*, 98(7), 1157-1160.

Rodriguez, E., Azevedo, R., Fernandes, P., & Santos, C. A. (2011). Cr (VI) induces DNA damage, cell cycle arrest and polyploidization: a flow cytometric and comet assay study in *Pisum sativum*. *Chemical Research in Toxicology*, 24(7), 1040-1047.

Ruhe, J. E., Streit, S., Hart, S., Wong, C. H., Specht, K., Knyazev, P., & Wong, W. (2007). Genetic alterations in the tyrosine kinase transcriptome of human cancer cell lines. *Cancer Research*, 67(23), 11368-11376.

Saelens, X., Festjens, N., Walle, L. V., Van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene*, 23(16), 2861.

Said, H. M., Polat, B., Hagemann, C., Anacker, J., Flentje, M., & Vordermark, D. (2009). Absence of GAPDH regulation in tumor-cells of different origin under hypoxic conditions in–vitro. *BMC Research Notes*, *2*(1), 8.

Sakaue-Sawano, A., Kobayashi, T., Ohtawa, K., & Miyawaki, A. (2011). Drug-induced cell cycle modulation leading to cell-cycle arrest, nuclear mis-segregation, or endoreplication. *BMC Cell Biology*, 12(1), 2-12.

Salvesen, G. S., & Dixit, V. M. (1997). Caspases: intracellular signaling by proteolysis. *Cell*, 91(4), 443-446.

Senderowicz, A. M., & Sausville, E. A. (2000). Preclinical and clinical development of cyclin-dependent kinase modulators. *Journal of the National Cancer Institute*, 92(5), 376-387.

Sherr, C. J. (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research*, 60(14), 3689-3695.

Sherr, C. J. (2004). Principles of tumor suppression. *Cell*, 116(2), 235-246.

Shiao, Y. H. (2003). A new reverse transcription-polymerase chain reaction method for accurate quantification. *BMC Biotechnology*, *3*(1), 22.

Siddique, H. R., Chowdhuri, D. K., Saxena, D. K., & Dhawan, A. (2005). Validation of *Drosophila melanogaster* as an in vivo model for genotoxicity assessment using modified alkalinecomet assay. *Mutagenesis*, 20(4), 285-290.

Singh, R. (2013). Cytotoxicity and gene expression of selected apoptotic markers in the human laryngeal carcinoma cell line (HEp-2) by *Bulbine* spp. fractions. *PhD Thesis. Durban University of Technology, South Africa*, 7-84.

Smith, C. C., O'donovan, M. R., & Martin, E. A. (2006). HOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis*, 21(3), 185-190.

Sporn, M. B. (1976). Approaches to prevention of epithelial cancer during the preneoplastic period. *Cancer Research*, 36(7), 2699-2702.

Suganuma, M., Kawabe, T., Hori, H., Funabiki, T., & Okamoto, T. (1999). Sensitization of cancer cells to DNA damage-induced cell death by specific cell cycle G2 checkpoint abrogation. *Cancer Research*, 59(23), 5887-5891.

Suliman, A., Lam, A., Datta, R., & Srivastava, R. K. (2001). Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and-independent pathways. *Oncogene*, 20(17), 2122-2133.

Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., & Kroemer, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *Journal of Experimental Medicine*, 184(4), 1331-1341.

Sylvester, P. W. (2011). Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Drug Design and Discovery: Methods and Protocols, edited by Satyanarayanajois SD. USA: Humana Press*, 157-168.

Taylor, W. R., & Stark, G. R. (2001). Regulation of the G2/M transition by p53. *Oncogene*, *20*(15), 1803.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science*, *267*(5203), 1456-1462.

Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?. *Nature Reviews Drug Discovery*, *8*(7), 579-591.

Tsao, A. S., Kim, E. S., & Hong, W. K. (2004). Chemoprevention of cancer. *CA: A Cancer Journal for Clinicians*, *54*(3), 150-180.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M. M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological Interactions*, 160(1), 1-40.

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.

Vargo-Gogola, T., & Rosen, J. M. (2007). Modelling breast cancer: one size does not fit all. *Nature Reviews Cancer*, 7(9), 659-672.

Visconti, R., Della Monica, R., & Grieco, D. (2016). Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword. *Journal of Experimental & Clinical Cancer Research*, 35(1), 153.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature*, 408(2), 307-310.

Walczak, H., & Krammer, P. H. (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Experimental Cell Research*, 256(1), 58-66.

Wang, F. (2003). Cancer cell culture-methods and protocols. *In Vitro Cellular & Developmental Biology-Animal*, 39(10), 476-476.

Wang, J. Y., & Cho, S. K. (2004). Coordination of repair, checkpoint, and cell death responses to DNA damage. *Advances in Protein Chemistry*, 69(1), 101-135.

Wasson, G. R., McKelvey-Martin, V. J., & Downes, C. S. (2008). The use of the comet assay in the study of human nutrition and cancer. *Mutagenesis*, 23(3), 153-162.

Watson, A. (1999). The role of Fas in apoptosis induced by anticancer drugs. *Hepatology*, 29(1), 280-281.

Wattenberg, L. W. (1985). Chemoprevention of cancer. *Cancer Research*, 45(1), 1-8.

Wyllie, A. H., & Duvall, E. (1992). Cell injury and death. *Oxford textbook of pathology, edited by Mcgee JO'D, Isaacson PG, Wright NA. Newyork: Oxford University Press*, 141-193.

Yang, J. S., Chen, G. W., Hsia, T. C., Ho, H. C., Ho, C. C., Lin, M. W., & Chung, J. G. (2009). Diallyl disulfide induces apoptosis in human colon cancer cell line (COLO 205) through the induction of reactive oxygen species, endoplasmic reticulum stress, caspases casade and mitochondrial-dependent pathways. *Food and Chemical Toxicology*, 47(1), 171-179.

Yarla, N. S., Bishayee, A., Sethi, G., Reddanna, P., Kalle, A. M., Dhananjaya, B. L., & Duddukuri, G. R. (2016). Targeting arachidonic acid pathway by natural products for cancer prevention and therapy. *Seminars in Cancer Biology*, 40 (1), 48-81.

Yeh, Y. T., Yeh, H., Su, S. H., Lin, J. S., Lee, K. J., Shyu, H. W., & Su, S. J. (2014). Phenethyl isothiocyanate induces DNA damage-associated G2/M arrest and subsequent apoptosis in oral cancer cells with varying p53 mutations. *Free Radical Biology and Medicine*, 74(2), 1-13.

Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gómez-Monterrey, I., Castedo, M., & Kroemer, G. (1996). Mitochondrial control of nuclear apoptosis. *Journal of Experimental Medicine*, 183(4), 1533-1544.

Zhang, J., Campbell, R. E., Ting, A. Y., & Tsien, R. Y. (2002). Creating new fluorescent probes for cell biology. *Nature Reviews Molecular Cell Biology*, 3(12), 906-918.

.....ꕥ.....

Chapter 5

SUMMARY

Glycolipids, one of the important membrane components in prokaryotic and eukaryotic organisms, represent a valuable resource for biomedical research and drug discovery. Glycolipids from different natural sources were found to exhibit immense structural variations. Peculiarities in the structure of glycolipids diversify its application in medicine and industry. Important biological properties shown by these compounds were found to depend on structural features such as nature and anomeric configuration of sugar, glycosidic linkage position, length of fatty acyl chain etc. Glycoglycerolipids denote one of the most abundant classes of glycolipid because of its occurrence in thylakoid membranes of all photosynthetic organisms. They are found to involve in various cellular events and also depicted important biological activities. Several studies demonstrated significant inhibitory effects shown by various glycolipids on different human cell carcinomas.

Cyanobacteria are identified as an important source of bioactive glycolipids. They were found to have glycolipid composition similar to chloroplasts, but some cyanobacterial strains displayed structural

variations in glycolipids. The study entitled "Isolation and characterization of glycolipids from *Synechocystis* sp. and its cytotoxic potential against colon cancer" highlights structural characterization of major glycolipids from the cyanobacteria; *Synechocystis* sp. and their potential to inhibit proliferation of SW480 colon cancer cells. *Synechocystis* represents one of the widely studied classes of cyanobacteria and several bioactive compounds have been explored from different strains of *Synechocystis*. This genus is found to have ability to adjust with varying salt conditions and is also recognized as important source of glycolipids. The composition and biological properties possessed by these microorganisms are found to vary among different strains and also depending on the conditions of growth. Therefore, identification of different phenotypes of the cyanobacteria is particularly important and their morphology is found to change according to different environmental or culture conditions. Thus, one of the preliminary aims of the study was identification of cyanobacteria. Genomic identification is employed because morphological features of organism may change with different conditions and also may lead to misidentifications. 16S rRNA gene sequences phylogenetic analysis of the species under study showed 99% similarity to *Synechocystis* sp. (GenBank Accession No. MF444861). The peculiar taxonomic features of the cyanobacteria are further established through fatty acid analysis.

Chemotaxonomy based on primary metabolites such as cellular fatty acids serve as an important tool for classifying cyanobacteria since chain length and, number and position of double bonds of fatty acids are known to be genetically determined. Fatty acid composition analysis of *Synechocystis* sp., through GC-MS, revealed that relative abundances of

saturated and monounsaturated fatty acids (SFA and MUFA) are higher compared with polyunsaturated fatty acids. This is in agreement with the fatty acid profile of most of the cyanobacterial community. Palmitic (C16:0) acid was found to be the most prominent fatty acid followed by oleic (18:1 n-9), stearic (18:0) and palmitoleic (16:1 n-7) acids. Relative abundances of saturated and monounsaturated fatty acids were found to be 52.97 ± 0.05% and 29.81 ± 0.03 % respectively of the total fatty acid content. Small amounts of 3-hydroxy fatty acids were also detected which further supports taxonomy of cyanobacteria. Analysis of hydroxy fatty acid fraction, obtained through thin layer chromatography separation, had shown presence of 3-hydroxy tetradecanoate, 3-hydroxy hexadecanoate, and 3-hydroxy hexadec-9-enoate as the major components.

Synechocystis sp. was mass cultured under suitable conditions to obtain more yield for isolating major glycolipids. TLC staining and LC-MS guided fractionation of cyanobacterial extract yielded six glycolipid fractions (G-1 to G-6). G-5 and G-6 fractions were found to be more polar compared with other fractions. The functional group characterization based on FT-IR analysis affirms the glycolipid nature of all the six fractions. FTIR spectra of G-2 and G-4 fractions are found to have bands indicating presence of double bond in the structure. More intense bands at glycosidic linkage positions inferred from FTIR spectra of G-5 and G-6, can be attributed to the extra sugar units in those fractions and also to their high polar character. Sugar analysis of glycolipids by HPLC, in comparison with monosaccharide standards, indicated that glycolipid fractions G-1 to G-4 contain galactose whereas G-5 and G-6 contain xylose as the sugar unit. Each glycolipid fractions

were acetylated to prevent rapid degradation of compounds while performing various spectroscopic characterisations in different solvents. Molecular mass of the purified glycolipid fractions G-1 to G-6 were recorded on HRMS. Further structural characterizations of glycolipids G-2 and G-5, obtained in good yields, were achieved using NMR and mass spectroscopic studies. GC-MS analyses of the fragments were also done to obtain exact nature of fatty acyl chains.

Galactosyl glycolipid G-2 is found to be similar in structure with monogalactosyl glycolipids found in photosynthetic organisms. Molecular mass of G-2 was recorded as m/z 949.5289 on HRMS and deduced molecular formula as $C_{53}H_{88}O_{14}$. Through NMR, ESI MS/MS analysis structure of glycolipid G-2 is established is (2S)-2-O-9,12,15-octadecatrienoyl-1-O-octadecanoyl-3-O-[β-D-galactopyranosyl]-glycerol. Optical rotation of the compound was found to be -8°. Molecular weight of xylosyl glycolipid G-5 was obtained as m/z 1223.4576 through HRMS and the inferred molecular formula was $C_{55}H_{82}O_{30}$. Only a few xylosyl glycolipids of natural origin have been identified. Based on NMR and MS/MS analysis, structure of G-5 is concluded as (2S)-2-O-dodecanoyl-1,3-di-O-[β-D-xylopyranose(1=>4)β-D-xylopyranosyl]-glycerol. Optical rotation of the compound was found to be -22°. MS/MS analysis along with GC-MS studies of fatty acid fragments gave information on the structure of other isolated glycolipids. Glycolipid G-1 (m/z 926.6344) was assumed to be monogalactosyl diglyceride containing palmitic acid (16:0) and stearic acid (18:0) in the fatty acyl chain. G-3 (m/z 1131.4764) and G-4 (m/z 1213.5478) are identified as digalactosyl diglycerides. G-3 was shown to possess dodecanoic acid (12:0) and

palmitic acid (16:0) in the fatty acyl chains of diglyceride. Fatty acid analysis of G-4 showed presence of two fatty acids, palmitic acid (16:0) and oleic acid (18:1). The xylosyl glycolipid G-6 (m/z 1251.6785) showed comparable mass spectra as that of glycolipid G-5. Hence, it is assumed to have similar structure with that of G-5 and found to contain tetradecanoic acid (14:0) in the fatty acyl chain. Further spectroscopic characterization is needed to find exact structure of G-6.

Another important focus of the study is on the activity of major glycolipids against colon cancer. Colon cancer is one of the commonly diagnosed cancers in Western countries. Incidence rate of colon cancer is also found to increase in Asian Pacific region with regard to the modern lifestyle. Anticancer compounds from natural sources are found to get more significance as they have shown lesser side effects compared to synthetic chemotherapeutic agents. Many natural products are found to exhibit potent cytotoxic effects against colon cancer cells in, *in vitro* and *in vivo* studies, and clinical trials. Glycolipids have demonstrated considerable activity against several human cell carcinomas. But, its anti-proliferative effects on colon cancer cells are not well established. In the present study, major glycolipids from cyanobacteria, *Synechocystis* sp. were screened for their anti-proliferative activity against SW480 colon cancer cells. Apoptosis and cell cycle arrest induced by the most active fraction were further analysed using suitable molecular methods.

Anticancer activity screening of galactosyl glycolipid G-2 and xylosyl glycolipid G-5 using MTT assay showed that G-5 possess higher anti-proliferative property against SW480 colon carcinoma cells with an

IC_{50} of 17.88 µg/mL compared to G-2 (IC_{50} 52.26 µg/mL). More number of hydroxyl groups in G-5 may be responsible for the greater activity shown by G-5. DNA damage induced by the compound in SW480 cells was analysed through comet assay. Increase in tail length, tail moment and tail DNA percentage of SW480 colon cancer cells were observed after treatment with IC_{50} of xylosyl glycolipid G-5 which indicates considerable DNA damage induced by G-5. Cell cycle analysis using flow cytometry indicated that G-5 induces G2/M check point aboragation in SW480 cells. G2/M cell cycle arrest and sub-G1 accumulation in G-5 treated cells have given evidence for apoptosis induction by the glycolipid. Apoptosis in the cancer cells was also detected using fluorescent microscopic evaluation of AO/EB double stained cells. Increase in number of bright green and orange cells with nuclear condensation and chromatin coagulation, indicative of early and late apoptotic cells respectively, have provided confirmation for stimulation of apoptosis by glycolipid G-5 in SW480 cells. Oxidative stress induced by the glycolipid was inferred from fluorescence measurements by using specific fluorogenic dye (DCFH-DA) for reactive oxygen species (ROS) detection. Effect of xylosyl glycolipid G-5 on mitochondrial potential ($\Delta\psi_m$) to regulate intrinsic pathway of apoptosis was also analysed using flow cytometry. Decrease in mitrochondrial membrane potential indicative of mitochondrial dysfunction is evidenced through increase in depolarized cell count with the aid of a cationic, lipophilic mitopotential dye and a death cell marker 7-AAD.

Involvement of certain regulatory proteins in the glycolipid induced apoptosis activation and cell cycle arrest was assessed, by analysing relative gene expression through reverse transcriptase PCR method, using specific gene primers. Upregulation of tumour suppressor protein p53 was observed by the action of glycolipid compound G-5 which indicates potential of the compound to induce apoptosis through mitochondrial dysfunction and also occurrence of a p53 dependant G2/M arrest. The death receptor ligand, FasL which can induce apoptosis through either mitochondrial dependant or independent pathways, was also found to be upregulated by the action of the glycolipid. This data suggest that there can be a coexistence of intrinsic and extrinsic pathways of apoptosis regulated by specific proteins and caspases. It is therefore necessary to further examine the involvement of various regulatory proteins and caspases, for understanding different signalling and execution pathways of apoptosis completely. Results of the study are summarized in Figure 5.1.

Hence, the study supports potential aspects of glycolipids in prevention and treatment of cancer by limiting cell proliferation through alteration of cell cycle regulatory molecules. A new cyanobacterial glycolipid, having potent cytotoxic potential against colon cancer, is introduced in the study. This will be an addition to the known group of natural compounds having cytotoxicity against colon cancer and can be a drug candidate for further *in vivo* and clinical trials. However, cyanobacterial metabolites including glycolipids are still need to be explored owing to their vast structural diversities. Isolation and characterization of those metabolites might expand research in natural product chemistry, and their potential applications in medicinal field.

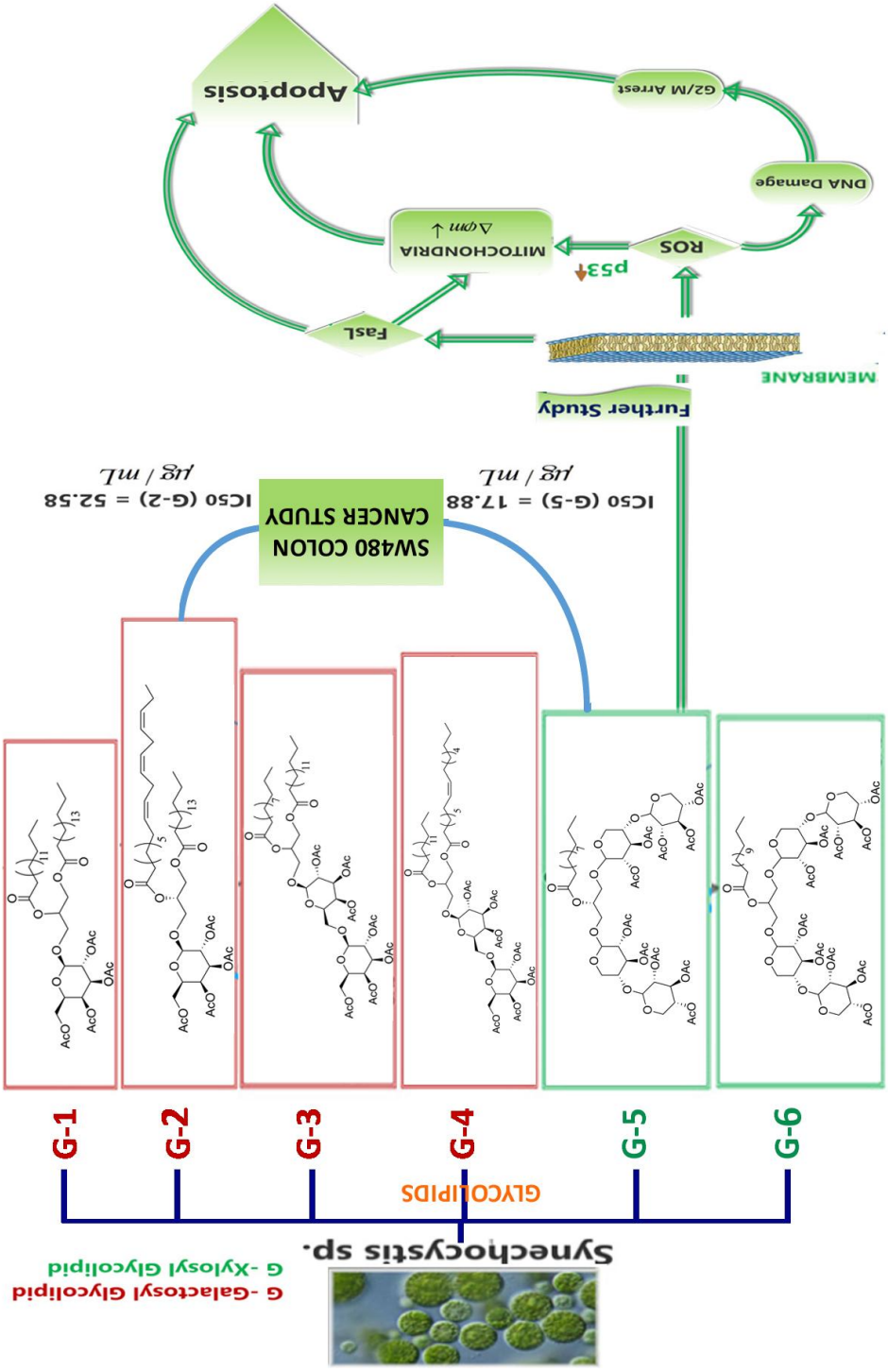


Figure 5.1 Graphical summary of the study

List of Publications

Saritha S., Kala, K. J., Prashob Peter, K. J, & Nair, S. M. (2017). *In vitro* antibacterial screening of fatty acid fractions from three different microalgae. *International Journal of Pharmacognosy and Phytochemical Research,* (accepted: 10132017PPRH).

Saritha S., Kala, K. J., Prashob Peter, K. J, & Nair, S. M. (2017). Antibacterial activity of hydroxy fatty acid fraction from *Synechocystis* sp. *Natural Product Research*, (under review: GNPL-2017-0987).

Suryakumari, S., **Saritha, S.,** Padma, P., Sheela, V. S., Gopinath, A., Jayalakshmy, K. V. & Nair, S. M. (2015). Chemometric assessment of water quality of a river using a major biochemical constituent. *International Journal of River Basin Management*, 13(2), 229-241.

Saritha, S., Nair, S. M., & Kumar, N. C. (2013). Nano-ordered cellulose containing Iα crystalline domains derived from the algae *Chaetomorpha antennina*. *BioNanoScience*, 3(4), 423-427.