

Bioactivity of marine organism-derived natural products

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DEDICATION

This dissertation is dedicated to my family members: my mom, dad, brother and sister. Thank you for all of your love, support, and sacrifice throughout my life.

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PREFACE

A section of the research presented in this thesis has been published in a peer-reviewed journal. A copy of the published manuscript has been attached for ease of reference. Additional manuscripts have been submitted for publication and are listed at the end of this section. Portions of this research have also been presented at an international conference.

Publication

- Ellithey M., Lall, N., Hussein, A.A., Meyer, D., 2013. Cytotoxic, Cytostatic and HIV-1 PR Inhibitory Activities of the Soft Coral *Litophyton arboreum*. *Marine Drugs* 2013, 11, 1-x manuscripts; doi: 10.3390/md110x000x. (Accepted November 2012).
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Conferences

Ellithey M. Lall, N., Hussein, A.A., Meyer, D. Poster presentation entitled: Isolation and Structure Elucidation of HIV-1 Protease inhibitors from the marine soft coral *Litophyton arboreum*. Presented at DART conference: Frontiers in drug development for antiretroviral therapies (4-7 December, 2012. San Diego, California).

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- Ellithey M. Lall, N., Hussein, A.A., Meyer, D. (2013). Seasonal evaluation of *Litophyton arboreum* extract constituents and biological activities extracts as well as its influence on the organism biology. Submitted to *Marine ecology*.

SUMMARY

Bioactivity of marine organism-derived natural products

By

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Background: HIV/AIDS is one of the most devastating diseases in the world with approximately 36 million people living with the virus in 2012 and approximately 2.7 million new infections in that same year. Antiretroviral therapy (ART) successfully reduce infection and decrease symptoms; but, the emergence of viral drug resistance due to drug induced mutations in viral genes can render treatment ineffective. Infection with HIV not only weakens the immune system leading to AIDS and increasing the risk of opportunistic infections, but also increases the risk of several types of cancer. These facts underscore an urgent need to develop new anti-HIV and cancer drugs with fewer or no side-effects. Research into drug discovery and development using natural products is increasingly becoming better established. Marine organisms as a source of natural products delivered numerous novel compounds with sensational multiple pharmacological properties. Thousands of novel compounds and their metabolites with diverse biological activities ranging from antiviral to anticancer have been isolated from various marine sources. With natural products, there are endless opportunities for discovering novel compounds that can be used as drugs or backbones of drug leads.

Methods: In this thesis, thirteen marine organisms were investigated for inhibitory properties against HIV-1 enzymes as well as for potential cytotoxicity.

Cytotoxicity (The ability to kill cancer cells) of the extracts was determined using tetrazolium dyes. Direct enzyme assays were used to determine the inhibitory properties of the extracts against HIV-1 protease (PR) and reverse transcriptase (RT).

The most active extract *Litophyton arboreum* was then subjected to silica gel chromatography in order to isolate, purify and identify the active compounds. These active compounds were then tested for cytotoxic and HIV-1 enzyme inhibitory activities. The cytotoxicity results were subsequently confirmed by real-time cell electronic sensing and the enzyme studies were supported by *in silico* analysis docking using MOE software. The most active compound (7 β -acetoxy-24-methylcholesta-5-24(28)-diene-3, 19-diol) also underwent an ecological study that demonstrated the role of this compound in the activity of the extract as well as the organism's seasonal distribution in its habitat. The HPLC qualitative profiles of the seasonal total extracts were performed and the concentration of the active compound in each extract was determined and related to the activities and seasonal distribution of the organism in the community.

Results and discussion: Cytotoxicity results of the 13 marine organism's extracts in (Hela, U937 and Vero cells) showed strong activity of *L. arboreum* against U-937 (IC₅₀; 6.5 μ g/ml \pm 2.3) with a selectivity index (SI) of 6.45, while *Sarcophyton trochliophorum* showed strong activity against HeLa cells (IC₅₀; 5.2 μ g/ml \pm 1.2) with an SI of 2.09. Other species showed moderate to weak cytotoxicity against both cell lines. Two extracts showed potent inhibitory activity against HIV-1 protease; these were *Cassiopeia andromeda* (IC₅₀; 0.84 μ g/ml \pm 0.1) and *Galaxura filamentosa* (2.6 μ g/ml \pm 1.3). It was interesting to note that the most active extracts against HIV-1 PR, *C. andromeda* and *G. filamentosa* showed no cytotoxicity in the three cell lines at the highest concentration tested (100 μ g/ml).

L. arboreum extract was the only extract that showed activities in all the bioassays tested in this study. The bioassay-guided fractionation using different chromatographic and spectroscopic techniques in the analysis of the Red Sea soft coral *L. arboreum* led

to the isolation of nine compounds; Further fractionation and purification of the active fractions resulted in the isolation and identification of nine known compounds (for which the structures are provided in Figure 4.2) sarcophytol M (**1**), alismol (**2**), 10-*O*-methyl alismoxide (**4**), alismoxide (**5**), (*S*)-chimyl alcohol (**6**), 7 β -acetoxy-24-methylcholesta-5-

24(28)-diene-3,19-diol (**7**), erythro-*N*-dodecanoyl-docosasphinga-(4*E*,8*E*)-dienine (**8**), and 24-methylcholesta-5,24(28)-diene-3 β ,7 β ,19-triol (**9**).

Compound 7 demonstrated strong cytotoxicity against HeLa cells (CC₅₀ 4.3 \pm 0.8 μ M), with a selectivity index of SI 8.1 which was confirmed by real time cell electronic sensing (RT-CES). Compounds 2, 7 and 8 showed strong inhibitory activity against HIV-1 PR at IC₅₀'s of 7.2 \pm 0.7, 4.85 \pm 0.2 and 4.80 \pm 0.9 μ M respectively. Docking studies gave comparable scores when comparing the binding mode of the active compounds to that of acetyl pepstatin, a known HIV-1 PR inhibitor. Interestingly, compound 8 showed potent HIV-1 PR inhibitory activity and did not demonstrate cytotoxicity against the cell lines used in the investigation. Also, compounds 2 and 5 showed cytostatic action in HeLa cells where the compounds inhibited the cells proliferation but did not kill the cancer cells; both compounds can be considered as potential leads in the development of virostatic cocktails that medicine - A drug which inhibits viral replication.

Changes in the biological activities of the seasonal extracts of the soft coral *L. arboreum* showed the highest activity in the autumn extract. The study also evaluated the concentration of the active triterpene erythro-*N*-dodecanoyl-docosasphinga-(4*E*,8*E*)-dienine) and its influence on the organism's maturation and distribution which showed a positive correlation between the compound's concentration and oocyte maturation as well as the dominance and distribution of the organism.

Conclusion: The findings presented in this thesis demonstrated that marine organisms remain one of the most interesting sources for the discovery of bioactive compounds against cancer and HIV. This study is the first to report the anticancer (cytotoxic behavior specific for cancer cell lines) and anti HIV activities of most of the screened organisms. It is also the first report for the metabolites isolation, identification and biological evaluation of *L. arboreum* collected from Sharm El sheikh, Red Sea. Some of the isolated

metabolites demonstrated potent anti-HIV-1 protease activity, with high safety margins. The detailed IC₅₀ study showed potent inhibitory activity of HIV-1 PR by compound 7β-acetoxy-24-methylcholesta-5-24(28)-diene-3,19-diol [7] (IC₅₀ 4.8 μM), in addition to compound 8 (4.86 μM), which also showed no toxicity against any of the cell lines under investigation. This work and many other published reports support the high cytotoxicity of polyhydroxylated sterols from marine organisms, which highlights the importance of this chemical skeleton in the discovery of potential lead compounds from marine sources.

This study demonstrated a seasonal change in the biological activities of the soft coral *L. arboreum* demonstrated the effect of the triterpene on the organism's maturation and distribution. The autumn season appear to be the best time for *L. arboreum* collection for biological purposes, where the highest distribution and highest intensity of the compounds were found.

The findings presented here suggest that the screened Red Sea marine organisms investigated could be an interesting source of bioactive compounds and deserve further bioassay-guided isolation procedures to determine the identity and structure of the active compounds. The biological activities of nine compounds isolated from *L. arboreum* delivered promising leads for further biological evaluation.

Compound	M. wt. (g/mol)	Cytotoxicity IC ₅₀ (μM)			SI Values		IC ₅₀ (μM)
		HeLa	Vero	U937	HeLa	U937	
							HIV-1 PR
1	290	27.5 ± 0.2	22 ± 0.2	31.7 ± 3.2	0.8	0.8	15.7 ± 0.1
2	220.3	30 ± 17.2	49	N/T	1.6	N/T	7.2 ± 0.7
3	398.6	48 ± 8.7	100 ± 1.2	N/T	2.1	N/T	N/T
4	252.3	38 ± 0.7	49.8 ± 0.5	50 ± 0.2	1.3	1.00	N/T
5	238.3	>100	>100	>100 ± 0.04	N/A	N/A	N/T
6	216	23.4 ± 5.8	60 ± 1.1	N/T	2.6	N/T	26.6 ± 2.6
7	742	5.3 ± 0.6	31.3 ± 14.0	10.6 ± 0.12	7.2	2.9	4.9 ± 0.2
8	535.8	38.2 ± 0.7	>100	N/T	2.6	3	4.8 ± 0.9
9	430.6	8 ± 0.5	11.4 ± 0.04	16.4 ± 1.25	1.4	1.3	N/T

Contents

SUBMISSION DECLARATION.....	II
PLAGIARISM DECLARATION	IV
DEDICATION	IV
ACKNOWLEDGMENT	V
PREFACE	XI
CONTENTS	XVI
LIST OF FIGURES	XXI
LIST OF TABLES.....	XXIII
LIST OF IMPORTANT ABBREVIATIONS	XXIV
CHAPTER 1	1
CHAPTER 2.....	4
2.1 THE HUMAN IMMUNODEFICIENCY VIRUS (HIV).....	4
2.1.1 HIV LIFE CYCLE.....	4
2.1.2 HIV ENZYMES.....	6
2.1.3 CLASSES OF ANTIRETROVIRAL AGENTS.....	6
2.1.4 COMMON SIDE EFFECTS OF ANTI-HIV DRUGS.....	8
2.2 CANCER	18
2.2.1 ORIGINS OF CANCER	18
2.2.2 TYPES OF CANCER	20
2.2.3 CANCER THERAPIES AND SIDE EFFECTS	20
• Radiation Therapy	21
• Targeted Therapies	22
• Transplantation	24
• Angiogenesis Inhibitors.....	25
• Biological Therapies for Cancer	26
• Cancer Vaccines.....	27
• Photodynamic therapy	27
• Cryosurgery	28
• Lasers.....	28
• Hyperthermia.....	29
• Complementary and Alternative Therapies	29

2.3. NATURAL PRODUCTS AS SOURCE OF BIOLOGICALLY ACTIVE COMPOUNDS.	30
2.3.1. Natural products and HIV treatment:	32
2.3.2. Natural products and cancer treatment	33
2.4 MARINE ORGANISMS AS NATURAL SOURCE FOR BIOACTIVE COMPOUNDS	34
2.4.1 Marine organisms as source of anti-HIV agents	35
2.4.2 Marine organisms as source of anti-cancer agents	41
2.5. SCREENING TECHNIQUE OF MARINE ORGANISMS	48
2.5.1 Sampling from different ecosystems	48
2.6. RED SEA ECOSYSTEM	49
2.6.1 Mangroves	49
2.6.1.1. Biological activities of sponges	51
2.6.1.2. Biological activities of Cnidarians	54
2.6.2. Seagrass beds	56
2.6.2.1. Biological activities of sea grasses	57
2.6.2.2. Biological activities of marine algae	58
2.6.2.3. Biological activities of Molluscs	60
2.6.3. Coral reef	61
2.6.3.1. Biological activities of the red algae	64
2.6.3.2. Biological activities of brown algae.....	65
2.6.3.3. Biological activities of soft corals	66
2.7. STRATEGIES FOR THE DISCOVERY OF MARINE DRUGS	69
2.8. THE ROLE OF LIPOPHILICITY IN DRUG DEVELOPMENT	71
2.9. COMMON DRUG DISCOVERY TECHNIQUES	72
2.10. Analytical techniques	73
2.10.1. Extraction of crude extracts as well as the isolation and identification of a bioactive compound	73
2.10.2. Bioassays and viability/cytotoxicity profiling	75
2.10.3. Molecular Docking	77
2.11. HYPOTHESIS	78
2.12. PURPOSE AND OBJECTIVE OF THE STUDY	78
Aims	78
2.13. SCREENING STRATEGY	79
2.14. OUTPUTS	81

CHAPTER 3.....	82
3.1. BACKGROUND.....	83
3.2. METHODS	85
3.2.1. Marine organisms	85
3.2.2. Extract preparation	86
3.2.3. Cell culture and cytotoxicity of extracts.....	86
3.2.4. HIV-1 direct enzyme assays	87
3.3. RESULTS AND DISCUSSION	88
3.3.1. Cytotoxicity of the extracts	88
3.3.2. Anti-HIV direct enzymes assays	91
3.4. CONCLUSION	95
3.5. AUTHORS' CONTRIBUTIONS.....	95
CHAPTER 4.....	96
4.1. INTRODUCTION.....	97
4.2. EXPERIMENTAL SECTION	99
4.2.1. General Experimental Procedures.....	99
4.2.3. Purification of the Active Constituents	99
4.2.4. HIV-1 Direct Enzyme Assays.....	102
4.2.5. Cytotoxicity	103
4.2.6. Real-Time Cell Electronic Sensing (RT-CES) xCELLigence.....	104
4.2.7. Molecular Docking	105
4.3.1. Bioassay guided isolation.....	106
4.3.2 Compounds isolation and structure elucidation	107
4.3.2. Cytotoxicity of the compounds.....	109
4.3.2.1. Real Time Cell Analysis	110
4.3.3. Inhibitory Activities of HIV-1 Enzymes	113
4.3.3.1. HIV-1 Enzymes direct assays.....	113
4.3.3.2. Molecular Docking	115
4.4. CONCLUSIONS	118
ACKNOWLEDGMENTS	119
CHAPTER 5.....	120
5.1. INTRODUCTION.....	120
5.2. AREA OF THE STUDY	122

5.3. METHODS	123
5.3.1. Seasonal ecological factors	123
5.3.2. Seasonal distribution of <i>L. arboreum</i>:	123
5.3.3. Sampling and Extraction	123
5.3.4. HPLC profiling	124
5.3.4.1. Qualitative Analysis.....	124
5.3.4.2. Quantitative profiling	124
5.3.5. Cell culture and Cytotoxicity of the extracts.....	125
5.4. RESULTS.....	126
5.4.1. Seasonal changes in the ecological factors	126
5.4.3. Biological evaluation of the seasonal extracts	128
5.4.3.1. Cell culture and Cytotoxicity	128
5.4.3.2. HIV-1 PR inhibitory activities	128
5.4.4.HPLC profiles	128
5.4.4.1. <i>Qualitative HPLC profiling for the seasonal extracts</i>	128
5.4.4.2. Quantitative profiling of the active triterpene.....	131
5.5. DISCUSSION	131
5.6. CONCLUSIONS	136
CHAPTER 6.....	137
6.1. OVERVIEW.....	137
6.2. CHAPTER 3. THE RANDOM SCREENING OF MARINE ORGANISMS	138
6.3. CHAPTER 4. ISOLATION AND BIOLOGICAL EVALUATION <i>L. ARBOREUM</i> METABOLITES:	139
6.4. CHAPTER 5: SEASONAL EVALUATION OF THE ACTIVE TRITERPENE ISOLATED FROM <i>L. ARBOREUM</i> AND ITS ECOLOGICAL CONSEQUENCES.....	141
6.5. REVISITING HYPOTHESIS	141
6.6. HYPOTHESIS ACCEPTED	142
6.7. FUTURE WORK.....	142
6.7.1- Further investigations for the potent untested marine organisms.	142
6.7.2- Structure activity relationship (SAR) approach.	143
6.8. OTHER INVESTIGATIONS	144
6.9. CONCLUSION	145
APPENDIX.....	166

8.1. ADDITIONAL INFORMATION FOR CHAPTER 3.....	166
8.1.1. Screening of marine organisms.....	166
8.1.2. The Reverse Transcriptase Assay	167
8.1.3. Principle XTT viability Assay.	169
8.2. ADDITIONAL INFORMATION FOR CHAPTER 4.....	170
8.2.1. Cytotoxic, Cytostatic and HIV-1 PR Inhibitory Activities of the Soft Coral <i>Litophyton arboreum</i> constituents.	170
8.2.2 Compound 5 crystallography.....	171
8.3. ADDITIONAL INFORMATION FOR CHAPTER 5.....	172
GLOSSARY	173

List of figures

Chapter 1

None

Chapter 2

Figure 2. 1: HIV-1 life cycle (Yasutsugu and Youichi Suzuki, 2011).	5
Figure 2. 2: Simplified model of suggested hypothesis about origin of the cancer stem cells.	19
Figure 2. 3: Mangrove swamps (Lieske and Myers, 2004).	49
Figure 2. 4 Sea grass bed, Red Sea (Lieske and Myers, 2004).	56
The coral reef ecosystem is a diverse collection of species that interact with each other and the physical environment. The sun is the initial source of energy for this ecosystem (Figure 2.5). Through photosynthesis, phytoplankton, algae, and other plants convert light energy into chemical energy (Kadow, 2001).	61
Figure 2. 6: Coral reef, Red Sea (lieske and myers, 2004).	61
Figure 2. 7: A typical drug discovery and development paradigm	73
Figure 2. 8: The principle of XTT assay (AppliChem GmbH, 2010)	76
Figure 2.9: Real time cell electronic sensing device and representation of data. BioTechniques, 2010. www.xcelligence.roche.com	77
Figure 2.10: Work plan followed in this study.....	80

Chapter 3

Figure 3. 1: Cytotoxicity of different marine extracts..	89
Figure 3. 2: HIV-1 PR inhibition percentages of different marine extracts.	92

Chapter 4

Figure 4. 1: Cytotoxicity of <i>L. arboreum</i> main fractions tested in U937 cells at 100 µg/mL.	107
Figure 4. 2: Chemical structure of <i>L. arboreum</i> isolated compounds.	108
Figure 4. 3: Comparison of RT-CES data of compounds 7 and 9.....	111
Figure 4. 4: The effect of compound 7 on the proliferation of HeLa cells using RT-CES analysis.....	112
Figure 4. 5: The effect of compound 2 at 16 µg/mL and compound 5 at 60 µg/mL in HeLa cells.	113
Figure 4. 6: Protease inhibition of <i>L. arboreum</i> isolated compounds at 100 µg/mL.....	114
Figure 4. 7: The effect of the active compound 8 on the proliferation of HeLa cells using RT-CES analysis.	115
Figure 4. 8: 3D Stereo-diagram of the ligand “acetyl pepstatin” combined with the HIV-1 protease receptor (5HVP) active site.	116
Figure 4. 9: Interaction of bioactive compounds with different amino acid moieties in HIV-1 protease receptor (5HVP) active site.....	117

Chapter 5

Figure 5. 1 Map shows the semi enclosed nature of Marsa Beria	122
Figure 5. 2: The seasonal change in <i>L. arboreum</i> percentage cover and dominance values.....	127
Figure 5.3: The seasonal change in <i>L. arboreum</i> total extract cytotoxic activities tested in HeLa and U937 cells at 100 µg/ml.	128
Figure 5.4: The seasonal change in HIV1-PR inhibitory activities of <i>L. arboreum</i> seasonal extracts tested at 100 µg/ml.....	128
Figure 5. 5: HPLC chromatograms of <i>L. arboreum</i> total extracts collected in different seasons. Arrows pointing to the active triterpene's peaks.	130
Figure 5. 6: 7β-acetoxy-24-methylcholesta-5-24 (28)-diene-3, 19-diol.....	131

Appendix

Figure 8. 1: Schematic illustration of collection and extracts preparation.....	166
Figure 8. 2: The principle of RT assay.....	167
Figure 8. 3. HIV-1 RT inhibitory activities of the marine extracts.....	168
Figure 8. 4: The principle of XTT assay	169
Figure 8. 5: Schematic illustration of isolation and purification of <i>Litophyton arboreum</i> constituents.	170
Figure 8. 6: Compound 5 crystallography	171
Figure 8. 7 Schematic illustration of the seasonal evaluation of the extracts as well as the active triterpene.....	172

List of Tables

Chapter 2

Table 2. 1: FDA approved Anti Retro Viruse drugs used in the treatment of HIV infection and their potential side-effects ¹	10
Table 2. 2 : Anti-HIV regimens multi-class combination products approved by FDA.....	17
Table 2. 3: Marine derived potential anti-HIV agents.....	37
Table 2. 4: Marine derived potential anticancer agent.	43
Table 2. 5: Current status of marine natural products in anticancer preclinical or clinical trials.	45
Table 2. 6: Samples collected from mangrove community*	51
Table 2. 7: Samples collected from seagrass bed*	57
Table 2. 8: Samples collected from the coral reef*	63

Chapter 3

Table 3. 1: The half maximal inhibition concentration (IC_{50}) of the lipophilic fraction of the marine organism extracts against HIV-1 PR is presented.....	93
---	----

Chapter 4

Table 4. 1: Cytotoxic inhibitory concentrations, selectivity indexes and HIV-1 PR inhibitory concentrations of the nine isolated compounds in different cell lines.	109
Table 4. 2: Hydrogen bond types and scores of bioactive compounds interacting with HIV-1 protease receptor (5HVP) active site.....	118

Chapter 5

Table 5. 1: The seasonal change in some the physic-chemical parameters of the water in Marsa Braykah (2011-2012).	127
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LIST OF IMPORTANT ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ARV	Antiretroviral
AZT	2,2"-Azinobis (3-ethylbenzthiazoline sulfonic acid)
CC₅₀	50% cytotoxic concentration
HeLa	Henrietta Lacks
PUFA	Polyunsaturated fatty acids
RMSD	Root-mean-square deviation values
CI	Cell Index
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
FDA	Food and drug administration
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HPLC	High performance liquid chromatography
IC₅₀	50% inhibitory concentration
IN	Integrase
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleos(t)ide reverse transcriptase inhibitors
PR	Protease
ART	Antiretroviral therapy
EIMS	Electron ionized mass spectrometry.
SI	Selectivity index
RT-CES	Real time cell electronic sensing
CI	cell index
AP	Acetyl pepstatin

CHAPTER 1

INTRODUCTION

HIV/AIDS is a major health problem worldwide. Infection with HIV weakens the immune system and reduces the body's ability to fight infections and increases the risk of several types of cancers. People infected with HIV are several thousand times more likely than uninfected people to be diagnosed with Kaposi sarcoma, at least 70 times more likely to be diagnosed with non-Hodgkin's lymphoma, and among women, at least 5 times more likely to be diagnosed with cervical cancer (Jiang *et al.*,2010). In addition, people infected with HIV are at higher risk of several other types of cancer including anal, liver, lung cancer, and Hodgkin's lymphoma. According to the National Cancer Institute (2012); 34,000 people with HIV developed one of these signature cancers in the previous five years.

AIDS and cancer are the leading causes of death in the world. Even though chemical antiretroviral drugs can bring about the suppression of serum load of the virus to undetectable levels; economical, commercial and political barriers have limited accessibility of these drugs to a large part of the population suffering from the disease, especially in developing countries (Matic *et al.*, 2006). The emergence of anti-retro viral treatments (ART) drugs resistance (Jiang *et al.*,2010) and the fact that, cancer cells have evolved multiple mechanisms to resist the induction of programmed cell death (apoptosis), (Fluda, 2010), are the major challenges that limits the utility of the available drugs and chemotherapy.

Natural products (NP) derived from marine organisms are excellent sources for the discovery of anti-HIV and anti-cancer agents. The numerous undiscovered unique metabolites in the marine environment are interesting sources to increase the numbers of novel drugs (Mayer *et al.*, 2010). Marine species comprise approximately one-half of the total global biodiversity, meaning that, the sea offers an enormous resource for novel compound identification (Aneiros and Garateix, 2004).

The main advantage of marine natural products is the diversity which is a source of very different kinds of substances because these organisms live in a very exigent, competitive and aggressive surrounding. Because this environment is very different in many respects from the terrestrial environment, there are demands on the organism for the production of quite specific and potent active molecules. These molecules or secondary metabolites have long been known to play an important role in the ecology of plants and animals

(Sondheimer and Simeone, 1970; Rice, 1984). Sammarco *et al.*, (1982) suggest that toxic compounds from soft corals serve both an anti-predator function and a role in competition for living space. Such secondary metabolites vary widely in type, concentration, and function. Thus far, they have been found to play roles in protecting the organism from predators. The effectiveness of compounds derived from individual species also varies widely with respect to interactions with other members of the community (Paul *et. al*, 1990). These effective marine secondary metabolites represent an interesting source for marine natural products that could eventually be developed into therapeutics for human use.

By 1974, two marine-derived natural products (cytarabine, Ara-C and vidarabine, Ara-A) were part of the pharmacopeia used to treat human disease. Since the approval in 2004 of ziconotide (Prialt1) from *Conus magus* for the treatment of moderate to severe pain, Yondelis1 derived from *Ecteinascidia turbinata* has received European approval in 2007 for the treatment of soft tissue sarcoma, and in 2009 for ovarian carcinoma. The marine environment serves as a source of functional materials, including polyunsaturated fatty acids (PUFA), polysaccharides, minerals and vitamins, anti-oxidants, enzymes and bioactive peptides (Wijesekara and Kim , 2010). Numerous other marine natural products or derivatives thereof are in different phases of clinical trials (**Mayer *et al.*, 2010**).

Although traditionally natural products have played an important role in drug discovery, in the past few years most Big Pharma companies have either terminated or considerably scaled down their natural product operations. This is despite a significant number of natural product-derived drugs being ranked in the top 35 worldwide selling ethical drugs in 2000, 2001, and 2002 (Butler , 2004). There were 15 new natural product-derived drugs launched from 2000 to 2003, as well as 15 natural product-derived compounds in Phase III clinical trials or registration at the end of 2003. Recently, there has been a renewed interest in natural product research due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas such as immunosuppression, anti-infectives, and metabolic diseases. To continue to be competitive with other drug discovery methods, natural product research needs to continually improve the speed of the screening, isolation, and structure elucidation processes, as well addressing the suitability of screens for natural product extracts and dealing with issues involved with large-scale compound supply (Butler, 2004).

This work presents our attempt at developing drug leads for marine organisms using quick and easy screening methodologies.

The next chapter 2 provides a literature review of topics relevant to this study. The hypothesis, purpose of the study, specific aims and screening strategy are provided at the end of chapter 2. The specific aims of the study are addressed in three experimental chapters. Chapter 3 present data on the effect of selected marine organisms crude extracts on HIV and cancer cell lines. The constituents of the most active extract were isolated, identified and the cytotoxicity and the inhibitory activities of these constituents were assessed (chapter 4). In chapter 5 the effect of different seasons on extract activity as well as triterpene quantity is presented. The experimental chapters are followed by an overall discussion of the findings, significance and limitations of the project as well as future recommendations (chapter 6). A comprehensive list of references is also provided. Supplementary data and a published manuscript on this work are provided in the appendix.

CHAPTER 2

LITERATURE REVIEW

2.1 The Human immunodeficiency virus (HIV).

Human immunodeficiency virus is a Lentivirus (slowly replicating retrovirus) that causes acquired immunodeficiency syndrome (Douek *et al*, 2009). It is a member of the genus Lentivirus, part of the family Retroviridae (International Committee on Taxonomy of Viruses, 2002). Lentiviruses have many morphological and biological properties in common (Lévy, 1993), and cause a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive. Infection with HIV occurs by the transfer of blood, semen, vaginal fluid, pre-ejaculate or breast milk. Within these bodily fluids, HIV is present as both free virus particles and virus within infected immune cells (Boundless, 2013).

HIV infects vital cells in the human immune system such as helper T cells (specifically CD4+ T cells), macrophages, and dendritic cells (Cunningham, 2010). HIV infection depletes CD4+ T cells through a number of mechanisms including: apoptosis of uninfected bystander cells (Garg, 2012), direct viral killing of infected cells, and killing of infected CD4+ T cells by CD8 cytotoxic lymphocytes that recognize infected cells (Kumar, 2012). When CD4+ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections (Janeway *et al*, 2001).

2.1.1 HIV life cycle.

The life cycle of HIV can be as short as about 1.5 days from viral entry into a cell, through replication, assembly and release of additional viruses, to the infection of other cells summarized in Figure 2.1. A brief description of the different steps of the life cycle follows.

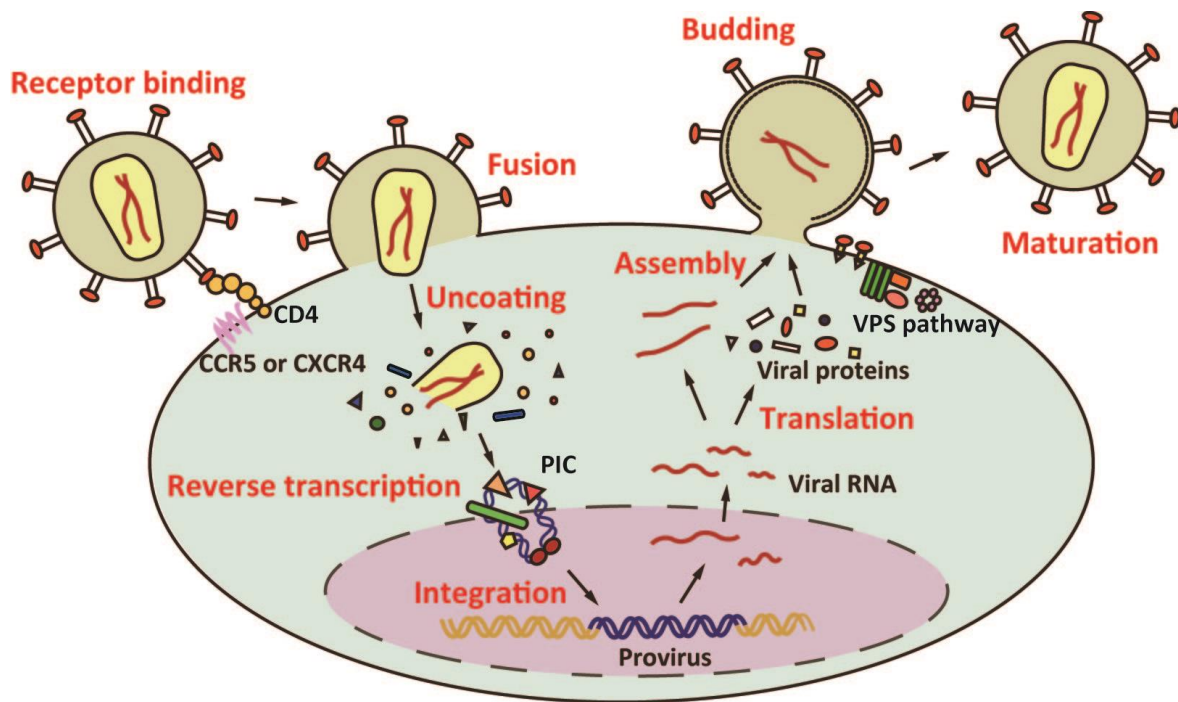


Figure 2. 1: HIV-1 life cycle (Yasutsugu and Youichi Suzuki, 2011).

Attachment and Fusion: HIV begins its life cycle when it attaches to a CD4 receptor and one of two co-receptors on the surface of a CD4 + T- Lymphocyte through its gp120 protein. The virus then fuses with the host cell. After fusion, the virus releases RNA, its genetic material, into the host cell. Reverse transcription occurs where an HIV enzyme called reverse transcriptase converts the single-stranded HIV RNA to double-stranded HIV DNA.

Integration: The newly formed HIV DNA enters the host cell's nucleus, where an HIV enzyme called integrase "integrates" the HIV DNA into the host cell's own DNA. The integrated HIV DNA is called a provirus. The provirus may remain inactive for several years, producing few or no new copies of HIV.

Transcription: When the host cell receives a signal to become active, the provirus uses a host enzyme called RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins.

Assembly: An HIV enzyme called protease cuts the long chains of HIV proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of HIV's RNA genetic material, a new virus particle is assembled.

Budding: The newly assembled virus pushes out ("buds") from the host cell. During budding, the new virus incorporates part of the cell's outer envelope. This envelope, which acts as a covering, is studded with protein and sugar combinations called HIV glycoproteins. These HIV glycoproteins are necessary for the virus to bind to CD4 and co-receptors. The new copies of HIV can now move on to infect other cells (Fass *et al.*, 2011) .

2.1.2 HIV Enzymes.

The HIV-1 genome encodes three essential enzymes for its replicative cycle; reverse transcriptase (RT), protease (PR) and integrase (IN). RT is the etiological agent of AIDS. It is a heterodimer consisting of a p66 (66 kDa) and a p51 (51 kDa) subunit (Arts *et al.*, 1994). and is a multifunctional enzyme showing RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H activities, all of which are required to convert the viral RNA genome into a double-stranded cDNA. Antiretroviral agents that inhibit the HIV-1 RT enzyme can be classified into two main groups; the nucleoside/nucleotide RT inhibitors (N(t)RTIs) which, following two or three phosphorylation steps, act as chain terminators and the nonnucleoside RT inhibitors (NNRTIs), that inhibit the enzyme in a noncompetitive manner by interacting with the allosteric binding site at the palm region of RT (Pomerantz *et al.*, 2003). PR belongs to the aspartyl protease class and functions as a dimer of 99 amino acids each. This enzyme cleaves the HIV gag and gag-pol polyprotein backbone at nine specific cleavage sites to produce shorter, functional proteins which are crucial in the life cycle of HIV-1 (Cheenpracha *et al.*, 2006). IN catalyzes the insertion and integration of viral DNA into the host genome. It is crucial for viral replication and has no counterpart in the host cell. HIV-1 IN is a 32 kDa protein comprised of three independently folded domains. The N-terminal domain (residues 1-50) is characterized by a conserved HHCC zinc binding motif while the dimeric catalytic core domain (residues 50-212) and the C-terminal domain (residues 213-288) are involved in non-specific DNA binding (Pommier *et al.*, 2005).

2.1.3 Classes of antiretroviral agents.

There are several classes of antiretroviral agents that act on different stages of the HIV life-cycle and its related enzymes.

Entry inhibitors (or fusion inhibitors) interfere with binding, fusion and entry of HIV-1 by blocking one of several targets. Maraviroc and enfuvirtide are the two currently available agents in this class. Maraviroc works by targeting CCR5, a co-receptor located on human helper T-cells. Caution should be used when administering this drug however due to a possible shift in tropism which allows HIV to target an alternative co-receptor such as CXCR4. In rare cases, individuals may have a mutation in the CCR5 delta gene which results in a nonfunctional CCR5 co-receptor and in turn, a means of resistance or slow progression of the disease. However as mentioned previously, this can be overcome if an HIV variant that targets CXCR4 becomes dominant (Quashie, 2013). To prevent fusion of the virus with the host membrane, Fuzeon (T20) can be used. Fuzeon is a peptide drug that must be injected and acts by interacting with the N-terminal heptad repeat of gp41 of HIV to form an inactive hetero six-helix bundle, therefore preventing infection of host cells (Lieberman-Blum, 2008).

Nucleoside reverse transcriptase inhibitors (NRTI) and nucleotide reverse transcriptase inhibitors (NtRTI) are nucleoside and nucleotide analogues which inhibit reverse transcription. NRTIs are chain terminators that once incorporated, work by preventing other nucleosides being incorporated because of the absence of a 3' OH group. Both act as competitive substrate inhibitors. Examples of NRTIs include deoxythymidine, zidovudine, stavudine, didanosine, zalcitabine, abacavir, lamivudine, emtricitabine and tenofovir (Das, 2013).

Non-nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcriptase by binding to an allosteric site of the enzyme. NNRTIs act as non-competitive inhibitors of reverse transcriptase. NNRTIs affect the handling of substrate (nucleotides) by reverse transcriptase by binding near the active site and causing "molecular arthritis". NNRTIs can be further classified into 1st generation and 2nd generation NNRTIs. First generation NNRTIs are more rigid in structure and resistance can quickly be developed against them. Because 2nd generation NNRTIs have a more flexible structure, they can adjust more readily and resist mutation more effectively. NNRTIs, for example, include nevirapine, delavirdine, efavirenz, and rilpivirine (Das, 2013).

Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. There are several integrase inhibitors currently under clinical trial, and raltegravir became the first to receive FDA approval in October 2007. Raltegravir has two metal binding groups that compete for substrate with

two Mg²⁺ ions at the metal binding site of integrase. Another clinically approved integrase inhibitor is Elvitegravir (Quashie, 2013).

Protease inhibitors block the viral protease enzyme necessary to produce mature virions upon budding from the host membrane. Particularly, these drugs prevent the cleavage of gag and gag/pol precursor proteins. Virus particles produced in the presence of protease inhibitors are defective and mostly non-infectious. Examples of HIV protease inhibitors are Lopinavir, Indinavir, Nelfinavir, Amprenavir and Ritonavir. Resistance to some protease inhibitors is high (Wensing, 2010).

Maturation inhibitors have a similar effect by binding to gag, but development of two experimental drugs in this class, Bevirimat and Vivecon, was halted in 2010 (Kalyan Das, 2013). Second generation drugs have been developed that are effective against otherwise resistant HIV variants (Wensing, 2010).

Twenty-one anti-HIV medications have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV. All of these drugs may cause negative side effects. Such side effects range from mild to life-threatening and are summarized in Table 2.1. and some are described below.

2.1.4 Common side effects of anti-HIV drugs.

According to the U.S. Department of Health and Human Services (2005) the side effects of anti-HIV medications can be:

- Hyperglycemia

Protease inhibitors (PIs) and infection with hepatitis C virus increase the risk of hyperglycemia and diabetes in people with HIV. The risk of developing hyperglycemia is about the same with all PIs. People who are older, overweight, have family members with diabetes, or are from certain ethnic groups are also at greater risk for developing hyperglycemia.

- Hyperlipidemia

Hyperlipidemia is an increase in the amount of fat (such as cholesterol and triglycerides) in the blood. These increases can lead to heart disease and pancreatitis. Some protease

inhibitors (PIs) can raise blood lipid levels. Some PIs, such as Norvir, are more likely to cause hyperlipidemia than other PIs.

- Lactic Acidosis

Nucleoside reverse transcriptase inhibitors (NRTIs) can cause hyperlactatemia by disrupting the function of the mitochondria. This is known as mitochondrial toxicity. When the mitochondria do not function efficiently, excess lactate is produced. NRTIs can also cause the liver to become fatty, a condition called hepatic steatosis. A fatty liver doesn't work well and cannot break down lactate efficiently. Severe hyperlactatemia leads to lactic acidosis. Lactic acidosis is a serious but very rare complication of treatment with NRTIs. Although all NRTIs are associated with hyperlactatemia and lactic acidosis, people taking Zerit (stavudine) and Videx (didanosine) seem to be at greater risk than people taking other NRTIs.

- Lipodystrophy

Early studies suggested that lipodystrophy was associated with the use of protease inhibitors (PIs), a class of commonly prescribed anti-HIV drugs. However, other studies have shown that lipodystrophy also occurs in people who have never taken PIs. Evidence now suggests that lipodystrophy is linked to taking nucleoside reverse transcriptase inhibitors (NRTIs) and PIs at the same time

- Skin Rash

Anti-HIV medications can cause mild skin rashes as well as serious, even life-threatening rashes. The vast majority of skin rashes are mild to moderate. However, some rashes can be serious. Skin rash may occur with medications from any of the three main HIV drug classes: NNRTIs, NRTIs, and PIs. NNRTIs cause the majority of skin rashes, with Viramune (nevirapine) causing the most severe rashes. Agenerase (amprenavir) and Aptivus (tipranavir) are the PIs most likely to cause skin rash.

Advanced management of HIV/AIDS normally includes antiretroviral therapy (ART) which involves taking a combination of anti-HIV medications (a regimen) shown in Table 2.2. A regimen contains three or more anti-HIV medications from at least two different drug classes. ART may reduce the risk of transmission of HIV but anti-HIV medications cannot cure HIV/AIDS (Dybul *et al.*, 2002).

Table 2. 1: FDA approved Anti Retro Viruse drugs used in the treatment of HIV infection and their potential side-effects¹.

Multi-class Combination products				
Brand Name	Generic Name	Dosage and Administration[‡]	Adverse Reactions	Approval Date
Atripla	Efavirenz Emtricitabine Tenofovir disoproxil fumarate	One tablet once daily taken orally on an empty stomach.	Lactic acidosis/severe hepatomegaly with steatosis, post treatment exacerbation of hepatitis B, diarrhoea, nausea, fatigue, headache, dizziness, depression, insomnia, abnormal dreams, and rash	12-Jul-06
Complera	Emtricitabine Rilpivirine Tenofovir disoproxil fumarate	One tablet taken once daily with a meal.	Lactic acidosis/severe hepatomegaly with steatosis, post treatment acute exacerbation of hepatitis B, diarrhoea, nausea, fatigue, headache, dizziness, depression, insomnia, abnormal dreams, and rash	10-Aug-11
Stribild	Elvitegravir Cobicistat Emtricitabine Tenofovir disoproxil fumarate	One tablet taken once daily with food.	Renal failure, lactic acidosis/severe hepatomegaly with steatosis, post treatment acute exacerbation of hepatitis B, nausea and diarrhoea	27-Aug-12
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)				
Brand Name	Generic Name	Dosage and Administration[‡]	Adverse Reactions	Approval Date
Combivir	Lamivudine Zidovudine	One tablet twice daily	Hematologic toxicity, myopathy, lactic acidosis, exacerbations of hepatitis B, headache, nausea, malaise and fatigue, nasal signs and symptoms, diarrhoea, and cough	27-Sep-97
Emtriva	Emtricitabine	One 200 mg capsule or 24 mL oral solution administered once daily	Lactic acidosis/severe hepatomegaly with steatosis, post treatment exacerbation of hepatitis B, headache, diarrhoea, nausea, fatigue, dizziness, depression, insomnia, abnormal,	02-Jul-03

			dreams, rash, abdominal pain, asthenia, increased cough, and rhinitis.	
Epivir	Lamivudine (3TC)	Dose of 300 mg/day oral solution administered with or without food	Lactic acidosis, post treatment exacerbations of hepatitis b in co-infected patients, headache, nausea, malaise, fatigue, nasal signs and symptoms, diarrhoea, fever and cough	17-Nov-95
Epzicom	Abacavir Lamivudine	One tablet daily	Drug hypersensitivity, lactic acidosis, severe hepatomegaly, exacerbations of hepatitis, insomnia, depression/depressed mood, headache/migraine, fatigue/malaise, dizziness/vertigo, nausea, and diarrhoea.	02-Aug-04
Hivid	Zalcitabine, dideoxycytidine, ddC (no longer marketed)	Tablet and solution discontinued	Clinical adverse reactions, severe peripheral neuropathy, pancreatitis, lactic acidosis, severe hepatomegaly with steatosis, hepatic failure and death.	19-Jun-92
Retrovir	Zidovudine Azidothymidine	Tablets, capsules and syrups 600 mg/day	Haematological toxicity, myopathy, lactic acidosis, headache, malaise, nausea, anorexia, vomiting, fever, cough, digestive disorders, anaemia and neutropenia.	19-Mar-87
Trizivir	Abacavir Zidovudine Lamivudine	One tablet twice daily	Hypersensitivity reactions, hematologic toxicity, myopathy, lactic acidosis and severe hepatomegaly, exacerbations of hepatitis B, nausea, headache, malaise and fatigue, and nausea and vomiting.	14-Nov-00
Truvada	Tenofovir disoproxil fumarate and emtricitabine	One tablet once daily taken orally with or without food.	Lactic acidosis/severe hepatomegaly with steatosis, post-treatment acute exacerbation of hepatitis B, risk of drug resistance, diarrhoea, nausea, fatigue, headache, dizziness, depression, insomnia, abnormal dreams and rash.	02-Aug-04
Videx EC	enteric coated didanosine (ddl EC)	Dosing based on body weight; 200-400 mg/day	Pancreatitis, lactic acidosis, hepatomegaly with steatosis, diarrhoea, peripheral neurologic symptoms/neuropathy, nausea, headache, rash, and vomiting.	31-Oct-00

Videx	Didanosine Dideoxyinosine (DDI)	Administered on an empty stomach at least 30 minutes before or 2 hours after eating. Dosing is based on body weight; 125-400 mg/day.	Pancreatitis, lactic acidosis, hepatomegaly with steatosis, diarrhoea, peripheral neurologic symptoms/neuropathy, abdominal pain, nausea, headache, rash, and vomiting.	09-Oct-91
Viread	Tenofovir disoproxil fumarate (TDF)	Dose of 300 mg/day taken orally without regard to food.	Lactic acidosis/severe hepatomegaly with steatosis, post treatment exacerbation of hepatitis, rash, diarrhoea, headache, pain, depression, asthenia, nausea, abdominal pain, insomnia, pruritus, vomiting, dizziness and pyrexia.	26-Oct-01
Zerit	Stavudine (d4T)	Dose of 30-40 mg taken every 12 h	Lactic acidosis, hepatomegaly with steatosis; pancreatitis, headache, diarrhoea, neuropathy, rash, nausea and vomiting.	24-Jun-94
Ziagen	Abacavir sulfate (ABC)	Dose of 600 mg/day	Hypersensitivity reactions, lactic acidosis, severe hepatomegaly, nausea, headache, malaise and fatigue, nausea and vomiting, dreams/sleep disorders, skin rashes, and ear/nose/throat infections.	17-Dec-98
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)				
Brand Name	Generic Name	Dosage and Administration²	Adverse Reactions	Approval Date
Edurant	Rilpivirine	One tablet taken once daily with a meal.	Fat redistribution or immune reconstitution syndrome, depression, insomnia, headache and rash.	20-May-11
Intelence	Etravirine	Dose of 200 mg taken twice daily following a meal.	Stevens-Johnson syndrome, hypersensitivity reaction, toxic epidermal necrolysis, erythema multiforme, rash, diarrhoea and peripheral neuropathy.	18-Jan-08

Rescriptor	Delavirdine (DLV)	Dose of 400 mg taken three times daily.	Abdominal cramps, abdominal distention, abdominal pain (localized), abscess, allergic reaction, chills, edema (generalized or localized), epidermal cyst, fever, infection viral, lip edema, malaise, susceptibility of <i>Mycobacterium tuberculosis</i> infection, neck rigidity, sebaceous cyst, and fat redistribution.	04-Apr-97
Sustiva	Efavirenz (EFV)	One capsule/tablet daily on an empty stomach.	Serious psychiatric symptoms, nervous system symptoms, hepatotoxicity, rash, convulsions, immune reconstitution syndrome, fat redistribution, rash, dizziness, nausea, headache, fatigue, insomnia and vomiting.	17-Sep-98
Viramune	Nevirapine (NVP)	One tablet daily	Life-threatening (including fatal) hepatotoxicity and skin reactions.	21-Jun-96
Viramune XR	Nevirapine (NVP)	One tablet daily	Life-threatening (including fatal) hepatotoxicity and skin reactions.	25-Mar-11
Protease Inhibitors (PIs)				
Brand Name	Generic Name	Dosage and Administration²	Adverse Reactions	Approval Date
Agenerase	Amprenavir (APV)	Capsule and oral solution discontinued	Mild to moderate gastrointestinal adverse events	15-Apr-99
Aptivus	Tipranavir (TPV)	Dose of 500 mg aptivus, co-administered with 200 mg ritonavir, twice daily	Hepatotoxicity, intracranial hemorrhage, diarrhoea, nausea, pyrexia, vomiting, fatigue, headache, rash and abdominal pain.	22-Jun-05
Crixivan	Indinavir (IDV)	Two capsules taken every 8 h	Nephrolithiasis/urolithiasis, haemolytic anaemia, hepatitis, hyperglycaemia, rhabdomyolysis, hyperbilirubinemia, tubulointerstitial nephritis, fat redistribution.	13-Mar-96

Fortovase	Saquinavir	Capsule and oral solution discontinued	Confusion, ataxia, weakness; acute myeloblastic leukemia; haemolytic anaemia; attempted suicide; Stevens-Johnson syndrome; seizures; severe cutaneous reaction; elevation of transaminases; thrombophlebitis; headache; thrombocytopenia; exacerbation of chronic liver disease, jaundice, ascites, quadrant abdominal pain; drug fever; bullous skin eruption and polyarthritis; pancreatitis leading to death; nephrolithiasis; thrombocytopenia and intracranial hemorrhage leading to death; peripheral vasoconstriction; portal hypertension; intestinal obstruction.	07-Nov-97
Invirase	Saquinavir mesylate, (SQV)	Dose of 1000 mg/day taken in combination with ritonavir 100 mg twice daily.	New onset or exacerbations of diabetes mellitus, hyperglycaemia, elevated cholesterol and/or triglyceride concentrations, fat redistribution, immune reconstitution syndrome, worsening liver disease, haemophilia, nausea, vomiting, diarrhoea, fatigue, pneumonia and abdominal pain.	06-Dec-95
Kaletra	Lopinavir and ritonavir, (LPV/RTV)	Three capsules taken twice daily with food.	Hypersensitivity, hepatotoxicity; new onset or exacerbations of diabetes mellitus, hyperglycaemia, immune reconstitution syndrome and fat redistribution; total cholesterol and triglycerides elevations, haemophilia, diarrhoea, nausea, abdominal pain, asthenia, vomiting, headache, and dyspepsia.	15-Sep-00

Lexiva	Fosamprenavir Calcium (FOS-APV)	Dose of 1,400 mg taken twice daily/1,400 mg plus ritonavir 200 mg once daily/1,400 or 700 mg plus ritonavir 100 mg once daily	Hypersensitivity, transaminase elevations, new onset or exacerbations of diabetes mellitus, hyperglycaemia, immune reconstitution syndrome, fat redistribution and elevated triglyceride and cholesterol concentrations; acute haemolytic anaemia, haemophilia, nephrolithiasis, diarrhoea, rash, nausea, vomiting, headache and neutropenia.	20-Oct-03
Norvir	Ritonavir (RTV)	Dose of 600 mg taken twice daily with meals.	Sedative hypnotics, antiarrhythmic, hypersensitivity, hepatic reactions, pancreatitis, total cholesterol and triglycerides elevations, new onset or exacerbations of diabetes mellitus, hyperglycaemia; immune reconstitution syndrome, fat redistribution, haemophilia, abdominal pain, asthenia, headache, malaise, anorexia, diarrhoea, dyspepsia, nausea, vomiting, paraesthesia, circumoral paraesthesia, peripheral	01-Mar-96
Prezista	Darunavir	Dose of 800 mg taken with ritonavir 100 mg once daily with food.	Drug-induced hepatitis, skin reactions, new onset diabetes mellitus or hyperglycaemia, fat redistribution, immune reconstitution syndrome, diarrhoea, nausea, rash, headache, abdominal pain and vomiting.	23-Jun-06
Reyataz	Atazanavir sulfate (ATV)	Dose of 300 mg taken with ritonavir 100 mg/day with food.	Cardiac conduction abnormalities, rash, hyperbilirubinemia, hepatotoxicity, nephrolithiasis, new onset or exacerbations of diabetes mellitus/hyperglycaemia, immune reconstitution syndrome and fat redistribution; haemophilia, nausea, jaundice/scleral icterus, abdominal pain, vomiting, insomnia, peripheral neurologic symptoms, dizziness, myalgia, diarrhoea, depression, and fever.	20-Jun-03

Viracept	Nelfinavir mesylate (NFV)	Dose of 1250 mg taken twice daily or 750 mg three times daily with a meal.	Diabetes mellitus/hyperglycaemia, haemophilia, fat redistribution, immune reconstitution syndrome, diarrhoea, leukopenia/neutropenia, nausea, rash, flatulence, anorexia and abdominal pain.	14-Mar-97
Fusion Inhibitors				
Brand Name	Generic Name	Dosage and Administration²	Adverse Reactions	Approval Date
Fuzeon	Enfuvirtide, T-20	Dose of 90 mg (1 mL) injected twice daily.	Injection site reaction, pneumonia, hypersensitivity, immune reconstitution syndrome, diarrhoea, nausea and fatigue.	13-Mar-03
Entry Inhibitors - CCR5 co-receptor antagonist				
Brand Name	Generic Name	Dosage and Administration²	Adverse Reactions	Approval Date
Selzentry	Maraviroc	Tablets: 150-600 mg taken twice daily	Hepatotoxicity, immune reconstitution syndrome, severe skin and hypersensitivity reactions, myocardial ischemia and/or infarction, respiratory tract infections, cough, pyrexia, rash and dizziness.	06-Aug-07
HIV integrase strand transfer inhibitors				
Brand Name	Generic Name	Dosage and Administration²	Adverse Reactions	Approval Date
ISENTRESS	Raltegravir	Dose: 400 mg film-coated tablet taken twice daily.	Severe, potentially life-threatening and fatal skin reactions; immune reconstitution syndrome, insomnia, headache, dizziness, nausea, fatigue, creatine kinase elevations, myopathy and rhabdomyolysis.	12-Oct-07

¹ This information is based on the U.S. Food and Drug Administration (FDA) <http://www.fda.gov>, 2012

² Dosage specified is for adults and adolescents. Dosage for pediatric patients is calculated based on body weight (kg) and does not exceed adult dose. Dosage is also adjusted in patients showing adverse reactions to Anti retro virus or demonstrating symptoms of other diseases/disorders.

Table 2. 2 : Anti-HIV regimens multi-class combination products approved by FDA.

Brand Name	Generic Name	Manufacturer Name
Atripla	Efavirenz, emtricitabine and tenofovir disoproxil fumarate	Bristol-Myers Squibb and Gilead Sciences
Complera	Emtricitabine, rilpivirine, and tenofovir disoproxil fumarate	Gilead Sciences
Stribild	Elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate	Gilead Sciences

*This information is based on the U.S. Food and Drug Administration (FDA) <http://www.fda.gov>, 2012

HIV lacks proofreading enzymes to correct errors made when RNA is converted to DNA by reverse transcription. Its short life-cycle and high error rate cause the virus to mutate very rapidly, resulting in a high genetic variability. Most of the mutations either are inferior to the parent virus (often lacking the ability to reproduce at all) or convey no advantage, but some of them have a natural selection superiority to their parent and can enable them to slip past defenses such as the human immune system and antiretroviral drugs. The more active copies of the virus the greater the possibility that one resistant to antiretroviral drugs will be made. When antiretroviral drugs are used improperly, these multi-drug resistant strains can become the dominant genotypes very rapidly. Improper serial use of the reverse transcriptase inhibitors zidovudine, didanosine, zalcitabine, stavudine, and lamivudine can lead to the development of multi-drug resistant mutations. Some of these mutations were observed before protease inhibitors had come into widespread use. The mutants retained sensitivity to the early protease inhibitor saquinavir (Schmit *et al.*, 1996).

Because of the shortcomings of chemical drugs such as toxicity, lack of curative and multiple effects, the search for more and better anti-HIV agents have been focused on different sources including natural products. Many natural products have been shown to possess promising activities that could assist in the prevention and amelioration of AIDS. Most of these natural anti-HIV agents have other medicinal values as well, which afford them further prospective as novel lead compounds for the development of new drugs. These natural products should deal with both the virus and the various disorders that are caused by HIV (Jiang *et al.*, 2010).

2.2 Cancer

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems.

2.2.1 Origins of Cancer

Cancer stem cells may be able to answer some of the questions related to cancer growth; however, origin of the cancer stem cells is yet to be determined. To recognise the origin of cancer stem cells, two important factors need to be considered; (1) a number of mutations are required for a cell to be cancerous and (2) a stem cell needs to

overcome any genetic constraints on both self-renewal and proliferation capabilities. It is unlikely that all the mutations could occur in the lifespan of a progenitor/mature cell. Therefore, cancer stem cells should be derived from either the self-renewing normal stem cells or from the progenitor cells that have acquired the ability of self-renewal due to mutations as shown in Figure 2.2 (Sagar *et al.*, 2007).

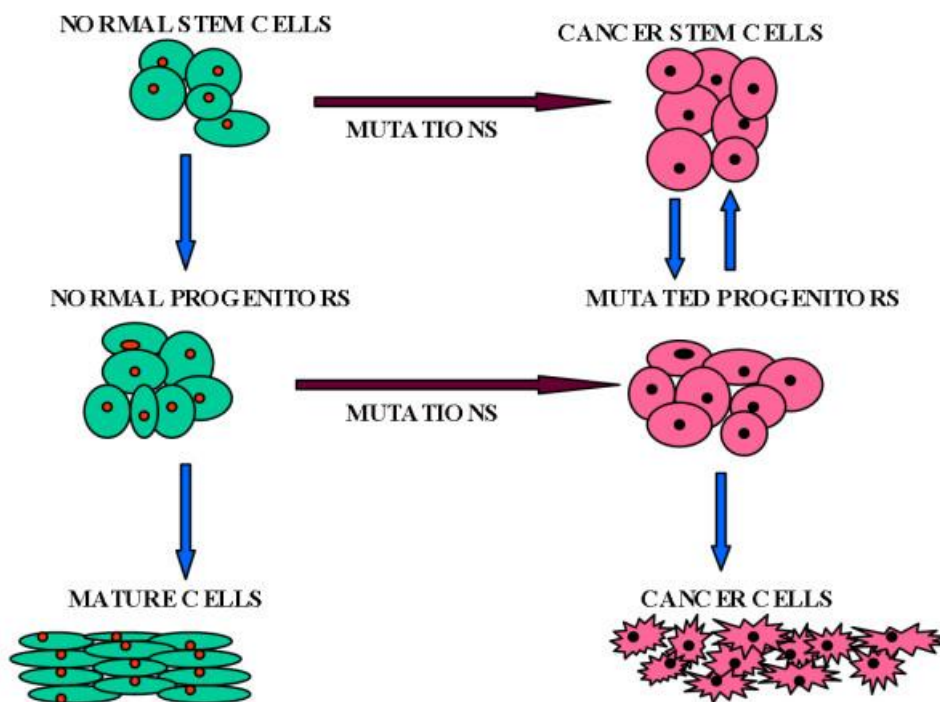


Figure 2. 2: Simplified model of suggested hypothesis about origin of the cancer stem cells.

The cancer stem cells may develop when self-renewing normal stem cells acquire mutations and are transformed by altering only proliferative pathways. It is also possible that the cancer stem cells originate by multiple oncogenic mutations in the restricted progenitor cells which acquire the capability of self-renewal (Jordan CT, 2006). The hypothesis that cancer stem cells are derived from normal stem cells rather than more committed progenitor cells have been addressed in the cases of AML where leukemia initiating cells (LIC) from various subtypes of AML with different stages of differentiation have been shown to share the same cell-surface markers with normal hematopoietic stem cells. However, some of the studies have suggested that cancer stem cells can be derived from the normal stem cells, as well as from the committed short-lived progenitors, giving rise to the tumors with comparable latencies, phenotypes and gene expression profiles. In the solid tumors, lack of the markers to characterize the tumor initiating cells (TIC) in the tumors has made it difficult to study the origins of the cancer stem cells, however there have been identification of cell-surface markers in the lung, brain and prostate which may allow the separation of the stem or progenitor cells with

the tumor initiating function (Sagar *et al.*, 2007).

2.2.2 Types of cancer

According to the National Cancer Institute 2013, there are more than 100 different types of cancer. Cancer types can be grouped into broader categories. The main categories of cancer include:

Carcinoma: Cancer that begins in the skin or in tissues that line or cover internal organs. There are a number of subtypes of carcinoma, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma.

Sarcoma: Cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.

Leukemia: Cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.

Lymphoma and myeloma: Cancers that begin in the cells of the immune system.

Central nervous system cancers: Cancers that begin in the tissues of the brain and spinal cord.

2.2.3 Cancer therapies and side effects

- Chemotherapy

According to NCI, Chemotherapy is a type of cancer treatment that uses drugs to destroy cancer cells. Chemotherapy works by stopping or slowing the growth of cancer cells. It can reduce a tumor size before surgery or radiation therapy (Neo-adjuvant chemotherapy), destroys the remaining cancer cells after surgery or radiation therapy (adjuvant chemotherapy) and it can help to destroy cancer cells that have come back (recurrent cancer) or spread to other parts of the body (metastatic cancer). Chemotherapy can also harm healthy cells that divide quickly, such as those that lines mouth and intestines or cause hair to grow. Damage to healthy cells may cause side effects. Often, side effects get better or go away after chemotherapy is over (Skeet Roland T, 2007).

Major side effects are anemia, appetite changes, bleeding problems, constipation, diarrhea, fatigue, hair loss, infection, memory changes, mouth and throat changes, nausea and vomiting, nerve changes, pain, sexual and fertility changes in men and women, skin and nail changes, swelling (Fluid Retention) and urination changes.

- Radiation Therapy

Radiation therapy uses high-energy radiation to shrink tumors and kill cancer cells. X-rays, gamma rays, and charged particles are types of radiation used for cancer treatment. The radiation may be delivered by a machine outside the body (external-beam radiation therapy), or it may come from radioactive material placed in the body near cancer cells (internal radiation therapy, also called brachytherapy). Systemic radiation therapy uses radioactive substances, such as radioactive iodine, that travel in the blood to kill cancer cells. About half of all cancer patients receive some type of radiation therapy sometime during the course of their treatment. Radiation therapy kills cancer cells by damaging their DNA. Radiation therapy can either damage DNA directly or create charged particles (free radicals) within the cells that can in turn damage the DNA. Cancer cells whose DNA is damaged beyond repair stop dividing or die. When the damaged cells die, they are broken down and eliminated by the body's natural processes (Lawrence *et al.*, 2008).

Radiation therapy can also damage normal cells, leading to both early (acute) and late (chronic) side effects. Acute side effects occur during treatment, and chronic side effects occur months or even years after treatment ends. The side effects that develop depend on the area of the body being treated, the dose given per day, the total dose given, the patient's general medical condition, and other treatments given at the same time.

Acute radiation side effects are caused by damage to rapidly dividing normal cells in the area being treated. These effects include skin irritation or damage at regions exposed to the radiation beams. Most acute effects disappear after treatment ends, though some can be permanent. Amifostine is the only drug approved by the FDA to protect normal tissues from radiation during treatment. This type of drug is called a radioprotector. Other potential radioprotectors are being tested in clinical trials. Fatigue is a common side effect of radiation therapy regardless of which part of the body is treated. Nausea with or without vomiting is common when the abdomen is treated and occurs sometimes when the brain is treated. Medications are available to help prevent or treat nausea and vomiting during treatment (Lawrence *et al.*, 2008).

Late side effects of radiation therapy may or may not occur. Depending on the area of the body treated, late side effects can include fibrosis (the replacement of normal tissue with scar tissue, leading to restricted movement of the affected area) damage to the

bowels, causing diarrhea and bleeding, memory loss and infertility (Lawrence *et al.*, 2008).

- Targeted Therapies

Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progress. By focusing on molecular and cellular changes that are specific to cancer, targeted cancer therapies may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells.

Targeted cancer therapies interfere with cancer cell division (proliferation) and spread in different ways. Many of these therapies focus on proteins that are involved in cell signaling pathways, which form a complex communication system that governs basic cellular functions and activities, such as cell division, cell movement, cell response to specific external stimuli and even cell death. By blocking signals that stimulate cancer cells to grow and divide uncontrollably, targeted cancer therapies can stop cancer progression. Targeted therapies also can cause cancer cell death directly by inducing apoptosis, or indirectly, by stimulating the immune system to recognize and destroy cancer cells and/or by delivering toxic substances to them. The development of targeted therapies requires the identification of targets that are known to play a key role in cancer cell growth and survival (Christy and Bojan, 2013).

According to NCI, targeted therapies have some limitations. Chief among these is the potential for cells to develop resistance to them. Targeted therapies may work best in combination, either with other targeted therapies or with more traditional therapies. The main categories of targeted therapy are small molecules and monoclonal antibodies.

Small molecules

- Imatinib mesylate (Gleevec, also known as STI-571) is approved for chronic myelogenous leukemia, gastrointestinal stromal tumor and some other types of cancer. Early clinical trials indicate that imatinib may be effective in treatment of dermatofibrosarcoma protuberans.
- Gefitinib (Iressa, also known as ZD1839), targets the epidermal growth factor receptor (EGFR) tyrosine kinase and is approved in the U.S. for non small cell lung cancer.

- Erlotinib (marketed as Tarceva). Erlotinib inhibits epidermal growth factor receptor, and works through a similar mechanism as gefitinib. Erlotinib has been shown to increase survival in metastatic non-small cell lung cancer when used as second line therapy. Because of this finding, Erlotinib has replaced gefitinib in this setting.
- Bortezomib (Velcade) is an apoptosis-inducing proteasome inhibitor drug that causes cancer cells to undergo cell death by interfering with proteins. It is approved in the U.S. to treat multiple myeloma that has not responded to other treatments.
- The selective estrogen receptor modulator tamoxifen has been described as the foundation of targeted therapy.
- Janus kinase inhibitors, e.g. FDA approved tofacitinib.
- ALK inhibitors, e.g. crizotinib.
- Bcl-2 inhibitors (e.g. obatoclax in clinical trials, ABT-263, and Gossypol.
- PARP inhibitors (e.g. Iniparib, Olaparib in clinical trials).
- PI3K inhibitors (e.g. perifosine in a phase III trial) • Apatinib is a selective VEGF Receptor 2 inhibitor which has shown encouraging anti-tumor activity in a broad range of malignancies in clinical trials. Apatinib is currently in clinical development for metastatic gastric carcinoma, metastatic breast cancer and advanced hepatocellular carcinoma ().
- AN-152, (AEZS-108) doxorubicin linked to [D-Lys (6)] - LHRH, Phase II results for ovarian cancer.
- BRAF inhibitors (vemurafenib, dabrafenib, LGX818) used to treat metastatic melanoma that harbors BRAF V600E mutation
- MEK inhibitors (trametinib, MEK162) are used in experiments, often in combination with BRAF inhibitors to treat melanoma
- CDK inhibitors, e.g. PD-0332991, LEE011 in clinical trials
- Hsp90 inhibitors, some in clinical trials

- salinomycin has demonstrated potency in killing cancer stem cells in both laboratory-created and naturally occurring breast tumors in mice.

Monoclonal antibodies

Several are in development and a few have been licenced by the FDA. Examples of licenced monoclonal antibodies include:

- Rituximab (marketed as MabThera or Rituxan) targets CD20 found on B cells. It is used in non-Hodgkin lymphoma.
- Trastuzumab (Herceptin) targets the Her2/neu (also known as ErbB2) receptor expressed in some types of breast cancer.
- Cetuximab (marketed as Erbitux) targets the epidermal growth factor receptor. It is used in the treatment of colon cancer and non-small cell lung cancer.
- Bevacizumab (marketed as Avastin) targets circulating VEGF ligand. It is approved for use in the treatment of colon cancer, breast cancer, non-small cell lung cancer, and is investigational in the treatment of sarcoma. Its use for the treatment of brain tumors has been recommended.

Many Antibody-drug conjugates (ADCs) are being developed. See also ADEPT (Antibody-directed enzyme prodrug therapy).

- Transplantation

Bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) are procedures that restore stem cells that have been destroyed by high doses of chemotherapy and/or radiation therapy. There are three types of transplants:

- In **autologous transplants**, patients receive their own stem cells.
- In **syngeneic transplants**, patients receive stem cells from their identical twin.
- In **allogeneic transplants**, patients receive stem cells from their brother, sister, or parent. A person who is not related to the patient (an unrelated donor) may also be used (Ryan, 2013).

Reason why BMT and PBSCT are used in cancer treatment is to make it possible for patients to receive very high doses of chemotherapy and/or radiation therapy. BMT and PBSCT are most commonly used in the treatment of leukemia and lymphoma. They are most effective when the leukemia or lymphoma is in remission (the signs and symptoms of cancer have disappeared). BMT and PBSCT are also used to treat other cancers such as neuroblastoma and multiple myeloma. Researchers are evaluating BMT and PBSCT in clinical trials for the treatment of various types of cancer. To minimize potential side effects, doctors most often use transplanted stem cells that match the patient's own stem cells as closely as possible (Ryan, 2013).

Because only a small amount of bone marrow is removed, donating usually does not pose any significant problems for the donor. The most serious risk associated with donating bone marrow involves the use of anesthesia during the procedure. Apheresis usually causes minimal discomfort. During apheresis, the person may feel lightheadedness, chills, numbness around the lips, and cramping in the hands. Unlike bone marrow donation, PBSC donation does not require anesthesia. The medication that is given to stimulate the mobilization (release) of stem cells from the marrow into the bloodstream may cause bone and muscle aches, headaches, fatigue, nausea, vomiting, and/or difficulty sleeping. These side effects generally stop within 2 to 3 days of the last dose of the medication. The major risk of both treatments is an increased susceptibility to infection and bleeding as a result of the high-dose cancer treatment. Patients who undergo BMT and PBSCT may experience short-term side effects such as nausea, vomiting, fatigue, loss of appetite, mouth sores, hair loss, and skin reactions. Potential long-term risks include complications of the pre-transplant chemotherapy and radiation therapy, such as infertility; cataracts (clouding of the lens of the eye, which causes loss of vision); secondary (new) cancers; and damage to the liver, kidneys, lungs, and/or heart (Ryan, 2013).

- Angiogenesis Inhibitors

Anti-angiogenic therapeutic drugs may act by inhibiting synthesis of antigenic proteins by cancer cells, neutralizing the angiogenic proteins, inhibiting the receptors of endothelia for angiogenic proteins, or directly inducing endothelial cell apoptosis (Chung Wu *et al.*, 2008).

Anti-angiogenic therapy most probably disturbs the tight endothelial cell-platelet interaction. Loss of vascular integrity will cause bleeding complications, gastrointestinal perforations and disturbed wound and ulcer healing. It can also increase the risk for arterial thrombosis (Kabbinavar *et al.*, 2003). Another side effect is the increase of thyroid-stimulating hormone and a decrease in the levels of the circulating thyroid hormones, indicative of hypothyroidism (Desai J *et al.*, 2006). The inhibition of angiogenesis can also cause severe skin toxicities (Robert C *et al.*, 2005). It is possible that inhibition of angiogenesis can cause leukopenia and lymphopenia, as well as thrombocytopenia (Ohm *et al.*, 2005).

- Biological Therapies for Cancer

Biological therapy is a type of treatment that works with the immune system. It can help fight cancer or help control side effects from other cancer treatments like chemotherapy. It can stop or slow the growth of cancer cells, make it easier for immune system to destroy, or get rid of, cancer cells or Keep cancer from spreading to other parts of the body. Biological therapy uses living organisms, substances derived from living organisms, or synthetic versions of such substances to treat cancer. Some types of biological therapy exploit the immune system's natural ability to detect and kill cancer cells, whereas other types target cancer cells directly. Biological therapies include monoclonal antibodies, cytokines, therapeutic vaccines, the bacterium bacillus Calmette-Guérin (commonly used as Mitab vaccine. Smith *et al.*, 2008), cancer-killing viruses, gene therapy, and adoptive T-cell transfer (Rosenberg *et al.*, 2008).

The side effects associated with various biological therapies can differ by treatment type. However, pain, swelling, soreness, redness, itchiness, and rash at the site of infusion or injection are fairly common with these treatments. Less common but more serious side effects tend to be more specific to one or a few types of biological therapy. For example, therapies intended to prompt an immune response against cancer can cause an array of flu-like symptoms, including fever, chills, weakness, dizziness, nausea or vomiting, muscle or joint aches, fatigue, headache, occasional breathing difficulties, and lowered or heightened blood pressure. Biological therapies that provoke an immune system response also pose a risk of severe or even fatal hypersensitivity reactions (Barry Leonard, 2004).

- Cancer Vaccines

Cancer vaccines are medicines that belong to a class of substances known as biological response modifiers. Biological response modifiers work by stimulating or restoring the immune system's ability to fight infections and disease. There are two broad types of cancer vaccines:

Preventive (or prophylactic) vaccines, which are intended to prevent cancer from developing in healthy people; and therapeutic vaccines, which are intended to treat an existing cancer by strengthening the body's natural defenses against the cancer (Lollini *et al.*, 2006). Vaccines intended to prevent or treat cancer appear to have safety profiles. However, the side effects of cancer vaccines can vary among vaccine formulations and from one person to another (Pazdur and Jones, 2007).

The most commonly reported side effect of cancer vaccines is inflammation at the site of injection, including redness, pain, swelling, warming of the skin, itchiness, and occasionally a rash, flu-like symptoms after receiving a cancer vaccine, including fever, chills, weakness, dizziness, nausea or vomiting, muscle ache, fatigue, headache, and occasional breathing difficulties. Blood pressure may also be affected. Cancer treatment vaccines may be most effective when given in combination with other forms of cancer therapy. In addition, in some clinical trials, cancer treatment vaccines have appeared to increase the effectiveness of other cancer therapies (Finn, 2008 and Emens, 2008).

- Photodynamic therapy

Photodynamic therapy (PDT) is a treatment that uses a drug, called a photosensitizer or photosensitizing agent, and a particular type of light. When photosensitizers are exposed to a specific wavelength of light, they produce a form of oxygen that kills nearby cells (Dolman *et al.*, 2003). Each photosensitizer is activated by light of a specific wavelength (Vrouenraets *et al.*, 2003 and Dougherty *et al.*, 1998). This wavelength determines how far the light can travel into the body (Vrouenraets *et al.*, 2003 and Gudgin *et al.*, 2002).

The treatment makes the skin and eyes sensitive to light for approximately 6 weeks after treatment (Emens, 2008). Thus, patients are advised to avoid direct sunlight and bright indoor light for at least 6 weeks. Photosensitizers tend to build up in tumors and the activating light is focused on the tumor. As a result, damage to healthy tissue is minimal.

However, PDT can cause burns, swelling, pain, and scarring in nearby healthy tissue (Vrouenraets *et al.*, 2003).

- Cryosurgery

Cryoablation therapy (or cryotherapy) uses liquid nitrogen (or the expansion of argon gas) to freeze and kill abnormal tissue. After numbing the tissue around the mass, a cryoprobe, which is shaped like a large needle, is inserted into the middle of the lesion. An ice ball forms at the tip of the probe and continues to grow until the images confirm that the entire tumor has been engulfed, killing the tissue (Cowen *et al.*, 2002). Cryoablation therapy (cryoablation or cryotherapy) is a method of treatment that involves destroying abnormal tissue by freezing. Cryoablation is “the in situ freezing and devitalization of tissues, which can be applied to produce predictable zone of necrosis that will destroy the target lesion as well as an appropriate margin of surrounding tissue” (Patel, 1996). An instrument probe (cryoprobe or cryoneedle) is placed in contact with the tissue to be frozen. When the probe is turned on, the cooling gas (usually argon or nitrogen) circulates through it, causing the tip to become extremely cold until an ice ball is formed. The whole process involved in cryotherapy takes about 10 – 20 minutes to complete (Okhai and Smith, 2009).

Cryosurgery does have side effects, although they may be less severe than those associated with surgery or radiation therapy. The effects depend on the location of the tumor. The acute side effects can be bleeding at the freeze site, blister formation, edema, headache (after treatment of facial lesions), pain, syncope (vasovagal; rare), excess granulation tissue formation (rare) and infection (rare). Protracted or permanent side effects are atrophy (rare), hair and hair follicle loss, and hypopigmentation while protracted but temporary side effects can be alteration of sensation, hyperpigmentation, hypertrophic scarring and Milia (Andrews, 2004).

- Lasers

Laser treatment has the potential to destroy primary breast tumors. This treatment can also seek and destroy cancer cells that have spread (metastasized) from the original tumor to other parts of the body. This is especially critical as metastasized cells are the primary cause of cancer death. In addition to being a potentially effective cancer treatment, laser-assisted immunotherapy appears to be able to act as a *cancer vaccine*, effectively preventing the same cancer from recurring. An ideal approach to treating

advanced cancers would accomplish eliminate the original tumor and empower the immune system to destroy the cancer cells. Then, instead of having to locate and treat every infiltrated lymph node and every hidden metastasis, the body's natural tumor surveillance system would do its work, root out the cancer, and prevent recurrence (Bromwell, 2012).

According to NCI, laser therapy has several limitations. Surgeons must have specialized training before they can do laser therapy, and strict safety precautions must be followed. Laser therapy is expensive and requires bulky equipment. In addition, the effects of laser therapy may not last long, so doctors may have to repeat the treatment for a patient to get the full benefit.

- Hyperthermia

Hyperthermia (also called thermal therapy or thermotherapy) is a type of cancer treatment in which body tissue is exposed to high temperatures (up to 113°F). Research has shown that high temperatures can damage and kill cancer cells, usually with minimal injury to normal tissues (van der Zee, 2002). By killing cancer cells and damaging proteins and structures within cells (Hildebrandt, 2002), hyperthermia may shrink tumors. Hyperthermia is under study in clinical trials and is not widely available.

- Complementary and Alternative Therapies

The use of complementary and alternative therapies is increasing, especially among patients with life-threatening diseases such as AIDS and cancer, and traditionally defined vulnerable populations, such as the elderly (Gerontol, 2001 and Rajendran *et al.*, 200). Prior studies have estimated that the proportion of cancer patients using CAM with higher figures among breast cancer patients, ranging from 67% to 83% (Boon *et al.*, 2000 and Richardson *et al.*, 2000). A recent study described CAM as a beneficial therapy for alleviating cancer pain, but the evidence levels were found to be low or moderate. It is also suggested the need of future large and rigor randomized controlled studies to confirm the benefits of CAM on adult cancer pain (Bao *et al.*, 2014). HIV positive people are more likely to use CAM than people with other serious illnesses such as cancer or arthritis (Hand 1989, Anderson *et al.* 1993). Researchers estimate that as many as two-thirds of people with HIV have tried CAM (Katz 2001). Additionally, use has not significantly declined since the widespread use of antiretroviral triple drug therapies (Onstott and Horn 1998, Katz 2001).

The complementary and alternative medicine (CAM) includes a wide variety of therapies, botanicals, nutritional products, and practices. These forms of treatment are used in addition to (complementary) or instead of (alternative) standard treatments (Foote-Ardah, 2003). Throughout history, natural products have afforded a rich source of anticancer and anti-HIV agents with diverse chemical structures and bioactivities. More than 50% of world marketed drugs have their origin of the nature. Natural products, of which structural diversity is so broad, are good sources for the effective discovery of anti-HIV agents with decreased toxicity (Jung *et al.*, 2013). Some technological and methodologic advances in structure elucidation, organic synthesis, and biological assay have resulted in the isolation and clinical evaluation of various novel anticancer agents such as, taxol, betulinic acid, camptothecin, resveratrol, podophyllotoxin and curcumin (E-Hu *et al.*, 2009).

2.3. Natural products as source of biologically active compounds.

Nature stands as an inexhaustible source of novel chemotypes and pharmacophores, and has been a source of medicinal agents for thousands of years. An impressive number of modern drugs find their origin in natural products. Natural product chemistry has experienced explosive and diversified growth, making natural products the subject of much interest and promise in the present day research directed towards drug design and discovery. It is noteworthy that natural products are a source of new compounds with diversified structural arrangements possessing interesting biological activities. It is a source of novel and active agents that may serve as the leads and scaffolds for elaboration into urgently needed efficacious drugs for a multitude of disease indications. Natural products have provided considerable value to the pharmaceutical industry over the past half century. In particular, the therapeutic areas of infectious diseases and oncology benefited much from numerous drug classes derived from the natural form and as templates for synthetic modification. About 40 new drugs launched on the market between 2000 and 2010, originating from terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Brahmachari, 2010).

Modern medicine has gradually developed over the years by scientific and observational efforts of scientists; however, the basis of its development remains rooted in traditional medicine and therapies, prevailing throughout the world for thousands of years, which continue to provide mankind with new remedies. Plant-based medicines initially dispensed in the form of crude drugs such as tinctures, teas, poultices, powders, and

other herbal formulations, now serve as the basis of novel drug discovery (Samuelsson, 2004).

Plant-based indigenous knowledge was passed down from generation to generation in various parts of the world throughout history and has significantly contributed to the development of different traditional systems of medicine. The use of natural products as medicine has invoked the isolation of active compounds; the first commercial pure natural product introduced for therapeutic use is generally considered to be the narcotic morphine, marketed by Merck in 1826 (Newman, 2000), and the first semi-synthetic pure drug aspirin, based on a natural product salicin isolated from *Salix alba*, was introduced by Bayer in 1899. These successes subsequently led to the isolation of early drugs such as cocaine, codeine, digitoxin, quinine, and pilocarpine, of which some are still in use today (Newman, 2000); (Kinghorn, 2001); (Buss, 2003) and (Mann, 2000).

A total of about 38 natural product-based drugs were approved and launched in the market during the period 2000 to 2010. Examples of these drugs are:

Arteether: (Artemotil®, Artecef ®), the semi-synthetic derivative of artemisinin (8), is a potent antimalarial drug. Artemisinin, a natural endoperoxide sesquiterpene lactone, is the active chemical constituent of *Artemisia annua*, a plant used in traditional Chinese (Cooper, 2004).

Caspofungin acetate: (Cancidas®, Merck, 2001) is a semi-synthetic antifungal lipopeptide compound derived from pneumocandin B0, a fermentation product of *Glarea lozoyensis* (Graul, 2002).

Ertapenem (Invanz™, Merck, 2001) is a new 1 β -methylcarbapenem antibiotic derived from thienamycin, isolated from *Streptomyces cattleya* (Bernardelli, 2002).

Cefditoren pivoxil: (Spectracef ®; TAP Pharmaceuticals, 2001) is an oral prodrug of cefditoren, a derivative of cephalosporin isolated from *Cephalosporium* species (Wellington, 2005).

Biapenem: (Omegacin®; Wyeth Lederle Japan, 2002) is a new analog of carbapenem based on thienamycin, isolated from *Streptomyces cattleya*; the antibacterial drug is found to be effective against both Gram-negative and Gram-positive bacteria including the species that produce β -lactamases (Malanoski, 1993).

Daptomycin: (Cubicin™; Cubist Pharmaceuticals, 2003), a cyclic lipopeptide antibacterial agent derived from *Streptomyces roseosporus* (Woodworth, 1992).

Telithromycin: (Ketek®; Aventis, 2004) is a semi-synthetic derivative of the 14-membered macrolide, erythromycin A, isolated from *Saccharopolyspora erythraea*, and retains the macrolactone ring as well as a D-desosamine sugar moiety (Cragg, 2001 and Bernardelli, 2002).

Fumagillin: (Flisint®; Sanofi-Aventis, 2005) was first isolated in 1949 from *Aspergillus fumigatus* and used shortly thereafter to treat intestinal amoebiasis; this antimicrobial agent is capable of inhibiting the proliferation of endothelial cells (McCowen, 1951).

Anidulafungin: (Eraxis™ in US/Ecalta™ in Europe, Pfizer, 2006), finds use against invasive and esophageal candidiasis and candidemia (Pfaller, 2001); the drug is a semi-synthetic derivative of the fungal metabolite echinocandin B (Torre, 2007).

Retapamulin: (Altabax™) in the US and (Altargo™) in Europe. GlaxoSmithKline, 2007, received FDA approval in April 2007 and the European Medicines Agency (EMA) approval in June 2007 for its topical use as 1% retapamulin ointment against bacterial infections (Rittenhouse, 2006 and Jacobs, 2007). The drug is a semi-synthetic derivative of the fungal metabolite pleuromutilin (Daum, 2007).

Telavancin: (Vibativ™; Theravance/Astellas Pharmaceuticals, 2009), was discovered by Theravance as an antibacterial agent, and was developed in partnership with Astellas.304–307 Telavancin, a semi-synthetic derivative of vancomycin (Leadbetter, 2004).

2.3.1. Natural products and HIV treatment:

Over the past 20 years, progress has been made in screening and developing natural products and chemically synthesized compounds as medication for HIV infection (Vlietinck *et al.*, 1998; De Clercq, 2000; Kong *et al.*, 2003; Wang *et al.*, 2004, 2008; Hupfeld and Efferth, 2009). Natural compounds such as flavonoids, polyphenols, alkaloids, coumarins, terpenoids, and peptides showed inhibitory activities on RT, PR and IN. Most of these natural products are flavonoids and polyphenols, followed by terpenoids. Many flavonoids and polyphenol compounds show efficient inhibition on RT, PR and IN at the same time. Comparing to flavonoids and polyphenols, alkaloids inhibitors are fewer. Terpenoids are also an important source of anti HIV inhibitors, in which diterpene and triterpene are the main two kinds of inhibitors. Fungi, plants and marine organisms are the main reservoir of these types of compounds (Lee *et al.*, 2010).

Natural inhibitors of RT have been studied more extensively than inhibitors of PR and IN. Nearly all the inhibitors to HIV-1 protease used clinically were produced by chemical synthesis. Initial inhibitors created in the laboratory were peptide derivatives which could bond with HIV-1 PR active sites. Later, designers shifted from creation of symmetric inhibitors of this inherently symmetric enzyme to their ultimate conversion to asymmetric compounds. Most of these chemically synthetic drugs were toxic and could not be used at high enough doses which soon engendered the resistant mutant of PR. In recent years, natural low-molecular-weight molecules compounds have drawn the attention of researchers (Jiang *et al.*, 2010).

2.3.2. Natural products and cancer treatment.

The plant based drug discovery resulted mainly in the development of anticancer agents including plants (vincristine, vinblastine, etoposide, paclitaxel, camptothecin, topotecan and irinotecan), marine organisms (citarabine, aplidine and dolastatin 10) and micro-organisms (dactinomycin, bleomycin and doxorubicin). Beside this there is numerous agents identified from fruits and vegetables can used in anticancer therapy. The agents include curcumin (turmeric), resveratrol (red grapes, peanuts and berries), genistein (soybean), diallyl sulfide (allium), S-allyl cysteine (allium), allicin (garlic), lycopene (tomato), capsaicin (red chilli), diosgenin (fenugreek), 6-gingerol (ginger), ellagic acid (pomegranate), ursolic acid (apple, pears, prunes), silymarin (milk thistle), anethol (anise, camphor, and fennel), catechins (green tea), eugenol (cloves), indole-3-carbinol (cruciferous vegetables), limonene (citrus fruits), beta carotene (carrots), and dietary fiber (Bhanot *et al.*, 2011).

Numerous phytochemicals derived from dietary and medicinal plants have been reported to inhibit, retard, or reverse a specific stage of the carcinogenic process. A wide array of molecules and events are involved in relaying intracellular signals to maintain cellular homeostasis (Surh, 2003).

Over 60% of the current anticancer drugs have their origin in one way or another from natural sources. Natural metabolites can target different strategies in cancer treatment. Flavonoids from natural sources have been identified as a one of the major classes of natural anticancer agents exerting antineoplastic activity via cell cycle arrest as a major mechanism in various types of cancer cells (Tumbas *et al.*, 2010). Phenolic and flavonoid compounds have been proven to have promising antioxidant activities (Bocco *et al.*, 1998; Li *et al.*, 2006). Flavonoids such as naringin, hesperidin, naringenin, hesperitin,

rutin, nobiletin, dan tangeretin found to inhibit carcinogenesis (Aranganathan and Nalini, 2009).

Apoptosis, programmed cell death, is a biological key pathway for regulating homeostasis and morphogenesis. Apoptolidin is a natural product originally isolated from a *Nocardioopsis* species. It can selectively induce apoptosis in several cancer cell lines (Daniel *et al.*, 2006).

Betulinic acid is a pentacyclic triterpenoid, which naturally occurs, for example, in the bark of white birch trees and has been identified to stimulate the mitochondrial apoptosis pathway preferentially in cancer cells (Fulda *et al.*, 1998). Resveratrol is another natural compound that is present in several dietary items, e.g., in grapes and red wine. Chemically, resveratrol belongs to the group of polyphenolic phytoalexins and has been described to interfere with mitochondrial functions by inhibiting mitochondrial ATP synthesis (Gledhill *et al.*, 2007). Vitamin E analogues, for example, α -tocopheryl succinate (α -TOS), have also been reported to selectively trigger mitochondrial apoptosis in tumor cells (Constantinou *et al.*, 2008). Gossypol, a polyphenolic aldehyde that naturally occurs in the cotton plant (Lynn and Jones, 2007), has been demonstrated to simultaneously antagonize several anti-apoptotic Bcl-2 proteins, which interfere with mitochondrial outer membrane permeabilization (Azmi and Mohammad, 2009).

2.4 Marine organisms as natural source for bioactive compounds

Marine organisms are a rich source of natural products (Pomponi, 1999). In recent time, advancement in deep-sea collection and aqua culture technology produced significant numbers of compounds derived from marine organisms entering preclinical and early clinical evaluation as potential anticancer agent (Schwartsmann, 2000 and Schwartsmann *et al.*, 2011). Overall, more than 3000 new substances have been identified from marine organisms that demonstrate great potential as a source of novel chemical classes (Schweitzer *et al.*, 1991). Marine compounds belong to very diverse structural classes including polyketides, terpenes, steroids and peptides. The organisms yielding these bioactive marine compounds include invertebrate animals, algae, fungi and bacteria (Rinehart, 2000).

The marine environment has been shown to be the source of chemically diverse structures with promising biological activities. Besides the chemical novelty associated

with these compounds, some of them possess novel mechanisms of action as well. To date, eight marine drugs have been approved by the FDA or EMEA registered, they are Cephalosporin C, Cytarabine (Ara-C), Vidrabine (Ara-A), Ziconotide (Prialt), omega-3-acid ethyl esters (Lovaza), ET-743 (Yondelis), E7389 (Halaven), Brentuximab vendotin (SGN-35) (Mayer et al., 2010 and Gerwick and moore, 2012).

The global marine preclinical pharmaceutical pipeline remains very active. During the period 1998-2008, the global marine pharmaceutical preclinical pipeline included 592 marine natural products that showed antitumor and cytotoxic activity and 666 additional chemicals which demonstrated other pharmacological activities (i.e. antibacterial, anticoagulant, anti-inflammatory, antifungal, anthelmintic, antiplatelet, antiprotozoal, and antiviral activities;) with actions on the cardiovascular, endocrine, immune, and nervous systems; and other miscellaneous mechanisms of action (Mayer *et al.*, 2011).

The solidness of the marine pharmaceuticals pipeline is evident, clinical trials with Soblidotin (TZT 1027), Tasidotin, Synthadotin (ILX-651), Bryostatin 1, Hemiasterilin (E7974), and Pseudopterosin have been completed, a compound (Plitidepsin) in Phase III trials, six compounds [(DMXBA(GTS-21), Plinabulin (NPI 2358), PM00104, Elisidepsin, PM01183, CDX-011)] in Phase II trials, and four compounds [Marizomib (Salinosporamide A Code, NPI-0052)], PM060184, SGN-75, ASG-5ME) in Phase I trials have been processed with numerous marine natural products being investigated preclinical as clinical candidates (Yonghong, 2012).

2.4.1 Marine organisms as source of anti-HIV agents

A great deal of interest has been expressed regarding marine-derived anti-HIV agents such as phlorotannins, sulfated chitooligosaccharides, sulfated polysaccharides, lectins and bioactive peptides (Vo and Kim, 2010). Some of marine derived potential anti-HIV agents are shown in Table 2.3 according to Tziveleka *et al.*, 2003.

For the first time, (Ahn *et al.* 2010) reported that 8,8'-bieckol and 8,4''-dieckol, which were isolated from the brown algae *Ecklonia cava* KJELLMAN, showed an inhibitory effect on HIV-1 reverse transcriptase and protease. The inhibition of reverse transcriptase by 8,8'-bieckol with a biaryl linkage (IC_{50} 0.5 μ M) was 10-fold greater than that of 8,4''-dieckol with a diphenyl ether linkage (IC_{50} 5.3 μ M). Although these two phlorotannis are dimers of eckol. The authors suggested that the steric hindrance of the hydroxyl and aryl groups near the biaryl linkage of 8,8'-bieckol caused the potent

inhibitory activity. Moreover, 8,8'-bieckol selectively inhibited reverse transcriptase over protease, and the inhibitory effect was comparable to the positive control nevirapine (IC_{50} , 0.28 μ M).

Lee and Maruyama, 1998 considered that oysters produced antiviral and antibacterial substances for preventing infectious diseases. Thus, they searched for HIV-1 protease inhibiting substances from the oyster *Crassostrea gigas*. They observed and isolated two peptides, Leu-Leu-Glu-Tyr-Ser-Ile and Leu-Leu-Glu-Tyr-Ser-Leu, which inhibited HIV-1 protease from thermolysin hydrolysate of oyster protein. The peptides 1 and 2 showed strong inhibition of HIV-1 protease at IC_{50} values of 20 and 15 nM, respectively. Moreover, these peptides behaved as competitive inhibitors for HIV-1 protease with K_i values of 13 and 10 nM, respectively. Lee and Maruyama, 1998 confirmed that the presence of C-, N-terminal hydrophobic amino acids and the length of the amino acid sequence in these peptides were important for their inhibitory activity.

The ethyl acetate extract of the sponge *Xestospongia muta*. 47 were fractionated to yield seven straight-chain unsaturated, polyacetylenic and brominated acids. These acetylenic acids were the first metabolites capable of inhibiting HIV protease. The mentioned metabolites inhibited HIV-1 protease having IC_{50} between 6 and 12 mM (Tziveleka *et al.*,2003).

Table 2. 3: Marine derived potential anti-HIV agents(Table adapted from Tziveleka *et al.*, 2003)

Organism	Group	Chemical class	Target
<i>Adocia</i> sp.	Sponge	Protein	viral coat protein gp120 and to the cellular receptor CD4
<i>Agardhiella tenera</i>	Alga	Sulfated polysaccharides	RT of HIV-1
<i>Aphanocapsa pulchra</i>	Micro alga	Extract	RT of HIV-1
<i>Aphanothece clathrata</i>	Micro alga	Extract	RT of HIV-1
<i>Aphanothece nidulans</i>	Micro alga	Extract	RT of HIV-1
<i>Asparagopsis armata</i>	Alga	Polysaccharides	RT of HIV-1
<i>Asterias amurensis</i>	Starfish	Sulfated sterols	anti-HIV <i>in vitro</i>
<i>Batzella</i> sp.	Sponge	Guanidine Alkaloids	inhibition of binding of HIV gp120 to CD-4
<i>Callipelta</i> sp.	Sponge	Depsipeptide	RT of HIV-1
<i>Clathria</i> sp.	Sponge	Steroid sulfates	RT of HIV-1
<i>Cochlodinium polykrikoides</i>	Micro alga	Sulfated polysaccharides	anti-HIV <i>in vitro</i>
<i>Corticium</i> sp.	Sponge	Steroid alkaloids	inhibit syncytia formation after HIV infection of a MT-4 cell line
<i>Coscinoderma</i> sp.	Sponge	Indole alkaloids	anti-HIV <i>in vitro</i>
<i>Dercitus</i> sp.	Sponge	Acridine alkaloids	antiviral activity against both a DNA and a RNA virus
<i>Dictyota dichotoma</i>	Alga	Diterpenes	HIV-1RT inhibitors (patent)
<i>Dictyota patens</i>	Alga	Diterpenes	HIV-1RT inhibitors (patent)
<i>Didemnum guttatum</i>	Tunicate	Lipid trisulfate	HIV-1 integrase inhibitor
<i>Didemnum molle</i>	Tunicate	Sulfated polysaccharide	preliminary anti-HIV
<i>Didemnum</i> sp.	Tunicate	Terpenoid	HIV-1 protease
<i>Dycidea cinerea</i>	Sponge	Hydroquinone	HIV-1 RT
		Sesquiterpenes	HIV-1 RT

<i>Dygenea simplex</i>	Alga	Sulfated polysaccharides	inhibiting HIV-1 cytopathic effects
<i>Echinaster brasiliensis</i>	Starfish	Sulfated sterols	inhibiting HIV-1 cytopathic effects
<i>Euchema cottonii</i>	Alga	Sulfated polysaccharides	anti-HIV <i>in vitro</i>
<i>Euryspongia</i> sp.	Sponge	Hydroquinone	anti-HIV <i>in vitro</i>
		Sesquiterpenes	anti-HIV <i>in vitro</i>
<i>Fascaplysinopsis reticulata</i>	Sponge	Alkaloids and	HIV-1 RT
		Alkaloid –sesterterpene salts	HIV-1 RT
<i>Celerina heffernani</i>	Starfish	Guanidine alkaloids	cytotoxic to CEM 4 cells infected by HIV-1
<i>Fromia monilis</i>	Starfish	Guanidine alkaloids	cytotoxic to CEM 4 cells infected by HIV-2
<i>Fucus vesiculosus</i>	Alga	Polysaccharide	HIV induced syncytium (giant cell) formation
<i>Fusarium heterosporum</i>	Marine Fungus	Terpenoids	HIV-1 INT
<i>Gigardina aciculaire</i>	Alga	Sulfated polysaccharides	anti-HIV <i>in vitro</i>
<i>Gigardina pistillata</i>	Alga	Sulfated polysaccharides	anti-HIV <i>in vitro</i>
<i>Gigardina tenella</i>	Algae	Sulfated lipids	HIV-1 RT
<i>Halicortex</i> sp.	Sponge	Bromoindole alkaloid	anti-HSV-1 and anti-HIV-1
<i>Haslae ostrearia</i>	Micro alga	Extract	delay the HIV-1 induced syncytia formation on MT-4 cells
<i>Hippospongia</i> sp.	Sponge	Acetylenic fatty acid derivatives	HIV-1 RT
<i>Hyrtios cf. erecta</i>	Sponge	Alkaloids and	HIV-1 RT
		Alkaloid –sesterterpene salts	HIV-1 RT
<i>Ircinia</i> sp.	Sponge	Prenylated hydroquinones	HIV-1, HIV-2 RT and murine leukaemia virus
<i>Limulus polyphemus</i>	Horse shoe crab	Peptides	HIV-induced cytopathogenicity
<i>Lobophytum</i> sp.	coral	Cembranoids	anti-HIV <i>in vitro</i>
<i>Lyngbya lagerheimii</i>	Micro alga	Sulfated lipids	HIV-1 RT
<i>Lyngbya majuscula</i>	Micro alga	Peptide	HIV-1 INT
<i>Mixylla rosacea</i>	Sponge	Sulfated polysaccharide	anti-HIV <i>in vitro</i>
<i>Monostroma latissimum</i>	Alga	Polysaccharides	later steps of viral replication in host cells

<i>Nostoc elipsosporum</i>	Micro alga	Protein	anti-HIV <i>in vitro</i>
<i>Nothogenia fastigiata</i>	Alga	Sulfated polysaccharides	HIV-cell fusion inhibitors
<i>Ophiaracna incrassate</i>	Ophiuroid	Sulfated sterols	cytopathic effects of HIV-1 infection in CEM-SS
<i>Ophiartum elegans</i>	Ophiuroid	Sulfated sterols	cytopathic effects of HIV-1 infection in CEM-SS
<i>Ophioderma longicaudum</i>	Ophiuroid	Sulfated sterols	cytopathic effects of HIV-1 infection in CEM-SS
<i>Ophiosphate gigaes</i>	Ophiuroid	Sulfated sterols	cytopathic effects of HIV-1 infection in CEM-SS
<i>Ophiotrix fragilis</i>	Ophiuroid	Sulfated sterols	cytopathic effects of HIV-1 infection in CEM-SS
<i>Oscillatoria acutissima</i>	Micro alga	Extract	HIV-1 RT
<i>Oscillatoria amoena</i>	Micro alga	Extract	HIV-1 RT
<i>Oscillatoria animalis</i>	Micro alga	Extract	HIV-1 RT
<i>Oscillatoria limnetica</i>	Micro alga	Glyco lipids	HIV-1 RT
<i>Petrosia</i> sp.	Sponge	Polyacetylenes	HIV-1 RT
<i>Petrosia weinbergi</i>	Sponge	Steroid sulfate	anti-HIV <i>in vitro</i>
<i>Peyssonelia</i> sp	Alga	Sesquiterpene hydroquinones	HIV-1, HIV-2 RT
<i>Phoma</i> sp.	Marine Fungus	Terpenoids	HIV-1 INT
<i>Phormidium corium</i>	Micro alga	Extract	HIV-1 RT
<i>Phormidium molle</i>	Micro alga	Extract	HIV-1 RT
<i>Phormidium tenue</i>	Micro alga	Sulfated lipids	HIV-1 RT
<i>Phormidium tenue</i>	Micro alga	Glyco lipids	HIV-1 RT
<i>Phormidium valderianum</i>	Micro alga	Extract	HIV-1 RT
<i>Phyllospongia lamellosa</i>	Sponge	Scalarane sesterterpenes	HIV-1 fusion inhibitor
<i>Polycitor</i> sp.	Tunicate	Aromatic alkaloid	HIV-1 RT
<i>Pseudoaxinnissa digitata</i>	Sponge	Steroid sulfates	HIV-1 cytopathicity
<i>Sargassum horneri</i>	Alga	Polysaccharides	Anti HIV fusion
<i>Schizothrix pallida</i>	Micro alga	Extract	HIV-1 RT
<i>Schizymenia pacifica</i>	Alga	Polysaccharides	HIV-1 RT

<i>Scytonema</i> sp.	Micro alga	Sulfated lipids	HIV-1 RT
<i>Sidonops microspinosa</i>	Sponge	Depsipeptide	cytopathic effect of HIV-1
<i>Spongia oceania</i>	Sponge	Phosphate diester	anti-HIV <i>in vitro</i>
<i>Styracaster caroli</i>	Starfish	Sulfated sterols	anti-HIV <i>in vitro</i>
<i>Synechococcus eleongatus</i>	Micro alga	Extract	HIV-1 RT
<i>Tachypleus tridentatus</i>	Horse shoe crab	Peptides	anti-HIV <i>in vitro</i>
<i>Theonella mirabilis</i>	Sponge	Depsipeptide	cytopathic effect of HIV-1
<i>Toxiclona toxius</i>	Sponge	Prenylated Hydroquinones	HIV-1 RT
<i>Tremaster novaecaledonia</i>	Starfish	Sulfated sterols	cytopathic effect of HIV-2
<i>Trikentrion loeve</i>	Sponge	Pyrrrole derivative	cytopathic effect of HIV-3
Unidentified	Micro alga	Amides	anti-HIV <i>in vitro</i>
Unidentified	Marine bacterium	Macrolide	anti-HIV <i>in vitro</i>
Unidentified acidian	Tunicate	Pyrrrole alkaloids	HIV-1 INT
<i>Verongia</i> sp.	Sponge	Quinolones	HIV-1, HIV-2 RT
<i>Verongida</i> order	Sponge	Bromotyramine lipids	anti-HIV <i>in vitro</i>
<i>Xestospongia muta</i>	Sponge	Brominated polyacetylenic acids	HIV protease
<i>Xestospongia</i> sp.	Sponge	Steroid sulfates	HIV-1 INT
<i>Xestospongia</i> sp.	Sponge	Polyoxygenated sterols	HIV-1 INT
<i>Xylaria</i> sp.	Marine Fungus	Terpenoid	HIV-1 INT

2.4.2 Marine organisms as source of anti-cancer agents

The first anticancer product didemnin B, a cyclic depsipeptide isolated from the tunicate *Trididemnum solidum* from marine source enter in clinical trials. Preliminary results showed a partial activity against non-Hodgkin's lymphoma (Chun *et al.*, 1986). It can inhibit protein synthesis and arrest G1 phase of cell-cycle. Another depsipeptide Aplidine appear to be more active as comparison with didemnin B in preclinical trial and does not produce life-threatening neuromuscular toxicity. Preclinical data indicate that aplidine is active against several tumors through blockade of cell-cycle progression at G1 phase (Geldof *et al.*, 1999). There are number of ecteinascidins have been isolated from the marine source tunicate *Ecteinascidia turbinata*. One of these ecteinascidins (ET-743) was selected for clinical trials and antitumor effects have been observed in phase I studies (Demetri *et al.*, 2000). ET-743 is a tetrahydroisoquinilone alkaloid and they acts by selective alkylation of guanine residues in the DNA minor groove (Erba *et al.*, 2001) and also interacts with nuclear proteins (Damia *et al.*, 2001). In Europe and the United States ET-743 was in phase II clinical trials in 2000 (Demetri *et al.*, 2000). The dolastatins are a class of peptides obtained from the Indian Ocean, *Dolabella auricularia* which have cytotoxic activity. Dolastatin10 and dolastatin15 of this class have received the greatest clinical interest. Dolastatin10 has entered in Phase I and Phase II clinical trials, after showing significant antitumor activity in preclinical models (Poncet, 1999). Its mechanism of action involves inhibition of microtubule assembly ultimately result in cell-cycle arrest in metaphase (Bai *et al.*, 1990 and Pathak *et al.*, 1998). 20 macrocyclic lactones isolated from *Bugula neritina* and other marine bryozoan, these macrocyclic compounds have shown significant activity against lymphocytic leukemia cell line (Pettit, 1991). Bryostatin1 has recently entered phase II clinical trials for the treatment of melanoma, non-Hodgkin's lymphoma, renal cancer and colorectal cancer (Pagliaro *et al.*, 2000; Varterasian *et al.*, 2000 and Zonder *et al.*, 2000) and continues to be evaluated in phase I clinical trials. Bryostatin1 has been found to promote the normal growth of bone marrow progenitor cells, to provide *in vivo* protection against normally lethal doses of ionizing radiation and to serve as an immune stimulant, enhancing the normal production of interleukin2 and interferons (Ahmad *et al.*, 2000). The potential of bryostatin was investigated as an HIV inhibitor and latent activator. Bryostatin revealed antiviral activity against R5- and X4-tropic viruses in receptor independent and partly via transient

CHAPTER 2
LITRATURE REVIEW

decrease in CD4/CXCR4 expression. Further, bryostatin at low nanomolar concentrations robustly reactivated latent viral infection in monocytic and lymphocytic cells via activation of Protein Kinase C (PKC) - α and - δ , because PKC inhibitors rottlerin and GF109203X abrogated the bryostatin effect (Mehla *et al.*, 2010). A number of compounds have been isolated from marine organisms as potential anti-cancer agents and some of these have reached clinical and preclinical studies as shown Table 2.4 (Bhanot *et al.*, 2011) and Table 2.5 (Newman *et al.*, 2011). Most of the references used to determine clinical trial status of compounds are relatively old and in many cases no new information could be obtained, making it difficult to know how many of the compounds mentioned progressed to viable drugs.

Table 2. 4: Marine derived potential anticancer agent.
 (Table adapted from Bhanot *et al.*, 2011)

Organism	Group	Chemical class	Target
<i>Lyngbya majuscula</i>	Sponge	Alkaloid	Induction of p21 and G2/M cell cycle arrest
<i>Orticium simplex</i>	Sponge	Alkaloid	Selective inhibitor of angiogenesis
<i>Acadia mucilage</i>	Ascidian	Depsipeptide	Oxidation and inactivation of low molecular weight-protein tyrosine phosphatase activity
<i>Lanthella quadrangulata</i>	Sponge	Alkaloid	Inhibition of angiogenesis in vitro and <i>in vivo</i> involves apoptosis
Most of the members	Ascidian	Carotenoid	Induction of apoptosis
<i>Spisula polynyma</i>	Mollusk	Alkaloid	ErbB3 protein and PI3K- Akt pathway involved in necrosis induction
<i>Clavularia viridis</i>	Soft coral	Prostanoid	G1 cell cycle arrest and apoptosis
<i>Latrunculia magnifica</i>	Sponge	Peptide	Disorganization of actin filaments
<i>Amphimedon</i> sp.	Sponge	Sesterterpene	G1 phase inhibition and apoptosis induction

<i>Streptomyces</i> sp.	Bacterium	Cyclic peptides	Increased polyploidy by putative topoisomerase II alterations
<i>Leptoshaeria</i> sp.	Fungus	Alkaloid	DNA topoisomerase I and II inhibition and apoptosis induction
<i>Theonella swinhoei</i>	Sponge	Polyketide	Protein synthesis inhibition
Most of the members	Sea cucumber	Saponin	Inhibition of angiogenesis and receptor tyrosine kinases
<i>Stylissa massa</i>	Sponge	Alkaloid	Inhibition of cyclin-dependent kinases and apoptosis induction
<i>Didemnidae</i> species	Ascidian	Depsipeptide	Induction of apoptosis with concomitant G1 arrest and G2 blockage
<i>Cystodytes</i> sp.	Ascidian	Alkaloid	Direct iminoquinone reduction and reactive oxygen species generation
Most of the members	Sponge	Alkaloid	Induction of eythroid differentiation and cell cycle arrest
<i>Spongia officinalis</i>	Sponge	Fatty acid	Induction of apoptosis via mitochondrial signaling pathway
Most of the members	Mollusc	Peptide	Binds to amino-terminal peptide of β -tubulin containing cysteine
<i>Pseudaxinyssa cantharella</i>	Sponge	Alkaloid	Induction of G2/M cell cycle arrest and p53 proteasome recruitment
<i>Halichondria okadai</i>	Sponge	Macrolide derivative	Induction of mitotic blockage and apoptosis

<i>Lissoclinum patella</i>	Ascidian	Fatty acid	G2/M cell cycle arrest
<i>Xestospongia</i> sp.	Sponge	Alkaloid	Induction of topoisomerase II α -mediated catenation of DNA

Table 2. 5: Current status of marine natural products in anticancer preclinical or clinical trials.

(Table adapted from Newman *et al.*, 2011).

Organism	Group	Chemical class	Target	Current status
<i>Ecteinascidia turbinata</i>	(tunicate)	Tetrahydroisoquinolone alkaloid	Tubulin	Phase II
<i>Dolabella auricularia</i>	(sea hare)	Linear peptide	Tubulin	Phase II
<i>Bugula neritina</i>	(bryozoan)	Macrocyclic lactone	PKC	Phase II
<i>Elysia rufescens</i>	(sea slug)	Cyclic depsipeptide	Lysosomes/erbB pathway	Phase II
<i>Squalus acanthias</i>	(shark)	Aminosteroid	Phospholipid bilayer	Phase II
<i>Trididemnum solidum</i>	(tunicat)	Cyclic depsipeptide	Ornithine decarboxylase	Phase II
<i>Trididemnum solidum</i>	(tunicate)	Cyclic depsipeptide	FK-506 bp	Phase II
<i>Dolabella auricularia</i> <i>/Symploca</i> sp.	(synthetic analogue)	Linear peptide	Tubulin	Phase II
<i>Dolabella auricularia</i> <i>/Symploca</i> sp.	(synthetic analogue)	Linear peptide	Tubulin	Phase I
<i>Halichondria okadae</i>	(sponge, synthetic)	Macrocyclic polyether	Tubulin	Phase I

<i>Discodermia dissolute</i>	(sponge)	Lactone	Tubulin	Phase I
<i>Cymbastella</i> sp.	(sponge metabolite)	Linear peptide	Tubulin	Phase I
<i>Jaspis digonoxea</i>	(sponge, synthetic)	q-Lactam peptide derivative	Methionine aminopeptidase	Phase I
<i>Agelas mauritianus</i>	(sponge, synthetic)	α -Galacosylceramide	Va24 + NKT cell activation	Phase I
<i>Lyngbya majuscula</i>	(cyanobacterium)	Thiazole lipid	Tubulin	Preclinical
<i>Lyngbya majuscula</i>	(cyanobacterium)	Cyclic depsipeptide	Tubulin	Preclinical
<i>Salinospora</i> sp.	(bacterium)	Bicyclic g-lactam-h lactone	20S proteasome	Preclinical
<i>Cacospongia mycofijiensis</i>	(sponge)	Macrolide	Tubulin	Preclinical
<i>Didemnin cucliferum</i>	(tunicates)	Cyclic peptide	Tubulin	Preclinical
<i>Diazona angulata</i>	(tunicate)	Cyclic peptide	Tubulin	Preclinical
<i>Eleutherobia</i> sp.	(soft corals)	Diterpene glycoside	Tubulin	Preclinical
<i>Sarcodictyon roseum</i>	(sponge)	Diterpene	Tubulin	Preclinical
<i>Mycale hentscheli</i>	(sponge)	Macrocyclic lactone	Tubulin	Preclinical
<i>Haliclona</i> sp.	(sponge)	Polyketide	Vo-ATPase	Preclinical
<i>Micromonospora marina</i>	(bacterium)	Depsipeptide	DNA-polymerase	Preclinical
<i>Didemnum</i> sp.	(sponge)	Aromatic alkaloid	Caspase- 2/mitochondria	Preclinical

<i>Kirkpatrickia variolosa</i>	(sponge)	Heterocyclic alkaloid	Cdk	Preclinical
<i>Lamellaria</i> sp.	(mollusc and various soft corals)	Pyrrrole alkaloid	Topoisomerase I /mitochindria	Preclinical
<i>Dictyodendrilla verongiformis</i>	(sponge)	Pyrrrolocarbazole derivatives	Telomerase	Preclinical

2.5. Screening technique of marine organisms

The chemical and biological diversity of the different marine evolutionary group is endless and therefore, this is an amazing resource for the discovery of new anticancer drugs. Comprising 34 of the 36 Phyla of life, marine ecosystems are indeed our last genetic diversity and biotechnological boundary; terrestrial systems possess only 17 Phyla (Chakraborty *et al.*, 2009).

2.5.1 Sampling from different ecosystems

The Red sea represents one of the most promising areas as a source of medicinal and nutritional natural products. Many collections made on the Red Sea reefs led to numerous comprehensive studies on the taxonomy of Red Sea soft corals (Benayahu, 1990, Verseveldt *et al.*, 1983 and Benayahu *et al.*, 1987). In terms of biodiversity, marine environments are among the richest and most complex ecosystems. Harsh chemical and physical conditions in the environment have been important drivers for the production of a variety of molecules with unique structural features. These marine molecules or secondary metabolites exhibit various types of biological activities (Jain *et al.*, 2008).

Secondary metabolites are essential for basic cellular and physiological life processes. Ample evidence now shows that marine secondary metabolites can mediate ecological and behavioral interactions among organisms. Some compounds function in chemical defense against potential predators and competitors. This is especially important for soft bodied organisms that cannot move away from their predators such as marine algae, sponges, ascidians (tunicates, or sea squirts), and octocorals, which use secondary metabolites to deter predatory fishes, to compete for space, and to control settlement and growth of bacteria and other fouling organisms. Secondary metabolites are also important in reproduction as sex pheromones and sperm-attractant molecules (Williams, 2005).

Chemical ecology of marine organisms relates very closely to biotechnology by exploring these secondary metabolites which has high diversity of complex structures to develop drugs to treat various life threatening diseases (Ortlepp, 2008).

2.6. Red sea Ecosystem

Red Sea ecosystems are connected in varying degree to each others, such as mangrove forests, seagrass beds, and the open ocean. Mangroves and seagrass beds interrupt freshwater discharge, are sinks for organic and inorganic materials, and can generate an environment with clear, nutrient poor water that promotes the growth of coral reefs offshore (Ku"hlmann, 1988; Ogden, 1988), but Szmant (1997) hypothesizing that reefs may have the ability to utilize and benefit from higher nutrient fluxes than the present paradigms imply. Coral reefs in turn serve as physical buffers for oceanic currents and waves, creating, over geologic time, a suitable environment for seagrass beds and mangroves. In addition to these physical interactions there are several biological and biogeochemical interactions between these interconnected ecosystems. In our study samples were randomly collected from these different ecosystems.

2.6.1 Mangroves



Figure 2. 3: Mangrove swamps (Lieske and Myers, 2004).

The term “mangrove” (Figure 2.3) refers to an assemblage of tropical trees and shrubs that grows in the intertidal zone. Mangroves include approximately 16 families and 40 to 50 species (Madhukar, 2013). In the Red Sea coastal region of Egypt, established mangrove communities are uniquely different from mangrove forests in other parts of the world because of their low biodiversity and harsh habitat of arid and highly saline conditions. Therefore mangrove forests in this area appear in patchy and scattered patterns at mouths of the valley or in sheltered lagoons with rare and irregular flooding.




Most of them are pure forests of *Avicennia marina*, occasionally mixed with *Rhizophora mucronata* in the southern part of the Red Sea (Yoshikawa *et al.*, 2011).

Mangroves are tropical species their latitudinal limits worldwide vary depending on air and water temperatures (Tomlinson 1986; Waisel 1972; Sherrod *et al.*, 1986; Sherrod & McMillan 1985). The abundance of mangroves is also affected by aridity, and development is much greater along coasts that have high inputs of rainfall (Macnae 1968; Golley *et al.* 1975). Salt is generally not a requirement for growth, since most mangroves can grow in freshwater (Tomlinson 1986; Ball 1988). However, they do not develop in strictly freshwater habitats because of competition from freshwater species. Salinity is thus important in eliminating other vascular plant species that are not adapted for growth in a saline habitat. Mangroves must exchange gases, and thus have lenticels and spongy tissue in their roots and modified branches for this purpose.

Generally, mangroves grow best in depositional sediments where wave action is minimal. It represents a highly nourished habitat for different species. All local species grow on a variety of substrates including sand, mud, rock and peat. Fine-grained muds of silt or clay with lots of organic matter are optimal (Ball, 1980). Mangrove communities produce much of the essential nutrients to support the organisms comprising the low end of the food chain, and support both large and small food webs. They also stabilize and develop shoreline, and over time, produce land (John Booker *et al.*, 1998).

Submerged roots of mangroves serve as a substrate that is dominated by sponges. Mangrove bottom is rich in dissolved organic matter (DOM) (de Goeij *et al.* 2008) and it has been hypothesized that DOM leaching from mangrove roots plays an important role in structuring mangrove sponge community composition (Hunting *et al.*, 2010).

Table 2. 6: Samples collected from mangrove community*

Kingdom	Animalia		
Phylum:	Porifera		Cnidaria
Class:	Demospongiae		Scyphozoa
Order:	Haplosclerida	Dictyoceratida	Rhizostomeae
Suborder:	Haplosclerina		Kolpophorae
Family:	Chalinidae	Spongiidae	Cassiopidae
Genus:	Haliclona	Spongia	Cassiopea
	<i>Haliclona rubens</i> (sensu Duchassaing & Michelotti, 1864)	<i>Spongia officinalis</i> (Linnaeus, 1759)	<i>Cassiopea andromeda</i> (Forskål, 1775)
			

* Pictures were taken by M. Ellithey for marine natural products laboratory, NRC, Egypt

Two sponges *Haliclona rubens* (sensu Duchassaing & Michelotti, 1864) and *Spongia officinalis* (Linnaeus, 1759) and one scyphozoan *Cassiopea andromeda* (Forskål, 1775) were collected.

2.6.1.1. Biological activities of sponges

Sponges are simple, multicellular, sessile invertebrates with no true tissue layers or organ¹, and are the most primitive invertebrates that are frequently exposed to intense predation as well as tissue infection by microorganisms. Sponge secondary metabolites obviously present a defensive role against predation, infections and fouling (Darah *et al.*, 2011).

Marine sponges have been considered as a gold mine during the past 50 years, with respect to the diversity of their secondary metabolites. The biological effects of new metabolites from sponges have been reported in hundreds of scientific papers (Sipkema *et al.*, 2005).

Sponges have the potential to provide future drugs against important diseases, such as cancer, a range of viral diseases, malaria, and inflammations (Tan *et al.*, 1999). Although the molecular mode of action of most metabolites is still unclear, for a substantial number of compounds the mechanisms by which they interfere with the pathogenesis of a wide range of diseases have been reported. This knowledge is one of the key factors necessary to transform bioactive compounds into medicines. Sponges produce a plethora of chemical compounds with widely varying carbon skeletons, which have been found to interfere with pathogenesis at many different points. The fact that a particular disease can be fought at different points increases the chance of developing selective drugs for specific targets (Sipkema *et al.*, 2005).

Pharmaceutical interest in sponges was aroused in the early 1950s by the discovery of nucleosides spongothymidine and spongouridine in the marine sponge *Cryptotethia crypta* (Bergmann and Feeney, 1950, 1951). These nucleosides were the basis for the synthesis of Ara-C, the first marine-derived anticancer agent, and the antiviral drug Ara-A (Proksch *et al.*, 2002). Ara-C is currently used in the routine treatment of patients with leukemia and lymphoma. One of its fluorinated derivatives has also been approved for use in patients with pancreatic, breast, bladder, and lung cancer (Schwartzmann, 2000). More than 15,000 marine products have been described thus far (MarinLit, 1999; Faulkner, 2000, 2001, 2002). Sponges, in particular, are responsible for more than 5300 different products, and every year hundreds of new compounds are being discovered (Faulkner 2000, 2001, 2002).

Most bioactive compounds from sponges can be classified as anti-inflammatory (Tan *et al.*, 1999), antitumor (Bradshaw *et al.*, 1993; Yoshiji *et al.*, 1999), immunosuppressive or neurosuppressive (Griffith and Gross, 1996), antiviral (Ford *et al.*, 1999), antimalarial (Bwijo *et al.*, 2003), antibiotic (Burkholder and Ruetzler, 1969), or antifouling (Holmes, 1970; Houghton, 1978). The chemical diversity of sponge products is remarkable. In addition to the unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives (which are frequently halogenated) have been described from sponges (Sipkema *et al.*, 2005).

Sponges also seem to produce the largest number and diversity of secondary metabolites, even though the functions of these secondary metabolites are unknown. Sponge research is yielding more than 200 new pharmacologically active metabolites per

year at present, and several compounds that are derived from sponges or from sponge-associated bacteria are in different phases of clinical trials (Turk *et al.*, 2013).

Sponges of the genus *Haliclona*, which also include polar species, are known to contain antibacterial 3-alkylpyridinium alkaloids (Timm *et al.*, 2010 and Turk *et al.*, 2008). Triterpenoid hydroquinones from *Haliclona* species were found to be Kinesin motor protein inhibitors (Blackburn *et al.*, 1999). Another unknown compound found to be v-ATPase inhibitor (Erickson *et al.*, 1997). Manzamine A proved to be Antimalarial agent (Ang *et al.*, 2001). In the study by Hattori *et al.*, 1998 study, the C22 ceramide found to have antifouling properties was described.

Eight novel cyclic bis-1,3-dialkylpyridiniums, as well as two known compounds from the cyclostelletamine class, were isolated from the sponge *Haliclona* sp. from Korea and found to show moderate cytotoxic and antibacterial activities against A549 cell-line and Gram-positive strains, respectively (Lee *et al.*, 2012).

Haliclona rubens

Haliclona rubens produces toxins for chemical defense; one of these toxins has been shown to cause potent depolarization of the end-plate membrane of frog skeletal muscle (Wang, 1973).

Two new acetylenic brominated derivatives were isolated from a Red Sea *Haliclona rubens* and were evaluated for their cytotoxicity employing four cancer cell lines. The compounds showed potent selective antitumor activity towards MCF-7 cells with IC50 values of 32.5 and 50.8 microM, respectively (Alarif *et al.*, 2013).

Spongia officinalis

Species of order Dictyoceratida was noted for the presence of its unusual secondary metabolite chemistry (Sanders and Soest, 1996). *Spongia officinalis* at species level is not extensively studied except for one study that demonstrated that its extract and fractions were a source of significant numbers of compounds with antiproliferative and anti-inflammatory activities, which may be useful for developing potential chemo preventive substances (Dellai *et al.*, 2012).

2.6.1.2. Biological activities of Cnidarians

The phylum Cnidaria is a large, diverse and ecologically important group of marine invertebrates that includes over 11,000 extant species (Daly *et al.*, 2007). Over 3000 marine natural products have been described from this phylum alone, mostly in the last decade (Rocha *et al.*, 2011).

The production of natural toxins is an interesting aspect, which characterizes the physiology and the ecology of a number of marine species that use them for defense/offence purposes. Cnidarians are of particular importance from this point of view; venoms produced by these species are contained in specialized structures called the nematocysts, which after mechanical or chemical stimulation inject the venom in the prey or in the attacker. Nematocysts, consisting of a capsule of proteinaceous nature containing a tightly wrapped and spiralized thread, which after mechanical or chemical stimulation is quickly extruded injecting the venom in the prey or in the attacker (Fautin, 2009).

Some bioactive substances were discovered in Cnidarians, such as prostaglandins (15R)-PGA₂ in the gorgonian *Plaxaura homomalla* (Weinheimer *et al.*, 1969), the Palytoxin local anesthetic and vasoconstrictive agent discovered in the zoanthid *Palythoa toxica* (Moore *et al.*, 1971), Pseudopterosin (Kohl *et al.*, 2003), Sarcodictyins and Eleutherobin. Cytolytic and antitumoral substances have also been found: for example, prostanoid compounds from the Anthozoan *Clavularia viridis* were shown to inhibit the growth of HL-60 leukemic cells (Honda *et al.*, 1985), the incidence and growth of Sinonasal cancer (SNC) tumors induced by N-Ethyl-N-Nitrosourea were shown to be affected by the crude venom of the scyphozoan *Cassiopea xamachana* (Orduña-Novoa *et al.*, 2003), while the growth of Ehrlich ascites tumors grafted in mice was inhibited by crude extracts of tissues from jellyfish and soft corals (Tabrah *et al.*, 1972). A novel endogenous antibacterial peptide, aurelin, which exhibited activity against Gram-positive was isolated from the Jellyfish *Aurelia aurita* (Ovchinnikova *et al.*, 2006). Equinatoxin extracted from *Actinia equina* showed antitumoral activity on cultured cells (Giraldi *et al.*, 19756); the palytoxin was shown to induce ion currents (channels permeable to Na⁺ and K⁺ and slightly permeable to Ca²⁺, choline and tetramethylammonium) in mouse neuroblastoma cells (Rouzair-Dubois *et al.*, 1990).

Cassiopeia andromeda

Cassiopeia andromeda is distributed in the Red Sea and in the Indo-Pacific Ocean; in the Mediterranean Sea it is found along the Lebanon and Israeli coasts coming from the Suez Canal (Galil *et al.*, 1990). *Cassiopeia Andromeda* was the first known lessepsian and the first Erythrean scyphozoan jellyfish found in the Eastern Mediterranean after the opening of the Suez Canal (Çevik *et al.*, 2006 and Galil *et al.*, 1990). It was reported in the Suez Canal already in the late 19th century; the first record in the Mediterranean was from Cyprus. Subsequently, it was occasionally reported in some areas of the Eastern Mediterranean as far as the Aegean Sea (Maas, 1903).

C. andromeda is a venomous species and its nematocysts have been studied. The crude venom produced pain when applied to human lips, resulted in lethality to mice at a dose of 0.21 mg protein kg⁻¹ mouse and 50 µg proteins caused vasopermeability and dermonecrosis after injection into mouse skin; furthermore, crude venom was shown to have phospholipase A2 activity and to induce lysis of mouse lymphocytes. Dosages of 1 µg protein lysed 50% of treated human erythrocytes (Radwan *et al.*, 2001).

2.6.2. Seagrass beds




Seagrasses provide an important habitat to a number of organisms. Some use seagrass beds as nursery areas; others seek shelter there for the duration of their lives (Figure 2.4). Larger animals such as manatee and sea turtles feed on animals that live in the seagrass beds. It is a unique group of flowering plants that have adapted to exist fully submersed in the sea profoundly influence the physical, chemical, and biological environments in coastal waters, acting as ecological engineers (*sensu*Wright and Jones 2006) and providing numerous important ecological services to the marine environment (Costanza *et al.*, 1997).



Figure 2. 4 Sea grass bed, Red Sea (Lieske and Myers, 2004).

Seagrasses alter water flow, nutrient cycling, and food web structure (Hemminga and Duarte 2000). They are an important food source for mega herbivores such as green sea turtles, dugongs, and manatees, and provide critical habitat for many animals, including commercially and recreationally important fishery species (Beck *et al.*, 2001). It also stabilizes sediments and produce large quantities of organic carbon. However, seagrasses and these associated ecosystem services are under direct threat from a host of anthropogenic influences (Orth *et al.*, 2006).

Table 2. 7: Samples collected from seagrass bed*

Kingdom	Plantae	Plantae	Animalia
phylum	Tracheophyta	Chlorophyta	Mollusca
Infraphylum	Spermatophytae		
Superclass	Angiospermae		
Class:	Monocots	Ulvophyceae	Gastropoda
Subclass			
Order:		Bryopsidales	Cephalaspidea
Suborder:			
Family:	Cymodoceaceae	Caulerpaceae	Bullidae
Genus:	Cymodocea	Caulerpa	Bulla
	<i>Cymodocea rotundata</i> (Ehrenberg & Hemprich ex Ascherson 1870)	<i>Caulerpa prolifera</i> (Forsskål J.V. Lamouroux 1809)	<i>Bulla ampulla</i> (Linnaeus, 1758)
			

* Pictures were taken by M. Ellithy for marine natural products laboratory, NRC, Egypt.

The sea grass *Cymodocea rotundata* and a green algae *Caulerpa prolifera* and the gastropod *Bulla ampulla* were collected from the sea grass bed.

2.6.2.1. Biological activities of sea grasses

In folk medicine, seagrasses have been used for a variety of remedial purposes, e.g. for the treatment of fever and skin diseases, muscle pains, wounds, stomach problems, remedy against stings of different kinds of rays and tranquillizers for babies (de la Torre-Castro & Rönnbäck, 2004). In India, seagrasses are used as medicine (treatment of

heart conditions, seasickness), food (nutritious seeds), fertilizer (nutrient rich biomass). Seagrasses produce antimicrobial compounds that may act to reduce or control the microbial growth and there are many reports describing antibacterial (Kannan *et al.*, 2012), antiviral (Rowley *et al.*, 2002), anti-inflammatory (Hua *et al.*, 2006), antidiabetic (Gokce & Haznedaroglu, 2008) and antioxidant activities (Samy *et al.*, 2010).

Cymodocea rotundata

The phytochemical analysis of *C. rotundata* revealed the presence of tannins, saponins, proteins, resins, reducing sugar, acidic compounds, alkaloids, cardiac glycosides and Terpenoids (Ergene *et al.*, 2006). Phytoconstituents like phenolics (tannin and phenol) have been implicated as antioxidants in the scavenging of radicals like NO and H₂O in algae and terrestrial plants (Gulcin *et al.*, 2004 and Badami *et al.*, 2005). The phytochemical compounds viz., glycoside, saponins, tannins, flavonoids, terpenoids and alkaloids have antimicrobial activity. The antibacterial activity exhibited by the marine plant parts could be due to the presence of phytochemicals like alkaloids, tannins, flavonoids and sugars present in the plant extracts (Fennel *et al.*, 2004). *C. rotundata* proved to contain strong antibacterial and antioxidant activities. In Mani *et al.*, 2012 study, the ethanol extract of *Cymodocea rotundata* showed strong antibacterial activities against 10 human pathogens. Maximum activity was against *Shigella* (7mm) and *P. fluorescens* (7mm).

Antioxidant activity from marine resources is not yet fully explored (Adhikari, & Menon, 2006). (Kannan *et al.*, 2012) discussed reports that have revealed seagrasses as a rich source of antioxidant compounds. Seagrasses used as human food especially by the coastal populations (Hemminga & Duarte, 2000). 2,2-diphenyl-1-picrylhydrazyl (DPPH) strong reducing power was noticed in the extract of *C. rotundata* which exhibited significantly (P 0.05) higher DPPH radical scavenging activity of 70.30%, which was higher than those of the positive control ascorbic acid (57.28%) and gallic acid (55.75%); (Kannan *et al.*, 2012).

2.6.2.2. Biological activities of marine algae

Seaweeds offer a wide range of therapeutic possibilities both internally and externally. The term seaweed in this case refers only to macrophytic marine algae, both wild and cultivated and growing in saltwater. All essential minerals are provided by dietary

seaweeds. No land plant even remotely approaches seaweeds as sources of metabolically-required minerals (Bergner, 1997). Marine algae, also known as marine vegetables, are naturally rich in polysaccharides, minerals, polyunsaturated fatty acids, vitamins and bioactive molecules. Their nutritional value is markedly higher than that of terrestrial vegetables. The bioactive compounds in marine algae have been reported to possess strong antihypertensive (Bocanegra *et al.*, 2011; Blokhuis and Arts, 2011), antitumor (Ahmed *et al.*, 2011), anti-inflammatory (Yuvaraj *et al.*, 2012), antidiabetic (Xu *et al.*, 2012 and Lee *et al.*, 2004) and anticoagulant properties (Albuquerque *et al.*, 2004 and Silva *et al.*, 2005). In addition, the prebiotic health potential of polysaccharides from seaweeds has been increasingly studied in recent years. Seaweeds can provide minerals often absent from freshwater and food crops grown on mineral-depleted soils. The single most important element provided by seaweeds, iodine. Seaweeds have the unique ability to concentrate iodine from the ocean, with certain types of brown seaweed accumulating over 30,000 times the iodine concentration of seawater (Küpper *et al.*, 1998). Seaweeds also have significant amounts (1-3%) of Omega-3 fatty acids. Nori, in particular has 3% omega-3 fatty acids and large amounts of vitamins A and C (Ryan, 2008).

Caulerpa prolifera

The genus *Caulerpa* has been widely studied and the structures of many new compounds such as triterpenoids and squalene derivatives, the nitrogen-containing compounds caulerpin and caulerpicin and the diterpenoid alcohol caulerpol, have been described. As *Caulerpa* species are usually found in abundance and highly exposed in areas of significant herbivore populations, it seems likely that these uncalcified algae possess chemical deterrents to reduce predation (Paul and Fenical 1985). The major secondary metabolite of *Caulerpa* species, caulerpenyne, is a novel lipoxygenase inhibitor that can easily be obtained in high quantities from the abundant algae. Members of the genus *Caulerpa* might be considered a natural source of caulerpenyne with its inhibitory effect on xanthine oxidase. Caulerpenyne inhibits xanthine oxidase with an IC₅₀ value of 5 µM, this shed the light on the potential of *Caulerpa* species as a natural remedy for gout. (Cengiz *et al.*, 2011); this type of caulerpenyne inhibition on xanthine oxidase was observed as an irreversible inhibition.

2.6.2.3. Biological activities of Molluscs

Molluscs are widely distributed throughout the world and have many representatives such as slugs, whelks, clams, mussels, oysters, scallops, squids and octopods in the marine and estuarine ecosystem. Many classes of bioactive compounds exhibiting anti-tumor, anti-leukemic, antibacterial and antiviral activities have been extracted from molluscs and reported worldwide (Kamiya *et al.*, 1984; Anand *et al.*, 1997 and Rajaganapathy *et al.*, 2000). Among the molluscs extracts some have pronounced pharmacological activities or other properties which are useful in the biomedical arena. It is surprising to find that some of the pharmacological activities are attributed to the presence of polysaccharides particularly sulphated muco polysaccharide. Antimicrobial peptides are important in the first line of the host defense system of many animal species (Boman *et al.*, 1995). The value of these molecules in innate immunity lies in their ability to function without either high specificity or memory. Moreover, these molecules are synthesized without dedicated cells or tissues and they can rapidly diffuse to the point of infection (Periyasamy *et al.*, 2012). Nothing about the biological activities of the genus *Bulla ampulla* were reported before.

2.6.3. Coral reef

Coral reefs are among the most productive and biologically diverse ecosystems on Earth (Odum and Odum, 1955).

The coral reef ecosystem is a diverse collection of species that interact with each other and the physical environment. The sun is the initial source of energy for this ecosystem (Figure 2.5). Through photosynthesis, phytoplankton, algae, and other plants convert light energy into chemical energy (Kadow, 2001).

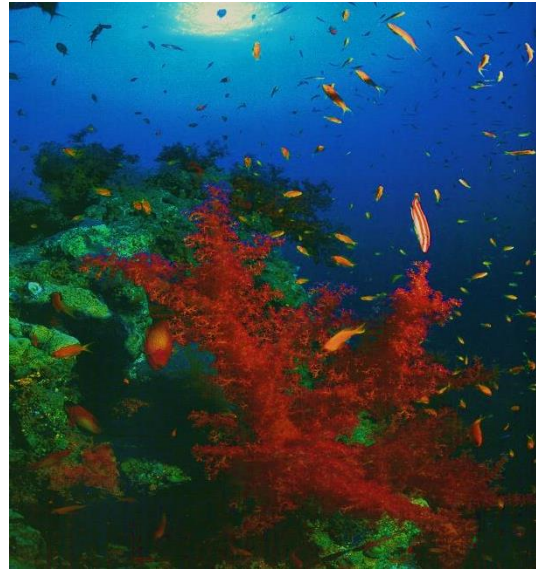


Figure 2. 6: Coral reef, Red Sea (Ileske and Myers, 2004).








A coral polyp is a tubular sac-like animal with a central mouth surrounded by one or more rings of gelatinous tentacles. Depending on the species, coral polyps measure anywhere from a few millimeters to several centimeters in diameter. Those small animals are extremely simple, composed of just two layers of cells. Corals often live in colonies, which also vary in size. Small colonies of only 25 cm exist alongside larger coral colonies reaching a height of 3-4 meters. The end opposite the tentacles, called the base, is attached to a substrate. The tentacles contain microscopic stinging capsules called nematocysts. A nematocyst is a globular structure containing a venom-filled thread with a minute barb at its tip. A tiny sensor projects outside the nematocyst. When the sensor is stimulated physically or chemically, the capsule explodes and ejects the thread with considerable force and speed. The barb penetrates the victim's skin and injects the venom. The external stimulus causes the mouth to open and the food particles are swept into the stomach cavity by the nematocyst filament, tentacles, or cilia (Jones *et al.*, 1973).

Most reef-building corals have a mutually beneficial relationship with microscopic unicellular algae called zooxanthellae that live within the cells of the coral's gastrodermis. As much as 90% of the organic material the algae manufacture photo synthetically is transferred to the host coral tissue. The mutualistic relationship between corals and their

algal endosymbionts is a key factor in the evolutionary success of hermatypic (reef building) corals (Muller-Parker and D'Elia, 1997).

Reefs are considered "medicine cabinets" of the future. it Considered "medicine cabinets" of the future, coral reef organisms hold great promise for pharmaceuticals including anti-cancer and anti-inflammatory drugs. The pharmaceutical industry has discovered potentially useful substances with anticancer, AIDS inhibiting, antimicrobial, anti-inflammatory and anticoagulating properties among the seaweeds, sponges, molluscs, corals soft-corals (order Alcyonacea) and gorgonians (order Gorgonacea) and sea anemones of the reefs (Sorokin, 1993; Carte', 1996; Birkeland, 1997a). It has been claimed that the discovery of prostaglandins in many of the gorgonians in the early 1970s was responsible for the expansion of marine natural products (Carte', 1996). Many species of seaweed are collected from reefs to be used in the production of agar and carrageenan (Birkeland, 1997a) and as manure (Craig *et al.*, 1990), and coral skeletons have proven to be promising in bone graft operations (Spurgeon, 1992).

Table 2. 8: Samples collected from the coral reef*

Kingdom	Plantae		Animalia	
Phylum:	Rhodophyta	<u>Heterokontophyta</u>	Cnidaria	
Class:	Florideophyceae	Phaeophyceae	Anthozoa	
Order:	Nemaliales	Fucales	Alcyonacea	
Family:	Galaxauraceae	Sargassaceae	Alcyoniidae	Nephtheidae
Genus:	<i>Galaxaura filamentosa</i> (W.R. Taylor 1945)	<i>Turbinaria turbinata</i> (Linnaeus Kuntze 1898)	<i>Sarcophyton trocheliophorum</i> (Von Marenzeller, 1886)	<i>Litophyton arboreum</i> (Forskål, 1775),
				
Kingdom	Animalia			
Phylum:	Cnidaria			
Class:	Anthozoa			
Order:	Alcyonacea			
Family:	Alcyoniidae			
Genus:	<i>Sinularia heterospiculata</i> (Verseveldt, J. 1976)	<i>Sinularia maxima</i> (Verseveldt, J. 1976)	<i>Sinularia polydactyla</i> (Verseveldt, J. 1976)	
				

* Pictures were taken by M. Ellithey for marine natural products laboratory, NRC, Egypt.

Two marine algae were collected from the reef edge the red algae *Galaxaura filamentosa* and the brown algae *Turbinaria turbinata*. Five soft corals were also

collected, *Litophyton arboreum* from family Nephtheidae and four from family Alcyoniidae, *Sarcophyton trocheliophorm* with three different sinularia species *Sinularia heterospiculata*, *Sinularia maxima* and *Sinularia polydactyla*.

2.6.3.1. Biological activities of the red algae

Some red algae species exhibit both ant nociceptive and anti-inflammatory effects. For example, a methanol extract of *Bryothamnion triquetrum* (Cavalcante-Silva *et al.*, 1992) had both antinociceptive and anti-inflammatory properties in experiments that used Swiss mice. Antinociceptive activity was examined using an acetic acid-induced writhing test, a hot-plate test, and glutamate-/formalin-induced nociception. Anti-inflammatory effects were assessed by zymosan A-induced peritonitis analysis. Antinociceptive and anti-inflammatory activities have also been reported for a sulfated polysaccharide fraction from *Gracilaria caudate* (Chaves *et al.*, 2013), a galactan from *Gelidium crinale* (de Sousa *et al.*, 2011), a mucin-binding agglutinin from *Hypnea cervicornis* (Bitencourt *et al.*, 2008), and a lectin from *Pterocladia capillacea* (Silva *et al.*, 2010) .

More recent research on extracts of red marine algae suggest that specific carbohydrates (sulfated polysaccharides) may inhibit both the DNA and RNA of viral infections and may operate both outside and within our infected cells (Baba *et al.*, 1988, Mitsuya *et al.*, 1988, Ueno and Kuno, 1987.) Work done in this area has shown that sulfated polysaccharide compounds suppressed retroviral replication and inhibited viral reverse transcriptases (Solomon *et al.*, 1966, Schaffrath *et al.*, 1976). A study done by Neushul (1990) showed that nearly all 39 species of marine red algae, including the family Halymeniaceae, also contained and exhibited an inhibitory substance that suppressed retroviral replication and inhibited viral reverse transcriptases. Studies by Nakashima *et al.*, (1987, 1988) support the hypothesis that a common immunomodulatory cell wall carbohydrate, like carrageenan, is a type of heparin receptor molecule, binding to a cell and triggering a specific cellular response sequence. Carrageenan may also be internalized into infected cells, thus inhibiting the virus. It also may inhibit fusion between infected cells Neushul (1990), Gonzales *et al.*, (1987) suggesting that sulfated polysaccharides inhibit a step in viral replication subsequent to viral internalization but prior to the onset of late viral protein synthesis.

Galaxaura filamentosa

Galaxaura filamentosa on the genus level was subjected to a study for the isolation and identification of its antineoplastic constituents. A new isolated compounds named Galaxamide was remarkably active against the human renal cell carcinoma GRC-1 and human hepatocellular carcinoma HepG2 cell lines with corresponding IC₅₀ values of 4.26 µg/mL and 4.63 µg/mL. (Liang *et al.*, 2010).

2.6.3.2. Biological activities of brown algae

In a study investigated the protective effect of fucoidan extracted from *Turbinaria conoides* against isoproterenol induced myocardial injury in rats. Biochemical assessment of myocardial injury was done by measuring the activities of creatine kinase, lactate dehydrogenase, membrane bound triphosphatases and minerals, which were significantly altered in isoproterenol administered rats. In addition, the enzyme mapping assay on the size of the myocardial infarct also correlated with these biochemical parameters. Thus, the observed protective effects of sulfated polysaccharides of *T. conoides* against ISO induced myocardial injury are due to membrane stabilizing properties which may be due to decreased lipid peroxidation. These results should trigger a renewed interest in the use of *T. conoides* fucoidan for myocardial injury (Krishnamurthy *et al.*, 2012).

Turbinaria conoides was also recognized as having mild-to-moderate cytotoxicity in HeLa cell. Oxygenated steroids exhibited cytotoxicity against HeLa cells with CC₅₀ values ranging from 60.9 µg/mL to >100 µg/mL (Kumar, 2012). A new steroid 14,15,18,20-diepoxyturbinarin, isolated from the cyclohexane extract of *Turbinaria conoides* could be developed as a new lead antifungal agent (Kumar, 2012).

Another *Turbinaria* species, *Turbinaria ornata* was found to contain the most active natural antioxidants in a study where the total antioxidant activity of organic extracts of 37 algal samples was determined. This algae exhibited significant antioxidant activity, a property that could lead to its application in one of many useful healthcare or related products as well as in chemoprevention of a variety of diseases including cancer (Kelman, 2012). Another study on the aqueous extract of *Turbinaria ornata* revealed anti-inflammatory activities that could be due to its potential antioxidant and free radical scavenging properties (Ananthi *et al.*, 2011).

Turbinaria turbinata

Turbinaria turbinata on the species level was found to possess promising *in vitro* activity against *L. mexicana promastigotes* (LC₅₀ values ranging from 10.9 to 49.9 µg/ml). No toxicity of algal extracts against *Artemia salina* was observed with LC₅₀ ranging from 119 to ≥ 1000 µg/ml (Freile-Pelegrin *et al.*, 2008).

The aqueous and organic extracts of 29 species of marine algae (14 species of Rhodophyta, 7 species of Phaeophyta, and 8 species of Chlorophyta) collected from the Gulf of Mexico and Caribbean coast of the Yucatan Peninsula (Mexico) were evaluated for their antiprotozoal activity *in vitro* against *Trypanosoma cruzi* trypomastigotes as one of the most important parasitic diseases worldwide. The toxicity of these extracts was evaluated using brine shrimp (*Artemia salina*). The cytotoxicity on mammalian cells was also assessed by the MTT viability assay. The organic extracts from *Dictyota caribea* (Horning & Schnetter), *Lobophora variegata* (J.V. Lamouroux) Womersley, *Turbinaria turbinata* (Linnaeus), and *Laurencia microcladia* (Ktzing) possess promising *in vitro* activity against *T. cruzi* trypomastigotes. The highest cytotoxicity was exhibited by *T. turbinata* (León-Deniz *et al.*, 2009).

2.6.3.3. Biological activities of soft corals

Soft corals are an essential component of the coral reef ecosystem, and are an important source of secondary metabolites exhibiting diverse biological properties (Faulkner, 1999; Bowden *et al.*, 1980; Simmons *et al.*, 2005; Blunt *et al.*, 2003; Blunt *et al.*, 2009; Chanmethakul *et al.*, 2010). Many soft corals (Coelenterata: Alcyonacea) possess an extensive range of unique organic molecules, the majority of which fall into the terpene class of compounds (Tursch *et al.*, 1978; Coll *et al.*, 1980; Coll, 1981). A number of these marine terpenoids have been shown to be distasteful (Lucas *et al.*, 1979) or toxic (Ne'eman *et al.*, 1974; Weinheimer *et al.*, 1977). It has been suggested that these secondary metabolites may function as chemical defenses against predation, fouling, and parasitism, conferring a selective advantage on some soft corals (Tursch *et al.*, 1978).

Order Alcyoniicae are among the major benthic components occupying space in the tropical Indo-Pacific reefs (DINESEN, 1983; HUSTON, 1985), as well as in the coral reefs of the Northern Red Sea (BENAYAHU and LOYA, 1977). Their evolutionary

success in areas of high levels of predation has been attributed to their production of significant amounts of secondary metabolites (SAMMARCO and COLL, 1988; 1992). It has been proven to be rich sources of natural terpenoid derivatives (Fraga, 2011; Blunt *et al.*, 2011) and terpenoids analogues are often found in large amounts in marine invertebrates, and represent the largest percentage of natural products isolated from marine organisms (Harper *et al.*, 2001).

The soft coral genus *Sinularia* is one of the most widely distributed soft corals. It constitutes a dominant portion of the biomass in the tropical reef environment. *Sinularia* elaborates a rich harvest of secondary metabolites including sesquiterpenes, diterpenes, polyhydroxylated steroids, and polyamine compounds. These metabolites were recently shown to possess a range of biological activities such as antimicrobial, anti-inflammatory, and cytotoxic activities. During the past decade, *Sinularia* has yielded many new structures with novel skeletons. Several of the previously published secondary metabolites have been reexamined for their pharmacological properties, and the results strongly support further investigations (Kamel and Slattery, 2005).

The new cembranoid diterpene danielid (1) along with 3 α -ethoxyfuranocembranoid 2, pukalide (3), 13 α -acetoxypukalide (4), furanocembranoid 5, and furanosesquiterpene 6 have been isolated from the soft coral *Sinularia asterolobata*. Compounds 2, 4, and 5 showed good antiproliferative activities against the cell lines L-929 and K-562, and weak cytotoxic effects on HeLa cells (Grote *et al.*, 2008).

Sinularia heterospiculata

Extracts of *Sinularia heterospiculata* and *Sinularia variabilis* showed the highest and potent widest spectrum of antifouling activity in a study screening the anti-fouling activity of 5 common Red Sea soft corals (Ali and Soliman, 2009). No data about any other biological activities of this coral was reported.

Sinularia maxima

Chemical investigation of the hybrid soft coral *Sinularia maxima* × *Sinularia polydactyla* yielded four new cembranolide diterpenes. One of it showed strong cytotoxicity on the

breast cancer SK-BR3 cell line and cervix cancer cell lines HeLa and HeLa-Apl cell lines with GI_{50} values of 0.039, 0.48, and 0.56 μM , respectively (Kamel and Slattery, 2005).

In a screening study of the crude aqueous methanol extracts of 31 species of various marine organisms (flora and fauna), for their antifungal activity against food poisoning strains of *Aspergillus*; *Sinularia maxima*, *Subergorgia suberosa*, *Echinogorgia pseudorassopo* and *Sabellaria cementifera* were mild (inhibition zone of 1–2 mm) to moderate (inhibition zone of 2–3 mm) active against the respective strains (Bhosale *et al.*, 1999).

Sinularia polydactyla

A specimen of *S. polydactyla* (Ehreberg) from Hainan contained a novel sesqui terpenoid with a guaiene structure; polydactin A, which was reported to possess moderate cytotoxic activities against human oral epidermoid carcinoma cell lines (KB) and human breast carcinoma (MCF) cell lines *in vitro*, with IC_{50} values of 13.0 and 14.0 mg/mL (Zhang *et al.*, 2008).

In another study, the defensive compounds pukalide and 11 β -acetoxypukalide were found in *S. polydactyla* eggs and larvae at adult level and three-fold lower than adult-level concentrations, respectively. These compounds provided some predator deterrent and antimicrobial protection against an ecologically relevant omnivorous fish *Canthigaster solandri* and a sympatric microbe *Vibrio* sp. (Starmer, 1999).

Sarcophyton trocheliophorum

A new compound, 7 β ,8 α -dihydroxydeepoxysarcophine (5), and for sarcophine (6), and 16-deoxysarcophine (7), isolated from the soft coral *Sarcophyton trocheliophorum* showed selective cytotoxicity (Duh and Hou, 1979). Novel fatty acid derivatives containing one γ -lactone ring and unusual unsaturated chains were isolated from *Sarcophyton trocheliophorum* collected in the Red Sea, gave positive results in a brine shrimp toxicity assay (Řezanka and Dembitsky, 2002).

The methylene chloride extract of *Sarcophyton trocheliophorum*, produced a known diterpenoid, (+)-isosarcophine. This compound exhibited cytotoxicity against the P388 cancer cell line (Jung, Sheng-Ge, 2000).

A detailed investigation of the South China Sea soft coral *Sarcophyton trocheliophorum* (Marenzeller) yielded six known terpenes. Some of the isolated compounds showed significant inhibitory activity against human protein tyrosine phosphatase 1B enzyme, a key target in the treatment of type-II diabetes and obesity, and some preliminary structure–activity relationships have been drawn (Liang, 2013).

The methanolic extract of *S. trocheliophorum* was found to show significant cytotoxicity *in vitro* in tissue culture cells in human KB, A549 lung carcinoma and HT-29 colon tumor, as well as in murine P-388 lymphocytic leukemia. (chang, 1992).

Litophyton arboreum

There are not enough studies on the biological activities of this coral. Fatty acid derivatives of *Lithophyton arboreum*, collected in the Red Sea, gave positive results in a brine shrimp toxicity assay (Řezanka and Dembitsky, 2002). A new cembrane diterpene (3E,11E)-cembra-3,8(19),11,15-tetraene-7 α -ol (1), nephthenol (2), and all-trans-peridinol (3) have been isolated from this organism. Some of the terpenes were tested for antiproliferative activity against the cell lines HUVEC and K-562 and for cytotoxicity against the cell line HeLa, and showed moderate activities (Shaker, 2010).

The furanocembranoid diterpene 11 β ,12 β -epoxypukalide and the sesquiterpene (-)-bicyclogermacrene have been obtained from this soft coral. The compounds showed weak antiproliferative activities against the cell lines L-929 and K-562, and weak cytotoxic effects on HeLa cells (Grote *et al.*, 2008).

Chapter 2 of this study evaluates the secondary metabolites isolated from *L. arboreum* which showed the best biological activities during the screening study. The isolation study focused on the organism's lipophilic secondary metabolites and the anti HIV and anti-cancer activities of the isolated metabolites.

2.7. Strategies for the discovery of marine drugs

According to Yasuhara-Bell, 2010, there are two main strategies for the discovery of marine drugs:

a) Targeting the marine organism

A characteristic example giving is the case of the marine snails of the genus *Conus*. The plethora of compounds within a single species and the presence of over 300 species of Conidae have led to the discovery of peptidic venoms that exhibit actions specific for the skeletal muscle Na⁺ channel and the N-methyl-D-aspartate (NMDA) receptors. *Conus* species (approximately 500 in number) will each use a distinctive assortment of peptides and that the pharmacological diversity in *Conus* venoms may be ultimately comparable to that of plant alkaloids or secondary metabolites of microorganisms (Olivera *et al.*, 1990).

b) Targeting the disease

This approach receives more attention (Yasuhara-Bell, 2010). It takes advantage of mechanistic, *in vitro* assays to broaden the search for therapeutic compounds from marine organisms through screening of a large number of different species (Grabley and Thiericke, 1999). The probability of finding useful active metabolites is obviously dependent on the number of samples screened, so that the selection of the lead molecules will be based on both the level of activity and the availability factors. Another advantage of the *in vitro* assays is that they are able to be adapted to allow high-throughput screening (HTPS). HTPS facilities offer the potential to readily screen hundreds of thousands of extracts per annum in parallel against numerous therapeutic targets. Frequently the active constituents are present in the extracts only in minute quantities and significant amounts of the organisms are needed to afford the necessary quantities for even the first stages of the pharmaceutical evaluation. To optimize the yield of the bioactive compounds selective extraction and purification protocols have been designed and developed (Smith, 2010). The recently developed spectroscopic techniques and experiments such as HPLC-MS / H-NMR analytics, C13-NMR and HPLC-ELSD allow the structural elucidation of complex structures isolated even in milligram quantities from obscure organisms (Donot *et al.*, 2013).

During the screening of natural products derived from numerous species, significant antiviral activity against HIV was observed (Rahman, 2010). Systematic screening started in late 1987 when the National Cancer Institute began an extensive evaluation of natural product extracts derived from microorganisms, plants, marine invertebrates and

algae for HIV inhibitory activity. In the following years, nearly 40,000 crude aqueous and organic solvent extracts were tested in a primary anti-HIV screen. A surprisingly large number (15%) of the aqueous extracts of terrestrial and marine organisms exhibited some activity in the screen (Laport, 2009).

2.8. The role of lipophilicity in drug development

The role of lipophilicity in drug discovery and design is a critical one. Lipophilicity is a key physicochemical property that plays a crucial role in determining ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties and the overall suitability of drug candidates. There is increasing evidence to suggest that control of physicochemical properties such as lipophilicity, within a defined optimal range, can improve compound quality and the likelihood of therapeutic success (Arnott and Planey, 2012).

Lipophilicity is a physicochemical property of crucial importance in medicinal chemistry. On the molecular level it encodes information on the network of inter- and intramolecular forces affecting drug transport through lipid structures as well as drug's interactions with the target protein. In result, on the organism level, lipophilicity is an important factor defining pharmacokinetics and pharmacodynamics of a drug substance. The ability of a drug to penetrate various biological membranes, tissues and barriers is a primary factor in controlling the interaction of drugs with biological systems (Rutkowska *et al.*, 2013).

The third part of this study (Chapter 5) evaluates the seasonal changes of the active metabolites of the active extract. This evaluation is to help understand the importance of these metabolites in the organism's life and also helps in identifying the best season for collection for further studies.

Several marine organisms are sessile and soft bodied; these delicate looking simple sea creatures protect themselves from predators and pathogens by chemical weapons (secondary metabolites). Intensive evolutionary pressure from competitors, that threaten by overgrowth, poisoning, infection or predation have armed these organisms with an arsenal of potent chemical defense agents. They have evolved the ability to synthesize these chemical weapons or to obtain them from marine microorganisms. These

compounds help them to deter predators, keep competitors at bay or paralyze their prey. Investigations in their chemical ecology have revealed that the secondary metabolites not only play various roles in the metabolism of the producer but also in their strategies in the given environment. The diversity of secondary metabolites produced by marine organisms has been highlighted in several reviews (Munro *et al*, 1999; Faulkner, 2002; Proksch *et al*, 2002; Haefner, 2003; Jimeno *et al*, 2004; Jha & Zi-rong, 2004). They range from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids to aliphatic cyclic peroxides and sterols. There is ample evidence documenting the role of these metabolites in chemical defense against predators (Schupp *et al*, 1999; Pisut & Pawlik, 2002) and epibionts (Wahl *et al*, 1994; Thakur & Anil, 2000; Thakur, 2001; Thakur *et al*, 2003).

2.9. Common drug discovery techniques

Drug discovery usually takes place in academic institutions and pharmaceutical companies. Figure 2.6 illustrates the processes involved in the discovery and development of drugs before they reach the market. The entire process usually takes about 10-15 years to just develop one new medicine. The next subsections will look into techniques and bioassays commonly used in drug discovery from natural products, some of which were incorporated in the present study.

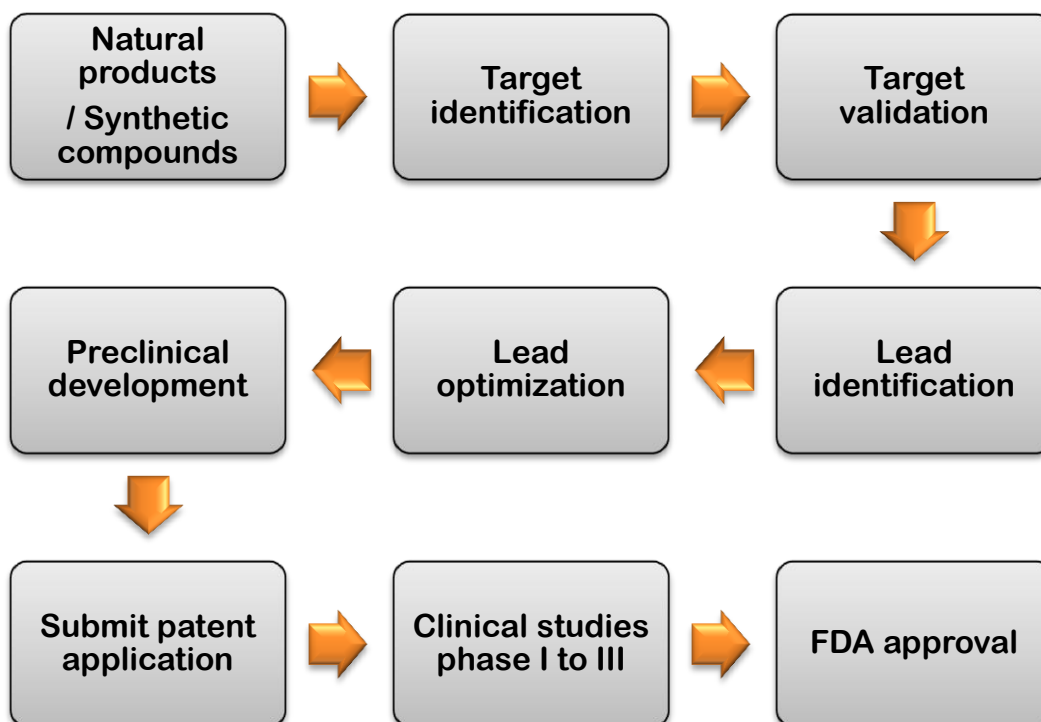


Figure 2. 7: A typical drug discovery and development paradigm
(Adapted from Choudhary and Khan, 2011; Erhardt, 2002).

In the first chapter of this study, 13 marine organisms from different ecosystems were screened for their anti-cancer and anti HIV-1 activities. Chapter 2 discusses the isolation and purification of active compounds from the most active species. The third chapter explains the seasonal changes experienced by *L. arboreum* and these effects on the organism seasonal distribution and the biological activities of seasonal extracts.

2.10. Analytical techniques

2.10.1. Extraction of crude extracts as well as the isolation and identification of a bioactive compound

The isolation and identification of a bioactive compound from a natural product requires a series of analytical techniques. Sample collection is the first step in organism analyses, followed by sample preparation. One of the popular ways of preparing crude extracts from natural material is by solvent extraction of macerated marine organism materials (t'Kindt *et al.*, 2009). Different solvents, depending on their polarity, yield different compounds from marine organisms' material. One of the preliminary basic techniques

used to screen crude natural compounds is thin layer chromatography (TLC). It is an official method prescribed in some herbal monographs of many pharmacopoeias and it is mostly used to characterize the first fingerprints of a crude extract which is usually made up of many different components (Tomczyk, Bazylo, & Staszewska, 2010). The solvent system used to separate crude extracts on a TLC plate depends on the polarity of the solvent that was initially used to extract the organisms material (Poole & Dias, 2000). TLC is fast, easy and it does not require pretreatment of samples. Large amounts of samples can be analyzed at the same time using TLC (Martelanc, Vovk, & Simonovska, 2009).

The detection and confirmation of separated components on a TLC plate can be observed by visualizing in daylight for colored substances, inspection under an ultraviolet (UV) light source for fluorescent components or by using selective detection (spray) reagents. UV visualization is normally done at 254 or 366nm (Kamatou *et al.*, 2008). TLC data provides an idea of the compounds/substances present in a crude extract, and can be done qualitatively or quantitatively (Martelanc *et al.*, 2009).

For complete separation and structural identification of a bioactive component from an organism's material, TLC will need to be combined with other analytical techniques. These other techniques are column chromatography (CC), high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) (Martelanc *et al.*, 2009; Tringali, 2001). HPLC analysis is used in separation and identification of non-volatile constituents of organisms extracts by comparing their retention times to standard substances/compounds (Bienvenu, Amabeoku, Eagles, Scott, & Springfield, 2002; Kamatou, Viljoen, van Vuuren, & van Zyl, 2006; Manojlović, Vasiljević, Jusković, Slobodan, & Milenković-andjelković, 2010). HPLC is further used for bioassay-guided fractionation procedures of crude marine extracts which helps in targeting and isolation of active compounds (Su *et al.*, 2005; Tringali, 2001).

In CC, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure) (Bhalla, Nayak, & Dev, 1967). CC is generally used as a purification technique to isolate desired organic compounds from a

mixture (Hussein, Meyer, & Jimeno, 2007; Macías, Lacret, Varela, Nogueiras, & Molinillo, 2008; Wang et al., 2009).

NMR is an excellent tool that gives detailed structural information of organic molecules. It is able to provide a structural difference between compounds with the same molecular mass or formula (Wu, Wang, & Simon, 2004). Proton (^1H) NMR and carbon-13 (^{13}C) NMR are the most commonly used NMR techniques. ^1H NMR identifies hydrogen atoms/protons in an organic molecule while ^{13}C NMR allows the identification of carbon atoms in organic molecules. There is a universal occurrence of protons in organisms metabolites, this makes ^1H NMR a great starting tool in structural elucidation of unknown constituents of bioactive crude extracts (Banci, Bertini, Luchinat, & Mori, 2010; Fan & Lane, 2008).

2.10.2. Bioassays and viability/cytotoxicity profiling

Bioassays are used in the investigation of natural products as part of the screening methods in drug development. The main objective of bioassays is to determine whether samples under study have any desired (or detrimental) biological activity. Screening with bioassays helps in the selection of marine organism materials for secondary testing and are also incorporated in guided fractionation of crude extracts in order to isolate the bioactive component (Tringali, 2001). In order to determine the number of viable cells Cell Proliferation Kit XTT employs 2, 3-Bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT). Only in living cells mitochondria are capable to reduce XTT to form an orange colored water soluble dye. Therefore, the concentration of the dye is proportional to the number of metabolically active cells the principle of the assay described in Figure 2.8.

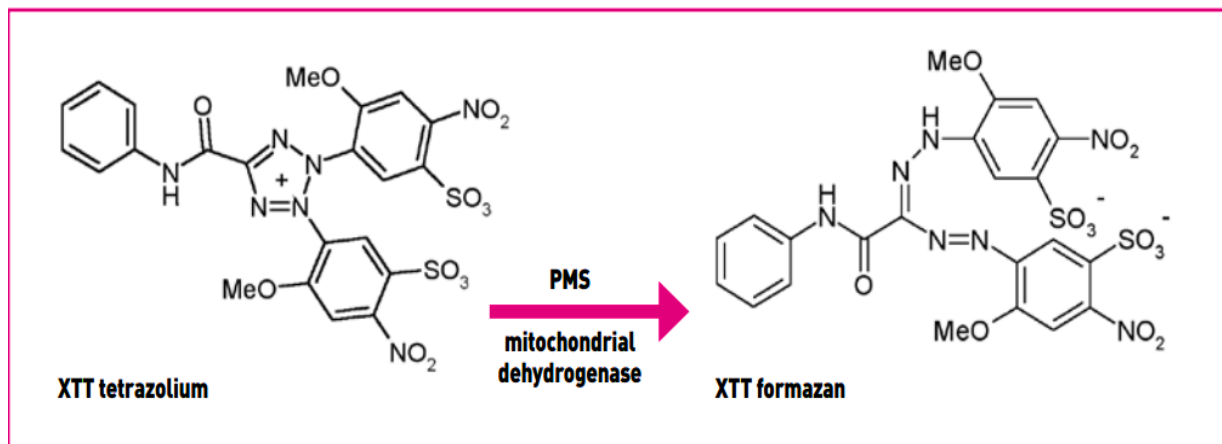


Figure 2. 8: The principle of XTT assay (AppliChem GmbH, 2010)

The assay is based on the cleavage of the tetrazolium salt XTT in the presence of an electron-coupling reagent, producing a soluble formazan salt. This conversion only occurs in viable cells as shown in (Figure 2.7). Cells grown in a 96-well tissue culture plate are incubated with the XTT labeling mixture for 2 - 20 hours. After this incubation period, the formazan dye formed is quantitated using a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells. More information about this assay is provided in the appendix.

In normal cell viability assays and techniques such as XTT, the effect of a specific compound or extract is assessed at a single time point. This however does not explain the mechanism of action that takes place from the time point of sample addition (treatment) to the end-point for viability/toxicity analysis. These techniques overlook the possible multiple and kinetic effects that a certain extract/compound might possess (Abassi *et al.*, 2009).

To overcome this limitation, a live cell morphological profiling process has been devised which is label/dye-free. It monitors the effects of substance addition to cells in real time. Measurement is done continuously, evaluating and displaying the data during the whole procedure (Abassi *et al.*, 2009). The instrument is called real time cell analyzer (RTCA) or real time cell electronic sensor (RT-CES) and it has many cell based applications including cell proliferation, measuring cytotoxicity/apoptosis, cell adhesion and spreading, and cell migration and invasion (Figure 2.8). The major limitation of RTCA is that it only works with adherent cell lines, cells that can attach to the surface of a special tissue culture plate (Atienza *et al.*, 2006).

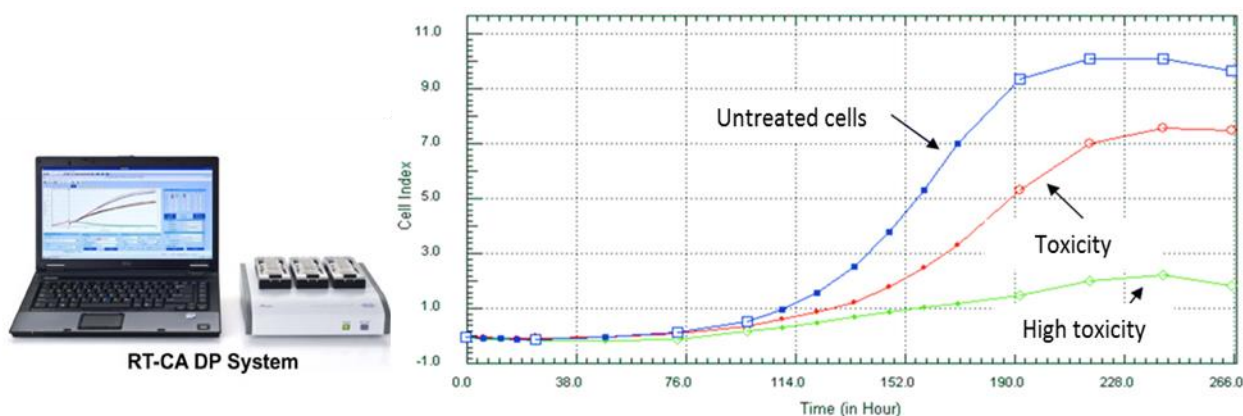


Figure 2.9: Real time cell electronic sensing device and representation of data.

BioTechniques, 2010. www.xcelligence.roche.com

RTCA uses microelectronic plates (E-plates) integrated with gold micro-electrode arrays on glass substrate in the bottom of the wells. Hence, the monitoring of the cells is microelectronic. The electrodes cover about 80% of the well bottom and a small electric field between electrodes is produced in the presence of media and a very low alternating current (AC) voltage of 10mV (Roche Diagnostics, Mannheim, Germany). This electric field is impeded by the presence of adherent cells and the degree of impedance is directly proportional to the number of cells attaching to the bottom of the well (Abassi *et al.*, 2009; Atienza *et al.*, 2006). Impedance values are converted to Cell Index (CI) values by the RTCA Software and these values appear on the y-axis on the generated results output chart (Figure 2.8). CI values reflect the number of cells, as well as morphological parameters such as size, shape and degree of cell attachment to the plates (Abassi *et al.*, 2009; Atienza *et al.*, 2006).

2.10.3. Molecular Docking

For the purpose of lead optimization and to find out the interaction between the compound and the HIV-1 protease receptor, molecular modeling calculations and local docking were done using MOE-Dock software. This was done to evaluate the binding free energies of this inhibitor into the target HIV-1 protease receptor, and to find out interactions between ligand and receptor, also, to compare affinities of the isolated bioactive compounds to the target HIV-1 protease receptor. For the docking calculations,

the protein structure (Protein data bank PDB code: 5HVP) was first separated from the inhibitor molecule and refined using molecular minimization with added hydrogen. Docking calculations were carried out using standard default variables for the MOE program. The binding affinity was evaluated by the binding free energies (S-score, kcal/mol), hydrogen bonds, and the root-mean-square deviation (RMSD) values. The compounds were docked into the same groove of the binding site of the native co-crystallized ligand. The Dock scoring was performed with the London dG scoring function and has been enhanced using two different refinement methods, which were capable of sampling conformational changes in the backbone structure. We allowed rotatable bonds; the best 10 poses were retained and analyzed for the binding poses best score. Energy minimization was done through. There are other bioassays not detailed in this chapter used as screening strategies such as direct enzyme assays (HIV-1 PR and RT) are explained in detail in various methodology sections of this thesis.

2.11. Hypothesis

For this study, it was hypothesized that organisms collected from the Red Sea, Egypt can be a source of unique bioactive compounds, (because these organisms live in a semi-enclosed environment under pressure of high salinity and low circulation). These organisms should be a source of metabolites with anticancer and HIV-1 enzyme inhibitory activities. To investigate this hypothesis, the following objective was formulated and investigated through the described aims.

2.12. Purpose and objective of the study

The purpose of this study was to investigate extracts and where promising activity existed, isolate active compounds from 13 marine organisms. The cytotoxic and the HIV-1 enzyme inhibitory activities of the extracts and the isolated compounds were to be investigated as well.

Aims

- The cancer and anti-HIV activities of thirteen marine organisms were investigated (Chapter 3). Marine organisms reportedly have different therapeutic uses including potential antiviral and anticancer therapy.

- In Chapter 4, research into one of the marine organisms, the soft coral *Litophyton arboreum*, was undertaken. This chapter also includes the bioassay-guided fractionation and the identification of Limit *L. arboreum* constituents. Limited reports exist in literature on *L. arboreum* total extract and its isolated compounds (only two studies which reported its cytotoxicity in different cell lines exist (Shaker *et al.*, 2010 and Grote *et al.*, 2008), but nothing was reported on its anti HIV-1 activities making this investigation novel.
- Chapter 5 was aimed at evaluating the seasonal changes in the biological activities of *L. arboreum* seasonal extracts. The seasonal changes of a major compound found in the crude extracts (7 β -acetoxy-24-methylcholesta-5-24(28)-diene-3, 19-diol) and reporting the role of this compound in the biology of the organism was also done.

2.13. Screening strategy

The overall methodology for this study is summarized in the schematic illustration shown in Figure 2.9. In the figure, thirteen marine organisms were collected from the Red Sea, Egypt. Organic extraction was carried out on freshly collected material to produce crude extracts and detailed explanations of the extraction procedure are provided in Chapter 3 and the appendix (Figure 8.1). Extracts were first screened for inhibitory properties against HIV-1 enzymes in direct enzyme assays and then for cytotoxicity. Most of the extracts had strong to weak cytotoxicity and inhibitory properties in the PR direct enzyme assay; two extracts with promising PR inhibitory activities were identified. All of the extracts showed weak inhibitory properties in the RT direct enzyme assay. Two soft corals showed promising cytotoxic activities with high selectivity indexes.

L. arboreum extract was found to have activities in all the assays used. This organism was there for selected for bioassay-guided purification. Purification yielded compounds that exhibited cytotoxic and HIV-1 PR inhibitory activity (detail in Chapter 4, section 8.2 of the appendix).

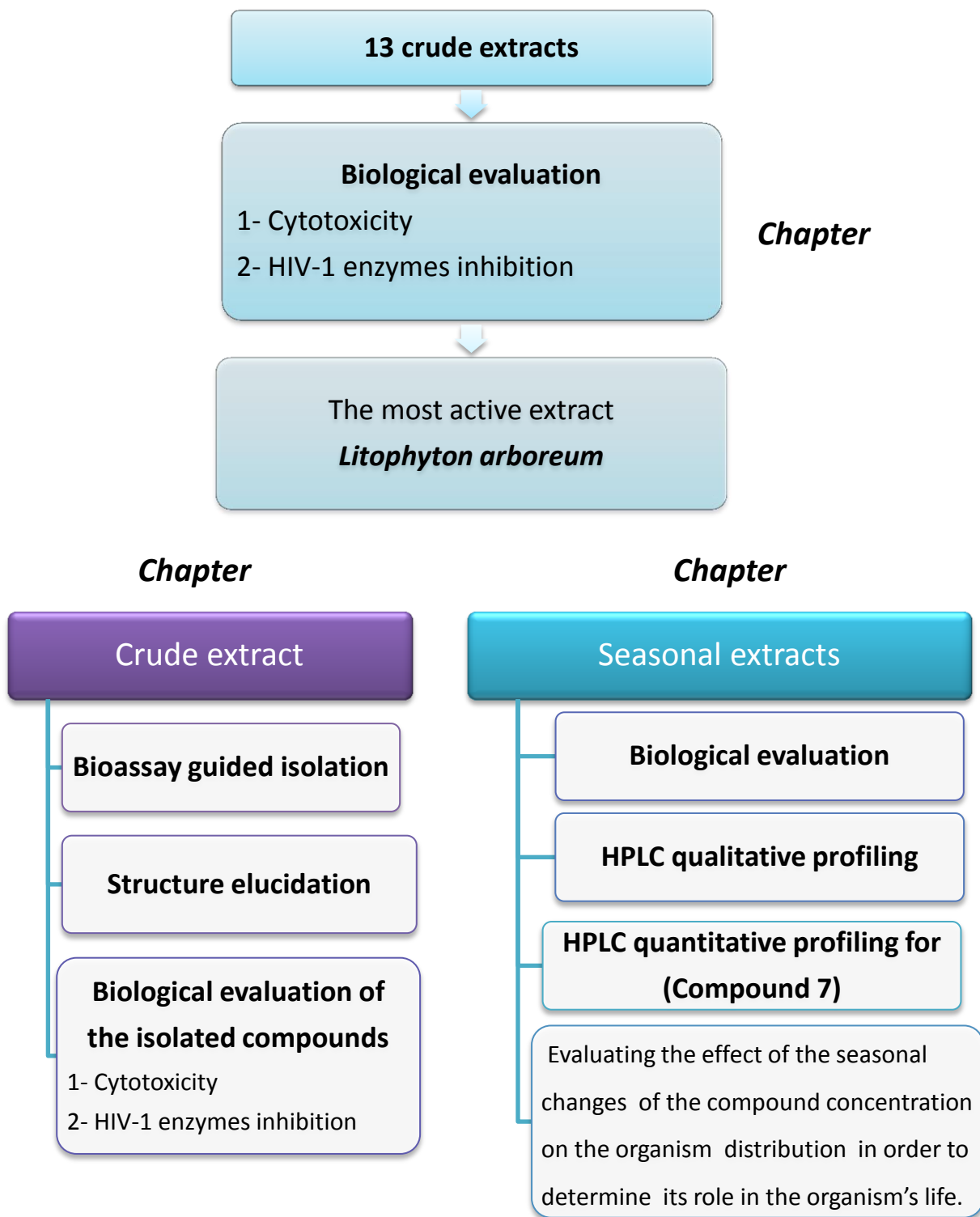


Figure 2.10: Work plan followed in this study.

2.14. Outputs

The research presented in this thesis was presented at DART, 2012 San Diego, California. Publications was generated from the work done on *L. arboreum* (Ellithey *et al.*, 2013) and study chapters have been submitted for publication. These are indicated in the preface and on the front pages of the different data chapters.

Accepted

- Ellithey M., Lall, N., Hussein, A.A., Meyer, D., 2013. Cytotoxic, Cytostatic and HIV-1 PR Inhibitory Activities of the Soft Coral *Litophyton arboreum*. *Marine Drugs* 2013, 11, 1-x manuscripts; doi: 10.3390/md110x000x. (Accepted November 2012).
- Ellithey M., Lall, N., Hussein, A.A., Meyer, D., 2013. Cytotoxic and HIV-1 enzyme inhibitory activities of Red Sea marine organisms. (Accepted December 2013).

Submitted

- Ellithey M. Lall, N., Hussein, A.A., Meyer, D. (2013). Seasonal evaluation of *Litophyton arboreum* extract constituents and biological activities extracts as well as its influence on the organism biology. Submitted to *Marine ecology*.

CHAPTER 3

CYTOTOXIC AND HIV-1 ENZYME INHIBITORY ACTIVITIES OF RED SEA MARINE ORGANISMS

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Abstract

Background: Cancer and HIV/AIDS are two of the greatest public health and humanitarian challenges facing the world today. Infection with HIV not only weakens the immune system leading to AIDS and increasing the risk of opportunistic infections, but also increases the risk of several types of cancer. The enormous biodiversity of marine habitats is mirrored by the molecular diversity of secondary metabolites found in marine animals, plants and microbes which is why this work was designed to assess the anti-HIV and cytotoxic activities of some marine organisms of the Red Sea.

Methods: The lipophilic fractions of methanolic extracts of thirteen marine organisms collected from the Red Sea (Egypt) were screened for cytotoxicity against two human cancer cell lines; leukaemia (U937) and cervical cancer (HeLa) cells. African green monkey kidney cells (Vero) were used as normal non-malignant control cells. The extracts were also tested for their inhibitory activity against HIV-1 enzymes, reverse transcriptase (RT) and protease (PR).

Results: Cytotoxicity results showed strong activity of the Cnidarian *Litophyton arboreum* against U-937 (IC₅₀; 6.5µg/ml ±2.3) with a selectivity index (SI) of 6.45, while the Cnidarian *Sarcophyton trochliophorum* showed strong activity against HeLa cells (IC₅₀; 5.2 µg/ml ±1.2) with an SI of 2.09. Other species showed moderate to weak cytotoxicity against both cell lines. Two extracts showed potent inhibitory activity against HIV-1 protease; these were the Cnidarian jelly fish *Cassiopeia andromeda* (IC₅₀; 0.84 µg/ml ±0.05) and the red algae *Galaxura filamentosa* (2.6 µg/ml ±1.29). It is interesting to note that the most active extracts against HIV-1 PR, *C. andromeda* and *G. filamentosa* showed no cytotoxicity in the three cell lines at the highest concentration tested (100 µg/ml).

Conclusion: The strong cytotoxicity of the soft corals *L. arboreum* and *S. trochliophorum* as well as the anti-PR activity of the jelly fish *C. andromeda* and the red algae *G. filamentosa* suggests the medicinal potential of crude extracts of these marine organisms.

Keywords: Red Sea, Marine organisms, cytotoxicity, HIV-1 protease and HIV-1 reverse transcriptase.

3.1. Background

Life-threatening illnesses such as cancer and the acquired immunodeficiency syndrome (AIDS) presents patients and their families with considerable burdens. For many sufferers both cancer and AIDS have evolved from acutely terminal conditions into chronic illnesses characterized by complex psychosocial and physical issues.

According to the World health Organization, Global Health Sector Strategy on HIV/AIDS, The number of people living with HIV/AIDS in 2010 was estimated to be around 34 million. On the other hand according to the American cancer society (2012), total of 1,638,910 new cancer cases and 577,190 deaths from cancer were determined to have occurred in the United States in 2012. Lower CD4 (white blood cell) counts and a weakened immune system are risk factors for pre-cervical and cervical cancer. Additionally, women with HIV are more likely to have a recurrence of pre-cervical cancer and antiretroviral therapy did not protect HIV-positive women from the development of pre-cervical cancer (Zaporozhan *et al.*, 2010; Lodi *et al.*, 2010 and Abraham *et al.*, 2010).

In order to combat both cancer and HIV/AIDS, colossal amounts of money, manpower, time and energy have been dedicated to research on novel compounds which can be developed as therapeutic agents. Anti-cancer drugs and HIV treatment are related since nucleotide analogues can be used for the treatment of both diseases. Using natural products to manufacture drugs is an ancient and well established practice that has yielded familiar products such as morphine, digitalis, penicillin, and aspirin (Newman *et al.*, 2010).

Mankind has known for thousands of years that marine organisms contain substances capable of potent biological activity which has recently also been demonstrated against different types of cancer and HIV/AIDS (Simmons *et al.*, 2005). Natural product screening has been a component of drug-lead development for the better part of 20 years. Novel pharmaceuticals, such as taxol, have been discovered through the screening of extracts from plants, microorganisms and marine organisms (John *et al.*, 2001), while *in vitro* inhibition of key enzymes of the viral life cycle serves as a first step in HIV/AIDS drug development.

The studies on the antiviral activities of marine natural products are presently attracting more and more attention worldwide. Marine derived compounds have been shown to have a variety of bioactivities such as antiviral, anticoagulant, antioxidant and other medicinal properties (Wei *et al.*, 2012). HIV-1 enzymes RT and PR were identified early on as potential drug targets. The discovery and development of inhibitors of these enzymes are an unqualified success of modern pharmacology and structural biology (Francesca *et al.*, 2012). The emergence of drug-induced mutations of HIV-1 enzymes leads to rapid loss of the potency of existing drugs and the need to develop new candidates (Mark *et al.*, 2012).

The Red sea represents one of the most promising areas as a source of medicinal natural products. Most of the investigations into the biological activities of organisms from the Red Sea was conducted either in the northern part of gulf Aqaba (Bergman *et al.*, 2012 and Kelman *et al.*, 2001), or the southern part of the sea and reported on the organisms' free radical scavenging and cancer growth inhibition activities (Diaa *et al.*, Alarif *et al.*, 2013 and Elamir *et al.*, 2012). These areas are subject to tourism and human impact, which can extensively affect the marine community. As reported by "Zalul," an

Israeli environmental lobby group, 'over the past five years the most northerly part of the Red Sea coral reef is now 70 % dead'.

The study area investigated in the current report, Sharm El-Sheikh, is located in the connection point of the south Suez Gulf and south Aqaba Gulf. Most of this region is protected by the Egyptian Ministry of State For Environmental Affairs. Despite its high water quality, there are no published reports for the biological activities of marine organisms from Sharm El-Sheikh which prompted the present investigation.

This study was designed to investigate the potential cytotoxic and anti-HIV activities of thirteen marine organisms collected from Sharm El-Sheikh, Red Sea (Egypt).

3.2. Methods

3.2.1. Marine organisms

Thirteen of the most abundant marine organisms from different families were collected from Nabq and Ras Mohammed protected areas (Sharm el-Sheikh, Red Sea, Egypt) in the period between March and April 2010. Fifty gram (50g) of each sample was collected according to the sampling and preservation protocol of Kathrina Fabricius following identification done by Mona Ellithey using the Red sea invertebrates' reference guide (Ewald *et al.*, 2004). Samples were collected from different marine environments. Two sponges; *Spongia officinalis* (Linnaeus, 1759), *Haliclona rubens* (sensu Duchassaing & Michelotti, 1864) and the jelly fish *Cassiopea andromeda* (Forskål, 1775) were collected from 0.6-1m depth of mangrove swamps. The sandy bottom was used for collection of the algae *Cymodocea rotundata* (Ehrenberg & Hemprich ex Ascherson 1870), *Caulerpa prolifera* (Forsskål J.V. Lamouroux 1809) and the gastropod *Bulla ampulla* (Linnaeus, 1758), from depths of 30-60 cm. From the stony bottom of the coral reef the algae *Galaxaura filamentosa* (W.R. Taylor 1945), the soft corals *Litophyton arboreum* (Forskål, 1775), *Sarcophyton trochliophorum* (Von Marenzeller, 1886), *Sinularia heterospiculata*, *Sinularia maxima*, *Sinularia polydactyla* (Verseveldt, J. 1976) and *Turbinaria turbinata* (Linnaeus Kuntze 1898) were collected at depths of 1-3 m .

3.2.2. Extract preparation

Fresh marine organisms (50 g each) were homogenized directly after collection with 150 ml of 90% methanol (Merck, Germany). After filtration the solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) and the residues re-suspended in water and partitioned with ethyl acetate in order to get rid of the salts and the high molecular weight hydrophilic compounds. The lipophilic fractions obtained by ethyl acetate were dried out by Rota vapor and were then tested for their *in vitro* cytotoxicity against two human cancer cell lines (U937) and (HeLa), and the African green monkey kidney cell line (Vero) as representative of a normal, non-cancerous cell line. The extracts were also tested for inhibitory activity in direct enzyme assays against HIV-1 reverse transcriptase and HIV-1 protease.

3.2.3. Cell culture and cytotoxicity of extracts

Chemicals and reagents; all cell lines, media, trypsin-EDTA, fetal bovine serum (FBS) and antibiotics (penicillin, streptomycin and fungizone) were purchased from Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA).

The ethyl acetate extracts were each dissolved in DMSO to a stock solution of 20 mg/mL and added to the microtitre plate. Serial dilutions were made to range from a concentration of (400 µg/mL to 3.12 µg/mL) for each extract. The negative control wells included cells exposed to 2% DMSO. And the positive control Actinomycin D with concentrations ranging between 0.5 µg/mL and 0.002 µg/mL. The microtitre plate was incubated for a further 72 h. and were tested for their cytotoxic activity using XTT, (Sodium 3-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) Colorimetric assay. The assay is based on the ability of live cells to reduce the yellow water soluble XTT into an insoluble formazan product (Zheng *et al.*, 2001). The marine extracts were first screened for their *in vitro* cytotoxicity at a concentration of 100 µg/mL against HeLa and U937 cells. Extracts which reduced > 50% of the cell proliferation of both cell lines were further tested at concentrations that ranged from (400 µg/mL to 3.12 µg/mL). A third non-cancerous cell line (Vero) was also included for the most active extracts in order to determine selectivity indices which represent the overall activity of the extract. Selectivity Indexes (SI) values were calculated as follows; the 50% inhibitory concentration (IC₅₀) of the extract tested in Vero cell line was divided

by the IC_{50} of the extract tested in a cancer cell. Higher SI values indicate the more selective extracts.

Cells were maintained in culture flasks in complete medium supplemented with 10% heat-inactivated FBS and antibiotic cocktail (100 U/mL penicillin, 100 g/L streptomycin and 250 g/L fungizone). Cells were cultured and maintained in a humidified atmosphere at 37 °C and 5% CO₂. Cytotoxicity was measured by the XTT method using the Cell Proliferation Kit II (F. Hoffmann-La Roche Ltd.). Cells (100 µL) were seeded (concentration 1×10^5 cells/mL) into a microtitre plate and incubated for 24 h to allow the cells to adhere. Following the evaporation of ethyl acetate, the extract powder residue was dissolved in DMSO and then serially diluted (3.12- 400 µg/mL), added to the plates and incubated for 72 h. A positive control for cytotoxicity, Actinomycin D and a negative control of cells with 2% DMSO were also included. After a 72 h incubation, XTT was added to a final concentration of 0.3 mg/mL and the cells incubated for 2–3 h. Absorbance of the developed colour was spectrophotometrically determined using a multi-well plate reader which measured the optical density at 450 nm with a reference wavelength of 690 nm. Mean IC_{50} is the concentration of extracts which reduces cell growth by 50% under the experimental conditions and is the average of at least three independent reproducible measurements. The IC_{50} values were reported at \pm 95% confidence intervals (\pm 95% CI). This analysis was performed using Graph Pad Prism (San Diego, USA). T test analysis was used to determine the significance of the extracts cytotoxicity

3.2.4. HIV-1 direct enzyme assays

Reverse transcriptase (RT) inhibitory activity of the crude extracts against a purified recombinant HIV1-RT (Merck, Darmstadt, Germany) was determined by using the Roche Diagnostics (Mannheim, Germany) colorimetric kit. The assay was performed as previously described as in Fonteh *et al.*, 2011.

HIV-1 protease enzyme (Bachem Bioscience Inc. King of Prussia, PA, UK) and the substrate (a synthetic peptide that contains a cleavage site Tyr-Pro for HIV protease as well as two covalently modified amino acids for the detection of cleavage). The assay was performed according to procedures by Lam *et al.*, 2000, in black 96 well assay plates

obtained from Corning Incorporated, (Corning, New York, USA). The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a synergy microplate spectrofluorometer (BioTek, Analytical & Diagnostic products, South Africa). Acetyl pepstatin (AP) was used as a positive control for HIV-1 PR inhibition. The blank treatment consisted of assay buffer with only the substrate and an untreated control of enzyme and substrate was also included. The percentage inhibition was calculated based on the formula: $100 - [(Test\ reagent\ RFU - background\ RFU) / (untreated\ control\ RFU - blank) \times 100]$ where RFU = relative fluorescence units.

All the marine organism materials were screened at 100µg/mL for inhibition of HIV-1 enzymes. IC₅₀ values of the most active extracts were calculated and compared to known HIV-1 PR and HIV-1 RT inhibitors. All the experiments were done 3 times in order to ensure the precision and accuracy of the data.

3.3. Results and discussion

3.3.1. Cytotoxicity of the extracts

The cytotoxicity of the soft corals *L. arboreum*, *S. polydactyla*, *S. maxima* and *S. heterospiculata* were tested for the first time in U937 cells. Figure 3.1 showing the cytotoxicity of extracts at 100µg/ml indicating that these soft corals showed potent cytotoxicity by killing more than 90% of the cells. Moderate cytotoxicity (<60% of cell death) was demonstrated by the soft coral *S. trocheliophorum*, the green algae *C. prolifera*, the sponges *S. officinalis* and *H. rubens*, the sea grass *C. roncata* and the brown algae *T. turbinata*. The lowest cytotoxicity in U937 cells was observed from the mollusc *B. ampulla*, the jelly fish *C. andromeda* and the red algae *G. filamentosa* where 60% of the cells were still viable values of the active extracts were significantly different ($P < 0.05$) from the values for negative controls.

This is also the first report for the cytotoxicity of the mollusc *B. ampulla* and the soft coral *S. polydactyla* in HeLa cells. As shown in Figure 3.1, these organisms showed potent cytotoxicity by killing more than 90% of the cells when tested at 100µg/mL. The soft corals *L. arboreum*, *S. maxima* and *S. trocheliophorum* showed strong cytotoxicity and killed more than 80% of the cells. *C. prolifera* and *S. heterospiculata* showed moderate toxicity (<60%). The lowest cytotoxicity in HeLa cells was observed for *S. officinalis*, *T.*

turbinata, *C. andromeda*, *H. rubens* and *C. rotundata* where more than 60% of the cells were still viable.

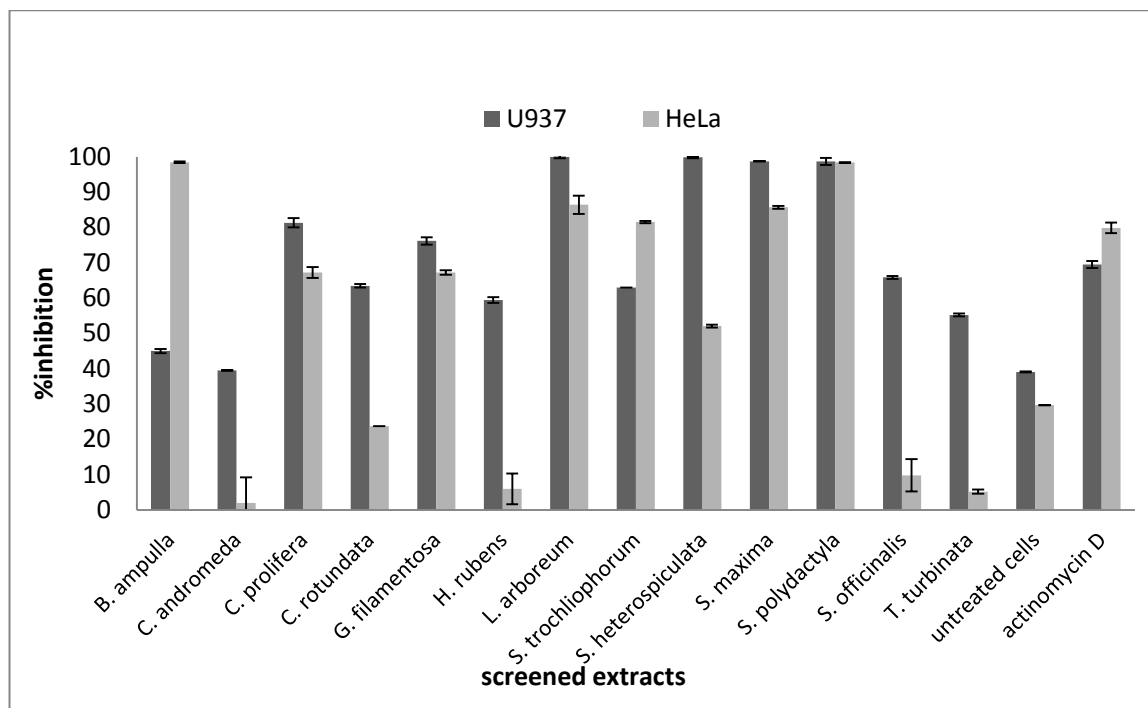


Figure 3. 1: Cytotoxicity of different marine extracts. Samples were dissolved in DMSO and diluted with medium to a final concentration of 100µg/ml.

Following these observations only potent and the strongly active extracts were subjected to detailed study to determine their half maximal inhibitory concentrations (IC_{50}) and selectivity indices. Once the IC_{50} values were determined, responses of the marine extracts were characterized according to the guidelines of the National Cancer Institute (NCI) where IC_{50} values of 10-20 µg/ml of a total/crude extract is viewed as cytotoxic, $20 \leq 50$ µg/ml as moderately cytotoxic and < 10 µg/ml as strongly cytotoxic. As the selectivity index value of an extract increase the ability of the extract to target the cancer cells rather than normal body cells also increases.

As shown in Table 3.1, the soft coral *L. arboreum* presented very strong cytotoxicity and selectivity (IC_{50} ; 6.5 µg/ml, SI; 6.45) in U937 cells which is reported here for the first time. It also showed a moderate activity in HeLa (IC_{50} ; 28.10 µg/ml, SI; 1.46) as compared to the cytotoxicity positive control, Actinomycin D (IC_{50} 7.8µg/ml, SI 1.5) in U937 cells and (IC_{50} ; 17.7 µg/ml, SI; 1.5) in HeLa. The observed toxicity can be due to the terpenoids which are extensively reported in soft corals from the family Alcyoniidae (Cheng *et al.*, 2010). Terpenoids, mainly macrocyclic cembrane-type diterpenoids and their derivatives,

represent important chemical defense tools for these animals against their natural predators (Li *et al.*, 2009). Results presented here are supported by current literature reporting that *L. arboreum* demonstrated moderate cytotoxicity against HUVEC, K-562 and HeLa (Shaker *et al.*, 2010), L-929 and K-562 (Grote *et al.*, 2008) as well as strong antibacterial activity (Kelman *et al.*, 2006).

The soft coral, *Sarcophyton trocheliophorum* showed promising cytotoxicity with a selectivity index higher than the positive control (IC₅₀ 5.2 µg/ml, SI; 2) in HeLa cells. In a similar study polyhydroxy sterols from *S. trocheliophorum* obtained in Singapore showed potent cell growth inhibitory activity against different human HL60 leukaemia (the present study used U937), M14 skin melanoma, and MCF7 breast carcinoma cells with EC₅₀ values of 2.8, 4.3, and 4.9 µg/ml respectively detected by the MTT assay (a related, not identical tetrazolium dye was used here), and exhibited minimal toxicity to normal human peripheral blood lymphocytes (Dong *et al.*, 2002). These data suggests that *S. trocheliophorum* from any marine environment may be producing sterols which when isolated or present in an extract retain their potency and may yet prove useful as anti-cancer drugs.

Previous studies showed that a hybridization of *Sinularia polydactyla* and *Sinularia maxima* species had strong cytotoxicity in the following cell lines; the breast cancer SK-BR3 cell line, cervical and HeLa-Apl cell lines (Kamel *et al.*, 2007). In the current study, when the original organisms (not hybrids) were tested, *S. polydactyla* exhibited moderate cytotoxicity (IC₅₀; 20 µg/ml, SI; 0.34) in HeLa cells. *S. maxima* had moderate toxicity (IC₅₀; 24.µg/mL, SI; 1.8) in U937 and in HeLa cells (IC₅₀; 30 µg/mL, SI; 1.46). In addition, another species that is reported here for the first time, *S. heterospiculata*, exhibited low cytotoxicity (IC₅₀; 45µg/ml, SI; 0.99) in U937 and no activity in HeLa cells.

The green algae *C. prolifera* showed weak to moderate cytotoxicity in U937 and HeLa cell lines (IC₅₀; 52 µg/ml, 32µg/ml and SI; 2.73, 4.4 respectively). Caulerpenyne (Cau), a metabolite from *Caulerpa taxifolia* has previously been shown to be cytotoxic against KB cells and fibroblasts from hamsters. Cau along with 6 other drugs representative of the major classes of anticancer products were tested against 8 cancer cell lines of human origin. Cau demonstrated growth-inhibitory effects in all cases with some variability between cell lines. It was also shown to induce inhibition of SK-N-SH tumor cell proliferation with an IC₅₀ of 10 +/- 2 µ M (Fischel *et al.*, 1995; Barbier *et al.*,

2001 and Cavas *et al.*, 2006). It is there for possible that the cytotoxicity demonstrated by the crude extract of *C.prolifera*, may increase if compounds like Cau is isolated from it. All other tested species showed moderate to weak activity against both cell lines.

The main goal of cancer chemotherapy research is to discover safe and efficient agents that target cancer cells specifically but are innocuous to normal cells. However, many anticancer drugs fail to meet this criterion being unable to discriminate between cancer and normal cells. The development of novel cancer chemotherapeutic agents with a higher potency and specificity against cancer cells is urgently needed. The selectivity index of *L. arboreum* in U937 and *S. trocheliophorum* in HeLa compares very well and even better than the positive control used in this study and is worthy of further investigation.

3.3.2. Anti-HIV direct enzymes assays

All the extracts were tested at 100µg/mL for the inhibition of HIV-1 RT and PR enzymes. The experiment was repeated at least 3 times. Extracts with percentages of inhibition $\geq 50\%$ were considered to be active while the extracts demonstrating $>90\%$ inhibition were considered potent.

Unfortunately, none of the screened extracts showed any inhibitory activity of HIV-1 RT (all inhibitions were $<50\%$). In cases where marine organisms were able to inhibit this enzyme, it was always pure compounds which were hydrophilic in nature, suggesting that for activity against this enzyme, more aqueous extracts should be prepared (Schaffrath *et al.*, 1976). On the other hand most of the extracts inhibited HIV-1 PR enzyme in the direct assays. These data is presented in Figure 3.2 demonstrating that strong inhibitory activity ($>80\%$) were obtained by *S. maxima*, *L. arboreum*, *B. ampulla*, *C. andromeda*, *G. filamentosa*, and *S. heterospiculata*. while, *S. trocheliophorum* and *S. polydactyla* was active but to a lesser extent demonstrating inhibitory activities of 65-80%. *C. rotundata* and *C. prolifera* exhibited very weak inhibitory activity ($<50\%$). Only the extracts with strong inhibitory activity were subjected to detailed study using serial dilutions of each extract in order to determine their IC_{50} values. Following IC_{50} assessments, the extract activities were classified as follows; potent activities were assigned to $IC_{50} < 10 \mu\text{g/ml}$, while $IC_{50} 10 \leq 20 \mu\text{g/mL}$ was considered strong, and IC_{50}

20 ≤ 50 µg/mL as moderate. Extracts demonstrating IC₅₀ values of >50 µg/ml were considered weak.

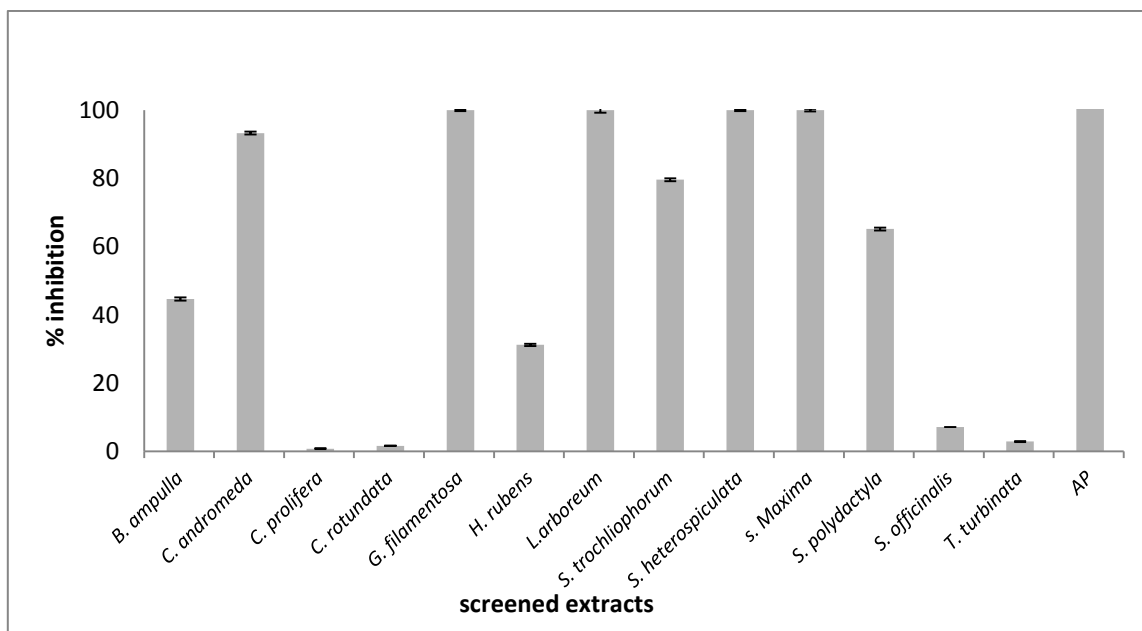


Figure 3. 2: HIV-1 PR inhibition percentages of different marine extracts. Samples were dissolved in DMSO and diluted with the assay buffer to a final concentration of 100µg/ml.

The detailed IC₅₀ study presented in Table 3.1, showed potent inhibitory activity of HIV-1 PR by the Cnidarian jelly fish *C. andromeda* (IC₅₀ 0.84 µg/ml). Phylum Cnidaria was reported to have antimicrobial (Radwan *et al.*, 2001), antioxidant (Ovchinnikova *et al.*, 2006) and anti-inflammatory (Marino *et al.*, 2008) activities. Nothing has been reported on the anti HIV-1 or the cytotoxicity of *C. andromeda* until now.

Table 3. 1: The half maximal inhibition concentration (IC₅₀) of the lipophilic fraction of the marine organism extracts against HIV-1 PR is presented.

Phylum	Marine organism	Cytotoxicity IC ₅₀			SI values		IC ₅₀
		(µg/mL)					(µg/mL)
		U937	HeLa	Vero	U937	HeLa	HIV-1 PR
Mollusca	<i>B. ampulla</i>	N/T ^a	77	111	N/A	1.4	N/T ^b
Rhodophyta	<i>G. filamentosa</i>	N/T ^a	N/T ^a	N/T ^a	N/A	N/A	2.6
Chlorophyta	<i>C. prolifera</i>	52	32	143	2.7	4.4	N/T ^b
Phaeophyta	<i>T. turbinata</i>	N/T ^a	N/T ^a	N/T ^a	N/A	N/A	N/T ^b
Tracheophyta	<i>C. rotundata</i>	N/T ^a	N/T ^a	N/T ^a	N/A	N/A	N/T ^b
Porifera	<i>H. rubens</i>	N/T ^a	N/T ^a	N/T ^a	N/A	N/A	N/T ^b
Porifera	<i>S. officinalis</i>	N/T ^a	N/T ^a	N/T ^a	N/A	N/A	N/T ^b
Cnidaria	<i>C. andromeda</i>	N/T ^a	N/T ^a	N/T ^a	N/A	N/A	0.84
Cnidaria	<i>S. heterospiculata</i>	45	n/a	44	0.99	n/a	8.6
Cnidaria	<i>S. maxima</i>	24	30	44	1.8	1.5	13
Cnidaria	<i>S. polydactyla</i>	89	20	7	0.1	0.3	N/T ^b
Cnidaria	<i>L. arboreum</i>	6.5	28	42	6.4	1.5	12.0
Cnidaria	<i>S. trocheliophorum</i>	N/T ^a	5.2	11	N/A	2.1	N/T ^b
Actinomycin D ^c		7.8	18	12	1.5	0.7	N/T ^b
Acetylpepstatine ^d							5.7

Not tested (N/T_a) samples with inhibitory activities less than 50% at 100 µg/mL

Not tested (N/T_b) samples with inhibitory activities less than 80% at 100 µg/mL

Subscript C indicates the anticancer drug used as positive control for the cytotoxicity assay.

Subscript D indicates the positive control used in the PR inhibition assay.

In this study, the red algae *G. filamentosa* showed potent HIV-1 PR inhibitory activity (IC_{50} 2.6 $\mu\text{g/mL}$). Many studies have discussed the biological importance of marine algae. Some of the studies in search of anti-herpetic substances have been particularly interesting. The use of these organisms as botanical agents to treat viral infection resulted in four patents (Ehresmann *et al.*, 1977 and 1979). The first two patents used aqueous extracts of *Neodilsea americana* and *N. integra* (Richards *et al.*, 1978) while the third patent is particularly well documented and involves the use of *Cryptosiphonia woodii* (Nonomura, 1985). For the latter, clinical efficacy was clearly demonstrated. The fourth patent is for the use of carrageenan and other sulfated polysaccharides for the treatment of diseases (including AIDS) caused by retroviral infection (Neushul, 1988). In another study on the hydrophilic metabolites (sulfated oligo- or poly-saccharide compounds) from red algae, retroviral replication was suppressed and viral reverse transcriptase inhibited (Schaffrath *et al.*, 1976).

It is important to mention that our study is the first report of the promising HIV-1 protease inhibitory activity of *C. andromeda* (IC_{50} ; 0.84 $\mu\text{g/ml}$) and *G. filamentosa* (IC_{50} ; 2.6 $\mu\text{g/mL}$) while the IC_{50} of the positive control (acetyl pepstatin) for enzyme inhibition in the assay was 5.7 $\mu\text{g/mL}$. These extracts showed no cytotoxicity against the three cell lines at the highest concentration (100 $\mu\text{g/mL}$) tested.

Moderate HIV-1 PR inhibitory activities were shown by the soft corals *S. heterospiculata* (8.6 $\mu\text{g/ml} \pm 3.4$), *L. arboreum* (12 $\mu\text{g/ml} \pm 2.4$) and *S. maxima* (13 $\mu\text{g/ml} \pm 2.3$). Metabolites from soft corals found to have cytotoxicity against selected cancer cell lines, antiviral activity against human cytomegalo virus (HCMV), anti-inflammatory activity and antibacterial activity against five selected bacterial strains, have been reported (Cheng *et al.*, 2010). To date, no anti-HIV activity have been reported in literature for the three soft corals tested here. Enzymatic assays are an important first step when identifying potential anti-HIV leads, however screening extracts and especially pure compounds in virus infected cell cultures are essential next steps to ensure that only the most promising of compounds able to inhibit whole virus replication progresses in the drug development pipe line.

3.4. Conclusion

The present study demonstrated that marine organisms remain one of the most interesting sources for the discovery of bioactive leads that can help in the treatment of cancer and HIV. This study is the first to report the anticancer and anti HIV activities of *B. ampulla*, *C. rotundata*, *G. filamentosa*, *H. rubens*, *S. trochliophorum*, *S. heterospiculata*, *S. officinalis* and *T. turbinata*. This is also the first report on the HIV-1 PR inhibitory activities of extracts of *C. andromeda*, *C. prolifera* and *L. arboreum*, *S. maxima*, *S. polydactyla*

Although the cytotoxicity of some of the extracts reported on here, have previously been tested in the HeLa cell line (for *C. prolifera*, *L. arboreum*, *S. maxima* and *S. polydactyla*), this study is the first to report cytotoxicity of these extracts in a leukaemia cancer cell line (U937).

The bio-activity evaluation of the extracts resulted in two marine extracts demonstrating strong anticancer activity; *L. arboreum* in U937 cells and *S. trochliophorum* in HeLa with high safety margins compared to Actinomycin D. *C. andromeda* showed potent anti-PR activity with an IC₅₀ 6 times lower than the positive control with no cytotoxicity in the different cell lines while *G. filamentosa* extract showed double the activity of the positive control with higher safety margins. These findings suggest that these species could be an interesting source of bioactive compounds and deserve further bioassay-guided isolation procedures to determine the identity and structure of the active compounds.

3.5. Authors' contributions

AH, DM and NL designed the study. AH and MSE was responsible for collection, identification and extraction of the samples; DM, NL and MSE performed the viability and the HIV enzymatic assays. MS wrote the first draft of the article, DM, AH; NL and MSE provided editorial and data interpretation information, all authors read and approved the final manuscript.

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CHAPTER 4

**CYTOTOXIC, CYTOSTATIC AND HIV-1 PR INHIBITORY ACTIVITIES OF THE SOFT
 CORAL *LITOPHYTON ARBOREUM***

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Abstract: Bioassay-guided fractionation using different chromatographic and spectroscopic techniques in the analysis of the Red Sea soft coral *Litophyton arboreum* led to the isolation of nine compounds; sarcophytol M (**1**), alismol (**2**), 24-methylcholesta-5,24(28)-diene-3 β -ol (**3**), 10-O-methyl alismoxide (**4**), alismoxide (**5**), (S)-chimyl alcohol (**6**), 7 β -acetoxy-24-methylcholesta-5-24(28)-diene-3,19-diol (**7**), erythro-N-dodecanoyl-docosasphinga-(4E,8E)-dienine (**8**), and 24-methylcholesta-5,24(28)-diene-3 β ,7 β ,19-triol (**9**). Some of the isolated compounds demonstrated potent cytotoxic- and/or cytostatic activity against HeLa and U937 cancer cell lines and inhibitory activity against HIV-1 protease (PR). Compound **7** was strongly cytotoxic against HeLa cells (CC₅₀ 4.3 \pm 0.8 μ M), with selectivity index of SI 8.1, which was confirmed by real time cell electronic sensing (RT-CES). Compounds **2**, **7**, and **8** showed strong inhibitory activity against HIV-1 PR at IC₅₀s of 7.2 \pm 0.7, 4.9 \pm 0.2, and 4.8 \pm 0.9 μ M

respectively. *In silico* docking of most compounds presented comparable scores to that of acetyl pepstatin, a known HIV-1 PR inhibitor. Interestingly, compound **8** showed potent HIV-1 PR inhibitory activity in the absence of cytotoxicity against the cell lines used. In addition, compounds **2** and **5** demonstrated cytostatic action in HeLa cells, revealing potential use in virostatic cocktails. Taken together, data presented here suggest *Litophyton arboreum* to contain promising compounds for further investigation against the diseases mentioned.

Keywords: Red Sea; *Litophyton arboreum*; HIV-1 protease; HIV-1 reverse transcriptase; cytotoxicity; real time cell analysis

4.1. Introduction

HIV/AIDS is one of the most devastating diseases in the world with approximately 34 million people living with the virus in 2010 and approximately 2.7 million new infections in that same year. Antiretroviral therapy (ART) successfully reduces infection and decreases symptoms; but, the emergence of viral drug resistance, due to drug-induced mutations in viral genes, renders treatment ineffective. This underscores an urgent need to develop new anti-HIV drugs (Logsdon *et al.*, 2004 and Leonardo *et al.*, 2012) with fewer side-effects to improve patient compliance.

The use of anti-HIV drugs as cancer treatments is not new. Azidothymidine was studied as an antineoplastic in the 1990s, but despite promising *in vitro* data, clinical trials showed little antitumor activity. HIV protease inhibitors were developed in the early 1990s, and their subsequent incorporation into highly active antiretroviral therapy (HAART) has profoundly changed the natural history of HIV infection. The potential antitumor properties of these drugs have been investigated because of their success in treating HIV-related Kaposi's sarcoma. HAART's effects on Kaposi's sarcoma did not always correlate with immune reconstitution, and activity against other solid and haematological malignancies has been established. Inhibition of tumor-cell invasion and angiogenesis were properties first ascribed to HIV protease inhibitors; these drugs have

pleiotropic antitumor effects, including inhibition of inflammatory cytokine production, proteasome activity, cell proliferation and survival, and induction of apoptosis. HIV protease inhibitors are thus a new class of anticancer drugs with multiple effects, and other anti-HIV drugs might hold similar promise (Chow *et al.*, 2009).

Marine organisms as a source of natural products delivered numerous novel compounds with sensational multiple pharmacological properties. During the past 20 years, thousands of novel compounds and their metabolites with diverse biological activities ranging from antiviral to anticancer have been isolated from various marine sources. The use of marine natural products as anti-HIV agents has also been described (Gochfeld *et al.*, 2003) with a number of potential lead compounds identified. In computational science, natural products have long captured the attention of medicinal chemists due to the diversity of their chemical scaffolds, potentially lower toxicities and bioactive substructures (Zhong *et al.*, 2012).

The Red Sea still contains a large number of uninvestigated organisms (flora and fauna). Exploration of untapped regions of this unique resource for the discovery of bioactive natural compounds is an urgent task due to the impending environmental changes that can occur to the wild flora as human encroachment continues. *Litophyton arboreum* is a common octocoral, widely distributed on the Red Sea coral reefs. A previous chemical study of *L. arboreum* growing in different parts of the Red Sea showed the presence of cembranoide diterpenes (Shaker *et al.*, 2010), which had moderate cytotoxicity in HeLa cells. Collections of this soft coral from other parts of the world showed different metabolites of this soft coral, e.g., furanocembranoides, which demonstrated antiproliferative activities against the cell lines L-929 and K-562 (Grote *et al.*, 2008), sesquiterpenes, sterols, and fatty acid derivatives (Rezanka *et al.*, 2001 and Li *et al.*, 1994) all believed to contain medicinal properties. Herein, we report on the isolation and identification of nine compounds from *L. arboreum* collected from Sharm El-Sheikh, Red Sea. The anti-HIV and anti-cancer potential of some of the purified compounds from the soft coral is reported here for the first time.

4.2. Experimental Section

4.2.1. General Experimental Procedures

The NMR spectra were recorded on Bruker spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C , and/or Varian “Mercury” 200 MHz, for ^1H and 50 MHz for ^{13}C in CDCl_3 with TMS as internal standard. Chemical shifts are given in δ (ppm) and coupling constants in Hertz. EIMS were recorded by a Shimadzu Qp-2010 plus. Cytotoxicity plates red by BIOTEK Power-Wave XS multiwell plate reader was used for the cytotoxicity assays. Pre-coated Si gel plates (Merck, Johannesburg, South Africa, Kieselgel 60 F254, 0.25 mm) were used for analytical TLC analyses. Silica gel 60 (Merck, Johannesburg, South Africa, 230–400 mesh) and Sephadex LH-20 from Pharmacia Fine Chemicals AB(Uppsala, Sweden) were used for column chromatography. Methanol, Dichloromethane, Ethyl acetate and DMSO, were obtained from Merck (Johannesburg, South Africa). All cell lines, media, trypsin-EDTA, fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin, and fungizone) were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, South Africa). A purified recombinant HIV1-RT (Merck, Darmstadt, Germany) was used with the cell free reverse transcriptase colorimetric kit (Roche Diagnostics. Mannheim, Germany). The HIV-1 protease enzyme (Bachem Bioscience Inc., King of Prussia, PA, USA) and the substrate (a synthetic peptide that contains a cleavage site Tyr-Pro for HIV protease, as well as two covalently modified amino acids for the detection of cleavage) were used.

4.2.2. Animal Material

The soft coral *L. arboreum* was collected at Sharm El-Sheikh (Red Sea, Egypt), in May 2010, at a depth of 2–3 m, the tissue was kept in MeOH and extracted in situ. The material was collected and identified by Mona Ellithey, co-author of this article.

4.2.3. Purification of the Active Constituents

The sliced bodies of *Litophyton arboreum* (2 kg, fresh material) were blended with MeOH, after filtration the residue was washed twice with fresh MeOH. The total extracts

were concentrated under a vacuum, and partitioned between EtOAc and H₂O. The solvent-free EtOAc fraction (25 g) was subjected to column chromatography on silica gel and eluted with mixtures of increasing polarity from hexane and EtOAc (0–100%) to yield 43 fractions, which were pooled together according to the TLC profile to twenty main fractions.

The twenty fractions (1–20) were tested for their cytotoxic activity on U937 cell line at 100 µg/mL. The main fractions 5–8, 11, 12, 14, 15, and 20 showed potent cytotoxicity (Figure 3.1) and were subjected to chromatographic purification. Fraction 5 was chromatographed (on silica gel using hexane/EtOAc (98:2) producing compound **1** (60 mg). Fraction 6 under the same conditions yielded pure **2** (750 mg). Fraction 7 was chromatographed (on silica gel using hexane/EtOAc (95:5) producing compound **3** (40.2 mg). Fractions 8 was chromatographed [on silica gel] and produced transparent oily pure compound **4** (650 mg). Fraction 11 on standing at room temperature for three weeks produced crystals, which were washed with a mixture of hexane and EtOAc and produced pure compound **5** (1.2 g). Fraction 12 was chromatographed (on silica gel using gradient mixture of hexane/EtOAc (80:20 to 70:30) producing compound **6** (200 mg). Fraction 14 was chromatographed (on silica gel using mixture of hexane/EtOAc (75:25) producing compounds **7** (50 mg) and **8** (75 mg). Fraction 20 was chromatographed (on silica gel using hexane/EtOAc (70:30) producing compound **9** (20 mg).

Nuclear Magnetic Resonance data for the isolated compounds

Sarcophytol M (**1**): Oil, $[\alpha]_{D}^{20} +34$ (c.0.76 CHCl₃); ¹H NMR, (200 MHz, CDCl₃) δ_H 5.25 (1H, t, *J* = 6.8 Hz, H-3); 4.91 (1H, t, *J* = 7.8 Hz, H-11); 4.89 (1H, t, *J* = 6.8 Hz, H-7); 1.61 (3H, s, Me-20); 1.59 (3H, m, H-19), 1.56 (3H, s, Me-18); 0.96, 0.94 (3H each, d, *J* = 6.8; Me-16, 17). ¹³C-NMR (50 MHz, CDCl₃) δ_C 136.7 (C-4), 135.5 (C-12), 133.3 (C-8), 125.9 (CH, C-7), 123.2 (CH, C-11), 120.9 (CH, C-3), 76.9 (C-1), 39.9 (CH₂, C-9), 39.5 (CH₂, C-5), 35.0 (CH₂, C-14), 34.8 (CH₂, C-2), 34.6 (CH, C-15), 33.5 (CH₂, C-13), 24.8 (CH₂, C-6), 23.7 (CH₂, C-10), 16.9 (CH₃, C-17), 16.6 (CH₃, C-16), 16.2 (CH₃, C-20), 15.4 (CH₃, C-19), 15.2 (CH₃, C-18) [12].

Alismol (**2**): Oil; $[\alpha]_{D}^{20} +4.9$ (c.0.2 CHCl₃); ¹H NMR (200 MHz, CDCl₃): δ_H 5.56 (1H, s, H-6), 4.69, 4.64 (1H each, s, H2-14); 1.17 (3H, s, Me-15), 0.93, 0.92 (3H each, d, *J* = 6.6 Hz, Me-13, 14); ¹³C NMR (50 MHz, CDCl₃): δ_C 153.9 (C-10), 149.3 (C-7), 121.4 (CH, C-6), 106.4 (CH₂, C-15), 80.4 (C-4), 54.6 (CH, C-1), 47.0 (CH, C-5), 40.0 (CH₂, C-3), 37.3 (CH, C-11), 36.9 (CH₂, C-9), 29.8 (CH₂, C-8), 24.6 (CH₂, C-2), 23.8 (CH₃, C-14), 21.3 (CH₃, C-13), 21.1 (CH₃, C-12).

24-Methyl-cholesta-5,24(28)-diene-3 β -ol (**3**): $[\alpha]_{D}^{25} +7.2^{\circ}$ (c 0.3 CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ_{H} 0.65 (3H, br s, H-18), 0.92 (3H, d, $J = 6.18$, H-21), 0.97 (3H, br s, H-19), 1.00 (3H, d, $J =$ Hz, H-27), 0.98, 1.00 (3H, d, $J =$ Hz, H-26), 3.51 (1H, br s, H-3), 4.63 (1H, s, H-28a), 4.69 (1H, s, H-28b), 5.32 (1H, s, H-6). ¹³C NMR (100 MHz, CDCl₃) data: δ_{C} 157.3 (C-24), 141.2 (C-5), 122.1 (CH, C-6), 106.4 (CH₂, C-28), 72.1 (CH, C-3), 57.2 (CH, C-14), 56.4 (CH, C-17), 50.5 (CH, C-9), 42.6 (C-13), 42.8 (CH₂, C-4), 40.1 (CH₂, C-12), 37.7 (CH₂, C-1), 36.2 (C-10), 35.7 (CH, C-20), 35.1 (CH₂, C-23), 34.2 (CH, C-25), 32.3 (CH, C-8), 32.0 (CH₂, C-7), 32.0 (CH₂, C-2), 31.4 (CH₂, C-22), 28.6 (CH₂, C-16), 24.7 (CH₂, C-15), 22.4 (CH₃, C-27), 22.3 (CH₃, C-26), 21.5 (CH₂, C-11), 19.8 (CH₃, C-19), 19.1 (CH₃, C-21), 12.3 (CH₃, C-18).

10-*O*-Methyl alismoxide (**4**): Oil, $[\alpha]_{D}^{20} +5.9^{\circ}$ (c 0.3 CHCl₃). ¹H NMR (200 MHz, CDCl₃) δ_{H} 5.41 (1H, br s, H-6), 3.12 (OMe); 1.13, 1.14 (3H each, s, Me-14, 15); 0.93, 0.90 (3H each, d, $J = 6.6$ Hz, Me-12, 13); ¹³C NMR (50 MHz, CDCl₃): δ_{C} 149.5(C-7), 121.2 (CH, C-6), 80.1 (C-4), 79.2(C-10), 50.0 (CH, C-5), 48.6 (OCH₃), 47.8 (CH, C-1), 40.4 (CH₂, C-3), 37.1(CH, C-11), 35.4(CH₂, C-9), 24.5(CH₂, C-8), 22.3(CH₃, C-14), 21.6 (CH₂, C-2), 21.5 (CH₃, C-13), 21.2 (CH₃, C-12), 17.9 (CH₃, C-15).

Alismoxide (**5**): Colorless crystals, mp 138–141 °C; $[\alpha]_{D}^{20} +9.3$ (c 0.9 CHCl₃), ¹H NMR (400 MHz, CDCl₃) δ_{H} 5.44 (1H, br d, $J = 3.0$ Hz, H-6), 0.98, 1.0 (3H each, d, $J = 6.9$ Hz, H-12, 13), 1.25, 1.28 (3H each, s, H-14,15); ¹³C NMR (100 MHz, CDCl₃): δ_{C} 149.4(C-7), 121.3 (CH, C-6), 80.0 (C-4), 75.2 (C-10), 50.5 (CH, C-1), 50.1 (CH, C-5), 42.5 (CH₂, C-9), 40.3 (CH₂, C-3), 37.2 (CH, C-11), 25.0 (CH₂, C-8), 22.4 (CH₃, C-14), 21.4 (CH₂, C-2), 21.3 (CH₃, C-15), 21.2 (CH₃, C-13), 21.1 (CH₃, C-12).

Chimyl alcohol (**6**): $[\alpha]_{D}^{20} +7.2^{\circ}$ (c 0.3 CHCl₃); ¹H NMR (CDCl₃, 200 MHz) δ_{H} , 3.83 (1 H, m, H-2), 3.72, 3.64 (2H, m, H-1), 3.45 (4H, m, H-1', 3), 1.52 (2H, m, 2'-H2), 1.26 (26H, br s, CH₂), 0.84 (3H, t, $J = 6.6$ Hz, CH₃); ¹³C NMR (50 MHz, CDCl₃): δ_{C} 72.4 (CH₂-1'), 71.8 (CH₂-1), 70.5 (CH-2), 64.2 (CH₂-3), 31.9 (CH₂-13'), 29.7-29.3 (CH₂'s), 26.0 (CH₂-3'), 22.7 (CH₂-14'), 14.1 (CH₃). HRESIMS: 317.3045 [M + H]⁺ corresponding to C₁₉H₄₁O₃, calculated for 317.3057.

7 β -Acetoxy-24-methyl-cholesta-5,24(28)-diene-3 β ,19-diol (**7**): Amorphous powder, $[\alpha]_{D}^{20} +6.7^{\circ}$ (c 0.5 CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ_{H} 5.56 (1H, t, $J = 2$ Hz, H-6), 4.95 (1H, dt, $J = 2.2, 2.2, 8.5$ Hz, H-7 α), 4.70 (1H, t, $J = 1.3$ Hz, H_A-28), 4.64 (1H, dd, $J = 1.3$ and 1.7 Hz, H_B-28), 3.87 (1H, d, $J = 11.5$ Hz, H_A-19), 3.64 (1H, d, $J = 11.5$ Hz, H_B-19), 3.59 (1H, tt, $J = 11.2, 4.7$ Hz, H-3 α), 2.38 (1H, ddd, $J = 13.5, 4.7, 2.4$ Hz, H-4 α), 2.01 (3H, s, OAc), 1.01 (3H, d, $J = 6.8$ Hz, Me-26), 1.00 (3H, d, $J = 6.8$ Hz, Me-27), 0.93 (3H, d, $J = 6.8$ Hz, Me-21), 0.73 (3H, s, Me-18). ¹³C NMR (100 MHz, CDCl₃): δ_{C} 171.4 (MeCOO-), 156.7 (C-24), 140.1 (C-5), 126.6 (CH, C-6), 106.0 (CH₂, C-28), 75.2 (CH, C-7), 70.8 (CH, C-3), 62.8 (CH₂, C-19), 56.5 (CH, C-14), 55.2 (CH, C-17), 48.5 (CH, C-9), 43.1 (C-13), 41.6 (CH₂, C-4), 41.4 (qC, C-10), 39.7 (CH₂, C-12), 37.8 (CH, C-8), 35.6 (CH, C-20), 34.6 (CH₂, C-22), 33.7 (CH, C-25), 33.1 (CH₂, C-1), 31.7 (CH₂, C-2), 30.9 (CH₂, C-23), 28.3 (CH₂, C-16), 24.9 (CH₂, C-15), 22.0 (CH₃, C-26), 21.8 (CH₃, C-27), 21.7 (CH₃, CH₃COO), 21.6 (CH₂, C-11), 18.7 (CH₃, C-21), 12.1 (CH₃, C-18).

Erythro-*N*-dodecanoyl-docosasphinga-(4*E*,8*E*)-dienine (**8**): ¹H NMR (400 MHz, CDCl₃): δ_{H} 6.25 (1H, d, $J_{NH,2} = 7.6$ Hz; H-NH), 5.78 (1H, dtd, $J = 15.4, 6.4, 1.2$ Hz; H-5), 5.54 (1H, ddt, $J = 15.4, 6.3, 1.3$ Hz; H-4), 5.42 (1H, dt, $J = 15.3, 6.3$ Hz; H-9), 5.36 (1H, dt, $J = 15.3, 5.9$ Hz; H-8), 4.32 (1H, br, apparently triplet, $J = 4.9, 6.3, 1.2$ Hz; H-3 (please note that the J values were measured in the other coupled protons)), 3.95 (1H, dd, $J = 11.0, 3.2$ Hz; H_A-1), 3.90 (1H, dddd, $J = 3.2, 3.2, 4.9$ Hz,

$J_{2,NH} = 7.6$ Hz; H-2), 3.70 (1H, dd $J = 11.0, 3.2$ Hz; H_B-1), 2.23 (2H, t, $J = 7.4$ Hz; 2H-2'), 2.12 (2H, m; 2H-6), 2.08 (2H, m; 2H-7), 1.96 (2H, q, $J = 6.4, 6.4$ Hz; 2H-10), 1.64 (4H, m; 2H-3' and 2H-of other position), 1.25 (-(CH₂)_n-), 0.88 (6H, t, $J_{vic} = 7.0$ Hz; Me-22 and Me-16'); ¹³C NMR (100 MHz, CDCl₃): δ_c 173.9 (C-1'), 133.5 (CH, C-5), 131.6 (CH, C-9), 129.2 (CH, C-4), 129.0 (CH, C-8), 74.7 (CH, C-3), 62.5 (CH₂, C-1), 54.4 (CH, C-2), 36.8 (CH₂, C-2'), 32.6 (CH₂, C-10), 32.3–31.9 (all CH₂), 29.7–29.1 (all CH₂), 25.8 (2CH₂, C-3' and other CH₂), 22.7 (2CH₂, C-21 and C-15'), 14.1 (2CH₃, C-22 and C-16'). HRESIMS: found 536.5040 [M + H]⁺, calculated for C₃₄H₆₆NO₃ 536.50458.

24-Methyl-cholesta-5,24(28)-diene-3 β ,7 β ,19-triol (**9**): Amorphous powder, $[\alpha]_{D}^{20} +5.2^\circ$ (c 0.2 MeOH). ¹H-NMR [200 MHz, CDCl₃/CD₃OD (1:3)] δ_H 5.44 (1H, s, H-6), 4.67, 4.61 (1H each, br s, H2-28) 3.54, 3.80 (1H each, d, $J = 11.8$ Hz, H-19), 3.60 (1H, br s, H-7), 3.42 (1H, m, H-3), 0.94 (6H, d, $J = 7.0$ Hz, Me-26, 27), 0.92 (3H, d, $J = 6.6$, Me-21), 0.72 (3H, br s, Me-18),. ¹³C-NMR [50 MHz, CDCl₃/CD₃OD (1:3)] δ_c 157.7 (C-24), 140.0 (C-5), 131.0 (CH, C-6), 106.9 (CH₂, C-28), 73.3 (CH, C-7), 72.0 (CH, C-3), 63.4 (CH₂, C-19), 58.6 (C-14), 56.8 (CH, C-17), 49.8 (CH, C-9), 44.3 (C-10), 42.7 (CH₂, C-4), 42.4 (CH, C-8), 42.4 (C-13), 41.5 (CH₂, C-12), 37.0 (CH, C-20), 36.0 (CH₂, C-22), 34.9 (CH, C-25), 34.0 (CH₂, C-1), 32.6 (CH, C-2), 32.1 (CH₂, C-23), 29.7 (CH₂, C-16) 27.2 (CH₂, C-15), 22.9 (CH₂, C-11), 22.5 (CH₃, C-26), 22.3 (CH₃, C-27), 19.3 (CH₃, C-21), 12.6 (CH₃, C-18).

4.2.4. HIV-1 Direct Enzyme Assays

Reverse transcriptase (RT) inhibitory activity of the crude extracts against a purified recombinant HIV1-RT (Merck, Darmstadt, Germany) was determined by using the Roche Diagnostics (Mannheim, Germany) colorimetric kit. The assay was performed as previously described [34]. HIV-1 protease enzyme was purchased from Bachem Bioscience Inc. King of Prussia, PA, UK and the substrate (a synthetic peptide that contains a cleavage site Tyr-Pro for HIV protease as well as two covalently modified amino acids for the detection of cleavage). The assay was performed according to procedures by Lam *et al.*, 2000 [39], in black 96 well assay plates obtained from Corning Incorporated, (Corning, New York, NY, USA). The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a synergy microplate spectrofluorometer (BioTek, Analytical & Diagnostic products, South Africa). Acetyl pepstatin (AP) was used as a positive control for HIV-1 PR inhibition. DMSO was included in the enzyme control (1% DMSO). Enzyme remains active after this addition as observed from the fluorescent values obtained. The percentage inhibition was calculated based on the formula: $100 - [(Test\ reagent\ RFU - background\ RFU)/(untreated\ control\ RFU - blank) \times 100]$ where RFU = relative fluorescence units.

All of the isolated compounds were screened for the inhibition of HIV-1 enzymes at 100 µg/mL. IC₅₀ values of the most active extracts were calculated and compared to known HIV-1 PR and HIV-1 RT inhibitors as well as the untreated enzymes activities. All the experiments were repeated three times in order to ensure the precision and accuracy of the data.

4.2.5. Cytotoxicity

All cell lines, media, trypsin-EDTA, fetal bovine serum (FBS), phosphate buffer saline (PBS) and antibiotics were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, South Africa).

HeLa, U937, and Vero cell lines were maintained in culture flasks containing Eagle's Minimum Essential Medium supplemented with 10% heat-inactivated FBS and 1% antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL fungizone). The cells were grown at 37 °C in a humidified incubator set at 5% CO₂. Cells were subcultured after they formed a monolayer on the flask. The cells were detached by treating them with trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 minutes and then by adding complete medium to inhibit the reaction. Cytotoxicity was measured by the XTT method using the Cell Proliferation Kit II. The method described by Zheng *et al.* 2001, was used to perform the assay. Cells were seeded (100 µL) in a 96-well microtiter plate (concentration 1 × 10⁵ cells/mL). The plate was then incubated for 24 h at 37 °C and 5% CO₂ to allow the cells to attach to the bottom of the wells. The compounds were each prepared to a stock solution of 20 mg/mL and added to the microtiter plate. Serial dilutions were made to range from a concentration of 0.7-100 µg/mL for each compound. The microtiter plate was incubated for a further 72 h. The control wells included vehicle-treated cells exposed to 2% DMSO and the positive control Actinomycin D, with concentrations ranging between 0.5 µg/mL and 0.002 µg/mL. After the 72 h incubation period, the XTT reagent (50 µL) was added to a final concentration of 0.3 mg/mL and the plate was then further incubated for another 2 h. After the incubation the absorbance of the color complex was read at 490 nm with a reference wavelength set at 690 nm. Mean IC₅₀ is the concentration of compound, which reduces cell growth

by 50% under the experimental conditions and is the average of at least three independent reproducible statistically significant measurements. The IC₅₀ values were reported at ±95% confidence intervals (±95% CI). The results were analyzed using the Graph Pad Prism 4 program.

4.2.6. Real-Time Cell Electronic Sensing (RT-CES) xCELLigence

The xCELLigence system was used according to the instructions of the supplier (Roche Diagnostics, Mannheim, Germany and ACEA Biosciences San Diego, CA, USA). HeLa cell line, media, trypsin-EDTA, fetal bovine serum (FBS), phosphate buffer saline (PBS), and antibiotics were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, South Africa). xCELLigence system was used according to the instructions of the supplier Roche Diagnostics, 2008. Mannheim, Germany and ACEA Biosciences San Diego, CA, USA).

The electronic impedance of sensor electrodes that connect the E plate to the instrument is measured to allow monitoring and detection of physiological changes of the cells on the electrodes. The impedance measured between electrodes in an individual well depends on electrode geometry, ion concentration in the well and whether or not cells are attached to the electrodes. In the absence of cells, electrode impedance is mainly determined by the ion environment, both at the electrode/solution interface, and in the bulk solution. In the presence of cells, cells attached to the electrode sensor surfaces will act as insulators and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance. Thus, the more the cells grow on the electrodes, the larger the value of electrode impedance. This assay has been described for the measurement of cytotoxicity (Wan *et al.*, 2001 and Atienza *et al.*, 2005) and can be used to determine other cellular parameters such as cell proliferation, cytotoxicity start time, cell recovery, and cell response patterns (Giaever *et al.*, 1993) in real time.

HeLa cells were grown and expanded in tissue-culture flasks. After reaching ~75% confluence, the HeLa were washed with PBS, and afterwards detached from the flasks by a brief treatment with trypsin. Subsequently, 50 µL of cell culture media at room temperature was added into each well of E-plate. After this the plate was connected to the system and checked in the cell culture incubator for proper electrical-contacts and the background impedance was measured over 24 h. Meanwhile, the cells were re-

suspended in cell culture medium and adjusted to 2×10^5 , 1×10^5 , 5×10^4 , and 2.5×10^4 cells/mL. 100 μ L microliters of each cell suspension was added to the 50 μ L medium containing wells on the E-plate, in order to determine the optimum cell concentration. After 30 min incubation at room temperature, E-plates were placed into the cell culture incubator. Finally, adhesion, growth, and proliferation of the cells was monitored every 30 min for a period of up to 18 h via the incorporated sensor electrode arrays of the E-Plate. The electrical impedance was measured by the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed Cell index (CI). After determining the optimal concentration for HeLa cells proliferation, cells at the optimal concentration were seeded in 100 μ L in each well of the E-plate, the proliferation, attachment, and spreading of the cells was monitored every 30 min by the xCELLigence system. Approximately 30 h after seeding, when the cell index was 1.5 (in the log growth phase), cells were then treated with the compounds at the IC_{50} obtained by XTT. The positive control Actinomycin D, with concentrations ranging between 0.5 μ g/mL and 0.002 μ g/mL, was also tested and just cells growing in the culture medium were included as (untreated cells) or negative control. All experiments were run for 72 h after adding the compounds. All calculations were obtained using the RTCA-integrated software of the xCELLigence system.

4.2.7. Molecular Docking

For the purpose of lead optimization and to find out the interaction between the compound and the HIV-1 protease receptor, molecular modeling calculations and local docking were done using MOE. This was done to evaluate the binding free energies of this inhibitor into the target HIV-1 protease receptor, and to find out interactions between ligand and receptor, also, to compare affinities of the isolated bioactive compounds to the target HIV-1 protease receptor. For the docking calculations, the protein structure (PDB code: 5HVP) (Fitzgerald *et al.*, 1990) was first separated from the inhibitor molecule and refined using molecular minimization with added hydrogen.

Docking calculations were carried out using standard default variables for the MOE program. The binding affinity was evaluated by the binding free energies (S-score, kcal/mol), hydrogen bonds, and RMSD values. The compounds were docked into the same groove of the binding site of the native co-crystallize ligand. The Dock scoring was

performed with the London dG scoring function and has been enhanced using two different refinement methods, which were capable of sampling conformational changes in the backbone structure. We allowed rotatable bonds; the best 10 poses were retained and analyzed for the binding poses best score. Energy minimization was done through Force-field MMFF94x Optimization using gradient of 0.0001 for determining low energy conformations with the most favorable (lowest energy) geometry.

4.3. Results and Discussion

4.3.1. Bioassay guided isolation

When screening several Red Sea marine organisms for biological activity, an ethyl acetate fraction of *L. arboreum* demonstrated very strong cytotoxicity in U937 (IC_{50} 6.5 ± 2.3 $\mu\text{g/mL}$) and moderate cytotoxicity (IC_{50} 28.1 ± 1.2 $\mu\text{g/mL}$) in HeLa cell lines. The fraction also showed strong HIV-1 PR inhibitory activity (IC_{50} 12 ± 1.3 $\mu\text{g/mL}$). These results provided justification for further chemical investigation of the lipophilic extract. Activities of the extract were characterized as discussed in Le Roux *et al.*, 2011.

The chromatographic process yielded 20 major fractions. The cytotoxicity of the extract in U937 cells was used to direct the bioassay-guided fractionation. U937 is one of the most resistant cancer cell lines. Anti-cancer drugs and HIV treatment are related since nucleotide analogs can be used for the treatment of both medical conditions making it possible for the same extracts to contain compounds active against both. Figure 4.1 shows that, between the twenty fractions, only fractions 5–8, 12, 14, 15, and 20 were 100% cytotoxic in U937 cell line when tested at a concentration of 100 $\mu\text{g/mL}$. Fractions 16–19 did not show any toxicity in the cells.

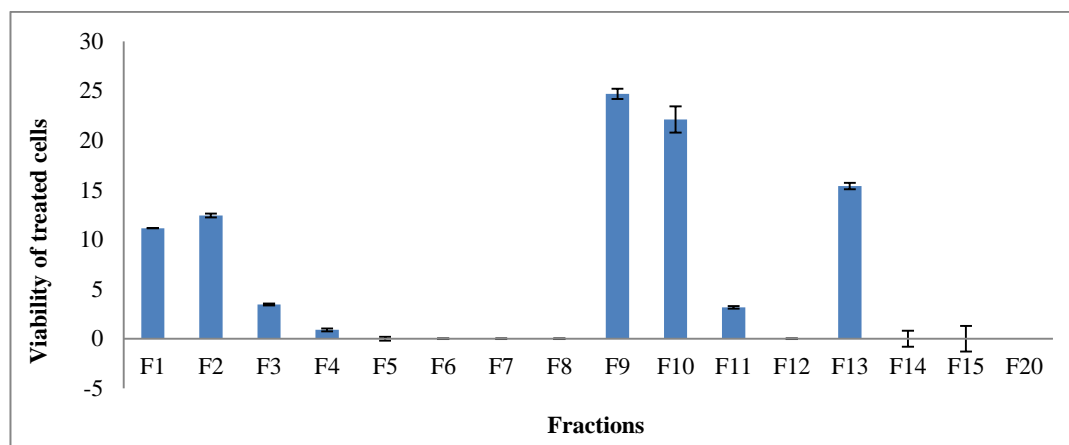


Figure 4. 1: Cytotoxicity of *L. arboreum* main fractions tested in U937 cells at 100 µg/mL.

4.3.2 Compounds isolation and structure elucidation

Further fractionation and purification of the active fractions resulted in the isolation and identification of nine known compounds (for which the structures are provided in Figure 4.2) sarcophytol M (**1**) isolated for the first time from this organism (Pardhy and Bhattacharya, 1978), alismol (**2**) (Oshima *et al.*, 1983 and Penga *et al.*, 2003), 24-methylcholesta-5,24(28)-diene-3 β -ol (**3**) (Ahmed *et al.*, 2006), 10-*O*-methyl alismoxide (**4**) (Penga *et al.*, 2003 and Jin *et al.*, 2012), alismoxide (**5**) (Oshima *et al.*, 1983 and Penga *et al.*, 2003), (*S*)-chimyl alcohol (**6**) (Chao *et al.*, 2007), 7 β -acetoxy-24-methylcholesta-5,24(28)-diene-3, 19-diol (**7**) (Faheem *et al.*, 2012), erythro-*N*-dodecanoyl-docosasphinga-(4*E*,8*E*)-dienine (**8**) (Chebaane *et al.*, 1986), and 24-methylcholesta-5,24(28)-diene-3 β ,7 β ,19-triol (**9**) (Bortolotto *et al.*, 1976).

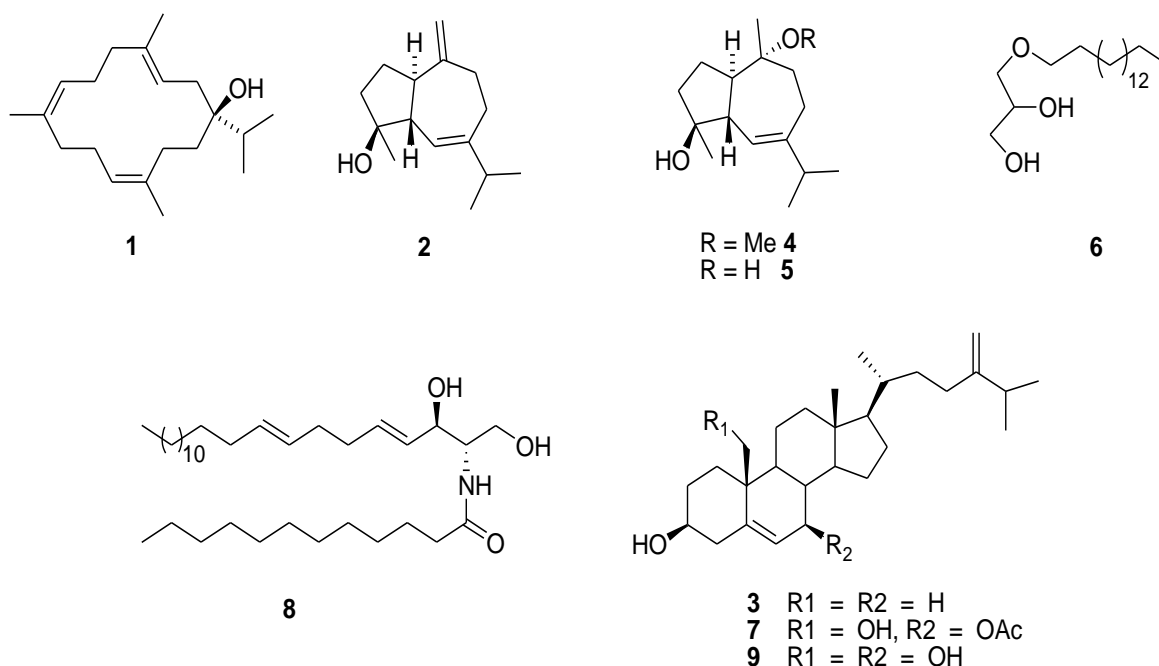


Figure 4. 2: Chemical structure of *L. arboreum* isolated compounds.

Compound **1** known as sarcophytol M (or serratol), was isolated for the first time in a high yield from *Boswellia serrata* (Pardhy and Bhattacharya, 1978) and it showed activity against *Trypanosoma brucei* and *Plasmodium falciparum* (Jin *et al.*, 2012). Compounds **2**, **4**, and **5** are rare metabolites, identified as active constituents found in extracts from *Alismatis* rhizome (Pardhy and Bhattacharya, 1978; Penga *et al.*, 2003 and Jin *et al.*, 2012). Compound **2** was found to inhibit the vascular contraction of rabbit thoracic aorta through increasing Ca²⁺ retention (Yamahara *et al.*, 1986 and Matsuda *et al.*, 1987). It demonstrated antihypertensive potential (Yamahara *et al.*, 1989), and showed promising inhibitory effects on INF- γ -induced nitric oxide production in murine macrophage RAW264.7 cells (Xu *et al.*, 2011). Compound **3** was previously isolated from the soft coral *Sinularia gibberosa* of Kenting coast, Taiwan but there are no reports on its biological activities. This study is the first report on the isolation of compound **4** and the second report of compounds **2** and **5** from marine resources (Grote *et al.*, 2008), and it is the first report of the HIV-1 enzyme inhibitory activities of most of these compounds, as well as the cytotoxicity in HeLa and U937 cancer cell lines.

Table 4. 1: Cytotoxic inhibitory concentrations, selectivity indexes and HIV-1 PR inhibitory concentrations of the nine isolated compounds in different cell lines.

Compound	Cytotoxicity IC ₅₀ (µM)			SI Values		IC ₅₀ (µM)
	HeLa	Vero	U937	HeLa	U937	HIV-1 PR
1	27.5 ± 0.2	22 ± 0.2	31.7 ± 3.2	0.8	0.8	15.7 ± 0.1
2	30 ± 17.2	49	N/T	1.6	N/T	7.2 ± 0.7
3	48 ± 8.7	100 ± 1.2	N/T	2.1	N/T	N/T
4	38 ± 0.7	49.8 ± 0.5	50 ± 0.2	1.3	1.00	N/T
5	>100	>100	>100 ± 0.04	N/A	N/A	N/T
6	23.4 ± 5.8	60 ± 1.1	N/T	2.6	N/T	26.6 ± 2.6
7	5.3 ± 0.6	31.3 ± 14.0	10.6 ± 0.12	7.2	2.9	4.9 ± 0.2
8	38.2 ± 0.7	>100	N/T	2.6	3	4.8 ± 0.9
9	8 ± 0.5	11.4 ± 0.04	16.4 ± 1.25	1.4	1.3	N/T
Positive control						
Actinomycin D	5.1 ± 0.1	8.8 ± 2.5	1.9 ± 0.9	1.7	4.7	
Acetyl pepstatin						8.5 ± 0.7

N/T: Not tested compounds (inhibitory % < 60% for PR inhibition and <80% cytotoxic).

4.3.2. Cytotoxicity of the compounds

The responses of the isolated compounds were characterized according to P. Prayong *et al.*, (2008), where an IC₅₀ value of <5 µg/mL for a pure compound is viewed as strongly cytotoxic. A selectivity index >3 indicates high selectivity (Mahmoud *et al.*, 2012). SI values were calculated as follows: IC₅₀ of the extract tested in the Vero cell line/IC₅₀ of the extract tested in the cancer cell. The inhibitory concentrations and selectivity indices of the isolated nine compounds shown in Table 4.1 showed that, steroids (7β-acetoxy-24-methylcholesta-5-24(28)-diene-3,19-diol (**7**) and 24-methylcholesta-5,24 (28)-diene-3β,7β,19-triol (**9**) showed the highest cytotoxicity among the isolated compounds. Compound **7** demonstrated strong cytotoxicity and selectivity (IC₅₀ 5.3 ± 0.60 µM (4.3

µg/mL), SI 7.2) in HeLa cells and high selectivity with moderate cytotoxicity (IC₅₀ 10.6 ± 0.12 µM (7.8 µg/mL), SI 2.9) in U937 cells. Compound **9** showed similar results with strong cytotoxicity (IC₅₀ 8 ± 0.5 µM (3.4 µg/mL) but moderate selectivity (SI 1.4) in HeLa cells and moderate cytotoxicity (IC₅₀ 16.4 ± 1.25 µM and moderate selectivity (SI 1.3) in U937 cells.

7β-acetoxy-24-methylcholesta-5-24(28)-diene-3,19-diol (**7**) from the soft coral *Nephthea chabroli* and compound **9** from the soft coral *Litophyton virides* showed sp. comparable cytotoxic activity for tests against different human cancer cell lines; the compounds showed cytotoxicity against human prostate cancer cell line LNCaP with IC₅₀ of 15.5 and 4.9 µg/mL (Zhang *et al.*, 2003), A549 cancer cell lines (0.8, 0.7 µg/mL), HT-29 (0.9, 0.7 µg/mL), KB (0.4, 0.6 µg/mL), P-388 (0.4, 0.2 µg/mL) (Duh *et al.*, 1998) (activities for compound **7** are presented first followed by activity of compound **9**). Activity against *Mycobacterium tuberculosis* was reported for compound **9** (El Sayed *et al.*, 2009), as was an immunosuppressive action (Hu *et al.*, 1998). In addition, 24-methylcholesta-5, 24(28)-diene-3β, 7β, 19-triol (**9**) showed preventive action against lipid peroxidation when mixed with different tissue homogenates *in vivo* (Xu *et al.*, 1997). The changing of functional groups of compounds **7** and **9**, especially at C-19 in addition to the 5-ene B ring, which is normally observed in an 8β, 9 α-half-chair conformations (Duax *et al.*, 1980), contributed strongly to the final activity and the mode of interaction with the cell membranes. However, there are small differences in the activity of these compounds which stem from the blocking of the hydroxyl group at C-7 (compound **7**). Thus increases the lipophilicity character of the compound. This supports the effect of lipophilicity on the biological activities as it controls the ability of a drug to penetrate various biological membranes, tissues, or barriers and represents a primary factor in controlling the interaction of drugs with biological systems (Emil *et al.*, 2004).

Moderate cytotoxicity was shown by compound **1** (IC₅₀ 27.5 ± 0.2 µM (8.1 µg/mL), SI 0.8). The rest of the compounds showed weak cytotoxicity (>10 µg/mL) compared to Actinomycin D IC_{50S} (5.1 ± 0.1 µM for HeLa, 8.8 ± 2.5 µM for Vero, and 1.9 ± 0.87 µM for U937).

4.3.2.1. Real Time Cell Analysis

Compound cytotoxicity was investigated further by confirmatory assays in HeLa cells, involving no-label real time cell analysis. Figure 4.3 shows the real time cell analyses for compounds **7** and **9**, which confirmed their cytotoxic effects when tested at IC₅₀s obtained by the end point assay XTT in the same cell line (4.3 μ M and 8 μ M, respectively).

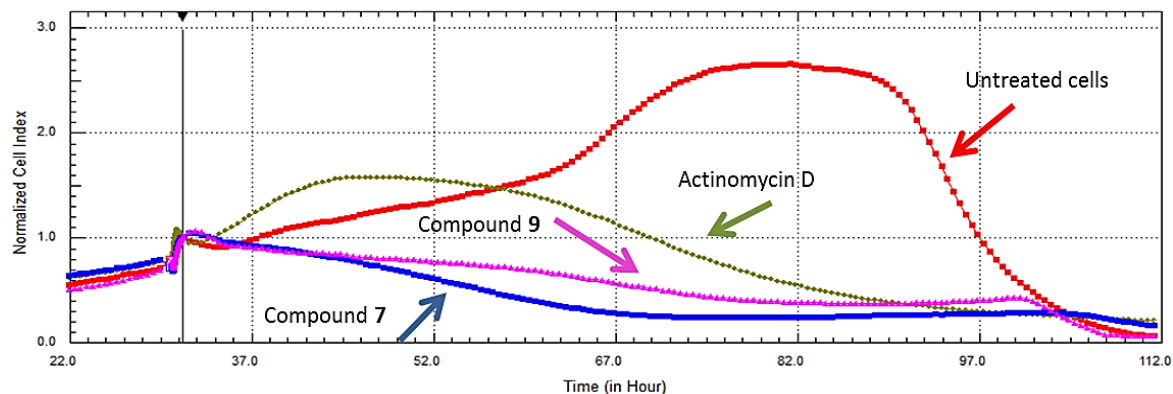


Figure 4. 3: Comparison of RT-CES data of compounds7 and 9.

7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3,19-diol (**7**) and 24-methylcholesta-5,24(28)-diene-3 β ,7 β ,19-triol (**9**) showing cytotoxicity profiles in HeLa cells. The compounds were more toxic than the positive control, Actinomycin D.

Further dose effects of compound **7** in HeLa cells (shown in Figure 4.4) were evaluated by testing the compound's cytotoxic effect at 10, 5, and 2.5 μ g/mL (13.4, 6.73, 3.36 μ M). At 10 and 5 μ g/mL, the compound initially underwent an uptake phase and then presented a dose dependent cytotoxic response for the duration of the experiment. While at a lower concentration (2.5 μ g), the cells maintained a dose dependent cytostatic response for the duration of the experiment. Cytostatic drugs do not kill cancer cells but instead stop cells from proliferating. This is the first report of the promising selectivity and cytotoxicity of compound **7** for which data was collected using a viability dye and confirmed with RT-CES; this holds a great promise for use of this compound in chemopreventive and chemotherapeutic strategies.

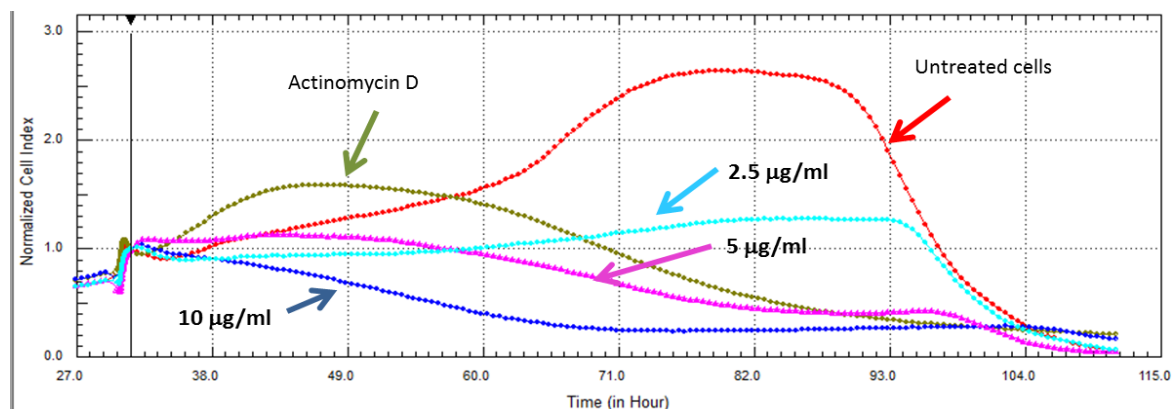


Figure 4. 4: The effect of compound 7 on the proliferation of HeLa cells using RT-CES analysis.

The compound displayed a dose dependent cytotoxic tendency at the IC₅₀ obtained by *in vitro* viability assay, and twice that amount. The lowest concentration, half of IC₅₀ (2.5 µg/mL), displayed cytostatic effects when compared to the untreated cells grown in culture medium only. Actinomycin D (5.6 µM) was used as a positive control for cell death.

Alismol (**2**) exhibited moderate cytotoxicity while Alismoxide (**5**) showed no toxicity *in vitro*, but data collected with xCELLigence showed the cytostatic effect of the compounds when tested at 30 µg/mL and 100 µg/mL.

As shown in Figure 4.5, it is clear that the compounds inhibited 50% of the cell proliferation compared to the untreated cells. The RT-CES diagram shows uptake of the compounds by the cells during the first h of incubation, later untreated cells proliferate (higher cell indices—CI) while cells incubated with compounds **2** and **5** do not show an increased CI, which can be interpreted as these compounds being cytostatic (anti-proliferative). This result supports the fact that RT-CES data is more reliable as it is not subjected to the shortcomings of the end point assays, such as sensitivity to environmental conditions or dependence on the cells' metabolism of formazan (Fonteh *et al.*, 2011).

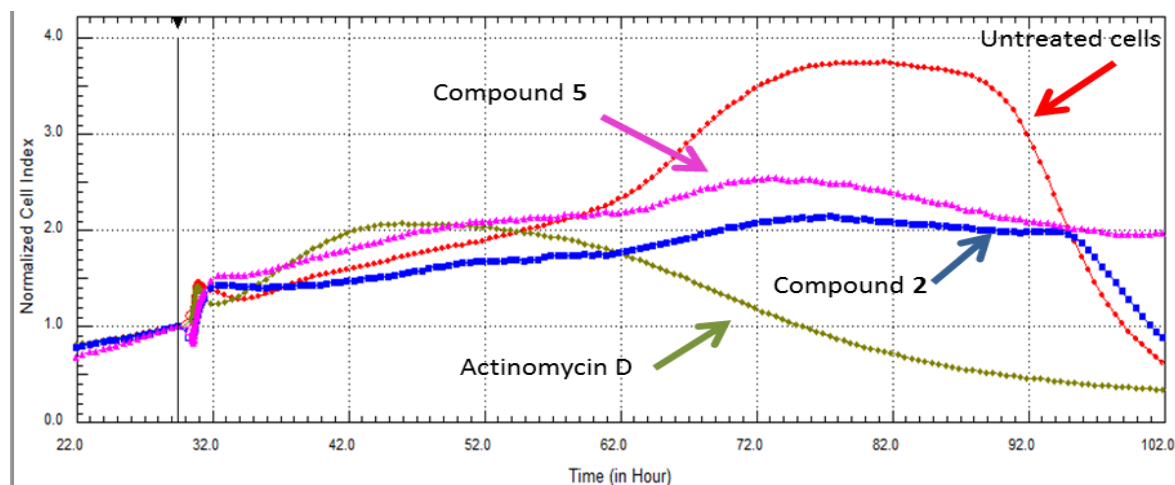


Figure 4. 5: The effect of compound 2 at 16 $\mu\text{g}/\text{mL}$ and compound 5 at 60 $\mu\text{g}/\text{mL}$ in HeLa cells.

The effects were compared to the untreated cells (culture media only) and the positive control Actinomycin D. The compounds demonstrated cytostatic behavior (in the presence of these compounds, cells did not proliferate, nor did they die).

4.3.3. Inhibitory Activities of HIV-1 Enzymes

4.3.3.1. HIV-1 Enzymes direct assays

The isolated compounds were screened for their inhibitory activity against HIV-1 RT and HIV-1 PR enzymes at 100 $\mu\text{g}/\text{mL}$. None of the compounds inhibited the HIV-1 RT enzyme, while, as shown in Figure 4.6 for HIV-1 PR, compounds Sarcophytol M (1), Chimyl alcohol (6) and Erythro-*N*-dodecanoyl-docosasphinga-(4*E*,8*E*)-dienine (8) showed 100% inhibition. Compound 2 showed inhibition of 96.2% while 7 demonstrated moderate inhibition of 71.5%.

The fifty percent inhibitory concentrations (IC_{50} s) were determined only for the compounds that inhibited more than 50% of the enzyme activity. The efficacy of the compounds was defined as potent for compounds with $\text{IC}_{50} < 5.6 \mu\text{M}$ as described by Cheenpracha, *et al.*, 2006 (Cheenpracha *et al.*, 2006), weak compounds with IC_{50} s $> 10 \mu\text{M}$ as described in Marastoni *et al.*, 1997 (Marastoni *et al.*, 1997).

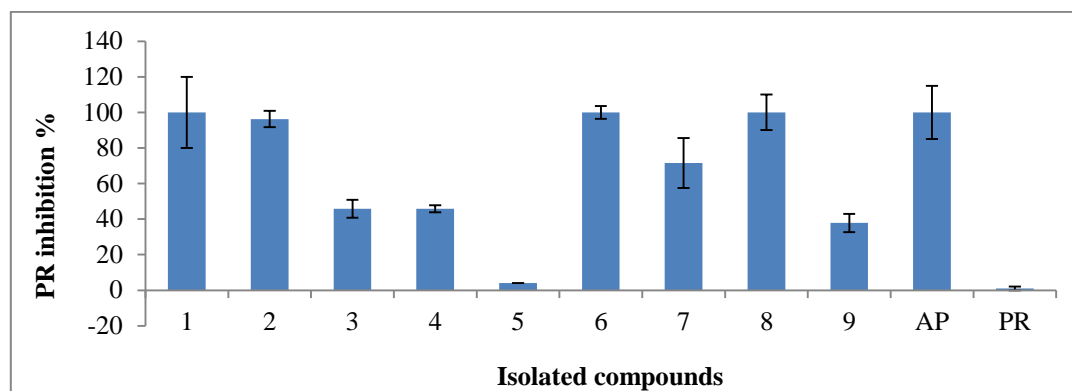


Figure 4. 6: Protease inhibition of *L. arboreum* isolated compounds at 100 µg/mL.

Fractions are indicated by number while the known inhibitor used as positive control, acetyl pepstatin is abbreviated AP.

The detailed IC_{50} results expressed in Table 4.1, showed potent inhibitory activity of HIV-1 PR by compounds **7** ($4.85 \pm 0.18 \mu\text{M}$), and **8** ($IC_{50} 4.80 \pm 0.92 \mu\text{M}$) compared to the positive control, which had IC_{50} of $8.5 \pm 0.72 \mu\text{M}$. Compound **2** showed moderate inhibitory activities with IC_{50} ($7.2 \pm 0.70 \mu\text{M}$). Sarcophytol M (**1**) and the Chimyl alcohol (**6**) showed weak inhibitory activities ($15.7 \pm 0.103 \mu\text{M}$ and $26.6 \pm 2.6 \mu\text{M}$).

The cleavage of host cell proteins by HIV-1 PR results in cell death via several necrotic and apoptotic pathways, possibly leading to depletion of CD4 + T-cells. Thus, protease inhibitors are particularly useful, not only because they inhibit viral replication but also because they rescue host immune cells (Yang *et al.*, 2012). Potent PR inhibition were found in our bioassay for some of the tested compounds, compound **7** demonstrated activity at ($IC_{50} 4.85 \mu\text{M}$). At this concentration, the compound showed 50% cell death in HeLa and 20% cell death in U937 cells while it had no effect in Vero (normal cells) as the IC_{50} in these cells was $31.3 \mu\text{M}$. On the other hand it was very interesting to find that, compound **8** (erythro-*N*-dodecanoyl-docosasphinga-(4*E*,8*E*)-dienine) was not toxic in any of the tested cell lines with IC_{50} s $71 \mu\text{M}$ in HeLa, $>100 \mu\text{M}$ in Vero cell lines and $>100 \mu\text{M}$ in U937 and its effects in HeLa cells was confirmed with real time cell analysis as shown in Figure 4.7. Cells treated with a very high concentration (100 µg/mL) of compound **8** were only affected at long incubation times compared to the untreated cells. The mechanism of action of compound **8** is not known yet. The compound could be a very promising candidate for designing new lead compounds

targeting HIV-1 infection, as the lipid rafts of the host cells primarily consist of sphingolipids and cholesterol and these have been implicated in the infectious route of HIV-1 entry. As lipid rafts are recognized sites for budding and entry of HIV-1, modulating the composition/structure of lipid rafts can influence the life cycle of HIV-1 inhibiting its replication (Verma *et al.*, 2009).

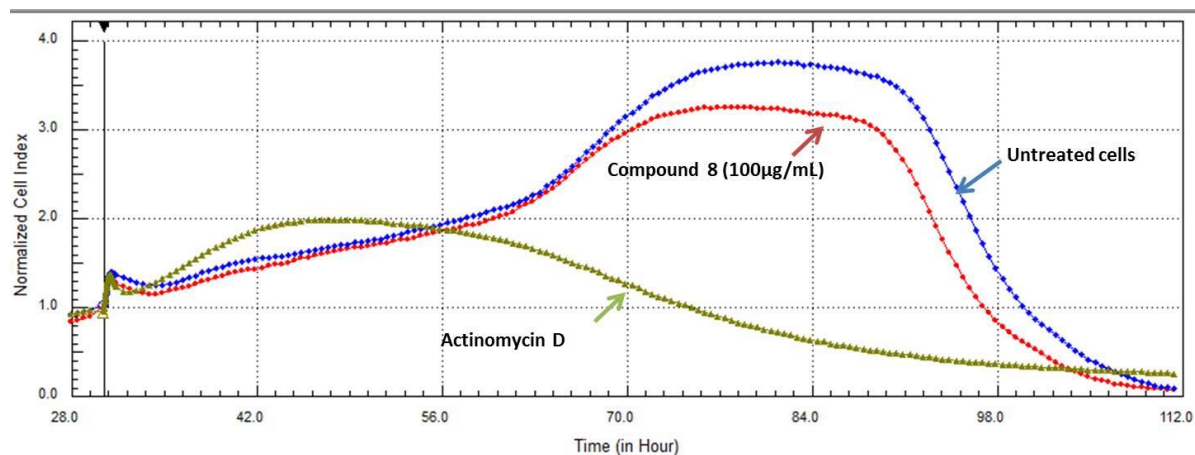


Figure 4. 7: The effect of the active compound 8 on the proliferation of HeLa cells using RT-CES analysis.

Cells treated with a high concentration (100 µg/mL) of compound 8 showed low level cytotoxicity compared with the untreated cells grown in culture medium only and the positive control Actinomycin D.

4.3.3.2. Molecular Docking

In the 3D stereo-diagram of acetyl pepstatin bound to the HIV-1 protease active site shown in Figure 4.8, the X-ray structure clearly reveals that most of the amino acid moieties surrounding the inhibitor are hydrophobic. The figure was based on the X-ray crystal structure 5HVP available from the Protein Data Bank. The moieties that make up these pockets (among others) are Asp 25, Gly 27, Asp 29, Gly 48, Ile 50, and Thr 80, in each monomer, and as seen from the green color in the figure, which indicate the hydrophobic nature of the receptor pocket.

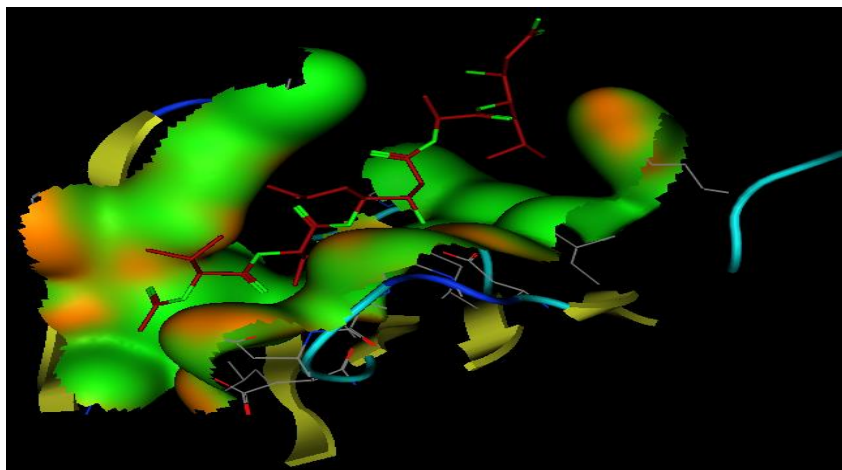


Figure 4. 8: 3D Stereo-diagram of the ligand “acetyl pepstatin” combined with the HIV-1 protease receptor (5HVP) active site.

The interaction of the ligand and the bioactive compounds (1, 2, 6, 7, and 8) Shown in Figure 4.9. The ligand (acetyl pepstatin) interacts through different hydrogen bonds with different amino acid moieties as shown in Table 4.2 and Figure 4.9 and scoring -24.79 . In addition, Table 4.2 also shows the interaction mode of bioactive compounds in the active site with different amino acid moieties with the corresponding measured bond distances. Interestingly, the compound containing an alkyl and an alkenyl chain gave a higher score, e.g., compound 6 (with long hydrocarbon chain) scored -17.16 followed by compound 8 (containing two fatty chains) scored -15.92 and compound 1 (with a lipophilic backbone containing only a hydroxyl group) scored -14.44 . In addition, this trend is very clear from the higher activity of compound 7 when compared to compound 9, which had only an extra acetyl group and a more lipophilic character. The data above indicated the importance of the hydrophobic part of different ligands when interacting in the binding pocket, and confirms the contribution of the hydrophobicity of inhibitors on HIV protease. Surprisingly, most of the nucleoside drugs in use are hydrophilic. Results presented here support the existing treatment strategies of using a combination of (hydrophilic and hydrophobic) drugs in the treatment of HIV.

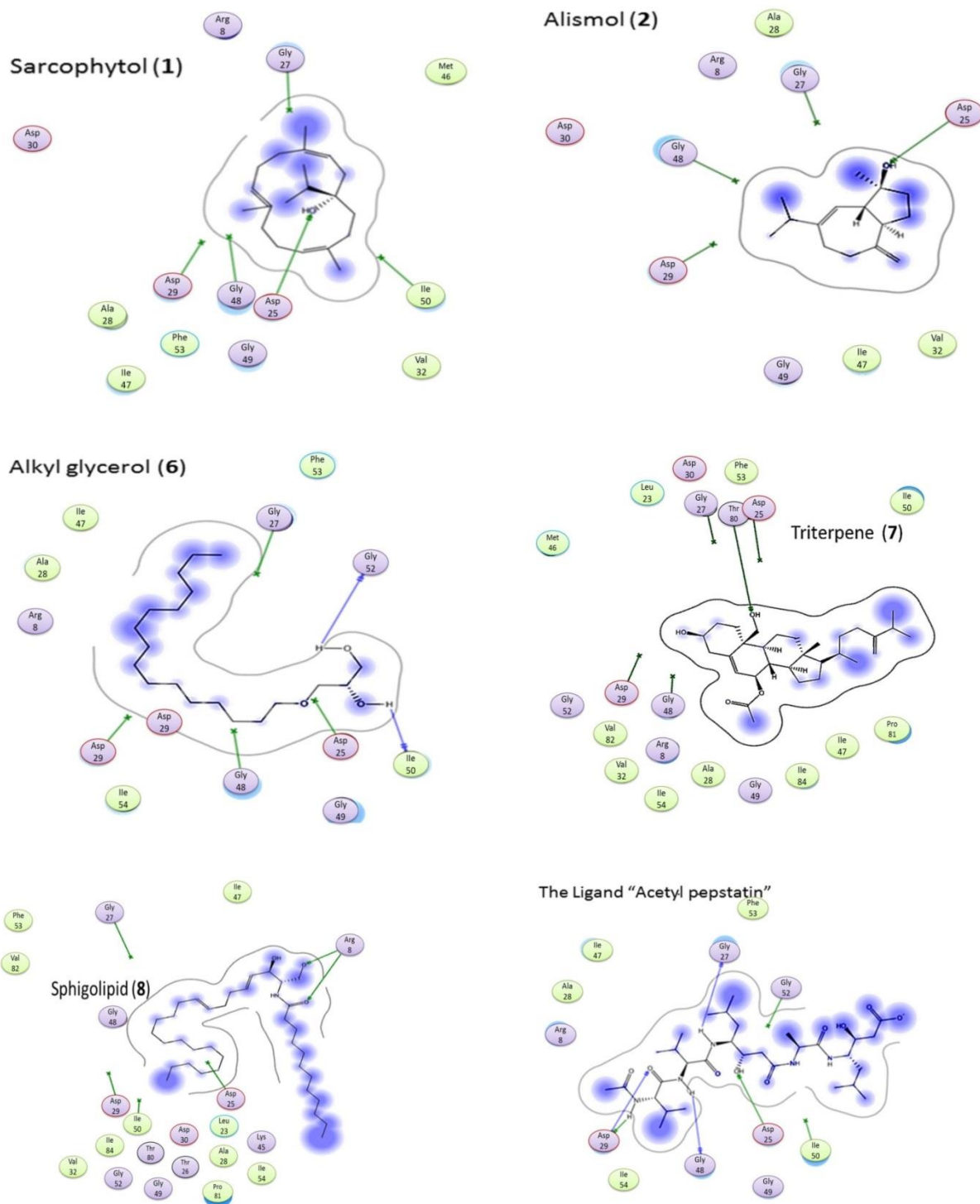


Figure 4. 9: Interaction of bioactive compounds with different amino acid moieties in HIV-1 protease receptor (5HVP) active site.

Table 4. 2: Hydrogen bond types and scores of bioactive compounds interacting with HIV-1 protease receptor (5HVP) active site.

Ligand/Compound	Residue	Type	Distance	Score
Ligand	ASP 25	H-don	1.99	-24.79
	ASP 25	H-don	2.72	
	GLY 27	H-don	2.16	
	ASP 29	H-don	1.95	
	GLY 48	H-don	1.94	
	ASP 25	H-acc	1.99	
	ASP 25	H-acc	2.72	
	ASP 29	H-acc	2.81	
	ASP 25	H-don	2.45	
	ASP 25	H-acc	2.45	
1	ASP 25	H-don	2.37	-11.14
2	ASP 25	H-acc	2.37	
6	ILE 50	H-don	1.50	-17.16
	GLY 52	H-don	1.65	
7	THR 80	H-don	2.75	-18.36
	THR 80	H-acc	2.75	
8	ARG 8	H-Acc	2.61	-15.92
	ARG 8	H-acc	3.01	

4.4. Conclusions

In this study, we report for the first time, the isolation, identification and biological evaluation of metabolites from *L. arboreum* collected from Sharm El sheikh (Red Sea). *L. arboreum* extract collected from the southern part of the Red Sea showed more promising cytotoxic metabolites than that of the same organism collected from the South China Sea (Li *et al.*, 1994). A possible reason for this could be spatial and temporal variations in secondary metabolites from organisms from different geographical regions. Potencies of the metabolites produced by an organism may also differ based on the

stress experienced by the organism in a particular environment (Fitzgerald *et al.*, 1990). This report is also the first to provide data on *L. arboreum* extract and the purified compounds demonstrating anti HIV-1 activities where some of the isolated metabolites exhibited potent anti-HIV-1 protease activity with low or no cytotoxicity. Some of the isolated metabolites demonstrated cytostatic activities, which suggest further investigation of their use in concert with anti-viral drugs where the cytostatic compound arrests cell growth while the antiviral drug inhibits viral replication. It was mostly the polyhydroxylated-isolated metabolites that showed strong cytotoxicity and high selectivity in the cancer cells used in this study. The high cytotoxicity of polyhydroxylated sterols from marine organisms is well reported and supported by data presented here and highlights the importance of this skeleton in developing potential lead compounds from marine sources. Taking into consideration the advantage of the chemical structure similarity of these compounds to the human sterols, polyhydroxysterols can be considered as potential lead compounds for the development of new, safe, and effective medicines. Further studies on this type of active compound scaffold using structure activity relationship (SAR) modeling, could lead to the production of new compounds with even better activities and drug-like properties.

The biological activities of the *L. arboreum* metabolites have not been extensively studied meaning these metabolites may yet demonstrate additional biological activities. The compounds isolated from *L. arboreum* have been isolated from terrestrial organisms as well but appear to demonstrate more varied functionalities when isolated from a marine environment. If further investigation strongly indicates clinical value, the supply of active materials will have to be addressed by synthesis rather than continued isolation from live organisms, which is why SAR studies are crucial.

Acknowledgments

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CHAPTER 5
SEASONAL VARIATION IN THE CHEMICAL CONSTITUENTS AND
ASSOCIATED BIOLOGICAL ACTIVITY OF THE SOFT CORAL
LITOPHYTON ARBOREUM

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Abstract

L. arboreum is a soft coral commonly found in the northern part of the Red Sea, for which recent studies reveals the presence of many bioactive compounds including the triterpene, 7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3, 19-diol. The effects of seasonal changes on the bioactivities of *L. arboreum* seasonal extracts collected from northern part of the red sea were evaluated. The concentration of the triterpene 7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3, 19-diol the major metabolite in *L. arboreum*, varied considerably with significant differences among seasonal samples. Implications of these variations in secondary metabolites are discussed.

5.1. Introduction

Despite more than three decades of intense interest in secondary metabolites of soft corals, and the isolation of numerous novel compounds with potent biological activity (Faulkner, 1998), little is known about within-and between-ecological variability in the levels and types of compounds in these species. Such information is important for our understanding of the factors affecting the production of chemical defenses, as well as to

provide insight into the processes that shape the evolution of chemical defenses (Harvell *et al.*, 1993).

In the marine environment, however, chemical variation has received less attention. Although several studies have reported the quantification of secondary metabolites of benthic marine organisms, the lack of information on intraspecific variation in the production of chemical defenses and its ecological consequences is notable, and the importance and need for such studies have been emphasized (Hay, 1996).

Secondary metabolites have long been known to play an important role in the ecology of plants and animals (Sondheimer and Simeone, 1970; Rice, 1984). Sammarco *et al.* (1982) suggested that, toxic compounds from soft corals serve both an anti-predator function and a role in competition for space. Terpenoids are commonly found in alcyonacean soft corals and found to be highly variable in their distribution within this order. Such secondary metabolites vary widely in type, concentration, and function. These compounds were reported to Thus far, they have been found to play roles in predator defense. The effectiveness of compounds derived from individual species also varies widely with respect to interactions with other members of the community (Paul *et al.*, 1990).

L. arboreum (Forsk., 1775) is a common encrusting alcyonacean soft coral on Red Sea reefs (Benayahu and Loya, 1977). It is 80 cm. pale Olive – green to yellow or grey with clusters of finger –like branches on tall stalks. It grows on seaward reef slopes, upright on hard bottoms and its range between red sea to the Pacific Ocean.

The chemical constituents of *L. arboreum* were evaluated before (Shaker *et al.*, 2010), but nothing was reported neither on this metabolites nor on its role in the organism biology.

In a recent study by our group, *L. arboreum* showed strong cytotoxic and the HIV-1 PR inhibitory activities and the organism's metabolites were isolated and identified, one of the isolated compounds is 7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3,19-diol (Figure 5.4) which showed the highest cytotoxic and HIV-1 protease inhibitory activities among the isolated compounds (Ellithey *et al.*, 2013).

The aim of this study was to determine the seasonal changes of *L. arboreum* cytotoxic and HIV-1 PR inhibitory activities and its relation to the triterpene concentrations in each seasonal extract as well as the influence of these results on the seasonal distribution of the organism.

This study was conducted in the period of (January 2010- November 2011). The seasons were defined in this study according to the stability of the water column, following Lindell and Post (1995). "Winter" refers to December \pm March, "spring" to April and May, "summer" to June \pm September, and "fall" to October and November.

5.2. Area of the study

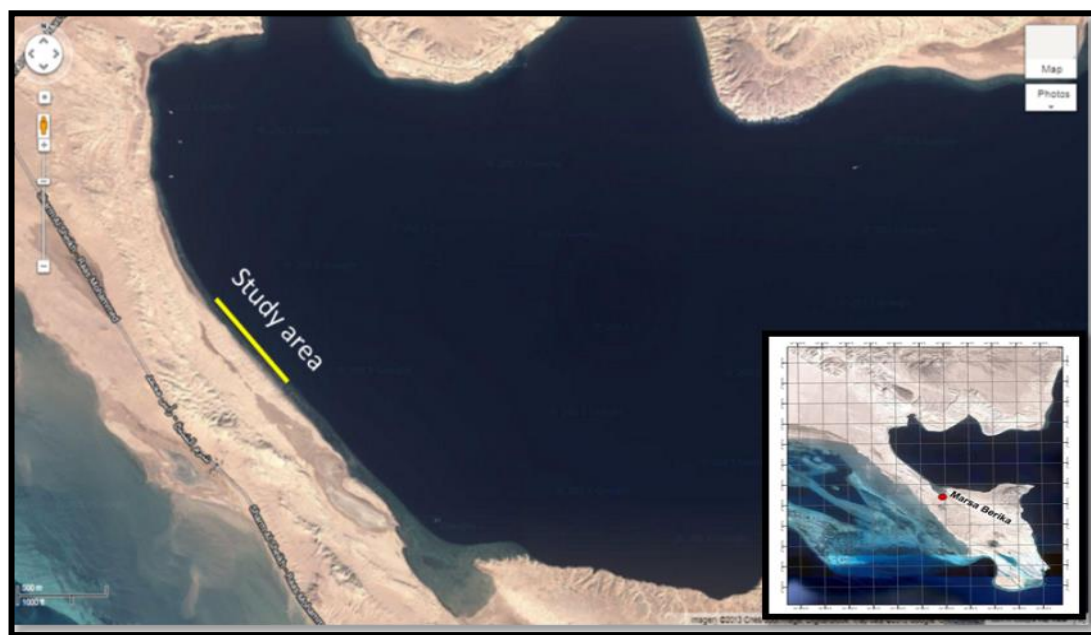


Figure 5. 1 Map shows the semi enclosed nature of Marsa Berika
Latitude: 27.774722 / Longitude: 34.2325.

The study was done along 200 m of the reef edge of Marsa Berika which is a small Gulf on the southern part of Sharm El-Sheikh, Egypt. Latitude: 27.774722 / Longitude: 34.2325. This area is semi enclosed and located in the point separating between Gulf of Aqaba and Gulf of Suez shown in Figure 5.1. This area characterized by calm water movements most of the year very large reef flat. From field observations this area also has a very strong tide in July that it could be totally exposed to air during midday and covered again with high tide at the afternoon. It also subjected to an extensive tidal range variation in winter where the low tide could last for two or three days. Seasonal water parameters and seasonal distribution of *L. arboreum* were determined. Seasonal samples were collected and identified by M. Ellithy, co-author of the article, according to coral reef guide (Red Sea to Gulf of Aden, south Oman) Ewald Lieske, 2004. Satellite pictures were obtained by Dr. A. Hassan, National center of remote sensing, Egypt.

5.3. Methods

5.3.1. Seasonal ecological factors

Ecological parameters such as temperature, pH, salinity and dissolved oxygen were seasonally recorded in the study area for two years.

The air temperature as well as the temperature of the sea water was measured during the collection of samples using a digital thermometer, the salinity of the water samples were measured using refractometer (ATAGO, Inc. USA), the dissolved), Dissolved oxygen content of the water samples was measured using Winkler method (Winkler, 1888) and pH values of the sea water was measured by pH-200: Waterproof pH Meter (HM Digital, Inc. USA).

5.3.2. Seasonal distribution of *L. arboreum*:

Quadrat – transect method was used for the seasonal quantitative assessment of *Litophyton arboreum* seasonal percentage cover over 200 m of the reef flat. The data collection was done by the use of (1mX1m) frame quadrat that divided to 25 squares (20cm each) laid along 200m transect that marked every 2m and attached from both sides to landmarks.

A snorkeling survey was done *in situ* by recording the percentage cover of the organism within each quadrat along the guiding transects. Data were analyzed using Bouchon, 1981 method.

$$\text{Percentage cover} = \frac{\text{Sum of the organism dominances of all the quadrates}}{\text{Total number of quadrates (50)}} \times 100$$

5.3.3. Sampling and Extraction

50 gm. of *L. arboreum* representative samples were collected seasonally according to sampling and preservation protocol. Fresh samples were collected from the field and kept in methanol. Samples were homogenized directly after collection with 150 ml of methanol (Merck, Germany). After filtration the solvent was distilled off under reduced pressure using a rotary evaporator (Buchi, Switzerland) and the residues re-suspended

in water and partitioned with ethyl acetate. The lipophilic fractions obtained by ethyl acetate were dried out and kept at 4 °C for further chemical and biological tests.

5.3.4. HPLC profiling

5.3.4.1. Qualitative Analysis

An Agilent 1100 HPLC system (Agilent Technologies, USA) consisted of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and ultraviolet detector coupled with an analytical workstation and Luna® C18 column, 5 µm, 150 mm × 4.6 mm i.d. (Phenomenex, Torrance, CA, USA) The HPLC coupled with evaporative light scattering detector (ELSD). Flow rate was 1.0 ml/min and sample injection volume was 10 µL. ELSD was optimized for triterpene detection with nebulization air flow at 1.1 L/min at 30 °C and evaporative chamber temperature at 30°C.

5.3.4.2. Quantitative profiling

Standard solution of the triterpene was dissolved in dimethyl sulphoxide (DMSO) with a ratio of 1 mg/ml. Different volumes were injected (2, 4, 8, 16, 32 and 64 µl), and a standard curve was established using concentration versus peak area. Stock solutions of different samples were prepared by dissolving the compound in DMSO with a ratio of 20 mg/ml. Mobile phase contained deionized water-acetic acid (A; 100:0.01, v/v) and acetonitrile-acetic acid (B; 100:0.01, v/v). The gradient elution was as follows: 30-50% B (0-10 min); 50 to 100% B (10.01 - 45.0 min); 100-100% B (45.01-60 min).

5.3.5. Cell culture and Cytotoxicity of the extracts.

Chemicals and reagents; all cell lines, media, trypsin-EDTA, fetal bovine serum (FBS) and antibiotics (penicillin, streptomycin and fungizone) were purchased from Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA).

All the seasonal extracts were tested for their cytotoxic activity using the 2, 3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, Na²) (XTT) colorimetric assay Kit II (F. Hoffmann-La Roche Ltd.). The assay is based on the ability of living cells to reduce the yellow water soluble XTT into an insoluble formazan product (Zheng *et al.*, 2001). The seasonal extracts were tested for their *in vitro* cytotoxicity at a concentration of (100 µg/ml) against HeLa and U937 cells.

Cells were maintained in culture flasks in complete medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic cocktail (100 U/mL penicillin, 100 g/L streptomycin and 250 g/L fungizone). Cells were cultured and maintained in a humidified atmosphere at 37 °C and 5% CO₂. Cytotoxicity was measured by the XTT

method using the Cell Proliferation Kit II (F. Hoffmann-La Roche Ltd.). Cells (100 μ l) were seeded (concentration 1×10^5 cells/mL) into a microtitre plate and incubated for 24 h to allow the cells to adhere. Extracts were diluted in medium (1.563–100 g/mL), added to the plates and incubated for 72 h. A positive control for cytotoxicity, XTT was added to a final concentration of 0.3 mg/mL and the cells incubated for 2–3 h. Absorbance of the developed colour was spectrophotometrically determined using a multi-well plate reader which measured the optical density at 450 nm with a reference wavelength of 690 nm. Each sample was tested in triplicate and every experiment repeated at least 3 times. The cytotoxic concentration that kills 50% of the cell population (CC_{50} values) was defined as the concentration of the sample at which absorbance was reduced by 50%. The results were statistically analyzed with Graph Pad Prism 4 soft-ware.

5.3.5. Cell culture and Cytotoxicity of the extracts

HIV-1 direct enzyme assays: HIV-1 protease enzyme (Bachem Bioscience Inc. King of Prussia, PA, UK) and the substrate (a synthetic peptide that contains a cleavage site Tyr-Pro for HIV protease as well as two covalently modified amino acids for the detection of cleavage) was initially presented by Matayoshi, 1990. The assay was performed according to procedures by Lam *et al.*, 2000 in black 96 well assay plates obtained from Corning Incorporated, (Corning, New York, USA). The fluorescence quantity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a synergy microplate spectrofluorometer (BioTek, Analytical & Diagnostic products, South Africa). Acetyl pepstatin (AP) was used as a positive control for HIV-1 PR inhibition. DMSO was included in the enzyme control (1% DMSO). Enzyme remains active after this addition as observed from the fluorescent) IC_{50} values of *L. arboreum* seasonal extracts were calculated and compared to known HIV-1 PR inhibitor.

The fluorogenic substrate of the HIV-1 PR assay was dissolved in DMSO to make a 1mM stock. The stock fluorogenic substrate was diluted to 16 μ M using assay buffer (0.1M sodium acetate, 1M NaCl, 1m MEDTA, 1mM dithiothreitol and 1 mg/mL bovine serum albumin, pH4.7). An aliquot of the substrate (16 μ M, 49 μ L) and 1 μ l of HIV-1 PR solution (1 μ g/ μ L; Bachem, Switzerland) were added to the reaction mixture in an assay buffer in the presence or absence (untreated control) of the extract to a final volume of 100 μ L (serial concentrations of 100 – 1.3 μ g/ml) of the extract. This mixture was incubated at 37 °C for 1h in Costar black 96 well fluorescence assay plates (Scientific group, South Africa). A 10 μ g/ml of acetyl pepstatin (Bachem-UK) was used as a positive

control for inhibition of HIV-1 protease. The fluorescence quantity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Synergy micro plate spectrofluorometer (BioTek, Analytical & Diagnostic Products, South Africa). A blank treatment consisted of assay buffer only. Every extract in buffer, an untreated control of enzyme and substrate was also included. Data was analyzed using the Ascent software (Biotek) and the percentage inhibition calculated based on the formula: $100 - [(Test\ reagent\ RFU / untreated\ control\ RFU) * 100]$ Where RFU= relative fluorescence units.

5.4. Results

5.4.1. Seasonal changes in the ecological factors

Table 5.1, shows the measured values of different ecological parameters under study, water temperature recorded high value in summer and spring and the lowest values in winter an autumn. The dissolved oxygen (DO) determined according to Winkler method and showed high levels in summer and autumn and low values in winter and spring. The salinity was stable with an increase in summer season. The pH values were almost constant though out the year (7.4) and increased in winter (8). This is almost a four fold decrease (generaly a change of one pH unit corresponds to a ten-fold change in hydrogen ion concentration).

$$pH = 7.4$$

$$[H+] = 10^{-pH}$$

$$[H+] = 10^{-7.4}$$

$$[H+] = 3.9 \times 10^{-8} \text{ M}$$

$$pH = 8$$

$$[H+] = 10^{-pH}$$

$$[H+] = 10^{-8}$$

$$[H+] = 1 \times 10^{-8} \text{ M}$$

All the values were still in the preferred range for coral growth.

Table 5. 1: The seasonal change in some the physic-chemical parameters of the water in Marsa Braykah (2011-2012).

Season	Salinity ppm(‰)	Air temperature °C	Water temperature °C	pH	DO mg/L
Winter	39	18	20	8	7
Spring	40	34	36	7.4	6.2
Summer	41	41	41	7.4	7.7
Autumn	40	25	23	7.4	7.7

5.4.2. Seasonal distribution of *Litophyton arboreum*

The seasonal distribution of *L. arboreum* percentage cover and the abundance densities using quadrat – transect were seasonally determined. The results (Figure 5. 2) showed that, the organism had the highest percentage cover and dominance values in summer and spring. While the organism showed the lowest distribution and dominance in winter season.

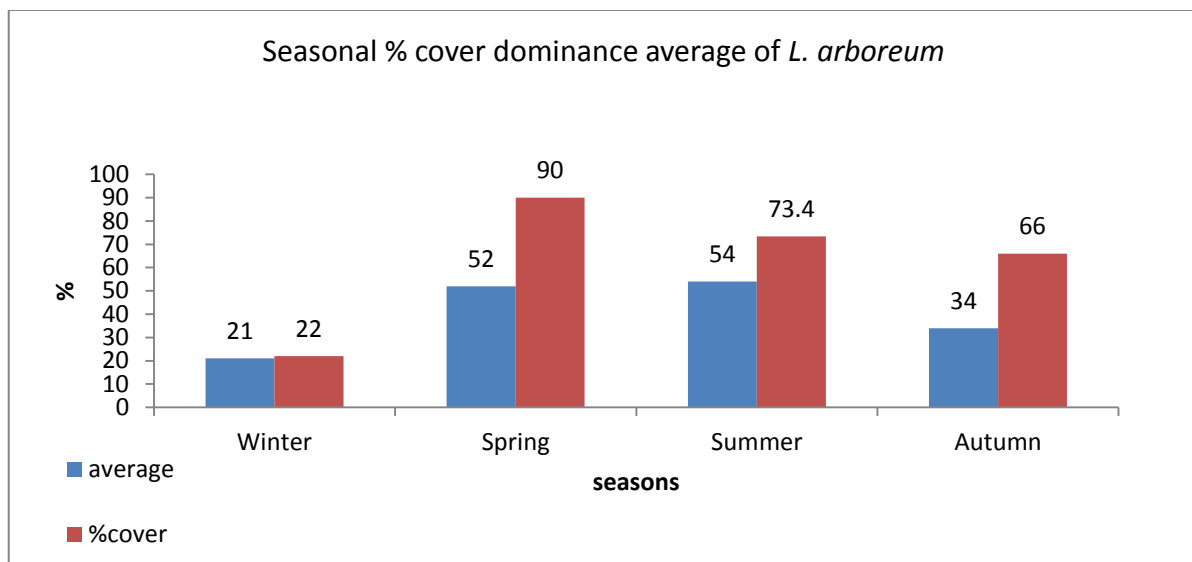


Figure 5. 2: The seasonal change in *L. arboreum* percentage cover and dominance values.

5.4.3. Biological evaluation of the seasonal extracts

The seasonal extracts were evaluated for their cytotoxic and anti HIV-1 PR inhibitory activities.

5.4.3.1. Cell culture and Cytotoxicity

The cytotoxic effect of *L. arboreum* extracts tested in HeLa and U937 cell lines at 100 µg/ml. The cytotoxicity significantly varied along seasons. As shown in Figure 5.3, the autumn extract showed the highest cytotoxicity in both cell lines tested. While in summer and spring seasons the cytotoxicity of the organism's extract decreased and was the lowest in winter extract.

5.4.3.2. HIV-1 PR inhibitory activities

Figure 5.4 shows that *L. arboreum* showed strong inhibitory of HIV-1 PR enzyme activities in all the seasonal extracts where its exhibited 100% inhibition of the enzyme, except for the winter extract where the extract lost its inhibitory activity and only inhibited less than 10% of the enzyme activity.

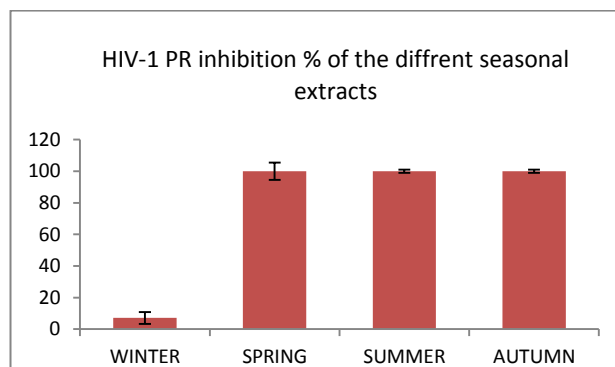
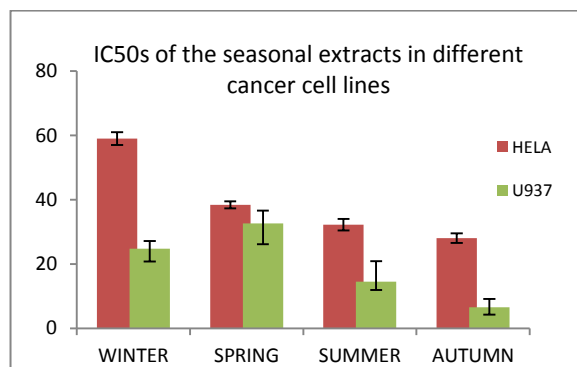


Figure 5.3: The seasonal change in *L. arboreum* total extract cytotoxic activities tested in HeLa and U937 cells at 100 µg/ml.

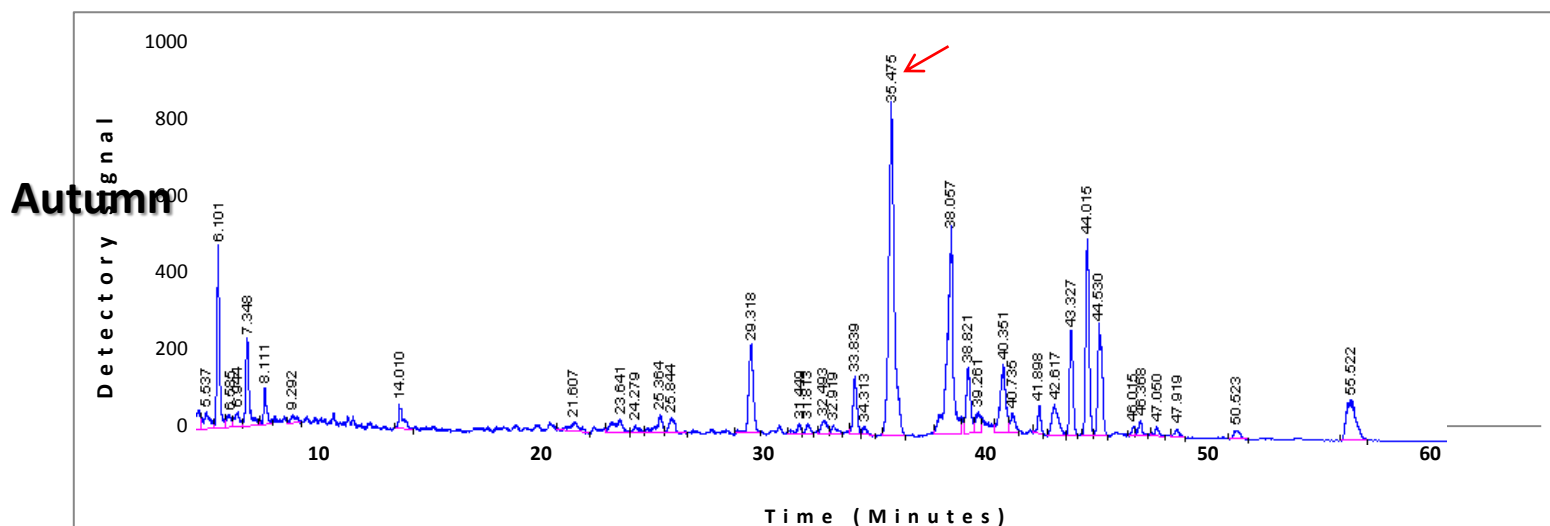
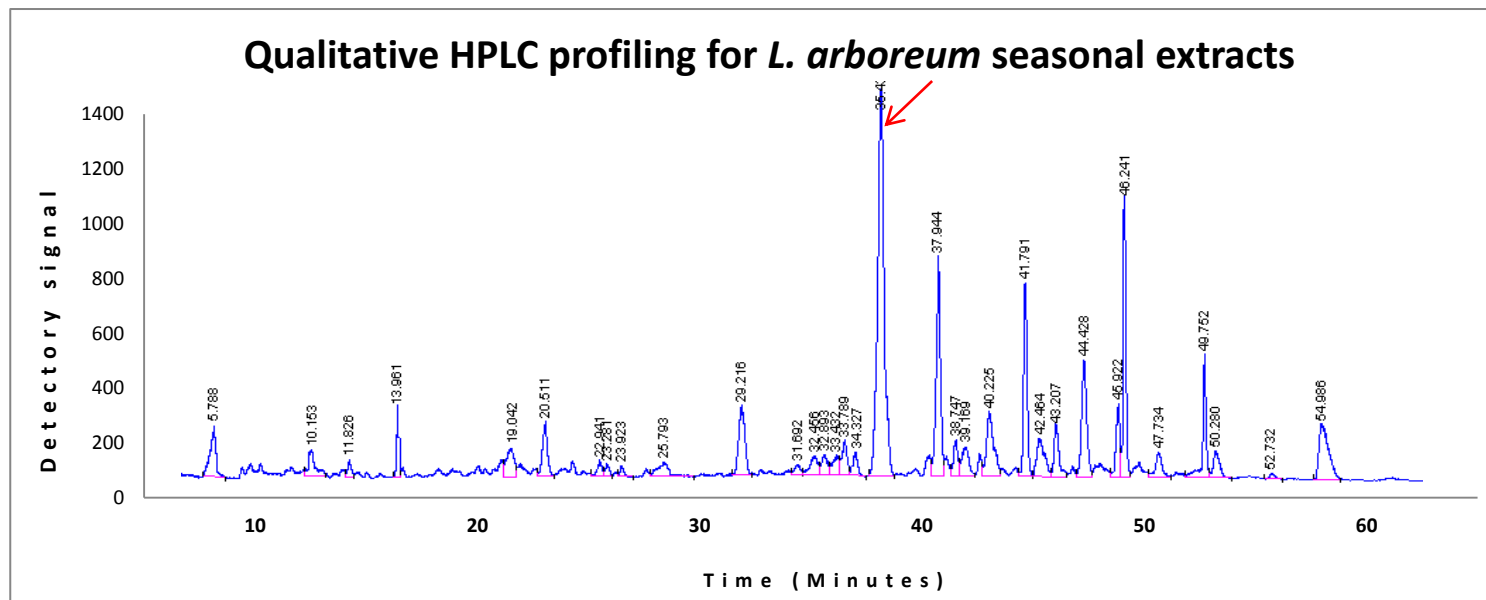
Figure 5.4: The seasonal change in HIV1-PR inhibitory activities of *L. arboreum* seasonal extracts tested at 100 µg/ml.

5.4.4. HPLC profiles

5.4.4.1. Qualitative HPLC profiling for the seasonal extracts

The qualitative HPLC profiling for the *L. arboreum* total extract showed notable differences in the compounds concentrations from season to season (Figure 5.5). These results indicate that the seasonal change in the ecological parameters can affect the compounds quantity inside the organism.

The profiles show that, the lowest quantity of the compounds was in the winter total extract while the highest quantity of the compounds found in autumn extract.



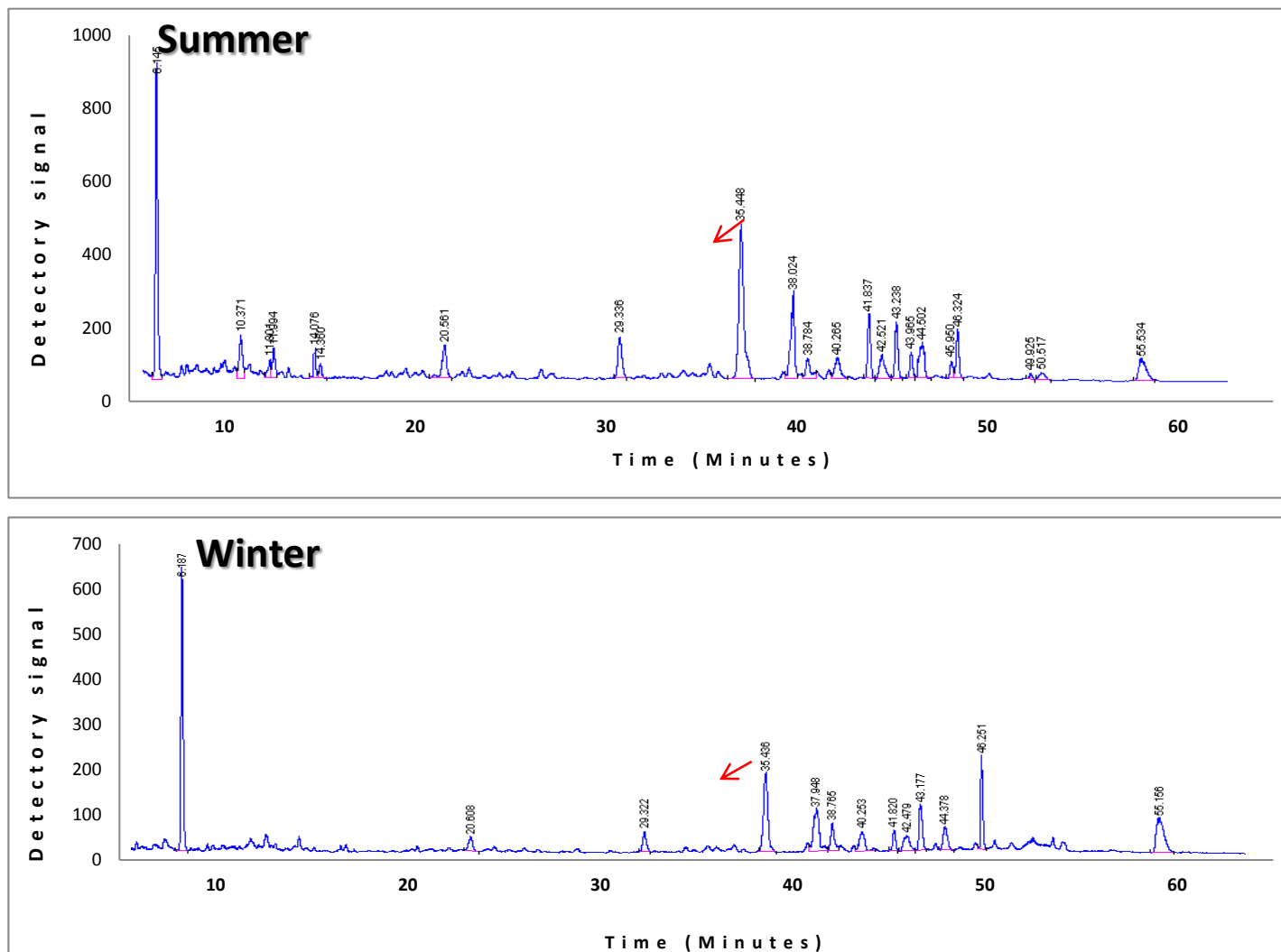


Figure 5. 5: HPLC chromatograms of *L. arboreum* total extracts collected in different seasons. Arrows pointing to the active triterpene's peaks.

5.4.4.2. Quantitative profiling of the active triterpene.

The evaporative light scattering detector (ELSD) is a general detector and recommended for compounds lacking the chromophores and cannot be detected by UV detector, also, ELSD is considered to be more advantageous considering the relative baseline instability and it showed compatibility with wide range of solvents.

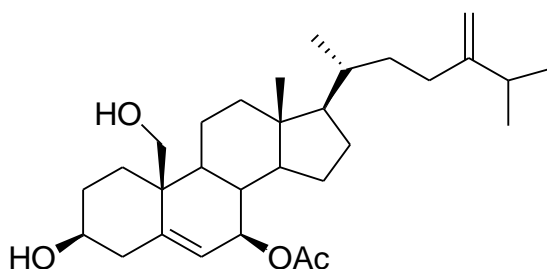


Figure 5. 6: 7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3, 19-diol

The active triterpene (Figure 5.6.) concentrations were determined by the coupling system of HPLC-ELSD which offers accurate method for determination the percentage of the triterpene. The developed method was used to determine the seasonal difference of the triterpene concentration HPLC quantitative profiling. The quantitative profiling of the compound concentration in each seasonal extract showed that the triterpene appeared at ~35.6 min. by tracking this compound at the same time, it was reported that, the compound concentrations significantly changed along seasons.

The quantity percentages of the compound were the lowest (0.264%) of the winter extract, (0.873%) of the summer extract, (0.979%) of the spring extract and the highest (2.314%) of the autumn extract.

5.5. Discussion

Marine organisms are adapted to live in specific temperature ranges as the temperature has direct effects on physiological processes such as organ function (Rahmstorf, 2003). According to our results, the major changing factor was the water temperature. In summer season (June-July), the temperature was double where it was in winter (December-January). Winter season in this area is characterized by strong wind and high water turbidity. This drastically change in water temperature has influenced some of the

other physicochemical parameters tested. The rapid change in temperature, density or salinity is referred to as a cline and it strongly affects the planktonic ecosystems and primary producers (Rahmstorf, 2003). A seasonal thermocline is formed when surface water is cooled, and sinks to the bottom resulting in a mixing of the layers. Another thermocline is created in the summer when the increase in sunlight results in less mixing and a “bloom” in phytoplankton. The increase in the number of phytoplankton dissolving inorganic nutrients causes an increase in chlorophyll biomass. Herbivorous zooplankton biomass also begins to increase, providing food for an entire food web. (Rahmstorf, 2003).

According to UNEP, 1997. Assessment of Land-based Sources and Activities Affecting the Marine Environment in the Red Sea and Gulf of Aden. Salinity is usually 35 ppt (parts per thousand), but can range from 28-41 ppt and is highest in the northern Red Sea. The salinity of the Red Sea is greater than the world average. This is due to several factors; High rate of evaporation and very little precipitation and the lack of significant rivers or streams draining into the sea as well as the limited connection with the Indian Ocean, which has lower water salinity. Seasonal salinity values recorded in our study. Table 5.1 showed the highest in summer (41 ppt) while the lowest value was in winter (39 ppt).

Our results showed lower pH values in the hot season (7.4, April - October) while it slightly increased in lower temperature (8, in winter). The reason that temperature affects water's pH is that water molecules have a slight tendency to break down into their constituents, hydrogen and oxygen, as temperature increases. As temperatures increase, a larger proportion of water molecules break up, releasing a few more hydrogen ions, this then decreases the pH of the water (Zeeber R, 1998).

The oxygen saturation concentration depends on temperature and salinity (Weiss, 1970). In addition to these conservative parameters, the dissolved oxygen concentration depends on the photosynthetic rate and subsequently on nutrient concentrations. High temperature and salinity cause the oxygen to be relatively low (Badran, 2001): the higher the temperature, the lower the solubility of oxygen in seawater. However, in our results, higher oxygen concentrations were recorded in hot seasons (April - October) than in winter season (November-January), despite the high temperatures (Table 5. 1). This may be attributed to a higher photosynthetic rate (Wheeler *et al.*, 2003) due to long day time

with sufficient strong light that represents energy source for primary producers and less mixing of water layers as discussed before. This exceptional finding also came matching with previous study by (Manasrah *et al.*, 2006). The dissolved oxygen was homogeneous in the study area; the dissolved oxygen in the present study was close to those reported in other parts of the Gulf (Badran *et al.*, 2005; Manasrah *et al.*, 2006; El-Sherbiny *et al.*, 2007).

Direct effects of changes in ocean temperature and chemistry may alter the physiological functioning, behavior, and demographic traits (e.g., productivity) of organisms, leading to shifts in the size structure, spatial range, and seasonal abundance of populations. Soft corals obtain energy from a variety of sources. Most are filter feeders, since the individual polyps are too small to catch larger prey. Most species feed on plankton and the larvae of other invertebrates. Many soft coral species contain symbiotic dinoflagellates, but some are obligate heterotrophs. The zooxanthellate species get much of their energy from photosynthesis, and can starve if the dinoflagellates die or if their light source is blocked for long periods of time (Scott *et al.*, 2012).

In this study, the distribution and the percentage cover of *L. arboreum* changed according to seasons. As presented in Figure 5.2, the lowest values were recorded in winter, which can be due to the poor water quality and less nutrients caused by insufficient light of winter short day times in this area which is important for the photosynthesis of the symbiotic zooxanthelle (Kuhl *et al.*, 1995). This was also supported by the DO results, as it was the lowest in winter as evidence of low photosynthesis. Or it can be due to the vigorous wave action caused by the wind and brief torrents in Red Sea and Gulf of Aden Region that can block the polyps or even shredder the large organisms. This came matching with our field observations, where only small patches of strong small individuals found in winter season. The poor distribution of *L. arboreum* can also be due to the unusual tidal range occurs in winter (Behairy 1992), that can totally exposes the reef flat. These factors can destroy the community and threat its survival.

In the Red Sea natural conditions, *L. arboreum* breeds in the period between July and August (Benayahu *et al.* 1992). Gametogenesis development occurs during the early spring and summer when planktonic food sources are most abundant (Hartnoll 1975). Prior to the breeding season in spring (April-June) and after the torrents season, the coral community starts to recover the stressful conditions. This period is characterized by

the increase of water temperature and the longer day- times. These factors lead to the increase of the phytoplankton levels and chlorophyll biomass which is essential for the coral feeding (Kuhl *et al.*, 1995). And *L. arboreum* individuals complete its maturation (Benayahu *et al.* 1992). This was confirmed by our results presented in Figure 5.2, as *L. arboreum* showed the highest percentage cover 90% and high abundance 52% in the spring season. According to field observations, the community showed small size and healthy colonies. As stated by (Benayahu *et al.* 1992) *L. arboreum* in this period is ready for breeding.

In summer season, water quality changes. Phytoplankton blooms forms due to the high temperatures which form high levels of nutrient. According to the field observations, the colonies were a mixture of strong and healthy small and large colonies. As supported by results obtained in this study, high percentage cover (73%) and the densest abundance (54%) were reported for this season. This also can be due to calm conditions of water movement which protect the organism and the high content of nutrients. While in autumn season (October-December), the water temperature decreased and the windy season started to develop. Despite of these stressing conditions, the percentage cover of the organism was just slightly decreased (66%). During the field observations, the *L. arboreum*'s community consisted of ruptured large colonies and healthy and strong ones.

The ecology of coral reef systems and changes in the community structure with respect to stress factors in the environment is one of the fundamental problems in environmental analyses (Morelock *et al.*, 1979). The reef community is a complex assemblage of organisms, but the response of the coral assemblage is the fundamental factor in survival or loss of the entire community under stress conditions; the resistance of the total community to environmental stresses cannot exceed the survival ability of the coral framework.

In this study, the qualitative HPLC profiling for the *L. arboreum* total extract showed notable differences in the compounds profiles from season to season (Figure 5. 3). These results indicate that the seasonal change in the ecological parameters can affect the compounds quantity of the organism.

Summer and spring's extracts showed notable increase in the compounds detector signals than winter (1200 and 1000) respectively (Figure 5.3 B and C). This can be a

result of the breeding season that occurs in this period where the organism is mature and strong, the reduction of the eggs is energetically costly as seen by Harrison and Wallace (1990). The high energy of the organism was reflected on its high distribution and wide percentage cover in these seasons. Summer season water conditions seemed to be better for the organism than spring represented in higher dissolved oxygen level (Table 5.1) that reflects the good quality of the water which resulted in the highest distribution and percentage cover of the organism in these seasons (Figure 5.2).

Autumn extract qualitative profile showed higher detector signals of the organism metabolites (1400.36) (Figure 5.3 D) followed by the summer extract. And slightly decrease in the percentage cover of only large mature individuals which could be due to the gradual decrease of water temperature and the beginning of the windy season. Despite of these ecological stresses, the autumn season showed a stable good water quality represented in high DO content.

Many soft corals (Coelenterata: Alcyonacea) possess an extensive range of unique organic molecules, the majority of which fall into the terpenoids class of compounds (Tursch *et al.*, 1978; Coll *et al.*, 1980 and 1981). A number of these marine terpenoids have been shown to be distasteful (Lucas *et al.*, 1979) or toxic (Ne'eman *et al.*, 1974; Weinheimer *et al.*, 1977). In this study, the determination of the active triterpene concentration in the seasonal extracts showed significant seasonal change. Terpenoids are known as a soft coral ant predator (coral fishes), the amount of which depends on the water quality, this came matching with the drastically decrease of the percentage cover in winter season when the compound showed the lowest concentration in the total extract which suggests that, the active triterpene (7 β -acetoxy-24-methylcholesta-5-24(28)-diene-3,19-diol) may be used by the organism as anti-predatory metabolite and it also explains the high percentage covers of the organism in the hot seasons, where the active triterpene showed higher concentrations. This compound could also be vital for the coral reproduction as suggested by (Sammarco and Coll, 1990), this fact can give a better explanation for the maximum concentration of the compound found in autumn extract which could be due to the oocyte maturation (Yamazato *et al.*, 1981). It also explains the high concentrations of the compound in spring and summer when the organism breed, as it found to be selectively concentrated in the organism oocytes (Benayahu *et al.* 1992). The lowest concentration of the compound in winter extract

could be due to that this extract was mostly composed of just the immature individuals found in the community due to the damage of the adult ones. The biological evaluation of the seasonal extracts was directly proportional to the triterpene concentration, the highest activities was reported for autumn extract where the highest concentration of the compound was found followed by summer and spring extracts and the lowest activities were reported for the winter extract where the lowest concentration of the extract was reported. This could suggest that the triterpene 7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3, 19-diol) was the compound that responsible for the extract activities.

5.6. Conclusions

This study demonstrated the seasonal change in the biological activities of the soft coral *L. arboreum* seasonal extracts where it was the highest in the autumn extract. The study also evaluated the concentration of the active triterpene 7 β -acetoxy-24-methylcholesta-5-24 (28)-diene-3, 19-diol) and its influence on the organism maturation and distribution which showed a positive relation between the compound concentration and maturity of the organism as well as the organism dominance and distribution. The study also suggested that this compound is the responsible for the total extract strong biological activity.

CHAPTER 6

CONCLUDING DISCUSSION AND FUTURE WORK

6.1. Overview

Presented in this thesis is, the anti HIV and anti-cancer activities of different marine extracts and the isolated compounds of the most active extract obtained from *L. arboreum* as well as the seasonal variation of the active compounds quantities in the total extract from the same organism and the effect on the dominance of the organism in the ecosystem.

Natural products (NPs) from marine organisms and fungi have potential for use in the treatment of various diseases. A wide spectra of compounds derived from these sources were found to have many applications in the fields of medicine, pharmacy and general biology. The enormous structural diversity of NPs and their medicinal significance has led researchers to predict that screening natural resources will generate new lead compounds. It is well established that structural analogs with greater pharmacological activity and fewer side effects can be generated by molecular modification of the functional groups of such lead compounds. The compounds derived from various NP sources have been a basis for the development of clinically important agents active against various diseases including taxol, vinblastine, vincristine and topotecan, which are important anticancer agents in widespread clinical use. A number of other promising agents such as flavopiridol, combretastatin, betulinic acid, and silvestrol are in clinical or preclinical development. A large number of anti-infective agents in clinical use are also derived from NPs.

Adding to screening as a strategy for selecting marine active compounds, chemical ecology now represents one of the new strategies for understanding and investigating the biological role of an organism's metabolites. For the past 4 decades, marine organisms have provided natural product chemists with a remarkable source of novel secondary metabolites (Faulkner 2000, and reviews cited therein). Because these compounds are often structurally complex, or present at high concentrations, chemists generally assumed that secondary metabolites provided some ecological function, often without any observational or experimental evidence. At the same time, ecologists were

observing patterns of the distributions of marine organisms, or direct interactions of predators and prey or of competitors, and often assuming that chemistry played a specific role. Beginning in the 1980s collaborations began between chemists and ecologists who resulted in an increasing number of studies in which up-to-date techniques of chemical isolation and identification were paired with ecologically relevant laboratory and field experiments. Since then, marine chemical ecology has developed rapidly, as evidenced by the number of pertinent reviews (e.g. Bakus *et al.* 1986, Hay & Fenical 1988, 1996, Hay & Steinberg 1992, Paul 1992, Pawlik 1992, 1993, Fenical 1993, Hay 1996, McClintock & Baker 1997). For these reasons strategies for marine drug discovery such as, screening of different organisms as well as the chemical ecology effects of their metabolites should provide the drugs library with new or more potent candidates by increasing the chemical space and the diversity of the sample collection (Zhang and Demian, 2005).

Drug-resistance and therapy failure due to drug-drug interactions are the main challenges in current treatment against Human Immunodeficiency Virus (HIV) infection (Martinez *et al.*, 2013). As cancer cells have evolved multiple mechanisms to resist the induction of programmed cell death (apoptosis), the modulation of apoptosis signaling pathways by natural compounds has been demonstrated to constitute a key event in these antitumor activities. As such, there is a continuous need for the development of new and more potent anti-cancer and anti-HIV drugs.

Marine compounds have high diverse structural classes including polyketides, terpenes, steroids and peptides which have been proven to be highly biologically active. These compounds can be found in sponges, algae and coelenterates. These groups of organisms were the subject of the first part of this study.

6.2. Chapter 3. The random screening of marine organisms

Thirteen organisms (mainly sponges, algae and coelenterates), were randomly selected from three different ecosystems in the Red Sea. Out of thirteen screened organisms, the coral reef ecosystem had the most diversity of active extracts, especially soft corals. Among the screened extracts, only the extracts of the soft coral *Sarcophyton trochiophilium* showed the highest cytotoxic activity in HeLa cells followed by *L.*

arboreum extract. While in U937 *L. arboreum* extract showed the highest cytotoxicity with very low IC₅₀ and high SI values which is reported for the first time in this study and was accepted for publication by journal of complementary and alternative medicine in December 2010.

For HIV-1PR inhibitory activities, the extracts of *Litophyton arboreum*, *Sinularia maxima Sinularia*, *heterospiculata* and *Galaxaura filamentosa* showed a strong inhibitory activity against the enzyme. While the cnidarian *Cassiopeia Andromeda* extract showed potent and promising inhibitory activity with very low IC₅₀ and high safety margins (cytotoxicity selectively targeted the cancer cells and showed very low cytotoxicity in normal cell lines). The HIV-1 PR inhibitory activities for the thirteen organisms were reported for the first time in this study.

The soft coral *L. arboreum*'s extract was active in all the assays used in this study. *L. arboreum* presented very strong cytotoxicity and selectivity in U937 cells which is reported here for the first time as well as it also showed moderate activity in HeLa compared to the cytotoxicity positive control used. This is also the first report for the HIV-1 PR inhibitory activity of *L. arboreum*. These findings supported the further investigation of this organism for the 2nd part of the study where we isolated and evaluated its active metabolites.

6.3. Chapter 4. Isolation and biological evaluation *L. arboreum* metabolites:

Alcyonacea are rich in bioactive compounds that have a role in defence against predators, competition for space, antifouling, and reproduction enhancement (Coll *et al.*, 1982; Sammarco and Coll, 1992; Kelman *et al.*, 1999). Most of these compounds are lipid-soluble, with terpenoids (di- and sesquiterpenes), and sterols predominating (Blunt *et al.*, 2012).

In this study 9 compounds were isolated from the lipophilic fraction of the soft coral extract. The compounds were mainly sterols and terpenoids. The HIV-1 PR inhibitory activities of the isolated compounds were never reported before and this is also the first report for the cytotoxic activities of most of these compounds in HeLa and U937 cells.

One of the isolated triterpenes showed potent cytotoxic effects in HeLa cells and strong cytotoxic effect in U937 cells with high selectivity indexes, which was confirmed by RT-CES. This triterpene had also shown a very strong HIV-1 PR inhibitory activity which was confirmed with *in silico* docking. Findings presented here suggest that HIV-1 protease inhibitors can suppress proliferation and induce differentiation of human myelocytic leukemia cells (also seen by Takayuki Ikezoe, 2007). Triterpene showed promise as HIV-1 PR inhibitor and as selective anticancer agent targeting U937 cells.

Another compound (a sphingolipids) showed potent HIV-1 PR inhibitory activity and did not demonstrate cytotoxicity against the cell lines used in the investigation. Sphingolipids have recently been shown to act as signaling molecules in many cellular functions and to play crucial roles in the regulation of path biological processes such as cancer, cardiovascular disease and neurodegenerative disorders and inflammation or infectious diseases. Because of the lack of cytotoxicity, this compound is a promising candidate not only for further investigation as potential protease inhibitor but also for involvement in other cellular processes in instances of disease.

An alkyl glycerol isolated in this study, showed cytostatic action in HeLa cells with strong inhibitory activity against HIV-1 PR. This compound can be considered a potential lead in the development of antiproliferative and virostatic drug to inhibit HIV replication. Virostatic drugs are characterized by the combination of a drugs directly inhibiting virus production (viro), and indirectly inhibiting the virus by reducing cellular proliferation (static) (Lori *et al.*, 2007). The 3-azido-3-deoxythymidine (AZT, Zidovudine) is an antiproliferative and virostatic drug widely used in human immunodeficiency virus type 1(HIV -1) infection treatment. AZT-based antiretroviral therapy has been proven to significantly reduce rates of certain AIDS-defining cancers (most dramatically, that of Kaposi's sarcoma and central nervous system lymphoma) (Biggar *et al.*, 2007). Early long-term higher-dose therapy with AZT was initially associated with side effects that sometimes limited therapy, including anemia, neutropenia, hepatotoxicity, cardiomyopathy, and myopathy. All of these conditions were generally found to be reversible upon reduction of AZT dosages (Sun *et al.*, 2010). Alismoxide and alismol isolated in this study, showed the required two effects of a virostatic drug.

6.4. Chapter 5: seasonal evaluation of the active triterpene isolated from *L. arboreum* and its ecological consequences.

Although several studies have reported the quantification of secondary metabolites from benthic marine organisms, the lack of information on the production of chemical defence and its ecological consequences is notable and the importance and need for such studies have been emphasized (Hay, 1996). In this study, the effect of seasonal changes on the total extract of *L. arboreum* was determined as well as the effect of different seasons on the amount of the active triterpene (7 β -acetoxy-24-methylcholesta-5-24(28)-diene-3,19-diol) isolated from the soft coral and the influence of these changes on the seasonal distribution of the organism in the community.

HPLC qualitative profiles for *L. arboreum* seasonal total extracts changed in the different seasons suggesting that, the concentrations of soft coral's defensive metabolites can significantly change due to the ecological temporal changes. This was also seen by Slattery *et al.*, 2001).

HPLC profiling also showed seasonal fluctuation in the active terpene concentrations. The highest concentration of the compound was found in the autumn season. Triterpenes are known to be used by the organism for chemical defenses and found also to be selectively stored or synthesized and concentrated in the organism's oocytes (Bowden *et al.*, 1985). According to the percentage cover results of *L. arboreum* determined in this study, the highest percentage cover was found in summer and spring not in autumn which suggest that this terpene was not used for the mature coral chemical defence but it could be used for the oocyte protection or coral spawn as suggested by Bowden *et al.*, 1985 and Rodriguez and Lasker, 2004. Such information is important for our understanding of the factors affecting the production of chemical metabolites used for defence.

6.5. Revisiting hypothesis

It was hypothesized that Red Sea marine organisms represents a unique source of bioactive natural compounds that can perhaps produce promising candidates for the treatment of cancer and HIV. This was proved by the results obtained in this study where

most of the organisms showed better biological activities than the activities showed by the same organisms from different habitats, adding to the native organisms that only exist in the Red Sea. In order to investigate this hypothesis several activities were undertaken. The screening technique used in this study, presented a reliable technique for quick discovery of biologically active hits. This technique helped in the discovery of the strong cytotoxicity of the soft corals as well as the first report of the anti-HIV activities of the screened organisms. The metabolites of the soft coral *L. arboreum* (the extract that showed activities in all of the assays used) showed promising cytotoxic and anti-HIV activities.

6.6. Hypothesis accepted

Based on the overall study, the hypothesis could therefore be converted into the following fact, “Red Sea marine organisms represent an unexplored source of bioactive compounds that inhibit HIV replication through inhibiting one of the key enzymes in the viral life cycle. These compounds also have cytotoxic and cytostatic activities which can provide the anticancer and anti HIV drug libraries with new, more effective candidates with fewer side effects.”

6.7. Future work

6.7.1- Further investigations for the potent untested marine organisms.

The first chapter of this study showed the screening of thirteen marine organisms and most of them showed potent and strong activities in the different assays used.

The work plan of this study allowed us to only test the most active extract and isolate its compounds. *L. arboreum*'s response in all the assays was superior to the other organisms for which potent responses were detected in the selected assay. For future work some of the untested extracts can be investigated further. For example, the soft coral, *Sarcophyton trocheliophorum* showed promising cytotoxicity and selectivity in HeLa cells. Other studies (Shaker et al., 2010 and Grote *et al.*, 2008) evaluated the cytotoxicity of this coral in different cancer cell lines. Meaning that data presented here

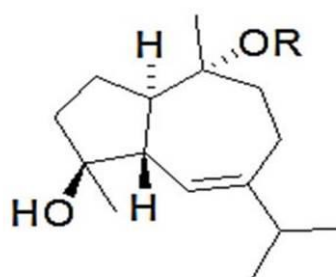
support the possibility that *S. trocheliophorum* may also contain useful anti-cancer compounds with multiple effects in different cancer cell lines.

This study is the first report for the potent HIV-1 PR inhibitory activity of the Cnidarian jelly fish *C. Andromeda*. In addition, the red algae *G. filamentosa* which showed potent HIV-1 PR inhibitory activity and interestingly, these extracts showed no cytotoxic activities in all the cell lines tested. This suggests the potential of *C. andromeda* and *G. filamentosa* as HIV-1 PR inhibitor with minimum side effects. This should be further explored by identifying the active components of these extracts.

6.7.2- Structure activity relationship (SAR) approach.

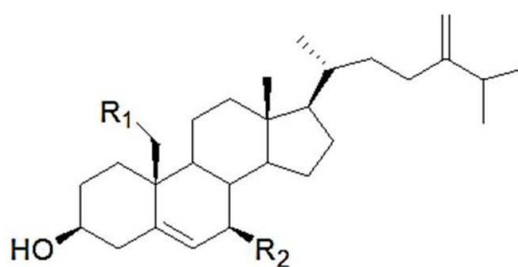
Structure-activity studies are critical to designing a pharmaceutical with the greatest potency and least side effects.

In this study 2 alismoxides were isolated (10-O-methyl alismoxide, (4) and alismoxide (5), having the same main structure but in compound 4, one of the hydrogen atoms was substituted by a methyl group. Compound 4 showed more activities than compound 5 which suggest that, the methyl group has an important role in the observer cytotoxicity.



Compound 4, R = Methyl group
Compound 5, R = H

Figure6. 1. alismoxides isolated from the soft coral *L. arboreum*.



Compound 3, R1= R2= H
Compound 7, R1= OH, R2= Acetoxy group
Compound 9, R1=R2=OH

Figure.6 2. The scaffold compound 3, hydroxylated triterpene (compound 9) and hydroxylated with Acetoxy group triterpene (compound 7).

By comparing the biological activities of the three compounds we found that, the scaffold had the lowest activities while the hydroxylated compound 9 and 7 showed better cytotoxic activities which suggest the important role of hydroxylation in the development of anticancer drugs.

For the HIV-1 PR inhibitory activities, compound 7 with acetoxy group showed potent inhibition compared to compound 9 that lacked this group. This finding supports the important role of the acetoxy group in the HIV-PR inhibitory activities.

By understanding the SAR or the isolated compounds, the activities can be improved upon and the side effects can be minimized and undesired physical properties of the active compounds in the drug discovery process prevented.

6.8. Other investigations

The effect of the isolated compounds against *Mycobacterium tuberculosis* (strain no. H37R) was also tested. A rapid radiometric method was used and isonicotinic acid hydrazide was included as positive control (Palomino *et al* 2002). A minimum inhibitory concentration (MIC) of 320 μ M was obtained for all the compounds tested which was an indication of very weak activity against *Mycobacterium tuberculosis*. Individuals infected with HIV are at higher risk of developing tuberculosis compared to those individuals not infected with the virus (UNAIDS, 2012). *M. tuberculosis* /HIV co infected individuals has to cope with two microorganisms.

6.9. Conclusion

The rich diversity of marine biota with its unique physiological adaptations to the harsh marine environment provides a fruitful source for the discovery of life saving drugs. With the implementation of scuba diving tools and the development of the sophisticated instruments for the isolation and elucidation of structures of natural products from marine organisms, a new and exciting vista is open for the exploration of precious drugs.

A total of 13 marine organisms were investigated and two were singled out as promising cytotoxic candidates and another two were potentially active against HIV-1 PR enzyme with high safety margins.

The total extract of *L. arboreum* showed cytotoxic and HIV-PR inhibitory activities while its isolated compounds produced promising cytotoxic, cytostatic and HIV-1 PR inhibitory activities. The chemical ecology of the active compounds was also reported for the first time in this study. Data presented here faraurs further investigation of Red Sea marine organisms for the development of useful medicines.

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APPENDIX

8.1. Additional information for chapter 3

8.1.1. Screening of marine organisms.

The effect of selected marine organisms' crude extracts on HIV and cancer cell lines were evaluated.

The first picture shows the Red Sea where samples were collected. Picture 2 shows the SCUBA diving collection which done by the student. (3) Shows examples of the collected organisms, (4) Samples were blended in methanol and (5) soaked overnight. In picture 6, the evaporation of the solvents and concentrating the extracts is shown. (7) Final product of the crude extract. Picture (8) shows the biological evaluation of the different extracts.

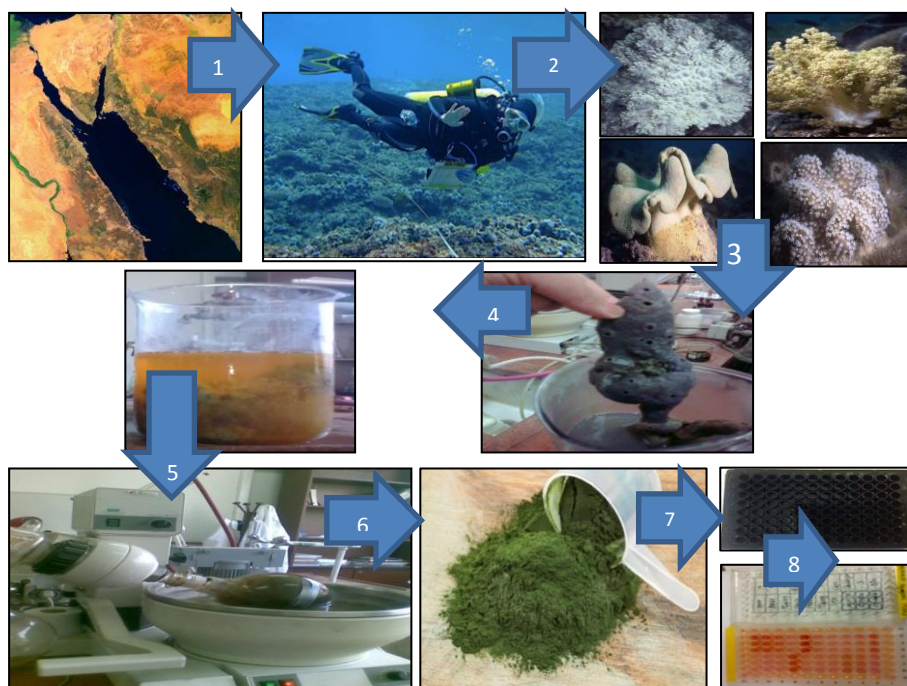


Figure 8. 1: Schematic illustration of collection and extracts preparation.

8.1.2. The Reverse Transcriptase Assay

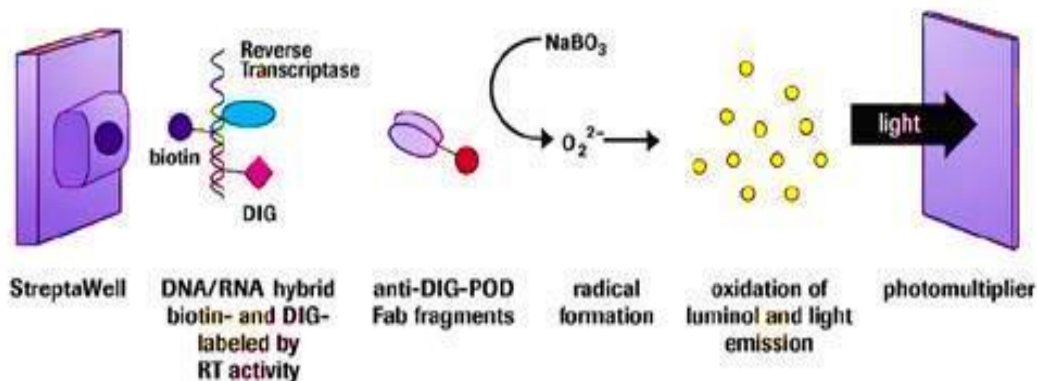


Figure 8. 2: The principle of RT assay

The colorimetric principle takes advantage of the ability of reverse transcriptase to synthesize DNA using the hybrid poly (A) x oligo (dT)₁₅ as a template and primer. It avoids the use of [³H] - or [³²P]-labeled nucleotides that are employed in standard RT assays. In place of radiolabeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into the same DNA molecule by the RT activity. A template/primer hybrid is supplied, but the flexibility of the assay allows the use of a template of choice (e.g., a viral template). The detection and quantification of the synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol: biotin-labeled DNA binds to the surface of streptavidin-coated microplate modules. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG- POD), is added and bound to the digoxigenin-labeled nucleotides (Licensed by Institute Pasteur). In the final step, the peroxidase substrate ABTS is added. The peroxidase enzyme catalyzes the cleavage of the substrate to produce a colored reaction product. The absorbance of the samples is determined using a microplate (ELISA) reader, and is directly correlated to the level of RT activity in the sample.

Figure 8.3 shows the inhibitory activities of the screened crude extracts which were lower than accepted (<50 %).

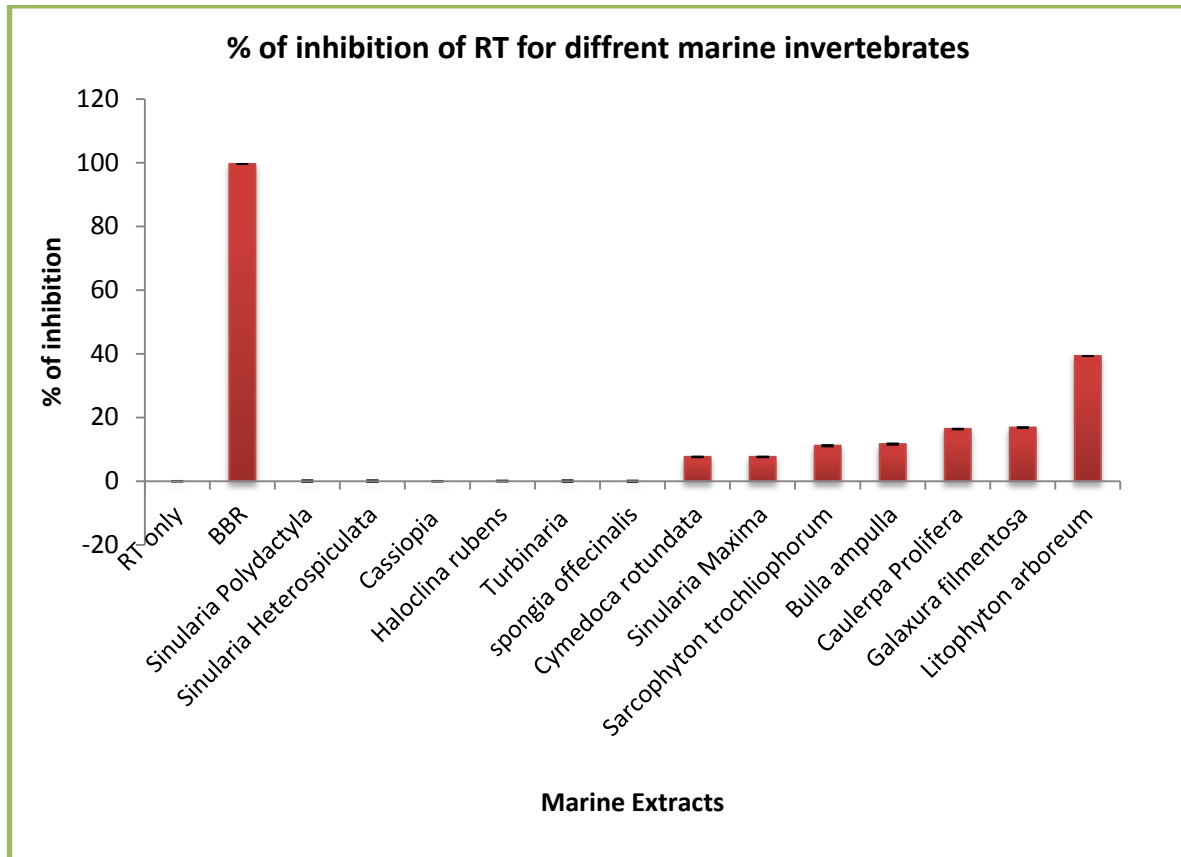


Figure 8. 3. HIV-1 RT inhibitory activities of the marine extracts

8.1.3. Principle XTT viability Assay.

Most eukaryotic cells have mitochondria. Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy. In addition to supplying cellular energy, mitochondria are involved in other tasks such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth. Tetrazolium salts are cleaved to formazan by the succinate-tetrazolium reductase system in the presence of an electron-coupling reagent, producing a soluble formazan salt. This conversion only occurs in viable cells because of the respiratory chain of the mitochondria, and is only active in metabolically intact cells.

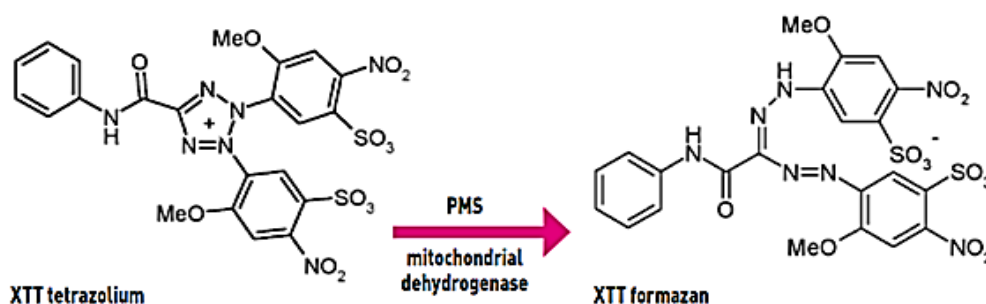


Figure 8. 4: The principle of XTT assay

The electron transport chain comprises an enzymatic series of electron donors and acceptors. Each electron donor passes electrons to a more electronegative acceptor, which in turn donates these electrons to another acceptor, a process that continues down the series until electrons are passed to oxygen, the most electronegative and terminal electron acceptor in the chain. Passage of electrons between donor and acceptor releases energy, which is used to generate a proton gradient across the mitochondrial membrane by actively "pumping" protons into the intermembrane space, producing a thermodynamic state that has the potential to do work. The entire process is called oxidative phosphorylation, since ADP is phosphorylated to ATP using the energy of hydrogen oxidation in many steps.

Cells grown in a 96-well tissue culture plate are incubated with the XTT labeling mixture for 2 - 20 hours. After this incubation period, the formazan dye formed is quantitated using a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells which also reflects the cytotoxicity of the compounds while cytostatic compounds should show a reduction of the

viable cell number compared to the untreated cells (shift from proliferation) which can be considered as low – moderate cytotoxicity.

8.2. Additional information for chapter 4

8.2.1. Cytotoxic, Cytostatic and HIV-1 PR Inhibitory Activities of the constituents of the Soft Coral *Litophyton arboreum* constituents.

(1) Collection of *Litophyton arboreum* collection. (2) Soaking and blending in methanol and preparation of the crude extract. (3) Silica gel column chromatography for the total extract, fractions collection. (4) Mixing similar fractions and concentrating the combined fractions. *L. arboreum* fractionation led to 20 different fractions shown in the TLC plates (5). Further purification for each fraction was done in order to obtain clean compounds as in (6). Further purification using HPLC chromatography (7) was used to further purify of each compound. NMR was used for structure elucidation of the pure compounds (8).

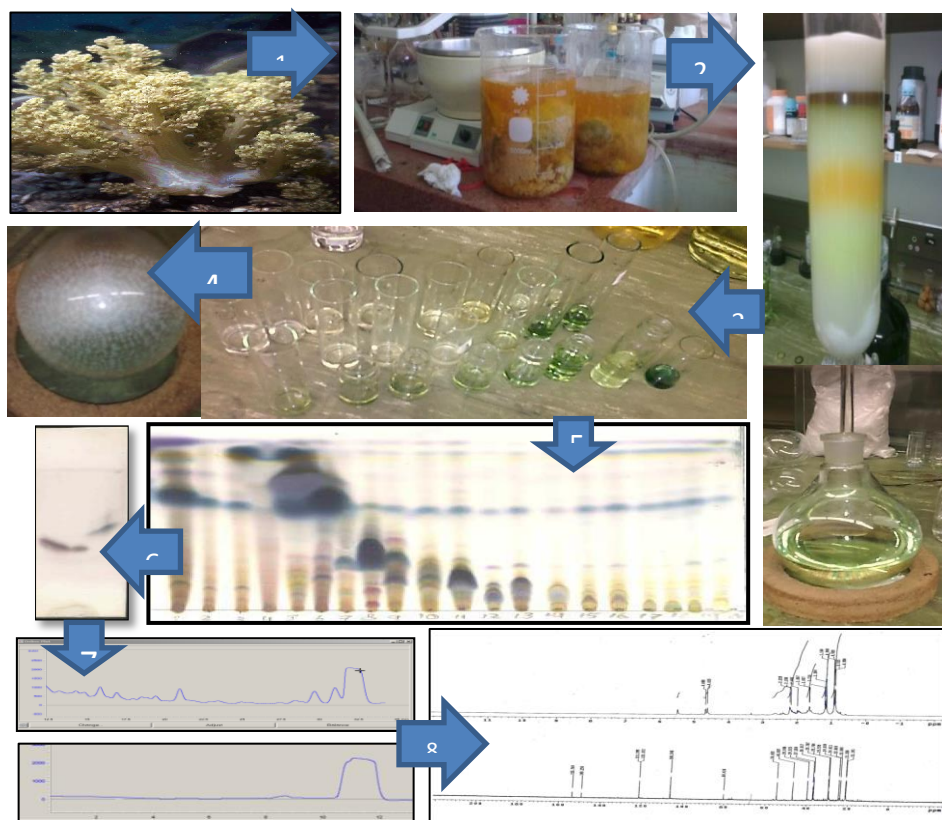


Figure 8. 5: Schematic illustration of isolation and purification of *Litophyton arboreum* constituents.

8.2.2 Compound 5 crystallography



Compound 5,
Formula: C₁₅ H₂
Description: Transparent crystals

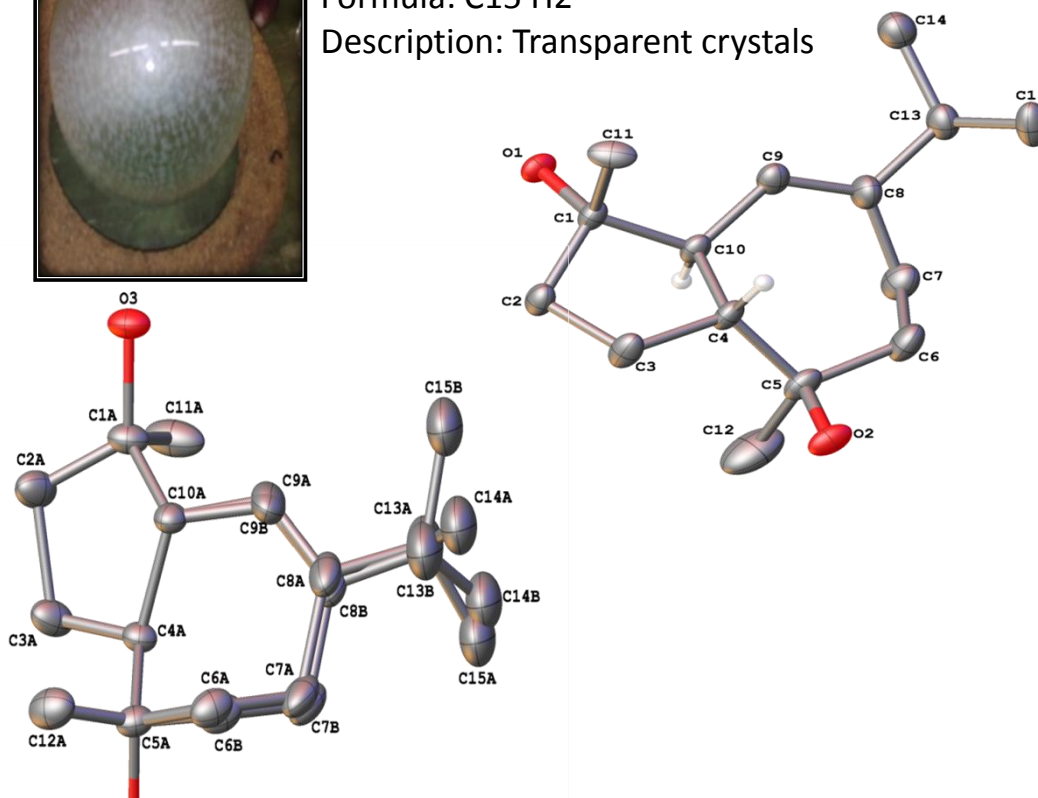


Figure 8. 6: Compound 5 crystallography

The compound obtained through the main column chromatography of the lipophilic extract of *L. arboreum*.

Crystal structure elucidation was done by Prof. Iliia A. Guzei, Director of Crystallography Chemistry Department University of Wisconsin-Madison.

The structure was deposited at the Cambridge Crystallographic Data Centre. The data have been assigned the following details

- Deposition numbers. CCDC 949268
- Unit Cell Parameters: a 16.326(4) b 16.326(4) c 32.266(9) I41/a

The structure can be accessed in the following link

https://www.ccdc.cam.ac.uk/services/structure_deposit/

8.3. Additional information for chapter 5

The seasonal changes in the concentration of the active Triterpene (compound 7) and its influence on the biological activities of *L. arboreum* seasonal extracts.

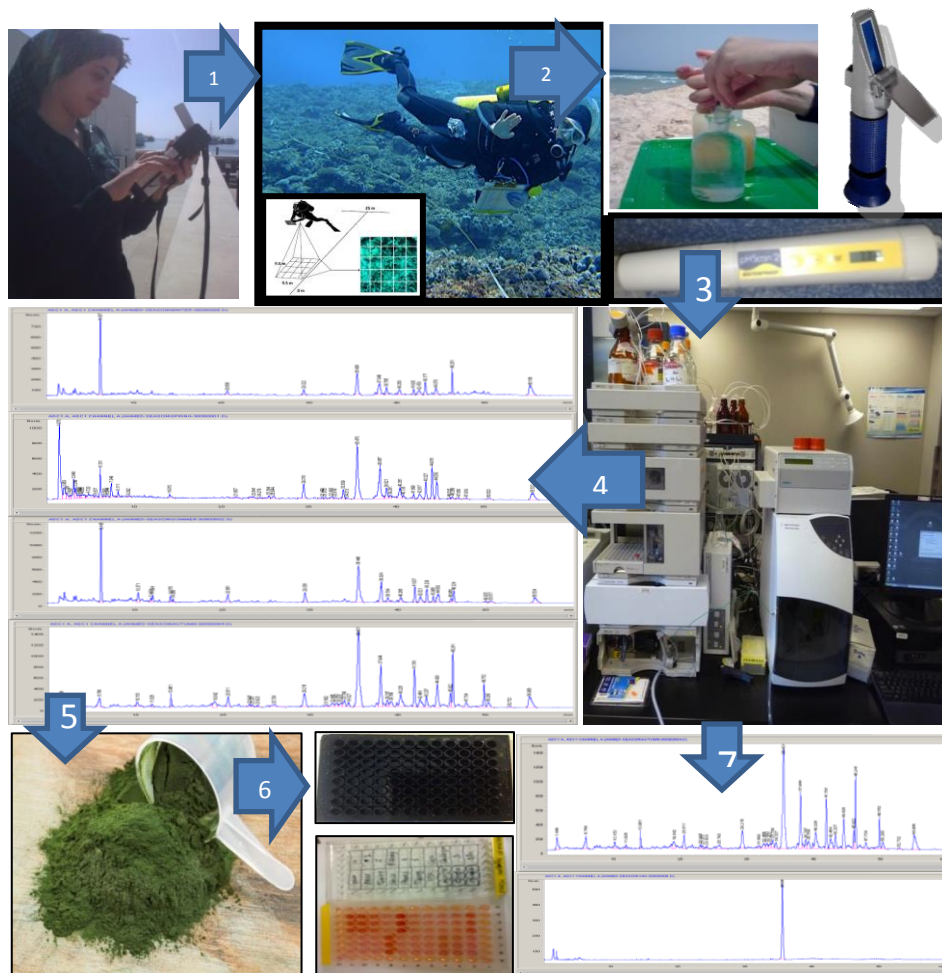


Figure 8. 7 Schematic illustration of the seasonal evaluation of the extracts as well as the active triterpene.

(1) Shows the location determination by GPS. (2) The seasonal distribution of *L. arboreum* were determined by quadrat transect method. (3) The determination of the seasonal ecological parameters. (4) HPLC qualitative profiling for the seasonal extracts that showed differences among seasons. (5), (6) Biological evaluation of the seasonal extracts. (7) Quantitative profiling for the triterpene in order to determine its concentration in each season.

GLOSSARY

The following is a list of important terminologies used in this thesis, adapted from Basson, 2005 and Fonteh, 2011.

Acquired immunodeficiency syndrome (AIDS): AIDS is the advanced stage of HIV infection. It is marked by characteristic opportunistic infections and malignancies as well as a loss of helper T-lymphocytes.

Active site: Asymmetric pocket on or near the surface of a macromolecule that promotes chemical catalysis when the appropriate sample or substrate binds.

Antibody: A class of large protein molecules that are produced in response to, and interact with, specific target molecules known as antigens.

Cell index: Unit for measuring cellular impedance or resistance on a real time cell electronic device, xCELLigence. The value is directly related to the ability of adherent cells to attach to electrode plates.

Cell line: A population of cultured cells that have been subjected to chemical or viral transformation. Cell lines can be propagated indefinitely.

Colorimetric: Is a method of determining the concentration of a chemical element or chemical compound in a solution with the aid of a colour reagent.

Cytokine: Small cell signalling proteins that influence the activity and behaviour of other cells. Some cytokines increase the intensity of an immune response, while others suppress it.

Crude extract: A concentrated form of an herb that is derived when the crude herb is mixed with water, alcohol, or another solvent and distilled or evaporated. Extracts may be either fluid or solid.

Human immunodeficiency virus (HIV): A retrovirus that causes AIDS.

Immunomodulatory: An immunological change in which one or more immune system molecules are altered through suppression or stimulation.

Impedance: Resistance

In silico: Virtual study performed using a computer and specially developed software.

In vitro: Refers to laboratory experiments performed outside the living organism.

Informed consent: The voluntary permission given by a person before participating in an experimental program after being informed of its purpose, procedures, risks and benefits.

Protease: Any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

Replication: The process by which a virus produces new virus particles or virions.

Reverse transcriptase: An enzyme found in retroviruses and few other viruses that enable them to make a DNA copy of their RNA genome.

TZM-bl: Is a genetically modified HeLa cell line that expresses CD4 and CCR5. The TZM-bl cell line is highly sensitive to infection with diverse isolates of HIV-1.

Virus particle: Complete, free-floating viral units as found.