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Biological activity of *Sinularia notanda*

by

Sidhika Hariparsad

Student number: 12213099

Submitted in partial fulfilment of the degree

***Magister Scientiae* Biotechnology**

Faculty of Natural and Agricultural Science

University of Pretoria

Pretoria

South Africa

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Preface

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Summary

Biological activity of *Sinularia notanda*

by

Sidhika Hariparsad

Supervisor: **Prof. Debra Meyer**
Co-supervisor: **Prof. Bavesh Kana**
Department: **Biochemistry**
Degree: **MSc Biotechnology**

In 1963 the study of marine natural products was just beginning and there were only a handful of public records with only one of these publications reporting a new compound. There has since been 9220 papers published, reporting on 24 662 new compounds. Annually a variety of cembrane diterpenoids with a range of biological activities are reportedly isolated from marine soft coral, and most notably these compounds possess anticancer properties. The investigation of a marine soft coral, *Sinularia notanda*, for novel bioactive compounds is presented here. The aim was to identify compounds active against key HIV enzymes as well as against cervical cancer, an opportunistic malignancy affecting many HIV positive women in Sub-Saharan Africa.

A methanol extract of *S. notanda* was prepared and the cytotoxicity of the crude extract tested against a cervical cancer (HeLa) cell line using sodium 2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide (XTT). The crude extract was also tested for HIV-1 protease inhibition using a direct enzyme assay, and antioxidant activity determined using 1,1-diphenyl-2picrylhydrazyl (DPPH). Bioassay guided fractionation, column chromatography and thin layer chromatography were used to identify active fractions from the total extract as well as to isolate biologically active compounds. The isolated compounds were tested for cytotoxic activity against four different cell lines. Nuclear magnetic resonance spectroscopy and x-ray crystallography were used for structure determination.

The crude ethyl acetate fraction of *S. notanda* had a 50% cytotoxic concentration (CC_{50}) of 33.82 µg/ml, exhibited moderate inhibition of HIV-1 protease (between 40 and 60% inhibition), was unable to inhibit reverse transcriptase and showed antioxidant activity at a concentration (IC_{50}) of 76.15 µg/ml. In comparison, the crude methanol fraction had a

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CC_{50} of 145.80 µg/ml, showed significantly lower protease inhibition ($p < 0.05$), and showed antioxidant activity at an IC_{50} of 27.16 µg/ml. Extracts from natural sources including soft coral are routinely screened for free radical scavenging ability and the DPPH assay is one of the fastest methods to do so. Free radicals are known to induce oxidative damage to biomolecules which can eventually lead to diseases such as cancer. Oxidative damage can also be caused by HIV infection and oxidative stress levels may be exacerbated by antiretroviral treatment. Free radical scavenging antioxidants can provide protection against the damage caused by reactive oxygen species and are therefore considered as important nutraceuticals.

Structural elucidation of a crystal isolated from *S. notanda* using NMR and x-ray crystallography confirmed a unique cembrane diterpenoid structure. The identified compound [(1R,3R,5S,12R,13S,E)-12-hydroxy-5,9,13-trimethyl-16-methylene-4,14-dioxatricyclo[11.3.2.03,5]octadec-8-en-15-one] showed moderate HIV-1 protease inhibition. This compound was abbreviated as CPD1. A second isolated compound designated E7 was found to be toxic to human leukemic monocyte lymphoma (U937) cells at 25 µg/ml resulting in cell viability of less than 10%. Structure elucidation of E7 by 1D and 2D NMR as well as mass spectrometric analysis confirmed this compound to be structurally identical to the one isolated as a crystal and it also exhibited similar cytotoxic behaviour. Although this is not the first time CPD1 has been isolated from a coral of the *Sinularia* genus, data presented in this dissertation represents the first time that the compound was isolated from *S. notanda*. In *Sinularia flexiblis* CPD1 was isolated as a ketone (carbon-oxygen double bond at C12) while in the current study it was isolated from *S. notanda* as an alcohol (hydroxyl group at C12). Ketones are produced from the oxidation of secondary alcohols and the change in functional groups at C12 of CPD1 could be attributed to different organic solvents being used for initial extraction. A third compound was isolated and showed < 50% inhibition of HeLa cell growth and > 90% inhibition of U937 cell growth at 50 µg/ml. Structure elucidation data identified the compound as 3-caffeoylquinic acid. This compound is not synthesised by the coral itself but is produced by algae that the coral ingested.

The cembrane diterpenoid isolated from *S. notanda* in this study showed moderate inhibition of HIV-1 protease and selective cytotoxicity towards the U937 lymphoma cell line (selectivity index > 2). These responses are not unusual as cembrane diterpenoids with anti-cancer potential are increasingly being isolated from soft corals.

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List of Important Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
Anti-DIG-POD	Digoxigenin conjugated to peroxidase
ARV	Antiretroviral
AZT	3’-azido-3’-deoxythymidine
cDNA	Complementary deoxyribonucleic acid
DABCYL	40-dimethylaminoazobenzene-4-carboxylate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Effective concentration of 50% of treated population
EDANS	5-(2-aminoethylamino)-1-napthalene sulfonate
ELISA	enzyme-linked immunosorbent assay
Env	Viral envelope protein
FRAP	Ferric reducing antioxidant power
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
IC_{50}	Inhibition concentration of 50% of treated population
KS	Kaposi sarcoma
LTR	Long terminal repeat
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
OI	Opportunistic infections
ORAC	Oxygen radical absorption capacity
PBMC	Peripheral blood mononuclear cells
PMS	Phenazine methosulphate
PR	Protease
RNA	Ribonucleic acid
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
TB	Tuberculosis
XTT	sodium 2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide

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1. Introduction

Ever since the first description of the Acquired Immune Deficiency Syndrome (AIDS), the disease has been related to depletion of CD4+ T helper lymphocytes and other cells (such as tissue macrophages) becoming infected by the Human Immunodeficiency Virus (HIV) (Weiss, 2012). HIV has numerous effects on the immune system, some of which can be exacerbated by Highly Active Antiretroviral Therapy (HAART) which is used to manage the progression of HIV infection. HAART can also be a major contributor to the development of oxidative stress in patients. The destruction of infected CD4 cells leads to defects in the immune system which causes a disruption of cytokines and chemokines and also results in oxidative stress. An increase in reactive oxygen species combined with the detrimental effects HIV has on the immune system predisposes infected individuals to various malignancies. HIV positive women in Sub-Saharan Africa have a higher prevalence of cervical squamous intra-epithelial lesions and invasive cervical cancer than HIV positive women in other regions of the world.

The effectiveness of drugs used to treat viral infections and cancer is influenced by the emergence of clinical resistance and has become an important factor in the development of new treatment (Nyce, Leonard, Canupp, Schulz, & Wong, 1993; Ueda, Cardarelli, Gottesman, & Pastan, 1987). Therefore, the search for potential drug candidates containing higher inhibitory activity against various HIV strains is increasing in the pharmaceutical industry. In this regard, natural bioactive compounds and their derivatives are great sources for the development of new generation anti-HIV therapeutics which are more effective with fewer side-effects (Matthée *et al.,* 1999; Vo & Kim, 2010). Over the last thirty years natural products from plant and marine organisms have provided an array of biologically active compounds which have provided new leads for drug discovery (Yang *et al.,* 2001). A number of the medically active compounds derived from marine organisms are terpene based as these are naturally used by the coral as a defense mechanism.

There are approximately 90 known species of the genus *Sinularia*, a hardy coral that has been reported to be one of the most toxic genera of all soft coral (Partin 2001). These coral can be found in many different locations, but is most commonly found in shallower waters. Of the many *Sinularia* species, approximately 36 have already been chemically studied and reported to produce secondary metabolites, the majority of which fall under the class of metabolites known as terpenes (Venkateswarlu *et al.,* 1999). Cembrane diterpenoids have

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been isolated from a multitude of soft corals such as *Lobophytum*, *Pachyclavularia* as well as from *Sinularia* (Ellithey *et al.,* 2014; Rashid *et al.,* 2000; Inman & Crews 1989). Almost half of these isolated compounds have been reported to have activity against various cancerous cell lines (Khalesi *et al.,* 2008). Many species of *Sinularia* have been extensively reported on, such as *S. flexibilis*, *S. gibberosa* and *S. crassa*, however, there has only been two previous reports on *Sinularia notanda*, the first of which was published by Tursch *et al.,* in 1978 and the second by Ahmed in 2007.

In the research presented here, the biological activity of *S. notanda* was assessed for the first time. The anti-HIV screening focused mainly on direct enzyme inhibition of HIV protease, while antioxidant activity and anti-cancer properties were assessed using a free radical scavenging assay and a tetrazolium dye assay respectively. The ethyl acetate fraction of *S. notanda* showed a higher biological activity than the methanol fraction providing evidence that active compounds were present in the ethyl acetate fraction of the soft coral. Bioassay guided fractionation was used to isolate three compounds, all of which exhibited selective toxicity towards a lymphoma cell line. These results showed that *S. notanda* contained compounds that could be used to develop novel therapeutics to treat opportunistic infections such as cervical cancer as well as AIDS related lymphoma in HIV infected individuals.

The dissertation content is presented as follows: the literature review in the next section provides information on how natural products have been used in the treatment of various diseases as well as the potential for isolation of therapeutically useful compounds from marine organisms. Background information on the life cycle of HIV as well as the opportunistic infections associated with viral infection is also included. The literature review concludes with the hypothesis as well as the aims investigated to prove this hypothesis. The methodologies used in this study precedes the results and discussion which report on the biological activity of the crude extracts of *Sinularia notanda,* and isolation of biologically active compounds. Finally, the conclusion presents the highlights of the study as well as areas for future investigation, followed by a comprehensive reference list. An appendix, containing results not regarded as part of the primary findings of this study, is included.

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2. Background and Literature Review

2.1 Medicinal Properties of Natural Products and Terpenoids

Nature has always provided a source of drugs for various ailments. The bioassay guided fractionation of crude extracts from natural sources has provided lead molecules for the discovery of drug candidates against numerous diseases (Figure 1) (Yang *et al.,* 2001). In the relatively short span of three decades, marine organisms have yielded an array of structures exhibiting a range of biological activities and several marine natural products, which have shown anti-HIV and anti-cancer activities (Singh *et al.,* 2005).

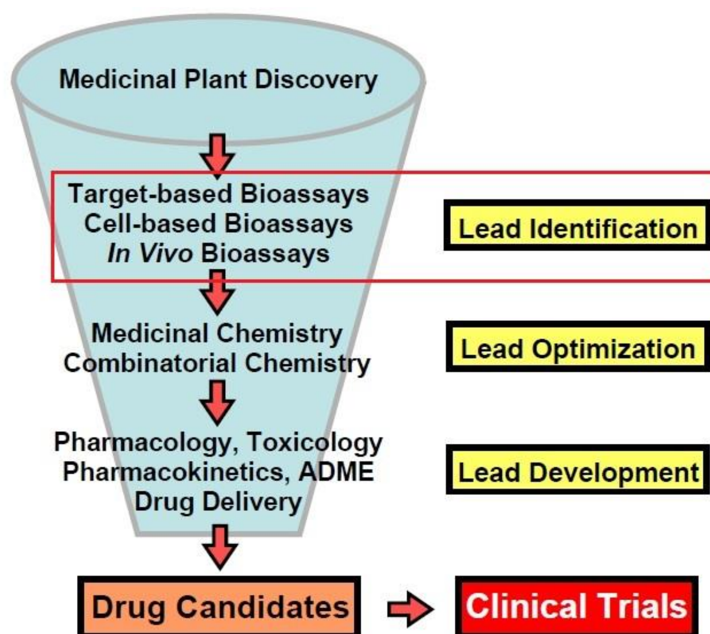

Figure 1: Diagramatic representation of the drug discovery process. Research conducted in this project fits under the lead identification section.

(Balunas & Kinghorn 2005).

Since the use of nitrogen mustard (mechlorethamine) as an anticancer agent in the 1940s, the United States Food and Drug Administration (FDA) approved 87 anticancer drugs for use in clinical trials up to 1994, 62% of which were derived from natural products directly or semi-synthetically (Kim & Park 2002). Of the 300 potential new anticancer drugs, 61% originated from terrestrial plants, marine and microbial resources (Cragg *et al.,* 1997).

Agaricus blazei, the Brazilian mushroom, has been used for the prevention of cancer and/or as an adjuvant with cancer chemotherapy drugs after the removal of malignant tumours. Animal studies and clinical experience demonstrated that *Agaricus blazei* displays anti-tumour activity, immunological enhancement, and was effective in treating Acquired Immunodeficiency Syndrome (AIDS), diabetes, hypotension and hepatitis (Huang & Mau

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2006). According to Manosroi and colleagues (2012) "the search for anti-cancer agents derived from natural sources started with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins." The first group to advance into clinical use was the vinca alkaloids isolated from the Madagascar periwinkle, *Catharantus roseus* G. These drugs were used in combination with other cancer drugs for the treatment of a variety of cancers including leukaemia, lymphomas, breast and lung cancers as well as Kaposi's sarcoma. Paclitaxel (Taxol) was initially isolated from the bark of the Pacific Yew, *Taxus breviflora* Nutt and has been used successfully in the treatment of breast, ovarian, non-small cell lung cancer and Kaposi's sarcoma (Manosroi *et al.,* 2012).

2.1.1 Terpenoids: The largest group of Natural Compounds

Terpenoids (isoprenoids) make up one of the largest families of natural products and are responsible for more than 40 000 individual compounds of both primary and secondary metabolism (Rocha 2013). Several terpenoids have been shown to be available for pharmaceutical applications, for example, artemisinin as an antimalarial drug and taxol to treat cancer. A terpene is defined as a hydrocarbon molecule while a terpenoid is a terpene that has been modified, for example, by the addition of an oxygen (Zwenger & Basu 2008). Terpenes are vital for life in most organisms where it exerts metabolic control and mediates inter and intra species interactions such as defense mechanisms. Many terpenes in plants are reported to act as toxins, growth inhibitors or deterrents to microorganisms (Devappa *et al.,* 2011).

Terpenes are classified based on the number and structural organization of carbons formed by the linear arrangement of isoprene units followed by cyclization and rearrangements of the carbon skeleton (Devappa *et al.,* 2011). The biosynthetic pathway of terpenoids has been elucidated in Figure 2. Isoprene or Isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal five carbon precursors of all terpenoids (Rocha 2013). In plants, terpenoid biosynthesis occurs by two different pathways to synthesize the main building block IPP. This is either via the Mevalonic acid pathway (HMG-CoA reductase pathway) that occurs in the cytosol and produces IPP for sesquiterpenoids, or the Methylerythritol phosphate/1-deoxy-D-xylulose (MEP/DOX) pathway which forms IPP in the chloroplast for mono and diterpenoids. After the discovery of the mevalonate (MVA) pathway in yeast and animals, it was assumed that IPP was

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synthesized from acetyl-CoA via MVA and then isomerized to DMAPP in all eukaryotes and some Gram-positive prokaryotes (Rocha 2013).

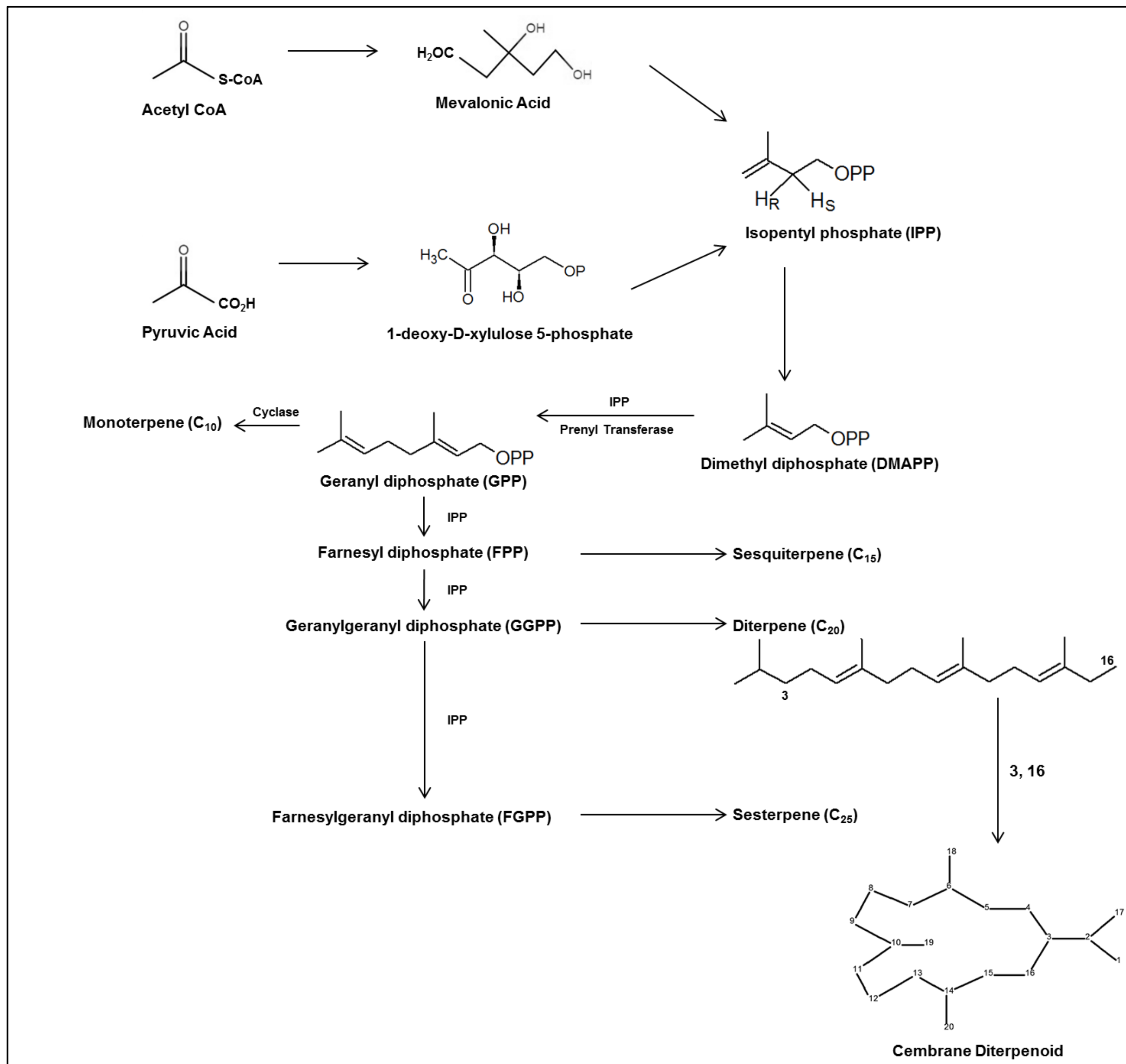

Figure 2: Biosynthetic pathway of cembrane diterpenoids in marine organisms (adapted from Zhang, Guo & Gu 2006; Wang, Tang & Bidigare 2005)

Many structurally distinct monocyclic and bicyclic terpenes arise from cyclisation and rearrangement of geranyl diphosphate (GPP) and the larger precursors, farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP) and farnesylgeranyl diphosphate (FGPP) give rise to an even larger number of terpene carbon skeletons (Wang *et al.,* 2005). Monoterpenoids ($C_{10}H_{16}$), made up of two isoprene units, are the smallest and simplest type of terpenoids; sesquiterpenoids ($C_{15}H_{24}$) are made up of three units; diterpenoids ($C_{20}H_{32}$),

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triterpenoids($C_{30}H_{48}$) and tetraterpenoids ($C_{40}H_{64}$) are made up four, six and eight units, respectively. The diterpene compounds are derived from GGPP and are further classified according to their biogenetic origin such as acyclic phytanes, bicyclic labdanes, tricyclic pimaranes, tetracyclic trachlobanes, macrocyclic cembrane diterpenes and mixed compounds (Devappa *et al.,* 2011). These compounds are classified according to the number and the cyclisation patterns of the skeletal structure. Terpene cyclases are responsible for the biosynthesis of thousands of natural terpenoids and are found in terrestrial and marine organisms (Wang *et al.,* 2005). A terpenoid cyclase binds and chaperones a linear prenyl diphosphate through a cyclisation cascade. The cyclase controls the reaction and provides a template for cyclisation and rearrangement.

For plants and marine organisms, secondary metabolites including terpenes play an important role in the continued competition for space and reproduction, maintenance of an undisturbed surface and the deterrence of predation (Gross & König 2006). Diterpenoid cembranes such as flexibilide and dihydroflexibilide are found in the sea water surrounding the soft coral *Sinularia flexibilis*. These compounds serve as a chemical defence as they are toxic to fish, cause tissue necrosis on nearby corals (eg *Acropora tinius*) and destroys fertilised eggs of hard corals (Aceret *et al.,* 1995).

Three cembrane diterpenoids were isolated from *Lobophytum* sp. that inhibited the cytopathic effect of *in vitro* HIV-1 infection in a cell based assay (Rashid *et al.,* 2000).Two cembrane diterpenoids, Sicrassarine A and B, were isolated from *Sinularia crassa* however these compounds were not toxic when tested at 20 µg/ml (Lin *et al.,* 2012).Other soft coral species from which cembrane diterpenoids have been isolated with anticancer activity include *Lobophytum michaelae* (Wang & Duh 2012), *Sinularia arborea* (Chen *et al.,* 2013), *Lobophytum crassum* (Lin *et al.,* 2011), *Pachyclavularia violacea* (Inman & Crews 1989; Xu *et al.,* 2000), *Sinularia gibberosa* (Li *et al.,* 2005), *Sinularia gaveli* and *Sinularia flexiblis* (Hu *et al.,* 2013; Yen *et al.,* 2012). These compounds have been tested against, amongst others; colorectal carcinoma (HCT-116), mammary gland adenocarcinoma (MDA-MB-231), acute promyelocytic leukemia (HL-60), cervical cancer (HeLa) and mouse lymphoma (P388) cell lines.

Sesquiterpene quinones and hydroquinones (sesquiterpenes) represent a small group of still expanding C15 – C16 metabolites with notable medical applications such as antitumour, antibacterial and anti-HIV activities. Due to their potential antitumour and anti-

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HIV activities as well as the novelty of their structures, the exploration of natural sesquiterpene quinones has increased (Abad *et al.,* 2011). Sesterpenes (C25) are the smallest and therefore the rarest subclass of terpenoids. These terpenoids frequently occur in marine organisms. Sponges can be considered as one of the prime sources of these C25 terpenoid compounds but they are not exclusively found in the marine environment (Gross & König 2006). Sesquiterpenes show a series of pharmacological properties including cytotoxicity, antimicrobial activity and platelet aggregation inhibition, however the anti-inflammatory activity is the dominating feature in this class of compounds (Blunt *et al.,* 2004; Keyzers & Davies-Coleman 2005).

Natural products were expected to play an important role as a major sources of new drugs due to the fact that they have unique structural diversity and relatively small dimensions (<2000 Da) (Sticher 2008). Extraction methods are used as a pre-purification step to selectively remove interfering compounds as well as to isolate the active compounds (Sticher 2008) . If a high polarity solvent is used for the first extraction step the next solvent partition has a finer separation into different polarity fractions. Extraction with low polarity solvents yields more lipophilic compounds and ethanolic solvents extract a larger variety of non-polar and polar compounds. Although the discovery of natural products started with investigations into medicinal plants providing a wealth of new and improved drugs, marine organisms have proved to be just as valuable in process of drug discovery.

2.2 Marine Organisms and the types of Compounds Produced

Over the past 50 years marine organisms have provided key structures and compounds that demonstrate their potential for industrial development as cosmetics, nutritional supplements, fine chemicals, agrochemicals and therapeutic agents for a variety of diseases (Yasuhara-Bell & Lu 2010). In the last 20 years there has been a more rigorous search to discover new drugs from the sea. Most studies have focused on chemical evaluations of marine invertebrates and have shown these organisms to be an important source of new biochemical leads (evident by the number of compounds currently in clinical trials). However it is difficult to obtain adequate, reliable, renewable supplies of these compounds from nature (Kobayashi 2000). Among the many phyla found in the oceans, the best sources of pharmacologically active compounds are bacteria (including cyanobacteria), fungi, certain groups of algae, sponges, soft corals and gorgonians, sea hares and nudibranchs, bryozoans, and tunicates. Some marine organisms such as

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dinoflagellates, echinoderms and some fish are well-known for their ability to produce potent toxins, but these are usually too toxic for medicinal use (Faulkner 2000).

With marine species comprising approximately one-half of the total global biodiversity, the sea offers an enormous resource for novel compounds. Very different substances have been obtained from marine organisms because they are living in a very competitive and aggressive environment, different in many ways from the terrestrial environment, a situation that demands the production of specific and potent active molecules (Vo & Kim 2010). It is probable that these chemical defence mechanisms evolved with the most primitive microorganisms but have been replaced in many more advanced organisms by physical defences and/or the ability to run or swim away and hide. Sessile, soft-bodied marine invertebrates that lack obvious physical defences are therefore prime candidates to possess bioactive metabolites (Faulkner 2000).

A large number of sulphated polysaccharides and other polyanionic substances have been found to be potent *in vitro* inhibitors of various viruses, including important human pathogens such as HIV, herpes simplex virus, human cytomegalovirus, dengue virus and respiratory syncytial virus (Queiroz *et al.,* 2008). Along with other marine invertebrates, sponges have been traditionally known as a source of novel bioactive peptides. The novel structural features and diverse biological activities of these peptidic metabolites have generated considerable interest (Plaza *et al.,* 2007).

2.2.1 Marine Invertebrates

Marine invertebrates are simple multicellular organisms attached to solid substrates in benthic habitats (habitats associated with the sea floor). Marine invertebrates in the family *Alcyoniidae* are a rich source of structurally diverse sesquiterpenes and diterpenes and soft corals such as those belonging to the genus *Lobophytum* and *Sinularia* have provided a number of interesting bioactive compounds against HIV (Rashid *et al.,* 2000; Ellithey *et al.,* 2014).

Pharmaceutical interest in marine organisms arose in the early 1950s with the discovery of the nucleosides spongothymidine and spongouridine in the marine sponge *Cryptotethia crypta* (Yasuhara-Bell & Lu 2010; Laport *et al.,* 2009; Bergmann & Feeney 1950). These nucleosides were the basis for the synthesis of Ara-C, the first marine derived anticancer agent which is being used in the treatment of patients with leukemia and lymphoma, and

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the antiviral drug Ara-A (vidarabine). Ara-A is a semisynthetic compound based on the arabinosyl nucleosides that inhibit viral DNA synthesis (Laport *et al.,* 2009; Bergmann & Feeney 1950). Due to the structural diversity of the secondary metabolites, marine sponges have been considered a "gold mine" with more than 15 000 marine products described so far (Sipkema *et al.,* 2005). Marine organisms have been excellent sources for natural products that are bioactive compounds, having activities such as enzyme inhibition, cell division inhibition, antiviral, antifungal, antimicrobial, anti-inflammatory, cytotoxic and cardiovascular properties (Lee *et al.,* 2001).

The chemical diversity of marine invertebrates products is significant with isolated compounds including unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides and amino acid derivatives (Sipkema *et al.,* 2005; Tziveleka *et al.,* 2003). The early appearance of soft coral, sponges and turnicates etc. in evolution has provided ample time for the development of an advanced chemical defence system. The synthesis of secondary metabolites is regulated depending on conditions that the marine invertebrate experiences. The large number of secondary metabolites discovered in marine invertebrates and the complexity of the compounds can be considered as an indication of the importance of these compounds for survival of the organism (Sipkema *et al.,*2005).

2.2.2 Octocorals and associated microorganisms

Bacteria and other microorganisms are ubiquitous in the marine environment and are taxonomically diverse, biologically active and colonise all marine habitats from the deep oceans to the shallow estuaries as well as coral reefs (Kelman *et al.,* 2006). The surface of living corals is covered by a thick layer of mucus which is colonised by bacteria, allowing for the establishment of a bacterial community that can be characteristic to a particular coral species. Some of these bacteria can be pathogenic to the coral and initiate disease while others could serve as beneficial symbionts or as benign associates (Kelman *et al.,* 2006).

Soft coral metabolites continue to dominate the reports of new compounds but there is an increasing interest in the possibility that associated microorganisms produce some of these metabolites (Kim & Park 2002). In soft coral as well as in sponges the role of the chemical constituents is unclear due to the complexity of the symbiotic relationship as many different bacterial species permanently inhabit soft coral and contribute to the total biomass. It has been suggested that the growth of useful microorganisms may be under the control of the host and serve as a source of food or supply other metabolic products (Sipkema *et al.,*

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2005). Various other microorganisms have been found in soft coral including a diverse range of archea, heterotrophic bacteria, cyanobacteria, green and red algae, dinoflagellates and diatoms. One host can have different symbionts. Bacteria collected from marine organisms have allowed isolation of antimicrobial and antiviral compounds, which suggests that these bacteria may play a role in the defence mechanism of these invertebrates (Lee *et al.,* 2001).

Antiviral compounds are of particular interest as viral diseases are a major health concern especially because viruses have the ability to rapidly develop resistance to existing drugs (Sagar *et al.,* 2010). The nucleoside spongothymidine and spongouridine were the first compounds to be isolated from a marine sponge *Tethya crypta* which led to the synthesis of the anti-cancer Ara-C and the first antiviral drug Ara-A. Ara-A was the only sponge derived compound which was approved by the US FDA as an antiviral drug, although its marketing was later stopped as it was found to be less efficient and more toxic than the newer drug acyclovir (Sagar *et al.,* 2010).

In the 19th and 20th centuries active ingredients from traditional medicines were purified and some of these pure compounds were used as drugs. Molecules such as morphine formed the basis for a new branch of organic chemistry. The marine environment may contain 80% of the world's plant and animal species and this large resource was left virtually unexplored until the mid to late 1960s when chemists from the United States, Europe and Japan began to study the chemical diversity of marine life (Chakraborty *et al.,* 2009). Marine organisms have developed complicated chemical means to survive in an environment that may have extreme temperature, pH changes and pressure.

Soft bodied sessile invertebrates such as soft coral often use a well developed chemical defence and have become the first target in screening programmes because of the potential to provide molecules of use in pharmacology. Octocorals were one of the first marine groups that were screened for secondary metabolites. Cembrane diterpenoids (Figure 3) were found to have an important function in chemical defence in competition for space (allelopathy), against fouling and for inhibiting the reproduction of other organisms such as fishes and some genera of hard coral (Khalesi *et al.,* 2008).

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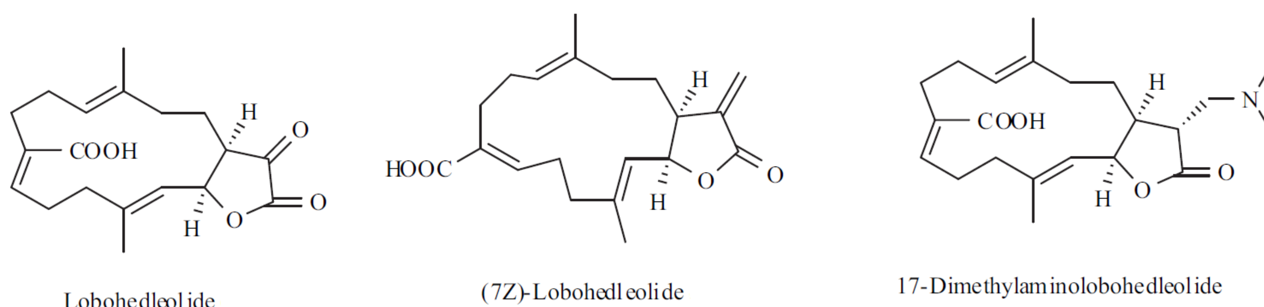

Figure 3: Cembrane diterpenes isolated from a Philippine soft coral, Lobophytum sp
(Gochfeld *et al.,* 2003)

The cembranoid diterpenes lobohedleolide, (7Z)-lobohedleolide and 17-dimethylaminolobohedleolide (Figure 3) were isolated from an anti-HIV extract of a Philippine soft coral.

2.3 **Sinularia *sp* and Biologically Active compounds Produced*.***

Most of the marine natural products which are available today are not general cytotoxic agents but are targeted towards specific cellular or biochemical events and therefore hold a strong potential as antimicrobial, anticancer or antiinflammatory agents (Arepalli *et al.,* 2009). Soft corals are territorial, stationary cnidarians which are a rich source of steroids, terpenoids and other type of secondary metabolites inhibiting the growth of neighbouring animals and algae. The genus *Sinularia* are very common with 90 known species, from these approximately 36 have been chemically examined and have produced several secondary metabolites including furano-sesquiterpenes, sesquiterpenes, cembrane derived diterpenoids and polyhydroxylated steroids. *Sinularia* has also been reported as one of the most toxic genera of all soft corals (Partin 2001). Consistent with this, soft corals belonging to the genus *Sinularia* have been well recognised as marine organisms containing various natural products that show interesting bioactivities. Numerous cytotoxic, anti-inflammatory and antiviral steroids have also been isolated from the *Sinularia* species in the last 10 years (Yen *et al.,* 2013). Among the plethora of secondary metabolites isolated from *Sinularia* are furanone diterpenes (*S.nanolobata*), glycosides (*S. cervicornis*), simple alkaloids (*S. capillosa*, S. fibrilla, S. polydactyla), sesquiterpenes (S. gibberosa), Norsesquiterpenes (*S. nanolobata*), polyoxygentaed sterols (*S. gyrosa* and *S. gibberosa*), cembranoids and norcembranoids (*S. gibberosa*, *S. microclavata*, *S. tenella*, *S. foeta*, *S. leptoclados*, *S. parva* and *S. conferta*) (Zhang *et al.,* 2006). Almost half of the compounds isolated from different *Sinularia* species have proven to be toxic towards a wide variety of cancerous cell lines.

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In 1975 the first cembrane diterpenoid isolated from *Sinularia* sp. was Sinulariolide isolated from *S. flexibilis*. This compound exhibited a broad range of biological activities but had limited cytotoxicity (Kamel & Slattery 2005). Aceret *et al.,* (1995) reported that flexibilide and dihydroflexibilide, two metabolites found in the tissue of *S. flexibilis*, were also found in the water surrounding the coral and in concert with other compounds were toxic to hard coral and were able to deter fish from feeding. Seven cembranoids were isolated from *S. giberossa,* four of which were novel and showed significant cytotoxicity towards lung carcinoma (A549), colorectal adenocarcinoma (HT-29), HeLa cells containing HPV (KB) and murine lymphoma (P388) cell lines (Duh & Hou 1996). *S. gyrossa* produced three novel diterpenoids, Gyrosanol A – C, showing no cytotoxic or antibacterial properties but which did exhibit antiviral activity against human cytomegalovirus and antiinflammatory activity by suppression of COX-2 (Chen *et al.,* 2010). Terpenoids isolated from *S. crassa* were tested against various cell lines and only exhibited cytotoxicity at concentrations above 20 µg/ml however, Crassarosterol A was toxic towards K562 and MOLT-4 leukemia cell lines (Lin *et al.,* 2012; Chen *et al.,* 2013). These studies indicate that soft coral of the genus *Sinularia* are a promising target for the isolation of new bioactive compounds to be used in the development of cancer chemotherapies.

Although many natural products from marine invertebrates are promising drugs or lead compounds, the difficulty in the supply of raw coral biomass has resulted in restrictions in the advance of biologically active products from corals (Khalesi *et al.,* 2008). A maintainable and cost effective supply method is required to overcome this problem. This can be done by aquaculture, cell culture, chemical synthesis and genetic manipulation (Khalesi *et al.,* 2008). Many bioactive marine natural products, especially those used in the pharmaceutical field, have complex structures and require a multi-step synthesis process that is not economically viable on an industrial scale (Ley *et al.,* 2002). Most active compounds are found at low concentrations in coral so large amounts of the coral are required to isolate sufficient compounds for clinical trials (Khalesi *et al.,* 2008). If these corals were to be harvested directly from the sea it would be environmentally destructive (Kleypas *et al.,* 1999). Corals have been successfully grown in hobby and public aquariums leading to the belief that aquaculture could be a solution for the supply of certain species of marine invertebrates. Culturing of marine organisms which have produced promising anticancer and antiviral compounds could be the answer to providing new lead compounds for the development of drugs to treat infections and diseases where existing treatment is no longer as effective. Infection with the Human Immunodeficiency Virus has plagued millions of

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people worldwide and the development of viral resistance to current treatment has made the search for new treatments a necessity.

2.4 Global Statistic of HIV Infection

Globally 34 million (31.4 – 35.9 million) people were living with Human Immunodeficiency Virus (HIV) during 2011. An estimated 0.8% of adults aged 15 – 49 years worldwide live with HIV although the burden of the pandemic varies between countries and regions. Sub-Saharan Africa remains the most severely affected with 1 in every 20 adults (4.9%) living with HIV. The number of adults and children that acquired HIV in 2011 (2.5 million) was 20% lower than in 2001 with the largest decreases seen in the Caribbean (42%) and Sub-Saharan Africa (25%), however the number of new infections in some parts of the world continues to increase. In 2011 1.7 million (1.5 – 1.9 million) people died from AIDS related causes worldwide showing a 24% decrease in AIDS related mortalities compared to 2005 (2.3 million). The number of AIDS-related deaths in Sub-Saharan Africa decreased by 32% from 2005 to 2011 but the region accounted for 70% of all people dying from AIDS in 2011. According to the UNAIDS World AIDS Day Report 2012, the HIV incidence rate in South Africa declined by 41% from 2001 (2.42 million) to 2011 (1.43 million) and the AIDS related deaths declined by 27% from 2005 (370 847 deaths) to 2011 (270 190 deaths) (UNAIDS 2012). Data published by UNAIDS in 2013 ranked South Africa first in the world with 6.3 million people living with HIV/AIDS with 340 000 new infections reported in 2013. South Africa is ranked second in the world having 200 000 AIDS related deaths in 2013 (decreased from 260 000 in 2012), second only to Nigeria (UNAIDS 2015).

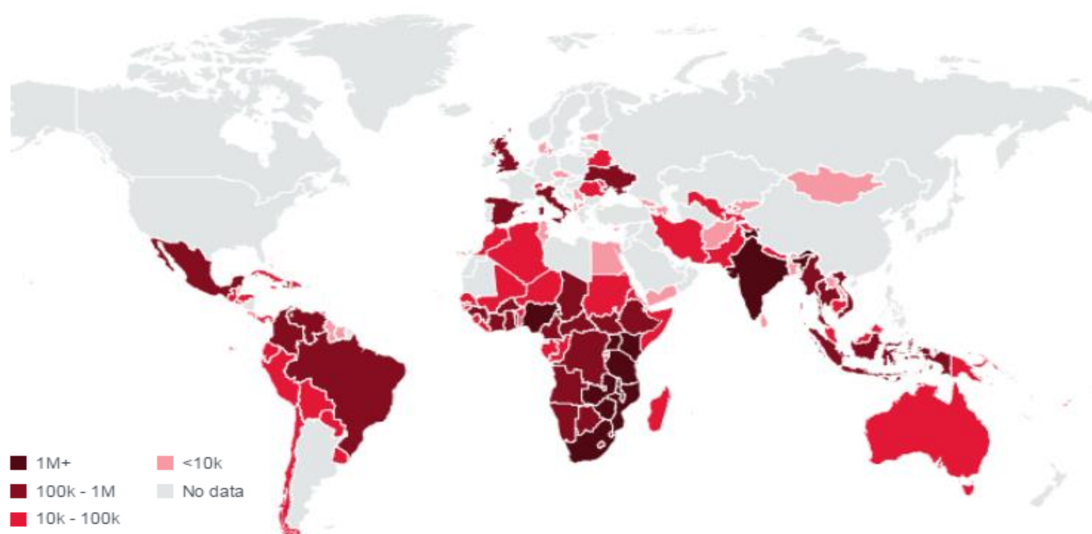

Figure 4: Number of people living with HIV in 2013 (UNAIDS, 2012)

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2.5 The Human Immunodeficiency Virus

AIDS is a clinical syndrome resulting from infection by HIV, a virus which causes profound immunosuppression (Singh *et al.,* 2005). HIV is classified in the family *Retroviridae*, subfamily *Lentivirinae* and genus *Lentivirus* (Shehu-Xhilaga & Oelrichs 2009) and is divided into two broad types, HIV-1 and HIV-2. HIV-1 is more commonly distributed and responsible for most of the infections worldwide. HIV-2 is confined to western central Africa and southern and western India but sporadic occurrences and transmission have been reported in countries such as the United States of America and Australia. HIV-1 is most similar to simian immunodeficiency virus (SIV) isolated from chimpanzees and HIV-2 to viral strains from the Sooty Mangabey (*Cercocebus torquatus atys*). HIV-2 results in a less virulent infection than HIV-1 with generally lower viral loads, lower rates of vertical transmission and slower progression of the disease to AIDS (Shehu-Xhilaga & Oelrichs 2009). HIV-1 is the cause of the worldwide pandemic and is most commonly referred to as HIV. It is a highly variable virus which mutates readily. There are many different strains of HIV-1 which are classified according to groups and subtypes; there are two groups, M and O. Within group M, there are currently known to be at least ten genetically distinct subtypes of HIV-1 (Singh *et al.,* 2005).

The defining property of retroviruses is their ability to assemble into particles that can leave producer cells and spread infection to susceptible cells and hosts (Sundquist & Kräusslich 2012). The structure of HIV follows the typical pattern of the retrovirus family, containing the single stranded, positive sense ribonucleic acid (RNA) genome of about 9.7 kilobases. There are two strands of HIV RNA and each strand has a copy of the virus's 9 genes (Shehu-Xhilaga & Oelrichs 2009). The RNA is surrounded by a cone shaped capsid consisting of around 2000 copies of the p24 viral protein, and surrounding the capsid is the viral envelope made up of a lipid bilayer from the cellular membrane of the host cell (Shehu-Xhilaga & Oelrichs 2009). The assembled virion packages all of the components required for infectivity (Figure 5) which includes two copies of viral RNA, cellular $tRNA^{Lys,3}$ molecules to prime complementary DNA (cDNA) synthesis, the viral envelope protein (Env), the Gag polyprotein, and the three viral enzymes protease, reverse transcriptase and integrase (Sundquist & Kräusslich 2012).

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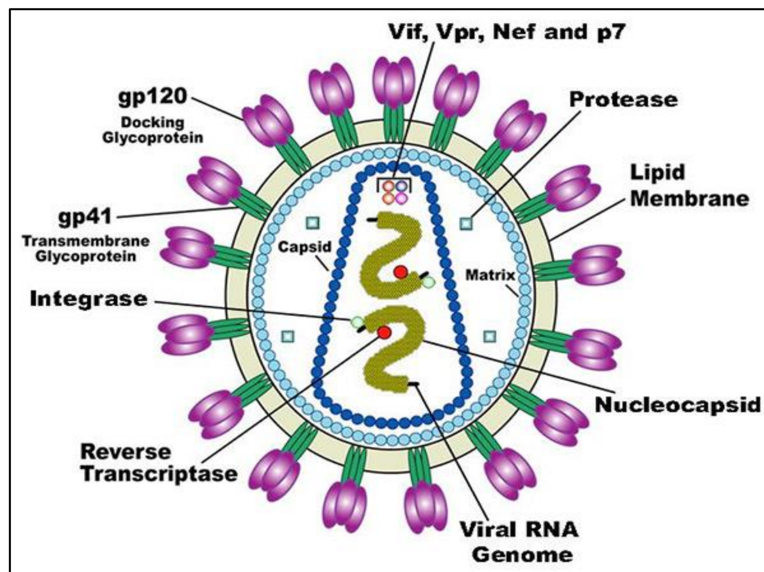

Figure 5: Diagrammatic structure of an HIV-1 virion

(Arnegard 2012)

2.6 Life cycle of HIV

The natural history and pathogenesis of HIV-1 infection are closely linked to the replication of the virus *in vivo* and the clinical stage is associated with all measures of virus load, including infectious virus titres in blood, viral antigen levels in serum, and viral nucleic acid content of lymphoreticular tissues, peripheral blood mononuclear cells (PBMCs) and plasma (Wei *et al.,* 1995). Ever since the first description, AIDS has been related to depletion of $CD4^{+}$ T helper lymphocytes in the blood. Other cells, such as tissue macrophages, become infected by HIV and there is a significant viral load in the lymph nodes in the absence of treatment (Weiss 2012).

During early infection, host cellular and humoral immune responses to HIV appear to be effective in limiting viral replication in peripheral blood cells (Weiss 2012). HIV uses a number of steps to enter the target cell and mechanisms by which this happens are understood in great molecular detail (Stolp & Fackler 2011). The primary receptor for HIV-1 is CD4 which is present on the surface of many lymphocytes, which are a critical part of the body's immune system (Singh *et al.,* 2005). This explains the propensity of the virus to infect certain T cells and macrophages (Levesque *et al.,* 2004). The first step of viral entry into target cells is the binding of a viral surface protein to receptors on the plasma membrane of the host cell (Permanyer *et al.,* 2010). Although CD4 binding is a prerequisite for HIV- 1 entry, attachment of the virus is mediated by molecules that serve to concentrate virus on the cell surface and increase the frequency of Env-receptor interactions (Doms &

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Trono 2000). HIV envelope glycoproteins gp120 and gp41 are the major inducers of neutralizing antibody responses and these proteins also govern the viral entry process.

Binding of gp120 to the CD4 receptor results in a conformational change in gp120 that exposes the binding site for one of the HIV-1 entry co-receptors CCR5 or CXCR4 (Stolp & Fackler, 2011). Most primary HIV-1 strains use the CCR5 chemokine receptor (R5 virus strain) and an absence of CCR5 due to a genetic polymorphism, is associated with a certain degree of resistance to viral infection (Doms & Trono 2000). In some individuals the virus evolves to use the related receptor CXCR4 either in place of (X4 strain) or in addition to CCR5 (R5X4 strains). The emergence of X4 virus types is associated with accelerated progression to AIDS (Doms & Trono 2000). Viral strains that bind to CCR5 infect macrophages and T cells and are characterised by less aggressive growth *in vitro*. Strains that recognise CXCR4 infect only T cells and T cell lines, with *in vitro* growth characterised by high viral titre and the presence of syncytial cells formed by the fusion of multiple infected cells (Shehu-Xhilaga & Oelrichs 2009).

Once the virus has entered cells, it replicates in the same way as other retroviruses. Briefly, one of the proteins carried in the virus particle is the viral reverse transcriptase (RT), which transcribes the viral RNA into a complementary DNA (cDNA) (Janeway *et al.,* 2001). The newly copied DNA is then integrated into the genome of the host cell, taking over the protein synthesis machinery to give rise to new virus particles. In the HIV-1 particle approximately 80 copies of RT are reported to be packaged inside the virus core. Reverse transcriptase shown in Figure 6 is a common name for an enzyme functioning as an RNA-dependent DNA polymerase to copy viral RNA to DNA before integrating the DNA into the host cell nucleus. RT activity includes RNA-dependent DNA polymerase activity to transcribe both single stranded RNA and single stranded DNA templates, DNA-dependent DNA activity and RNaseH activity to degrade RNA from the RNA-DNA hybrids.

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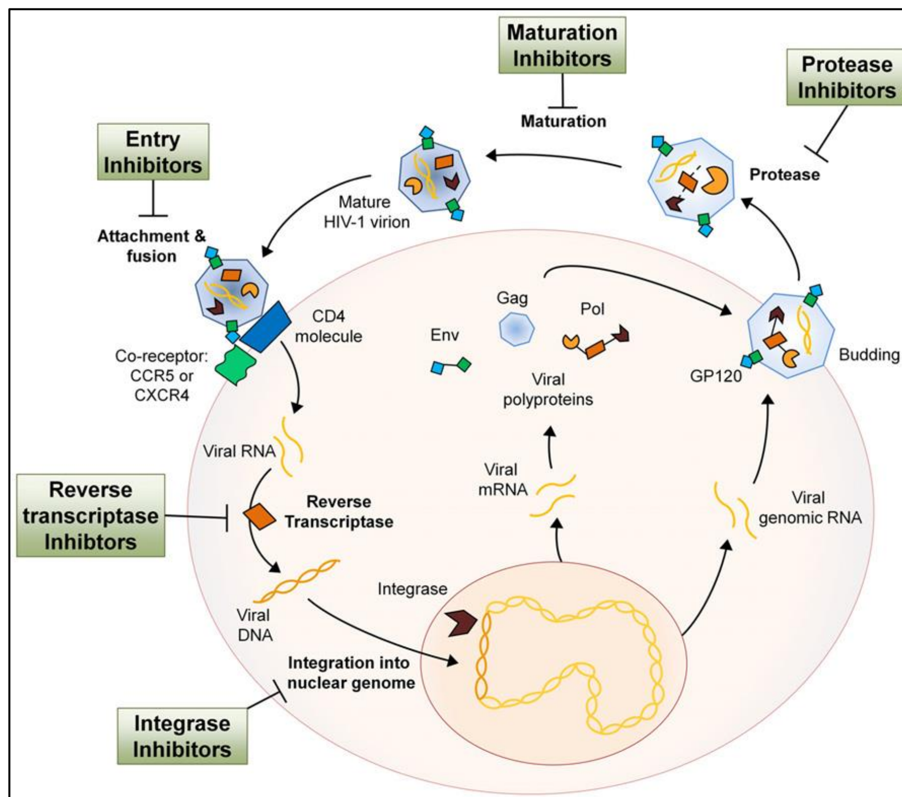

Figure 6: Replication cycle and potential drug targets of HIV-1
(reproduced with the permission of R. L. Smith, de Boer, Brul, Budovskaya, & van Spek, 2012).

Reverse transcription of DNA from RNA requires primer annealing at the 5' end of the genome. An antisense DNA strand is produced while RNaseH degrades the original RNA strand for antisense strand transfer and sense strand synthesis, which also generates polypurine tract (PPT) primers to initiate sense strand synthesis. RT does not have a proof reading mechanism causing it to incorporate incorrect bases into the DNA, which is one of the causes of the high mutation rate of HIV (Wisniewski *et al.,* 2000; Malmsten 2005).

The viral cDNA is then integrated into the host-cell genome by the viral integrase, which also enters the cell with the viral RNA. The integrated cDNA copy is known as the provirus. In activated CD4 T cells, virus replication is initiated by transcription of the provirus. However, HIV can, like other retroviruses, establish a latent infection in which the provirus remains dormant. This seems to occur in memory CD4 T cells and in dormant macrophages, and these cells are thought to be important reservoirs of infection (Janeway *et al.,* 2001).

The HIV genome (Figure 7) consists of nine genes flanked by long terminal repeat (LTR) sequences. The latter are required for the integration of the provirus into the host-cell DNA

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and contain binding sites for gene regulatory proteins that control the expression of the viral genes. Like other retroviruses, HIV has three major genes which are *gag*, *pol*, and *env*. The *gag* gene encodes the structural proteins of the viral core, *pol* encodes the enzymes involved in viral replication and integration, and *env* encodes the viral envelope glycoproteins. The *gag* and *pol* mRNAs are translated to produce polyproteins that are then cleaved by the viral protease (also encoded by *pol*) into individual functional proteins. The product of the *env* gene, gp160, has to be cleaved by a host-cell protease into gp120 and gp41, which are then assembled as trimers into the viral envelope. HIV has six other, smaller, genes encoding proteins that affect viral replication and infectivity in various ways. Two of these, Tat and Rev, perform regulatory functions that are essential for viral replication. The remaining four, Nef, Vif, Vpr, and Vpu, are essential for efficient virus production *in vivo* (Janeway *et al.,* 2001).

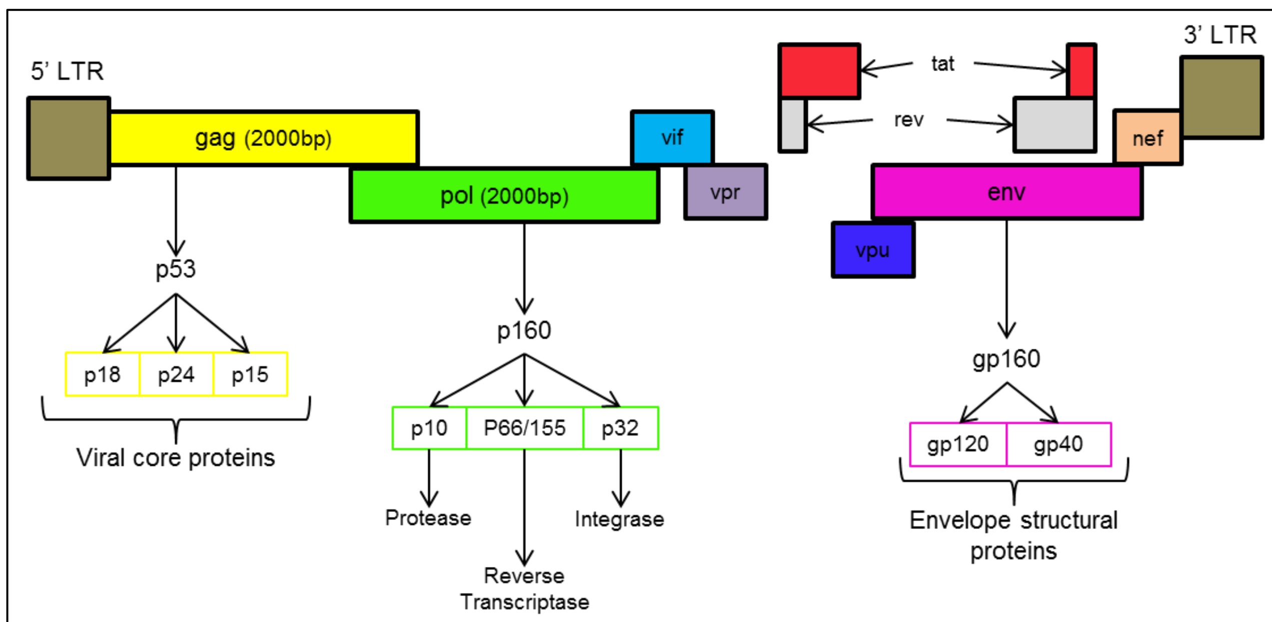

Figure 7: Diagrammatic representation of an HIV-1 genome

(adapted with permission from Hoffman & Rockstroh, 2012; Rubbert, Behrens, & Ostrowski, 2011)

Immature viral polypeptides are processed into their functional forms by the protease enzyme and assembled with HIV RNA transcripts into new viral particles. During budding from the plasma membrane, viral proteins within these particles are processed into their functional forms by HIV protease and rearranged into mature particles (Figure 8). The Vpu protein aids virion release from the cell membrane where it works with a host cell factor named tetherin, an interferon-alpha-induced human protein. Tetherin is a membrane associated protein that blocks release of viral particles. Without Vpu, HIV-1 particles

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become tethered to the cell membrane and cannot be released (Shehu-Xhilaga & Oelrichs 2009).

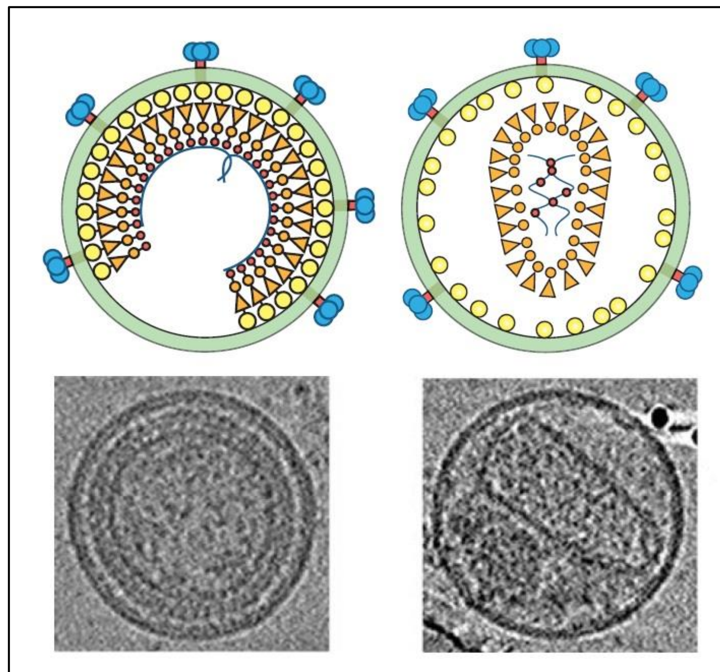

Figure 8: Diagrammatic representation and cryon-electron microscopic representation of an immature virion (left) and mature virus (right) particle

(reproduced with the permission of Sundquist & Kräusslich, 2012).

2.7 Stages of HIV infection

The acute phase of HIV infection is clinically characterized by an influenza like illness in up to 80% of cases, with a large amount of virus (viremia) in the peripheral blood and a significant decrease in the numbers of circulating $CD4^{+}$ T cells caused by cell death. Diagnosis at this stage is easily missed. The acute viremia is seen in almost all patients with the activation of $CD8^{+}$ T cells, which kill HIV-infected cells, and also with antibody production (seroconversion) (Johnston & Fauci 2007). The cytotoxic T-cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4 T-cell counts recover to around 800 cells per µl (Figure 9) (the normal value is around 1200 cells per µl) (Janeway *et al.,* 2001).

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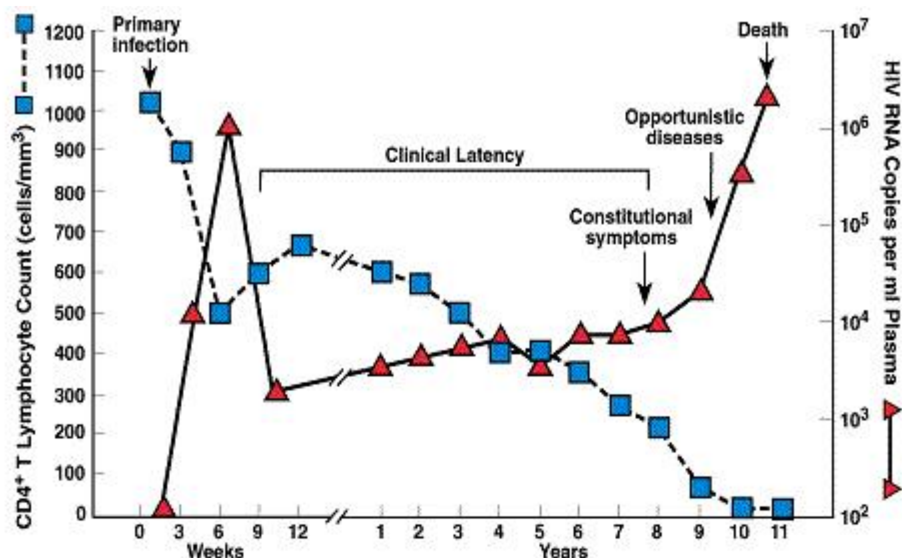

Figure 9: Time course of HIV infection

(Fauci 1988; Popovic *et al.,* 2009)

Primary infection is followed by a long period of clinical latency (up to 10 or more years) in which little or no viral replication is observed in the peripheral blood compartment (Poli *et al.,* 1993). The next stage is subclinical infection in which people are asymptomatic but have persistent generalised lymphadenopathy, which is an indication that immune suppression has begun. Subclinical infection is characterised by >500 $CD4^+$ T cells/µl or per mm^3 of blood (Dimmock *et al.,* 2001). During the pre-AIDS stage of infection (<200cells/µl) patients are asymptomatic but show signs of weight loss and may suffer from candidiasis, fever, diarrhoea lasting more than one month, or more than one episode of shingles. This is indicative of a defect in cell mediated immunity (Dimmock *et al.,* 2001). The final phase of infection is when opportunistic infections or tumours occur usually in conjunction with a re-emergence of HIV in the peripheral blood compartment (Poli *et al.,* 1993). AIDS is characterised as fewer than 200 CD4+ cells/µl blood and results in severe infections or cancers. If an AIDS patient does not die from infection with opportunistic microorganisms, HIV goes on to infect $CD4^-$ cells and causes diseases in the muscles and central and peripheral nervous systems. AIDS dementia or collapse of brain function is the final stage of infection. The time taken to progress to AIDS varies greatly between individuals and can be influenced by availability and adherence to treatment (Dimmock *et al.,* 2001). Naturally occurring polymorphisms of the CCR5 coreceptor plays an important role in the susceptibility of T cells to HIV-1 infection and progress of the disease. HIV pathogenesis involves both the response of cytotoxic T cells (cytotoxic response) to viral proteins and the release of β-chemokines by CD8+ T cells (suppression response) which are important in the anti-HIV response.

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2.8 The effect of HIV on the Immune System

HIV infection causes down-regulation of CD4 molecules expressed at the cell surface. Early in infection, the Nef accessory protein down-modulates CD4 molecules that are already present at the cell surface by speeding up their endocytosis and degradation in lysosomes. At later stages of infection, the envelope gp160 precursor retains newly synthesized CD4 in the endoplasmic reticulum. The accessory viral protein U (Vpu) regulates the half-life of CD4 by inducing the degradation of viral receptors complexed to Env-gp160 precursors in the endoplasmic reticulum, releasing gp160 and allowing its maturation and transport (Levesque *et al.,* 2004).

The CD4 antigen is a type I integral membrane glycoprotein of 55-kDa that is expressed at the cell surface of class II major histocompatibility complex (MHC-II)-restricted T lymphocytes, thymocytes, and cells of the macrophage-monocyte lineage. The CD4 molecule plays a key role in the development and activation of helper T cells. During the antigenic response, the physiological role of CD4 is to stabilize interactions between T cells and antigen-presenting cells and to transduce a signal, critical for antigen responsiveness of $CD4^{+}$ T cells, upon binding to nonpolymorphic regions of the MHC-II molecule (Levesque *et al.,* 2004; Dimmock *et al.,* 2001).

During the sexual transmission of HIV, dendritic cells (DC) such as Langerhans cells and immature myeloid cells at mucosal surfaces are proposed to be among the first target cells to encounter the virus. HIV attachment factors expressed by immature dendritic cells bind HIV and migrate to lymphoid tissues containing CD4+ T cells. Maturation of these dendritic cells result in the presentation of HIV antigens to T cells in the lymphoid tissues and initiate antiviral immune responses (Wu & Ramani 2006). Follicular dendritic cells (FDCs) which are found in germinal centres can trap large amounts of HIV at the cell surface providing a 'hideaway' for HIV (Wu & Ramani 2006). Without antiviral therapy HIV infects more cells in the FDC network leading to lymph node damage and this causes the release of more viral particles into the circulation (Mishra *et al.,* 2009).

Dendritic cells are the antigen-presenting cells which are important in linking innate and adaptive immunity. DC maturation can be induced by different stimuli like pro-inflammatory cytokines and bacterial products. Immature dendritic cells have pattern recognition receptors (PRRs) for microbes (Mayer *et al.,* 2013). One such receptor is the toll-like receptor (TLR) which has the ability to load antigens and then migrate to the lymph nodes,

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where they mature and present the proteasome-degraded antigens to naive T-cells. Mature DCs stop antigen-loading and enhance the expression of major histocompatibility complexes and accessory molecules, such as CD86, CD80 and CD40, and then produce cytokines and chemokines (Dimmock *et al.,* 2001; Lin *et al.,* 2013). The signalling cascade responsible for lipopolysaccharide-stimulated DCs is also involved in the TLR4 binding lipopolysaccharide, p38 mitogen-activated protein kinase (MAPK) activation and Nuclear Factor-kappa B (NF-κB) activation (Lin *et al.,* 2013).

NF-κB in the cytoplasm complexes with the inhibitor of κB (I-κB) repressor protein. Activation by stimulus such as lipopolysaccharides phosphorylates I-κB by I-κB kinase (IKK), and is subsequently degraded by ubiquitin-mediated proteasomal degradation. NF-κB is dissociated from I-κB in the cytoplasm and is translocated into the nucleus to activate the inflammatory cytokine and chemokine genes. Therefore, dendritic cells play a role in controlling infectious diseases, as well as cancers. However, they also play a part in the pathogenesis of chronic inflammation and autoimmunity. Stopping these diseases may be brought about by the suppression of DC activation (Lin *et al.,* 2013). HIV infection has been shown to affect peripheral cells such as monocytes and dendritic cells as well as the central nervous system leading to immune dysfunction (Samikkannu *et al.,* 2014).

2.8.1 HIV infection causes oxidative stress

HIV directly affects immune function causing a deficiency in antigen presentation. This is exhibited by a disruption of inflammatory cytokines, chemokines, and results in oxidative stress-induced reactive oxygen species (Samikkannu *et al.,* 2014). HIV infection activates the immune system and affects apoptotic pathways leading to cell death.

Oxidation is the transfer of electrons from one atom to another and is an essential part of metabolism as oxygen is the ultimate electron acceptor in the respiratory electron transport chain, which produces energy (ATP). A free radical is any species (atom, molecule or ion) containing at least one unpaired electron in its outermost orbital and which in turn is able to exist independently. Free radicals are generated when the electron flow becomes uncoupled (transfer of unpaired single electrons). The unpaired electron makes free radicals highly reactive and capable of causing oxidative damage. The major family of free radicals is the reactive oxygen species (ROS) (Ngondi *et al.,* 2006). Free radicals cause damage to different levels in the cell attacking lipids and proteins in the cell membrane resulting in the cell being unable to perform vital functions such as the transport of nutrients, waste disposal and cell division. Free radicals oxidise many biological structures causing

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oxidative damage which is a major cause of aging, cancer, atherosclerosis and chronic inflammatory processes. Antioxidants may delay or stop the formation of free radicals by donating hydrogen atoms or scavenging them. An antioxidant can be broadly defined as any molecule capable of preventing or delaying oxidation (loss of one or more electrons) from other molecules, usually biological substrates such as lipids, proteins or nucleic acids (Pérez & Aguilar, 2013). Under normal circumstances the body is protected from oxidative damage by a careful balance between pro-oxidants and antioxidants (Ngondi *et al.,* 2006). Certain foods are good sources of antioxidants and are sometimes included in the treatment of illnesses associated with oxidative stress.

ROS play an important role in several metabolic pathways such as redox-dependent signalling cascades, intracellular signalling after T cell activation, apoptotic and necrotic cell death pathways and chemotaxis, by increasing CCR5 and CXCR4 expression (Salmen & Berrueta 2012)

An increased oxidative stress condition has been repeatedly described in HIV infected patients. Evidence of this is based on elevated extracellular and intracellular ROS levels, reduction in glutathione (GSH) and thioredoxin concentrations, disturbance of mitochondrial membrane potential, and changes in the expression and activation status of cell death receptors, which may lead to host cell death (Salmen & Berrueta 2012). During apoptosis there is an increase in reactive oxygen species (ROS) leading to oxidative stress, which could further contribute to apoptotic cell death. An increased in HIV replication, induced by ROS, has been observed in reservoir cells (Salmen & Berrueta 2012). Increased ROS formation causes damage to proteins, lipids and nucleic acids. The viral Tat protein has been shown to increase the apoptotic index by increasing the production of intracellular reactive oxygen species. Antioxidants that are naturally present to protect the immune system are depleted in the process of protecting cells against reactive oxygen species-induced oxidative damage (Oguntibeju *et al.,* 2009).

HIV infection increases the oxidative stress process, which is then further increased by HAART usage (Sundaram *et al.,* 2008; Sharma 2014). HAART can also cause oxidative stress in patients by interfering with mitochondrial function. HIV also causes a reduction of glutathione (GSH) leading to oxidative damage. Insufficient GSH has been linked to an increase in HIV viral transcription and progression of the disease (Staal *et al.,* 1993; Aukrust *et al.,* 2003). GSH is natural antioxidant found in mammalian cells and functions to

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protect the body against oxidative stress by acting as a free radical scavenger. The antioxidant function of GSH is related to oxidation of the thiol group of the cysteine residue using GSH peroxidase, forming the disulphide GSSG. GSSG is catalytically reduced back to the thiol form (GSH) by glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) (Ciriolo *et al.,* 1997).

2.9 HIV and Associated Opportunistic Infections

HIV infection, by virtue of its severe repression of the immune system, predisposes infected individuals to malignancy (Tombes & Parpart 1998). The most common tumours in patients with AIDS are Kaposi sarcoma (KS) and non-Hodgkin lymphoma, but various other tumours, such as Hodgkin disease, alimentary tract tumours and anorectal cancers have been reported (Spano *et al.,* 2002). The natural history of HIV infection is variable. Some individuals live with HIV for many years without developing significant immunosuppression while others may develop AIDS and die within a year or two. The rate of progression may be affected by many factors such as the infecting viral strain, host susceptibility and immune function as well as external factors such as access to healthcare and exposure to coinfections (Lawn 2005).

The occurrence of oral lesions in HIV infected persons has been documented from the first reports of a group of young homosexual males presenting with *Pneumocystis carinii* pneumonia (PCP) in 1981 (Leigh *et al.,* 2004). Other opportunistic infections (OI) occurring orally in HIV infected patients are oral hairy leukoplakia (OHL), KS, oral warts and necrotising periodontal conditions. None of these oral infections are life threatening on their own but are all associated with significant morbidity caused by oral pain, reduced nutritional intake and associated wasting. Oropharyngeal candidiasis (OPC), OHL and KS occurred when blood CD4+ T cells dropped below 400 cells per µl (Leigh *et al.,* 2004; Margiotta *et al.,* 1999). The most commonly studied OI in HIV positive individuals is OPC caused by *Candida albicans*.

Immune suppression by HIV infection also appears to worsen the outcome of Human Papilloma Virus (HPV) infection. The most important known determinant of HPV persistence and progression to cancer is the viral type which is normally HPV 16. Women infected with HIV are at a significantly increased risk of invasive cervical cancer (Frisch *et al.,* 2000; Clifford *et al.,* 2006). Prior to the introduction of Highly Active Antiretroviral Therapy (HAART), the major causes of mortality among HIV positive individuals were

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opportunistic infections such as PCP and malignancies like KS and non-Hodgkins lymphoma. Although cervical cancer is included as an AIDS defining illness, the incidence and associated mortality rate from cervical cancer is low in countries with routine cervical cytologic screening (Palefsky 2003). HIV positive women have a higher prevalence of cervicovaginal HPV infection than age and risk matched HIV negative women. HPV prevalence increased levels for both low risk HPV types (6 and 11) as well as high risk types (16 and 18) in patients with progressively lower CD4+ levels (Palefsky 2003). Anal intra-epithelial neoplasia (AIN) was also shown to be more common among HIV positive men (Palefsky 2006). As with HPV infection, one of the most consistent risk factors for AIN was a lower CD4+ level suggesting that HIV-related immune suppression as reflected by CD4+ levels, was playing a role in disease pathogenesis (Palefsky 2006; Frisch *et al.,* 2000).

The relationship between HIV and cervical neoplasia is unique. Unlike other AIDS-related malignancies, the association is not explained solely on the basis of immunosuppression. Cervical cancer and HIV infection are both, in part, sexually transmitted diseases and share common etiologies involving sexual behavioural risk factors (Maiman *et al.,* 1993). HIV seropositivity may predispose women to cervical neoplasia which could adversely affect the prognosis of those with invasive cervical cancer (Boardman *et al.,* 2013). As in other AIDS-related malignant lesions, patients with massive cervical carcinoma have a more advanced stage of the disease and a poorer prognosis than HIV seronegative patients (Smith 2010). Maiman *et al.,* (1993) also states that patients with AIDS and KS have more widely distributed lesions and mucus membrane and lymph node involvement than patients with classic KS. Besides carcinomas and malignancies, opportunistic infection with other microorganisms which can also occur during HIV/AIDS, the most common in Sub-Saharan Africa being infection by *Mycobacterium tuberculosis* (Morens *et al.,* 2004). Additional examples of opportunistic infections are shown in Table 1 (Janeway *et al.,* 2001).

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Table 1: Table of possible opportunistic infections and AIDS associated diseases that may occur in HIV positive individuals (Janeway *et al.,* 2001)**.**

Infections	
Parasites	*Toxoplasma* spp. *Crytosporidium* spp. *Leishmania* spp. *Microsporidium* spp.
Intracellular Bacteria	*Mycobacterium tuberculosis* *Mycobacterium avium intracellulare* *Salmonella* spp.
Fungi	*Pneumocystis jirovecii* *Crytococcus neoformans* *Candida* spp. *Histoplasma capsulatum* *Coccidioides immitis*
Viruses	Herpes simplex Cytomegalovirus Herpes zoster
Malignancies	
Kaposi's Sarcoma - (HHV8)	
Non-Hodgkins lymphoma, including EBV-positive Burkitt's lymphoma	
Primary lymphoma of the brain	

Tuberculosis (TB) was a leading cause of death worldwide for centuries until 1945 when improved treatment and hygiene practices resulted in a steady decline in the death rate up to 1985. However from 1985 until 1992 in the US alone, there were over 50 000 new cases of TB (Kirschner 1999). TB has since been called the “main opportunistic disease for HIV” and the HIV/AIDS pandemic has led to a dramatic increase in the number of TB cases worldwide (Bertozzi *et al.,* 2005). Globally, 9% of all new TB cases (31% in Africa) in adults were attributed to HIV/AIDS, as were 12% of the deaths from TB in 2000 (Sharma *et al.,* 2005). The interaction between TB and HIV infection is complex and HIV infected patients who have a weakened immune system have an increased likelihood of reactivation, reinfection and progression of latent TB infection to active disease. Activation of mononuclear cells during the host response to TB leads to accelerated HIV replication, which may increase the HIV load at anatomical sites associated with TB (Foroughi *et al.,* 2011). HAART leads to dramatic reductions in the incidence of opportunistic infections including TB as pathogen specific immune responses are restored.

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2.10 Cervical Cancer

High risk HPV (hrHPV) infection is usually related to cancer of the cervix, vagina,vulva, anal canal, penis and oropharynx (Arbyn *et al.,* 2012). HPV is detectable in virtually all cervical cancer cases with HPV16 being the most common type. HPV 16 and HPV 18 account for approximately 71% of all cases of cervical cancer (Arbyn *et al.,* 2012). HPV is one of the most common sexually transmitted infections (Palefsky 2010). Cervical infections are the best understood HPV infection and cervical HPV persistence is the known necessary event for the development of cervical cancer (Mosciki *et al.,* 2012).

Approximately 530 000 new cases of cervical cancer were estimated for 2008 and it is reported that this number could increase to as much as 665 000 by 2020. Worldwide the ratio of mortality to incidence is 52% with an estimated 275 000 women dying in 2008. Cervical cancer is the third most common cancer in women worldwide and the second most common in developing regions. Approximately 47% of new annual cervical cancer cases are diagnosed in women older than 50. Less developed regions of the world account for 86% of the global burden where cervical cancer accounts for 13% of all cancers in women and is the most common cancer in woman in Sub-Saharan Africa, Central Asia and Melanesia (Arbyn *et al.,* 2012).

Invasive cervical cancer is the third most common cancer among women worldwide, however there are no standard guidelines on the optimal method to screen HIV infected women for cervical cancer in resource limited countries (RLC) where access to screening is limited by financial and personal capabilities (Firnhaber *et al.,* 2013). A screening programme with technically appropriate detection methods could reduce morbidity and mortality among HIV infected women. Alternative screening methods have been evaluated for the detection of high grade cervical intra-epithelial neoplasia (CIN 2 or 3) in Africa but a comparison of the three most common cervical cancer screening methods (Pap smear, visual inspection with 3-5% acetic acid; and HPV DNA testing) is not always conducted in HIV infected women in Africa. The decision on which screening modality to use will be influenced by cost, patient population, availability if skilled human resource and laboratory capacity.

HIV positive women in Sub-Saharan Africa have a higher prevalence of cervical squamous intra-epithelial lesions (SIL) and invasive cervical cancer than HIV positive women in other regions of the world. Women with a lower CD4 count consistently have higher rates of high

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grade squamous intra-epithelial lesions. The prevalence of hrHPV infection is high in Sub-Saharan Africa with infections persisting longer in HIV positive women. Limited access to cervical cancer screening programmes in this region of the world place the women at an increased risk for the development of cervical cancer (Firnhaber *et al.,* 2013).

HIV positive women in resource limited countries have better access to HAART and are living longer as death caused by opportunistic infections decline. The use of HAART has been shown to reduce the risk of opportunistic malignancies such as Kaposi sarcoma and some lymphomas. Firnhaber *et al.,* (2012) reported that HAART use was associated with a reduction in the rate of incidence and progression of cervical cancer lesions in HIV positive women, concluding that the integration of cervical cancer screening programmes into HIV treatment programmes should be a public health imperative in South Africa.

Table 2: Selected statistics from GLOBOCAN (2012)

All Cancer (excl. non-melanoma skin cancer)				
	Women		**Men**	
Region	Cases	Death	Cases	Death
Worldwide	6663	3548	7427	4653
More developed regions	2832	1287	3244	1592
Less developed regions	3831	2261	4184	3062
WHO Africa Region	381	250	265	205
Cervical Cancer				
Worldwide	528	266	-	-
More developed regions	83	36	-	-
Less developed regions	445	230	-	-
WHO Africa Region	92	57	-	-
WHO Americas Region	83	36	-	-
WHO South East Asia Region	175	94	-	-
WHO Western Pacific Region	94	43	-	-

Approximately 85% of the global cervical cancer burden is in less developed regions (along with liver cancer) where it accounts for 12% of all female cancers (Table 2). An age-standardised rate (ASR) is a summary measure of the rate that a population would have if it had a standard age structure. Standardization is necessary when comparing several

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populations that differ with respect to age because age has a powerful influence on the risk of dying from cancer. High risk regions with ASRs over 30 per 100 000 are Eastern Africa (42.7), Melanasia (33.3), Southern Africa (31.5) and Middle Africa (30.6). Lowest rates are seen in Australia/New Zealand (5.5) and Western Africa (4.4).

Cervical cancer caused 266 000 deaths in 2012, accounting for 7.5% of all female cancer deaths (GLOBOCAN 2012) and with the death of approximately 250 000 women each year worldwide, it remains one of the leading causes of cancer related mortality in women (Palefsky 2010; World Health Organisation 2013a). The peak rate of HPV infection is seen in women under the age of 25 with a decline that plateaus around 30 – 35 years, but in some countries there is a slight increase in women aged 50 years or older (Mosciki *et al.,* 2012). Approximately 87% of cervical cancer deaths occur in less developed regions. Mortality rates in Western Asia, Western Europe, Australia and New Zealand are less than 2 per 100 000 while the rates are greater than 20 per 100 000 in Melanesia (20.6), Middle Africa (22.2) and East Africa (27.6). In countries with limited screening the mortality from cervical cancer is greater than that of men with HPV-related disease, however in the developed world the number of HPV-related cancers in men is similar to that of cervical cancer in women (Palefsky 2010).

2.10.1 HIV and Lymphoma

Histiocytic lymphoma is the term that was previously used to describe what is now known as Non-Hodgkins Lymphoma (NHL) which is made up pf large abnormal lymphoid cells. Lymphoma is a type of cancer that develops in the lymphatic system and abnormal cells can be found in lymph tissue as well as in the blood and includes mature B call and T cell NHL. Many tumours that were once considered to be histiocytic lymphomas are now considered to be a type of large cell lymphoma (Khalili *et al.,* 2010). Other types of cancer such as chronic lymphocytic leukemia (CLL) present the same as small lyphocytic lymphoma (SLL) but patients with SLL do not have abnormal cells in the blood, therefore CLL is classified as a NHL. Lymphoma starts in the lymph system which is found throughout the body and can spread to the liver, into bone, the brain and the abdomen. A weakened immune system has been shown to increase the risk of developing lymphoma.

Ng and McGrath (2002) reported that the prevalence of lymphoma is the USA was 1.6% per year and without effective antiretroviral therapy (ART) it was estimated that 5 – 10% of all HIV infected individuals would have had lymphoma as either an initial or subsequent AIDS defining syndrome. The introduction of ART did not decrease HIV associated

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lymphoma in the same manner as seen with other opportunistic infections and Karposi Sarcoma, but potent ART did decrease the incidence of lymphoma. ART in combination with antineoplastic chemotherapy improved the outcome and survival of HIV infected patients. Carter (2012) reported that many HIV positive patients will start or continue their HIV treatment in order to boost their immune system, which can serve to improve the chances of treatment being successful.

HIV related lymphomas are fast growing and require immediate treatment. The current treatment is a combination of chemotherapy drugs often given with a monoclonal antibody drug such as Rituximab. Treating HIV infection with HAART is a crucial part of effectively treating lymphoma in HIV positive individuals (McMillan, 2014). The reason why NHL was more common in HIV infected individuals as apposed to other forms of immunosuppression is not clear but Falco and colleagues (2010) made the following hypotheses: (1) HIV could be targeting specific genes, (2) local immune dysregulation could cause an alteration in the cellular and cytokine profiles, (3) direct viral-viral interactions. HIV decreases cell mediated immunity and the failure of CD4 T cells to recognise abnormally proliferating cells leads to the development of AIDS associated malignancies (Falco *et al*,. 2010).

NHL is an AIDS defining condition, and the incidence rate has decreased since the introduction of ART. Rates were 4 – 23 times higher in the HIV positive population depending on the proportion of individuals receiving ART. NHL impacts the survival of HIV positive patients. Immune deficiency, oncogenic viruses such as Epstein Barr Virus (EBV), HIV viremia, immune activation and aging are all factors that can contribute to the development of NHL with respect to HIV infection. Achenbach and colleagues reported that HIV viremia is the cause immune dysfunction, B cell activation and therefore a higher risk for developing NHL. NHL was found to be higher when HIV viremia was above the detection limit (50 copies/mL) in a dose dependent relationship. Previous studies only showed that a high viremia, cumulative HIV viremia and lack of ART were predictive of NHL independent of the lowest CD4 count after infection (nadir) (Achenbach *et al.,* 2014).

HIV increases the risk of NHL independent of the effect on T cells. B cells are activated during HIV infection in the absence of treatment and biomarkers of this activation is associated with AIDS related NHL (AIDS-NHL). B cell activation upregulates a pro-mutagenic enzyme known as cytidine deaminase (AID) and this enzyme is central to the development of immunoglobulin heavy-chain gene class switch recombination or somatic

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hypermutation in germinal center B cells. Increased AID can be seen in patients prior to AIDS-NHL but can also be induced by EBV, Hepatitis C virus, HPV or HIV. The HIV envelope acquires the CD40 ligand (CD40L) from the host cell membrane and this ligand is a potent stimulator of B cells. CD40L positive virions were reported to induce AID gene expression while CD40L negative virions did not. Induction was mediated by a direct interaction between CD40L in the HIV envelop and CD40 receptors on B cells. This cascade of events showed that it was biologically possible for HIV virions to directly promoted the development of B cell NHL.

2.11 Treatment of HIV and Associated infections

The concept of treating HIV infection with HAART was introduced more than a decade after the discovery of HIV (Vogel *et al.,* 2010). HAART uses a combination of antiretroviral drugs giving rise to lasting suppression of HIV replication and preventing the consequences of uncontrolled HIV infection such as the loss of CD4-cell mediated immunity (Vogel *et al.,* 2010).

The three viral enzymes encoded by the pol gene (Figure 7) of HIV-1 have been regarded as the appropriate targets for antiretroviral agents since these enzymes play key roles in the viral replication cycle. HIV reverse transcriptase (RT), Integrase (IN) and Protease (PR) are not indigenous to the host and therefore represent attractive targets for anti-HIV agents (Ahn *et al.,* 2002). There are three major classes of antiretrovirals: nucleoside reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse-transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) (Clavel & Hance 2004).

HAART combines antiretroviral therapies with different modes of action and has significantly improved the prognosis of patients infected with HIV (Martin *et al.,* 2008). Drug therapies presently in use for the treatment of HIV infection and AIDS reduce viral replication and can slow the progression of the disease but cannot rid the body of HIV. Current and experimental drug therapies target several sites within the viral replication cycle which includes the initial binding and recognition of the viral envelope protein gp120 to CD4, viral RNA replication, integration into the host cell nucleus, transcription or translation, assembly and release of new viral particles (Beerenwinkel *et al.,* 2005). Patients are frequently affected by long term side effects of the treatment, drug toxicity and drug-drug interactions, all of which may have negative effects on the outcomes of the treatment. There is therefore a need for new or additional antiretrovirals that have improved tolerability

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and safety. The most commonly used antiretrovirals are nucleoside inhibitors and HIV protease inhibitors which prevent maturation of the provirus. Currently AIDS therapy relies on a triple combination of HIV-1 RT and PR inhibitors (Matthée *et al.,* 1999).

The indications for initiating Antiretroviral (ARV) therapy are based on a patient's clinical symptoms, immunological status ($CD4^+$ cell count) and viral load. ARV therapy is recommended for all symptomatic patients (those with a history of AIDS-defining illness) regardless of the immunological status as well as for patients with a $CD4^+$ count higher than 350 cells/mm^3 and ≤500 cells/mm^3 (Jain *et al.,* 2006; World Health Organisation 2013b). Antiretroviral therapy (ART) should be initiated regardless of the clinical stage of infection or CD4 cell count if patients are coinfected with HIV and active TB, in individuals coinfected with HIV and Hepatitis B with evidence of severe chronic liver disease, partners with HIV in serodiscordant couples (to reduce HIV transmission to uninfected partners) and in pregnant or breastfeeding women who are HIV positive (World Health Organisation 2013b).

Several nucleosides inhibit RT, the viral RNA- dependent DNA polymerase which catalyses the transcription of HIV genetic material from RNA to DNA (Gochfeld *et al.,* 2003). Newly identified RT inhibitors with novel structural features and different mechanisms of inhibiting the enzyme will help to delay the development of resistance and hopefully control HIV infection. One of the first potent RT inhibiting drugs developed was a nucleoside analogue 3'-azido-3'-deoxythymidine (AZT) but the development of HIV strains resistant to this drug and others required the search for new and alternate RT inhibitors (Fonteh *et al.,* 2009). HIV protease is an enzyme that modifies structural and regulatory polyproteins into their active forms. In the absence of this protease, viral enzymes, including reverse transcriptase, integrase and the protease itself, are not produced. Protease inhibitors block the activity of this enzyme and infectivity of the virus is severely diminished (Gochfeld *et al.,* 2003).

2.11.1 Side effects of HAART and Adherence to Treatment

Due to the complexity of HAART the emergence of drug resistance and social stigma associated with HIV in developing countries, the long term management of HIV infected patients is difficult (Aspeling 2006). Potent drugs may fail at a later stage because of chronic adverse side effects and the emergence and transmission of drug-resistant variants. This underlines the need for continuous efforts in the discovery of additional anti-HIV agents (Singh & Bodiwala 2010). More than 100 resistance-associated mutations, spanning the HIV-1 RT- and PR-coding regions, have been reported. Also an increasing

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number of variations of the virus have emerged with multiple or multidrug resistance-associated mutations (Hertogs *et al.,* 1998).

Combination therapy had profound effects for people living with HIV with most of the attention being focused on the benefits of the therapy. However the complexity of the drug regimens, the need for total adherence and the long term nature of the treatment has caused problems with patients adhering to the treatment (Chesney *et al.,* 2000). Non-adherence to treatment results in the therapy being less successful and drug resistant strains of the virus develop, which is not only dangerous for the patient but is also detrimental to public health (Chesney *et al.,* 2000).

According to Paterson and colleagues (2000) two groups of patients may experience clinical and virologic failure. The first consists of patients who have received various different antiretroviral drugs over a long period of time and may be infected predominantly with drug resistant viral strains. The second group consists of patients who adhere poorly to the treatment regimen. An acceptable level of adherence to therapy in chronic illness is consumption of more than 80% of the prescribed dose. Patients with 95% or higher adherence had a better virologic outcome, a greater increase in CD4 cell count and a lower hospitalisation rate than patients with lower levels of adherence (Paterson *et al.,* 2000). It is possible that patients with HIV tolerate side effects better than patients with “less severe” chronic disease because they are more aware of the adverse outcomes associated with poor adherence.

Zidovudine (ZDV) is a 3’-azido analogue of thymidine was one of the first nucleoside analogues found to be effective in inhibiting HIV reverse transcriptase (RT). Other RT inhibitors that followed ZDV are didanosine, stavudine and abacavir. Some of these drugs were also able to protect patients against AIDS dementia complex due to good penetration of the blood brain barrier. NRTI therapy also revealed important side effects such as mild myopathy to possibly fatal pancreatitis, liver failure and lactic acidosis. Decreased mitochondrial energy generating capacity was believed to be the reason for these side effects (Brinkman *et al.,* 1998).

Chesney and colleagues (2000) conducted a review of studies done from 1979 to 1998 focused on treatment adherence in patients with hypertension. One of the most common findings in these studies is that there is a direct correlation between adherence to treatment

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and complexity of the drug regimen, the number of different medications included in the regimen and the extent to which the regimen interferes with the patients daily life. The best adherence was seen when the medication relieved symptoms of the disease and the worst adherence was seen when the treatment produced side effects.

Intermittent adherence to HIV drug therapy can result in the failure of the treatment and emergence of drug resistant strains, in the case of protease inhibitors cross resistance can occur with short term non-adherence. As little as one week on non-adherence may be enough to cause a rapid rebound of plasma viremia leading to treatment failure (Weiser *et al.,* 2003). It was noted that gender could also play a role in treatment compliance as woman sometimes had a higher level of noncompliance in cases where the patient had to manage difficult family and child care responsibilities (Chesney *et al.,* 2000). It was postulated that patients who used illegal drugs or were of a lower socio-economic status would be at higher risk for noncompliance. Studies showed that although injection drug users accessed medical care later in the course of infection, once treatment was started, the patients were as compliant as other patients (Chesney *et al.,* 2000). Stress and depression has also been known to predict noncompliance in other diseases and is consistently associated with non-adherence to HIV therapy. A study of ARV adherence among semiurban South Africans living in extreme poverty showed that lower socioeconomic status was not a predictor of adherence for patients with fully subsidized therapy. Adherence levels was actually similar to or better than those found in industrialized countries (Weiser *et al.,* 2003).

Common but mild side effects occur early in most antiretroviral treatments and include gastrointestinal effects such as bloating, nausea and diarrhea, which may be brief but can also continue throughout therapy. Other common minor side effects are fatigue and headache caused by AZT and nightmares associated with EFV (Montessori *et al.,* 2004). Several uncommon but more serious adverse effects associated with antiretroviral therapy are AZT-associated anemia, d4T-associated peripheral neuropathy, PI-associated retinoid and NNRTI-associated hypersensitivity reactions. Other serious side effects of HIV treatment include lactic acidosis, hyperlactatemia, hepatotoxicity, hyperglycemia, fat maldistribution, hyperlipidemia, bleeding disorders, osteoporosis and skin rash (Montessori *et al.,* 2004).

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The potential lethality of NRTI-associated lactic acidosis means that it is very impotant to be aware of the signs and symptoms during treatment. This is characterized by complaints of malaise, nausea and vomiting, fatigue and tachypnea, followed by liver failure, cardiac dysrhythmias and death. Transaminitis and hepatotoxicity are associated with most of the antiretroviral agents. Hepatotoxicity was observed in 30% of patients who initiated treatment with ritonavir (RTV) but only 6% to 7% of those who initiated therapy with saquinavir, nelfinavir (NFV) or indinavir (IDV) experienced severe hepatotoxicity (Montessori *et al.,* 2004). The rate of severe hepatotoxicity associated with PI among patients with Hepatitis C was twice as high as among patients without Hepatitis C infection. Lipodystrophy is part of a metabolic syndrome that includes dyslipidemia, insulin resistance and accelerated bone loss. PI therapy has been most strongly linked to lipodystrophy although NRTIs, especially d4T, have also been associated with lipodystrophy.

2.11.2 Side effects caused by HAART

The use of combination therapy has proven to be effective in controlling the progression of HIV but the virus began to develop resistance to treatment almost from the beginning and since then, a number of drug resistant strains have evolved. These drug resistant strains have since been transmitted to individuals who have never received ART and this is known as transmitted drug resistance (Smith *et al.,* 2010).

Induced viral resistance can occur in patients by the formation of gene mutations during the reverse transcription of viral RNA to viral DNA which is known to be an error prone process (Jain *et al.,* 2006). If any of these mutations confer a selective advantage to the virus, the mutated virus will overtake other viral strains in the body. Viral resistance also develops during therapy and although these strains can be transmitted from patient to patient, some strains of HIV are naturally resistant to certain drugs, such as HIV type 2 which is resistant to most nonnucleoside reverse transcriptase inhibitors (Clavel & Hance 2004). This is known as primary resistance.

Nucleoside and nucleotide analogues lack a 3' hydroxyl group and arrest the synthesis of viral DNA by reverse transcriptase because no additional nucleotides can be attached to the analogue. Mutations in RT can promote resistance by damaging the ability of reverse transcriptase to incorporate the analogue into DNA. Removal of the analogue can also occur due to a different mutation which is known as the thymidine analogue mutation and has been reported to promote resistance against Tenofovir (Clavel & Hance 2004; Smith *et al.,* 2010). NNRTIs are small molecules that have a strong affinity for the hydrophobic

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pocket close to the catalytic site of RT. Mutations caused after the failure of NNRTI treatment are located in this pocket and reduce the affinity of the drug with RT causing resistance to drugs such as nevirapine. Resistance to PIs occurs due to amino acid substitutions either inside the substrate binding domain of protease or at distant sites and these amino acid changes modify the number and nature of the contact between the inhibitor and the protease (Clavel & Hance 2004).

The prevalence of drug resistant virus in patients not on treatment with established HIV infection was assessed. One study showed a lower prevalence of drug-resistant variants in established infection compared to newly infected patients in the same geographic regions, but another did not confirm this observation (Descamps *et al.,* 2005) These studies used different criteria for cut-off for key mutations associated with resistance in genotypic assays. If resistant mutants are initially present, in the absence of treatent, wildtype viruses can develop in an infected person and may become predominant (Hirsch *et al.,* 2000). Drug resistant variants at low levels in a patient might not be detected by current assays, but could develop rapidly when antiretroviral therapy is initiated. Transmission of resistant viruses has important clinical and public health implications, as it can compromise a patient's response to initial therapy (Descamps *et al.,* 2005). The use of antiretroviral resistance testing is recommended to determine the occurrence of circulating resistance strains in patients before starting treatment.

2.11.3 Vaccine development

An HIV vaccine has yet to be approved decades after HIV was identified as the causative agent of AIDS. Vaccine development normally takes a long time to develop however the normal strategies used have failed when applied to HIV (Burton *et al.,* 2012). Natural infection with acute viruses such as smallpox and polio lead to individuals who are immune to reinfection and vaccines can be developed to mimic natural infection without adverse pathological conditions. For persistent viruses immunity to infection is not easy to determine however in the case of human papillomavirus (HPV) a fairly straightforward method was used in which the vaccine elicit an IgG response. With a variable retrovirus such as HIV, the usual vaccine strategies have not been successful (Burton *et al.,* 2012). Live attenuated viruses (measles, mumps, yellow fever), inactivated viruses (poliovirus) or virus like particles (HPV) have been used to imitate natural infection and produce an immunological memory but this has been unsuccessful with HIV.

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The number of AIDS related deaths and new HIV infections has decreased since the increase in the use of ART. Improved approaches to the prevention of mother to child transmission has also decreased the number of new infections and even the death of children worldwide. Preexposure prophylaxis with antiretroviral medication has also decreased the risk of HIV infection and treating infected individuals with a combination of ARTs has reduced the likelihood of transmitting HIV to an uninfected partner. All this has happened without the development of a vaccine (Fauci & Marston 2014). The only way to completely control the AIDS pandemic would be a combination of a safe and effective vaccine with nonvaccine prevention methods.

Multiple approaches have been used in the attempt to develop a vaccine however to date only three have shown any promise of success. A recombinant gp120 protein and a recombinant adenovirus type 5 vaccine (containing HIV *gag, pol* and *nef* genes) both failed to control HIV infection. In 2009 the first promise of an effective vaccine was seen with a prime-boost (RV-144) using a canarypox vector prime and a monomeric gp120 boost (Koff *et al.,* 2013). The main challenges regarding vaccine development are the variability of HIV worldwide, the lack of validated animal models, the limited knowledge of which parts of the virus would be required for the immune response that would be required for prevention and control of the HIV, the lack of natural protective immunity against HIV and the fast integration of the HIV genome into the host genome (Koff *et al.,* 2013).

The development of more than 20 antiretroviral therapies has resulted in a decrease in morbidity and mortality associated with AIDS, especially in low and middle income countries where treatment has become more readily available. The nature of the interaction between HIV and the immune system is complex and the relevance of different immune responses is not completely understood (Johnston & Fauci 2007). Soluble forms of gp120, all or portions of the uncleaved gp160 protein and envelope peptides have all been tested for safety and immunogenicity in numerous clinical trials but subsequent experiments showed that the antiserum did not neutralise primary isolates of HIV grown and tested in peripheral blood mononuclear cells (Johnston & Fauci 2007).

Despite the general success of HAART many patients only benefit temporarily because of therapeutic failure caused by the emergence of drug resistant virus, cross resistance to drugs and cytotoxicity (Brito *et al.,* 2007). The effectiveness of drugs used to treat viral infections and cancer is also influenced by the emergence of clinical resistance and has

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become an important factor in the development of new treatment (Nyce *et al.,* 1993; Ueda *et al.,* 1987). Therefore, the search for potential drug candidates containing higher inhibitory activity against various HIV strains is increasing in the pharmaceutical industry. In this regard, natural bioactive compounds and their derivatives are great sources for the development of new generation anti-HIV therapeutics which are more effective with fewer side-effects (Vo & Kim 2010; Matthée *et al.,* 1999).

2.12 Hypothesis

Marine soft corals produce compounds with medicinal properties which may be inhibitory to HIV-1 enzymes and limiting to HIV-1 associated opportunistic co-morbidities.

2.13 Aims

To investigate the above-mentioned hypothesis, the following aims were investigated:

1. **Preparation of crude extracts of *Sinularia notanda*.**
   Soft coral were removed from the rock substrate and extraction solvents were chosen specifically to isolate terpenoids as these compounds have routinely been isolated from marine soft coral, and demonstrated biological activity.

2. **Testing of crude extracts for HIV-1 protease (and reverse transcriptase) inhibition, cytotoxicity and antioxidant potential.**
   Assays for inhibition of HIV-1 enzymes were chosen as these enzymes are essential to the viral life cycle. Also, soft coral extracts with activity against HIV enzymes have been reported (Slattery *et al.,* 2005). Cytotoxicity assays were performed using cancerous and non-cancerous cell lines to determine the selectivity of the extracts and compounds such that compounds with a low selectivity and high toxicity towards non-cancerous cell lines would not be studied further. Anti-cancer abilities of soft coral extracts or isolated compounds are better known than anti-viral activities.

3. **Bioassay guided fractionation of active crude extracts using conventional chromatographic techniques to isolate the bioactive compounds**
   Fractionation of extracts can be done using techniques such is high performance chromatography or more simply by using column chromatography. A “biomolecular interaction step” is necessary so that relevant fractions, and subsequent compounds,

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can be focused on (Weller 2012). Bioactivity based assays are therefore routinely used to provide valuable information on the biological effects of complex mixtures, and to aid in more efficient isolation of bioactive compounds.

4. **Structural elucidation of active compounds**.

   One and two dimensional Nuclear Magnetic Resonance Spectroscopy was used to determine the molecular structure of isolated compounds. X-ray crystallography and Mass Spectrometry were used to confirm the identity of the structure. These techniques are routinely used in the identification of unknown compounds.

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3. Methods

For the type of analysis presented in this dissertation, active materials were isolated from the soft coral using chromatography followed by the determination of biological activity using various assays. In this section the principles of the methods used will be provided as well as the protocol that was followed. Background will be provided on compound isolation techniques (chromatograph), compound structure determination techniques (Nuclear Magnetic Resonance Spectroscopy), cell proliferation and compound toxicity (tetrazolium dye) and other bioassays used to assess the activity of crude extracts as well as isolated compounds. A summary of the work flow is provided in Figure 16

3.1 Chromatography and Compound Isolation

Background: Chromatographic methods can be divided as being either adsorption or partition chromatography. Adsorption chromatography makes use of a stationary phase such as an ion exchange resin that has a finite number of binding sites for analytes, relying on specific interactions between analytes and binding sites on the surface of the sorbent (Boyer 2006). The attractive forces may be ionic, hydrogen bonding or hydrophobic interactions. Adsorptive techniques are best used for the separation of macromolecules including proteins and nucleic acids (Misra 2006; Boyer 2006). Partition chromatography is the distribution of an analyte between two liquid phases involving direct interaction using 2 liquids (Boyer 2006). This technique may also use a liquid immobilised on a solid support such as paper, thin layer and gas-liquid chromatography. For partition chromatography the sorbent consists of inert solid particles coated with liquid adsorbent (Boyer 2006). Partition chromatography has been widely used for the separation and identification of amino acids, carbohydrates and fatty acids. All chromatographic separation relies to some extent on adsorptive processes.

Column chromatography is the oldest form of chromatographic technique in which a glass column is packed with a solid stationary phase. The importance of column chromatography is mainly due to the fact that it has simple packing procedure, low operating pressure and low expense for instruments. Column chromatography is separated into two categories depending on how the solvent flows down the column. If the solvent is allowed to flow down the column by gravity it is called gravity column chromatography. If the solvent is forced down the column by positive air pressure, it is called flash chromatography. Silica gel (SiO_2) and alumina (Al_2O_3) are two adsorbents commonly used. These adsorbents are available in different mesh sizes which refers to the mesh of the sieve used to size the silica. Adsorbent

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particle size affects how the solvent flows through the column. Smaller particles (higher mesh values) are used for flash chromatography and larger particles (lower mesh values) are used for gravity chromatography. For example, 70–230 silica gels are used for gravity columns and 230–400 mesh for flash columns.

The sample mixture is applied to the top of the column and the mobile phase is allowed to move down through the column under gravity (Houghton & Raman 1998). The components of the mixture move through the column at different rates as bands. Once the bands reach the end of the column they are eluted in solution. The stationary phase of column chromatography is usually an adsorbent of high polarity such as silica. More recently other stationary phases such as reverse-phase silica, ion-exchange resins and exclusion chromatography stationary phases have been used. Elution with the mobile phase can be isocratic (one composition of eluent) or gradient (different compositions of mobile phase as a gradient of increasing polarity). Alcohols should be chosen if the analyte contains hydroxyl groups, acetone or esters would be used if the analyte contains carbonyl groups and hydrocarbons should be used if the analytes are non-polar (Wilson & Walker, 2010). Fractions can then be collected continuously as a measure of fixed volume or time. Similar fractions can then be pooled together based on the Thin Layer Chromatography (TLC) profiles.

TLC is a method for analysing mixtures by separating the compounds in the mixture. TLC can be used to help determine the number of components in a mixture, the identity of compounds, and the purity of a compound. TLC is an inexpensive and sensitive technique where microgram quantities can be analysed relatively quickly. Unknown samples may be applied to a plate alongside appropriate standards and a comparison of the retention factors may be used to identify the unknown sample. This chromatographic process consists of three steps which are spotting, development, and visualization. The sample to be analysed is usually dissolved in a volatile solvent and a small drop is applied using a micocapillary pipet, allowed to dry and the process is repeated over the same spot. A wide variety of solvent systems may be used for the development of a TLC plate and solvent systems can be screened by developing several small chromatography plates in small sealed bottles containing the different solvent systems. Unless the components of a sample are coloured, the detection of a particular compound may require visualisation under an ultraviolet light or the use of a staining agent. General detection methods such as a vanillin-sulphuric acid solution, which is sprayed onto the TLC plate and heated to 120°C, produce

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different coloured spots with organic compounds and can be used to detect phenols, essential oils, steroids and higher alcohols.

Protocol – ***Column Chromatography*****:** The following protocol was used for the initial fractionation of crude ethyl acetate extract of *S. notanda*. The solvent systems used for the elution of this column are presented in Table 3. The solvents used for the elution of subsequent silica columns used during this study are presented with the relevant results. Column chromatography separation and purification was performed on silica gel 60: 70 – 230 mesh (Merck, Darmstadt, Germany). Three grams of extract was dissolved in 1 – 2 ml of hexane after which dry silica was added to the dissolved compound at a ratio of 1 part extract to 2 parts silica. Ethyl acetate was then added to form a homogenous paste of extract and silica, and the mixture was left to dry. A glass chromatography column was washed with 100% methanol followed by 100% hexane and allowed to dry. The column was packed with silica gel and the prepared extract. Elution of the column was started with 100% hexane and eluted with increasing polarity, concluding with 100% ethyl acetate (Table 3). Fractions were collected every 500 mL and concentrated under reduced pressure. TLC profiles of each fraction was obtained and similar fractions were pooled.

Table 3: Elution solvents used for column chromatography of the crude ethyl acetate fraction of *S. notanda*

% Hexane	100	98	92	90	88	85	82	80	78	76	74	72	70	65	60	40	20	0
% Ethyl Acetate	0	2	8	10	12	15	18	20	22	24	26	28	30	35	40	60	80	100
Volume used	1L	1L	1L	1L	1L	1L	1L	1L	2L	2L	2L	2L	1L	1L	1L	1L	0.5 L	1L

Protocol – ***Thin Layer Chromatography*****:** Precoated silica gel 60 F524 plates (Merck) were used for thin layer chromatography analysis. The mobile phase was chosen based on the solvent system used for the elution of the fraction from the silica column and adjusted to provide adequate separation of the compounds. Samples were dissolved in approximately 500 µl of either hexane or ethyl acetate, 1 – 2 µl was applied 2 cm from the bottom of the plate and allowed to dry. Glass tanks containing the mobile phase were allowed to equilibrate for approximately 30 minutes with a glass lid placed on top. Plates were placed vertically in the mobile phase and allowed to develop until the solvent system reached three quarters of the way to the top of the plate. The TLC plate was removed from the developing tank, allowed to air dry and fluorescent compounds were visualized under 254 nm and 366 nm UV light. The plates were then stained with 3% vanillin-sulphuric acid, dried and gently heated to visualise the compounds.

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3.1.1 Procedure for bioassay guided fractionation

Fresh *Sinularia notanda* (260 g) was 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 residue (14.7 g) was re-suspended in ethyl acetate and partitioned with methanol in order to remove salts and the high molecular weight hydrophilic compounds. The lipophilic fractions obtained by ethyl acetate was dried by rotavapor and tested for *in vitro* cytotoxicity against the cervical cancer cell line (HeLa), and the African green monkey kidney cell line (Vero) as representative of a normal, non-cancerous cell line. The extracts were tested for inhibitory activity in direct enzyme assays against HIV-1 protease and for free-radical scavenging ability using an antioxidant assay. The ethyl acetate fraction was partitioned on silica gel using n-hexane and ethyl acetate (100:0 to 0:100) and 500 mL fractions were obtained. Similar fractions were pooled based on TLC profiles and all fractions were tested for cytotoxicity. Active fractions were further purified using silica gel chromatography. Crude extracts and compounds used in all biological assays were dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 20 mg/mL and dilutions were made to the various concentrations used for testing.

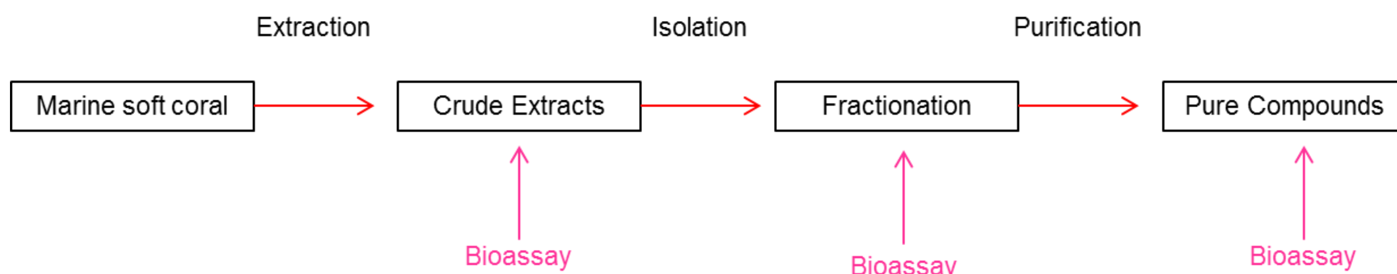

Figure 10: Procedure for Bioassay guided fractionation to obtain active compounds from medicinal plants

(Nguyen & Duez 2008).

3.2 Structural Determination Methods

3.2.1 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) was discovered by physicists Felix Bloch and Edward Purcell and their collaborators in 1946 (Pellecchia *et al.,* 2002). Subsequently high resolution NMR was quickly developed by analytical chemists as a powerful technique for the determination of molecular structures (Smith & Blandford 1995). Spectroscopy is the measurement of the frequency dependence absorption or emission of energy by a system.

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NMR Spectroscopy refers to the absorption and release of radio frequency (rf) energy by a nucleus in a magnetic field. Possession of charge and spin renders some nuclei magnetic and confers various properties on them which affect their behaviour in an external magnetic field (Bo) (Smith & Blandford 1995). Isotope labelling in macromolecular NMR spectroscopy can result in increased sensitivity and resolution, and in reduced complexity of the NMR spectra. The most commonly used isotopes are ^{2}H (deuterium), ^{13}C and ^{15}N. Knowledge of the three dimensional structure of drug targets (such as proteins) provides a starting point for structure based approaches to drug design by defining the features of the ligands and their target proteins (Blundell *et al.,* 2002). This information can assist in the discovery of compounds that have better interactions with the drug targets resulting in better activity and selectivity (Greer *et al.,* 1994). NMR spectroscopy is the best method for complete structure elucidation (including stereochemistry) of noncrystalline samples. For structure elucidation of secondary natural products (not proteins) only 1H-NMR and 13C-NMR spectroscopy, including combined methods such as 2-D NMR spectroscopy, are important because carbon and hydrogen are the most abundant atoms in natural products (Dzeroski *et al.,* 1998).

Protocol: NMR spectra presented in the results section of this dissertation were measured at 25^{0}C on a Bruker Avance III 400MHz instrument (Chemistry Department, University of Pretoria) operating at a frequency of 400.13 MHz. For each sample, 128 free induction decays (FIDs) were collected and 32 scans were measured per sample, with relaxation delay of 1.5s between pulses.

3.2.2 X-ray Crystallography

X-ray crystallography was performed at the Department of Chemistry, University of Johannesburg. Intensity data were measured on a Bruker Apex DUO using an Incoatec IµS microfocus X-ray source equipped with an Incoatec quazar multilayer mirror. Sample temperature was kept constant at 100(1) K using a Oxford Cryostream 700+. A total of 6122 reflections were collected up to a θmax of 65.27°. The structure was solved by direct methods and refined by a full-matrix least-squares on F^2 procedure. The refined structural model converged to a final R1 = 0.0388, wR2 = 0.0779 for 3032 observed reflection [$I > 2\sigma(I)$] and 223 variable parameters. The absolute configuration was determined by Flack's method with Flack's parameter determined as 0.0(2).

3.2.3 Mass Spectrometry

Background: NMR and Mass spectrometry (MS) are being used to study the biology of macromolecules and processes. NMR is especially useful because in addition to proton

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spectra it is also possible to measure the presence of 13C, 19F, 15N and 31P nuclei. MS is similar to NMR in that is has been of great value in the structural elucidation of relatively small organic biomolecules (molecular weight limit at approximately 1000) (Boyer 2006). The mass spectrometer consists of three components that are coupled together. These components are the ionisation device, the mass analyser and the ion detector. Mass spectrometry of proteins and other biopolymers was initially hindered because analytes are measured in the gas phase during analysis (Loudon 1995). Neutral molecules are ionised and their positively charged ion products are directed through an electric and/ or magnetic field where they are separated (analysed) based on their mass to charge ratio (*m/z*). The spectrum that is generated by MS displays ion intensity as a function of *m/z*.

Electron spray ionisation (ESI) is known as a soft ionisation method as it causes minimal damage to the sample during ionisation (Scripps Center for Metabolomics 2010). ESI is a method used with carbohydrates, small oligonucleotides, synthetic polymers, and lipids (Kind & Fiehn 2010), producing gaseous ionized molecules directly from a liquid solution (Scripps Center for Metabolomics 2010). A fine spray of highly charged droplets are created in the presence of an electric field. The tip of a metal nozzle is kept at a potential anywhere between 700 V to 5000 V and the nozzle disperses the solution into a fine spray of charged droplets (Boyer 2006; Scripps Center for Metabolomics 2010). Either dry gas, heat, or both are applied to the droplets at atmospheric pressure thus causing the solvent to evaporate from each droplet. As the size of the charged droplet decreases, the charge density on its surface increases. The emerging ions are directed through electrostatic lenses leading to the vacuum of the mass analyser. An accurate and precise mass measurement increases the certainty of the identification of the elemental formula of a compound. With increasing *m/z* ratio the number of possible formulae dramatically increase making identification more difficult (Webb *et al.,* 2004).

Protocol: Mass spectrometry analysis was carried out using a Synapt high resolution qTOF-MS (Waters, MA, USA). ESI was used in the range of m/z 100 to m/z 1000 in both positive and negative ion mode. Full scan mass spectra were acquired at a mass accuracy of 0.5 Da. A lock spray was used to ensure the accuracy and reproducibility of the analyses. A lock mass of leucine encephalin (556.3 µg/ml) was used via a lock spray interface at a flow rate of 0.4 mL/min. The scan time was set to 0.1 seconds with an interscan delay of 0.2 seconds. Nitrogen was used as both desolvation gas at a flow rate of 700 L/h, and cone gas at a flow rate of 50 L/h. The temperature at desolvation was set at

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120°C. The capillary voltage was set to 2.5 kV and the cone voltage was at 1600 V. Collision energy of 3eV was found to be optimal for efficient fragmentation of target compounds. Raw data was analysed using MassLynx software (Waters, MA, USA) (Hao *et al.,* 2012; Stražić *et al.,* 2014).

3.3 Cell Culture

All methods described below were performed under sterile conditions in a P2 bio-safety cabinet. HeLa and Vero cells were grown in a humidified environment at 37°C and 5% CO_2 in Minimum Essential Medium (Thermo Scientific, Utah) supplemented with 5% Fetal Bovine Serum (Thermo Scientific, Utah) and 1% Antibiotic/Antimycotic Solution (v/v) (Thermo Scientific, Utah). U937 and U1 cells were cultured in RPMI-1640 containing L-glutamine (Sigma-Aldrich, USA) supplemented with 2g/L NaHCO3, 5% HEPS buffer (v/v) (Gibco Life Technologies, USA), 10% FBS (v/v), 1% Gantamicin (v/v) (Sigma-Aldrich, USA) and antibiotic/antimycotic solution. The culture medium was renewed every two to three days when cells were approximately 80% confluent (Han *et al.,* 2011).

3.3.1 Thawing and culturing cells from frozen stock

Hela and Vero Cells

HeLa cells from a -70°C storage freezer were thawed in a 37°C water bath. The cells were placed in 9 ml of 10% complete medium (MEM + 10% Fetal Bovine Serum + 1% Antibiotic Solution) and centrifuged (BHG Hermle Z320 Centrifuge) for 5 minutes at 1200 revolutions per minute (rpm) at room temperature to wash off the Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, Germany) present in the freeze medium. The supernatant was poured off and the cells were resuspended in 15 ml 10% complete medium. The resuspended cells were slowly pipetted into a 75 cm^3 tissue culture flask (Nunc, Denmark) and placed in a 37°C incubator at 90% humidity and 5% CO_2. Cells were checked daily under a microscope. Once cells were 70 – 90% confluent, usually within two to three days, trypsinisation was carried out and the culture medium was replaced with 5% culture medium (ATCC 2012).

Vero cells from -70°C storage were thawed in a 37°C water bath. The cells were placed in 9 ml 20% complete medium (MEM + 20% FBS + 1% Antibiotic Solution) and centrifuged for 5 minutes at 1200 rpm at room temperature. The supernatant was poured off and the cells were resuspended in 15 ml 20% complete medium. The resuspended cells were pipetted into a 75 cm^3 tissue culture flask and placed in a 37°C incubator at 90% humidity and 5% CO_2. Cells were checked daily. Once cells were 70 – 90% confluent trypsinisation was

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carried out and the culture medium was replaced with 10% culture medium. After the second trypsinisation Vero cells were continually grown in 5% culture medium (Ammerman *et al.,* 2008).

U937 and U1 cells

U937 and U1 cells from liquid nitrogen storage were thawed in a 37°C water bath. The cells were placed in 9 ml 30% complete medium (RPMI + 30% FBS + 1% Antibiotic Solution) and centrifuged for 5 minutes at 1200 rpm at room temperature. The supernatant was poured off and the cells were resuspended in 15 ml 30% complete medium. The resuspended cells were pipetted into a 75 cm^3 tissue culture flask and placed in a 37°C incubator at 90% humidity and 5% CO_2. Cells were checked daily. Once cells were 70 – 90% confluent (surface area of culture flask occupied by cells) cells were split into two flasks and medium was replaced with 20% culture medium. Cells were continually grown in 10% culture medium.

3.3.2 Maintenance of cell cultures

Hela and Vero Cells

Once cell cultures had reached confluency, culture medium was removed from flasks. Cells were washed with 5 ml Phosphate Buffered Saline (1 X PBS) to remove residual culture medium. Two millilitres of trypsin (Thermo Scientific, Utah) was added to each flask and swirled around the flask for 30 seconds. The trypsin was removed and flasks were placed in a 37°C incubator for 3 minutes. Trypsinised cells were washed out of the flask with 10 ml 5% complete media and placed in a 15 ml centrifuge tube. Trypsinised cells were centrifuged for 5 minutes at 1200 rpm at room temperature and the supernatant was discarded. Cells for freezing were resuspended in 900 µl FBS and 100 µl DMSO (1×10^6 cells per ml) and the 1 ml solution was stored in a cryogenic vile at -70°C. Cells to be used for assays were resuspended in 5% complete media at 1×10^5 cells/ml.

U937 and U1 Cells

Cultures were maintained at a cell density of between 1 x 10^5 and 2 x 10^6 viable cells/mL by the addition of fresh medium. For the replacement of complete culture media, cells were removed from the culture flask and centrifuged in falcon tubes at 1200 rpm for 5 minutes. Cells were resuspended at concentration of 1 to 2 x 10^5 viable cells/mL and placed back in culture flasks. Fresh media was added every 3 to 4 days depending on cell density.

3.4 XTT Cell Viability Assay

Background: The enzymatic XTT assay is based on the fact that the internal environment of proliferating cells is more reduced than that of non-proliferating cells because the ratios

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of NADPH:NADP, FADH:FAD, FMNH:FMN and NADH:NAD increase during proliferation (de Souza *et al.,* 2002). Compounds such as tetrazolium salts and Alamar Blue which can be reduced by these metabolic intermediates, can be used to monitor cell proliferation. The reduction of a compound in a cell proliferation assay is accompanied by a measurable shift in wavelength with a corresponding colour change. The vast majority of cellular applications of tetrazolium dyes involve microplate assays that measure cell proliferation where it is assumed that dye reduction will be proportional to the number of viable cells in the exponential growth phase (Berridge *et al.,* 2005).

The most frequently used tetrazolium salts are MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], XTT [sodium 2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide] and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. The chemical structures of these tetrazolium salts can be seen in Figure 11. The unique chemical and biological properties of tetrazolium salts that have led to the widespread application in histochemistry, cell biology, biochemistry and biotechnology depend on the positively charged quaternary tetrazole ring core containing four nitrogen atoms (Berridge *et al.,* 2005). The reduction of tetrazolium salts from colourless or weakly coloured aqueous solutions to brightly coloured derivatives known as formazans is due to the disruption of the tetrazole ring and this has been the basis of their use as vital dyes in redox histochemistry and in biochemical applications for many years (Berridge *et al.,* 2005).

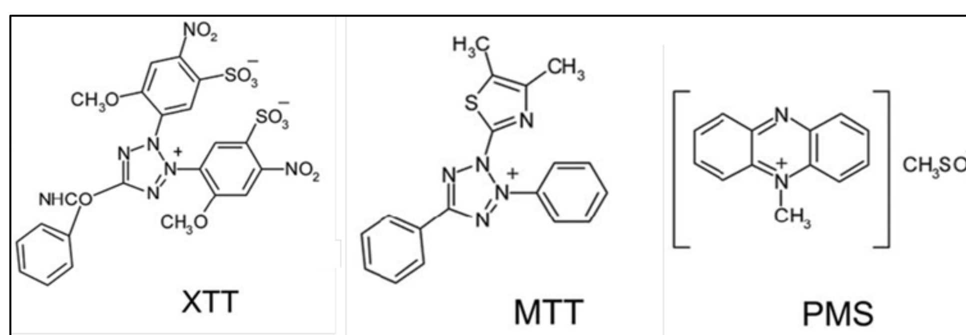

Figure 11: Chemical structure of tetrazolium salts XTT and MTT, and the intermediate electron acceptor PMS
(adapted from Berridge *et al.,* 2005)

XTT dye reduction occurs at the cell surface facilitated by trans-plasma membrane electron transport, indicated in Figure 12, where mitochondrial succinoxidases and cytochrome P450 systems contribute to the XTT response with their reductants being transferred to the plasma membrane (Kuhn *et al.,* 2003).

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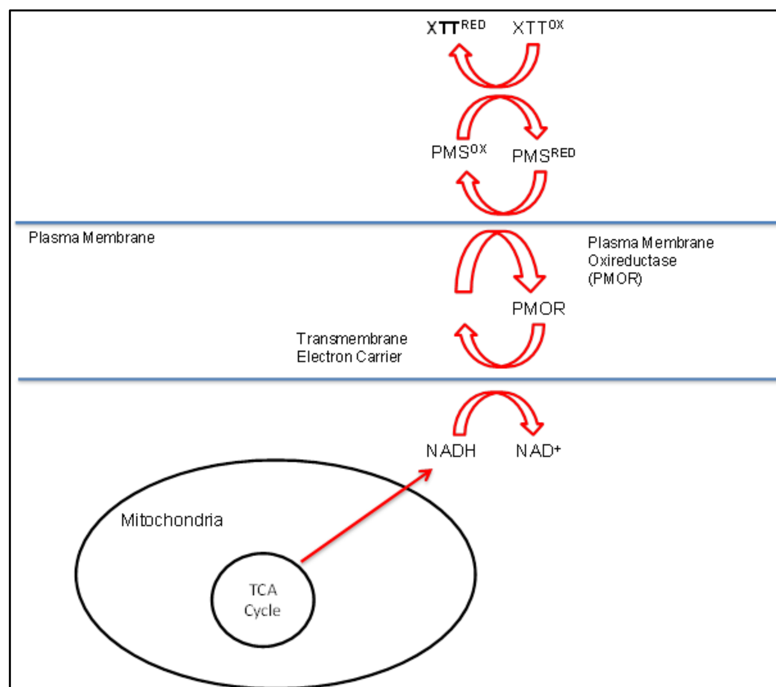

Figure 12: XTT reduction by cellular enzymes (ATCC 2011; Berridge *et al.,* 2005).

XTT can be used alone as a detection reaction but the results are not ideal. The XTT assay results are greatly improved when an intermediate electron acceptor phenazine methosulphate (PMS) is used with the dye. PMS mediates XTT reduction by picking up electrons at the cell surface, or at a site in the plasma membrane that is readily accessible, forming a reactive intermediate that then reduces XTT (Berridge *et al.,* 2005) (Figure 12). In most commercially available kits PMS is included. It has been found that XTT as well as the other tetrazolium salts do not give very reproducible results thus the results must be confirmed with another assay. The xCelligence Real Time Cell Analysis system was used to confirm results obtained using the XTT assay.

Protocol: HeLa or Vero cells were trypsinised as described in the methods above. For all cell lines used cells were counted using the Trypan Blue dye exclusion method and cells were resuspended in either 5% complete MEM (HeLa and Vero) or 10% complete RPMI (U937 and U1) in order to have a suspension of 1×10^5 cell/ml. To each 96 well plate, 200 µl incomplete media was added to all the outer wells (to stop evaporation of media from wells containing cells) and 100 µl of the cell suspension was added to the rest of the wells. Plates were incubated for 24 hours at 37°C at 5% CO_2 and 90% humidity, after which cells were treated with selected extracts.

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Extracts were dissolved in DMSO to a concentration of 20 mg/ml and then diluted in media to a concentration of 1000 µg/ml. These "stock" solutions were diluted with media to the various concentrations used for testing. One hundred microliters of each sample dilution was added in triplicate to the appropriate wells. Actinomycin D was used as the positive control for cell death and was diluted with media from 1000 µg/ml stock to 0.4 µg/ml. The dilutions were prepared from the 0.4 µg/ml dilution using media. The addition of actinomycin D to the wells was done in the dark as Actinomycin D is light and air sensitive. A control plate was also prepared and contained only 100 µl of media and 100 µl of each sample dilution in triplicate. Plates were incubated for 72 hours at 37°C at 5% CO_2 and 90% humidity.

XTT (Roche, Germany) was prepared according to the manufacturer's instructions. For every 1 ml of XTT 20 µl of PMS (Roche, Germany) was added. Sufficient solution was prepared to allow for 50 µl to be added to each well. After XTT was added the plates were incubated for 1 hour, after which the absorbance was read at 450 nm (reference wavelength at 690 nm) on a Thermo Labsystems Multiskan Ascent (AEC Amersham) 96 well plate reader to measure the colour change of XTT and PMS from light yellow to bright orange. An average of the triplicate values and the standard deviations were calculated. Percentage viability was calculated using the equation:

$$Percentage\ Control = \frac{Average\ triplicate\ of\ sample\ values}{Average\ triplicate\ of\ control\ values} \times 100$$

IC_{50} concentrations were calculated using GraphPad Prism 5 (California, USA) and Microsoft Excel 2010 (Washington, USA).

3.5 DPPH Antioxidant Assay

Background: Oxidation is one of the most important processes producing free radicals in food, chemicals and even in living organisms, where free radicals have an important role in processes of food spoilage, chemical material degradation and contributes to more than one hundred disorders in humans (Szabo *et al.,* 2007). Free radicals are a serious threat to human health because they are metabolised within organisms and attack biologically active substances such as fatty acid, proteins and nucleic acids. This can lead to cell damage, cancer or metabolic and neurological diseases (Wolosiak *et al.,* 2011). Antioxidants are substances that even at low concentrations significantly delay or prevent oxidation of easily oxidizable substrates. Natural antioxidants, particularly in fruits and vegetables, have gained interest due to epidemiological studies which have indicated that frequent

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consumption of natural antioxidants is associated with lower risk of cardiovascular disease and cancer (Thaipong *et al.,* 2006). The defensive effects of natural antioxidants are related to three major groups: vitamins, phenolics and carotenoids, where ascorbic acid and phenolics are known as hydrophilic antioxidants and carotenoids as lipophilic antioxidants (Thaipong *et al.,* 2006). Several assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2-azinobis(3-ethyl-benzothiazoline-6-sulphonic acid)], FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorption capacity) have been frequently used to estimate antioxidant properties in fruits and vegetables. According to Szabo *et al.,* (2007) the DPPH assay is one of the best known and frequently used methods to assess antioxidant activity. Plant extracts are routinely screened for antioxidant activity as plants are known to be high in phenolic compounds which have the ability to dismutate reactive oxygen species (Kapewangolo *et al.,* 2015). Metabolites such as phenolics and terpenoids have been isolated from various marine soft coral species and are reported to have antioxidant properties (Mohammed *et al.,* 2011; Rezai & Sheijoni 2011). Some of these compounds may also be used by coral species for protection against UV radiation (Khalesi *et al.,* 2008).

DPPH is a stable free radical because of its spare electron delocalisation over the whole molecule shown in Figure 13. The delocalisation causes a deep violet colour with a λmax around 520 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable non-radical form of DPPH is obtained with a simultaneous change of the violet colour to pale yellow (Szabo *et al.,* 2007).

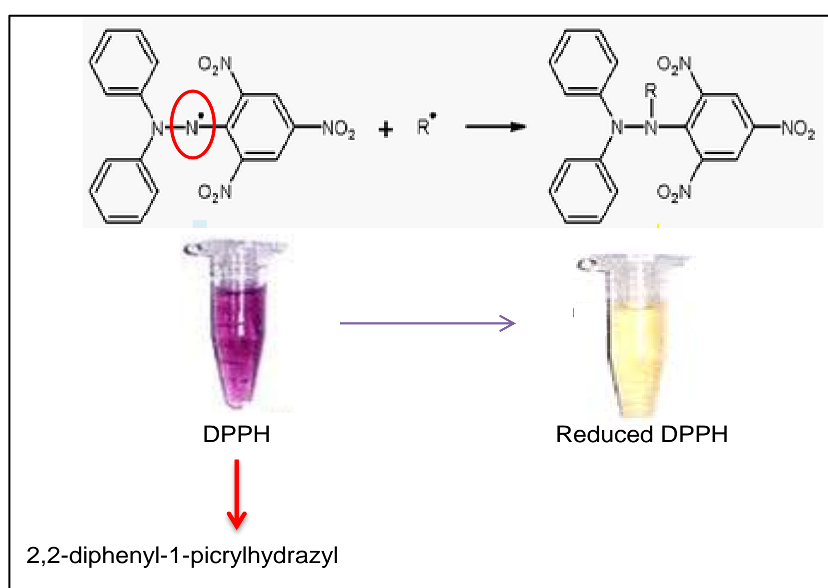

Figure 13: Depiction of the DPPH free radical (left) and the reduced form of the molecule (right) with the associated colour change
(Tam 2012)

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Protocol: Stock solutions of extracts were prepared at a starting concentration of 500 µg/ml using absolute ethanol. Vitamin C stock was prepared to a concentration of 100 µg/ml in absolute ethanol. Vitamin C was used as a positive control as it is a known antioxidant that inhibits the oxidation of other molecules within the body. Two hundred microliters of distilled water (dH_2O) was added to all the wells of the first row of the 96 well plate and 110 µl was added to the remainder of the wells. Extracts were dissolved in absolute ethanol and 20 µl was added to the first row of wells for Extracts (A) and Extract Controls (C) as depicted in Figure 14.

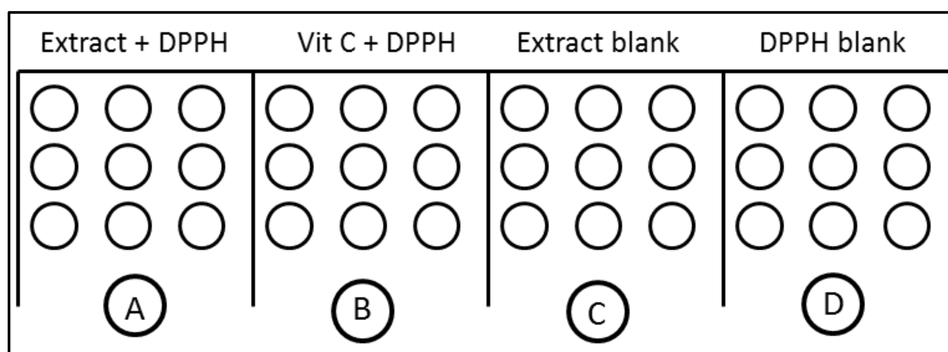

Figure 14: Layout of 96 well plate for DPPH assay

Twenty microliters of Vitamin C stock was added to the first row of B (Positive control) and 20 µl absolute ethanol was added to the first row of C (Solvent Control). A serial dilution of 110 µl from the first row of triplicates was done (from row A to H) and the final 110 µl from row H was discarded. When handling DPPH gloves were worn at all times. DPPH powder (35 µg/ml) was dissolved in absolute ethanol in a foil covered bottle to a final volume of 50 ml. Dissolving of DPPH and its addition to the test plate was done in a fume hood that had a charcoal filter and DPPH was exposed to as little light as possible. Ninety microliters of DPPH was added to all triplicate wells except to the wells for the extract control. Plates were covered in foil and incubated in the dark for 30 minutes. Plates were read at 492 nm on an ELISA plate reader. IC_{50} concentrations were calculated using GraphPad Prism 5 and Microsoft Excel 2010 (Tam 2012; Sharma & Bhat 2009). Scavenging of the DPPH radical was calculated using the equation:

$$Percentage\ free\ radical\ scavenging = \frac{Absorbance\ (control) - Absorbance\ (sample)}{Absorbance\ (control)} \times 100$$

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3.6 HIV-1 Protease Inhibition

Background: As stated earlier in the dissertation, HIV was identified as an organism for which novel treatment was required. This retrovirus encodes proteins that are initially synthesised as large polyprotein precursors and are later processed by posttranslational cleavage. HIV protease (PR) is a homodimeric aspartyl protease which is crucial for proper maturation of the infectious virion (Lam *et al.,* 2000). The inhibition of HIV PR is a common target for the treatment of AIDS, but treatment with current drugs (saquinavir, ritonavir, indinavir etc.) is at a high dose and long term use may result in drug resistance. Matayoshi and collegues (1990) developed a fluorescence assay based on the quenched fluorogenic substrate 1 (Figure 15) consisting of an octapeptide with a fluorescent donor 5-(2-aminoethylamino)-1-napthalene sulfonate (EDANS) attached at the $COOH^-$ terminal and a quenching acceptor 40-dimethylaminoazobenzene-4-carboxylate (DABCYL) attached at the NH_2- terminal.

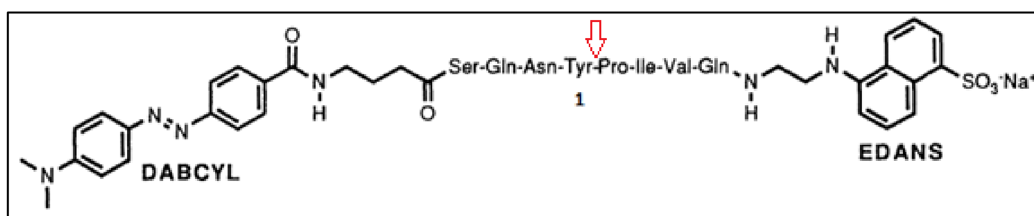

Figure 15: HIV Protease substrate 1
(Fonteh *et al.,* 2011; Matayoshi *et al.,* 1990)

The octapeptide sequence of substrate 1 (SQNYPIVQ) corresponds to the naturally occurring Pr55*gag* p17/p24 cleavage site for the HIV protease which cleaves at the Tyr-Pro bond. This assay is fast, accurate, reproducible and follows a standard ELISA protocol which avoids the use of harmful radioisotopes. The real time assay functions on the use of a substrate that exhibits a spectrophotometric change proportional to its rate of cleavage by the enzyme (Maggiora *et al.,* 1992).

Protocol: For the purpose of this research the procedure followed for the PR assay was based on that published by Fonteh *et al.,* (2009). The protease enzyme (0.1 mg/ml - Bachem, Switzerland) was diluted to a 10 µg/ml working stock using assay buffer (0.1 M Sodium acetate, 1 M Sodium chloride, 1 mM EDTA, 1 mM dithiothreitol and 1 mg/ml bovine serum albumin; pH 4.7). The fluorogenic protease substrate was dissolved in DMSO to prepare a 1 mM stock which was diluted to a 10 µM working stock using assay buffer. The crude extracts screened for PR inhibition were prepared to a final concentration of 200 µg/ml and 100 µg/ml (final assay concentration of 100 µg/ml and 50 µg/ml). Acetyl Pepstatin (Sigma, Germany), the positive control, was dissolved in DMSO (1 mg/ml) and

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diluted with assay buffer to 10 µg/ml working stock. Reactions were performed in a black 96-well plate (Table 4), wells for the buffer blank contained 100 µl of assay buffer. The fluorogenic substrate was added last and this was done in the dark. The plate was incubated for 1 hour at 37°C, the plate was covered in foil to protect the substrate from light. The fluorescence intensity was measured on a Flouroskan (Flouroskan Ascent, Thermo Lab Systems, USA) at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Enzyme inhibition was calculated as follows, where RFU represents Relative Fluorescence Units:

$$\%\ Activity = \left[\frac{(Extract\ RFU - Negative\ control) - (Extract\ Blank - Buffer\ Blank)}{DMSO\ control\ RFU - Negative\ control}\right] \times 100$$

$$\%\ Enzyme\ Inhibition = 100 - \%\ Activity$$

Table 4: Reaction content for HIV-1 protease inhibition assay

	Negative control (µl)	Positive control (µl)	DMSO control (µl)	Extract (µl)	Extract Blank (µl)
Buffer	51	39	39		51
Acetyl Pepstatin		10			
DMSO			10		
Prepared extract in assay buffer				49	49
Protease enzyme		2	2	2	
Protease flourogenic substrate	49	49	49	49	
Final volume	**100**	**100**	**100**	**100**	**100**

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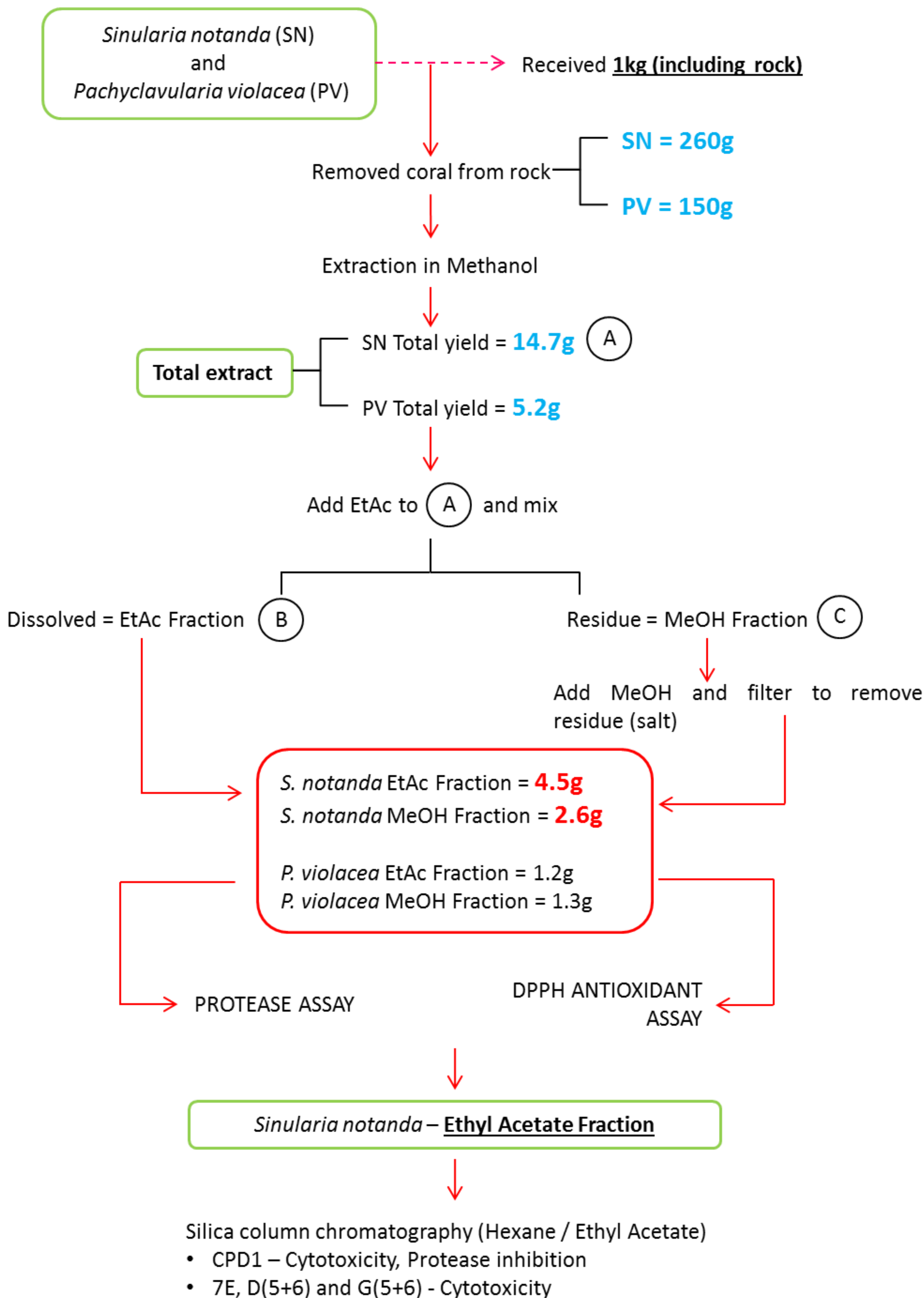

Figure 16: Summary of work flow

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4. Results

This dissertation presents the biological activity of *Sinularia notanda* crude extract and purified compounds. Due to the fact that bioassay guided fractionation was used, the activity of the crude extracts precedes information on pure compound structure determination and activities. Treatment of HIV is associated with severe side effects as well as failure of treatment due to the emergence of drug resistant virus. Marine natural products have proved to be a promising source for new antiviral and anticancer chemotherapy lead molecules. Methanol extraction and solvent partitioning is a commonly used practice and the first step in the isolation of active compounds from crude extracts. The results presented below represent the screening and compound isolation from *Sinularia notanda*. Two other soft coral species were pre-screened with *S. notanda*; the results for the screening of the crude extracts of *Pachyclavularia violacea* and *Discosoma sanctithomae* are presented in the Appendix.

4.1 Crude Extract Bioactivity

HIV-1 protease inhibition was measured using a direct enzyme assay. This assay involves a synthetic peptide with a cleavage site for HIV-1 protease. The peptide consists of an octapeptide with a fluorescent donor 5-(2-aminoethylamino)-1-napthalene sulfonate (EDANS) and a quenching acceptor 40-dimethylaminoazobenzene-4-carboxylate (DABCYL) attached at opposite ends of the peptide. Cleavage of the peptide by HIV protease resulted in measurable fluorescence at 490 nm. Inhibition of HIV protease prevents cleavage of the peptide allowing quenching to occur with a resulting decrease in fluorescence intensity. The total crude extracts of all three soft coral species were screened for HIV-1 protease inhibition (Figure 35). None of the extracts inhibited HIV-1 protease activity at 50% or more, however *S. notanda* showed moderate enzyme inhibition and was selected for further screening. Moderate inhibition was defined by Min *et al.,* (2001) as 40 – 60% inhibition. Methanol and ethyl acetate extracts of *Sinularia notanda* were subsequently screened for HIV-1 protease inhibition at 100 µg/ml and 50 µg/ml (Figure 17). Inhibition of HIV-1 protease by *S. notanda* ethyl acetate fraction (48.7% inhibition) was significantly greater ($p < 0.05$) than inhibition by the methanol fraction (29.1% inhibition) at 100 µg/ml and there was no significant difference in the inhibition at 50 µg/ml. Acetyl pepstatin, a known HIV-1 protease inhibitor, was used as the positive control and showed 95.4% protease inhibition at 10 µg/ml and a 50% inhibitory concentration (IC_{50}) of 1.37 ± 0.008 µg/ml.

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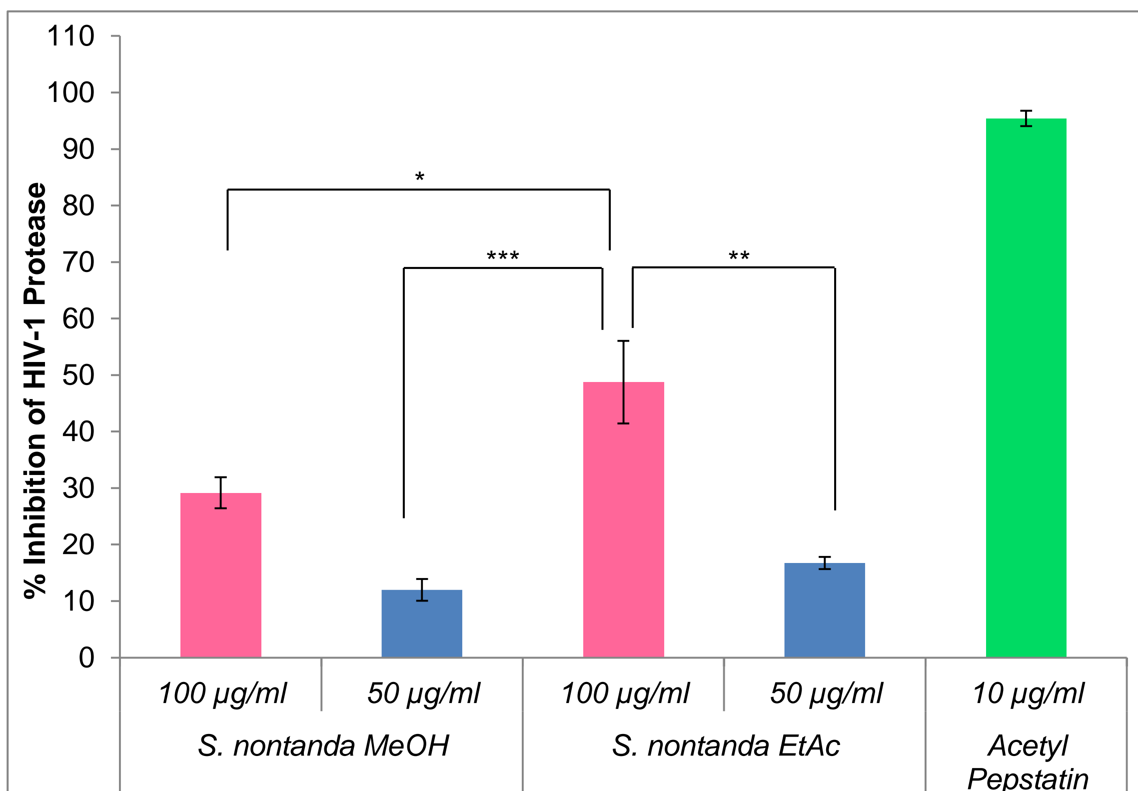

Figure 17: Screening of crude extracts of *S. notanda* for HIV-1 protease inhibition
Methanol and ethyl acetate crude extracts were tested at 100 µg/ml and 50 µg/ml. Acetyl pepstatin was used as a positive control at 10 µg/ml. Significance was evaluated using ANOVA (Tukey) multiple comparison test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ ($n = 4 \pm$ SEM)

Antioxidant activity of the crude extracts was assessed using the DPPH assay. This measured the ability of the extract to reduce the stable DPPH free radical (purple) to diphenylpicrylhydrazine (yellow). The colour change was measured spectrophotometrically at 490 nm. The concentration of the extracts used ranged from 100 µg/ml to 3.9 µg/ml. Vitamin C, a known natural antioxidant, was used as the positive control. Vitamin C showed a 75% free radical scavenging activity at the highest concentration of 10 µg/ml (Figure 18). Methanol and ethyl acetate fractions of *S. notanda* (Figure 18) were tested for free radical scavenging activity at eight different concentrations ranging from 500 µg/ml to 3.91 µg/ml. At concentrations from 250 µg/ml to 7.81 µg/ml the ethyl acetate extract of *S. notanda* had a significantly greater percentage of free radical scavenging than the methanol extract. The IC_{50} for the ethyl acetate fraction was 76.15 ± 2.93 µg/ml and the methanol fraction had an IC_{50} of 27.16 ± 0.59 µg/ml.

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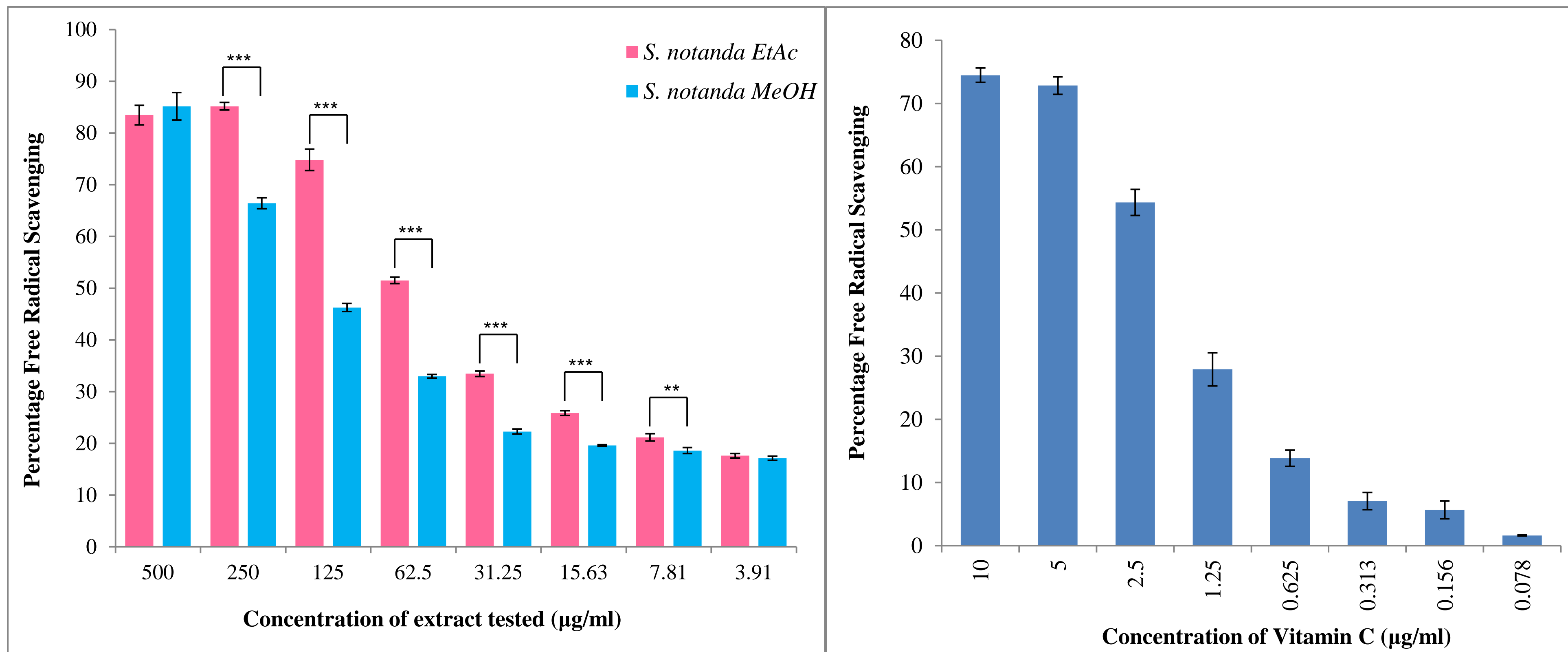

Figure 18: Screening of *S. notanda* crude extracts for DPPH free radical scavenging ability.
Methanol and ethyl acetate crude extracts were tested at eight different concentrations. Vitamin C was used as a positive control. Significance was evaluated using ANOVA (Tukey) where ** $p < 0.01$; *** $p < 0.001$ ($n = 2 \pm$ SEM)

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The XTT colorimetric assay was used to assess the viability of HeLa cells after treatment with *S. notanda* extracts. Crude extracts were tested at concentrations ranging from 200 µg/ml to 12.5 µg/ml and toxicity was observed in a dose dependent manner. Actinomycin D was used as a positive control for the XTT assay and had a CC_{50} of 0.0063 ± 0.0014 µg/ml (n = 3 ± SEM).

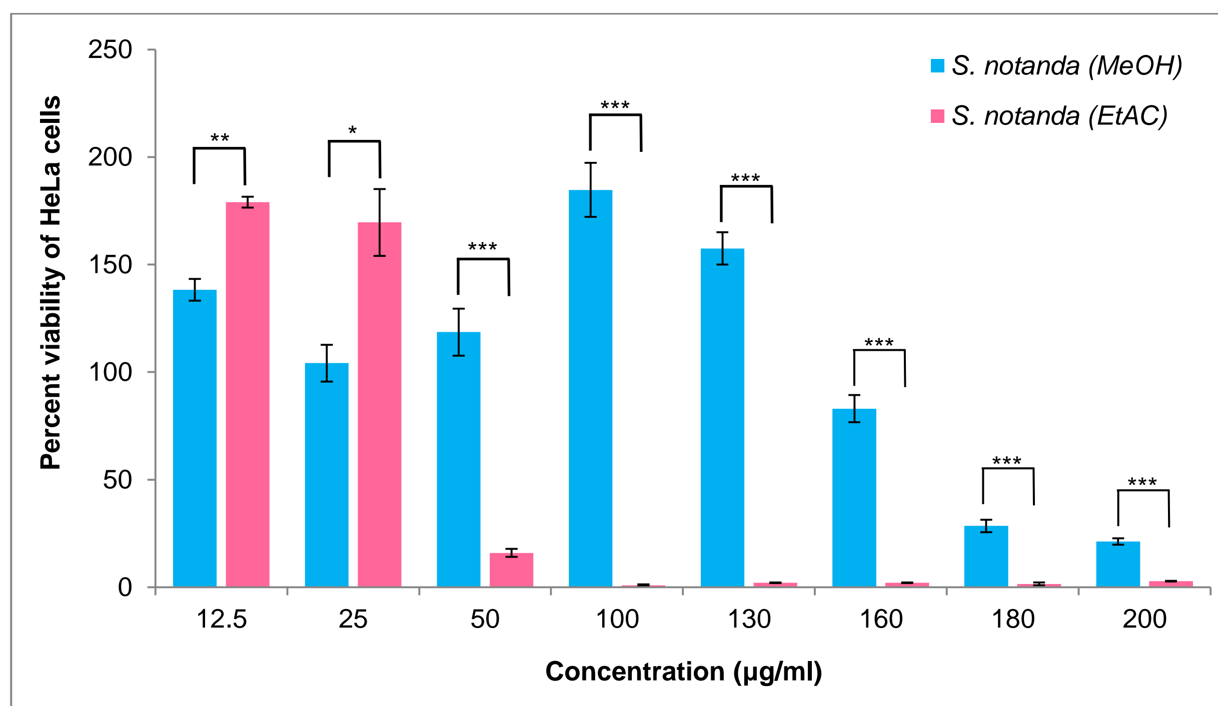

Figure 19: Representation of the dose response of crude extracts of *S. notanda* when screened for cytotoxicity against HeLa cells using the XTT cell viability assay
Significance was evaluated using an unpaired t-test where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (n = 2 ± SEM)

Methanol and ethyl acetate fractions of *S. notanda* (Figure 19) were tested for cytoxicity against HeLa cells at eight different concentrations. Initial screening was done at concentrations ranging from 400 µg/ml to 3.1 µg/ml, however no dose response was seen and the concentration range was decreased to 200 µg/ml to 12.5 µg/ml. The CC_{50} for the ethyl acetate fraction was 29.48 ± 3.55 µg/ml (n = 3 ± SEM) and the methanol fraction had a CC_{50} of 145.80 ± 0.59 µg/ml. From the results above it could be seen that the ethyl acetate fraction of *S. notanda* had significantly greater bioactivity than the methanol fraction. The results that follow therefore focused solely on the isolation of compounds from the ethyl acetate fraction.

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4.2 Compound Isolation, Structural Elucidation and Bioactivity

The ethyl acetate fraction was dissolved in ethyl acetate and mixed with silica, this was allowed to dry after which, it was added to a silica column washed with hexane (Figure 20 A and B). The extract was then partitioned on silica gel using n-hexane : ethyl acetate (100:0 to 0:100). Fractions were collected every 500 mL and concentrated by drying, using a Rotorvap.

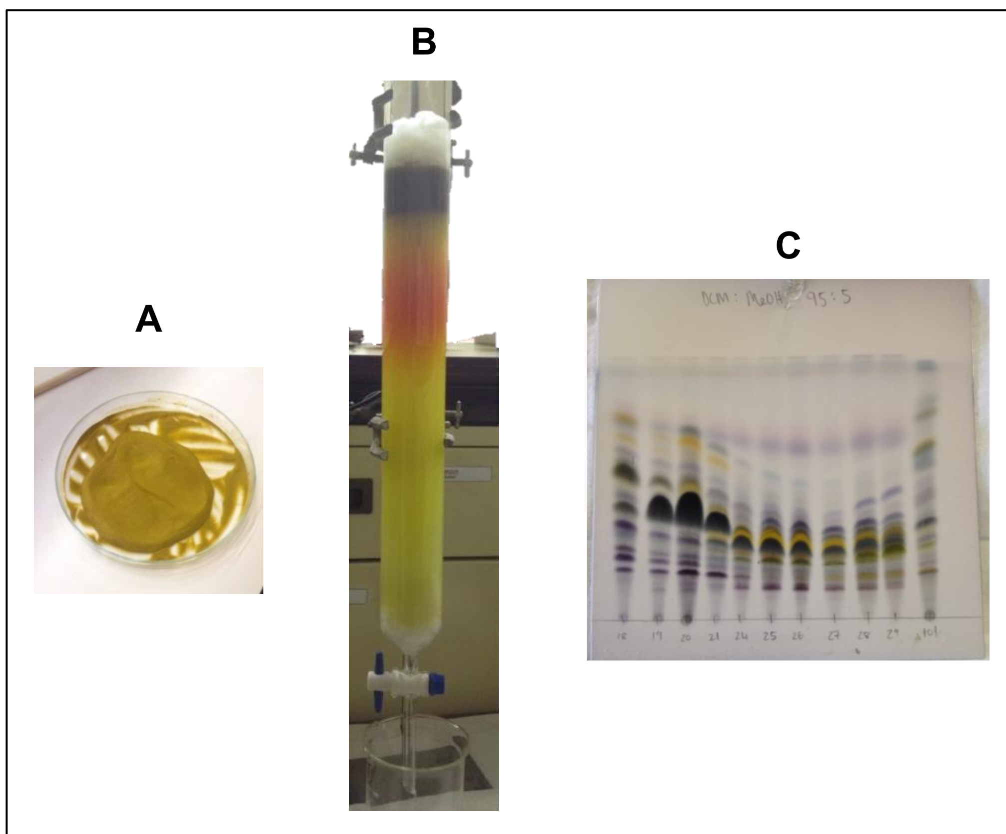

Figure 20: silica column chromatography of *S. notanda* ethyl acetate crude extract. Crude ethyl acetate fraction mixed with silica and dried (A) before application to the column (B). Thirty six fractions were collected and evapourated under reduced pressure. TLC profiles were obtained for each fraction (C)

Thirty five fractions were collected and the TLC profile of each fraction was obtained. Figure 20 C is a representation of the TLC profile of 10 collected fractions as well as the total ethyl acetate fraction (plate developed in 95:5 (v/v) dichloromethane : methanol and stained with vanillin-sulphuric acid solution). Upon drying of Fraction 20, crystals formed and were then removed from the soluble part of the fraction by filtration. The crystal, from here on referred to as CPD1, was submitted for structural elucidation by 1-dimensional and 2-dimensional NMR (Figure 21 to Figure 25) and X-Ray crystallography (Table 5 to Table 11). Selected fractions were screened for HIV-1 protease inhibition and these data is shown in the appendix (Figure 40). CPD1 and Fraction 11 exhibited moderate protease inhibition (40 – 55% inhibition).

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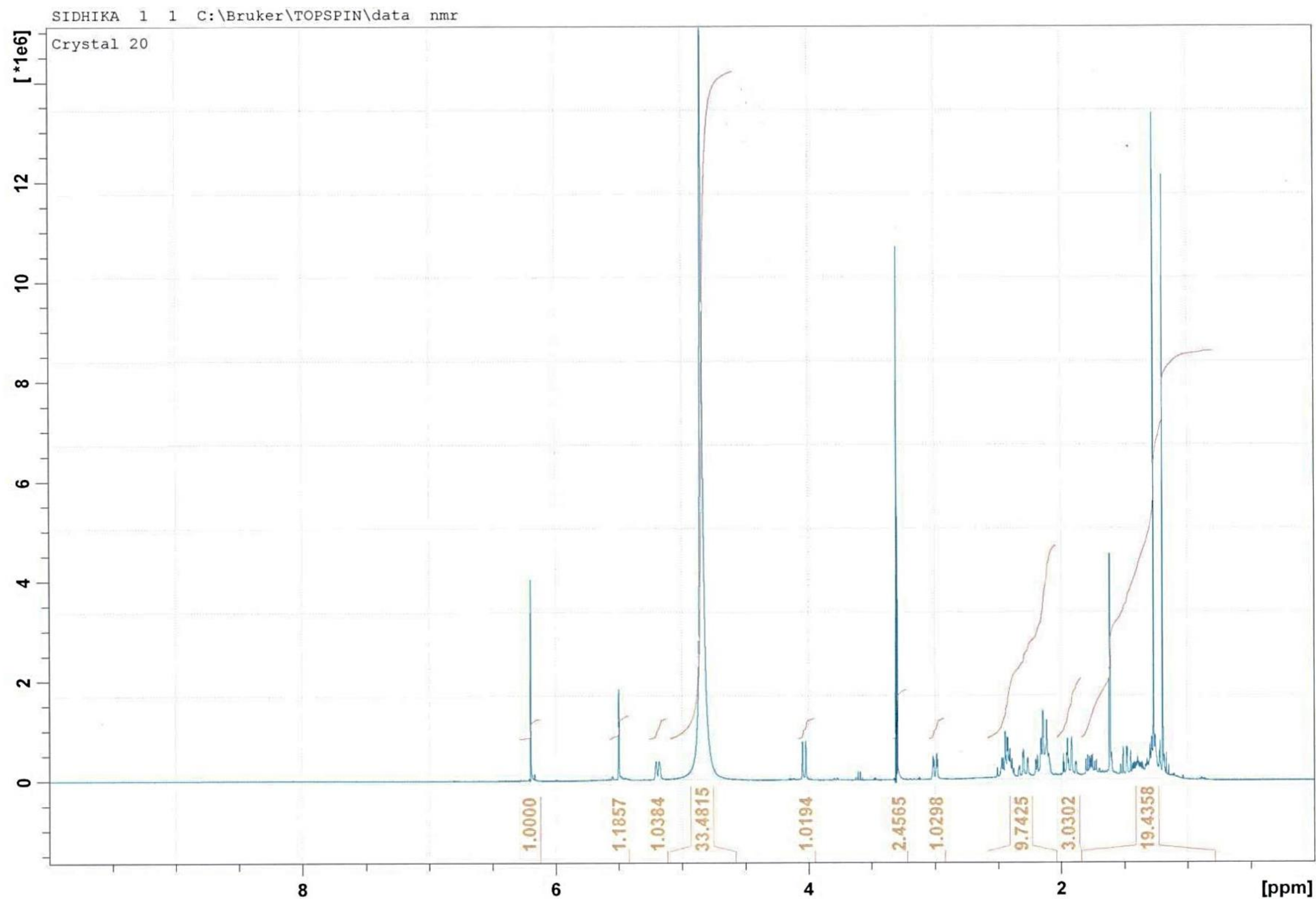

Figure 21: ^{1}H NMR spectrum for the isolated compound CPD1
Spectrum showing the chemical shift of protons. Methyl groups can be found at 0.9 ppm, methylene at 1.33 ppm, alkenes at 4.5 – 6.1 ppm and alkynes at 2.0 – 3.2 ppm.

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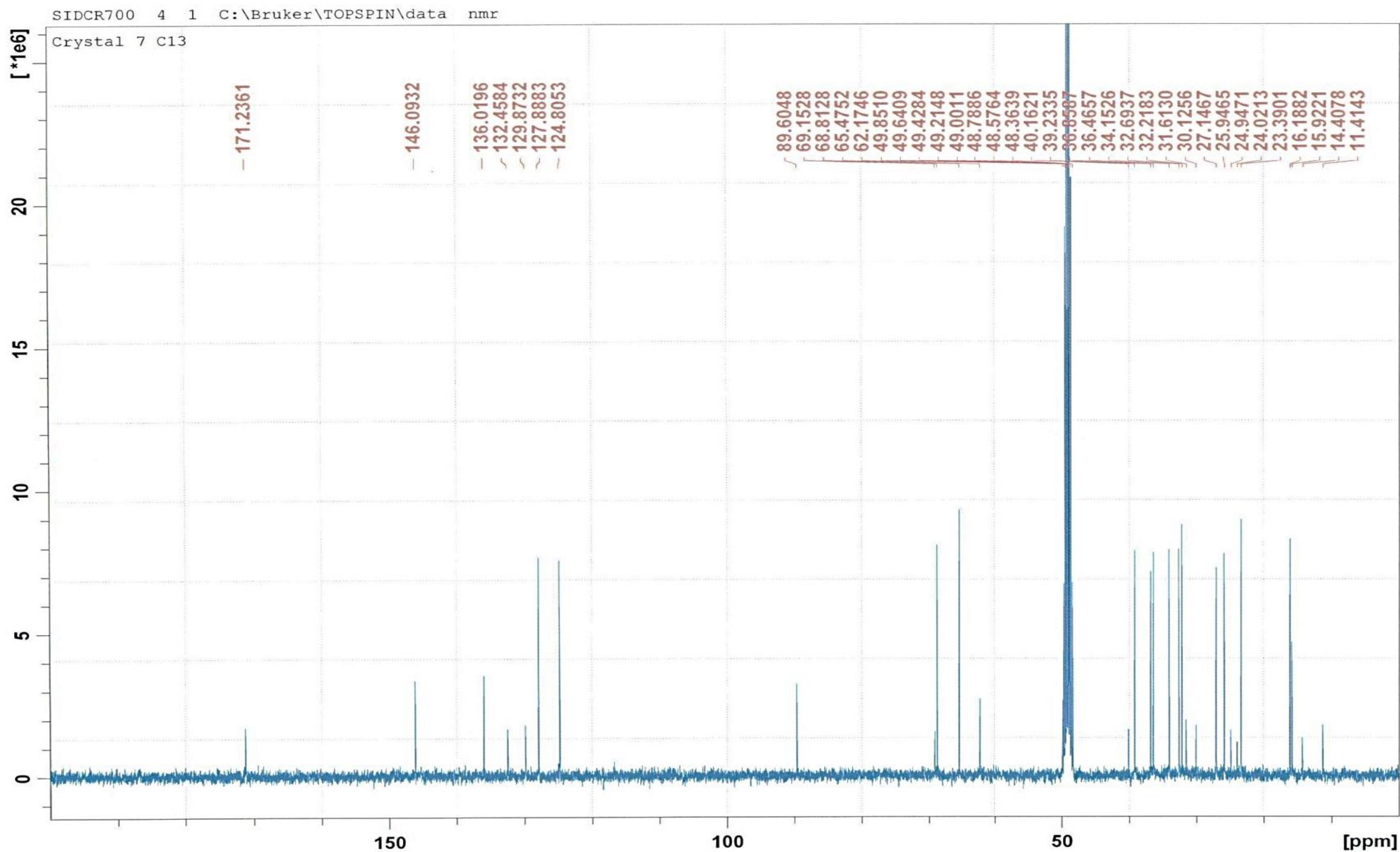

Figure 22: ^{13}C NMR spectrum for CPD1

Spectrum showing the chemical shift of carbon atoms: Methyl groups can be seen at 10 – 15 ppm, methylene groups at 16 – 25 ppm, primary alcohols at 50 – 65 ppm and alkenes at 115 – 140 ppm

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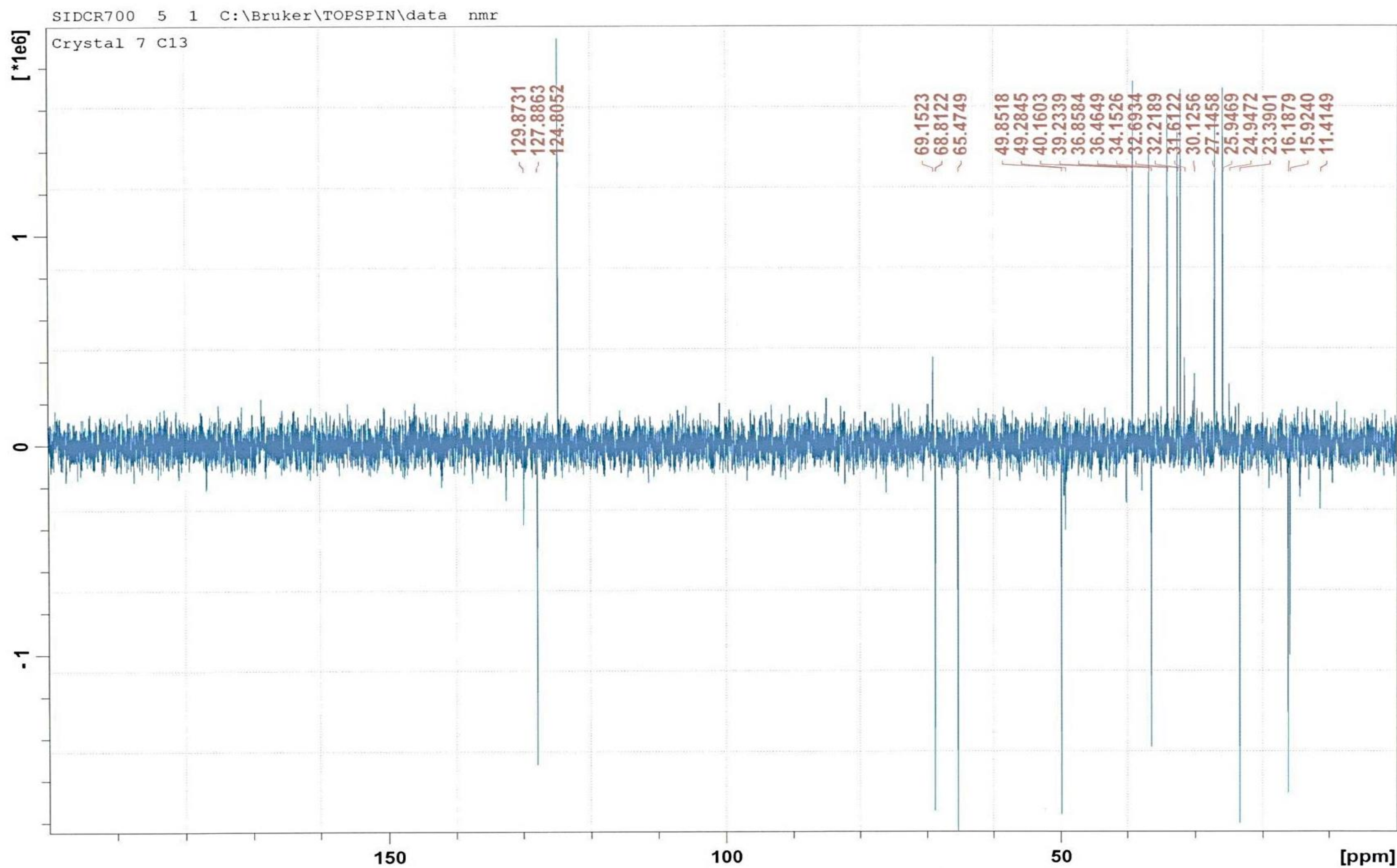

Figure 23: Dept 135 for CPD1

Distortionless Enhancement of Polarization Transfer (DEPT-135) shows information on attached protons: the positive range shows CH_3 and CH groups while the negative range shows signals for CH_2 groups.

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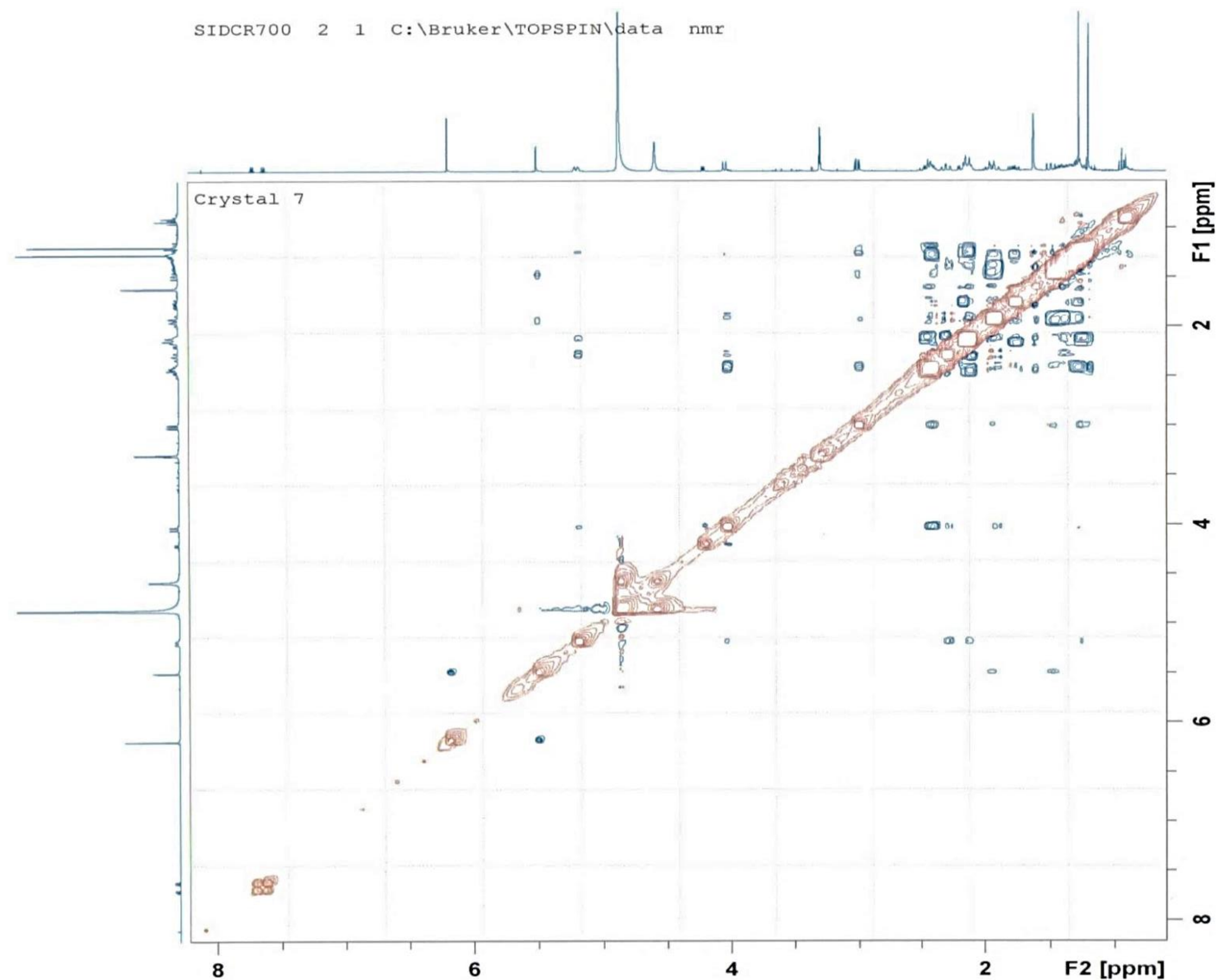

Figure 24: Noesy correlation for CPD1

NOESY: Nuclear Overhauser Effect Spectroscopy, 2D NMR method used to mapping NOE correlations between protons of CPD1

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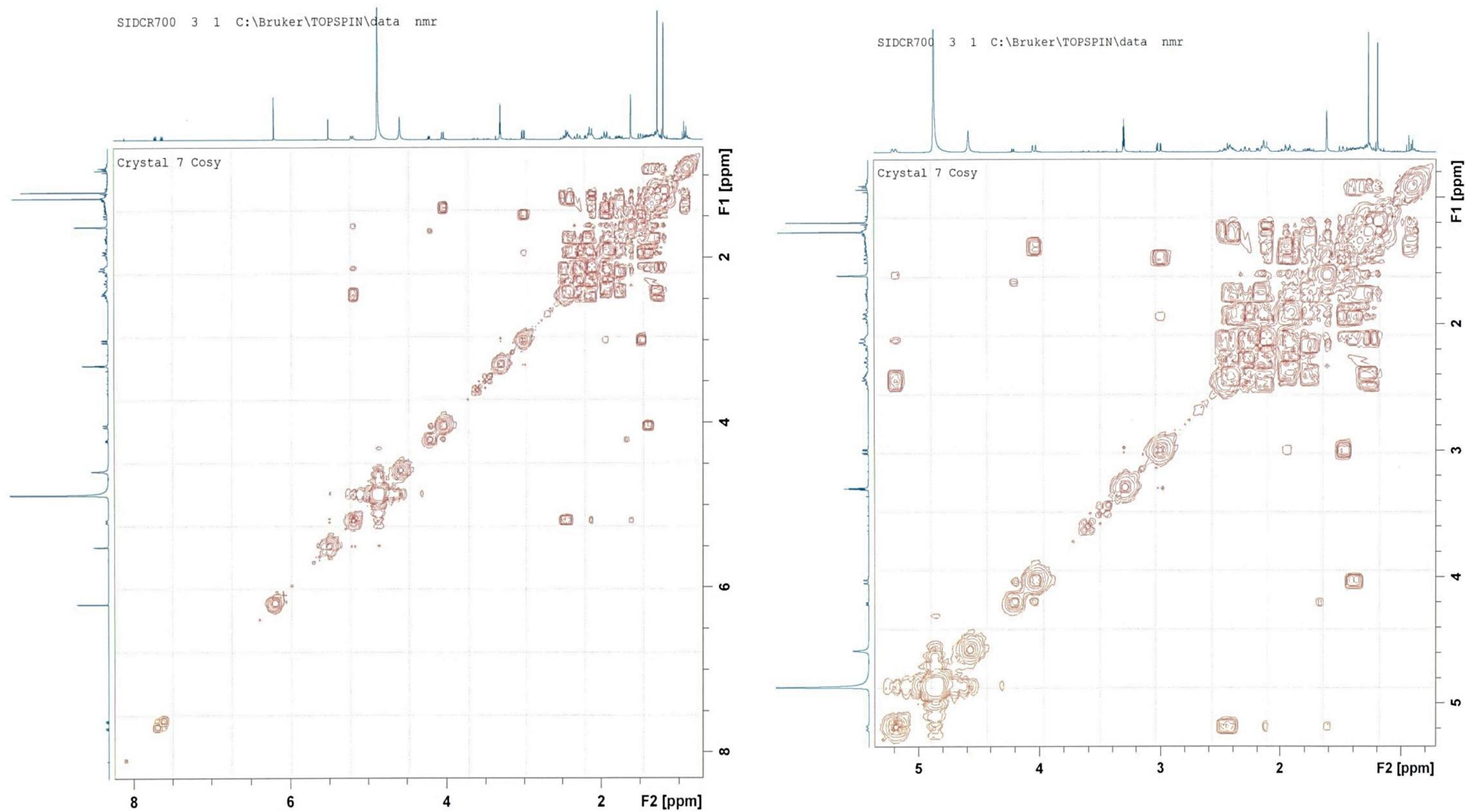

Figure 25: Cosy for CPD1

Representation of nuclei in CPD1 that share scalar (J) coupling. The coupling relationship is for protons within the structure that are two or three bonds apart.

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Table 5: Crystallographic data for compound CPD1

Empirical formula	C20 H30 O4	
Formula weight	334.44	
Temperature	100(2) K	
Wavelength	1.54178 Å	
Crystal System	Orthorombic	
Space Group	$P2_12_12_1$	
Unit cell dimension	a = 8.8817(5) Å	α = 90°
	b = 11.0893(5) Å	β = 90°
	c = 19.8361(9) Å	γ = 90°
Volume	1953.69(17) $Å^3$	
Z	4	
Density (calculated)	1.137 Mg/m^3	
Absorption coefficient	0.621 mm^{-1}	
F(000)	728	
Crystal size	0.287 x 0.176 x 0.120 mm^3	
Theta range for data collection	5.457 to 65.267°.	
Index ranges	-10<=h<=9, -13<=k<=5, -23<=l<=19	
Reflections collected	6122	
Independent reflections	3032 [R(int) = 0.0453]	
Completeness to theta = 67.679°	91.80%	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.7526 and 0.6619	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	3032 / 0 / 223	
Goodness-of-fit on F^2	1.03	
Final R indices [I>2sigma(I)]	R1 = 0.0388, wR2 = 0.0779	
R indices (all data)	R1 = 0.0508, wR2 = 0.0820	
Absolute structure parameter	0.0(2)	
Extinction coefficient	n/a	
Largest diff. peak and hole	0.121 and -0.148 e.$Å^{-3}$	

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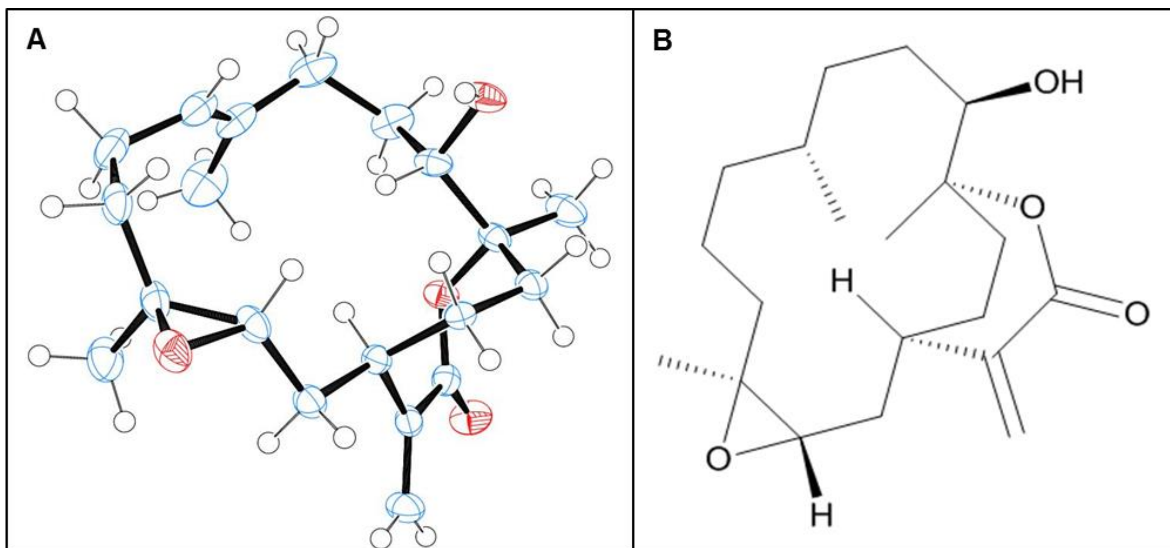

Figure 26: A – Ellipsoid plot constructed from X-Ray crystallography data (standard procedures followed); B – Interpretation of 1D and 2D NMR spectra of CPD1

A suitable colourless block-like specimen of CPD1 was used for X-ray crystallographic analysis. The crystal had a molecular formula of $C_{20}H_{30}O_4$ and relative molecular mass of 334.44. The orthorhombic crystal had the dimensions 0.12 mm x 0.18 mm x 0.29 mm and a calculated density of 1.137 Mg/m3 (Table 5).

X-ray crystallography of CPD1 produced an ellipsoid plot of a compound with no structural matches on the Cambridge Crystallographic Data Center (CCDC). NMR analysis confirmed the structure to be [(1R,3R,5S,12R,13S,E)-12-hydroxy-5,9,13-trimethyl-16-methylene-4,14-dioxatricyclo[11.3.2.03,5]octadec-8-en-15-one with a hydroxyl group at C12 and an epoxide at C4/C5. The alkene double bond could not be confirmed by x-ray crystallography however subsequent editing of the structure confirmed the double bond with a length of approximately 1.5Å.

The 35 fractions collected from silica column 1 were combined to 11 main fractions based on TLC profiles and tested for cytotoxicity against HeLa cells. Each fraction was screened at 50µg/ml and 100µg/ml and the percent viability of HeLa cells is presented in Figure 27. Each of the 11 fractions were weighed as only fractions with the highest cytoxicity as well as the highest yield were chosen to continue with further isolations. The cytoxicity data and weights for the 11 fractions are summarised in Table 6.

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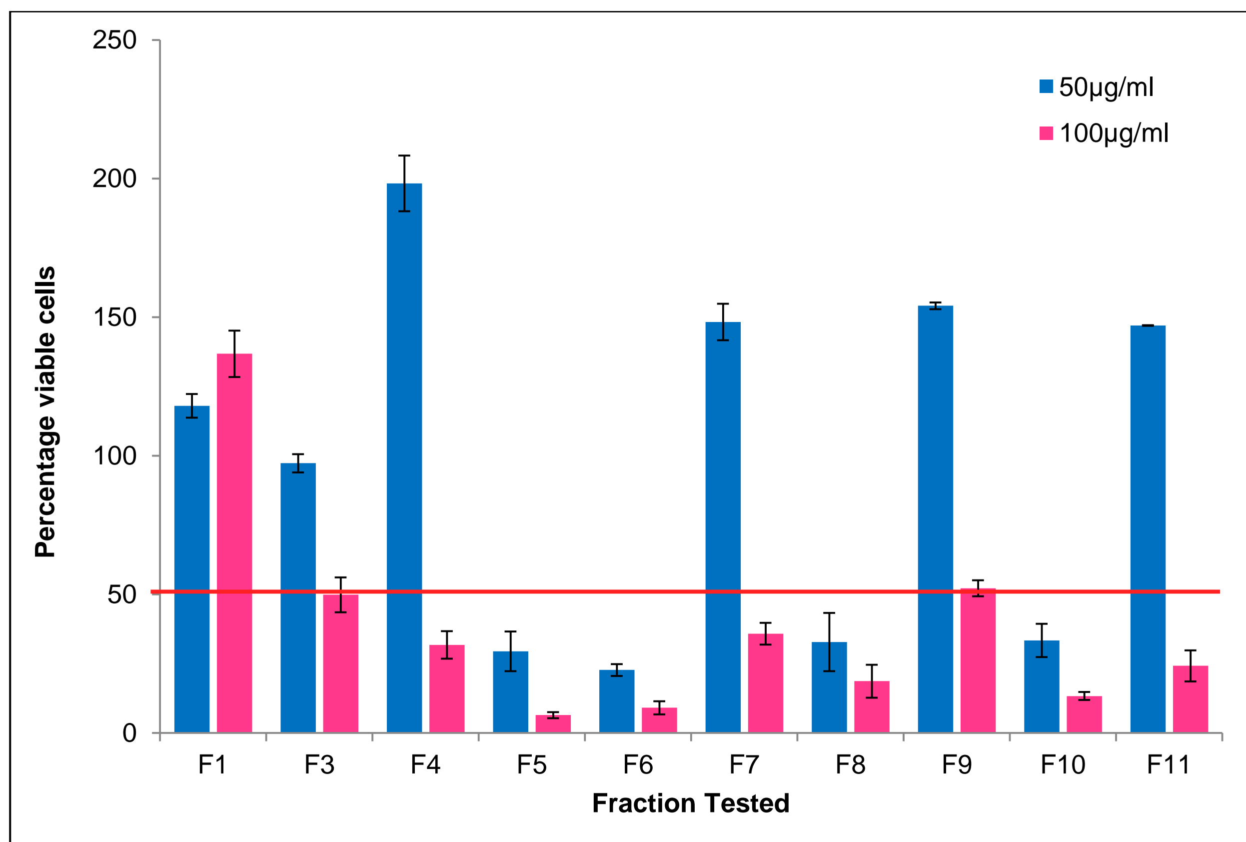

Figure 27: Percent viability of HeLa cells treated with fractions obtained from the first silica column of *S. notanda* ethyl acetate crude extract.

Fractions were tested against HeLa cells at 100 µg/ml and 50 µg/ml. A cut-off of 50% viability was used as indicated by the red line. Fractions resulting in a cell viability of less than 50% were considered for further purification (n = 2 ± SD).

Table 6: Weight of 11 main fractions and % viability of HeLa cells (using XTT)

Fraction 5 and 6 (yellow) were combined based on TLC profiles. Fraction 7 (green) had the greatest yield and was also identified as the fraction from which the crystal CPD1 was isolated. Fraction 7 was used to isolate CPD1 in non-crystalline form.

FRACTION	AMOUNT (mg)	% Viable cells at 50µg/ml	% Viable cells at 100µg/ml
F1	230	117.9	136.8
F2	20	Did not test	
F3	207	97.3	49.8
F4	92	198.3	31.8
F5	306	29.5	6.5
F6	260	22.7	9.1
F7*	515	148.3	35.8
F8	203	32.8	18.7
F9	154	154.1	52.2
F10	87	33.4	13.4
F11	254	146.9	24.3

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Fractions 5 and 6 exhibited the greatest inhibition of HeLa cell growth and were combined based on the TLC profiles (Figure 28). Subsequent isolation from the 11 main fractions had to take not only biological activity into account, but also the weight of the fractions (Table 6). Fraction 7 was identified as the main fraction that CPD1 was isolated from. This fraction was subjected to silica column partitioning (Column 2 - Figure 28) using the following solvent system:

90% Hexane : 10% Ethyl Acetate (200 ml)
85% Hexane : 15% Ethyl Acetate (800 ml)
80% Hexane : 20% Ethyl Acetate (200 ml)
75% Hexane : 25% Ethyl Acetate (200 ml)
70% Hexane : 30% Ethyl Acetate (1 L and column wash)

Fractions were collected every 100 mL and concentrated using a Rotorvap. These fractions were combined according to similar TLC profiles to obtain 9 final fractions. Fraction 7E and 7F showed the same TLC retention factor as CPD1 therefore 7F was submitted for 1D and 2D NMR analysis as well as Mass spectrometry analysis, which both confirmed that 7E had an identical structure to CPD1.

Silica column chromatography (Column 3) of Fraction (5+6) was performed using an isocratic solvent system of 80% Hexane and 20% Ethyl Acetate (v/v). Fractions were collected every 100 mL and concentrated using a Rotorvap. These fractions were combined according to similar TLC profiles to obtain 9 final fractions. Fractions D(5+6) and G(5+6) were identified as fractions from which a third pure compound could be isolated however this was not possible using prep-TLC as the sample loss would be too great. Fraction G(5+6) was subjected to prep-HPLC purification, following which structural elucidation by mass spectrometry identified the compound 3-caffeoylquinic acid as the major compound identified in the fraction (Figure 29). There were also traces of 4-caffeoylquinic acid identified in this fraction. Fractions D(5+6) and G(5+6) were tested for cytotoxicity against HeLa and U937 cells (Figure 30). Both fractions exhibited a greater toxicity towards U937 cells. Fraction D(5+6) did show greater inhibition of HeLa cells than G(5+6) at the lowest concentration of 25 µg/ml.

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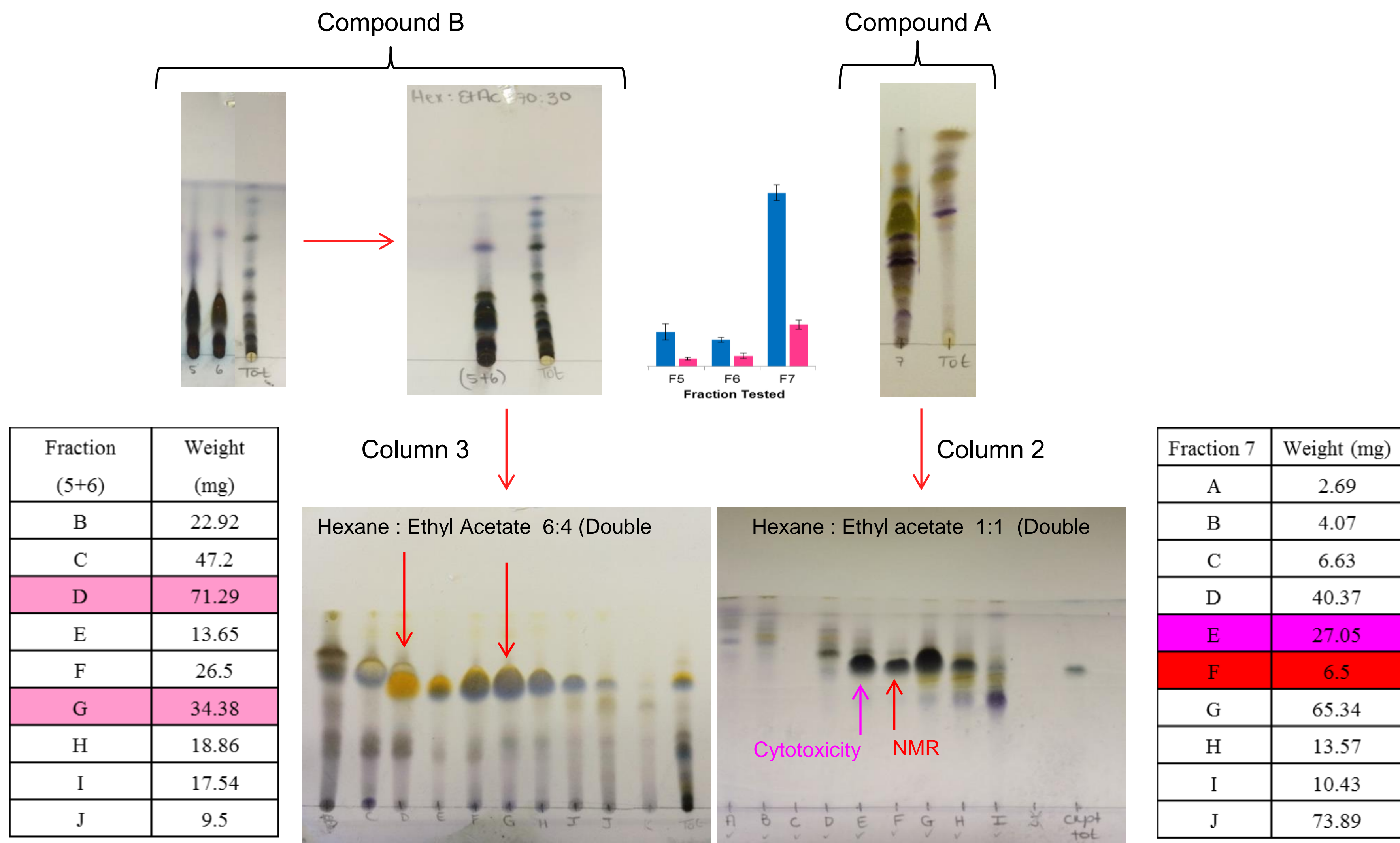

Fraction (5+6)	Weight (mg)
B	22.92
C	47.2
D	71.29
E	13.65
F	26.5
G	34.38
H	18.86
I	17.54
J	9.5

Fraction 7	Weight (mg)
A	2.69
B	4.07
C	6.63
D	40.37
E	27.05
F	6.5
G	65.34
H	13.57
I	10.43
J	73.89

Figure 28: Silica column chromatography of active fractions based on the XTT cell viability results of Figure 27

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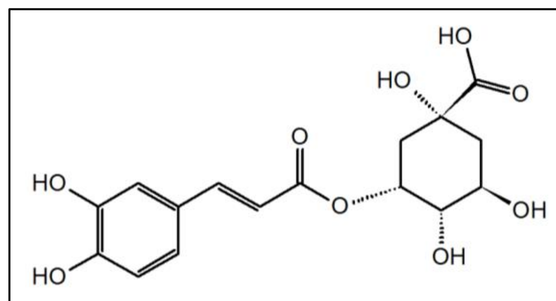

Figure 29: Prep-HPLC identified 3-caffeoylquinic acid as the major compound in Fraction G(5+6)

D - HeLa A
% Viability of HeLa cells
70 60 50 40 30 20 10 0
Concentration µg/ml 25 50 100

G - HeLa C
% Viability of HeLa cells
250 200 150 100 50 0
Concentration µg/ml 25 50 100

D - U937 B
% Viability of U937 cells
4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
Concentration µg/ml 25 50 100

G - U937 D
% Viability of U937 cells
9.0 8.0 7.0 6.0 5.0 4.0 3.0 2.0 1.0 0.0
Concentration µg/ml 25 50 100

Figure 30: Cytotoxicity of fraction (5+6)

A shows that the toxicity of D(5+6) against HeLa cells ten times greater than the toxicity of G(5+6) against HeLa cells (graph **C**). Graph **B** and **D** represent the toxicity of D(5+6) and G(5+6) respectively against U937 cells, where both fractions exhibited cell viability under 10% at all three concentrations.

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The XTT cell viability assay also showed that 7E exhibited a similar cytotoxicity profile towards HeLa cells as CPD1 (Figure 31). CPD1 was tested for cytoxicity against HeLa, Vero, U1 and U937 cell lines at concentrations ranging from 100 µg/ml to 0.78 µg/ml (Figure 32). Vero cells were used as a control cell line that represents normal mammalian cells. CPD1 did not show toxicity towards HeLa and U1 cells at concentrations lower than 50 µg/ml and inhibited Vero cells by 50% or lower at all concentrations. CPD1 exhibited the greatest toxicity towards U937 cells showing greater than 50% inhibition at 3.13 µg/ml and higher. Data presented in Figure 32 was used to calculate CC_{50} concentrations for CPD1 with respect to each cell line tested as well as CC_{50} concentrations for the positive control Actinomycin D (Table 7). U937 cells treated with CPD1 had a significantly lower CC_{50} concentration compared to the control cell line ($p < 0.05$) and a selectivity index of 2.54. For all other cell lines the CC_{50} concentration of CPD1 was significantly greater when compared to the CC_{50} concentration calculated against Vero cells ($p < 0.0001$).

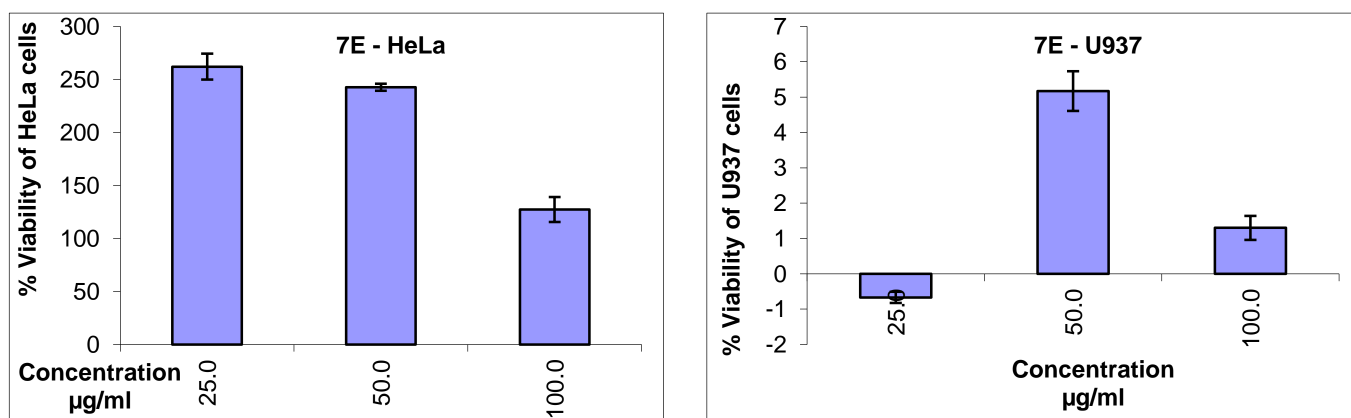

Figure 31: Cytotoxicity of compound 7E

Percent viability of HeLa and U937 cells when treated with compound 7E showed that this compound was not toxic to HeLa cells at the highest tested concentration of 100 µg/ml. The compound was extremely toxic to U937 cells at all three tested concentrations with a cell viability of less than 10%.

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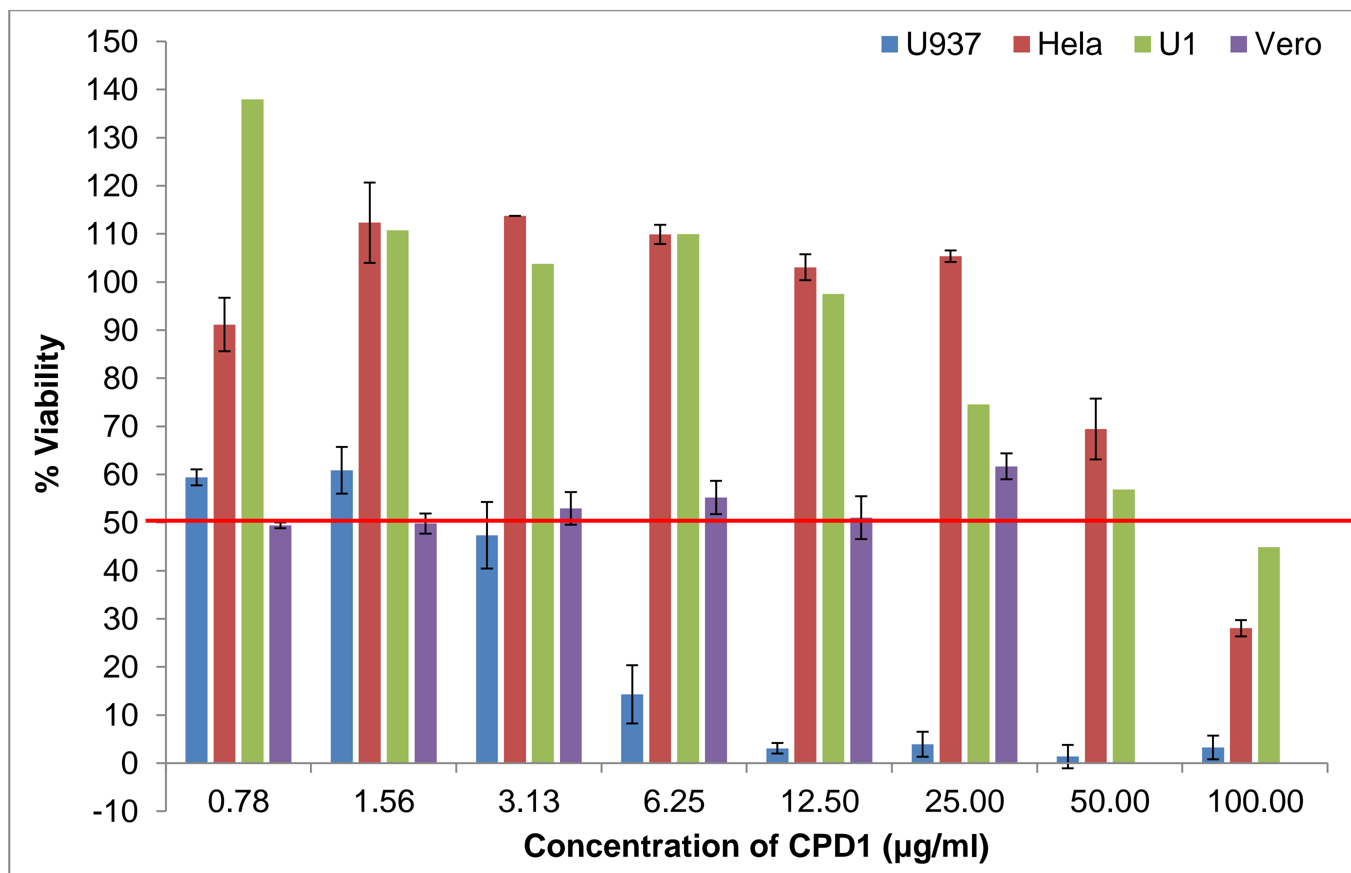

Figure 32: Cytotoxicity of CPD1 against HeLa, U937, U1 and Vero cell lines
A cut-off of 50% viability was used as indicated by the red line.

Table 7: Protease inhibition and antioxidant activity of crude extracts of *S. notanda* (SN) and *P. violacea* (PV); as well as CC_{50} concentrations of CPD1 when tested for cytoxicity against four different cell lines

	SN EtAc	SN MeOH	PV EtAc	PV MeOH
	% Inhibition of HIV-1 Protease			
100 µg/ml	43.0 ± 9.8	20.3 ± 9.1	35.0 ± 5.8	19.6 ± 9.5
50 µg/ml	24.3 ± 5.9	14.7 ± 5.5	33.7 ± 7.8	30.7 ± 15.2
	Antioxidant activity (DPPH) (IC_{50})			
	78.94 µg/ml	33.96 µg/ml	165 µg/ml	-
	Cytotoxicity of crude extract against HeLa cells (CC_{50})			
	29.48 ± 3.55 µg/ml	145.80 ± 0.59 µg/ml	-	-
	Cytotoxicity of CPD1 - SN EtAc (CC_{50})			Actinomycin D
	Cell Line	U1	32.73 ± 2.235 µg/ml	9.78E-03 ± 2.31E-04 µg/ml
		U937	3.91 ± 0.017 µg/ml	0.0487 ± 4.87E-03 µg/ml
		HeLa	61.12 ± 2.73 µg/ml	0.016 ± 5.6E-03 µg/ml
		Vero	9.947 ± 1.921 µg/ml	5.03E-02 ± 2.38E-03 µg/ml

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5. Discussion

Marine natural products have produced a variety of compounds in the field of anti-cancer chemotherapy as is evident by the number of compounds undergoing clinical trials (Chakraborty *et al.,* 2009; Dos Santos *et al.,* 2011). The majority of biomedical compounds are isolated from sponges and coelenterates (Blunt *et al.,* 2004) and the number of new compounds reported from *Cnidarians* has increased by 38% over the last 10 years (Blunt *et al.,* 2015). In this study crude extracts and purified material from a member of the Cnidarian family showed antioxidant and anticancer activity as well as moderate antiviral activity.

Soft corals of the genus Sinularia (*Alcyoniidae*) have been well recognized as a rich source of sesquiterpenes, diterpenes, polyhydroxylated steroids, and polyamine metabolites, with a number of these isolated metabolites exhibiting various biological properties such as cytotoxicity, anti-inflammatory, and antimicrobial activities (Cheng *et al.,* 2010; Blunt *et al.,* 2006; Srinivasan *et al.,* 2014). A specific terpenoid, namely a cembrane diterpenoid was identified here and demonstrated bioactivity; specifically this compound showed moderate to high cytoxicity (dependant on the cell line used) as well as antioxidant activity. NMR, X-ray crystallography and mass spectrometry confirmed the structure and purity of the compound.

Terpenoids from plants or marine organisms are widely considered as potential anticancer drug precursors, causing cell death, decreased proliferation and induction of apoptosis (Chen *et al.,* 2010). Cembrane diterpenoids have been reported in a few terrestrial organisms, such as tobacco leaves, pine oleoresins and termite secretions but are generally the major components of marine octocorals (Lin *et al.,* 2012). Cembranoids and their cyclized derivatives are produced as a defense against predators and settlement of microorganisms (Li *et al.,* 2005). In 1962 the first cembrane diterpene, (+)-cembrene was reported from pine oleoresin. In 1979 a new cembranoid diterpene sarcophytol (A) was isolated from the Okinawa soft coral *Sarcophyton glaucum*. *Sarcophyton* is among the most abundant soft coral genera on many coral reefs and is one of the better studied organisms (Yang *et al.,* 2015). Sarcophytol has attracted the most attention because of its strong inhibtory activity against tumour promoters. This was followed by the isolation of hundreds of cytotoxic cembranoids from plants and insects as well as of marine origin (Yang *et al.,* 2015). In previous studies octocorals belonging to the genus *Sinularia* have been proven to be rich sources of bioactive terpenoid analogues (Hu *et al.,* 2013; Rocha *et al.,* 2011)

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Cembrane based diterpenoids that contain lactones (cembranolides) have been isolated from various octocorals, but most notably from those belonging to the genera of *Sinularia*, *Sarcophyton* and *Lobophyton*. These metabolites have been shown to inhibit the growth of various cancer cell lines (Ahmed 2007; Li *et al.,* 2005). In the marine environment cembranoids usually exhibit cyclic ether lactone or furane moieties around the cembrane framework. From a biomedical perspective, cytotoxixicty is the most remarkable property of cembrane diterpenoids but these secondary metabolites also have neuroprotective, anti-inflammatory, antiarthritic, calcium-antagonistic and antimicrobial activity. A large number of highly functionalised cembranoid diterpenes containing an α-methylidene-γ-lactone moiety have been isolated and identified from marine soft coral especially from the genera *Lobophyton*, *Sarcophyton* and *Sinularia* (Yang *et al.,* 2015)

Alluri, Thameemulanasari & Reddy (2012) reported methanol extraction and dichloromethane extraction of a soft coral species, showing the methanol crude extract to be more toxic to HeLa cells than the dichloromethane extract. It was expected that the methanolic extract of *S. notanda* would show greater biological activity than the ethyl acetate extract. In this study the ethyl acetate fraction of *S. notanda* consistently showed higher biological activity than the methanol fraction with protease inhibition being significantly greater at the highest concentration, antioxidant activity was significantly higher at seven of the eight concentrations tested and the CC_{50} concentration was 4 times lower than that of the methanol fraction when tested against HeLa cells. However, once compounds were isolated from the ethyl acetate fraction, toxicity towards HeLa cells was lost. Compounds capable of inhibiting HeLa cells may have also been retained in the methanol fraction and can be confirmed by extracting larger quantities of coral in order to produce enough of the fraction for compound isolation. Sub-fraction D showed a higher toxicity towards both HeLa and U937 cells compared to CPD1. The TLC profile of this fraction revealed two compounds (one blue, one yellow) with almost identical retention factors. The toxicity of this sub-fraction is attributed to 3-caffeoylquinic acid, which was the major compound identified from this fraction. One cannot however rule out the possible synergistic effect of this compound with 4-caffeoylquinic acid which was also found in trace amounts.

It has also been suggested that marine extracts contain extremely labile compounds and decomposition of these compounds by heat, light, air and pH may occur at any stage of the extraction process (Shahbudin *et al.,* 2011). Depending on the type of compounds one

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expects to isolate, different extraction methods can be used. To test traditionally used plants for reported pharmaceutical action, water should be used in the same manner as the traditional medicines would be administered (cold or boiling water etc.). Water extracts have shown the potential to contain antioxidant compounds but have proven to be a disadvantage when attempting to extract compounds with anti-cancer and anti-HIV properties. To identify and isolate secondary metabolites methanol or ethanol should be used to extract most of the polar and semipolar metabolites and for lipophilic compounds solvent such as chloroform should be used. In a previous study by Tursch *et al.,* (1975) specimens of *S. flexiblis* were sundried, the coral was defatted in hexane and extracted with methylene chloride. Direct crystallisation of the evapourated extract from ether followed by silica column chromatography using hexane and acetone (9:1) and final recrystallization from benzene resulted in a compound structurally identical to CPD1. Hu *et al.,* (2013) extracted sliced *S. flexiblis* in ethyl acetate and separated on silica using hexane and ethyl acetate to obtain 11 main fractions, of which one was separated using normal phase HPLC with hexane and acetone (7:1) producing the ketone version of CPD1. Although this is not the first time CPD1 has been isolated from a coral of the *Sinularia* genus, this is the first time that the compound has been isolated from *S. notanda*. The initial extraction of the coral in methanol could be the reason why the reduced alcohol form of the compound was isolated in this study as opposed to the ketone isolated from *S. notanda* by Tursch *et al.,* (1978). The production of a secondary alcohol from a ketone is a basic reduction reaction as seen in Figure 33.

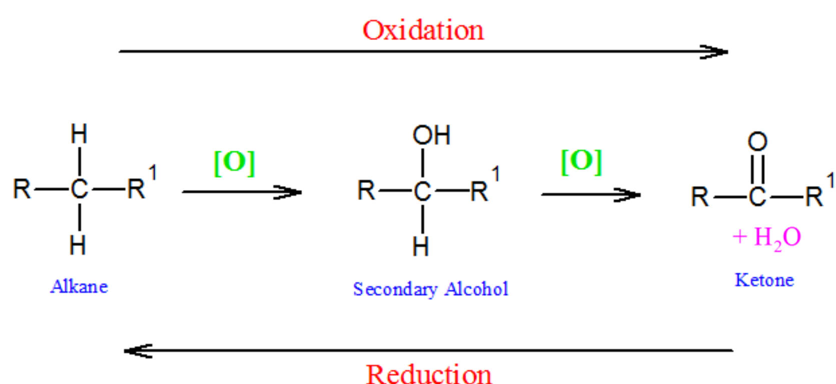

Figure 33: Oxidation and reduction reactions of a secondary alcohol

In general it is believed that the cyclisation of the geranylgeraniol derived precursor between carbon 1 and 14 generate a 14-membered diterpenoid (cembrane or thumbergane). The simple cembrane generally contains a varying 14-membered carbocyclic ring backbone and an isopropyl group, isopropenyl group or isopropyl/isopropenyl acid group at C-1. Li *et al.,* (2005) stated that the biogenesis of

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cambranes involves intramolecular cyclisation from geranylgeranyl diphosphate leading to the 14-membered ring hydrocarbon neocembrane, followed by selective enzymatic oxidations at the 14-membered ring. Geranylgeranyl pyrophosphate is considered to be the precursor that, through a series of ring cyclisations, dehydrogenations, epoxidation, hydroxylations, double-bond rearrangements, oxidated ring cleavages, and cyclic peroxidization, would yield the cembranoids isolated from soft coral of *Sinularia* (Li *et al.,* 2005). The 14-membered carbocyclic ring is commonly fused to a 5, 6 or 7 membered lactone ring, as can be seen in Figure 34. These compounds are involved in new ring formations as well as skeletal rearrangements. Many of the newly formed rings result from nucleophillic attacks of epoxide moieties by alkoxide with C2,C3 or C4 to form a lactone ring (Yang *et al.,* 2015). C18 and C2, C19 and C6, C20 and C10, and C20 and C1 can also be connected to the same oxygen atom to for a lactone ring.

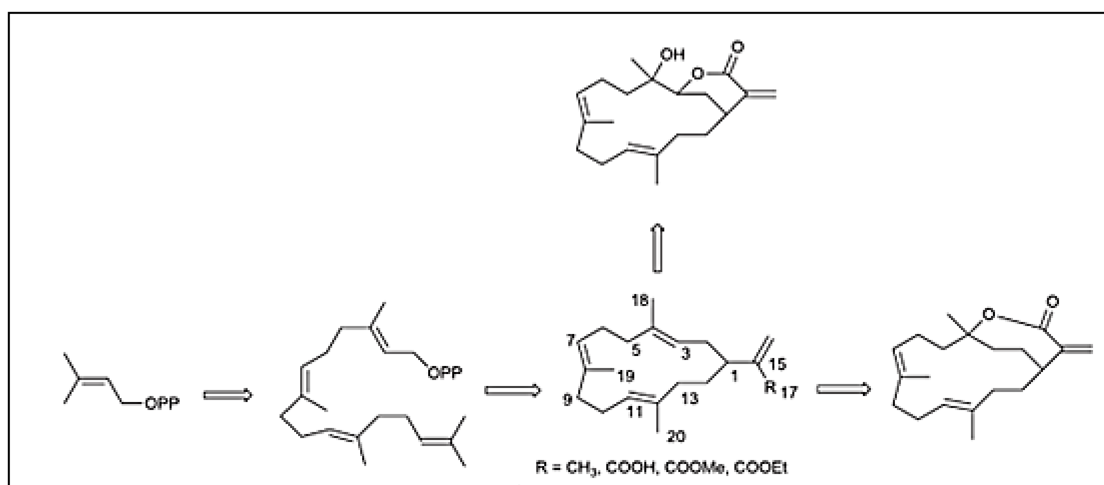

Figure 34: Biosynthetic pathway of cembrane diterpenoids
(adapted from Yang *et al.,* 2015)

Ahmed (2007) isolated a novel cembrane-based diterpenoid (notandolide) from *S. notanda*, containing α-methylene-ε-lactone. This compound is structurally similar to CPD1 but does not contain the epoxide that CPD1 has at C4/C5. Alkaline hydrolysis of the notandolide by Ahmed (2007) resulted in a related compound that inhibited 93.1 ± 2.1% cell growth of HepG2 (hepatocellular carcinoma) cells, 48.3 ± 6.0% growth of Hep3B (hepatocellular carcinoma, contains Hepatitis B) and 47.8 ± 1.9% growth of MCF-7 (breast cancer) cells at 30 µg/ml. Modification of the compounds disrupted the lactone ring and this led to the assumption that the lactone ring was essential to the toxicity of marine cembranolides. In a separate study, Yen *et al.,* (2012) isolated a norcembranoidal diterpene with a γ-lactone ring which exhibited *in vitro* cytotoxicity against various cancer cell lines (K562, MOLT-4, HTC-116, DLD-1, T-47D and MDA-MB-231) at an IC_{50} less than 5µg/ml. The second

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compound isolated (scabrolide D) differed from the norcembranoidal diterpene with the presence of an epoxide instead of OAc at C11. Scabrolide D did not inhibit cell growth in the cell lines when tested at 20µg/ml for 72 hours. From the two studies mentioned above one can assume that the epoxide present on CPD1 is not critical to the toxicity of the compound but the lactone ring is.

Arepalli *et al.,* (2009) evaluated the apoptotic effect of a furano-sesquiterpene isolated from *Sinularia kavarattiensis* on human monocytoid leukemia cells (THP-1) and found that it induced anti-proliferative effects involving a mitochondrial dependent pathway similar to the mechanism of action of doxorubicin and cisplatin. This compound showed an almost three times higher CC_{50} concentration towards HeLa cells (CC_{50} = 116.54 µg/ml) than towards U937 cells (CC_{50} = 41.64 µg/ml). Five Sinularolides were tested by Li *et al.,* (2005) against HeLa cells and reported moderate cytotoxicity, compounds with an CC_{50} greater than 10 µg/ml were considered nontoxic. This study has once again shown that a terpene, in this case a cembrane diterpenoid, exhibits selective cytotoxicity towards U937 cells. Both CPD1 and 3-caffeoylquinic acid showed a CC_{50} of less than 10 µg/ml against this cell line proving further that these compounds isolated from *S. notanda* are promising anti-cancer compounds. Li *et al.,* (2005) reported that the epoxide unit at C12/C13 was required for toxicity towards the cell lines tested. These findings contradict that of Ahmed (2007) and Yen *et al.,* (2012). In order to evaluate the importance of the epoxide and the lactone ring of CPD1, different forms of the compound must be synthesised to determine if the changes in structure improved or hindered the cytotoxicity of CPD1 against HeLa cells.

Cell death by necrosis leads to the loss of cell membrane integrity and uncontrolled release of the contents into the surrounding tissue causing inflammation and can be considered a toxic process in comparison to necrosis. It is therefore an advantage to identify anticancer agents that specifically induce apoptosis as opposed to necrosis (Edwards *et al.,* 2012). Apoptosis plays a vital role in controlling cell number in many developmental and physiological stages, tissue homeostasis and immune system regulation while inadequate apoptosis is an integral part of cancer development. Agents which supress or inhibit the development of malignant cells by inducing apoptosis, may represent a useful mechanistic approach to both chemoprevention and chemotherapy of cancer. Various molecular pathways have been associated with the anticancer action of terpenoids including the induction of apoptosis by ubiquitin-proteasome and inhibition of the NF-κB signalling pathway. The mechanism of cell death induction by the cembrane diterpenoid reported in

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this study is under investigation, as this class of compounds has been known to induce cell death by apoptosis rather than by necrosis.

In previous studies, many cembranes have been found to exhibit various biological activities, such as antitumor, anti-microbial and anti-inflammatory activities. Within those, the cembrane-type diterpenoids with anti-inflammatory activities may decrease the expression levels of iNOS or COX-2, and somewhat upregulate TGF-β in macrophages. Cembranolides isolated from soft coral can also suppress the activation and maturation of murine DCs. Pre-treatment of immature DCs with cembranolides (e.g., lobocrassin B) effectively inhibits TNF-α production and attenuates DC maturation after LPS stimulation. NF-κB nuclear translocation was also inhibited dose-dependently by lobocrassin B treatment. In particular, marine natural products have recently been recognized as a promising source of NF-κB inhibitors. The cembrane-type diterpenoids isolated from soft corals (Sarcophyton sp. and Sinularia sp.) have been shown to inhibit both TNF-α-induced NF-κB-DNA binding, as well as TNF-α-induced I-κB degradation and nuclear translocation of p50/p65 (Lin *et al.,* 2013). Since TNF-α was identified as a pro-inflammatory cytokine, several inhibitors of this cytokine have been approved for clinical use. NF-κB is known to activate the expression of many genes involved in the pathology of conditions such as arthritis and other inflammatory responses also linked to TNF-α (Abad *et al.,* 2008). A lot of attention has been placed on the potential benefits of antioxidants due to the role reactive oxygen species play in chronic and acute inflammation. Marine organisms have provided may antiinflammatory and antioxidant natural products, one of the best known coming from the filamentous unicellular alga *Spirulina* (Abad *et al.,* 2008). Two norditerpenes (norcembranolide and sinuleptolide) were isolated from an Okinawan *Sinularia* species and the compounds inhibited TNF-α production in LPS stimulated mouse macrophage like cells, and had an inhibitory effect on nitric oxide production (Takaki *et al.,* 2003). *Sinularia crassa* produced a cembrane diterpene (lobohedleolide) which exhibited antiinflammatory activity *in vitro* (Radhika *et al.,* 2005).

Cortés *et al.,* (2014) reported a weak antibacterial activity when compounds isolated from *Ceramium rubrum* were tested alone however as more compounds were added together from the same extract the antibacterial activity increased. Although this may not be a phenomenon that has been extensively documented in marine organisms, synergism between compounds have been reported for plants (Sökmen *et al.,* 2004). Major compounds in natural extracts may not be solely responsible for the observed bioactivity.

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Various minor compounds may contribute to the bioactivity when combined with major compounds (Lis-Balchin *et al.,* 1998; Patharakorn *et al.,* 2010; Cortés *et al.,* 2014).

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6. Conclusion and Future Perspectives

The genus of *Sinularia* has been extensively studied but the soft coral *Sinularia notanda* has not. *S. notanda* has the potential to produce compounds that are chemotherapeutic. The present study revealed a cembrane diterpenoid that has previously been isolated from *Sinularia flexiblis* but not from *S. notanda*. The compound did not show strong inhibition of HIV-1 enzymes but was found to be cytotoxic. This work corroborates previous studies of soft coral from the *Sinularia* genus where Sinulariolide (CPD1) has been isolated. This compound was isolated extensively from different species within this genus and it was surmised that this particular cembrane diterpene may serve as a chemotaxanomic marker of *Sinularia* soft coral species.

In this study it was hypothesised that "marine soft corals produce bioactive compounds with medicinal properties which may be inhibitory to HIV-1 enzymes and limiting to HIV-1 associated opportunistic co-morbidities." While investigating this hypothesis the following aims were completed:

Aim 1 and 2 – Preparation of crude extracts of *S. notanda* and testing of the crude extracts for HIV-1 protease (and reverse transcriptase) inhibition, cytotoxicity and antioxidant activity: The data obtained showed that the total crude extract prepared from *S. notanda* moderately inhibited HIV-1 protease and could not inhibit HIV-1 integrase. The crude ethyl acetate fraction of *S. notanda* showed significantly greater protease inhibition, antioxidant activity and cytoxicity than the crude methanol fraction.

Aim 3 and 4 – Bioassay guided fractionation of active crude extracts using chromatographic techniques to isolate bioactive compounds and structural elucidation of compounds: Bioassay guided fractionation focused solely on the ethyl acetate fraction of *S. notanda* and cytotoxicity was used to evaluate activity of sub-fractions and identify those containing active compounds. CPD1 was isolated in both crystalline and non-crystalline form, confirmed by NMR, and exhibited potent inhibition of U937 cells. A unique compound, 3-caffeoylquinic acid, was also isolated and the presence of this metabolite was attributed to the breakdown of algae ingested by the coral.

During this study the question arose as to whether aquarium culture of organisms such as soft coral would still produce the same compounds as coral grown in the wild, or if changes in microorganisms living on the coral as well as compounds produced as a chemical defense system may be altered when the coral is grown in a non-hostile environment. The isolation of Sinulariolide is evidence that the growth of coral in a controlled environment did

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not drastically changed the metabolites produced. This also serves as confirmation that the isolation protocol was correct as the previous studies regarding *S. notanda* did not use methanol and ethyl acetate in the preparation of the crude extracts.

Future work includes the synthesis of CPD1 for testing against different cell lines as well as to establish whether cell death is apoptotic or necrotic. Flow cytometry flourochromes are able to distinguish between these two types of cell death mechanisms. Modification of the epoxide or lactone ring of CPD1 will provide information on the functional group within the structure that is responsible for cytoxicity. These modifications will also be able to assess if the cytoxicity and selectivity of the compound towards HeLa cells will improve.

The methanol fraction of *S. notanda* as well as sub-fractions of the ethyl acetate fraction exhibited cytotoxicity towards HeLa cells. Further isolation from these fractions should produce compounds with the ability to inhibit the growth of Hela cells. Isolated compounds can also be tested further for *Mycobacterium tuberculosis* inhibition supporting the notion that marine organisms contain compounds that can be used to treat opportunistic infections in HIV positive patients.

Finally, the study of soft coral has produced compounds with medicinal properties and in this study *S. notanda* has proven its potential to be added to the list of marine organisms used to identify leads for potential drug candidates, supporting the original hypothesis of this dissertation.

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8. Appendix

This section contains additional data that supports the primary findings of the dissertation. The crude extracts of two other soft coral species, *Pachyclavularia violacea* and *Discosoma sanctithomae*, were also evaluated for bioactivity but results were not promising therefore the investigation into these two species was not carried out further during this study.

8.1 *Biological activity of* P. violacea *and* D. sanctithomae

The total crude extracts of all three soft coral species were screened for HIV-1 protease inhibition as two concentrations (Figure 35). None of the extracts inhibited HIV-1 protease activity at 50% or more. The total extract of *D. sanctithomae* showed moderate protease inhibition at 100 µg/ml however the low extract yield partition chromatography was not possible for the extract.

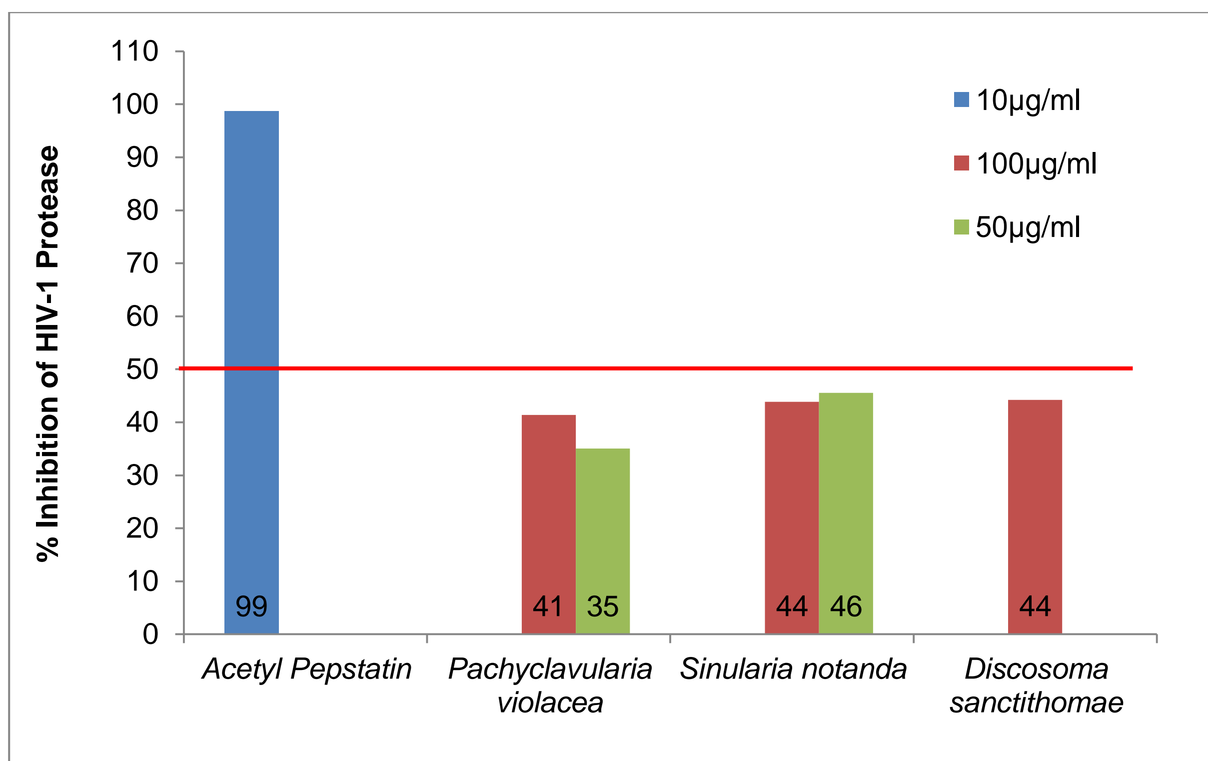

Figure 35: Screening of *P. violacea*, *S. notanda* and *D. sanctithomae* for HIV-1 Protease inhibition (n = 1)

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Methanol and ethyl acetate extracts of *P. violacea* was screened for HIV-1 protease inhibition at 100 µg/ml and 50 µg/ml (Figure 36). Inhibition of HIV-1 protease by *P. violacea* ethyl acetate fraction (39.64% inhibition) was not significantly greater than inhibition by the methanol fraction (27.74% inhibition) at 100 µg/ml. This moderate inhibition and low extract yield resulted in this soft coral species not being used for compound isolation. Acetyl pepstatin, a known HIV-1 protease inhibitor, was used as the positive control and showed 95.42% protease inhibition at 10 µg/ml.

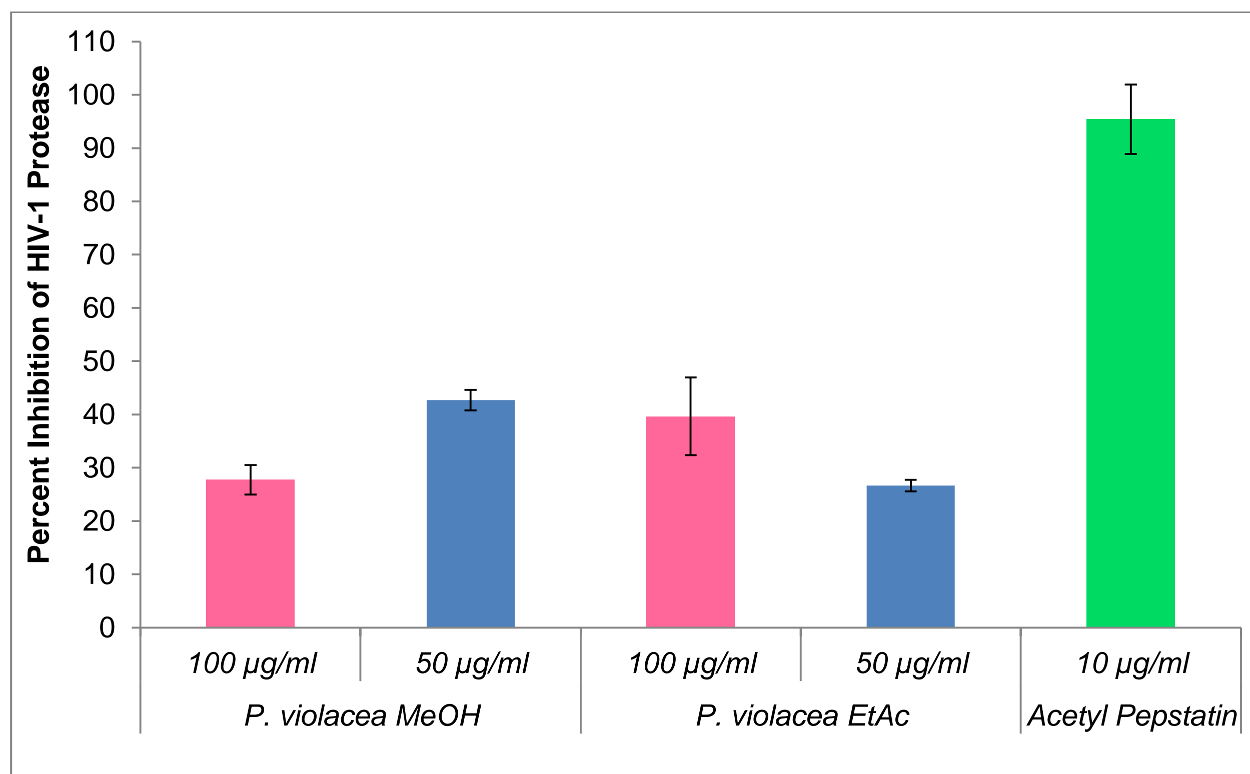

Figure 36: Screening of crude extracts of P. violacea for HIV-1 Protease inhibition
The difference in protease inhibition of the methanol and ethyl acetate fractions were not statistically significant (ANOVA, n = 3 ± SEM)

The total crude extract of *P. violacea* (Figure 37) was tested for free radical scavenging activity at eight different concentrations ranging from 500 µg/ml to 3.91 µg/ml. The calculated IC_{50} for the total crude extract of *P. violacea* was 35.9 ± 8.9 µg/ml. These data showed that *P. violacea* could contain compounds with antioxidant potential however isolation of these compounds was not possible with the small amount of extract available. The direct inhibition of HIV reverse transcriptase was evaluated for the soft coral species (Figure 34). All extracts showed very low inhibition of reverse transcriptase with the highest enzyme inhibition at 20% produced by the ethyl acetate fraction of *S. notanda* as well as by the total crude extract of *P. violacea.*

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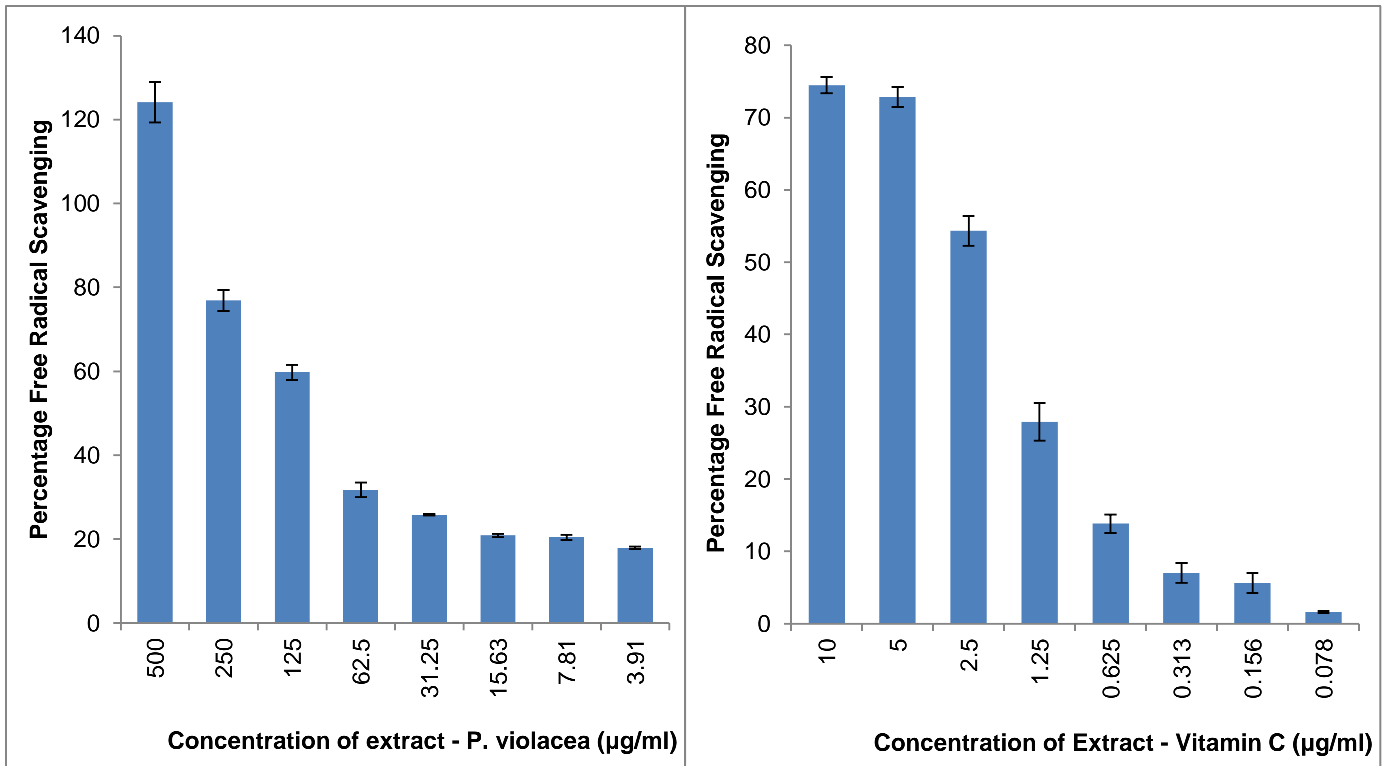

Figure 37: Screening of the *P. violacea* crude extract for DPPH free radical scavenging ability
Vitamin C was used as a positive control (n = 2 ± SEM)

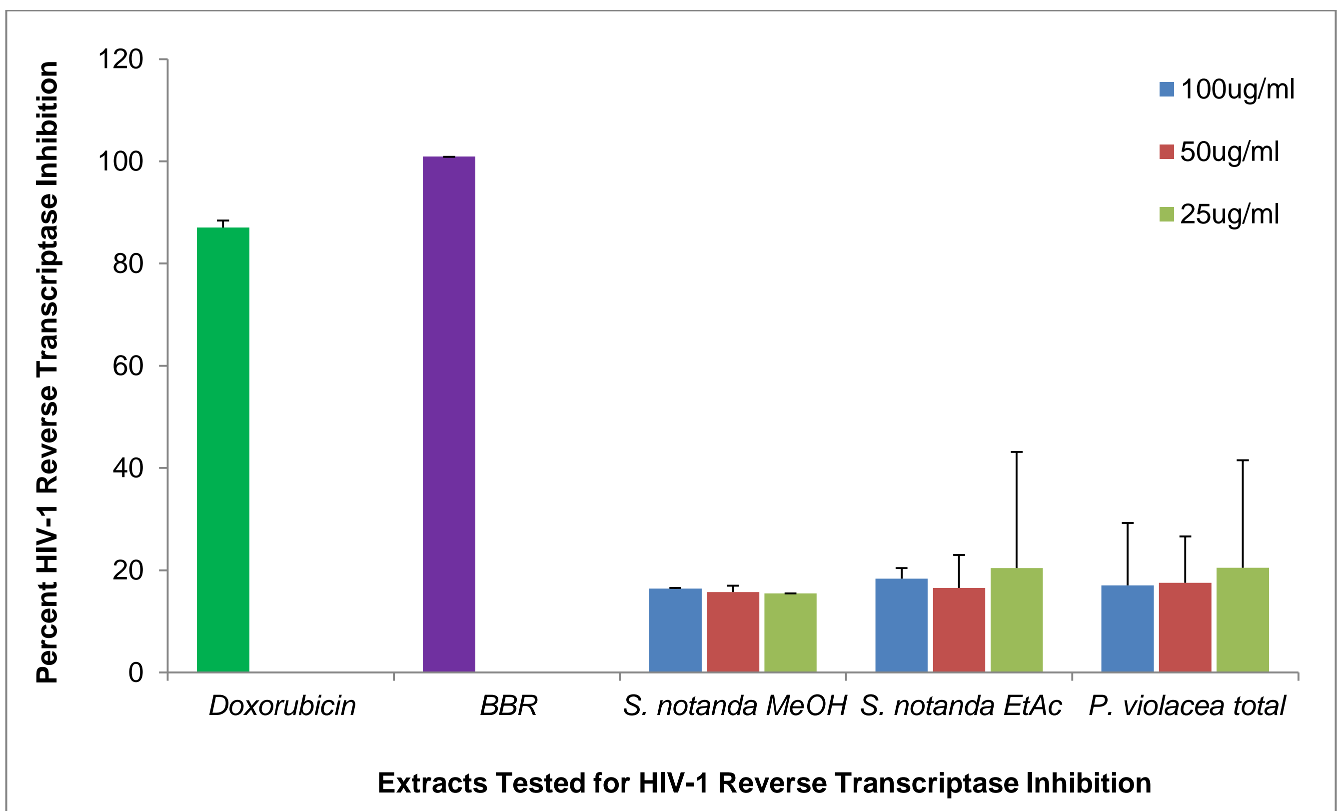

Figure 38: Screening of *S. notanda* and *P. violacea* crude extracts for HIV-1 Reverse Transcriptase inhibition

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Methanol and ethyl acetate fractions of the total crude extract of *P. violacea* (Figure 39) were tested for cytoxicity against HeLa cells at eight different concentrations. Initial screening was done at concentrations ranging from 400 µg/ml to 3.1 µg/ml, however no dose response was seen and the concentration range was decreased to 200 µg/ml to 12.5 µg/ml. This coral exhibited cytotoxicity towards HeLa cells at 100 µg/ml and higher.

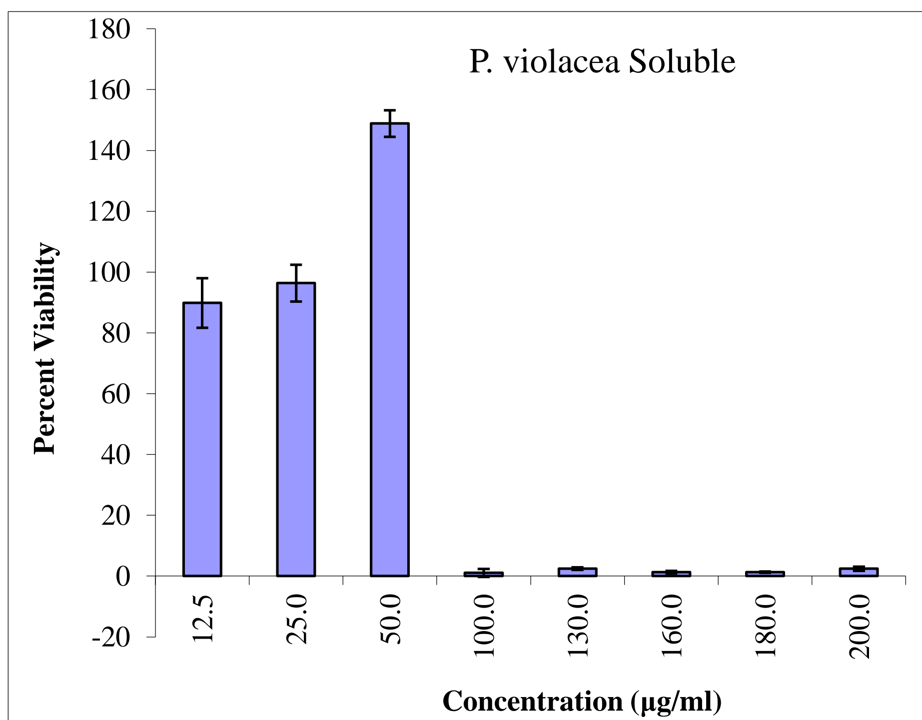

Figure 39: Dose response of *P. violacea* cytotoxicity against HeLa cells

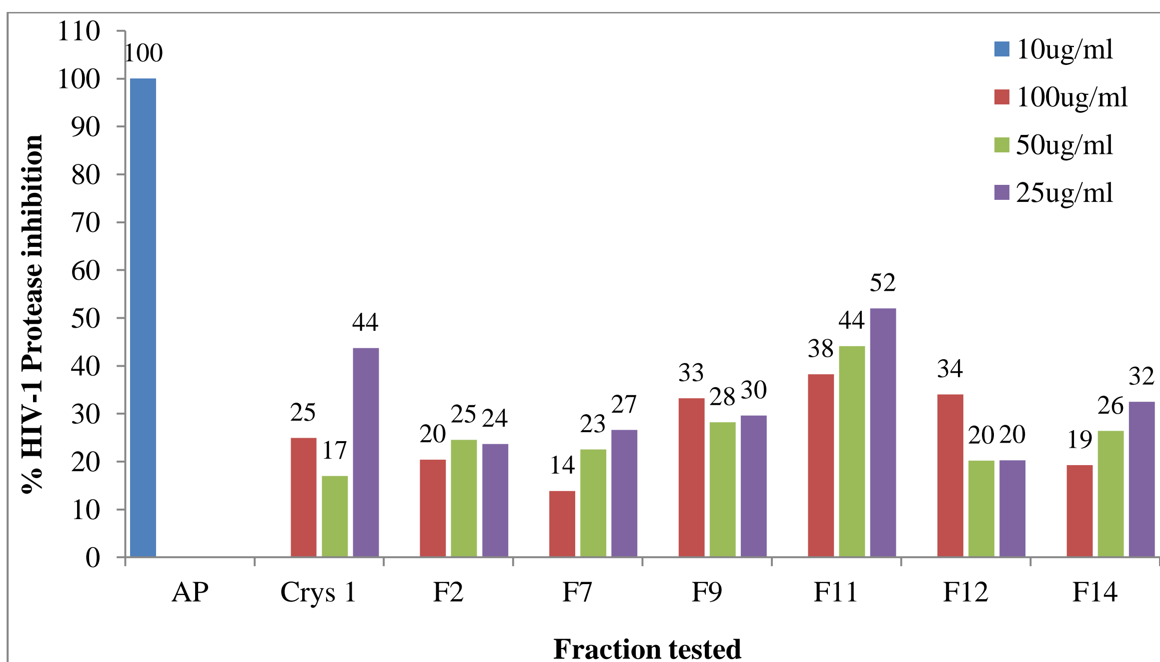

Figure 40: Selected fractions of *S. notanda* tested for HIV-1 Protease inhibition

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8.2 Atomic coordinates and bond lengths obtained for CPD1 using X-ray crystallography

Table 8: Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for CPD1.
U(eq) is defined as one third of the trace orthogonalized U^{ij} tensor

	x	y	z	U(eq)
O(1)	4712(2)	4654(2)	2412(1)	26(1)
O(2)	1532(2)	3576(2)	1396(1)	34(1)
O(3)	7683(2)	2693(2)	-275(1)	32(1)
O(4)	6415(2)	4520(2)	3225(1)	36(1)
C(1)	8321(3)	3140(3)	2418(2)	33(1)
C(2)	6982(3)	3479(3)	2202(1)	23(1)
C(3)	6030(3)	4214(3)	2670(1)	25(1)
C(4)	3437(3)	3836(3)	2248(1)	25(1)
C(5)	2813(3)	4298(3)	1572(1)	27(1)
C(6)	2372(4)	5617(3)	1567(2)	36(1)
C(7)	2255(4)	6099(3)	842(2)	42(1)
C(8)	3730(4)	6156(3)	466(2)	36(1)
C(9)	4908(5)	6986(3)	764(2)	53(1)
C(10)	3950(4)	5517(3)	-92(2)	33(1)
C(11)	5259(4)	5535(3)	-571(2)	38(1)
C(12)	6045(3)	4324(3)	-695(1)	32(1)
C(13)	7150(3)	3930(3)	-162(1)	29(1)
C(14)	6742(3)	2956(3)	308(1)	27(1)
C(15)	7429(3)	2773(3)	992(1)	26(1)
C(16)	6311(3)	3196(3)	1526(1)	22(1)
C(17)	2332(4)	3986(3)	2828(2)	38(1)
C(18)	3971(3)	2543(3)	2197(1)	25(1)
C(19)	5038(3)	2268(2)	1613(1)	22(1)
C(20)	8402(4)	4802(3)	1(2)	41(1)

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Table 9: Bond lengths and angles for compound CPD1

O(1)-C(3)	1.368(3)
O(1)-C(4)	1.487(3)
O(2)-C(5)	1.434(4)
O(2)-H(3)	0.79(4)
O(3)-C(14)	1.457(3)
O(3)-C(13)	1.468(4)
O(4)-C(3)	1.203(3)
C(1)-C(2)	1.319(4)
C(1)-H(1)	0.9500
C(1)-H(5)	0.9500
C(2)-C(3)	1.496(4)
C(2)-C(16)	1.501(4)
C(4)-C(18)	1.515(4)
C(4)-C(17)	1.521(4)
C(4)-C(5)	1.539(4)
C(5)-C(6)	1.514(4)
C(5)-H(30)	1.000
C(6)-C(7)	1.537(4)
C(6)-H(29)	0.9900
C(6)-H(28)	0.9900
C(7)-C(8)	1.508(5)
C(7)-H(26)	0.9900
C(7)-H(27)	0.9900
C(8)-C(10)	1.330(4)
C(8)-C(9)	1.513(5)
C(9)-H(25)	0.9800
C(9)-H(24)	0.9800
C(9)-H(2)	0.9800
C(10)-C(11)	1.501(4)
C(10)-H(23)	0.9500
C(11)-C(12)	1.534(5)
C(11)-H(21)	0.9900
C(11)-H(22)	0.9900
C(12)-C(13)	1.508(4)
C(12)-H(20)	0.9900
C(12)-H(19)	0.9900
C(13)-C(14)	1.471(4)
C(13)-C(20)	1.509(4)
C(14)-C(15)	1.502(4)
C(14)-H(4)	1.0000
C(15)-C(16)	1.526(4)
C(15)-H(14)	0.9900
C(15)-H(15)	0.9900
C(16)-C(19)	1.539(4)
C(16)-H(13)	1.0000
C(17)-H(6)	0.9800
C(17)-H(8)	0.9800
C(17)-H(7)	0.9800
C(18)-C(19)	1.527(4)
C(18)-H(12)	0.9900
C(18)-H(9)	0.9900
C(19)-H(11)	0.9900
C(19)-H(10)	0.9900
C(20)-H(17)	0.9800
C(20)-H(16)	0.9800
C(20)-H(18)	0.9800
C(3)-O(1)-C(4)	121.0(2)
C(5)-O(2)-H(3)	109(3)
C(14)-O(3)-C(13)	60.39(18)
C(2)-C(1)-H(1)	120.0
C(2)-C(1)-H(5)	120.0
H(1)-C(1)-H(5)	120.0
C(1)-C(2)-C(3)	117.6(3)
C(1)-C(2)-C(16)	126.1(3)
C(3)-C(2)-C(16)	116.3(2)
O(4)-C(3)-O(1)	119.1(3)
O(4)-C(3)-C(2)	124.1(3)

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O(1)-C(3)-C(2)	116.5(2)
O(1)-C(4)-C(18)	110.7(2)
O(1)-C(4)-C(17)	105.1(2)
C(18)-C(4)-C(17)	110.9(2)
O(1)-C(4)-C(5)	105.2(2)
C(18)-C(4)-C(5)	111.7(2)
C(17)-C(4)-C(5)	113.0(2)
O(2)-C(5)-C(6)	109.4(2)
O(2)-C(5)-C(4)	108.2(2)
C(6)-C(5)-C(4)	114.9(2)
O(2)-C(5)-H(30)	108.1
C(6)-C(5)-H(30)	108.1
C(4)-C(5)-H(30)	108.1
C(5)-C(6)-C(7)	111.1(2)
C(5)-C(6)-H(29)	109.4
C(7)-C(6)-H(29)	109.4
C(5)-C(6)-H(28)	109.4
C(7)-C(6)-H(28)	109.4
H(29)-C(6)-H(28)	108
C(8)-C(7)-C(6)	114.7(3)
C(8)-C(7)-H(26)	108.6
C(6)-C(7)-H(26)	108.6
C(8)-C(7)-H(27)	108.6
C(6)-C(7)-H(27)	108.6
H(26)-C(7)-H(27)	107.6
C(10)-C(8)-C(7)	121.1(3)
C(10)-C(8)-C(9)	123.2(3)
C(7)-C(8)-C(9)	115.7(3)
C(8)-C(9)-H(25)	109.5
C(8)-C(9)-H(24)	109.5
H(25)-C(9)-H(24)	109.5
C(8)-C(9)-H(2)	109.5
H(25)-C(9)-H(2)	109.5
H(24)-C(9)-H(2)	109.5
C(2)-C(16)-C(19)	109.4(2)

C(8)-C(10)-C(11)	129.3(3)
C(8)-C(10)-H(23)	115.3
C(11)-C(10)-H(23)	115.3
C(10)-C(11)-C(12)	116.3(3)
C(10)-C(11)-H(21)	108.2
C(12)-C(11)-H(21)	108.2
C(10)-C(11)-H(22)	108.2
C(12)-C(11)-H(22)	108.2
H(21)-C(11)-H(22)	107.4
C(13)-C(12)-C(11)	115.9(2)
C(13)-C(12)-H(20)	108.3
C(11)-C(12)-H(20)	108.3
C(13)-C(12)-H(19)	108.3
C(11)-C(12)-H(19)	108.3
H(20)-C(12)-H(19)	107.4
O(3)-C(13)-C(14)	59.41(18)
O(3)-C(13)-C(12)	111.9(2)
C(14)-C(13)-C(12)	119.8(2)
O(3)-C(13)-C(20)	113.2(3)
C(14)-C(13)-C(20)	121.1(3)
C(12)-C(13)-C(20)	116.4(3)
O(3)-C(14)-C(13)	60.20(18)
O(3)-C(14)-C(15)	117.2(2)
C(13)-C(14)-C(15)	124.8(2)
O(3)-C(14)-H(4)	114.5
C(13)-C(14)-H(4)	114.5
C(15)-C(14)-H(4)	114.5
C(14)-C(15)-C(16)	108.8(2)
C(14)-C(15)-H(14)	109.9
C(16)-C(15)-H(14)	109.9
C(14)-C(15)-H(15)	109.9
C(16)-C(15)-H(15)	109.9
H(14)-C(15)-H(15)	108.3
C(2)-C(16)-C(15)	115.2(2)

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C(15)-C(16)-C(19)	110.5(2)
C(2)-C(16)-H(13)	107.1
C(15)-C(16)-H(13)	107.1
C(19)-C(16)-H(13)	107.1
C(4)-C(17)-H(6)	109.5
C(4)-C(17)-H(8)	109.5
H(6)-C(17)-H(8)	109.5
C(4)-C(17)-H(7)	109.5
H(6)-C(17)-H(7)	109.5
H(8)-C(17)-H(7)	109.5
C(4)-C(18)-C(19)	115.7(2)
C(4)-C(18)-H(12)	108.4
C(19)-C(18)-H(12)	108.4
C(4)-C(18)-H(9)	108.4
C(19)-C(18)-H(9)	108.4
H(12)-C(18)-H(9)	107.4
C(18)-C(19)-C(16)	114.0(2)
C(18)-C(19)-H(11)	108.7
C(16)-C(19)-H(11)	108.7
C(18)-C(19)-H(10)	108.7
C(16)-C(19)-H(10)	108.7
H(11)-C(19)-H(10)	107.6
C(13)-C(20)-H(17)	109.5
C(13)-C(20)-H(16)	109.5
H(17)-C(20)-H(16)	109.5
C(13)-C(20)-H(18)	109.5
H(17)-C(20)-H(18)	109.5
H(16)-C(20)-H(18)	109.5

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C(4)-O(1)-C(3)-O(4)	-116.8(3)
C(4)-O(1)-C(3)-C(2)	69.6(3)
C(1)-C(2)-C(3)-O(4)	0.6(4)
C(16)-C(2)-C(3)-O(4)	-179.4(3)
C(1)-C(2)-C(3)-O(1)	173.7(3)
C(16)-C(2)-C(3)-O(1)	-6.2(4)
C(3)-O(1)-C(4)-C(18)	-17.6(3)
C(3)-O(1)-C(4)-C(17)	102.1(3)
C(3)-O(1)-C(4)-C(5)	-138.4(2)
O(1)-C(4)-C(5)-O(2)	-177.6(2)
C(18)-C(4)-C(5)-O(2)	62.2(3)
C(17)-C(4)-C(5)-O(2)	-63.6(3)
O(1)-C(4)-C(5)-C(6)	-55.1(3)
C(18)-C(4)-C(5)-C(6)	-175.3(2)
C(17)-C(4)-C(5)-C(6)	58.9(3)
O(2)-C(5)-C(6)-C(7)	-76.5(3)
C(4)-C(5)-C(6)-C(7)	161.7(3)
C(5)-C(6)-C(7)-C(8)	-67.0(4)
C(6)-C(7)-C(8)-C(10)	118.3(3)
C(6)-C(7)-C(8)-C(9)	-62.3(4)
C(7)-C(8)-C(10)-C(11)	173.5(3)
C(9)-C(8)-C(10)-C(11)	-5.8(5)
C(8)-C(10)-C(11)-C(12)	123.9(3)
C(10)-C(11)-C(12)-C(13)	-80.2(3)

C(14)-O(3)-C(13)-C(12)	-112.6(3)
C(14)-O(3)-C(13)-C(20)	113.6(3)
C(11)-C(12)-C(13)-O(3)	172.0(2)
C(11)-C(12)-C(13)-C(14)	105.7(3)
C(11)-C(12)-C(13)-C(20)	-55.7(4)
C(13)-O(3)-C(14)-C(15)	-116.5(3)
C(12)-C(13)-C(14)-O(3)	99.3(3)
C(20)-C(13)-C(14)-O(3)	-100.2(3)
O(3)-C(13)-C(14)-C(15)	104.1(3)
C(12)-C(13)-C(14)-C(15)	-156.6(3)
C(20)-C(13)-C(14)-C(15)	3.9(4)
O(3)-C(14)-C(15)-C(16)	175.3(2)
C(13)-C(14)-C(15)-C(16)	104.2(3)
C(1)-C(2)-C(16)-C(15)	-16.7(4)
C(3)-C(2)-C(16)-C(15)	163.2(2)
C(1)-C(2)-C(16)-C(19)	108.4(3)
C(3)-C(2)-C(16)-C(19)	-71.6(3)
C(14)-C(15)-C(16)-C(2)	-159.8(2)
C(14)-C(15)-C(16)-C(19)	75.6(3)
O(1)-C(4)-C(18)-C(19)	-66.3(3)
C(17)-C(4)-C(18)-C(19)	177.5(2)
C(5)-C(4)-C(18)-C(19)	50.6(3)
C(4)-C(18)-C(19)-C(16)	45.9(3)
C(2)-C(16)-C(19)-C(18)	45.1(3)
C(15)-C(16)-C(19)-C(18)	172.9(2)

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Table 10: Anistropic displacement parameters ($Å^2 \times 10^3$) for CPD1

The anistropic displacement factor exponent takes the form: $-2\pi^2[h^2 a^{*2}U^{11} + ... + 2 h k a^* b^* U^{12}]$

	U11	U22	U33	U23	U13	U12
O(1)	23(1)	30(1)	25(1)	-7(1)	-4(1)	0(1)
O(2)	22(1)	49(1)	30(1)	-14(1)	-4(1)	3(1)
O(3)	30(1)	44(1)	22(1)	-8(1)	7(1)	-2(1)
O(4)	41(1)	42(1)	26(1)	-11(1)	-8(1)	4(1)
C(1)	24(2)	50(2)	26(2)	-1(2)	-4(1)	2(1)
C(2)	22(2)	26(2)	21(1)	2(1)	1(1)	-4(1)
C(3)	27(2)	25(2)	23(2)	0(1)	-1(1)	-5(1)
C(4)	20(1)	33(2)	22(2)	-5(1)	0(1)	-4(1)
C(5)	21(1)	36(2)	24(2)	-7(1)	-2(1)	4(1)
C(6)	41(2)	37(2)	30(2)	-8(2)	-5(2)	11(1)
C(7)	50(2)	39(2)	36(2)	-3(2)	-11(2)	18(2)
C(8)	50(2)	27(2)	30(2)	5(1)	-12(2)	6(1)
C(9)	71(3)	42(2)	46(2)	-7(2)	-9(2)	-7(2)
C(10)	41(2)	31(2)	28(2)	5(2)	-9(1)	0(1)
C(11)	50(2)	38(2)	27(2)	10(2)	-7(2)	-8(2)
C(12)	35(2)	43(2)	17(2)	-1(1)	3(1)	-12(1)
C(13)	30(2)	36(2)	21(2)	-2(1)	5(1)	-9(1)
C(14)	22(2)	36(2)	22(1)	-8(1)	6(1)	-3(1)
C(15)	23(2)	32(2)	24(2)	-1(1)	2(1)	-1(1)
C(16)	20(1)	28(2)	18(1)	-1(1)	1(1)	-1(1)
C(17)	26(2)	58(2)	29(2)	-9(2)	2(1)	-1(1)
C(18)	21(1)	34(2)	21(1)	0(1)	-2(1)	-4(1)
C(19)	21(1)	24(2)	21(1)	2(1)	-3(1)	0(1)
C(20)	38(2)	51(2)	34(2)	3(2)	-2(2)	-19(2)

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Table 11: Hydrogen coordinate (x 10^4) and ionotropic displacement parameters (Å^2 x 10^3) for CPD1

	X	Y	Z	U(eq)
H(1)	8650	3364	2857	40
H(5)	8957	2672	2136	40
H(30)	3603	4173	1220	32
H(29)	3131	6090	1818	43
H(28)	1390	5716	1796	43
H(26)	1553	5578	586	50
H(27)	1815	6918	856	50
H(25)	5366	6601	1159	79
H(24)	4433	7746	901	79
H(2)	5686	7150	427	79
H(23)	3165	4971	-205	40
H(21)	6015	6111	-396	46
H(22)	4901	5849	-1010	46
H(20)	5262	3693	-740	38
H(19)	6583	4371	-1131	38
H(4)	5678	2670	267	32
H(14)	8375	3239	1029	32
H(15)	7666	1909	1060	32
H(13)	5841	3956	1355	26
H(6)	2795	3696	3246	56
H(8)	1419	3519	2735	56
H(7)	2071	4841	2877	56
H(12)	3078	2014	2154	30
H(9)	4485	2328	2623	30
H(11)	4447	2234	1190	27
H(10)	5490	1463	1685	27
H(17)	9170	4392	272	61
H(16)	7994	5485	255	61
H(18)	8855	5094	-419	61
H(3)	1740(50)	3180(40)	1080(20)	61

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8.3 NMR Spectra for compound 7E

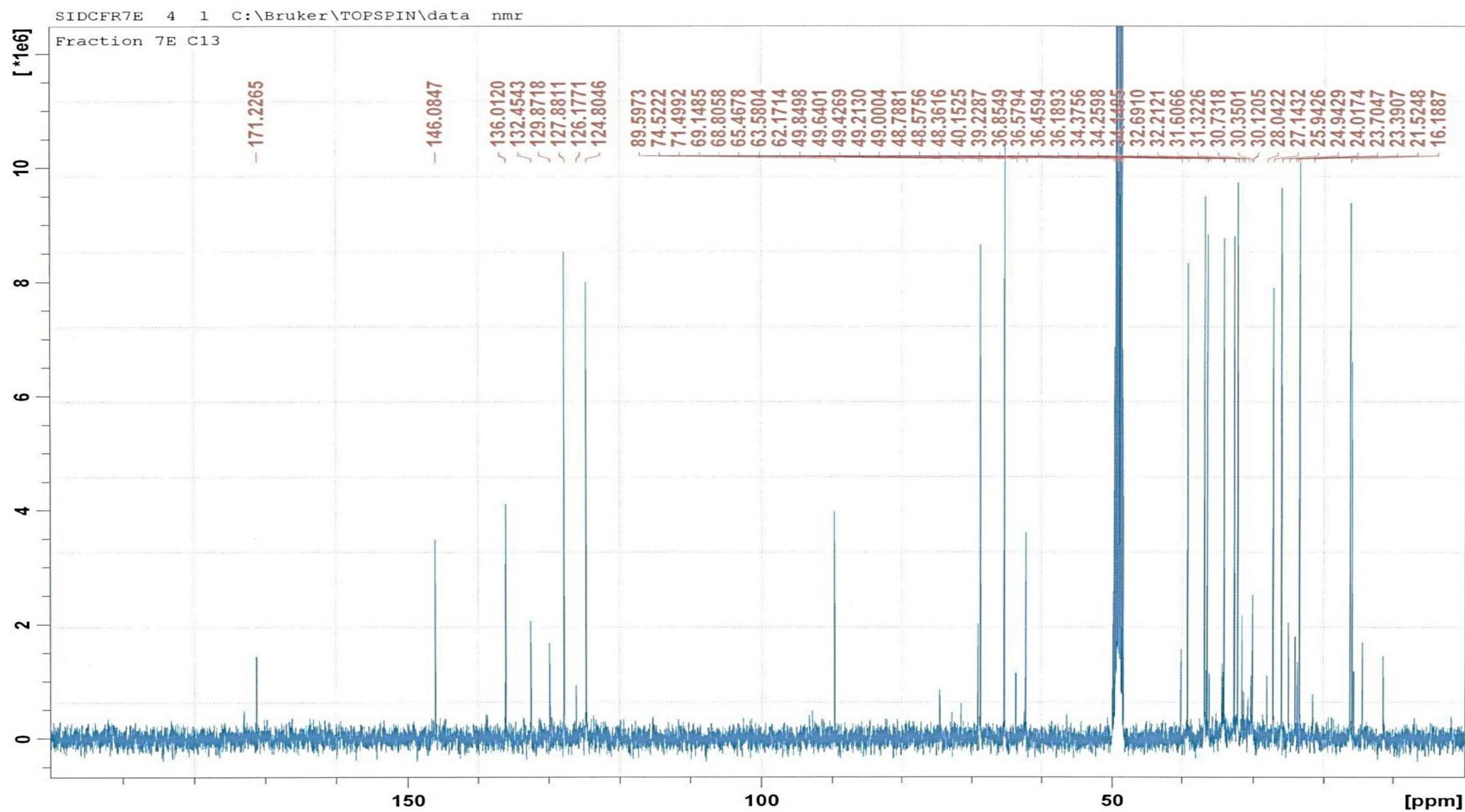

Figure 41: Compound 7E ^{13}C NMR Spectrum

Spectrum showing the chemical shift of carbon atoms: Methyl groups can be seen at 10 – 15 ppm, methylene groups at 16 – 25 ppm, primary alcohols at 50 – 65 ppm and alkenes at 115 – 140 ppm

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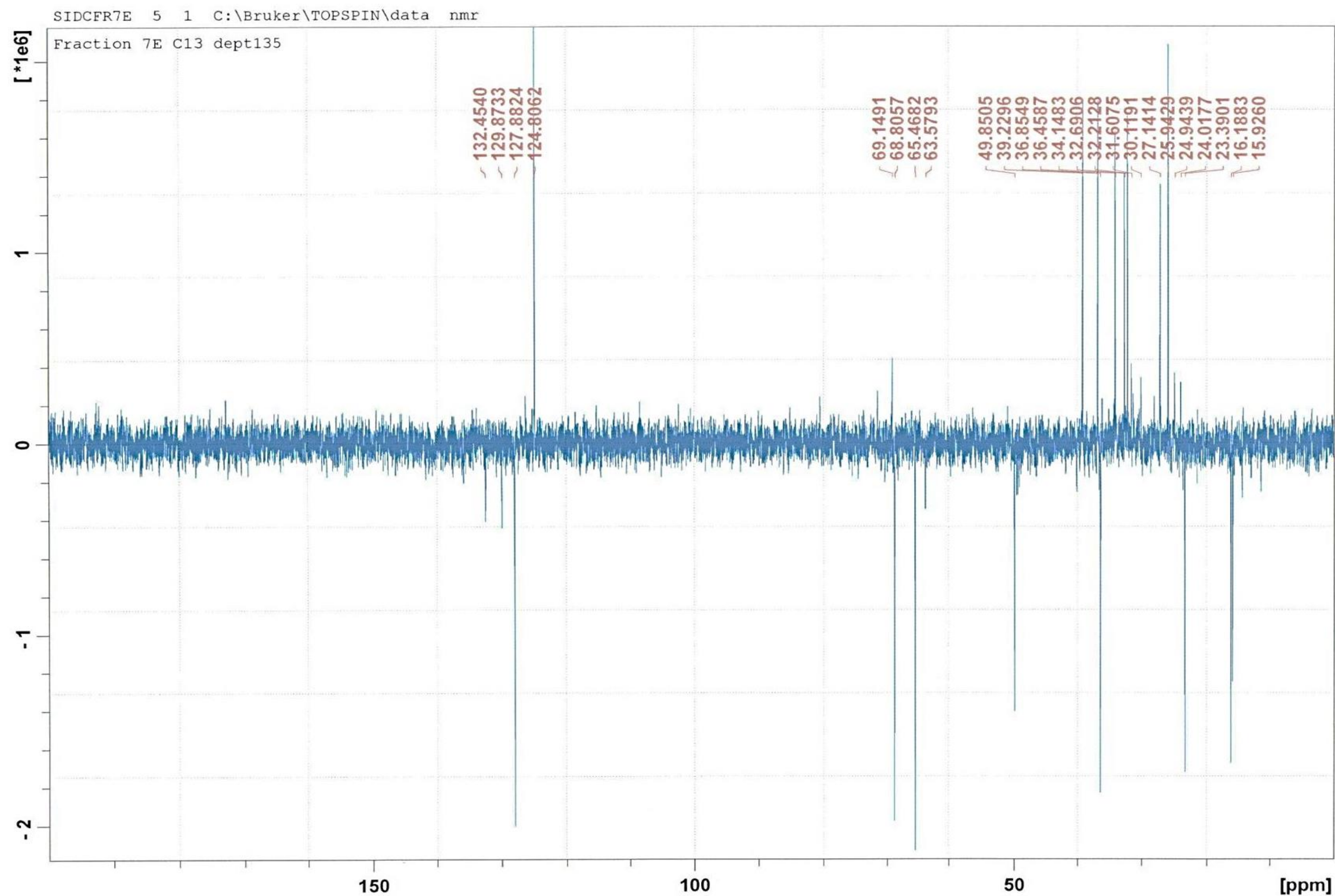

Figure 42: Compound 7E ^{1}H NMR Spectrum

Spectrum showing the chemical shift of protons. Methyl groups can be found at 0.9 ppm, methylene at 1.33 ppm, alkenes at 4.5 – 6.1 ppm and alkynes at 2.0 – 3.2 ppm.

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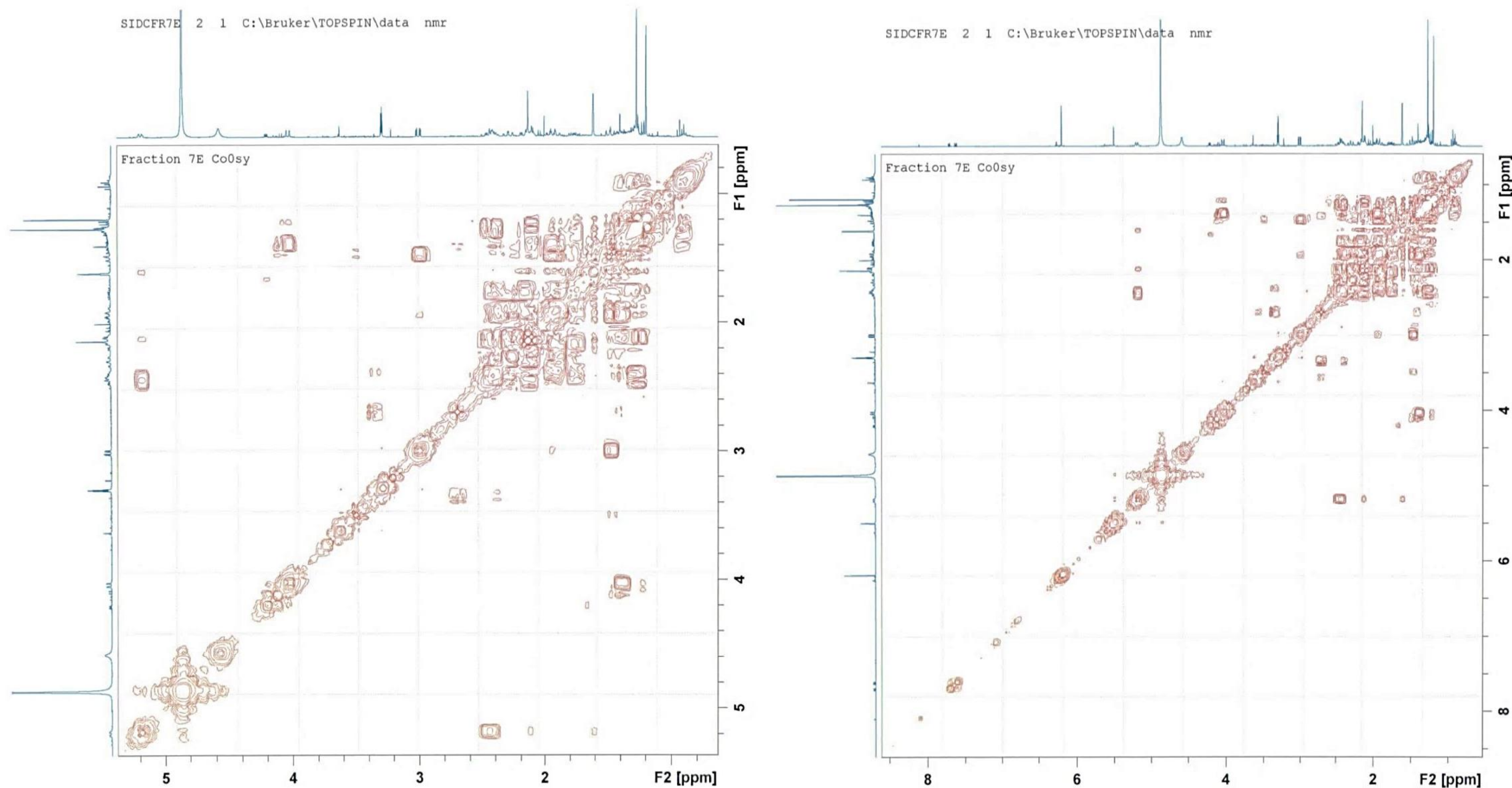

Figure 43: Compound 7E Cosy correlation spectrum

Representation of nuclei in 7E that share scalar (J) coupling. The coupling relationship is for protons within the structure that are two or three bonds apart.

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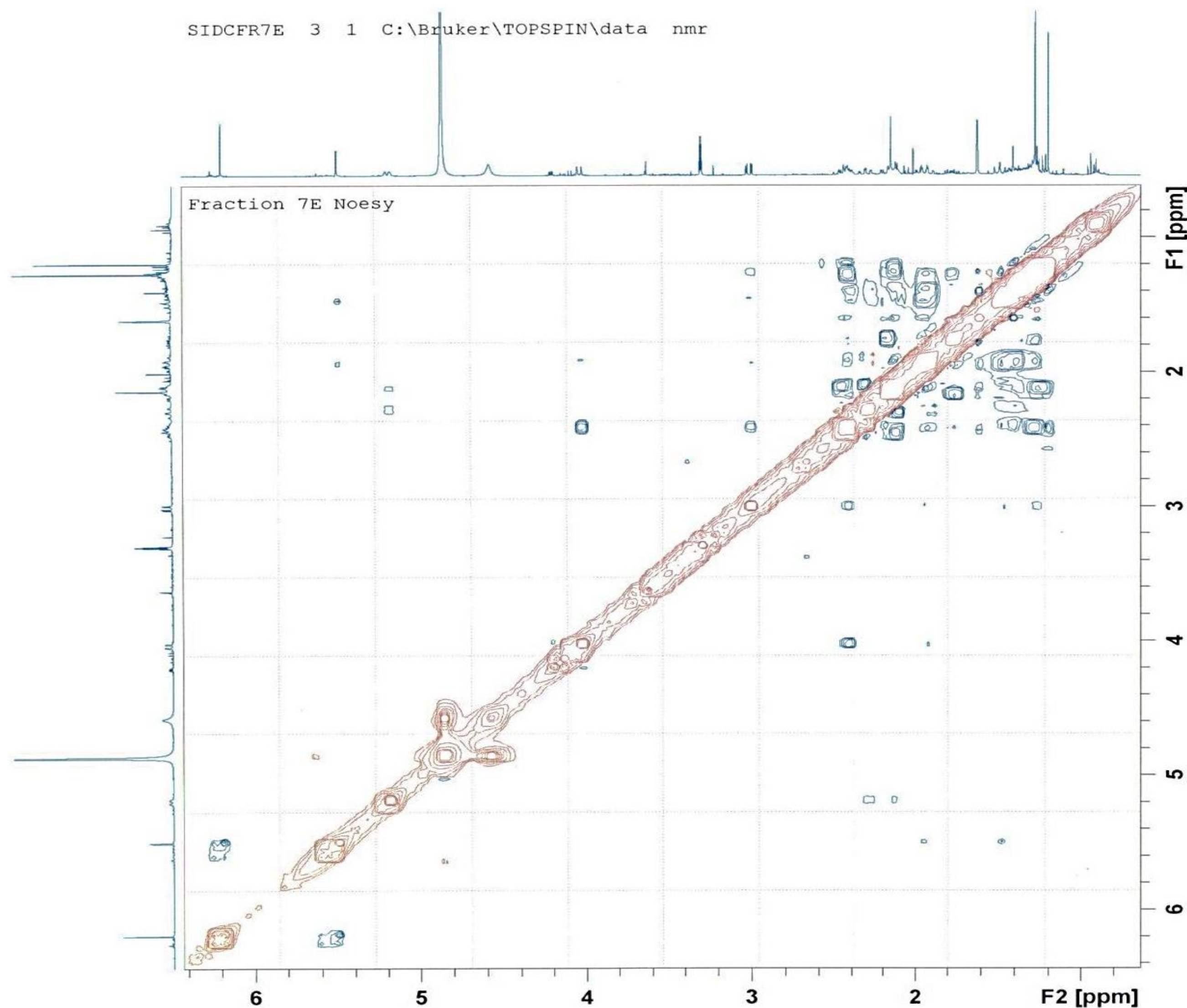

Figure 44: Compound 7E NOESY correlation spectrum

NOESY: Nuclear Overhauser Effect Spectroscopy, 2D NMR method used to mapping NOE correlations between protons of 7E

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9. Manuscript Submitted for Publication

A cembrane diterpenoid from *Sinularia notanda* with potent cytotoxic activity

Sidhika Hariparsad [1], Alfred Muller [2], Henok Kinfe [2], Bavesh Kana [3], Debra Meyer [4]*

[1] Department of Biochemistry, Faculty of Natural and Agricultural Sciences, University of Pretoria, Hatfield Campus, Pretoria, 0002, South Africa

[2] Department of Chemistry, Faculty of Science, University of Johannesburg, Auckland Park Kingsway Campus, Johannesburg, 2006, South Africa

[3] DST/NRF Centre of Excellence for Biomedical TB Research, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, National Health Laboratory Service, Johannesburg, South Africa

[4] Department of Biochemistry, Faculty of Science, University of Johannesburg, Auckland Park Kingsway Campus, Johannesburg, 2006, South Africa

*Author to whom correspondence should be addressed: Department of Biochemistry, Faculty of Science, University of Johannesburg, Auckland Park Kingsway Campus, Johannesburg, 2006, South Africa. Tel: +2711 559 2374/3826/2459 Fax: +2711 559 2307 Email: dmeyer@uj.ac.za

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Abstract: A large number of soft coral from the genus *Sinularia* have already been chemically studied and reported to produce secondary metabolites, the majority of which have been identified as terpenes (1). Cembrane diterpenoids have been isolated from various soft coral species as well as from *Sinularia* and almost half of these isolated compounds have been reported to have activity against various cancerous cell lines (2). A Sinulariolide (**1**) and chlorogenic acid, 3-caffeoylquinic acid (**2**) was isolated from the soft coral *Sinularia notanda*. The structures of these metabolites were determined using various spectroscopic methods and the cytotoxicity of the compounds evaluated against selected cell lines. Compound **1** showed a significantly greater cytoxicity towards the human leukemic cell line (U937) compared to the control cell line (Vero). Compound **2** in conjunction with

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an additional chlorogenic acid exhibited significantly higher cytoxicity ($p < 0.001$) towards U937 cells when compared to a cervical cancer (Hela) cell line.

Keywords: *Sinularia notanda*, cembrane diterpenoid, cytotoxicity

1. Introduction

In exploring new sources of bioactive natural products, the marine environment deserves considerable attention due to the wide variety of microorganisms and metabolites found there. The ocean is a complex environment and is home to a diverse collection of microorganisms that are able to survive under extreme temperature, pressure and salinity (3). Natural Products have proven to be a successful source of drug leads (3). Of the 175 anticancer drugs approved between 1940 and 2006, 42% were either natural products or compounds derived from natural products (4).

Marine natural products have produced a variety of compounds in the field of anti-cancer chemotherapy and a number of compounds are undergoing clinical trials (5,6). Soft corals of the genus *Sinularia* (Alcyoniidae) have been well documented as a source of sesquiterpenes, diterpenes, polyhydroxylated steroids, and polyamine metabolites, with a number of these metabolites having biological activity such as cytotoxicity, anti-inflammatory, and antimicrobial properties (7–9).

Marine-derived compounds have also proven to be a source of pharmacologically active substances used for the production of drugs for treatment against AIDS, inflammatory conditions and microbial diseases (10). Although only a few marine-derived products are currently on the market (e.g., Prialt® and Yondelis®), several new compounds are under clinical development (10,11). Cembrane based diterpenoids that contain lactones (cembranolides) have been isolated from various octocorals, but most notably from those belonging to the genera of *Sinularia*, *Sarcophyton* and *Lobophyton*. These metabolites have been shown to inhibit the growth of various cancer cell lines (12,13). In 1979 a new cembranoid diterpene sarcophytol (A) was isolated from the Okinawa soft coral *Sarcophyton glaucum* and has gained significant attention due to its potent inhibition of tumour promoters. This discovery has driven the isolation of numerous cytotoxic cembranoids of marine origin (14).

The soft coral genus *Sinularia* is one of the dominant inhabitants of shallow coral reef across the Indo-Pacific (15). This soft coral belongs to the phylum *Cnidaria* and the family *Alcyonaria*.

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Compounds isolated from this genus have demonstrated antimicrobial, anti-inflammatory and cytotoxic activities (16). The study of *Sinularia* has provided a wide variety of secondary metabolites such as diterpenes, polyhydroxylated sterols and polyamine compounds. Among the many compounds isolated from the Taiwanese soft coral *Sinularia nanolobata*, Furanones and three diterpene Nanolobatins were found to be cytotoxic against cancer cell lines (8,17). *Sinularia* has proven to be a rich sources of bioactive terpenoid analogues (10,18). Cembranoids and their cyclized derivatives are reportedly produced as a defense against predators and settlement of microorganisms (13). A cembrane based diterpenoid containing an α-methylene-ε-lactone ring and a norcembranoidal diterpene with a γ-lactone ring have shown promising cytotoxicity against various cancerous cell lines (19,20). Removal of either the lactone ring or epoxide from biologically active cembrane diterpenoids has been shown to alter the cytotoxicity of the compound. Of the many species of *Sinularia* that have been examined over the years, *Sinularia notanda* remains one of the few species within this genus yet to be studied. In this study, we address this by attempting to isolate bioactive compounds from *S. notanda* and found two metabolites with notable cytotoxic activity.

2. Results and Discussion

The cembrane diterpenoid isolated from *Sinularia notanda* in this study showed moderate to high cytoxicity (dependent on the type of cell line). NMR, X-ray crystallography and mass spectrometry confirmed the structure and purity of the compound.

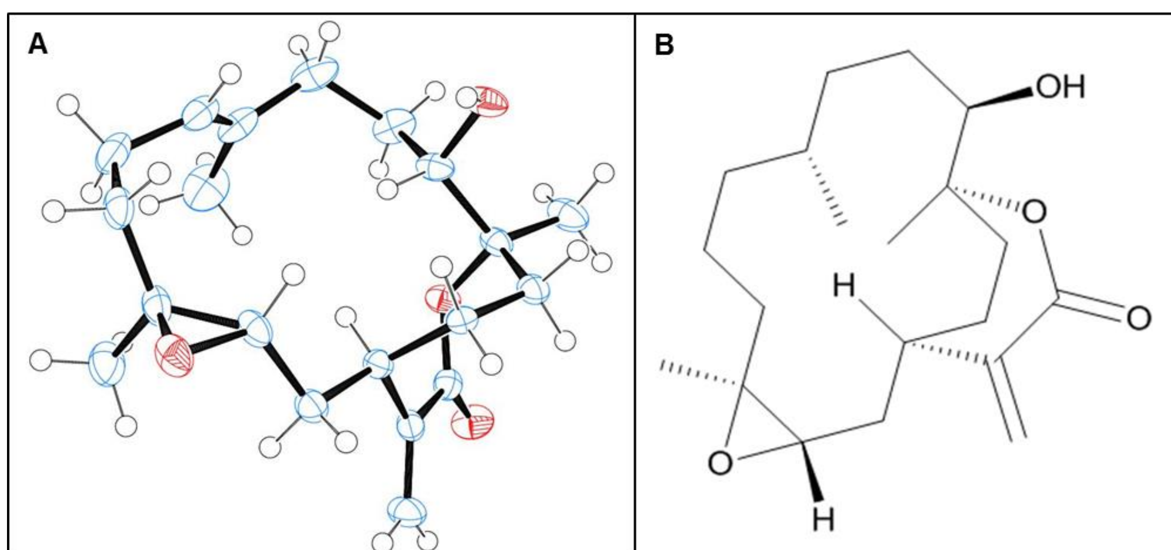

Figure 1: Ellipsoid plot (A) constructed from X-Ray crystallography data and structure obtained from 1D and 2D NMR spectra (B) of Sinulariolide (Compound **1**)

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A suitable colourless block-like specimen of Compound 1 was used for X-ray crystallographic analysis. The crystal had a molecular formula of $C_{20}H_{30}O_4$ and relative molecular mass of 334.44. The orthorhombic crystal had the dimensions 0.12 mm x 0.18 mm x 0.29 mm and a calculated density of 1.137 Mg/m3. X-ray crystallography of Compound **1** produced an ellipsoid plot of a compound with no structural matches on the Cambridge Crystallographic Data Center (CCDC) and structural refinement showed the crystal structure in to have a fit of less than 5%. The ellipsoid plot and structure of Compound **1** is given in Figure 1.

Compound **1**: (1*R*,3*R*,5*S*,12*R*,13*S*,*E*)-12-hydroxy-5,9,13-trimethyl-16-methylene-4,14-dioxatricyclo[11.3.2.03,5]octadec-8-en-15-one ^{1}H NMR (400 MHz, CD_3OD) δ: 6.20 (d, *J* = 0.8Hz, 1H, H-17a), 5.50 (t, *J* = 0.8Hz, 1H, H-17b), 5.26 – 5.12 (m, 1H, H-8), 4.04 (d, *J* = 9.6Hz, 1H, H-12), 3.01 (dd, *J* = 3.0Hz and 11.0Hz, 1H, H-4), 2.50 – 2.36 (m, 3H, H-2, H-7a, H-18a), 2.30 (dt, *J*=2.1 and 13.4 Hz, 1H, H-6a), 2.21 – 2.05 (m, 4H, H-7b, H-19a, H-10a, H-6b), 1.98 – 1.84 (m, 4H, H-3b, H-11b, H-18b, H-10b), 1.26 (s, 3H, H-20 (CH_3)), 1.23 (s, 3H, H-22 (CH_3)).

Compound **1** was previously isolated from *S. flexiblis* by Tursch *et al*. (1975) using hexane and methylene chloride, and a ketone isomer of Compound **1** was isolated by Hu *et al*. (2013) using ethyl acetate to prepare the crude extract. The present study made use of methanol for the preparation of the crude extract followed by separation on a silica-gel column chromatography using a mixture of hexane and ethyl acetate as an eluent which resulted in the isolation of the reduced form of the compound isolated by Hu and colleages. Methanol extraction and dichloromethane extraction of a soft coral species by Alluri, Thameemulanasari, & Reddy (2012) demonstrated that the methanol crude extract was more toxic to HeLa cells than the dichloromethane extract. Here Compound 1 was tested against three different cell lines and the toxicity was compared to the non-cancerous Vero cells, as the control cell line (Figure 2). At all tested concentrations the toxicity of Compound **1** was significantly greater ($p < 0.01$) against U937 cells when compared to U1 cells (a daughter cell line derived from U937 cells). The same held true when comparing the toxicity of Compound **1** against U937 and Hela cells, as indicated in Figure 2. At tested concentrations between 6.25 µg/ml and 25 µg/ml the cell viability of U937 cells was significantly lower ($p < 0.01$) than that of the control cell line indicating the selectivity of this compound towards cancerous cells.

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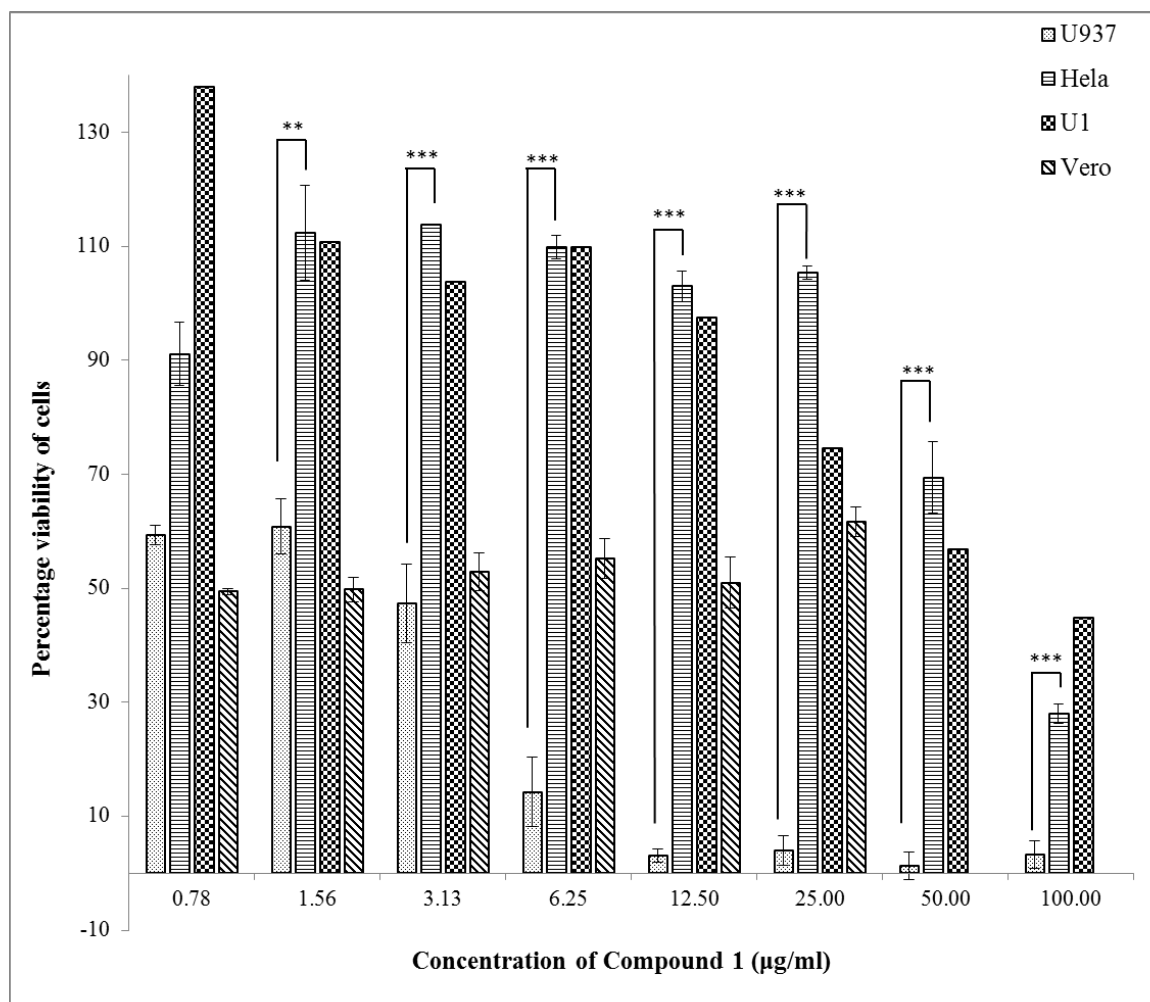

Figure 2: Compound 1 tested against HeLa, Vero, U937 and U1 cells. Significance was determined using one way ANOVA (Tukey) where $^{**}p<0.01$ and $^{***}p<0.001$

Using the cytotoxicity data, we calculated CC_{50} concentrations for Compound **1** with respect to each cell line and compared to CC_{50} values for the positive control Actinomycin D (Table 1). U937 cells treated with Compound **1** had a significantly lower CC_{50} concentration compared to the control cell line ($p < 0.05$) and a selectivity index (SI) of 2.54. Although the cytotoxic concentration of Compound **1** was eighty times larger than that of Actinomycin D against U937 cells, the selectivity index was still better than that of the positive control (SI = 0.9). For all other cell lines treated with Compound **1**, the CC_{50} value was significantly greater when compared to the CC_{50} value calculated against Vero cells as well as the CC_{50} value of Actinomycin D ($p < 0.001$). Compound **1** also showed an almost three times higher CC_{50} concentration towards HeLa cells than towards U937 cells.

Table 1: CC_{50} values ± SEM of Compound 1

Compound	CC_{50} (µg/mL)			
	U1	U937	HeLa	Vero
1	32.73 ± 2.235	3.91 ± 0.017	61.12 ± 2.73	9.94 ± 1.92
Actinomycin D	9.78E-03 ± 2.31E-04	0.0487 ± 4.87E-03	0.016 ± 5.6E-03	5.03E-02 ± 2.38E-03

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Five Sinularolides were tested by Li *et al*., (2005) against HeLa cells of which demonstrated moderate cytotoxicity. These authors hypothesized that the epoxide unit was required for the toxicity towards the cell lines tested. Hence, the importance of both the lactone ring and the epoxide unit, with respect to the toxicity of cembrane diterpenoids similar to Compound **1**, needs to be fully elucidated. Arepalli *et al.,* (2009) evaluated the apoptotic effect of a furano-sesquiterpene isolated from *Sinularia kavarattiensis* on human monocytoid leukemia cells (THP-1) and found that it induced anti-proliferative effects involving a mitochondrial dependent pathway similar to the mechanism of action of doxorubicin and cisplatin.

Compound **2**, 3-caffeoylquinic acid (Figure 3), was the major compound identified in certain fractions by mass spectrometry. There were also traces of 4-caffeoylquinic acid identified in these two fractions. The toxicity of these fractions was assessed against Hela and U937 cell lines using the XTT cell viability assay (Figure 4). These fractions showed a higher toxicity towards both cell lines when compared to the toxicity of Compound **1**. Cortés *et al*., (2014) reported a weak antibacterial activity when compounds isolated from *Ceramium rubrum* were tested alone however as more compounds were added together from the same extract the antibacterial activity increased. Although this phenomenon might not be extensively documented in marine organisms, synergism between compounds has been reported in plants (26). Major compounds in natural extracts may not be solely responsible for the observed bioactivity and the reported potency may be the result of combinatorial activity. A number of minor compounds may contribute to the bioactivity when used in conjunction with major compounds (25,27,28).

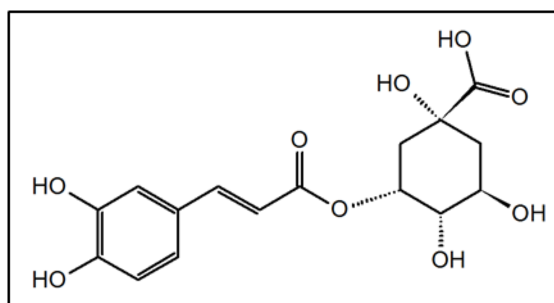

Figure 3: Compound 2 isolated from *S. notanda* identified as 3-caffeoylquinic acid.

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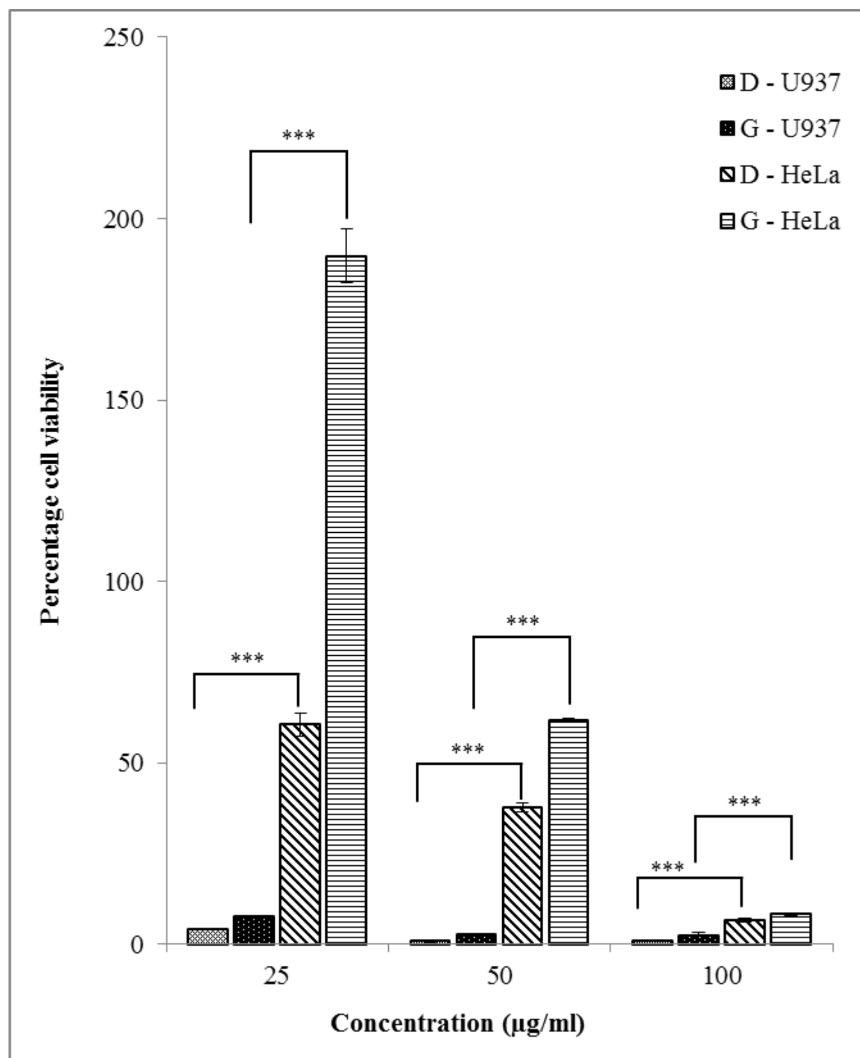

Figure 4: Cytotoxicity of fractions 5+6 (Compound **2**) – The toxicity of D(5+6) against HeLa cells was ten times greater than the toxicity of G(5+6) against HeLa cells and both D(5+6) and G(5+6) exhibited cell viability under 10% against U937 cells, at all three tested concentrations. Significance was determined using one way ANOVA (Tukey) where ***$p<0.001$.

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Experimental Section

2.1 General Experimental Procedures

^{1}H and ^{13}C NMR were recorded on Bruker 400 MHz (Massachusetts, USA) NMR spectrometer. The solvent used was methanol-d4. Column chromatography separation and purification were performed on silica gel 60: 70 – 230 mesh (Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich, Sweden). Precoated silica gel 60 F_{524} plates (Merck) were used for thin layer chromatography analysis and were visualized under UV light and by staining with vanillin-sulphuric acid followed by heating.

All ^{1}H NMR spectra were measured at $25^{0}C$ on a Bruker Avance III 400MHz instrument (Chemistry Department, University of Pretoria) equipped with cryogenic inverse triple resonance TXI probe head, operating at a frequency of 400.13 MHz

2.2 Extraction and Isolation

Fresh *Sinularia notanda* (260 g) was homogenized directly after collection with 150 ml of 90% methanol (Merck, Germany). Animal material was collected and identified by Dory Pets, Centurion and confirmation of identification was done by Dr. Mona Ellithey. After filtration the solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) and the residue (14.7 g) was re-suspended in ethyl acetate and partitioned with methanol in order to remove salts and the high molecular weight hydrophilic compounds. The lipophilic fractions obtained by ethyl acetate was dried out by rotavapor and tested for *in vitro* cytotoxicity against the cervical cancer cell line (HeLa), and the African green monkey kidney cell line (Vero) as representative of a normal, non-cancerous cell line. The extracts were tested for inhibitory activity in a direct enzyme assays against HIV-1 protease and for free-radical scavenging ability using the DPPH antioxidant assay. The ethyl acetate fraction was partitioned on silica gel using n-hexane and ethyl acetate (100:0 to 0:100) and 500 mL fractions were obtained. Similar fractions were pooled based on TLC profiles and all fractions were tested for cytotoxicity. Active fractions were further purified using silica gel chromatography. Compound 2 was obtained by elution using *N*-hexane and ethyl acetate (90:10 to 70:30).

*2.3 Single-Crystal X-ray Crystallography of (**1**)*

A suitable colorless block-like specimen of Compound 1 was used for X-ray crystallographic analysis. Crystal data and experimental details: C20H30O4, Mr = 334.44, crystal size 0.120 mm x 0.176 mm x 0.287 mm, crystal system orthorhombic, space group $P\ 2_{1}2_{1}2_{1}$ (Z = 4), with a = 8.881 (5) Å, b = 11.0893 (5) Å, c = 19.8361 (9) Å, V = 1953.69 (17) $Å^{3}$, D_{calcd} = 1.137 mg/m^{3}. Intensity data were measured on a Bruker Apex DUO using an Incoatec IμS microfocus X-ray

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source equipped with a Incoatec quazar multilayer mirror. Sample temperature was kept constant at 100(1) K using an Oxford Cryostream 700+. A total of 6122 reflections were collected up to a θ_{max} of 65.27°. The structure was solved by direct methods and refined by a full-matrix least-squares on F^2 procedure. The refined structural model converged to a final $R1 = 0.0388$, $wR2 = 0.0779$ for 3032 observed reflection [$I > 2\sigma(I)$] and 223 variable parameters. The absolute configuration was determined by Flack's method with Flack's parameter determined as 0.0(2).

2.4 Cell Culture and Cytotoxicity

All methods described below were performed under sterile conditions in a P2 bio-safety cabinet. Cells were grown in a humidified environment at 37°C and 5% CO_2. HeLa and Vero cells were cultured in Minimum Essential Medium (Thermo Scientific, Utah) supplemented with 5% Fetal Bovine Serum (FBS) (Thermo Scientific, Utah) and 1% Antibiotic/Antimycotic Solution (v/v) (Thermo Scientific, Utah). U937 and U1 cells were cultured in RPMI-1640 containing L-glutamine (Sigma-Aldrich, USA) supplemented with 2 g/L $NaHCO_3$, 5% HEPES buffer (v/v) (Gibco, Life Technologies, USA), 10% FBS (v/v), 1% Gentamicin (v/v) (Sigma-Aldrich, USA) and antibiotic/Antimycotic solution. The culture medium was renewed every two to three days when cells were approximately 80% confluent (29). Crude extract and compounds were dissolved in DMSO to stock concentrations of 20 mg/mL and serial dilutions were made to create a concentration range of 100 µg/mL to 0.78 µg/mL.

Cytotoxicity was measured using the formazan salt sodium 2,3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide (XTT) Cell Proliferation Kit II (F. Hoffmann-La Roche Ltd.). Cells (100 µl) were seeded at 1×10^5 cells/mL in a 96 well microtitre plate (Corning, USA) and incubated for 24 hours to allow the cells to attach. Extracts and compound were dissolved in DMSO and then serially diluted (100 µg/mL to 0.78 µg/mL), added to the plates (100 µL) and incubated for 72 hours. Control wells included a positive control for cytotoxicity, actinomycin D, a negative control of cells with DMSO and a blank control containing cells and media only. After 72 h incubation, XTT was added to the cells at a final concentration of 0.3 mg/mL with Phenazine methosulphate (PMS) and incubated for 1 hour. Absorbance of the developed colour was spectrophotometrically determined using a multi-well plate reader (Multisckan Ascent, Thermo Labsystems, USA) which measured the optical density at 450 nm with a reference wavelength of 690 nm. The fifty percent cytotoxic concentration (CC_{50}) of the extract/compound is the concentration which reduces cell growth by 50% when compared to the experimental controls and is the average of at least three independent experimental repeats. The CC_{50} values for the extract and compound were obtained using GraphPad Prism (Graphpad Software Inc., USA) (30).

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3. Conclusion

This study of *S. notanda* yielded the isolation of Sinulariolide (Compound 1), a cembrane diterpenoid previously reported in other species of *Sinularia* (10,16,31,32). A related ketone version of this compound, II-Dihydrosinulariolide was isolated from this soft coral by Tursch *et al*., (1978). The cytotoxicity (3.91 ± 0.017 μg/ml) and selectivity (2.54) of Sinulariolide against a human leukemic cell line is evidence that *S. notanda* also has the potential to produce chemotherapeutic compounds and warrants further investigation into the mode of action. This work corroborates previous studies of soft coral from the *Sinularia* genus where Sinulariolide was isolated. This compound has been isolated extensively from different species within this genus and this particular cembrane diterpene may serve as a chemotaxanomic marker of *Sinularia* soft coral species (33,34).

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Mr Eric Palmer (Department of Chemistry, University of Pretoria) and Dr Mellisa Makatini (Mintek, Randburg, South Africa) for assistance with NMR and Mass Spectrometry analysis respectively.

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5. Conflict of Interest

The authors declare no conflict of interest.

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