PURIFICATION OF PLANT-DERIVED MOLECULES THAT MODULATE SPHINGOLIPID ENZYMES IN CANCER AND INFLAMMATION

By

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DECLARATION

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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This thesis is dedicated to the soul of my cousin Fatma Al-Zahraa, who died at age nine with brain tumour. Her pain for three years, make my choice of my life goal to find cures and end peoples suffering and pain. Also, this is dedicated to my aunt who passed away before I accomplished my PhD, I knew how happy she will be finishing this journey. May both your souls rest in peace.

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Abbreviations

ABC294640	3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-lmethyl)amide
ABC	ATP binding cassette
aCKAR	aPKC-specific C Kinase Activity Reporter
APAF1	Apoptotic protease activating factor 1
APC	activated protein C
AP-1	activator protein
ATP	Adenosine 5'-triphosphate
Bax/Bak	pro-apoptotic members of Bcl2 family
Bcl-2	B-cell lymphoma 2
BSA	bovine serum albumin
CIB1	calcium- and integrin-binding protein 1
CNS	central nervous system
Cer	Ceramide
C1P	ceramide -1-phosphate
CerS	ceramide synthase
CerS1-6	Ceramide synthesis 1-6
CERT	ceramide transfer protein
CAPP	ceramide-activated protein phosphatase
C1P	Cermaide-1-phosphate
CLK1	CDC like kinase 1
Col1	collagen 1
C5a	compliment 5a
CTGF	connective tissue growth factor
COSY	Correlation Spectroscopy
cPLA2	Cytosolic phospholipase A2
CDK1	cyclin-dependent kinase 1
COX	cyclooxygenase
DAGK	diacylglycerol kinase
DAGKα	Diacylglycerol kinase α
DBD	DNA binding domain
Des1	dihydroceramide desaturase
Des2	dihydroceramide desaturase 2
DEPT	Distortionless Enhancement through Polarization Transfer
DIABLO	Direct Inhibitor of Apoptosis-Binding protein with Low pI
DT	dithiothreitol
Degs1	drosophila degenerative spermatocyte 1
dhCer	dihydroceramide
DMEM	Dulbecco's Modified Eagle's Medium
DMS	*N,N*-dimethylsphingosine
DNA	Deoxyribonucleic acid
DMSO	Deuterated dimethylsulphoxide

DPPH	2,2-diphenyl-1-picrylhydrazyl
ECG	Electrocardiogram
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EFCS	European Foetal Calf Serum
EGF	epithelial growth factor
ER	endoplasmic reticulum
ERK ½	Extracellular signal-regulated kinases 1 and 2
FADD	FAS-associated death domain
FN-1	fibronectin 1
G82D	dominant negative SK1 mutant
GPCRs	G-protein coupled receptors
GTPase	guanosine triphosphatase
HDAC1 and 2	histone deacetylases 1 and 2
HIV	human immunodeficiency virus
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HRP	Horseradish peroxidase
HSQC	heteronuclear single quantum coherence
hTERT	human telomerase reverse transcriptase
IFN-γ	interferon gamma
IGFBP3	IGF binding protein 3
IGF	insulin-like growth factor
IKK	IκB kinase
IκB	Inhibitor of NFKB
IL-6	Interleukin-6
IL-8	Interleukin-8
JNK	c-JUN N-terminal kinase
KDa	Kilodalton
K_i	inhibition constant
KLHL5	kelch-like protein 5
LBD	ligand binding domain
LCHRMS	Liquid chromatography high resolution spectroscopy
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MFS	; major facilitator superfamily
Mfsd2b	major facilitator superfamily 2b
Mg	miligram
MG132	carbobenzoxy-Leu-Leu-leucinal
MOMP	mitochondrial outer membrane permeabilization
MLK-1	mixed lineage kinase 1
mRNA	Messenger ribonucleic acid
MRPs	multidrug resistant proteins

MS	mass spectrometer
MS	multiple sclerosis
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NADH	reduced nicotinamide adenine dinucleotide
NF- κB	nuclear factor kappa B
NGF	nerve growth factor
NMR	nuclear magnetic resonance
NOESY	Nuclear Overhauser Enhancement SpectroscopY
PAF	platelet-activating factor
PAR-1	protease activated receptor 1
PARP	Poly (ADP-ribose) polymerase
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
(**PGC1)b**	peroxisome proliferator activated receptor-γ coactivator 1 b
PGE2	Prostaglandin E2
PKB	protein kinase B
PKCα	protein kinase C α
PKCδ	Protein kinase C δ
PKC ζ	protein kinase Cζ,
PKD	protein kinase D
PLC	phospholipase C
PMA	Phorbol myristate acetate
PPAR-γ	peroxisome proliferator activated receptor-γ
prep-TLC	Preparative thin layer chromatography
PP2A	protein phosphatase family member
RIP1	Receptor interacting protein 1
ROS	reactive oxygen species
ROCK	Rho-associated kinase
SDS-PAGE	One dimensional SDS polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
siRNA	Small interfering RNA
Smac	Second mitochondria-derived activator of caspase
SMases	Sphingomylinases
SMS1	sphingomyelin synthase 1
SMS2	Sphingomyline synthesis 2
SMPD 1-2	Sphingomyelin Phosphodiesterase 1-3
SK	sphingosine kinase
SK1	Sphingosine kinase 1
SK2	Sphingosine kinase 2
SKi	Sphingosine kinase inhibitor
SPL	S1P lyase
SPNS2	Spinster homologue protein
SPT	serine palmitoyl transferase

S1P	Sphingosine -1-phosphate
S1PP	Sphingosine-1-phosphate phosphatase: (S1PP)
S1P 1-5	Sphingosine-1-phosphate receptors 1-5
SYK	spleen tyrosine kinase
TBST	Tris buffer saline with Tween
TEMED	tetramethyl-ethylene-diamine
TLC	Thin layer chromatography
TNK2	Tyrosine Kinase Non-Receptor 2
TNFα	tumour necrosis factor
TRAF2	TNF receptor-associate factor 2
TRAIL-R1	TNF-related apoptosis-inducing ligand-R1
TRAIL-R2	TNF-related apoptosis-inducing ligand-R2
TSSK	testis-specific serine kinase
UUO	ureteral obstruction model
VEGF	vascular endothelial growth factor
XBP-1;	X-box DNA-binding protein-1s
α-SMA	α-smooth muscle actin

Abstract

There is a constant need to discover new anticancer and anti-inflammatory compounds that can be developed as medicines. Sphingosine kinase 1 (SK1) and dihydroceramide desaturase (Des1) have been demonstrated to have a key role in sphingolipid metabolism and are potential targets for anticancer/anti-inflammatory therapeutics. This study aims to screen a plant library collected during field work in Egypt and then to isolate new anticancer and anti-inflammatory compounds with activity against SK1 and/ or Des1. This aim was achieved using plant extracts tested on breast cancer cell viability, proliferation and SK1 and/or Des1 protein expression. Bio-assay guided fractionation and isolation of compounds using flash column, silica gel column chromatography and preparative TLC techniques, followed by structure elucidation using 1D and 2D spectroscopic analysis enabled identification of compounds that met the criteria above. Cell proliferation was determined using [^{3}H]-thymidine incorporation assay. Western blotting technique was used to determine the effect of isolated compounds on the targeted enzymes SK1/Des1 as well as the apoptotic pathway (PARP). There were three major findings. First, three plant species *Gomphocarpus sinaicus*, *Urginea maritima* and *Pancratium tortuosum* exhibited anticancer activity. Second, narciclasine was isolated for the first time from *P. tortuosum* and was demonstrated to inhibit cell proliferation ($p<0.05$) and to reduce SK1 and Des1 expression in MDA-MB-231 and MCF7-7L breast cancer cells. Narciclasine also induced PARP cleavage (a marker for apoptosis) and reduced expression of Ki67 and phosphorylated AKT levels (a marker for cell survival). The reduced expression of SK1 and Des1 in response to narciclasine was independent of the ubiquitin-proteasomal pathway, suggesting that this compound might affect the transcriptional/translational regulation of SK1 and Des1. Finally, narciclasine also exhibited ant-inflammatory activity as evidenced by its ability to prevent TNFα-stimulated degradation of IκB and to inhibit NFκB- and AP1-dependent transcriptional activity in keratinocytes. Third, a mixture of cardenolide glycosides were isolated from *G. sinaicus* and shown to inhibit cell proliferation ($p<0.05$), reduce expression of SK1 and Des1 and induce modest PARP cleavage in MDA-MB-231 and MCF-7L cells. This mixture contained humistratin and calactin and/or calotropin. These compounds

reduced SK1 expression by inducing its proteasomal degradation, while the reduction in Des1 expression may be *via* a transcriptional/translational mechanism and is independent of ubiquitin-proteasomal degradation pathway. The mixture also exhibited anti-inflammatory activity in inhibiting NFκB- and AP-1-dependent transcriptional activity in keratinocytes. Narciclasine and the cardenolide glycoside mixture are potential anticancer/anti-inflammatory compounds require further studies in order to establish whether these compounds might be usefully exploited to treat cancer and inflammatory disease.

CHAPTER 1:
GENERAL INTRODUCTION

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1.1 Natural products and drug discovery

For centuries, traditional medicines including plants, animal products and minerals have been used in different formulations for the treatment of disease. These practices are still common in many countries including in Africa and Asia. Many traditional medicines have been used for a long time and are well documented by their local communities. These include Iranian, Islamic, Chinese, Indian, Korean, and African traditional medicines (Builders, 2018, Wangchuk, 2018). The medicinal properties of extracts have provided the basis for the isolation and identification of novel therapeutically active compounds that can be formulated as drugs and used to treat many diseases (Zhang *et al.*, 2018a). For example, Taxol is a drug which was isolated from the plant *Taxus baccata* and which had been used historically, without scientific evidence, to cure cancer in people from Central Himalaya for many centuries (Wangkheirakpam, 2018).

The World Health Organization (WHO) states that traditional medicines could have dangerous effects, if used in inappropriate manner. Therefore, more research is required to establish the safety and efficacy of such medicinal extracts, medicinal plants and other natural products (Wangkheirakpam, 2018). Nevertheless, traditional medicinal plants have been a productive source of new drugs. For example, nearly 50% of the drugs currently sold on the market and approved since 1994 are originally from natural sources. In addition, more than 100 compounds are under clinical trial and the same number are in pre-clinical development (Butler, 2008, Harvey, 2008). Altogether, this provides a strong rationale for employing a drug discovery approach using natural products as the starting material.

Modern medicine based on crude natural products have been the source for medication in early nineteenth century, based on historical use through time. By the twentieth century, techniques had advanced sufficiently to purify the active components from these natural products (Ji *et al.*, 2009, Süntar, 2020). Examples include atropine, morphine, and cocaine and which are still currently used (Orhan, 2014). The purification of the active ingredient can significantly reduce the side effects associated with other molecules in the extract (Qian *et al.*, 2020). Purification of the active

ingredient also allows for standardised dose regimes to be employed. To achieve this, it is necessary to test crude extracts for their biological activity followed by chromatographic fractionation, purification, and identification of the active ingredient in the extract (Joshi, 2012).

In order to reduce side-effects of current drugs and to eliminate unmet medical need in diseases with no current cures, it is required to identify novel therapeutic targets and to isolate/develop new compounds that can be used as medicines with improved efficacy and reduced side-effects. To meet this, there is a need for extensive phytochemical and pharmacological studies to produce novel medicines (Frank *et al.*, 2014).

Natural products have been the starting point for drugs synthesis of new drugs. Of the 36% of purely synthetic drugs approved by Food and Drug Administration (FDA), 19% are pharmacophores derived from natural products (Newman, 2020). The rest are mimetics of naturally occurring bioactive compounds that bind to the active site of biological and disease relevant protein targets. For example, artemisinin isolated from *Artemisia annua* is the most famous anti-malarial drug (Klayman *et al.*, 1984). Another example is salicylic acid which was isolated from the willow tree. This was used by Egyptians and Babylonians in the 5th century BC to relieve pain during childbirth and to reduce fever. Arthur Eichengrun in 1897 discovered that the acetyl derivative of salicylic acid isolated from willow reduced bad taste and stomach irritation and acidity (Sneader, 2000). Subsequently, synthetic aspirin was produced, and today ~ 50 billion tablets are consumed each year. Aspirin was shown to inhibit cyclooxygenase (COX), which catalyses the first step in the production of prostaglandins and thromboxanes by acetylation (Pedersen and FitzGerald, 1984, Lecomte *et al.*, 1994).

Natural products are a useful starting point for drug discovery programmes using a medicinal chemistry approach for example, Taxol (Cragg and Newman, 2013, Lahlou, 2013). The reason is that these molecules are rich in oxygen atoms, contain a high proportion of sp3 hybridised carbon and exhibit diverse stereochemistry (Morrison and Hergenrother, 2013). Therefore, natural products have a huge chemical structural diversity with distinct scaffolds that exceed synthetic compounds. The improvement

in medicinal chemistry, structure elucidation and molecular engineering, such as gene silencing, has placed less reliance on drug discovery from natural products. Indeed, some companies have dropped their natural products programmes and replaced them with complimentary chemistry approaches. The pharmaceutical industry has taken the approach of combining complimentary chemistry with High-Throughput Screening [HTS] (Miyagawa *et al.*, 2009) in an attempt to discover new hits. Unfortunately, while HTS accelerates and increases the number of compounds screened, the number of new chemical agents discovered is low. Many chemical and combinatorial libraries have been established commercially but none have resulted in an approved drug (Fox and Spector, 1999, van Hilten *et al.*, 2019). Others have argued that complimentary chemistry should be used to support natural products drug discovery rather than simply replacing it (Paululat *et al.*, 1999, Barnes *et al.*, 2016, Simm *et al.*, 2018, Li *et al.*, 2019). The reliance on synthetic drug discovery approaches using structural guided medicinal chemistry has not produced adequately different or biologically active compounds. Therefore, some small pharmaceutical businesses have revived their natural products programmes (Koehn and Carter, 2005, Dias *et al.*, 2012, Atanasov *et al.*, 2021). Phytochemicals are chemically show more diversity than synthetic molecules (Henkel *et al.*, 1999) and natural products library has not been the target for HTS. Moreover, complimentary chemistry has not considered the naturally isolated compounds. Indeed, natural products have more hits, structure diversity and biological activities than the complementation libraries. Indeed, Breinbauer *et al.* (2002) have argued that it is the biological applicability and pharmacophore diversity that is important in the drug discovery process rather than the total number of compounds in a chemical library.

More natural products research is needed because of the attractive variety in chemical structures facilitated by the improvement in isolation, characterisation, and production technologies. In addition to plants, other organisms such as marine animals, ants and microbes provide a huge diversity of potential novel drugs (Orhan, 2014). Indeed, there is increasing interest in isolating natural products from endophytes (microorganism that colonise inside plants or soil, establishing a relationship with the host plant) and soil (Bacon and White, 2000). This is an attractive approach as there is an enormous diversity of micro-organisms, of which only a small sub-set have been

cultured and only some have been investigated for secondary metabolites. For example, 1 gram of soil contains 1000-10000 microorganisms. This new approach is important, as the anticipated number of fungal species is 5 times the number of plant species (Demain and Zhang, 2005), thereby increasing the potential source of new medicines.

A few companies have integrated natural products screening and genetic approaches in the search for new antibiotics, immunosuppressants and enzyme inhibitors. The change in screening philosophy has been complemented with the application of molecular biology to isolate bioactive agents that modulate cellular processes at the molecular level (Zhang and Demain, 2007, Wermuth *et al.*, 2015, Gill, 2016).

1.2 Plants used in this study

Egyptian Flora has a big diversity due to its geographical location. Selection of plant species had been done through, searching the flora of Egypt references, making a survey to document the traditional uses by local peoples, end up by choosing a list of plants to be collected in a field trip for further phytochemical investigation in search of compounds that have anticancer and/ or anti-inflammatory properties. The plants were selected based on their traditional uses and previous phytochemical and pharmacological characterisation (Table 1.1). Some background information about the distribution and taxonomy is included for each plant.

1.2.1 *Pancratium tortuosum*

Family: Amaryllidaceae

Synonym: *Pancratium tortifolium*

Common names: Zambak

Pancratium is the most widespread of all the genera of Amaryllidaceae. It is paleo-tropical genus consisting of 16 species distributed throughout Africa, tropical Asia, Macaronesia, and the Mediterranean basin. It has been introduced and cultivated in many countries (El-Hadidy *et al.*, 2012). *P. tortuosum* in particular is found in the Nubia Nile Land Mount Erkowit, Arabia and Egypt (Baker, 1898).

Many species of *Pancratium* are of high value economically and medicinally. Several species are known for their use to treat cancer. The extract contains diverse type of alkaloids. Other species are locally important in traditional medicine or folk practice. For instance, *P. tenuifolium* is used in Botswana, specifically in the 'coming-of-age' ceremony (El-Hadidy *et al.*, 2012).

Phytochemical studies on *P. tortuosum*

There has been no previous study on *P. tortuosum*, but some studies on other species of the same genus e.g. *P. maritimum* Viz. have led to isolation of four chromones and flavanes (Ali *et al.*, 1990). In addition, the flavanes, syzalterin, (-)- farrerol and (-)-liquiritigenin have been isolated from the Egyptian *P. maritimum*. Chemical characterisation of *P. maritimum* also led to isolation and elucidation of many alkaloids, such as lycorine, tazettine, pancracine, lycorenine, galanthamine and tripheridine (Abou-Donia *et al.*, 1991). Three chromone aglucones, one glucosyl and one glucosyloxy chromone and glucosyloxy alkaloids were isolated from *P. biflorum* (Ghosal *et al.*, 1984, Berkov *et al.*, 2004). In addition, Youssef *et al.*, (1998) reported the isolation of chromones, chromone-glucosides and glucosyloxy acetophenones from *P. biflorum*.

Pharmacological studies *P. tortuosum*

Pancratistatin was first isolated from the bulbs of *P. littorale* collected in Hawaii, as new phenanthridone biosynthetic products. Pancratistatin show effectiveness against the murine P-388 lymphocytic leukaemia and significantly reduce cell growth of the P-388 cell line *in vitro* and murine M-5076 ovary sarcoma *in vivo* (Pettit *et al.*, 1986).

1.2.2 *Urginea maritima*

Family: Asparagaceae

Synonyms: *Scilla maritima, Urginea scilla, Drimia maritima*.

Common names: Basal Elonsol

U. maritima is an onion-like Liliaceae distributed throughout the Mediterranean basin and is well adapted to this climate. The plant growing season is through autumn to

spring; during summer, the leaves get dry leaving the bulbs in dormancy (Pascual-Villalobos and Robledo, 1999).

U. maritima is a plant which has been known for its medicinal use to humankind since antiquity. The bulbs and their extracts have been used by ancient Egyptians in treating hydropsy, the major symptom being cardiac failure. The plant was the only resource in Egypt for the preparation of galenical and pharmaceutical products. The purified glycosides are used in the treatment of cardiac diseases and cases refractory to Digitalis and Strophanthus therapy (Sayed, 1980, Tuncok *et al.*, 1995).

Phytochemical studies on *U. maritima*

The chemistry of *U. maritima* has been extensively studied. Many bufadienolides have been isolated from the bulb of *U. maritima*. These include proscillaridin A, scillaren A, scilliroside, scillirosidine (Kopp *et al.*, 1996), 9-hydroxyscilliphaeoside (Krenn *et al.*, 1996), 11α-hydroxyscilliglaucoside (Krenn *et al.*, 2000), cardiac glycosides (Jha and Sen, 1981, Kopp *et al.*, 1996) and lignin glycoside (Iizuka *et al.*, 2001).

Pharmacological studies on *U. maritima*

Sixty-one plants have been tested for their cytotoxicity in human lymphoma U-937 glioblastoma multiforme (GBM) cells. The ethanol extract of *U. maritima* exhibits the greatest potency with almost complete cell death observed at the lowest concentration (1µg/ml) (El-Seedi *et al.*, 2013). The *U. maritima* extract was more cytotoxic than cisplatin, and gemcitabine (the most effective drugs in non-small cell lung cancer, NSCLC) (Bozcuk *et al.*, 2011) in A549 NSCLC cells. Also, the *U. maritima* extract has significant cytotoxic activity against PC-3 (prostate cancer) and U373 GBM cells (Mohamed *et al.*, 2014). In studies of plant disease, *U. maritima* extract exhibits slight genotoxic and cytotoxic activity compared with Vydate®, a chemical pesticide (Mert and Betül, 2008). In this regard, *U. maritima* bulbs used to be seen planted nearby the roots of fruit-trees to prevent ant infestations in Spain (Pascual-Villalobos and Robledo, 1999). In a study of the bulb extracts on 24 day old larvae, it caused 60-100% mortality (Sayed, 1980). Further studies report that *U. maritima* bufadienolides induces insecticide effects on *Tribolium castaneum* (Pascual-Villalobos, 2002).

1.2.3 *Gomphocarpus sinaicus*

Family: Apocynaceae

Synonyms: *Asclepias sinaica*

Common names: Hargal Gabaley

G. sinaicus is a genus of mostly shrubby herbs with non-tuberous root stocks distributed throughout Africa, Arabia and adjacent territories, south of the Dead Sea (Goyder and Nicholas, 2001). *G. sinaicus* is a woody, much branched shrub, l-l.5 m in height and is plentiful in the sandy mountainous regions in Sinai (El-Askary *et al.*, 1993). *G. sinaicus* is not endemic to the Sinai, being found also in Palestine and is widespread in Saudi Arabia (Newbold *et al.*, 2007)**.**

G. sinaicus has shown high toxicity for man and animals because of its high content of cardiac glycosides (El-Askary *et al.*, 1995a). *G. sinaicus* is one of the important plants in the Sinai ecosystem. It is inedible and is poisonous to grazing animals. Some other species of *Gomphocarpus* have been used in medicine for treatment of respiratory diseases, including asthma, although now it is little used as a herbal medicine (Semida *et al.*, 2006). The official name for this species now is *Gomphocarpus sinaicus*, *Asclepias sinaica* name is not acceptable name anymore (Govaerts, 1995).

Phytochemical studies on *G. sinaicus*

Gomphocarpus species are characterized to contain mainly 5-alpha-cardenolide glycosides, with a doubly linked sugar (El-Askary *et al.*, 1995a). Delta 5-cardenolides, nine cardenolide glycosides and one aglycone, as well as five new compounds have been separated from the aerial parts of the milkweed, *G. sinaicus* (Abdel-Azim *et al.*, 1996).

Pharmacological studies on *G. sinaicus*

Two cardenolide compounds have been isolated from the methanolic extract of *G. sinaicus* and each exhibits antifungal activity against both soil borne fungi, *R. solani, F. oxysporium*, and post-harvest fungi, *R. stolonifer, P. digtatum*, The aerial parts of *G. sinaicus* contain four flavonoids and the plant extract, applied after injection of

carrageenin- induced oedema in hind paw rat (an assay for anti-inflammatory drugs), all caused a significant reduction in the volume of rat paw ($p<0.001$) at 3 h of treatment for two dose levels. indicating considerable anti-inflammatory effects in rats (Batran *et al.*, 2005).

1.2.4 *Psoralea plicata*

Family: Fabaceae

Synonyms: *Cullen plicatum, Cullen plicata, Psoralea odorata*

Common names: Shajarat an-na'am, Marmid, Makka Buti

P. plicata grows in tropical and sub-tropical region of Pakistan and is found commonly in the Sind and Bunjab areas (Rasool *et al.*, 1990). It is also, widely distributed in the Alaqi area, south east of Aswan (Springuel *et al.*, 1997). The wild herb *P. plicata* has been used in folk medicine as a skin photosensitizer, anthelminthic, anti-pyretic, analgesic, anti-inflammatory, diuretic, and diaphoretic. It is also useful in bilious infection, in leprosy and in menstrual disorders (Rasool *et al.*, 1989, El-Abagy Elham *et al.*, 2012). Hamed *et al.* (1997) reported its medicinal use by Bedouins for different ailments. The roots are used as a tooth stick for the cleaning of teeth and the seeds are used as decoction and powder. It is medicinally used for skin diseases, such as psoriasis and is believed to be a blood purifier (Ahmed *et al.*, 2012).

Phytochemical studies on *Psoralea plicata*

Phyto-analysis of the whole plant yielded different types of compounds such as alkaloids, flavonoids, triterpenes (Rasool *et al.*, 1989), saponins and coumarins (Al-Yahya *et al.*, 1987). Recent phytochemical studies on the wild herb *P. plicata*, revealed the presence of other interesting compounds, including benzofuran glycosides (Hamed *et al.*, 1999), furocumarins, chromenes, isoflavonoids, phenolic cinnamates, cinnamate dimers (Hamed *et al.*, 1997), flavonoids, glycosides, monoterpenoids, triterpenoids and tocopherol (El-Abagy Elham *et al.*, 2012).

Pharmacological studies on *Psoralea plicata*

The *P. plicata* ethanol extract has been tested against *Culex pipiens*, a member of the mosquito family which carries multiple pathogens. The extract induced mortality and

reduced fecundity. Plicatin B, flavonoid mixtures and chromene isolated from *P. plicata* mixture exhibited the strongest insecticide activities against mosquitos (El-Abagy Elham *et al.*, 2012). Using 2 mg of *P. plicata* ethanol extract causes an inconsequential increase in the contraction force of isolated heart, while a 4 mg dose resulted in para sympathomimetic activity using smooth muscle preparation and a higher dose (20 mg) resulted in lowering blood pressure of anaesthetized rabbits accompanied with rapid respiration (Al-Yahya *et al.*, 1987).

1.2.5 *Heliotropium supinum*

Family: Boraginacea

Synonyms: *Lithospermum heliotropioides, Heliotropium malabarica, Heliotropium coromandelianum*

Common names: Qoddeih, Zorreiqa

Heliotropium genus is widely spread in the temperate and tropical zones of both hemispheres. It has had a variety of treatment uses since ancient times, including inflammatory diseases such as gout and rheumatism, skin diseases, menstrual disorder and as a cure against toxic insect bites (Ghori *et al.*, 2016).

Ethno-medicinal studies have shown that *H. supinum* has been used by healers in India as pounded aerial portions of the plant are applied onto snake bites, while 5 ml of juice is given orally at frequent intervals (Teklehaymanot and Giday, 2007). It has also been used in east Africa by women after childbirth (Schoental, 1970). In Namibia, people use it as a folklore medicine primarily by mixing pulped plant with water to treat tumours (Ghori *et al.*, 2016).

Phytochemical studies of *Heliotropium* genus

A variety of molecules have been separated and identified from different species of *Heliotropium genus*. Those compounds reported as bioactive phytochemicals with potential therapeutic effects. Also, have a wide range of compound types abundant in this genus, such as alkaloids (pyrrolizidine type), terpenes, phenolic compounds and quinones (Ghori *et al.*, 2016).

Pharmacological studies of *Heliotropium* genus

Pyrrolizidine alkaloids, terpenoids and flavonoids are bioactive compounds that have been isolated from the genus *Heliotropium*. Different species of genus *Heliotropium* extracts and ingredients have significant bio-activities, such as anti-microbial, antitumour, antiviral, anti-inflammatory activities, wound healing properties, cytotoxicity and phytotoxicity (Ghori *et al.*, 2016).

1.2.6 *Asphodelus fistulosus*

Family: Liliaceae

Common names: Onion weed

Asphodelaceae is one of the sub-families of liliaceae, which are distributed in tropical, subtropical, and temperate regions around the world. The main location is in southern Africa. Among these, *Asphodelus tenuifolius* is used by local population as a diuretic (Boatwright, 2012, Safder *et al.*, 2012). *A. fistulosus* (onion weed) is distributed through the Mediterranean region, extending through western Asia to India (Pitt *et al.*, 2006). The bulbs and roots of *A. microcarpus* are applied as a treatment for skin diseases (ectodermal parasites and psoriasis) and jaundice. It is also used as an anti-microbial cure by Bedouins (El-Seedi, 2007). *A. tenuifolius* seeds are generally taken for colds, hemorrhoids, as a febrifuge and diuretic agent. The seeds are also applied to healing wounds and ulcers and for inflamed regions of the body (Panghal *et al.*, 2011).

Phytochemical studies of *A. fistulosus*

Different species of *Asphodelus* genus have been studied and reported the presence of various type of chemicals such as, lipids, carbohydrates, coumarins and anthraquinones, have been reported and isolated from *A. microcarpus, A. fistulosus and A. ramosus* (Hammouda *et al.*, 1974, Ghoneim *et al.*, 2014), flavonoids from *A. globifera.* In addition, different types of terpenes, like sesquiterpens (*A. globifera, A. anatolica* and *A. damascene*), triterpenes and triterpene glycosides have been isolated (*A. microcarpus*) (Panghal *et al.*, 2011, Safder *et al.*, 2012).

Pharmacological studies of *A. fistulosus, A. microcarpus, A. aestivus* Brot.

Isolated compounds from *A. microcarpus* exhibit potent activity against methicillin-resistant *Staphylococcus aureus*. It also has anti-leishmanial activity (Ghoneim *et al.*, 2014). Both ethyl ether and ethyl acetate extracts of *A. aestivus* Brot. significantly scavenge DPPH (2,2- diphenyl-1-picrylhydrazyl) antioxidant activity. The extract also has cytotoxic effect against MCF-7 breast cancer cells in concentration and time-dependent manner and induces significant deoxyribonucleic acid damage (Shall and Gilbert, 2000, Aslantürk and Çelik, 2013).

1.2.7 *Glinus lotoides*

Family: Molluginaceae

Synonyms: *Glinus dictamnoides, Mollugo hirta, Mollugo glinus*

Common names: Ghobbeira, Damsees

Glinus lotoides is known as lotus sweet juices and is broadly found in tropical and subtropical zones of the world. In Ethiopia, where it is well-known as ‘metere’, the seeds have been used as an anthelminthic, laxative, antimicrobial and anti-diabetic agent. In India, it is used traditionally as medicine against diarrhea and bilious attacks. It is also applied as a purgative and for curing boils, wounds and pains (Demma *et al.*, 2013). It is commonly named in Arabic as Hashishet El-aqrab or Moghera (Hamed *et al.*, 1996).

Phytochemical studies of *Glinus lotoides*

The major constituents isolated and identified from *G. lotoides* seeds are triterpenoidal saponins (hopane-type) and *C*-glycoside flavones. Its cesticidal and pharmacological activity is accounted for these bioactive components. The hopane type saponins isolated from the *G. lotoides* seeds are; four glinusides F- I, six lotoidesides A-F and succulentoside B. Additionally, vicenin-2 and vitexin-2``-*O*-glucoside (flavonoids) (Biswas *et al.*, 2005). Hamed and El-Emary (1999) isolated one triterpenoidal saponin from its seeds. Furthermore, from the aerial parts of Egyptian *G. lotoides* var. *dictamnoides* confirmed the presence of the five hopane triterpenoidal saponins (glinusides A- E) (Endale *et al.*, 2005).

Pharmacological studies of *Glinus lotoides*

Methanolic extracts of *G. lotoides* have antitumour activity including against Dalton`s acsetic lymphoma in mice. In addition, these extracts have potential antimicrobial activity (Sathiyanarayanan *et al.*, 2006). Anticholesterolemic, hepatoprotective and antioxidant activities of an ethanolic extract are also described in rats (Demma *et al.*, 2013). An ethanolic extract of *G. lotoides* seeds is highly active *in vitro* against *Hymenolepis nana* worms and *in vivo* against *Taenia saginata* (Demma *et al.*, 2007). *G. lotoides* crude extract analysed in preliminary pharmacological studies, showed no effect on blood pressure, heart rate or the ECG of anesthetized rabbits. After oral administration there was no effect on bile production in guinea pigs or contractions of frog muscles isolated. The reported activities of *G. lotoides* seeds are attributed to saponins contents (Endale *et al.*, 2005). The hydroalcoholic extract of *G. lotoides* also has potential genotoxic effect *in vitro* at high concentrations (Demma *et al.*, 2009).

1.2.8 *Halocnemum strobilaceum*

Family: Amaranthaceae

Synonyms: *Salicornia strobiacea, Salicornia cruciata*

Common names: Hatab Ahmr

Phytochemical studies of *Halocnemum strobilaceum*

H. strobilaceum extracts contain fatty acids, triterpenoids and flavonoids (Cybulska *et al.*, 2014). The unsaponifiable plant fraction was found to be rich in palmitic acid. Additionally, the triterpenoids, campesterol, stigmasterol, betasitosterol and alpha-amyrin were also identified (Radwan and Shams, 2007). Flavonoids have characteristic antioxidant properties, mostly found and isolated from chloroform and ethyl acetate fractions. These mainly contain chrysoeriol, luteolin galactoside, quercetin rhamnoside and luteolin (Radwan and Shams, 2007).

Pharmacological studies of *Halocnemum strobilaceum*

The ethyl acetate extract from *H. strobilaceum* displays antioxidant activity (Radwan and Shams, 2007). Additionally, luteolin isolated from *H. strobilaceum*, shows anti-inflammatory activity and has been examined as a treatment for multiple sclerosis

(Theoharides, 2009). Scopoletin, a coumaric derivative has been isolated from these fractions (Radwan and Shams, 2007) showing antioxidant activity (Shaw *et al.*, 2003). Flavonoid and flavonoid glycosides were isolated from the epigeal part of *H. strobilaceum* (Miftakhova *et al.*, 1999). Further chemical analysis isolated the caffeic acid ester from the whole herb (Gibbons *et al.*, 1999). In addition, Miftakhova *et al.* (2001) isolated four coumarins from the aerial parts of *H. strobilaceum*.

1.2.9 *Crotalaria* sp.

Family: Fabaceae

Common names: Natash

Genus *Crotalaria* is one of the family of Fabaceae and is basically restricted to the tropical and subtropical areas of the world. *Crotalaria* sp. consist of a wide range of medicinal plants used in indigenous Indian medicine (Rao and Narukulla, 2007) as a purgative agent (Rao and Rao, 1999).

Phytochemical studies of *Crotalaria sp.*

The phytochemistry of this genus has importance in Indian traditional medicine. The plant contains several polyphenolic compounds including prenylated chalcone, flavone, chalcone, chromeno-dihydrochalcones and tri-methoxy-chalcone (Narender *et al.*, 2005, Rao and Narukulla, 2007). In addition, pyrrolizidine alkaloids has been isolated from *C. trifoliastrum* roots (Rao and Rao, 1999)

Pharmacological studies of *Crotalaria sp.*

Chalcone-type compounds exhibit inhibition of parasite maturation in the ring and schizont stages (Plasmodium falciparum (Strain NF-54)) (Narender *et al.*, 2005). In addition, chromeno-dihydrochalcones exhibit significant bioactivity against HIV, insecticidal, anti-inflammatory and antifeedant activity (Narender and Gupta, 2004).

Table 1. 1: Plant species used in current study

Plant	Family	Voucher No.*	Reason of choice
1.2.1 Pancratium tortuosum	Amaryllidaceae	10084	• locally important in traditional medicine. • high content of various alkaloid types of *Pancratium* genus.
1.2.2 Urginea maritima	Asparagaceae	DY-UM-2009	• the traditional use since antiquity for cardiac failure • high content of cardenolide glycosides.
1.2.3 Gomphocarpus sinaicus	Apocynaceae	-	• high content of cardiac glycosides.
1.2.4 Psoralea plicata	Fabaceae	4937	• used in folk medicine as a skin photosensitizer, anthelminthic, anti-pyretic, analgesic, anti-inflammatory, diuretic, and diaphoretic. • contain alkaloids, flavonoids, triterpenes and saponins.
1.2.5 Heliotropium supinum	Boraginacea	*11262*	• uses since ancient times, including inflammatory diseases • alkaloids (Pyrrolizidine type), terpenes.
1.2.6 Asphodelus fistulosus	Liliaceae	10099	• used locally as a diuretic, as an anti-microbial cure by Bedouins.
1.2.7 Glinus lotoides	Molluginaceae	10569	• used traditionally by Indians as medicine against diarrhea and bilious attacks. • contain triterpenoidal saponins (hopane-type) and *C*-glycoside flavones.
1.2.8 Halocnemum strobilaceum	Amaranthaceae	*4766*	• contain triterpenoids and flavonoids.
1.2.9 *Crotalaria* sp.	Fabaceae	-	• has importance in Indian traditional medicine.

* This plant species has been identified by prof. Magdi El-Sayed and Dr. Eman Atito, Botany Department, Aswan University, Egypt, using Herbarium specimens.

In the current study, specific plants were chosen based on their previous pharmacological properties, e.g., *U. maritima* as containing anticancer agents. These were screened for novel anticancer and/or anti-inflammatory compounds. Lastly, some plants have not fully been studied but have traditional uses which should be investigated e.g., *H. strobilaceum* in the future. Sphingolipids have been involved in various cancer types, breast, prostate, brain, and ovarian cancer. Therefore, the current study aimed to screen the selected plants on specifically two enzymes of sphingolipids metabolism, sphingosine kinase 1 (SK1) and dihydroceramide desaturase 1 (Des1). SK1 and Des1 play a role in cancer and inflammation diseases, inhibiting those enzymes will achieve the goal of the current study of discovery of new anticancer/anti-inflammatory compound/s.

1.3 Sphingolipids

Sphingolipids are a bioactive class of lipids which have received much attention recently due to their structural role and as signalling components of cell function in health and disease. Historically, the first sphingolipid, sphingosine was isolated from the brain and was named after the Greek mythical creature, the Sphinx. Sphingolipids are distributed mainly in the plasma membrane but also in lysosomes, endoplasmic reticulum, and Golgi apparatus. Sphingolipids are amphiphilic molecules that consist of a hydrophilic head group, such as saccharide, oligosaccharide (e.g. glycosphingolipids) or phosphocholine (e.g. sphingomyelin), linked to a hydrophobic long chain base (such as sphingosine) together with amide-linked fatty acids at C2 of the long chain base (Menaldino *et al.*, 2003, Sonnino *et al.*, 2006, Grassi *et al.*, 2019). Sphingolipids are bioactive molecules which respond to specific stimuli that regulate downstream effectors and targets. There are many bioactive sphingolipids, such as ceramide, ceramide 1-phosphate and sphingosine 1-phosphate (S1P) and these are the main actors in complex cellular processes that control, for instance, cell survival. Sphingolipids have crucial roles in membrane micro-domains, named lipid rafts (Gulbins and Kolesnick, 2003, Futerman and Hannun, 2004). These contain sphingolipids with different acyl chain length and distinct hydrophilic head groups that are organised to provide signalling platforms containing receptors and effectors (Brügger *et al.*, 2004).

Generally, ceramide functions to promote apoptosis and inhibit proliferation, whereas S1P supresses apoptosis and induces cell growth. This is known as the sphingolipid rheostat. Therefore, interconversion of ceramide, sphingosine and S1P can lead to apoptotic or survival programmes dependent on the position of the rheostat and which can be regulated by extracellular stimuli e.g., growth factors (Pyne and Pyne, 2000, Ogretmen and Hannun, 2004, Newton *et al.*, 2015). In addition, a nuanced view is that it is the ceramide driven biology versus that driven by S1P rather than the absolute level of these lipids that underpins the rheostat (Newton *et al.*, 2015).

Ceramide is synthesised by two different pathways (Figure 1.1). The first, is the *de novo* pathway and the second is termed the salvage pathway. The *de novo* biosynthesis pathway takes place at the cytoplasmic surface of the endoplasmic reticulum (ER) beginning with the condensation of L-serine and palmitoyl-CoA to form 3-*keto*-sphinganine, catalysed by serine palmitoyltransferase (SPT) (Merrill, 2002, Hanada, 2003, Hait *et al.*, 2006). The product of this reaction is reduced by 3-*keto*-sphinganine reductase to produce sphinganine, followed by acylation by a family of dihydroceramide synthases to form dihydroceramide. The addition of a *trans* 4, 5 double bond to dihydroceramide produces ceramide, catalysed by dihydroceramide desaturase (Des1) (Kolesnick and Hannun, 1999). Ceramide can then be transported by vesicular and non-vesicular mechanisms to the Golgi apparatus *via*, for instance, the ceramide transfer protein (CERT). Remarkably, CERT contains a pleckstrin homology (PH) domain, which allows its targeting to the Golgi apparatus in mammals (Venkataraman and Futerman, 2000). Complex sphingolipids derived from ceramide are synthesised in the Golgi apparatus and can be transported to other organelles (Spiegel and Milstien, 2003, Okada *et al.*, 2009). For example, ceramide can be glycosylated to form glucosylceramide at the Golgi cytoplasmic surface followed by conversion to complex glycosphingolipids after transferring to the Golgi lumen. Phosphorylation of ceramide by ceramide kinase produces ceramide-1-phosphate (C1P); and C1P can be converted back to ceramide by lipid phosphate phosphatase (Mandon *et al.*, 1992, Pitson, 2011). Alternatively, a phosphocholine headgroup added to ceramide by the Golgi lumen-localised sphingomyelin synthase (SMS1) produces sphingomyelin. In turn, sphingomyelin can be hydrolysed to ceramide by sphingomyelinase present at the plasma membrane (SMS2) and subsequently

converted to sphingosine by ceramidase action. The phosphorylation of sphingosine by sphingosine kinases (SK1 and SK2) produces S1P (Nagiec *et al.*, 1998, Hannun and Obeid, 2008a). S1P is a bioactive lipid and can be dephosphorylated to sphingosine by S1P phosphatase (S1PP) and the resulting sphingosine then reacylated to ceramide-by-ceramide synthase in the ER via the salvage pathway. Alternatively, S1P can be degraded to hexadecenal and phosphoethanolamine by S1P lyase (S1P lyase). These metabolites exit the sphingolipid metabolic pathway (Olivera and Spiegel, 2001, Deevska and Nikolova-Karakashian, 2017) but can be converted to phospholipids. This is the only known pathway of sphingolipids breakdown (Olson *et al.*, 2016).

Sphingomyelin and other sphingolipids are also found elsewhere in the cell. For example, a neutral ceramidase has been observed in mitochondria and sphingomyelin is present in the nucleus (El Bawab *et al.*, 2000). The relationship between sphingolipids metabolism in the endomembrane system organelles and others not connected to them by vesicular transport pathway are yet to be fully established. Although CERT transports ceramide from ER to Golgi, there might be a similar pathway that transports precursors to mitochondria or to the nucleus. Indeed, sphingosine easily transfer between organelles due to its enough aqueous solubility (Watanabe *et al.*, 2004).

The presence of multiple enzymes that are products of distinct genes with specific locations adds complexity to sphingolipid metabolism. For example, ceramide synthase (Tea *et al.*, 2020) is composed of distinct isoforms that are primarily localised in the ER but also detected at the nucleus, mitochondria, plasma membrane, Golgi and lysosome. The different isoforms of CerS exhibit differential preferences for fatty acids of various carbon chain lengths. It is accepted that sphingolipids will stay at the same localisation unless they are transported. S1P, C1P and glucosylceramide are specific transporters, have evolved in transporting ceramide (Hannun and Obeid, 2008b, 2018).

1.4 Ceramide

Ceramide sits at the centre of sphingolipid biosynthetic pathways. It is extremely hydrophobic and resides in the compartment where it is formed, unless transported to

other organelles by, for example, CERT. Ceramide is synthesised in ER through the *de novo* pathway and can be produced by the action of neutral and acidic sphingomyelinases and glucocerebrosidase at the plasma membrane, lysosome and in the mitochondria to produce compartment-specific ceramide (Mao *et al.*, 2000). Ceramide formed at the plasma membrane shows distinct and specific functions including stimulating the aggregation of the Fas receptor and activation of protein kinase Cζ (PKCζ) (Johnson *et al.*, 2002, Arana *et al.*, 2010). Ceramide is directly formed from the action of ceramide synthases (CerS1-CerS6) and dihydroceramide desaturase (Des1) and SMases. It is deacylated by ceramidase of which there are at least 5 isoforms (Fu *et al.*, 2018). The enzymes are variously localised in the plasma membrane, lysosome, mitochondria, Golgi, and ER. The existence of different enzymes, in distinct locations, suggest specificity in their mechanisms of regulation to enable spatial and temporal function of multiple ceramide species (Stiban *et al.*, 2010).

Mass spectroscopic analysis has revealed the existence of multiple species of ceramide thereby raising the concept that each performs specific pleiotropic functions in the cell. Ceramide species differ in the length of their fatty acid chain, saturation, or unsaturation, and/or modified functional group. For example, Des 1 introduces a *trans* 4.5 double bond to ceramides, whereas Des 2 introduces a hydroxyl at the 4-position. Also, fatty acid 2-hydroxylase introduces an α-hydroxy on the amide linked fatty acid. Together CerS1, fatty acid 2-hydroxylase and SPT3 catalyse the formation of α-hydroxy-$C_{18:1}$-ceramide with a C_{16}-sphingoid backbone (Zitomer *et al.*, 2009).

CerS are integral membrane proteins of the ER with their active site facing cytoplasm, although a subset of CerS have been partially purified from a mitochondria-enriched fraction (Hirschberg *et al.*, 1993). CerS1-6 catalyse the formation of dihydroceramides with different carbon chain lengths, which are substrates for the generation of multiple ceramide species ranging from C_{14} to C_{32} with varying degrees of saturation (Figure 1.2) (Saddoughi and Ogretmen, 2013). CerS1 is mainly expressed in brain with low levels in skeletal muscle and testis (Becker *et al.*, 2008, Park *et al.*, 2014b). CerS were originally known as the *LASS* (Longevity Assurance) gene based on yeast protein longevity assurance gene-1 (LAG1p). All CerS use acyl CoA to catalyse N-acylation

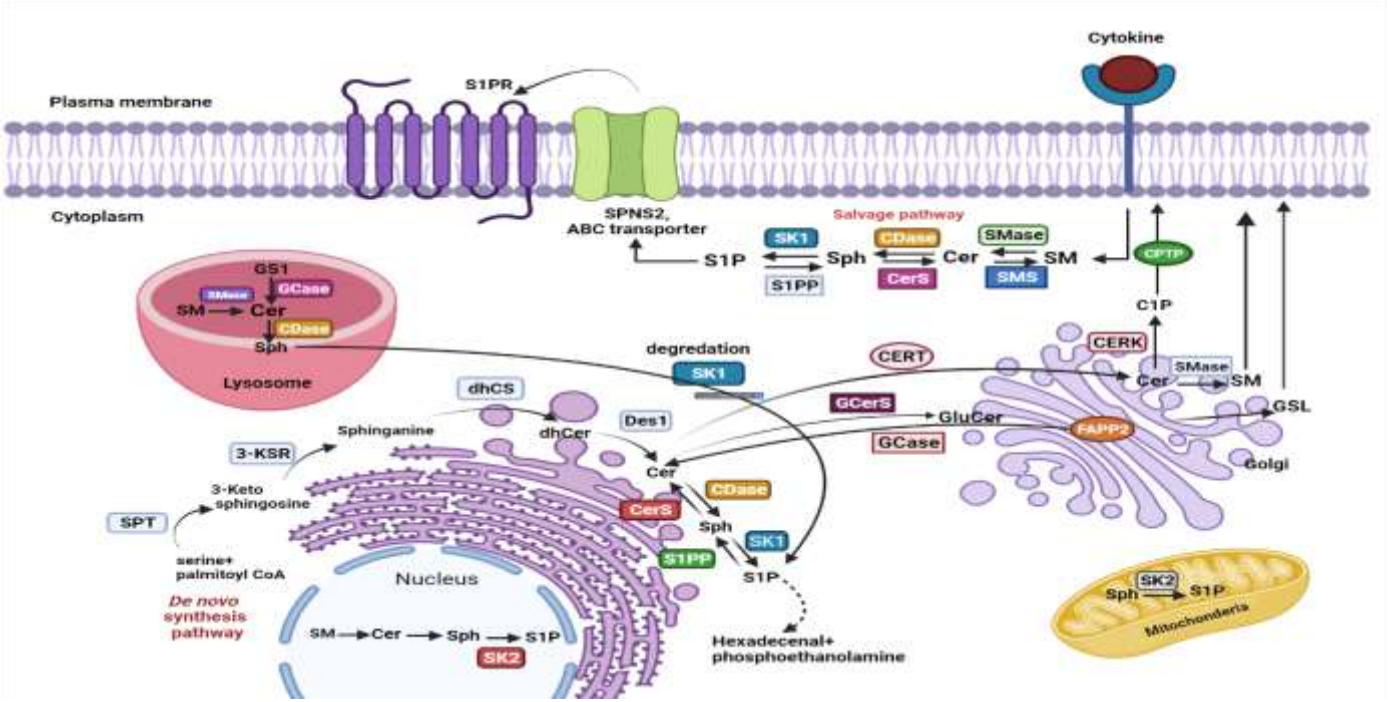

Figure 1. 1: Sphingolipid metabolism:

Sphingolipid metabolism and their structures are shown, along with the key enzymes involved in their interconversion. 3-KSR, 3-keto-sphinganine reductase; ABC, ATP binding cassette; C1P, ceramide-1-phosphate; CDase, ceramidase; Cer, ceramide; CERK, ceramide kinase; CerS, ceramide synthase; CERT, ceramide transfer protein; CPTP, C1P-specific transfer protein; Degs1, dihydroceramide desaturase; dhCer, dihydroceramide; dhCS, dihydroceramide synthase; FAPP2, four-phosphate adaptor protein 2; GCase, glycosidase; GCerS, glucosylceramide synthase; GluCer, glucosylceramide; GSL, glycosphingolipids; S1P, sphingosine-1-phosphate; S1PP, S1P phosphatases; S1PR, sphingosine 1-phosphate receptor; SK1, sphingosine kinase 1; SK2, sphingosine kinase 2; SM, sphingomyelin; SMase, sphingomyelinases; SMS, sphingomyelin synthase; Sph, sphingosine; SPL, S1P lyase; SPNS2, spinster homologue protein 2; SPT, serine palmitoyl transferase (Adapted from **(Maceyka and Spiegel, 2014).**

of dihydrosphingosine to produce dihydydroceramide. Each CerS isoform has high specificity different acyl chain lengths (Levy and Futerman, 2010).

CerS1 (*LASS*1) generates C-$_{18}$-dihydroceramide, which is subsequently converted to C-$_{18}$-ceramide. The enzyme has a role in controlling the growth of head and neck squamous cell carcinoma (HNSCC), based on data showing reduced C18-ceramide levels in most of HNSCC compared with normal tissues. Another important role of CerS1 is in managing the sensitivity to chemotherapeutic drugs. The enzyme can sensitize human embryonic kidney cells to various drugs such as cisplatin, doxorubicin, and vincristine (Min *et al.*, 2007).

CerS2 (LASS2) is abundant in many tissues including kidney and liver. It is involved in regulating body weight and food intake. Two studies have suggested a role for CerS2 in breast cancer. The first study showed that an increase in ceramides, particularly C_{16} and C_{24}, in malignant tumour tissue and this was correlated with increased mRNA for CerS2, CerS4 and CerS6 (Schiffmann *et al.*, 2009). The second study reported a significant correlation between CerS2 expression and poor prognosis (Erez-Roman *et al.*, 2010). This indicates the importance of CerS2 gene in breast cancer pathogenesis and diagnosis (Pewzner-Jung *et al.*, 2006, Levy and Futerman, 2010).

CerS3 (LASS3) is located mainly in skin (highly expressed in keratinocytes) and testis and can synthesise ceramides containing α-hydroxy-fatty acid. These assist in maintaining the water permeability barrier function of the skin. CerS3 has a role in sperm formation and production of androgen. CerS3 catalyses the formation of the longest chain $C_{26\ to}$ C_{32}-ceramides (Mizutani *et al.*, 2006).

CerS4 is mainly expressed in skin, heart, leukocytes, and liver. CerS4 makes both C_{18}-ceramide and C_{20}-ceramide. CerS5 predominates in lung epithelial cells and catalyses synthesis of C16-ceramide as does CerS6, which is abundant in intestine and kidney (Laviad *et al.*, 2008). CerS6 [LASS6] and CerS5 show high primary amino acid sequence homology and can also synthesise C_{12}- and C_{14}-ceramides to some extent. (Figure 1.2) (Mesicek *et al.*, 2010). CerS6 could play a crucial role in breast cancer as CerS6 expression is regulated by oestrogen (Weinmann *et al.*, 2005, Ruckhäberle *et al.*, 2008).

Many studies have suggested that the actions of ceramide are chain length dependent. In this regard, there are intracellular proteins to which ceramide binds to affect their activity. For example, CAPP (ceramide-activated protein phosphatase), is a cytosolic ceramide-binding protein which has been identified as a member of the PP2A protein phosphatase family (Dobrowsky and Hannun, 1992, Dobrowsky *et al.*, 1993). Ceramide also activates other kinases such as KSR (kinase suppressor of Ras) (Zhang *et al.*, 1997) and protein kinase C ζ (PKC ζ) which are involved in mitogenic signalling. Interestingly, CerS1 and CerS6 catalyse formation of C_{18} and C_{16}-ceramide respectively and which have opposite roles in regulating cell fate in head and neck cancer cells. CerS1/C_{18}-ceramide is implicated in promoting cell death and inhibition of cell growth in these cancer cells while C_{16}-ceramide stimulates their proliferation. However, other studies showed that C_{16}- ceramide is pro-apoptotic while C_{24}-ceramide promotes cell survival. Overall, ceramides appear to be context dependent with variable roles (Mesicek *et al.*, 2010, Galadari *et al.*, 2015).

Ceramides activate various tumour suppressive, metabolic, and anti-proliferative cellular programs, such as apoptosis, senescence, insulin resistance, inflammation, autophagy, and necrosis by initiating or suppressing key effectors (Merrill, 1991, Hannun and Obeid, 1995, 2002, Arana *et al.*, 2010, Galadari *et al.*, 2015). Ceramides induce apoptosis by inhibiting pro-survival Akt signalling and activating PP2A protein phosphatase (Truman *et al.*, 2014). This involves regulation of pro-apoptotic protein, Bax and Bcl2 and which leads to MOMP (mitochondrial outer membrane permeabilization), a key initiator of apoptosis. Indeed, CerS5 over-expression increases apoptosis, while CerS4 and CerS6 upregulation causes cell death in breast and colon cancer cells). Moreover, the effects of ceramide have been distinguished from those of dihydroceramide, e.g. in inducing apoptosis and other cell responses (Bielawska *et al.*, 1993). The modern concept is that distinct ceramide species in various cell compartments serve as mini hubs in the sphingolipid pathway.

In mammalian cells, there are three distinct classes of SMases which catalyse the production of ceramide from sphingomyelin. These can be distinguished by the effect of pH on their catalytic activity, i.e., acid, neutral and alkaline SMases. All 3 enzyme classes are involved in signal transduction processes, while the alkaline SMase is additionally involved in digestion of dietary SM in the intestine. In HT-29 colon

carcinoma cells, alkaline SMase has been linked with the inhibition of cell proliferation (Pewzner-Jung *et al.*, 2006). The SMPD1 gene encodes acid SMase, which is localized in lysosomes, the plasma membrane surface, and secretory vesicles (enabling export from the cell) due to glycosylation process of enzyme (Schissel *et al.*, 1998, Henry *et al.*, 2013). Neutral SMase is localised to the endoplasmic reticulum and/or mitochondrial membrane. Genes encoding neutral SMases include SMPD2 (neutral SMase1), SMPD3 gene (neutral SMase2) and SMPD4 (neutral SMase3) (Henry *et al.*, 2013).

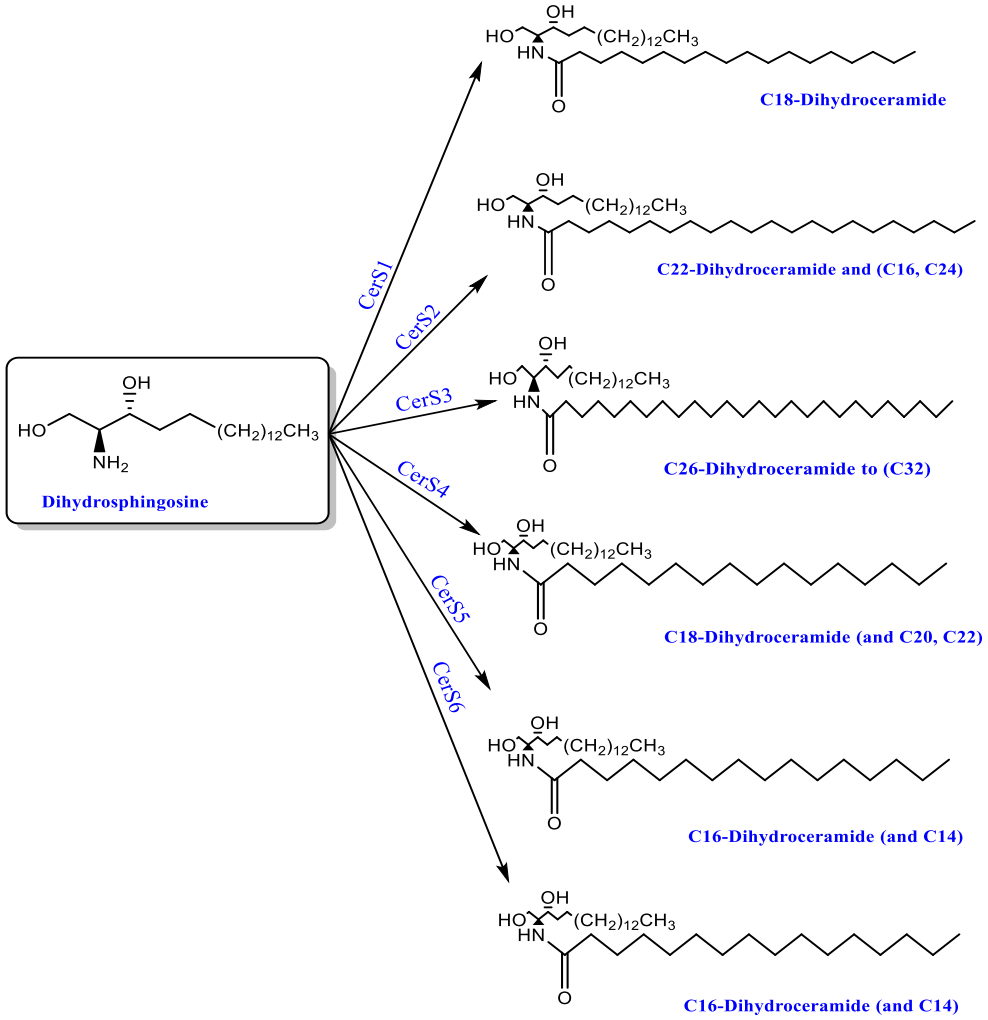

Figure 1. 2: Ceramide synthase specificity*:*

Distinct CerS isoforms catalyse the formation of ceramides with different fatty acid chain length (Adapted from *(*Cingolani *et al.*, 2016*)).*

1.5 Dihydroceramide and dihydroceramide desaturase

The formation of ceramide involves the desaturation of dihydroceramide (dhCer), catalysed by Des1 and Des2. However, there is some debate as to whether Des2 is a true dihydroceramide desaturase. Des1 adds a hydroxyl group to the C4 position of the dhCer backbone, followed by a dehydration reaction, with the use of NADPH or NADH as electron donors. Oxygen is the electron acceptor producing a double bond in the C4-C5 position of Cer (Geeraert *et al.*, 1997, Michel *et al.*, 1997). This reaction occurs at the cytosolic face of ER. Interestingly, 4-hydroxyceramide is the intermediate reaction product, which is also named phytoceramide. The latter is the main ceramide species in plants and yeast. The high expression of Des2 in the intestines, kidneys, and skin, is correlated with high abundance of phytoceramides. whereas Des1 is found in all tissues (Omae *et al.*, 2004, Gault *et al.*, 2010, Hernández-Corbacho *et al.*, 2017). In 1996, Degs1 named drosophila degenerative spermatocyte 1 after cloning the gene encoding Des1 from *Drosophila melanogaster* (Geeraert *et al.*, 1997). DhCers are found in different cell compartments such as, plasma membrane, ER, nucleus, and mitochondria. From a structural context, dhCers do not have transbilayer lipid mobility, which impact many biological processes. Ceramide and dhCers are different in their biophysical behaviour. Indeed, dhCers prevent formation of channels in planar membranes or mitochondria, while ceramides promote this process. Thus, dhCers block the permeabilization of the mitochondrial outer membrane induced by ceramides (Brockman *et al.*, 2004, Podbielska *et al.*, 2012). Therefore, it has been proposed that ceramide and dhCer elicit opposing functions in the cell (Stiban *et al.*, 2006). Nevertheless, in common with ceramides, dhCers are precursors for complex sphingolipids, such as dihydroglycosylceramides, dihydrosphingomyelin and dihydrogangliosides.

Early studies suggested that dhCers were biologically inactive. Therefore, whereas ceramides induce apoptosis and block cell growth, dhCers were ineffective. However, dhCer are implicated in regulating cell growth (Mitoma *et al.*, 1998), platelet aggregation (Simon and Gear, 1998), DNA damage (Ueda *et al.*, 1998), management of ion channels and inhibition of insulin signalling and glucose uptake (Summers *et al.*, 1998, Chik *et al.*, 1999, Siddique *et al.*, 2015). For instance, (Stiban *et al.*, 2006) was the first to show that dhCer inhibits channel formation induced by ceramide in

mitochondria, resulting in blockade of cytochrome c release, which is the first step in the intrinsic apoptotic response. In addition, (Merrill *et al.*, 2005) also demonstrated that fenretinide (4-HPR) increased dhCer levels in DU145 human prostate cancer cells and this was linked with cell death and provided mechanistic information relating to the clinical use of 4-HPR in oncology. DhCer has an essential role in inducing autophagy in cancer cells and is cytotoxic. 4-HPR is chemically similar to dhCer and inhibits Des1 by disrupting the electron transport necessary for the desaturation reaction (Zheng *et al.*, 2006). These discoveries provided impetus for the study of Des1 in a broad context of its biological roles.

4-HPR, C2- dhCer and Des1 inhibitors (section 1.6.1) were reported to induce the formation of autophagosomes. These findings revealed that inhibition of Des1 induces sensitivity to autophagic stimuli. In these studies, the stimulatory effect of dhCer on autophagy conferred resistance to apoptosis (Siddique *et al.*, 2013). However, this is a controversial area as sustained autophagy can lead to apoptosis and this might indeed, underlie some of the actions of dhCer.

Some studies involving pharmacological agents suggest that dhCer regulates cell proliferation. Curcumin, which was separated from turmeric spice, has been reported to inhibit Des1 to induce cell cycle arrest at G2/M phase and induce autophagy in malignant glioma cells (Aoki *et al.*, 2007, Fabrias *et al.*, 2012). In addition, the COX2 inhibitor celecoxib inhibits Des1 activity and promotes dhCer accumulation and growth arrest. These effects were blocked by the SPT inhibitor, myriocin, which prevents *de novo* synthesis of dhCer. Finally, the Des1 inhibitor, XM462 induced the increase of dhCer and this was correlated with delayed G1/S transition and inhibition of cell proliferation (Gagliostro *et al.*, 2012).

The role of dhCer in regulating apoptosis is controversial. Some groups have proposed that the cytotoxic effects of 4-HPR and resveratrol is *via* a dhCer-dependent mechanism. However, inhibiting Des1 activity using other pharmacological reagents, siRNA, or gene depletion confers resistance to apoptosis (Siddique *et al.*, 2012). For instance, in HNSCC, knockdown of Des1 decreases apoptosis in response to photodynamic therapy and reduced mitochondrial depolarisation, late apoptosis, and cell death. The treatment increased dhCer levels without a change in ceramide levels,

although this is rather surprising. The authors proposed that dhCer might block the effect of ceramide on mitochondrial channels. This occurs with very low concentrations of dhCer, implicating that the dhCer:Cer ratio may contribute to whether apoptosis occurs (Stiban *et al.*, 2006, Breen *et al.*, 2013, Siddique *et al.*, 2015). The activities of dhCer suggest that targeting Des1 inhibition may represent a useful strategy for cancer therapy. Other potential therapeutic opportunities of Des1 inhibition include to treat viral infections, obesity, and insulin resistance (Triola *et al.*, 2001, Munoz-Olaya *et al.*, 2008, Casasampere *et al.*, 2016, Pou *et al.*, 2017).

Recent studies report the polyubiquitination of Des1 in response to cells' treatment with the sphingosine kinase (SK) inhibitor, SKi, or Des1 inhibitor, 4-HPR. Polyubiquitinated forms of Des1 exhibit a "gain of function", which allows the activation of p38 MAPK, JNK and XBP-1s pro-survival pathways. In contrast, ABC294640, another SK inhibitor, fails to promote polyubiquitination of Des1 at concentrations that induce *de novo* ceramide synthesis and promote apoptosis via a native Des1-dependent mechanism. These findings are the first to indicate that there are opposing functions of native and polyubiquitinated Des1 in regulating cell survival (Alsanafi *et al.*, 2018).

1.6 Dihydroceramide desaturase (Des1) inhibitors

Many drugs have been reported to inhibit Des1 activity. The first group of compounds claimed to reduce Des1 activity were isolated from natural resources, such as, resveratrol, curcumin, Δ9-tetrahydrocannabinol, and celecoxib. The availability of pharmacological agents that target Des1 has enabled characterisation of the biological role of dhCer (Table 1.2) (Casasampere *et al.*, 2016).

1.6.1 Fenretinide (4-HPR)

4-HPR is a vitamin A analogue (Figure 1.3) and was examined for the prevention of cancer. 4-HPR induces apoptosis and suppresses cancer cell proliferation (Zheng *et al.*, 2006). It also decreases high-fat diet-stimulated obesity and insulin resistance. 4-HPR inhibits Des1 with IC_{50} of 1.68 µM, involving the phenolic group. This finding is supported by the lack of activity in all *trans*-retinoic acid, which has no phenolic group. 4-HPR cytotoxicity is linked with the redox state of the cells and the formation of reactive oxygen species (ROS). Indeed, its inhibitory effect on Des1 appears to be

indirect and involving oxidative stress (Idkowiak-Baldys *et al.*, 2010). 4-HPR induces apoptosis at high concentrations (> 5μM) whereas at lower doses induces G0/G1 cell cycle arrest.

1.6.2 Resveratrol

Resveratrol or (3,5,4`-trihydroxy-*trans*-stilbene) (Figure.1.3) has recognised antioxidant effects. In addition, it has anti-inflammatory, anti-fibrotic effects and anti-tumour activities and is protective in renal diseases, neurodegeneration, diabetes, and cardiovascular diseases. Resveratrol induces programmed death of myelogenous leukaemia and promyelocytic leukaemia cells via a ceramide-dependent mechanism. In most cell types tested, resveratrol blocks proliferation and induces G1/S cell cycle arrest. Resveratrol inhibits Des1 at 50 and 100 μM (Shin *et al.*, 2012) but also inhibits SK1 protein expression (Lim *et al.*, 2012a). In comparison with the Des1 inhibitors, GT-11 and 4-HPR, resveratrol inhibits Des1 in a different manner. Resveratrol induces cell cycle arrest in MDA-MB-231 cells (Dolfini *et al.*, 2007). whereas inhibiting Des1 with resveratrol induces autophagy in HGC27 cells (Signorelli *et al.*, 2009, Rodriguez-Cuenca *et al.*, 2015), The dhCer : Cer ratio could be important to determine cell fate. Resveratrol is known to induce cell death by increasing ceramide (via *de novo* synthesis) in many cancer cell types (Dolfini *et al.*, 2007).

1.6.3 Celecoxib

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-pyrazol-1-yl] (Figure 1.3) inhibits Des1 with IC_{50} of 80 μM. It also increases dihydroceramide while reducing ceramide levels in several cell lines. Inhibition of Des1 by celecoxib has an anti-proliferative effect in gastric cancer cells. It promotes cell cycle arrest (G1/G0 phase) and stimulates apoptosis and autophagy by abrogating the PI3K/AKT signalling pathway (Huang and Sinicrope, 2010, Casasampere *et al.*, 2016).

1.6.4 THC

Δ9-tetrahydrocannabinol (THC) is a bioactive compound isolated from *Cannabis sativa*. THC, HU-211, ajulemic acid (Figure 1.3) and other cannabinoid-based drugs are moderately effective in treating multiple sclerosis, arthritis, and traumatic brain injury. Cannabinoid-based drugs have also been reported to have significant anti-inflammatory effects in inflammatory bowel disease. Cannabinoids also have anti-

tumorigenic effect and inhibit migration and invasion of cancer cells and tumour neovascularization (Velasco *et al.*, 2007, Massi *et al.*, 2013). However, cannabinoids have a limited clinical use, due to their unwanted psychoactive side effects. A study of THC anti-tumour action revealed its mechanism in activating ER stress-regulated protein p38 and tribbles-related protein 3 (TRB3). The latter links ER stress to autophagy and then apoptosis in the mechanism of cannabinoid-induced anti-tumour activity. THC inhibition of Des1 is with IC_{50} of 23 μM in rat liver microsomes. More investigations to study the involvement of dhCer in THC anti-tumour activity is warranted (Klein and Newton, 2007, Velasco *et al.*, 2007).

1.6.5 γ-Tocopherol and γ-tocotrienol

Two natural compounds of vitamin E are γ-tocopherol ((2*R*)-2,7,8-trimethyl-2-[(4*R*, 8*R*)-4,8,12-trimethyltridecyl]-6-chromanol) and γ-tocotrienol ((R) γ-tocotrienol or [*R*-(E,E)]-3,4-dihydro-2,7,8-trimethyl-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-2-H-1-benzopyran-6-ol) (Figure 1.3). They have well known antioxidant activity, can lower blood cholesterol levels, inhibit cancer progression and are neuroprotective. Tocotrienols are cytotoxic (at 40 μM) in MCF7 and MDA-MB-231 human breast cancer cells and this is correlated with autophagy and ER stress, stimulating apoptosis. Tocotrienols are also effective in combination therapies and as radiosensitisers. There are links between γ-tocopherol and γ-tocotrienol and sphingolipids and autophagic-dependent apoptosis (Jiang *et al.*, 2012, Tiwari *et al.*, 2015). Inhibiting the *de novo* ceramide synthesis pathway with, myriocin (SPT inhibitor) or, fumonisin B1 (CerS inhibitor), blocks the effect of γ-tocopherol in inducing cell death, thereby suggesting that γ-tocopherol induces cell death via a dhCer-dependent mechanism. γ-Tocopherol has a lower inhibitory effect on Des1 with 60% inhibition at 100 μM for 36 hours in LNCaP cells (Zheng *et al.*, 2006).

1.6.6 Curcumin

Curcumin, isolated from *Curcuma longa* (turmeric), is also known as ((1 E,6E)-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (Figure 1.3). Curcumin exhibits anti-inflammatory, antioxidant, hypoglycemic and anticancer activities (Ghosh *et al.*, 2015). It is also a modulator of various signalling pathways such as transcription factors, tumour suppressor genes (with a beneficial effect in cancer),

diabetes, Alzheimer's disease and cardiovascular disease (Ghosh *et al.*, 2014). Curcumin inhibits Des1 in HGC27 (human gastric adenocarcinoma cells) (Fabrias *et al.*, 2012). Curcumin affects Des1 indirectly in two different ways: *via* the electron transport chain level or *via* modifying the redox status of the cell. No actions of curcumin have been linked with changes in dhCer levels. However, curcumin induces autophagy of U87-MG and U373-MG cancer cells and promotes cell cycle arrest at G2/M phase by inhibiting protein kinase B and activation of the extracellular signal regulated kinase (ERK-1/2) pathway (Kizhakkayil *et al.*, 2012) that might be linked with Des-dependent signalling.

1.6.7 SKI II

Many studies support the role of SKI II, named as (4-[[4-(4-chlorophenyl)-2-thiazolyl]-amino]-phenol) in inhibiting Des1 activity (Figure 1.3). It is a non-competitive inhibitor inducing a decreases Des1 activity via inhibition of NADH-cytochrome b5 reductase, which was supported by molecular modelling studies. Treatment of HGC27 cells with SKI II decreases S1P levels (consistent with SK inhibition) and increases dhCer levels (Cingolani *et al.*, 2014). There was no increase in ceramide levels despite SK inhibition. SKI II reduces cell proliferation, promotes autophagy, and arrests cells in the G0/G1 phase of the cell cycle (Cingolani *et al.*, 2014).

1.6.8 GT11

GT11 (C8-cyclopropenylceramide) is the first synthesised inhibitor of Des1 and is a sphingolipid analogue (Figure 1.3). Structure/activity studies have reported 2*S*, 3*R* stereochemistry, the presence of a free hydroxyl function at C1 and the cyclopropane replacing the ceramide double bond are required for inhibition of Des1 activity. The inhibitory effect of GT11 is diminished when the N-methyl is substituted or replaced with the amide with carbamate function. Des1 activity is significantly reduced in primary culture cerebellar neurons, when treated with GT11 (IC_{50} of 23 nM). Increasing the concentration to more than 5μM of GT11 enhanced long chain phosphates accumulation, suggesting that GT11 also inhibits S1P lyase activity. In turn, the accumulated S1P down-regulates SPT activity, hence reducing *de novo* sphingolipid biosynthesis (Triola *et al.*, 2004).

1.6.9 XM462

XM462 (5-thiahydroceramide) was synthetically designed with a sulphur atom in place of the C5 methylene group of the enzyme substrate (Figure 1.3). XM462 has an inhibitory effect on Des1 in human leukaemia Jurkat A3 cells (IC_{50} = 0.43 μM) and in rat liver microsomes (IC_{50} = 8.2 μM) (Munoz-Olaya *et al.*, 2008). Additionally, modifications of XM462 resulted in further Des1 inhibitors with slightly different potencies depending on the N-acyl moiety (Camacho *et al.*, 2012). XM462 promotes an increase of dhCer, reduces cyclin D1 expression and delays G1/S transition of cell cycle in human gastric cancer cells, HGC27 cells. This is thought to involve stimulation of ER stress and autophagic responses (Signorelli *et al.*, 2009, Gagliostro *et al.*, 2012).

Table 1. 2: Dihydroceramide desaturase (Des1) inhibitors

Inhibitor	Des1 inhibition (IC_{50})	Compound origin	Other activity
Fenretinide (4-HPR)	1.68 µM	A vitamin A analogue	▪ Induces apoptosis and suppresses cancer cell proliferation.
Resveratrol	50 & 100 µM	First isolated from white hellebore *Veratrum grandiflorum*	▪ Blocks proliferation and induces G1/S cell cycle arrest. ▪ Inhibits SK1 protein expression.
Celecoxib	80 µM	Synthetic	▪ Increases dihydroceramide while reducing ceramide levels in several cell lines. ▪ It promotes cell cycle arrest (G1/G0 phase) and stimulates apoptosis.
Δ9-tetrahydrocannabinol (THC)	23 µM	Isolated from *Cannabis sativa*.	▪ Anti-inflammatory. ▪ Inhibit migration and invasion of cancer cells and tumour neovascularization.
γ-Tocopherol & γ-tocotrienol	100 µM	Nature compounds of vitamin E	▪ inhibit cancer progression. ▪ Tocotrienols are cytotoxic, stimulating apoptosis.
Curcumin	-	Isolated from *Curcuma longa* (turmeric)	▪ Exhibits anti-inflammatory, antioxidant, and anticancer activities ▪ a modulator of various signalling pathways.
SKI II	-	Synthetic	▪ Decreases S1P levels . ▪ Increases dhCer levels in HGC27 cells. ▪ Promotes autophagy.
GT11	23 nM	Synthetic	▪ Inhibits S1P lyase activity.
XM462	0.43 µM	Synthetic	▪ Promotes an increase of dhCer, reduces cyclin D1 expression and delays G1/S transition of cell cycle in human gastric cancer cells, HGC27 cells.

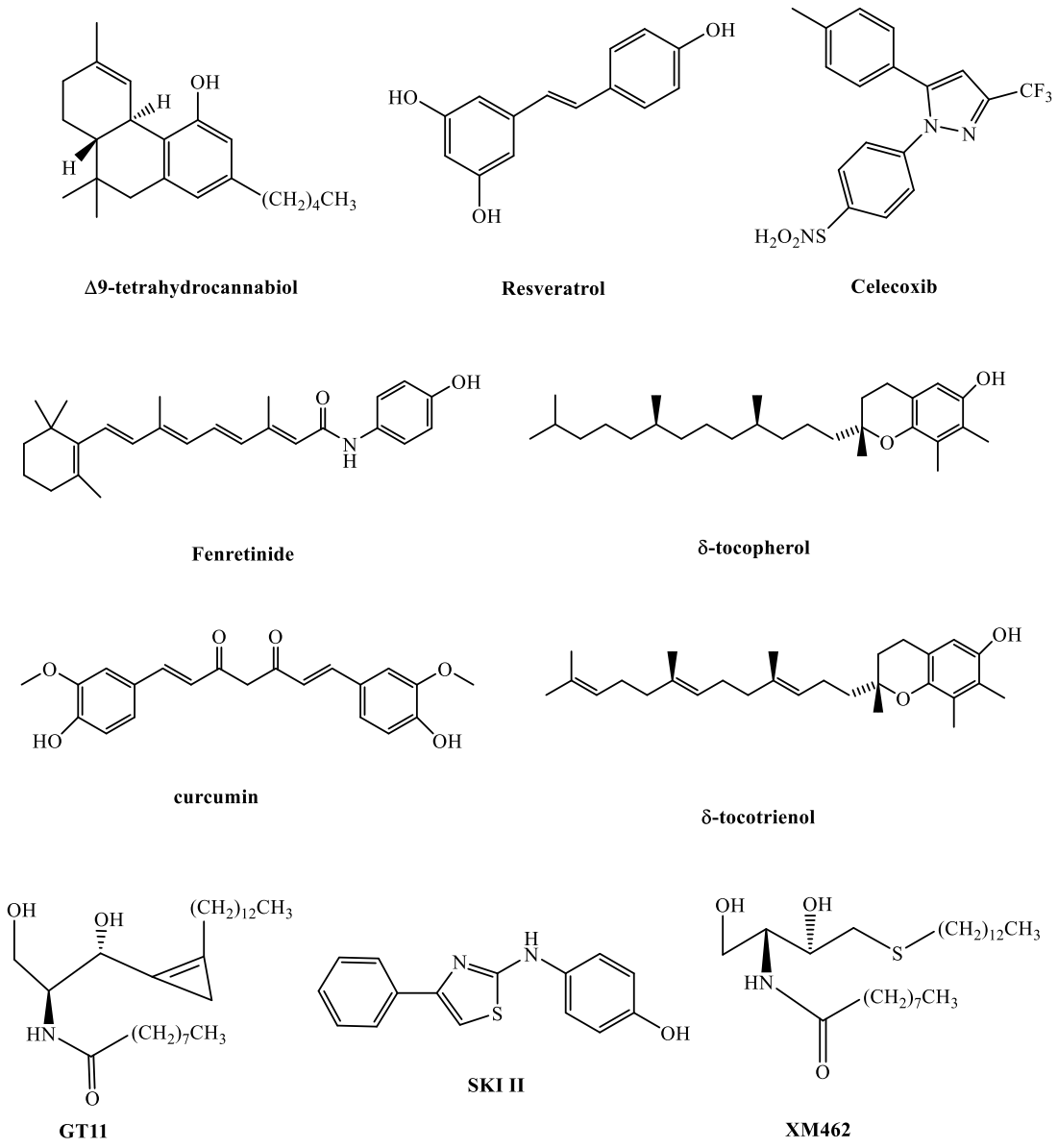

Figure 1. 3: Des1 inhibitors.
Chemical structures for Des1 inhibitors (Adapted from (Casasampere *et al*., 2016)).

1.7 Sphingosine-1-phosphate (S1P)

Sphingosine 1-phosphate (S1P) is a pleiotropic lipid that has a significant impact on many cellular physiology and pathology processes (Spiegel and Milstien, 2003, Bartke and Hannun, 2009). Sphingosine kinase (SK1 and SK2 isoforms), S1P phosphatase (S1PP) and S1P lyase (SPL) control S1P signalling events. SK and SPL are expressed ubiquitously (Wacker *et al.*, 2009). SK1 and SK2 catalyse the synthesis of S1P from sphingosine; S1PP catalyses the reverse dephosphorylation reaction. S1P lyase

irreversibly degrades S1P at the ER by cleavage at the C2-C3 bond, resulting in the formation of phosphoethanolamine and 2-hexadecenal ((Taha *et al.*, 2006b, Saddoughi *et al.*, 2008). SPL helps to determine the ceramide (pro-apoptotic) and S1P (pro-survival) balance: hence, these enzymes are necessary for appropriate cell fate responses and their de-regulation can be linked to pathophysiology of diseases, where cell growth and/or death is abnormal, as in cancer and degenerative diseases (Merrill, 2002).

The levels of extracellular S1P depend on specific cell and tissue types (Cyster and Schwab, 2012). Blood cells (mainly erythrocytes; platelets) and endothelial cells are the major sources of plasma and lymph S1P. This is exported from cells by specific transporters (Hasegawa *et al.*, 2010) to function as a lipid mediator. The intracellular level of S1P is low due to high SPL activity. However, S1PP and SPL are lacking in erythrocytes and platelets store and release S1P. Unlike sphingosine, S1P cannot freely diffuse from the cell, due to its polar nature (Hannun and Obeid, 2008b, Adada *et al.*, 2013). However, it may move between different membranes without being released from cells (Fyrst and Saba, 2010). The endothelial cell S1P transporter, named spinster homologue protein (SPNS2) (504 amino acids; predicted 12 transmembrane protein) is a type of major facilitator superfamily (MFS) transporter, first identified in zebra fish (Visentin *et al.*, 2006, Takabe and Spiegel, 2014). S1P transporters in erythrocytes belong to the ATP-binding cassette (ABC) transporter family including ABCA1, ABCC1 (also known as MRP1) and ABCG2 (Kobayashi *et al.*, 2006, Nishi *et al.*, 2014). ABC transporters were initially known in upregulated cancer cells after treatment with cytotoxic chemotherapeutic agents, as multidrug resistant proteins (MRPs). Recently, Mfsd2b (the major facilitator superfamily 2b), an orphan transporter, has been identified as S1P exporter in red blood cells and platelets. This was confirmed when S1P levels accumulated in mfsd2b knockout mice more than in wild type mice. The Mfsd2b pathway contributes approximately half of the plasma S1P pool (Vu *et al.*, 2017).

Intracellularly, S1P has a second messenger role. This is supported by the finding that sphingosine-induced calcium release is dependent on SK activity (Rosen *et al.*, 2009). Interestingly, S1P generated by SK1 in response to TNF receptor stimulation, binds directly to and activates the E3 ubiquitin ligase activity of TNF receptor-associate

factor 2 (TRAF2), which is an important component in NF-$_k$B signalling, that regulates transcription of pro-survival or anti-apoptosis genes (Alvarez *et al.*, 2010, Xia and Wadham, 2011). Nuclear S1P produced by ERK/MAPK-regulated SK2, is an endogenous inhibitor of histone deacetylases 1 and 2 (HDAC1 and 2) which controls epigenetic gene expression (Hait *et al.*, 2009, Maczis *et al.*, 2016), is another intracellular target of S1P, which regulates cytochrome-*c*-oxidase function and assembly in mitochondria (Strub *et al.*, 2011)). In addition, recent studies suggest the binding of S1P to human telomerase. The catalytic subunit of human telomerase reverse transcriptase (hTERT) is stabilised by phosphorylation. Binding between the hydroxyl group (C′3-OH) of S1P (generated by SK2) and hTERT (Asp^{684}) stabilises hTERT. Thus, hTERT stability is reduced by the inhibition of SK2 or mutation of the S1P binding site in hTERT (Panneer Selvam *et al.*, 2015). Also, S1P binds directly to the transcription factor peroxisome proliferator activated receptor (PPAR)-γ, via its ligand binding domain (LBD). Production of endogenous S1P also increases PPAR-γ expression. In endothelial cells, following S1P activation, PPAR-γ bound to the co-activator factor, peroxisome proliferator activated receptor-γ coactivator 1 (PGC1)b. The PPAR-γ/(PGC1)b complex reduces vascular development *in vitro* and *in vivo* (Parham *et al.*, 2015). S1P also controls the cellular activity of aPKC, as shown by using a genetically encoded reporter, aPKC-specific C Kinase Activity Reporter (aCKAR), Biochemical studies show that S1P directly binds to the kinase domain of aPKC to relieve auto-inhibitory activity. The S1P-dependent activation of aPKC suppresses apoptosis in HeLa cells (Kajimoto *et al.*, 2019). In addition, S1P specifically binds the N-terminal domain of GRP94 and HSP90α, which are heat shock proteins (Park and Im, 2017, Kobayashi *et al.*, 2018, Lidgerwood *et al.*, 2018).

When S1P is transferred out of cells, S1P acts in an autocrine or paracrine manner, termed ‘inside-out’ signalling by binding to specific G protein coupled receptors, S1P$_{1-5}$, previously called the endothelial differentiation gene receptor-1, 3, 5, 6 and 8, which couple through heterotrimeric G protein family members (G_i, G_q and $G_{12/13}$) to regulate signalling through adenylyl cyclase, phospholipase C, MAPK, PKC and Rho pathways (Figure 1.4) (Pyne and Pyne, 2000).

$S1P_1$ is an important receptor on lymphocytes, required for their trafficking (and that of hematopoietic progenitors) through blood, lymph, and peripheral tissues (Im *et al.*, 2000, Kimura *et al.*, 2008, Don *et al.*, 2014) and egress from secondary lymphoid organs (Matloubian *et al.*, 2004) $S1P_1$ is also located in brain, lung, spleen, heart/vasculature and kidney. It is pro-tumorigenic by promoting migration and invasion in many cancer cells (Li *et al.*, 2009b) and is critical for angiogenesis and vascular maturation (Yoon *et al.*, 2008). $S1P_2$ is widely expressed and plays a role in cancer progression. The receptor has different functions in cancer cells and is also essential for development of vestibular and hearing functions (Lee *et al.*, 1998). $S1P_{2/3}$ are useful in perinatal survival and play a major role in the cardiovascular system (Patmanathan *et al.*, 2017). $S1P_3$ is highly expressed in heart, lung, spleen, kidney, intestine, diaphragm, and certain cartilage enriched regions. $S1P_4$ receptors is expressed in the lymphoid system and $S1P_5$ is expressed in the white matter tracts of the central nervous system (CNS).

Activation of $S1P_1$ with spatially limited formation of S1P is essential for PDGF-directed cell movement. S1P receptors regulate various signalling pathways such as, Rho family (Rac and Rho) (Jo *et al.*, 2005), adenylyl cyclase, c-Jun N-terminal kinase (JNK), phospholipase C (PLC) and intracellular calcium, PI3K/AKT and ERK1/2 signalling (Pyne and Pyne, 2000, Pyne *et al.*, 2009, Patmanathan *et al.*, 2017). These are linked to cell survival, motility, and angiogenesis. De-regulation of these pathways is related to the development and progression of a variety of cancer types (Pyne *et al.*, 2014).

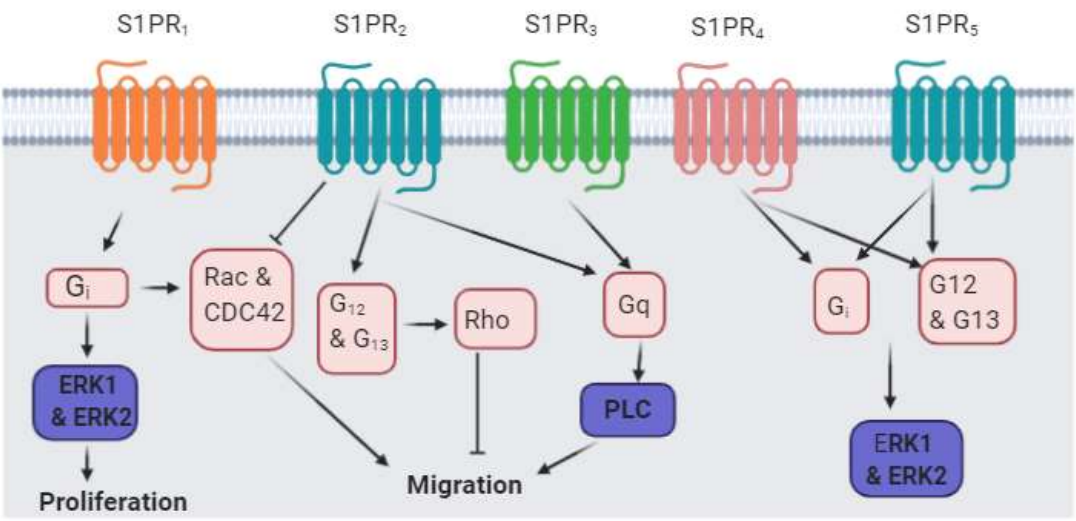

Figure 1. 4: S1P receptors.
Sphingosine-1‑phosphate (S1P) receptors in terms of regulating the motility, growth and survival of cancer cells are shown (Adapted from (Pyne and Pyne, 2010)).

S1P receptors couple to one or more G-proteins (Figure 1.4). $S1P_1$ is exclusively coupled to G_i. $S1P_2$ and $S1P_3$ couple to multiple G proteins, including G_i, $G_{12/13}$ and G_q and $S1P_4$ and $S1P_5$ signal through G_i or $G_{12/13}$ and G_i or G_{12} subunits, respectively (Susann and Bodo, 2010). S1P receptor complexity of coupling to different G-proteins increases the repertoire of signalling pathways that can be activated. For instance, the $S1P_1$ receptor activates Ras and the ERK1/2 pathway to promote proliferation. The receptor is also linked with the activation of PI3K/AKT signalling to promote cell survival. In addition, the stimulation of PI3K and the small GTPase Rac induce cell migration, increase endothelial barrier function, and induce vasodilation. The activation of PLC increases intracellular free calcium ($[Ca^{2+}]_i$), which is essential for several cellular responses including contraction of smooth muscle cells. $S1P_2$ receptor coupling to $G_{12/13}$ activates Rho (a small GTPase) and the Rho-associated kinase (ROCK) to decrease endothelial barrier function, induce vasoconstriction and inhibit migration (Brinkmann, 2007, Yester *et al.*, 2011, Takuwa *et al.*, 2012). Additionally,

stimulation of S1P receptors can contribute to protection against the apoptosis (Figure 1.4). Targeting S1P receptors for therapeutic benefit has been achieved, e.g. with Gilenya® for relapsing and remitting multiple sclerosis (Sanford, 2014).

S1P production and release through transporter proteins to the extracellular milieu has been established. However, its presence in a lipid microenvironment close to S1P receptors might allow effective binding of S1P to S1P receptors (Takabe *et al.*, 2008). Additionally, intracellular S1P and its balance with ceramide in the sphingolipid rheostat (Figure 1.5) regulates cell growth or death in response to cellular stimuli (Cuvillier *et al.*, 1996, Spiegel and Milstien, 2003). Ceramide has intracellular targets that activate its apoptotic effect (Section 1.4), whereas intracellular targets of S1P are associated with cell survival and proliferation. The sphingolipid rheostat regulates survival, apoptosis, chemoresistance and radioresistance of cancer cells *in vitro* in many ways. For example, siRNA knock down of SK1 expression decreases cell proliferation and enhances the ceramide/S1P ratio to induce apoptosis of prostate, pancreatic and leukaemia cells (Akao *et al.*, 2006, Pyne and Pyne, 2010). Also, siRNA knockdown of SK2 implicates this enzyme in proapoptotic effects in mouse embryonic fibroblasts (MEF) cells. This could be due to the BH3 domain in SK2 or due to the release of cytochrome c following TNF stimulation (Liu *et al.*, 2003). Therefore, the sphingolipid rheostat is a target for drugs that inhibit SK1 and/or SK2 to tilt the balance toward ceramide and to thereby induce cytotoxicity in cancer cells.

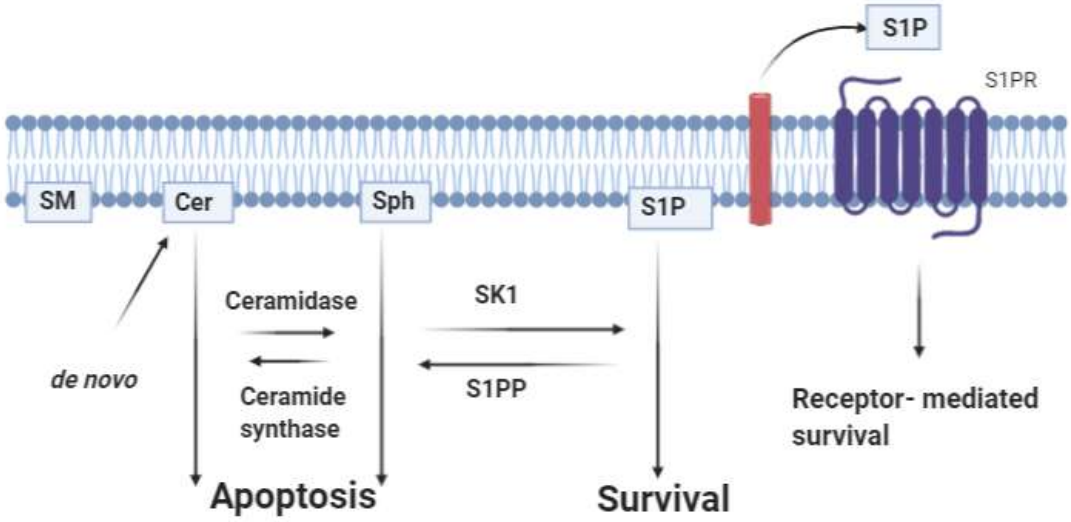

Figure 1. 5: The ceramide–sphingosine–S1P rheostat, 'inside-out' signalling and cancer cell survival.
(Adapted from (Pyne and Pyne, 2010)).

1.8 Sphingosine kinases

Two mammalian sphingosine kinases, SK1 and SK2, have been cloned and characterised (Sukocheva *et al.*, 2003, Lai *et al.*, 2008). There are three splice variant forms of SK1 (SK1a, SK1b and SK1c) and two variants of SK2 (Venkataraman *et al.*, 2006). SK1 (43 kDa) and SK2 (65 kDa) are encoded by different genes: the SK1 gene is on chromosome 17 (17q25.2) and that for SK2 on 19 (19q13.2). The two isoforms possess 80% amino acid sequence similarity (Pitson *et al.*, 2002). They share five highly conserved regions within their polypeptide sequence, termed C1–C5 domains. However, SK2 has an extended N-terminus and a distinct central region (Figure 1.6). Both enzymes catalyse phosphorylation of sphingosine to produce S1P (Kohama *et al.*, 1998). However, they are distributed differently in organs and tissues, with SK1 mRNA being most abundant in the brain, heart, thymus, spleen, kidney, and lung (Melendez, 2008), whereas SK2 mRNA is most abundant in the kidneys and liver (Pitson *et al.*, 2003, Liu *et al.*, 2012). SK1 and SK2 differ in their kinetic properties and subcellular localizations. Therefore, while the SKs share some similarities, they have distinct cellular functions and are differently regulated. For example, SK1 localises mainly in the cytoplasm and upon agonist-receptor stimulation can be

phosphorylated by ERK1/2 at Ser225. This enables the translocation of SK1 from the cytoplasm to the plasma membrane, which requires the protein CIB1 (calcium- and integrin-binding protein 1) (Jarman *et al.*, 2010). Interestingly, other phosphorylation-independent mechanisms of SK1 translocation to the plasma membrane have also been reported (Gault *et al.*, 2012) together with increased secretion of S1P from the cells allowing for the autocrine/paracrine signalling (Johnson *et al.*, 2002). SK1 is also found close to the nucleus (the perinuclear region) (Delon *et al.*, 2004) and in the nucleus itself (Kleuser *et al.*, 2001). This re-localisation of SK1 to the plasma membrane provides evidence for its signalling role in inducing cell proliferation and survival because it allows access to the substrate, sphingosine. SK1 activation appears essential for mediating oestrogen-dependent regulation of breast tumour cell growth and survival, as well as ERK1/2 activation (Sukocheva *et al.*, 2003). PDGF activates SK in both the cytosol and the nucleoplasm, with associated progression of cells through the cell cycle (Kleuser *et al.*, 1998). Therefore, the location and activation of SK both determine the specificity of signalling by these enzymes.

SK2 is often localised to the nucleus (Pitson *et al.*, 2003, Pitman *et al.*, 2016) but this depends on cell type and cell density (Maceyka *et al.*, 2005a, Leclercq and Pitson, 2006). In Hela cells, the enzyme is mainly nuclear, whereas in HEK293 cells the enzyme is cytosolic (Igarashi *et al.*, 2003). COS7 cells express high levels of SK2 in the cytoplasm at low confluence and high levels in the nucleus at high confluence. Moreover, SK2 translocation to the nucleus supresses DNA synthesis and may induce growth arrest. Phosphorylation of SK2 by protein kinase D (PKD) appears to regulate its nuclear-cytoplasmic shuttling under physiological conditions (Ding *et al.*, 2007).

Basal SK activity maintains low sphingosine levels in the cell to reduce cytotoxicity (Chan and Pitson, 2013). Its activity is regulated by both post-translational and transcriptional processes by growth factors (Maceyka *et al.*, 2012). Activators of SK include G-protein coupled receptors (GPCRs), small GTPases, tyrosine kinase receptors, pro-inflammatory cytokines and immunoglobulin receptors, calcium, and protein kinases (Schnute *et al.*, 2012). The activation of SK2 can be induced by epithelial growth factor (EGF) and ERK1/2-dependent phosphorylation (Hait *et al.*, 2007). Fc-receptor engagement in mast cells also activates SK2 (Hait *et al.*, 2005, Olivera *et al.*, 2006).

SK1 affects cell transformation and tumour growth and is involved in Ras-mediated oncogenesis in NIH3T3 cells (Xia *et al.*, 2000). Over-expression of SK1 occurs in carcinomas of the breast, such as MCF-7 breast cancer cells, and increases tumorigenesis in nude mice, possibly by promoting cell growth and neovascularisation of the tumour (Nava *et al.*, 2002). This also occurs in prostate, colon, oesophagus, and lung, as well as other cancers (Aurelio *et al.*, 2016). Indeed, melanoma cells that are resistant to therapy show high levels of S1P to ceramide and express more SK1. Overexpression of anti-apoptotic protein, B-cell lymphoma 2 (Bcl-2) is associated with increased SK1 levels (Bektas *et al.*, 2005, Sordillo *et al.*, 2016). Therefore, SK1 has anti-apoptotic effects and protects cells from TNFα, ionising radiation or anticancer drugs (Song *et al.*, 2011). In contrast, SK1 inhibition with *N*, *N*-dimethylsphingosine (SK inhibitor) (Endo *et al.*, 1991), or G82D (dominant negative SK1 mutant) (Pitson *et al.*, 2000), markedly inhibited cell growth and supressed Ras-induced cell transformation in various tumour cells (Pitson *et al.*, 2000). It has also been stated that siRNA-induced down-regulation of SK1, but not SK2, supresses the ability of TNFα to induce COX-2 expression and to produce prostaglandin E2 (PGE2) in L929 murine fibrosarcoma and A549 human lung carcinoma cells (Pettus *et al.*, 2003). Conversely, siRNA targeting of SPL and S1PP increased TNFα-induced COX-2 expression, followed by an augmented production of PGE2 (Sano *et al.*, 1995). COX-2 expression and PGE2 production have been implicated in humans and rodent colon carcinogenesis. Undeniably, COX-2 inhibition results in reduction in development of colon cancer, whereas administration of PGE2 enhances colon carcinogenesis (Kawamori *et al.*, 2003, Kawamori *et al.*, 2006). Interestingly, SK1 facilitates the GPCR-dependent transactivation of the EGFR signalling, suggesting the key role of SK1 in directing mitogenic signalling in cancer cell growth (Wang *et al.*, 2005). Furthermore, under stress conditions, such as starvation, SK1 promotes cells survival and growth (Le Scolan *et al.*, 2005). SK1 is associated with chemo-resistance (Baran *et al.*, 2007). Increased expression of SK1 has been reported in a broad range of human solid cancers and haematological malignancies (French *et al.*, 2003). Therefore, SK1 is a potential therapeutic anticancer target.

While the role of SK2 in cancer is less well known, its high expression in human non-small cell lung cancer has lately been associated with poor survival rates of patients

(Song *et al.*, 2011). Numerous SK2-selective inhibitors have anticancer effects (French *et al.*, 2010). Indeed, the SK2 inhibitor, ABC294640 (Yeliva/Opaganib) has progressed to clinical trials, in the treatment of cholangiocarcinoma. Additionally, siRNA knockdown studies also show the value of targeting SK2 in some cancers, including acute lymphocytic leukaemia (ALL) (Wallington-Beddoe *et al.*, 2014b) multiple myeloma, glioblastoma (Kummetha Venkata *et al.*, 2014, Tea *et al.*, 2020). Indeed, knock down of SK2 in U-1242 MG cells was more effective than SK1 knockdown in blocking proliferation of U-87 glioblastoma cells (Van Brocklyn *et al.*, 2005). Knockdown of SK1and SK2 in A498 cancer cells resulted in induction in the percentage of cells in G1 phase and cell cycle arrest in the S and G2-M phases (Gao *et al.*, 2012). The effect of SK2 knockdown was greater than that of SK1 knockdown. Thus, while both SK isoforms are implicated in cancer, different cancer types appear to involve only one or other isoform.

The SK1 crystal structure has accelerated progress in the SK field (Wang *et al.*, 2014). This provided deep knowledge of the binding sites, substrate binding pocket and other binding domains, to facilitate the development of SK inhibitors. Additionally, the sequence similarity of SK1 with SK2 and molecular modelling of SK2 has presented molecular insights to distinguish between these two enzymes in their sphingosine binding and ATP binding sites (Wang *et al.*, 2013, Pitman *et al.*, 2015), allowing the discovery of isoform-selective inhibitors. Undeniably, the latest identified selective inhibitors have revealed the major roles of SK1 and SK2 in regulating cellular processes. Notably, one SK1 inhibitor, PF-543, was unable to induce apoptosis in cancer cells, even though significantly decreasing cellular S1P levels (Schnute *et al.*, 2012), With SK2 inhibitors, there are some inconsistency in that they either increase (Kharel *et al.*, 2015) or decrease S1P levels (Beljanski *et al.*, 2011). In mice. Therefore, there is a need of re-evaluate the inhibitors' mechanisms to assess on- and off-target effects. Certainly, crucial 'off targets' have been described for ABC294640, such as Des1 (Venant *et al.*, 2015, McNaughton *et al.*, 2016) and the oestrogen receptor (Antoon *et al.*, 2011).

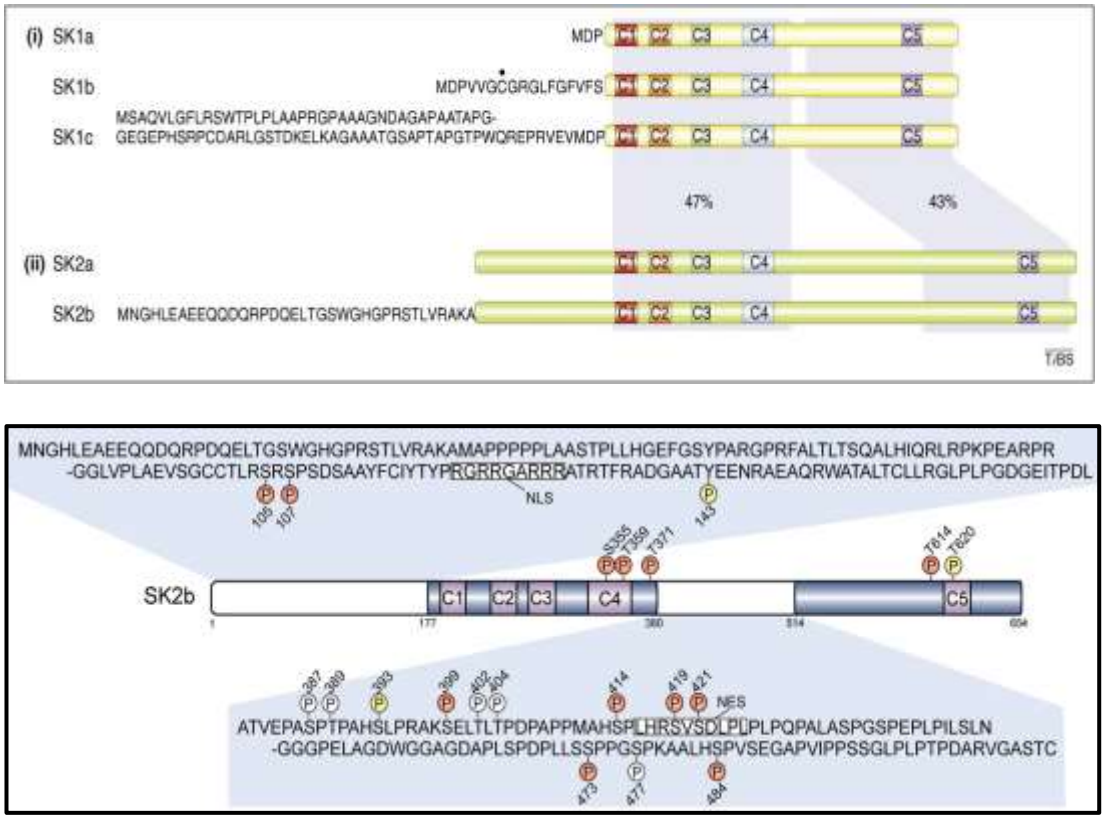

Figure 1. 6: Human SKs isoforms.
Regions of homology and difference between SK1 and SK2. SK2 has an N terminal extension, additional central region with a nuclear export sequence (NES) and a putative transmembrane domain (Adapted from (Neubauer and Pitson, 2013)).

1.8.1 The role of sphingosine kinase in cancer

Cancer hallmarks organised into eight major hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming energy metabolism and evading immune response and enabling genome instability and mutation, and tumour-promoting inflammation (Hanahan and Weinberg, 2000, Fouad and Aanei, 2017). SK1 and S1P are engaged in many of the hallmarks of cancer cells. Cancer cells develop a "non-oncogene addiction" for SK1, as no activating mutations have been identified in the SK1 gene (Vadas *et al.*, 2008). It has been documented that SK1 is able to protect tumour cells against apoptotic stimuli (e.g. TNF, Fas ligand, serum deprivation, radiation, anticancer drugs) (Kane *et al.*, 1999). For instance, stimulating normal human endothelial cells by TNF, activates SK1 to protect against apoptosis. Indeed, a natural transformation of C11 (endothelial cell line) could not

activate SK1 in response to TNF and enhance TNF-dependent apoptosis (Xia *et al.*, 1999). SK1 block apoptosis is related to inhibition of caspases 3, 6 and 7 (Cuvillier *et al.*, 1998) and changes in Bcl2 family pro- and anti-apoptotic proteins (Limaye *et al.*, 2005). This leads to release of cytochrome c and Smac/ DIABLO from mitochondria (Cuvillier *et al.*, 2001). Thus, SK1protects against apoptosis via both the intrinsic and extrinsic pathways (Vadas *et al.*, 2008) (see section 1.9). It has been noted that knockdown of SK2 results in compensatory increases in expression of SK1 (Liang *et al.*, 2013) adding further complexity to the system. Since SK2, but not SK1, could be harmful in some inflammatory diseases and some cancers, it can be advantageous to target both SKs to counter effects by both enzymes.

SK1 knockdown results in a reduction of S1P with induction in cellular ceramides, dihydroceramides and sphingosine (Van Brocklyn *et al.*, 2005), as well as an alteration in the molecular ceramide species composition, dependent on cell type. For example, there is an increase in pro-apoptotic long chain (C24) ceramides in 'heavy membranes' (isolated by fractionation of SK1 knockdown cells) in comparison to whole cell extracts. This indicates that SK1 affects ceramides subcellular distribution (Taha *et al.*, 2006a). In contrast, ceramide levels were only slightly affected via SK2 knockdown (Gao and Smith, 2011), with variations enhanced only by exogenous addition of sphingosine (Maceyka *et al.*, 2005b). Additionally, in A498 kidney cancer cells, the overall ceramide levels are not affected by SK2 knockdown whereas S1P levels increased, accompanied by upregulation of SK1. However, knockdown of both SK enzymes in these cells blocked SK1 compensation, resulting in reduction in S1P levels and significant increase in long chain ceramides (≤ C22) levels (Gao *et al.*, 2012).

Overexpression of SK1 decreases apoptosis and increases proliferation in cells, consistent with a role for this enzyme in oncogenesis (Olivera *et al.*, 1999). Other studies support a role for SK1 in naturally occurring tumours. Thus, SK1 mRNA levels are elevated in many human tumours (French *et al.*, 2003). Indeed, high SK1 expression is correlated with poor prognostic survival of patients in several cancer types.

Initial studies described pro-apoptotic (Mullen and Obeid, 2012) effects of forced overexpression of SK2. Consistent with this, endogenous SK2 knockdown using

siRNA in HEK293, or mouse embryonic fibroblasts suppressed TNF-α–dependent apoptosis (Chipuk *et al.*, 2012). However, several studies demonstrate a pro-cancer role for SK2. Notably, inhibiting SK2 either with pharmacological agents or its elimination by siRNA in various cancer cell lines, has an anticancer effect (Van Brocklyn *et al.*, 2005). *In vivo* studies using genetic deletion of SK2 in MCF-7 breast tumour xenografts have also shown a significant reduction in tumour growth (Weigert *et al.*, 2009) Pharmacological inhibition of SK2 in several mouse tumour models, including breast, kidney, pancreatic, liver and colon cancer also reduces cancer growth (French *et al.*, 2010). Furthermore, SK2 has a key role in inducing migration *via* transforming growth factorβ (TGFβ) in oesophageal cancer cells and EGF in breast cancer cells, indicating a potential role for SK2 in metastasis (Hait *et al.*, 2005, Miller *et al.*, 2008).

The elevation of SK1 expression in many cancers could be of prognostic value (Facchinetti *et al.*, 2010, Heffernan-Stroud and Obeid, 2013). Indeed, expression of SK1 is high in ER negative breast tumours, and this correlates with shorter survival times (Ruckhäberle *et al.*, 2008). Furthermore, high expression of SK1 in non-Hodgkin lymphomas, astrocytoma, gastric cancer, salivary gland carcinoma, oesophageal carcinoma, non-small-cell-lung and HNSCC also correlates with shorter disease-specific survival time (Li *et al.*, 2008, Li *et al.*, 2009c, Facchinetti *et al.*, 2010, Pan *et al.*, 2011).

Indeed, the overexpression of SK1 in prostate cancer cells can promote resistance to chemotherapy by reducing the ceramide/S1P ratio (Pchejetski *et al.*, 2005). SK1 over-expression also reduces the sensitivity of A-375 melanoma cells to Fas- and ceramide-dependent apoptosis, while siRNA knockdown of SK1 decreases apoptotic resistance in Mel-2a cells (Bektas *et al.*, 2005, Heffernan-Stroud and Obeid, 2013). Furthermore, it has been shown that dasatinib and resveratrol induce apoptosis by down-regulating SK1 and up-regulating CerS genes to increase the ceramide/S1P ratio in K562 chronic myeloid leukaemia cells (Gencer *et al.*, 2011, Kartal *et al.*, 2011). This opposing regulation of CerS and SK1 has also been observed in primary breast cancer samples (Erez-Roman *et al.*, 2010).

SK1/S1P signalling is associated with drug resistance, while lately SK2/S1P signalling has been reported to confer chemo-resistance to cancers. Thus, using pharmacological agents to inhibit SK2 sensitises cancer cells to chemotherapy. For example, ABC294640 (SK2 inhibitor) reduces (ER)-positive breast cancer tumour growth by ~68 % in comparison with vehicle-treated tumours (Selvam and Ogretmen, 2013), and blocks proliferation of MDA-MB-231 endocrine therapy-resistant and chemo-resistant MCF-7TN-R cells (Antoon *et al.*, 2011). Furthermore, hypoxia enhances SK2 activity followed by S1P secretion and action via $S1P_1$/$S1P_3$ to stimulate ERK1/2 signalling and to thereby grant resistance to etoposide-induced apoptosis of A549 lung cancer cells (Schnitzer *et al.*, 2009). Thus, both SK1 and SK2 are involved in numerous cancer types.

1.8.2 The role of sphingosine kinases in inflammation

SK1 and SK2 are also involved in inflammation and could serve as potential therapeutic targets in inflammatory diseases. For example, they participate in neutrophil priming and macrophage responses (Ibrahim *et al.*, 2004, Baumruker *et al.*, 2005) and in vascular endothelial cell adhesion molecule expression. SK1 and SK2 have been involved in asthma, being related to determining the allergic response of mast cells and also, inducing constriction of airway smooth muscle cells SK1 also has a role in hypertension, by controlling of vascular smooth muscle cell contraction (Bolz *et al.*, 2003).

SK1 has a pro-inflammatory role in rheumatoid arthritis, while exerting a protective role in neuro-inflammation (Baker *et al.*, 2010, Nayak *et al.*, 2010, Grin'kina *et al.*, 2012, Pyne *et al.*, 2016b). Therefore, its inflammatory role appears to have a cellular-specific context. In the immune system cells, SK activation occurs in response to crosslinking of immunoglobulin surface receptors during immune cell activation (Jolly *et al.*, 2002). SK is also activated in response to pro-inflammatory cytokines and inflammatory regulated growth factors, such as PDGF (Suzuki *et al.*, 2007, Hannun and Obeid, 2008b), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), insulin-like growth factor (IGF), IGF binding protein 3 (IGFBP3), lysophosphatidic acid (LPA), lipopolysaccharide (LPS), compliment 5a (C5a), TNFα, and IL-1β (Snider *et al.*, 2010). In addition, SK is involved in regulating the stimulation

of many proteins relevant to inflammation, such as COX2 and monocyte chemoattractant protein-1 (MCP-1) (Pettus *et al.*, 2003).

Patient samples and mouse models of ulcerative colitis (UC) and inflammatory bowel disease (IBD) are associated with an increase in the SK/S1P pathway (Snider *et al.*, 2009). Pharmacological inhibition of SK1 (Maines *et al.*, 2008) decreases disease parameters in these inflammatory/immune diseases. SK1/S1P and the S1P receptors also regulate a variety of immune cell types implicated in inflammatory diseases. In platelets, activation and release of S1P from granular stores occurs upon activation of the coagulation cascade in acute inflammation In bronchial epithelial cells, S1P plays a role in activation, survival and adherence of macrophages to the external matrix through integrins (Alemany *et al.*, 2007), while SK1 knockdown reduces TNFα, IL-6, and IL-8 in macrophages (Venkataraman *et al.*, 2006).

SK1 is involved in NF_KB regulation via TNFα, which drives pro-inflammatory responses. For example, SK1 binds to TRAF-2 and S1P binds to and stimulates TRAF-2 E3 ligase to polyubiquitinate RIP1 (lysine(K)-63-linked polyubiquitination). RIP1 has a functional role in regulating I_KB kinase phosphorylation, thereby inducing I_KB degradation and NF_KB activation. Therefore, the regulation of K63 polyubiquitination by SK1/S1P represents is a new signalling pathway in inflammation (Alvarez *et al.*, 2010, Pyne *et al.*, 2016a). Given the key role that SK/S1P in the inflammatory pathology, it is important to develop SK inhibitors to target inflammatory disease (Baker *et al.*, 2013, Gandy and Obeid, 2013b).

Pharmacological inhibition of SK can prevent inflammatory diseases, such as arthritis and atherosclerosis in two ways: by inhibiting lymphocyte egress and reducing cytokine signalling. The emergence of FTY720 (Gilenya®) and other S1P receptor modulators indicates that S1P receptors are involved in autoreactive T-cell pathology (Allende *et al.*, 2004, Matloubian *et al.*, 2004). Thus, S1P recruits' lymphocytes to local areas of inflammation and spreads the inflammatory signalling response. For example, downregulation of SK in a collagen-induced arthritis model in mice decreases disease severity and plasma levels of TNFα, IL-6, IFN-γ and S1P (Lai *et al.*, 2008). FTY720 (a sphingosine mimic and pro-drug, active as FTY720-phosphate) reduces the number of mature circulating lymphocytes which are retained in the

thymus and secondary lymphoid tissues due to down-regulation of $S1P_1$ in response to FTY720-phosphate (Chiba *et al.*, 2006). This immunomodulation has triggered the study of FTY720 treatment in various inflammatory diseases where SK1/S1P is involved including colitis, arthritis, and asthma (Snider *et al.*, 2010). SK1 also has a vital role in the production of pro-inflammatory agents from human macrophages in response to anaphylatoxins. SK1 also regulates these cells' motility, consistent with SK1 being a prospective target for therapeutic use in the treatment of inflammatory and autoimmune diseases (Baumruker *et al.*, 2005).

Binding of S1P to its receptor $S1P_1$ improves vascular barrier integrity and endothelial barrier resistance (Garcia *et al.*, 2001). Also, S1P reduces vascular dysfunction in response to thrombin (Schaphorst *et al.*, 2003) and vascular permeability in response to VEGF (Sanchez *et al.*, 2003). In addition, activated protein C (APC) protects the endothelial barrier by stimulating SK1 through a protease activated receptor-1 (PAR-1)-dependent mechanism, thus increasing S1P production and $S1P_1$ activation (Feistritzer and Riewald, 2005).

Neutrophils cause tissue damage in the early acute inflammatory response, when highly stimulated or when avoiding apoptosis. Neutrophil priming agents, such as platelet-activating factor (PAF) and TNFα, rapidly induce an increase in SK activity. Moreover, the SK inhibitor *N,N*-dimethylsphingosine (DMS) inhibits this activation and PAF-induced calcium signalling, suggesting that the intracellular calcium elevation by these agents is SK-dependent. SK1 is also necessary for catestatin-stimulated migration of monocytes (Egger *et al.*, 2008). Finally, macrophages treated with LPS have increased SK1 activity, leading to generation of S1P and induction of COX2 (Hammad *et al.*, 2008).

1.9 Sphingolipids and programmed cell death

Cancer cells are immortal and do not undergo the normal life-death process. However, they can be forced to die by chemotherapeutic agents. There are many different types of death including apoptosis, necrosis, autophagic cell death and caspase-dependent and independent cell death (Leist and Jäättelä, 2001, Wyllie and Golstein, 2001, Bröker *et al.*, 2005). Cancer cells resist cell death through resistance mechanisms such as, up-regulation of the expression of anti-apoptotic genes (e.g., Bcl-2 (B cell

lymphoma 2) family members, which improve membrane stability of mitochondria and ER) or through inactivation of pro-apoptotic genes (e.g., p53, BAX and APAF1 (Apoptotic Protease Activating Factor 1). Regarding pro-apoptotic genes, different mutations resulting in the reduction or loss of protein function or expression have been found in many cancer cells. Cancer cells also use immunosuppressive strategies to escape cytotoxic CD8+ T cells and NK cells (Igney and Krammer, 2002).

Anti-apoptotic proteins work as firewalls towards chemotherapy, radiotherapy, as well as ceramide and its metabolites (Decaudin *et al.*, 1997). The anti-apoptotic mechanism prevents the re-localisation of pro-apoptotic mitochondrial proteins and thereby limits release of cytochrome c (Yang *et al.*, 1997) and calcium into the cytoplasm (Lam *et al.*, 1994).

1.9.1 Apoptosis

Programmed cell death (PCD) regulates normal cell growth/death in various organisms such as aging, and tissue homeostasis. Apoptosis is one of several PCD mechanisms, in which complex molecular signalling systems are triggered (Fuchs and Steller, 2011). Morphological changes shown during apoptosis include cell rounding, shrinkage of pseudopods, plasma membrane blebbing, decreased cellular volume, chromatin condensation and nuclear fragmentation. In the body, apoptotic cells are ingested and degraded by phagocytes.

PCD involves three steps: initiation, commitment, and execution. First, initiation occurs at or in the cellular compartment where a stress occurs (Feng and Hannun, 1998). For example, disruption of calcium homeostasis at the ER results in the activation of calcium-mediated cell death. Initiation is followed by activation of specific biochemical pathways involving for instance, PI3K/AKT signalling. The commitment step occurs at the mitochondrion level is the no return point (Ferri and Kroemer, 2001). Loss of the mitochondrial outer membrane permeabilisation (MOMP) releases numerous proteins from the intermembrane space into the cytoplasm. This includes cytochrome c, which is most examined. This is followed by its oligomerisation with the adapter molecule APAF1 and the triggering of caspase 9 activity. The latter activates caspases 3 and 7, execution caspases that progress programmed cell death (Wolf and Green, 1999). This pathway is the intrinsic apoptotic

process. The mitochondria have other proteins which, when released, drive caspase-independent cell death. After MOMP takes place, mitochondrial apoptosis inducing factor (Matrone *et al.*, 2017) and endonuclease G (Endo G) move to the nucleus and enhance apoptosis-like PCD, which involves chromatin condensation (Hegde *et al.*, 2002). The intrinsic apoptotic pathway can occur as a consequence of ER stress or in response to ultraviolet radiation, free radicals or cytotoxic drugs, which prompt DNA damage (Soengas *et al.*, 1999). DNA damage and ER stress activate Bax/Bak (pro-apoptotic members of the Bcl-2 family) and induce MOMP, eventually resulting in caspase-dependent/independent apoptosis (Lakhani *et al.*, 2006).

The extrinsic apoptotic pathway is started by stimulation of cell surface death receptor (Fas, DR5, TNF-R1) by their ligands (FasL, TRAIL, and TNF). Both pathways involve caspase activation that results in apoptotic cell death (Figure 1.7) (Schmitz *et al.*, 2000). Crosstalk between the intrinsic/extrinsic pathways also occurs.

Extrinsic pathway death receptors include members of the tumour necrosis factor (TNF) receptor superfamily (CD95, TRAIL-R1 (TNF-related apoptosis-inducing ligand-R1) and TRAIL-R2 (TNF-related apoptosis-inducing ligand-R2). These proteins are characterised by an intracellular 'death domain' (Yu *et al.*, 1999). In contrast, a non-signalling receptors of TNF superfamily, called decoy receptors, are closely similar to death receptors but lack the death domain and have lower death receptor function (Ashkenazi, 2002).

The intracellular protein FADD (FAS-associated death domain) forms a complex with the death domain of activated death receptors which then activates caspases 8 and 10 (Sprick *et al.*, 2000). Sometimes this pathway fails to initiate apoptosis, which demands the engagement of mitochondria via the intrinsic pathway. Hence, caspases 8 and 10 cleaved BID (a BCL-2 family protein) moves to the mitochondria. In the cytoplasm, a complex form between cytochrome c and APAF1 (apoptotic inactive initiator caspase activating factor 1) and inactive pro-caspase 9 in a complex called the "apoptosome". This results in mitochondrial initiator caspase (caspase-9) activation (Zamzami and Kroemer, 2001), which results in cleavage of different substrates like cytokeratins, poly-(ADP-ribose) polymerase (PARP) and plasma membrane cytoskeletal proteins (alpha fodrin), which consequently provoke morphological and

biochemical changes associated with apoptosis (Ghavami *et al.*, 2009). Other target proteins that are proteolysed are lamins, the 70-kDa protein of the U1 snRNP, topoisomerase I and II and the retinoblastoma protein, fodrin (Rosen and Casciola-Rosen, 1997).

Cleavage of PARP to an 89 kDa fragment (Lazebnik *et al.*, 1994) is an early event in apoptosis, occurring after chromatin DNA fragmentation but before inter-nucleosomal fragmentation (Lazebnik *et al.*, 1994, Greidinger *et al.*, 1996). However, it is not evident in some models of apoptosis, e.g. in Hep3B cells induced to apoptosis by camptothecin (Adjei *et al.*, 1996). PARP is a nuclear enzyme which has a DNA binding domain ((DBD) containing two Zn2+ fingers and a helix-turn-helix motif) at the N-terminus, a catalytic domain at the C-terminus and an intervening auto-modification domain. PARP is involved in DNA repair and thereby cell homeostasis. It recognises DNA breaks and binds, via its Zn2+ fingers, before catalysing repair by forming ADP-ribose polymers (both long and branched) using NAD as a substrate (Lindahl *et al.*, 1995, Shall and Gilbert, 2000).

Sphingolipids directly affect and regulate the extrinsic pathway of apoptosis (Figure 1.7). For example, SMase produces ceramides which bind to and activate the lysosomal protease cathepsin D. This cleaves the protein BID to activate the apoptotic pathway (Heinrich *et al.*, 2004). Ceramide activates protein kinase Cζ (PKCζ), which, in turn, activates c-Jun NH2-terminal kinase 1 (JNK1) and inhibits protein kinase B (PKB or Akt) to induce apoptosis (Bourbon *et al.*, 2000, Bourbon *et al.*, 2002). In contrast, S1P suppresses ceramide-mediated activation of JNK1 but activates the Akt/mTOR complex 1 (mTORC1), ERK1/2, and NFκB (Cuvillier *et al.*, 1996) signalling pathway. Thus, regulators of the extrinsic pathway of apoptosis are differentially affected by the various sphingolipids (Figure 1.7).

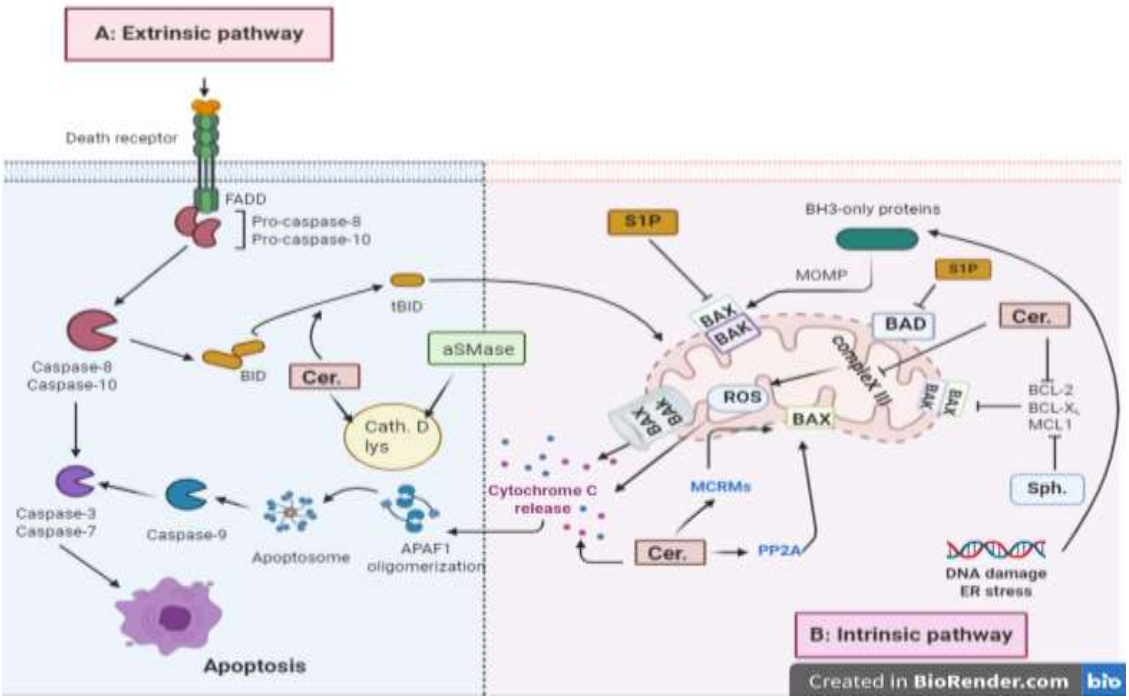

Figure 1. 7: Schematic representation of key events in the apoptotic pathway and regulation of apoptosis by sphingolipids.
(Adapted from (Lee *et al*., 2015))

Sphingolipids also affect intrinsic apoptosis (Figure 1.7). Ceramides induce cytochrome c release by the creation of channels in the mitochondria outer membrane to induce activation of caspase-9 (Schenck *et al*., 2007). Ceramide also inhibits mitochondrial complex III to produce reactive oxygen species (ROS) that also stimulate the intrinsic pathway (García-Ruiz *et al*., 1997) whereas S1P inhibits apoptosis via BAD inactivation (Stoica *et al*., 2003) and the reduction of Bcl-2 and Bcl-xL, which are anti-apoptotic proteins (Lee *et al*., 2015).

1.10 Sphingosine kinase inhibitors

Nearly all known SK inhibitors have inhibitory action by binding to the sphingosine binding sites of these enzymes. SK inhibitors are either analogues of sphingosine (e.g. *N*,*N* dimethylsphingosine (DMS)) (Edsall *et al.*, 1998), non-lipid inhibitors (e.g. PF-543) (Schnute *et al.*, 2012) or inhibitor of ATP binding (MP-A08) (Pitman *et al.*, 2015) (Table 1.3). Despite their usefulness, there is complexity in interpretation of data

obtained using SK inhibitors due to their potential action on other sphingolipid metabolising enzymes or sphingolipid-regulated proteins. For example, DMS is a sphingosine analogue and inhibitor of SK but is also an activator of sphingosine activated enzymes (PKD and 14-3-3) (French *et al.*, 2006) and an inhibitor of PKC. Thus, it is hard to be precise that the effect of this inhibitor is attributed solely to SK inhibition or is due to 'off-target' (French *et al.*, 2006, Vadas *et al.*, 2008). More recently, and with the discovery of the SK1 crystal structure and homology modelling of SK2, more potent and isoform selective inhibitors have been developed. Despite this, inhibitors targeting the sphingosine binding site might also bind to other sphingolipid metabolising enzymes or other proteins that use chemically similar substrates or ligands. Similarly, ATP-competitive inhibitors need to be selective for SKs and not affect any other kinase enzyme, all of which use ATP. Therefore, siRNA or genetic knockdown of SK1 or SK2, as appropriate, can provide evidence of targeting specificity and validation of new chemical inhibitors to these enzymes.

1.10.1 SK1 inhibitors

1.10.1.1 SK1-I

SK1-I (BML-258) ((*2R,3S,4E*)-N-methyl-5-(4-pentylphenyl)-2-aminopent-4-ene-1,3-diol), was the first SK inhibitor that demonstrated selectivity for SK1 over SK2. This water-soluble analogue of sphingosine (Figure 1.8), discovered in 2008, has been widely used, including *in vivo*, to assess the important role of SK1. This is a sphingosine competitive inhibitor (K_i of 10 μM) and selective for SK1 over SK2 and CERK (as well as 11 protein kinases). In U937 human monocytes, SK1-I reduced S1P and increased ceramide with no change in sphingosine or dihydrosphingosine. It also induced apoptosis via downregulation of ERK1/2 and Akt signalling pathway. The inhibitor was not cytotoxic in normal peripheral blood mononuclear cells (Paugh *et al.*, 2008, Zhang *et al.*, 2008). SK1-I exhibits good solubility, facilitating *in vivo* delivery in saline, but is not soluble in polyethylene glycol and DMSO. In a study using a syngeneic breast cancer model in mice, SK1-I reduced tumour burden and metastasis, lowered tumour S1P levels, enhanced tumour apoptosis and decreased haemangiogenesis and lymphangiogenesis (Nagahashi *et al.*, 2012). Also, SK1-I reduces tumour burden in a mouse xenograft squamous cell carcinoma model and improves the efficacy of doxorubicin. Therefore, SK1-I is a chemo-sensitising agent

(Hazar-Rethinam *et al.*, 2015). Moreover, combining SK1-I and bortezomib (a proteasome inhibitor), synergistically increased apoptosis, decreased colony formation, and induced down-regulation of BCR/ABL and Mcl-1 in human leukaemia cells that were either imatinib-resistant or imatinib-sensitive (Li *et al.*, 2011, Hazar-Rethinam *et al.*, 2015). In addition, low cytotoxicity against normal cells indicates that these molecules might have some therapeutic potential for treating cancer (Paugh *et al.*, 2008).

1.10.1.2. PF-543

PF-543 ((*R*)-(1-(4-((3-methyl-5-(phenyl-sulfonylmethyl)-phenoxy)-methyl)-benzyl) pyrrolidin-2-yl)-methanol) is a highly potent inhibitor of SK1 (K_i = 3.6 nM) with 130-fold selectivity over SK2), while also failing to affect other 40 lipid kinases (including phosphatidylinositol kinases) and protein kinases (Figure 1.8). However, it has a slight inhibitory effect against mixed lineage kinase-1 (MLK-1) (~ 50% at 10 μM) (Schnute *et al.*, 2012). PF-543 reduces S1P generation and induces proteasomal degradation of SK1 (Byun *et al.*, 2013). However, it was unable to promote apoptosis in different cancer cells (Schrecengost *et al.*, 2015). This finding is contrary to most SK1 knockdown studies, where tumour growth is inhibited. More recently, PF-543 when used in higher concentrations 2.5-10-fold than needed to reduce S1P in cells, was found to stimulate necrosis in colorectal cancer cell lines (Ju *et al.*, 2016). Interestingly, PF-543 significantly decreases S1P and accumulates sphingosine levels. However, there was no expected increase in cellular ceramide levels, which may account for its lack of potency in inducing apoptosis (Schnute *et al.*, 2012). The explanation for this could be that PF-543 might modulate other sphingolipid enzymes that negate the effect of the inhibitor on ceramide levels in certain cancer cells. Subsequently, PF-543 was shown to increase in dihydroceramides, ceramide monohexosides and lactosylceramides in HGC 27 cells (Cingolani *et al.*, 2014). The ability of PF-543 to decrease S1P levels with no cell death induction may provide prospective therapeutic use in other diseases, such as inflammatory disorders (Schnute *et al.*, 2012). In addition, in the study by Schnute *et al.* (2012), the ATP concentration was not saturating (in excess) meaning that the assay conditions involved two-substrate kinetics. Despite this, PF-543 was extremely selective for SK1, inhibit SK2

at higher concentrations of 50 µM three times the inhibition at lower concentration of 5 µM.

Mapping the two SK isoforms from the available SK1 crystal structure, suggests that the differences between the two enzymes is in the foot of the lipid binding "J-channel". Recent analysis by Adams *et al.* (2019), predicts that SK1 *vs* SK2 selectivity by PF-543 is due to accommodation of the inhibitor's sulphonyl group in the heel of the deeper J channel in SK1 whereas the more limited space at the heel of the J channel in SK2 indicates that the sulphonyl cannot be accommodated. Other SK1 *vs* SK2 structural differences in the J channel sub-pocket where the methyl group of PF-543 sits might also contribute to its selectivity for SK1 (Adams *et al.*, 2019).

This recent study designed and synthesised isoform-selective and dual SK1/SK2 inhibitors using PF-543 as a starting point, exploring and exploiting the variations in the 'heel and toe' of the foot of J channel of SK1 and SK2. Linkers within PF-543 were replaced and various halogen substitutions of the phenyl ring were made to generate a series of nanomolar potent inhibitors which ranged from 100-fold SK1 selective (PF-543; (Schnute *et al.*, 2012) through an equipotent SK1/SK2 inhibitor (compound 49) to a 100-fold SK2 selective inhibitor (compound 55; see section 1.10.2.7) (Adams *et al.*, 2019).

1.10.1.3 SK1-5c (CAY 10621)

SK1-5c named (2,2-dimethyl-4S-(1-oxo-2-hexadecyn-1-yl)-1,1-dimethylethyl-ester-3-oxazolidinecarboxylic acid) or CAY10621, inhibits SK1 (IC_{50} of 3.3 µM) with selectivity over SK2 (Figure 1.8) (Wong *et al.*, 2009). Others report K_i values of 15 µM for SK1and 46 µM for SK2 (Kharel *et al.*, 2012). It slightly reduces cell proliferation in U937 cells but has a marked effect on cell viability of colon cancer cells, especially when combined with inactivation of AKT signalling (Tan *et al.*, 2014). SK1-5c reduced proliferation of MDA-MB-231 and MCF-7 breast cancer cell lines, *in vitro* colony formation and enhanced their cell death. SK1-5c also weakened MDA-MB-231 tumour growth in a mouse xenograft model and chemo-sensitised these breast cancer cells (Datta *et al.*, 2014) and a panel of colon cancer cell lines. These effects were phenocopied using siRNA knockdown of SK1. Hence, further studies are

required to describe SK1-5c inhibitory effects toward other sphingolipid metabolising enzymes, protein kinases and lipids.

1.10.1.4 SKI-178

Modification of SK1-I resulted in new inhibitor named SKI-178, chemically known as (N′-[1-(3,4-dimethoxyphenyl)-ethylidene]-3-(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide). SKI-178 is approximately 20-fold selective for SK1 (K_i of 1.3 μM), over SK2, (Figure 1.8) (Hengst *et al.*, 2010). It reduced S1P and increased ceramide levels in HL-60 cells. SKI-178 induced apoptosis in some cancer cell types (with IC_{50} = 0.1–1.8 μM) (Dick *et al.*, 2015). For example, in Acute myeloid leukaemia (AML) cell line, HL-60, SKI-178 enhanced apoptosis in a prolonged mitosis through a mechanism of activating cyclin-dependent kinase 1 (CDK1), which in turn causes phosphorylation and degradation of Bcl-2, BCL-XL and Mcl-1. This agrees with a role for SK1 in mitotic exit in AML cells and was confirmed by SK1 genetic knockdown in a human pancreatic cell line (Kotelevets *et al.*, 2012, Dick *et al.*, 2015). Significantly, SKI-178 was effective against a multi-drug resistant AML line. These findings indicate that SKI-178 could be a valuable chemo-sensitising agent in AML. Furthermore, modification of SKI-178 by adding methyl and methoxy groups enhanced its pharmacological activities and selectivity for SK1. SKI-178 was the first SK1-selective non-lipid small molecule inhibitor (Hengst *et al.*, 2010).

1.10.1.5 Compound 82 (Amgen 82)

Compound 82 also known as Amgen 82, was developed through modification of SKI-II (French *et al.*, 2003), using a structure-based design (Figure 1.8) (Gustin *et al.*, 2013). Amgen 82 is a competitive dual SK1/SK2 inhibitor, with an IC_{50} value of 20 nM for human SK1 and an IC_{50} value of 100 nM for human SK2. Moreover, it reduces S1P formation in human melanoma cell line (WM266.4) with IC_{50} of 63 nM while in breast cancer line (MDA.MB.231) with IC_{50} of 90 nM (Rex *et al.*, 2013). Interestingly, compound 82 showed an inhibitory effect on mouse SK1 (IC_{50} of 70 nM), while no significant inhibitory effect of mouse SK2 was seen at 10 μM (Gustin *et al.*, 2013). These findings indicate there are differences in the sphingosine binding sites between human and mouse SK2 isoforms. Compound 82 reduced S1P levels in human breast and melanoma cells, but only exhibited growth inhibitory effects at 100-fold higher

concentration than for SK1 (Micromolar concentrations). Since compound 82 is an analogue of SKI-II, which inhibits Des1 (Cingolani *et al.*, 2014), it should be tested for activity against Des1.

1.10.1.6 Amidine inhibitors

Amidine based compounds exhibit selectivity and potency for SK1 and SK2. The inhibitory effect toward SK relies on the electrostatic properties of the basic amidine group which directly binds with ATP-γ-phosphate (Kharel and Sellmyer, 2011). Compound 1a known as (*S*)-1-(4-dodecylbenzoyl)-pyrrolidine-2-carboximidamide) (Figure 1.8), is a SK1-specific inhibitor with high potency, showing a K_i of 0.1 μM. The initial compound, VPC94075 (Figure 1.8) showed an inhibitory effect on both SK isoforms with IC_{50} values of 55 and 20 μM for SK1 and SK2, respectively (Mathews *et al.*, 2010). VPC94075 decreased S1P levels with anti-proliferative effects in cells, despite being only moderately potent. Compound 1a has an amidine functional group with an extended tail and shows specificity toward SK1(Kennedy *et al.*, 2011). Compound 1a is a competitive inhibitor for sphingosine with no effect towards other lipid kinases at 30-folds the concentration of the K_i. Subsequently, two inhibitors were developed. Compound 1 is a dual SK inhibitor, known as N-(1-carbamimidoylcyclopropyl)-4-dodecylbenzamide and the other compound 2 is a SK1-selective inhibitor, known as 1-carbamimidoyl-N-(4-dodecylphenyl) cyclopropane carboxamide. These improved compounds display sub-micromolar IC_{50} values: compound 1 - SK1 IC_{50} of 0.2 μM, SK2 IC_{50} of 0.5 μM; compound 2 - SK1 IC_{50} of 0.3 μM, SK2 IC_{50} of 6 μM. There was no inhibitory effect on human diacylglycerol kinase (DAGK) or PKCα. The selectivity for SK1 over SK2 was found to be due to the amide functional group and longer tail length (C12 vs C8) (Mathews *et al.*, 2010). *In vitro* administration of compound 1a to U937 cells, decreases S1P levels within 10 minutes and the decrease is sustained for 24 hours (Kharel *et al.*, 2012). Interestingly, compound 1a prompts a doubling of sphingosine and dihydrosphingosine but only markedly increased ceramide levels when used at 10 μM, which is 100 times higher concentration than that required for inhibiting SK1(Kharel and Sellmyer, 2011). Compound 1a was efficient at reducing S1P accompanied by moderate increases in sphingosine and C16:0 ceramide levels in U937 cells. Compound 1a also inhibits pro-survival ERK1/2 and AKT signalling and induces PARP cleavage, a marker of

apoptosis. This only occurs after 16 hours of treatment at 10μM. In mice, 50% reduction in S1P levels was observed. At one-hour post-administration, the compound was cleared from the bloodstream (Kharel and Sellmyer, 2011). Notably, much higher concentrations are required to reduce cellular levels of S1P and to show an anti-proliferative effect on U937 cells.

1.10.1.7 CB5468139

As a result of screening a small library, CB5468139 has been identified as an ATP-competitive SK1 inhibitor (Figure 1.8). It has a K_i of 0.28 μM with no noted inhibition of SK2 below concentration 100 μM. In A498 kidney adenocarcinoma cells, CB5468139 inhibits cell growth with an EC_{50} of 10–15 μM and moderately decreases S1P and increases ceramide levels. Cell cycle progression was not affected but the compound induced autophagy (Gao *et al.*, 2012). Off targets of CB5468139 include several protein kinases, such as CLK1, Met, PIM2, SYK and TNK2, which are inhibited with IC_{50} of 2 μM, indicating that the compound lacks specificity at the ATP binding site of kinases (Gao *et al.*, 2012).

1.10.1.8 FTY720

FTY720 (2-amino-2-[2-(4-octylphenyl) ethyl] propane-1, 3-diol) (Figure 1.8), with a structure similar to sphingosine, was developed from the compound ISP-1 (myriocin) that was isolated from the fungus Is*aria sinclairii* (Adachi and Chiba, 2007). Also known as Fingolimod, or Gilenya™ in the market, FTY720 is currently in clinical use to arrest symptoms and decelerate the development of multiple sclerosis (MS) (Chun and Hartung, 2010). In its phosphorylated state, FTY720 binds four types of S1P receptor ($S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$, but not $S1P_2$). It acts as an agonist in the short term but a functional antagonist of $S1P_1$ (which underlies, in part, its therapeutic effect) in the longer term due to the internalisation and subsequent degradation of $S1P_1$ receptors (Antoon *et al.*, 2011).

FTY720 is a sphingosine-competitive inhibitor of SK1 with a K_i around 2μM (Tonelli *et al.*, 2010). It also induces proteasomal degradation of SK1, which likely contributes to its SK inhibitory effect in the longer term (Lim *et al.*, 2011b). FTY720 also activates PP2A (Saddoughi and Ogretmen, 2013) and inhibits cPLA2 and PKCδ (Payne *et al.*, 2007, Hung *et al.*, 2008). Moreover, it has also been noted that FTY720 inhibits SK1

to increase radiation sensitivity of prostate cancer tumour xenografts to reduce tumour growth and metastasis in mice (Pchejetski *et al.*, 2010). Modified forms of FTY720, such as (*S*)-FTY720 vinyl phosphonate, inhibit SK1 and similarly induce its proteasomal degradation and the apoptosis of breast and prostate cancer cells (Tonelli *et al.*, 2010), as well as preventing rearrangement of actin in response to S1P stimulation of MCF-7 cells (Lim *et al.*, 2011a).

1.10.1.9 *D, L*-threo-dihydrosphingosine

D, L-threo-dihydrosphingosine (DHS) is the earliest discovered inhibitor of SK. It was synthesised from the naturally occurring *D*-erythro-dihydrosphingosine (or sphinganine) (Figure 1.8). DHS is a sphingosine competitive SK1 inhibitor (K_i of approximately 3-6 μM) with a high degree of SK stereoselectivity. It acts as a SK2 substrate and, after integrating into sphingolipid metabolic pathway, it can be further metabolised. Though moderately potent for SK1. *L*-threo-DHS (known as safingol) also has inhibitory effects towards other kinases. For example, it is used as an inhibitor of PKC-α in the clinic and research settings. These findings indicate its limited usefulness as a SK inhibitor (Coward *et al.*, 2009).

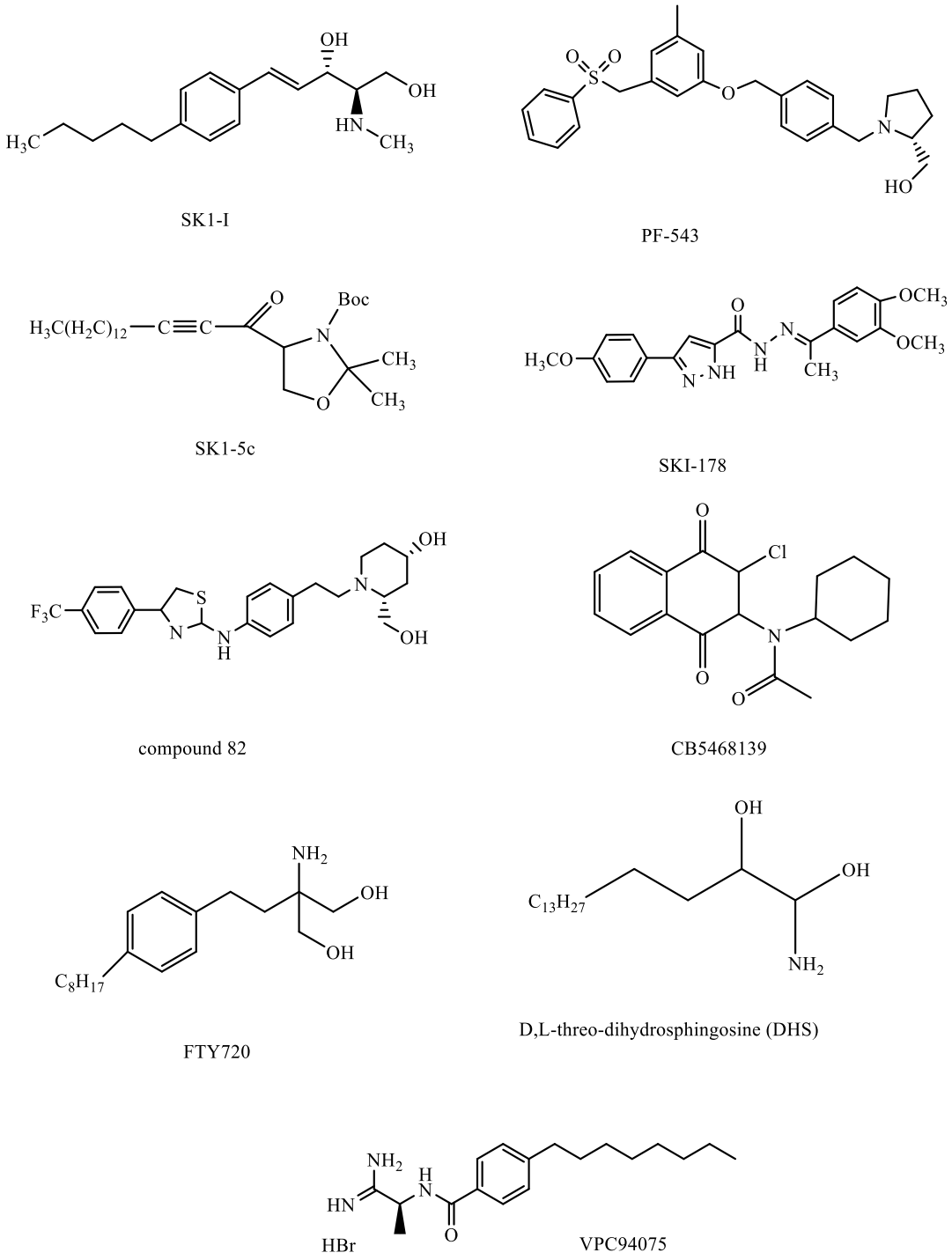

Figure 1. 8: SK1 inhibitor structures.

1.10.2 SK2-selective inhibitors

1.10.2.1 ABC294640

ABC294640 (Yeliva/Opaganib) is a non-lipid SK2 inhibitor with a K*i* of ~10 μM. ABC294640 (Figure 1.9) has been widely used to invoke a role for SK2 in many diseases. It accumulates intracellular sphingosine and ceramide species with a reduction of S1P levels and abrogates cell proliferation (French *et al.*, 2010). Notably, however, ABC294640 has off-target effects: it behaves as a weak oestrogen receptor (ERα) antagonist at low micromolar concentration and promotes the proteasomal degradation of Des1 (leading to an increase in dihydroceramide levels) and SK1 (McNaughton *et al.*, 2016). Despite this, ABC294640 is in phase I/II trials (as YELIVA™ or Opaganib) for refractory/relapsed patients diffuse large B-cell lymphoma and virus-induced lymphoma as well as has lately accomplished phase I trials for using in later stage solid tumours (Lin *et al.*, 2015). Indeed, ABC294640 sensitises cancer cells to chemotherapy (Antoon *et al.*, 2011), Bcl-2 inhibitors, the Bcr-Abl inhibitor, Imatinib (Wallington-Beddoe *et al.*, 2014a) and the proteasome inhibitor, Bortezomib (Venkata *et al.*, 2014). ABC294640 blocks AKT and ERK1/2 signalling to stimulate autophagy of tumour cells causing non-apoptotic cell death (Sheng *et al.*, 2014). It also promotes proteasomal degradation of c-Myc, Mcl-1 (Venkata *et al.*, 2014) and inhibits NF_KB-mediated chemo resistance in breast cancer cells (Liu *et al.*, 2012).

1.10.2.2 K145

K145 named [3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione] (Figure 1.9), is a selective inhibitor for SK2 (Ki of 6.4 μM) with a sphingosine-like structure. K145 exhibits selectivity towards SK2 (no inhibition of SK1 below 10 μM), with no activity against CERK and 11 other kinases, even though it has a modest inhibition effect against AKT and calmodulin kinase Iiβ at 10 μM. K145 reduces total cellular levels of S1P with no effect on ceramide levels (Liu *et al.*, 2013), compatible with its inhibitory effect against SK2. It also prevented FTY720 phosphorylation in cells (Liu *et al.*, 2013), which is catalysed by SK2. U937 cells treated with K145 have reduced proliferation and enhanced apoptosis via a mechanism involving inhibition of ERK1/2 and Akt signalling (Liu *et al.*, 2013).

1.10.2.3 SG12 and SG14

SG12 and SG14 (Figure 1.9) are sphingosine analogues synthesised from a *p*-octyl-phenyl backbone. SG-12 is both a selective SK2 inhibitor but also a substrate for SK2, exhibiting a Km of 5.5 μM, similar to sphingosine (Gao *et al.*, 2012). Notably, phosphorylation of SG12 by SK2 is crucial for apoptosis induction in the A20/2J (the murine B lymphoma-derived cell line) (Hara-Yokoyama *et al.*, 2013). Additional research is needed to identify the apoptotic pathway and whether this may include an effect of SG-12-phosphate on S1P receptors.

1.10.2.4 ROMe ((R)-FTY720-OMe)

SK2 generates *(S)*-FTY720-phosphate by phosphorylation of *(S)*-FTY720 (fingolimod/Gilenya) on the pro-chiral hydroxyl group (Figure 1.8). Alternatively, hydroxyl group replacement with a methoxy group generated (*R*)-FTY720-OMe (ROMe, Figure 1.9). ROMe inhibits SK2 with a K_i = 17 μM, with a slight SK1 inhibition at 50 μM. In HEK293 cells, ROMe inhibits SK2 activity while, in MCF7 breast cancer cells, it decreases DNA synthesis and blocks rearrangement of actin in response to S1P stimulation (Lim *et al.*, 2011a). ROMe enhanced autophagy T cell leukaemia cell lines (Evangelisti *et al.*, 2014), accumulated sphingosine and reduced S1P in LNCaP prostate cancer cells, but was without effect on ceramide levels (Watson *et al.*, 2013). This may suggest that ROMe affects CerS (a known FTY720 target) (Berdyshev *et al.*, 2009, Park *et al.*, 2014a). ROMe limits cell growth by preventing SK2-derived S1P growth-stimulating effects of on $S1P_4$ and $S1P_2$ receptors, in MDA-MB-231 breast cancer cells (Ohotski *et al.*, 2014). ROMe improves pulmonary endothelial vascular integrity through $S1P_1$ receptor signalling (Camp *et al.*, 2016). Interestingly, ROMe reduces expression of SK2 independently of lysosomal or proteasomal pathways in HEK293 cells (Lim *et al.*, 2011b).

1.10.2.5 SLR080811

SLR080811 was synthesised by modification of the amidine SK1 inhibitor, compound 1a. Retention of the pyrrolidine ring and substitution of guanidine for the amidine yielded SLR080811 (Figure 1.9), which is known as ((S)-2-(3-(4- octylphenyl)-1,2,4-oxadiazol-5-yl) pyrrolidine-1-carboximidamide) (Kennedy *et al.*, 2011). SLR080811 inhibits both SK isoforms, with K_i of 1.3 for SK2 and 12 μM for SK1, thus exhibiting

~10-fold selectivity for SK2. It has no inhibitory activity at 3 μM against either CERK or DAGKα (Kharel *et al.*, 2012). Selectivity for SK2 over SK1 was also demonstrated using knockdown of SK1 and SK2: SLR080811 shows a reduction effect in intracellular S1P levels in SK1 knocked-down cells, but no reduction with SK2 knocked-down cells. SLR080811 reduced the levels of S1P and dihydroS1P in Jurkat, U937 and SKOV3 cancer cells. Further analysis validated that SLR080811 enhances sphingosine, dihydrosphingosine and C16 ceramide levels but without effects on other ceramide species of U937 cells.

Lately, two compounds based on SLR080811 have been developed. These are LM6041434 and SLC5111312, which exhibited inhibitory effect in mouse and rat SK2 (K_i values of 0.4–1.1 μM). Notably, SLC5111312 was observed to downregulate both SK1 and SK2 activity in rat, while being selective for SK2 in mouse (Kharel *et al.*, 2015).

1.10.2.6 SLP120701

SLP120701 is known as ((S)-2-(3-(4-octylphenyl)-1,2,4-oxadiazol-5-yl)-azetidine-1-carboximid-amide hydrochloride). It selectively inhibits SK2 with K_i of 1.2 μM and with ~ 10-fold selectivity over SK1 (Patwardhan *et al.*, 2015). SLP120701 (Figure 1.9) reduced S1P and enhanced sphingosine levels in U937 cells but without being cytotoxic in U937 cells after 24 hours incubation.

1.10.2.7 Compound 55 (HWG-35D)

HWG-35D is a potent SK2 selective inhibitor (Figure 1.9), synthesised by substituting a methyleneoxy linker for the sulphonyl linker in PF-543 and modifying the terminal phenyl ring with a para-chloro group (Adams *et al.*, 2019). HWG-35D was 100-fold more selective for SK2 over SK1. It also showed markedly improved potency than the prevailing SK2 inhibitor, ABC294640. It only inhibits SK2 without showing any effect on SK1. In a comparative study between HWG-35D and ABC294640 in psoriasis-like skin disease, both improve symptoms of a psoriasis-like skin condition (Shin *et al.*, 2020). This supports a pro-inflammatory role for SK2. Additionally, it was demonstrated that HWG-35D had no off-target effect on SK1 and Des1, i.e., expression of these proteins was unaffected. In a further *in vivo* model, HWG-35D was investigated for its therapeutic potential on tubulointerstitial fibrosis prompted by

unilateral ureteral obstruction model (UUO) in mice (Schwalm *et al.*, 2021). This study proved the ability of HWG-35D to attenuate the kidney fibrotic response induced by UUO, limiting expression of fibrotic markers (e.g. collagen-1 (Col1), fibronectin-1 (FN-1), connective tissue growth factor (CTGF) and α-smooth muscle actin (α-SMA)) and collagen accumulation. This was accompanied by an induction Smad7 expression (a negative regulator of pro-fibrotic TGFβ/Smad signalling) and a significant increase in sphingosine.

1.10.2.8 Compound VT-20dd

Compound 20dd was developed from another SK1- selective inhibitor (SLP7111228). (Figure 1.9). It is 100-fold selective for SK1 over SK2 and lowers S1P levels in human leukaemia U937 cells (Childress *et al.*, 2017). Compound 20dd was synthesised as a para-substituted biphenyl derivative. The introduction of large substituent at the para position produced a potent and selective SK2 inhibitor (K_i 90 nM).

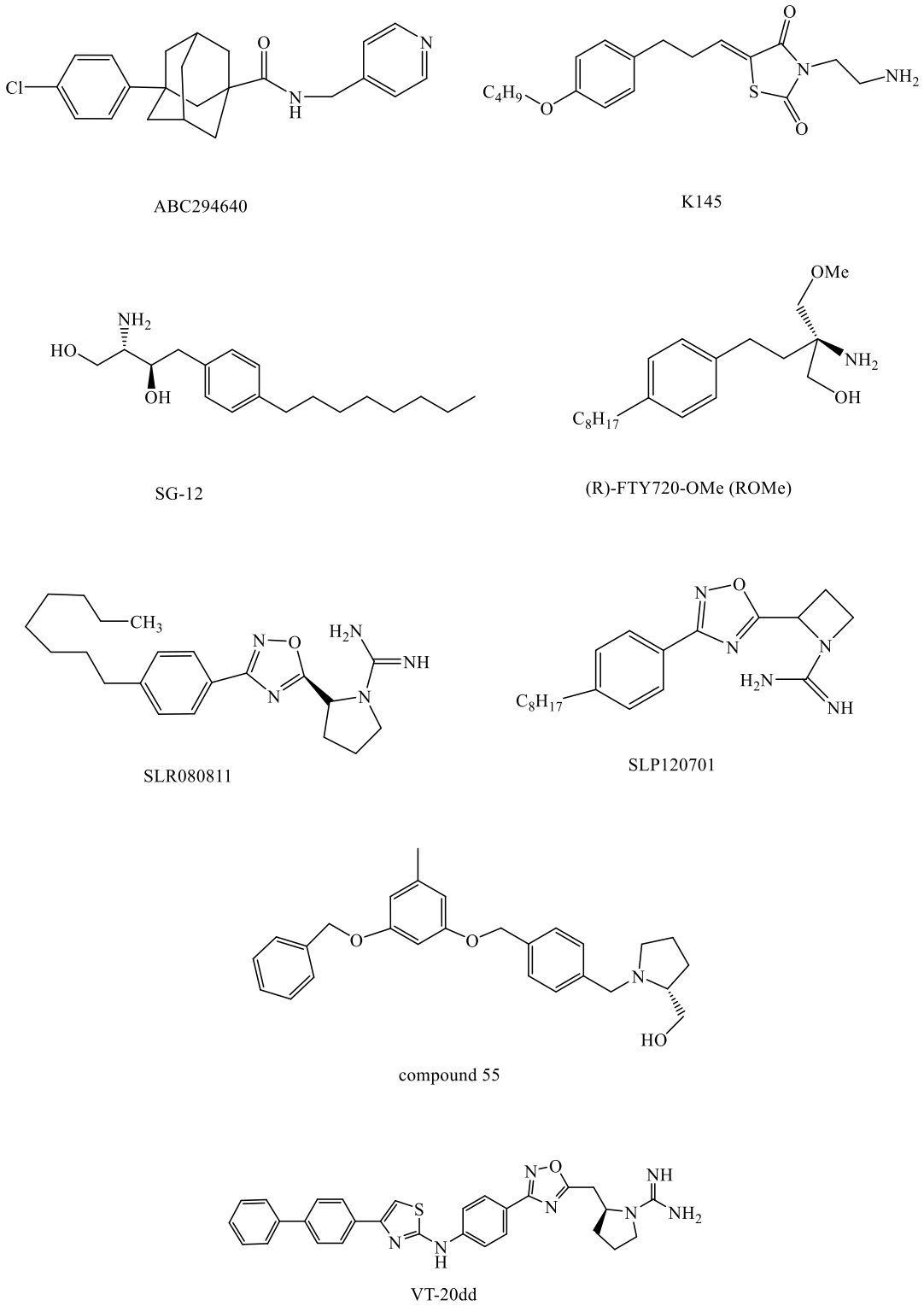

Figure 1. 9: Sphingosine kinase 2 inhibitors

1.10.3 Dual SK1/SK2 inhibitors

1.10.3.1 SKI-II

SKI-II (or SKi) has chemical name 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino]-phenol (Figure1.10) (French *et al.*, 2003). It is a SK1/2 dual inhibitor with SK1 with K_i value of 16 μM and SK2 with K_i of 8 μM (Aurelio *et al.*, 2016). Indeed, it showed inhibitory effect toward SK1 with K_i value of 16 and for SK2 with K_i of 8 μM (Gao *et al.*, 2012). This suggests that SKI-II is slightly more SK2-potent inhibitor than SK1. It exhibits mixed inhibition of SK1, with K_i (competitive) of 17 μM and K_i (uncompetitive) of 48 μM (Lim *et al.*, 2011a). It also inhibits Des1 (see 1.6.7). SKi decreases cellular levels of S1P and increase ceramides and sphingosine in LNCaP prostate cancer cells to induce their apoptosis (Loveridge *et al.*, 2010). SKi does not directly inhibit ERK1/2, PKC or PI3K (French *et al.*, 2003). In addition, SKi sensitises glioblastoma cell lines to temozolomide (Noack *et al.*, 2014), chemo-resistant MCF7-TN-R cells to doxorubicin (Antoon *et al.*, 2012) and HNSCC lines to cetuximab (an EGF receptor inhibitor) (Schiefler *et al.*, 2014). In addition, SKi provokes ubiquitin-proteasomal and lysosomal degradation of SK1 (Loveridge *et al.*, 2010, Ren *et al.*, 2010). The proteasomal degradation of SK1 involves its ubiquitination at Lys183, and KLHL5 (kelch-like protein 5) is an important adaptor linking SK1 to the Cul3 E3 ubiquitin ligase complex that promotes SK1 degradation (Powell *et al.*, 2019). In contrast, SKi does not induce degradation of SK2 (Watson *et al.*, 2013). Notably, SKi increased proteasomal degradation of SK1 may occur in two ways: a direct effect of inhibitor binding to SK1, as well as due to an indirect effect on the proteasome (Loveridge *et al.*, 2010, McNaughton *et al.*, 2016).

SKi also induces degradation of Des1 *via* the proteasome (McNaughton *et al.*, 2016). and inhibits NADH-cytochrome b5 reductase, an upstream Des1 activator, resulting in loss of Des1 activity (Cingolani *et al.*, 2014). It may also act by stimulating oxidative stress in cells, which may inhibit redox-sensitive Des1 (Idkowiak-Baldys *et al.*, 2010). Inhibition of Des1 decreases flux *via* the sphingolipid pathway and increases dihydroceramides (Loveridge *et al.*, 2010).

1.10.3.2. SKI-I

SKI-I termed N-[(2-hydroxy-1-naphthyl)-methylene]-3-(2-naphthyl)-1H-pyrazole- 5-carbohydrazide (Figure 1.10), competitively inhibits SK1 with an IC_{50} of 1.2 μM (French *et al.*, 2003). It also inhibits SK2 activity with similar potency (Hengst *et al.*, 2010). Also, SKI-I inhibits ERK1/2 with an IC_{50} of 11 μM (French *et al.*, 2003). This dual SK inhibitor has not been proven to have any effects against other lipid kinases such as CERK or DAGK or on cellular dihydrosphingolipids. Treatment of melanoma cells with SKI-I reduced S1P, increased ceramides (long chain and very long chain) but, unexpectedly, reduced sphingosine (Madhunapantula *et al.*, 2012). The latter may indicate another target within the sphingolipid pathways, such as ceramidase. Bladder cancer cells undergo apoptosis (caspase-dependent), whereas mouse embryonic fibroblasts undergo autophagy (Young *et al.*, 2012) in response to SKI-I treatment. Low micromolar concentrations (0.4-5 uM) reduced proliferation in various cancer cell types, including chemo-resistant breast cancer cells (Sharma *et al.*, 2010).

1.10.3.3 MP-A08

The first highly selective ATP-competitive SK1/SK2 inhibitor is MP-A08 (Figure 1.10). It shows slight or no inhibition of CERK and DAGK. In silico docking of MP-A08 to SK1 at the ATP-binding pocket has been performed (Pitman *et al.*, 2015). MP-A08 does not inhibit Des1. Screening of 140 human protein kinases at high MP-A08 concentrations (250 μM) revealed a partial inhibition of testis-specific serine kinase (TSSK). However, this is well above the concentration required to inhibit either hSK or mSK isoforms (IC_{50} values: hSK1, 27 μM; hSK2, 7 μM). In cancer cells, it increases sphingosine and ceramide, reduces S1P and induces apoptosis. However, it fails to induce proteasomal degradation of SK1, which is a property of sphingosine-competitive SK inhibitors. *In vivo*, MP-A08 inhibits tumour growth and angiogenesis and promotes apoptosis of tumour cells (Pitman *et al.*, 2015).

1.10.3.4 Dimethylsphingosine (DMS)

DMS, *N,N*-dimethylsphingosine is both a PKC inhibitor, a sphingosine-competitive inhibitor of SK1 with K_i of 5 μM, and non-competitive inhibitor of SK2 with a K_i of 12 μM (Figure 1.10) (Endo *et al.*, 1991, Pyne and Pyne, 2010). DMS induces ubiquitin-proteasomal degradation of SK1 (Lim *et al.*, 2011a) and has limited use due to its

known ‘off target’ effects on PKC and CERK (Sugiura *et al.*, 2002) as well as SRC kinases, ERK1/2, casein kinase II and EGFR. It is also an activator of SDK1 (sphingosine-dependent protein kinase 1). DMS has concentration-dependent effects including, at low concentrations, the induction of SK activity (Jin *et al.*, 2006, Gandy and Obeid, 2013a).

1.10.3.5 Compound Pfizer- 27c

Compound Pfizer-27c was synthesised following a high-throughput screening study against SK1 from which two chemotypes were identified, of which compound 12 (an aminobenzimidazole) and compound 20a (a benzylpyrrolidine) were identified. An optimisation strategy was followed to generate Pfizer-27c, which was synthesised from compound 12 by replacement of phenyl methylsulfone in the tail region with a heteroaryl group making this compound a dual inhibitor of SK1 and SK2 with IC_{50} values of 25 and 2.4 nM, respectively (Schnute *et al.*, 2017).

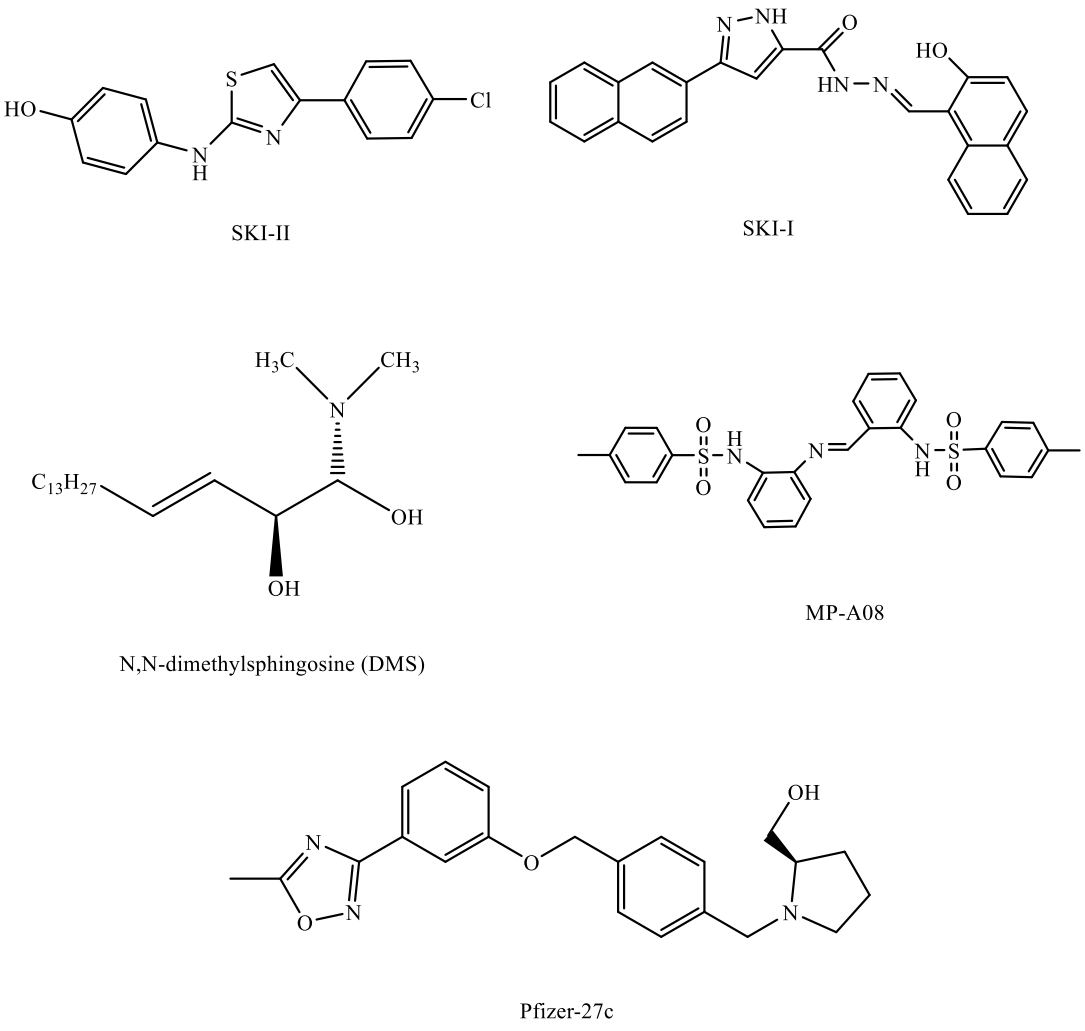

Figure 1. 10: Dual sphingosine kinase inhibitors

1.10.4 Other SK inhibitors

In the past few years, many novel SK inhibitor compounds have been discovered. For example, LCL351 (Figure1.11), sphingosine with guanidine analogue has been modified as an SK1 inhibitor. It exhibits nanomolar potency for SK1 (IC_{50} of 40 nM) with 7-fold selectivity over SK2 (Sharma, 2011). Compound 51 (IC_{50} of 58 nM) and 54 (IC_{50} of 10 nM) are two additional SK1-selective inhibitors that are sphingosine

analogues (Figure 1.11). These compounds demonstrate moderate oral bioavailability in rats with blood half-lives that suggest they could be useful *in vivo* (Xiang *et al.*, 2009, Xiang *et al.*, 2010).

Compound C2 (Figure 1.11) was identified after optimisation of a hit from high throughput screening using a novel yeast complementation assay (using genetically modified *S. cerevisiae*). It has selectivity for SK1 (500-fold over SK2) and IC_{50} of 63 nM without any effect on 62 human protein and lipid kinases assays at 3µM (Kashem *et al.*, 2016). Studies have also explored analogues of FTY720 (Raje *et al.*, 2012) and SLR080811 (Congdon *et al.*, 2015). Due to the interest in finding more potent and selective inhibitors for SK2, a few compounds were synthesised. These include VT-ME6 which contains a quaternary ammonium group. Indeed, a positive charge is necessary for the engagement between the key amino acid and the enzyme binding site. This compound has 3-fold selectivity for SK2 over SK1 (Congdon *et al.*, 2015). A diversity-oriented synthesis employing a novel scaffold and head group identified quaternary ammonium salts as SK inhibitors with low micromolar K*i* values (Raje *et al.*, 2012).

Moreover, analogues of new inhibitors extracted from natural products, such as resveratrol (the anticancer agent) and its dimer derivatives, ampelopsin A and belanocarpol (Figure 1.11), have been found to inhibit SK1 and decrease its expression but not that of SK2 in HEK293 cells, while reducing proliferation and inducing apoptosis (Lim *et al.*, 2012a). In agreement with these findings, high concentrations of resveratrol induce apoptosis, while low concentrations halt cancer cell growth (Nakagawa *et al.*, 2001).

The SK2 inhibitor (2*S*,3*S*,4*R*)-pachastrisamine has also been reported (Figure 1.11) (Yoshimitsu *et al.*, 2011, Lim *et al.*, 2012b). Additionally, the AKT inhibitor, BI-69A11 was shown to reduce NF_KB signalling by inhibiting SK1 activity to reduce melanoma growth (Feng *et al.*, 2011). B-5354 was separated from *Trichopezizella barbata* culture broth. It inhibits SK1 and SK2 with a K_i of approximately 3µM and is a non-competitive inhibitor regarding sphingosine. B-5354 sensitises prostate cancer cells to chemotherapeutics (Kono *et al.*, 2002). F-12509a (Figure 1.11) is a sesquiterpene quinone isolated from cultured broths of a discomycete, *Trichopezizella*

barbata. SANK 25395. F-12509a competitively inhibits SK1 with Ki of 4 µM. It enhances apoptosis of cancer cells by activating the release of the two major pro-apoptotic proteins, cytochrome c and SMAC/Diablo (Bonhoure *et al.*, 2006). F-12509a inhibits SK1 activity, decreases S1P levels and increases ceramide levels in HL-60 cells. Finally, S-15183a/b is a natural compound separated from the fungus *Zopfiella inermis*.

Many of these compounds need to be further validated as SK inhibitors and anticancer agents and for further drug optimisation. All of these results indicate that SK inhibitors could be a beneficial addition to the chemotherapeutic strategies employed for treating various types of tumours (Cuvillier, 2007, Pyne *et al.*, 2011, Heffernan-Stroud and Obeid, 2013).

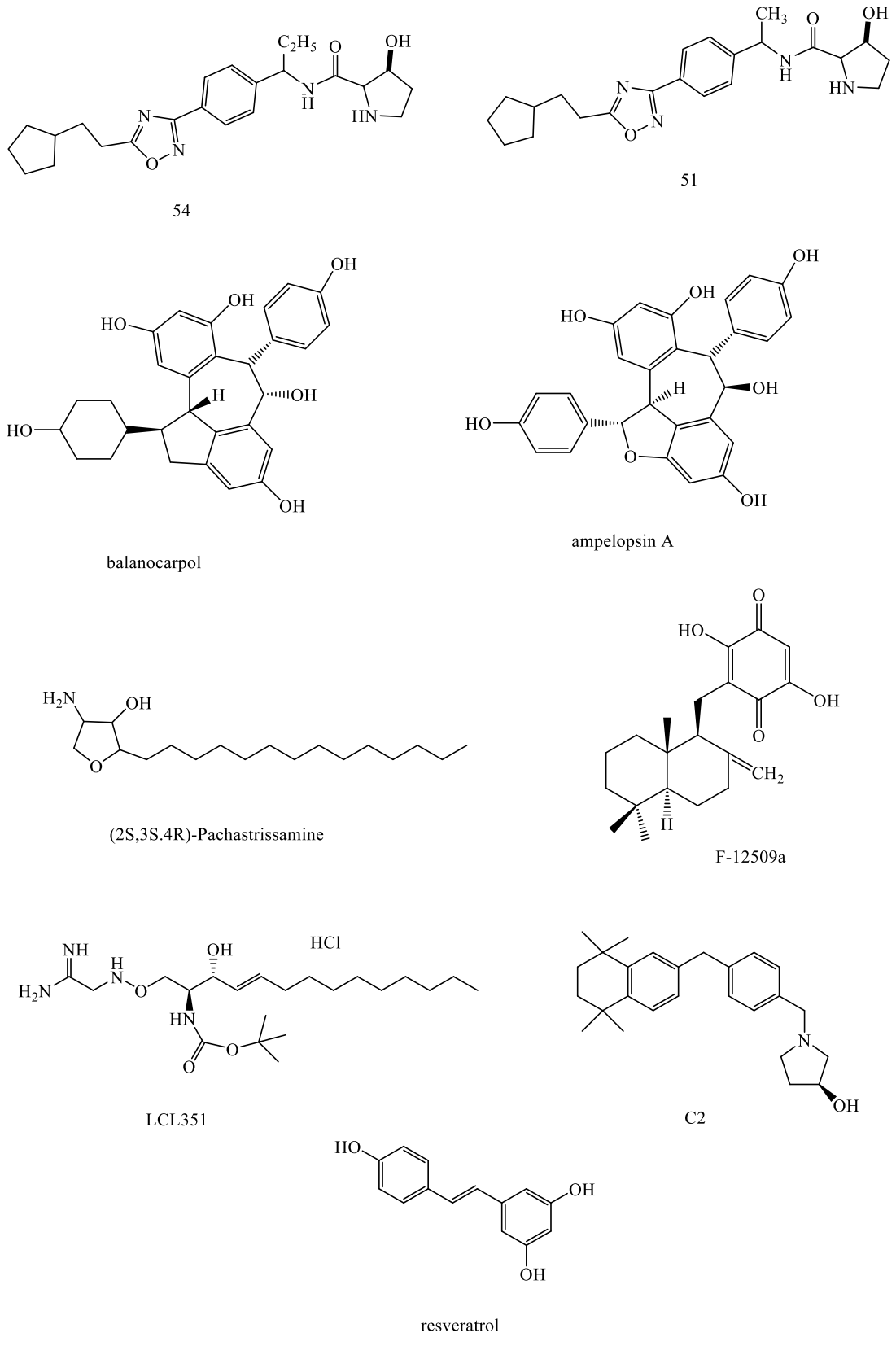

Figure 1. 11: Other sphingosine kinase structures.

Table 1. 3: SK1 inhibitors

Inhibitor type	Inhibitor name	Potency (Ki)	
		SK1	SK2
SK1-selective	SK1-I	10 μM	-
	PF-543	3.6 nM	-
	SK1-5c (CAY 10621)	15 μM	-
	SKI-178	1.3 μM	-
	Compound 82 (Amgen 82)	90 nM	-
	Compound 1a	0.1 □M	-
	VPC94075	55 μM	-
	CB5468139	0.28 μM	-
	FTY720	2μM	-
	D,L-threo-dihydrosphingosine (DHS)(safingol)	3-6 μM	-
SK2-selective	ABC294640	-	~10 μM
	K145	-	6.4 μM
	SG12 and SG14	-	5.5 μM
	ROMe ((R)-FTY720-OMe)	-	17 μM
	SLR080811	-	1.3 μM
	SLP120701	-	1.2 μM
	Compound 55 (HWG-35D)	-	41 nM
	Compound VT-20dd	-	90 nM
Dual-SK1/SK2	SKI-II	16 μM	8 μM
	SKI-I	1.2 μM	1.2 μM
	MP-A08	27 μM	7 μM
	Dimethylsphingosine (DMS)	5 μM	12 μM
	Pfizer- 27c	25 nM	2.4 nM

1.11 Project aim

A weight of evidence supports the crucial role of the two enzymes, SK1 and Des1 in cancer and inflammation. Hence, these enzymes are targets for new anticancer/anti-inflammatory therapeutics. A number of compounds, of natural origin or synthesised, have been identified, but none have reached the clinical trial phase I/II. Therefore, there is a need to discover more SK1/or Des1 inhibitors. This study aims to extract, isolate, and identify anticancer and anti-inflammatory compounds from various plants and to test whether they are SK1/or Des1 inhibitors. The plants selected were based on their traditional uses in local community in Egypt against cancer/inflammation. Compounds isolated from the plant extracts will be screened for their ability to reduce DNA synthesis (a measure of cell proliferation), expression of SK1 and Des1 and PARP cleavage (as an apoptosis marker). The isolated compounds will also be assessed as anti-inflammatory agents by modulation of NFκB signalling. In conclusion the isolated compounds which meet these criteria could be considered potential anticancer or anti-inflammatory agents.

CHAPTER 2:

Material & Methods

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2.1 Materials

2.1.1 Cell culture

❖ Invitrogen Ltd. (Paisley, UK).
• Dulbecco's Modified Eagle's Medium (DMEM). • Penicillin/streptomycin (10,000 units/ml penicillin; 10,000 µg/ml streptomycin). • Trypsin (0.25% with EDTA 4Na).
❖ Sera Laboratories International Ltd. (through BioIVT, West Sussex, UK).
• European Foetal Calf Serum (EFCS)
❖ The European Collection of Animal Cell cultures (through Public Health England, Salisbury, UK).
• Breast cancer cell lines, MCF7/L and MDA-MB-231 cells
❖ Gifted by Prof G. Tigyi (Memphis, USA).
• MEF cells (Mouse Embryonic Fibroblast)
❖ Gifted by Dr A. Paul (University of Strathclyde).
• Reporter cells, NCTC-AP-1 and NCTC-NF_KB, which are keratinocytes that have been genetically modified to express either an AP-1-binding promoter-driven or an NF_KB-binding promoter-driven luciferase gene, were

2.1.2 Antibodies

Antibody	**Company**
Horseradish peroxidase (HRP)-linked anti-mouse IgG,	Sigma-Aldrich (Cat. # A9044)
Horseradish peroxidase (HRP)-linked anti-rabbit IgG.	Sigma-Aldrich (Cat. # A0545)
DEGS1	Abcam (Cambridge, UK) (cat# Ab185237)

ERK2	BD BIOSCIENCES (Wokingham, UK) (cat# 610104)
GAPDH	Santa Cruz Biotechnology (Insight Biotechnology, Wembley, UK) (cat# SC-47724)
IkB α (c-21)	Santa Cruz Biotechnology (cat# SC-371)
JNK1 (2C6)	Cell Signaling (cat# 3708S)
JNK2 (D-2)	Santa Cruz Biotechnology (cat# SC-7345)
Ki67	Sigma-Aldrich (cat# WH0004288M1_100UG)
Phospho-AKT (T308)	Cell Signaling (cat#2965S)
PARP	Cell Signaling (cat# 9542S)
Phospho-ERK1/2 (E-4)	Santa Cruz Biotechnology (cat# SC-7383)
Phospho-JNK (T183/Y185)	Cell Signaling (cat# 4671S)

SK1: synthesised by Abgent according to the method detailed in Huwiler *et al.* (2006).

2.1.3 Inhibitors and agonists

Inhibitor/agonist	**Company**
Sphingosine kinase inhibitor (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole)	Calbiochem (Millipore UK Ltd, Watford, UK) (Cat# 567731)
MG132 (Z- Leu-Leu-Leu-al)	Sigma-Aldrich (Cat# C2211)

2.1.4 Radioisotopes

[^{3}H] Thymidine (25 Ci/mmol), from GE Healthcare (Hatfield, UK).

2.1.5 Solvents

All solvents used in the extraction and purification of active compounds were of HPLC grade.

Solvent	Company
• n-hexane	VWR (Lutterworth, UK)
• ethyl acetate	VWR (Lutterworth, UK)
• methanol	VWR (Lutterworth, UK)
• deuterated DMSO (DMSO-*d6*) (99.9 atom % D)	Sigma-Aldrich (Poole, UK)
• deuterated methanol (CD3OD)	Sigma-Aldrich (Poole, UK)

2.1.6 Other chemicals used

Chemicals	Company
• Thin layer chromatography (TLC) grade silica gel coated aluminum sheets.	Merck (Gillingham, UK)
• TLC grade silica gel 60 H	Merck (Gillingham, UK)
• Flash column grade silica gel particle size 230-440 mesh size	Sigma-Aldrich (Poole, UK)
• Column grade silica gel particle size 40-75 um	Sigma-Aldrich (Poole, UK)
• Vanillin powder	Sigma-Aldrich (Poole, UK)
• *p*-Anisaldehyde reagent	Sigma-Aldrich (Poole, UK)
• Anti- bumping granules	Sigma-Aldrich (Poole, UK)
• Methylthiazolyldiphenyl-tetrazolium bromide	(Sigma-Aldrich)

2.1.7 General reagents

Reagent	Company
• Hybond™ ECL™ Nitrocellulose membrane	GE Healthcare (UK)

- Kodak LX 24 developer — Christiansen-Linhart (Munich, Germany)
- Kodak industrex fixer — Christiansen-Linhart (Munich, Germany)
- CEA RP New X-ray film — Christiansen-Linhart (Munich, Germany)

All biochemical reagents were purchased from (including pre-stained molecular weight markers (SDS-7B)) or from Fisher Scientific (Loughborough, UK), unless otherwise stated.

2.1.8 Stimuli

Stimuli	Company
Phorbol myristate acetate (PMA)	Thermo Fisher Scientific (Loughborough, UK) (cat# 10061403)
Tumor necrosis factor alpha (h-TNFα)	R&D Systems (Abingdon, UK) (cat# 210-TA1CF)

2.1.9 Plant materials

In this study, a number of plants were collected for further phytochemical investigation in search of anticancer or anti-inflammatory compounds. Choosing plants was based on known traditional uses, previous published phytochemical, and pharmacological studies on each plant. All plant materials were collected from different places in Egypt, during field work, in year 2015-2016 (Table 2.1).

Table 2. 1: Plants list, location, and collection time and weight.

No	Plant Latin name	Location	Part used	GPS points	Season (month)	dry weight
1	*Gomphocarpus sinaicus*	St Katherine, Sinai	aerial parts	28.5433 N, 33.9330E	February 2016	900 g
2	*Asphodelus fistulosus*	Aswan, Wadi Kherat,	aerial parts, roots	24.0401N, 34.7127E	December 2015	172 g
		Mount Elba, Wadi Yahmeeb	aerial parts, roots	22.2376N, 36.3596E	December 2015	40 g
3	*Psoralea plicata*	Aswan University campus	aerial parts	23.9981N, 32.8615E	January 2016	593 g
4	*Halocnemum strobilaceum*	Burg ElArab, west Alexandria	aerial parts, roots	30.9245 N, 29.5252E	February 2016	494 g
5	*Crotalaria spp.*	Mount Elba, Wadi Adeeb	aerial parts	22.2256 N, 36.3947E	December 2015	18 g
6	*Heliotropium supinum*	West Sehail village Aswan	aerial parts	24.0589N, 32.8643 E	February 2016	425 g
7	*Urginea maritima*	Haraz Market	bulb	----------	June 2015	50 g
8	*Pancratium tortuosum*	Mount Elba, Wadi Adeeb	Bulb, and leaves	22.2261N, 36.394E	December 2015	357 g
		Mount Elba, Wadi Yahmeeb	Bulb, leaves	22.2408N, 36.3573E	December 2015	62 g
9	*Glinus lotoides*	Wadi Alaqi & Lake Nasser, Aswan	aerial parts	22.7273 N, 33.2253E, 23.9622 N, 32.8667E	October 2015, February 2016	410 g, 172 g

2.2 Methods

2.2.1 Plant extraction

Fresh plant material was air dried and grounded to a fine powder. The grounded material (500 g) was extracted using Soxhlet apparatus with ethanol (in total 2.5 litre). About half a litre used daily for extraction. Each daily collected extract was evaporated under vacuum, using a rotary evaporator to be checked with TLC, for an indication of extraction process end. The extract residue was stored in sealed ampules at -20°C until used (Fig. 2.1).

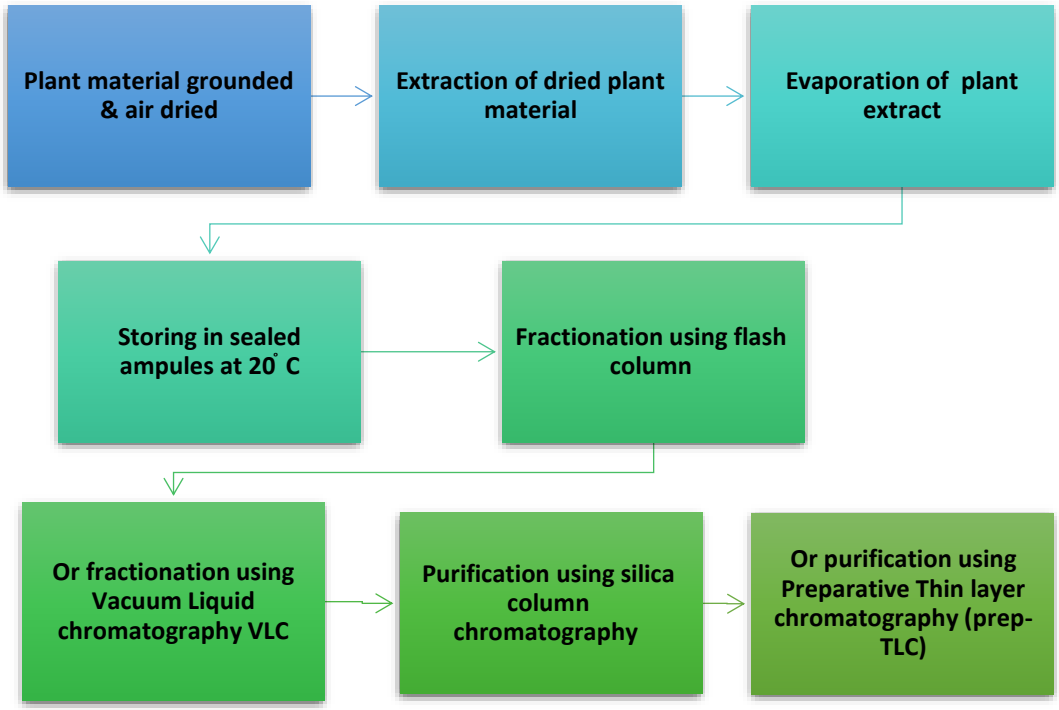


Figure 2. 1: Flowchart of plant extraction process.

2.2.2 Flash column chromatography

Plant extracts were fractionated using Flash column technique. Firstly, the plant extract was dissolved in a suitable solvent, usually methanol (HPLC grade), to which silica gel (of particle size 200-440 mesh) was added to make a slurry. The mixture was left until dryness to evaporate any excess solvent. Secondly, the extract slurry was loaded after dissolving in minimum suitable solvent, into a glass column (1 litre, 2.9 cm diameter) on top of a layer of silica gel (200-440 mesh size) of about twice the slurry

volume. The solvent systems used in this technique, are summarised in Table 2.2. Each solvent system (about 1- 2 litre of each solvent system) was collected separately and evaporated using a rotary evaporator until almost dry, then collected and left to evaporate to dryness before further use. In most cases, a freeze dryer was used to totally remove any excess solvent.

Table 2. 2*:* Solvent system used in Flash column chromatography & Vacuum liquid chromatography.

Composition	Ratio
n-Hexane	100
n-Hexane-EtOac	50:50
EtOac	100
EtOac: MeOH	50:50
MeOH	100

2.2.3 Vacuum liquid chromatography

Column grade silica gel (200 g) was dry packed under vacuum in a Büchner funnel with a sintered glass disc. The plant extract was then applied on top of the packed VLC (Erdreich-Epstein *et al.*) column (the height of the packed layer was ~5 cm) Similar to Flash chromatography, gradient elution with solvent systems of increasing polarity was performed (as in Table 2.2). Collected fractions were then dried using rotary evaporator (freeze-dried if necessary) and stored at -20ºC.

2.2.4 Silica gel column chromatography

Plant extract fractions were purified using a silica gel column (of particle size 70-230 mesh size), which was packed in a dry way or a wet way, to separate and purify the compounds after fractionation using flash column technique. In the dry packing method, a glass column was used (size depends on the amount of sample to be loaded, e.g., 150 g silica gel in 3.6 cm in diameter). Dry silica gel was loaded to two thirds of the height of the column, followed by the solvent system to be used for purifying the fraction, until the packed silica was wet and with no trapped air. This was followed by loading the fraction sample (dissolved in a minimal amount of the solvent). The

column was eluted using the solvent systems listed in Table 2.3 and in the same sequence (from low polarity to high polarity), thereby separating and purifying compounds in a polarity-dependent manner. For the wet packing method, the column was prepared by loading the silica gel after re-suspending it in the starting solvent of the solvent system. Thereafter, all steps were similar to the dry packing method.

Table 2. 3: Solvent system for column chromatography, silica gel (70-230 mesh size):

Composition	Ratio
Hexane: EtOac: MeOH	7: 4 0.25
Hexane: EtOac: MeOH	7: 4: 0.5
Hexane: EtOac: MeOH	7: 4: 1
Hexane: EtOac: MeOH	7: 4: 1.5
Hexane: EtOac: MeOH	7: 4: 2
Hexane: EtOac: MeOH	7: 4: 3

2.2.5 Thin layer chromatography (TLC)

TLC reagent vanillin-H_2SO_4 was prepared by mixing 5 g of vanillin with 100 ml methanol, 10 drops of glacial acetic acid and 15 drops of concentrated H_2SO_4. This reagent is multi-purpose and enables the detection of most functional groups with different colouration. For example, terpenes will give blue spots whereas coumarin give red spots, saccharides will give yellow spot turn to black when TLC plate gets cold, alkaloid will give orange spot and phenolic compounds will give purple spots. Therefore, TLC of the Flash column chromatography or VLC fractions was performed as a preliminary step for compound detection. Fractions were applied to a TLC plate which was placed in a TLC tank for development using a solvent system (Hexane: Ethyl acetate: Methanol 6:3:0.5). Non-destructive detection of natural compounds was achieved by placing the developed TLC plate under a UV lamp using short (254 nm) and long (366 nm) wavelengths. If recovery of the compounds was not necessary, the TLC plate was sprayed with *p*-Anisaldehyde reagent, which was prepared by mixing 0.5 ml of *p*-Anisaldehyde with 50 ml of methanol, 5 ml of concentrated H_2SO_4 and 5 ml of glacial acetic acid.

2.2.6 Preparative thin layer chromatography (prep-TLC)

Prep-TLC was used to separate compounds, by applying 10 mg - 1 g of sample material to silica gel coated glass (Whatman LK5D glass TLC plates, pre-coated with Partisil K5 (150Å silica) as a long streak. The loaded plate was placed in a TLC tank for development using Hexane: Ethyl acetate: Methanol 6:3:0.5 (v/v). Non-destructive detection of natural compounds was initially achieved by placing the developed TLC plate under a UV lamp using short (254 nm) and long (366 nm) wavelengths. For more accuracy in locating compounds, this part of the TLC plate was sprayed with *p*-Anisaldehyde reagent with the rest of the plate covered. Then, the compounds were recovered by scraping the relevant area of plate and eluting in 200 ml MeOH for 24 hours. This was followed by solvent evaporation using a rotary evaporator until dryness.

2.2.7 Structure elucidation with nuclear magnetic resonance (NMR)

All NMR experiments were performed by technician Craig Irving, with a JEOL (JNM LA500) spectrometer operating at 600 and 500 (^{1}H) and 125 (^{13}C) MHz using deuterated DMSO solvents and residual solvent peaks as internal reference. Typically, 10-20 mg and 20-50 mg of samples were dissolved in deuterated solvents for ^{1}H and ^{13}C NMR respectively. NMR sample tubes made of borosilicate glass were used for the measurement of all spectra except when the compound was less than 10 mg which required the use of a Norell micro-tube.

^{1}H-NMR was performed on all samples to establish an initial impression of the type and number of compounds present in the sample. Further structural analysis was assisted with two-dimensional NMR experiments such as COSY (COrrelation SpectroscopY), HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence). ^{13}C and DEPT (Distortionless Enhancement through Polarization Transfer). NMR experiments were performed when samples were in sufficient pure amount to obtain a good signal to noise ratio. Spatial structural information was obtained with NOESY (Nuclear Overhauser Enhancement SpectroscopY).

2.2.8 Liquid chromatography high resolution-mass spectrometry (LCHRMS).

LCHRMS was used to establish the molecular weight of the purified samples of interest. Around 1 mg of samples was dissolved in methanol (HPLC grade) to get a concentration of 1mg/ml and 20µl of the sample solution was injected into the LC77 mass spectrometer (MS). The high-resolution mass spectra were obtained by using an LTQ Orbitrap MS (Thermo Orbitrap mass spectrometer) in negative ion mode with a needle voltage of -4.0 kV.

The separation was performed on an ACE-C18 column (150.3 mm, 3 µm) from HiChrom UK with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The binary mobile phase gradient at a flow rate of 300µL/min for 46 minutes as shown below (Table 2.4) Samples were submitted to the mass spectroscopy services at Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS). LC-MS was run by MS technicians.

Table 2. 4: Gradient elution program applied for C18 in LC-MS analysis

Time (min)	Mobile Phase A	Mobile Phase B	Flow rate (ml/min)
0	95	5	0.3
30	0	100	0.3
35	0	100	0.3
36	95	5	0.3
46	95	5	0.3

2.2.9 Cell culture maintenance

Aseptic technique was maintained in all cell culture work which was carried out in a laminar flow hood. All incubations were performed in a humidified incubator maintained at 5% CO_2 and 37°C. DMEM was used as a basic medium to maintain cell cultures, typically in 75 cm^2 flasks. MEF cells (Mouse Embryonic Fibroblast), MCF7/L and MDA-MB-231 breast cancer cells, reporter cells NCTC-AP1 and NCTC-NFκB were grown in DMEM containing 10% (v/v) EFCS, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium).

2.2.10 Trypsinising and passaging cells

Cells were passaged when they were approaching a confluent monolayer. Briefly, the adhering cell monolayer was washed with serum free DMEM (37°C) to remove any residual EFCS-containing medium which may inhibit the action of trypsin. Then, 2 ml of trypsin solution (37°C) was added to the 75cm^2 flask to cover the adhering cells. After 2 mins (at 37°C), the flask was tapped gently to dislodge the cells. Next, 8 ml of complete medium was added to the flask to inactivate any trypsin activity. To each new culture in a 75 cm^2 flask, 1 ml of this cell suspension was added with 9 ml complete medium. After 3 or 4 days, the medium was removed from the sub confluent cultures and replaced with 10 ml fresh complete medium. Confluent secondary culture was passaged by repeating all steps described above. Cells were plated into 12, 24 or 96 well plates for experiments, as required.

2.2.11 Freezing cells

Confluent cell lines were frozen for long term storage to preserve cells. Freezing medium for MEF, MCF7/L, MDA-MB-231, NCTC-AP1 and NCTC-NFκB cells was made of DMEM supplemented with 20% (v/v) EFCS and 10% (v/v) DMSO. Confluent cells were trypsinised as described in Section 2.2.9. Next, the cell suspension was centrifuged for 3 mins at 180 ***g***. The supernatant was removed, and the cell pellet re-suspended in 4 ml 4°C freezing medium. 0.5 ml aliquots of cell suspension were added into 2 ml cryogenic vials. The vials were placed at -80°C freezer overnight before being transferred to liquid nitrogen freezer for long term storage.

2.2.12 Thawing and recovering cells

Cryogenic vials containing frozen cells were removed from liquid nitrogen freezer and placed in 37°C water bath immediately for rapid thawing. Thawed cells were drawn into a Pasteur pipette to break up cell clumps before being transferred into a new flask with the addition of 10 ml complete medium (37°C). After 24 h, cultures were checked to ensure cell attachment to the flask and the medium was replaced with fresh complete medium.

2.2.13 Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

The plant extracts were tested for their cytotoxicity using MTT reagent with MEF cell, MCF7/L and MDA-MB-231. Cells were plated at 10 x10^3 cells/well (in 50 µl) in 96-well plates and maintained overnight in complete media. Afterwards, plant extracts were added for 24 h (in 50 µl) in two concentrations (1 and 5 µg/ml final) or vehicle control of DMSO (0.05 µg/ml). Subsequently, MTT reagent (250 mg MTT in 50 ml phosphate buffered saline (PBS)) was added (50 µl/well) for 2 h. Then, the MTT reagent was aspirated and MTT stop reagent added (4 mM HCl, 0.1% NP-40 in isopropanol) (100 µl/well), followed by mechanical shaking of the plate for 30 min. The optical density reading at 620 nm was measured using a plate reader. Each treatment was performed with four replicates and the whole experiment was repeated three times.

2.2.14 [^{3}H]-thymidine incorporation assay

The ability of plant extracts to inhibit DNA synthesis was assessed by the incorporation of [^{3}H]-thymidine into newly synthesised DNA by MEF, MCF7/L and MDA-MB-231 cells. Cells were plated at 10 x 10^3 cells/well (1 ml) in 24-well plates and maintained overnight in complete medium. Subsequently, complete medium was removed and serum free medium (1 ml) was added to each well for another 24 h. Then cells were treated with two concentration of plant extracts (1 and 5 µg/ml), or with the vehicle control (DMSO, 0.05 µg/ml). Cells were then incubated for another 16-18 h before pulsing with [^{3}H]- thymidine (0.25 kBq/well; added as 10 µl) for 5 h. Then, reaction was terminated by washing 3 times with 1ml ice cold 10% (w/v) trichloroacetic acid (10 min/wash; maintained on ice). Radionucleotides incorporated into DNA were harvested with 0.25 ml 0.1% (w/v) sodium dodecyl sulphate (SDS) with 0.3 M NaOH. Samples were transferred to vials for quantification by liquid scintillation counting with 2ml scintillation cocktail (Optiphase HiSafe III, Perkin Elmer, Seer Green, UK). Each treatment was performed in triplicate and the whole experiment was repeated three times.

2.2.15 Preparation of cell lysates for Western blotting

Cells were grown in DMEM (10% EFCS) on 12 well plates for 24 h. Upon reaching 70-80% confluence, the medium was aspirated and replaced with DMEM (serum free

medium) for 24 h. This was followed by adding the treatments (5µg/ml extract or 0.05 µg/ml DMSO vehicle control) for the next 24 h. After appropriate treatment, cell lysates were prepared by aspirating the medium and adding 200 µl sample buffer/ per well (containing 62.5 mM Tris Base (pH 6.7), 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 12.5% (v/v) glycerol, 1.25% (w/v) SDS, 50 mM dithiothreitol (DTT) and 0.05% (w/v) bromophenol blue). The sample was passed up and down for three times using a gauge 23 needle and 1ml syringe to homogenise and shear DNA. Prepared cell lysates were collected in 1 ml vial tubes and stored at -20°C if not processed by one dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) the same day.

2.2.16 One dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)

A 10 x 10 cm polyacrylamide gel (1 mm depth) was cast between two glass plates, with a lower resolving gel topped by a stacking gel. The resolving gel was made from 10% (v/v) acrylamide: bis-acrylamide (29:1) 0.375 M Tris-Base (pH 8.8), 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate and 0.1% (w/v) TEMED (tetramethyl-ethylene-diamine); the stacking gel was made from 4% (v/v) acrylamide: bis-acrylamide (29:1), 0.125 M Tris-Base (pH 6.7), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.2% TEMED. A comb (15 or 10 well, as required) was immediately inserted to create ‘wells’ for sample loading. The set gel was secured in an electrophoresis apparatus filled with a running buffer containing 25 mM Tris Base, 0.19 M glycine and 0.1% (w/v) SDS and the comb removed. Equal volumes of samples (typically 15 – 25 µl) were loaded using a Hamilton syringe into the gel, in parallel with a lane containing 3 µl prestained molecular weight markers. Gels were run at 140V for 90 min until the bromophenol blue tracking dye reached the bottom of the resolving gel.

2.2.17 Immunoblotting

After running the gel, resolved proteins were transferred to a nitrocellulose membrane, with cooling, in a tank transfer system (Bio-Rad Mini Trans-Blot kit) at 140V for 1 h, in transfer buffer containing of 25 mM Tris-base, 0.19 M glycine and 20% (v/v) methanol. This was followed by incubating the membrane in 20 ml blocking buffer containing either 3% (w/v) skimmed milk or bovine serum albumin (BSA) in Tris-

buffered saline with Tween (TBST), which was made with 10 mM Tris-base (pH 7.4), 100 mM NaCl and 0.1% (v/v) Tween-20 for 1 h, by agitating on a rocker at room temperature. The membrane then incubated with the primary antibody (various, as shown below in Table 2.5) overnight at 4°C with gentle agitation. After overnight incubation with the primary antibody, the blot was washed three times for 7 min in TBST, then incubated in secondary antibody (horseradish peroxidase-conjugated anti-Ig (mouse or rabbit-specific, as required) (1:40000 diluted in 1% (w/v) BSA in TBST) for 1 h at room temperature with gentle agitation. Finally, the blot had three washes for 7 min each with TBST.

In dark room, the membrane was soaked for 3 min in developing solution for detection by enhanced chemiluminescence (ECL). The ECL solution was prepared by mixing equal volumes of two reagents: reagent 1 containing 2.5 mM luminol, 0.1 M Tris base (pH 8.5) and 1,1 mM *p*-coumaric acid and reagent 2 containing 0.02% (v/v) H_2O_2 and 0.1 M Tris-Base (pH 8.5). The membrane was briefly dried (to remove excess ECL solution) and immediately placed between two plastic sheets in a metal X-ray cassette and exposed to X-ray film. Exposure time varied from a few seconds to 5 min (depending on the antibody used). The X-ray film was developed by passing it through an X-Omat machine to get a chemiluminescent signal from the immunoreactive proteins. Membranes were routinely stripped and re-probed for other proteins using other antibodies; this could be repeated several times. Stripping was by incubating the membrane, with rocking, at 60°C in stripping buffer contain 62.5 mM Tris base (pH 6.7), 2% (w/v) SDS and 100 mM β-mercaptoethanol for 30 min. Finally, the membrane was washed three times for 7 min in TBST before incubation with another primary antibody.

Table 2. 5: Antibodies used in immunoblotting

1st Antibody	Preparation 1:1000	Blocking	2nd Antibody 1% BSA	Fresh or reprobe blot
ACTIN (rabbit)	1% BSA	1% BSA	0.5:20000	Reprobe
DEGS1 (rabbit)	1% BSA	1% BSA	0.5:20000	Fresh or reprobe
Erk2 (mouse/rabbit)	3% BSA	3% BSA	0.5:20000	Fresh
GAPDH (Mouse)	1% BSA	1% BSA	0.5:20000	Re-probe
IkB α (rabbit)	1%BSA	1% BSA	1:20000	Fresh
JNK (mouse)	3% BSA	3% BSA	0.5:20000	Fresh
Ki67 (mouse)	1%BSA	1% BSA	1:20000	Fresh
P-AKT (T308) (rabbit)	5% BSA	3% BSA	0.5:20000	Fresh
PARP (rabbit)	1% BSA	1% skimmed milk	0.5:20000	Fresh
P-ERK-1/2 (mouse)	3% BSA	3% BSA	0.5:20000	Fresh
P-SAPK/JNK (T183/Y185) (rabbit)	3% BSA	3% BSA	0.5:20000	Fresh
SK1 (rabbit)	3% BSA	3% BSA	0.5:20000	Fresh

2.2.18 Luciferase assay

Reporter cells, NCTC-AP-1 or NCTC-NF_KB, were seeded in black-walled 96 well plates at 10000 cells per well, in 200 µl of complete DMEM medium. These were incubated overnight for 24 h. Cells were quiesced with phenol red-free DMEM

medium (50 µl/well) for 24 h. The cells were then pre-treated with inhibitors at certain concentrations (see figure legends 3.26, 3.27 and 4.11) for 10 min followed by the addition of h-TNFα (15 ng/ml) or PMA (1µM) respectively and incubation at 37°C for 4 h. Medium was removed before the addition of 100 µl/well of complete lysis buffer [25 mM tris Base (pH7.8), 8 mM $MgCl_2$, 1% triton X 100, 15% (v/v) glycerol] at room temperature, supplemented with [1 mM ATP, 1% BSA, 1 mM DTT, and 0.2 mM luciferin] on the day of the experiment (whereas stock lysis buffer could be prepared and stored at 4°C). The plate, with complete lysis buffer, was maintained in dark at room temperature for five minutes before luminescence measurements were taken using plate reader Victor Iso96l um (560 nm).

2.2.19 Statistics

All numerical data are presented as means +/- standard deviations for at least three independent measurements unless otherwise stated. The data were usually normalised to allow combination of data between different experiments. Data was analysed statistically using Prism 5 software GraphPad Software (La Jolla California USA, www.graphpad.com) by Student's t test or by One-Way ANOVA, as indicated. Differences were considered statistically significant at $p<0.05$. Dunnett's post-hoc test was used to make comparison between treated samples and the control. Bonferroni's post-hoc test was used to compare treatments with each other.

Chapter 3:

Preliminary screening and Bioassay-guided isolation of anticancer/anti-inflammatory compounds

CHAPTER 3: Preliminary screening and bioassay-guided isolation of anticancer/anti-inflammatory compounds

3.1 Introduction

After collecting plants from different areas around Egypt, eleven plant crude extracts were selected, based on their traditional uses, and previously isolated compounds, for preliminary screening against sphingosine kinase, dihydroceramide desaturase, PARP cleavage and cancer cell growth. All the plants have shown interesting previous biological activity including anticancer effects. For example, *U. maritima*, known as an onion-like Liliaceae was studied for cytotoxicity with the A549 NSCLC (non-small cell lung cancer) cell line. In this regard, the *U. maritima* extract was more cytotoxic than cisplatin and gemcitabine (the most active drugs in NSCLC (Bozcuk *et al.*, 2011). The *U. maritima* extract also shows significant activity against PC-3 (prostate cancer) and U373 GBM (glioblastoma) cell lines (Mohamed *et al.*, 2014). *U. maritima* represents a plant which has been known for its medicinal use to mankind since antiquity. For instance, the *U. maritima* bulbs extract has been used by the ancient Egyptians for treating cardiac failure. In addition, the purified glycosides from *U. maritima* are used in the treatment of cardiac diseases in unmanageable cases that no longer respond to Digitalis and Strophanthus drugs (Sayed, 1980, Tuncok *et al.*, 1995). In addition, pancratistatin (Figure 3.1) is a natural product that was isolated for the first time from the spider lily *Pancratium littorale* in 1984. It induces apoptosis in several cancer cell lines including lymphoma cells (Jurkat), breast cancer cells (MCF-7), and rat hepatoma cells (5123tc) at low concentrations (Pandey *et al.*, 2005).

Ethyl ether and ethyl acetate extracts from *Asphodelus aestivus* Brot. have significant antioxidant DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity. In addition, ethyl ether, ethyl acetate, water, and methanol extracts of *A. aestivus* Brot. exhibited cytotoxic activity on MCF-7 cells inducing significant deoxyribonucleic acid (Shall and Gilbert) damage in a time- and concentration-dependent manner (Aslantürk and Çelik, 2013). Pyrrolizidine alkaloids, terpenoids and flavonoids are the most bioactive components in the genus *Heliotropium*, which exhibited significant cytotoxicity,

phytotoxicity and anti-microbial, anti-tumour, anti-viral, anti-inflammatory activities with wound healing properties (Ghori *et al.*, 2016).

Amaryllidaceae is distinctive in the presence of showy lily-like epigynous flowers as in common onion. According to Meerow and Snijman (1998), there are about 800 species in 59 genera. Its regions of diversity are in South America (28 genera) and Africa (19 genera). Eight genera are growing in Mediterranean and temperate regions of Asia.

In Egypt, Amaryllidaceae is represented by two genera *Pancratium* and *Narcissus*. The latter is more widespread and common than the former. In the search for small molecules with potential anticancer activity, the Amaryllidaceae family in particular has been fruitful. Indeed, more than 100 structurally diverse alkaloids have been isolated from Amaryllidaceae species, possessing a wide range of biological activities. For example, lycorine (Figure 3.1) was the first member to be isolated from this family in 1877. Amarbellisine, cardamine, galanthine, pseudolycorine and ungeremine (Figure 3.1) are other examples of alkaloids based on the pyrrol-[de]phenanthridine skeleton, which are derived from natural or synthetic origin. Also, the lycorenine-type alkaloid is another large group of compounds isolated from Amaryllidaceae family but, in this case, based on the benzopyrano-[3,4-g]-indole skeleton. Nobilisitine B and clivonine (in hydrochloride form) belong to the pretazettine group while tazettine is the prototype (Figure 3.1). Lastly, alkaloids based on the 5,10β-ethano-phenanthridine skeleton belong to the crinine-type of compounds such as, ambelline, buphanamine, buphanisine, haemanthamine, and haemanthidine (Figure 3.2) (Cedrón *et al.*, 2010).

Some of the isolated Amaryllidaceae alkaloids possess anti-proliferative properties. It has been proposed that these compounds are active by disrupting eukaryotic protein biosynthesis. Alkaloids of non-basic isocarbostyrils from the Amaryllidaceae species demonstrate cytostatic activity and reduce cancer cell proliferation and migration by disrupting the actin cytoskeleton. Meanwhile, alkaloids of the isocarbostyrils such as, narciclasine (Figure 3.2) and pancratistatin (Figure 3.1) induce apoptosis in cancer cells of epithelial origin, but not in glioma cells, only at high concentrations, i.e., one log higher than their *in vitro* growth inhibitory IC_{50} values (Van Goietsenoven *et al.*, 2013).

3.1.1 *Pancratium tortuosum*

Pancratium genus comprises 16 palaeotropical species distributed in Macaronesia, the Mediterranean basin, and throughout Africa to tropical Asia. It has also been introduced and cultivated in many countries (El-Hadidy *et al.*, 2011). *Pancratium* can adapt extreme climates from extreme dry and sandy areas. Taxonomically, the *Pancratium* species are geophytic monocots, bulbous herbaceous perennials producing showy white fragrant flowers with a straight perianth tube and conspicuous corona formed by the basal connection of the staminal filaments (El-Hadidy *et al.*, 2012). *P. tortuosum* in particular is found in Nubia Nile Land, Mount Erkowit, Arabia, Egypt (Baker, 1898).

Complex structural type of alkaloids with significant therapeutical properties have been isolated from the *Pancratium* genus. For example, in early investigation on the Egyptian *P. maritimum* resulted in the isolation of lycorine, tazettine, pancracine, galanthamine, homolycorine, haemanthidine, haemanthamine, pseudolycorine and 11-hydroxyvittatine (Figure 3.2 and Figure 3.3). Moreover, two 2-oxyphenathridinium betaine type alkaloids were isolated, ungeremine and zefbetaine (Figure 3.3), which are known by their biological activities such as, cytotoxic, antibiotic, and plant growth-regulatory activities (Kornienko and Evidente, 2008).

Many *Pancratium* species are of high economical and medicinal value. Some species are cultivated due to their unique alkaloid content, which can be used in cancer treatment. Others are used locally in traditional and folk practices. For example, *P. tenuifolium* used in the coming-of-age ceremony in Botswana (El-Hadidy *et al.*, 2012). In Hawaii, *P. littorale* bulbs were found to contain pancratistatin, a phenanthridone biosynthetic product, which proved to be effective against the murine P-388 lymphocytic leukaemia and markedly inhibited growth of the P-388 cell line *in vitro* and *in vivo* of murine M-5076 ovary sarcoma (Pettit *et al.*, 1986). In addition, pancratistatin has significant apoptotic activity and supresses growth of the cancer cell line HT-29 in xenograft tumours. Interestingly, it did not induce an apoptotic effect in non-cancerous human colon fibroblast (CCD-18Co) cells (Pettit *et al.*, 1986).

There has been no previous study on the *P. tortuosum*, but some studies on other species of the same genus have led to isolation of four chromones and flavans from

the *P. maritimum* Viz. (Ali *et al.*, 1990). Also, earlier chemical investigation of *P. maritimum* led to the isolation of a variety of alkaloids, such as lycorine, tazettine, pancracine, *O*-methyllycorenine and galanthamine (Figure 3.2 and Figure 3.3) (Jin, 2003). From *P. biflorum*, three chromone aglucones, one glucosyl and one glucosyloxy chromones were isolated, in addition to glucosyloxy alkaloids (Ghosal *et al.*, 1984, Berkov *et al.*, 2004). Youssef *et al.* (1998) reported the isolation of chromones, chromone-glucosides and glucosyloxy, acetophenones from *P. biflorum*. In addition, the flavanes syzalterin, (-)- farrerol and (-)-liquiritigenin (Figure 3.3) have been isolated from the Egyptian *P. maritimum*.

Results presented in this Chapter outline the preliminary screening of several plant extracts before following the sub-fractionation of bioactive components of *P. tortuosum* and isolation of narciclasine.

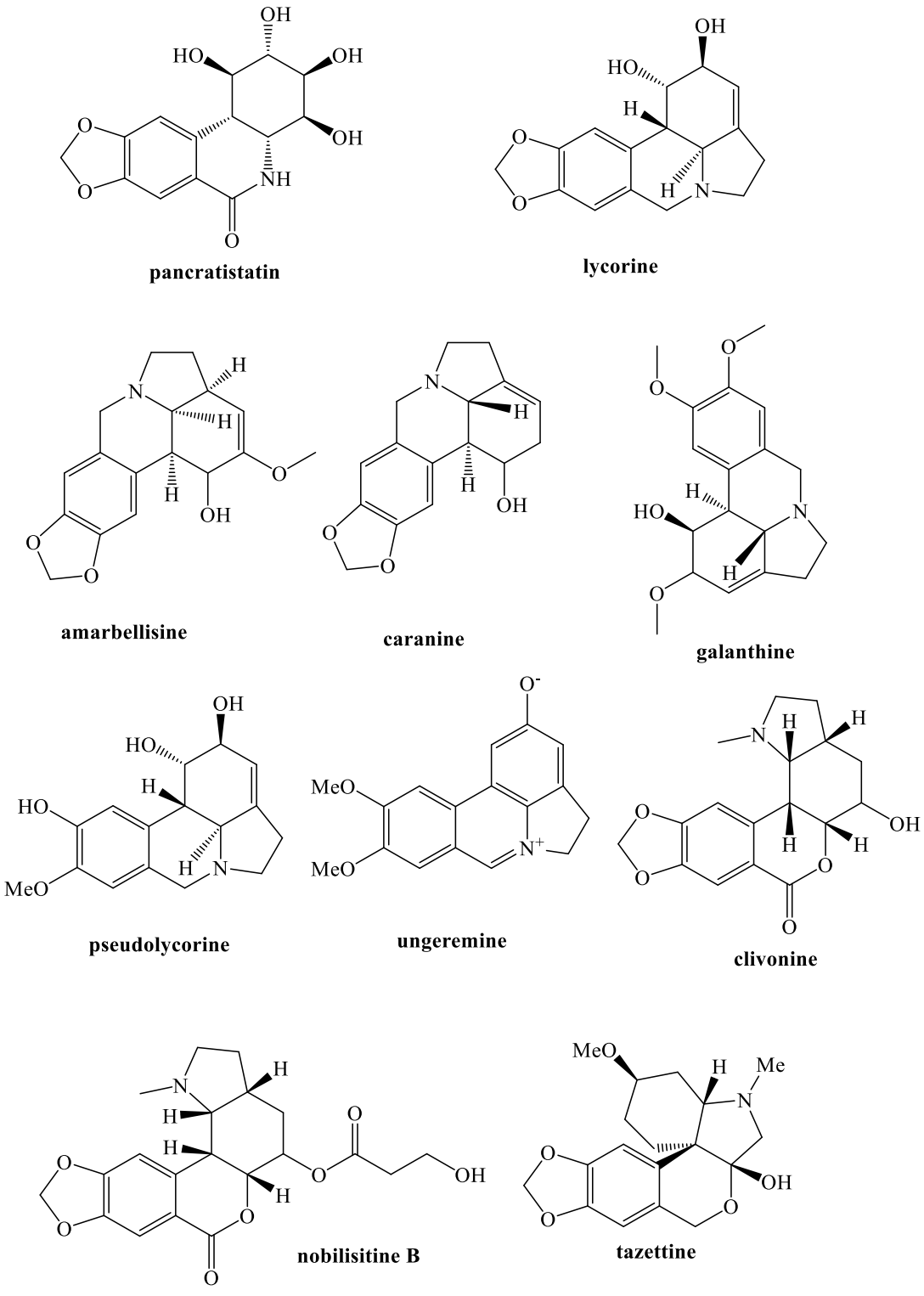
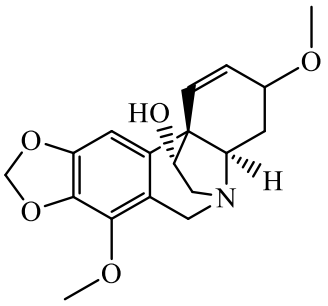
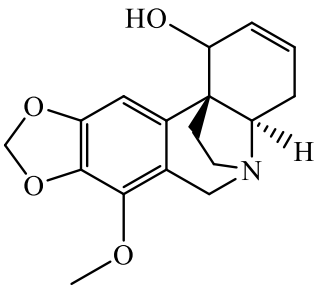


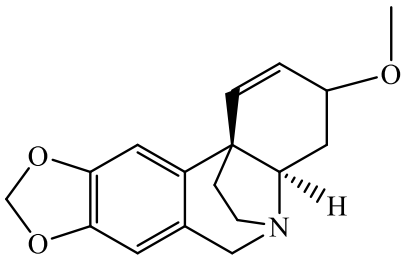
Figure 3. 1: Alkaloid structures previously isolated from *Pancratium* genus.



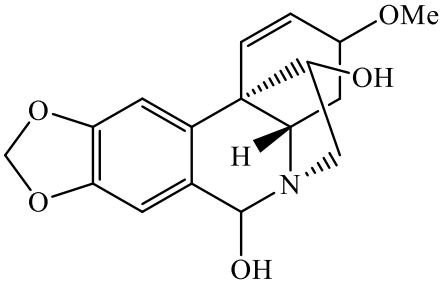
ambelline



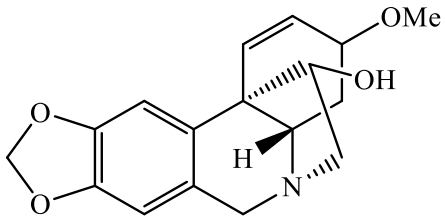
buphanamine



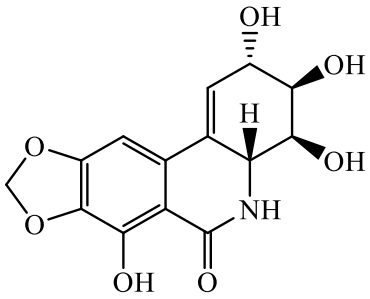
buphanisine



haemanthidine



haemanthamine



narciclasine

Figure 3. 2: Alkaloid structures previously isolated from *Pancratium* genus.

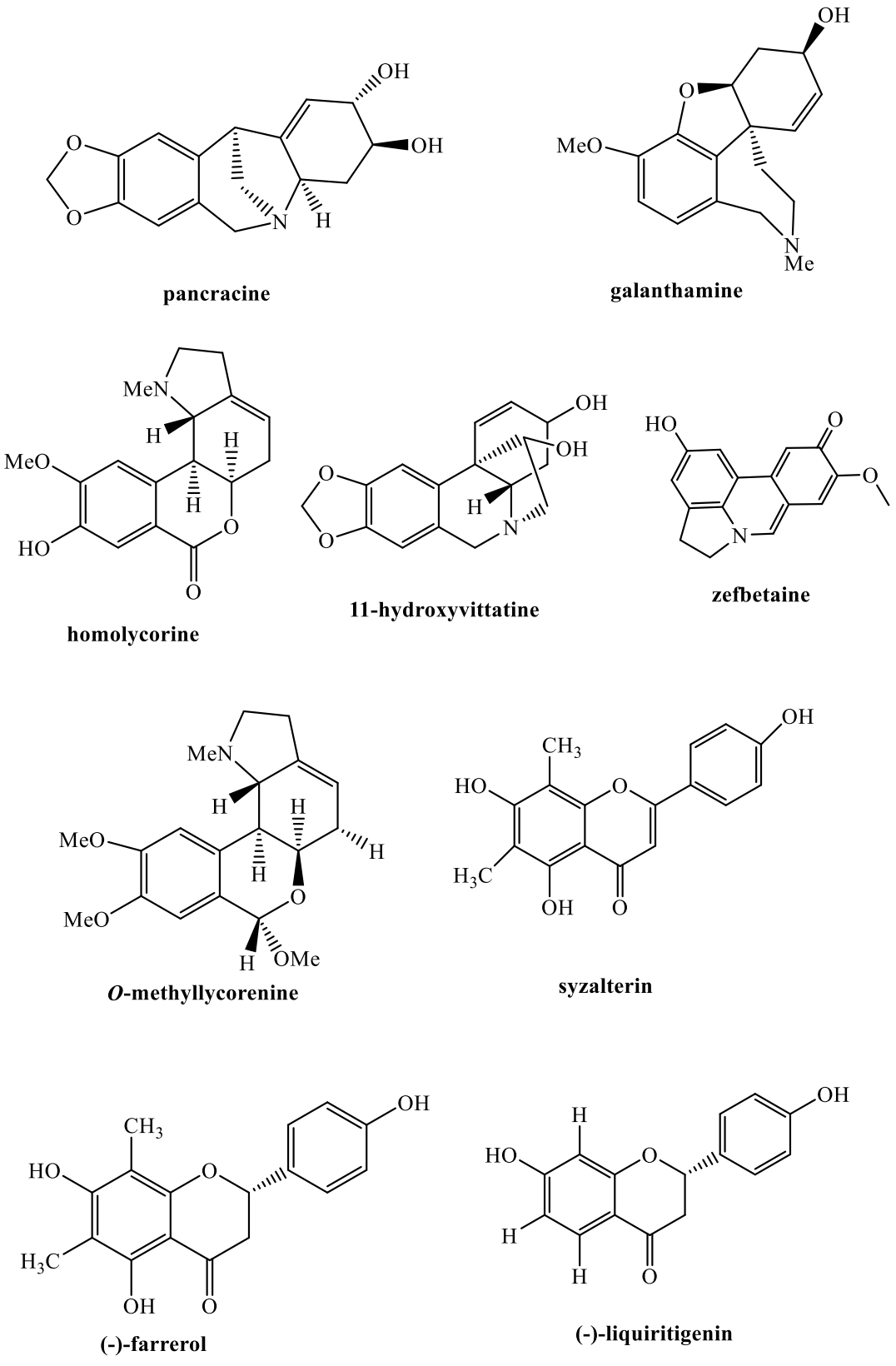


Figure 3. 3*:* Alkaloid structures previously isolated from *Pancratium* genus.

3.2 Results

3.2.1 Effect of plant crude extracts on cell viability, DNA synthesis and protein markers

Initially, preliminary screening of the selected plants against cellular viability was performed using the tetrazolium salt MTT to evaluate potential detrimental effects of the extracts on cellular metabolic activity. In this study, two breast cancer cell lines, MDA-MB-231 (a triple negative type) and MCF7-L (oestrogen receptor positive type) and normal mouse embryo fibroblast (MEFs) were used. Cells were grown to ~ 70-80% confluence and treated with and without plant crude extracts for 24 h. The plant crude extracts were used at 1 and 5 µg/ml (concentrations used based on previous study of King Lim, PhD thesis 2010). All plant crude extracts at 1 µg/ml or 5 µg/ml exhibited no significant effect on MDA-MB-231 breast cancer cell viability (Figure 3.4 A and B respectively). All the plant crude extracts at 1 µg/ml showed no significant effect on MCF-7 cell viability (Figure 3.5 A), while two plant crude extracts, *H. strobilasium* and *Crotolaria* sp., at 5 µg/ml, reduced MCF-7 cell viability ($p<0.05$ *versus* control) (Figure 3.5 B). None of the plant crude extracts had a cytotoxic effect at 1 µg/ml in MEFs. In contrast, there was significant cytotoxic effects (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs control) for all the extracts on MEF cell viability at 5 µg/ml (Figure 3.6 A and B).

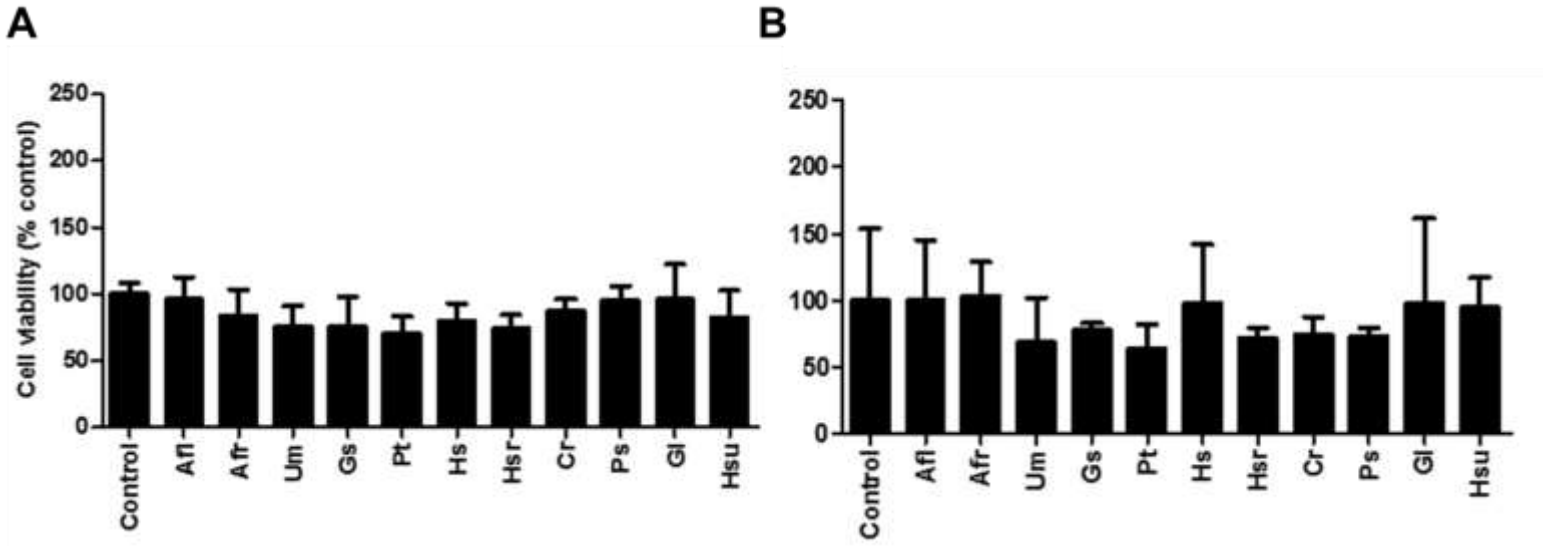


Figure 3. 4: Effect of plant crude extracts on cell viability of MDA-MB-231 cell.

MDA-MB-231 cells were plated for 24 h before being treated with vehicle control (0.05 % DMSO) or (**A**) plant crude extracts (1 µg/ml), or (**B**) plant crude extracts (5 µg/ml), for 24 h and then assayed for viability by MTT assay. Data are expressed as percentage of control and represent mean +/- SD for n = 3 experiments, each performed in triplicate. Abbreviations: *Afl, A. fistulosus; Afr, A. fistulosus roots; Um, U. maritima; Gs, G. sinaicus; Pt, P. tortuosum; Hs, H. strobilaceum; Hsr, H. strobilaceum roots; Cr, Crotalaria sp.; Ps, P. plicata; Gl, G. lotoides; Hsu, H. supinum.*

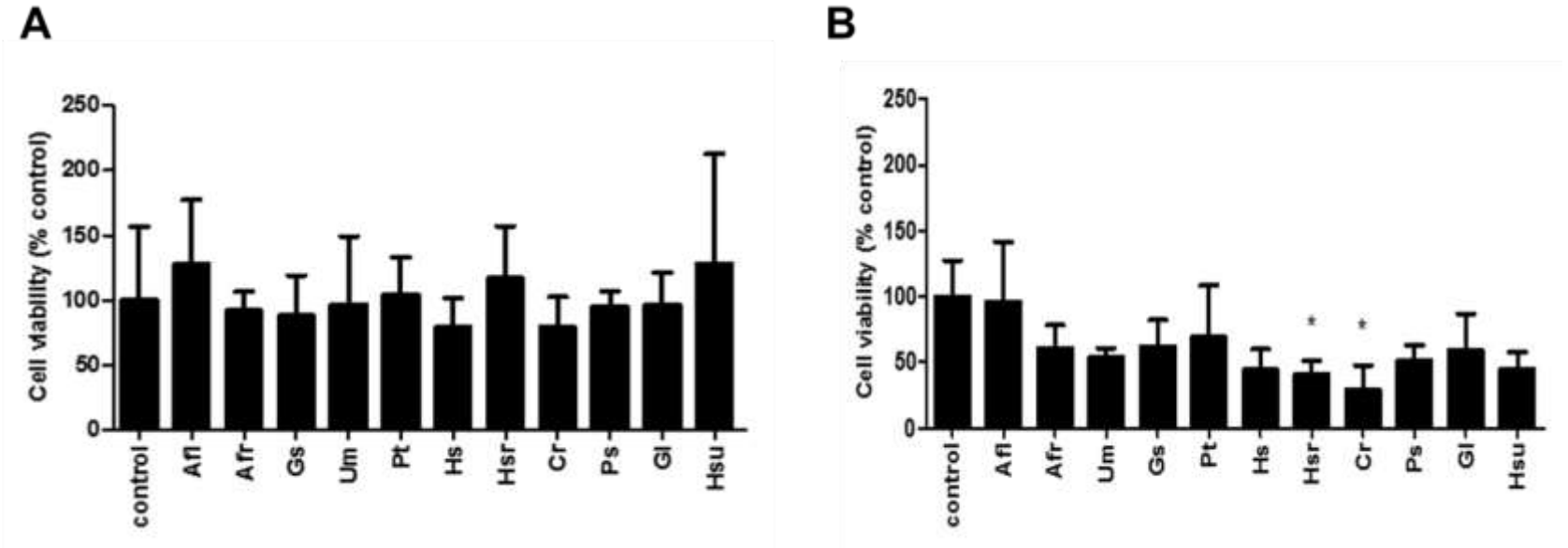


Figure 3. 5: Effect of plant crude extracts on cell viability of MCF7/L cells.

MCF7/L cells were plated for 24 h before being treated with vehicle control (0.05 % DMSO) or (**A**) plant crude extracts (1 µg/ml), or (**B**) plant crude extracts (5 µg/ml), for 24 h and then assayed for viability by MTT assay. Data are expressed as percentage of control and represent mean +/- SD for n = 3 experiments, each performed in triplicate. * $p<0.05$ vs control (one-way ANOVA with Dunnett's post-hoc test). Abbreviations: *Afl, A. fistulosus; Afr, A. fistulosus roots; Um, U. maritima; Gs, G. sinaicus; Pt, P. tortuosum; Hs, H. strobilaceum; Hsr, H. strobilaceum roots; Cr, Crotalaria sp.; Ps, P. plicata; Gl, G. lotoides; Hsu, H. supinum.*

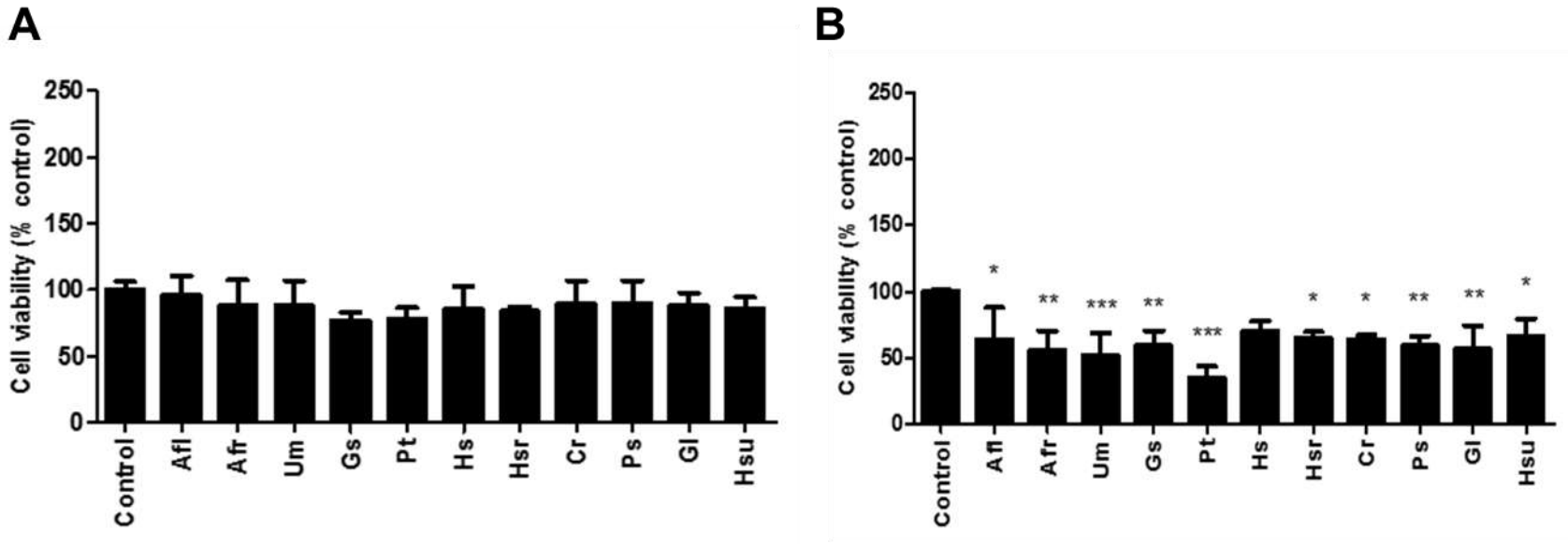


Figure 3. 6: Effect of plant crude extracts on cell viability of MEF cells.

MEF cells were plated for 24 h before being treated with vehicle control (0.05 % DMSO) or (**A**) plant crude extracts (1 µg/ml), or (**B**) plant crude extracts (5 µg/ml), for 24 h and then assayed for viability by MTT assay. Data are expressed as percentage of control and represent mean +/- SD for n = 3 experiments, each performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). Abbreviations: *Afl, A. fistulosus; Afr, A. fistulosus roots; Um, U. maritima; Gs, G. sinaicus; Pt, P. tortuosum; Hs, H. strobilaceum; Hsr, H. strobilaceum roots; Cr, Crotalaria sp.; Ps, P. plicata; Gl, G. lotoides; Hsu, H. supinum.*

There are many ways to assess cell growth inhibition by exogenously added agents. For example, the inhibition of the cell cycle, and therefore, DNA synthesis can be used. To confirm the inhibition of DNA synthesis by selected plant crude extracts, [^{3}H]-thymidine incorporation into newly synthesised DNA was measured. At the same time, certain protein markers were assessed by Western blotting to provide further insight into which plant extracts may contain anti-proliferative or pro-apoptotic compounds. The proteins assessed were SK1, Des1 and PARP whereas GAPDH was used as a protein loading control. The SK inhibitor, SKi (2-(p-hydroxyanilino-4-p-chlorophenyl) thiazole) was used as a positive control, as it induces proteasomal degradation of SK1 in MCF7/L cells and Des1 in LNCaP-AI prostate cancer cells, thereby reducing their expression and removing these proteins from the cells (Loveridge *et al.*, 2010, McNaughton *et al.*, 2016, Pyne *et al.*, 2016a). Sphingosine binding site inhibitors of SK1 have a common feature of inducing ubiquitin-proteasomal degradation of SK1 in cancer cell lines (Loveridge *et al.*, 2010, Lim *et al.*, 2011b). Therefore, SKi can be used to compare mechanisms of action with active components of the plant extracts.

A comparison was made between two breast cancer cell lines, MDA-MB-231 and MCF7-L cells and normal MEFs Cells were grown to ~70% confluence and then incubated in the presence and absence of plant crude extracts at 1 and 5 µg/ml. All the crude extracts, except for *P. tortuosum* (5 µg/ml) had no effect on DNA synthesis in MEF cells (Figure 3.7 A and B). There was a slight effect on MEF cells with *P. tortuosum* extract at 5 µg/ml (Figure 3.7 B).

None of the plant extracts at 1µg/ml had an effect on DNA synthesis in MDA-MB-231 breast cancer cells (Figure 3.8 A). However, two plant crude extracts *P. tortuosum* and *G. sinaicus* ($p<0.05$) reduced DNA synthesis in MDA-MB-231 cells (Figure 3.8 B). Only SK1a was detected in lysates of MDA-MB-231 cells (Figure 3.8 C). Although treatment of MDA-MB-231 cells with SKi reduced SK1a expression, there was little or no effect of the various plant extracts. Similarly, there was little effect of the extracts on Des1 expression. The treatment of these breast cancer cells with crude extracts Gs, or Um or Pt did induce a small increase in PARP cleavage over and above the control, suggesting that these extracts might induce apoptosis of MDA-MB-231 cells (Figure 3.8 C).

All the plant crude extracts reduced DNA synthesis at 5 µg/ml in MCF-7 cells (Figure 3.9 B). At 1 µg/ml, *P. tortuosum, G. sinaicus, U. maritima, P. plicata, Crotalaria sp*., *H. supinum* and *H. strobilaceum* induced a significant reduction in DNA synthesis (Figure 3.9 A). The 3 splice variants of SK1 were detected in MCF-7L cells corresponding to SK1a-c (Figure 3.9 C). Treatment of MCF-7L cells with SKi induced a reduction in the expression of SK1a, SK1b and SK1c and this was recapitulated by Gs, Um and Pt. It is notable that while SKi had no effect on Des1 expression, all the plant extracts were able to abolish Des1 expression. Treatment of the cells with SKi or crude extracts Gs or Um or Pt also induced PARP cleavage, suggesting that the removal of SK1a-c, and not Des1 was associated with increased apoptosis (Figure 3.9 C). This was also correlated with the ability of Gs, Um and Pt to reduce DNA synthesis in MCF-7L cells (Figure 3.9 A and B).

These findings demonstrate that the extracts of *P. tortuosum, G. sinaicus* and *U. maritima* selectively affect cancer cells with little or no effect on normal MEFs. Therefore, the plant crude extracts of *P. tortuosum* (Pt) and *G. sinaicus* (As) were selected to be fractionated to identify the compounds that are responsible for the pharmacological effects on DNA synthesis inhibition and on the expression of SK1, Des1 and PARP cleavage.

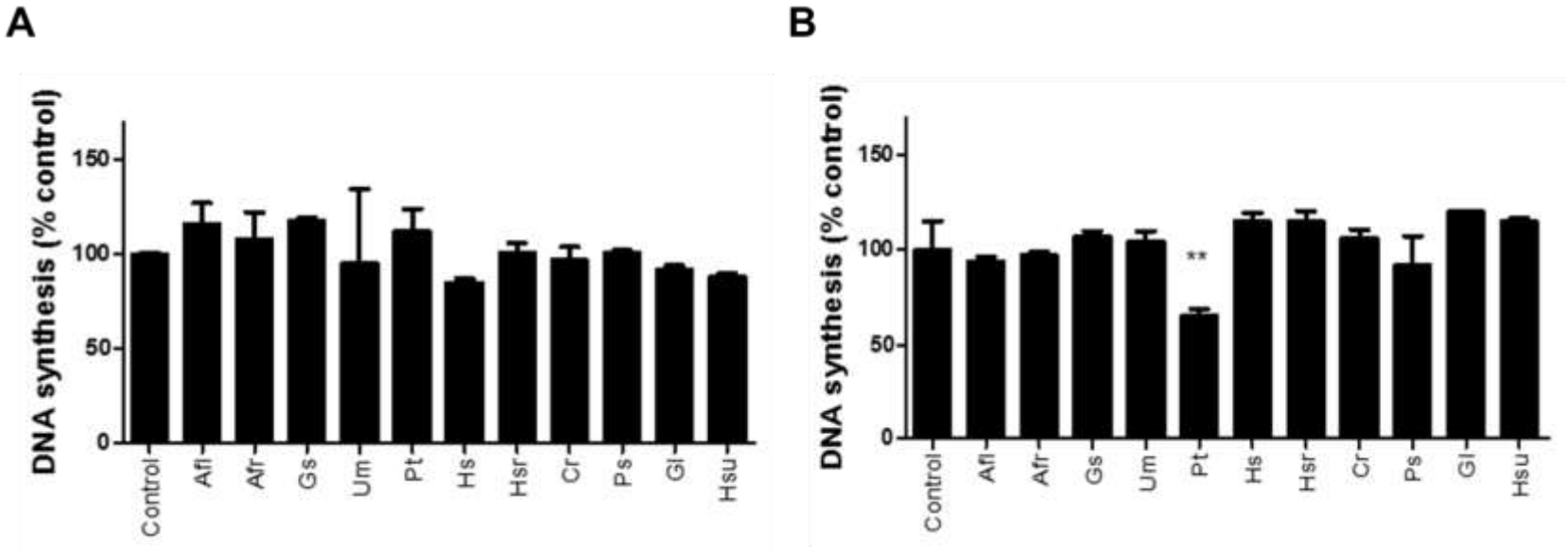


Figure 3. 7*:* Effect of plant crude extracts on DNA synthesis of MEF cells.

Quiescent MEF cells were treated (**A**) with 1 µg/ml of plant crude extracts, (**B**) with 5 µg/ml or vehicle control (0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H]-thymidine into newly synthesised DNA was measured as described under Methods (section 2.2.13). Data are expressed as percentage of control and represent means +/- SD of combined data from 3 experiments. **$p<0.01$ vs control (one way ANOVA with Dunnett's post-hoc test). Abbreviations: *Afl, A. fistulosus; Afr, A. fistulosus roots; Um, U. maritima; Gs, G. sinaicus; Pt, P. tortuosum; Hs, H. strobilaceum; Hsr, H. strobilaceum roots; Cr, Crotalaria sp.; Ps, P. plicata; Gl, G. lotoides; Hsu, H. supinum.*

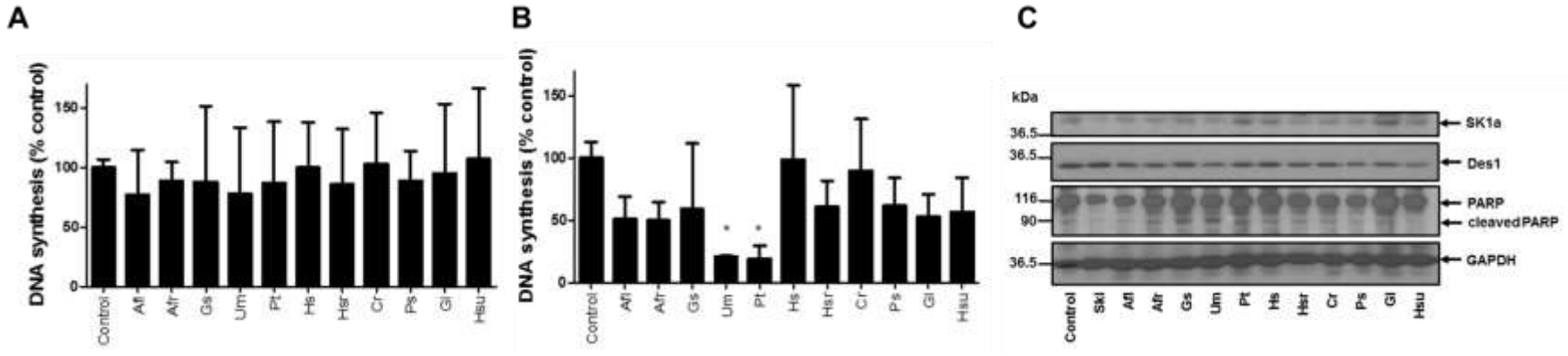


Figure 3. 8: Effect of plant crude extracts on DNA synthesis and key proteins in MDA-MB-231cells.

Quiescent MDA-MB-231 cells were treated with (**A**) with plant crude extracts (1 µg/ml), (**B**) with plant crude extracts (5 µg/ml) or vehicle control (0.05% (v/v) DMSO), for 16 h and then [^{3}H] thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described under Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments. *$p<0.05$ vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) Effect of plant crude extracts on SK1, Des1 and PARP: quiescent MDA-MB-231 cell were treated with vehicle control (0.1% (v/v/) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each plant crude extract for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.16). Blots were then stripped and re-probed for GAPDH to check for similar protein loading. Abbreviations*: Afl, A. fistulosus; Afr, A. fistulosus roots; Um, U. maritima; Gs, G. sinaicus; Pt, P. tortuosum; Hs, H. strobilaceum; Hsr, H. strobilaceum roots; Cr, Crotalaria sp.; Ps, P. plicata; Gl, G. lotoides; Hsu, H. supinum.*

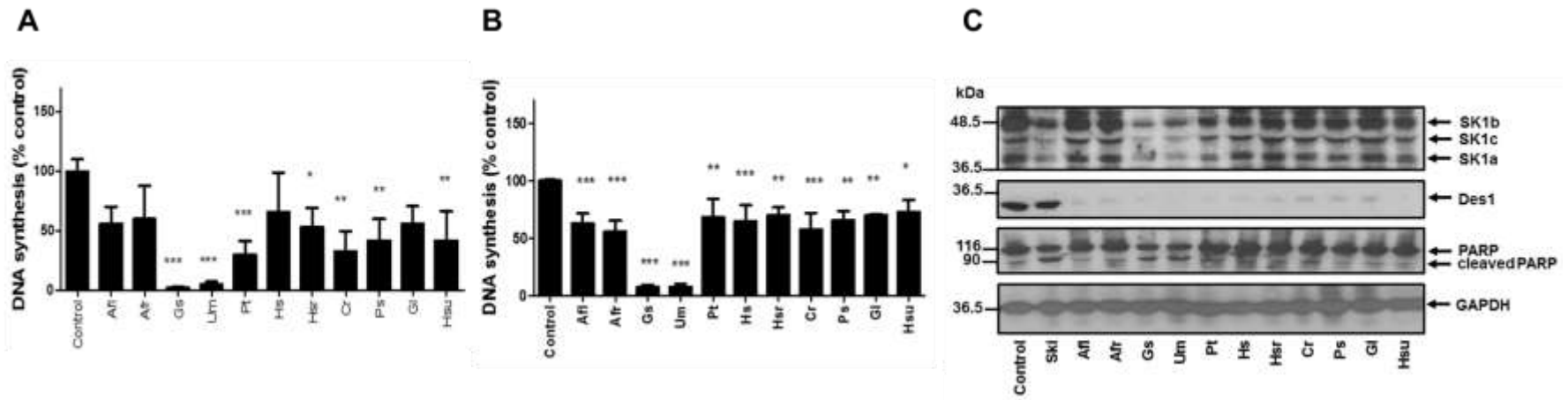


Figure 3. 9: Effect of plant crude extracts on DNA synthesis and key proteins in MCF7L cells.

Quiescent MCF7 cells were treated with (**A**) plant crude extracts (1 µg/ml), (**B**) plant crude extracts (5 µg/ml) or vehicle control (0.05% (v/v) DMSO) for 16 h, and then [^{3}H] thymidine (0.5 µCi/ml) for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods sections (2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments. *p<0.05, **p<0.01, ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) Effect of plant crude extracts on SK1, Des1 and PARP: quiescent MCF7/L cell were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each plant crude extract for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.16). Blots were then striped and re-probed for GAPDH to check for similar protein loading. Abbreviations: *Afl, A. fistulosus; Afr, A. fistulosus roots; Um, U. maritima; Gs, G. sinaicus; Pt, P. tortuosum; Hs, H. strobilaceum; Hsr, H. strobilaceum roots; Cr, Crotalaria sp.; Ps, P. plicata; Gl, G. lotoides; Hsu, H. supinum.*

3.2.2 Fractionation of plant crude extract from *P. tortuosum*

P. tortuosum crude extract were fractionated by flash chromatography as described in Methods (section 2.2.2), yielding four different fractions (50% ethyl acetate, 100% ethyl acetate, 50% methanol and 100 % methanol), after washing with hexane 100% for defatting. The dry weight of starting material for *P. tortuosum* was 7.8 grams. The yields after flash chromatography are given in Table 3.1.

Table 3. 1: *P. tortuosum* fractions dry weight:

Pt fractions	Solvent	Dry weight g
Pt 2	50% (v/v) ethyl acetate	0.192
Pt 3	100% (v/v) ethyl acetate	0.109
Pt 4	50% (v/v) methanol	3.0507
Pt 5	100% (v/v) methanol	3.002

3.2.3 Effect of *P. tortuosum* fractions on DNA synthesis and protein markers

Fractionation of *P. tortuosum* crude extracts, yielded four fractions (Pt 2 (50% (v/v) ethyl acetate), Pt 3 (100% (v/v) ethyl acetate), Pt 4 (50% (v/v) methanol), Pt 5 (100% (v/v) methanol) (Table 3.1). 1 µg/ml and 5 µg/ml of each fraction was tested for their effect on DNA synthesis. Treatment of MDA-MB-231 cells with Pt 3 (100% ethyl acetate) was more effective at reducing DNA synthesis compared with Pt 4 (50% (v/v) methanol) at 1µg/ml (Figure 3.10 A), while both fractions almost abolished DNA synthesis at 5 µg/ml (Figure 3.10 B), indicating that the effects are concentration-dependent. The fractions had very little effect, if any, on the expression of SK1a or Des1 in MDA-MB-231 cells. However, Pt 3 and SKi did induce significant PARP cleavage, indicating the Pt 3 induces apoptosis independently of SK1 and Des1 expression. There was some effect of Pt 4 and Pt 5 on PARP cleavage, albeit less than Pt 3 (Figure 3.10 C).

At 1µg/ml, Pt 3 induced a marked reduction in DNA synthesis in MCF7/L cells (Figure 3.11 A), while the other fractions, *with the exception of* Pt 5 had no effect. At 5µg/ml,

all the fractions were active in reducing DNA synthesis in MCF7/L cells (Figure 3.11 B). Only SKi and Pt 3 induced a reduction in SK1a expression and Des1 and this was associated with increased PARP cleavage (Figure 3.11 C).

In summary, the Pt 3 fraction showed a clear decrease in DNA synthesis, induced PARP cleavage in both breast cancer cell lines and downregulated SK1a and Des1 protein expression in MCF-7L cells. Pt 3 was therefore subjected to further purification. Although Pt 3 activates apoptosis in MDA-MB-231 cells, the effects are apparently independent of SK1 and Des1 expression.

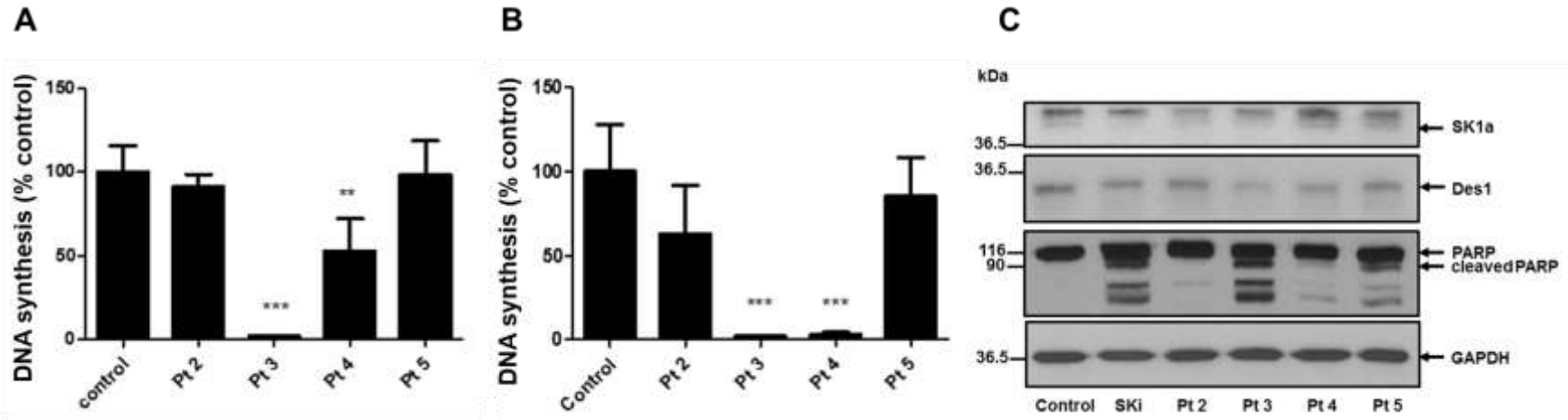


Figure 3. 10: Effect of *P. tortuosum* fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with (**A**) *P. tortuosum* fractions (1 µg/ml) or (**B**) *P. tortuosum* fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesized DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined date from 3 experiments. **p<0.01, ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1, Des1 and PARP cleavage detection: quiescent MDA-MB-231 cell were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.16). Blots were then striped and re-probed for GAPDH to check for similar protein loading.

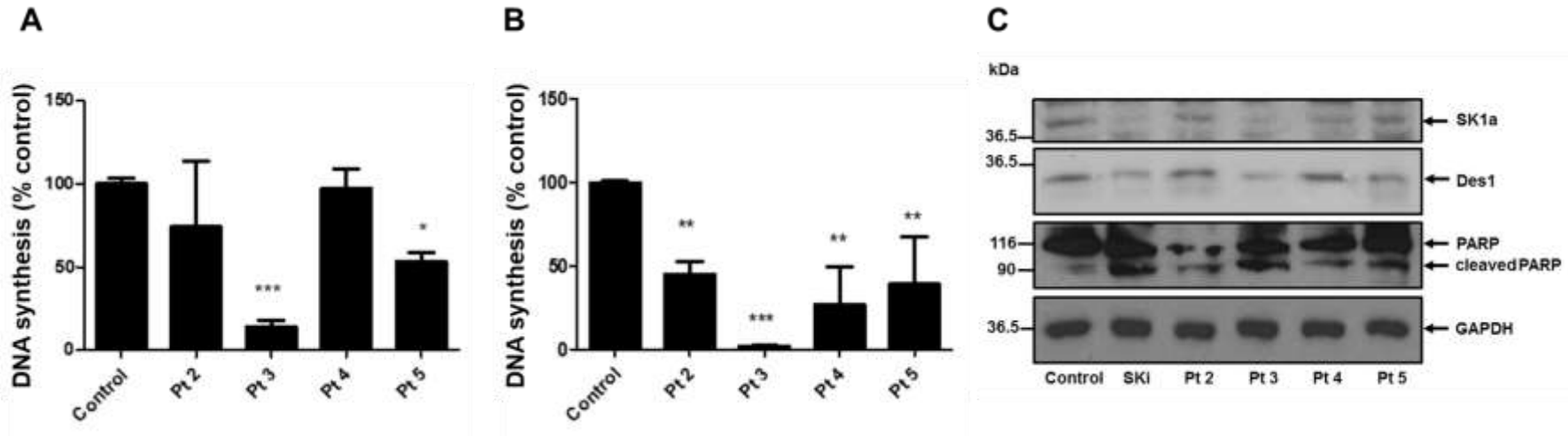


Figure 3. 11: Effect of *P. tortuosum* fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MCF7/L cells.

Quiescent MCF7/L cells were treated with (**A**) *P. tortuosum* fractions (1 µg/ml) or (**B**) *P. tortuosum* fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments. **p<0.01, ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1, Des1 and PARP cleavage detection: quiescent MCF7L cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.16). Blots were then striped and re-probed for GAPDH to check for similar protein loading.

Based on these results Figures 3.10 and 3.11), sub-fractionation and separation of Pt 3 (100% ethyl acetate) fraction was performed to further purify active compounds. Using silica column chromatography as described in Methods (section 2.2.4), using solvent mixture hexane: ethyl acetate: methanol (6:3:0.5). Pt 3 yielded seven sub-fractions named: Pt 3.1, Pt 3.2, Pt 3.3, Pt 3.4, Pt 3.5, Pt 3.6, and Pt 3. 8 (Table 3.2). These were then tested for their effect on DNA synthesis (at 1 and 5 µg/ml) and on SK1, Des1 expression and PARP cleavage at 5 µg/ml in both MDA-MB-231 and MCF7/L cells.

Table 3. 2: *P. tortuosum* sub-fractions (Pt 3) samples dry weight:

Pt sample	Dry weight / µg
Pt 3.1	1.2
Pt 3.2	0.9
P. t 3.3	3.2
Pt 3.4	1.0
Pt 3.5	3.4
Pt 3.6	0.1
Pt 3.8	0.1

Treatment of MDA-MB-231 cells with each Pt sub-fraction (1 µg/ml) reduced DNA synthesis (Figure 3.12 A) and this inhibitory effect was more pronounced at 5 µg/ml (Figure 3.12 B). None of the sub-fractions produced a convincing reduction in SK1a or Des1 although Pt 3.3 did induce PARP cleavage as well as, other sub-fractions; Pt 3.2, Pt 3.5, Pt 3.6 (Figure3.12 C). These data are consistent with previous findings showing less than robust effects on SK1a and Des1.

Treatment of MCF-7L cells with the Pt sub-fractions at 1µg/ml was largely ineffective at inhibiting DNA synthesis with the exception of Pt 3.3 (Figure 3.13 A). However,

other sub-fractions were effective at 5 µg/ml (Figure 3.13 B). At this concentration, Pt 3.3 and Pt 3.8 induced a reduction in the expression of SK1a-c and this was associated with increased PARP cleavage (Figure 3.13 C). Pt 3.2-Pt.3.6 also induced the loss of Des1 and this was also associated with PARP cleavage (Figure 3.13 C). Therefore, with the exception of Pt 3.8, it is unclear whether PARP cleavage is associated with SK1 and/or Des1 or either.

Therefore, Pt 3.3 is a potential modulator of SK1 and/or Des1 expression. Pt 3.3 was therefore subjected to 1D-2D spectroscopic analysis to identify the active molecule.

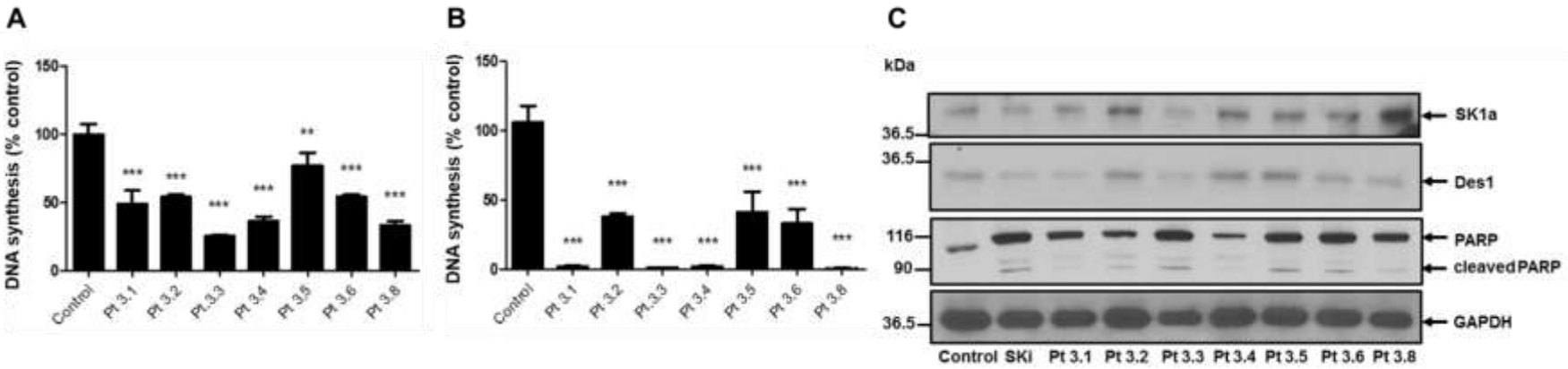


Figure 3. 12: Effect of *P. tortuosum* sub-fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with (**A**) *P. tortuosum* sub-fractions (1 µg/ml) or (**B**) *P. tortuosum* sub-fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments **p<0.01, ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1, Des1 and PARP cleavage detection: quiescent MDA-MB-231 cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.16). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

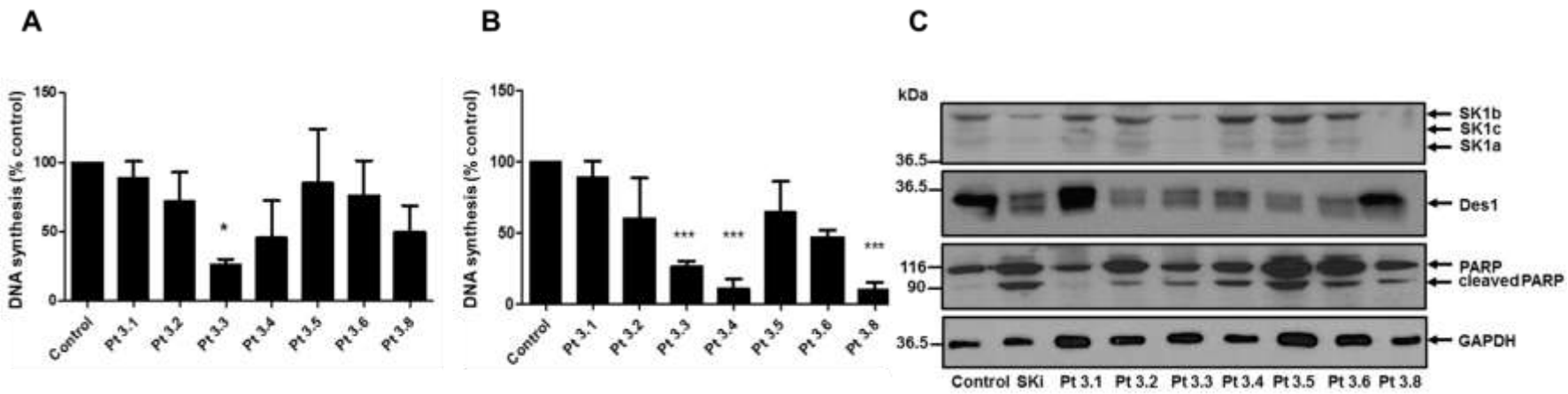


Figure 3. 13: Effect of *P. tortuosum* sub-fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MCF7/L cells.

Quiescent MCF7/L cells were treated with (**A**) *P. tortuosum* sub-fractions (1 µg/ml) or (**B**) *P. tortuosum sub-fractions* (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments. *$p<0.05$, ***$p<0.001$ vs control (one-way ANOVA with Dunnett's post-hoc test) (**C**) SK1, Des1 and PARP cleavage detection: quiescent MCF7/L cell were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.16). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

3.2.4 Structure elucidation of Pt 3 samples:

Structure elucidation of compound in fraction Pt 3.3 was achieved using 1D and 2D NMR spectroscopic analysis. Due to the low amount of the sample after freeze-drying (~5.0 mg). The NMR spectra were obtained using Norell micro-tubes to improve signal to noise ratio during acquisition. The compound in fraction Pt 3.3 under LCHRMS yielded a molecular ion peak $[M+H]^+$ at *m/z* 308.0760, corresponding to the molecular formula $C_{14}H_{13}NO_7$ (Figure 3.14). The proton spectrum showed a highly de-shielded proton at δ_H 13.26 (s), indicating the presence of a H-bonded hydroxyl proton, (identified as 7-OH). Additionally, an amine proton appeared at δ_H 7.87 (NH), (Figure 3.15- 3.16). In addition, there were three hydroxyl groups; two at δ_H 5.22 (brs) (2-OH and 4-OH), which showed correlations in ^{1}H-^{1}H COSY with proton H-2 at δ_H 4.03 and another at δ_H 5.03 (3-OH) (brs), which correlated with H-3 at δ_H 3.71 (Table 3.3).

In the ^{1}H-^{1}H COSY spectrum (Figure 3.17), the presence of cross peaks indicates spin-spin coupled protons that are neighbouring. Protons bonded to the same carbon are usually identical with no coupling. However, two-bond coupling or geminal coupling ($^2J_{HH}$) can be observed when two protons bonded to the same carbon are not identical (also known as diastereotopic). These protons are not in the same chemical environment because of other substituents in close proximity to chiral centres. A different phenomenon was observed in this molecule due to the dissociation nature of H-11 (show as a doublet) (Figure 3.17). Definable cross-peaks in the COSY spectrum were observed for $^3J_{H1,H2}$, $^3J_{H2,H3}$ (weak), $^3J_{H2,2\text{-}OH}$, $^3J_{H3,H4}$, $^3J_{H3,3\text{-}OH}$, $^4J_{H1,H4a}$ (weak), with a larger coupling and strong correlation observed between H4 and H4a (Figure 3.17).

The DEPT-q spectrum (Figure 3.18) showed that the compound in fraction Pt 3.3 was relatively pure, and all carbon signals were distinct and clearly observable due to acquisition with a good signal-to-noise ratio (Figure 3.18). The DEPT-q data revealed the presence of fourteen carbon atoms. These were resolved from HSQC spectroscopic analysis (Figure 3.19) into, one methylene carbon (CH_2), six methines (CH) and seven quaternary carbons. The connections between each proton and carbon present in the molecule were obtained with two-dimensional HSQC and HMBC spectra. All protons

were appropriately assigned to their corresponding carbons based on correlations in the HSQC spectrum (Figure 3.19). Of note, H-11 did not correlate with any carbon in the spectrum confirming its identity as an O-CH2-O and not CH. In addition, extensive long-range correlations from protons to carbons with long distance were observed in the HMBC spectrum allowing the connection between different spin systems to be confirmed (Figure 3.20). For example, H-10 (δ 6.86) shows typical two (^{2}J CH) bond couplings to neighbouring carbons: H10/C9, H10/C6a, and H10/C10a. Other significant correlations included the N-H proton, which showed ^{3}J CH bond couplings to neighbouring carbons NH/C6a and NH/C10b. There were also ^{2}J CH bond coupling with neighbouring carbon C-4a. H-1 exhibited three-bond coupling with two neighbouring carbons, H11/C9 and H11/C10a. Information concerning the relative stereochemistry of the compound was obtained. Magnetic nuclei can interact through a nuclear Overhauser effect (Jacobs *et al.*, 2004) they are close to each other in space and the NOESY spectrum can provide such information. As shown in (Figure 3.21), H-3 interacted with H-4. This indicates that they interact "see" each other through space and are in a *cis* configuration. In addition, NOEs were weakly observed between H-2 and H-3 and H-4, indicating that H-2 is a *trans* configuration. Moreover, very weak NOE was observed between H-4 and NH which in turn interact weakly with H-4 suggesting that they are also in a *trans* configuration.

Therefore, HMBC allows the linkage of the different spin systems in the molecule, supporting the proposed structure to be narciclasine. Narciclasine has previously been isolated from *Narcissus* species (Van Goietsenoven *et al.*, 2013). All chemical shifts of protons and carbons in narciclasine were comparable to those reported by others (Dumont *et al.*, 2007, McNulty *et al.*, 2011), where the spectra were obtained in DMSO-d_6 (Table 3.3).

Table 3. 3: ^{1}H-NMR (500 MHz) & DEPT-q spectrum (100 MHz) of Pt 3.3, in DMSO-*d_6*

No.	**δ*C* ppm**	**δ*H* ppm**	**HMBC**
1	124.8	6.15 br t	
2	69.2	4.03	
3	72.4	3.71	C-1
4	68.9	3.81	C-4a
4a	52.9	4.19	C-2, C-4, C-1, C-10b
N-H	-	7.87 s	C-6, C-10b, C-6a, C-4, C-4a
6	169	-	
6a	105.6	-	
7	144.9	-	
8	132.5	-	
9	152.4	-	
10	95.9	6.86	C-9, C-8, C-10b, C-6a
10a	132.1	-	
10b	129.3	-	
11	102.1	6.09	C-9, C-8
7-OH	-	13.26 s	C-7, C-8, C6a
2-OH	-	5.22 br s	
3-OH	-	5.03 br s	
4-OH	-	5.22 br s	

s = singlet, br t = broad triplet

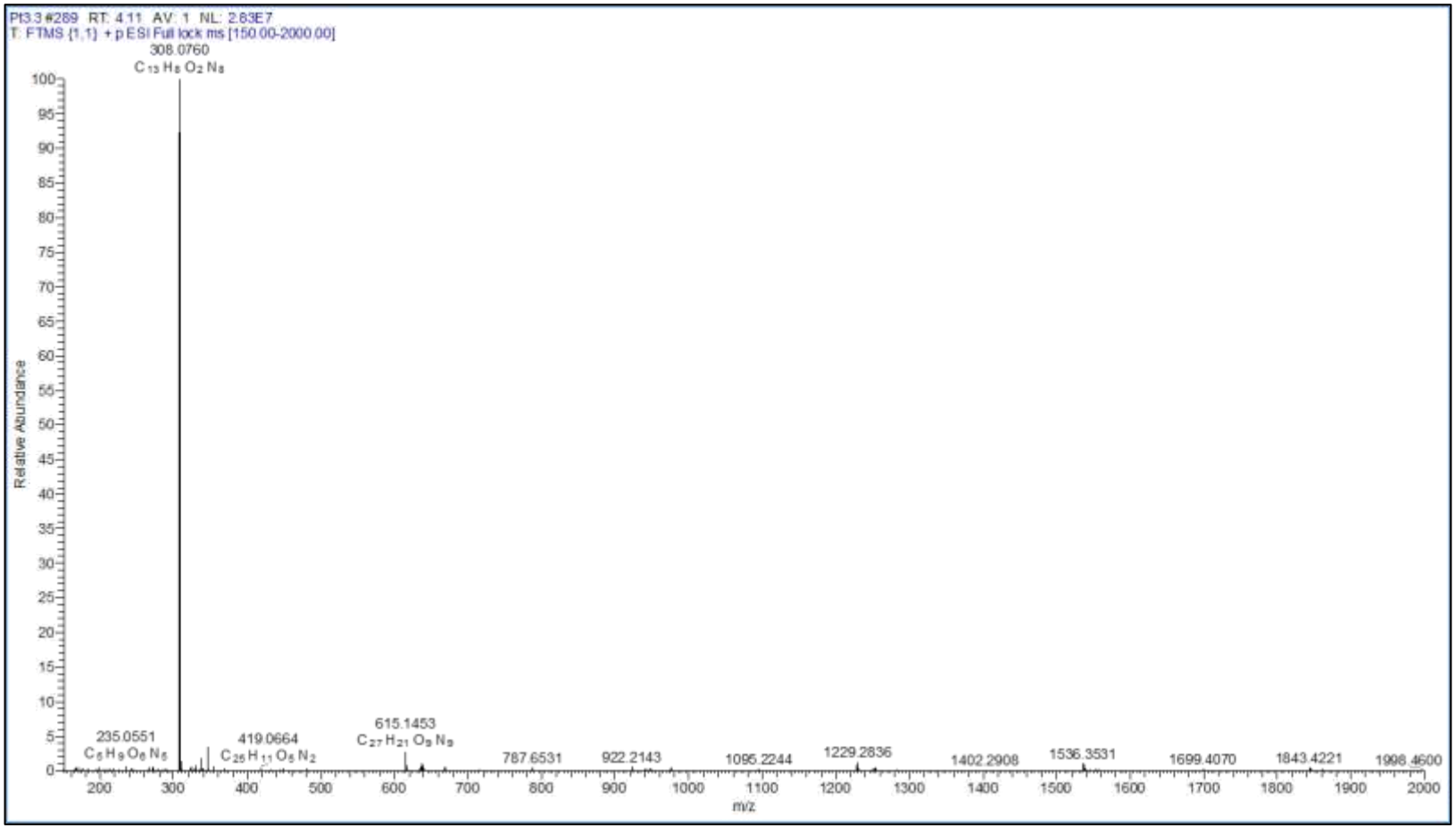


Figure 3. 14: LCHRMS of Pt 3.3.

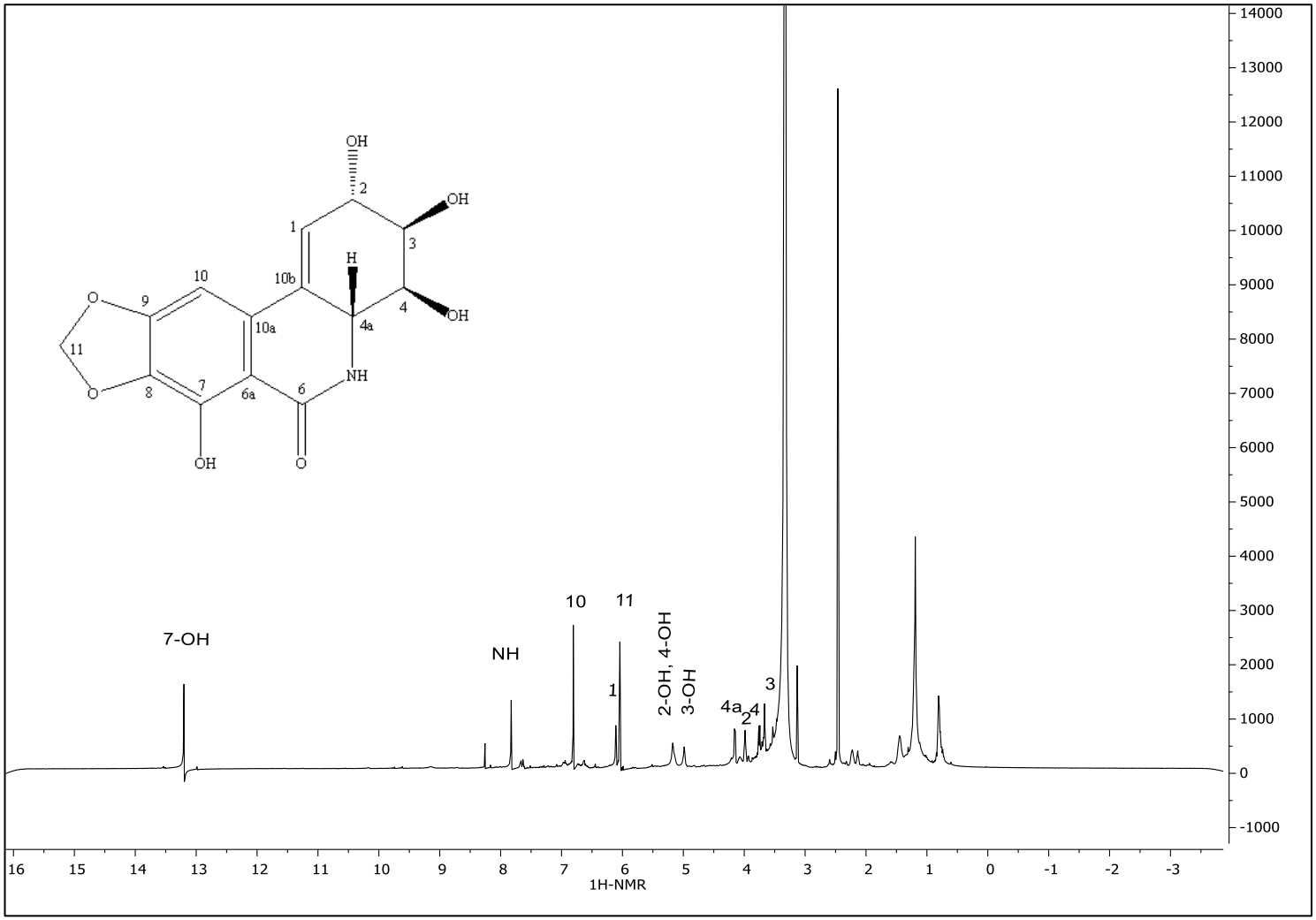


Figure 3. 15: ^{1}H-NMR spectroscopic analysis of Pt 3.3 in DMSO-*d*$_6$

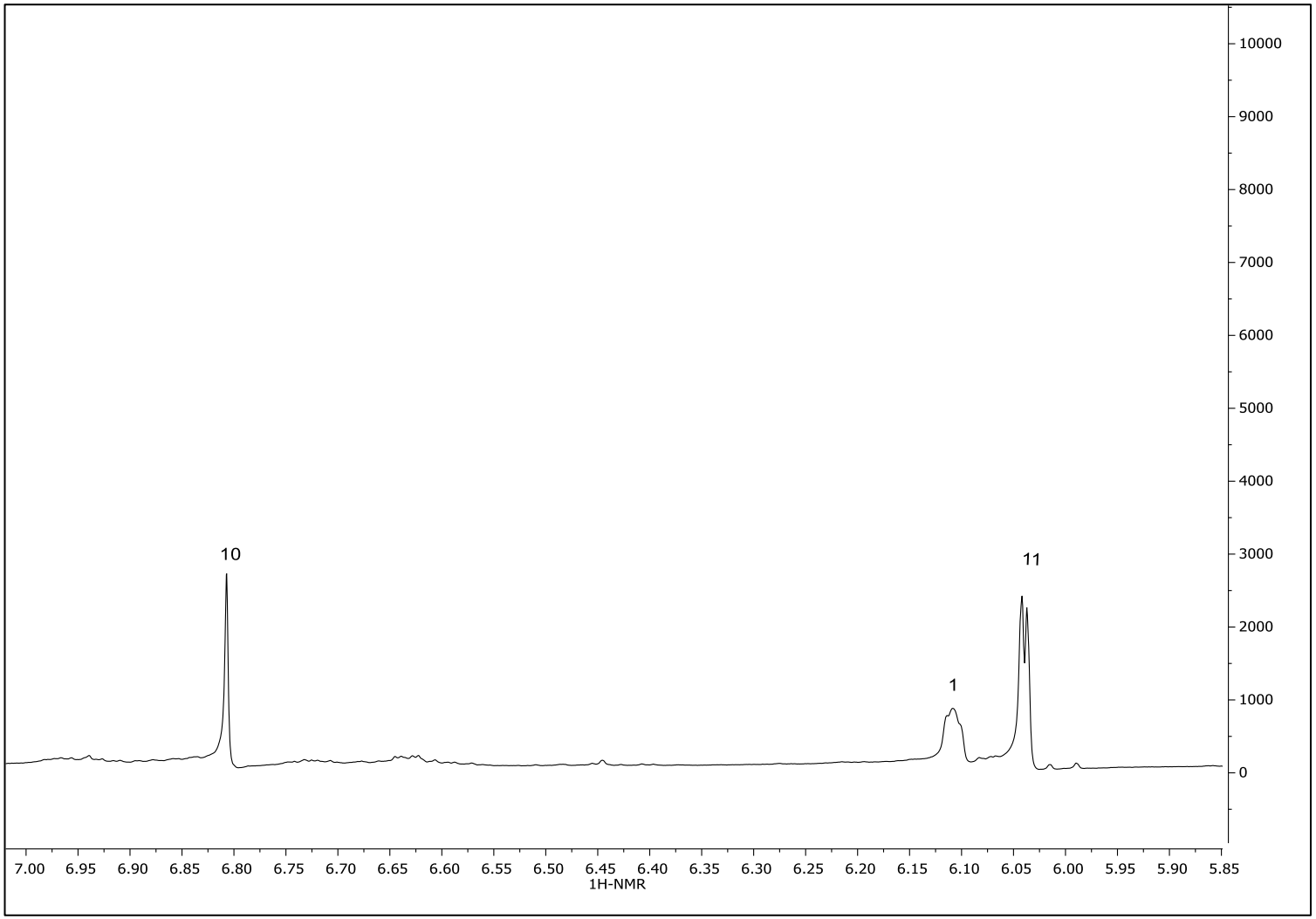


Figure 3. 16 : ^{1}H-NMR spectroscopic analysis of Pt 3.3 in DMSO-*d6*- expansion

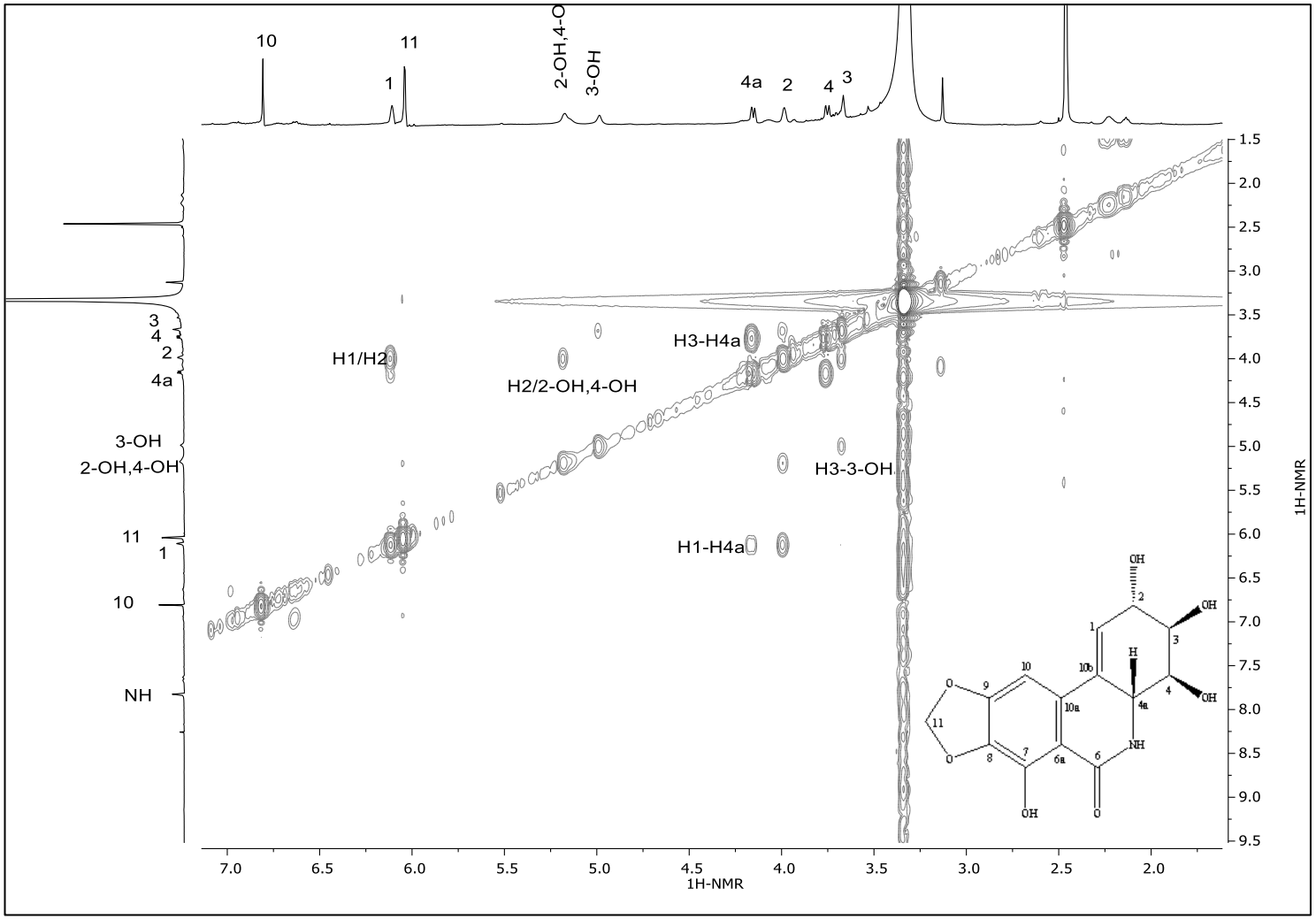


Figure 3. 17: ^{1}H-^{1}H-COSY spectroscopic analysis of Pt 3.3 in DMSO-*d*$_6$

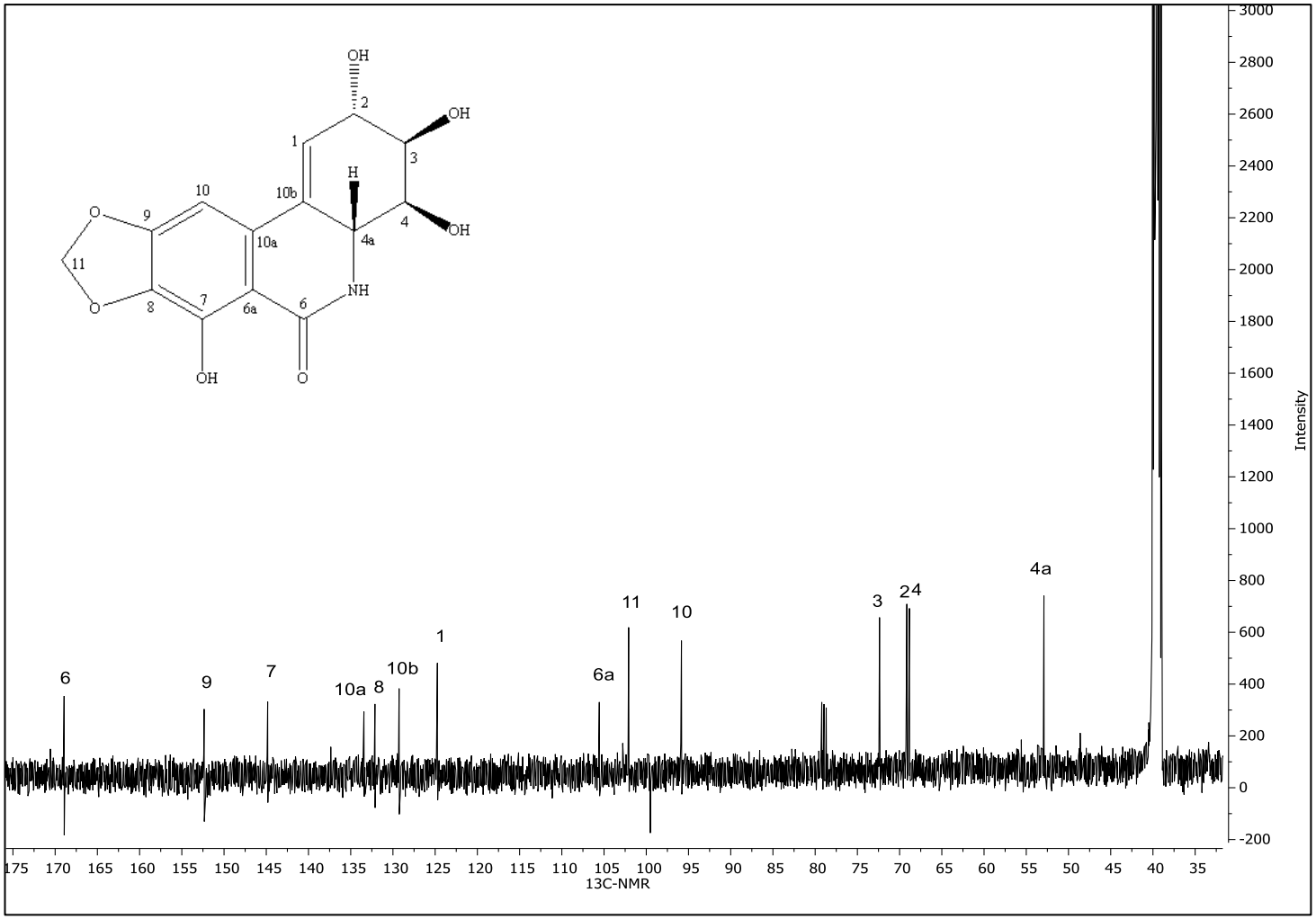


Figure 3. 18: DEPT-q spectroscopic analysis of Pt 3.3 in DMSO-d_6

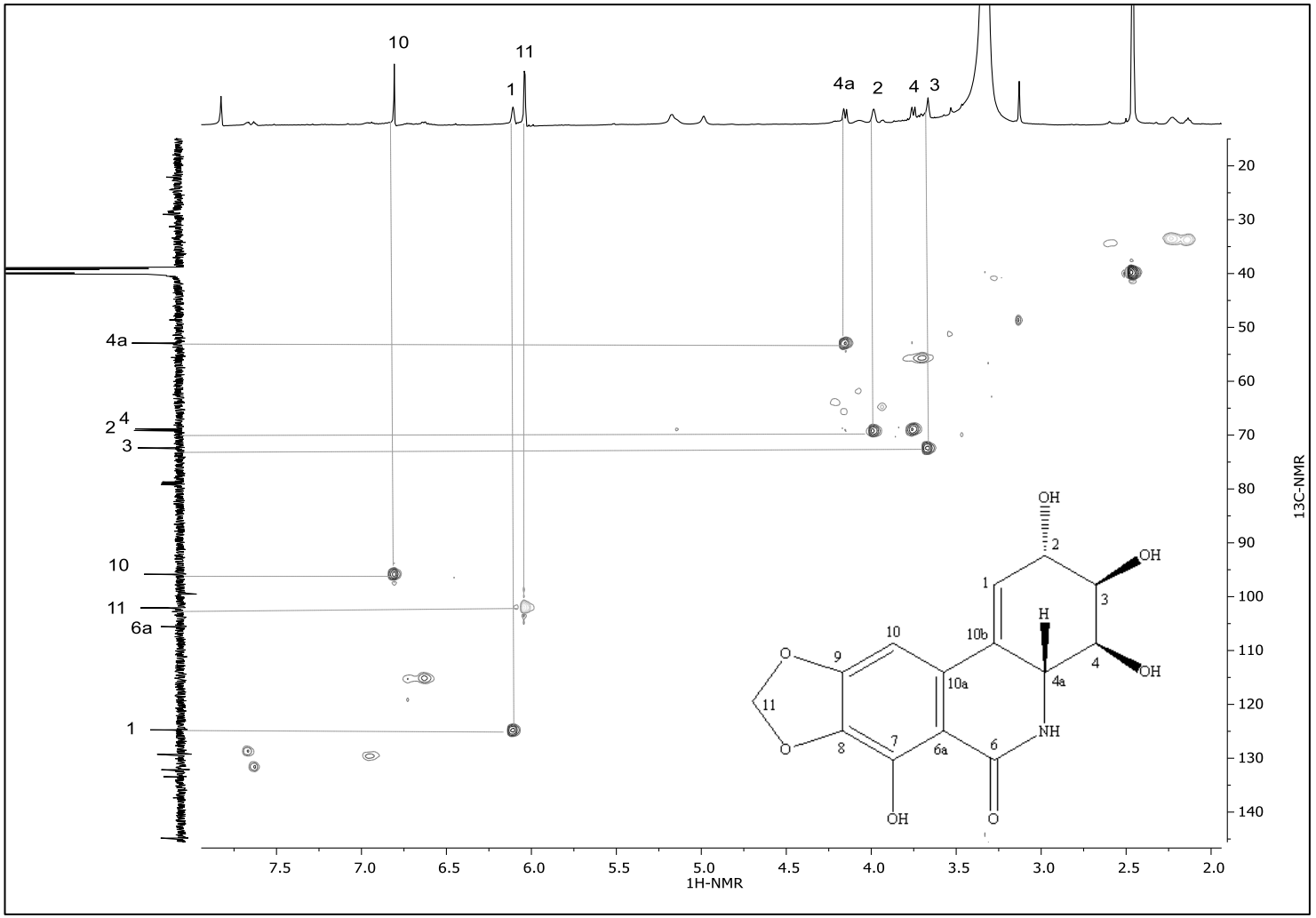


Figure 3. 19: HSQC spectroscopic analysis of Pt 3.3 in DMSO-*d*$_6$.

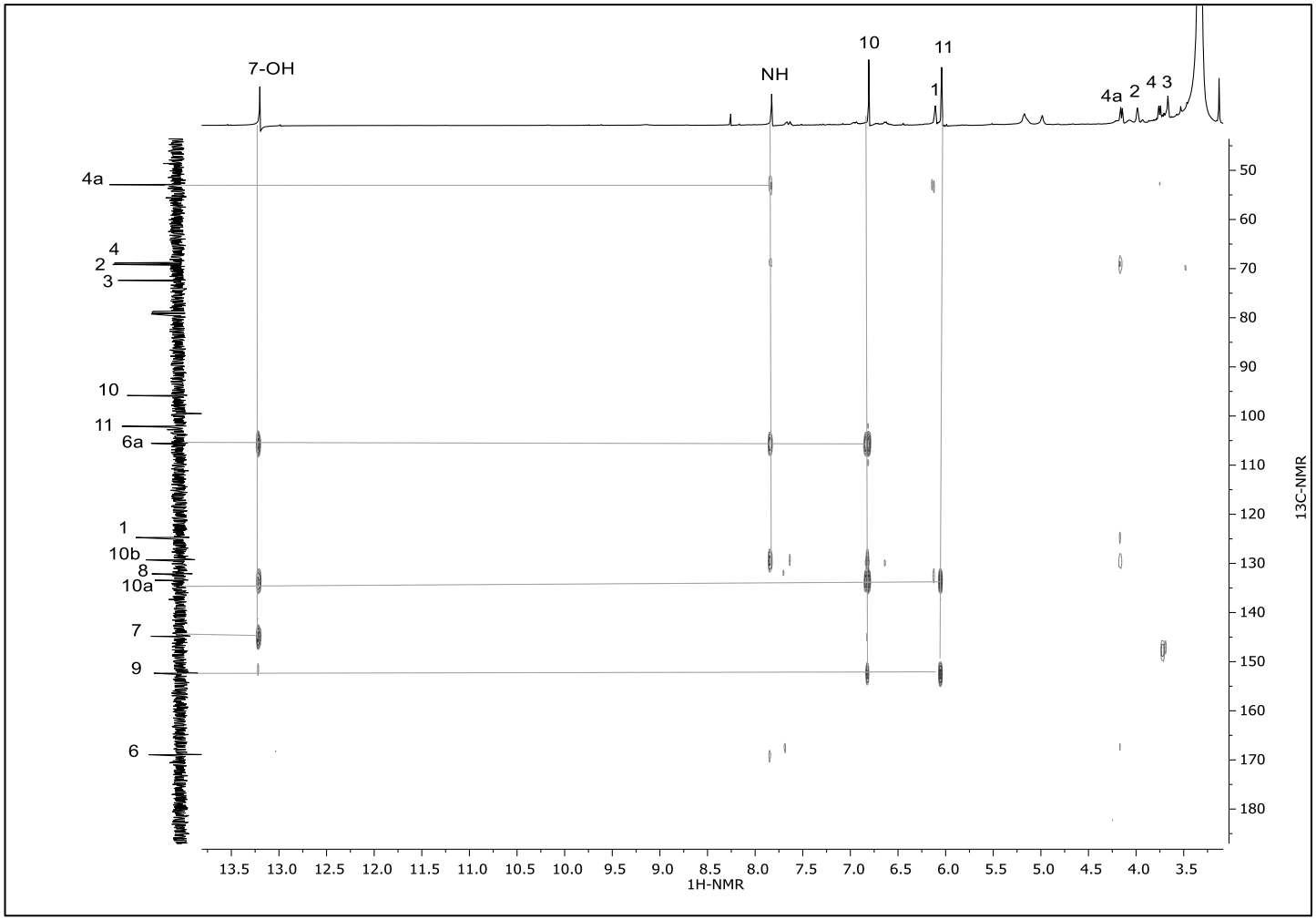


Figure 3. 20: HMBC spectroscopic analysis of Pt 3.3 in DMSO-d_6.

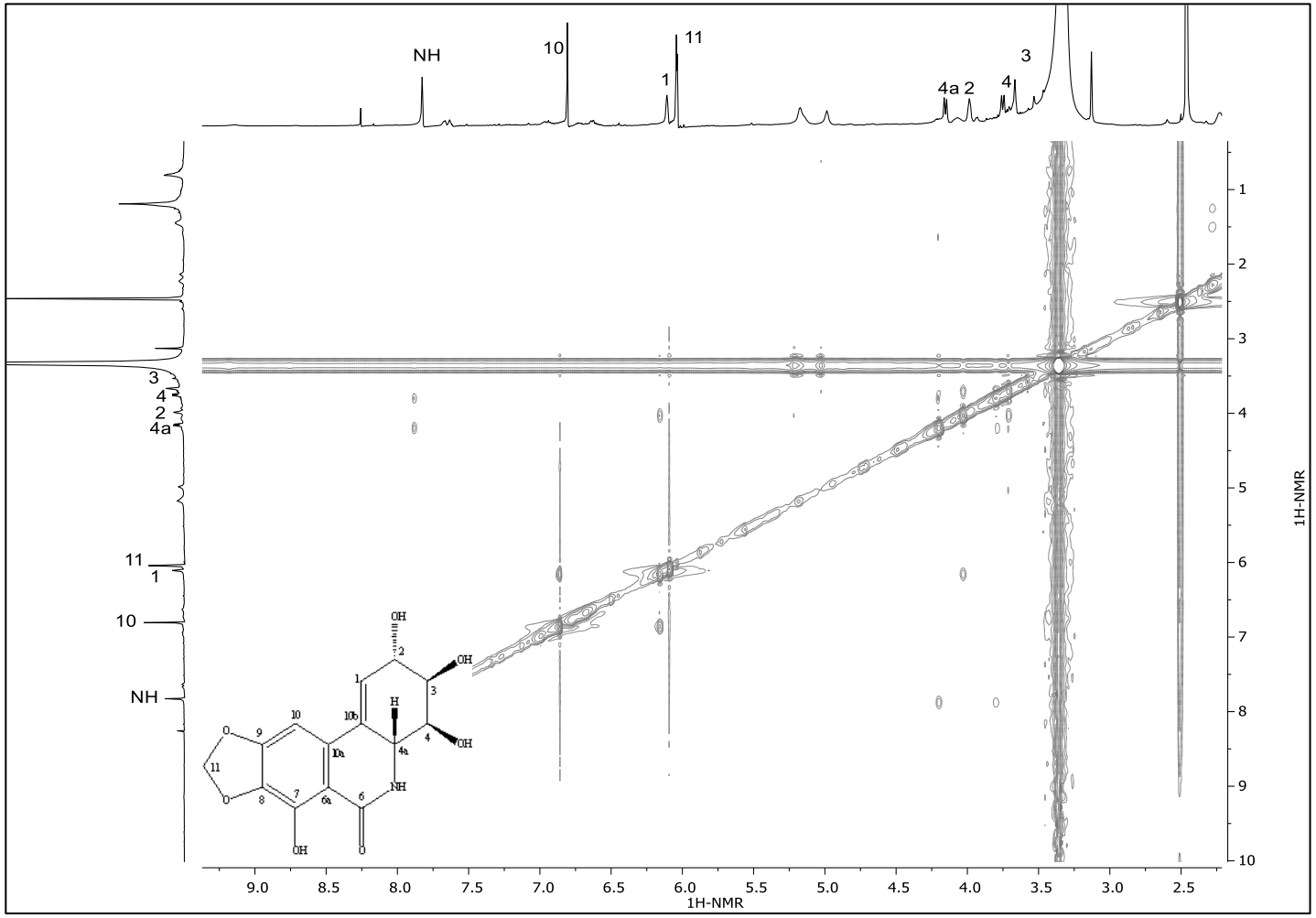


Figure 3. 21: NOESY spectroscopic analysis of Pt 3.3 in DMSO-d_6.

3.2.5 Comparison of effects between narciclasine/Pt 3.3 and SKi

SKi was used in this study for comparison with Pt 3.3 and commercially sourced narciclasine in combination with the proteasomal inhibitor MG132, using the two-breast cancer cell lines; MDA-MB-231 and MCF7-L. This enables analysis of whether the compound isolated in the Pt 3.3 fraction behaves similarly to commercially sourced narciclasine and whether it has similar effects to a sphingosine binding site inhibitor of SK1. SK1 inhibitors, including SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole)), have a common feature of inducing ubiquitin-proteasomal degradation of SK1 in cancer cell lines (Loveridge *et al.*, 2010, Lim *et al.*, 2011b), that is the SKi-induced degradation of SK1 can be prevented by pre-treatment of the cells with MG132.

In this regard, MDA-MB-231 cells were treated with SKi (10 µM) and Pt 3.3 (5 µg/ml) or narciclasine (4 µM) for 24 h, either with or without pre-treatment with MG132 (10 µM, 30 min). The concentration of narciclasine used in current study experiments based on the calculation of Pt 3.3 used (w/v), after identification of Pt 3.3 as narciclasine. The treatment of MDA-MB-231 cells with SKi reduced the expression of SK1a. but this was not clearly prevented by pre-treatment with MG132. Pt 3.3 and narciclasine also induced a reduction in SK1a expression that was not prevented by MG132 (Figure 3.22 A). SKi had no significant effect on Des1 expression, while both Pt 3.3 and narciclasine reduced the expression of Des1 in a MG132-insensitive manner, i.e., independent of the proteasome (Figure 3.22 A).

Similar treatment of MCF-7L cells with SKi (10 µM) or Pt 3.3 (5 µg/ml) or narciclasine (4 µM) for 24 h reduced SK1a, levels which was reversed by pre-treatment with MG132 (10 µM, 30 min) only in the case of SKi. Narciclasine and Pt 3.3 also reduced Des1 expression in MCF7/L cells, while SKi induced a less robust reduction. In each case, the decrease in Des1 expression was not reversed by pre-treatment with MG132 (Figure 3.24 A).

Downregulation of SK1 expression in many cancer cell lines causes cell growth arrest (Taha *et al.*, 2006a). Ki67 is extensively used as a prognostic and predictive marker for cell growth in cancer. Ki67 is present during the cell cycle (G1, S, G2 and M),

while its expression decreases sharply in the later phases of mitosis. Ki67 expression is associated with the high proliferative activity of intrinsic cell populations in tumours. correlated with the worse survival in patients (Hooghe *et al.*, 2008, Shirendeb *et al.*, 2009). In addition, the PI3K/AKT pathway is involved in promoting cell survival (Zheng and Quirion, 2004). Therefore, it was of interest to examine the effect of narciclasine on phospho-AKT, indicating activation of the PI3K pathway, and Ki67, indicating an active cell cycle.

Thus, MDA-MB-231 breast cells were treated with SKi and narciclasine and Ki67 expression and AKT phosphorylation measured by western blot analysis. SKi induced a small reduction in Ki67 expression in MDAMB-231 cells (Figure 3.22 C), whereas there was a marked reduction of Ki67 upon treatment with narciclasine. In addition, the treatment of MDA-MB-231 cells with narciclasine reduced phosphorylated AKT levels, while SKi was without effect (Figure 3.22 C).

In MCF-7L cells, in contrast to MDA-MB-231 cells, Ki67 expression was slightly reduced with narciclasine, while SKi had no effect (Figure 3.24 C) in contrast. treatment of 231 and MCF-7L cells with narciclasine reduced phosphorylated AKT levels (Figure 3.24 C).

Of the three MAPK pathways, the ERK1/2 pathway is most involved in breast cancer signalling (Jo *et al.*, 2007). Several studies have analysed the importance of ERK1/2 pathway in cell survival and growth. For example, a study used the MEK inhibitor PD98059 (25 µM) against MCF7 cells to show a suppression and inhibition of the pathway at 3 h and throughout the entire time course. When PD98059 was used at 50 µM, there was an inhibition of ERK1/2 pathway through the entire time course with cell death (Ripple *et al.*, 2005). The involvement of JNK activity differs between cancer types. In study by Wang *et al.* (2013) 14 normal human and breast cancer tissues were investigated for the expression and activity of MAPK. While there was overexpression of ERK1/2 in all breast cancer tissues, the expression of JNK1 but not JNK2 increased in breast cancer tissue compared to the normal tissues of the same patient (Bode and Dong, 2007). Treatment of MDA-MB-231 cells with SKi reduced phosphorylated ERK levels, indicating inhibition of its activation, while narciclasine had no effect. It was notable that MG132 also reduced phosphorylated ERK levels

(Figure 3.23). In contrast, both SKi and narciclasine increased phosphorylated JNK levels in the presence of MG132 (Figure 3.23). Thus, under conditions of proteasome inhibition with MG132, which induces ER stress (Meusser *et al.*, 2005, Park *et al.*, 2011), SKi and narciclasine enhance activation of the JNK pathway.

Similar results were obtained with narciclasine in MCF-7L cells (Figure 3.25). These findings indicate that narciclasine exhibits both overlapping and non-overlapping mechanism of action with SKi. Nevertheless, Pt 3.3 and narciclasine induce apoptosis as demonstrated by increase in PARP cleavage in both breast cancer cell lines (Figure 3.22 B, Figure 3.24 B).

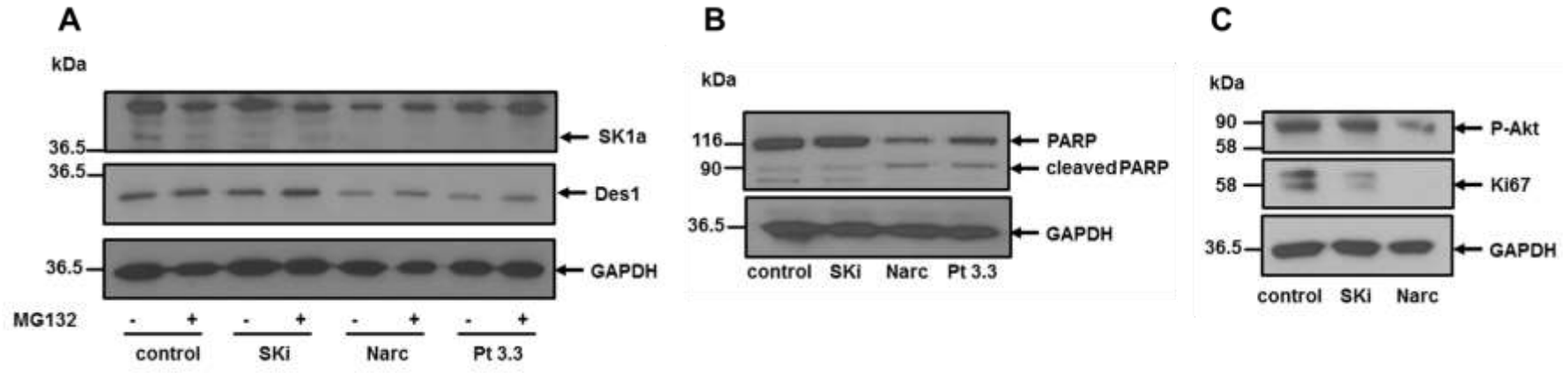


Figure 3. 22: Comparison of effects of SKi, narciclasine and Pt 3.3 on SK1, Des1, PARP, phospho-Akt and Ki67 in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM), Narciclasine (4 µM) or Pt 3.3 (5 ug/ml) for 24 h after pre-treatment with or without MG132 (10 µM, 30 min). Cell lysates were separated by SDS-PAGE and immunoblotted for (**A**) SK1 and Des1, (**B**) PARP or (**C**) P-AKT and Ki67 according to methods (section 2.2.15- 2.2.17). Blots were then stripped and re-probed for GAPDH to ensure similar protein loading.

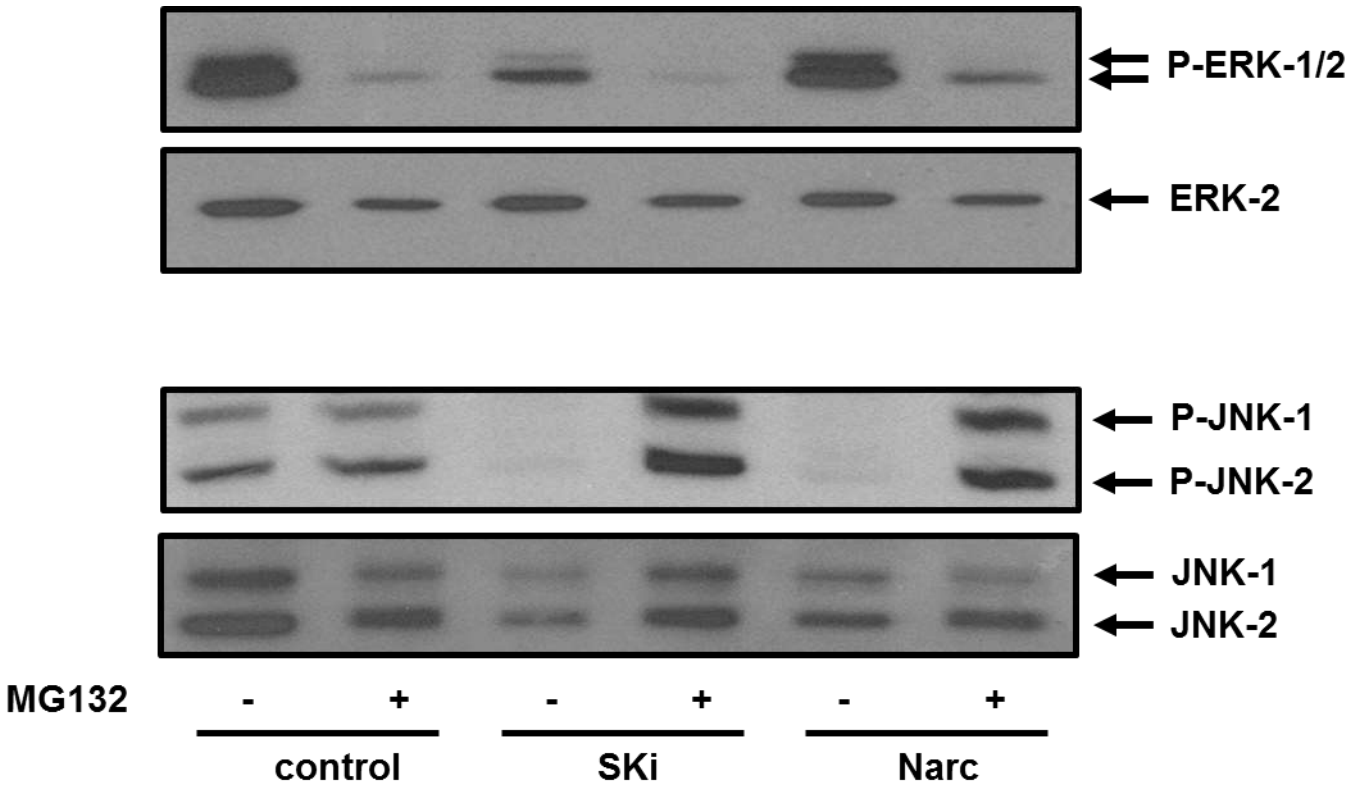


Figure 3. 23: Comparison of effects of SKi and narciclasine on phospho-ERK-1/2 and phospho-JNK in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or Narciclasine (4 µM) for 24 h after pre-treatment with or without MG132 (10 µM, 30 min). Cell lysates were separated by SDS-PAGE and immunoblotted for P-ERK1/2, and P-JNK. Blots were then stripped and re-probed for ERK2 and JNK to check for similar protein loading.

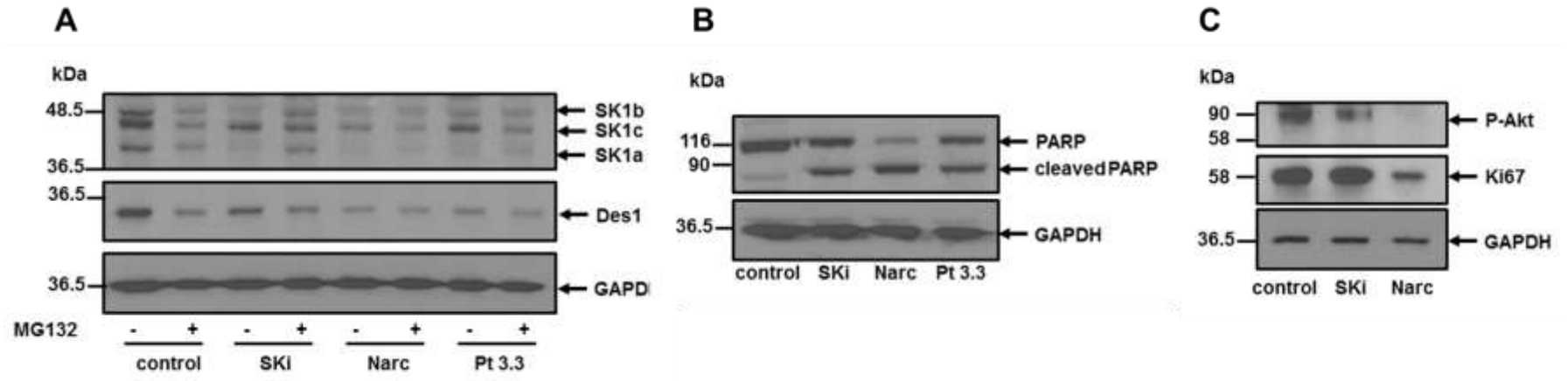


Figure 3. 24: Comparison of effects of SKi, narciclasine and Pt 3.3 on SK1, Des1, PARP, phospho-Akt and Ki67 in MCF-7/L cells.

Quiescent MCF-7/L cells were treated with vehicle control (0.05% DMSO), inhibitor SKi (10 µM), Narciclasine (4 µM) or Pt 3.3 (5 ug/ml) for 24 h after pre-treatment with or without MG132 (10 µM, 30 min). Cell lysates were separated by SDS-PAGE and immunoblotted for (**A**) SK1 and DEGS1, (**B**) PARP or (**C**) P-AKT and Ki67according to methods (section 2.2.15-2.2.17). Blots were then stripped and re-probed with GAPDH to ensure similar protein loading.

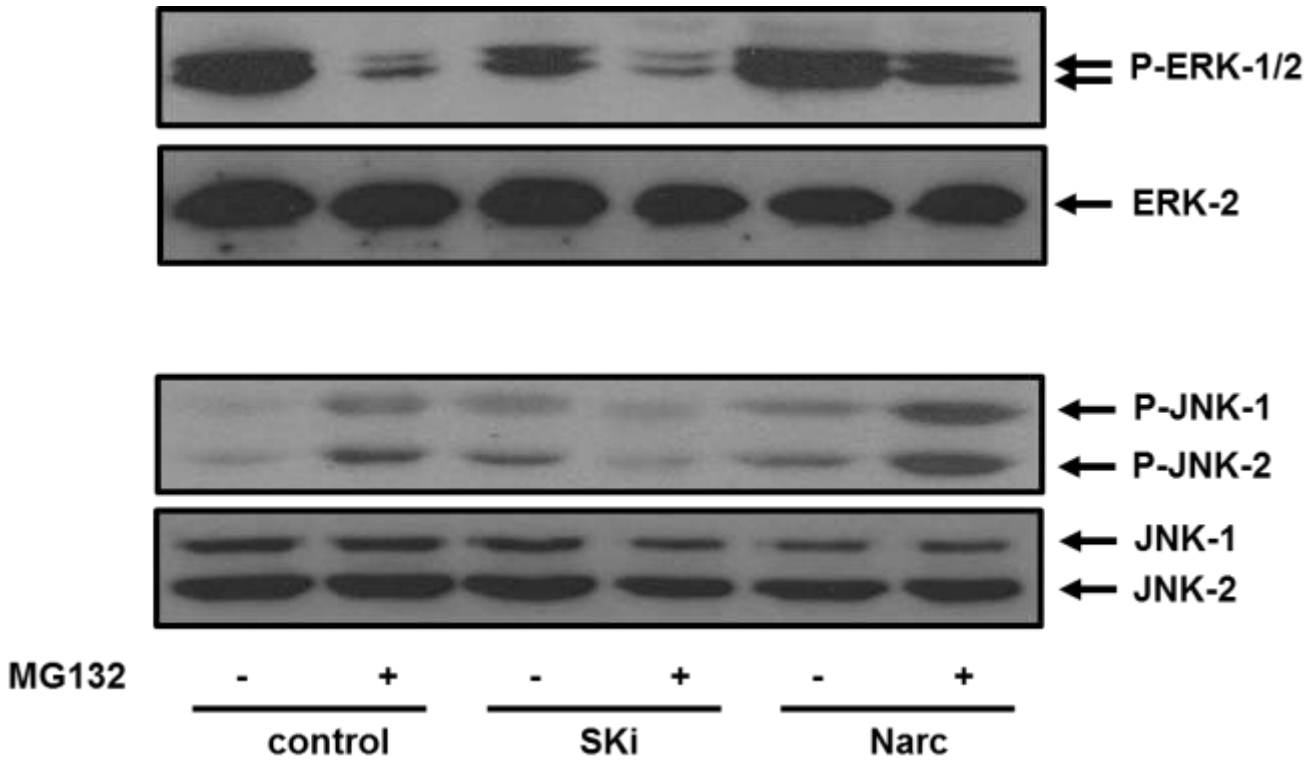


Figure 3. 25: Comparison of effects of SKi and narciclasine on phospho-ERK-1/2 and phospho-JNK in MCF-7/L cells.

Quiescent MCF-7/L cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or Narciclasine (4 µM) for 24 h after pre-treatment with or without MG132 (10 µM, 30 min). Cell lysates were separated by SDS-PAGE and immunoblotted for P-ERK1/2, and P-JNK. Blots were then stripped and re-probed for ERK2 and JNK to check for similar protein loading.

3.2.6 Effect of narciclasine on NF_KB signalling and transcriptional activity in NCTC-NF_KB reporter keratinocytes

SK1 has an important pro-inflammatory role in many diseases (Neubauer and Pitson, 2013). For instance, pro-inflammatory TNFα activates SK1 (Adada *et al.*, 2013). Therefore, it was of interest to test the effects of narciclasine on inflammatory signalling. This was achieved using NCTC/NFκB keratinocytes. These cells have been genetically modified by the introduction of a NFκB-binding promoter-regulated luciferase gene. Initially, the effect of TNFα on NFκB-driven transcriptional activity was measured using a luciferase reporter assay. The reporter cells were treated with TNFα (15 ng/ml), which induced NFκB-dependent transcriptional activity in a time-dependent manner, which was significant at 4 hours (Figure 3.26, left graph). A comparison was made of the effects of narciclasine, Pt 3.3 and a SK2 inhibitor (K145) and an inhibitor of NFκB signalling, BMS345541. Pre-treatment of the cells with narciclasine (4 µM, 30 min) or Pt 3.3 (5 ug/ml, 30 min) or NFκB inhibitor, BMS345541 (20 µM, 30 min) significantly reduced TNFα-stimulated NFκB transcriptional activity. In contrast, the SK2 inhibitor, K145 (10 µM, 10 min) was without effect (Figure 3.26, right graph). Narciclasine and TNFα were also evaluated for their effect on IκB degradation, which is an early and transient event in the activation of the NFκB signalling (Ramakrishnan *et al.*, 2004). As expected, TNFα induced a time-dependent reduction in IκB as the NFκB signalling pathway was activated (Figure 3.27, left panel). In contrast, narciclasine alone was without effect (Figure 3.27, middle panel). However, pre-treatment of the cells with narciclasine blocked subsequent TNFα-stimulated degradation of IκB, suggesting that the molecular basis for the anti-inflammatory activity of narciclasine is the blockade of NFκB activation by TNFα (Figure 3.27, right panel).

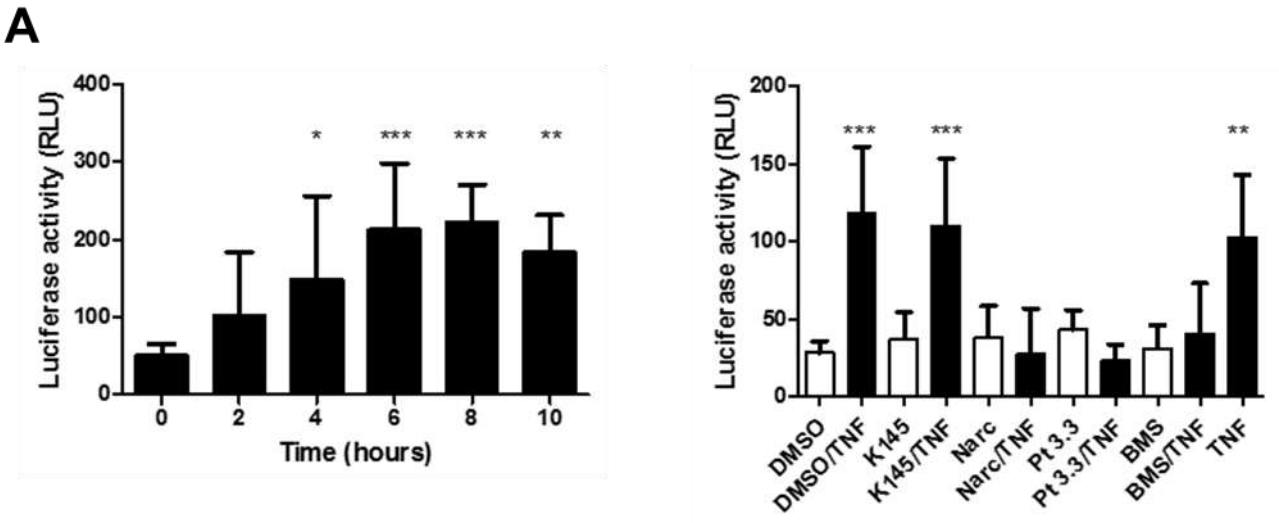


Figure 3. 26: Effect of Narciclasine and Pt 3.3 on TNFα-stimulated NFκB signalling. Luciferase reporter assay:

Quiescent NCTC-NF-κB reporter cells were treated with TNFα (15 ng/ml) for 0-120 min. (left panel) or pre-treated with BMS345541 (10 μM for 30 min) or SK2 inhibitor, K145 (10 μM for 10 min) or narciclasine (4 μM for 30 min) or Pt 3.3 (5 μg/ml for 30 min) or vehicle alone (DMSO 0.05% (v/v)) prior to stimulation with TNFα (15 ng/ml) for 4 h. Luciferase expression was measured by luminescence activity according to methods (section 2.2.18). Data are expressed as a % of control ± SEM for n=3 or more experiments. left panel, *p<0.05, **p<0.01 and ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test); lower panel, ***p<0.001 vs control; *** p<0.001 and ns (not significant) vs DMSO/TNF sample (one-way ANOVA with Bonferroni's post-hoc test).

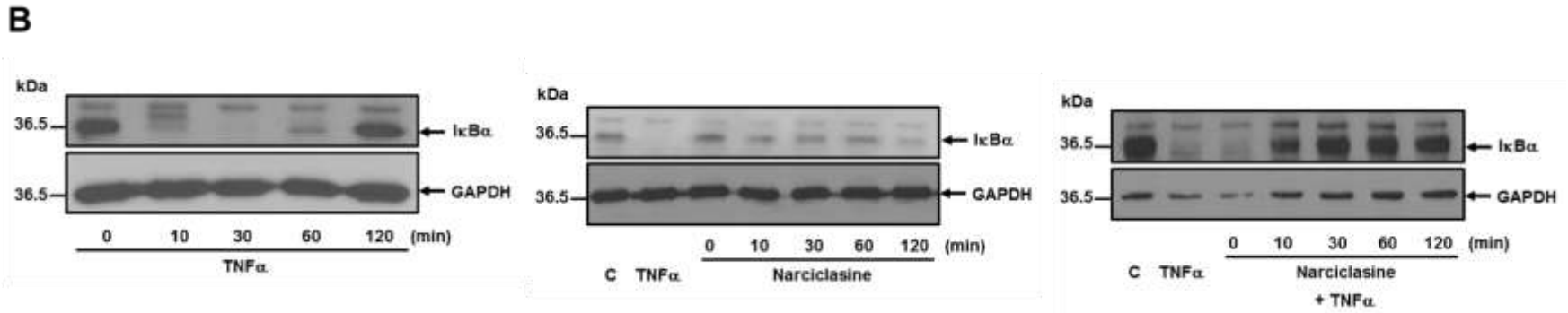


Figure 3.2727: Effect of narciclasine and Pt 3.3 on TNFα-stimulated NFκB signalling. NFκB signalling, indicated by IκB degradation: Quiescent NCTC-NFκB reporter cells were treated with (left panel) TNFα (15 ng/ml) for 0-120 min. Middle panel – cells were treated with narciclasine (4 μM) for 0-120 min with for TNFα (15 ng/ml, 30 min, positive control). right panel – cells were treated with narciclasine (4 μM) for 0-120 min prior to addition of TNFα (15 ng/ml, 30 min) or with TNFα alone. Vehicle is DMSO (0.05% (v/v)). Cell lysates were separated by SDS-PAGE and immunoblotted with anti-IκB-α antibody. Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

3.2.7 Effect of narciclasine on AP-1 transcriptional activity and ERK/JNK signalling in NCTC-AP-1 reporter keratinocytes

A second luciferase reporter cell line was used to investigate the effect of narciclasine against the transcriptional regulation of AP-1 in NCTC-AP-1 reporter keratinocytes. These cells have been genetically modified by the introduction of an AP-1-binding promoter-regulated luciferase gene. NCTC-AP-1 cells were treated with phorbol 21-myristate 13-acetate (PMA) at 100 nM. PMA, which is a direct activator of protein kinase C (PKC), an indirect activator of the transcription factor, AP-1, induced a time-dependent increase in AP-1-dependent transcriptional activity, with significant luciferase expression and activity detected after 4 hours (Figure 3.28 A). PMA also provoked a transient increase in JNK activation (evident at 30 and 60 min) and a sustained stimulation of ERK-1/2 (from 10 -120 min) (Figure 3.28 B). Pre-treatment of the NCTC-AP1 cells with narciclasine (4 µM, 10 min) or Pt 3.3 (5 µg/ml, 10 min) reduced subsequent PMA-stimulated AP1 transcriptional activity. In contrast, the SK2 inhibitor, K145 (10 µM, 10 min) was without effect (Figure 3.28 C).

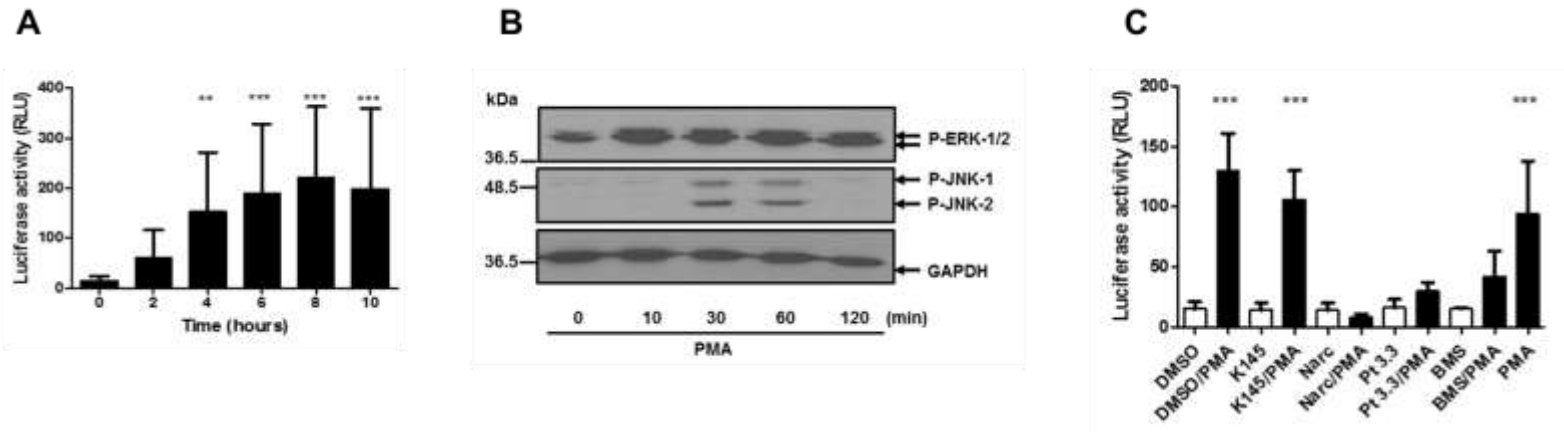


Figure 3. 28: Effect of Narciclasine and Pt 3.3 on PMA-stimulated AP-1- signalling.

(A) Luciferase reporter assay: Quiescent NCTC-AP-1 reporter cells were treated with PMA (100 nM) for 0-10 h before luminescence activity was measured according to methods (section 2.2.18). Data are expressed as a % of control ± SEM for n=3 or more experiments. **p<0.01 and ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). **(B) Effect of PMA on ERK and JNK signalling.** Quiescent NCTC-AP-1 reporter cells were treated with PMA (100 nM) for 0-120 min. Cell lysates were separated by SDS-PAGE and immunoblotted for P-JNK, P-ERK. Blots were then stripped and re-probed for GAPDH to check for similar protein loading. (**C**) **Effect of Narciclasine, Pt 3.3 and K145 on AP-1-driven luciferase expression.** Quiescent NCTC-AP-1 reporter cells were pre-treated with K145 (10 µM, 10 min), narciclasine (4 µM for 30 min. or Pt 3.3 (5 µg/ml for 30 min. or with vehicle alone (DMSO 0.05% (v/v)) prior to stimulation with PMA (100 nM) for 4 h before luminescence activity was measured. Data are expressed as a % of control ± SEM for n=3 or more experiments. ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test); $^{+++}$ p<0.001 and ns (not significant) vs DMSO/PMA sample (one-way ANOVA with Bonferroni's post-hoc test).

3.3 Discussion

3.3.1 Screening of plant crude extract

Many strategies have been published for selecting plants as a candidate for drug discovery programs; this includes random selection followed by phytochemical screening. The focus is on known bioactive compounds such as alkaloids, flavonoids, and triterpenes; used in both *in vitro* and *in vivo* preclinical animal models of disease. Other screening approaches are based on the ethnomedical use of various plants (Fabricant and Farnsworth, 2001). All these strategies contribute to health care worldwide by enabling identification of novel compounds for medicinal use. All the selected plants for this study were collected based on these strategies. The objective of the study was to identify novel anticancer and anti-inflammatory agents from plant species, by establishing activity against SK1, Des1 and PARP cleavage as a marker for apoptosis (cytotoxicity) and NFκB and AP-1 as markers of inflammation-based signalling.

Eleven plant crude extracts were screened from a total of nine plants because some extracts prepared from different parts of the same plant. This allows better characterisation of the constituents produced by different parts of the plants. Preliminary data indicated that screening all plant crude extracts had no effect on metabolic viability of both breast cancer cell lines, MDA-MB-231 and MCF7-L cells, except for *H. strobilaceum* and *Crotalaria* sp., which reduced metabolic viability of MCF7-L cells at high concentration. There was also an effect on the metabolic viability of MEF cells at high concentration by all plant crude extracts (Figure 3.4-Figure. 3.6).

The use of the DNA synthesis assay to monitor growth demonstrated that three out of the eleven plant crude extracts, namely, *G. sinaicus, U. maritima* and *P. tortuosum* significantly reduced cell proliferation (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs control) in both breast cancer cell lines at 5 µg/ml (Figure 3.7 – Figure 3.9). These findings suggest that these plants contain anti-proliferative agents, this is in agreement with previous literature (Pettit *et al.*, 1986, Batran *et al.*, 2005, El-Seedi *et al.*, 2013). All the plant crude extracts were screened for their effect (at high concentration 5 µg/ml) on SK1, Des1 and PARP in both breast cancer cell lines. This narrowed the search to

three plant crude extracts, *G. sinaicus, U. maritima* and *P. tortuosum*. These had little or no effect on SK1 or Des1 protein expression in MDA-MB-231 cells, but did induce PARP cleavage, indicative of apoptosis. In contrast, both *G. sinaicus* and *U. maritima* reduced the expression of SK1a-c while the effect of *P. tortuosum* was weaker in MCF-7L cells. The three plant crude extracts reduced Des1 expression and increased induction of PARP cleavage. Since only crude plant extracts were examined, it is difficult at this stage, to establish whether the effects are due solely to a single cytotoxic constituent and whether the effects of the extracts on SK1/ Des1 expression are linked to the induction of PARP cleavage and apoptosis. Indeed, plants containing many compounds might have mutually exclusive effects on SK1, Des1 and apoptosis. For example, several cytotoxic compounds have been isolated from *Pancratium littorale* and these compounds were active against the growth of murine P-388 lymphocytic leukaemia and murine M-5076 ovary sarcoma (Pettit *et al.*, 1986). Similarly, *U. maritima* also produces cardiac-glycoside compounds which are cytotoxic against several tumour cell lines (El-Seedi *et al.*, 2013). Therefore, it was necessary to isolate the active compounds, and this was undertaken in the current study. I was particularly interested in plant crude extracts which inhibited DNA synthesis, and reduced SK1 or Des1 protein expression and promoted PARP cleavage. However, some extracts were active in reducing DNA synthesis but did not affect SK1 nor Des1 expression.

3.3.2 *P. tortuosum*

The main finding of this study that *P. tortuosum*, contains compounds which exhibit anticancer activity *in vitro*. This is in agreement with previous studies on other species of the same *Pancratium* genus and with the Amaryllidaceae family (Pettit *et al.*, 1986).

1D and 2D spectroscopy enabled the determination of the absolute structure of the active compound in *P. tortuosum* in the 100% (v/v/) ethyl acetate fraction. This was identified as narciclasine (1,3,4,5-tetrahydro-2,3,4,7-tetrahydroxy[1-,3]-dioxolo-[4,5-j]-phenanthridin-6(2H)-one. The structure is in agreement with studies published by Ceriotti (1967) and Ingrassia *et al.* (2009). Narciclasine is secondary metabolite since the nitrogen is amidic in character. However, it has a structure related to the Amaryllidaceae lycorine type alkaloids. The narciclasine structure was under

extensive chemical characterisation in particular studying its stereochemistry by an X-ray analysis of the corresponding tetraacetate (Immirzi and Fuganti, 1972).

Narciclasine and pancratistatin were reported as the most important isocarbostyril constituents of Amaryllidaceae, which are responsible for the therapeutic activities of these plants in traditional medicine treatment of cancer (Kornienko and Evidente, 2008). Indeed, previous studies using narciclasine isolated from different varieties of *Narcissus* bulbs have demonstrated potent anti-mitotic activity (Ceriotti, 1967). The anticancer activity of narciclasine has attracted attention toward the isolation of its naturally occurring analogues from different Amaryllidaceae species (Kornienko and Evidente, 2008).

Narciclasine and pancratistatin have emerged as interesting anti-tumour drugs in the NCI database (National Cancer Institution). The first evaluation of narciclasine bioactivity is strong mitosis-blocking activity, as it significantly inhibited the wheat grain radicals as well as inhibiting the growth of murine sarcoma cells *in vivo*. In fact, Carrasco *et al.* (1975) proposed the mechanism of narciclasine action is due to the blockade of peptide bond formation at the ribosome by inhibiting protein synthesis in rabbit reticulocytes and in a yeast-derived cell-free system. Recently, McLachlan *et al.* (2005) demonstrated that pancratistatin, which is also a lycorine type alkaloid like narciclasine (Figure 3.2), increased apoptosis induction in SHSY-5Y neuroblastoma cells. Similarly, narciclasine, at concentrations 1 μM, induced apoptosis-mediated cytotoxic effects in human carcinoma cells but not in normal fibroblasts via the extrinsic apoptotic pathway (Dumont *et al.*, 2007). In agreement with this, the present study showed that narciclasine reduced DNA synthesis in MCF7/L and MDA-MB-231 breast cancer cell lines (Figure 3.12 and 3.13) and induced apoptosis as indicated by enhanced PARP cleavage (Figures 3.12, 3.13, 3.22 and 3.24).

SK1 inhibitors have been shown to induce proteasomal degradation of SK1 in MCF7 cells and LNCAP-AI cells (Lim *et al.*, 2011b, McNaughton *et al.*, 2016). In this study it has been observed that narciclasine also reduced the expression of SK1. However, this was insensitive to the proteasome inhibitor, MG132, suggesting that unlike SK1 inhibitors, the reduction in expression is not related to ubiquitin-proteasomal degradation, and suggests an alternative mechanism of action. This might include the

inhibition of transcriptional/translational regulation of SK1 expression by narciclasine. The findings suggest that narciclasine is not a direct inhibitor of SK1.

Further evidence that the narciclasine effect on apoptosis might not be related to effects on SK1 or Des1 is the finding that some of the actions of SKi are not recapitulated by narciclasine. For instance, in MCF7/L cells, SKi stimulated PARP cleavage (Figure 3.24), and this is associated with a reduction in phosphorylation state of ERK1/2 (Figure 3.25), while narciclasine and Pt 3.3 had no effect on the phosphorylation state of ERK1/2 in MCF-7L cells (Figure 3.25). Both SKi and narciclasine activated JNK (Figure 3.25), but narciclasine had a stronger negative effect on Ki67 expression and phospho-AKT levels compared with SKi in MCF7/L cells (Figure 3.24). Ki67 is a nuclear non-histone protein that is useful as a proliferative marker. It is also considered as a prognostic marker in early breast cancer (De Azambuja *et al.*, 2007, Yerushalmi *et al.*, 2010). Indeed, high Ki67 expression correlates with higher tumour grade in breast cancer (Soliman and Yussif, 2016, Hashmi *et al.*, 2019). The inhibitory effect of narciclasine on phospho-AKT is significant because this protein suppresses apoptosis via the BAD/Bcl2 pathway (Liu *et al.*, 2015).

Narciclasine also reduced Des1 expression via a mechanism that was independent of the ubiquitin-proteasomal degradation pathway (Figure 3.22 and 3.24). Indeed, in MCF-7 cells, SKi also reduced Des1 expression via a mechanism insensitive to MG132. These finding indicate subtle differences in the regulation of Des1 in different cancer cell types. The effect on Des1 by narciclasine is significant as others have shown that inhibition of Des1 induces anticancer activity (Aurelio *et al.*, 2016).

The pro-inflammatory cytokine TNFα has been involved in many cellular processes and triggers signalling pathways which activate NFκB and AP-1 pathways. When the IKK pathway is stimulated with TNFα, IKK is activated, which in turn results in phosphorylation and degradation of IκB. IκB is in a complex with NFκB and inhibits its activity. Subsequently, IκB degradation enables the release of NF-κB, which then translocate from the cytoplasm to nucleus to induce transcriptional gene programmes. TNFα binding to its receptors also activates the PKC/JNK pathway which leads to the activation of AP-1 (Jiang *et al.*, 2003). Phorbol 12-myristate 13-acetate (PMA) activates members of the PKC family by binding a cysteine-rich region which is

physiologically recognised by DAG, leading to the activation of the MAPK pathway and AP-1-dependent transcriptional programmes. Thus, agents able to suppress AP-1 activation have the potential to suppress inflammation and show therapeutic potential.

In this study, narciclasine appears to be associated with NF-κB-dependent transcriptional regulation in keratinocytes. This is supported by the finding that pre-treatment of keratinocyte reporter cells with narciclasine reduced subsequent TNFα-stimulated degradation of IκB (Fig. 3.27). These important findings suggest that narciclasine has potential as an anti-inflammatory agent. This finding is supported by the study of Furst (2016) which reported that narciclasine has anti-inflammatory action *in vivo*. Narciclasine also inhibited transcriptional regulation by AP-1 (Fig. 3.28).

In conclusion, narciclasine was isolated for the first time from *P. tortuosum. Also,* was identified as the active component. It is shown to have both anticancer and anti-inflammatory activity, which can be attributed to the importance of the *Pancratium* species and their contribution to chemistry and pharmacological activity.

Chapter 4:

Bio-assay guided isolation of anticancer/ anti-inflammatory compounds from *Gomphocarpus sinaicus*

CHAPTER 4: Bio-assay guided isolation of anticancer/anti-inflammatory compounds from *Gomphocarpus sinaicus*

4.1 Introduction

4.1.1 Apocynaceae family

Apocynaceae is one of the largest flowering plant families comprising tropical trees, shrubs, and vines. One of the distinctive features of this family is the production of milky saponins (glycosides in which sugars are conjugated to a steroid or a triterpenoid moiety) from almost all its species. This family has five sub-families called Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae and Asclepiadoideae (known as Asclepiadaceae) and contains approximately 424 genera and more than 2000 species (Lawrence, 1951). The family is primarily distributed in the tropics and sub-tropical zones (Ping-tao *et al.*, 1996).

This family is one of the most important families in the plant kingdom due to its multi-medicinal uses and is a rich source of food, poisons, and drugs. The family has been used traditionally and in conventional medicine. In Indian, Chinese, and Thai folklore medicine, species of this family are used to treat various of diseases such as, gastrointestinal ailments, fever, malaria, pain, diabetes, skin, and ecto-parasitic diseases. Species of this family have been reported to possess anticancer properties. These include *Catharanthus*, *Nerium*, *Plumeria*, *Tabernaemontana*, *Ichnocarpus* and *Catharanthus roseus* (El-Sayed and Cordell, 1981, Noble, 1990, Gajalakshmi *et al.*, 2013). Other uses of species of this family are for timber and as ornamentals.

Extensive phytochemical work has been undertaken on many species of the Apocynaceae family. These studies have reported an abundance of a variety of compounds type, mostly are alkaloids, terpenoids, steroids, flavonoids, glycosides, simple phenols, lactones, and hydrocarbons (Fu *et al.*, 2005, Wong *et al.*, 2013, Zhang *et al.*, 2019). The types of alkaloids that have been reported and isolated from different plant species are indole, iboga and vinca alkaloids. For example, eleven indole alkaloids extracted from leaves, flowers and stems of *Tabernaemontana divaricata*

have been evaluated and shown to possess anti-bacterial activity (Arambewela and Ranatunge, 1991, Kam *et al.*, 1993). In addition, well over a hundred alkaloids from different parts of *Catharanthus roseus* have been reported to possess anticancer and anti-hypertensive activities (van Doorn *et al.*, 2004). The alkaloids, reserpine and rescinnamine (Figure 4.1) have been isolated from this family and have been used for hypertension. Cardiac glycosides have also been isolated (Lemieux *et al.*, 1956, Fife *et al.*, 1960, Lucky and Islam, 2019). Terpenes and their derivatives are present in many members of the family. For example, the *Carissa* genus contains high amounts of different terpenoids, including mono-, sesqui- and triterpenoids. Among all the genus species, *Carissa carandas* has the highest terpenoids contents, with the flowers containing an abundance of monoterpenes (Zaki *et al.*, 1981), while the roots and flowers are rich in the sesquiterpenes (Singh and Rastogi, 1972). In contrast, the leaves, fruits, and flowers are rich in triterpenes (Pakrashi *et al.*, 1968, Naim *et al.*, 1985, Naim *et al.*, 1988). Carrisone is a sesquiterpene whereas lupeol and oleanolic acid are triterpenes which exhibit anti-inflammatory activity against nitric oxide, tumour necrosis factor-α and interlukin-1β (Itankar *et al.*, 2011). On the other hand, carandinol, an isohopane-type triterpenoid isolated for the first time from *Carissa carandas* leaves, showed a significant cytotoxic effect against HeLa, 3T3, and PC-3 cancer cell lines (Begum *et al.*, 2013).

Cardiac glycosides are a major class of glycosides that are broadly used in the treatment of heart failure. The two genera *Thevetia* and *Nerium* are reported to contain the highest levels of glycosides. Kaneroside and neriumoside are two cardiac glycosides (Figure. 4.1) that were isolated from the leaves of *Nerium oleander*. Cardenolides, a sub-class of cardiac glycosides, were also found in *Nerium oleander* and *Thevetia peruviana*. The cardiac glycosides isolated from *N. oleander* include odoroside H, neridiginoside, nerizoside and neritaloside and have been reported to exhibit central nervous system-depressant activity in mice (Begum *et al.*, 1999). In addition, the isolated cardiac glycoside from *T. peruviana* including neriifolin, peruvoside and thevefolin (Figure 4.1) have been screened for their ability to overcome TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) resistance, which is a feature of cancer cells. These studies demonstrated that thevefolin (Figure 4.2) was

effective against TRAIL resistance in human gastric adenocarcinoma cells, thereby enabling effective cytotoxic activity against cancer cells (Miyagawa *et al.*, 2009).

4.1.2 *Gomphocarpus sinaicus (Asclepias sinaica*)

The South Sinai Mountain range contains the largest biodiversity in Egypt and a large area of this region was declared the Saint Katherine Protectorate in 1996. Due to its geomorphologic formations and the wide variation in climate, it has a unique type of vegetation. Indeed, 44% of Egypt's endemic plants are present in the Saint Katherine Protectorate making it the most unique floristically diverse spot in the Middle East. Approximately 1261 species have been recorded in Sinai (Boulos, 1995) of which 472 plant species grow in south Sinai (Shaltout *et al.,* 2004) and of these 19 species are endemic (Boulos, 1995).

Sinai Bedouins use desert plants widely in their habitats. Several hundred medicinal plant species found in the Sinai Peninsula have been identified and reported their usages in the ethnobotanical literature (Bailey and Danin, 1981, Boulos, 1983). Their documented medicinal uses by Sinai Bedouins are as anti-bacterial and anti-fungal agents, and for curing aliments such as wounds, cuts, sores, colds, coughs and diarrhoea (Khafagi and Dewedar, 2000).

G. sinaicus is a perennial shrub and the only Middle Eastern member of the mainly African genus *Gomphocarpus* (Goyder and Nicholas, 2001) and also placed as the well-known New World genus *Asclepias* (Boulos, 2000). It occurs in the south Sinai governorate in Egypt and is a relatively common plant in the bottom of Wadi Arbaein in the Saint Katherine Protectorate where it is patchily distributed along the wadi floor at low densities. It occurs in some wadis but not others and is largely absent from the highest wadis.

G. sinaicus is known for its toxicity to man and animals, owing to the high concentration of cardiac glycosides (El-Askary *et al.*, 1995a). It was stated that insects of several orders segregate cardenolides from their milkweed food plants, where the stored compounds act as a source of protection for these insects against predators (Seiber *et al.*, 1978, Elbanna *et al.*, 2009). In Saudi Arabia, the whole plant *G. sinaicus* is used traditionally as a decoction in haemorrhagic (bleeding) conditions including

rhinorrhagia (nosebleed) and metrorrhagia (irregular menstruation) (Youssef, 2013). The juices of *G. sinaicus* in Egypt are also used externally to heal skin diseases (El-Seedi *et al.*, 2013).

The main cardenolide glycoside in all plant parts is reported to be 5, 6-dehydrocalotropin. Similar concentrations of this glycoside are found in the various plant tissues, although seeds and roots have a lower concentration (El-Askary *et al.*, 1995b). The cardenolide glycoside and cardenolide genin with predicted structures, 15-hydroxyl-3,4,5,6-dehydrocalotropin and 3,4,5,6-dehydrocalotropin, respectively (Figure 4.3 and Figure 4), were isolated from *G. sinaicus* (Abbassy *et al.*, 2012). Both compounds have been assessed for their anti-fungal activity and exhibit potential as fungicides. Activity-guided fractionation of *G. sinaicus* methanol extract yielded six cardenolides, included: 7,8-dehydrocalotropin, calotropin, coroglaucigenin, 3-(6-deoxy-β-allopyranoside)-19-acetate and frugoside-19-acetate. Other cardenolides such as, 15*β*-hydroxy-5,6-dehydrocalotropin, coroglaucigenin and 3-(6-deoxy-β-allopyranoside)-19-acetate, were isolated by chloroform extraction of the stems of *G. sinaicus*. 5,6-dehydrocalotropagenin and 16α-hydroxy-5,6-dehydrocalotropin were also isolated and identified (El-Askary *et al.*, 1995a). In addition, a methanol extract of *G. sinaicus*, revealed the presence of flavonoids (Heneidak *et al.*, 2006) including quercetin glycoside (quercetin-*O*-*di*-rhamnosyl-hexoside, quercetin-3-*O*-pentosyl-hexoside, quercetin-3-*O*-rutinoside, rutin, kaempferol, 3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside. The de-fatted ethanolic extract of *G. sinaicus* caused a significant reduction in the rat paw volume (***$p<0.001$) indicating that this extract exhibits anti-inflammatory activity. In addition, the administration of the *G. sinaicus* de-fatted ethanolic extract at high concentration enhanced glucose, liver enzyme and lipid components in diabetic rats (El-Batran *et al.*, 2006).

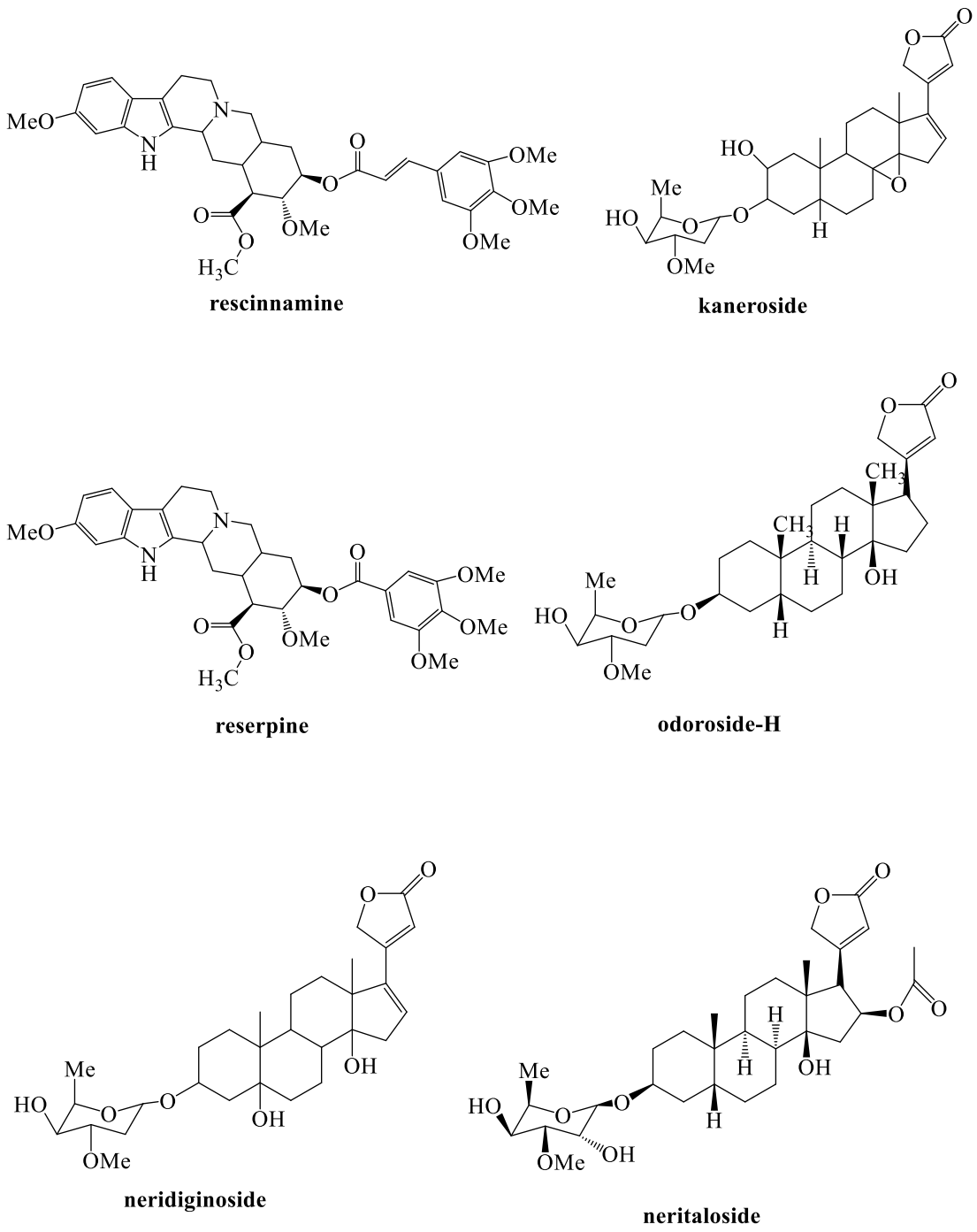


Figure 4. 1: Structures of some isolated compounds from Apocynaceae and *G. sinaicus.*

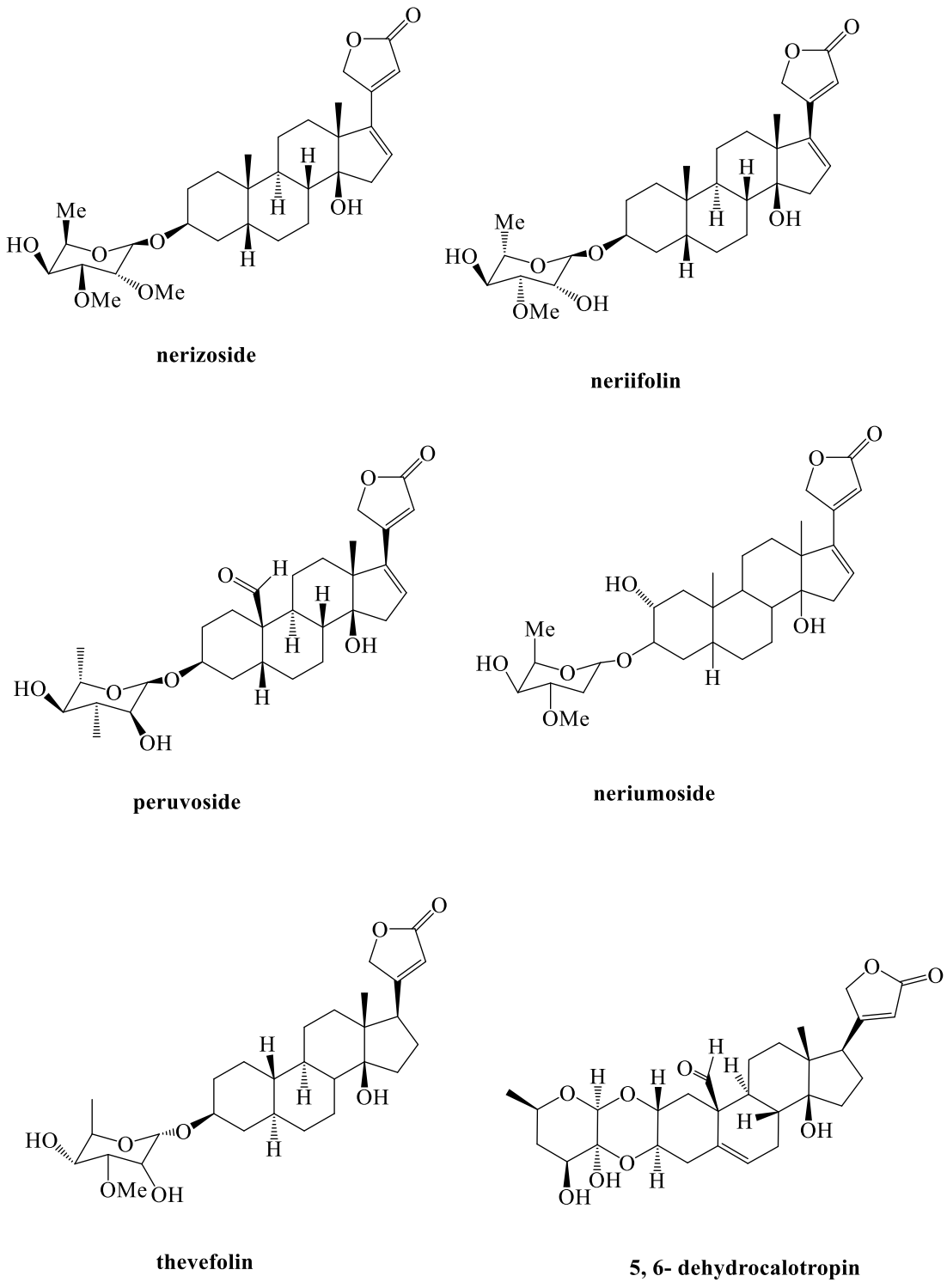
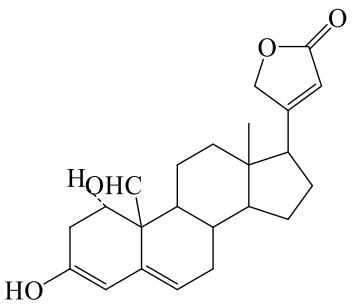
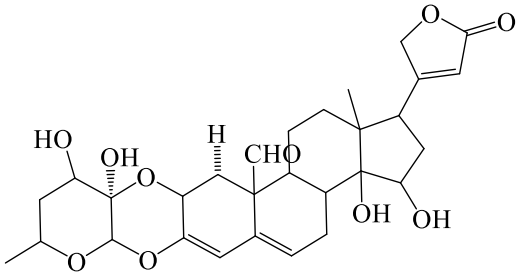


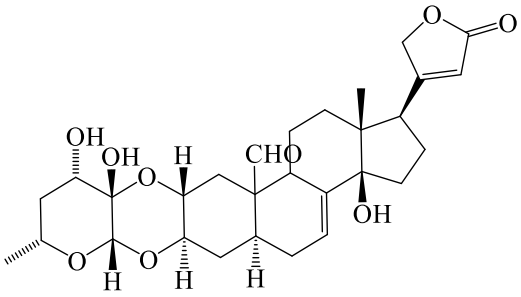
Figure 4. 2: Structures of some isolated compounds from Apocynaceae and *G. sinaicus.*



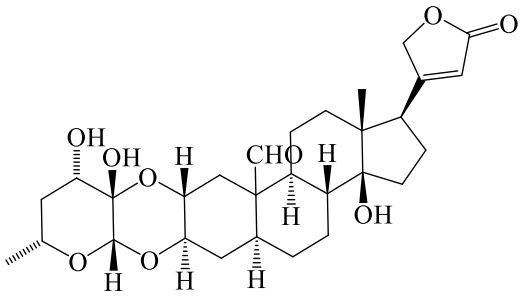
3,4,5,6-dehydrocalotropin



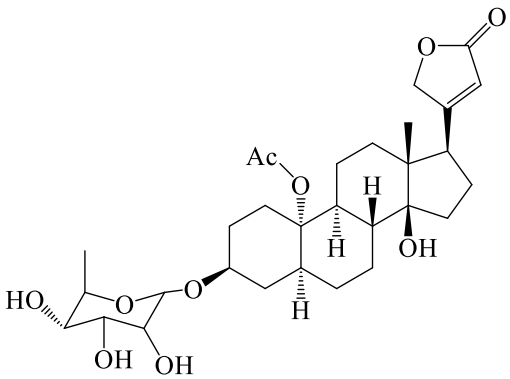
15-hydroxyl-3,4,5,6-dehydrocalotropin



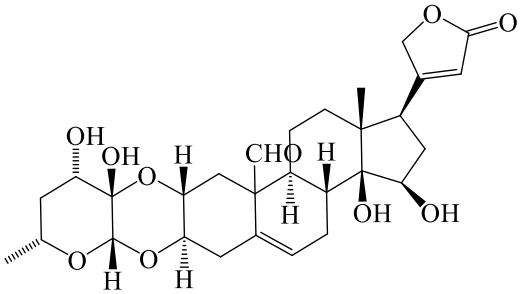
7,8-dehydrocalotropin



calotropin

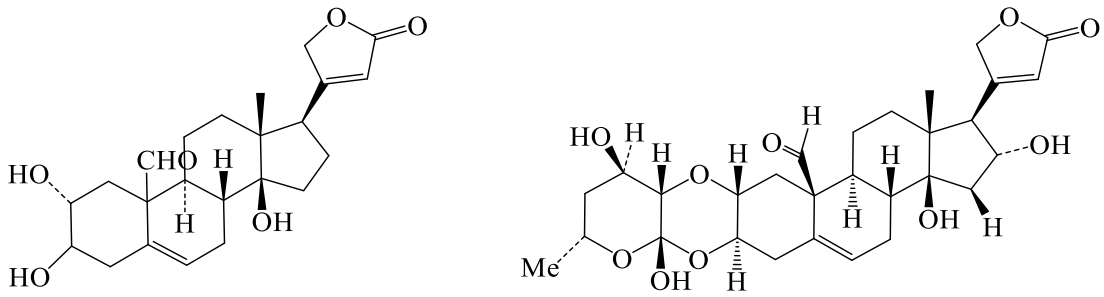


coroglaucigenin 3-(6-deoxy-b-allopyranoside) -19-acetate (frugoside 19-acetate)



15b-hydroxy-5,6-dehydrocalotropin

Figure 4. 3: Structures of some isolated compounds from Apocynaceae and *G. sinaicus.*



5,6-dehydrocalotropagenin

16a-hydroxy-5,6-dehydrocalotropin

Figure 4. 4: Structures of some isolated compounds from Apocynaceae and *G. sinaicus.*

4.2 Results

Previous screening of the various plant crude extracts revealed that *G. sinaicus* contains potential anticancer compound(s) against MDA-MB-231 and MCF7-L breast cancer cell lines. This was evidenced by the ability of the crude extract to reduce DNA synthesis, modulate SK1 and Des1 protein expression and induce apoptosis; indicated by PARP cleavage (Figures 3.8 and 3.9). Therefore, the extract was subjected to fractionation in order to isolate the active component(s) with respect to these activities in cancer cells.

4.2.1 Fractionation of *G. sinaicus (*Gs*)* crude extract

The *G. sinaicus* crude extract was fractionated using flash column chromatography as described in the methods (section 2.2.2). This yielded four fractions that contained compounds with different polarity in 50% (v/v) ethyl acetate (Gs2), 100% (v/v) ethyl acetate (Gs3), 50% (v/v) methanol (Gs4) and 100% (v/v) methanol (Gs5). The dry weight of starting material for *G. sinaicus* was 30 g. The yields and fraction weight and dryness are presented in Table 4.1.

Table 4. 1: *G. sinaicus* fractions dry weights:

***G. sinaicus* fraction**	**Solvent**	**Dry weight/g**
Gs 2	50% (v/v) ethyl acetate	7.9701
Gs 3	100% (v/v) ethyl acetate	6.3049
Gs 4	50% (v/v) MeOH	5.1456
Gs 5	100% (v/v) MeOH	9.9156

4.2.2 Effect of *G. sinaicus* fractions on DNA synthesis and protein markers

The Gs fractions (Gs 2– Gs5) were assessed for their ability to inhibit [^{3}H]-thymidine incorporation into DNA. As in previous experiments, two concentrations, 1µg/ml and 5µg/ml were used and compared with the vehicle control (0.05% (*v/v*) DMSO). Treatment of MDA-MB-231 cells with Gs3 fraction (100% (v/v) ethyl acetate) at 1 µg/ml was the most effective at reducing DNA synthesis compared with the other fractions (Figure 4.5 A) and this was concentration-dependent. In contrast, the Gs5 (100% (v/v) MeOH) fraction had no effect with increasing the concentration (Figure 4.5 B). Treatment of MDA-MB-231 cells with either Gs2, Gs3 or Gs4 reduced SK1a and Des1 expression and Gs3 was the most effective at inducing PARP cleavage (Figure 4.5C). Gs5 removed Des1 but was without effect on SK1.

All four Gs fractions induced inhibition of DNA synthesis in MCF-7L cells with Gs3 (100% (v/v) ethyl acetate) fraction being the most effective and which almost abolished DNA synthesis at 5 µg/ml concentration (***$p<0.001$) (Figure 4.6 A and Figure 4.6 B). Inhibition of DNA synthesis was concentration-dependent (Figure. 4.6 A and 4.6 B). The fractions had little effect on SK1b expression but, with the exception of *Gs*5, reduced the expression of SK1a and SK1c, while the Gs3 fraction also reduced Des1 expression and induced PARP cleavage. There was a minor effect of Gs2, Gs4 and Gs5 on PARP cleavage (Figure 4.6 C), indicating that the effects on PARP cleavage by Gs3 might be linked to removal of both Des1 and SK1a, b.

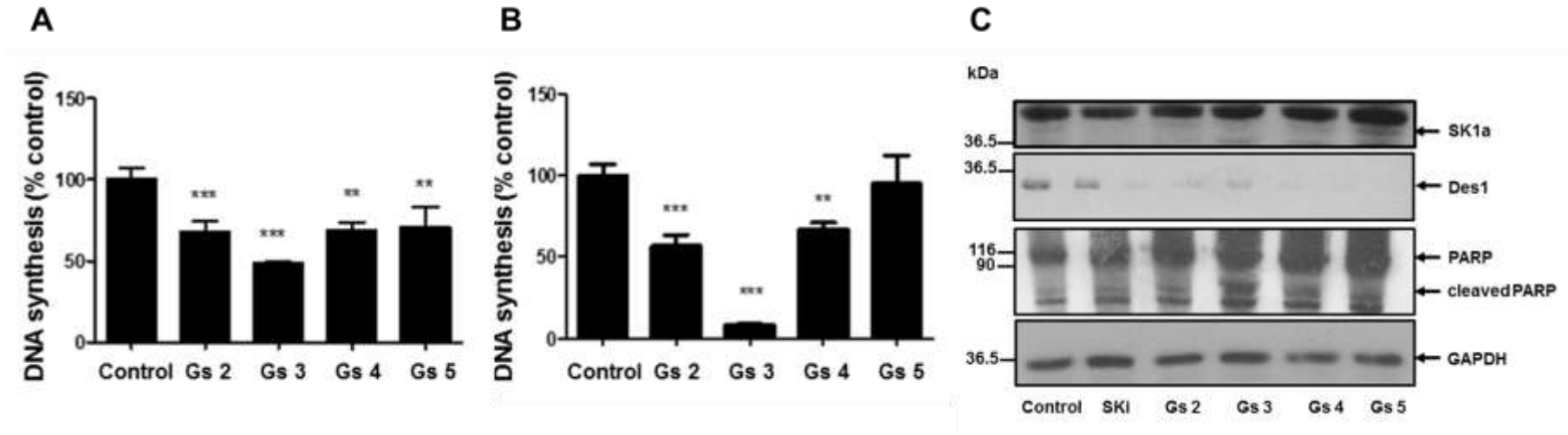


Figure 4. 5*:* Effect of *G. sinaicus* fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with (**A**) *G. sinaicus* fractions (1 µg/ml) or (**B**) *G. sinaicus* fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined date from 3 experiments. ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1, Des1 and PARP cleavage detection: quiescent MDA-MB-231 cell were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.15- 2.2.17). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

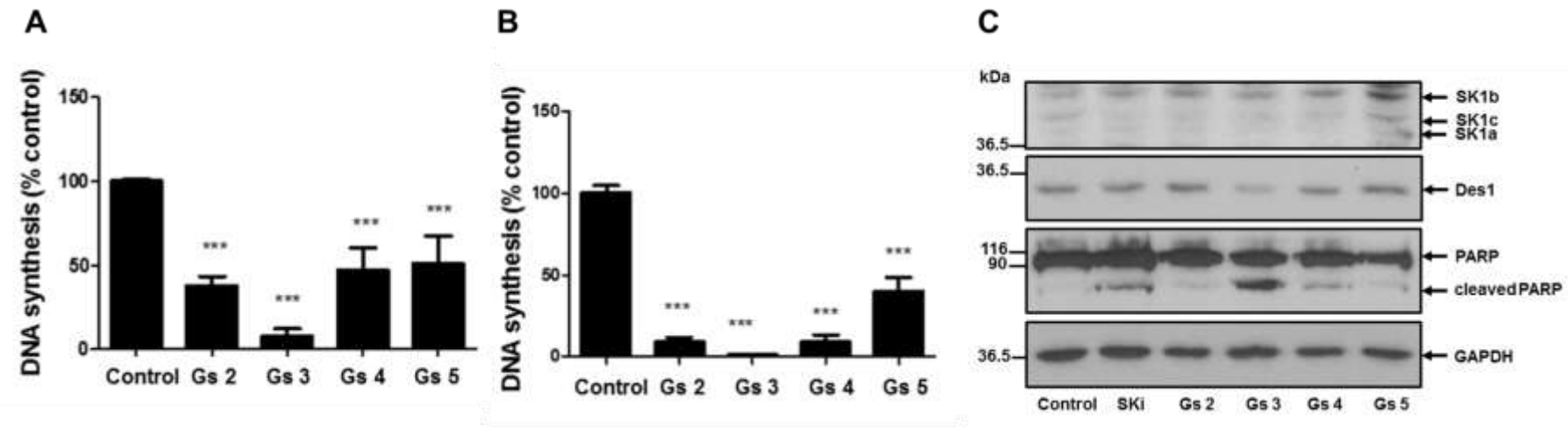


Figure 4. 6: Effect of *G. sinaicus* fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MCF7/L cells.

Quiescent MCF7/L cells were treated with (**A**) *G. sinaicus* fractions (1 µg/ml) or (**B**) *G. sinaicus* fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments. ***$p<0.001$ vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1, Des1 and PARP cleavage detection: quiescent MCF7L cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.15 - 2.2.17). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

4.2.3 *G. sinaicus* fraction 3 sub-fractionations: effect of Gs 3 sub-fractions on DNA synthesis and protein markers.

Based on previous results of *G. sinaicus* fractions, sub-fractionation, and separation of Gs3 (100% (v/v) ethyl acetate) fraction was performed to further purify active compounds. Using silica column chromatography as described in Methods (section 2.2.4), Gs3 yielded six sub-fractions named; Gs3.1, Gs3.2, Gs3.3, Gs3.4, Gs3.5 and Gs3.6. These were then tested for their effect on DNA synthesis (at 1 and 5 µg/ml) and on SK1 and Des1 expression and PARP cleavage at 5 µg/ml in both MDA-MB-231 and MCF7/L cell lines.

Treatment of MDA-MB-231 cells with Gs3.1, Gs3.2, Gs3.3 and Gs3.4 sub-fractions (1 and 5 µg/ml) reduced DNA synthesis (Figure 4.7 A) in a concentration-dependent manner (Figure 4.7 B). There was a small reduction with Gs3.5 fraction at 5 µg/ml and no effect of Gs3.6. Treatment of MDA-MB-231 cells with; Gs3.1, Gs3.2, Gs3.3 and Gs3.4 reduced SK1a expression, while Gs3.5 and Gs3.6 had no effect. All the fractions reduced Des1 expression, while only Gs3.1, Gs3.2, Gs3.3 and Gs3.4 induced PARP cleavage (Figure 4.7 C).

Treatment of MCF-7L cells with Gs3.1, Gs3.2, Gs3.3 and Gs3.4 sub-fractions (1 and 5 µg/ml) reduced DNA synthesis in a concentration-dependent manner (Figure 4.8 A and B). Gs3.1- Gs3.3 had little effect on SK1 expression but did reduce Des1 expression (Figure 4.8 C) and had a modest effect on PARP cleavage thereby suggesting that the active compounds in the sub-fractions can act as anticancer or anti-inflammatory agents.

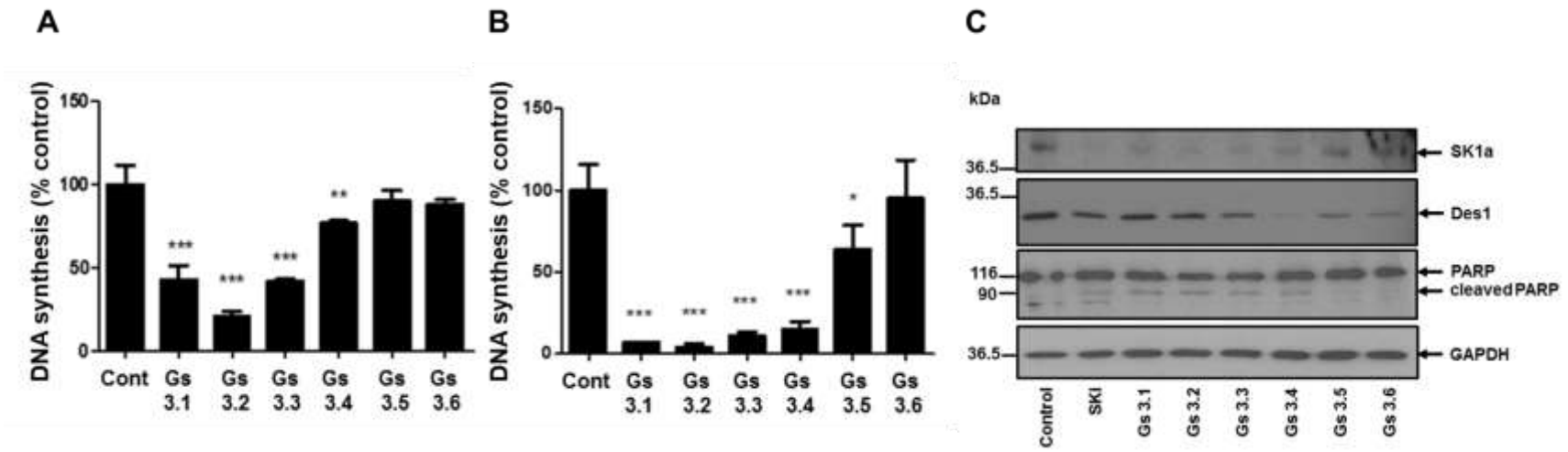


Figure 4. 7*: Effect of *G. sinaicus* sub-fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with (**A**) *G. sinaicus* sub-fractions (1 µg/ml) or (**B**) *G. sinaicus* sub-fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments *P<0.01, **p<0.001, ***p<0.0001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1, Des1 and PARP cleavage detection: quiescent MDA-MB-231 cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.15 - 2.2.17). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

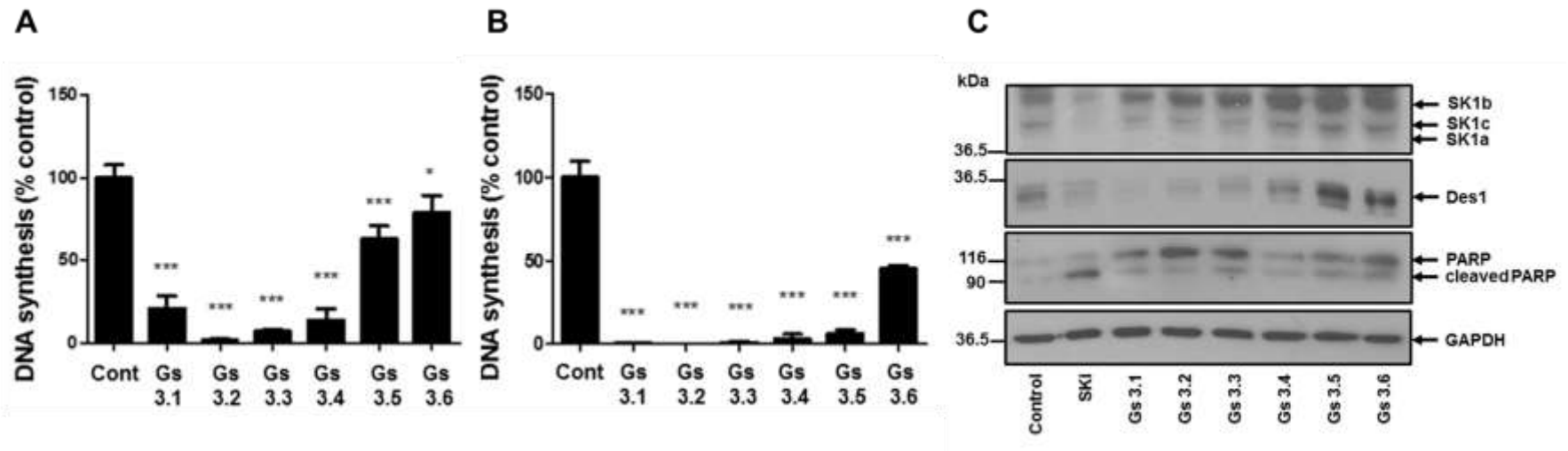


Figure 4. 8*:* Effect of *G. sinaicus* sub-fractions on DNA synthesis, SK1, Des1 and PARP cleavage in MCF7/L cells.

Quiescent MCF7/L cells were treated with (**A**) *G. sinaicus* sub-fractions (1 µg/ml) or (**B**) *G. sinaicus* sub-fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments. *p<0.05, ***p<0.0001 vs control (one-way ANOVA with Dunnett's post-hoc test) (**C**) SK1, Des1 and PARP cleavage detection: quiescent MCF7/L cell were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each fraction for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted for SK1, Des1 and PARP according to methods (section 2.2.15 - 2.2.17). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

4.2.4 Purification of Gs3.2 sub-fraction: effect of Gs3.2 sub-fractions on DNA synthesis and protein markers

From previous experiments, Gs3.2 has shown the greatest inhibition of DNA synthesis with inducing PARP cleavage against both breast cancer cell lines. Hence, purification and separation of the Gs3.2 sub-fraction was performed to isolate the active compound(s) using preparative TLC as described in Methods (section 2.2.6) with solvent system (ethyl acetate: MeOH 3: 0.5). This process yielded four sub-fractions named, Gs3.2.1, Gs3.2.2, Gs3.2.3 and Gs3.2.4 as shown below (Table 4.2).

Table 4. 2: *G. sinaicus, Gs* 3.2 sub-fractions dry weights*:*

Gs 3.2 sub-fractions	Dry weight/ µg
Gs 3.2.1	46.2
Gs 3.2.2	23.1
Gs 3.2.3	7
Gs 3.2.4	2

Treatment of MDA-MB-231 cells with the four Gs3.2 sub-fractions reduced DNA synthesis at both 1 µg/ml and 5 µg/ml (Figure 4.9 A and 4.9 B). Their effects on SK1 and Des1 protein levels were also examined. In this study, SKi was used for comparison with Gs3.2 sub-fractions, in combination with the proteasomal inhibitor, MG 132, which limits protein degradation.

MDA-MB-231 cells were treated with SKi (10 µM) and each of the Gs3.2 sub-fraction (5 µg/ml) for 24 h, either with or without pre-treatment with MG132 (10 µM, 30 min). Treatment of MDA-MB-231 cells with SKi reduced SK1a expression, which was prevented by pre-treatment with MG132 (Figure 4.9 C). Gs3.2.1, Gs3.2.3 or Gs3.2.4 also induced a reduction in SK1a expression, which was prevented by pre-treatment with MG132. Gs3.2.2 had no effect on SK1 expression. All the fractions induced a reduction in Des1 expression, but this was not prevented by MG132 (Figure 4.9C).

Treatment of MCF7-L cells with all Gs3.2 sub-fractions reduced DNA synthesis at both concentrations (1 µg/ml and 5 µg/ml) (Figure 4.10 A and Figure 4.10 B).

Treatment of MCF-7L cells with Gs3.2.1, Gs3.2.2 or Gs3.2.3 induced a clear decrease in SK1a-c expression and this reduction was prevented by pre-treatment with MG132. These three fractions also induced a reduction on Des1 expression, but pre-treatment with MG132 did not prevent this (Figure 4.10 C). Gs3.2.4 had little effect on SK1 or Des1 expression in MCF7-L cells.

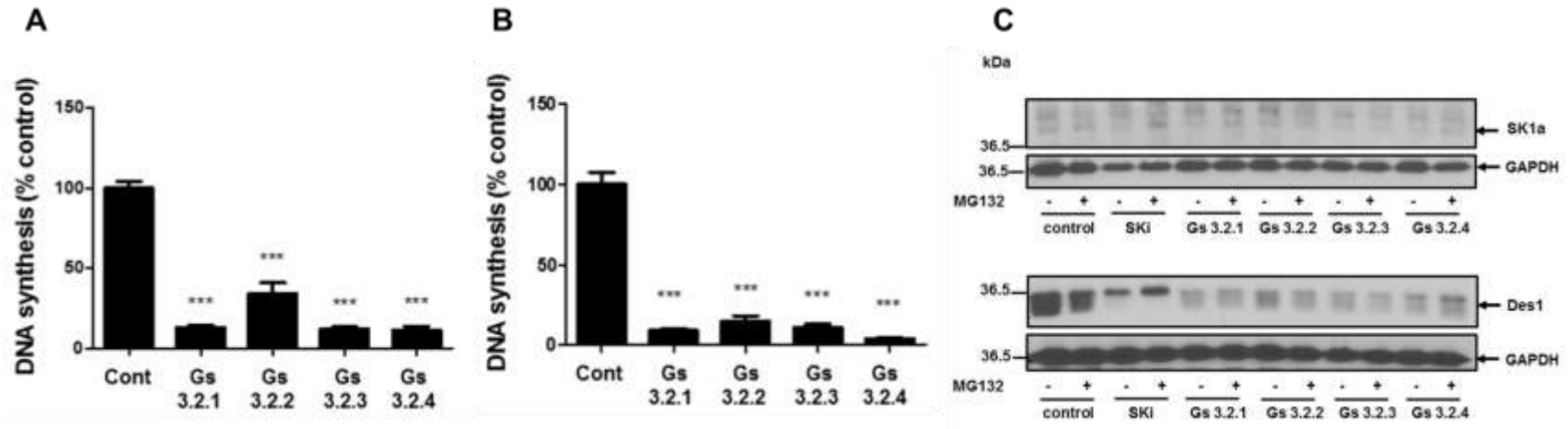


Figure 4. 9: Effect of Gs 3.2 sub-fractions on DNA synthesis, SK1 and Des1 in MDA-MB-231 cells.

Quiescent MDA-MB-231 cells were treated with (**A**) Gs3.2 sub-fractions (1 µg/ml) or (**B**) Gs3.2 sub-fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments ***P<0.0001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1 and Des1 detection: quiescent MDA-MB-231 cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each sub-sub-fraction for 24 h after pre-treatment with or without MG132 (10 µM, 30 min). Cell lysates were separated by SDS-PAGE and immunoblotted for SK1and Des1 according to methods (section 2.2.15 - 2.2.17). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

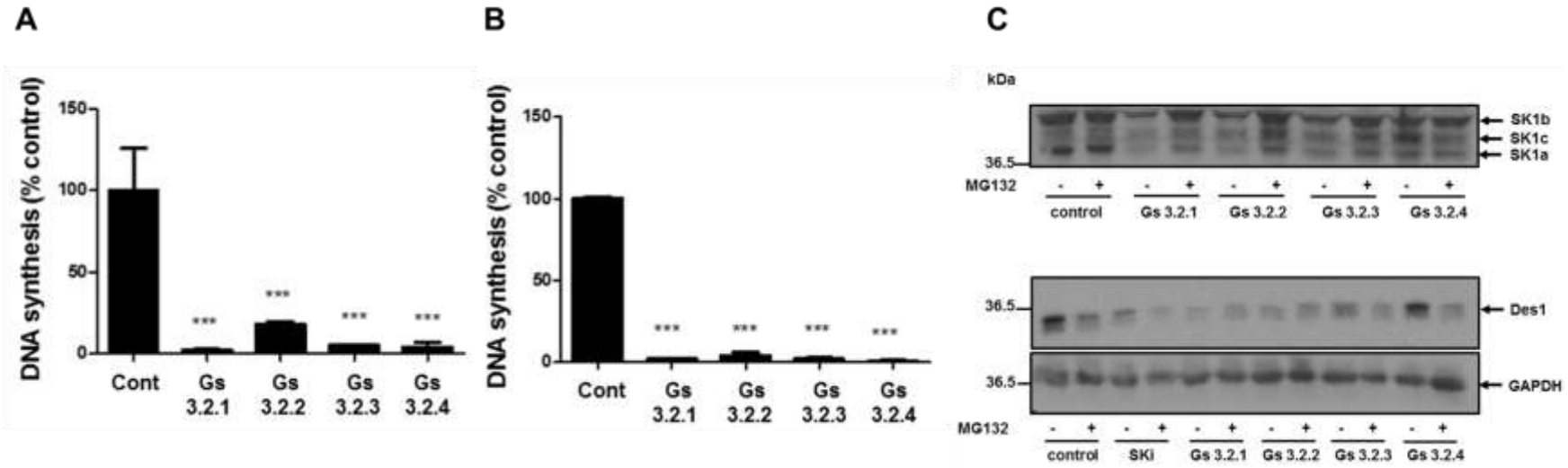


Figure 4. 10*:* Effect of Gs3.2 sub-fractions on DNA synthesis, SK1 and Des1 in MCF7-L cells.

Quiescent MCF7-L cells were treated with (**A**) Gs3.2 sub-fractions (1 µg/ml) or (**B**) Gs3.2 sub-fractions (5 µg/ml) (vehicle control 0.05% (v/v) DMSO) for 16 h and then [^{3}H]-thymidine (0.5 µCi/ml) added for a further 5 h. Incorporation of [^{3}H] thymidine into newly synthesised DNA was measured as described in Methods (section 2.2.14). Data are expressed as percentage of control and represent mean +/- SD of combined data from 3 experiments ***P<0.0001 vs control (one-way ANOVA with Dunnett's post-hoc test). (**C**) SK1 and Des1 detection: quiescent MCF7-L cells were treated with vehicle control (0.05% (v/v) DMSO), inhibitor SKi (10 µM) or 5 µg/ml of each sub-sub-fraction for 24 h after pre-treatment with or without MG132 (10 µM, 30 min). Cell lysates were separated by SDS-PAGE and immunoblotted for SK1and Des1 according to methods (section 2.2.15 - 2.2.17). Blots were then stripped and re-probed for GAPDH to check for similar protein loading.

4.2.5 Effect of Gs 3.2.1 and Gs 3.2.2 on NF_KB and AP-1 transcriptional activity

Gs 3.2.1 and Gs 3.2.2 were also assessed for their effect on inflammatory signalling. This was achieved using NCTC/NF_KB reporter keratinocytes. Initially, the effect of TNFα on NF_KB-driven transcriptional activity was measured using a luciferase reporter assay. A comparison was made of the effects of Gs 3.2.1 and Gs 3.2.2. Pre-treatment of the cells with Gs 3.2.1 (5 ug/ml, 30 min) significantly reduced TNFα-stimulated NF_KB transcriptional activity (***p<00.1). In contrast, pre-treatment of the cells with Gs 3.2.2 (5 ug/ml, 30 min) had no effect (Figure 4.11 A).

A second luciferase reporter cell line was used to investigate the effect of Gs 3.2.1 and Gs 3.2.2 on the transcriptional regulation of AP-1 in NCTC-AP-1 reporter keratinocytes. NCTC-AP-1 cells were treated with PMA at 100 nM. PMA, which is a direct activator of PKC that promotes AP-1 transcriptional activity. Pre-treatment of the NCTC-AP1 cells with Gs 3.2.1 (5 µg/ml, 30 min) or Gs 3.2.2 (5 µg/ml, 30 min) reduced PMA-stimulated AP-1 transcriptional activity (Figure 4.11 B).

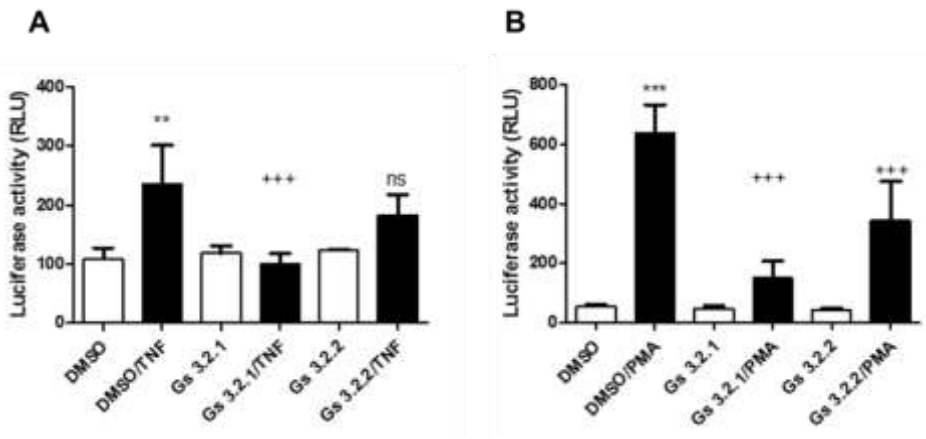


Figure 4. 11*:* Effect of Gs 3.2.1 and Gs 3.2.2 on TNFα-stimulated NFκB signalling and on PMA-stimulated AP-1- signalling.

Luciferase reporter assay (**A**) Quiescent NCTC-NFκB reporter cells were pre-treated with Gs 3.2.1 (5 µg/ml for 30 min) or Gs 3.2.2 (5 µg/ml for 30 min) or vehicle alone (DMSO 0.05% (v/v)) prior to stimulation with TNFα (15 ng/ml) for 4 h. Data are expressed as a % of control ± SEM for n=3 or more experiments. **p<0.01 vs control (one-way ANOVA with Dunnett's post-hoc test); +++p<0.001 and ns (not significant) vs DMSO/TNFα sample (one-way ANOVA with Bonferroni's post-hoc test). (**B**) Quiescent NCTC-AP-1 reporter cells were pre-treated with Gs 3.2.1 (5 µg/ml for 30 min), Gs 3.2.2 (5 µg/ml for 30 min) or with vehicle alone (DMSO 0.05% (v/v)) prior to stimulation with PMA (100 nM) for 4 h before luminescence activity was measured. Data are expressed as a % of control ± SEM for n=3 or more experiments. ***p<0.001 vs control (one-way ANOVA with Dunnett's post-hoc test); +++p<0.001 vs DMSO/PMA sample (one-way ANOVA with Bonferroni's post-hoc test).

4.2.6 Structure elucidation of Gs 3.2.1 sub-fraction:

NMR analysis of Gs 3.2.1 sub-fraction revealed that this sample was a mixture of two principal components that were similar in structure and identified (as detailed below) as humistratin (compound **1**) and calotropin (compound **2**) or its stereoisomer, calactin (compound **3**) (Figure 4.34). Based on ^{1}H-NMR integrals, the ratio of the two compounds in the sample is approximately 1.5: 1.00. The DEPT q spectrum indicated four carbonyl carbons at δ_C 177.57, 176.92, 208.23 and 209.42. There are also 16 oxygenated carbons ranging from 69.35 to 97.65 ppm and four methyl carbons at δ_C 16.19, 16.40 and 21.32 (2X) ppm. In addition, there are six olefinic carbons at δ_C 177.57, 117.89, 176.92, 118.21, 121.67 and 141.00 ppm. The rest of the signals are of methylene carbons which overlapped between δ_C 27.40 to 53.95 ppm (Figure 4.12-4.15). The connections between the carbons and directly attached protons were elucidated using the HSQC spectrum (Figure 4.16-4.20). The structures were further probed with heteronuclear multi-bond correlations and homonuclear ^{1}H-^{13}C correlations in 2D spectra (HMBC, Figures 4.24–4.29; and ^{1}H-^{1}H-COSY, Figure 4.30-4.32). From the ^{1}H-NMR spectrum, 6 proton signals were identified for the **E** ring of the two compounds [δ 5.05, 4.94 (H-21a/b) and 5.90 (H-22) in compound **1**; δ 5.10, 4.98 (H-21a/b) and 5.96 (H-22) in compound **2/3**] (Figure 4.18-4.20). Furthermore, each of the 3 protons of each ring showed cross peaks to neighbouring carbons (H-21a,b/C-22 and H-22/C-21, H-22,H-21/C-23) in the long rang coupling of the HMBC spectrum (Figure 4.21- 4.26). In addition, each showed correlations between H-21a/H-22 and H-21b/H-22 in the long rang coupling ^{1}H-^{1}H-COSY (Figure 4.21-4.223) which confirmed the presence of two α, β-unsaturated γ-lactone moieties. Each moiety labelled as Ring **E** (Figure 4.34) belongs to two different compounds (**1** and **2/3**), and these account for four of the six alkene resonances, therefore. The remaining two olefinic carbons, giving signals at δ_C 141.00 and 121.67, are due to the presence of an additional trisubstituted double bond at the connection of rings **B** and **C**, and this is unique to compound **1**. In addition, the ^{1}H-NMR spectrum showed two highly deshielded protons at δ_H 10.02 and 9.78 (identified as 19-OCH, in compound **1** and **2/3** respectively), which indicated the presence of an aldehyde functional group in both compounds. These resonances correspond to the two most downfield CH signals in the

DEPT q spectrum (δ_C 208.23 and 209.42), the chemical shift of which is also highly characteristic for aldehydes.

Significantly, the ^{1}H-NMR spectrum suggested the presence of two anomeric centres giving rise to singlet resonances, at δ_H 4.46 and 4.45 for compound **1** and compound **2/3** respectively, and these were shown from the HSQC spectrum to be directly attached to C-1` carbons with resonances at $\delta_C$ 97.39 and 97.31 for the two compounds. Multi-bond correlations from the H-1` resonances to closely paired deshielded ^{13}C resonances for C-2` ($\delta_C$ 92.65/92.68), C-3` (δ_C 73.91/73.96) and C-5` (coincident for the two compounds at δ_C 69.35) were observed in the HMBC spectrum. These resonances were consistent with the presence of a 2-oxopyranose carbohydrate-derived ring **G** structure. Additional multi-bond correlations observed in the HMBC spectrum were consistent the proposed ring **G** structure, notably for the following cross peaks: C-1`/H-5`, C-2`/H-6`, C-2`/H-3`, C-3`/H-5`, C-5`/H-3` (Figure 4.27-4.29). Cross-ring connectivity was established through a multi-bond C-3/H-1` correlation and additional cross peaks were observed consistent with the Ring **A** structure: C-2/H-3, C-3/H-1b, C-2/H-1a,b, C-2/H-5, C-3/H-6b (Figure 4.17). For the oxygenated methine resonances — CH-2, CH-3, CH-1`, CH-3` and CH-5` — it is interesting to note that the hydrogens exhibit very close correspondence for the two compounds except for the two H-2 signals (δ_H 4.21 and 3.92). This may potentially reflect a difference in the ring **G** stereochemistry at C-3` or/and the stereochemistry at the junction of rings **F** and **G**.

Cross-ring connectivity was also established from the HMBC spectrum for rings **E** and **D**. Thus, in ring **E** correlations were seen from C-20 to the hydrogens of the CH_2-16 and CH-17 centres in ring **D** (Figure 4.26). Additional connections were deduced for the ring junction quaternary carbons between rings **C** and **D**. In this case, correlations were observed from C-13 (δ_H 51.59/50.66) to H-17 (δ_H 2.82/2.92) and from C-14 (δ_H 85.65/85.74) to H-17 (δ_H 2.821/2.92) in both compounds, thereby fixing the position of the carbinol centre at the ring **C**/**D** fusion (Figure 4.24 and 4.29). Correlation was also observed from the C-13 centres to the hydrogens of the attached bridgehead methyl at position-18 (Figure 4.27). In the case of compound **1**, the C-14 carbinol carbon was also clearly correlated with the olefinic H-7 resonance (δ_H 6.11), while C-

7 (δ_C 121.67) and C-8 (δ_C 141.00) correlated with H-9 (δ_H 2.40) (Figure 4.27). For both compounds, correlations were observed from the aldehydic carbon centres (C-19; δ_C 208.23/209.42) to both hydrogens of the CH_2-1 methylenes (ca. δ_H 2.4 and 1.1 for the H-1a and H-1b resonances in both compounds) (Figure 4.24-4.29). The latter protons, as well as the aldehydic protons correlated to the respective quaternary C-10 centres at δ_C 53.39 and 54.01 for the two compounds.

Taken together, the structural elucidation establishes the identity of compound **1** as the cardenolide glycoside, 7,8-dehydro-calotropin (humistratin, Figure 4.34). The analysis was reinforced by acquisition of a ^{1}H-^{1}H COSY spectrum (Figure 4.30-4.32) from which selected correlations are shown in Table 4.3. The stereochemical assignment in the structure is made on the basis of literature precedent (Kupchan *et al.*, 1964, Singh and Rastogi, 1969, Nishio *et al.*, 1982, Cheung *et al.*, 1989, Abdel-Azim *et al.*, 1996), however, and further evaluation is needed from NOESY spectroscopic analysis of the pure compound to conclusively prove this.

The second compound is clearly closely related to humistratin (**1**) but lacks the 7,8-double bond, and this gives rise to some differences in the chemical shifts of the carbons and protons of rings **A**, **B** and **C** (Table 4.4). This compound is likely to be calotropin (Figure 4.34, **2**) itself or its stereoisomer, calactin (Figure 4.34, **3**). Again, NOESY spectroscopic analysis of a pure sample is needed to confirm the exact identity (**2** vs **3**) for this compound.

The structure elucidation of the two principal compounds in the Gs 3.2.1 sample, as humistratin (**1**) and calotropin /calactin (**3**) (Helbig *et al.*, 2003), was further supported by LCHRMS, which revealed the presence of the appropriate molecular ions (531.2593 (**1**), 533.2749 (**2**/**3**) (Figure 4.32). The NMR spectra for the sample do reveal the presence of a number of additional minors, unidentified substances, but the bulk of the sample comprises the compounds **1** and **2**/**3**, which are present in 1.5: 1.0 ratio (based on integration of the aldehydic resonances in the ^{1}H-NMR spectrum).

Table 4. 3: **Spectral data summary for Compound 1 in Gs 3.2.1: humistratin.**

No.	δH ppm	δC ppm	HMBC	^{1}H-^{1}H COSY
1	1.14, 2.47	35.95	H-19, H-10, H-9	
2	3.85	70.28	H-3, H-1b, H-5	H-1a, H-1b, H-3
3	3.92	73.24	H-1b, H-6b, H-1`	H-5, H-4b, H-1a
4	1.19, 1.81	34.30	H-5	H-5b
5	1.91	39.93	H-9, H-11	
6	1.34, 2.32	30.42	H-4a, H-6a	H-5
7	6.11	121.67	H-6b	H-6b
8	-	141.00	H-6b	
9	2.40	45.55	H-1b, H-15a	
10	-	54.01	H-1a, b	
11	1.64	38.06	H-18	H-12a
12	1.62, 1.59	39.76	H-18	
13	-	51.59	H-17, H-16a, b, H-12b, H-9, H-11, H-18	
14	-	85.65	H-17, H-16b, H-9, H-18	
15	1.65,1.40	34.40		
16	2.23, 2.00	28.54		
17	2.84	51.78	H-22, H-16a, b- H-18	H-16a, H-12b, H-16b
18	0.80	16.14		
19	10.02	209.42	H-1a, H-6b	H-1
20	-	177.89	H-16a, H-21, H-22	
21	4.94, 5.05	75.25	H-22, H-17	
22	5.90	117.89	H-21a, b, H-17	
23	-	177.57	H-21a, b, H-22, H-17, H-15b	
1`	4.46	97.39	H-5`	
2`	-	92.65	H-1`, H-3`, H-4`b	
3`	3.60	73.91	H-1`, H-5`, H-4`a, H-6`	
4`	1.59, 1.75	39.51	H-3`, H-6`	H-4`a
5`	3.67	69.35	H-1`, H-6`, H-4`b	H-4`a
6`	1.24	21.39	H-5`, H-4`a	

s=singlet, brs= broad singlet, d = doublet, dd= doublet of doublet, t= triplet, q=quartet, m= multiplet signals. It is hard to measure the J value cause of the overlapping of the compound's protons.

Table 4.4: Spectral data summary for Compound 2 in Gs3.2.1: calotropin/calactin

No.	δH ppm	δC ppm	HMBC	^{1}H-^{1}H COSY
1	2.48, 1.11	36.76	H-19, H-10	H-5
2	4.21	69.94	H-3, H-1b, H-5, H-4a	H-1a, b
3	3.92	72.65	H-1b, H-4a, H-5, H-1`	H-1a, H-4b, H-5
4	1.75, 1.58	39.59	H-6	
5	1.63	42.57	H-4a	H-4a
6	1.75, 2.18	26.35	H-6a, H-7a	
7	1.96, 2.16	27.95		
8	2.71	49.79	H-6a, H-1a	H-6a
9	2.41	45.63	H-5, H-11a, b, H-1a	H-1a
10	-	53.59	H-1a, b, H-9, H-4a, H-11a	
11	1.40, 1.48	33.48		
12	1.90, 1.10	43.28	H-11b, H-16a	
13	-	50.66	H-22	
14	-	85.74	H-18, H-17, H-16b	
15	1.70, 2.11	32.80		
16	2.27, 2.00	28.71	H-17	
17	2.92	51.60	H-22, H-12b, H-16b, H-18	H-12b, H-16a, H-15b
18	0.82	16.56		
19	9.78	208.23	H-1a, H-6b	H-1
20	-	178.22	H-21, H-22	
21	5.10, 4.98	75.25	H-22, H-17	
22	5.96	118.21	H-21a, b- H-17	
23	-	176.92	H-21a, b – H-22, H-17	
1`	4.46	97.31	H-5`	
2`	-	92.65	H-1`, H-3`, H-4`a	
3`	3.60	73.91	H-1`, H-5`, H-4`a, b, H-6`	H-4`a
4`	1.92, 1.67	39.82	H-3`, H-6`	
5`	3.68	69.43	H-1`, H-6`, H-4`b	
6`	1.24	21.39	H-5`, H-4`a	

S=singlet, brs= broad singlet, d = doublet, dd= doublet of doublet, t= triplet, q=quartet, m= multiplet signals . It is hard to measure the J value cause of the overlapping of compounds protons.

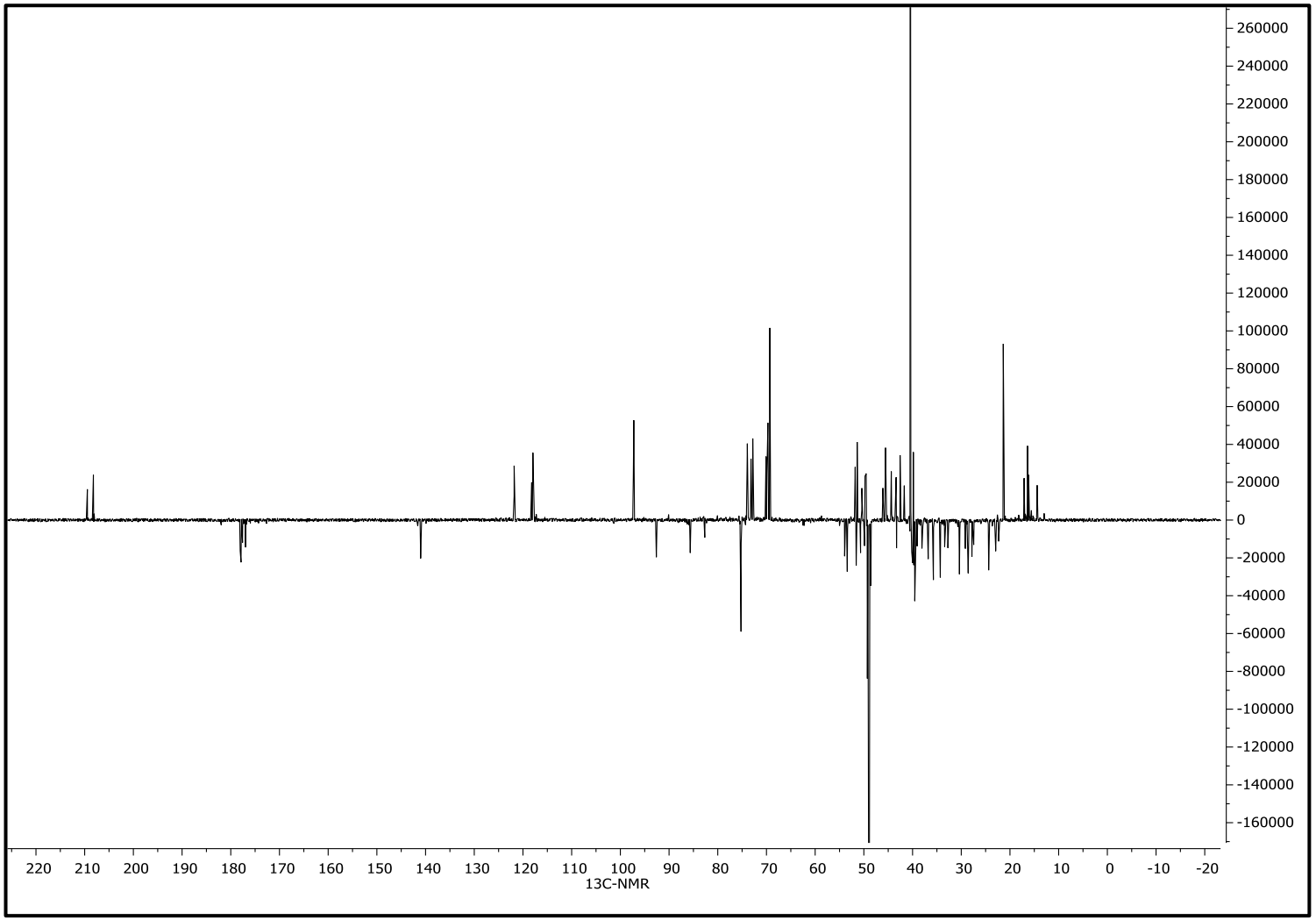


Figure 4. 12: DEPT q spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD

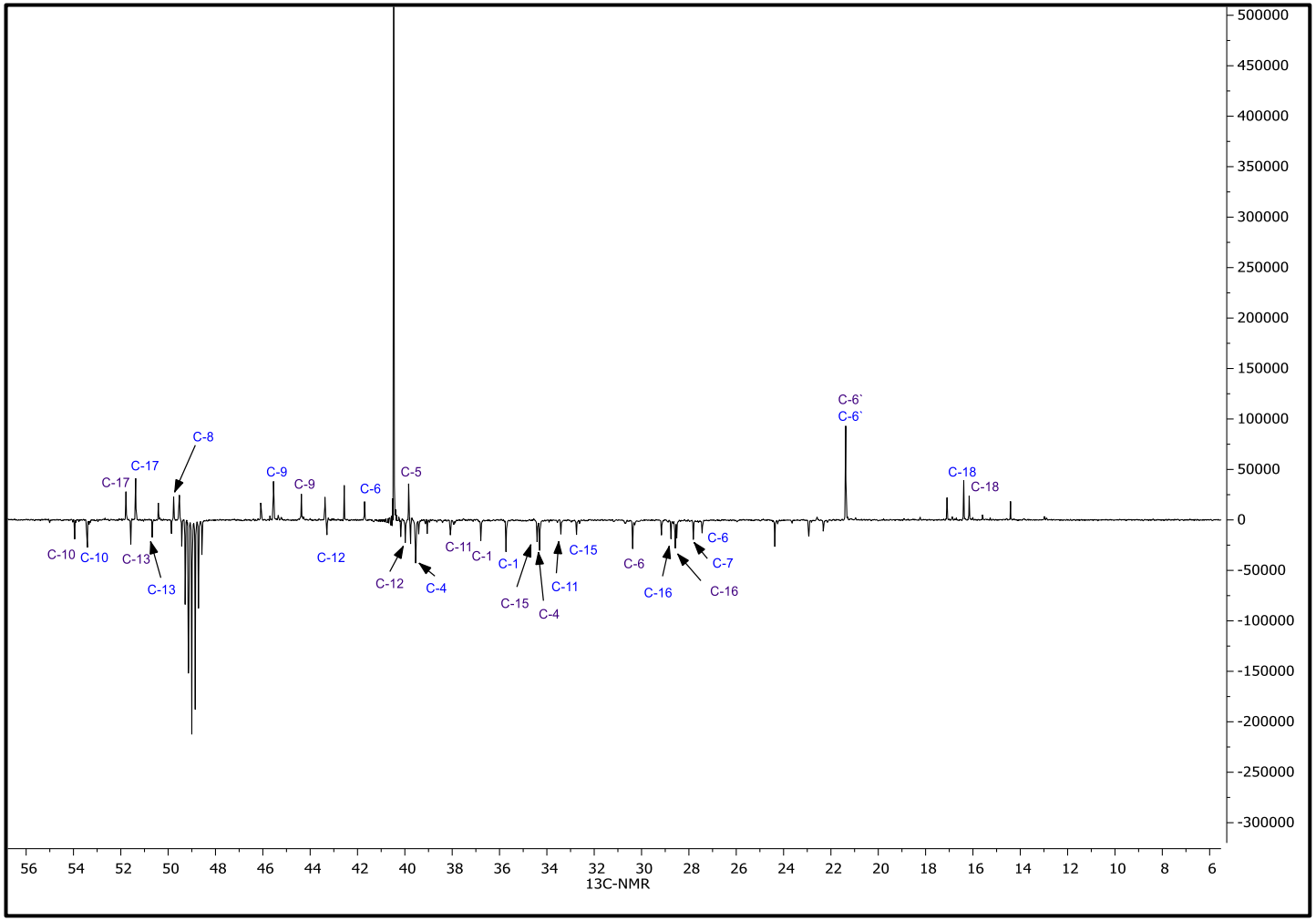


Figure 4. 13: DEPT q spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-1

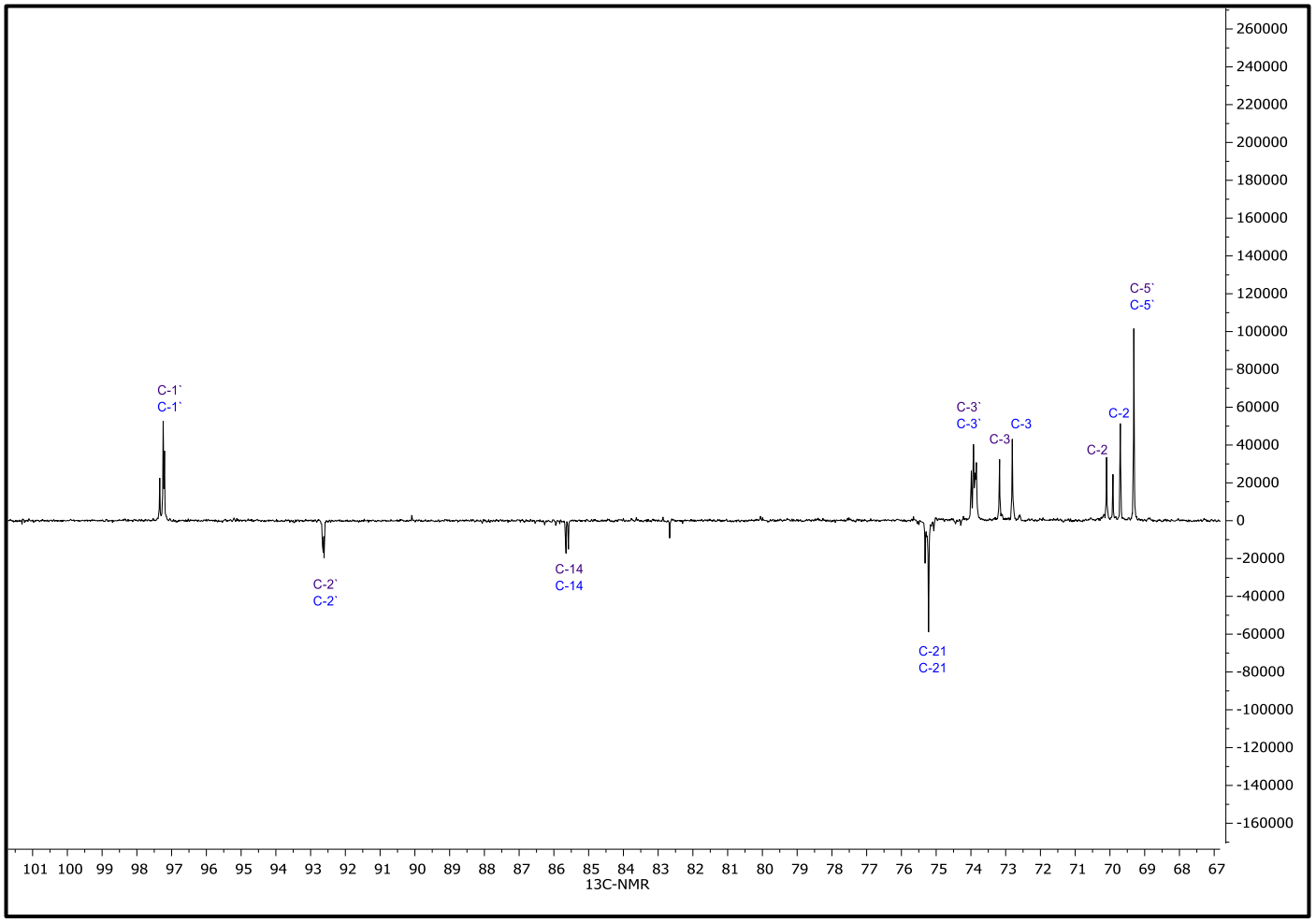


Figure 4. 14: DEPT q spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-2

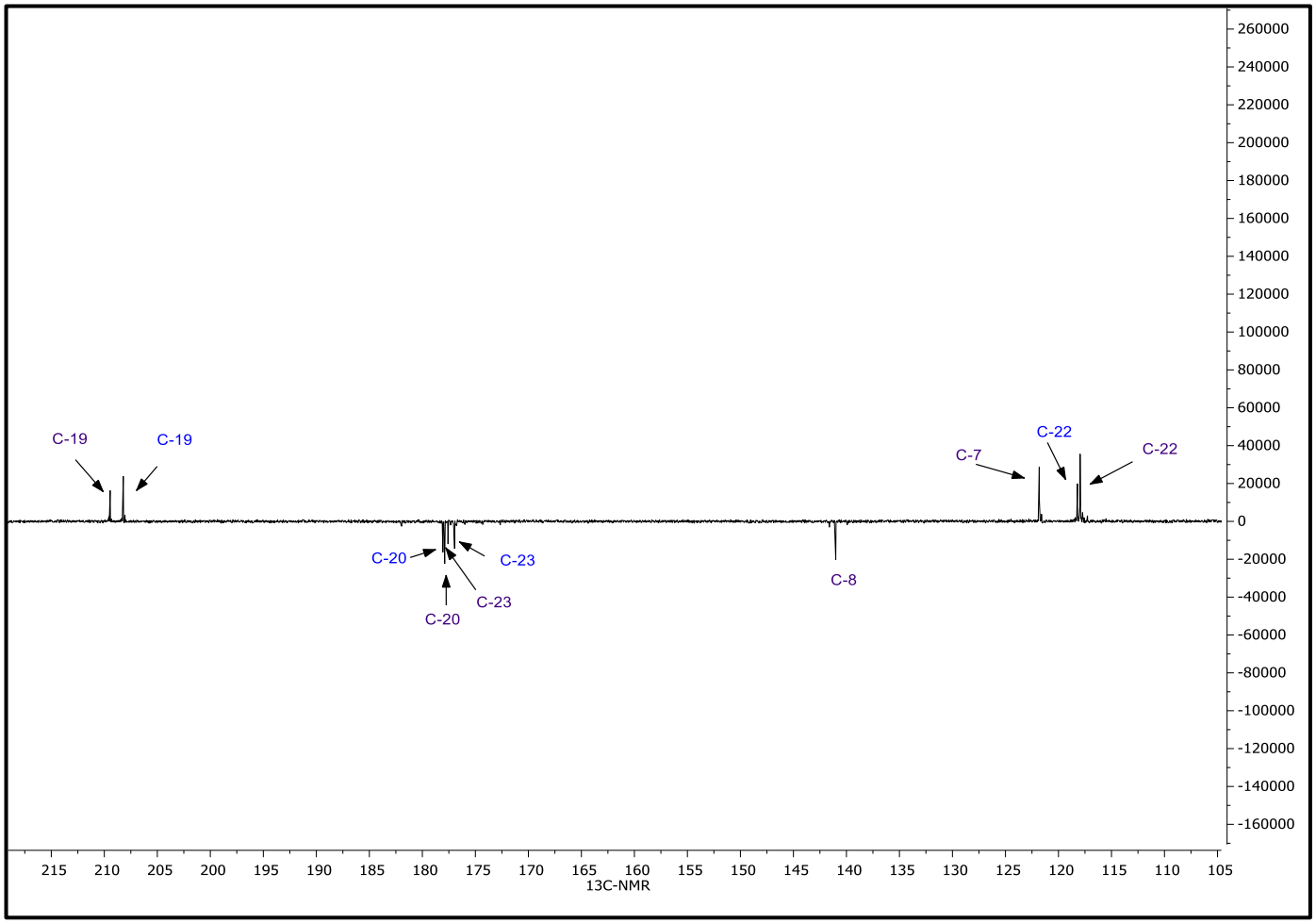


Figure 4. 15: DEPT q spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-3

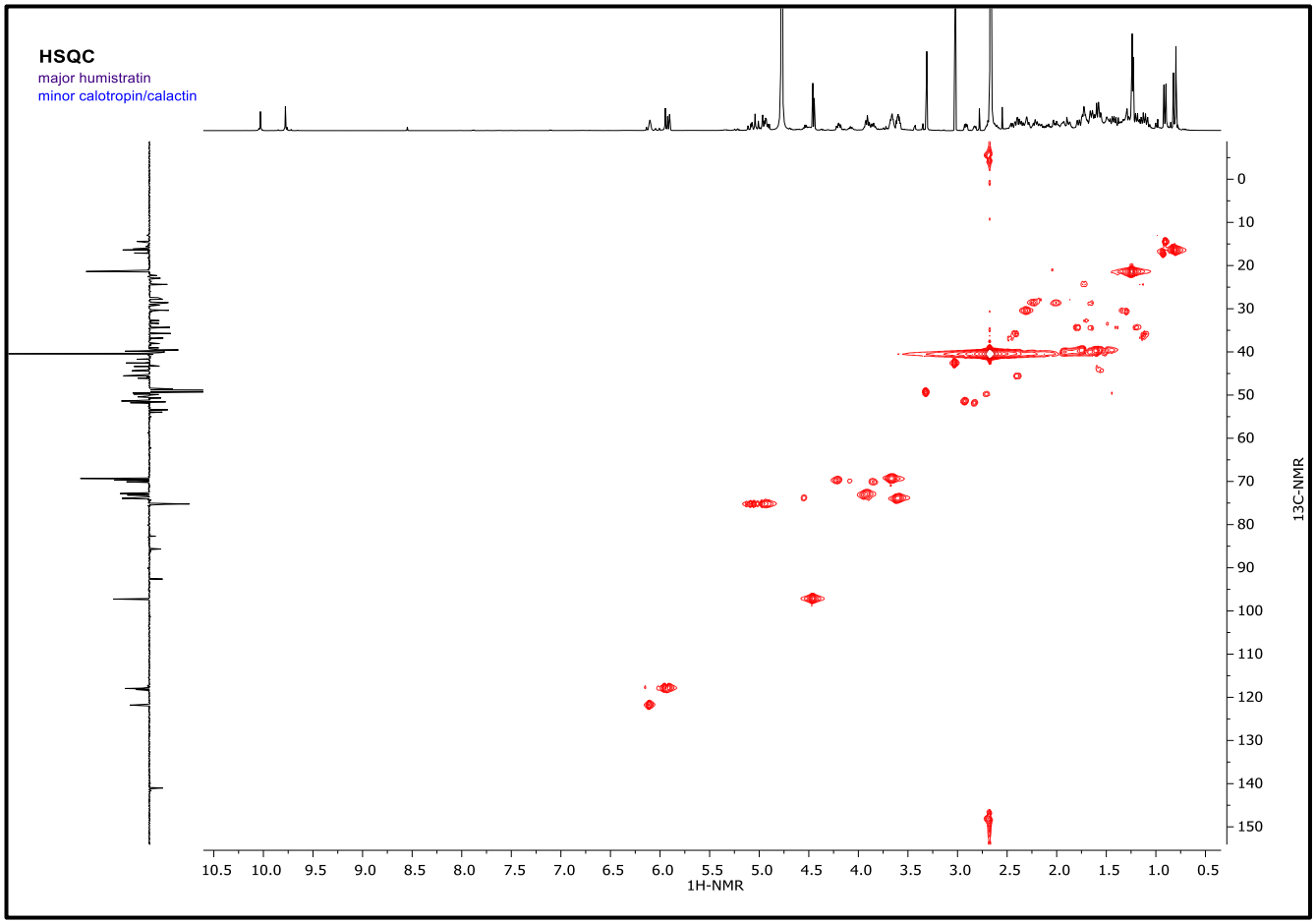


Figure 4. 16: HSQC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD

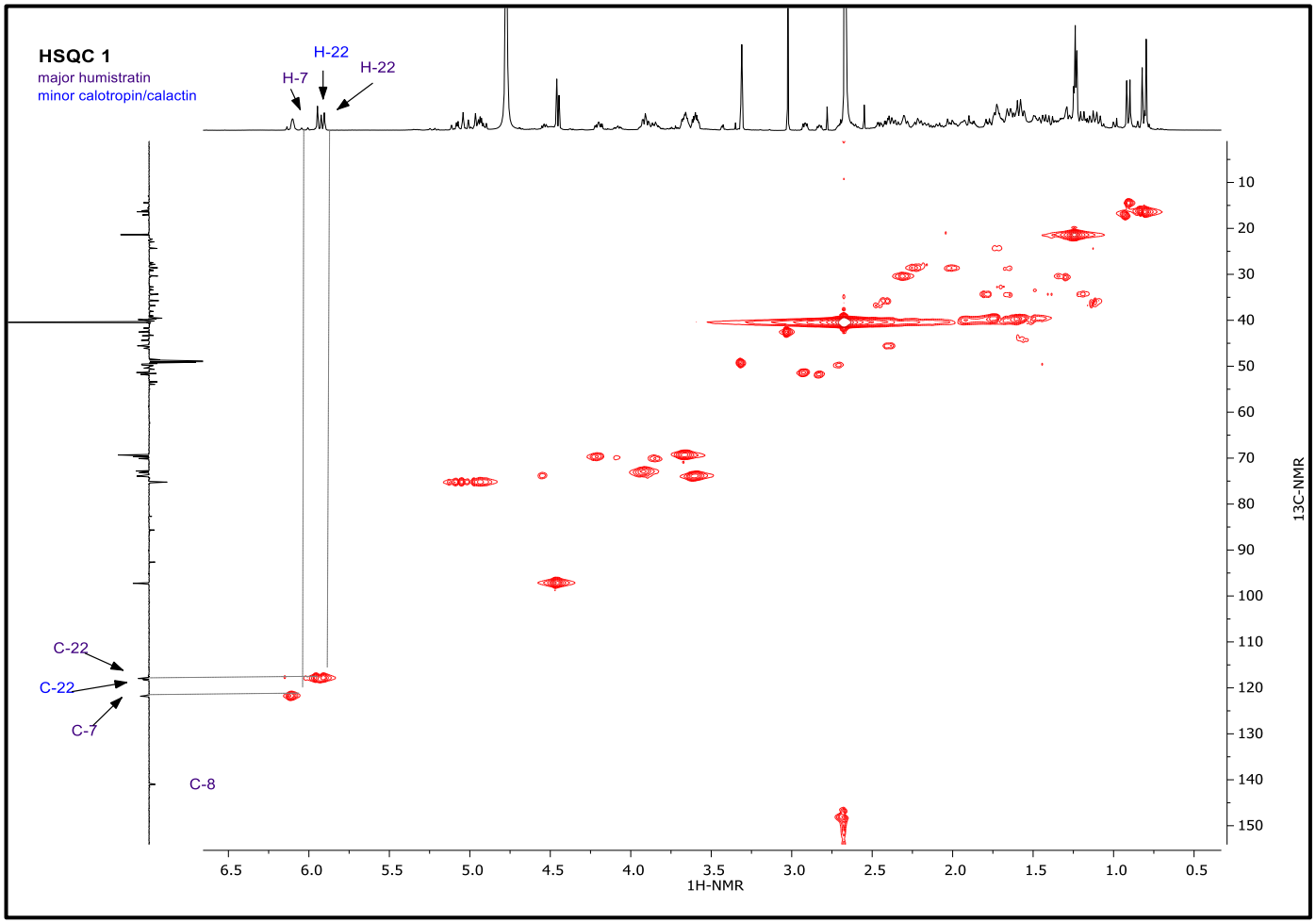


Figure 4. 17: HSQC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-1

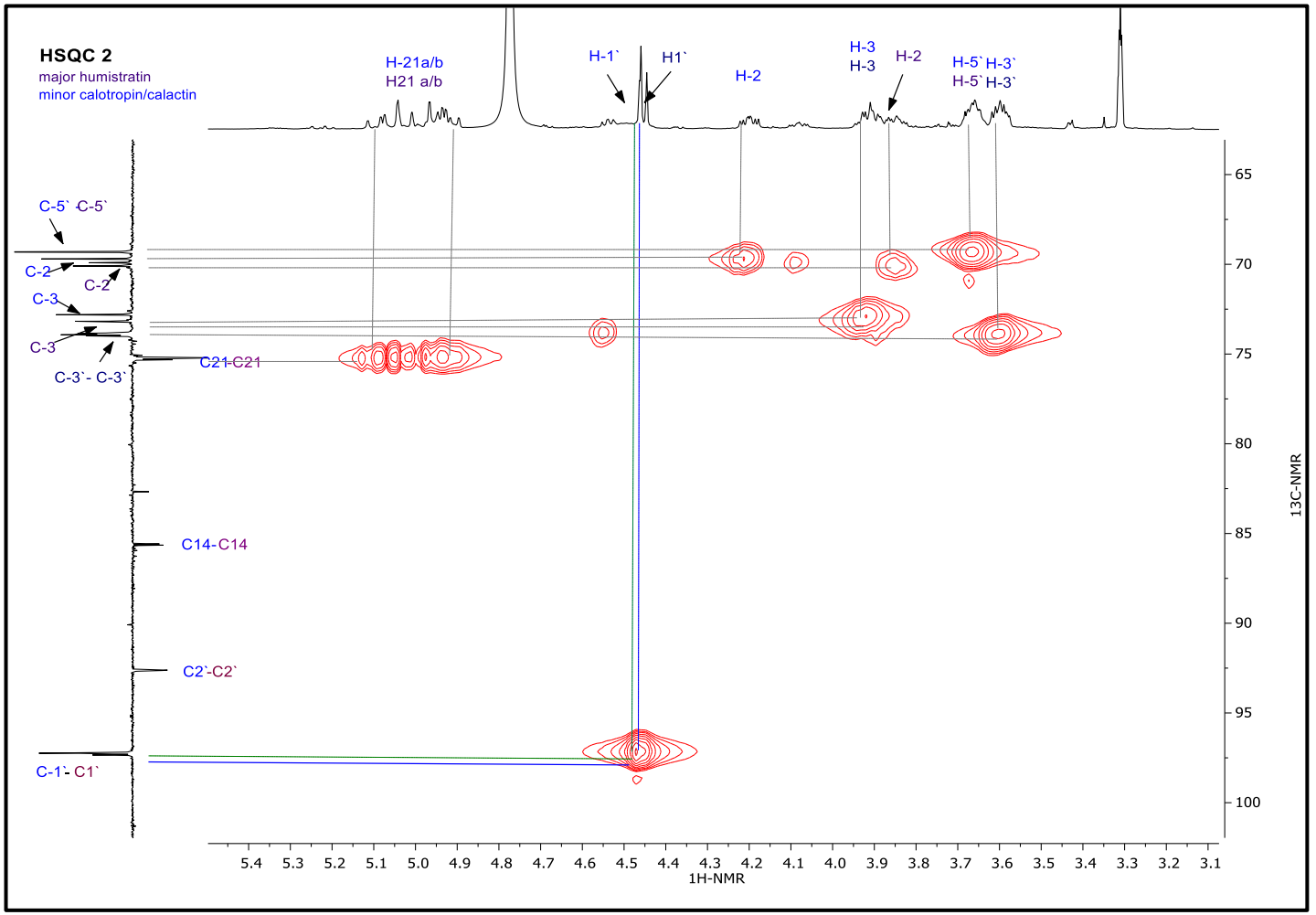


Figure 4. 18: HSQC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-2

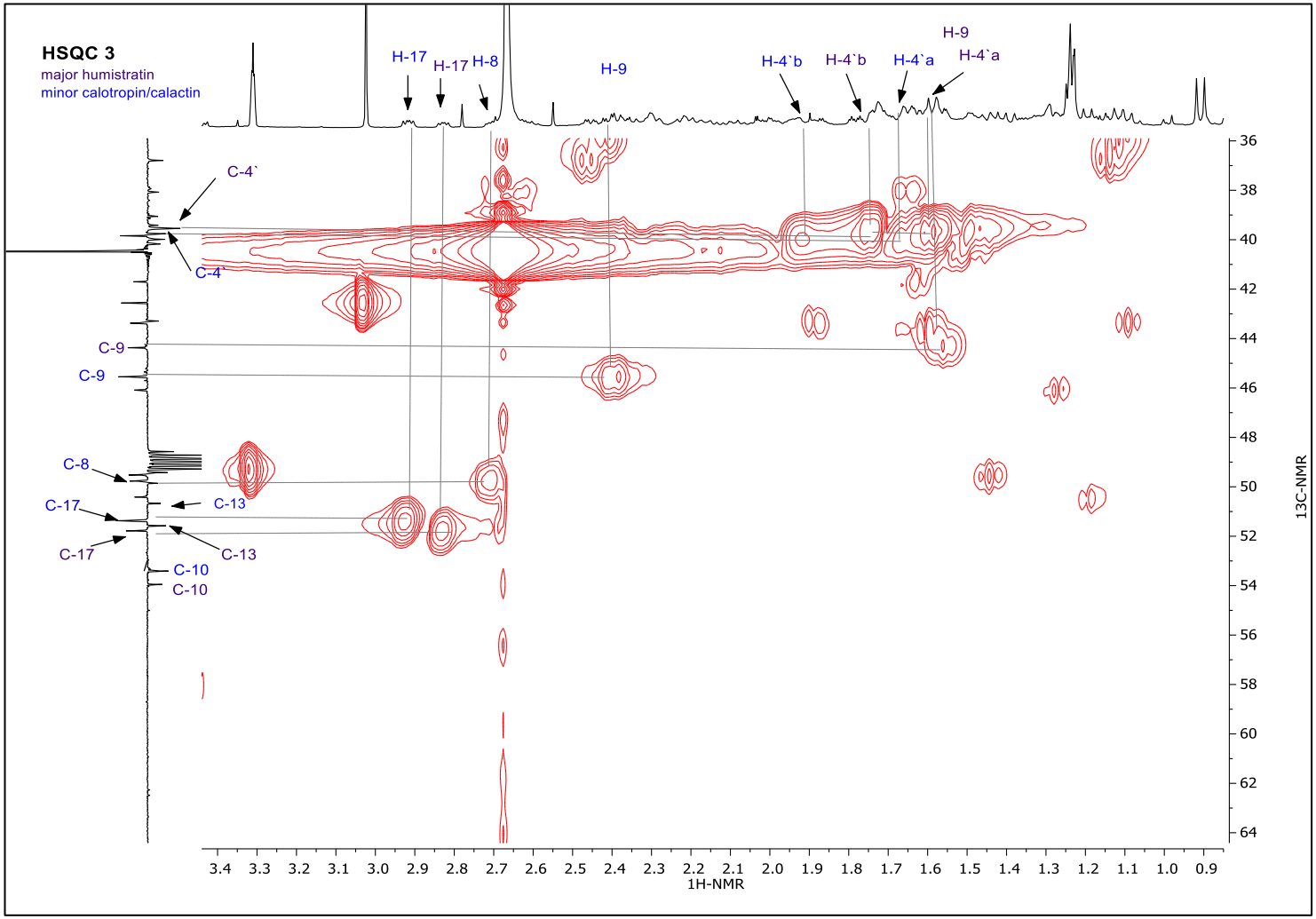


Figure 4. 19: HSQC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-3

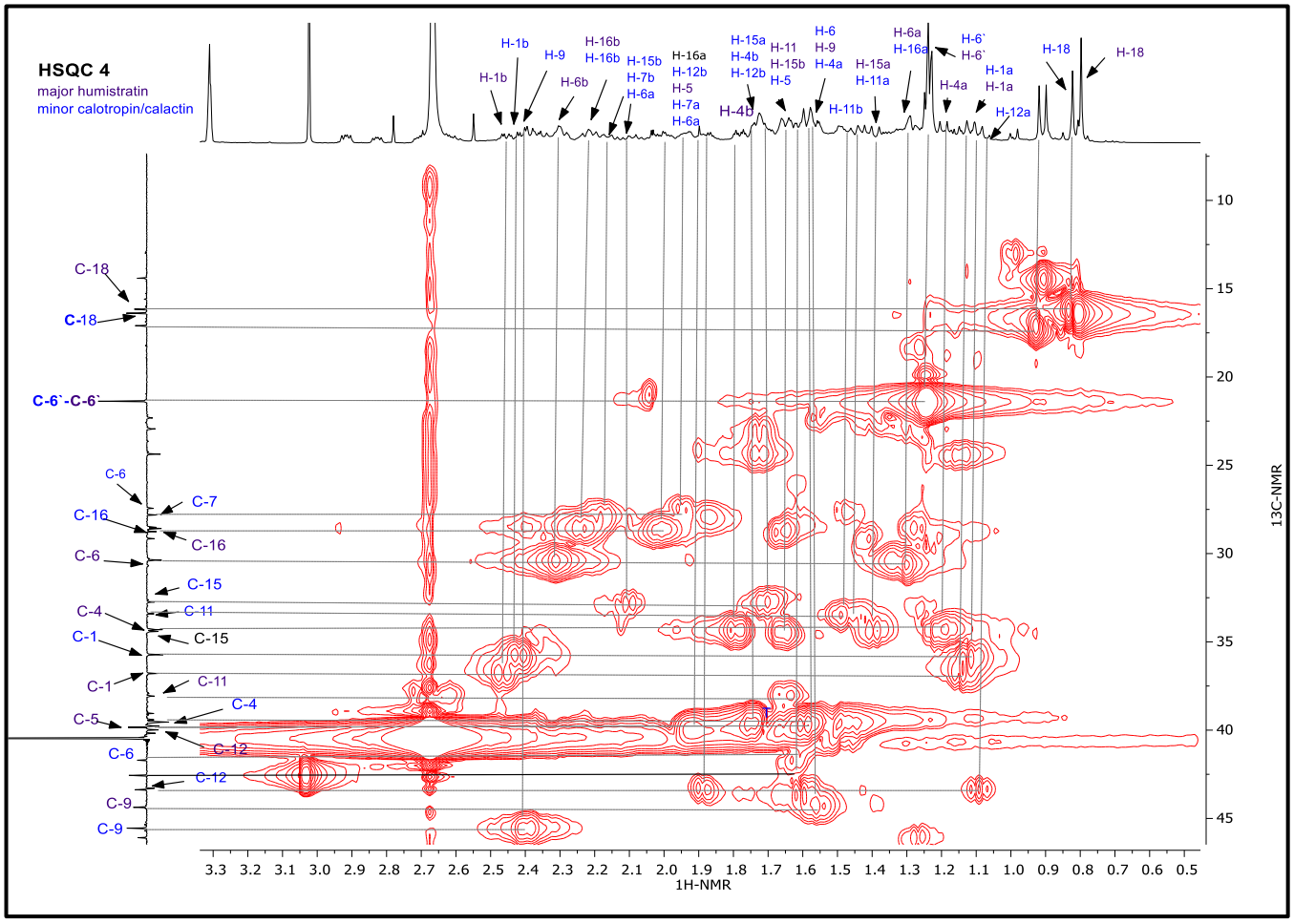


Figure 4. 20: HSQC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD- expansion-4

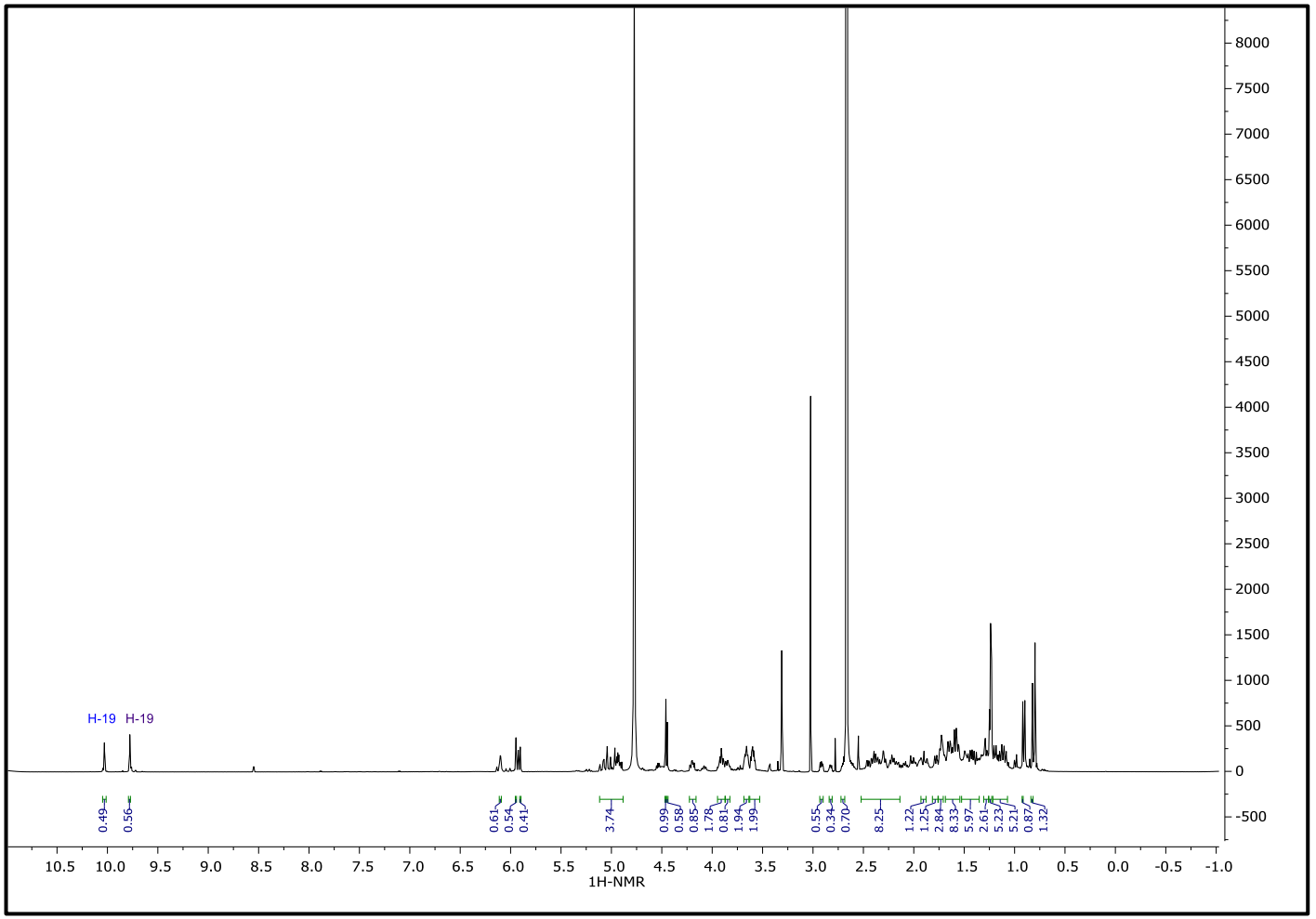


Figure 4. 21: ^{1}H-NMR of Gs 3.2.1 sub-fraction in CD_3OD

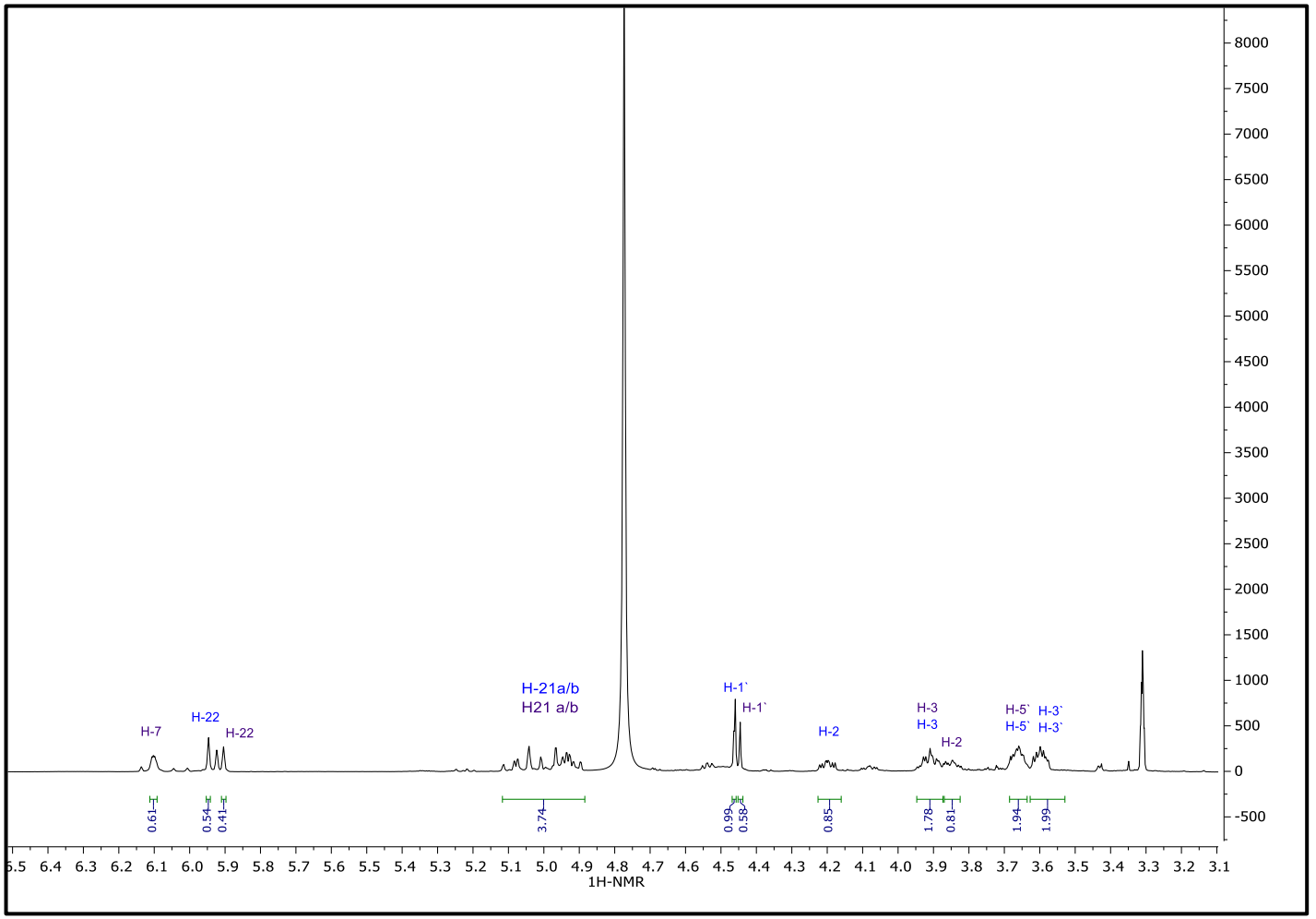


Figure 4. 22: ^{1}H-NMR of Gs 3.2.1 sub-fraction in CD_3OD-expansion-1

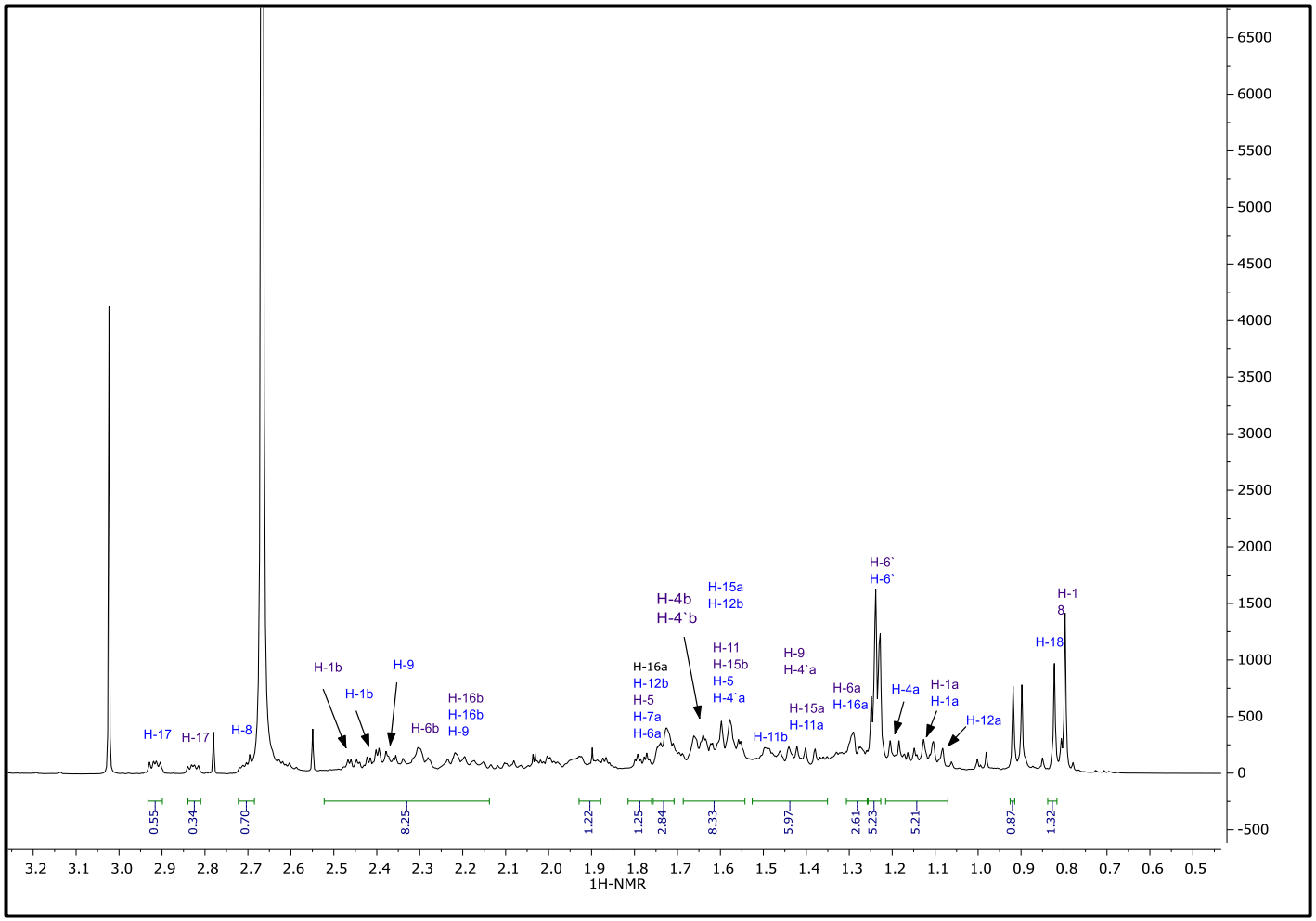


Figure 4. 23: ^{1}H-NMR of Gs 3.2.1 sub-fraction in CD_3OD-expansion-2

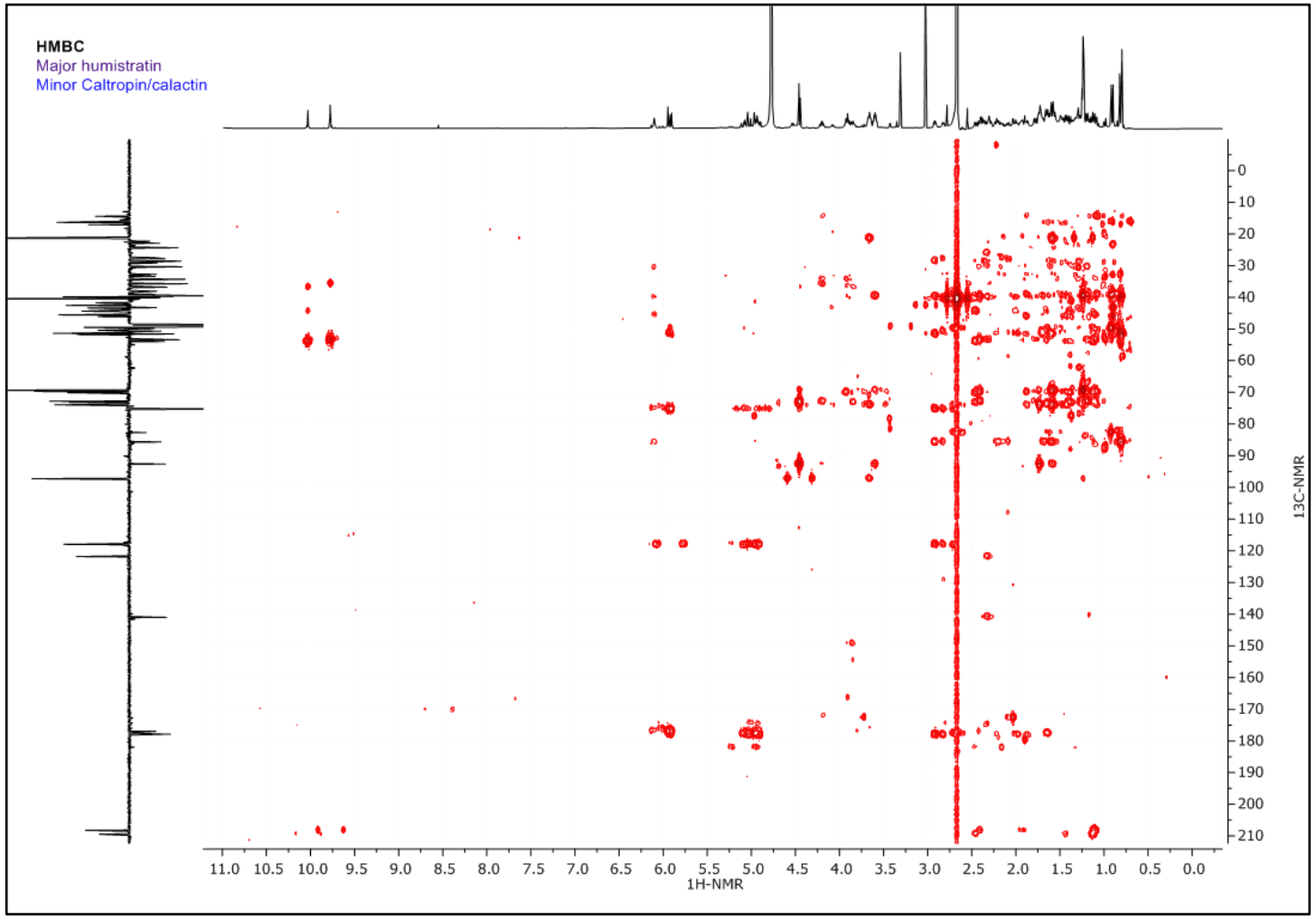


Figure 4. 24: HMBC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD

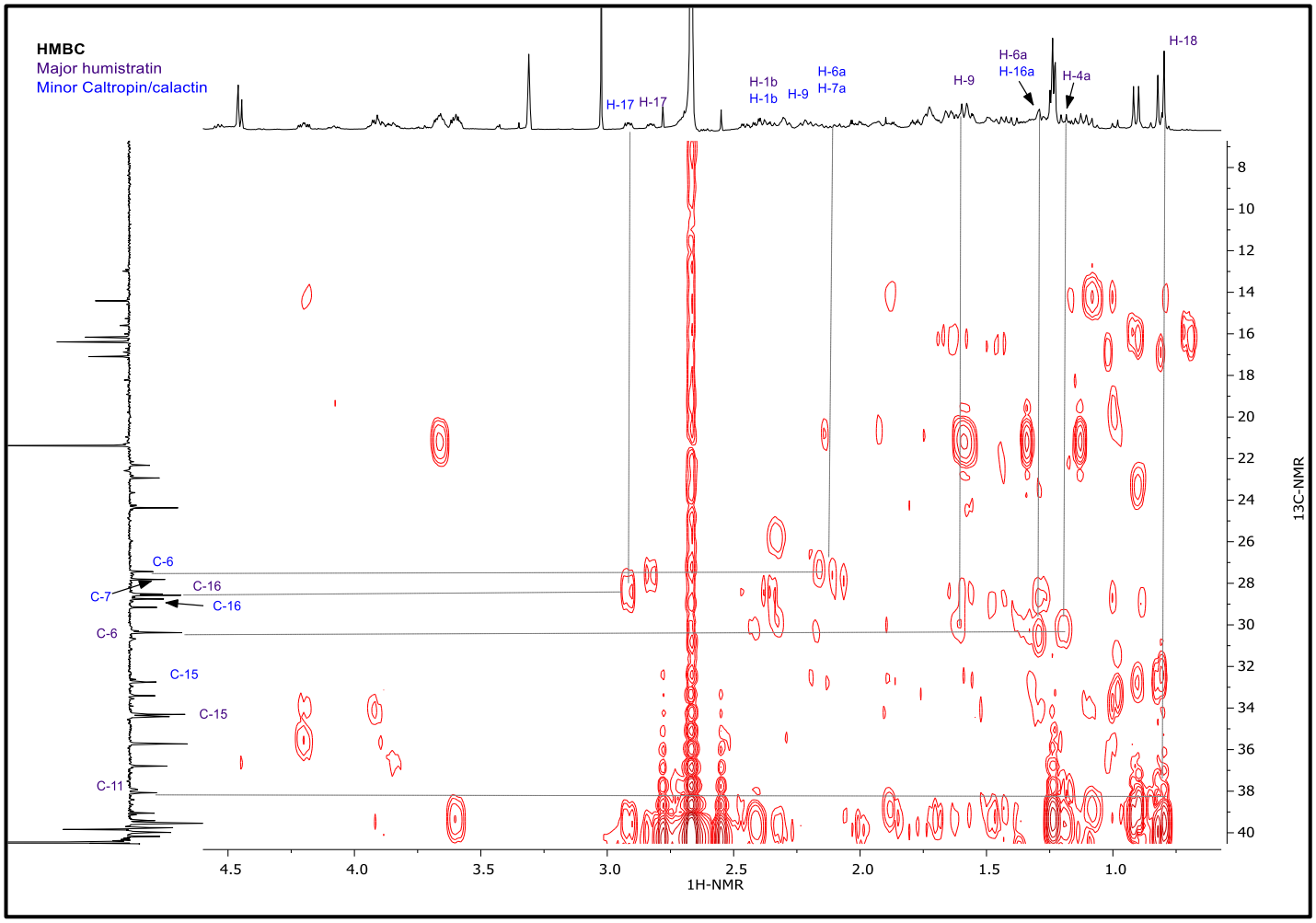


Figure 4. 25: HMBC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD – expansion- 1

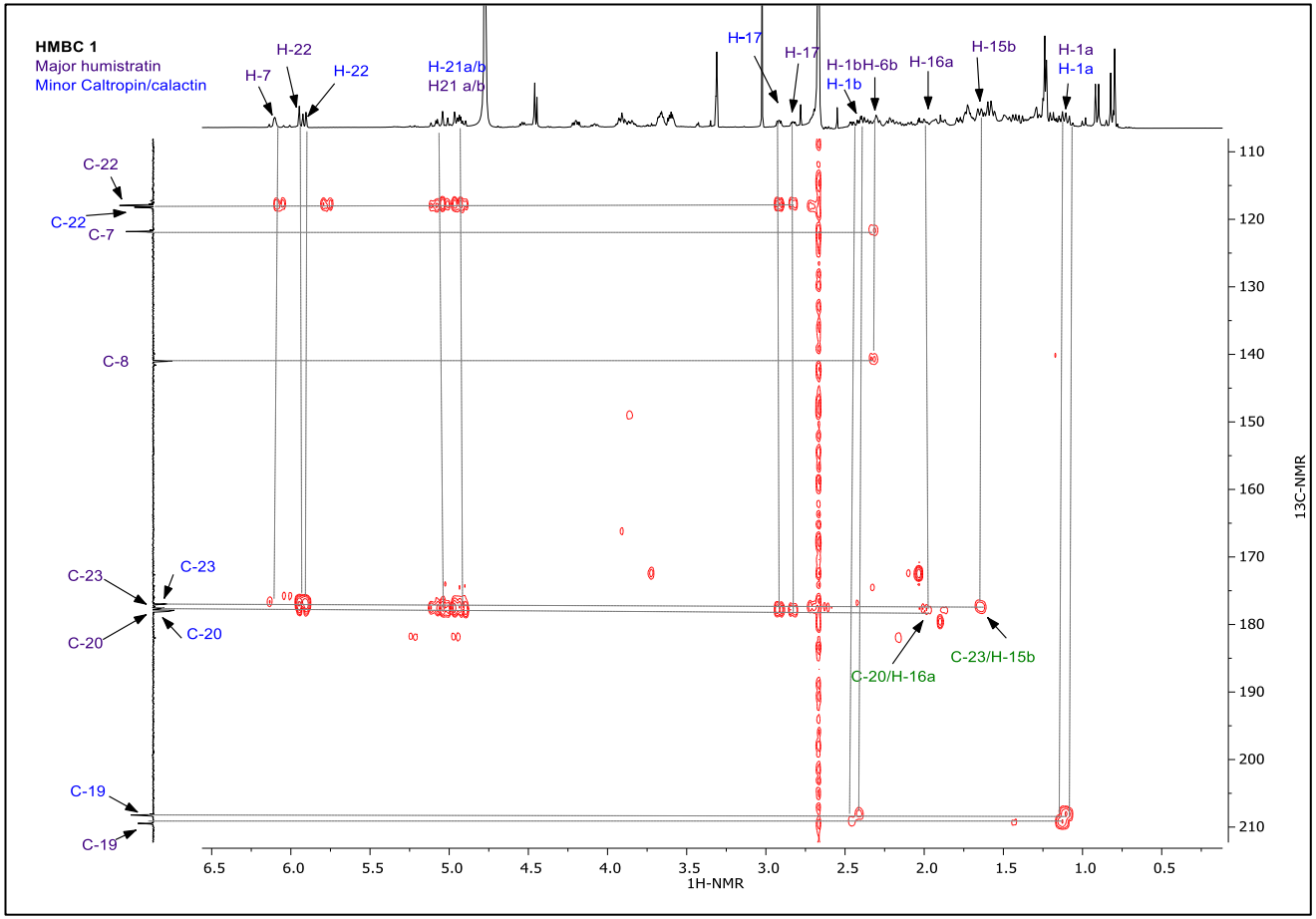


Figure 4. 26: HMBC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD – expansion- 2

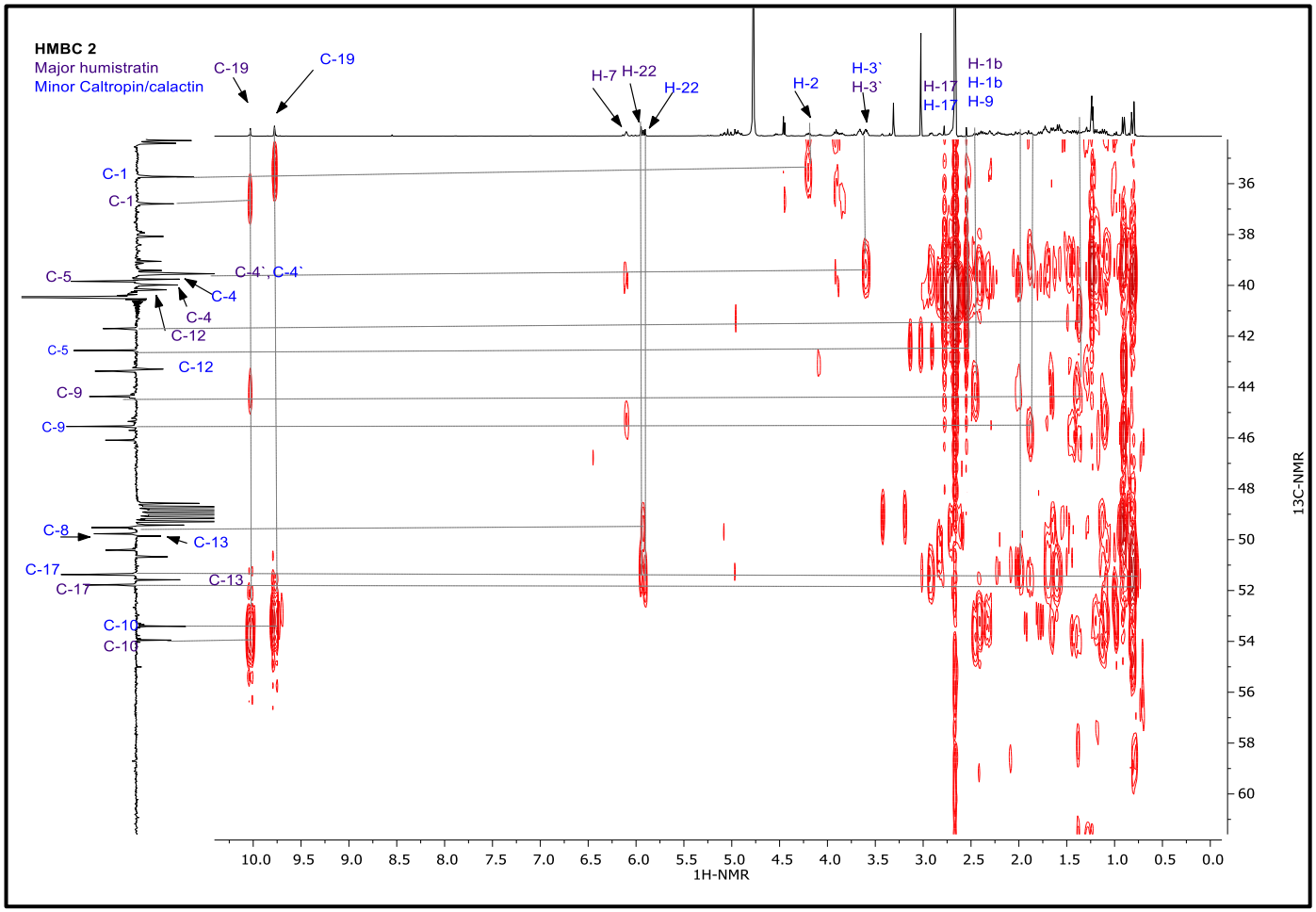


Figure 4. 27: HMBC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD – expansion- 3

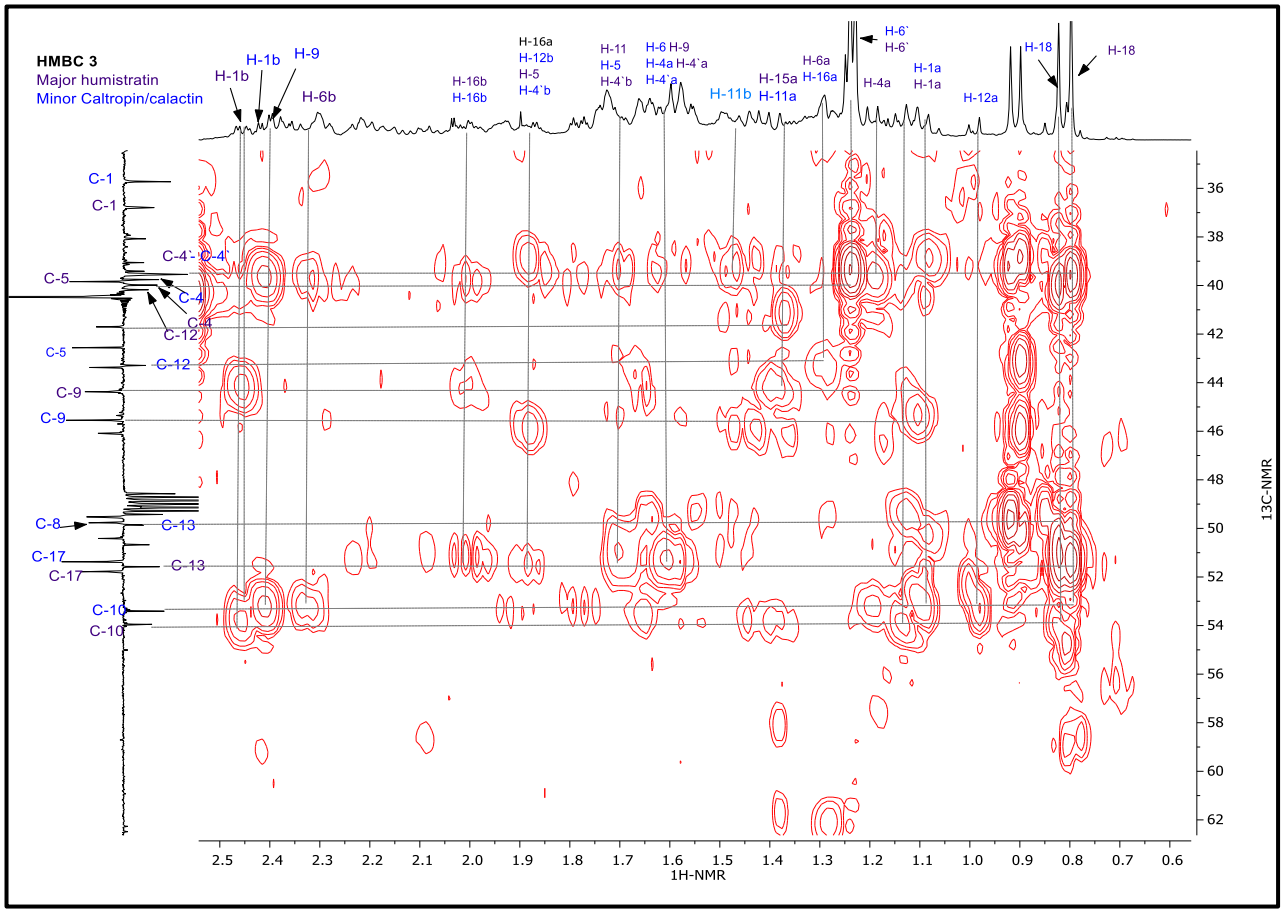


Figure 4. 28: HMBC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD – expansion- 4

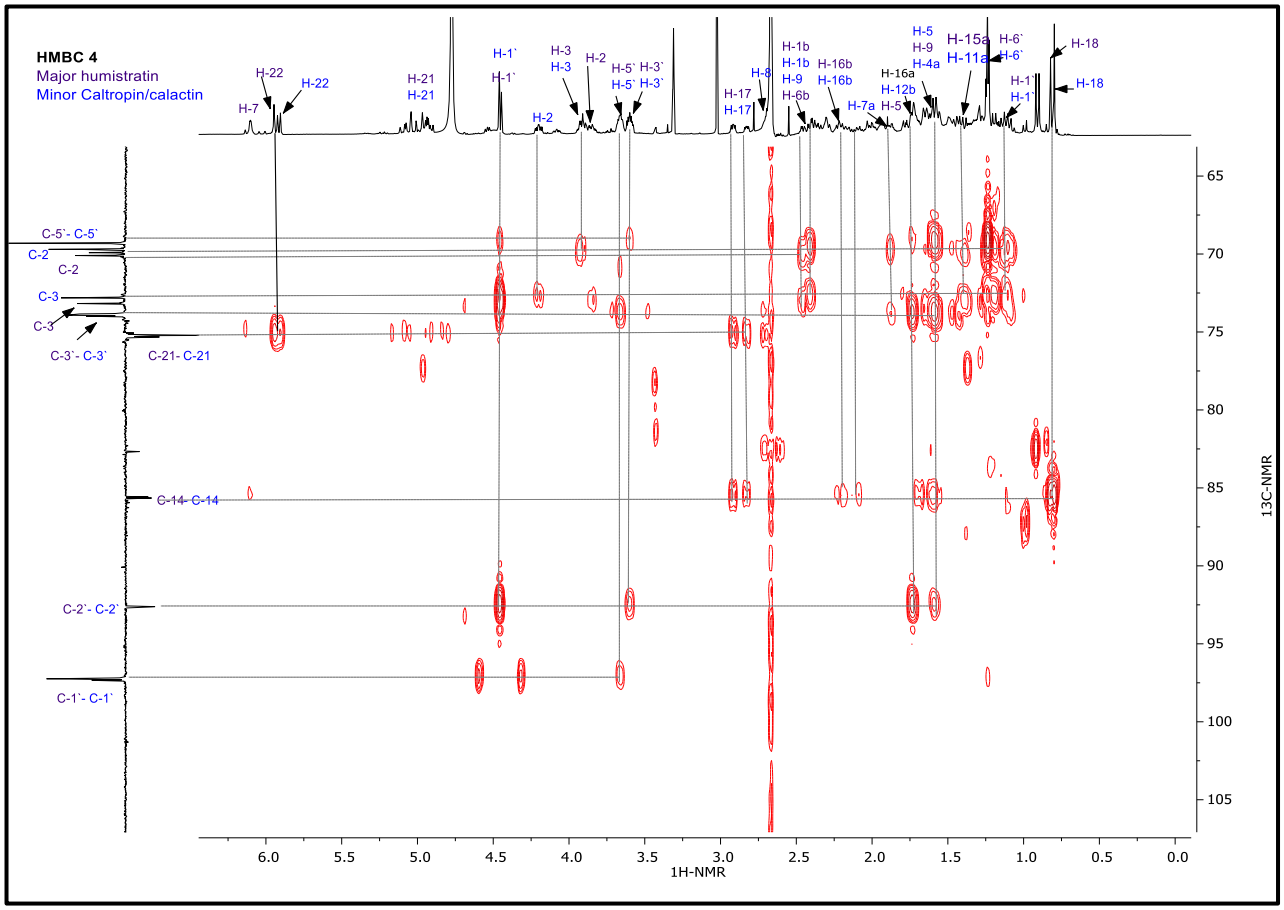


Figure 4. 29: HMBC spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD – expansion- 5

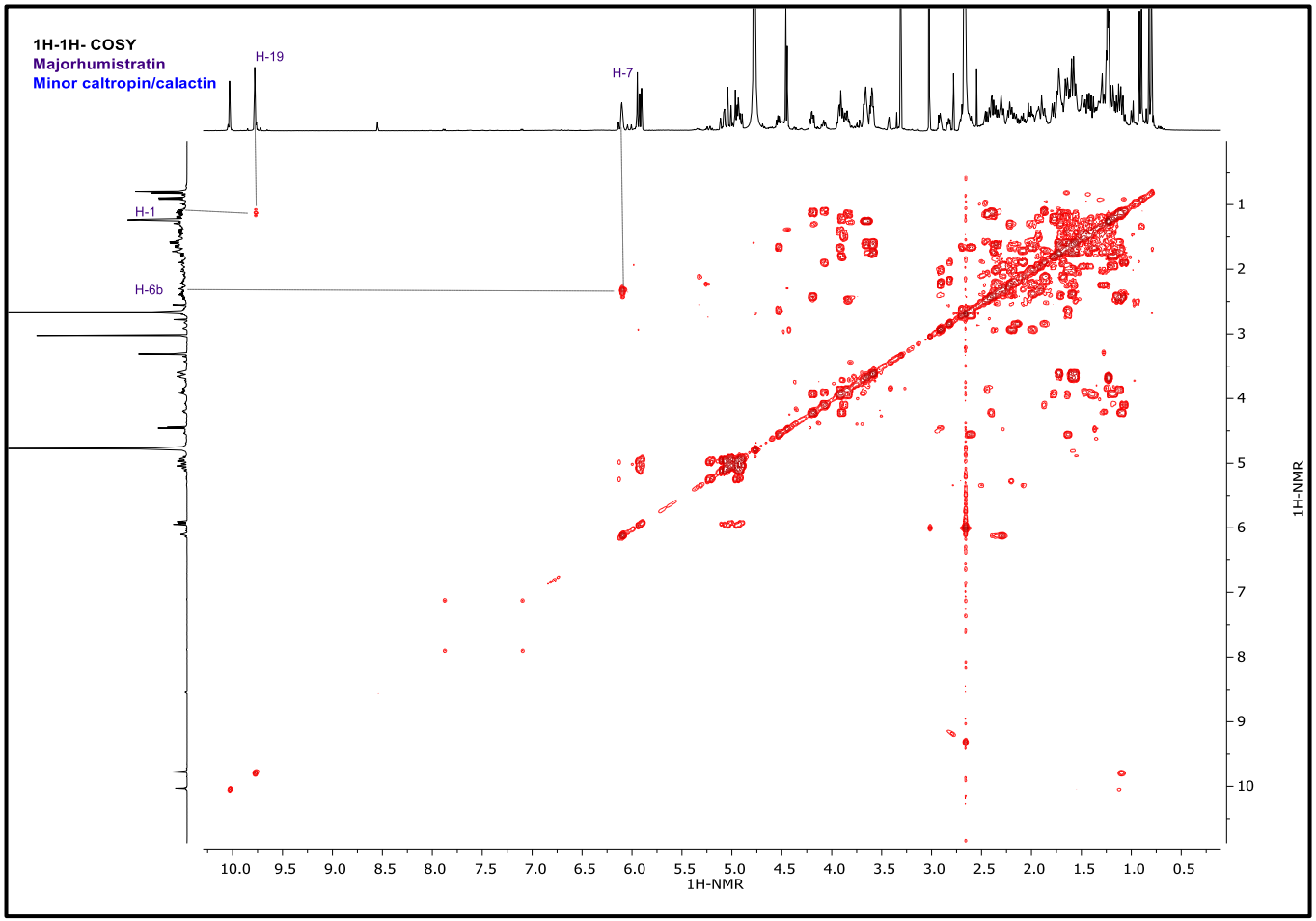


Figure 4. 30: ^{1}H-^{1}H COSY spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD

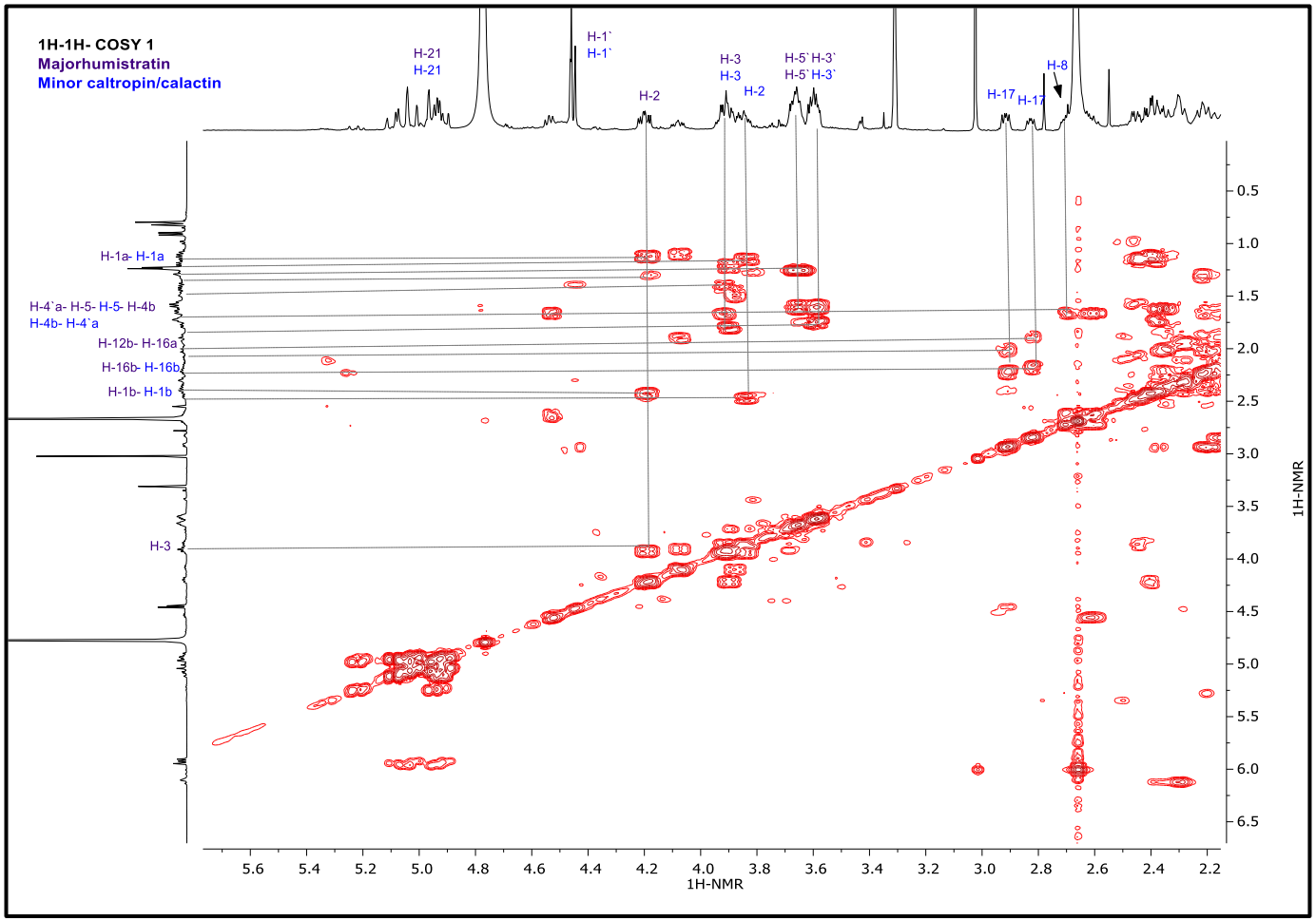


Figure 4. 31: ^{1}H-^{1}H COSY spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD-expansion-1

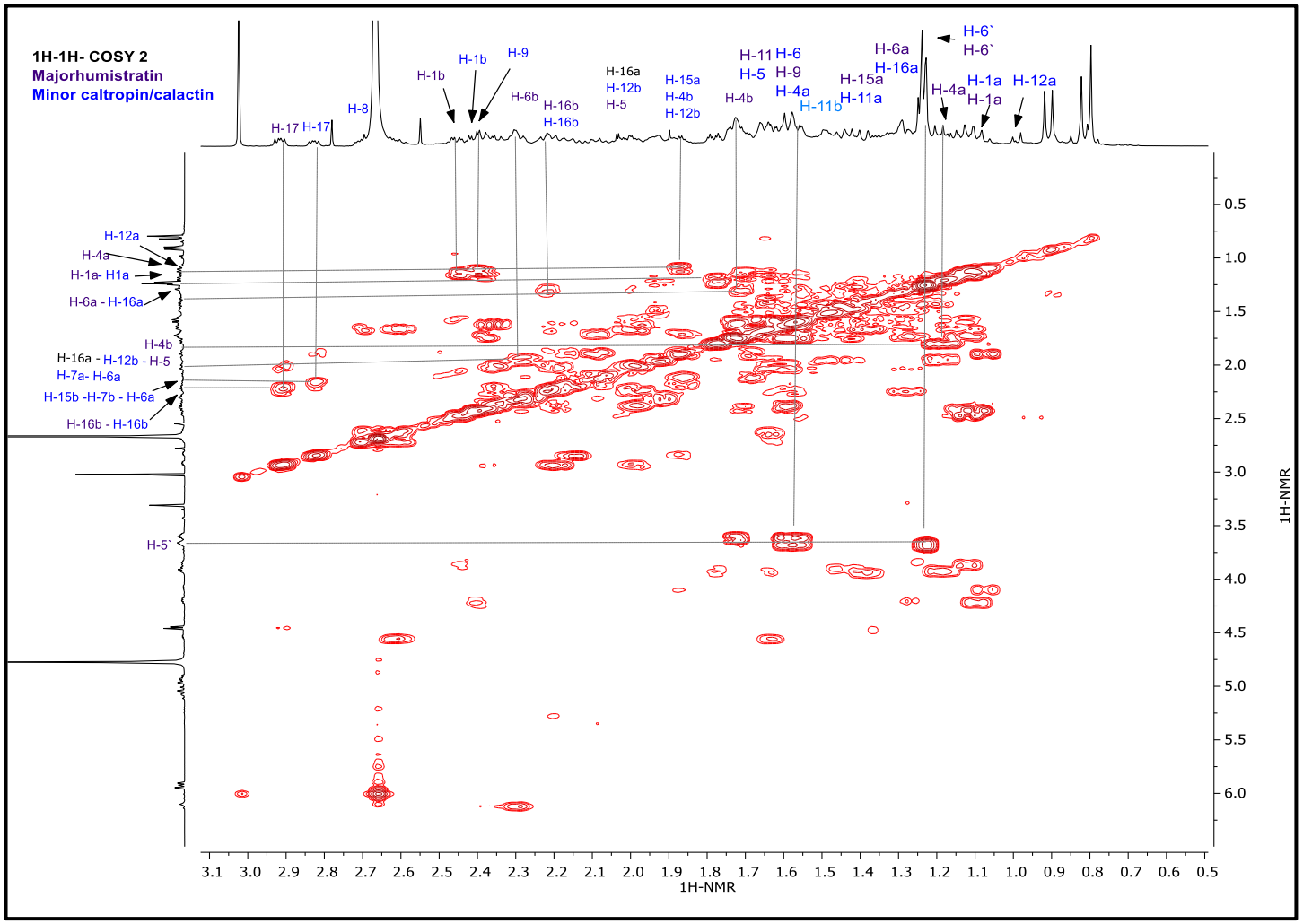


Figure 4. 32: ^{1}H-^{1}H COSY spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD-expansion-2

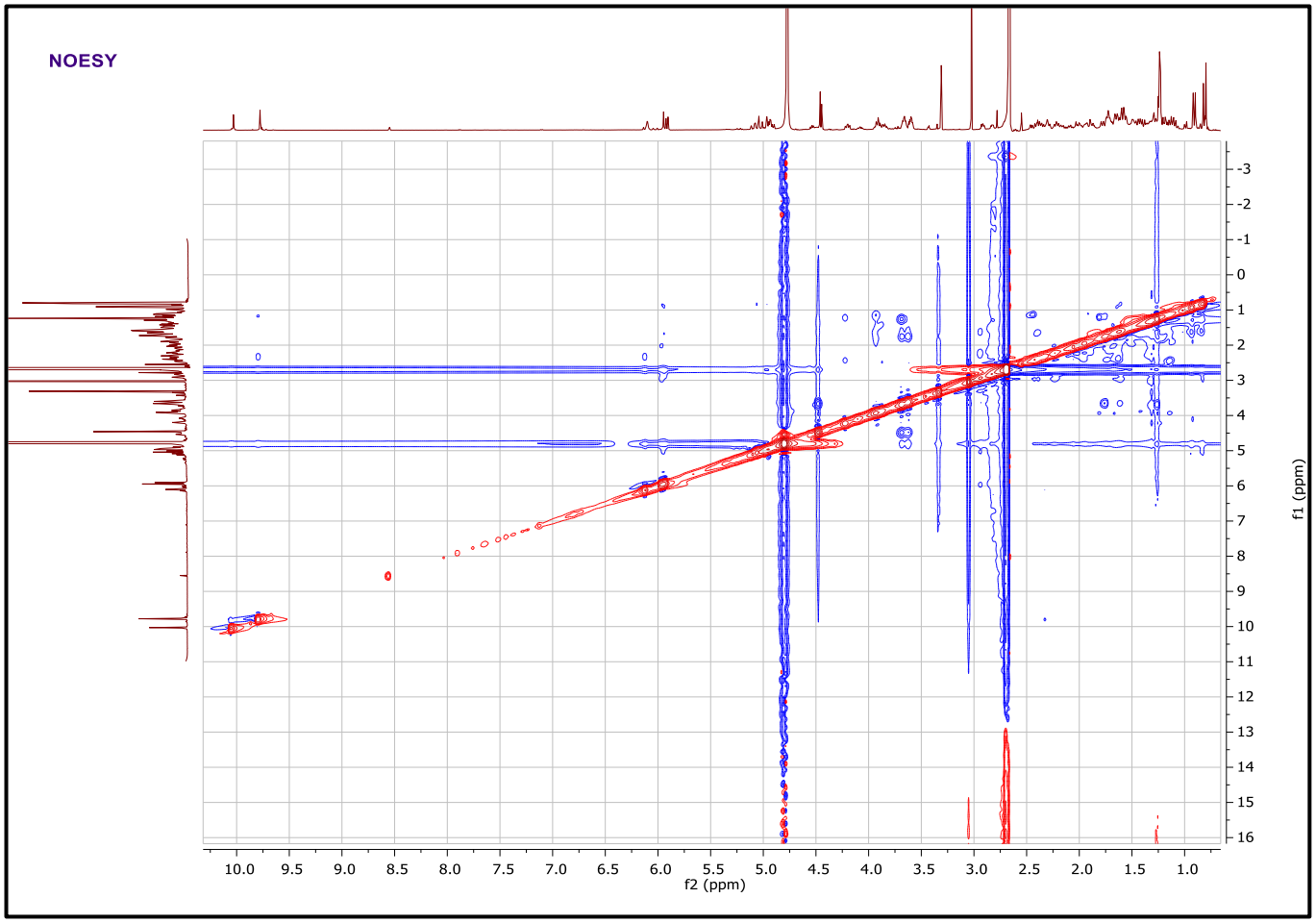
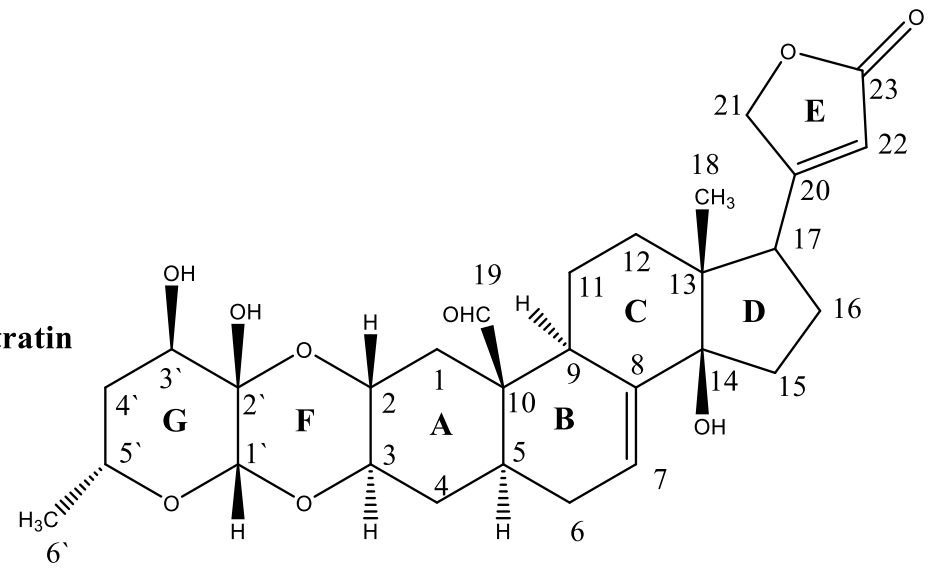
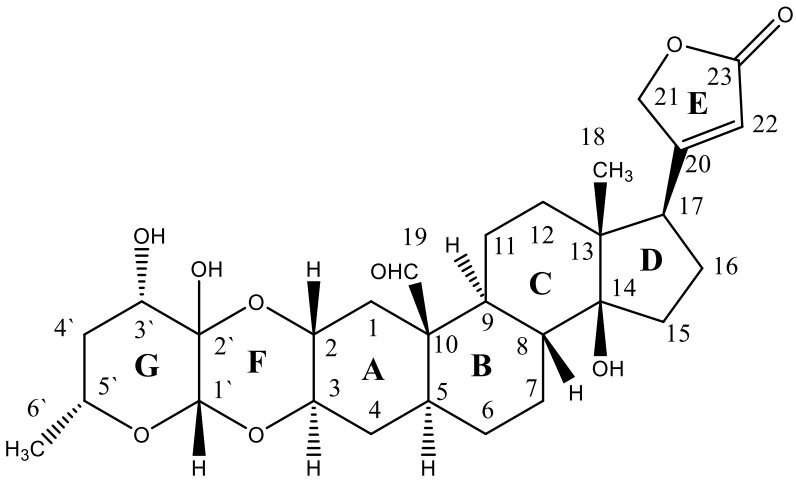


Figure 4. 33: NOESY spectroscopic analysis of Gs 3.2.1 sub-fraction in CD_3OD

(1) Humistratin



(2) Calotropin



(3) Calactin

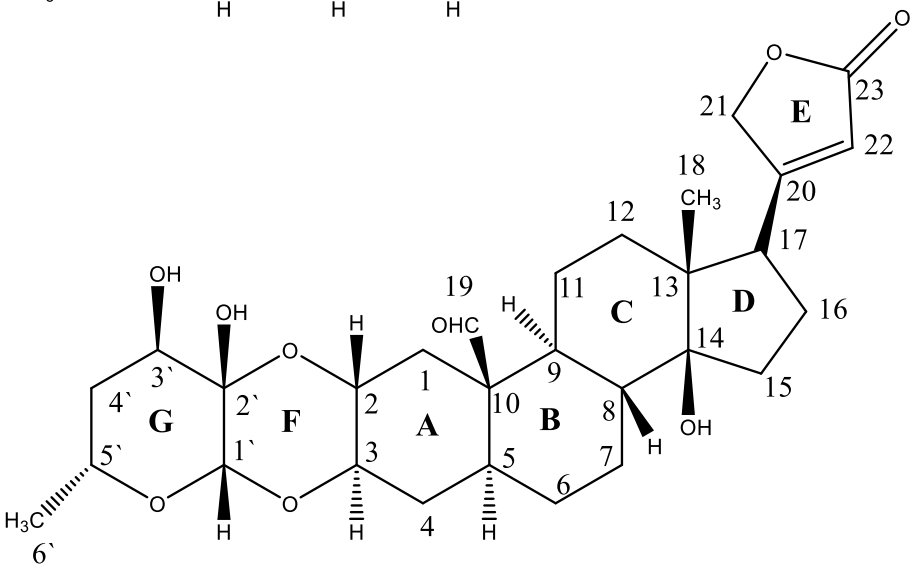


Figure 4. 34: Compounds isolated from Gs 3.2.1 sub-fraction

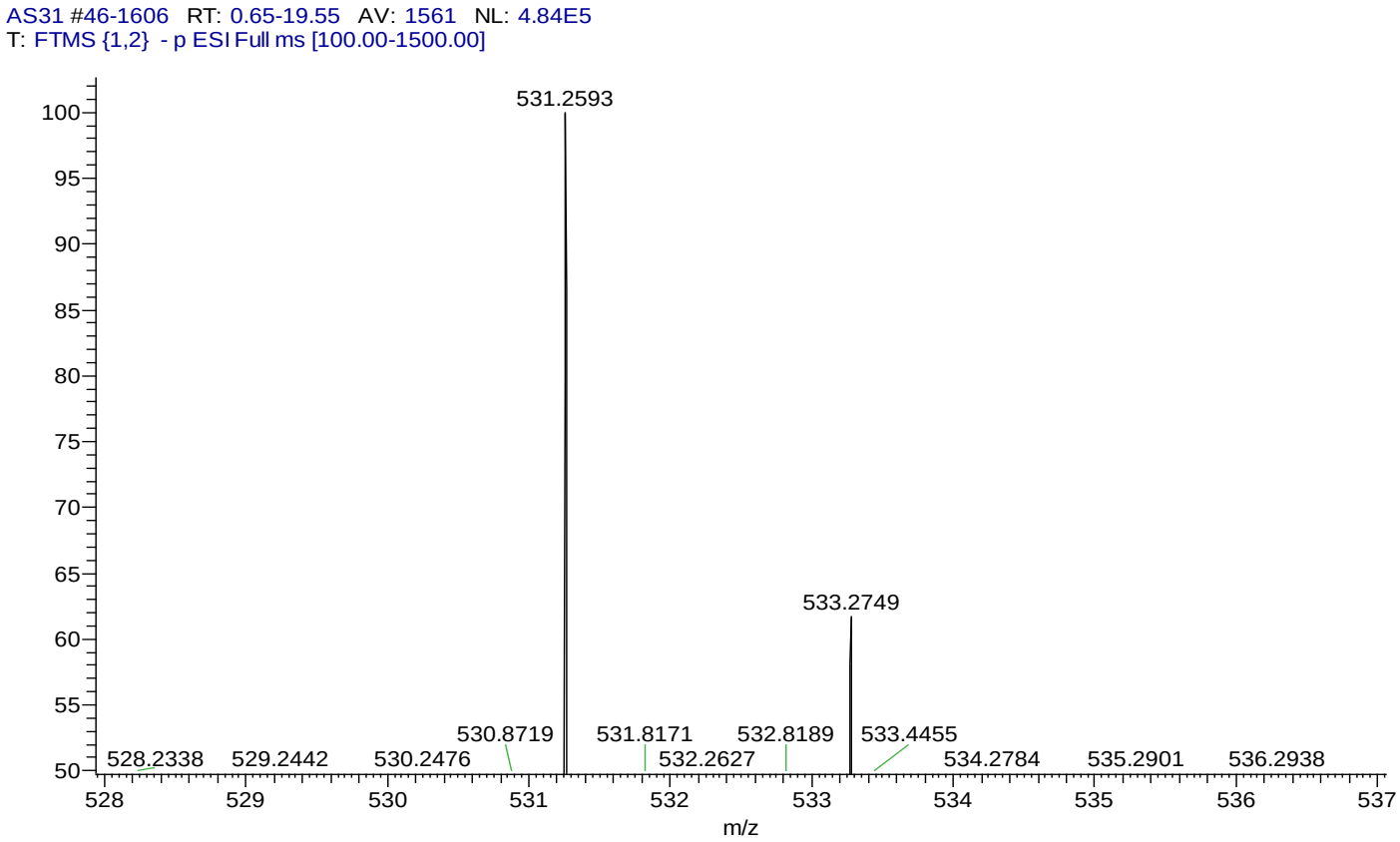


Figure 4. 35: LCHRMS of Gs 3.2.1 sub-fraction

4.3 Discussion

The main finding of this study is that *G. sinaicus* and its fractions exhibit anti-proliferative activity against MDA-MB-231 and MCF7-L breast cancer cells, suggesting that they contain compounds with anticancer activity. This agrees with previous studies of the same genus. The main compounds in Gs 3.2.1 are humistratin and calotropin, members of the cardenolide glycoside family. Indeed, previous studies have shown that cardenolide glycosides isolated from *Asclepias curassavica* exhibit strong cytotoxic activity against HepG2 and Raji cell lines (Li *et al.*, 2009a). In addition, previous studies have utilised bio-guided fractionation of *Asclepias subulata* Decne (Rascon-Valenzuela *et al.*, 2015) to measure the anti-proliferative activity on three human cancer cell lines (A549, LS180 and PC-3), one murine cancer cell line (RAW264.7) and one human normal cell line (ARPE-19). These studies demonstrated highly effective anti-proliferative activity of the four isolated cardenolide glycosides, namely 12,16-dihydroxicalotropin, calotropin, corotoxigenin-3-O-glucopyrano-side and desglucouzarin (Rascon-Valenzuela *et al.*, 2015).

1D and 2D spectroscopic analysis enabled the determination of the potential structures of the active components of Gs 3.2.1 sub-fraction. This analysis identified a mixture of four cardenolide glycosides. Two major compounds have been elucidated and are humistratin (compound **1**) and calotropin (compound **2**) or calactin (compound **3**). Calotropin and calactin have the same structure with only stereochemistry differences. Confirmation of their structures could be resolved using NOESY of the pure compound. The structures are of agreement with studies published by Abdel-Azim (Abdel-Azim *et al.*, 1996).

The present study showed that the active mixture of Gs 3.2.1 sub-fraction, which has been identified as containing cardenolide glycosides, reduced DNA synthesis in MCF7-L and MDA-MB-231 breast cancer cell lines (Figure 4.9 A &B and Figure 4.10 A & B). The Gs 3.2 sub-fraction (from which Gs 3.2.1 was derived) induced apoptosis as indicated by its ability to promote PARP cleavage (Figure 4.7 C and Figure 4.8 C). Many previous studies have isolated cardenolide glycosides either from Asclepiadaceae or other different plant families and have reported that cardenolide glycosides possess strong cytotoxic activity against different cancer cell types. For

example, toxicarioside E, F, G, and H are cytotoxic cardenolide glycosides with activity against SGC-7901 and SMMC-7721 cancer cell lines and which were isolated from the latex of *Antiaris toxicaria* (Dong *et al.*, 2011). In addition, the dichloromethane extract of *Calotropis gigantea* Linn. leaves showed significant cytotoxic activity against many cancer cell types including non-small cell lung carcinoma (A549), colon carcinoma (HCT 116) and hepatocellular carcinoma (Hep G2). Further purification of this extract enabled identification of six cardenolide glycosides. Uscharin was the most active compound against all cancer cell types (Jacinto *et al.*, 2011). Furthermore, three cardenolide glycosides isolated from *Calotropis gigantea* Linn bark also exhibited high inhibitory effect against A549 and HeLa cell lines (Van Khang *et al.*, 2014). In addition, five cardenolide glycosides were isolated from the roots of *Pergularia tomentosa*, which belong to family Asclepiadaceae. These compounds induced a dose-dependent reduction in cell growth of Kaposi's sarcoma (KS) cells (Hamed *et al.*, 2006). Therefore, many isolated cardenolide glycosides demonstrate anticancer properties, and this includes digoxin, which has anticancer activity in human breast cancer cell lines, MCF7 and MDA-MB-231 cells (Winnicka *et al.*, 2008) and increased apoptosis of a prostate cancer cell line *in vitro*. It is therefore significant that digoxin has been reported in clinical studies to be therapeutically effective against breast and prostate cancers (Platz *et al.*, 2011).

SK1 inhibitors have been reported to induce proteasomal degradation of SK1 in MCF7 cells and LNCAP-AI cells (Lim *et al.*, 2011b, McNaughton *et al.*, 2016). In this study, it has been observed that the cardenolide glycoside mixture of Gs 3.2.1 reduced the expression of SK1, which was sensitive the proteasome inhibitor MG132. This indicates that cardenolide glycosides can induce the ubiquitin-proteasomal degradation of SK1. There are two mechanisms by which compounds induce the ubiquitin-proteasomal degradation of SK1. First, direct inhibitors bind to SK1 and induce a conformational change that enables ubiquitination by Kelch-Cul3 E3 ligase (Pyne *et al.*, 2016a, Powell *et al.*, 2019). Second, compounds can induce a direct activation of the proteasome and increased turnover of SK1. This is probably related to ER stress and ER associated proteasomal degradation. Further work is required to determine whether the effects of cardenolide glycosides on the ubiquitin-proteasomal degradation of SK1 is *via* a direct or indirect effect.

The cardenolide glycoside mixture of Gs 3.2.1 also reduced Des1 expression in both MDA-MB-231 and MCF-7 cells via a mechanism that was independent of the ubiquitin-proteasomal degradation pathway (Figure 4.9 C and Figure 4.10 C). Indeed, in MDA-MB-231 and MCF7-L cells, SKi also reduced Des1 expression via a mechanism insensitive to MG132. The effect on Des1 by the Gs 3.2.1 mixture is significant as others have shown that inhibition of Des1 induces anticancer activity (Aurelio *et al.*, 2016). It remains to be determined whether the loss of Des1 is due to enhanced lysosomal protease action or altered gene transcriptional activity or post-translational mRNA stability changes.

Calotropin together with other isolated cardenolide glycosides from *A. subulata*, 12,16-dihydroxycalotropin, corotoxigenin, 3-O-glucopyranoside and desglucouzarin, have been shown to highly induce anti-proliferative activity against lung adenocarcinoma cell line (A549), prostate cancer cell line (PC-3) and colon cancer cell line (LS180). The mechanism of cell death induced by cardenolides has been elucidated by Rascón-Valenzuela (Rascon-Valenzuela *et al.*, 2016). These workers reported that the compounds induce apoptosis via the extrinsic pathway (activation of caspase- 8). However, others have shown that calotropin activates other signalling pathways involving casein kinase 1 α in colon cancer cells and this involves inhibition of Wnt signalling (Park *et al.*, 2014a). Calotropin also induces cell cycle arrest by upregulating the expression of p27 and downregulating the G2/M proteins and cyclins A and B which leads to the inhibition of human chronic myeloid leukaemia K562 cell growth. In addition, calotropin induces downregulation of anti-apoptotic signalling and survival pathways, leading to caspase-3 activation and the induction of apoptosis (Wang *et al.*, 2009). Calotropin also induces cell cycle arrest by decreasing the expression levels of CDK1 and CDK2 and promoting apoptosis by regulating the cytotoxic T lymphocyte-associated antigen – mediated TGFβ/ERK signalling pathway in lung cancer cells (Tian *et al.*, 2018). These findings are consistent with the findings of the current study showing that calotropin inhibits DNA synthesis and promotes apoptosis of breast cancer cells.

YAP (Yes-associated protein) has become a promising therapeutic target, as it has an important role in cell proliferation and survival. Indeed, de-regulation of YAP is correlated with many cancer types, such as breast and colon cancer. Recently,

calotropin has been shown to inhibit proliferation of colorectal cancer cells *in vivo* and *in vitro*, and activation of YAP by inducing down-regulation of LATS1, which subsequently blocks the Hippo pathway, and which is considered a key player in tumour biology (Zhou *et al.*, 2019). It might also be significant that S1P can activate YAP (Cheng *et al.*, 2018, Pyne and Pyne, 2020) and thus proteasomal degradation of SK1 by calotropin might impact YAP signalling *via* this mechanism.

Additional evidence supporting anticancer activity by cardenolide glycosides was demonstrated with convallatoxin and peruvoside, which induced cytotoxic effect on MCF7 cells with a loss of colony formation and cell cycle arrest at G0/G1. Also, both compounds demonstrated concentration- and time-dependent differential effects in MDA-MB-468 on MDA-MB-231 cell lines. Both convallotoxin and peruvoside induce downregulation of p-Akt in both cell lines. However, they exert a differential effect on p-ERK, with activation in MCF7 cells and inhibition in MDA-MB-468 cells. Finally, both compounds enhance PARP cleavage and increase the expression of pro-apoptotic protein phospho-p53, with a decrease in Bcl2 and XIAP (cell survival proteins) (Kaushik *et al.*, 2017).

An important finding of this study is that Gs 3.2.1 and Gs 3.2.2 have potential of anti-inflammatory activity. This is supported by the finding that pre-treatment of keratinocyte reporter cells with Gs 3.2.1 reduced TNFα-stimulated NFκB transcriptional activity (Figure 4.11). This finding is supported by the study of Sreenivasan *et al.* (2003), and which is supported by a previous study where oleandrin was shown to inhibit NFκB and AP-1, which stimulated with different anti-inflammatory stimuli, as well as its inhibitory effect on JNK and MEK activation (Manna *et al.*, 2000). Ouabain and digitoxin, another two cardenolide glycosides, were reported to block activation of TNF α/ NFκB pathways (Manna *et al.*, 2000). Treatment of A549 NSCLC tumour cells with UNBS1450, a hemi-synthesised cardenolide for several hours deactivated NFκB activity, via degradation of IκBα (Mijatovic *et al.*, 2006). activity similar effect was reported for human leukaemia cells (Juncker *et al.*, 2011). The mechanism of oleandrin and digitoxin-induced blocking of the TNFα/ NFκB pathway was investigated in HEK239 cells. Pre-treatment of cells transfected with Flag-tagged TNFR1 and HA-tagged TRADD with oleandrin and

digitoxin blocked the formation of Flag-TNFR1/HA-TRADD complexes whereas there was no effect of these two cardiac glycosides on the TNFα /TNFR1 interaction (Yang *et al.*, 2005). Thus, both oleandrin and digitoxin have an anti-inflammatory effect on NFκB and JNK pathways by blocking the initial upstream between TNFα-stimulated TNFR1 and TRADD.

In conclusion, *G. sinaicus* extracted cardenolides are highly active with anticancer and anti-inflammatory activities. The current study adds new findings about the potential activity of humistratin as an anti-inflammatory and anticancer agent as well as its effect against SK1 and Des1 expression proteins for the all the identified cardenolide glycosides. However, further purification of other active compounds is required.

CHAPTER 5:
GENERAL DISCUSSION

Chapter 5: General discussion

This current study involved screening and compound identification using plants sourced from Egypt during field work. First, screening plant species was undertaken to identify extracts with anticancer activity against breast cancer cell lines. Second, a fractionation, compound isolation and identification strategy were undertaken to identify potential anticancer/anti-inflammatory compounds. This was guided by various bioassays of marker proteins, such as SK1/Des1. In recent years, sphingolipid metabolism has been extensively studied and shown to be de-regulated in many diseases, including cancer (Hannun and Obeid, 2008b, Pyne and Pyne, 2010). Des1, SK1 and SK2 are three vital enzymes involved in regulating the levels of bioactive signalling sphingolipids, including ceramide and S1P that have been implicated in disease pathogenesis. For instance, SK1 is overexpressed in cancers including breast, ovary, kidney and lung cancer as well as haematological cancers such as leukaemia (Pyne and Pyne, 2010). The role of Des1 is somewhat more controversial because it has been implicated in regulating autophagy and it is unclear what determinants govern autophagic-induced apoptosis or cell survival (Siddique *et al.*, 2015). Nevertheless, both SK1 and Des1 are therapeutic targets for intervention in disease. Toward this end, potential anticancer/anti-inflammatory agents that modulate Des1/SK1 expression were isolated from the plant extracts.

5.1 Anticancer activity

In chapter 3, the main finding is the identification of plant species with anticancer activity and these included *P. tortuosum*, *U. maritima* and *G. sinaicus*. Narciclasine was isolated from *P. tortuosum* (Chapter 3) and shown to reduce the expression of SK1 and Des1 in two breast cancer cell lines. The anticancer activity of narciclasine is consistent with other published literature. For instance, narciclasine inhibits proliferation and induces apoptosis in breast cancer cells that involves the cleavage of PARP. In this case, narciclasine inhibited the proliferation of seven breast cancer cell lines, including MCF7, MDA-MB-231, MDA-MB-468, BT-483, BT-549, HCC-1937 and MCF-10A (Cao *et al.*, 2018) and activated caspase-9 and PARP cleavage in HCC-1937 and MDA-MB-231 cells. Narciclasine also induced LC3B-I/II processing, increased beclin-1 expression and promoted p62 degradation suggesting that

narciclasine promotes autophagy-dependent-apoptosis in these cancer cell types. Indeed, the treatment of these cell lines with the autophagy inhibitor 3-methyladenine (3-MA, which inhibits phosphoinositide 3-kinase) blocked caspase 9-dependent apoptosis. In the current study, narciclasine induced a reduction in SK1 and Des1 expression *via* a mechanism that is independent of the ubiquitin-proteasomal pathway. This was evident as the reduction in SK1 and Des1 expression was not blocked by the proteasome inhibitor, MG-132. Thus, narciclasine might modulate the expression of these proteins by a mechanism that involve another degradative pathway, such as cathepsin-dependent regulation (e.g. lysosomal) (Ren *et al.*, 2010). In this regard, Ren *et al.* (2010) reported that SKi induced a reduction of SK1 protein expression *via* lysosomal degradation in pericytes, using chloroquine (lysosomal inhibitor) and lactacystin (proteasomal inhibitor). SKi did not affect TGFβ stimulated SK1 mRNA which indicates that SKi mediates its effect via a post-translational mechanism.

The current study has not addressed whether narciclasine might also affect transcriptional and/or translational regulation to reduce SK1 and/or Des 1 expression. This can be confirmed by assessing the effect of narciclasine on SK1 and Des1 mRNA expression and/or stability (using QPCR) or by measuring *de novo* protein synthesis using [^{35}S] methionine incorporation into SK1 and Des1 under conditions where degradation has been inhibited (e.g., with cycloheximide). Lysosomal degradation can also be assessed using inhibitors of cathepsin D (e.g. Pepstatin) (Gacko *et al.*, 2007). It is noteworthy that narciclasine does not directly inhibit SK1 activity (Susan Pyne, unpublished data) assessed using a radiometric assay with purified SK1.

Narciclasine also reduced Ki67 expression, a marker for proliferation. However, currently it is unclear whether the modulation of SK1 and Des1 expression accounts for the effects of narciclasine on Ki67 expression. It has been reported that Ki67 is expressed during the active phases of cell cycle division (G_1, G_2, S and M) and is not expressed during the resting phase (G_0 phase) in both normal and tumour cells (Gerdes *et al.*, 1984, Scholzen and Gerdes, 2000). However, Ki67 is degraded by the proteasome in G0 and G1 (Sobecki *et al.*, 2017).

It is well established that Ki67 is higher in cancerous tissue compared to normal tissue and that is associated with cancer metastasis and worse clinical outcomes. Targeting

Ki67 arrests proliferation and it has been suggested that Ki67 may be an appropriate target for cancer therapy (Li *et al.*, 2015). Therefore, it would be of interest to test whether narciclasine induce cell cycle arrest by, for example, FACS analysis of propidium iodide-stained cells (control vs narciclasine treated). Additionally, detection of Ki67 in fixed cells using antibodies and immunohistochemistry staining could be used to address further test depletion of ki67 expression in response to narciclasine.

The main finding of chapter 4 is that a mixture of cardenolide glycosides including humistratin and calotropin/calactin were isolated from *G. sinaicus* and shown to possess anticancer activity and to promote the ubiquitin-proteasomal degradation of SK1 and to reduce the expression of Des1. A mixture of these compounds isolated in fraction Gs 3.2.1, were found to inhibit cell proliferation and induce apoptosis via cleavage of PARP in both breast cancer cell lines. Additional studies are required to establish whether these compounds that commercially available in pure form, bind to SK1 or whether they act indirectly, via a sensor, to promote ubiquitin-proteasomal degradation of SK1. The modulation of Des1 expression, which was insensitive to MG132, might involve alterations in its transcriptional/translation regulation of Des1 expression, which could be further investigated using QPCR.

There are several studies which have reported that cardenolide glycosides exhibit anticancer activity including in breast, colon, prostate, and skin cancer. For example, digitoxin induced apoptosis in pancreatic cancer cells through distinct kinase and interferon signalling networks, while inhibiting proliferation in oesophageal adenocarcinoma EAC cell lines *via* downregulation of p38 MAP Kinase 6, Also, proscillaridin A is another cardenolide glycoside which induces apoptosis in NSCLC cells and decreases cell growth *via* calcium-induced death receptor 4 (DR4) upregulation (Sreenivasan *et al.*, 2003, Wang *et al.*, 2009, Krishna *et al.*, 2015, Schneider *et al.*, 2016, Schneider *et al.*, 2017, Li *et al.*, 2018).

A key issue is to understand the molecular mechanism by which SK1 expression is increased in cancer and determine the mechanism by narciclasine and cardenolide glycosides can reverse the increase in SK1 expression to inhibit cancer progression. Many factors that are increased in cancer and inflammatory disease modulate the

expression of SK1. These include, TGF-β (transforming growth factor-β) (Yamanaka *et al.*, 2004) and TNFα (Xia *et al.*, 1998). In addition, leptin was found to induce SK1 activity and expression in MDA-MB-231 and BT-549 cells (Alshaker *et al.*, 2014). Other pharmacological stimuli such as the synthetic phorbol ester, PMA, in MEG-O1 (human megakaryoblastic leukaemia cell line) increase SK1 expression and a PKC inhibitor prevents PMA-stimulated SK1 activity (Nakade *et al.*, 2003). In addition, different transcriptional factors are involved in regulating SK1 expression. For example, Sp1 (specificity protein 1) is a transcription factor that is involved in the regulation of SK1 in response to nerve growth factor (NGF) in PC-12 cells (Sobue *et al.*, 2005). This is important since Sp1 is over expressed in many cancers, such as lung, pancreatic, breast and glioma. Indeed, there is a correlation between Sp1 and SK1 in terms of disease severity, neovascularisation and metastasis (Beishline and Azizkhan-Clifford, 2015). AP2 is another transcription factor that is involved in the regulation of SK1, for instance, in human neuroblastoma cells in response to GDNF (glial line-derives neurotrophic factor) (Sobue *et al.*, 2005). E2F transcriptional family members, E2F1 and E2F7 also regulate SK1 expression in head, neck and liver cancer (Lu *et al.*, 2016). Moreover, SK1 is regulated in response to hypoxia in many cancer cells, brain, renal and endothelial cells. This involves von Hippel-Lindau (VHL), which is also called hypoxia inducible factors (HIFs) and which is a sensor for low oxygen that promotes cancer cell survival (Kim and Kende, 2004). In clear cell renal cell carcinoma (ccRCC), 786-O, HIF2α increases SK1 expression through the loss of VHL protein, while knockdown of HIF2α by siRNA reduced SK1 expression, thereby suggesting an amplification loop between SK1/S1P and HIFs in response to low oxygen (Anelli *et al.*, 2008). Anelli also showed that hypoxia induces SK1, HIF1α and HIF2α expression and knockdown of HIF2α decreases SK1 expression in glioma cells. Also, HIF2α but not HIF1α is bound to SK1 gene, while in another study by Bouquerel *et al.* (2016), it was reported that SK1 regulates the expression of HIF2α expression in lung and ccRCC cells in response to hypoxia. Moreover, Bouquerel *et al.* (2016) proposed that this regulation is due to SK1-dependent downregulation of the AKT/mTOR signalling pathway. In addition, LMO2 (LIM-domain-only protein 2) is another transcription factor that regulates SK1 expression. Knockdown of LMO2 decreased SK1 levels, while overexpression of LMO2 increased SK1 expression in

human umbilical vein endothelial cells (HUVECs) (Matrone *et al.*, 2017). It has been recently shown that SFMBT1 (Scm-like with four malignant brain tumour domains 1) limits transcription of SK1 (Liu *et al.*, 2020). Further studies of these transcription factors and regulation of SK1 expression could be performed to establish whether narciclasine and cardenolide glycosides can exert anticancer activity by modulation of these transcriptional mechanisms.

Another way that SK1 regulated is through its proteasomal degradation. Most of inhibitors targeting SK1, have been reported to degrade SK1 at protein level. Cardenolide glycosides from *G. sinaicus* utilise this mechanism and further work is required to more fully characterise whether cardenolide glycosides activate an E3 ligase that can catalyse polyubiquitination of SK1 to drive proteasomal degradation of the enzyme (Powell *et al.*, 2019). The ultimate result is to reduce the production of S1P, preventing cancer cells from having pro-growth signalling from S1P.

It has been reported that Des1 levels are controlled in post-translational process *via* ubiquitination and proteasome-dependent degradation in response to UV light, chemotherapeutic drugs and DTT (Sridevi *et al.*, 2009, Alsanafi *et al.*, 2018). Both of Hand2 and NFATC1 proteins demonstrate strong binding with the DES1 promotor. Maximum activation was observed with the co-expression of these two proteins, which indicates that both are required for Des1 expression (Azzam *et al.*, 2013). Therefore, narciclasine and other isolated cardenolide glycosides mixture might affect these transcriptional factors.

Many different micro-RNA molecules can limit SK1 expression in different cancer types. These include mi-RNA-124 (ovarian cancer), mi-RNA-125b, mi-RNA-613 (bladder cancer), mi-RNA-659-3p (colon cancer), mi-RNA-506 (hepatocellular carcinoma), miRNA-101 (colorectal cancer) and miRNA-330-3p (gastric cancer), which are downregulated in these cancer cell types. When re-expressed, they reduce SK1 expression. For example, overexpression of miR-124 in ovarian cancer led to SK1 downregulation and consequently, reduce invasion and migration of cancer cells (Zhang et al., 2013). Micro-RNAs participate in fine-tuning protein expression. This is achieved by them forming a complex with ribonuclear proteins, collectively termed the RNA-induced silencing complex (RISC). The RNA component of RISC

specifically hybridises with mRNA molecules which are then either degraded (and therefore not translated to protein) or become translationally repressed (reduced protein expression). Currently, it is not known which miRNAs regulate the expression of Des1. Investigation of the effect of narciclasine on transcriptional and translational regulation of SK1 and Des1 expression would be of value (as proteasomal degradation of SK1 and Des1 protein does not contribute to a reduction in their expression in cells treated with narciclasine),

5.2 Anti-inflammatory activity

The current study also demonstrated that narciclasine (chapter 3) is an anti-inflammatory agent as it blocked TNFα-stimulated IκBα degradation and inhibited NFκB-dependent and AP-1-dependent transcriptional activity. TNFα is a cytokine that induces apoptosis via several signalling pathways. The most familiar is by activation of TNFR1, which associates with TRADD and then activates caspase 8, initiating the caspase cascade resulting in apoptosis (Baud and Karin, 2001), TNFα induced apoptosis is observed in breast cancer cells such as MCF7 and in HER2-positive cell types. Indeed, blockade of TNFα signalling has been suggested as a strategy for treating HER2 positive breast cancer types in combination with existing anti-HER2 treatments (Mercogliano *et al.*, 2020). In this regard, many cancers rise from sites of infections, inflammations, or chronic irritation. Moreover, other studies found proinflammatory signalling to be correlated with the aggressiveness of breast cancers (Qiao *et al.*, 2016). Therefore, it would be interesting to also investigate the effects of narciclasine on a HER2-positive breast cancer cell line, e.g., MDA-MB-453 cells and in a wider inflammatory context.

Another finding of chapter 4 is the anti-inflammatory effect of the cardenolide mixture, which reduces TNFα-stimulated NFκB transcriptional activity and inhibits AP-1-dependent transcriptional regulation. More research is required to determine the mechanism(s) of inhibition of NFκB/AP1 signalling by these cardenolide glycosides.

Ectopic over-expression of Des1 using several cancer cells, including liver cancer (HepG2), lung cancer (A549), oesophageal cells (Eca 109) and breast cancer (MCF7 cells), promotes the transition of cells from G0/G1 to S phase. This was associated

with increased cyclin D1 protein levels with no change in other cyclin protein levels. Interestingly, NFκB activation has been linked with increased Des1 expression which in turn induce cyclin D1 (Zhou *et al.*, 2009). This is of particular interest given that narciclasine and the cardenolide glycosides mixture modulate NFκB transcriptional activity in keratinocytes in the current study.

For decades, many natural products have been known to be effective against many diseases because of their antioxidant, anti-inflammatory, anti-viral and anticancer effects. Quercetin was studied along other plant derived natural products (ursolic acid, capsaicin, DL-α tocopherol acetate, citral, limonin, vanillin and simvastatin) to evaluate its inhibitory effect against SK1 (Gupta *et al.*, 2019). Using isothermal titration calorimetry, molecular docking, MD simulation and ATPase inhibition assay, suggested a strong interaction between quercetin and SK1. Analysis of this complex revealed that quercetin occupies the same position of D-sphingosine (Gupta *et al.*, 2019). This interaction appears important as quercetin is reported to inhibit the S1P/SK1 pathway signalling and to reduce pulmonary fibrosis *in vivo* (Zhang *et al.*, 2018b). Moreover, other high-throughput screening studies of natural products have been undertaken to find and isolate compounds with SK1 inhibitor activity (Jairajpuri *et al.*, 2020). Two compounds, ZINC05434006 and ZINC04260971, were found to be potent inhibitors of SK1 and which bind directly to SK1 to inhibit activity. In addition, both compounds exhibit anticancer activity, and this provides impetus for further optimisation to produce new medicines.

5.3 Future directions

The isolated compounds in present study, are lead compounds to develop new therapeutic drugs. More work needed to assess the effect of narciclasine and cardenolide mixture compounds (humistratin, calotropin and calactin) on transcriptional and translational regulation of SK1 and Des1 expression, and to assess whether narciclasine and cardenolide glycoside mixture induce cell cycle arrest. Using computational methods with the availability of the 3D structure of the target protein (SK1) (Adams *et al.*, 2016, Adams *et al.*, 2019) will enable the investigation to understand the molecular basis of the interaction between the lead compounds and the protein target. It can be focused on the development of SK-selective inhibitors. The

three cardenolide glycosides could be studied applying a molecular docking approach and MD simulation. This will provide more data of the binding mode of each compound to SK1. Such studies might guide optimisation of the compounds to produce highly effective SK1 inhibitors with drug-like properties.

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